Full Paper

2′-Chloro-4′-aminoflavone Derivatives Selectively Targeting Hepatocarcinoma Cells: Convenient Synthetic Process, G_2/M Cell Cycle Arrest and Apoptosis Triggers

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A series of 2′-chloro-4′-nitroflavone and 2′-chloro-4′-aminoflavone derivatives were synthesized by a convenient synthetic process. The *in vitro* anti-proliferation ability of these compounds was evaluated against hepatocarcinoma cells (HepG2), breast adenocarcinoma cells (MCF-7), and human chronic myelogenous leukemia cells (K562). Most of synthetic compounds possessed notable anti-proliferation activity against HepG2 cells and little activity against MCF-7 cells and K562 cells. In particular, compounds **4c** and **4e** exhibited high anti-proliferation activity against HepG2 cells with IC_{50} at about 2.0 μM. Further toxicity screening toward normal human hepatocytes indicated that some compounds had low toxicity against normal liver cells, among which **4e** displayed very weak effects on QSG7701 and HL7702 cells, with IC_{50} values >100 and 50 μM, respectively. Compound **4c**, with the best anti-proliferation activity in amino-substituted flavones (IC_{50} = 2.0 μM), was selected for further evaluation of its effects on apoptosis and the cell cycle. HepG2 cells were exposed to this compound at 10 μM, which induced nuclear disassembly and DNA fragmentation. Flow cytometry analysis suggested that the population of apoptotic cells greatly increased in the **4c**-treated HepG2 cells, and the cell cycle was arrested at the G_2/M phase.

Keywords: Anti-tumor / Apoptosis / Cell cycle arrest / Cell selectivity / Flavonoids

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Introduction

Flavonoids are widely distributed among plant species, and consist of more than 9000 natural compounds [1]. Owing to their various biological activities, the study of flavonoids has been a research focus in the past decades [2, 3]. A subset of flavonoids, flavones, contain a C_6–C_3–C_6 carbon framework and possess many biological activities such as anti-inflammatory, antioestrogenic, antimicrobial, anti-allergic, antioxidant, antitumor, and cytotoxic activities [4]. The antitumor activity of flavones was embodied by arresting cell cycle and inducing cell apoptosis [5–8]. Apoptosis, also named programmed cell death [9], plays a vital role for eliminating cells in a damaged or infected situation, and is an effective and new cancer treatment strategy [10, 11]. Consequently, the design and synthesis of novel flavone derivatives as apoptosis triggers is a promising research field [12–14]. In recent years, amino-substituted flavones have attracted an increasing interest [15–19], because the amino group can act as a hydrogen donor or hydrogen bonding acceptor for binding with target proteins. For example, 2′-aminothioflavones were reported as specific inhibitors of the ERK-MAP kinase signal-

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ing pathway [18], 3′-aminoflavones as activators of apoptosis [19], and 4′-aminoflavones are potential inhibitors of protein tyrosine kinases [20]. Most aminoflavone derivatives are derived from synthetic chemistry [21–23]. The Baker–Venkataraman routine is the most commonly used method to synthesize various substituted flavone derivatives [24], but highly toxic pyridine and step-by-step synthetic operations are needed [25, 26].

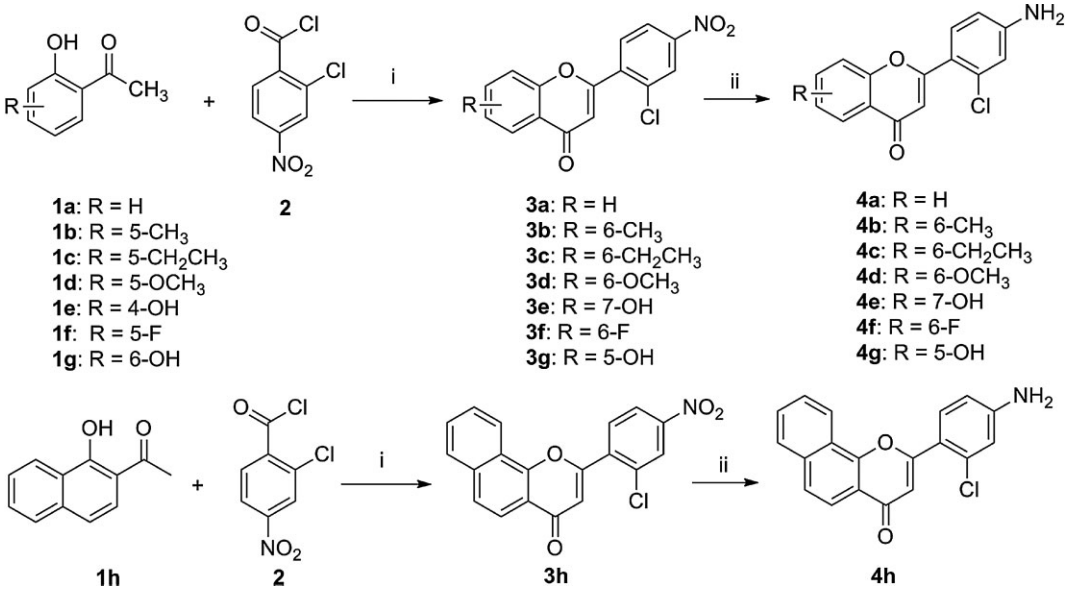
In our previous study, we reported the synthesis and bioactivity evaluation of some novel flavone derivatives with 6-chlorine or 6-isopropyl substitutions [27]. Those compounds showed good inhibition affect on HepG2 cells probably by induction of the apoptosis pathway, and the structure–activity relationship of B-ring substitutions was discussed. Herein, we continued that study and used a convenient synthetic process to synthesize 2′-chloro-4′-nitroflavones, which were then reduced to corresponding 2′-chloro-4′-aminoflavones. Some of these novel synthetic flavone derivatives were discovered to selectively inhibit HepG2 cell growth. Structure–activity relationships at the A-ring were disclosed. The most active compound in amino-substituted flavones, **4c**, arrested the cell cycle at the G_2/M phase, and some typical morphological changes of apoptotic cells were observed in **4c**-treated HepG2 cells.

