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Preliminary communication

A novel [1,2,4] triazolo [1,5-*a*] pyrimidine-based phenyl-linked steroid dimer: Synthesis and its cytotoxic activity

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ABSTRACT

A novel [1,2,4] triazolo [1,5-*a*] pyrimidine-based phenyl-linked steroid dimer was designed, synthesized and evaluated for its cytotoxic activity against five human cancer cell lines and the cytotoxicity against human normal liver cell L-02. Compound **3** showed excellent cytotoxic activity and good selectivity between cancer and normal cells. Further mechanistic studies revealed that treatment of EC109 cells with compound **3** caused an obvious G2/M arrest in a concentration- and time-dependent manner and induced apoptosis probably through the mitochondrial pathway accompanied with the decrease of mitochondrial membrane potential, activations of caspase-9/-3, cleavage of MDM2 as well as up-regulation of the expressions of p53 and Bax.

1. Introduction

Steroids are a class of important multi-cyclic compounds which exhibit diverse biological activities in living organisms. It is proved that a number of biologically important properties of modified steroids are dependent upon structural features of the steroid ring system [1] and side chain [2]. Chemical modifications of the steroid ring system and side chain provide a way to alter the functional groups, and numerous structure activity relationships have been established by such synthetic alterations [3].

Dimeric steroids are a special group of compounds which have recently received significant attention [4,5]. There is evidence that dimerization of steroid skeleton offers some unique characteristics that are applicable in different areas, especially in pharmacology [6–8]. Among them, natural products such as cephalostatins, ritterazines and crellastatins (Fig. 1) are classical dimeric steroids with potent cytotoxicity [9,10]. As the natural supply of dimeric steroids with any direct pharmacological action is extremely limited, the synthesis of them has drawn wide attention among medicinal chemists. Up to now, there have been a number of steroid dimers synthesized and studied for their pharmacological activities [11,12].

On the other hand, [1,2,4] triazolo [1,5-*a*] pyrimidines (TPs), a subtype of purine analogs, have been widely investigated and identified to possess diverse pharmacological properties. For example, the most widely known triazolopyrimidine derivative is the simple molecule of Trapidil, which acts as a platelet-derived growth factor antagonist and as a phosphodiesterase inhibitor [13]. Recently, our group reported the synthesis and bioactivity of novel 7′-aryl-androstano[17,16-*d*][1,2,4]triazolo[1,5-*a*]pyrimidines derivatives, showing that some of these compounds had significant inhibitory activity against some human cancer cell lines [14,15]. In view of the pharmacological importance of dimeric steroids and triazolo [1,5-*a*] pyrimidine functional group and in continuation of our previous work in developing new biologically active modified steroids [16–20], we herein represent the synthesis of a novel [1,2,4] triazolo [1,5-*a*] pyrimidine-based phenyl-linked steroid dimer and its cytotoxic activity against human cancer cell lines in vitro and human normal liver cell (L-02). Besides, we also explore the effects toward the cell cycle and possible mechanisms of inducing apoptosis.

2. Results and discussion

2.1. Chemistry

The protocol for the synthesis of [1,2,4] triazolo [1,5-*a*] pyrimidine-based phenyl-linked steroid dimer **3** was very simple

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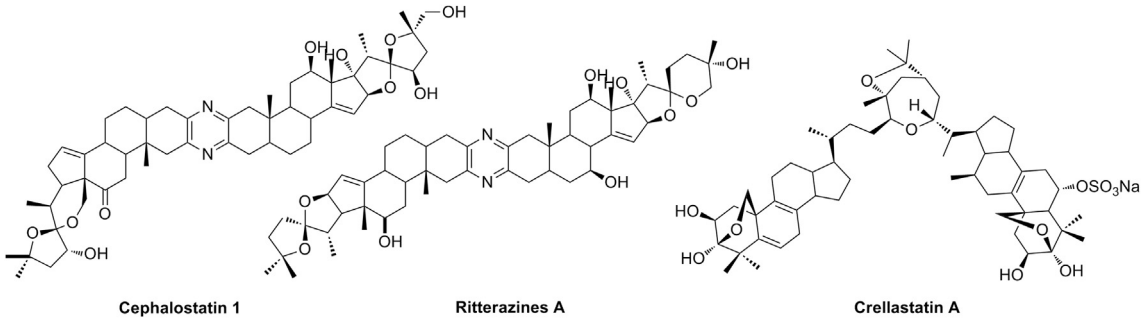


Fig. 1. Three classical natural occurring steroid dimers.

and straightforward as shown in Scheme 1. The protocol involved the condensation reaction of dehydroepiandrosterone (DHEA, **1**) with isophthalaldehyde via Claisen–Schmidt condensation catalyzed by KF/Al_2O_3 in ethanol [21], affording a novel phenyl-linked ketosteroid dimer **2** in 90% yield, which was subjected to Aza-Michael addition reaction and intramolecular cyclization reaction with 3-amino-1,2,4-triazole (3-AT) subsequently in the presence of *t*-BuOK and finally gave the target compound **3**. Besides, we also investigated the effects of catalysts (Lewis acids and Lewis bases), solvents (EtOH, *n*-BuOH) and the molar ratio towards the reaction between compound **2** and 3-AT under reflux, revealing that the optimized reaction condition was **2** (1.0 mmol), 3-AT (4.0 mmol) and *t*-BuOK (4.0 mmol) in *n*-BuOH (20 mL) under reflux.

