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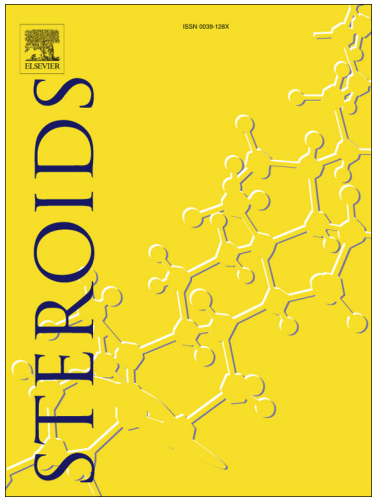

Design, synthesis and biological evaluation of novel 5α, 8α-endoperoxide steroidal derivatives with hybrid side chain as anticancer agents

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Design, synthesis and biological evaluation of novel 5α, 8α-endoperoxide steroidal derivatives with hybrid side chain as anticancer agents

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Abstract

A series of novel 5α, 8α-endoperoxide steroidal hybrid derivatives containing isatin or indole substituents on the C-17 side chain were synthesized and characterized. The preliminary anti-proliferative activity of the compounds against HepG2, MCF-7, HT-29 and HeLa cell lines were investigated. Compounds **7g** and **7l** displayed significant anti-proliferative activity *in vitro* against HepG2 and Hela cells, with IC_{50} values lower than 8 μM. Furthermore, the biological functions of **7g** were examined by flow cytometry and colony analysis. The results showed that **7g** could induce HepG2 cell apoptosis, inhibited cell cycle progression, and colony growth. The studies indicated that structural modification at C-17 position could be a promising launch point for design steroidal anticancer agents.

Keywords: ergosterol peroxide, isatin, indole, hybrids, antiproliferative activity.

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1. Introduction

Nowadays, natural products have attracted extensive attention in disease treatment and human health promotion. Natural product-based drug discovery is a major route leading to developing potent drugs for diseases [1]. Natural endoperoxides are cyclic organic compounds, such as artemisinin, talaperoxides B, schinalactone A and gracilioethers A, which all have a peroxide bridge in their structures (**Figure 1**) [2, 3]. A plenty of natural endoperoxides have been proved with a range of bioactivities, such as anticancer, antifungal and antiviral activity [4-7].

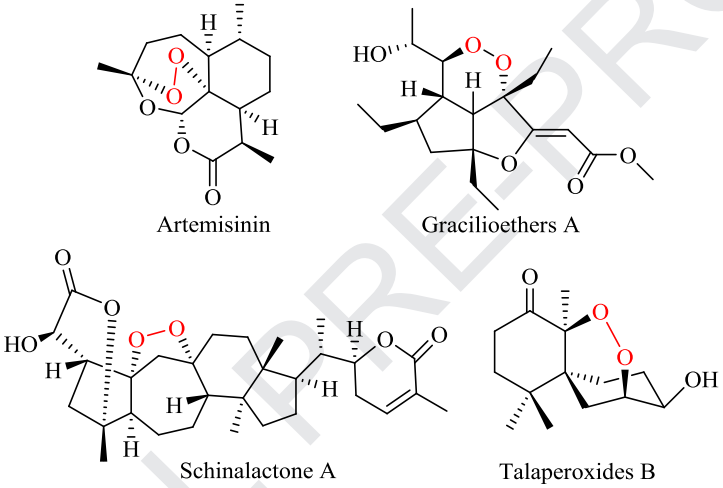

Figure 1. Structures of natural endoperoxides with bioactivities.

Ergosterol peroxide (5α,8α-epidioxyergosta-6,22-diene-3β-ol, EP), is one metabolite of series natural sterol endoperoxide derivatives, has been continuously isolated from many kinds of medicinal fungi (**Figure 2**) [8, 9]. A plenty of literatures have been reported that EP can inhibit cancer cells growth by cytotoxicity [10-13]. In our previous study, we proved that EP could induce Foxo3a activity by inhibiting pAKT to induce cancer cell death [14].

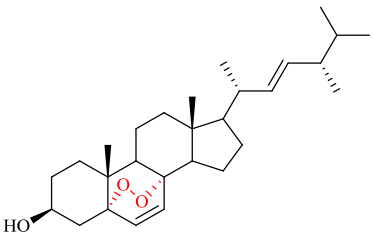

Figure 2. Structure of ergosterol peroxide (EP).

Isatin is well known as an important natural product, and which is also a

common scaffold in various drugs, agrochemicals and dyes [15-17]. Many isatin derivatives exhibit various pharmacological activities. It is noteworthy that, some isatin derivatives have been successfully developed and used as anticancer drugs such as semaxanib, intedanib and sunitinib (**Figure 3**). Isatin moiety has been proved as an attractive pharmacophore in the design and development of new drugs [18-20]. In addition, the indole structure is also found in plenty of natural products, and which is also considered to be a potential scaffold for new drugs design and development [21].

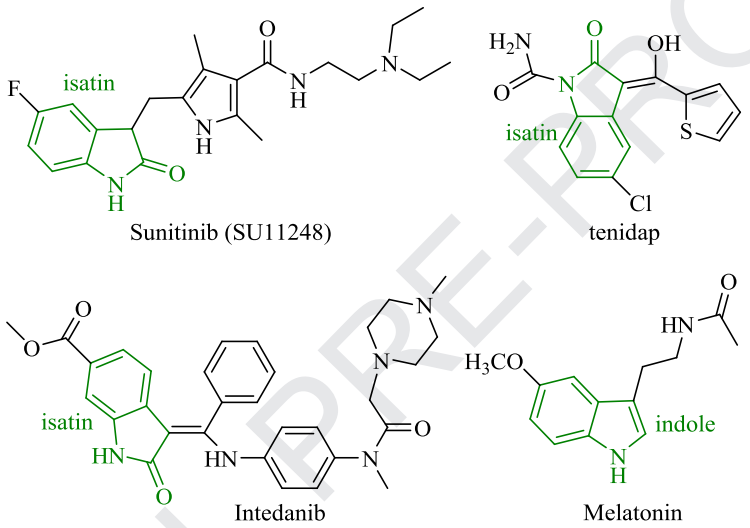

Figure 3. Structures for some anticancer drugs derived from isatin/indole.

Steroidal compounds have always been widespread application in medicine field, such as anti-inflammatory, contraceptive, diuretic, and anticancer agents [22-24]. The special skeleton features of steroidal provide additional fascination for us to design and synthesis new entities, which with the potential and prospect to become new drugs [25, 26].

In addition, hydrazine and its analogues have also been demonstrated to exhibit wide activities including antibacterial, antivirus and antitumor for a long time [27]. Especially, steroidal hydrazones have attracted the great attention of many researchers in recent years, and some novel steroidal hydrazones analogues (**Figure 4**) also presented obviously pharmaceutical activities such as antibacterial, antivirous, and cytotoxicity activities [17, 28].

