https://dx.doi.org/10.21577/0103-5053.20210140

J. Braz. Chem. Soc., Vol. 33, No. 3, 227-237, 2022

Article

Synthesis and Biological Evaluation of Novel Betulin Derivatives with Aromatic Hydrazone Side Chain as Potential Anticancer Agents

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A series of novel betulin-28-hydrazone derivatives (**7a**-**7o**) were synthesized. All compounds were evaluated for their *in vitro* cytotoxicities in four human carcinoma cells (HepG2, MCF-7, HCT-116 and A549). Among them, compound **7l** displayed the most potent cytotoxicity with an IC_{50} (concentration of the tested compound that inhibits 50% of cell growth) value of 7.37 ± 0.38 μM against MCF-7 cells. The preliminary cellular mechanism studies indicated that compound **7l** could induce MCF-7 cells apoptosis. The above findings indicated that compound **7l** may be used as a lead compound for antitumor agents with improved efficacy.

Keywords: betulin derivatives, hydrazone, heterocycles, antitumor, apoptosis

Introduction

Nowadays, cancer has become the second leading cause of death worldwide.[1] The most effective of therapies used in cancer treatment continue to be traditional cytotoxic agents.[2] It is worth noting that the discovery of potent anticancer drugs from natural sources is still one of the important directions in the field of drug research.[3-5] Betulin (lup-20(29)-ene-3β,28-diol, BE, **1**) is an important natural lupine-type triterpenoid widely distributed in plenty of plants, especially abundant in the bark of birch trees (Figure 1).[6] Betulin has been shown to exert various pharmacological and biological activities, such as antibacterial, anti-HIV (human immunodeficiency virus) and anti-inflammatory properties.[7-15] Recently, a plenty of studies[16-26] have reported that betulin and its derivatives present significant antitumor activities against kinds of human cancer cell lines, such as colorectal carcinoma (HT29, HCT116), lung (A549), liver (SK-HEP-1, HepG2), breast (MCF-7, MDA-MB231), prostate (PC3), as well as cervical (HeLa) and leukemia (HL-60, K562, U937). However, the high hydrophobicity of betulin hampers its further development as cytotoxic drug. There are three active positions in betulin structure, namely the isopropenyl side chain at C-19, and two hydroxyl groups at C-3 and C-28. It is quite possible to make chemical modification of these positions to obtain novel betulin derivatives with desired biological properties.

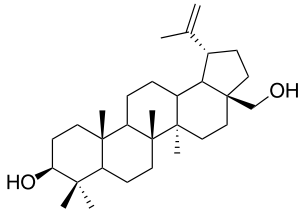

Figure 1. Structure of betulin (BE, **1**).

It is well known that hydrazine, hydrazone and their derivatives are series of highly active molecules, which have attracted considerable attention of medicinal chemists for the development of new anti-cancer drugs.[27-32] An overall survey of the structure of a hydrazone shows that it has (*i*) nucleophilic imine and amino-type nitrogens, (*ii*) an imine carbon that has both electrophilic and nucleophilic character, (*iii*) configurational isomerism stemming from the intrinsic nature of the C=N double bond, and (*iv*) in most cases an acidic N–H proton (Figure 2). These features give the hydrazone group its physical and chemical properties, in addition to playing a crucial part in determining the range of applications it can be involved in.[33] In addition, heterocycles are important structural units present in many drugs. 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.[34-37]

Thus, inspired by good biological property of hydrazone and aromatic heterocycle, in view of the potential medicinal research value of betulin and in continuation of an ongoing program aiming at developing

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Editors handled this article: Teodoro S. Kaufman and Brenno A. D. Neto (Associate)

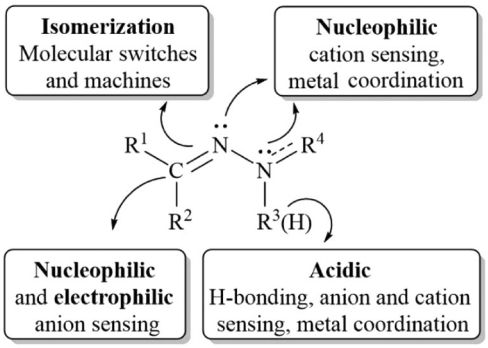


Figure 2. The structural and functional diversity of the hydrazone group.

more potential anticancer drugs, in the present study fifteen betulin derivatives modified at C-28 position with aromatic heterocycles were designed and synthesized. We hope to obtain more useful information about the influence of heterocycles at the C-28 position on cytotoxic activity in the group of betulin and their underlying mechanisms of antitumor effect.

