New synthetic flavone derivatives induce apoptosis of hepatocarcinoma cells

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ABSTRACT

Natural flavonoids have broad biological activity, including anticancer. In this study, a series of novel flavone derivatives were synthesized with the substitutions of chlorine, isopropyl, methoxy, and nitro groups on the benzene ring of flavone skeleton to develop effective anticancer agents. Antiproliferative assays showed that the synthesized chemicals possess notable activity against hepatocarcinoma cells (HepG-2); in particular, the compound **6f** with chlorine and dimethoxy modifications at the two benzene rings showed an IC_{50} at 1.1 μM to HepG-2. The **6f** also displayed marked anticancer activity towards a panel of cancer cells, including nasopharyngeal carcinoma cells (CNE-2 and CNE-1), breast adenocarcinoma cell (MCF-7), and epithelial carcinoma cells (Hela). Exposing HepG-2 cells to compound **6f** at 10 μM induced chromatin condensation, nuclear disassembly, and DNA fragmentation. In **6f**-treated HepG-2 cells, the sub-G_0 population was remarkably increased; and in these cells, both caspase-8 and caspase-9 activity was significantly increased, which in turn activated caspase-3. In addition, proapoptotic Bax was upregulated by compound **6f** while the antiapoptotic Bcl-2 was downregulated. Taken together, our data suggest that the new flavonoid derivative **6f** triggers apoptosis through both death-receptor and mitochondria-dependent intrinsic pathways, being a potent therapeutic agent against hepatocarcinoma.

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1. Introduction

Apoptosis is essential for normal development and the maintenance of homeostasis.[1] It plays a necessary role to protect against carcinogenesis by eliminating damaged cells.[2] Many studies have demonstrated that the dysregulation of apoptosis results in cancer and this provides an approach to develop therapeutic agents via inducing apoptosis. Design and development of small molecules that can induce cancer cells to apoptosis is an attractive approach for the development of new anticancer agents.[3–6]

Flavonoids are an extensive group of polyphenolic compounds which have been found in plants and dietary components, such as fruits, soy beans, vegetables, and red wine.[7–9] A large number of biological effects have been attributed to these compounds, including anticancer,[10–14] antifungal,[15,16] antiviral,[17,18] anti-inflammatory,[19] antioxidant,[19,20] anti-osteoporotic,[21,22] and anticarcinogenic activities.[23] In particular, flavonoid compounds in the diet may act as chemopreventive agents against the development of cancers in the alimentary tract.[24,25] In this regard, a number of flavone derivatives have been synthesized and their antiproliferative effects and antitumor mechanisms have been investigated.[26–28] Many lines of evidence have shown that flavonoid compounds are able to induce apoptosis in various human cancer cell lines.[27,29] However, the mechanism of flavonoids' antitumor effects is not fully understood because flavonoids with different structures display diverse biological potency and mechanisms of action.

In this study, we synthesized a series of new flavonoid derivatives with different substitutional groups and studied the structure–activity relationship. The results indicated that most of the new synthetic derivatives displayed noticeable antiproliferative activity against hepatocarcinoma cells (HepG-2) and the most active compound **6f** was selected to investigate the mechanisms of action. Results showed that the synthetic new flavonoid derivatives are able to induce cancer cell apoptosis via a caspase-dependent pathway.

2. Results and discussion

2.1. Synthesis of new flavone derivatives

The synthesis route and structures of new flavone derivatives investigated in this study were illustrated in Scheme 1. Synthetic reactions involved an acetylation, a Fries rearrangement, a modified Schotten–Baumann reaction, and a Baker–Venkataraman rearrangement, followed by cyclization catalyzed by acid.[30] A

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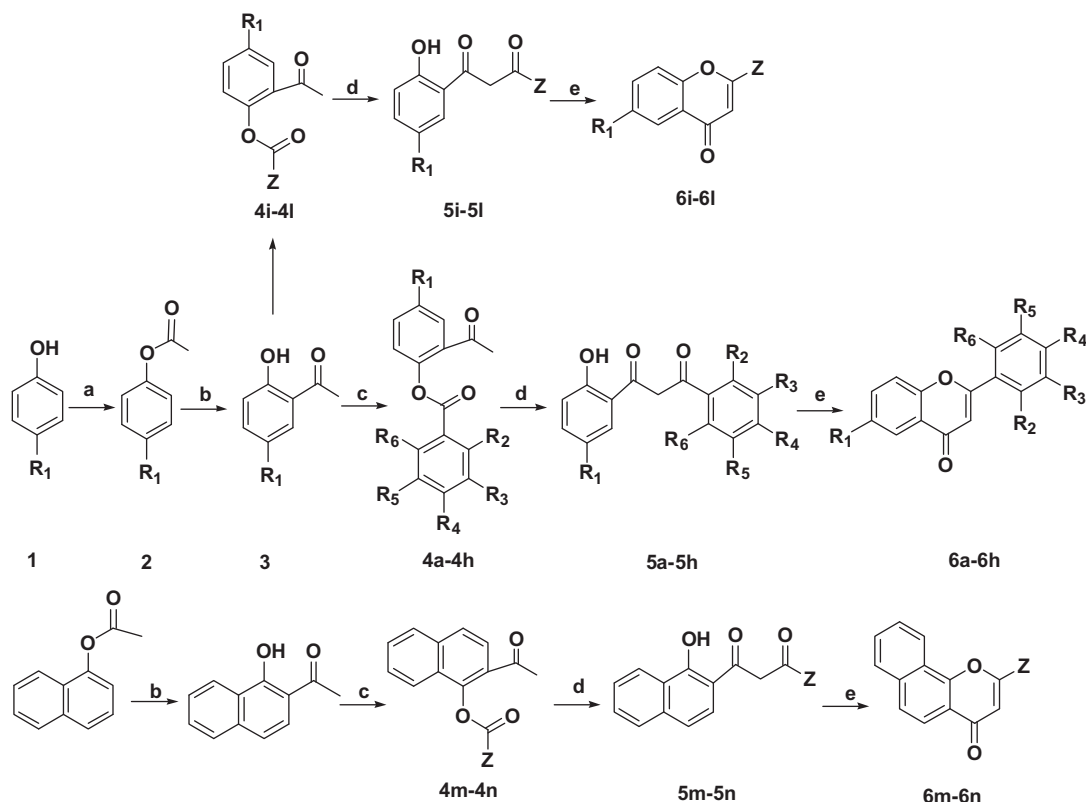
Scheme 1. R_1–R_6, A and Z are defined in Tables 1 and 2. Reagents: (a) Ac_2O, H_2O, NaOH. (b) $AlCl_3$, NaCl. (c) Substituted or non-substituted benzoyl chloride, pyridine. (d) KOH, pyridine. (e) H_2SO_4, AcOH.

