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SYNTHESIS AND CYTOTOXIC EVALUATION OF STEROIDAL ENDOPEROXIDE DERIVATIVES WITH HYDRAZIDE SIDE CHAIN AS ANTICANCER AGENTS

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Abstract – A series of novel steroidal 5 α ,8 α -endoperoxide derivatives bearing hydrazide side chain were synthesized and evaluated for their cytotoxicities in four human cancer cell lines (HepG2, HCT-116, MCF-7, and A549) using the MTT assay *in vitro*. The results showed that compound **6k** exhibited significant cytotoxic activity against HepG2 cells (IC₅₀ = 5.60 μ M). Further cellular mechanism studies in HepG2 cells indicated that compound **6k** triggered the mitochondrial-mediated apoptosis by decreasing mitochondrial membrane potential (MMP) which was associated with up-regulation of Bax, down-regulation of Bcl-2.

INTRODUCTION

The incidence and mortality of cancer are rapidly growing worldwide. Current studies showed that chemotherapy was still one of the most effective methods for cancer treatment. However, undesirable side effects seriously impede its clinical applications. The discovery of potent anticancer drugs from natural products is one of the important directions in drug research. Nowadays, it's still more than 50% of drugs used in the areas of cancer and infectious diseases are based on natural origins.¹ Natural endoperoxides are cyclic organic compounds, which all have a distinct peroxide bond (-O-O-) in their structures, such as artemisinin, talaperoxides B and gracilioethers A, etc. (Figure 1).² Most of natural endoperoxides have been proved with antiviral, anticancer or antifungal activity.³ The most representative discovery of endoperoxide from natural products is artemisinin by Tu (2015 Nobel Award winner).⁴

Ergosterol peroxide (5 α ,8 α -epidioxyergosta-6,22-dien-3 β -ol, EP) is a member of a class of fungal secondary metabolites of sterol 5 α ,8 α -endoperoxide derivatives (Figure 1). A number of biological activities have been attributed to EP, including antitumor, anti-inflammatory, antiviral and immunomodulatory activities and antioxidant activities.⁵ As an important active lead compound in drug discovery, EP is well known for its 5 α ,8 α -peroxy moiety. During the last years, our research group has been working on the modifications of steroids to obtain more active compounds as potential antitumor agents.⁶ We speculate that the difference of side chains at C-17 position and 5 α ,8 α -peroxy group would provide synergistic effect for the bioactivity.

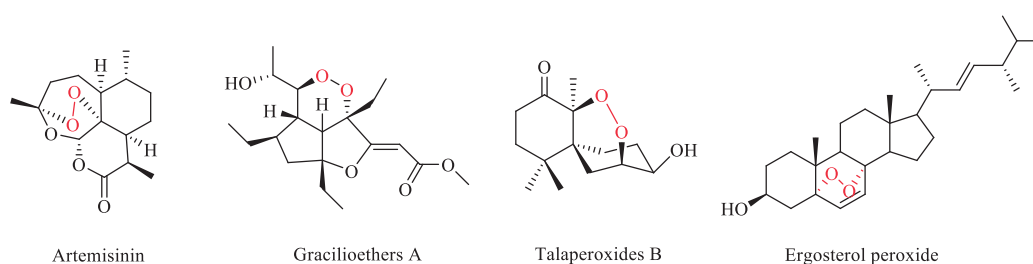
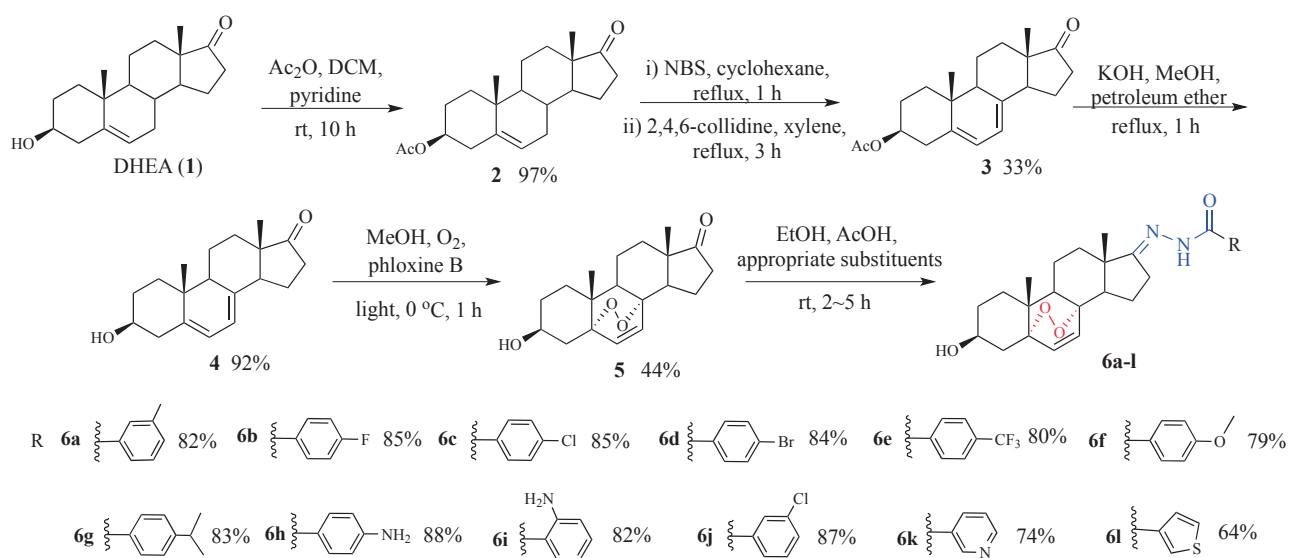


