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Novel Naphthalimide–Benzoic Acid Conjugates as Potential Apoptosis-Inducing Agents: Design, Synthesis, and Biological Activity

Aibin Wu[1,2,*], Ping Mei[1], Yufang Xu[2] and Xuhong Qian[2]

[1]*School of Chemistry and Environmental Engineering, Yangtze University, Jingzhou 434023, China*

[2]*Shanghai Key Laboratory of Chemical Biology, School of Pharmacy, East China University of Science and Technology, Shanghai 200237, China*

**Corresponding author: Aibin Wu, abwu@yangtzeu.edu.cn*

A series of novel naphthalimide derivatives with 4-[4-(3,3-diphenylallyl)piperazin-1-yl]benzoic acid as side chain were designed and synthesized. Their antitumor activities were evaluated against a variety of cancer cell lines *in vitro*. Preliminary results showed that most of the derivatives had cytotoxic activity comparable with that of amonafide, with IC_{50} values of 10^{-6}–10^{-5} M. Interestingly, compound 12e had the unique antitumor activity against MCF-7 among the cancer cell lines tested. More importantly, flow cytometric analysis indicated that compared with amonafide, the target compounds could effectively induce G_2/M arrest and progress to apoptosis in HL-60 cells after double staining with annexin V–FITC and propidium iodide. The present work provided a novel class of naphthalimide-based derivatives with potential apoptosis-inducing and improved antitumor activity for further optimization.

Key words: apoptosis, benzoic acid, cell cycle arrest, conjugate, cytotoxicity, naphthalimide

Abbreviations: A549, human lung cancer cell line; HeLa, human cervical carcinoma cell line; P388, murine leukemia cell line; HL-60, human promyelocytic leukemia cell line; MCF-7, human caucasian breast adenocarcinoma cell line; HCT-8, human ileocecal adenocarcinoma cell line; A375, human melanoma cell line; MTT, microculture tetrazolium-formazan; FACS, fluorescence-activated cell sorting; NMR, nuclear magnetic resonance; FTIR, Fourier transform infrared spectrum; HRMS (ESI), high-resolution mass spectrometry (electrospray ionization); DMSO, dimethyl sulfoxide; TMS tetramethylsilane; $CDCl_3$, deuterated chloroform; CD_3COCD_3, deuterated acetone; m.p., melt point.

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The development of highly efficient chemotherapeutic agents remained an important endeavor (1). Naphthalimide-based anticancer drugs have attracted great attention and constituted an indispensable part in the development of antitumor agents (2), because of its significantly wide range of applications in photodynamic therapeutics (3), DNA intercalator (2), inhibitor of topoisomerase and telomerase (4), as well as analgesics and fluorescent markers in medicine and biology (5). Therefore, numerous naphthalimide derivatives have been synthesized and evaluated as antitumor agents (6–8). The continuing success of naphthalimide-based anticancer drugs (9) and recent introduction of its several derivatives into clinical trials [Amonafide **1** (10), Azonafide **2** (11), and DMP-840 **3** (12), Figure 1] demonstrated that naphthalimide derivatives represented a group of promising anticancer drugs.

However, this conventional cancer chemotherapy was primarily inadequate because of the lack of selectivity of the largest majority of drugs for targeting cancer cells over their non-cancerous counterparts (13). Moreover, drug-resistant and multidrug-resistant malignancy could escape treatment (14,15) by a variety of biochemical mechanisms (16,17). These defects made current anticancer therapies less effective, leading ultimately to the failure (18). Furthermore, there were some problems on the poor solubility and complicated synthesis of the derivatives. Therefore, it was necessary to attempt neotype structural modification of naphthalimide with alternative mechanism of action.

In parallel with the growth of knowledge concerning side effects of this medicine, activation of apoptosis pathways has become an alternative and promising method of cancer treatment (19,20). Apoptosis was an evolutionarily conserved and highly regulated process, which was used to eliminate defective and unnecessary cells (21,22). Meanwhile, apoptosis was a cellular process critical to normal development and homeostasis of multi–cellular organisms (18). There was a wealth of evidence that disorder of this process was strongly associated with cancer, and induction of apoptosis was undoubtedly the most potent defense against cancer (15,19). Therefore, design and discovery of highly efficient apoptotic inducers, which could specifically target the abnormal cell death pathway found in cancer cells, have been paid great attention (15,20,23–26). The representative reported that apoptotic inducer **4** (23,24), characterized by 4-[4-(3,3-diphenylallyl)piperazin-1-yl] benzoic acid moiety, was illustrated in Figure 1. These inspired us to assume that naphthalimide conjugated with this specific functional group might have some improved or different biological activity (27–29).