Results and discussion

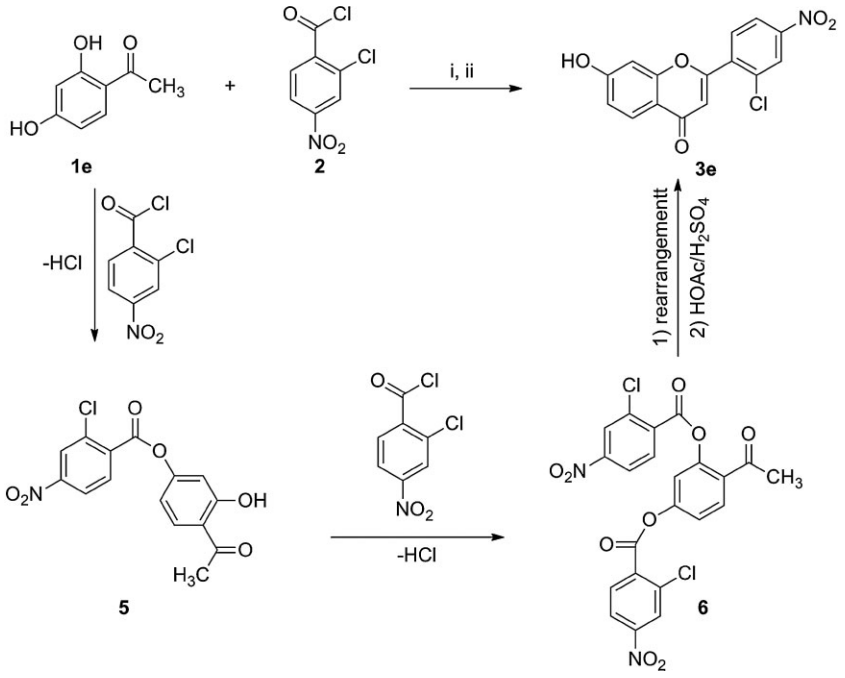
Synthesis of 2′-chloro-4′-aminoflavone derivatives

The synthetic route of target flavone derivatives is illustrated in Scheme 1. Briefly, *0*-hydroxyacetophenone derivatives and 2-chloro-4-nitrobenzoylchloride were employed as starting materials, which were refluxed in dry acetone for 15 h with anhydrous K_2CO_3 as catalyst. Acetone was then removed after the reaction by evaporation. Without further purification, glacial acetic acid and concentrated H_2SO_4 were added into the resulting mixture to reflux for another 2 h. Inorganic salts were then removed by hot filtration, and the resulting solution was allowed to cool down to room temperature to give the desired products as crystals. To obtain the corresponding amino products, these nitro derivatives were further treated with 4 equiv. of $SnCl_2$ under reflux in anhydrous ethanol for 2 h, after which the final products were purified by column chromatography.

Selective protection of hydroxyl group on *0*-hydroxyacetophenone derivatives is usually used to synthesize the A-ring hydroxyl substituted flavones. Some commonly used protection groups are tetra-butyl dimethylsilyl (TBS) [28], methyl [25, 29, 30], methoxymethyl (MOM) [31], benzoyl (Bz) [32], etc. It is noteworthy to point out that in our synthetic route, 7-hydroxyl-2′-chloro-4′-nitroflavone (**3e**) was conveniently obtained by a one-pot reaction of 2,4-dihydroxylacetophenone **1e** and 2-chloro-4-nitrobenzoylchloride **2** without protection/deprotection steps. As shown in Scheme 2, only product **5** is obtained when 1 equiv. of **2** reacted with **1e**. When 2 equiv. of **2** reacted with **1e**, the main product is **3e** in about 26% yield. A mechanism was proposed by which the hydroxyl group on compound **5** after the first nucleophilic substitution reacted with excess **2** to give compound **6**, followed by ring-closure and rearrangement to form the final product **3e**.



Scheme 1. Synthesis of compounds **3a**–**3h** and **4a**–**4h**. Regents and conditions: (i) dry K_2CO_3, acetone, reflux for 15 h; then conc. H_2SO_4, CH_3COOH, reflux for 2 h; (ii) anhydrous $SnCl_2$, anhydrous CH_3CH_2OH, reflux for 2 h.



Scheme 2. The consecutive reaction mechanism and intermediate products in the synthetic processes of **3e**.

Antiproliferative activity and tumor cell selectivity of synthetic flavones

The anti-proliferation ability of the synthesized compounds 2′-choro-4′-nitroflavone derivatives (**3a**–**3h**) and 2′-choro-4′-aminoflavone derivatives (**4a**–**4h**) were evaluated against HepG2 cells *in vitro* by MTT assay (MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), and the results are summarized in Table 1. Most compounds exhibited obvious anti-proliferation activity against HepG2 cells. Comparison of the activity of 4′-nitroflavone derivatives and their reduction products 4′-aminoflavone derivatives indicated that the latter had more promising activities against HepG2 cells, except for compound **3b**. Introduction of alkyl groups on the A-ring, for example, methyl and ethyl groups as weak electron-donating groups, provided better anti-tumor activities. However, electron-withdrawing groups and strong electron-donating groups have less effect on anti-tumor activities. The reason might be attributed to better solubility and bioavailability of alkyl-substituted compounds than fluoro- and methoxy-substituted flavone derivatives [33].

The most effective compound, **4c**, was chosen to investigate its inhibition on colony formation against HepG2 cells. As shown in Fig. 1, HepG2 cells were strongly inhibited at concentrations as low as 2.5 μM. Thus, both MTT and colony formation inhibition results demonstrated the inhibition effect of our synthetic compounds against HepG2 cells.