series of α-naphthalin compounds were synthesized in parallel (Scheme 1). Products were purified by column chromatography and characterized by NMR and mass spectral analysis.

2.2. Antiproliferative activity of new flavone derivatives

The antiproliferative activity of all newly synthesized flavone derivatives were evaluated with HepG-2 cells in vitro. Viable cells were determined using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay after exposure for 72 h.[33] As summarized in Tables 1 and 2, most chemicals displayed promising antiproliferative activity against HepG-2 cells, except for **6g** and **6h** (IC_{50} >50 μM). A structure–activity analysis revealed several findings as follows: (1) flavone antiproliferative activity depended on the backbone of 2-substituent group, as suggested by the results of compounds **6a**, **6i**, and **6k**; (2) number and position of substituent methoxy groups greatly affected the antiproliferative potency of flavones. Among compounds **6b**, **6c**, and **6d**, the **6c** with

Table 1
Cytotoxic activity of compounds **6a–6h** against HepG-2 cells

Compd	R_1	R_2	R_3	R_4	R_5	R_6	IC_{50}^a (μM)
6a	$CH(CH_3)_2$	H	H	H	H	H	6.4 ± 1.8
6b	$CH(CH_3)_2$	OCH_3	H	H	H	H	5.4 ± 1.7
6c	$CH(CH_3)_2$	H	OCH_3	H	H	H	2.1 ± 0.7
6d	$CH(CH_3)_2$	H	H	OCH_3	H	H	9.9 ± 1.9
6e	$CH(CH_3)_2$	H	OCH_3	OCH_3	OCH_3	H	3.6 ± 0.6
6f	Cl	H	OCH_3	H	OCH_3	H	1.1 ± 0.3
6g	$CH(CH_3)_2$	H	NO_2	H	H	H	>50
6h	$CH(CH_3)_2$	H	H	NO_2	H	H	>50

[a] Cell viability was determined by the MTT assay, IC_{50} denotes the concentration of drug required for 50% inhibition of cell growth (HepG-2). Results are the means of three independent determinations.

Table 2
Cytotoxic activity of compounds **6i–6n** against HepG-2 cells

Compd	R_1	Z	IC_{50}^a (μM)
6i	$CH(CH_3)_2$	Cyclopentyl	8.6 ± 1.1
6j	Cl	Cyclopentyl	13.4 ± 4.7
6m	—	Cyclopentyl	6.1 ± 3.8
6k	$CH(CH_3)_2$	Cyclohexyl	18.0 ± 5.2
6l	Cl	Cyclohexyl	20.0 ± 7.6
6n	—	Cyclohexyl	2.9 ± 0.9

[a] Viable cells were determined by MTT proliferation assays after incubation with tested compound for 72 h.

an –OMe group at 3′-position showed the most antiproliferative activity. It is probably due to the more prominent resonance effect than 4′-position and 2′-position. It is noteworthy that the introduction of two more methoxyl groups to the compound **6d**, forming compound **6e**, enhanced up to three folds of the antiproliferative activity, compared to the parental compound **6d**; (3) use of a nitro group as a substituent on the benzene ring (compounds **6g** and **6h**) inactivated the flavone antiproliferative activity against HepG-2, which suggests that electron-negativity in the benzene ring greatly affected the compound activity; (4) finally, not only the substituent groups, but also the backbone ring (benzene vs naphthalene) played significant influence on the antiproliferation activity of compounds, as indicated by the comparative data of compounds (**6m** vs **6i/6j** and **6n** vs **6k/6l**). Therefore, the naphthalene group may improve the antiproliferation activity, which provides a new idea for the development of novel flavone derivatives.

Among the 14 compounds, compound **6f** showed most promising antiproliferative activity against HepG-2 with an IC_{50} at 1.1 μM, and thus was tested for its activity towards a panel of other four tumor cell lines. As shown in Figure 1A, compound **6f** showed significant cytotoxicity to all tested cell lines in a dose-dependent
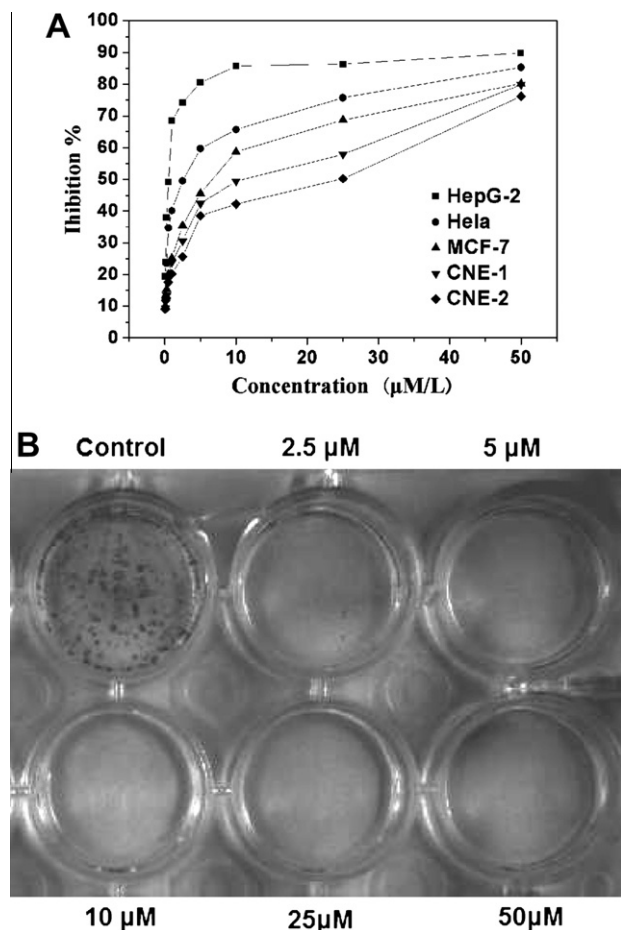
Figure 1. Antiproliferative activity of compound **6f**. (A) Effect of **6f** on the viability of HepG-2 (■), Hela (●), MCF-7 (▲), CNE-1 (▼), and CNE-2 (♦) cells. (B) Effect of **6f** on colony formation of HepG-2 cells.

