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Original article

Novel naphthalimide derivatives as potential apoptosis-inducing agents: Design, synthesis and biological evaluation

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ABSTRACT

A series of novel naphthalimide derivatives with flexible alkyl/aryl moieties were designed and synthesized. Their antitumor activities were evaluated against HeLa, A549, P388, HL-60, MCF-7, HCT-8 and A375 cancer cell lines in vitro. The preliminary results showed that most of the derivatives had comparable antitumor activities over Amonafide with the IC_{50} values of 10^{-6} to 10^{-5} M. More importantly, flow cytometric analysis indicated that the derivatives could effectively induce G_2/M arrest and progress to apoptosis in HL-60 cell line after double staining with annexin V–FITC and propidium iodide. The present work provided a novel class of naphthalimide-based derivatives with potent apoptosis-inducing and antitumor activities for further optimization.

1. Introduction

The design and synthesis of highly efficient antitumor agents had attracted a lot of attention in organic and medicinal chemistry. Naphthalimide-based anticancer drugs constituted an indispensable part in the development of antitumor agents. Although this kind of chemotherapeutic agent was the mainstay for cancer therapy for decades [1–4], the medical need was largely unmet due to its unexpected toxicity such as Amonafide (**1**, Fig. 1), which limited the use and development of this kind of drug [5]. Another crucial limitation was that malignant stem cells could escape treatment of this medicine [6]. These defects conferred cancer cells resistance to therapeutic agents and made current anticancer therapies less effective, leading ultimately to their failure [7]. So it was necessary for us to attempt alternative structural modification of naphthalimide to achieve favorable or different biological activities.

In parallel with the growth of knowledge concerning the side effects of this medicine, the activation of apoptosis pathways has become an alternative and promising method of cancer treatment [8,9]. Apoptosis was a cellular process critical to normal development and homeostasis of multicellular organisms [7]. Meanwhile, apoptosis was also an evolutionarily conserved and highly regulated process [10], which used to eliminate defective and unnecessary cells [11]. It was recognized that dysfunction of the apoptosis machinery was a hallmark of cancer [12–14], and induction of apoptosis was arguably the most potent defense against cancer [5]. Therefore, much attention was paid to the design and discovery of apoptotic inducers during recent years [15–34], and promising results have been obtained in this way. Compounds **2** [5] and **3** [34] were the representative reported apoptotic inducers (Fig. 1), and had the characteristics of a conformational flexible aryl moiety. These inspired us to assume that naphthalimide derivatives with alkyl/aryl moiety linked by various chains might have some improved or different biological activities.

As shown in Scheme 1, the naphthalimide scaffold was utilized as key prototype structural unit, and the aliphatic amine and aryl functional groups were conjugated to the naphthalene ring. Introduction of basic hydrophilic amine chain to the position-2 of naphthalimide ring might contribute to increase its cytotoxicity against tumor cell lines [35]. The amino substituents introduced to the position-6 of naphthalimide ring were difficult to be acetylated [36] and might involve arrest of cell cycle [37]. Various linkers were designed between naphthalimide scaffold and flexible aryl moiety in order to investigate their effects on the biological activities. Therefore, the target compounds **5a–c**, **7a–e** and **9a–b** were prepared, and their in vitro cytotoxicities and apoptosis-inducing activities were also evaluated.

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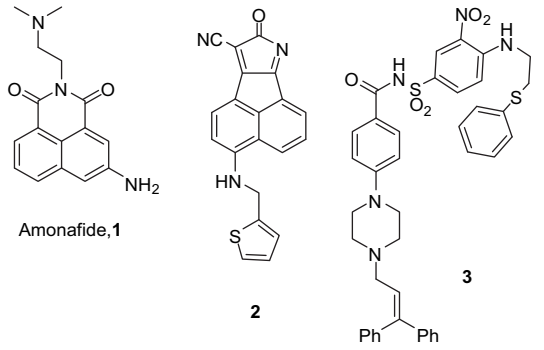


Fig. 1. The structures of some reported compounds.

2. Results and discussion

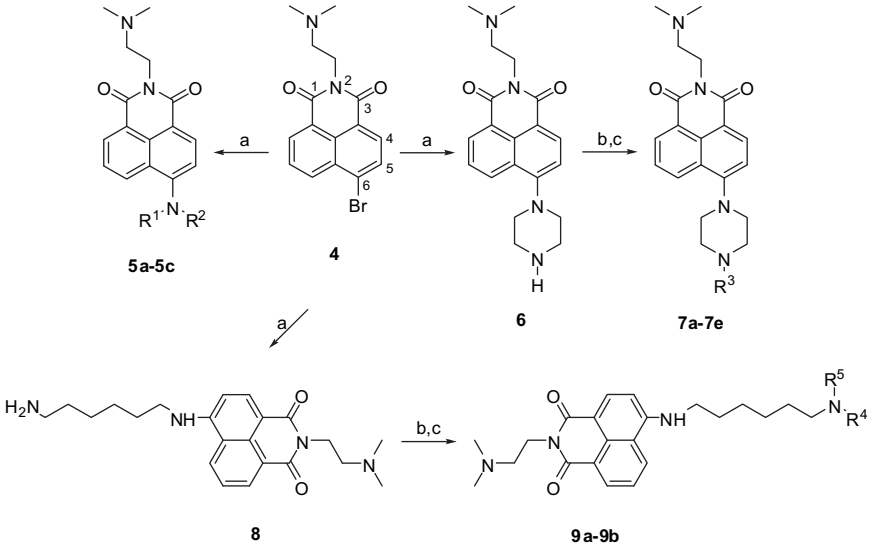
2.1. Chemistry

The synthetic routes of the designed compounds 2-(2-(dimethylamino)ethyl)-6-(substituted amino)-1*H*-benzo[*de*]isoquinoline-1,3(2*H*)-dione derivatives **5a–c**, **7a–e** and **9a–b** were shown in Scheme 1. 6-Bromo-2-(2-(dimethylamino)ethyl)-1*H*-benzo[*de*]isoquinoline-1,3(2*H*)-dione **4** [38] was treated with various aliphatic amines in $CH_3OCH_2CH_2OH$, affording the target compounds **5a–c** with moderate yields of 40–60% and intermediates **6** and **8**, respectively. The subsequent nucleophilic addition/reduction reaction of **6** and **8** with substituted cinnamaldehyde led to the target compounds **7a–d** and **9b** with satisfied yields of 85–90%. Compounds **7e** and **9a** were prepared by the condensation reaction of **6** and **8** with 4-(4-(3,3-diphenylallyl)piperazin-1-yl)benzoic acid [34]. The structures of all the newly synthesized compounds were well identified by ^{1}H NMR, ^{13}C NMR, HRMS and IR spectra.