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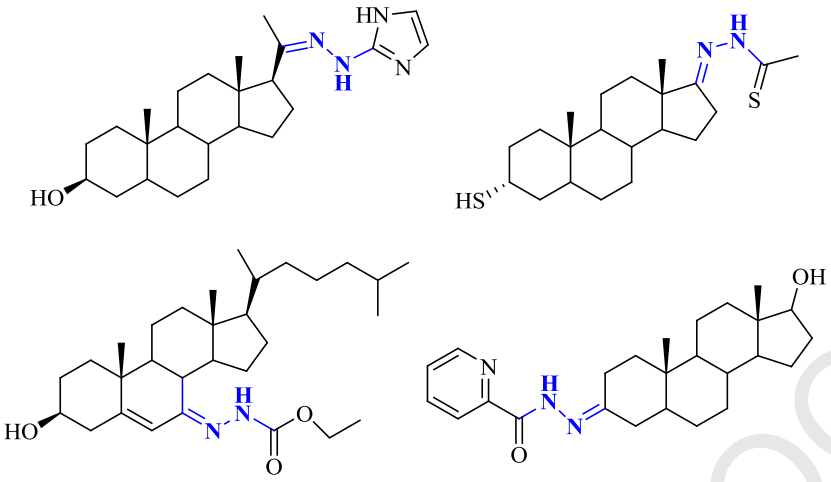

Figure 4. Structure of some steroidal hydrazones derivatives.

As an important active lead compound, EP is well known for its 5α, 8α-peroxybridge. In our previous studies, we have synthesized series of sterol 5α, 8α-endoperoxides from available natural sterols and found that several compounds displayed significantly cytotoxic activity against kinds of human tumor cells [29, 30]. As we known, pharmacophores hybridization of natural products is the most efficient approach for discovery of novel bioactive agents in the field of medicinal chemistry. In the present study, we integrated the structural features of ergosterol peroxide, hydrazone, and isatin/indole unit to design and synthesize a new series of steroidal hybrids as shown in **Figure 5**, wishing to find novel functional steroidal molecules with significant inhibited activity to tumor cells.

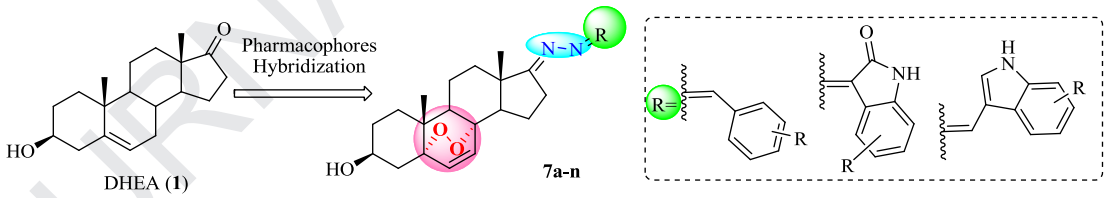

Figure 5. Design strategy of novel derivatives.

2. Experimental section

2.1. Chemistry

2.1.1. General

Reagents were used without further purification. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were measured on a Bruker Avance DRX400 spectrometer with tetramethylsilane (TMS) as internal standards. Chemical shifts were given as δ ppm values. Melting points were determined on an MP120 auto point apparatus. Mass spectra of all compounds were tested on an Esquire 6000 mass spectrometer in ESI

mode. Flash chromatography in the experiments was performed using silica gel (400 mesh).

*2.1.2. Synthesis of 3β-acetoxyandrosta-5-en-17-one (**2**)*

To a suspension of DHEA (**1**) (14.4 g, 0.05 mol) in CH_2Cl_2-pyridine 4∶1 (80 mL) was added Ac_2O (6 mL, 0.07 mol) over 15 min. The mixture was stirred for 8 hat room temperature, and then water (50 mL) was added. The resulting mixture extracted with EtOAc (2 × 80 mL). The combined organic phase was washed with $NaHCO_3$ saturated aqueous (2 × 50 mL) and brine (2 × 50 mL) and dried over anhydrous Na_2SO_4. Evaporation of solvent gave crude **2** as a white solid (16 g, 97.6% yield). mp: 168-170 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.40 (1H, d, J = 5.0 Hz), 4.65-4.54 (1H, m), 2.45 (1H, m), 2.34 (2H, d, J = 7.8 Hz), 2.11 (2H, m, 2H), 2.05 (3H, s), 1.93 (1H, d, J = 6.2 Hz), 1.90 (1H, d, J = 3.2 Hz), 1.87-1.81 (2H, m), 1.71-1.62 (4H, m), 1.55 (1H, m), 1.49 (1H, m), 1.34-1.25 (2H, m), 1.19-1.09 (1H, m), 1.03(3H, s), 1.00 (1H, d, J = 3.8 Hz), 0.90 (3H, s). MS *m/z*: 353.9 $[M+Na]^+$.

*2.1.3. Synthesis of 3β-acetoxyandrosta-5,7-diene-17-one (**3**)*

To a solution of intermediate **2** (16 g, 0.05 mol) in cyclohexane (70 mL) was heated to 70 °C for 20 min, and then was added NBS (12.5 g, 0.07 mol). The mixture was refluxed for 1.5 h. The mixture was diluted with 150 mL water. The precipitate was collected and washed with water. Then the solid was dissolved in DCM and washed with brine, dried over anhydrous Na_2SO_4. Evaporationof solvent gave a light brown solid (17.5 g, 85%). The mixture of the crude bromide (17.5 g, 0.043 mol) prepared above was added into tetrabutylammonium fluoride (45 mL 1.0 M solution in THF, 0.07 mol, 1.5 equiv). The resulting brown solution was stirred for 10 h, and then followed by rotary evaporation under 45 °C to a yellow solid. The solid was diluted with DCM (100 mL) and washed with water (2 × 50 mL), dried over anhydrous Na_2SO_4.Purified by chromatographic column to give a pale yellow solid as compound **3** (5.8 g). Yield: 35%, mp: 112-115 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.62 (1H, d, J = 3.0 Hz), 5.58 (1H, d, J = 3.2 Hz), 4.70 (1H, m), 2.56-2.49 (2H, m), 2.38 (1H, d, J = 12.5 Hz), 2.24-2.17 (2H, m), 2.07 (3H, s), 1.97-1.92 (2H, m), 1.90 (1H, d, J = 3.8 Hz), 1.74 (2H, d, J = 4.8 Hz), 1.70(1H, d, J = 5.0 Hz), 1.58 (2H, s), 1.40-1.34 (2H, m), 1.27 (1H, s), 0.99 (3H, s), 0.83 (3H, s). MS *m/z* 351.7 $[M+Na]^+$.