manner. Although the antiproliferative activity varied with cell lines, the effective IC_{50} is at approximate 10 μM or less, except for CNE-2 cells (Table 3).

Antiproliferative activity of compound **6f** was further confirmed by the clonogenic growth of HepG-2 cells, a critical feature of tumor cells (Fig. 1B). Results showed that compound **6f** at a concentration of low up to 2.5 μM greatly decreased the colony formation capability of HepG-2 cells. Together these data suggest that **6f** is a potent anticancer agent.

2.3. Compound 6f induces HepG-2 cell apoptosis

HepG-2 cells were most sensitive to the compound **6f**, and thus were used to investigate the molecular mechanism of action. Apoptosis is a programmed cell death, characterized with chromaqtin condensation and internucleosomal DNA fragmentation.[31] To understand whether compound **6f** induces apoptosis of HepG-2 cells, we examined cell morphology and DNA fragmentation when exposed to compound **6f** at 10 μM. As shown in Figure 2A, compound **6f** led to significant chromatin condensation, nuclear deformation, and disassembly, compared to control cells. Next,

Table 3
Growth inhibition compound **6f** on different tumor cells

Cell line	Hela	MCF-7	CNE-1	CNE-2
IC_{50}^{a} (μM)	3.0	7.4	10.4	12.9

[a] Viable cells were determined by MTT proliferation assays after incubation with tested compound for 72 h.

the endonucleolytic DNA cleavage was checked by agarose gel electrophoresis to further confirm **6f**-induced cell apoptosis. Cleavage of DNA at the internucleosomal linker sites yielding DNA fragments in multiples fragments (180–200 bp) was regarded as a biochemical characteristic of apoptosis.[32] As shown in Figure 2B, DNA fragmentation occurred in a dose–response manner in **6f**-treated cells, whereas no obvious DNA ladders were observed in the control cells.

Compound **6f**-induced apoptosis of HepG-2 cells was further proven by a FACScan analysis. In the flow cytometry analysis, the sub-G_0 cell population is considered as apoptotic cells.[33] With propidium iodide (PI) staining for DNA-content histogram, we assessed the sub-G_0 populations of HepG-2 cells in the presence of the compound **6f** at different concentrations. The results demonstrated that the sub-G_0 phase of the cell cycle in HepG-2 cells consisted of 3.41%, 21.54%, 34.54%, 55.83%, and 79.32% at 0, 5, 10, 25, and 50 μM of compound **6f**, respectively; accordingly, cells in the G_1, S, and G_2 phases were concomitantly decreased. Together these data suggested that the compound **6f** efficiently induced apoptosis of HepG-2 cells.