Experimental

Chemistry

All materials were purchased from commercial suppliers and used without further purification (Energy, Shanghai, China). Reaction progress was real-time monitored by thin-layer-chromatography (TLC) using F254 silica gel plates (Biohonor, Guangzhou, China). The intermediates and target compounds were purified by flash column chromatography using 300 mesh silica gel (Yinlong, Qingdao, China). Melting points (mp) were determined using an MP120 melting point apparatus (Haineng, Fujian, China) and were not corrected. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded with Bruker AVANCE NEO600 spectrometer (tetramethylsilane (TMS) as internal standard) (Bruker, Berlin, Germany). The chemical shifts were expressed in ppm. Low resolution mass spectra (MS) were recorded on Esquire 6000 mass spectrometer (Bruker, Berlin, Germany). High-resolution mass spectra (HRMS) were obtained using an Agilent 6250 mass spectrometer (Agilient, San Francisco, USA). The values of MS were recorded in a positive ion mode with electrospray ionization (ESI) source.

3-*O*,28-*O*-Acetyl-betulin (**2**)

To a solution of betulin (**1**, 3.10 g, 7.0 mmol) in pyridine (80 mL), it was added 4-dimethylaminopyridine (DMAP, 40 mg, 0.3 mmol) and acetic anhydride (Ac_2O, 2 mL, 24 mmol) at 0 °C. The reaction was stirred at room temperature for 6 h. The solution was evaporated and then redissolved with CH_2Cl_2 (100 mL), washed with sat. $NaHCO_3$. The combined solution was washed with brine twice and dried over anhydrous Na_2SO_4. The solvent was evaporated and purified by silica gel column chromatography (ethyl acetate/petroleum ether = 1/50) to give **2** (2.95 g, 80%). White solid; mp 216.0-217.5 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 4.69 (s, 1H, –C=CH), 4.59 (s, 1H, –C=CH), 4.47 (dd, 1H, *J* 10.6, 5.7 Hz, AcO–CH), 4.25 (d, 1H, *J* 10.9 Hz, CH_2–O), 3.85 (d, 1H, *J* 11.0 Hz, CH_2–O), 2.44 (td, 1H, *J* 11.1, 5.8 Hz, H3), 2.07 (s, 3H, CH_3–COO), 2.04 (s, 3H, CH_3–COO), 1.68 (s, 3H, CH_3–C=C), 1.03 (s, 3H, –CH_3), 0.97 (s, 3H, –CH_3), 0.87-0.82 (m, 9H, –CH_3 × 3), 0.78 (d, 1H, *J* 9.3 Hz); ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.6, 171.0, 150.2, 109.9, 80.9, 62.8, 55.4, 50.3, 48.8, 47.7, 46.3, 42.7, 40.9, 38.4, 37.8, 37.6, 37.1, 34.5, 34.1, 29.7, 29.6, 27.9, 27.1, 25.2, 23.7, 21.3, 21.1, 20.8, 19.1, 18.2, 16.5, 16.2, 16.0, 14.7; MS (ESI) *m/z*, $[M + H]^+$: 527.4.

3-*O*-Acetyl-betulin (**3**)

To a solution of 3-*O*,28-*O*-acetyl-betulin (**2**, 3.41 g, 6.4 mmol) in isopropyl alcohol (*i*-PrOH, 160 mL), it was added titanium propoxide (Ti(*i*-PrOH)$_4$, 10 mL, 35 mmol). The reaction temperature was increased to 85 °C and stirred for 5 h. The solution was evaporated and then added CH_2Cl_2 (50 mL) and water (50 mL). The filtration was washed with brine twice and dried over anhydrous Na_2SO_4. The solvent was evaporated and purified by silica gel column chromatography (ethyl acetate/petroleum ether = 1/7) to give **3** (2.40 g, 76%). White solid; mp 256.1-257.8 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 4.68 (d, 1H, *J* 1.8 Hz, –C=CH), 4.58 (s, 1H, –C=CH), 4.47 (dd, 1H, *J* 11.0, 5.4 Hz, AcO–CH), 3.85-3.74 (m, 1H, CH_2–O), 3.33 (d, 1H, *J* 10.8 Hz, CH_2–O), 2.38 (td, 1H, *J* 11.0, 5.8 Hz), 2.04 (s, 3H, CH_3–COO), 1.69 (s, 3H, CH_3–C=C), 1.02 (s, 3H, –CH_3), 0.97 (s, 3H, –CH_3), 0.88-0.81 (m, 9H, –CH_3 × 3); ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.0, 150.5, 109.7, 80.9, 60.6, 55.4, 50.3, 48.7, 47.8, 47.8, 42.7, 40.9, 38.4, 37.8, 37.3, 37.1, 34.2, 34.0, 29.7, 29.2, 27.9, 27.0, 25.2, 23.7, 21.3, 20.8, 19.1, 18.2, 16.5, 16.2, 16.0, 14.7; MS (ESI) *m/z*, $[M + H]^+$: 485.4.