All the synthesized compounds were characterized by ^1H, ^{13}C, IR and mass spectra. In the ^1H NMR spectrum of compound **3**, the 2′-H signals (N–C<u>H</u>=N, s, 2H) appeared at 8.37 ppm as a sharp singlet while the signals of the olefinic protons (C=C<u>H</u>–C_6H_4–) of compound **2** disappeared. In addition, the other four characteristic aromatic protons, two 3α-H and two 6-H protons showed their singlets at 8.42–7.82, 3.59–3.53 and 5.40–5.39 ppm, respectively. The ^{13}C NMR spectra of compound **3** exhibited the characteristic signals of 3a′-C at 181.07 ppm and the absence of 17-C carbonyl carbon signals. The signals of other carbons existed in phenyl ring, [1,2,4] triazolo [1,5-*a*] pyrimidine ring system and double bond of B-ring were also remarkable. The IR spectrum of **3** revealed the complete disappearance of the carbonyl groups (C-17) and showed a broad peak at 3420 cm^{-1} due to the 3β-OH. Furthermore, the presence of a molecular ion peak at m/z = 803.4757 ($[M + H]^+$) in the mass spectra (calcd. 803.4761) further confirmed the structure of the final product **3**.

A possible mechanism for the formation of [1,2,4] triazolo [1,5-*a*] pyrimidine-based phenyl-linked steroid dimer **3** was proposed in Scheme 2. Under basic condition, 3-AT existed as the anion and attacked the β-carbon of aromatic α, β-unsaturated ketone unit (**2**) via Aza-Michael addition reaction to afford the intermediate **A**. Subsequently, the amino group attacked the 17-ketone to form the heterocycle **B** via an intramolecular cyclization reaction followed by elimination of H_2O to give the dihydrotriazolopyrimidine **C**. Finally, the target compound **3** was formed by automatic aromatization of intermediate **C** under the same condition. In order to demonstrate the reaction mechanism clearly, the sequence involving 1 M of **2** reacting with 1 M of 3-AT was shown in Scheme 2.

2.2. Biological evaluation

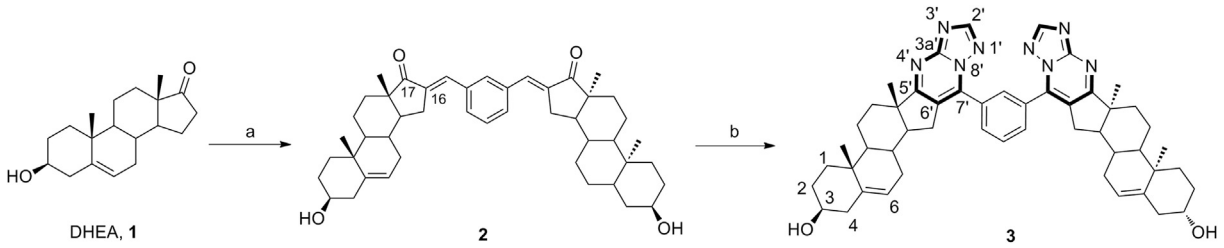
2.2.1. Cytotoxic activity

The IC_{50} values (concentration required to inhibit tumor cell proliferation by 50%) for the synthesized compounds against five human cancer cell lines including human gastric cancer cell line (MGC-803), human breast cancer cell line (MCF-7), human liver cancer cell line (SMMC-7721), human prostate cancer cell line (PC-3) and human esophageal cancer cell line (EC-109) were determined using the MTT assay. Besides, we also evaluated the cytotoxicity of compound **3** against human normal liver cell (L-02) and the cell viability at different concentrations of compound **3**. The results are showed in Table 1 and Fig. 2.

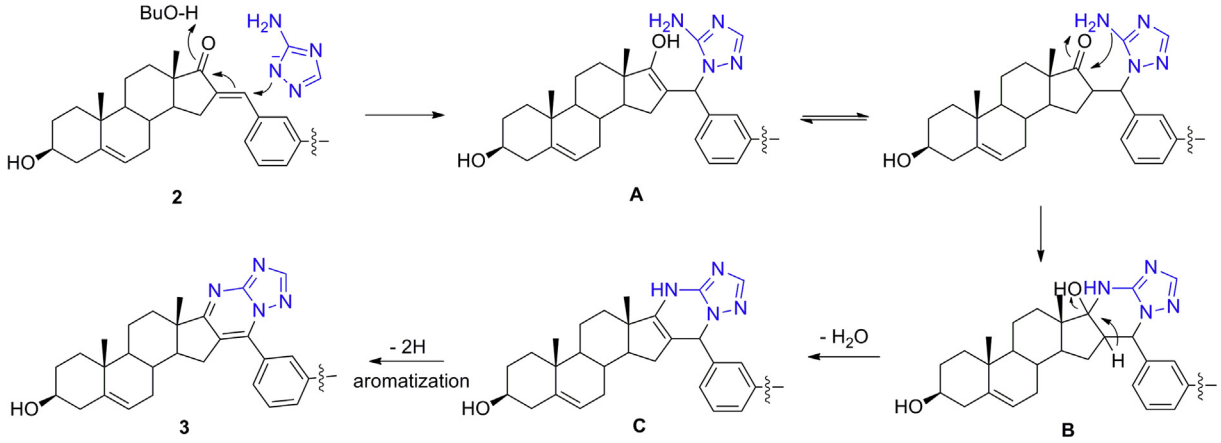
As shown in Table 1, compound **3** represented excellent cytotoxic activity with the IC_{50} values of 2.36 ± 0.35, 0.13 ± 0.12, 1.68 ± 0.16, 3.18 ± 0.51 and 1.19 ± 0.25 μM against EC109, MGC-803, SMMC-7721, PC-3 and MCF-7, respectively. Besides, compound **3** had an IC_{50} value of 82.95 ± 2.27 μM against human normal liver cells (L-02), 49-fold less toxic than that against SMMC-7721 cancer cells. This showed that our designed compound **3** had good selectivity between cancer and normal cells. As demonstrated in Fig. 2, the cancer cell viability decreased significantly with the increase of the concentration of compound **3**. However, the normal liver cell viability dropped slowly. The viability of human normal liver cell stood at around 70% even at 20 μM of **3**.

