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Synthesis, characterization and antitumor activities of some steroidal derivatives with side chain of 17-hydrazone aromatic heterocycle

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ABSTRACT

Here a series of dehydroepiandrosterone-17-hydrazone and estrone-17-hydrazone derivatives possessing various aromatic heterocycle structures in 17-side chain of their steroidal nucleus were synthesized and their structures were evaluated. The antiproliferative activity of synthesized compounds against some cancer cells was investigated. The results have demonstrated that some dehydroepiandrosterone-17-hydrazone derivatives show distinct antiproliferative activity against some cancer cells through inducing cancer cell apoptosis, and compound **8** with a quinoline structure in 17-side chain displays excellent antiproliferative activity in vitro against SGC 7901 cancer cell (human gastric carcinoma) with an IC_{50} value of 1 μM. In addition, estrone-17-hydrazone derivatives having a key feature of indole group in the structure showed a special obvious cytotoxicity against HeLa cells, but almost inactive against other cells. The information obtained from the studies is valuable for the design of novel steroidal chemotherapeutic drugs.

1. Introduction

Heterocycles are ubiquitous in drug molecules because they possess hydrogen bond donors and acceptors in a rigid framework, and they can therefore effectively interact with target enzymes and receptors via hydrogen bond interactions. They can enhance binding affinity and improve in vitro potency. Heterocycles can modulate lipophilicity of the drug molecules or improve aqueous solubility of the compounds, thus providing desired pharmacokinetic properties and pharmaceutical properties [1]. The compounds containing heterocycles had been widely applied to pharmaceutical and pesticide field for its good biological activity. The versatility of heterocycles in modern anticancer drug discovery is amply demonstrated in various anticancer new drugs, such as axitinib [2], ponalinib [3], imatinib [4], gefitinib [5], lapatinib [6], etc.

It is well known that steroids play an important biological role in life. They can regulate a variety of biological processes and have been widely used in medicine as essentials of anti-inflammatory, anabolic, anticancer and contraceptive drugs. Scientists have found that introducing some heterocycles into the steroids [7–14], changing the steroidal side chain or substitution of the steroidal skeleton, introducing heteroatom or replacing one or more carbon atoms in steroidal molecule with heteroatom may result in change of its biological activities [15–19]. So far, the steroids containing heterocycles had been widely researched and reported [20–22]. Literatures suggested that such compounds displayed distinct cytotoxicity against cancer cell lines [23–27].

In our previous studies, we synthesized some novel steroidal oximes, hydrazones, lactone and lactams, and investigated their cytotoxic activity against different types of cancer cells [28–34]. The results showed that some steroidal oximes, hydrazones, A-homo or B-homo steroidal lactams possessing a cholesteric side chain and the 3-, 6- or 7-hydroxyl or hydroximino group displayed distinct cytotoxic activity against some cancer cells. In order to obtain biologically potent compounds with diverse structures, therefore in this paper, a series of steroidal compounds containing heterocycle attached to the steroidal 17-sidechain by a hydrazone structure were designed and synthesized using dehydroepiandrosterone and estrone as staring materials. Furthermore, the antiproliferative activity of the compounds against some cancer cells was evaluated in vitro.

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2. Result and discussion

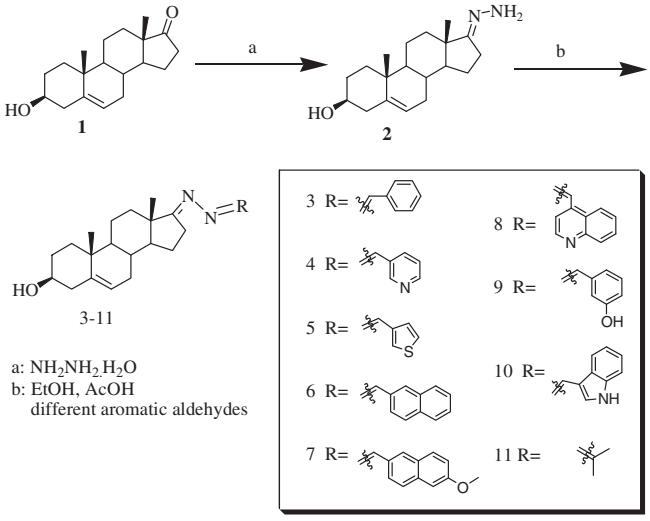
2.1. Chemistry

2.1.1. Synthesis of dehydroepiandrosterone-17-hydrazone aromatic heterocycle derivatives

Scheme 1 outlines the synthetic procedures of compounds **3–11**. First, the dehydroepiandrosterone was converted to the corresponding dehydroepiandrosterone-17-hydrazone (**2**) via reaction with hydrazine hydrate in anhydrous ethanol. After crystallized, the reaction of pure steroidal hydrazone with appropriate aromatic aldehydes gave steroidal hydrazone derivatives **3–11**. Their structures were confirmed by IR, NMR and HRMS spectrum. In the ^{1}H NMR spectrum the resonances showing of C22-H at 8.310 ppm (s) and 22-C at 151.2 ppm demonstrate a formation of 17=N—N=CAr bond in **4.** The downfield chemical shifts of Ar—H at 7.350, 8.115, 8.630, 8.883 ppm and Ar—C at 123.7, 130.5, 134.5, 149.8, 154.3 ppm show the presence of pyridine ring in compound **4**.

2.1.2. Synthesis of estrone-17-hydrazone aromatic heterocycle derivatives

To determine the effect of A-ring's structure in steroidal nucleus to the cytotoxicity, we synthesized compounds **14–18** (Scheme 2). Compounds **14–18** were prepared similarly as the procedures for the synthesis of compounds **3–11** and their structures had been confirmed by IR, NMR and HRMS spectra.



Scheme 1. Synthesis of compounds **3–11**.