On the basis of above consideration, we focused on modifications in positions 2 and 6 of naphthalimide (Figure 2). In molecular

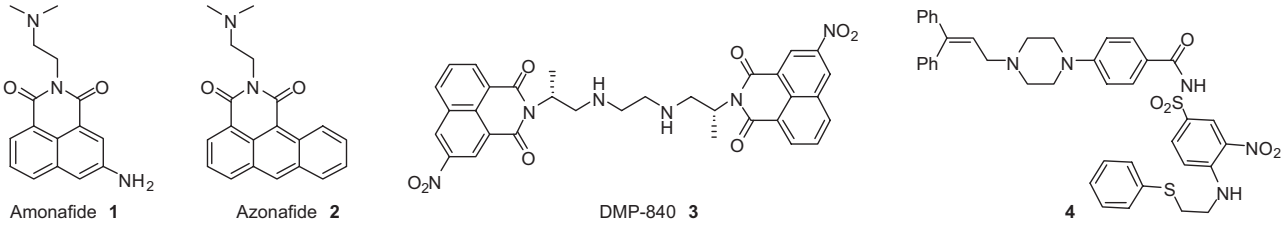

Figure 1: Structures of some naphthalimide derivatives **1–3** in clinical trials and reported apoptotic inducer **4**.

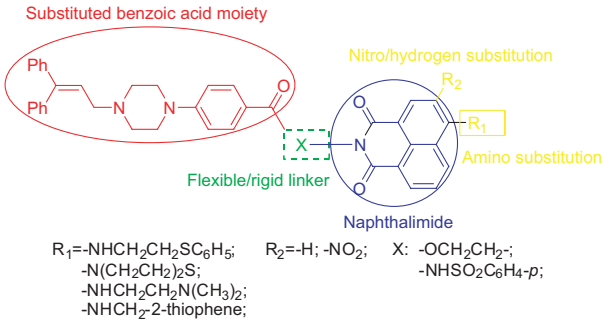

Figure 2: The design strategy of novel naphthalimide–benzoic acid conjugates.

design, naphthalimide scaffold was utilized as key prototype structural unit, and substituted benzoic acid and aliphatic amine functional groups were conjugated to naphthalimide. Amino substituent introduced to position 6 of naphthalimide was difficult to be acetylated (30) and might involve arrest of cell cycle (31). Different types of linkers, ethanolamine and sulfanilamide (32,33), connected naphthalimide with substituted benzoic acid to investigate their effects on biological activity. As shown in Figure 3, the target compounds **12a**–**e** and **13a**–**d** were prepared. Their *in vitro* cytotoxicities, effects on cell cycle, and apoptosis-inducing activities were also investigated.

Materials and Methods

General

All the reagents were of the commercial quality and were used without purification. ^{1}H and ^{13}C NMR were obtained with AV-400 spectrometer (Bruker Co., Fällanden, Switzerland) with chemical shifts reported as ppm (in $CDCl_3$/DMSO-d_6/CD_3COCD_3, TMS as internal standard). IR was obtained using 2000 FT-IR spectrometer (PerkinElmer Co., Boston, MA, USA). HRMS was obtained on HPLC-Q-Tof MS spectrometer (Micromass Co., Manchester, UK). Melting points were determined by an X6 micromelting point apparatus, which were corrected by standard process. Column chromatography was performed using silica gel 200–300 mesh.

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 assay. Growth inhibitory effects on the cancer cell lines were measured by MTT assay (34).

Cell cycle analysis

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 of 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 of PBS (100 μL/1 $\times$ 10^5 cells) and treated with 0.5 μL of 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, USA). Cell cycle distribution was analyzed using the Modifit's program.

Annexin V-FITC staining

Extent of apoptosis was measured through Annexin V–FITC apoptosis detection kit (Invitrogen Co., Grand Island, NY, USA) as described by the manufacture's instruction. Briefly, HL-60 cells were collected 36 h after the target compounds treatment, washed twice with PBS, and then resuspended in 400 μL 1$\times$ 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$\times$ binding buffer was added into each tube, the stained cells were analyzed by flow cytometry.