Figure 1. Representative compounds of natural endoperoxides with bioactivities

Derivatization of steroids has gain attention of the drug designing scientists for the treatment of cancer.⁷ The association of steroids with heterogeneous rings improves their biological activities. In addition, it is worth to notice that there are a lot of reports referring to anticancer activity of hydrazide derivatives.⁸ Inspired by good biological property of hydrazide derivatives, and in continuation with our previous work on the synthesis of novel steroidal endoperoxide derivatives, we here report the design and synthesis of a series of novel peroxide steroidal derivatives with hydrazide side-chain and their underlying action mechanisms of anticancer activity, wishing to find novel steroidal molecules with potent anticancer activity.

RESULTS AND DISCUSSION

Chemistry. The general procedure for the synthesis of derivatives is shown in Scheme 1. Dehydroepiandrosterone (DHEA, **1**) was reacted with acetic anhydride in the presence of pyridine and dichloromethane to give compound **2**. Compound **2** further underwent bromination with *N*-bromosuccinimide (NBS) and debromination with 2,4,6-collidine to afford compound **3**. Compound **3** was reacted with potassium hydroxide for deacetylation to give compound **4**. Subsequently, compound **4** was reacted with oxygen to give endoperoxide **5**, using phloxine B as photosensitizer. At last, the endoperoxide **5** was reacted with different hydrazine substituents to obtain target novel 5 α ,8 α -endoperoxide steroidal derivatives **6a-l**. The structures of all compounds were characterized by HRMS, ¹H NMR and ¹³C NMR spectra.



Scheme 1. Synthetic route of the target compounds **6a-l**













Biology. All of the newly synthesized endoperoxide steroidal derivatives **6a-l** were investigated *in vitro* for their anti-proliferative activities against human hepatocellular carcinoma cells (HepG2), human breast carcinoma cells (MCF-7), human colorectal cells (HCT-116), and human lung carcinoma cells (A549) by the MTT assay. Mitomycin was used as positive reference drug. The results of MTT assay were summarized as IC₅₀ values in Table 1.

Studies have shown increased biological activities of the C-17 conjugates of various hydrazines as compared to ergosterol peroxide. Especially, most of derivatives displayed significant cytotoxic activities against HepG2 cell lines. For HepG2 cell line, compounds **6k** and **6h** were the most active compounds with IC₅₀ values of 5.60 and 8.57 μM , respectively. Compound **6k** was 4-fold more potent than ergosterol peroxide. For MCF-7 cell line, compounds **6h** and **6k** were the most active compounds with IC₅₀ values of 16.35 and 11.37 μM , respectively. For HCT-116 cell line, compounds **6c** and **6k** showed similar cytotoxicity to ergosterol peroxide.

Besides, it is notable that the type of substituent at C-17 position provided opportunity to further increase the inhibitory activity. Compound **6k** with nicotinic hydrazide was more potent than other compounds with aromatic substituents. The data showed that the incorporation of pyridine ring at side chain of peroxy steroidal led to significant improvement in cytotoxic activity when compared with aryl rings. In addition, compounds **6b**, **6c**, **6d**, **6e**, **6h**, **6i** and **6j** exhibited more obvious cytotoxic activity as compared to compounds **6a** and **6f**, which probably means the electron withdrawing nature of the substituent present on above compounds contributes additional effects on cancer cell growth inhibition. It is disappointing that the activity of compound **6l** with 2-thiophenecarboxylic hydrazide markedly reduced.

The above preliminary results accounted for the effectiveness of design strategy by integrate hydrazide side-chain into peroxide steroidal, and the novel hybrids might be used as an active scaffold for further optimization of potential anticancer agents. To further investigate the cellular mechanism of these new compounds, **6k** was chosen for subsequent biological functions experiments in HepG2 cells.

Table 1. *In vitro* antiproliferative activity data of compounds **6** [IC_{50} (μ M)^{a,b}]

Compound	R	HepG2	HCT-116	MCF-7	A549
6a		65.23	>100	83.29	-
6b		35.40	62.14	52.32	>100
6c		18.32	33.45	36.70	64.48
6d		14.35	36.44	27.82	76.32
6e		23.74	52.75	37.72	44.30
6f		>100	-	-	>100
6g		39.62	>100	44.66	61.33
6h		8.57	37.56	16.35	32.92
6i		11.35	40.13	19.22	58.69
6j		20.42	69.79	23.60	67.31
6k		5.60	21.04	11.37	40.17
6l		47.64	>100	-	-
EP	-	21.02	31.28	18.10	19.60
Mitomycin	-	29.44	12.08	17.33	10.72

^aData represent the mean values of three independent determinations. ^b“-” not active.

To further demonstrate whether **6k** could induce apoptosis in HepG2 cells, **6k**-treated HepG2 cells were doubly stained with Annexin V-FITC and propidium iodide (PI). As shown in Figure 2, the percentage of total apoptotic and necrotic cells increased to 24.51% and 45.78% in a dose-dependent manner after treatment with **6k** at the concentrations of 6 and 12 μ M, respectively. The results suggested that **6k** exerted its inhibitory effects on proliferation by inducing HepG2 cells apoptosis.