2.2.2. Apoptosis assay

Due to the excellent cytotoxic activity against all tested human cancer cell lines and low cytotoxicity against human normal liver cell, compound **3** was chosen to be further investigated regarding its mechanism of action. In order to better characterize the mode of



Scheme 1. Protocol for the synthesis of [1,2,4] triazolo [1,5-*a*] pyrimidine-based phenyl-linked steroid dimer (**3**). Reagents and conditions: (a) isophthalaldehyde, KF/Al_2O_3, EtOH, reflux; (b) 3-amino-1,2,4-triazole, *t*-BuOK, *n*-BuOH, reflux.



Scheme 2. Proposed mechanism for the formation of steroid dimer **3**.

cell death induced by compound **3**, we performed a biparametric cytofluorimetric analysis using propidium iodide (PI) and Annexin V-FITC in EC109 cells. After treatment with compound **3** for 12 or 24 h at different concentrations (0, 2.5, 5, 10, 20, 40 μM), EC109 cells were labeled with the two dyes, and the resulting red (in the web version) (PI) and green (FITC) fluorescence was monitored by flow cytometry. It can be observed from Fig. 3 that compound **3** caused the early and late apoptosis. Specifically, after treatment for 12 h, when the concentration was 2.5 μM, the early apoptosis rate was 26.9%, and for the high concentration group (40 μM), the early and late apoptosis rates were 12.2% and 65.3%, respectively (6.1% and 1.4% for the control group) (Fig. 3A). Similarly, the early and late apoptosis rates for the high concentration group (40 μM) were 12.0% and 78.4% after treatment for 24 h. The results showed that compound **3** markedly increased the cellular apoptosis in a concentration- and time-dependent manner.

2.2.3. Cell cycle analysis

To better understand the cytotoxic activity of compound **3**, a cell-cycle cytotoxicity assay was performed by treating EC109 cells at different concentrations of compound **3** (0, 2.5, 5.0, 10 μM). After treatment EC109 cells for 12 h, it was observed that the percentage of cells in G2/M phase at different concentrations were 13.72%, 17.94%, 20.79% and 28.32%, respectively (Fig. 4A), whereas when treatment for 24 h, the percentage of cells in G2/M phase were 6.31%, 14.85%, 19.43% and 25.12%, respectively (Fig. 4B). The results suggested that **3** caused an obvious G2/M arrest pattern in a concentration- and time-dependent manner with a concomitant decrease in terms of the number of cells in other phases of the cell cycle.

2.2.4. Morphological studies

After treatment with **3** at the indicated concentrations for 24 h, morphology changes of EC109 cells were recorded using an inverted microscope. 0.1% DMSO was used as a control (Fig. 5A). Besides, nuclear morphological changes at the corresponding concentration were also recorded and qualitatively evaluated by means of Hoechst 33258 fluorescent staining after incubation for 24 h. Typical apoptotic markers, including nuclear condensation and nuclear fragmentation, were detected, especially at the highest concentrations of **3** (Fig. 5B).

2.2.5. Detection of mitochondrial membrane potential and related protein expression

After EC109 cells were incubated with compound **3** (0, 5.0, 10 μM) for 24 h, the cells were stained with JC-1 and detection by flow cytometer (Fig. 6), the percentage of cells with green fluorescence was 8. 1%, 40.0% and 76.7% respectively, revealing that **3** could decrease mitochondrial membrane potential (MMP) in a concentration-dependent manner. Meanwhile, caspase-3/-9 activation and changes in MDM2, p53 and Bax expression levels in EC109 cells were also observed after treatment for 24 h with compound **3** at three different concentrations, which indicated that compound **3** up-regulated p53 via suppressing MDM2, then activated the expression of Bax, and induced cytochrome *c* release from the mitochondria, and finally activated caspase-3/-9 (Fig. 7).

3. Conclusions

In summary, we first report the efficient synthesis and cytotoxic activity of [1,2,4] triazolo [1,5-*a*] pyrimidine-based phenyl-linked steroid dimer **3**. The results exhibited that compound **3** had excellent anticancer activities against EC109, MGC-803, PC-3, MCF-7 and SMMC-7721 with the IC_{50} values of 2.36 ± 0.35, 0.13 ± 0.12, 3.18 ± 0.51, 1.68 ± 0.16 and 1.19 ± 0.25 μM, respectively. Besides, compound **3** represented low cytotoxicity against human normal liver cell L-02 (IC_{50} = 82.95 ± 2.27 μM). Besides, investigations indicated that compound **3** caused the early and late cellular apoptosis in a concentration- and time-independent manner and an obvious G2/M arrest with a concomitant decrease of cells in other phases of the cell cycle. Morphology changes of EC109 cells were also observed. Further study indicated that compound **3** induced EC109 cells apoptosis probably through mitochondrial pathway. Specifically, Compound **3** up-regulates p53 via suppressing MDM2, then activates the expression of Bax, and induces cytochrome *c* release from the mitochondria, and finally activates caspase-3/-9. Synthesis of more analogs, SAR studies and further mechanism investigations are under way and will be reported in due course.