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*2.1.4. Synthesis of 3β-hydroxyandrosta-5,7-diene-17-one (**4**)*

To a suspension of intermediate **3** (11 g, 0.034 mol) in MeOH (100 mL) was added 25% (wt) NaOMe in MeOH (10 mL). The mixture was reflux for 1 h. 100 mL of water was added into the mixture over 0.5 h, and the mixture was continually stirred for 1 h. The precipitate was collected and washed with water (50 mL), and dried under high vacuum at 40 °C to get a brown solid crude product. Purified by chromatographic column to give a pale solid as intermediate **4** (9.3 g). Yield: 96%, mp: 157-159 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.00 (1H, d, J = 9.8 Hz), 5.70 (1H, d, J = 9.8 Hz), 4.30 (1H, t, J = 7.9 Hz), 3.76-3.60 (1H, m), 2.67-2.41 (2H, m), 1.03 (3H, s), 0.96 (3H, s). MS (ESI) *m/z* 309.9 $[M+Na]^+$.

2.1.5. Synthesis of 5α,8α-cyclicobioxygen-6-vinyl-3β-DHEA (**5**)

To a solution of intermediate **4** (140 mg) in pyridine (20 mL) was added eosin (1 mg) in a quartz tube. The mixture kept in a water-cooled bath and vigorously stirred by bubbling into the oxygen. At the same time, the mixture was irradiated with an iodine tungsten lamp (220 V 500 W) for 0.5 h. The solution was poured into ice-water (20 mL) and extracted with ethyl acetate (2 × 50 mL). The combined organic phase was washed with brine (2 × 50 mL) and dried over anhydrous Na_2SO_4. Evaporated of solvent and then purified by chromatographic column (40% EA in PE) to give **5** as white needles (101.4 mg, 63%), m.p. 167.0-168.1 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.53 (1H, d, J = 8.5 Hz), 6.35 (1H, d, J = 8.5 Hz), 4.01 (1H, m), 2.58-2.47 (1H, m), 2.24-2.12 (2H,m), 2.08-2.00 (1H, m), 1.98-1.94 (1H, m), 1.88 (1H, m), 1.86-1.81 (2H, m), 1.72 (1H, m), 1.67-1.62 (1H, m), 1.58-1.54 (4H, m), 1.54-1.49 (1H, m), 1.38-1.22 (2H, m), 1.00 (3H, s), 0.92 (3H, s). ^{13}C NMR (100 MHz, $CDCl_3$) δ 218.3, 136.5, 129.8, 82.5, 78.9, 66.3, 51.5, 48.9, 47.7, 37.0, 36.8, 35.6, 34.7, 31.6, 29.9, 22.7, 19.0, 18.3, 15.0. MS (ESI) *m/z* 319.19 $[M+H]^+$.

2.1.6. Synthesis of 5α,8α-epidioxy-17-hydrazonoandrostan-3β-ol (**6**)

To a solution of intermediate **5** (2.0 g, 6.7mmol) in EtOH (50 mL) was added 85% hydrazine hydrate (3 mL). The mixture was heated to 45 °C and stirred for 2 h. The white solid was collected by filtrating. Intermediate **6** was obtained as white solid (2.1 g) after recrystallizing in MeOH. LC-MS (ESI) *m/z*: 333.1 $[M+H]^+$.

2.1.7. Synthesis of novel derivatives **7a-n**

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To a suspension of intermediate **6** (0.2 mmol) in anhydrous EtOH (30 mL) was added different substituent (0.2 mmol). The mixture was continually stirred under 50 °C for 2~3 h until no starting material. Then the solvent was evaporated by rotary evaporation. The residue was purified by flash chromatography to afford compounds **7a-n**.

*2.1.7.1. 5α,8α-Epidioxy-17-(4-fluoro-benzylidene)hydrazonoand-rost-3β-ol **(7a)***. White powder, yield 70%, mp 72.3-74.8 °C, ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.27 (1H, s), 7.78-7.74 (2H, dd, *J* = 8.5, 5.5 Hz), 7.13-7.09 (2H, t, *J* = 8.6 Hz), 6.55 (1H, d, *J* = 8.2 Hz), 6.33 (1H, d, *J* = 8.0 Hz), 4.01 (1H, m), 2.78-2.63 (2H, m), 2.15 (1H, m), 2.05-2.04 (1H, m), 1.96-1.93 (2H, m), 1.89-1.86 (2H, m), 1.77-1.63 (4H, m), 1.63-1.56 (2H, m), 1.36-1.27 (2H, m), 1.13 (3H, m), 1.05 (1H, s), 0.94 (3H, s). ^{13}C NMR (100 MHz, $CDCl_3$) δ 181.2, 165.5, 160.8, 156.7, 136.1, 130.1, 115.8, 115.7, 82.3, 79.1, 66.2, 51.5, 49.1, 45.9, 37.1, 36.9, 34.7, 33.9, 30.1, 29.7, 27.8, 23.0, 20.0, 18.1, 16.7. MS (ESI) *m/z*: 439.2 $[M+H]^+$.

*2.1.7.2. 5α,8α-Epidioxy-17-(benzylidene)hydrazonoandrost-3β-ol **(7b)***. White powder, yield 72%, mp 75.4-76.7 °C, ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.28 (1H, s), 7.74 (1H, m), 7.54 (2H, d, *J* = 5.7 Hz), 7.42 (2H, m), 6.54 (1H, d, *J* = 8.4 Hz), 6.32 (1H, d, *J* = 8.2 Hz), 3.98 (1H, dd, *J* = 11.3, 5.6 Hz, C3-αH), 2.83-2.60 (2H, m), 1.11 (3H, m), 0.93-0.88 (3H, m). ^{13}C NMR (100 MHz, $CDCl_3$) δ 182.0, 155.7, 138.5, 136.2, 132.4, 129.4, 128.4, 118.4, 113.8, 71.6, 53.7, 50.4, 44.3, 42.2, 37.2, 36.8, 33.9, 31.6, 31.5, 31.3, 28.0, 23.3, 20.6, 19.5, 16.5. MS (ESI) *m/z*: 421.2 $[M+H]^+$.