Results and Discussion

Synthesis

The synthetic route of the designed compounds 2-[1,3-dioxo-6-(substituted amino)-1*H*-benzo[*de*]isoquinolin- 2(3*H*)-yl]ethyl 4-[4-(3,3-diphenylallyl)piperazin-1-yl]benzoate (**12a**–**e**) and *N*-[4-(1,3-dioxo-6-[substituted amino]-1*H*-benzo[*de*]isoquinolin-2[3*H*]-yl)phenylsulfonyl]-4-[4-(3,3-diphenylallyl)piperazin-1-yl]benzamide (**13a**–**d**) was shown in Figure 3. 6-Nitrobenzo[*de*]isochromene-1,3-dione **5** (35) was treated with ethanolamine in EtOH for 1 h or treated with sulfanilamide in glacial acetic acid for 5 h, respectively, affording intermediates **7** or **8** with satisfactory yield. Nucleophilic substitution of **7** or **8** with aliphatic amines gave the key intermediates **10**

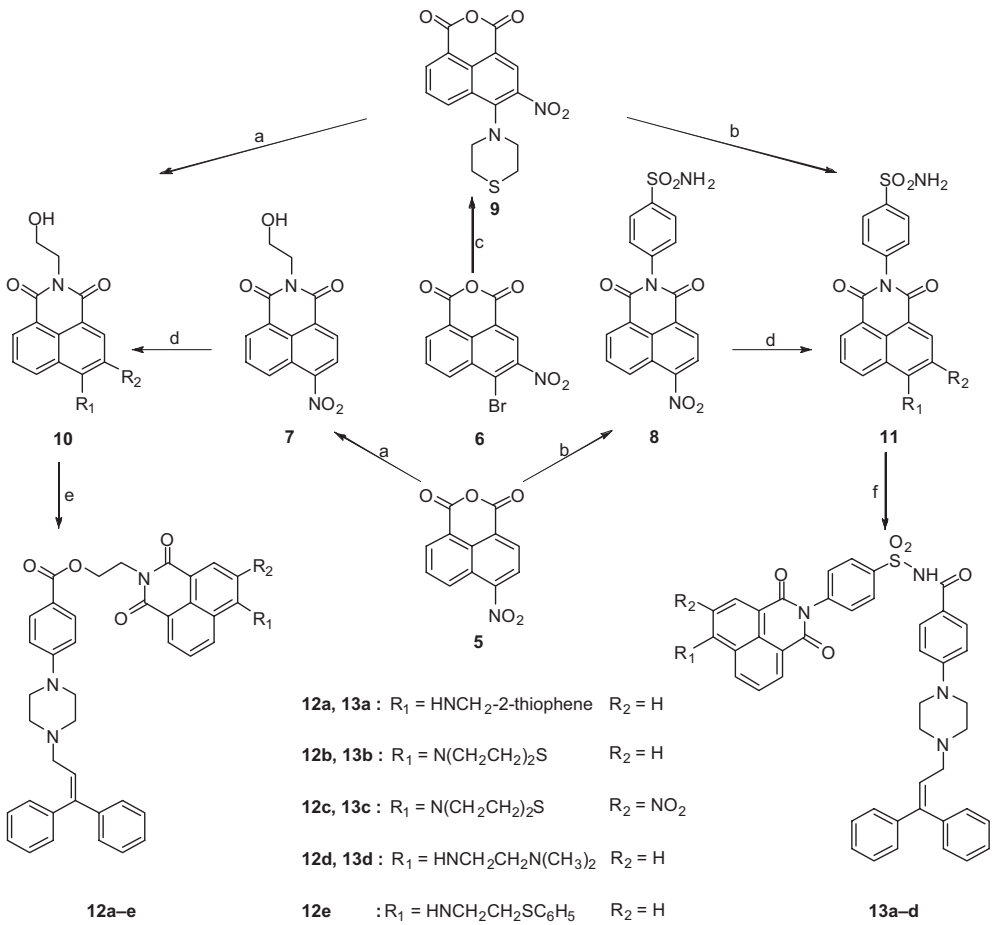

Figure 3: Reagents and conditions: (A) ethanolamine, EtOH, reflux 1 h, 95% yield; (B) sulfanilamide, HOAc, reflux 5 h, 75% yield; (C) thiomorpholine, EtOH, reflux 1 h, 85% yield; (D) corresponding amine, DMF, r.t. 24–36 h, 40–60% yield; (E) 4-[4-(3,3-diphenylallyl)piperazin-1-yl]benzoic acid (23), $CHCl_3$, EDCI, DMAP, r.t. 24 h, 55–65% yield; (F) 4-[4-(3,3-diphenylallyl)piperazin-1-yl]benzoic acid, DMF, EDCI, DMAP, r.t. 48 h, 50–60% yield.