2.2. Biological results and discussion

2.2.1. Cell culture and assay for cell viability

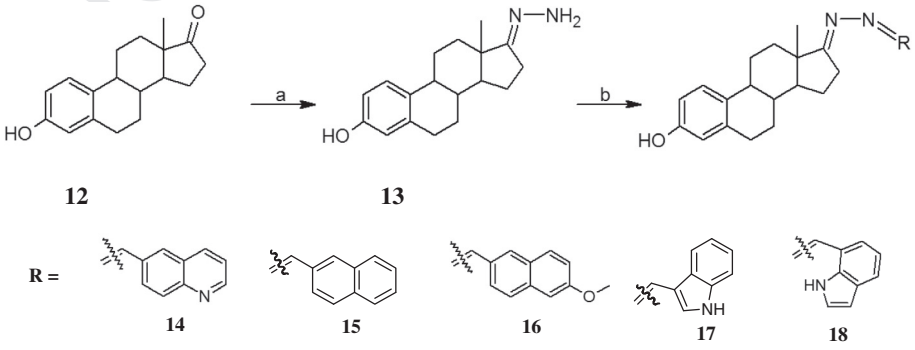
To evaluate the antiproliferative activity of the compounds, we determined their IC_{50} values on HeLa (human cervical carcinoma), HT-29 (human colon carcinoma), Bel 7404 (human liver carcinoma) and SGC 7901 (human gastric carcinoma) cancer cells using a MTT assay. MTT is a compound that can be taken up by viable cells and reduced by a mitochondrial dehydrogenase forming a formazan product in living cells. The absorbance of the formazan product at 492 nm is in linear proportion to cell numbers. The results were summarized as IC_{50} values in μmol/L in Table 1.

As showed in Table 1, dehydroepiandrosterone-17-hydrazone aromatic heterocycle compounds exhibit distinct antiproliferative activity, but compound **11** with a structure of propan-2-ylidenehydrazone is almost inactive against these cancer cells. The compounds **8–10** with 17-quinoline-4′-methanylidenehydrazone, 17-(3′-hydroxy)benzylidenehydrazone and 17-indol-3′-methanylidenehydrazone showed better cytotoxicity. Thereinto, compound **8** with the structure of a quinoline was found to be the most potent compound as anticancer agent. It displayed a better antiproliferative activity than cisplatin did (a positive control) against the tested cancer cells and owns an IC_{50} value of 1.0 μM on SGC-7901 cell. However, after the A-ring in the steroidal nucleus of compounds **6–8** was transformed into a benzene ring, compounds **14–16** obtained resulted in a dramatic decrease of the cytotoxicity. This showed the importance of the structure of steroidal nucleus in steroids.

Interestingly, compounds **17** and **18** having a key feature of indole group in their structures showed a special cytotoxicity against HeLa cells with an IC_{50} value of 5.0 μM and was almost inactive against other cells. It shows that compounds **17** and **18** are better potent compounds to the proliferate inhibition of HeLa tumor cell line, therefore, the further structural modification and antitumor activity study in vivo would be in progress.

2.2.2. Annexin V assay

To further disclose the antiproliferative mechanism of compounds, the HeLa and Bel-7404 cells were treated with compound **8**, and Annexin V assay was performed. The translocation of membrane phospholipid phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane is an early event of cell apoptosis. Annexin V is a 35–36 kD Ca^{2+} dependent, phospholipid-binding protein that has a high affinity for PS. Therefore, FITC-conjugated Annexin V is commonly used to determine apoptotic cells at an early stage. As shown in Fig. 1, treatment with different concentration of compound **8** resulted in different amounts of PI/Annexin V double-labeled apoptotic cells (control: 0.0%) after 24 h incubation (the lower right quadrant and the upper right quadrant which contains early and late apoptotic cells, respectively), suggesting compound **8** is a potent apoptotic inducer in these carcinoma cells.



Scheme 2. Synthesis of compounds **14–18**.

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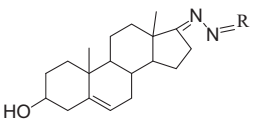
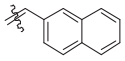
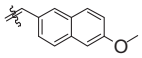
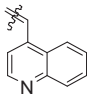
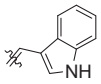
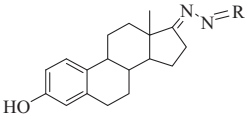
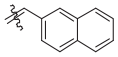
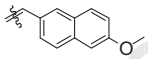
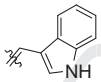
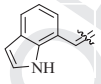
Table 1
In vitro[a] antiproliferative activities (IC_{50} in μmol/L) of 17-hydrazone aromatic heterocycle steroids.

Substrate	R	No.	HeLa	HT-29	Bel-7404	SGC-7901
		**3**	69.2	12.8	15.3	15.3
		**4**	43.5	20.5	33.2	21.9
		**5**	35.4	29.2	33.9	40.4
		**6**	20.0	17.2	23.2	13.6
		**7**	46.8	12.7	33.2	46.8
		**8**	6.6	5.9	13.6	1.0
		**9**	18.8	21.3	23.8	17.1
		**10**	12.7	13.9	16.3	11.4
		**11**	>100	87.7	>100	>100
		**14**	>80	ND	>80	21
		**15**	>80	ND	67	18
		**16**	>80	ND	>80	>80
		**17**	5.0	ND	>80	>80
		**18**	<5	ND	>80	>80
Cisplatin			10.1	ND	23.2	6.7

ND: not determined.
[a] Data represent the mean values of three independent determinations.

3. Conclusion

We have prepared a series of dehydroepiandrosterone-17-hydrazone and estrone-17-hydrazone derivatives possessing various aromatic heterocycle structures. The antiproliferative activity of the synthesized compounds against HeLa (human cervical carcinoma), HT-29 (human colon carcinoma), Bel 7404 (human liver carcinoma) and SGC 7901 (human gastric carcinoma) cancer cells was investigated. The results had demonstrated that some dehydroepiandrosterone-17-hydrazone derivatives showed distinct antiproliferative activity through inducing cancer cell apoptosis. The dehydroepiandrosterone quinoline-4-methanylidenehydrazone (**8**) with a quinoline structure in 17-sidechain displays better antiproliferative activity in vitro against tested cells than cisplatin did. In addition, dehydroepiandrosterone-17-hydrazone derivatives possess a better antiproliferative activity than estrone-17-hydrazone derivatives against tested cells. However, estrone-17-hydrazone derivatives having a key feature of indole group in its structure showed a special cytotoxicity against HeLa cells, but almost inactive against other cells. Our findings provide new evidences showing the relationship between the chemical structure and biological function. The information obtained from the studies is valuable for the design of novel steroidal chemotherapeutic drugs.