4. Experimental section

4.1. General

Reagents and solvents were purchased from commercial sources and were used without further purification. Thin-layer chromatography (TLC) was carried out on glass plates coated with silica gel (Qingdao Haiyang Chemical Co., G60F-254) and visualized by UV light (254 nm). The products were purified by column chromatography over silica gel (Qingdao Haiyang Chemical Co., 200–300 mesh). Melting points were determined on a X-5 micromelting apparatus and are uncorrected. All the NMR spectra were recorded

Table 1
Preliminary cytotoxic activities of compound **3** against five human cancer cell lines and human normal cell L-02.

Compounds	IC_{50} (μM)[a]					
	EC109	MGC-803	SMMC-7721	PC-3	MCF-7	L-02
3	2.36 ± 0.35	0.13 ± 0.12	1.68 ± 0.16	3.18 ± 0.52	1.19 ± 0.25	82.95 ± 2.27
5-Fu	10.61 ± 1.08	6.92 ± 0.35	9.78 ± 0.99	24.76 ± 3.44	7.54 ± 0.70	nd[b]

[a] Inhibitory activity was assayed by exposure for 72 h to substances and expressed as concentration required to inhibit tumor cell proliferation by 50% (IC_{50}). Data are presented as the means ± SDs of three independent experiments.
[b] Not determined.

with a Bruker DPX 400 MHz spectrometer with TMS as internal standard in $CDCl_3$. Chemical shifts are given as δ ppm values relative to TMS (Most of the peaks due to the steroidal skeleton are merged and could not be differentiated. Thus δ values of only those peaks that distinguish the product and could easily be differentiated are reported). High-resolution mass spectra (HRMS) were recorded on a Waters Micromass Q-T of Micromass spectrometer by electrospray ionization (ESI).

4.2. Preparation of the KF/Al_2O_3 catalyst

To a solution of KF (58 g) in water (100 mL) was added Al_2O_3 (100 g) with stirring. The mixture was stirred for 3 h at 80 °C, then the solvent was evaporated and the solid was dried for 4 h at 120 °C to give KF/Al_2O_3 catalyst.

4.3. Synthesis of phenyl-linked 16-methylene-17-ketosteroid dimer (**2**)

A mixture of dehydroepiandrosterone (DHEA, **1**) (2.0 mmol), isophthalaldehyde (1.1 mmol) and KF/Al_2O_3 (1.0 mmol) in EtOH (20 mL) was heated under reflux for about 1 h. After completion of the reaction as evident from TLC (petroleum ether/ethyl acetate = 3/1), the slurry was filtered and the residue was washed thoroughly with CH_2Cl_2. The filtrate was condensed under reduced pressure, and the solid obtained was crystallized from EtOH to yield the phenyl linked 16-methylene-17-ketosteroid dimer **2**. White solid, yield 90%, mp 222.9–224.0 °C. IR (KBr, cm^{-1}) *v*: 3439, 2932, 2899, 2858, 1717, 1628, 1453, 1374, 1265, 1096, 1057, 1009, 797, 559; ^1H NMR (400 MHz, $CDCl_3$): δ 7.72 (s, 1H, Ar–H), 7.55 (d, *J* = 7.8 Hz, 2H, Ar–H), 7.52–7.48 (m, 1H, Ar–H), 7.46 (s, 2H, Ar–CH=), 5.41 (d, *J* = 4.8 Hz, 2H, 6-H), 3.61–3.50 (m, 2H, 3α-H), 1.18–1.05 (m, 10H), 1.01 (s, 6H, 18-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 209.53, 141.19, 136.72, 136.08, 132.43, 131.90, 130.84, 129.08, 120.75, 71.59, 50.32, 49.78, 47.39, 42.22, 37.14, 36.76, 31.59, 31.56, 31.27, 31.04, 29.51, 20.40, 19.46, 14.24; HRMS (ESI): *m*/*z* calcd for $C_{46}H_{58}O_4Na$ $(M + Na)^+$, 697.4233; found, 697.4235.

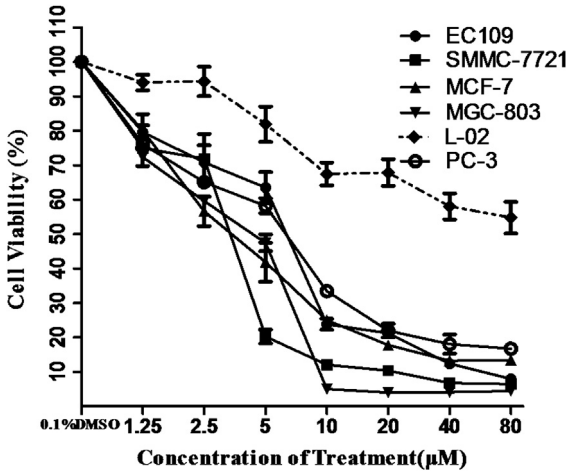


Fig. 2. Effects of **3** on the growth of Carcinoma cell lines and human normal liver cell. 8×10^3 Carcinoma cells were plated in 96-well culture plates. After 24 h, the cells were changed with fresh medium and treated with **3** at the indicated concentrations for 72 h, respectively. Culture medium with 0.1% DMSO was used as controls. Normal human liver cells L-02, as well as Carcinoma cell were plated and treated under the same conditions with **3** at the indicated concentrations for 72 h. Cell viability was measured by MTT assay. Data are means ± SD. Each sample was counted in duplicate. The IC_{50} values were calculated by Graphpad prism software.