*2.1.7.3. 5α,8α-Epidioxy-17-(3-trifluoromethyl-benzylidene)hydra-zonoandrost-3β-ol **(7c)***. White powder, yield 78%, mp 80.1-82.2 °C, ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.32 (1H, s), 8.03 (1H, s), 7.93 (1H, d, *J* = 7.5 Hz), 7.69 (1H, d, *J* = 7.6 Hz), 7.55 (1H, t, *J* = 7.7 Hz), 6.56-6.54 (1H, d, *J* = 8.4 Hz), 6.35-6.33 (1H, d, *J* = 8.4 Hz), 4.05-3.99 (1H, m), 2.81-2.65 (2H, m), 2.18-2.14 (1H, m), 2.08-2.05 (1H, m), 1.99-1.91 (2H, m), 1.89-1.82 (2H, m), 1.77-1.69 (4H, m), 1.67-1.56 (2H, m), 1.37-1.26 (2H, m), 1.15 (3H, s), 1.06 (1H, s), 0.95 (3H, s). ^{13}C NMR (100 MHz, $CDCl_3$) δ 181.7, 161.0, 156.6, 136.2, 131.8, 131.2, 130.0, 129.4, 129.2, 124.6, 124.5, 82.3, 79.1, 66.3, 51.5, 49.1, 46.0, 37.2, 36.8, 34.7, 33.9, 30.1, 27.8, 23.0, 20.3, 18.3, 18.0. MS (ESI) *m/z*: 489.2 $[M+H]^+$.

2.1.7.4.

5α,8α-Epidioxy-17-(3-methoxy-4-hydroxy-benzylidene)hydrazonoandrost-3β-ol ***(7d)***. White powder, yield 84%, mp 95.3-96.2 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.20 (1H, s), 7.43 (1H, d, J = 1.4 Hz), 7.28 (1H, s), 7.14 (1H, dd, J = 8.1, 1.5 Hz), 6.94 (1H, d, J = 8.1 Hz), 6.54 (1H, d, J = 8.5 Hz), 6.32 (1H, d, J = 8.5 Hz), 6.06 (1H, s), 4.00 (1H, m), 3.95 (3H, m), 2.76-2.71 (2H, m), 2.04 (1H, m), 1.92-1.86 (1H, m), 1.80-1.72 (4H, m), 1.63-1.52 (3H, m), 1.36-1.27 (2H, m), 1.14-1.11 (3H, m), 1.04 (1H, s), 0.94 (3H, s). ^{13}C NMR (100 MHz, $CDCl_3$) δ 180.2, 157.7, 148.5, 147.0, 136.1, 130.1, 129.7, 123.9, 114.4, 108.4, 82.3, 79.1, 66.2, 55.3, 51.5, 49.2, 45.9, 37.2, 36.8, 34.5, 34.0, 30.1, 27.8, 23.0, 20.3, 18.3, 18.0. MS (ESI) *m/z*: 451.2 $[M+H]^+$.

2.1.7.5. 5α,8α-Epidioxy-17-(isatin)hydrazonoandrost-3β-ol ***(7e)***. Bright yellow powder, yield 74%, mp 198-200 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.78 (1H, s), 7.77 (1H, d, J = 7.0 Hz), 7.37 (1H, d, J = 7.2 Hz), 7.01 (1H, d, J = 7.1 Hz), 6.88 (1H, d, J = 7.4 Hz), 6.53 (1H, d, J = 8.5 Hz), 6.31 (1H, d, J = 7.6 Hz), 4.59 (1H, m), 2.41-2.39 (2H, m), 2.01-1.86 (5H, m), 1.76-1.23 (10H, m), 1.08 (3H, s), 0.88 (3H, s). ^{13}C NMR (100 MHz, DMSO-d_6) δ 177.0, 164.7, 147.2, 145.1, 136.5, 133.6, 130.2, 128.6, 122.5, 117.0, 111.1, 82.2, 80.9, 78.7, 65.1, 55.3, 51.5, 48.9, 46.9, 46.1, 37.1, 36.0, 30.4, 22.8, 20.3, 18.4, 18.2. MS (ESI) *m/z*: 462.3 $[M+H]^+$.

2.1.7.6. 5α,8α-Epidioxy-17-(5-fluoro-isatin)hydrazonoandrost-3β-ol ***(7f)***. Yellow powder, yield 75%, mp 169-170 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.82 (1H, s), 7.60-7.50 (1H, dd, J = 2.4, 2.0 Hz), 7.28-7.24 (1H, t, J = 7.9 Hz), 6.89 (1H, dd, J = 8.5, 4.2 Hz), 7.26 (1H, m), 6.89 (1H, dd, J = 8.5, 4.3 Hz), 6.54 (1H, d, J = 8.5 Hz), 6.31 (1H, d, J = 8.5 Hz), 2.48-2.36 (2H, m), 2.04-1.23 (16, m), 1.11 (3H, s), 0.85 (3H, s). ^{13}C NMR (100 MHz, DMSO-d_6) δ 179.1, 164.7, 156.6, 147.2, 141.4, 136.6, 130.2, 120.5, 117.5, 115.3, 112.2, 82.2, 80.9, 78.7, 78.6, 65.1, 51.9, 48.8, 46.7, 37.1, 35.9, 34.8, 30.4, 22.8, 20.3, 18.4, 18.2. MS (ESI) *m/z*: 480.2 $[M+H]^+$.

2.1.7.7. 5α,8α-Epidioxy-17-(5-chloro-isatin)hydrazonoandrost-3β-ol ***(7g)***. Yellow powder, yield 69%, mp 171-172 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.93 (1H, s), 7.79 (1H, s), 7.43 (1H, d, J = 8.3 Hz), 6.90 (1H, d, J = 8.4 Hz), 6.53 (1H, d, J = 8.5 Hz), 6.30 (1H, d, J = 8.4 Hz), 4.58 (1H, m), 2.40-2.32 (2H, m), 2.04-1.85 (5H, m), 1.73-1.15 (10H, m), 1.06 (3H, s), 0.86 (3H, s). ^{13}C NMR (100 MHz, DMSO-d_6) δ 178.8, 164.4, 146.9, 143.8, 136.6, 133.0, 130.1, 128.2, 126.1, 118.2, 112.7, 82.2, 80.9,

78.6, 65.1, 51.6, 48.9, 46.8, 46.3, 37.1, 35.9, 34.8, 28.4, 22.8, 20.4, 18.3, 18.2. MS (ESI) *m/z*: 496.2 $[M+H]^+$.

*2.1.7.8. 5α,8α-Epidioxy-17-(5-bromo-isatin)hydrazonoandrost-3β-ol (**7h**).* Bright yellow powder, yield 74%, mp 156-158 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.94 (1H, s), 7.94 (1H, s), 7.56 (1H, dd, *J* = 8.3, 1.9 Hz), 6.86 (1H, d, *J* = 8.3 Hz), 6.53 (1H, d, *J* = 8.5 Hz), 6.31 (1H, d, *J* = 8.5 Hz), 4.58 (1H, m), 2.41 (1H, m), 1.98 (1H, m), 1.86-1.75 (2H, m), 1.71-1.20 (13H, m), 1.11 (3H, s), 0.85 (3H, s). ^{13}C NMR (100 MHz, DMSO-d_6) δ 178.5, 164.2, 146.6, 144.1, 136.6, 135.8, 130.9, 130.1, 118.6, 113.6, 82.2, 80.9, 78.6, 65.1, 51.6, 48.8, 46.7, 37.3, 37.1, 34.0, 30.4, 28.0, 26.8, 22.9, 20.4, 18.4, 18.2. MS (ESI) *m/z*: 540.2 $[M+H]^+$.