Breast adenocarcinoma cells (MCF-7) and human chronic myelogenous leukemia cells (K562) were also selected to evaluate the anti-proliferation activities of the synthetic compounds. The results are shown in Table 1. An interesting result was that all compounds displayed no effect against MCF-7 cells (with IC_{50} >50 μM), and only a few 4′-aminoflavone derivatives show weak activities against K562 cells, while 4′-nitroflavone analogues had no effect against K562

Table 1. Anti-proliferation activity of compounds against HepG2 cells, K562 cells, and MCF-7 cells

Compound	IC_{50} (μM)[a)]		
	HepG-2	K562	MCF-7
3a	7.4 ± 0.7	>50	>50
4a	2.5 ± 0.4	17.0 ± 0.6	>50
3b	1.6 ± 0.2	>50	>50
4b	2.5 ± 1.2	20.4 ± 2.3	>50
3c	6.1 ± 0.3	>50	>50
4c	1.8 ± 0.3	10.8 ± 1.5	>50
3d	nd	nd	nd
4d	10.2 ± 0.8	12.3 ± 1.4	>50
3e	>50	>50	>50
4e	2.1 ± 0.5	>50	>50
3f	13.7 ± 1.4	>50	>50
4f	7.2 ± 1.0	36.7 ± 1.8	>50
3g	14.5 ± 2.7	>50	>50
4g	7.3 ± 0.4	>50	>50
3h	>50	>50	>50
4h	19.4 ± 2.1	>50	>50
Colchicine	1.8 ± 0.4	nd	nd
Imatinib	nd	4.9 ± 0.8	nd

nd, not determined.
[a)] All values are means of three experiments.

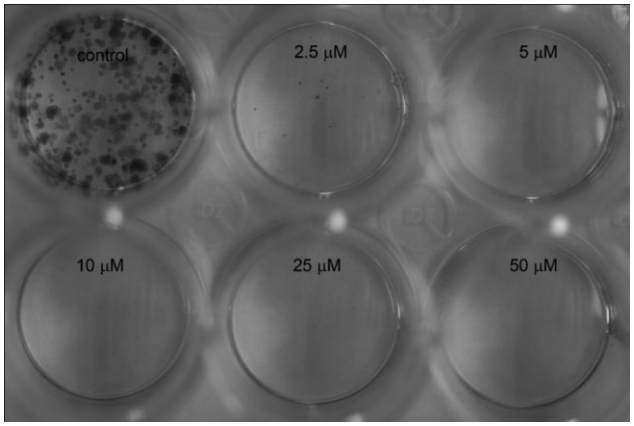


Figure 1. Effect of **4c** on colony formation of HepG2 cells.

cells. These results indicated that our synthetic compounds might selectively target HepG2 cells.

To further identify the selectivity of synthetic compounds, we evaluated the toxic activities of synthetic compounds against two normal human liver cells, QSG7701 and HL7702 cells. As shown in Table 2, compound **4e** exhibited less cytoxicity to QSG7701 cells with the inhibition rate of 21 and 15% at the concentration of 100 and 50 μM, respectively. In addition, compounds **3c**, **3e**, **3f**, and **4h** also displayed very weak cytoxicity against the normal human liver cells.

Table 2. Toxicity evaluation of compounds against QSG7701 cells and HL7702 cells

Compound	Inhibition rate[a] (100 μM)		Inhibition rate[a] (50 μM)	
	QSG7701	HL7702	QSG7701	HL7702
3a	67.6%	51.3%	54.1%	34.9%
4a	59.9%	57.2%	37.3%	46.2%
3b	48.8%	65.7%	47.0%	46.1%
4b	60.7%	63.9%	54.2%	47.5%
3c	34.6%	46.2%	17.9%	35.9%
4c	96.4%	99.2%	72.3%	56.6%
3d	nd	nd	nd	nd
4d	96.1%	85.2%	63.9%	63.3%
3e	26.5%	26.3%	33.5%	26.6%
4e	21.3%	54.4%	15.2%	37.9%
3f	37.8%	56.8%	34.5%	52.9%
4f	59.0%	63.2%	57.9%	55.9%
3g	nd	nd	nd	nd
4g	88.2%	84.3%	84.1%	72.9%
3h	nd	nd	nd	nd
4h	58.8%	48.6%	37.8%	39.0%

nd, not determined.
[a] All values are means of three experiments.

6-Ethyl-2′-chloro-4′-aminoflavone 4c induced apoptosis of HepG2 cells

Apoptosis is regulated by genetic mechanisms and is principally characterized by morphological and biochemical changes in the nucleus including chromatin condensation and internucleosomal DNA fragmentation [34, 35]. In order to confirm the existence of apoptosis in HepG2 cells treated with 6-ethyl-2′-chloro-4′-aminoflavone **4c** at 10 μM, typical apoptotic morphological study and flow cytometry analysis were carried out.

HepG2 cells were treated with 10 μM of **4c** and **4d** for 24 h and then stained with Hoechst 33258 for fluorescence microscopy observation. The observation showed some characteristic morphological changes of apoptosis, including membrane blebbing, nuclear condensation and fragmentation, chromatin condensation, and the formation of apoptotic bodies as shown in Fig. 2. Another typical feature of cells undergoing apoptosis was the loss of small DNA fragments which could be observed by agarose gel electrophoresis. As shown in Fig. 2, there were no obvious DNA segments in control cells. However, different concentrations of compound-treated cells displayed DNA laddering in a dose-dependent manner.

The Annexin-V/PI binding assay was used to further confirm the effect of compound **4c** induced apoptosis. Annexin-V conjugated with the fluorochrome FITC serves as a marker for apoptotic cells because it has a strong binding affinity to phosphatidylserine (PS), which re-distributes from the inner to the outer layer of the plasma membrane in apoptotic cells. The dual parametric dot plots show four quadrants as follows: (1) the lower left quadrant represents the viable cell population (annexin-V negative and PI negative); (2) the upper right represents apoptotic cells undergoing secondary necrosis at the last stage or dead cells (annexin-V and PI double positive); (3) the lower right represents the early stage apoptotic cell population (annexin-V positive and PI negative). As shown in Fig. 3, only about 8.29% of the untreated HepG2 cells were apoptotic cells either in early or secondary stage. However, the apoptotic cell population increased after the treatment with **4c** at different concentrations. The dose-dependent effect can also be observed as the concentration of **4c** increased from 5 to 50 μM, in which the apoptotic cells increased from 11.9 to 56.3%. The data also suggested that an increasing number of apoptotic cells progressed from the early stage to the late stage resulting in either death or secondary necrosis under the effect of **4c** at higher concentrations, which confirmed again that **4c** induced the apoptosis of HepG2 cells.