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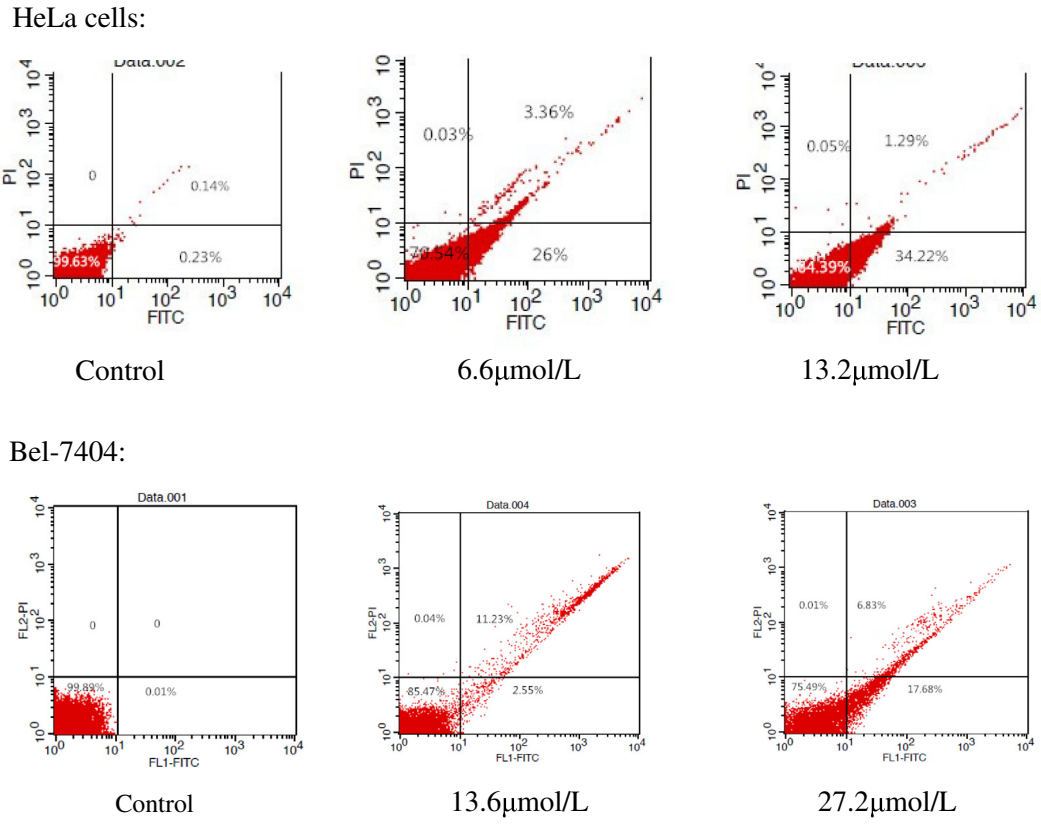

Q5 **Fig. 1.** Tumor cells were double-stained with Annexin V/PI and analyzed by flow cytometry. Treatment with compound **8** for 24 h induced apoptosis of cells.

4. Experimental

4.1. Chemistry

The sterols were purchased from the Sinopharm Chemical Reagent Co., Ltd., Shanghai, China. All chemicals and solvents were analytical grade and solvents were purified by general methods before being used. Melting points were determined on an X_4 apparatus and were uncorrected. Infrared spectra were measured with a Nicolet FT-360 Spectrophotometer. The ^1H and ^{13}C NMR spectra were recorded in $CDCl_3$ on a Bruker AV-600 spectrometer at working frequencies 600 and 150 MHz, and a Bruker AV-300 spectrometer at working frequencies 300 and 75 MHz, respectively. Chemical shifts are expressed in parts per million (δ) values and coupling constants (J) in Hertz. HREIMS was measured on an Agilent 6210 TOFMS instrument. The cell proliferation assay was undertaken by a MTT method using 96-well plates on MLLTISKAN MK3 analysis spectrometer.

4.1.1. Synthesis of dehydroepiandrosterone-20-hydrazone (**2**)

Dehydroepiandrosterone (3.0 g, 10 mmol) was dissolved in 40 mL of ethanol. The mixture was heated to 45 °C, and 4 mL of 85% hydrazine hydrate was added. The solution was stirred for 2 h until no starting material was observed. The reaction was terminated and some white solid was separated out. The solid was filtrated and recrystallized using MeOH as solvent. Compound **2** was obtained as white solid (2.1 g) after drying. Yield: 67%, m.p.: 190–192 °C. IR (KBr) ν/cm^{-1}: 3358, 3244, 2958, 2937, 2892, 2855, 2831, 1666, 1621, 1470, 1454, 1433, 1376, 1049, 743; ^1H NMR (300 MHz, $CDCl_3$)δ: 0.89 (3H, s, 18-CH_3), 1.05 (3H, s, 19-CH_3), 2.37–2.19 (4H, m, C4—H and C16—H), 3.60–3.48 (1H, m, C3—αH), 4.77 (2H, br s, —NH_2), 5.38 (1H, d, J = 5.1, C6—H); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 17.4 (19-C), 19.6 (18-C), 20.8 (11-C), 23.5 (15-C), 24.7 (16-C), 31.3 (8-C), 31.4 (2-C), 31.9 (7-C), 34.8 (12-C), 36.7 (1-C), 37.4 (10-C), 42.7 (13-C), 43.5 (4-C), 50.6 (9-C), 54.2 (14-C), 70.4 (3-C), 120.7 (6-C), 141.9 (5-C), 161.8 (17-C).

4.1.2. General procedure for the synthesis of compounds **3**–**11**

After 0.2 mmol of compound **2** was dissolved in 25 mL of anhydrous ethanol, 0.2 mmol of aromatic aldehyde was added. Then the mixture was heated to 50 °C and stirred continually at the temperature until no starting material. Then the reaction was terminated and solvent was evaporated under reduced pressure. The residue was purified by recrystallization or flash chromatography on silica gel (300–400 mesh) to afford the corresponding target products **3**–**11**.