3-*O*-Acetyl-betulinicaldehyde (**4**)

To a solution of 3-*O*-acetyl-betulin (**3**, 300 mg, 0.6 mmol) in CH_2Cl_2 (20 mL), it was added pyridinium chlorochromate (PCC, 400 mg, 1.8 mmol). The reaction mixture was stirred at 35 °C for 1 h. Then silica gel (1.50 g) was added into mixture and concentrated to a dry powder. The crude product was purified by silica gel column chromatography to give **4** (240 mg, 80%). White solid; mp 270.2-271.8 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 9.67 (d, 1H, *J* 1.1 Hz, –CHO), 4.76 (d, 1H, *J* 0.9 Hz, C=C–H), 4.63 (s, 1H, C=C–H), 4.47 (dd, 1H, *J* 10.9, 5.5 Hz, AcO–CH),

2.86 (td, 1H, *J* 11.2, 5.9 Hz), 2.04 (s, 3H, CH_3–COO), 1.70 (s, 3H, –CH_3), 0.97 (s, 3H, –CH_3), 0.92 (s, 3H, –CH_3), 0.84 (d, 9H, *J* 8.5 Hz, –CH_3 × 3); ^{13}C NMR (150 MHz, $CDCl_3$) δ 206.7, 171.0, 149.7, 110.2, 80.9, 59.3, 55.4, 50.4, 48.1, 47.6, 42.6, 40.8, 38.7, 38.4, 37.8, 37.1, 34.3, 33.2, 29.8, 29.2, 28.8, 27.9, 25.5, 23.7, 21.3, 20.7, 19.0, 18.2, 16.5, 16.2, 15.9, 14.2; MS (ESI) *m/z*, $[M + H]^+$: 483.4.

Betulinicaldehyde (**5**)

The solution of 3-*O*-acetyl-betulinicaldehyde (**4**, 200 mg, 0.4 mmol) in 2% NaOH-MeOH (10 mL) was stirred for 2 h at 80 °C. The solution was evaporated and then redissolved with CH_2Cl_2 (100 mL), washed with brine twice and dried over anhydrous Na_2SO_4. The solvent was evaporated and purified by silica gel column chromatography (ethyl acetate/petroleum ether = 1/10) to give **5** (116 mg, 65%). White solid; mp 285.3-287.2 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 9.68 (d, 1H, *J* 1.3 Hz, –CHO), 4.76 (s, 1H, C=C–H), 4.63 (s, 1H, C=C–H), 3.18 (dd, 1H, *J* 11.5, 4.7 Hz, O–C–H), 2.86 (td, 1H, *J* 11.2, 5.9 Hz, C=C–CH–), 1.70 (s, 3H, CH_3–C=C), 0.97 (s, 3H, –CH_3), 0.95 (s, 3H, –CH_3), 0.92 (s, 3H, –CH_3), 0.82 (s, 3H, –CH_3), 0.75 (s, 3H, –CH_3); ^{13}C NMR (150 MHz, $CDCl_3$) δ 206.7, 149.7, 110.2, 79.0, 59.3, 55.3, 50.5, 48.1, 47.5, 42.6, 40.8, 38.8, 38.7, 38.7, 37.2, 34.3, 33.2, 29.9, 29.3, 28.8, 28.0, 27.4, 25.5, 20.8, 19.0, 18.3, 16.1, 15.9, 15.4, 14.3; MS (ESI) *m/z*, $[M + H]^+$: 441.4.