2.2. Cytotoxic effects

The in vitro antitumor activities of the target compounds were evaluated by examining their cytotoxic effects using sulforhodamine B (SRB) assay [39] against A549 and MTT tetrazolium dye assay [40] against HeLa, P388, HL-60, MCF-7, HCT-8 and A375, respectively. The IC_{50} represented the drug concentration (μM) required to inhibit cell growth by 50% and the results were summarized in Table 1.

From Table 1, it could be seen that the target compounds showed comparable cytotoxicities over Amonafide against tested cancer cell lines except for HCT-8 with the IC_{50} values of 10^{-6} to 10^{-5} M. Compounds **5b** and **7a** exhibited the highest cytotoxicities against HeLa, A549 and P388, MCF-7 cell lines among each group with IC_{50} values of 6.06, 1.27 and 4.53, 8.65 μM, respectively. For A375 and HL-60 cell lines, the highest cytotoxic compounds were **7d** and **5a**, respectively. Among them, the IC_{50} values of compounds **5a** and **5b** were 2.1 and 10.2-fold lower than the values found for Amonafide against HL-60 and A549 cell lines, respectively. In most cases, the cytotoxicities increased in sequence of **9**, **7** and **5**, which indicated that the magnitude and conformation of alkyl/aryl substituents had intense influence on the cytotoxic activities of these compounds. For example, compound **7a** was more cytotoxic than compound **9b** but less cytotoxic than compound **5b**. Moreover, the linkers in position-6 influenced its cytotoxicity. We found that compounds **5c** and **9a** bearing flexible alkyl linker showed a striking contrast compared to compound **7e** bearing semi-rigid piperazin linker. Additionally, the cytotoxic activities of compounds **7a–d** were also influenced by the substituents on aryl moiety.



5a : R^1 = -H; R^2 = -CH_2-2-thiophene

5b : R^1 , R^2 = -$CH_2CH_2SCH_2CH_2$-

5c : R^1 = -H; R^2 = -$CH_2CH_2SC_6H_5$

9a : R^4 = -H; R^5 = -COC_6H_4-*p*-$N(CH_2CH_2)_2NCH_2CH{=}C(C_6H_5)_2$

9b : $R^4 = R^5$ = -$CH_2CH{=}C(C_6H_5)_2$

7a : R^3=-$CH_2CH{=}C(C_6H_5)_2$

7b : R^3=-$CH_2CH{=}CH(C_6H_4$-*p*-CN)

7c : R^3=-$CH_2CH{=}CH(C_6H_4$-*p*-$N(CH_3)_2)$

7d : R^3=-$CH_2CH{=}CHC_6H_5$

7e : R^3=-COC_6H_4-*p*-$N(CH_2CH_2)_2NCH_2CH{=}C(C_6H_5)_2$

Scheme 1. Reagents and conditions: (a) corresponding amine, $HOCH_2CH_2OCH_3$, reflux 3 h, 40–88% yield; (b) corresponding cinnamaldehyde, $NaBH(OAc)_3$, $ClCH_2CH_2Cl$, r.t. 3 h, 85–90% yield; (c) 4-(4-(3,3-diphenylallyl) piperazin-1-yl)benzoic acid, $CHCl_3$, EDCI, DMAP, r.t. 48 h, 60% yield.

Table 1
Cytotoxicities of naphthalimide derivatives against HeLa, A549, P388, HL-60, MCF-7, HCT-8 and A375 cell lines.

Compound	Cytotoxicity (IC_{50}, μM)						
	HeLa	A549	P388	HL-60	MCF-7	HCT-8	A375
Amonafide	1.40	13.00	4.56	17.96	11.88	9.26	7.51
5a	12.59	6.24	26.71	8.55	13.02	>50	46.10
5b	6.06	1.27	>50	16.99	16.04	43.79	11.45
5c	6.89	3.37	7.23	37.63	16.37	>50	6.66
7a	7.64	>50	4.53	22.09	8.65	>50	11.93
7b	6.89	11.73	6.61	10.86	16.77	>50	7.41
7c	>50	4.88	11.29	>50	>50	>50	>50
7d	11.4	>50	9.12	12.21	12.21	>50	6.62
7e	>50	25.58	13.81	>50	20.68	>50	>50
9a	7.19	36.25	7.67	8.65	21.74	>50	6.81
9b	36.01	22.01	24.45	31.66	>50	>50	36.90

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

When the substituents were phenyl and nitrile groups, relatively favorable cytotoxic activities were obtained. Therefore, manipulation of naphthalimide derivatives to achieve potent antitumor activities, seemed to be closely dependent on structures and conformation of the aryl groups and the linkers, which played important roles in the chemical and biological functions [41].

2.3. Cell cycle profile and apoptosis in HL-60 cells

In the search of a possible mechanism of action responsible for antitumor activity of the target compounds, we have investigated their effects on the cell cycle by fluorescence activated cell sorting (FACS) [17–19,30,31]. HL-60 cell line was used in the assay. Cells were treated with these compounds, and after 24 h were fixed and labeled with propidium iodide. The different phases of cell cycle were analyzed by flow cytometry and the results were summarized in Table 2.

As shown in Table 2, the target compounds were found to effectively induce G_2/M arrest and progress to apoptosis in HL-60 cell line [42]. After incubation with IC_{50} concentration of these compounds, the sub-G_1 portions were increased from 9.59% to 19.30% or from 7.62% to 17.33% versus the untreated control or the control treated with Amonafide, respectively. Meanwhile, the G_2/M population increased from 13.29% in the control to 13.73–33.08% in cells treated with the target compounds. Moreover, the compounds treatment HL-60 cell line could notably induce morphological changes into rounding form (data not listed), which was a primary indication of apoptosis and cell death [21]. The results in Tables 1 and 2 indicated that there was no obvious relationship between cytotoxicity and apoptosis-inducing activity. Cell cycle distribution of HL-60 cell line in the presence of representative compounds **5b**, **7b** and **7e** were displayed in Fig. 2.

To further verify their pro-apoptotic function, FACS analysis was carried out after double staining cells with propidium iodide and annexin V–FITC [7,21,29]. As shown in Fig. 3, representative compound **5b** was very effective in induction of apoptosis in a dose–dependent manner. Treatment of HL-60 cell line by 4.3, 8.5 and 17 μM of **5b** for 36 h resulted in 48.92%, 85.05% and 90.88% of apoptotic cells, respectively, as compared to 2.84% of apoptotic cells in an untreated control. Also, compounds **7b** and **7e** had significant effects on induction of apoptosis, and the data were summarized in Table 3. (Early apoptosis corresponded to annexin V single-positive cells and late apoptosis/necrosis corresponded to double-positive cells.) These results collectively suggested that the target compounds might inhibit the growth of HL-60 cell line by induction of apoptosis. Detailed interaction mechanism, structure–antitumor activity relationships and further biological experiments to distinguish late apoptosis from necrosis, were well under way in our laboratory.