*2.1.7.9. 5α,8α-Epidioxy-17-(5-nitro-isatin)hydrazonoandrost-3β-ol (**7i**).* Yellow powder, yield 72%, mp 177-179 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.49 (1H, s), 8.62 (1H, s), 8.29 (1H, d, *J* = 8.7 Hz), 7.06 (1H, d, *J* = 8.6 Hz), 6.54 (1H, d, *J* = 8.4 Hz), 6.32 (1H, d, *J* = 8.3 Hz), 4.63 (1H, s), 2.43 (2H, m), 2.05 (1H, m), 1.86-1.75 (5H, m), 1.72-1.25 (9H, m), 1.15 (3H, s), 0.87 (3H, s). ^{13}C NMR (100 MHz, DMSO-d_6) δ 178.6, 164.8, 150.4, 145.9, 142.4, 136.6, 130.1, 129.7, 123.8, 116.8, 111.3, 82.2, 78.7, 65.1, 51.6, 48.8, 46.8, 37.3, 34.9, 33.9, 30.4, 28.1, 26.8, 22.9, 20.4, 18.4, 18.2. MS (ESI) *m/z*: 507.3 $[M+H]^+$.

*2.1.7.10. 5α,8α-Epidioxy-17-(5-methoxy-isatin)hydrazonoandro-st-3β-ol (**7j**).* Yellow powder, yield 71%, mp 156-159 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.59 (1H, s), 7.41 (1H, d, *J* = 2.0 Hz), 6.98 (1H, dd, *J* = 8.4, 2.3 Hz), 6.80 (1H, d, *J* = 8.5 Hz), 6.53 (1H, d, *J* = 8.4 Hz), 6.31 (1H, d, *J* = 8.5 Hz), 4.63 (1H, s), 3.70 (3H, s), 2.44-2.33 (1H, m), 1.99 (1H, m), 1.95-1.50 (10H, m), 1.48-1.22 (5H, m), 1.11 (3H, s), 0.85 (3H, s). ^{13}C NMR (100 MHz, DMSO-d_6) δ 177.5, 164.8, 155.0, 147.1, 138.7, 136.6, 130.2, 119.3, 117.5, 114.1, 111.8, 82.2, 78.7, 65.1, 56.0, 51.6, 48.9, 46.6, 37.3, 37.1, 34.9, 34.1, 30.4, 28.0, 22.9, 20.4, 18.4, 18.2. MS (ESI) *m/z*: 492.2 $[M+H]^+$.

*2.1.7.11. 5α,8α-Epidioxy-17-(5-trifluoromethoxy-isatin)hydrazo-noandrost-3β-ol (**7k**).* Yellow powder, yield 70%, mp 148-150 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.99 (1H, s), 7.77 (1H, s), 7.40 (2H, d, *J* = 7.5 Hz), 6.97 (2H, d, *J* = 8.5 Hz), 6.52 (1H, d, *J* = 8.5 Hz), 6.30 (1H, d, *J* = 8.4 Hz), 3.66-3.49 (1H, m), 2.35 (1H, m), 2.03-1.50 (10H, m), 1.48-1.22 (6H, m), 1.07 (3H, s), 0.86 (3H, s). ^{13}C NMR (100 MHz, DMSO-d_6) δ

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179.4, 164.6, 147.2, 144.0, 143.2, 136.6, 130.2, 126.7, 121.2, 117.5, 112.3, 82.2, 78.7, 65.1, 51.6, 48.9, 46.7, 37.3, 37.1, 34.9, 33.9, 30.4, 28.0, 26.8, 22.9, 20.3, 18.4, 18.1. MS (ESI) *m/z*: 546.2 $[M+H]^+$.

*2.1.7.12. 5α,8α-Epidioxy-17-(5-fluoro-indole)hydrazonoandrost-3β-ol (**7l**).* Yellow powder, yield 68%, mp 151-153 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.98 (1H, s), 8.56 (1H, d, J = 6.2 Hz), 7.83 (2H, m), 7.48 (1H, dd, J = 8.7, 4.5 Hz), 7.07 (1H, d, J = 9.1 Hz), 6.55 (1H, d, J = 6.0 Hz), 6.18 (1H, d, J = 6.0 Hz), 3.78 (1H, m), 2.83-2.61 (2H, m), 191-1.52 (11H, m), 1.48-1.24 (4H, m), 1.01 (3H, s), 0.93 (3H, s). ^{13}C NMR (100 MHz, DMSO-d_6) δ 180.4, 158.9, 156.6, 154.2, 133.7, 133.5, 125.1, 124.9, 113.1, 112.1, 110.7, 106.8, 75.4, 72.9, 65.2, 57.3, 49.2, 45.0, 43.6, 33.8, 32.3, 30.9, 27.5, 26.7, 22.0, 20.0, 19.7, 16.6. MS (ESI) *m/z*: 478.2 $[M+H]^+$.

*2.1.7.13. 5α,8α-Epidioxy-17-(5-chloro-indole)hydrazonoandrost-3β-ol (**7m**).* Yellow powder, yield 70%, mp 143-145 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.98 (1H, s), 8.56 (1H, s), 8.19 (1H, s), 7.93 (1H, d, J = 7.0 Hz), 7.50 (1H, d, J = 6.7 Hz), 7.24 (1H, d, J = 7.6 Hz), 6.56 (1H, d, J = 7.2 Hz), 6.18 (1H, d, J = 7.4 Hz), 4.31 (1H, s), 2.74 (1H, m), 2.04-1.78 (2H, s), 1.76-1.50 (10H, m), 1.49-1.25 (4H, m), 1.01 (3H, s), 0.94 (3H, s). ^{13}C NMR (100 MHz, DMSO-d_6) δ 180.1, 154.5, 153.5, 137.7, 132.1, 128.3, 127.5, 127.2, 125.2, 115.1, 111.8, 105.3, 75.4, 72.9, 65.2, 59.7, 57.3, 49.3, 45.0, 43.6, 33.8, 32.3, 30.9, 27.4, 26.7, 22.0, 20.0, 19.7, 16.6, 14.1. MS (ESI) *m/z*: 494.2 $[M+H]^+$.