*4.1.2.1. Dehydroepiandrosterone benzylidenehydrazone (**3**).* White solid, yield: 86%, m.p.: 62–63 °C. Compound **3** was a mixture of (22-*E*) and (22-*Z*)-**3** (ratio: *E*: *Z* ≈ 3.3:1). IR (KBr) ν/cm^{-1}: 3428, 2933, 2856, 2345, 1650, 1450, 1368, 1058; ^1H NMR (300 MHz, $CDCl_3$) δ: 0.89 (0.7H, s, 18-CH_3, *Z*-), 1.00 (2.3H, s, 18-CH_3, *E*-), 1.03 (0.7H, s, 19-CH_3, *Z*-), 1.06 (2.3H, s, 19-CH_3, *E*-), 3.09 (1H, br s, —OH), 3.59–3.49 (1H, m, C3—αH), 5.37 (1H, d, J = 4.8, C6—H), 7.47–7.38 (3H, m, Ar—H), 7.77–7.74 (1.5H, m, Ar—H), 7.87–7.84 (0.5H, m, Ar—H), 8.30 (0.7H, s, C22—H, *E*-), 8.68 (0.2H, s, C22—H, *Z*-); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 16.5 (19-C), 19.5 (18-C), 20.7 (11-C), 23.3 (15-C), 28.0 (16-C), 31.3 (8-C), 31.4 (2-C), 31.6 (7-C), 33.9 (12-C), 36.7 (1-C), 37.2 (10-C), 42.2 (13-C), 44.3 (4-C), 50.4 (9-C), 53.6 (14-C), 71.6 (3-C), 121.0 (6-C), 128.1 (2′- and 6′-Ar—C, *E*-), 128.5 (2′- and 6′-Ar—C, *Z*-), 128.7 (3′- and 5′-Ar—C, *E*-), 128.8 (3′- and 5′-Ar—C, *Z*-), 130.6 (4′-Ar-C), 134.6 (1′-Ar—C), 141.1 (5-C), 157.3 (22-C, *E*-), 162.2 (22-C, *Z*-), 183.1 (17-C); HRESI-MS (m/z): 391.2750 $[M+H]^+$ (calcd for $C_{26}H_{35}N_2O$, 391.2749).

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*4.1.2.2. Dehydroepiandrosterone pyridine-3-methanylidenehydrazone (**4**).* Faint yellow crystals, yield: 74%, m.p.: 195–197 °C; IR (KBr) ν/cm^{-1}: 3399, 3277, 2958, 2921, 2843, 1732, 1646, 1418, 1364, 1324, 1258, 1058, 1037, 964, 800; ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.00 (3H, s, 18-CH_3), 1.06 (3H, s, 19-CH_3), 2.67–2.61 (2H, m, C16-H), 3.59–3.49 (1H, m, C3—αH), 5.38 (1H, d, J = 5.1, C6—H), 7.35 (1H, dd, J = 7.8, 4.8, 5′-Ar—H), 8.12 (1H, d, d, J = 7.8, 4′-Ar—H), 8.31 (1H, s, C22—H), 8.63 (1H, dd, J = 4.8, 1.2, 6′-Ar—H), 8.88 (1H, d, J = 1.2, 2′-Ar—H); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 16.5 (19-C), 19.5 (18-C), 20.7 (11-C), 23.3 (15-C), 28.0 (16-C), 31.3 (8-C), 31.5 (2-C), 31.6 (7-C), 33.8 (12-C), 36.7 (1-C), 37.2 (10-C), 42.3 (13-C), 44.4 (4-C), 50.3 (9-C), 53.6 (14-C), 71.5 (3-C), 121.0 (6-C), 123.7 (5′-Ar—C), 130.5 (4′-Ar—C), 134.5 (3′-Ar—C), 141.1 (5-C), 149.8 (2′-Ar—C), 151.2 (22-C), 154.3 (6′-Ar—C), 183.8 (17-C); HRESI-MS (m/z): 392.2719 $[M+H]^+$ (calcd for $C_{25}H_{34}N_3O$, 392.2702).

*4.1.2.3. Dehydroepiandrosterone thiophene-3-methanylidenehydrazone (**5**).* Faint yellow crystals, yield: 80%, m.p.: 195–196 °C; IR (KBr) ν/cm^{-1}: 3428, 2933, 2897, 2852, 1728, 1642, 1446, 1364, 1238, 1057; ^{1}H NMR (300 MHz, $CDCl_3$) δ: 0.97 (3H, s, 18-CH_3), 1.04 (3H, s, 19-CH_3), 2.65–2.58 (2H, m, C16-H), 3.56–3.46 (1H, m, C_3—αH), 5.35 (1H, d, J = 4.8, C6—H), 7.31 (1H, dd, J = 4.8, 2.7, 4′-Ar—H), 7.54 (1H, d, J = 4.8, 5′-Ar—H), 7.59 (1H, d, J = 2.7, 2′-Ar—H), 8.30 (1H, s, C22—H); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 16.5 (19-C), 19.5 (18-C), 20.7 (11-C), 23.3 (15-C), 27.9 (16-C), 31.3 (8-C), 31.5 (2-C), 31.6 (7-C), 33.9 (12-C), 36.7 (1-C), 37.2 (10-C), 42.2 (13-C), 44.3 (4-C), 50.4 (9-C), 53.6 (14-C), 71.5 (3-C), 121.0 (6-C), 125.6 (4′-Ar—C), 126.5 (5′-Ar—C), 128.4 (2′-Ar—C), 138.1 (5-C), 141.1 (3′-Ar—C), 152.1 (22-C), 182.8 (17-C); HRESI-MS (m/z): 397.2332 $[M+H]^+$ (calcd for $C_{24}H_{33}N_2OS$, 397.2314).