Table 2
Cell cycle distribution of HL-60 cell line in the presence of IC_{50} concentration of the target compounds.

Compound	Concentration (μM)	Cell cycle distribution (%)			
		G_1	S	G_2/M	sub-G_1
Control	0	52.86	33.86	13.29	0.72
Amonafide	18	52.26	36.68	11.06	2.69
5a	9	54.34	27.76	17.90	11.70
5b	17	30.64	42.54	26.81	13.20
5c	38	55.52	27.70	16.78	10.31
7a	22	52.36	32.68	14.96	20.02
7b	11	37.07	29.84	33.08	12.41
7c	50	34.93	51.34	13.73	18.77
7d	12	40.33	41.06	18.61	14.52
7e	50	52.04	25.34	22.62	13.20
9a	9	51.15	32.41	16.44	17.98
9b	32	66.73	0.3	32.97	17.31

3. Conclusion

In summary, a series of novel naphthalimide derivatives with flexible alkyl/aryl moiety were designed and synthesized, and their antitumor activities were evaluated against a variety of cancer cell lines in vitro. The preliminary results showed that most of the derivatives had comparable antitumor activities over Amonafide with the IC_{50} values of 10^{-6} to 10^{-5} M. More importantly, flow cytometric analysis indicated that these derivatives could effectively induce G_2/M arrest and progress to apoptosis in HL-60 cell line after double staining with annexin V and propidium iodide. This suggested that the target compounds might inhibit the growth of HL-60 cell line by induction of apoptosis. The present work demonstrated that incorporating the biological active unit of naphthalimide and flexible alkyl/aryl moiety might be able to result in a novel class of lead compounds with potential apoptosis-inducing and antitumor activities. Further structural optimization and detailed biological studies on the molecular mechanism of action about the designed naphthalimide derivatives were under the way.

4. Experimental protocols

All reagents were of the commercial quality and were used without purification. ^{1}H and ^{13}C NMR were obtained with a Bruker AV-400 spectrometer with chemical shifts reported as ppm (in $CDCl_3$/DMSO-d_6/CD_3COCD_3, TMS as internal standard). IR was obtained using a Perkin–Elmer 2000 FTIR instrument. High-resolution mass spectra (HRMS) were obtained on a HPLC-Q-Tof MS (Micro) spectrometer. Melting points were determined by an X-6 micro-melting point apparatus and uncorrected. Column chromatography was performed using silica gel 200–300 mesh.

4.1. Synthesis of naphthalimide derivatives

4.1.1. General procedure for the synthesis of *6*, *8* and *5a–5c*

Compound **4** (50 mg, 0.144 mmol), prepared by using the previously reported method [38], was dissolved in ethylene glycol monomethyl ether (2 mL), then piperazin or corresponding amine (1.44 mmol) was added. The solution was stirred and refluxed under nitrogen for 3 h, cooled, and concentrated under vacuum. The residue was subjected to column chromatography on silica gel. Compounds **6**, **8** and **5a–5c** were separated with CH_2Cl_2/CH_3OH 50:1 (v/v) as orange solids, respectively.

*4.1.1.1. 2-(2-(Dimethylamino)ethyl)-6-(piperazin-1-yl)-1H-benzo[de] isoquinoline-1,3(2H)-dione (**6**).* Orange solid. Yield: 88%; m.p. 99.1–101.1 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ_H: 8.26–8.22 (m, 2H, Ar–H), 8.18 (d, J = 8.0 Hz, 1H, Ar–H), 7.62 (t, J = 8.0 Hz, 1H, Ar–H), 7.10