28-Hydrazonomethyl-betulin (**6**)

To a solution of betulinicaldehyde (**5**, 1.55 g, 3.5 mmol) in ethanol (80 mL), it was added 85% hydrazine hydrate (2 mL). The reaction mixture was stirred at 40 °C for 5 h. The solvent was evaporated and purified by silica gel column chromatography (ethyl acetate/petroleum ether = 1/1) to give **6** (1.25 g, 70%). White solid; mp 243.5-245.0 °C; ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.25 (d, 2H, *J* 22.3 Hz, NH_2), 5.14 (s, 1H, N=CH), 4.71 (s, 1H, C=C–H), 4.59 (s, 1H, C=C–H), 3.18 (dd, 1H, *J* 11.2, 4.2 Hz, OH), 1.69 (s, 3H, CH_3), 0.98 (t, 9H, *J* 9.4 Hz, CH_3 × 3), 0.93-0.88 (m, 1H), 0.82 (s, 1H, CH_3), 0.76 (s, 3H, CH_3), 0.68 (d, 1H, *J* 9.3 Hz); ^{13}C NMR (150 MHz, $CDCl_3$) δ 150.3, 150.1, 109.8, 79.0, 55.3, 50.4, 50.2, 49.3, 48.0, 42.8, 40.9, 38.9, 38.7, 38.4, 37.3, 37.2, 34.3, 32.7, 30.0, 28.0, 27.9, 27.4, 25.3, 20.9, 19.2, 18.3, 16.1, 16.1, 15.4, 14.7; MS (ESI) *m/z*, $[M + H]^+$: 445.4.

General procedure for synthesis of compounds **7a**-**7o**

To a solution of 28-hydrazonomethyl-betulin (**6**, 1 mmol) in ethanol (20 mL), it was added aldehyde substituent (2 mmol) and five drops of acetic acid. The reaction mixture was stirred at 30 °C for 5-10 h till no material. The solvent was evaporated and purified by silica gel column chromatography (dichloromethane/methanol) to get pure target compounds **7a**-**7o**.

Betulin-28-(benzylidene)hydrazone (**7a**)

White solid; 73%; ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.50 (s, 1H), 8.12 (s, 1H), 7.82 (m, 2H), 7.45 (m, 2H), 4.74 (s, 1H), 4.62 (s, 1H), 3.18 (dd, 1H, *J* 11.5, 4.6 Hz, OH), 2.66 (m, 1H, H3), 2.22-2.13 (m, 1H), 2.02 (s, 1H), 1.94 (m, 1H), 1.87 (m, 1H), 1.73 (s, 3H, CH_3), 1.69-1.10 (m, 18H), 1.00 (s, 3H, CH_3), 0.97 (m, 6H, CH_3), 0.87 (m, 2H), 0.81 (s, 3H, CH_3), 0.75 (s, 3H, CH_3), 0.68 (d, 1H, *J* 11.2 Hz); ^{13}C NMR (150 MHz, $CDCl_3$) δ 169.8 (C-28), 160.5 (C=N), 150.0 (C-20), 134.1 (C-Ar), 131.0 (C-Ar), 128.8 (C-Ar), 128.7 (C-Ar), 128.6 (C-Ar), 128.4 (C-Ar), 109.9 (C-29), 79.0 (C-3), 55.3 (C-5), 50.4 (C-9), 49.5 (C-19), 47.9 (C-17), 46.8 (C-18), 42.9 (C-14), 40.9 (C-8), 38.8 (C-4), 38.7 (C-1), 37.3 (C-13), 37.2 (C-10), 34.8 (C-7), 34.3 (C-22), 32.2 (C-21), 29.9 (C-16), 28.1 (C-23), 27.5 (C-2), 27.2 (C-15), 25.3 (C-12), 20.8 (C-11), 19.2 (C-30), 18.3 (C-6), 16.1 (C-25), 16.0 (C-26), 15.4 (C-24), 14.7 (C-27); HRMS (ESI) *m/z*, calcd. for $C_{37}H_{54}N_2O$ $[M + H]^+$: 543.4314, found: 543.4312.