*4.1.2.4. Dehydroepiandrosterone naphthalen-2-methanylidenehydrazone (**6**).* White crystals, yield: 95%, m.p.: 110–111 °C; IR (KBr) ν/cm^{-1}: 3387, 2929, 2896, 2855, 2831, 1732, 1646, 1576, 1511, 1454, 1429, 1372, 1331, 1061, 800, 767; ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.05 (3H, s, 18-CH_3), 1.06 (3H, s, 19-CH_3), 2.83–2.70 (2H, m, C16—H), 3.57–3.47 (1H, m, C_3—αH), 5.37 (1H, d, J = 3.6, C6—H), 7.63–7.49 (3H, m, Ar—H), 7.96–7.87 (3H, m, Ar—H), 8.95 (1H, s, 1′-Ar—H), 8.98 (1H, s, C22-H); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 16.6 (19-C), 19.5 (18-C), 20.7 (11-C), 23.4 (15-C), 28.5 (16-C), 31.4 (8-C), 31.5 (2-C), 31.6 (7-C), 34.0 (12-C), 36.7 (1-C), 37.3 (10-C), 42.3 (13-C), 44.4 (4-C), 50.4 (9-C), 53.7 (14-C), 71.5 (3-C), 121.0 (6-C), 125.0 (9′-Ar—C), 125.3 (7′-Ar—C), 126.1 (3′-Ar—C), 127.2 (1′-Ar—C), 128.7 (4′-Ar—C), 129.3 (5′-Ar—C), 130.2 (6′-Ar—C), 131.2 (8′-Ar—C), 131.3 (2′-Ar—C), 133.9 (10′-Ar—C), 141.1 (5-C), 157.9 (22-C), 183.4 (17-C); HRESI-MS (m/z): 441.2930 $[M+H]^+$ (calcd for $C_{30}H_{37}N_2O$, 441.2906).

*4.1.2.5. Dehydroepiandrosterone 6-methoxynaphthalen-2-methanylidenehydrazone (**7**).* White crystals, yield: 77%, m.p.: 100–101 °C; IR (KBr) ν/cm^{-1}: 3423, 2929, 2829, 1621, 1593, 1474, 1380, 1331, 1262, 1241, 1196, 1164, 1053, 1021, 857; ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.01 (3H, s, 18-CH_3), 1.05 (3H, s, 19-CH_3), 2.72–2.67 (2H, m, C16—H), 3.57–3.49 (1H, m, C3—αH), 3.914 (3H, s, Ar—O—CH_3), 5.36 (1H, d, J = 3.9, C6—H), 7.14 (1H, s, 1′-Ar—H), 7.16 (1H, d, J = 8.4, Ar—H), 7.74 (1H, d, J = 9.3, 8′-Ar—H), 7.77 (1H, d, J = 9.3, 7′-Ar—H), 7.96 (1H, s, 5′-Ar—H), 8.00 (1H, d, J = 8.4, 4′-Ar—H), 8.43 (1H, s, C22-H); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 16.7 (19-C), 19.5 (18-C), 20.7 (11-C), 23.4 (15-C), 28.1 (16-C), 31.3 (8-C), 31.5 (2-C), 31.6 (7-C), 33.9 (12-C), 36.7 (1-C), 37.2 (10-C), 42.3 (13-C), 44.3 (4-C), 50.4 (9-C), 53.6 (14-C), 55.4 (Ar—O—C), 71.5 (3-C), 106.1 (1′-Ar—C), 119.2 (3′-Ar—C), 121.1 (9′-Ar—C), 124.3 (6-C), 127.3 (8′-Ar—C), 128.5 (7′-Ar—C), 129.8 (5′-Ar—C), 130.1 (4′-Ar—C), 130.3 (6′-Ar—C), 136.0 (10′-Ar—C), 141.1 (5-C), 157.6 (22-C), 158.7 (2′-Ar—C), 183.0 (17-C); HRESI-MS (m/z): 471.3015 $[M+H]^+$ (calcd for $C_{31}H_{39}N_2O_2$, 471.3012).

*4.1.2.6. Dehydroepiandrosterone quinoline-4-methanylidenehydrazone (**8**).* White crystals, yield: 95%, m.p.: 207–208 °C; IR (KBr) ν/cm^{-1}: 3350, 2933, 2852, 1646, 1573, 1507, 1462, 1368, 1062, 764; ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.05 (3H, s, 18-CH_3), 1.07 (3H, s, 19-CH_3), 2.76–2.69 (2H, m, C16—H), 3.61–3.51 (1H, m, C3—αH), 5.38 (1H, d, J = 4.8, C6—H), 7.64 (1H, t, J = 7.5, 7′-Ar—H), 7.76 (1H, d, J = 7.5, 6′-Ar—H), 7.78 (1H, d, J = 4.8, 3′-Ar—H), 8.18 (1H, d, J = 8.1, 8′-Ar—H), 8.74 (1H, d, J = 8.1, 5′-Ar—H), 8.91 (1H, s, C22—H), 8.98 (1H, d, J = 4.8, 2′-Ar—H); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 16.5 (19-C), 19.5 (18-C), 20.7 (11-C), 23.4 (15-C), 28.5 (16-C), 31.3 (8-C), 31.5 (2-C), 31.6 (7-C), 33.8 (12-C), 36.7 (1-C), 37.3 (10-C), 42.3 (13-C), 44.6 (4-C), 50.3 (9-C), 53.6 (14-C), 71.6 (3-C), 120.9 (3′-Ar—C), 121.0 (6-C), 124.5 (9′-Ar—C), 125.7 (5′-Ar—C), 127.6 (6′-Ar—C), 129.5 (7′-Ar—C), 130.1 (8′-Ar—C), 137.9 (10′-Ar—C), 141.1 (4′-Ar—C), 148.9 (5-C), 150.1 (2′-Ar—C), 155.0 (22-C), 184.6 (17-C); HRESI-MS (m/z): 442.2859 $[M+H]^+$ (calcd for $C_{29}H_{36}N_3O$, 442.2858).

*4.1.2.7. Dehydroepiandrosterone 3-hydroxybenzylidenehydrazone (**9**).* White solids, yield: 51%, m.p.: 136–138 °C; IR (KBr) ν/cm^{-1}: 3419, 2929, 2851, 2357, 2340, 1723, 1654, 1450, 1380, 1258, 1217; ^{1}H NMR (300 MHz, $CDCl_3$) δ: 0.91 (3H, s, 18-CH_3), 0.99 (3H, s, 19-CH_3), 2.60–2.43 (2H, m, C16—H), 3.40–3.25 (1H, m, C3-αH), 4.62 (1H, s, —OH), 5.30 (1H, d, J = 4.5, C6—H), 6.87–6.84 (1H, m, 2′-Ar—H), 7.30–7.15 (3H, m, 4′,5′,6′-Ar—H), 8.21 (1H, s, C22—H), 9.62 (1H, s, Ar—OH); ^{13}C NMR (75 MHz, DMSO) δ: 16.9 (19-C), 19.7 (18-C), 20.8 (11-C), 23.4 (15-C), 27.9 (16-C), 31.2 (8-C), 31.5 (2-C), 31.9 (7-C), 34.2 (12-C), 36.7 (1-C), 37.4 (10-C), 42.7 (13-C), 44.4 (4-C), 50.4 (9-C), 53.4 (14-C), 70.4 (3-C), 114.0 (6′-Ar—C), 118.3 (6-C), 119.8 (4′-Ar—C), 120.6 (2′-6-C), 130.2 (5′-Ar—C),136.1 (3′-Ar—C), 141.9 (5-C), 157.4 (22-C), 158.0 (1′-Ar—C), 181.8 (17-C); HRESI-MS (m/z): 407.2697 $[M+H]^+$ (calcd for $C_{26}H_{35}N_2O_2$, 407.2699).