2.4. Compound 6f-induced apoptosis is mediated via caspase-dependent pathways
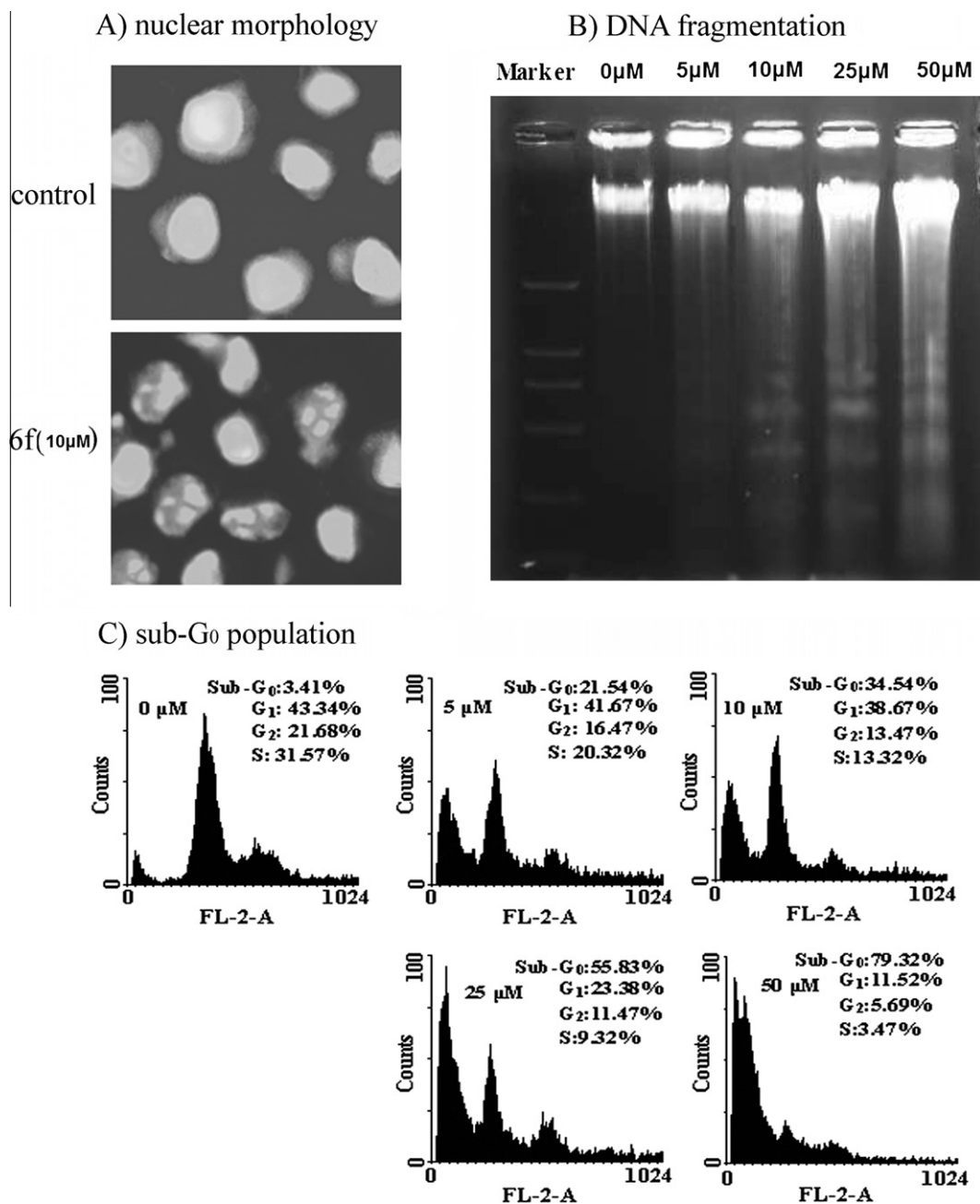
Apoptosis is triggered by either intrinsic mitochondrial or death-receptor pathways; the mitochondrial pathway includes caspase-dependent or independent apoptosis.[34,35] Caspases, a family of cystein proteases, are capable of cleaving essential cellular substrates after aspartic residues and are critical for the initiation and execution phases of apoptosis.[36–38] To understand the underlying pathway(s) of compound **6f**-induced apoptosis, we examined caspase-3 activity via measuring Ac-DEVD-pNA cleavage of HepG-2 cell lysates collected at 0, 4, 8, 12, 16, and 24 h after compound **6f** treatment. As shown in Figure 3A-a, the activity of caspase-3 was increased with time and peaked at 12 h after treatment. Thereafter, caspase-3 activity gradually decreased, which may be due to massive loss of cells. These results indicated that the activation of caspase-3 was involved in the compound **6f**-induced cell apoptosis.

It is understood that caspase-8 is involved in the death-receptor pathway while caspase-9 mediates the mitochondrial pathway.[39] Once activated, both caspase-8 and caspase-9 activates downstream caspase-3, triggering cell apoptosis. Therefore, we measured the activity of caspase-8 and caspase-9, respectively, and the results showed that both caspase-8 and caspase-9 were activated dramatically with an activity peak at 8 h after treatment with the compound **6f**, suggesting that both mitochondrial and death-receptor pathways were involved in the **6f**-induced apoptosis (Fig. 3A-b). Nevertheless, compared to caspase-8, the activity of caspase-9 was higher and lasted longer in response to the **6f**, suggesting that the apoptosis proceeded mainly via a caspase-dependent intrinsic mitochondria pathway.

Bcl-2 family genes have a central role in controlling the mitochondrial pathway.[4] Therefore, we examined the expression of Bcl-2, an apoptotic inhibitor, and Bax, an apoptotic inducer using semi-quantitative RT-PCR. The results indicated that the compound **6f** led to a dose-dependent suppression of Bcl-2 expression, accompanied by concomitant increase of Bax expression (Fig. 3B). These results suggest that compound **6f** induces apoptosis of HepG-2 cells via both death-receptor and the mitochondria-dependent apoptotic cascade.

3. Conclusion

We have designed and synthesized a series of new flavone derivatives with potent antiproliferative activity against HepG-2

A) nuclear morphology

control

6f(10µM)

B) DNA fragmentation

Marker 0µM 5µM 10µM 25µM 50µM

C) sub-G0 population

0 µM — Sub-G0:3.41% G1: 43.34% G2: 21.68% S: 31.57%

5 µM — Sub-G0:21.54% G1: 41.67% G2: 16.47% S: 20.32%

10 µM — Sub-G0:34.54% G1:38.67% G2:13.47% S:13.32%

25 µM — Sub-G0:55.83% G1:23.38% G2:11.47% S:9.32%

50 µM — Sub-G0:79.32% G1:11.52% G2:5.69% S:3.47%

Figure 2. Apoptosis of HepG-2 cells induced by compound **6f**. (A) Fluorescent staining of nuclei by Hoechst 33258. (B) DNA fragmentation. HepG-2 cells were treated with compound **6f** at 0, 5, 10, 25, and 50 µM for 36 h and DNA fragments were prepared. (C) Dose-dependent effects of **6f** on cell cycle phase distributions. Cells were treated with 0, 5, 10, 25, and 50 µM **6f** for 36 h and analyzed by flow cytometry.

cells in vitro. The cytotoxic activity of the new synthetic flavone derivatives was dependent on the nature and position of the substituents on the two benzene rings. Among the synthetic compounds, the **6f** is the most active, inducing death-receptor and mitochondrial-dependent apoptosis via triggering the caspase cascade. Therefore, the compound **6f** may be a potent lead compound for further optimization as a chemotherapeutic agent for hepatocarcinoma therapy.