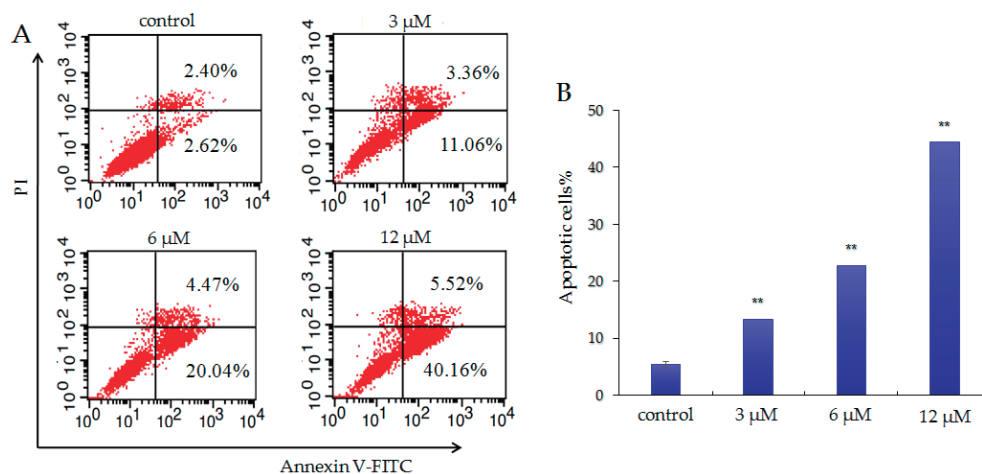


Figure 2. Compound **6k** induced HepG2 cells apoptosis. (A) Apoptotic HepG2 cells were detected with Annexin V-FITC/PI staining. Quantitative analysis of apoptosis rates by flow cytometry. (B) The bar graph represents the apoptotic cell population at various concentrations. Data were expressed as mean \pm SEM, * $P < 0.05$, ** $P < 0.01$ versus control.

Mitochondria dysfunction plays an important role in inducing apoptosis in cancer cells. The loss of MMP ($\Delta\Psi_m$) has been indicated as an early hallmark of mitochondrial dysfunction in apoptotic cells. The cationic dye JC-1 is an ideal MMP-sensitive probe, which can be used to detect the changes of MMP by flow cytometry. As shown in Figure 3, **6k** induced a doses-dependent increase in depolarized cell population from 5.57% of control to 20.59%, 34.26% and 57.50% at the concentrations of 3, 6 and 12 μM , respectively. The results indicated that the apoptosis of HepG2 cells induced by **6k** was associated with the intrinsic mitochondrial-mediated pathways.

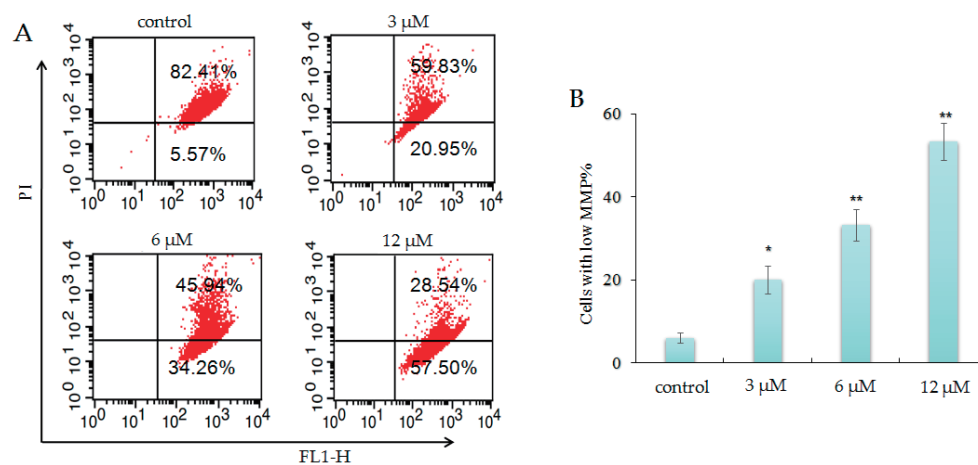


Figure 3. Compound **6k** induced mitochondrial depolarization of HepG2 cells. (A) The changes of MMP were detected by flow cytometry. (B) The bar graph represents the low MMP cell population at various concentrations. Data were expressed as mean \pm SEM, * $P < 0.05$, ** $P < 0.01$ versus control.

The mitochondria-dependent apoptotic pathway, also called the intrinsic pathway, is modulated by the Bcl-2 family of proteins, including Bax (pro-apoptotic) and Bcl-2 (anti-apoptotic protein), which could induce cytochrome *c* (cyto-*c*) released into the cytosol, resulting in the activation of the caspase-9, -3, finally triggering the execution of apoptosis.⁹ To further provide the molecular mechanistic insight into how compound **6k** induces apoptosis in HepG2 cells, we adopted quantitative real-time polymerase chain reaction (RT-PCR) to examine the ability of compound **6k** on the expression of apoptotic proteins Bax and Bcl-2. As shown in Figure 4, the results indicated that compound **6k** significantly suppressed the levels of Bcl-2 expression, but increased the expression levels of Bax in a dose-dependent manner.