or **11**. **10c** and **11c** were prepared using **6** (36) as starting material. Subsequent condensation of **10** or **11** with 4-[4-(3,3-diphenylallyl)piperazin-1-yl]benzoic acid (23) afforded the target compounds **12a**–**e** or **13a**–**d** with moderate yields of 50–65%, respectively. The structures of all the newly synthesized compounds were confirmed by ^{1}H NMR, ^{13}C NMR, HRMS, and FTIR (see Figures S1–S27, Supporting Information).

Cytotoxic effects

In vitro antitumor activity of the target compounds was evaluated by examining their cytotoxic effects using MTT assay (34) against HeLa, A549, P388, HL-60, MCF-7, HCT-8, and A375 cancer cell lines. The IC_{50} represented the drug concentration (μM) required to inhibit cell growth by 50%. The results were summarized in Table 1.

As shown in Table 1, the target compounds had cytotoxicities comparable with those of amonafide against the cancer cell lines tested except for HCT-8, with IC_{50} values of 10^{-6}–10^{-5} M. Compound **13d** had the highest cytotoxicity against HeLa, P388, and A375 cell lines among each group with IC_{50} values of 6.70, 10.54, and 6.63 μM. For A549, HL-60, and MCF-7 cell lines, the highest cytotoxic compounds were **12d**, **12b,** and **13a**. Among these, IC_{50} values of compounds **12d**, **12b**, **13a**, and **13d** were factors of 10.8, 1.6, 1.2, and 1.1 lower than those of amonafide against A549, HL-60, MCF-7, and A375 cell lines. In most cases, cytotoxicity increased in the sequence of **13** and **12**, which indicated that the magnitude and conformation of the linkers had substantial effect on the cytotoxicity and selectivity of these compounds. For example, compounds **12a** and **13a**, respectively, showed the highest cytotoxicity against A375 and MCF-7 cell lines. Moreover, the substituent in position 6 as well as nitro group in position 5 of naphthalimide also affected their cytotoxicity. We found that compounds **12a**–**d** and **13a**–**d** bearing different substituents showed a striking contrast to each other. It was interesting that compound **12e** with phenylthioethylamino group had exclusive antitumor activity against MCF-7 (IC_{50} = 12.21 μM), whereas for the other cancer cell lines tested, it had no activity (IC_{50} > 50 μM). This was in accordance with our previous reported results, indicating that position 6 of naphthalimide was a crucial active site (37), and

Table 1: Cytotoxicity of the target compounds against HeLa, A549, P388, HL-60, MCF-7, HCT-8, and A375 cancer cell lines

	Cytotoxicity (IC_{50}, μM)[a]						
Compound	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
12a	12.60	>50	47.53	11.94	11.23	>50	7.24
12b	7.00	2.51	30.91	11.51	11.92	>50	7.13
12c	>50	>50	39.42	>50	28.14	>50	>50
12d	>50	1.22	43.81	>50	32.33	>50	>50
12e	>50	>50	>50	>50	12.21	>50	>50
13a	21.00	>50	39.82	28.11	10.06	>50	22.04
13b	12.30	57.4	37.87	38.36	24.25	>50	19.51
13c	7.30	>50	21.33	12.62	20.37	>50	6.75
13d	6.70	>50	10.54	11.92	18.92	>50	6.63

[a]Cytotoxicity values were means of three experiments.

introduction of appropriate substituent to the position could obviously improve the antitumor selectivity (38). Additionally, it was worth noting that the target compounds without *N,N*-dimethylethylenediamine substituent in position 2 of naphthalimide, which was considered to be important for cytotoxicity (2), still had good activity. Therefore, manipulation of naphthalimide derivatives to achieve potent antitumor activity seemed to be closely dependent on structures and conformation of the substituents and the linkers, which played important roles in their chemical and biological functions.

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 FACS (26). HL-60 cells were used in the assay. Cells were treated with the target compounds and fixed and labeled with propidium iodide. The different phases of cell cycle were analyzed by flow cytometry. The results were summarized in Table 2.