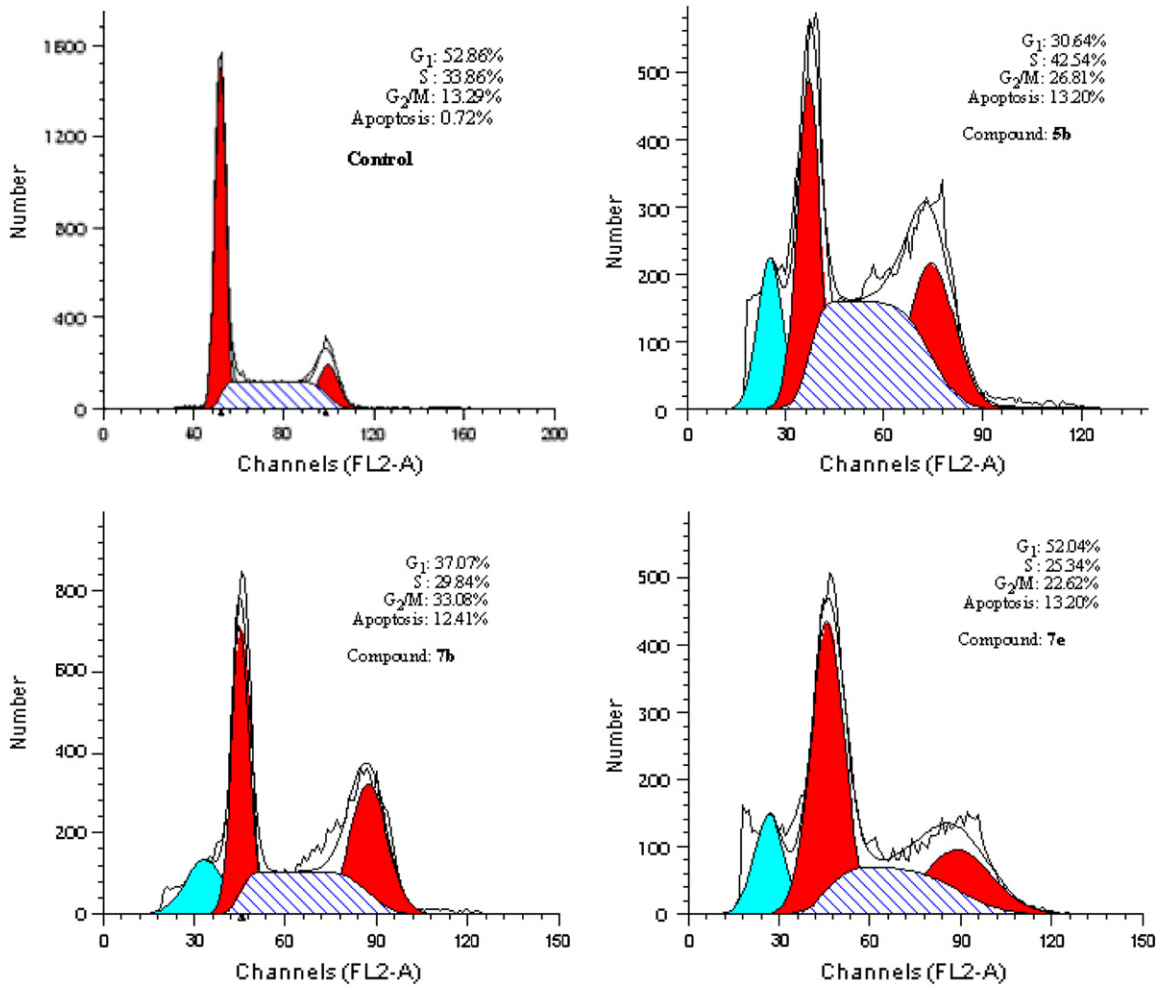


Fig. 2. Cell cycle distribution of HL-60 cell line in the presence of representative compounds **5b**, **7b** and **7e**, respectively. HL-60 cell line was treated with IC_{50} concentration of the compounds or with 0.1% DMSO. Cells were harvested, fixed and stained with propidium iodide. 20,000 stained cells were then subjected to FACScalibur analysis to determine the distribution of cells.

(d, $J = 8.0$ Hz, 1H, Ar–H), 4.02 (t, $J = 6.8$ Hz, 2H, NCH_2), 3.04 (s, 4H, CH_2NCH_2), 2.97 (s, 4H, CH_2NHCH_2), 2.41 (t, $J = 6.8$ Hz, 2H, $CH_2N(CH_3)_2$), 2.16 (s, 6H, $N(CH_3)_2$); MS (EI) *m/z*: 352.2 $(M)^+$.

*4.1.1.2. 6-(6-Aminohexylamino)-2-(2-(dimethylamino)ethyl)-1H-benzo[*de*]isoquinoline-1,3(2H)-dione (**8**).* Orange solid. Yield: 60%; m.p. 141.9–143.9 °C; ^1H NMR ($CDCl_3$, 400 MHz) δ_H: 8.56 (d, $J = 7.2$ Hz, 1H, Ar–H), 8.44 (d, $J = 8.4$ Hz, 1H, Ar–H), 8.09 (d, $J = 8.0$ Hz, 1H, Ar–H), 7.60 (t, $J = 8.0$ Hz, 1H, Ar–H), 6.70 (d, $J = 8.8$ Hz, 1H, Ar–H), 5.35 (s, br, 1H, ArNH), 4.32 (t, $J = 7.2$ Hz, 2H, NCH_2), 3.41 (q, $J_1 = 6.8$ Hz, $J_2 = 12.4$ Hz, 2H, $ArNHCH_2$), 2.75 (t, $J = 6.4$ Hz, 2H, CH_2N), 2.66 (t, $J = 6.4$ Hz, 2H, $CH_2N(CH_3)_2$), 2.38 (s, 6H, $N(CH_3)_2$), 1.84–1.83 (m, 2H, CH_2CH_2N), 1.56–1.43 (m, 6H, $CH_2CH_2CH_2$); MS (EI) *m/z*: 382.2 $(M)^+$.

*4.1.1.3. 2-(2-(Dimethylamino)ethyl)-6-(thiophene-2-ylmethylamino)-1H-benzo[*de*]isoquinoline-1,3(2H)-dione (**5a**).* Orange solid. Yield: 60%; m.p. 154.9–156.9 °C; ^1H NMR ($CDCl_3$, 400 MHz) δ_H: 8.46 (d, $J = 7.6$ Hz, 1H, Ar–H), 8.35 (d, $J = 8.4$ Hz, 1H, Ar–H), 8.06 (d, $J = 8.4$ Hz, 1H, Ar–H), 7.53 (t, $J = 8.0$ Hz, 1H, Ar–H), 7.27 (d, $J = 4.4$ Hz, 1H, thiophene-H), 7.11 (d, $J = 3.2$ Hz, 1H, thiophene-H), 7.02–7.00 (m, 1H, thiophene-H), 6.70 (d, $J = 8.4$ Hz, 1H, Ar–H), 5.98 (t, $J = 5.2$ Hz, 1H, Ar–NH), 4.76 (d, $J = 5.2$ Hz, 2H, $NHCH_2$-thiophene), 4.34 (t, $J = 6.8$ Hz, 2H, CH_2N), 2.74 (t, $J = 6.8$ Hz, 2H, $CH_2N(CH_3)_2$), 2.42 (s, 6H, $N(CH_3)_2$); ^{13}C NMR ($CDCl_3$, 100 MHz) δ_C: 164.63, 164.07, 148.76, 140.28, 134.17, 131.13, 129.52, 127.21, 126.14, 126.08, 125.37, 124.80, 122.88, 120.28, 110.87, 104.84, 57.22, 45.69, 42.94, 37.60; FTIR (KBr, cm^{-1}): 3288, 2940, 2770, 1673, 1643, 1580, 1547, 1387, 1365, 1320, 1287, 1238, 1120, 1090, 774, 693; HRMS (ES+) calcd for $C_{21}H_{22}N_3O_2S$ $([M + H])^+$ 380.1433, found 380.1404.

*4.1.1.4. 2-(2-(Dimethylamino)ethyl)-6-thiomorpholino-1H-benzo[*de*]isoquinoline-1,3(2H)-dione (**5b**) [43].* Orange yellow solid. Yield: 40%; m.p. 122.5–124.5 °C; ^1H NMR ($CDCl_3$, 400 MHz) δ_H: 8.58 (d, $J = 6.8$ Hz, 1H, Ar–H), 8.51 (d, $J = 8.0$ Hz, 1H, Ar–H), 8.36 (d, $J = 8.4$ Hz, 1H, Ar–H), 7.70 (t, $J = 7.6$ Hz, 1H, Ar–H), 7.23 (d, $J = 8.0$ Hz, 1H, Ar–H), 4.33 (t, $J = 6.8$ Hz, 2H, CH_2N), 3.50 (s, 4H, CH_2NCH_2), 2.97 (s, 4H, CH_2SCH_2), 2.69 (t, $J = 2.4$ Hz, 2H, $CH_2N(CH_3)_2$), 2.39 (s, 6H, $N(CH_3)_2$); ^{13}C NMR ($CDCl_3$, 100 MHz) δ_C: 164.43, 163.95, 156.74, 132.48, 131.23, 129.99, 129.89, 126.55, 125.93, 123.24, 117.18, 115.95, 56.88, 55.55, 45.58, 37.79, 28.14; FTIR (KBr, cm^{-1}): 2970, 2911, 2814, 2762, 1691, 1654, 1584, 1509, 1446, 1391, 1339, 1283, 1231, 1127, 1038, 949, 852, 778, 756; HRMS (ES+) calcd for $C_{20}H_{24}N_3O_2S$ $([M + H])^+$ 370.1589, found 370.1588.