4.4. Synthesis of [1,2,4] triazolo [1,5-a] pyrimidine-based phenyl-linked steroid dimer (**3**)

The phenyl-linked 16-methylene-17-ketosteroid dimer **2** (1.0 mmol) was dissolved in *n*-BuOH (20 mL). To the solution was added 3-amino-1,2,4-triazole (4.0 mmol) and *t*-BuOK (4.0 mmol). The resulting mixture was refluxed for about 24 h. The solvent was removed and CH_2Cl_2 was added. The insoluble *t*-BuOK was filtered and washed thoroughly with CH_2Cl_2. After removal of the solvent, the residue was purified by silica gel chromatography using dichloromethane/methanol (15/1) as the eluent to give the corresponding product **3**. White solid, yield 69%, mp > 300 °C. IR (KBr, cm^{-1}) *v*: 3420, 2929, 2855, 1628, 1542, 1522, 1436, 1376, 1274, 1133, 1058, 1040, 789, 658. ^1H NMR (400 MHz, $CDCl_3$): δ 8.42 (s, 1H, Ar–H), 8.37 (s, 2H, 2′-H), 8.02 (d, *J* = 7.8 Hz, 2H, Ar–H), 7.84 (t, *J* = 7.8 Hz, 1H, Ar–H), 5.40 (d, *J* = 4.2 Hz, 2H, 6-H), 3.62–3.50 (m, 2H, 3α-H), 1.22 (s, 6H, 18-H), 1.20–1.09 (m, 10H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 181.07, 155.91, 154.74, 141.47, 141.41, 132.00, 131.25, 129.78, 128.98, 123.25, 120.48, 71.52, 55.96, 50.30, 46.50, 42.21, 37.11, 36.78, 33.02, 31.57, 31.25, 31.12, 29.06, 20.50, 19.47, 17.12; HRMS (ESI): *m*/*z* calcd for $C_{50}H_{59}N_8O_2$ $(M + H)^+$, 803.4761; found, 803.4757.

4.5. Cell culturing

Human cancer cell lines and human normal liver cell line were maintained in minimal essential medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. Cancer cells were maintained in RPMI1640 medium; human normal liver cell line (L-02) was maintained in DMEM high Glucose medium. All cell lines were purchased from the China Center for Type Culture Collection (CCTCC, Shanghai, China). For pharmacological investigations, 10 mM stock solutions of the tested compounds were prepared with dimethyl sulfoxide (DMSO). The highest DMSO concentration of the medium (0.1%) did not have any substantial effect on the determined cellular functions.

4.6. Cytotoxic activity assays

Exponentially growing cells were seeded into 96-well plates at a concentration of 5×10^3 cells per well. After 24 h incubation at 37 °C, the culture medium was removed and replaced with fresh medium containing the candidate compounds in different concentrations. The cells were incubated for another 72 h. Then,