*2.1.7.14. 5α,8α-Epidioxy-17-(5-methoxy-indole)hydrazonoandro-st-3β-ol (**7n**).* Yellow powder, yield 71%, mp 153-155 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.56 (1H, s), 8.56 (1H, s), 7.77 (2H, d, J = 11.6 Hz), 7.35 (1H, d, J = 8.8 Hz), 6.84 (1H, d, J = 8.8 Hz), 6.55 (1H, s), 6.14 (1H, s), 3.90-3.68 (3H, m), 2.88-2.56 (2H, m), 1.94-1.91 (2H, m), 1.79-1.50 (11H, m), 1.48-1.25 (4H, m), 1.01 (3H, s), 0.93 (3H, s). ^{13}C NMR (100 MHz, DMSO-d_6) δ 180.1, 154.6, 154.5, 132.1 131.9, 125.3, 112.5, 112.3, 111.9, 103.9, 75.4, 72.9, 65.2, 61.3, 57.4, 55.0, 49.3, 45.0, 43.5, 33.9, 32.3, 30.9, 27.4, 26.7, 22.1, 20.1, 19.7, 16.6, 15.1. MS (ESI) *m/z*: 490.2 $[M+H]^+$.

2.2. Biological Evaluation

2.2.1. MTT Cytotoxicity Assay.

Cytotoxicity activities of all synthesized compounds were tested in the human HepG2, HT-28, MCF-7 and HeLa cancer cell lines by MTT assay. Compounds were

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solubilized in DMSO at gradient concentrations from 5 μM to 60 μM. Cells were inoculated into 96-well plates for 24 h. The cells were treated with gradient concentrations of compounds for 48 h and then were added with 10 μL of MTT for 2 h. The formazan dye product was measured by the absorbance at 490 nm on a Spectra Max 340 microplate reader. The IC_{50} values of compounds were derived by SPSS nonlinear regression analysis.

2.2.2. Flow Cytometry Analysis.

Cell cycle analysis was detected by a cell cycle staining (PI) kit purchased from BD Pharmingen according to the instructions. HepG2 cells were inoculated into 6-well plates at a concentration of 8×10^4 cells/mL to attach overnight. The cells were treated with different concentrations of compound **7g** for 24 h. The cells were collected, washed twice with PBS, and centrifuged (300 g, 5 min). To detect cell cycle analysis, the collected pellets were incubated (10^6 cells/mL) with propidium iodide staining solution (50 μg/mL PI) at room temperature for 1 h. The cells were analyzed by flow cytometry (BD Biosciences).

2.2.3. Colony Analysis.

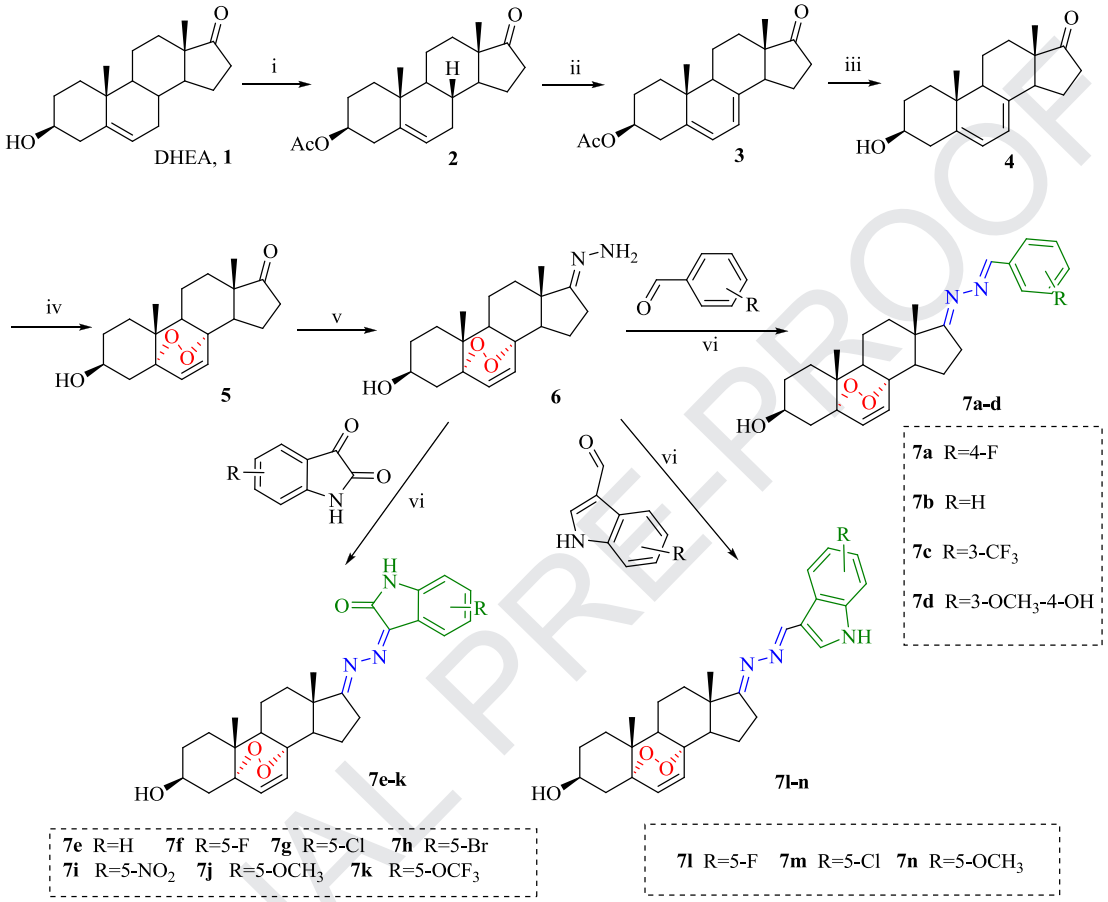
HepG2 cells were inoculated into 6-well plates at a concentration of 500 cells/well. Then 15 μM of compound **7g** were added into each plate and mixed with low melting (0.25%) agarose gel. The 6-well plates were maintained in incubator for 14 days. The formation of colonies were counted, stained, and analyzed under a microscope.

3. Results and Discussion

3.1 Chemistry.

The general procedure for the synthesis of steroidal hybrids derivatives is shown in **Scheme 1**. Dehydroepiandrosterone (DHEA, **1**) as starting material, was acetylize with acetic anhydride to give compound **2**, which then underwent bromination and debromination with NBS, *n*-Bu_4NBr and *n*-Bu_4NF to afford $_{\Delta}^{5,7}$-diene acetate **3**. Subsequently, compound **3** was converted to compound **4** via deacetylation reaction with potassium hydroxide in 30% overall yield from **1**. The key compound **4** was converted to endoperoxide **5** through photooxidation reaction with eosin as

photosensitizer. Then, the reactions of compound **5** with hydrazine hydrate yielded the corresponding steroidal hydrazine **6**. At last, the reaction of steroidal hydrazine **6** with different substituent gave our target compounds **7a-n**.The structures were characterized by MS, ^{1}H NMR and ^{13}C NMR spectrum.