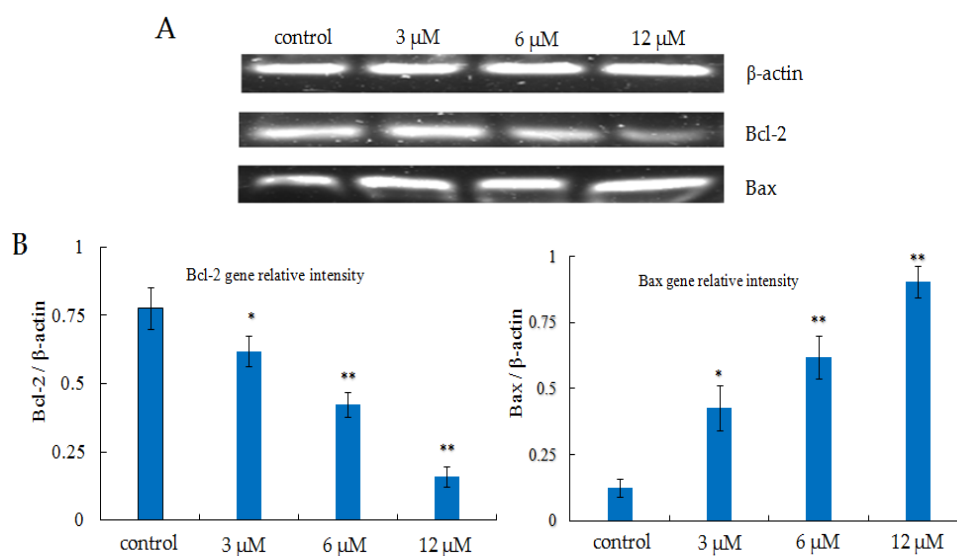


Figure 4. Compound **6k** changed the expression of apoptosis-related protein Bax and Bcl-2 in HepG2 cells. (A) The results was analyzed by Real-time PCR. (B) The bar graph represents the Bcl-2 and Bax protein relative intensity at various concentrations. * $P < 0.05$, ** $P < 0.01$ versus control.

In summary, we have successfully prepared a series of novel 5 α ,8 α -endoperoxide steroidal derivatives with hydrazide side-chain on the C-17 position. The *in vitro* anti-proliferative activity of the compounds against HepG2, A549, MCF-7, and HCT-116 cell lines were evaluated. Among them, compound **6k** was the most cytotoxic against HepG2 cells with an IC₅₀ value of 5.60 μ M. Furthermore, the cellular mechanism studies exhibited that **6k** could increase the percentage of apoptotic cells, triggered mitochondrial membrane potential (MMP) depolarization in HepG2 cells. What's more, RT-PCR indicated that **6k** could modulate up-regulation of apoptosis protein Bax and down-regulation of antiapoptotic protein Bcl-2. The above findings indicated that compound **6k** may be used as a promising skeleton for antitumor agents with improved efficacy.

EXPERIMENTAL

General methods. Reagents were used without further purification. NMR spectra were recorded on a Bruker Avance III HD 600 at ambient temperature. ^1H NMR (600 MHz) and ^{13}C NMR (150 MHz) were recorded on a Bruker Avance DRX400 instrument (Bruker, USA), using tetramethylsilane (TMS) as internal standards. MS-ESI mass spectra were obtained on an Esquire 6000 mass spectrometer. HRMS data were measured using a Bruker APEX IV Fourier transform ion cyclotron resonance mass spectrometer (Bruker, USA). Melting points were determined on an MP120 auto point apparatus (Haineng, China). Reactions were monitored by thin-layer chromatography (TLC) on a precoated silica gel 60 F254 plate (layer thickness 0.2 mm; E. Merck, Darmstadt, Germany) and detected by staining with a solution containing 5% H_2SO_4 in EtOH (100 mL), followed by heating. Flash column chromatography was performed on silica gel 60 (200-300 mesh, Qingdao Haiyang Chemical Co. Ltd.).

3β -Acetoxyandrost-5-en-17-one (2), 3β -acetoxyandrosta-5,7-dien-17-one (3) and 3β -hydroxyandrosta-5,7-dien-17-one (4) were prepared as described in the literature.^{6a}

Synthesis of $5\alpha,8\alpha$ -cyclicbioxygen-6-vinyl- 3β -DHEA (5). Intermediate 4 (1.0 g) in MeOH (150 mL) was added phloxine B (5 mg) in a 250 mL round-bottom flask. The mixture kept in a water-cooled bath and stirred by bubbling into high purity oxygen. While, the mixture was lighted with a 500 W iodine tungsten lamp (220 V) for 3 h. After completion of reaction, the solids were removed by filtration. The MeOH was evaporated, and then the crude product was purified by chromatographic column (EtOAc/petroleum ether, 1:5) to give 5 as white needles (450.0 mg), mp 166.8-167.9 °C. ^1H NMR (600 MHz, CDCl_3) δ 6.49 (d, $J = 8.2$ Hz, 1H), 6.35 (d, $J = 8.0$ Hz, 1H), 3.97 (s, 1H), 2.60-2.49 (m, 1H), 2.25-2.11 (m, 2H), 2.07-1.99 (m, 1H), 1.96 (m, 1H), 1.86 (m, 1H), 1.84-1.80 (m, 2H), 1.71 (m, 1H), 1.65-1.60 (m, 1H), 1.59-1.55 (m, 4H), 1.55-1.48 (m, 1H), 1.39-1.24 (m, 2H), 1.02 (s, 3H), 0.94 (s, 3H). ^{13}C NMR (150 MHz, CDCl_3) δ 217.9, 136.6, 130.0, 82.6, 78.8, 66.2, 52.0, 48.7, 47.6, 37.2, 36.7, 35.5, 34.8, 31.3, 29.8, 22.8, 19.0, 18.4, 15.1. MS (ESI) m/z : 319.2 $[\text{M}+\text{H}]^+$.