6-Ethyl-2′-chloro-4′-aminoflavone (4c) arrested cell cycle in G_2/M phase

The cell cycle is a vital process strictly regulated by transcriptional signaling pathways [36–38], deregulation of which

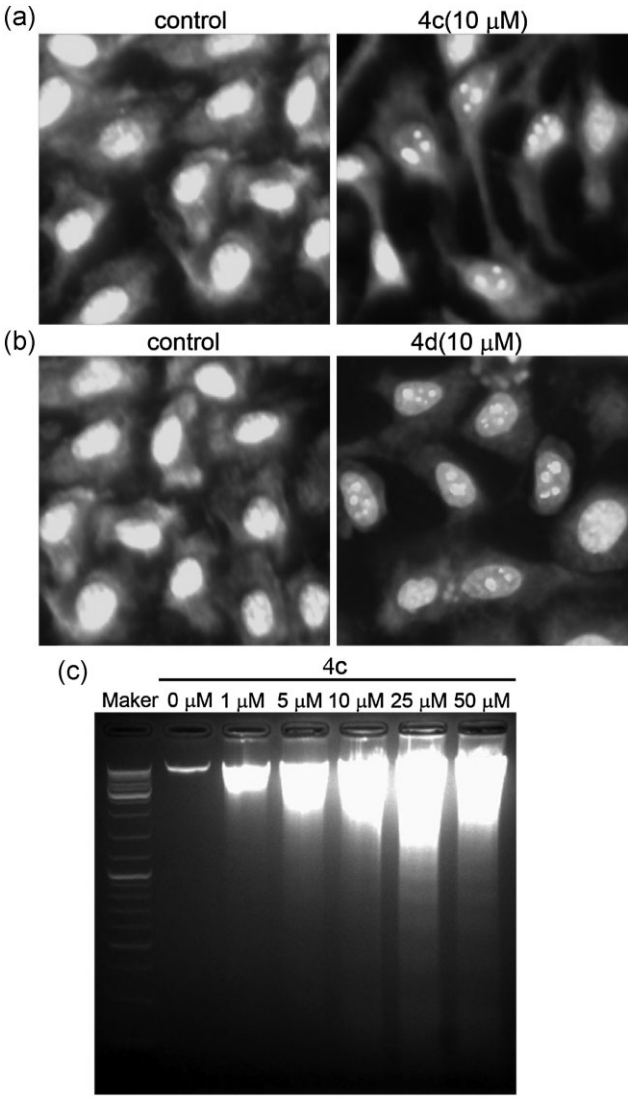


Figure 2. Fluorescent staining of nuclei by Hoechst 33258: (a) left: control cells; right: 10 μM **4c**-treated HepG2 cells; (b) left: control cells; right: 10 μM **4d**-treated HepG2 cells. DNA fragmentation induced by **4c**: (c) HepG2 cells were treated by **4c** at 0, 1, 5, 10, 25, and 50 μM for 30 h, then DNA was extracted for analysis.

concerns many diseases including human cancer. The cell cycle consists of four distinct phases: G_1 phase, S phase, G_2 phase, and M phase [39], the progress of which is monitored and regulated by cell cycle checkpoints so as to ensure repair of DNA damage and completion of each phase. Therefore, affecting the cell cycle has been one robust cancer therapy strategy, and some compounds exerted potent antitumor activity through cell cycle arrest [40, 41]. Herein, we investigated the effect of **4c** on the cell cycle and results showed that this compound arrested the cell cycle at the G_2/M phase in a dose-dependent manner. As shown in Fig. 4, the percent of cells at the G_2/M phase increased from 16 to 31% when the concentration was raised from 5 to 50 μM.

Conclusion

A convenient synthetic process was employed to synthesize 2′-chloro-4′-nitroflavone and 2′-chloro-4′-aminoflavone derivatives. These novel synthetic flavone derivatives possessed notable anti-proliferation activity with selectivity against HepG2 cells, and structure–activity relationships at the A-ring were disclosed. The introduction of alkyl group into the A-ring effectively improved their anti-proliferation ability. Compound **4c** demonstrated the strongest inhibition activity against HepG2 cell lines in amino-substituted flavone derivatives. Further study showed that the cell cycle was arrested at the G_2/M phase and induced apoptosis in HepG2 cells. This compound could be considered as a potential lead compound for developing new therapeutic agents against hepatocarcinoma.

Experimental

Chemistry

Melting points were measured with a SGW X-4 electrothermal melting apparatus, and data were uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded with a Bruker spectrometer at 400 MHz for ^{1}H NMR, at 101 MHz for ^{13}C NMR, with TMS as an internal standard in $CDCl_3$, or in [D_6] DMSO. High-resolution mass spectra were recorded with a Waters Q-Tof Premier mass spectrometer. Acetone was dried according to the standard process. All other reagents and analytical grade solvents commercially available were used without further purification.

*General procedure for the synthesis of derivatives of 2′-chloro-4′-nitroflavones (**3a–3h**)*

To a 100 mL round-bottomed flask add 2-chloro-4-nitrobenzoylchloride (0.01 mol, 2.20 g) and equimolar *O*-hydroxyacetophenone derivatives, dry K_2CO_3 (5.52 g, 0.040 mol), 20 mL dry acetone, and reflux the mixture for 15 h. Then, acetone was removed by vacuum distillation, and then 20 mL ice acetate acid and 0.50 mL concentrated sulfuric acid were added to the above resulting solids. The solutions were refluxed for 2 h to obtain white solids and clear solutions. The white solids were separated from the solutions by hot filtration. The filtrates were allowed to cool to room temperature, and crystals of derivatives of 2′-chloro-4′-nitroflavones were obtained.

*2′-Chloro-4′-nitroflavone (**3a**)*

Yield 55%; white solid, mp: 143–144°C; Ms (EI) 301.00. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.43 (d, J = 2.2 Hz, 1H), 8.28 (ddd, J = 7.9, 4.6, 1.9 Hz, 2H), 7.87 (d, J = 8.5 Hz, 1H), 7.75 (ddd, J = 8.7, 7.2, 1.7 Hz, 1H), 7.59–7.45 (m, 2H), 6.72 (s, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 177.53, 160.13, 156.55, 149.20, 137.56, 134.41, 134.37, 131.55, 126.00, 125.97, 125.83, 123.91, 122.05, 118.20, 113.87.