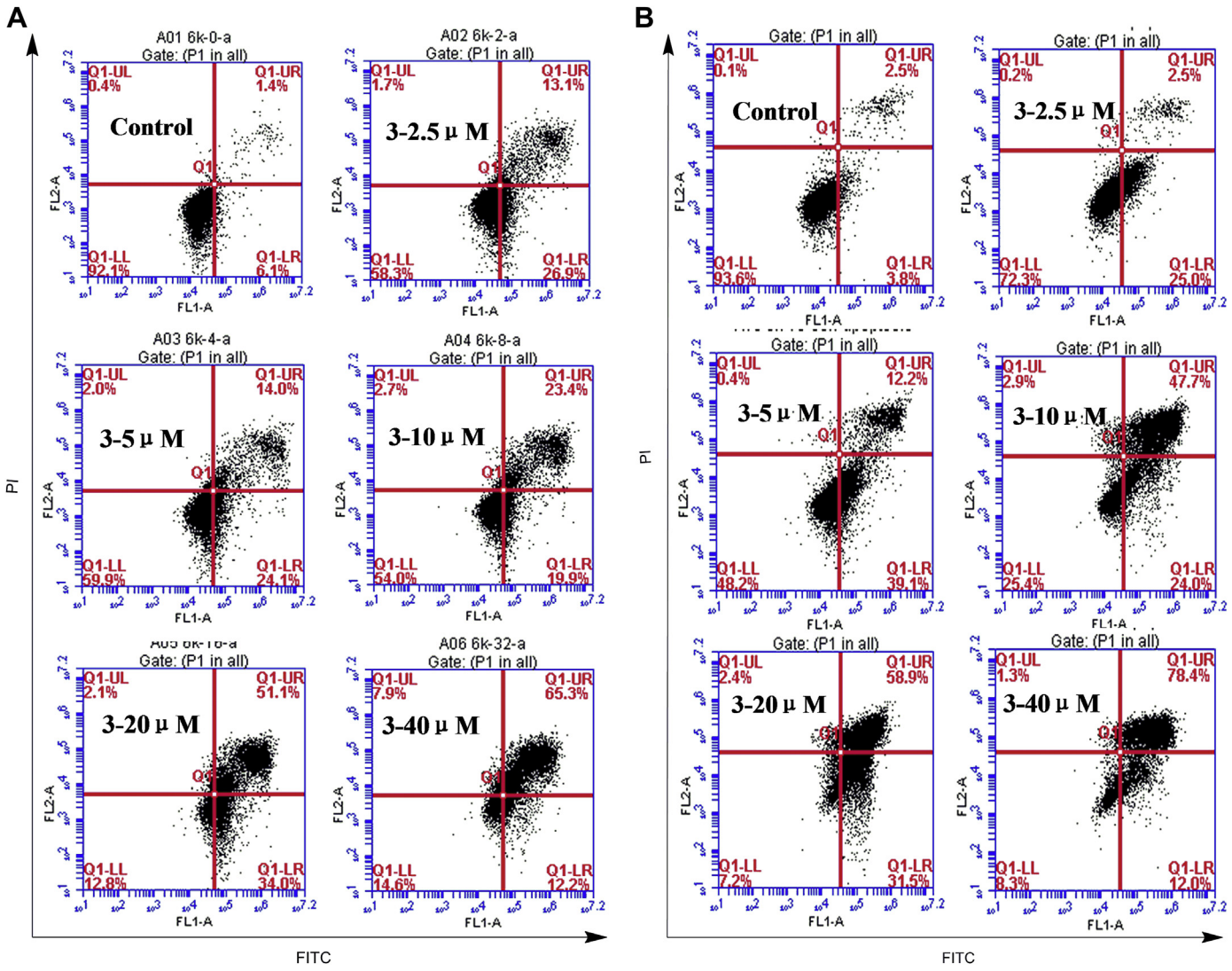


Fig. 3. Apoptosis effect on human EC109 cell line induced by compound **3**. Apoptotic cells were detected with Annexin V-FITC/PI double staining after incubation with compound **3** (0, 2.5, 5, 10, 20, 40 μmol/L) for 12 h or 24 h. (A) Incubated for 12 h; (B) incubated for 24 h. The lower left quadrants represent live cells, the lower right quadrants are for early/primary apoptotic cells, upper right quadrants are for late/secondary apoptotic cells, while the upper left quadrants represent cells damaged during the procedure. The experiments were performed three times, and a representative experiment is shown.

20 μL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (5 mg/mL) was added to all wells and incubated for 4 h at 37 °C. Discarded the suspension and added 150 μL of dimethyl sulfoxide (DMSO) to each well and shook the plates to dissolve the dark blue crystals (formazan); the absorbance was measured using a microplate reader at a wavelength of 490 nm. Each concentration was analyzed in triplicate and the experiment was repeated three times. The average 50% inhibitory concentration (IC_{50}) was determined from the dose–response curves according to the inhibition ratio for each concentration.

4.7. Analysis of cellular apoptosis

EC109 cells were plated in 6-well plates (5.0×10^6 cells/mL) and incubated at 37 °C for 12 or 24 h. Exponentially growing cells were then incubated for 12 or 24 h with complete medium (blank) or with the compound **3**. Cells were then harvested and the Annexin-V-FITC/PI apoptosis kit (Biovision) was used according to the manufacturer's instructions to detect apoptotic cells. Ten thousand events were collected for each sample and analyzed by Accuri C6 flow cytometer.

4.8. Flow cytometric analysis of cell cycle distribution

For flow cytometric analysis of DNA content, 5.0×10^6 EC109 cells in exponential growth were treated with different concentrations of the test compounds for 12 or 24 h. After an incubation period, the cells were collected, centrifuged and fixed with ice-cold ethanol (70%). The cells were then treated with buffer containing RNAse A and 0.1% Triton X-100 and then stained with PI. Samples were analyzed on Accuri C6 flow cytometer (Becton, Dickinson). Data obtained from the flow cytometer was analyzed using the FlowJo software (Tree Star, Inc., Ashland, OR, USA).

4.9. Hoechst 33258 staining

Near-confluent EC109 cells were seeded into a 6-well plate (5.0×10^6 cells/well). After incubation for 24 h with the test compound, Hoechst 33258 was added to the culture medium to give final concentrations of 5 μg/mL, respectively. The cells were incubated with the staining mixture for 30 min at 37 °C and then the cells were observed under a fluorescence microscope (Leica Microsystems AG, Wetzlar, Germany). Apoptotic cells were examined and identified