*4.1.1.5. 2-(2-(Dimethylamino)ethyl)-6-(2-(phenylthio)ethylamino)-1H-benzo[*de*]isoquinoline-1,3(2*H*)-dione (**5c**).* Orange red solid. Yield: 55%; m.p. 142.3–144.3 °C; ^1H NMR ($CDCl_3$, 400 MHz) δ_H: 8.54 (d, $J = 7.2$ Hz, 1H, Ar–H), 8.39 (d, $J = 8.4$ Hz, 1H, Ar–H), 7.94

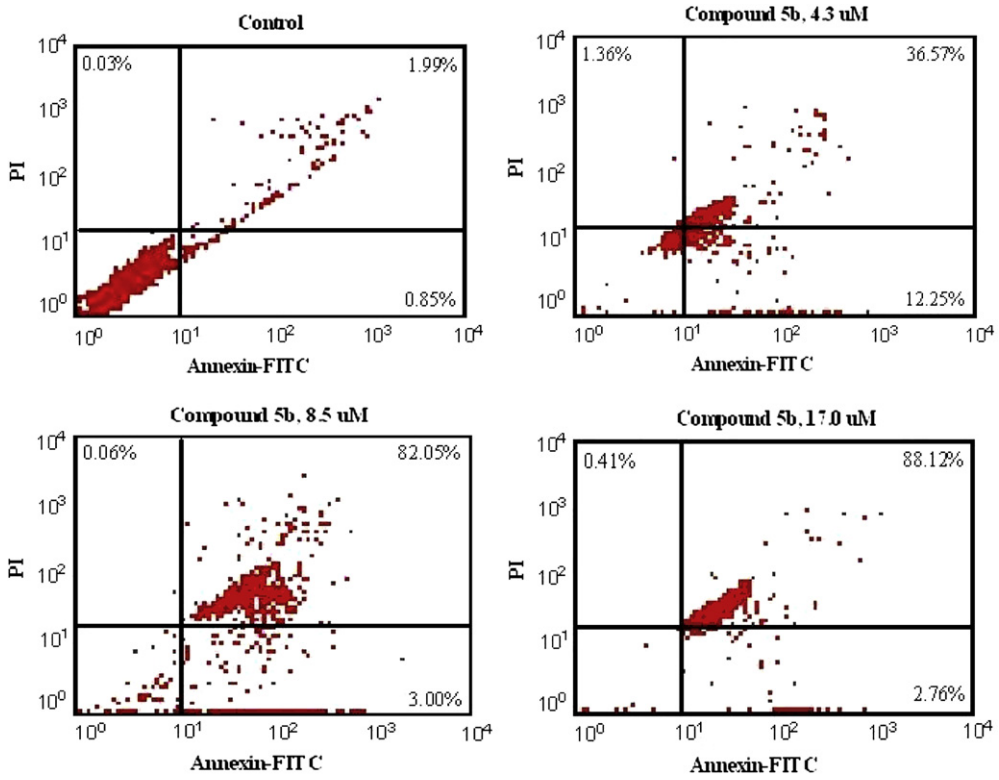


Fig. 3. Compound **5b** induced apoptosis of HL-60 cell line. HL-60 cell line was treated with 4.3, 8.5 and 17.0 μM of **5b** or with vehicle solvent (0.1% DMSO) for 36 h and stained with annexin V–FITC and propidium iodide. Stained cells then were subjected to FACScalibur analysis to determine the distribution of cells.

(d, $J = 8.4$ Hz, 1H, Ar–H), 7.53 (t, $J = 8.0$ Hz, 1H, Ar–H), 7.47 (d, $J = 7.2$ Hz, 2H, Ar–H), 7.35–7.29 (m, 3H, Ar–H), 6.57 (d, $J = 8.4$ Hz, 1H, Ar–H), 5.91 (s, br, 1H, Ar–NH), 4.44 (t, $J = 6.8$ Hz, 2H, CH_2N), 3.60 (q, $J_1 = 6$ Hz, $J_2 = 12$ Hz, 2H, $NH\underline{CH_2}$), 3.33 (t, $J = 6$ Hz, 2H, $\underline{CH_2}N(CH_3)_2$), 3.02 (s, br, 2H, CH_2S), 2.64 (s, 6H, $N(CH_3)_2$); ^{13}C NMR ($CDCl_3$, 100 MHz) δ_C: 164.91, 164.10, 134.57, 134.34, 131.33, 130.76, 129.91, 129.32, 127.15, 124.71, 120.40, 104.15, 56.14, 44.27, 42.43, 33.05, 29.69; FTIR (KBr, cm^{-1}): 3296, 3051, 2918, 2844, 1680, 1632, 1576, 1547, 1394, 1361, 1290, 1246, 1179, 1112, 771, 752, 698; HRMS (ES+) calcd for $C_{24}H_{26}N_3O_2S$ $([M+H])^+$ 420.1746, found 420.1730.

4.1.2. General procedure for the synthesis of **7a–7d** and **9b**

Compound **6**/**8** (0.142 mmol) was dissolved in 1,2-dichloroethane (2 mL) in the presence of $NaBH(OAc)_3$ (36 mg, 0.171 mmol), then the corresponding cinnamaldehyde (0.171 mmol) was added. The solution was stirred at room temperature under nitrogen for 3 h, then diluted with saturated $NaHCO_3$ solution (15 mL) and extracted twice with ethyl acetate (30 mL). The combined organic phase was washed twice with saturated NaCl solution and dried over sodium sulfate and evaporated to dryness. The residue was purified on silica gel column chromatography by using CH_2Cl_2/CH_3OH 50:1 (v/v) as the eluent, affording **7a–7d** and **9b** as bright yellow–khaki solids.