4. Experimental section

4.1. Chemistry

^{1}H NMR and ^{13}C NMR spectra were obtained in CDCl$_3$ at 300 MHz or 500 MHz for ^{1}H NMR and 75 MHz or 125 MHz for

^{13}C NMR with tetramethylsilane as the internal standard. Melting points were determined by the open capillary method on a SGW X-4 electrothermal melting apparatus and are uncorrected. Reaction progress was monitored using analytical thin layer chromatography (TLC) on pre-coated silica gel plates and the spots were detected under UV light (254 nm). High-resolution mass spectra were obtained with Waters micromass Q-Tof Premier mass spectrometer. All other reagents and analytical grade solvents were bought from commercial sources and used without further purification unless indicated. Pyridine was distilled from anhydrous potassium hydroxide (KOH) immediately prior to use.

4.1.1. Synthesis of acetic acid 4-isopropyl-phenyl ester (2)

10 mmol of **1** (4-isopropyl-phenol) was added into the NaOH (15 mmol) of ice water solution keeping rapidly stirring, then the
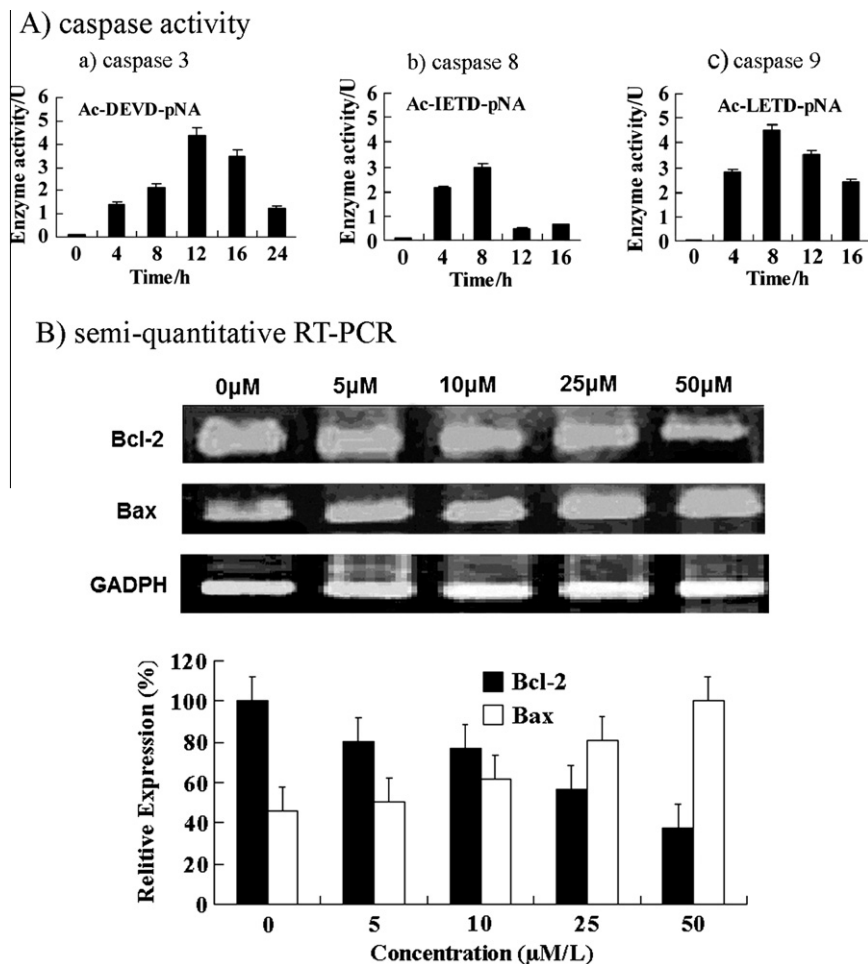
Figure 3. Mechanism of compound **6f**-induced apoptosis. (A) Effect of **6f** on caspase activity. (a) Caspase-3 activity was detected by Ac-DEVD-pNA at 0, 4, 8, 12, 16, and 24 h after treated by **6f** at 50 μM. (b) Caspase-8 and (c) capase-9 activities detected by Ac-IETD-pNA and Ac-LEHD-pNA at 0, 4, 8, 12, and 16 h of compound **6f** treatment at 50 μM. (B) Effects of **6f** on Bcl-2 and Bax expression. Cells were treated with 0, 5, 10, 25, and 50 μM for 26 h and then harvested. The total RNA was extracted, and then cDNA was synthesized from total RNA (1 μg) which was amplified using the sets of primers for examining the expression of Bcl-2 and Bax.

acetic anhydride was dropped into foregoing solution at ice bath, keeping the dropped in an appropriative velocity. The mixture was stirred for 3 h and extracted with EtOAc, the organic layer was collected, dried over anhydrous NaSO₄, and evaporated under vacuum to obtain the desired crude product **2**, which was used without further purification. Yield: 90%.

4.1.2. Synthesis of 1-(2-hydroxy-5-isopropyl-phenyl)-ethanone (3)

In a 100-ml round-bottomed flask, 5 mmol compound **2**, 10 mmol NaCl, and 10 mmol anhydrous AlCl₃ were added. The temperature of the reaction mixture was heated and maintained at 110 °C for 1.5 h while being stirred continuously. Then ice was added into flask slightly. The mixture was extracted with 20 ml EtOAc, the organic layer was dried over anhydrous NaSO₄ and the dried solution was concentrated to get the crude product, which on purification by column chromatography afforded pure compound **3**. Yield: 67%.

4.1.3. General synthesis of compounds 5[30]

Compound **4** (1.0 mmol) was dissolved in 5 ml of anhydrous pyridine containing finely powdered potassium hydroxide (84 mg, 1.5 mmol) and stirred at 50 °C until TLC indicated completion of reaction. After the mixture was cooled to room temperature and 25 ml of 10% acetic acid (aq) was added, the

precipitate formed was collected by filtration and purified by chromatography.

4.1.4. Synthesis of compounds 6a–6n

All of the compounds in the **6** series were synthesized according to the literature as outlined in Scheme 1.[30,40] Concentrated sulfuric acid (0.2 ml), compound **5** (0.5 mmol) and 5 ml of glacial acetic acid were stirred at 80 °C for 2 h. After the reaction mixture was cooled to room temperature, 25 ml ice water was slowly added with rapidly stirring. The crude products were collected by filtration, washed with water, and purified by chromatography.