General procedure for synthesis of novel derivatives 6a-l. Intermediate 5 (1 mmol) reacted with different hydrazide substituents (2 mmol) in the presence of EtOH (20 mL) and five drops of acetic acid for 5~8 h until no starting material. On completion of reaction, the solvent was evaporated by rotary evaporation. The solid compounds were purified by flash chromatography with CH_2Cl_2 and MeOH, and recrystallized in MeOH to get pure target compounds 6a-l.

3-Methyl- N^1 -(3β -hydroxy- $5\alpha,8\alpha$ -epidioxyandrost-6-en-17-ylidene)benzohydrazide (6a). White solid (yield 82%), mp 151.4-152.6 °C. ^1H NMR (600 MHz, $\text{DMSO}-d_6$) δ 10.17 (s, 1H, NH), 7.59 (d, $J = 15.5$ Hz, 2H, Ar-H), 7.35 (s, 2H, Ar-H), 6.52 (d, $J = 8.0$ Hz, 1H, C6-H), 6.29 (d, $J = 8.3$ Hz, 1H, C7-H), 3.59 (d, $J = 4.9$ Hz, 1H, C3-OH), 2.71 (dd, $J = 17.7, 8.2$ Hz, 1H), 2.48-2.43 (m, 1H), 2.36 (s, 3H, Ar- CH_3), 1.90-1.59 (m,

10H), 1.42 (d, $J = 9.9$ Hz, 3H), 1.23 (s, 2H), 0.99 (s, 3H, C19-CH₃), 0.83 (s, 3H, C18-CH₃). ¹³C NMR (150 MHz, DMSO-*d*₆) δ 172.7, 164.0, 138.0, 136.4, 134.6, 132.3, 130.3, 128.6, 125.3, 82.1, 78.7, 65.1, 55.4, 51.6, 49.1, 46.5, 37.3, 37.1, 34.9, 34.3, 30.4, 27.1, 22.9, 21.4, 20.4, 18.5, 18.4. HRMS (ESI) m/z calcd for C₂₇H₃₅N₂O₄ [M+H]⁺ 451.2641, found 422.2639.

4-Fluoro-*N*¹-(3 β -hydroxy-5 α ,8 α -epidioxyandrost-6-en-17-ylidene)benzohydrazide (6b). White solid (yield 85%), mp 148.2-149.4 °C. ¹H NMR (600 MHz, CDCl₃) δ 8.63 (s, 1H, NH), 7.50 (dd, $J = 7.8$ Hz, 2H, Ar-H), 7.03 (t, $J = 8.5$ Hz, 2H, Ar-H), 6.49 (d, $J = 8.5$ Hz, 1H, C6-H), 6.35 (d, $J = 8.5$ Hz, 1H, C7-H), 3.75-3.60 (m, 1H, C3-OH), 2.81-2.56 (m, 2H, C16-H), 0.97 (s, 3H, C19-CH₃), 0.91 (s, 3H, C18-CH₃). ¹³C NMR (150 MHz, CDCl₃) δ 171.8, 168.2, 136.3, 130.9, 129.7, 128.8, 121.7, 115.8, 115.6, 82.4, 78.7, 72.8, 66.2, 51.3, 49.6, 45.9, 36.8, 34.6, 34.0, 30.0, 26.9, 26.1, 22.8, 20.3, 18.7, 18.2. HRMS (ESI) m/z calcd for C₂₆H₃₂FN₂O₄ [M+H]⁺ 455.2319, found 455.2317.

4-Chloro-*N*¹-(3 β -hydroxy-5 α ,8 α -epidioxyandrost-6-en-17-ylidene)benzohydrazide (6c). White solid (yield 85%), mp 150.3-151.1 °C. ¹H NMR (600 MHz, DMSO-*d*₆) δ 10.41 (s, 1H, NH), 7.84 (d, $J = 7.5$ Hz, 2H, Ar-H), 7.54 (d, $J = 7.8$ Hz, 2H, Ar-H), 6.52 (d, $J = 8.3$ Hz, 1H, C6-H), 6.29 (d, $J = 8.4$ Hz, 1H, C7-H), 3.58 (m, 1H, C3-OH), 2.73 (dd, $J = 18.4, 8.5$ Hz, 1H), 2.49-2.42 (m, 1H), 1.89-1.57 (m, 10H), 1.44-1.22 (m, 5H), 0.99 (s, 3H, C19-CH₃), 0.87 (s, 3H, C18-CH₃). ¹³C NMR (150 MHz, DMSO-*d*₆) δ 167.4, 163.0, 136.4, 133.3, 132.2, 130.1, 129.1, 128.7, 82.1, 78.7, 67.8, 65.0, 51.6, 49.1, 46.5, 37.3, 37.1, 34.9, 30.4, 28.8, 23.7, 22.8, 20.4, 18.5, 18.4. HRMS (ESI) m/z calcd for C₂₆H₃₂ClN₂O₄ [M+H]⁺ 471.2148, found 471.2142.