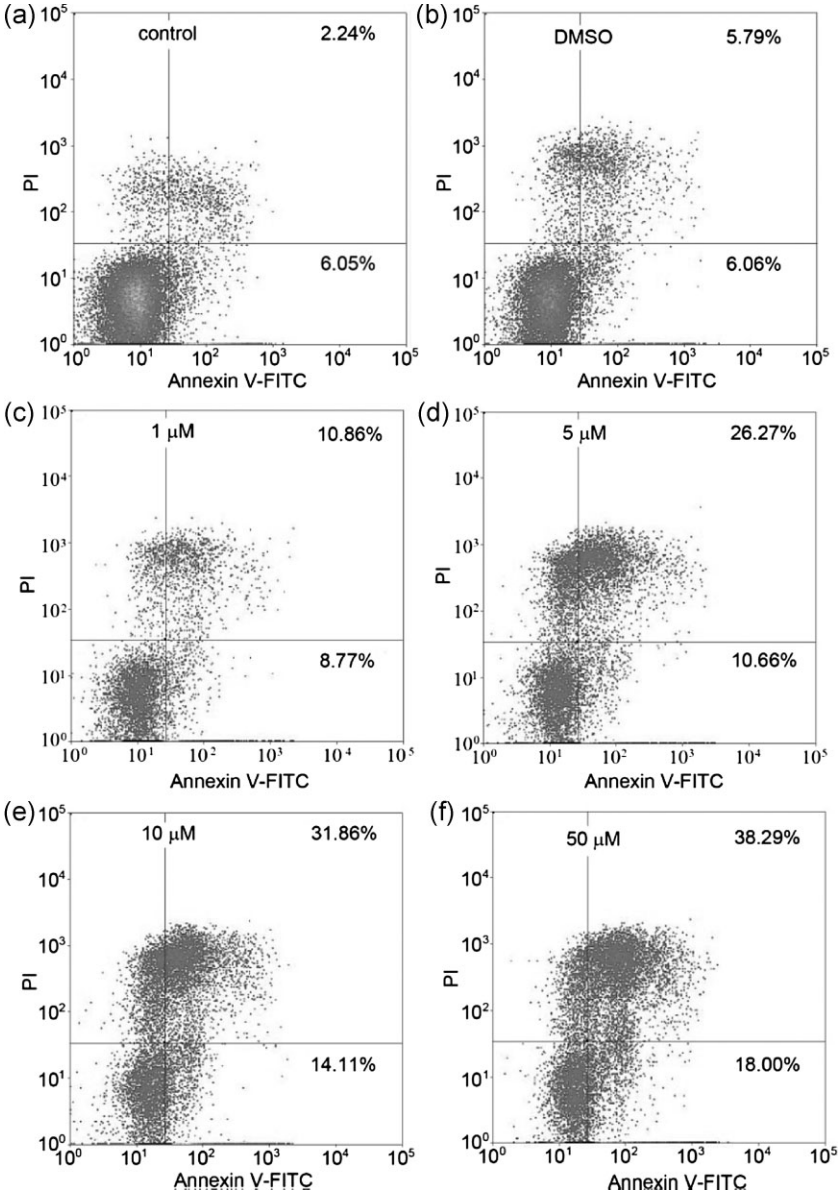


Figure 3. Flow cytometry analysis of cell apoptosis induced by **4c**. HepG2 cells were treated by **4c** at 0, 1, 5, 10, and 50 μM for 36 h.

*6-Methyl-2′-chloro-4′-nitroflavone (**3b**)*

Yield 62%; white solid, mp: 179–180°C; Ms (EI) 315.01. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.42 (d, J = 1.38 Hz, 1H), 8.27 (dd, J = 8.94, 1.38 Hz, 1H), 8.04 (s, 1H), 7.87 (d, J = 8.94 Hz, 1H), 7.56 (dd, J = 8.94, 1.38 Hz, 1H), 7.43 (d, J = 8.94 Hz, 1H), 6.70 (s, 1H), 2.50 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 177.79, 160.01, 154.85, 149.10, 137.69, 136.02, 135.70, 131.64, 126.04, 125.26, 123.53, 122.11, 118.04, 113.73, 21.10.

*6-Ethyl-2′-chloro-4′-nitroflavone (**3c**)*

Yield 60%; white solid, mp: 152–153°C; Ms (EI) 329.04. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.42 (d, J = 2.2 Hz, 1H), 8.27 (dd, J = 8.5, 2.2 Hz, 1H), 8.08 (d, J = 2.1 Hz, 1H), 7.86 (d, J = 8.5 Hz, 1H), 7.59 (dd, J = 8.6, 2.2 Hz, 1H), 7.46 (d, J = 8.6 Hz, 1H), 6.70 (s, 1H), 2.80 (q, J = 7.6 Hz, 2H), 1.31 (t, J = 7.6 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 177.87, 160.04, 155.03, 149.16, 142.35, 137.75, 134.70, 134.44, 131.62, 126.07, 124.12, 123.69, 122.10, 118.14, 113.74, 28.51, 15.58.

*6-Methoxy-2′-chloro-4′-nitroflavone (**3d**)*

Yield 35%; white solid, mp: 208–209°C; Ms (EI) 331.00. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.42 (d, J = 2.2 Hz, 1H), 8.27 (dd, J = 8.5, 2.2 Hz, 1H), 7.86 (d, J = 8.5 Hz, 1H), 7.62 (d, J = 3.1 Hz, 1H), 7.48 (d, J = 9.2 Hz, 1H), 7.34 (dd, J = 9.2, 3.1 Hz, 1H), 6.71 (s, 1H), 3.93 (s, 3H). ^{13}C NMR (101 MHz,$CDCl_3$) δ 177.67, 159.99, 157.57, 151.48, 149.19, 137.67, 134.44, 131.65, 126.09, 124.66, 124.56, 122.12, 119.76, 113.05, 104.99, 56.14.