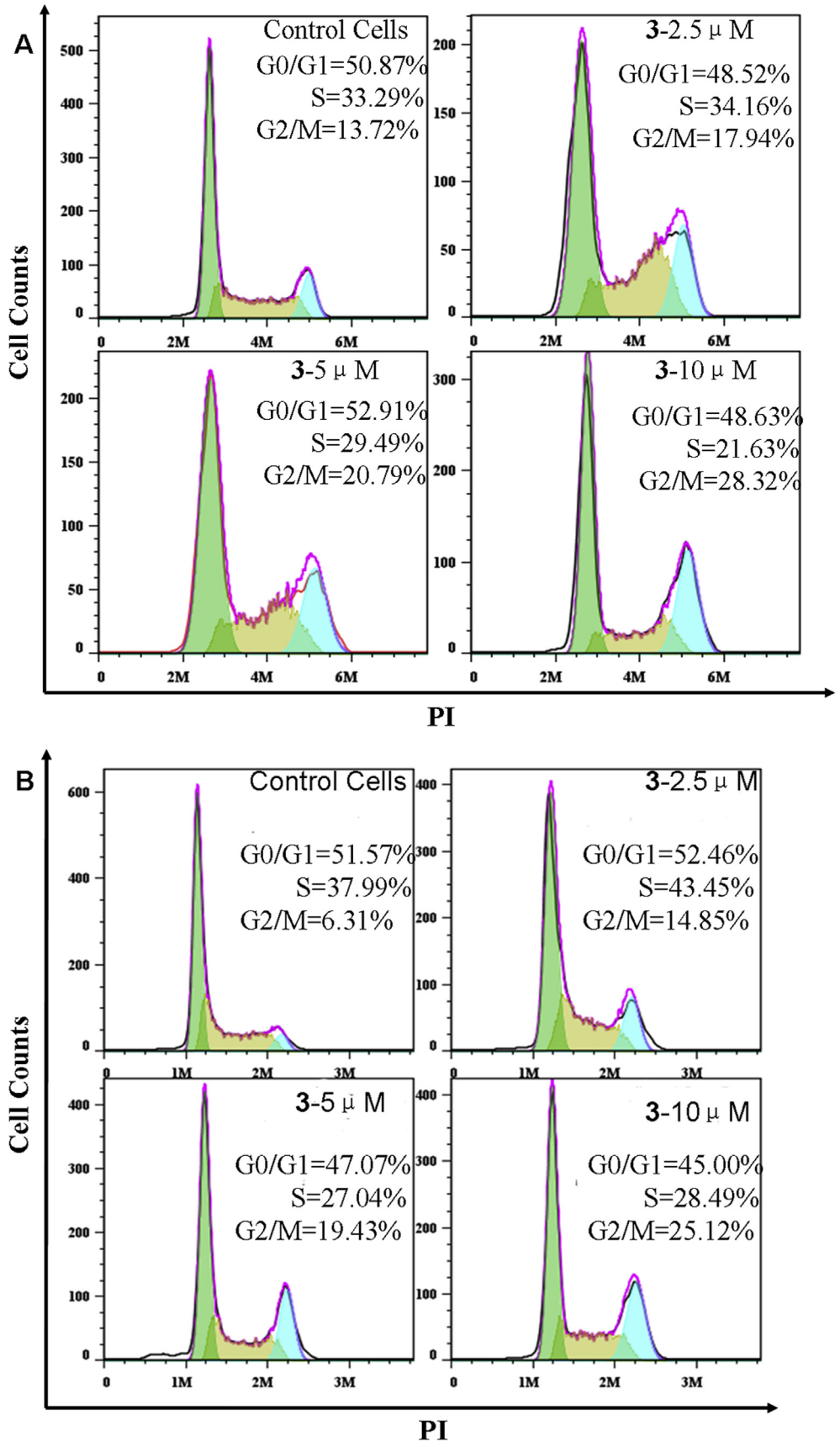


Fig. 4. Effect of compound **3** on the cell cycle distribution of EC109 cells. Cells were treated with different concentrations (0, 2.5, 5, 10 μmol/L) for 12 h or 24 h. Then the cells were fixed and stained with PI to analyze DNA content by flow cytometry. (A) Incubated for 12 h; (B) incubated for 24 h. The experiments were performed three times, and a representative experiment is shown.

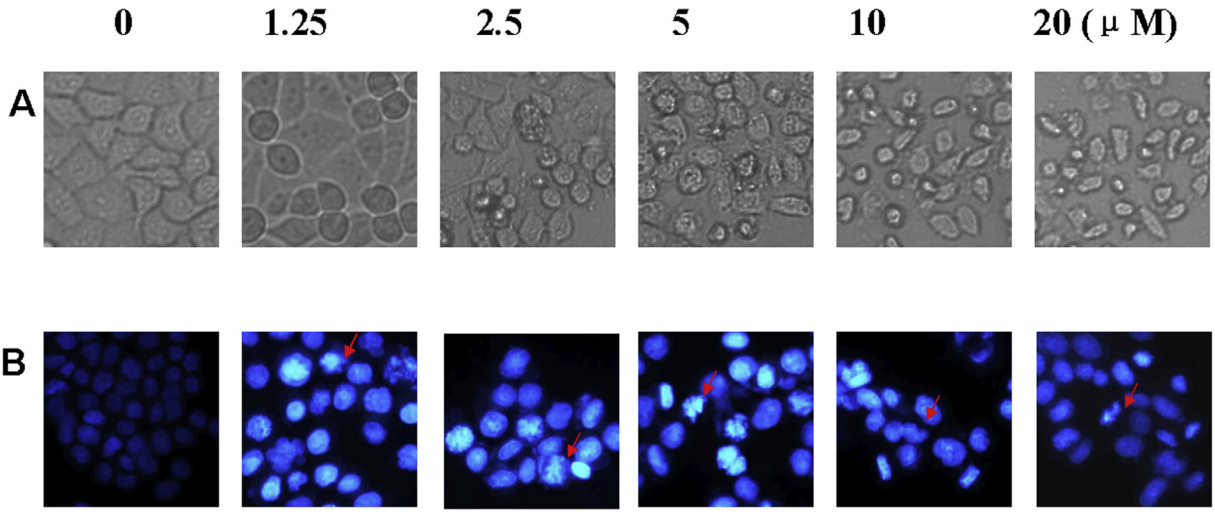


Fig. 5. EC109 cells treated by **3** showed typical apoptotic morphologies. (A) Morphology changes in EC109 cells treated with **3** at the indicated concentrations. After exposure to **3** for 24 h, cells were photographed using inverted microscope. 0.1% DMSO was used as a control (magnification 200×). (B) Effects of **3** on the nuclear morphology of EC109 cells were assayed by Hoechst 33258 staining. After treatment with **3** for 24 h, EC109 cells were stained with Hoechst 33258 and photographed under a fluorescence microscope (magnification 200×).

according to the condensation and fragmentation of their nuclei by fluorescence microscopy. Hoechst 33258 permeates all the cells and makes the nuclei appear blue. Apoptosis was revealed by nuclear changes such as chromatin condensation and nuclear fragmentation.