4-Bromo-*N*¹-(3 β -hydroxy-5 α ,8 α -epidioxyandrost-6-en-17-ylidene)benzohydrazide (6d). White solid (yield 84%), mp 144.8-146.0 °C. ¹H NMR (600 MHz, DMSO-*d*₆) δ 10.40 (s, 1H, NH), 7.76 (d, $J = 7.8$ Hz, 2H, Ar-H), 7.68 (d, $J = 7.9$ Hz, 2H, Ar-H), 6.51 (d, $J = 8.4$ Hz, 1H, C6-H), 6.29 (d, $J = 8.4$ Hz, 1H, C7-H), 3.57 (m, 1H, C3-OH), 2.73 (m, 1H), 2.49-2.39 (m, 1H), 1.83-1.62 (m, 10H), 1.46-1.34 (m, 3H), 1.23 (m, 2H), 0.99 (s, 3H, C19-CH₃), 0.83 (s, 3H, C18-CH₃). ¹³C NMR (150 MHz, DMSO-*d*₆) δ 173.8, 163.2, 136.4, 133.5, 131.7, 130.4, 130.3, 125.4, 82.1, 78.7, 65.1, 51.6, 49.1, 46.5, 37.3, 37.1, 34.9, 34.2, 30.4, 27.3, 23.7, 22.8, 20.4, 18.5, 18.4. HRMS (ESI) m/z calcd for C₂₆H₃₂BrN₂O₄ [M+H]⁺ 515.1591, found 515.1578.

4-Trifluoromethyl-*N*¹-(3 β -hydroxy-5 α ,8 α -epidioxyandrost-6-en-17-ylidene)benzohydrazide (6e). White solid (yield 80%), mp 157.2-158.7 °C. ¹H NMR (600 MHz, DMSO-*d*₆) δ 10.61 (s, 1H, NH), 8.01 (d, $J = 7.8$ Hz, 2H, Ar-H), 7.85 (d, $J = 7.9$ Hz, 2H, Ar-H), 6.52 (d, $J = 8.4$ Hz, 1H, C6-H), 6.29 (d, $J = 8.4$ Hz, 1H, C6-H), 3.59 (s, 1H, C3-OH), 2.74 (dd, $J = 18.8, 8.8$ Hz, 1H), 2.49-2.43 (m, 1H), 1.88-1.61 (m, 10H), 1.42 (dm, 3H), 1.23 (m, 2H), 1.00 (s, 3H, C19-CH₃), 0.84 (s, 3H, C18-CH₃). ¹³C NMR (150 MHz, DMSO-*d*₆) δ 170.8, 168.5, 138.3, 136.5, 132.0, 130.1, 129.1, 122.1, 115.8, 82.1, 78.5, 72.9, 65.5, 51.5,

49.6, 45.7, 37.3, 37.0, 34.8, 34.1, 30.5, 26.2, 22.8, 20.3, 18.7, 18.3, 14.0. HRMS (ESI) m/z calcd for $C_{27}H_{32}F_3N_2O_4$ $[M+H]^+$ 505.2355, found 505.2346.

4-Methoxy- N^1 -(3 β -hydroxy-5 α ,8 α -epidioxyandrost-6-en-17-ylidene)benzohydrazide (6f). White solid (yield 79%), mp 122.6-123.5 °C. 1H NMR (600 MHz, $CDCl_3$) δ 7.90 (s, 1H, NH), 7.50 (d, J = 7.8 Hz, 2H, Ar-H), 6.87 (d, J = 7.8 Hz, 2H, Ar-H), 6.49 (d, J = 7.5 Hz, 1H, C6-H), 6.32 (d, J = 7.5 Hz, 1H, C7-H), 4.03-3.85 (m, 1H, C3-OH), 3.81 (s, 3H, OCH_3), 0.99 (s, 3H, C19- CH_3), 0.92 (s, 3H, C18- CH_3). ^{13}C NMR (150 MHz, $CDCl_3$) δ 173.8, 163.2, 136.4, 133.5, 131.7, 130.4, 130.3, 125.4, 82.1, 78.7, 65.1, 51.6, 49.1, 46.5, 37.3, 37.1, 34.9, 34.2, 30.4, 27.3, 23.7, 22.8, 20.4, 18.5, 18.4. HRMS (ESI) m/z calcd for $C_{27}H_{35}N_2O_5$ $[M+H]^+$ 467.2553, found 467.2549.

4-Isopropyl- N^1 -(3 β -hydroxy-5 α ,8 α -epidioxyandrost-6-en-17-ylidene)benzohydrazide (6g). White solid (yield 83%), mp 166.5-166.7 °C. 1H NMR (600 MHz, $CDCl_3$) δ 7.90 (s, 1H, NH), 7.43 (d, J = 7.4 Hz, 2H, Ar-H), 7.21 (d, J = 7.4 Hz, Ar-H), 6.49 (d, J = 8.5 Hz, 1H, C6-H), 6.48 (d, J = 8.5 Hz, 1H, C7-H), 4.03-3.94 (m, 1H, C3-OH), 2.73-2.55 (m, 2H, C16-H), 1.22 (s, 3H, $CH(CH_3)_3$), 1.24 (s, 3H, $CH(CH_3)_3$), 1.08 (s, 3H, C19- CH_3), 0.96 (s, 3H, C18- CH_3). ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.7, 168.2, 145.3, 136.3, 134.8, 130.9, 129.7, 128.8, 124.0, 120.1, 82.4, 78.7, 72.9, 66.2, 65.6, 51.3, 49.6, 45.9, 37.1, 36.8, 34.6, 34.0, 33.6, 30.0, 26.1, 22.9, 20.3, 18.7, 18.2, 13.7. HRMS (ESI) m/z calcd for $C_{29}H_{39}N_2O_4$ $[M+H]^+$ 479.2962, found 479.2957.