4.1.4.1. 6-Isopropylflavone (6a).
Yield: 67%; slight yellow solid, mp: 83–86 °C; ^{1}H NMR (CDCl₃, 300 MHz) δ 1.30 (d, J = 6.90 Hz, 6H), 3.03 (m, 1H), 6.82 (s, 1H), 7.50 (m, 5H), 7.89 (m, 2H), 8.07 (m, 1H); ^{13}C NMR (CDCl₃, 75 MHz) δ 23.93, 33.78, 107.33, 117.96, 122.38, 123.63, 126.22, 128.99, 131.50, 131.81, 132.73, 146.14, 154.68, 163.23, 178.69; HRMS m/z calcd for $[C_{18}H_{17}O_2]^+$: 265.1229; found: 265.1219.

4.1.4.2. 6-Isopropyl-2′-methoxylflavone (6b).
Yield: 76%; slight yellow oil; ^{1}H NMR (CDCl₃, 500 MHz) δ 1.30 (d, J = 6.90 Hz, 6H), 3.03 (m, 1H), 3.91 (s, 3H), 7.01 (m, 1H), 7.07 (m, 1H), 7.13 (s, 1H), 7.43 (m, 2H), 7.53 (m, 1H), 7.87 (m, 1H), 8.08 (m, 1H); ^{13}C NMR (CDCl₃, 125 MHz) δ 23.79, 33.64, 55.49, 111.64, 112.29, 117.75,

120.56, 120.83, 122.15, 123.39, 129.09, 132.14, 132.29, 145.63, 154.80, 157.83, 160.59, 178.90; HRMS m/z calcd for $[C_{19}H_{19}O_3]^+$: 295.1334; found: 295.1329.

4.1.4.3. 6-Isopropyl-3′-methoxylflavone (6c). Yield: 81%; white solid, mp: 114–116 °C; ^{1}H NMR (CDCl$_3$, 500 MHz) δ 1.31 (d, J = 6.90 Hz, 6H), 3.04 (m, 1H), 3.90 (s, 3H), 6.82 (s, 1H), 7.07 (m, 1H), 7.43 (m, 2H), 7.50 (m, 2H), 7.58 (m, 1H), 8.80 (m, 1H); ^{13}C NMR (CDCl$_3$, 75 MHz) δ 23.95, 33.82, 55.47, 107.64, 111.74, 117.10, 117.99, 118.72, 122.42, 123.70, 130.11, 132.75, 133.28, 146.22, 154.71, 160.02, 163.08, 178.72; HRMS m/z calcd for $[C_{19}H_{19}O_3]^+$: 295.1334; found: 295.1336.

4.1.4.4. 6-Isopropyl-4′-methoxylflavone (6d). Yield: 78%; white solid, mp: 134–136 °C; ^{1}H NMR (CDCl$_3$, 300 MHz) δ 1.32 (d, J = 6.90 Hz, 6H), 3.03 (m, 1H), 3.90 (s, 3H), 6.78 (s, 1H), 7.03 (m, 2H), 7.49 (m, 1H), 7.58 (m, 1H), 7.90 (m, 2H), 8.07 (m, 1H); ^{13}C NMR (CDCl$_3$, 75 MHz) δ 23.96, 33.81, 55.50, 106.01, 114.43, 117.84, 122.39, 123.63, 124.14, 127.96, 132.51, 146.01, 154.63, 162.33, 163.27, 178.66; HRMS m/z calcd for $[C_{19}H_{19}O_3]^+$: 295.1334; found: 295.1336.

4.1.4.5. 6-Isopropyl-3′,4′,5′-trimethoxylflavone (6e). Yield: 55%; brown oil; ^{1}H NMR (CDCl$_3$, 500 MHz) δ 1.32 (d, 6H, J = 6.95 Hz), 3.05 (m, 1H), 3.94 (s, 3H), 3.96 (s, 6H), 6.81 (s, 1H), 7.14 (s, 2H), 7.52 (d, 1H, J = 8.60 Hz), 7.58 (m, 1H), 8.07 (d, 1H, J = 2.152 Hz); ^{13}C NMR (CDCl$_3$, 125 MHz) δ 23.76, 33.63, 56.19, 60.84, 103.66, 106.99, 117.77, 122.23, 123.44, 126.92, 132.55, 141.08, 146.08, 153.42, 154.50, 162.98, 178.49; HRMS m/z calcd for $[C_{21}H_{23}O_5]^+$: 355.1546; found: 355.1538.

4.1.4.6. 6-Chloro-3′,5′-dimethoxylflavone (6f). Yield: 62%; slight yellow solid, mp: 175–179 °C; ^{1}H NMR (CDCl$_3$, 300 MHz) δ 3.87 (s, 6H), 6.62 (m, 1H), 6.78 (s, 1H), 7.01 (m, 2H), 7.52 (m, 1H), 7.64 (m, 1H), 8.18 (m, 1H); ^{13}C NMR (CDCl$_3$, 75 MHz) δ 55.62, 103.68, 104.51, 107.84, 119.87, 124.92, 125.16, 131.25, 133.28, 134.01, 154.53, 161.23, 163.50, 171.22; HRMS m/z calcd for $[C_{17}H_{14}ClO_4]^+$: 317.0581; found: 317.0571.