Betulin-28-(4-fluoro-benzylidene)hydrazone (**7b**)

White solid; 77%; ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.48 (s, 1H), 8.12 (s, 1H), 7.80 (dd, 2H, *J* 8.7, 5.5 Hz), 7.12 (t, 2H, *J* 8.6 Hz), 4.74 (s, 1H), 4.62 (s, 1H), 3.19 (dd, 1H, *J* 11.5, 4.7 Hz, OH), 2.66 (m, 1H, H3), 2.16 (m, 1H), 2.00 (m, 1H), 1.94 (m, 1H), 1.86 (m, 1H), 1.75 (s, 1H), 1.73 (s, 3H, CH_3), 1.68-1.10 (m, 18H), 1.00 (d, 3H, *J* 7.7 Hz, CH_3), 0.97 (s, CH_3), 0.91 (m, 1H), 0.81 (s, 3H, CH_3), 0.76 (s, 3H, CH_3), 0.68 (d, 1H, *J* 11.2 Hz); ^{13}C NMR (150 MHz, $CDCl_3$) δ 170.1 (C-28), 159.3 (C=N), 150.0 (C-20), 145.0 (C-Ar), 130.4 (C-Ar), 130.3 (C-Ar), 116.0 (C-Ar), 115.8 (C-Ar), 110.0 (C-29), 78.9 (C-3), 55.3 (C-5), 50.4 (C-9), 49.5 (C-19), 47.9 (C-17), 42.9 (C-14), 40.9 (C-8), 38.8 (C-4), 38.7 (C-1), 37.3 (C-13), 37.2 (C-10), 34.8 (C-7), 34.3 (C-22), 32.1 (C-21), 29.7 (C-16), 28.1 (C-23), 27.4 (C-2), 27.2 (C-15), 25.3 (C-12), 20.8 (C-11), 19.1 (C-30), 18.3 (C-6), 16.1 (C-25), 16.0 (C-26), 15.4 (C-24), 14.7 (C-27); HRMS (ESI) *m/z*, calcd. for $C_{37}H_{53}FN_2O$ $[M + H]^+$: 561.4220, found: 561.4217.

Betulin-28-(4-chloro-benzylidene)hydrazone (**7c**)

Pale yellow solid; 75%; ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.76 (s, 1H), 8.11 (s, 1H), 8.05 (m, 1H), 7.44 (m, 1H), 7.25-7.18 (m, 1H), 7.12 (dd, *J* 20.6, 10.4 Hz, 1H), 4.74 (s, 1H), 4.62 (s, 1H), 3.19 (dd, 1H, *J* 11.5, 4.7 Hz), 2.65 (m, 1H), 2.24-2.14 (m, 1H), 2.09-2.00 (m, 1H), 1.99-1.91

(m, 2H), 1.89-1.85 (m, 1H), 1.75 (m, 1H), 1.73 (d, 3H, *J* 7.6 Hz, CH_3), 1.70-1.07 (m, 17H), 1.01 (d, 3H, *J* 8.3 Hz, CH_3), 0.99-0.95 (s, 6H, CH_3), 0.94-0.87 (m, 1H), 0.81 (s, 3H, CH_3), 0.76 (s, 3H, CH_3), 0.71-0.65 (m, 1H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 170.2 (C-28), 153.7 (C-20), 145.0 (C-Ar), 132.6 (C-Ar), 127.6 (C-Ar), 124.4 (C-Ar), 116.0 (C-Ar), 110.0 (C-29), 78.9 (C-3), 55.3 (C-5), 50.4 (C-9), 49.5 (C-19), 47.9 (C-17), 42.8 (C-14), 41.0 (C-8), 38.8 (C-4), 38.7 (C-1), 37.3 (C-13), 37.2 (C-10), 34.8 (C-7), 34.3 (C-22), 32.1 (C-21), 29.9 (C-16), 28.1 (C-23), 27.4 (C-15), 27.2 (C-2), 25.3 (C-12), 20.8 (C-11), 20.7 (C-30), 19.2 (C-30), 18.3 (C-6), 16.1 (C-25), 15.9 (C-26), 15.4 (C-24), 14.7 (C-27); HRMS (ESI) *m/z*, calcd. for $C_{37}H_{53}ClN_2O$ $[M + H]^+$: 577.3925, found: 577.3921.