4-amino- N^1 -(3 β -hydroxy-5 α ,8 α -epidioxyandrost-6-en-17-ylidene)benzohydrazide (6h). White solid (yield 88%), mp 169.2-170.5 °C. 1H NMR (600 MHz, $DMSO-d_6$) δ 9.74 (s, 1H, NH), 7.56 (d, J = 8.3 Hz, 2H, Ar-H), 6.55 (d, J = 8.4 Hz, 2H, Ar-H), 6.51 (d, J = 8.5 Hz, 1H, C6-H), 6.28 (d, J = 8.5 Hz, 1H, C7-H), 5.70 (s, 2H, NH_2), 3.64-3.55 (m, 1H, C3-OH), 2.70 (dd, J = 18.4, 8.7 Hz, 1H), 2.43 (dd, J = 18.3, 8.9 Hz, 1H), 1.89-1.54 (m, 10H), 1.50-1.36 (m, 3H), 1.28-1.15 (m, 2H), 0.97 (s, 3H, C19- CH_3), 0.83 (s, 3H, C18- CH_3). ^{13}C NMR (150 MHz, $DMSO-d_6$) δ 172.7, 164.0, 136.4 (2), 130.3(2), 120.6 (2), 112.9, 82.1, 78.7, 65.1, 51.6, 49.2, 46.3, 37.3, 37.1, 34.9, 34.3, 30.4, 26.7, 22.9, 20.4, 18.6, 18.4. HRMS (ESI) m/z calcd for $C_{26}H_{34}N_3O_4$ $[M+H]^+$ 452.2541, found 452.2537.

2-Amino- N^1 -(3 β -hydroxy-5 α ,8 α -epidioxyandrost-6-en-17-ylidene)benzohydrazide (6i). White solid (yield 82%), mp 163.5-165.0 °C. 1H NMR (600 MHz, $DMSO-d_6$) δ 10.01 (s, 1H, NH), 7.44 (d, J = 7.7 Hz, 1H, Ar-H), 7.15 (t, J = 7.6 Hz, 1H, Ar-H), 6.74 (d, J = 2.2 Hz, 1H, Ar-H), 6.72 (d, J = 2.4 Hz, 1H, Ar-H), 6.54 (d, J = 2.8 Hz, 1H, C6-H), 6.51 (d, J = 3.6 Hz, 1H, C7-H), 6.20 (s, 2H, NH_2), 3.63-3.52 (m, 1H, C3-OH), 2.70 (dd, J = 18.7, 8.9 Hz, 1H), 2.47-2.38 (m, 1H), 1.86-1.57 (m, 10H), 1.45-1.33 (m, 3H), 1.22 (m, 2H), 0.97 (s, 3H, C19- CH_3), 0.83 (s, 3H, C18- CH_3). ^{13}C NMR (150 MHz, $DMSO-d_6$) δ 173.0, 163.5, 149.9, 136.4 (2), 130.3(2), 116.6, 115.2, 82.1, 78.7, 65.1, 51.6, 49.1, 46.4, 37.3, 37.1, 34.9, 34.3, 30.4, 26.9, 22.9, 20.4, 18.6, 18.4. HRMS (ESI) m/z calcd for $C_{26}H_{33}N_3O_4$ $[M+H]^+$ 452.2541, found 452.2535.

3-Chloro- N^1 -(3 β -hydroxy-5 α ,8 α -epidioxyandrost-6-en-17-ylidene)benzohydrazide (6j). White solid

(yield 87%), mp 161.8-163.3 °C. ¹H NMR (600 MHz, CDCl₃) δ 9.04 (s, 1H, NH), 7.71 (m, 1H, Ar-H), 7.65 (d, *J* = 7.8, 1H, Ar-H), 7.53 (m, 1H, Ar-H), 7.35, 7.15 (d, *J* = 5.7, 3.3 Hz, 0.5H, Ar-H), 6.47 (d, *J* = 8.5, 1H, C6-H), 6.31 (d, *J* = 8.5, 1H, C7-H), 4.02-3.84 (m, 1H, C3-OH), 2.81-2.52 (m, 2H), 1.07 (s, 3H, C19-CH₃), 0.90 (s, 3H, C18-CH₃). ¹³C NMR (150 MHz, CDCl₃) δ 171.9, 168.4, 136.3, 134.6, 132.2, 130.9, 128.8, 124.6, 119.9, 82.4, 78.7, 72.8, 65.6, 51.3, 49.5, 45.9, 36.7, 34.6, 34.0, 30.5, 30.0, 26.1, 22.8, 20.3, 18.7, 18.2. HRMS (ESI) *m/z* calcd for C₂₆H₃₂ClN₂O₄ [M+H]⁺ 471.2136, found 471.2143.