4.1.4.7. 6-Isopropyl-3′-nitroflavone (6g). Yield: 76%; white solid, mp: 183–185 °C; ^{1}H NMR (CDCl$_3$, 300 MHz) δ 1.33 (d, J = 6.90 Hz, 6H), 3.07 (m, 1H), 6.91 (s, 1H), 7.61 (m, 1H), 7.74 (m, 1H), 8.06 (m, 1H), 8.23 (m, 1H), 8.39 (m, 1H), 8.82 (s, 1H); ^{13}C NMR (CDCl$_3$, 75 MHz) δ 23.91, 33.83, 108.66, 118.05, 121.25, 122.57, 123.65, 125.82, 130.25, 131.72, 133.29, 133.80, 146.80, 148.81, 154.61, 160.36, 178.29; HRMS m/z calcd for $[C_{18}H_{16}NO_4]^+$: 310.1079; found: 310.1086.

4.1.4.8. 6-Isopropyl-4′-nitroflavone (6h). Yield: 70%; slight yellow solid, mp: 212–216 °C; ^{1}H NMR (CDCl$_3$, 500 MHz) δ 1.33 (d, J = 6.90 Hz, 6H), 3.07 (m, 1H), 6.91 (s, 1H), 7.54 (d, 1H, J = 8.60 Hz), 7.63 (d, J = 8.20 Hz, 1H), 8.10 (m, 3H), 8.38 (m, 2H); ^{13}C NMR (CDCl$_3$, 125 MHz) δ 23.75, 33.69, 109.32, 117.87, 122.46, 124.08, 127.06, 133.16, 137.67, 146.71, 149.28, 154.52, 160.29, 172.20, 178.19; HRMS m/z calcd for $[C_{18}H_{16}NO_4]^+$: 310.1079; found: 310.1080.

4.1.4.9. 2-Cyclopentyl-6-isopropyl-chromen-4-one (6i). Yield: 72%; white oil; ^{1}H NMR (CDCl$_3$, 500 MHz) δ 1.29 (d, J = 6.90 Hz, 6H), 1.77 (m, 6H), 2.07 (m, 2H), 3.00 (m, 2H), 6.20 (s, 1H), 7.36 (m, 1H), 7.51 (m, 1H), 8.03 (m, 1H); ^{13}C NMR (CDCl$_3$, 75 MHz) δ 23.97, 25.55, 31.13, 33.77, 44.19, 108.06, 117.77, 122.27, 123.46, 132.33, 145.69, 154.99, 172.65, 178.76; HRMS m/z calcd for $[C_{17}H_{21}O_2]^+$: 257.1542; found: 257.1548.

4.1.4.10. 2-Cyclopentyl-6-chloro-chromen-4-one (6j). Yield: 64%; white solid, mp: 65–68 °C; ^{1}H NMR (CDCl$_3$, 300 MHz) δ 1.43 (m, 3H), 1.78 (m, 1H), 1.88 (m, 2H), 2.04 (m, 2H), 2.53 (m, 1H), 6.17 (s, 1H), 7.39 (m, 1H), 7.57 (m, 1H), 8.13 (m, 1H); ^{13}C NMR (CDCl$_3$, 125 MHz) δ 25.87, 30.44, 42.83, 107.91, 119.66, 124.79, 125.10, 130.77, 133.62, 154.85, 173.82, 177.46; HRMS m/z calcd for $[C_{14}H_{14}ClO_2]^+$: 249.0682; found: 249.0688.

4.1.4.11. 2-Cyclohexyl-6-isopropyl-chromen-4-one (6k). Yield: 59%; white oil; ^{1}H NMR (CDCl$_3$, 300 MHz) δ 1.29 (d, J = 6.90 Hz, 6H), 1.40 (m, 5H), 1.77 (m, 1H), 1.87 (m, 2H), 2.03 (m, 2H), 2.53 (m, 1H), 3.02 (m, 1H), 6.20 (s, 1H), 7.37 (m, 1H), 7.53 (m, 1H), 8.03 (m, 1H); ^{13}C NMR (CDCl$_3$, 125 MHz) δ 23.79, 25.62, 25.73, 30.46, 33.63, 42.76, 107.54, 117.55, 122.25, 123.35, 132.18, 145.63, 154.85, 173.25, 178.90; HRMS m/z calcd for $[C_{18}H_{23}O_2]^+$: 271.1698; found: 271.1705.

4.1.4.12. 2-Cyclohexyl-6-chloro-chromen-4-one (6l). Yield: 64%; white solid, mp: 101–105 °C; ^{1}H NMR (CDCl$_3$, 300 MHz) δ 1.76 (m, 8H), 2.08 (m, 2H), 3.01 (m, 1H), 6.21 (s, 1H), 7.38 (m, 1H), 7.56 (m, 1H), 8.10 (m, 1H); ^{13}C NMR (CDCl$_3$, 75 MHz): δ 25.53, 31.01, 44.15, 108.18, 119.62, 124.67, 124.97, 130.66, 133.51, 154.81, 173.17, 177.20; HRMS m/z calcd for $[C_{15}H_{16}ClO_2]^+$: 263.0839; found: 263.0842.

4.1.4.13. 2-Cyclopentyl-benzo[h]chromen-4-one (6m). Yield: 70%; white solid, mp: 85–87 °C; ^{1}H NMR (CDCl$_3$, 300 MHz) δ 1.85 (m, 6H), 2.18 (m, 2H), 3.16 (m, 1H), 6.40 (s, 1H), 7.68 (m, 3H), 7.91 (m, 1H), 8.13 (m, 1H), 8.44 (m, 1H); ^{13}C NMR (CDCl$_3$, 125 MHz) δ 25.69, 31.40, 44.15, 109.63, 119.96, 120.85, 122.14, 124.04, 124.84, 126.97, 128.12, 129.04, 135.82, 153.80, 171.74, 178.32; HRMS m/z calcd for $[C_{18}H_{17}O_2]^+$: 265.1229; found: 265.1226.