Betulin-28-(4-bromo-benzylidene)hydrazone (**7d**)

Pale yellow solid; 72%; ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.44 (s, 1H), 8.11 (s, 1H), 7.69 (m, 2H), 7.58 (dd, 2H, *J* 14.5, 8.4 Hz), 4.74 (s, 1H), 4.62 (s, 1H), 3.20 (m, 1H), 2.70-2.61 (m, 1H), 2.20 (m, 1H), 2.02 (m, 1H), 1.94 (m, 1H), 1.85 (m, 1H), 1.73 (s, 3H, CH_3), 1.67-1.10 (m, 18H), 1.00 (s, 3H, CH_3), 0.97 (s, 6H, CH_3), 0.89 (m, 2H), 0.81 (s, 3H, CH_3), 0.76 (s, 3H, CH_3), 0.71-0.67 (m, 1H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 170.4 (C-28), 159.3 (C=N), 149.9 (C-20), 133.0 (C-Ar), 132.1 (C-Ar), 132.0 (C-Ar), 129.9 (C-Ar), 129.7 (C-Ar), 125.4 (C-Ar), 110.0 (C-29), 78.9 (C-3), 55.3 (C-5), 50.4 (C-9), 49.5 (C-19), 47.9 (C-17), 42.8 (C-14), 40.9 (C-8), 38.8 (C-4), 38.7 (C-1), 37.2 (C-13), 37.1 (C-10), 34.8 (C-7), 34.3 (C-22), 32.1 (C-21), 29.9 (C-16), 28.1 (C-23), 27.4 (C-2), 27.2 (C-15), 25.3 (C-12), 20.8 (C-11), 19.2 (C-30), 18.3 (C-6), 16.1 (C-25), 16.0 (C-26), 15.3 (C-24), 14.7 (C-27); HRMS (ESI) *m/z*, calcd. for $C_{37}H_{53}BrN_2O$ $[M + H]^+$: 623.3420, found: 623.3396.

Betulin-28-(4-methyl-benzylidene)hydrazone (**7e**)

White solid; 80%; ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.48 (s, 1H), 8.14 (s, 1H), 7.70 (d, 2H, *J* 8.0 Hz), 7.27-7.21 (m, 2H), 4.74 (s, 1H), 4.62 (s, 1H), 3.23-3.15 (m, 1H), 2.71-2.61 (m, 1H), 2.40 (s, 3H, Ar-CH_3), 2.16 (s, 1H), 2.02 (s, 1H), 1.99-1.92 (m, 2H), 1.87 (m, 1H), 1.75 (s, 1H), 1.71 (s, 3H, CH_3), 1.66-1.05 (m, 18H), 1.00 (s, 3H, CH_3), 1.00-0.93 (m, 6H, CH_3), 0.91-0.83 (m, 2H), 0.81 (s, 3H, CH_3), 0.75 (d, 3H, *J* 6.0 Hz, CH_3), 0.71-0.64 (m, 1H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 169.7 (C-28), 160.7 (C=N), 150.0 (C-20), 141.5 (C-Ar), 129.6 (C-Ar), 129.5 (C-Ar), 128.6 (C-Ar), 128.5 (C-Ar), 109.9 (C-29), 79.0 (C-3), 55.3 (C-5), 50.4 (C-9), 49.5 (C-19), 47.8 (C-17), 42.8 (C-14), 40.9 (C-8), 38.8 (C-4), 38.7 (C-1), 37.2 (C-13), 37.1 (C-10), 34.8 (C-7), 34.3 (C-22), 32.1 (C-21), 29.9 (C-16), 28.0 (C-23), 27.4 (C-2), 27.2 (C-15), 25.3 (C-12), 20.8 (C-11), 19.2 (C-30), 18.2 (C-6), 16.0 (C-25), 15.9 (C-26), 15.5 (C-24), 14.7 (C-27); HRMS (ESI) *m/z*, calcd. for $C_{38}H_{56}N_2O$ $[M + H]^+$: 557.4471, found: 557.4468.

Betulin-28-(4-methoxy-benzylidene)hydrazone (**7f**)