*4.1.2.8. Dehydroepiandrosterone indol-3-methanylidenehydrazone (**10**).* White crystals, yield: 72%, m.p.: 234–236 °C; IR (KBr) ν/cm^{-1}: 3395, 3224, 2929, 2851, 2823, 2361, 1654, 1589, 1568, 1527, 1450, 1388, 1241, 1053; ^{1}H NMR (300 MHz, $CDCl_3$)δ: 1.03 (3H, s, 18-CH_3), 1.08 (3H, s, 19-CH_3), 2.90–2.80 (2H, m, C16—H), 3.62–3.51 (1H, m, C3—αH), 5.41 (1H, d, J = 4.8, C6—H), 7.32–7.26 (1H, m, 6′-Ar—H), 7.41 (1H, dd, J = 6.3, 3.0, 5′-Ar—H), 7.49 (1H, d, J = 2.7, 7′-Ar—H), 8.39–8.38 (1H, m, 4′-Ar—H), 8.65 (1H, s, C22—H), 8.80 (1H, s, —NH); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 16.6 (19-C), 19.5 (18-C), 20.8 (11-C), 23.4 (15-C), 28.3 (16-C), 31.4 (8-C), 31.5 (2-C), 31.6 (7-C), 34.1 (12-C), 36.6 (1-C), 37.2 (10-C), 42.3 (13-C), 44.1 (4-C), 50.4 (9-C), 53.8 (14-C), 71.7 (3-C), 111.2 (7′-Ar—C), 114.0 (3′-Ar—C), 121.2 (6-C), 121.5 (5′-Ar—C), 122.8 (6′-Ar—C), 123.5 (4′-Ar—C), 125.1 (8′-Ar—C), 129.3 (2′-Ar—C), 136.9 (9′-Ar—C), 141.0 (5-C), 153.7 (22-C), 182.5 (17-C); HRESI-MS (m/z): 430.2889 $[M+H]^+$ (calcd for $C_{28}H_{36}N_3O$, 430.2858).

*4.1.2.9. Dehydroepiandrosterone propan-2-ylidenehydrazone (**11**).* White crystals, yield: 81%, m.p.: 171–172 °C; IR (KBr) ν/cm^{-1}: 3427, 2929, 2888, 2855, 1662, 1450, 1425, 1368, 1241, 1053, 1033, 851, 800, 735; ^{1}H NMR (300 MHz, $CDCl_3$)δ: 0.92 (3H, s, 18-CH_3), 1.02 (3H, s, 19-CH_3), 1.81 (3H, s, 23-CH_3), 1.98 (3H, s, 24-CH_3), 3.53–3.43 (1H, m, C3—αH), 5.34 (1H, d, J = 4.8, C6—H); ^{13}C NMR (75 MHz, $CDCl_3$) δ: 16.6 (19-C), 17.6 (18-C), 19.5 (24-C), 20.7 (11-C), 23.3 (23-C), 25.0 (15-C), 27.1 (16-C), 31.3 (8-C), 31.5 (2-C), 31.6 (7-C), 34.0 (12-C), 36.6 (1-C), 37.3 (10-C), 42.2 (13-C), 44.1 (4-C), 50.5 (9-C), 53.7 (14-C), 71.4 (3-C), 121.0 (6-C), 141.2

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(5-C), 159.3 (22-C), 175.1 (17-C); HRESI-MS (*m/z*): 343.2729 $[M+H]^+$ (calcd for $C_{22}H_{35}N_2O$, 343.2749).

*4.1.2.10. Synthesis of estrone-20-hydrazone (**13**).* Compound **13** was prepared similarly as the procedure for the synthesis of **2**, but from compound **12**. White solid, yield: 95%, m.p.: 250–253 °C; IR (KBr) ν/cm^{-1}: 3376, 2945, 2863, 1659, 1611, 1501, 1452, 1369, 1235, 1078, 906, 861, 826, 788, 734; ^1H NMR (300 MHz, DMSO) δ: 0.77 (3H, s, 18-CH_3), 2.75–2.67 (2H, m, C16—H), 5.314 (2H, br s, NH_2), 6.44 (1H, d, J = 2.1, C4—H), 6.51 (1H, dd, J = 8.4, 2.1, C2—H), 7.04 (1H, d, J = 8.4, C1—H), 9.02 (1H, s, —OH); ^{13}C NMR (75 MHz, DMSO) δ: 17.7 (18-C), 23.2 (15-C), 24.8 (11-C), 26.5 (7-C), 27.3 (16-C), 29.6 (6-C), 34.8 (12-C), 44.0 (9-C), 44.2 (13-C), 52.9 (14-C), 113.2 (2-C), 115.4 (4-C), 126.4 (1-C), 130.8 (10-C), 137.6 (5-C), 155.4 (3-C), 162.2 (17-C). HRESI-MS (*m/z*): 285.1978 $[M+H]^+$ (calcd for $C_{18}H_{25}N_2O$, 285.1961).