Scheme 1. Synthesis of compounds **7a-n**. Reagents and conditions: (i) Ac_2O, pyridine, DCM, rt; (ii) Cyclohexane, NBS, reflux, 1 h; (iii) NaOMe, MeOH, reflux, 1 h; Bu_4NF, THF, rt, 12 h; (iv) pyridine, O_2, light, 0 ℃, 0.5 h; (v) NH_2NH_2-H_2O, EtOH, 45 ℃, 1 h; (vi) EtOH, AcOH, appropriate substituents, rt, 1~2 h.

3.2. Biological evaluation

3.2.1. In vitro anti-proliferative activity assays of **7a-n**

The newly synthesized compounds **7a-n** and EP were investigated for their anti-proliferative activities against human hepatocellular carcinomacells (HepG2), human breast carcinoma cells (MCF-7), human colon carcinoma cells (HT-29), and human cervical carcinoma cells (HeLa) by the MTT assay. Cisplatin was used as positive control drug. The anti-proliferative activity data of compounds were summarized as IC_{50} values (μM) in Table 1.

Results of the MTT assay indicated that most of synthesized compounds showed anti-proliferative activity against all the tested adherent cancer cell lines. Compounds

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7g and **7l** exhibited the most prominent inhibitory activity against the tested cell lines with IC_{50} values of 7.97 μM and 6.46 μM for HepG2 cells, 5.69 μM and 7.43 μM for Hela cells, respectively. The results suggested the following rough structure-activity relationships (SAR) considerations. Compound **5**, which has only one carbonyl group at the C-17 position, showed hardly any inhibitory activity (IC_{50} values more than 60 μM). On the contrary, most of compounds with hybrid side-chain exhibited inhibitory activity against the tested cell lines. The results indicated that the introduction of hybrids to the C-17 position resulted in increased inhibitory activity. In addition, compared to EP, some of compounds exhibited better antitumor activity. It appeared that chemcial structural modification to the C-17 position could be an opportunity for development of ergosterol peroxide analogues with more remarkable bioactivity.

Besides, it is notable that the type of substituent at C-17 position provided opportunity to further increase the inhibitory activity. Compounds **7e-n** with isatin or indole substituents on the side chain exhibited more obvious cytotoxic activity as compared to compounds **7a-d** that with aromatic substituents. Especially, most of hybrids **7e-n** displayed significant cytotoxic activities against HepG2 and HeLa cell lines. The IC_{50} values for compound **7g** bearing a 5-Cl-isatin substituent is up to 7.97 and 5.69 μM against HepG2 and Hela cell lines, respectively. In addition, compound **7l** bearing a 5-F-indole substituent is up to 6.46 and 7.43 μM against HepG2 and HeLa cell lines, respectively. The preliminary results accounted for the strategy that combined isatin/indole and peroxide steroidal was effective, and the novel hybrids might be used as an active scaffold for further optimization of potential anticancer agents.

Table 1. *In vitro* anti-proliferativeactivities of compounds.

Compd	IC_{50} (μM)[a]			
	HepG2	HT-29	MCF-7	HeLa
5	>60	>60	>60	>60
7a	44.30	32.21	37.40	>60
7b	>60	>60	>60	>60
7c	32.50	>60	>60	36.47
7d	23.02	>60	37.20	27.62
7e	13.23	25.30	15.10	12.58
7f	10.04	19.60	14.35	11.46
7g	7.97	20.42	9.05	5.69
7h	9.22	19.65	16.83	13.90
7i	21.42	41.10	24.50	16.43
7j	13.12	31.93	15.90	15.54
7k	17.11	40.05	23.02	12.72
7l	6.46	17.50	10.80	7.43
7m	9.27	22.28	>60	10.64
7n	15.45	>60	26.33	19.42

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EP	23.35	26.42	18.00	19.54
Cisplatin	3.04	4.52	6.56	5.68

[a] Data represent the mean values of three independent determinations.

Among the compounds under study, most of compounds showed more obvious anti-proliferative activity against HepG2 cell lines. Compound **7g** and HepG2 cell lines were further chosen for more biological functions experiments.

*3.2.2. Effect of **7g** on the cell cycle progression.*

The effect of synthesized compound **7g** on tumor cell cycle progression was examined by flow cytometry. The HepG2 cells were treated with **7g** at a very low concentration (3 μM) for 24 h. As shown in Figure 6A, the number of cells in the G1 phase increased significantly, suggesting a distinct inhibitory effect of **7g** on cell cycle progression. On the other hand, as shown in Figure 6B, we also detected that the number of HepG2 cells decreased in S-G2 phases when the cells were treated with **7g**.

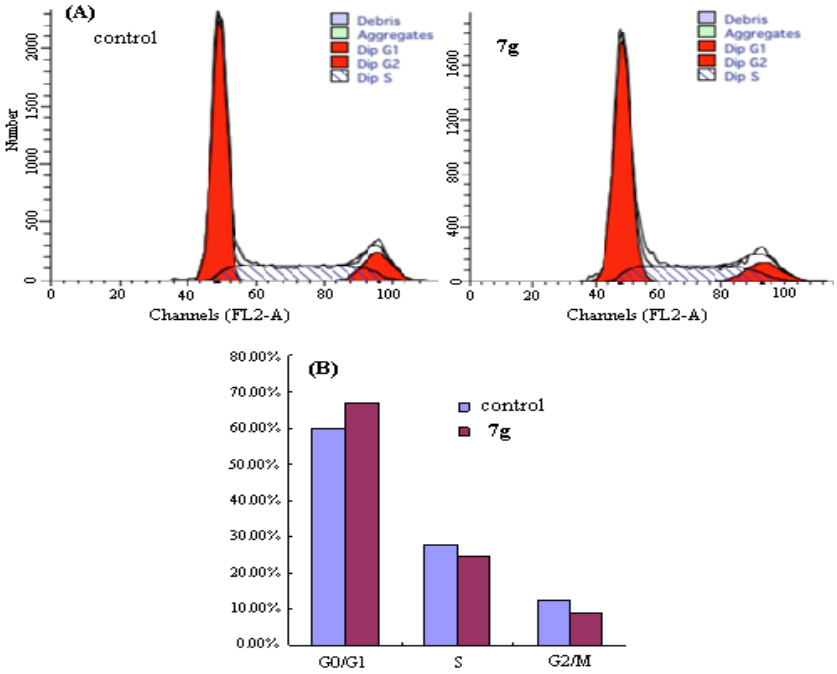

Figure 6. Compound **7g** inhibited HepG2 cell cycle progression. (A) Typical distribution of cell cycles treated with or without **7g**. (B) Statistical analysis of cells distributed in each phase of cell cycles when the cells were treated with or without **7g**.