4.1.4.14. 2-Cyclohexyl-benzo[h]chromen-4-one (6n). Yield: 75%; white solid, mp: 117–121 °C; ^{1}H NMR (CDCl$_3$, 500 MHz) δ 1.29 (m, 1H), 1.45 (m, 2H), 1.57 (m, 2H), 1.80 (m, 1H), 1.92 (m,2H), 2.13 (m, 2H), 2.67 (m, 1H), 6.32 (s, 1H), 7.66 (m, 3H), 7.88 (m, 1H), 8.12 (m, 1H), 8.46 (m, 1H); ^{13}C NMR (CDCl$_3$, 125 MHz) δ 25.81, 25.91, 30.74, 42.73, 109.20, 119.98, 120.82, 122.16, 124.06, 124.80, 126.92, 128.08, 129.01, 135.81, 153.70, 172.27, 178.41; HRMS m/z calcd for $[C_{19}H_{19}O_2]^+$: 279.1385; found: 279.1377.

4.2. Bioassay

4.2.1. Cell culture and reagents

Caspase-3, caspase-8, and caspase-9 activity assay kits were purchased from Beyotime. Monoclonal antibody for β-actin was purchased from Beyotime.

The human cell lines used in the present study included HepG-2 (hepatocellular carcinoma), Hela, CNE-1, CNE-2, and MCF-7 cells. All cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 100 μg/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO$_2$.

4.2.2. Cell proliferation assay and colony formation assay

Cells were seeded at a density of $5 \times 10^3/100$ μl medium in 96-well microtiter plate and treated with the synthesized compounds at different concentrations, ranging from 0.5 to 50 μM. Viable cells were incubated with MTT (5 mg/ml) for 5 h and formazan precipitate was dissolved in 100 μl DMSO and the absorbance at 490 nm was measured by Multimode Detector DTX880 (Beckman Coulter).

For colony formation assays, HepG-2 cells at the exponential phase were plated into 24-well culture plate (200–300 cells/well) and allowed to adhere for 10 h before treatment. Culture medium containing compound **6f** ranging from 0 to 50 μM was added to

cells and incubated for 14 days. Cells were then rinsed with PBS, stained with 1.0% crystal violet and photographed with a digital camera (Bio-Rad).

4.2.3. Fluorescent morphological assay

HepG-2 cells were seeded into 24-well culture plates and treated with 10 μM compound **6f** for 24 h. Cells were washed with PBS, fixed in MeOH–HAc (3:1, v/v) for 10 min at 4 °C, and stained with Hoechst 33258 (5 μg/ml) for 5 min at room temperature and then examined under a fluorescent microscope.

4.2.4. DNA fragmentation assay

The isolation of fragmented DNA was performed in accordance to a standard procedure. HepG-2 cells (1×10^6) were seeded in 6-well plate and treated with 0, 5, 10, 25, and 50 μM **6f** for 36 h. The cells were harvested by trypsinization, washed with PBS, centrifuged, and then lysed in buffer [10 mM Tris–HCl (pH 7.4), 10 mM EDTA (pH 8.0), 0.5% Triton X-100, and Proteinase K (200 μg/ml)]. DNA was extracted through organic solvent extraction. The extracted DNA was separated by electrophoresis in 2% agarose gel and visualized with ethidium bromide staining.

4.2.5. Capase-3, -8 and -9 assays

HepG-2 cells treated with **6f** for 0, 2, 4, 8, 14, and 24 h were collected by trypsinization, rinsed twice with PBS, resuspended in lysis buffer (caspase-8 and caspase-9 activity assay kit) and incubated on ice for 15 min. The lysate was centrifuged at 4 °C for 15 min at 20,000*g* for 15 min. The supernatant was used for protein concentration determination by the Bradford method with BSA as a standard, and 0.1 mg of total protein was used for caspase activity assay with Ac-DEVD-*p*NA, Ac-IETD-*p*NA, and Ac-LEHD-*p*NA as substrate for caspase-3, caspase-8, and caspase-9, respectively. Abs 405 nm agitated by the production of *p*NA were continuously recorded by Multimode Detector DTX880 (Beckman Coulter) after incubation for 2 h at 37 °C.

4.2.6. Flow cytometry analysis

Compound **6f**-treated and control cells were collected and washed twice with ice-cold PBS. The cells were fixed with 70% ethanol at 4 °C overnight, and resuspended at 5×10^5/ml cells of propidium iodide [50 μg/ml in 0.1% sodium citrate plus 0.03% (v/v) Nonidet P-40] and incubated on ice for 30 min. The tubes were packed with tin foil on ice until used for flow cytometry analysis using a FACscan flow cytometry (Bedtin-Dickinsom) with Cell-Quest software.

4.2.7. Semi-quantitative RT-PCR analysis

Total RNA was extracted from cells using Trizol reagent (Invitrogen) according to the supplier's instruction. RNA was quantitated by optical density measurements at 260 and 280 nm using ultraviolet spectrophotometer. One microgram of purified RNA was used for reverse transcription. Primers were obtained from invitrogen and their sequences were: 5′-ATTGTGGCCTTCTTTGAGTTC-3′ (sense) and 5′-TGGCAGTAAATAGCTGATTCG-3′ (antisense) for Bcl-2, 5′-TTTGCTTCAGGGTTTCATC-3′ (sense) and 5′-AAGATGGTCACG GTCTGC-3′ (antisense) for Bax, and 5′-CAACGTGTCAGTGGTGGA CCTG-3′ (sense) and 5′-TTACTCCTTGGAGGCCATGTGG-3′ (antisense) for GADPH. An aliquot (1 μl) of RT product was used for PCR amplification in a total volume of 25 μl. Amplification of all genes using a Bio-Rad thermal cycler was comprised of 30 cycles (98 °C for 15 s, 57 °C for 10 s, 72 °C for 35 s for Bcl-2 and Bax), while GADPH control was amplified in 30 cycles (98 °C for 15 s, 62 °C for 10 s, 72 °C for 35 s). PCR products (8 μl) were analyzed by electrophoresis on 1.2% (w/v) agarose gel and were visualized by ethidium bromide staining under ultraviolet light. Densitometric analysis of the images was conducted with Quantity One software. Results were presented as normalized fold changes in relation to control.

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