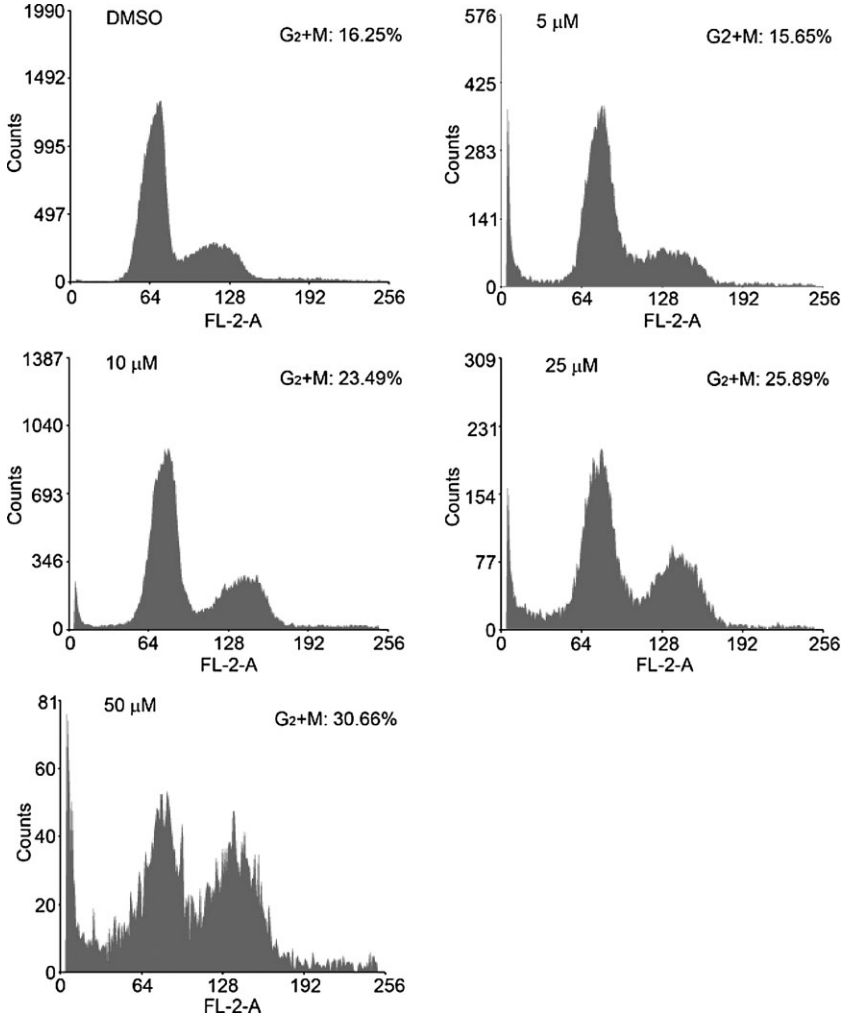


Figure 4. Analysis of cell cycle phase distribution induced by **4c**. HepG2 cells were treated by **4c** at 0, 5, 10, 25, and 50 μM for 36 h, and then analyzed by flow cytometry.

*7-Hydrox-2′-chloro-4′-nitroflavone (**3e**)*

In its special synthetic processes, twofold equivalents 2-chloro-4-nitrobenzoylchloride (0.02 mol, 4.40 g) were needed. The other operations were the same as the general procedure. Yield 26%; white solid, mp >300°C; Ms (EI) 316.99. ^{1}H NMR (400 MHz, DMSO) δ 10.96 (s, 1H), 8.48 (d, J = 1.7 Hz, 1H), 8.34 (dd, J = 8.5, 1.7 Hz, 1H), 8.09 (d, J = 8.5 Hz, 1H), 7.94 (d, J = 8.7 Hz, 1H), 6.98 (dd, J = 8.7, 1.6 Hz, 1H), 6.90 (s, 1H), 6.63 (s, 1H). ^{13}C NMR (101 MHz, DMSO) δ 175.61, 163.12, 159.50, 157.72, 148.96, 136.90, 132.79, 132.48, 126.69, 125.18, 122.52, 115.93, 115.52, 112.77, 102.38. Intermediate product (**5**), white solid, ^{1}H NMR (400 MHz, DMSO) 12.20 (s, 1H), 8.48 (m, 1H), 8.37 (m, 2H), 8.04 (d, J = 8.40 Hz, 1H), 6.98–7.03 (m, 2H), 2.67 (s, 3H).

*6-Fluoro-2′-chloro-4′-nitroflavone (**3f**)*

Yield 50%; white solid, mp: 191–192°C; Ms (EI) 319.02. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.43 (d, J = 2.2 Hz, 1H), 8.28 (dd, J = 8.5, 2.2 Hz, 1H), 7.90 (dd, J = 8.0, 3.0 Hz, 1H), 7.86 (d, J = 8.5 Hz, 1H), 7.55 (dt, J = 9.6, 4.8 Hz, 1H), 7.47 (ddd, J = 9.2, 7.5, 3.1 Hz, 1H), 6.70 (s, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 110.53, 110.764, 112.839, 120.11 (J = 8.08 Hz), 121.816, 122.34 (J = 25.65 Hz), 124.86 (J = 7.37 Hz), 125.772, 131.28, 134.17, 149.03, 152.46, 158.44, 160.52 (J = 78.17 Hz), 176.49.

*5-Hydroxy-2′-chloro-4′-nitroflavone (**3g**)*

Its synthetic procedure was more convenient than the general procedure. Equimolar *0*-hydroxyacetophenone and 2-chloro-4-nitrobenzoyl chloride, and 4 equiv. of dry K_2CO_3 were refluxed for 15 h, acetone was removed by evaporation, and the resulting brown solid was purified by rinsing with hot water. Yield 30%; light brown solid, mp: 193–194°C; Ms (EI) 317.03. ^{1}H NMR (400 MHz, DMSO) δ 12.39 (s, 1H), 8.52 (d, J = 2.1 Hz, 1H), 8.43–8.34 (m, 1H), 8.12 (d, J = 8.5 Hz, 1H), 7.74 (t, J = 8.4 Hz, 1H), 7.15 (d, J = 8.4 Hz, 1H), 6.99–6.84 (m, 2H). ^{13}C NMR (101 MHz, DMSO) δ 182.59, 161.61, 159.82, 156.16, 149.28, 136.50, 136.22, 132.88, 132.72, 125.24, 122.56, 111.91, 111.42, 110.16, 107.53.