4.10. Detection of mitochondrial membrane potential ($\Delta\Psi m$)

Mitochondria membrane potential was determined by the fluorescent dye JC-1. After incubation with different concentrations of compound **3** for 24 h, the cells were stained with 10 μg/mL JC-1 and incubated at 37 °C for 30 min. The fluorescence intensity of cells was determined by flow cytometer.

4.11. Measurement of cytochrome c release from the mitochondria

EC109 cells were cultured with different concentrations of compound **3** for 24 h, the mitochondria and cytosol were separated using the cell mitochondria isolation kit. Cells were briefly incubated in 200 mL ice cold mitochondrial lyses buffer for 10 min. The cell suspension was then taken into a glass homogenizer and homogenized for 30 strokes using a tight pestle on ice. The homogenate was subjected to centrifuging at 600× *g* for 10 min to remove nuclei and unbroken cells. Then the supernatant was collected and centrifuged again at 12,000× *g* for 30 min at 4 °C to obtain the cytosol (supernatant) and the mitochondria (pellet) fraction. Samples of the cytosol and the mitochondria were dissolved in lyses buffer and proteins were subjected to Western blot analysis.

4.12. Western blot analysis

EC109 cells were cultured with different concentrations of compound **3** for 24 h, both adherent and floating cells were collected, and then Western blot analysis was performed. Briefly, Cells were washed with cold PBS and lysed in cell lysis buffer [1% NP-40, 0.1% sodium dodecyl sulfate (SDS), 150 mM NaCl, 25 mM Tris–HCl, 1% deoxycholic acid sodium salt, 1% PMSF] for 30 min on ice. Total protein contents were measured by protein assay reagent. The protein lysates were separated by electrophoresis in 10% SDS polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was blocked with 5% skim milk for 2 h at room temperature and then incubated with the indicated primary antibodies against cytochrome *c*, caspase-9, caspase-3, MDM2, p53, and Bax overnight at 4 °C. After that, the membrane was incubated with secondary antibodies, goat anti-rabbit and goat anti-mouse IgG conjugated with peroxidase (HRP). Visualization of bands by an enhanced chemiluminescence kit was performed according to the manufacturer's instructions. GAPDH was used as loading control.

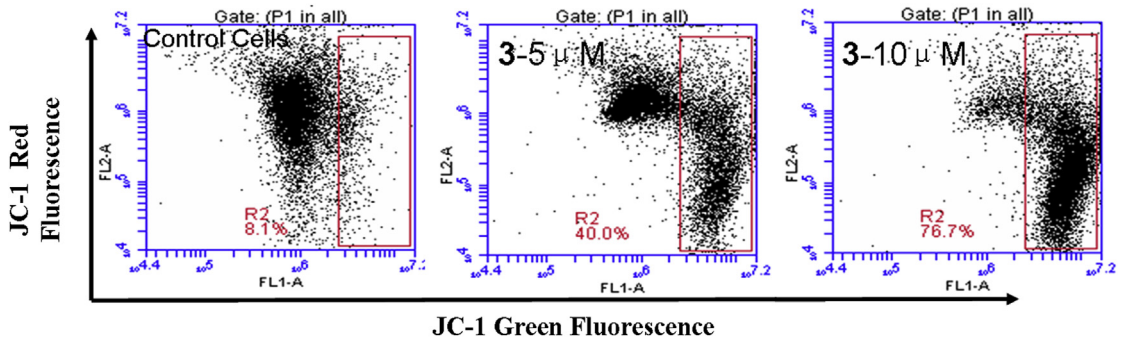


Fig. 6. Compound **3** decreased mitochondrial membrane potential. EC109 Cells were incubated with **3** at three different concentrations for 24 h. The cells were stained with JC-1 and detection by flow cytometer.

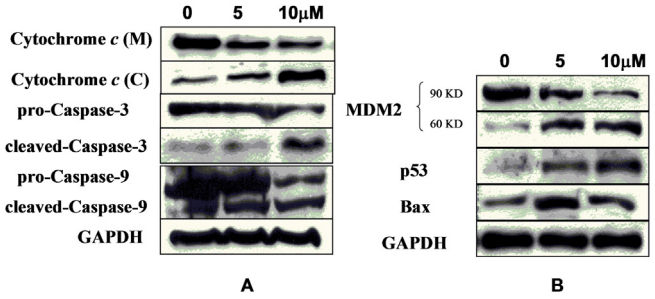


Fig. 7. Compound **3** regulated related protein expression. (A) Caspase-3/-9 activation and changes in cytochrome *c* in EC109 cells after **3** treatment for 24 h; (B) Changes in MDM2, p53 and Bax expression levels in EC109 cells after **3** treatment for 24 h. Equal amounts of protein from whole-cell extracts were separated by SDS-PAGE. Western blot analysis was performed with specific antibodies against MDM2, p53, Bax, cytochrome *c*, caspase-3/-9, respectively.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.08.029.

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