*4.1.2.1. 2-(2-(Dimethylamino)ethyl)-6-(4-(3,3-diphenylallyl)piperazin-1-yl)-1H-benzo[*de*]isoquinoline-1,3(2H)-dione (**7a**).* Bright yellow solid. Yield: 90%; m.p. 160.6–161.9 °C; ^1H NMR ($CDCl_3$, 400 MHz) δ_H: 8.57 (d, $J = 7.2$ Hz, 1H, Ar–H), 8.52 (d, $J = 8.0$ Hz, 1H, Ar–H), 8.37 (d, $J = 8.4$ Hz, 1H, Ar–H), 7.67 (t, $J = 8.0$ Hz, 1H, Ar–H), 7.45–7.37 (m, 3H, Ar–H), 7.29–7.28 (m, 5H, Ar–H), 7.23–7.22 (m, 3H, Ar–H), 6.29 (t, $J = 6.8$ Hz, 1H, C=CH), 4.33 (t, $J = 7.2$ Hz, 2H, CH_2N), 3.31 (s, 4H, CH_2NCH_2), 3.25 (d, $J = 6.8$ Hz, 2H, $CH\underline{CH_2}N$), 2.79 (s, 4H, CH_2NCH_2), 2.66 (t, $J = 7.2$ Hz, 2H, $\underline{CH_2}N(CH_3)_2$), 2.37 (s, 6H, $N(CH_3)_2$); ^{13}C NMR ($CDCl_3$, 100 MHz) δ_C: 164.52, 164.03, 155.98, 144.64, 141.91, 139.57, 132.61, 131.11, 130.29, 129.94, 129.80, 128.27, 128.20, 127.43, 127.32, 127.27, 126.18, 125.57, 123.23, 116.68, 114.93,

Table 3
Apoptosis data of representative compounds **7b** and **7e** treatment HL-60 cells after double staining with annexin V–FITC and propidium iodide.

Compound	Concentration (μM)	Viable cells (%)	Early apoptosis (%)	Late apoptosis and necrosis (%)
7b	0	95.33	0.51	3.92
	2.7	28.11	64.60	5.98
	5.5	0.28	70.49	27.68
	11.0	1.16	18.43	79.03
7e	0	95.33	0.51	3.92
	12.5	49.16	43.69	5.71
	25.0	0.34	79.60	18.64
	50.0	0.16	40.18	58.07

Extent of apoptosis was measured through annexin V–FITC apoptosis detection kit (Invitrogen, USA) as described by the manufacture's instruction. Early apoptosis corresponded to annexin V single-positive cells and late apoptosis/necrosis corresponded to double-positive cells.

57.28, 56.99, 53.21, 53.10, 45.70, 37.95; FTIR (KBr, cm^{-1}): 3422, 2938, 2814, 2762, 1688, 1647, 1584, 1450, 1383, 1235, 1135, 1045, 786, 756, 696; HRMS (ES+) calcd for $C_{35}H_{37}N_4O_2$ $([M+H])^+$ 545.2917, found 545.2917.

*4.1.2.2. (E)-4-(3-(4-(2-(2-(Dimethylamino)ethyl)-1,3-dioxo-2,3-dihydro-1H-benzo[*de*]isoquinolin-6-yl)piperazin-1-yl)prop-1-enyl)benzonitrile (**7b**).* Orange solid. Yield: 90%; m.p. 128.2–130.2 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ_H: 8.58 (d, $J=7.2$ Hz, 1H, Ar–H), 8.52 (d, $J=8.0$ Hz, 1H, Ar–H), 8.41 (d, $J=8.4$ Hz, 1H, Ar–H), 7.69 (t, $J=8.0$ Hz, 1H, Ar–H), 7.62 (d, $J=8.4$ Hz, 2H, Ar–H), 7.49 (d, $J=8.4$ Hz, 2H, Ar–H), 7.23 (d, $J=8.0$ Hz, 1H, Ar–H), 6.64 (d, $J=16.0$ Hz, 1H, C=CH), 6.51–6.44 (m, 1H, Ar–H), 4.33 (t, $J=7.2$ Hz, 2H, CH_2N), 3.36–3.33 (m, 6H, $CH\underline{CH_2}N$ and $\underline{CH_2}NCH_2$), 2.85 (s, 4H, CH_2NCH_2), 2.67 (t, $J=7.2$ Hz, 2H, $\underline{CH_2}N(CH_3)_2$), 2.37 (s, 6H, $N(CH_3)_2$); ^{13}C NMR ($CDCl_3$, 100 MHz) δ_C: 164.50, 164.01, 155.84, 141.22, 132.59, 132.47, 131.62, 131.15, 130.59, 130.21, 129.95, 126.82, 126.19, 125.64, 123.28, 118.85, 116.83, 114.97, 110.93, 60.73, 56.96, 53.32, 53.02, 45.66, 37.93; FTIR (KBr, cm^{-1}): 2933, 2814, 2762, 2214, 1695, 1651, 1584, 1569, 1450, 1368, 1231, 1131, 1038, 975, 778; HRMS (ES+) calcd for $C_{30}H_{32}N_5O_2$ $([M+H])^+$ 494.2556, found 494.2542.

*4.1.2.3. (E)-2-(2-(Dimethylamino)ethyl)-6-(4-(3-(4-(dimethylamino)-phenyl)allyl)piperazin-1-yl)-1*H*-benzo[*de*]isoquinoline-1,3(2*H*)-dione (**7c**).* Orange red solid. Yield: 85%; m.p. 125.7–127.7 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ_H: 8.58 (dd, $J_1=7.2$ Hz, $J_2=0.8$ Hz, 1H, Ar–H), 8.52 (d, $J=8.0$ Hz, 1H, Ar–H), 8.42 (dd, $J_1=8.4$ Hz, $J_2=0.8$ Hz, 1H, Ar–H), 7.68 (q, $J_1=7.6$ Hz, $J_2=0.8$ Hz, 1H, Ar–H), 7.32 (d, $J=8.8$ Hz, 2H, Ar–H), 7.22 (d, $J=8.0$ Hz, 1H, Ar–H), 6.70 (d, $J=8.8$ Hz, 2H, Ar–H), 6.52 (d, $J=15.6$ Hz, 1H, C=CH), 6.16–6.09 (m, 1H, Ar–H), 4.33 (t, $J=7.2$ Hz, 2H, CH_2N), 3.33–3.29 (m, 6H, $CH\underline{CH_2}N$ and $\underline{CH_2}NCH_2$), 2.97 (s, 6H, Ar–$N(CH_3)_2$), 2.84 (s, br, 4H, CH_2NCH_2), 2.66 (t, $J=7.2$ Hz, 2H, $\underline{CH_2}N(CH_3)_2$), 2.37 (s, 6H, $N(CH_3)_2$); ^{13}C NMR ($CDCl_3$, 100 MHz) δ_C: 164.54, 164.04, 150.19, 133.63, 132.64, 131.11, 130.37, 129.96, 127.31, 126.18, 125.56, 125.25, 123.22, 121.35, 116.61, 114.92, 112.45, 61.34, 56.97, 53.12, 53.09, 45.68, 40.49, 37.94; FTIR (KBr, cm^{-1}): 2933, 2807, 2755, 1688, 1654, 1584, 1521, 1446, 1346, 1231, 1116, 1042, 964, 782, 756; HRMS (ES+) calcd for $C_{31}H_{38}N_5O_2$ $([M+H])^+$ 512.3026, found 512.3026.