As shown in Table 2, the target compounds could effectively induce G_2/M arrest and progress to apoptosis in HL-60 cell line. After incubation with IC_{50} concentration of the target compounds, the sub-G_1 portions were increased from 6.44% to 20.68% or from 4.47% to 18.71% 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 14.85–34.10% in cells treated with the target compounds. Moreover, on compounds treatment, HL-60 cells could notably induce morphological changes into rounding form (data not listed), which was a primary indication of apoptosis and cell death (39). The results in Table 1 and Table 2 indicated that there was no obvious relationship between cytotoxicity and apoptosis-inducing activity. Cell cycle distribution of HL-60 cells in the presence of representative compounds **12b** and **13d** was displayed in Figure 4.

To further confirm their pro-apoptotic function, FACS analysis was carried out after double staining cells with propidium iodide and annexin V–FITC (18,26,39). Early apoptosis corresponded to annexin V single-positive cells and late apoptosis/necrosis corresponded to double-positive cells. As shown in Figure 5, representative compounds **12b** and **13d** were very effective in induction of apoptosis in a dose-dependent manner. Treatment of HL-60 cell line by 3.0, 6.0, and 12.0 μM of **12b** resulted in 54.38%, 42.30%, and 2.10% of apoptotic cells as compared to 0.81% of apoptotic cells in an untreated control. Also, compound **13d** had significant effects on

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

		Cell cycle distribution (%)[a]			
Compound	Concentration (μM)	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
12a	12	62.00	23.15	14.85	9.32
12b	12	63.47	21.14	15.40	9.18
12c	50	61.31	8.25	30.45	10.11
12d	50	68.64	0.20	31.16	11.74
12e	50	62.02	3.87	34.10	7.16
13a	28	65.39	1.93	32.68	21.40
13b	38	71.20	0.08	28.80	7.20
13c	13	64.95	19.44	15.60	10.43
13d	12	59.56	24.11	16.32	10.11

[a]Cell cycle distribution values were means of three experiments.

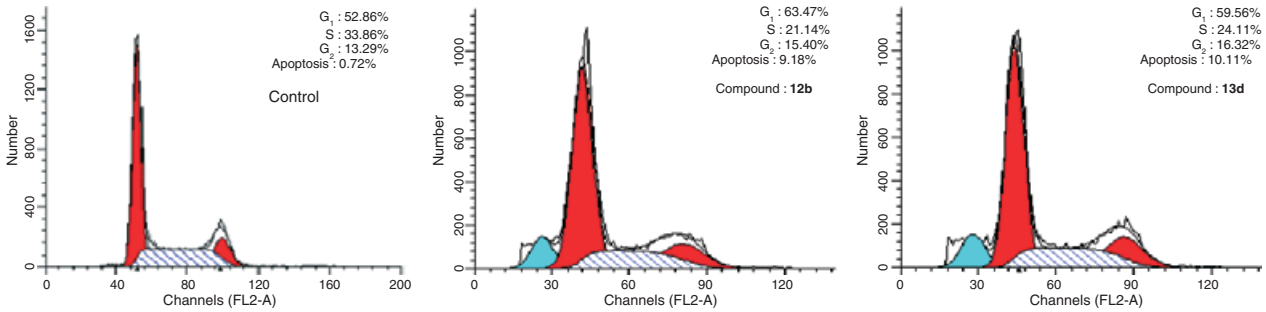

Figure 4: Cell cycle distribution of HL-60 cells in the presence of representative compounds **12b** and **13d**, respectively. HL-60 cells were treated with IC_{50} concentration of the compounds or with 0.1% DMSO. Cells were harvested, fixed, and stained with propidium iodide. 20 000 Stained cells were then subjected to FACScalibur analysis to determine the distribution of cells.