Compounds **14–18** were prepared similarly as the procedures for the synthesis of compounds **3–11.**

*4.1.2.11. Estrone quinoline-6-methanylidenehydrazone (**14**).* Faint yellow solid, yield: 82%, m.p.: 236–238 °C; IR (KBr) ν/cm^{-1}: 3441, 2925, 1651, 1601, 1566, 1504, 1444, 1285, 1230, 756; ^1H NMR (600 MHz, $CDCl_3$) δ: 1.07 (s, 3H, 18-CH_3), 2.86–2.78 (4H, m, C6- and C16-H), 6.51 (1H, br s, —OH), 6.63 (1H, s, C4—H), 6.69 (1H, s, C2—H), 7.18 (1H, s, C1—H), 7.65 (1H, s, Ar—H), 7.79 (2H, d, J = 10.2, Ar—H), 8.20 (1H, s, Ar—H), 8.73 (s, 1H, C21—H), 8.97 (2H, d, J = 15.9, Ar-CH); ^{13}C NMR (150 MHz, $CDCl_3$) δ: 17.0 (18-C), 23.2 (15-C), 26.5 (11-C), 27.3 (16-C), 28.7 (7-C), 29.7 (6-C), 34.1 (12-C), 38.5 (8-C), 44.2 (9-C), 45.2 (13-C), 52.5 (14-C), 113.2 (2-C), 115.6 (4-C), 121.1 (3′-Ar—C), 124.8 (5′-Ar—C), 126.0 (6′-Ar—C), 126.6 (1-Ar—C), 127.8 (9′-C), 129.8 (7′-Ar—C), 130.0 (8′-Ar—C), 132.0 (10-C), 138.2 (10′-Ar—C), 138.3 (5-C), 148.8 (4′-Ar—C), 150.0 (2′-Ar—C), 154.2 (21-C), 155.2 (3-C), 185.1 (17-C); HRESI-MS (*m/z*): 424.2398 $[M+H]^+$ (calcd for $C_{28}H_{30}N_3O$, 424.2389).

4.1.2.12. Estrone naphthalen-2-methanylidenehydrazone (15). Faint yellow solid, yield: 66%, m.p.: 192–194 °C; IR (KBr) ν/cm^{-1}: 3385, 2928, 1725, 1648, 1606, 1496, 1449, 1352, 1283, 1240,1156, 1061, 915, 798, 776, 728; ^1H NMR (600 MHz, $CDCl_3$) δ: 1.07 (3H, s, 18-CH_3), 2.88–2.77 (4H, m, C6- and C16-H), 6.52 (1H, br s, —OH), 6.62 (1H, s, C4—H), 6.69 (1H, dd, J = 3.0, 8.4, C2—H), 7.17 (1H, d, J = 8.4, C1—H), 7.56–7.51 (2H, m, Ar—H), 7.60–7.57 (1H, m, Ar—H), 7.94–7.89 (3H, m, Ar—H), 8.86 (1H, d, J = 8.4, Ar—H), 9.01 (1H, s, C21—H); ^{13}C NMR (150 MHz, $CDCl_3$) δ: 17.0 (18-C), 23.2 (15-C), 26.4 (11-C), 27.3 (16-C), 28.8 (7-C), 29.7 (6-C), 34.1 (12-C), 38.4 (8-C), 44.1 (13-C), 45.1 (9-C), 52.5 (14-C), 113.1 (2-C), 115.6 (4-C), 125.0 (10′-Ar—C), 125.4 (6′-Ar—C), 126.2 (2′-Ar—C), 126.6 (1-C), 127.4 (9′-Ar—C), 128.8 (7′-Ar—C), 129.4 (5′-Ar-C), 130.1 (8′-Ar—C), 131.3 (4′-Ar—C), 131.5 (3′-Ar—C), 132.1 (10-C), 134.0 (1′-Ar—C), 138.1 (5-C), 154.0 (21-C), 158.4 (3-C), 184.5 (17-C); HRESI-MS (*m/z*): 423.2443 $[M+H]^+$ (calcd for $C_{29}H_{31}N_2O$, 423.2436).

*4.1.2.13. Estrone 6-methoxynaphthalen-2-methanylidenehydrazone (**16**).* Faint yellow solid, yield: 62%, m.p.: 224–226 °C; IR (KBr) ν/cm^{-1}: 3424, 2940, 1626, 1604, 1499, 1437, 1232, 1172, 1015, 858, 813; ^1H NMR (600 MHz, $CDCl_3$) δ: 1.03 (3H, s, 18-CH_3), 2.20 (1H, dt, J = 13.2, 3.6, C9—αH), 2.29 (1H, td, J = 10.8, 3.6, C15—βH), 2.44–2.40 (1H, m, C9—αH), 2.77–2.74 (2H, m, C6—H), 2.89–2.84 (2H, m, C16—H), 3.94 (3H, s, —OCH_3), 5.00 (1H, br s, —OH), 6.59 (1H, d, J = 2.4, C4—H), 6.65 (1H, dd, J = 8.4, 2.4, C2—H), 7.18–7.15 (2H, m, Ar—H), 7.19 (1H, d, J = 8.4, C1—H), 7.75 (1H, d, J = 9.0, 4′-Ar—H), 7.78 (1H, d, J = 9.0, 7′-Ar—H), 7.96 (1H, s, 5′-Ar—H), 8.00 (1H, d, J = 9.0, 8′-Ar—H), 8.44 (1H, s, C21—H); ^{13}C NMR (150 MHz, $CDCl_3$) δ: 17.0 (18-C), 23.2 (15-C), 26.5 (11-C), 27.3 (16-C), 28.2 (7-C), 29.7 (6-C), 34.2 (12-C), 38.5 (8-C), 44.2 (13-C), 45.0 (9-C), 52.5 (14-C), 55.5 (—OCH_3), 106.2 (5′-Ar—C), 112.9 (2-C), 115.4 (4-C), 119.4 (10′-Ar—C), 124.4 (9′-Ar—C), 126.7 (4′-Ar—C), 127.4 (2′-Ar—C), 128.7 (7′-Ar—C), 130.0 (1-C), 130.3 (8′-Ar—C), 130.4 (10-C), 132.6 (3′-Ar—C), 136.2 (1′-Ar—C), 138.3 (5-C), 153.6 (21-C), 157.9 (6′-Ar—C), 158.9 (3-C), 183.2 (17-C); HRESI-MS (*m/z*): 453.2535 $[M+H]^+$ (calcd for $C_{30}H_{33}N_2O_2$, 453.2542).