*4.1.2.4. (E)-6-(4-Cinnamylpiperazin-1-yl)-2-(2-(dimethylamino)ethyl)-1*H*-benzo[*de*]isoquinoline-1,3(2*H*)-dione (**7d**).* Khaki solid. Yield: 85%; m.p. 164.6–166.6 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ_H: 8.58 (dd, $J_1=7.6$ Hz, $J_2=0.8$ Hz, 1H, Ar–H), 8.52 (d, $J=8.0$ Hz, 1H, Ar–H), 8.42 (dd, $J_1=8.8$ Hz, $J_2=0.8$ Hz, 1H, Ar–H), 7.69 (q, $J_1=8.4$ Hz, $J_2=0.8$ Hz, 1H, Ar–H), 7.42 (d, $J=7.2$ Hz, 2H, Ar–H), 7.36–7.32 (m, 2H, Ar–H), 7.28–7.22 (m, 2H, Ar–H), 6.62 (d, $J=16$ Hz, 1H, C=CH), 6.38–6.31 (m, 1H, Ar–H), 4.33 (t, $J=7.2$ Hz, 2H, CH_2N), 3.34–3.32 (m, 6H, $CH\underline{CH_2}N$ and $\underline{CH_2}NCH_2$), 2.85 (s, br, 4H, CH_2NCH_2), 2.66 (t, $J=7.2$ Hz, 2H, $\underline{CH_2}N(CH_3)_2$), 2.37 (s, 6H, $N(CH_3)_2$); ^{13}C NMR ($CDCl_3$, 100 MHz) δ_C: 164.52, 164.03, 155.98, 136.77, 133.52, 132.62, 131.12, 130.31, 129.95, 128.62, 127.66, 126.36, 126.18, 126.08, 125.59, 123.24, 116.69, 114.94, 61.04, 56.97, 53.21, 53.07, 45.68, 37.94; FTIR (KBr, cm^{-1}): 2962, 2807, 2762, 1688, 1651, 1584, 1509, 1450, 1391, 1365, 1246, 1120, 1042, 971, 782, 760, 693; HRMS (ES+) calcd for $C_{29}H_{33}N_4O_2$ $([M+H])^+$ 469.2604, found 469.2600.

*4.1.2.5. 6-(6-(Bis(3,3-diphenylallyl)amino)hexylamino)-2-(2-(dimethylamino)ethyl)-1H-benzo[*de*]isoquinoline-1,3(2H)-dione (**9b**).* Viscous orange solid. Yield: 85%; m.p. 76.8–78.8 °C; ^{1}H NMR (CD_3COCD_3, 400 MHz) δ_H: 8.56 (dd, $J_1=0.8$ Hz, $J_2=8.4$ Hz, 1H, Ar–H), 8.46 (dd, $J_1=1.2$ Hz, $J_2=7.6$ Hz, 1H, Ar–H), 8.34 (d, $J=8.8$ Hz, 1H, Ar–H), 7.61 (dd, $J_1=1.2$ Hz, $J_2=8.4$ Hz, 1H, Ar–H), 7.39–7.31 (m, 6H, Ar–H), 7.26–7.22 (m, 6H, Ar–H), 7.17–7.13 (m, 8H, Ar–H), 6.97 (t, $J=5.2$ Hz, 1H, Ar–NH), 6.78 (d, $J=8.8$ Hz, 1H, Ar–H), 6.17 (d, $J=6.8$ Hz, 2H, 2C=CH), 4.23 (t, $J=6.8$ Hz, 2H, CH_2N), 3.45 (q, $J=6.4$ Hz,, 2H, Ar–$NH\underline{CH_2}$), 3.18 (d, $J=6.8$ Hz, 4H, $2N\underline{CH_2}CH$), 2.56 (t, $J=6.8$ Hz, 2H, $\underline{CH_2}N(CH_3)_2$), 2.45 (d, $J=6.8$ Hz, 2H, $\underline{CH_2}N(CH_2CH{=})_2$), 2.27 (s, 6H, $N(CH_3)_2$), 1.83–1.76 (m, 2H, CH_2), 1.62–1.59 (m, 2H, CH_2), 1.49–1.43 (m, 4H, CH_2CH_2); ^{13}C NMR (CD_3COCD_3, 100 MHz) δ_C: 164.01, 163.24, 150.40, 143.36, 142.36, 139.74, 134.08, 130.48, 129.90, 129.74, 128.13, 128.09, 127.37, 127.17, 127.10, 124.20, 122.82, 120.52, 109.06, 103.78, 57.02, 53.79, 52.67, 45.16, 43.35, 37.44, 31.77, 27.00, 26.88, 24.76, 22.47, 13.52; FTIR (KBr, cm^{-1}): 2925, 2844, 1680, 1643, 1580, 1535, 1439, 1361, 1342, 1242, 1123, 767, 696; HRMS (ES+) calcd for $C_{52}H_{55}N_4O_2$ $([M+H])^+$ 767.4325, found 767.4337.

*4.1.3. General procedure for the synthesis of **7e** and **9a***

To a solution of compound **6**/**8** (0.142 mmol) in $CHCl_3$ (2 mL), 4-(4-(3,3-diphenylallyl)piperazin-1-yl)benzoic acid (68 mg, 0.170 mmol), prepared by using the previously reported method [27,34,44], EDCI (65 mg, 0.340 mmol) and DMAP (41 mg, 0.340 mmol) were added. The solution was stirred at room temperature under nitrogen for 48 h and then concentrated. The residue was purified on silica gel column chromatography by using CH_2Cl_2/CH_3OH 50:1 (v/v) as the eluent, affording **7e**/**9a** as bright yellow–orange red solids.