*2-(2-Chloro-4-nitrophenyl)-4H-benzo[h]chromene-4-one (**3h**)*

Yield 32%; white solid, mp: 280–282°C; Ms (EI) 351.00. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.50 (dd, J = 9.3, 5.3 Hz, 2H), 8.34 (dd, J = 8.5, 2.2 Hz, 1H), 8.21 (d, J = 8.7 Hz, 1H), 7.97 (dd, J = 10.8, 8.2 Hz, 2H), 7.86 (d, J = 8.7 Hz, 1H), 7.73 (dtd, J = 16.4, 7.0, 1.2 Hz, 2H), 6.89 (s, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 177.45, 159.62, 154.14, 149.30, 137.56, 136.26, 134.47, 131.77, 129.74, 128.34, 127.54, 126.21, 126.05, 124.00, 122.34, 122.24, 120.65, 120.35, 115.07.

General procedure for the synthesis of derivatives of 2′-chloro-4′-aminoflavones

Derivatives of 2′-chloro-4′-nitroflavones and fourfold equivalents anhydrous $SnCl_2$ were refluxed in the anhydrous ethanol for 2 h, ethanol was removed by evaporation, and the resulting solids were purified by column chromatography, dichloromethane/methanol = 20:1 v/v as flush.

*2′-Chloro-4′-aminoflavone (**4a**)*

Yield 90%; yellow solid, mp: 147–148°C; HRMS (ESI) m/z calcd for $[M+H]^+$ 272.0479, found 272.0477. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.24 (dd, J = 8.0, 1.6 Hz, 1H), 7.68 (ddd, J = 8.7, 7.1, 1.7 Hz, 1H), 7.54–7.45 (m, 2H), 7.45–7.39 (m, 1H), 6.80 (d, J = 2.3 Hz, 1H), 6.72–6.62 (m, 2H), 4.07 (s, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 178.36, 163.04, 156.55, 149.52, 133.98, 133.61, 131.76, 125.69, 125.06, 123.84, 121.08, 118.11, 116.14, 113.11, 111.92.

*6-Methyl-2′-chloro-4′-chloroaminoflavone (**4b**)*

Yield 92%; yellow solid, mp: 165–166°C; HRMS (ESI) m/z calcd for $[M+H]^+$ 286.0636, found 286.0639. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.02 (s, 1H), 7.45–7.47 (m, 2H), 7.40 (d, J = 4.44 Hz, 1H), 6.79 (s, 1H), 6.65 (m, 3H), 4.06 (s, 2H), 2.36 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 178.50, 163.00, 154.82, 149.68, 135.01, 134.85, 133.87, 131.69, 124.93, 123.40, 120.88, 117.86, 116.09, 113.09, 111.61, 20.94.

*6-Ethyl-2′-chloro-4′-aminoflavone (**4c**)*

Yield 88%; yellow solid, mp: 96–97°C; HRMS (ESI) m/z calcd for $[M+H]^+$ 300.0792, found 300.0788. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.05 (d, J = 2.0 Hz, 1H), 7.52 (dd, J = 8.6, 2.2 Hz, 1H), 7.44 (dd, J = 14.4, 8.5 Hz, 2H), 6.79 (d, J = 2.3 Hz, 1H), 6.69–6.62 (m, 2H), 4.07 (s, 2H), 2.77 (q, J = 7.6 Hz, 2H), 1.30 (t, J = 7.6 Hz, 3H). ^{13}C NMR (101 MHz, CDCl3) δ 178.34, 162.72, 154.77, 149.25, 141.16, 133.74, 133.62, 131.53, 123.61, 123.35, 121.01, 117.75, 115.92, 112.90, 111.53, 28.21, 15.37.

*6-Methoxy-2′-chloro-4′-aminoflavone (**4d**)*

Yield 80%; yellow solid, mp: 142–143°C; HRMS (ESI) m/z calcd for $[M+H]^+$ 302.0585, found 302.0580. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.60 (d, J = 3.0 Hz, 1H), 7.50–7.41 (m, 2H), 7.28 (d, J = 3.1 Hz, 1H), 6.79 (d, J = 2.3 Hz, 1H), 6.70–6.62 (m, 2H), 4.09 (s, 2H), 3.91 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 178.21, 162.82, 156.93, 151.41, 149.47, 133.93, 131.73, 124.39, 123.71, 121.15, 119.54, 116.13, 113.12, 111.14, 104.78, 55.97.

*7-Hydroxy-2′-chloro-4′-aminoflavone (**4e**)*

Yield 72%; yellow solid, mp: 281–283°C; HRMS (ESI) m/z calcd for $[M+H]^+$ 288.0428, found 288.0451. ^{1}H NMR (400 MHz, DMSO) δ 10.79 (d, J = 15.6 Hz, 1H), 7.89 (t, J = 8.0 Hz, 1H), 7.47 (d, J = 8.5 Hz, 1H), 7.01–6.81 (m, 2H), 6.74 (d, J = 2.0 Hz, 1H), 6.63 (dd, J = 8.5, 2.1 Hz, 1H), 6.42 (d, J = 17.3 Hz, 1H), 6.08 (s, 2H). ^{13}C NMR (101 MHz, DMSO) δ 175.93, 162.53, 162.31, 157.57, 152.26, 132.24, 131.75, 126.42, 116.93, 115.84, 114.83, 114.13, 112.26, 109.95, 102.25.