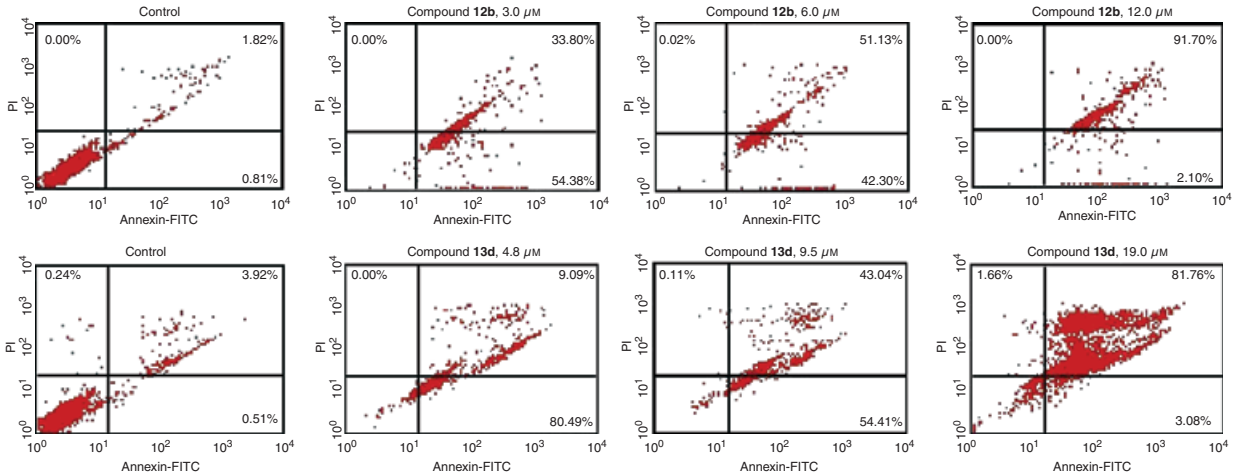

Figure 5: Compounds **12b** and **13d** induced apoptosis of HL-60 cells. Cells were treated with corresponding concentration of the compounds or with vehicle solvent (0.1% DMSO), and then stained with annexin V–FITC and propidium iodide. Stained cells were subjected to FACS analysis to determine the distribution of cells. Early apoptosis corresponded to annexin V single–positive cells and late apoptosis/necrosis corresponded to double–positive cells.

induction of apoptosis. These results collectively suggested that the target compounds might inhibit the growth of HL-60 cell line by induction of apoptosis.

However, the search of the reasonable targets of the synthesized compounds still remained endeavor. Assays for DNA intercalation and Top II inhibition of the compounds had been carried out. It was surprising that the target compounds had less interaction with DNA and Top II inhibition than amonafide did (data not listed). Maybe, Bcl-2/x_L binding or other factors contributed its unique activity? This section of work was under investigation in our laboratory. We hope that in near future, we could find the answer.

Conclusions

A series of novel naphthalimide–benzoic acid conjugates were designed and synthesized. Their antitumor activities were evaluated against a variety of cancer cell lines *in vitro*. Preliminary results showed that most of the derivatives had cytotoxic activity comparable with that of amonafide with IC_{50} values of 10^{-6}–10^{-5} M. It was interesting that compound **12e** exhibited the selective cytotoxicity against MCF-7 among the cancer cell lines tested. 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 compared with amonafide. The present work demonstrated that conjugating the biological active units of naphthalimide and 4-[4-(3,3-diphenylallyl)piperazin-1-yl]benzoic acid might be able to result in a novel class of lead compounds with potential apoptosis-inducing and improved antitumor activity. Further structural optimization and detailed biological studies on the mechanism of action about the designed naphthalimide derivatives were under way.

Acknowledgments

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. 1H NMR spectrum of **12a**.

Figure S2. 13C NMR spectrum of **12a**.

Figure S3. HRMS spectrum of **12a**.

Figure S4. 1H NMR spectrum of **12b**.

Figure S5. 13C NMR spectrum of **12b**.

Figure S6. HRMS spectrum of **12b**.

Figure S7. 1H NMR spectrum of **12c**.

Figure S8. 13C NMR spectrum of **12c**.

Figure S9. HRMS spectrum of **12c**.

Figure S10. 1H NMR spectrum of **12d**.

Figure S11. 13C NMR spectrum of **12d**.

Figure S12. HRMS spectrum of **12d**.

Figure S13. 1H NMR spectrum of **12e**.

Figure S14. 13C NMR spectrum of **12e**.

Figure S15. HRMS spectrum of **12e**.

Figure S16. 1H NMR spectrum of **13a**.

Figure S17. 13C NMR spectrum of **13a**.

Figure S18. HRMS spectrum of **13a**.

Figure S19. 1H NMR spectrum of **13b**.

Figure S20. 13C NMR spectrum of **13b**.

Figure S21. HRMS spectrum of **13b**.

Figure S22. 1H NMR spectrum of **13c**.

Figure S23. 13C NMR spectrum of **13c**.

Figure S24. HRMS spectrum of **13c**.

Figure S25. 1H NMR spectrum of **13d**.

Figure S26. 13C NMR spectrum of **13d**.

Figure S27. HRMS spectrum of **13d**.