*3.2.3. Effect of **7g** on the cell apoptosis.*

Furthermore, to test the induction of apoptosis, HepG2 cells treated with **7g** for 24 h was doubly stained with annexin V/PI. As shown in Figure 7, compound **7g** could

effectively induce apoptosis of HepG2 cells. At the 8 μM concentration **7g** causes 35.81% late apoptosis annexin V-positive and PI-positive cells, suggesting compound **7g** is a potent apoptotic inducer in HepG2 cells.

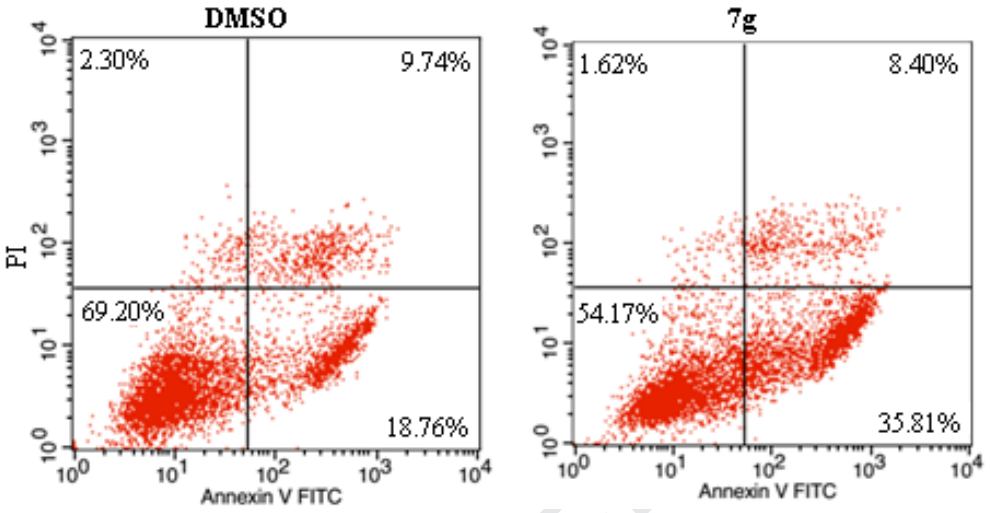

Figure 7. Compound **7g** induced HepG2 cell apoptosis. Typical distribution of cell apoptosis treated with or without **7g** analyzed by annexin V/PI staining.

*3.2.4. Effect of **7g** on the cell colony.*

As we known, HepG2 cell is a non-tumorigenic cell line, which would be difficult for us to test the inhibitory effect of compound **7g** on tumor growth *in vivo*. Hence, we evaluated the effect of compound **7g** on cancer colony formation *in vitro*. The HepG2 cells were treated with **7g** at a concentration of 8 μM for 20 days. Then the colony formation was examined under light microscopy. As shown in Figure 8A and 8B, the formed number of colonies of HepG2 cells treated with **7g** distinctly lower than the untreated cells.

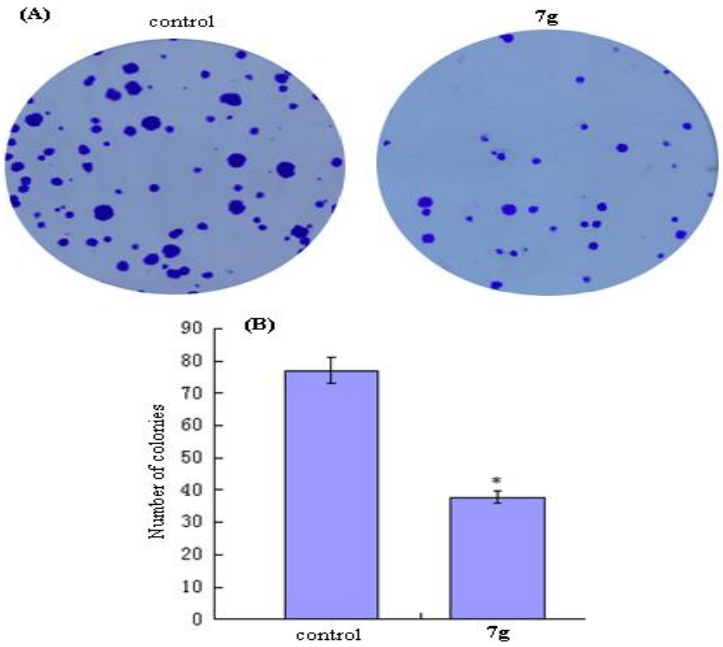

Figure 8. Compound **7g** inhibited cell colony formation. (A) Representative images of HepG2 cells colonies after treatment with **7g**. The plates were stained with Coomassie Blue. (B) Data are presented as the mean ± SD of three experiments. **p*<0.01.

4. Conclusions

In summary, we have successfully prepared a series of 5α, 8α-endoperoxidesteroidal hybrid derivatives possessing various substituents on the C-17 side chain. The *in vitro* anti-proliferative activities of all synthesized compounds were investigated. Some of the synthesized compounds exhibited significant anticancer activities against all the four tested cell lines. In particular, compound **7g** and **7l** were the most promising derivatives, with IC_{50} values lower than 8 μM against HepG2 and HeLa cell lines. Furthermore, the biological experiments showed that compound **7g** inhibited cycle progression of HepG2 cells, induced cell apoptosis, and colony growth. It appeared that chemical structural modification of this type to the C-17 position could be an opportunity for development of novel steroidal anticancer agents.

Supplementary data

Supplementary data: the ^{1}H NMR and ^{13}C NMR spectra of new compounds associated with this article can be found, in the online version, at

Supplementary data

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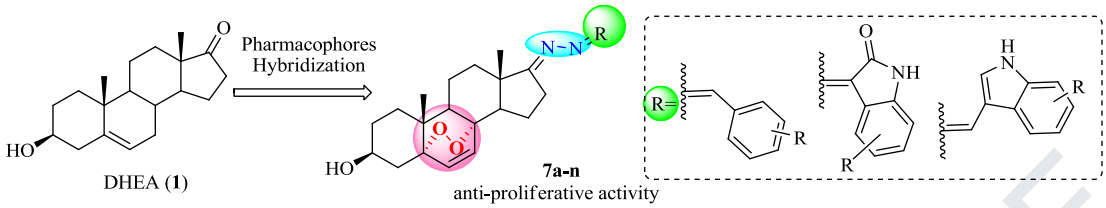
Graphical Abstract



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