*6-Fluoro-2′-chloro-4′-aminoflavone (**4f**)*

Yield 85%; yellow solid, mp: 176–177°C; HRMS (ESI) m/z calcd for $[M+H]^+$ 289.0306, found 289.0303. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.87 (dd, J = 8.2, 3.1 Hz, 1H), 7.51 (dd, J = 9.1, 4.2 Hz, 1H), 7.46 (d, J = 8.5 Hz, 1H), 7.40 (ddd, J = 9.1, 7.7, 3.1 Hz, 1H), 6.80 (d, J = 2.3 Hz, 1H), 6.70–6.62 (m, 2H), 4.09 (s, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) 177.53 (J = 2.22 Hz), 163.39, 159.60 (J = 247.65 Hz), 152.78, 149.77, 134.04, 131.76, 125.05 (J = 7.37 Hz), 121.75 (J = 25.55 Hz), 120.48 (J = 53.53 Hz), 116.18, 113.14, 111.16, 110.70, 110.47.

*5-Hydroxy-2′-chloro-4′-aminoflavone (**4g**)*

Yield 70%; yellow solid, mp: 167–169°C; HRMS (ESI) m/z calcd for $[M+H]^+$ 288.0428, found 288.0425. ^{1}H NMR (400 MHz, DMSO) δ 12.73 (s, 1H), 7.66 (d, J = 8.6 Hz, 1H), 7.54 (d, J = 8.4 Hz, 1H), 7.07 (d, J = 8.2 Hz, 1H), 6.80 (d, J = 2.2 Hz, 1H), 6.76 (d, J = 2.4 Hz, 1H), 6.63–6.66 (m, 2H), 6.23 (s, 2H). ^{13}C NMR (101 MHz, DMSO) 183.14, 165.09, 160.39, 156.57, 153.47, 136.32, 133.17, 132.75, 116.38, 114.92, 112.88, 111.35, 110.32, 109.04, 107.83.

*2-(2-Chloro-4-aminophenyl)-4H-benzo[h]chromene-4-one (**4h**)*

Yield 81%; light yellow solid, mp: 238–241°C; HRMS (ESI) m/z calcd for $[M+H]^+$ 322.0636, found 322.0635. ^{1}H NMR (400 MHz, DMSO) δ 8.54 (d, J = 7.5 Hz, 1H), 8.17–8.08 (m, 1H), 8.01 (d, J = 8.7 Hz, 1H), 7.94 (d, J = 8.7 Hz, 1H), 7.87–7.74 (m, 2H), 7.64 (d, J = 8.5 Hz, 1H), 6.81 (d, J = 2.0 Hz, 1H), 6.78–6.66 (m, 2H), 6.18 (br, 2H). ^{13}C NMR (101 MHz, DMSO) δ 177.00, 163.11, 153.57, 153.18, 135.91, 132.89, 132.87, 130.04, 128.77, 128.21, 125.76, 124.01, 122.59, 120.55, 119.87, 117.12, 114.95, 113.10, 111.75.

Biological assays

Cell culture and reagents

All human cell lines, including HepG2 cells, K562 cells, MCF-7 cells, QSG7701 cells, and HL7702 cells were obtained from Cell Resources Center of Shanghai Institutes for Biological Science, Chinese Academy of Science. They were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, 100 μg mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin at 37°C in a humidified atmosphere of 5% CO_2.

Cell growth inhibition assay

The synthesized compounds were dissolved in DMSO, and then diluted with culture medium to the final concentrations ranging from 0.5 to 50 μM (the final DMSO concentration was <1%) for tumor cell assays, 50 and 100 μM for normal liver cell assays. Hundred microliters of cell solution with the concentration of 5×10^5 cells mL^{-1} was seeded to each well of a 96-well plate and incubated for 24 h at 37°C in a 5% CO_2 incubator. The medium was then removed from the 96-well plate. The test compound solution was added to each well for the treatment of maintained cells in triplicate per concentration, and incubated at 37°C in a 5% CO_2 incubator for 48 h. After this treatment, 10 μL MTT

solution (5 mg mL^{-1}) was then added to each well and incubated for 4 h at 37°C. The formazan precipitate was dissolved in 100 μL DMSO and the absorbance at 495 nm was determined using a Multimode Detector DTX880 (Beckman Coulter).

For colony formation assays, approximately 300 HepG2 cells at the exponential phase were seeded into individual wells of a 24-well plate and incubated for 12 h to allow for adhesion. Culture medium containing compound **4c** ranging from 0 to 50 μM was added to each well and incubated for 14 days. Cells were then washed with PBS and stained with 1.0% crystal violet. The photograph was taken by a Canon digital camera.

Cell morphological and DNA fragmentation assay

For the cell morphological assay, HepG2 cells were seeded to each well of a 24-well plate and treated with 10 μM compound **4c** and **4d** for 24 h in a 5% CO_2 incubator at 37°C. Cells were then rinsed with PBS and fixed with MeOH–HAc (3:1 v/v) for 10 min at 4°C. Hoechst 333258 staining solution (5 μg mL^{-1}) was added to each well and incubated for 5 min at 37°C, and then detected under a fluorescence microscope.

For the DNA fragmentation assay, the isolation of fragmented DNA was performed according to a standard procedure. 1×10^6 HepG2 cells/well were seeded in a 6-well plate and treated with 0, 1, 5, 10, 25, and 50 μM compound **4c** for 30 h. The cells were then trypsinized with 0.025% trypsin-EDTA, rinsed in PBS, centrifuged at 800 rpm and 4°C for 10 min, and then lysed on ice for 10 min in buffer A (0.5% Triton X-100, 10 mM KCl, 50 mM Tris–HCl pH 7.5, 10 mM EDTA). The extracted DNA was finally separated by electrophoresis in 2% agarose gel and visualized with ethidium bromide staining.

Flow cytometry assay

For the apoptosis assay, untreated and drug-treated cells were collected by centrifugation and washed twice with ice-cold PBS. Surface exposure of PS in apoptotic cells was measured by the Annexin VFITC/PI apoptosis detection kit (Beyotime Company) according to the protocol described using flow cytometry (Moflo XDP, Beckman Coulter) [42]. For the cell cycle profile assay, the collected cells were resuspended in propidium iodide (50 μg mL^{-1} PI in 0.1% sodium citrate plus 0.03% v/v NP-40) and incubated for 30 min in the dark. The cell cycle profile was analyzed with flow cytometry (Moflo XDP, Beckman Coulter).

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Supplementary data

^1H NMR and ^{13}C NMR spectra of synthetic flavones in this article can be found.

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