*4.1.2.14. Estrone indol-3-methanylidenehydrazone (**17**).* Faint yellow solid, yield: 48%, m.p.: 198–200 °C; IR (KBr) ν/cm^{-1}: 3391, 2922, 1646, 1591, 1494, 1452, 1240, 1098, 921, 811, 741; ^1H NMR (300 MHz, DMSO) δ: 0.92 (3H, s, 18-CH_3), 2.82–2.63 (1H, m, C6—H and C16—H), 6.47 (1H, d, J = 2.4, C4—H), 6.53 (1H, dd, J = 8.4, 2.4, C2—H), 7.06 (1H, d, J = 8.4, C1—H), 7.23–7.12 (2H, m, 5′- and 6′-Ar—H), 7.46 (1H, d, J = 7.5, 7′-Ar—H), 7.82 (1H, d, J = 2.7, 2′-Ar—H), 8.22 (1H, d, J = 7.5, 4′-Ar—H), 8.59 (1H, s, C21—H), 9.05 (1H, s, —OH), 11.62 (1H, s, —NH); ^{13}C NMR (75 MHz, DMSO) δ: 17.3 (18-C), 23.1 (15-C), 26.5 (11-C), 27.3 (7-C), 28.2 (16-C), 29.6 (6-C), 34.6 (12-C), 38.5 (8-C), 44.1 (13-C), 44.6 (9-C), 52.4 (14-C), 112.3 (7′-Ar—C), 112.7 (2-C), 113.3 (4-C), 115.4 (3′-Ar—C), 121.1 (5′-Ar—C), 122.6 (6′-Ar—C), 123.1 (4′-Ar—C), 125.2 (8′-Ar—C), 126.5 (1-C), 130.8 (2′-Ar—C), 132.2 (10-C), 137.6 (5-C and 9′-Ar—C), 154.7 (3-C), 155.4 (21-C), 180.8 (17-C); HRESI-MS (*m/z*): 412.2392 $[M+H]^+$ (calcd for $C_{27}H_{30}N_3O$, 412.2389).

*4.1.2.15. Estrone indol-7-methanylidenehydrazone (**18**).* Faint yellow solid, yield: 65%, m.p.: 192–194 °C; IR (KBr) ν/cm^{-1}: 3409, 2917, 1646, 1497, 1429, 1342, 1242,1118, 896, 756; ^1H NMR (600 MHz, $CDCl_3$) δ: 1.04 (3H, s, 18-CH_3), 2.92–2.81 (3H, m, C16—H and C6—H), 4.90 (1H, br s, —OH), 6.59 (1H, s, C4—H), 6.65 (1H, d, J = 7.8, C2—H), 7.20 (1H, d, J = 8.4, 3′-Ar—H), 7.24 (1H, d, J = 7.8, C1—H), 7.35–7.31 (2H, m, 2′- and 5′-Ar—H), 7.40 (1H, d, J = 7.2, 6′-Ar—H), 7.49 (1H, d, J = 7.8, 4′-Ar—H), 8.37 (1H, s, —NH), 8.70 (1H, s, C21—H); ^{13}C NMR (150 MHz, $CDCl_3$) δ: 17.0 (18-C), 23.2 (15-C), 26.5 (11-C), 27.3 (16-C), 28.7 (7-C), 29.8 (6-C), 34.3 (12-C), 38.5 (8-C), 44.2 (13-C), 44.9 (9-C), 52.6 (14-C), 104.3 (3′-Ar—C), 112.9 (2-C), 113.8 (4-C), 115.4 (6′-Ar—C), 121.9 (5′-Ar—C), 123.9 (4′-Ar—C), 125.6 (8′-Ar—C), 125.6 (2′-Ar—C), 126.70 (1-C), 126.73 (7′-Ar—C), 132.7 (10-C), 136.4 (5-C), 138.3 (9′-Ar—C), 153.6 (21-C), 159.6 (3-C), 183.4 (17-C); HRESI-MS (*m/z*): 412.2395 $[M+H]^+$ (calcd for $C_{27}H_{30}N_3O$, 412.2389).

4.2. Biological assays

4.2.1. Materials

Stock solutions of the compounds were prepared in sterile dimethyl sulfoxide (DMSO) (Sigma) at a concentration of 10 mg/mL and afterward diluted with complete nutrient medium (RPMI-1640) supplemented with 10% heat inactivated fetal bovine serum and 0.1 g/L penicillin G + 0.1 g/L streptomycin sulfate.

4.2.2. Cell culture

SGC-7901, HT-29, Bel-7404 and HeLa cancer cells were grown in the medium (RPMI-1640) supplemented with 10% heat inactivated fetal bovine serum and 0.1 g/L penicillin G + 0.1 g/L streptomycin sulfate in a humidified atmosphere of 5% CO_2 at 37 °C.

4.2.3. Assay for cell viability

The anticancer activity in vitro was measured using the MTT assay. Briefly, cells ($1–2 \times 10^4$ cells per well) were seeded in 96-wells plates for 24 h. Different concentrations of the test compound were added to the cells. An equal amount of DMSO was added to the cells used as negative controls. Triplicate wells were prepared for each individual dose. After reincubated for 72 h, the cells were washed with sterile phosphate buffer saline (PBS). 190 μL of RPMI-1640 and 10 μL of the tetrazolium dye (MTT) (5 mg/mL) solution were added to each well, and the cells were

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incubated for additional 4 h. After the supernatant was discarded, 200 μL of DMSO was added to dissolve the purple formazan crystals formed. The absorbance values (*A*) at 492 nm were determined using a MLLTISKAN MK3 analysis spectrometer (Thermo Scientific Co.). The IC_{50} values were calculated as the concentration of drug yielding 50% cell survival.

4.2.4. Annexin V staining assay

Apoptosis was detected with an Annexin V-FITC kit purchased from BD Pharmingen (San Diego, CA) according to the manufacturer's instructions. HeLa or Bel-7404 cells were seeded in 35 mm culture dishes and allowed to attach overnight. The cells were treated with different concentrations of compound **8** for 24 h respectively, collected, and washed twice with PBS. To detect early and late apoptosis, both adherent and floating cells were harvested together and resuspended in Annexin V binding buffer at a concentration of 10^6 cells/mL. Subsequently, 5 μL of FITC-conjugated Annexin V and 5 μL of propidium iodide were added to 100 μL of the cell suspension (10^5 cells). The cells were incubated for 15 min at room temperature in the dark. Finally, 400 μL of Annexin V binding buffer was added to each tube, and cells were analyzed by a two color cytometry using FACS Calibur (BD Biosciences).

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