N¹-(3β-Hydroxy-5α,8α-epidioxyandrost-6-en-17-ylidene)nicotinohydrazide (6k). White solid (yield 74%), mp 175.7-177.1 °C. ¹H NMR (600 MHz, CDCl₃) δ 9.15 (d, *J* = 107.0 Hz, 1H, NH), 8.63 (d, *J* = 110.7 Hz, 2H, Ar-H), 8.30 (d, *J* = 56.7 Hz, 1H, Ar-H), 7.48 (s, 1H, Ar-H), 6.49 (s, 1H, C6-H), 6.32 (d, *J* = 8.4 Hz, 1H, C7-H), 3.97 (m, 1H, C3-OH), 2.55 (s, 1H), 2.38 (s, 1H), 2.15-1.72 (m, 10H), 1.56 (m, 2H), 1.26 (s, 3H), 1.07 (s, 3H, C19-CH₃), 0.90 (s, 3H, C18-CH₃). ¹³C NMR (150 MHz, CDCl₃) δ 172.2, 162.1, 150.8, 136.4 (2), 130.3 (2), 122.9, 121.9, 82.1, 78.7, 65.1, 51.6, 49.1, 46.5, 37.3, 37.1, 34.9, 34.2, 30.4, 27.6, 22.9, 20.4, 18.5, 18.4. HRMS (ESI) *m/z* calcd for C₂₅H₃₂N₃O₄ [M+H]⁺ 4382.2458, found 438.2463.

N¹-(3β-Hydroxy-5α,8α-epidioxyandrost-6-en-17-ylidene)thiophene-2-carbohydrazide (6l). Faint yellow (yield 64%), mp 136.6-138.1 °C. ¹H NMR (600 MHz, CDCl₃) δ 8.30 (s, 1H, NH), 7.61 (d, 1H, *J* = 2.4 Hz, Ar-H), 7.54 (d, 1H, *J* = 4.8 Hz, Ar-H), 7.34 (d, 1H, *J* = 3.6 Hz, Ar-H), 6.53 (d, 1H, *J* = 8.4 Hz, C6-H), 6.31 (d, 1H, *J* = 8.4 Hz, C7-H), 4.04-3.89 (1H, m, C3-αH), 2.90-2.51 (2H, m), 1.09 (s, 3H, C19-CH₃), 0.92 (s, 1H, C18-CH₃). ¹³C NMR (150 MHz, CDCl₃) δ 180.8, 152.6, 136.1, 131.0, 130.1, 128.8, 126.6, 125.5, 82.4, 79.1, 71.8, 66.2, 51.5, 49.1, 45.9, 37.1, 34.7, 34.0, 30.1, 27.8, 23.0, 20.3, 19.2, 18.0. HRMS (ESI) *m/z* calcd for C₂₄H₃₁N₂O₄S [M+H]⁺ 443.2097, found 443.2089.

MTT Cytotoxicity Assay. HepG2, MCF-7, A549 and HCT-116 cancer cell lines were cultured in DMEM medium supplemented with 10% FBS, 100 units/mL penicillin and 100 µg/mL streptomycin at 37 °C in a humidified atmosphere of 5% CO₂. All reagents were purchased from HyClone (Utah, USA). Cytotoxicity activities of all synthesized compounds were tested by MTT assay. Compounds were solubilized in DMSO at gradient concentrations from 1 µM to 100 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 IC₅₀ values of compounds were derived by SPSS nonlinear regression analysis.

Apoptosis analysis by flow cytometry. Cell apoptosis analysis was detected by an Annexin V-FITC/PI apoptosis detection kit (Beyotime, Shanghai, China). HepG2 cells were seeded into 6-well plates at a concentration of 7 × 10⁴ cells/mL for overnight. The cells were treated with compound **6k** (0, 3, 6 and 12 µM) for 24 h. The cells were collected, washed twice with PBS. The cells were resuspended in the

Annexin V-FITC/PI staining solution for 15 min. The cells apoptosis rates were analyzed by flow cytometry (BD Biosciences, San Jose, CA, USA).

Analysis of mitochondrial membrane potential. HepG2 cells were seeded in a 12-well plate (1×10^6 cells/well). Then cells were treated with different doses of **6k** (0, 3, 6 and 12 μM) for 24 h. After treatment, cells were treated with 5 μM cationic dye JC-1 (KGA601, KeyGEN Biotech) for 30 min. Finally, cells were harvested and washed with PBS, and then analyzed by flow cytometer (BD Biosciences, San Jose, CA, USA).

Real-Time Polymerase Chain Reaction (RT-PCR). The HepG2 cells were incubated with compound **6k** at the concentrations of 0, 3, 6 and 12 μM for 24 h at 37 °C. RNA samples were reverse transcribed to cDNA and the PCR reactions were performed using TaKaRa SYBR Green Master Mix carried out in ABI 7500 Real-Time PCR instrument (ThermoFisher). The program for amplification was 1 cycle of 95 °C for 2 min followed by 40 cycles of 95 °C for 10 s, 60 °C for 30 s, and 95 °C for 10 s. Real-time RT-PCR experiments were performed in triplicate. The primers we used were as follows: β -actin: forward: 5'-CTCCTGAGCGCAAGATCTCC-3', reverse: 5'-GTCACCTTCACCGTTCCAGT-3', 299 bp. Bcl-2: forward: 5'-ACAGGTGTAAGCCACCGAAC-3', reverse: 5'-GTTGCAGTGAGCCAAGATCA-3', 299 bp. Bax: forward: 5'-TGGTGAAACCTTGTCTGCAC-3', reverse: 5'-CTTTGCCTTCTGGGTTCAAG-3', 297 bp.

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