*4.1.3.1. 2-(2-(Dimethylamino)ethyl)-6-(4-(4-(4-(3,3-diphenylallyl)piperazin-1-yl)benzoyl)piperazin-1-yl)-1H-benzo[*de*]isoquinoline-1,3(2*H*)-dione (**7e**).* Bright yellow solid. Yield: 60%; m.p. 99.5–101.5 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ_H: 8.61 (d, $J=7.2$ Hz, 1H, Ar–H), 8.54 (d, $J=8.0$ Hz, 1H, Ar–H), 8.43 (d, $J=8.4$ Hz, 1H, Ar–H), 7.73 (t, $J=8.0$ Hz, 1H, Ar–H), 7.43–7.34 (m, 5H, Ar–H), 7.32–7.28 (m, 5H, Ar–H), 7.25–7.22 (m, 1H, Ar–H), 7.18 (d, $J=6.8$ Hz, 2H, Ar–H), 6.91 (d, $J=8.8$ Hz, 2H, Ar–H), 6.26 (t, $J=7.2$ Hz, 1H, C=CH), 4.33 (t, $J=7.2$ Hz, 2H, CH_2N), 3.98 (s, 4H, CH_2NCH_2), 3.30–3.27 (m, 8H, $N(CH_2CH_2)_2N$), 3.15 (d, $J=6.8$ Hz, 2H, $CH\underline{CH_2}N$), 2.67 (t, $J=7.2$ Hz, 2H, $\underline{CH_2}N(CH_3)_2$), 2.61 (s, 4H, CH_2NCH_2), 2.37 (s, 6H, $N(CH_3)_2$); ^{13}C NMR ($CDCl_3$, 100 MHz) δ_C: 170.94, 164.40, 163.94, 155.28, 153.44, 144.55, 141.89, 139.53, 132.44, 131.28, 129.96, 129.84, 129.76, 129.18, 128.25, 128.19, 127.41, 127.28, 127.25, 126.28, 126.03, 125.84, 124.96, 123.87, 117.52, 115.38, 114.57, 57.28, 56.92, 53.23, 52.92, 48.19, 45.72, 38.00; FTIR (KBr, cm^{-1}): 2933, 2814, 1695, 1654, 1584, 1509, 1387, 1368, 1279, 1235, 1135, 1019, 786, 756, 696; HRMS (ES+) calcd for $C_{46}H_{49}N_6O_3$ $([M+H])^+$ 733.3866, found 733.3851.

*4.1.3.2. N-(6-(2-(2-(Dimethylamino)ethyl)-1,3-dioxo-2,3-dihydro-1H-benzo[*de*]isoquinolin-6-ylamino)hexyl)-4-(4-(3,3-diphenylallyl)piperazin-1-yl)benzamide (**9a**).* Orange red solid. Yield: 60%; m.p. 85.6–87.6 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ_H: 8.69 (d, $J=8.4$ Hz, 1H, Ar–H), 8.40 (d, $J=7.2$ Hz, 1H, Ar–H), 8.23 (d, $J=8.8$ Hz, 1H, Ar–H), 8.14 (t, $J=5.2$ Hz, 1H, Ar–H), 7.74 (t, $J=4.2$ Hz, 1H, Ar–H), 7.70–7.62 (m, 3H, Ar–H), 7.41 (t, $J=7.2$ Hz, 2H, Ar–H), 7.35–7.22 (m, 4H, Ar–H), 7.21–7.19 (m, 2H, Ar–H), 7.14–7.12 (m, 2H, Ar–H), 6.88 (d, $J=8.8$ Hz, 2H, Ar–H), 6.72 (d, $J=8.8$ Hz, 1H, Ar–H), 6.18 (t, $J=6.4$ Hz, 1H, C=CH), 4.10 (t, $J=7.2$ Hz, 2H, CH_2N), 3.35 (d, $J=6.4$ Hz, 2H, $CONH\underline{CH_2}$), 3.24–3.21 (m, 6H, $\underline{CH_2}NCH_2$ and $NH\underline{CH_2}$), 3.00 (d, $J=6.8$ Hz, 2H, $N\underline{CH_2}CH$), 2.46–2.45 (m, 6H, $\underline{CH_2}NCH_2$ and $\underline{CH_2}N(CH_3)_2$), 2.18 (s, 6H, $N(CH_3)_2$), 1.71–1.65 (m, 2H, CH_2), 1.55–1.51 (m, 2H, CH_2), 1.50–1.42 (m, 4H, CH_2CH_2); ^{13}C NMR (DMSO-d_6, 100 MHz) δ_C: 166.24, 164.21, 163.32, 153.03, 151.13, 143.62, 142.04, 139.62, 134.77, 131.11, 129.92, 129.85, 129.08, 128.80, 128.75, 127.77, 127.37, 126.76, 124.62, 124.42, 122.26, 120.55, 113.95, 107.87, 104.19, 57.12, 52.98, 47.69, 45.82, 43.25, 29.73, 28.26, 26.83, 26.73; FTIR (KBr, cm^{-1}): 3280, 3044, 2925, 2844, 1680, 1639, 1576, 1543, 1502, 1450, 1391, 1361, 1290, 1238, 1120, 1005, 771, 695; HRMS (ES+) calcd for $C_{48}H_{55}N_6O_3$ $([M+H])^+$ 763.4336, found 763.4332.

4.2. Cytotoxic evaluation in vitro

The target compounds were submitted to the Chinese National Center for Drug Screening and School of Pharmacy in East China University of Science and Technology for in vitro antitumor activity assays. Growth inhibitory effects on the cell lines HeLa, P388, HL-60, MCF-7, HCT-8 and A375 were measured by using MTT assay [40]. For A549 cell line, the growth inhibition effect was tested by sulforhodamine B (SRB) assay [39].

4.3. Cell cycle ananlysis

HL-60 cells were incubated with different concentration of the compounds. After centrifugation at 1000 rpm for 5 min at room temperature, the supernatant was removed. And then, the cells were washed twice with PBS solution and fixed with 300 μL PBS and 700 μL of ice cold 75% EtOH overnight. Fixed cells were harvested by centrifugation at 1000 rpm for 10 min at room temperature and washed twice with PBS. Collected cells were resuspended in 1 mL PBS (100 μL/1 × 10^5 cells) and treated with 0.5 μL RNase A at 37 °C for 30 min. Propidium iodide was then added to a final concentration of 50 μg/mL for DNA staining, and 20,000 fixed cells were analyzed on a FACScalibur (Becton Dickinson, San Jose, CA). Cell cycle distribution was analyzed using the Modifit's program (Becton Dickinson).

4.4. Annexin V–FITC staining

Extent of apoptosis was measured through annexin V–FITC apoptosis detection kit (Invitrogen, USA) as described by the manufacture's instruction. Briefly, HL-60 cells were collected 36 h after the target compounds treatment, and washed twice with PBS, then resuspended in 400 μL 1 × binding buffer (10 mM HEPES/NaOH, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4). Cells (100 μL) were transferred to a 5-mL culture tube containing 5 μL of annexin V–FITC and 10 μL of propidium iodide, and then incubated for 15 min at room temperature in the dark. After 1 × binding buffer was added into each tube, the stained cells were analyzed by flow cytometry.

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