White solid; 82%; ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.47 (s, 1H), 8.15 (s, 1H), 7.76 (d, 2H, *J* 8.4 Hz), 6.99-6.92 (m, 2H), 4.74 (s, 1H), 4.62 (s, 1H), 3.91-3.82 (m, 3H, OCH_3), 3.19 (dd, 1H, *J* 11.5, 4.7 Hz), 2.65 (m, 1H), 2.20-2.13 (m, 1H), 2.01 (m, 2H), 1.97-1.91 (m, 2H), 1.87 (m, 1H), 1.74 (s, 3H, CH_3), 1.70-1.07 (m, 17H), 1.00 (s, 3H, CH_3), 0.96 (m, 6H, CH_3), 0.93-0.87 (m, 2H), 0.81 (s, 3H, CH_3), 0.76 (s, 3H, CH_3), 0.68 (d, 1H, *J* 11.1 Hz); ^{13}C NMR (150 MHz, $CDCl_3$) δ 169.8 (C-28), 160.3 (C=N), 150.0 (C-20), 132.2 (C-Ar), 130.2 (C-Ar), 116.0 (C-Ar), 114.8 (C-Ar), 114.3 (C-Ar), 114.2 (C-Ar), 109.9 (C-29), 79.0 (C-3), 55.5 (OCH_3), 55.3 (C-5), 50.4 (C-9), 49.5 (C-19), 47.9 (C-17), 42.8 (C-14), 40.9 (C-8), 38.8 (C-4), 38.7 (C-1), 37.3 (C-13), 37.2 (C-10), 34.8 (C-7), 34.3 (C-22), 32.2 (C-21), 29.9 (C-16), 27.9 (C-23), 27.4 (C-2), 27.2 (C-15), 25.3 (C-12), 20.8 (C-11), 20.2 (C-30), 18.3 (C-6), 16.1 (C-25), 16.0 (C-26), 15.4 (C-24), 14.7 (C-27); HRMS (ESI) *m/z*, calcd. for $C_{38}H_{56}N_2O_2$ $[M + H]^+$: 573.4420, found: 573.4417.

Betulin-28-(4-nitro-benzylidene)hydrazone (**7g**)

Pale yellow solid; 79%; ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.70 (s, 1H), 8.34 (s, 1H), 8.15 (m, 1H), 8.10 (s, 1H), 8.08 (d, 1H, *J* 7.7 Hz), 7.62 (m, 1H), 4.76 (s, 1H), 4.63 (s, 1H), 3.19 (dd, 1H, *J* 11.5, 4.6 Hz), 2.73-2.61 (m, 1H), 2.22-2.14 (m, 1H), 2.05 (s, 1H), 1.97-1.91 (m, 2H), 1.85 (m, 1H), 1.76 (m, 1H), 1.73 (s, 3H, CH_3), 1.70-1.06 (m, 18H), 1.02 (s, 3H, CH_3), 0.96 (s, 6H, CH_3), 0.93-0.87 (m, 2H), 0.81 (s, 3H, CH_3), 0.76 (s, 3H, CH_3), 0.69 (d, 1H, *J* 11.2 Hz); ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.6 (C-28), 160.5 (C=N), 149.8 (C-20), 135.9 (C-Ar), 133.8 (C-Ar), 129.7 (C-Ar), 125.2 (C-Ar), 122.9 (C-Ar), 110.1 (C-29), 78.9 (C-3), 55.3 (C-5), 50.4 (C-9), 49.5 (C-19), 47.9 (C-17), 42.9 (C-14), 40.9 (C-8), 38.9 (C-4), 38.8 (C-1), 37.2 (C-13), 37.1 (C-10), 34.8 (C-7), 34.3 (C-22), 32.1 (C-21), 29.9 (C-16), 28.1 (C-23), 27.4 (C-2), 27.2 (C-15), 25.3 (C-12), 20.8 (C-11), 20.7 (C-30), 18.3 (C-6), 16.1 (C-25), 16.0 (C-26), 15.4 (C-24), 14.7 (C-27); HRMS (ESI) *m/z*, calcd. for $C_{37}H_{53}N_3O_3$ $[M + H]^+$: 588.4165, found: 588.4162.

Betulin-28-(3-trifluoromethyl-benzylidene)hydrazone (**7h**)

Pale yellow solid; 82%; ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.45 (s, 1H), 8.06 (s, 1H), 8.03 (s, 1H), 7.87 (d, 1H, *J* 7.7 Hz), 7.62 (d, 1H, *J* 7.8 Hz), 7.49 (t, 1H, *J* 7.8 Hz), 4.68 (s, 1H), 4.56 (s, 1H), 3.12 (dd, 1H, *J* 11.5, 4.7 Hz), 2.60 (td, 1H, *J* 11.1, 5.2 Hz), 2.15-2.06 (m, 1H), 1.98-1.92 (m, 1H), 1.90-1.84 (m, 2H), 1.79 (s, 1H), 1.68 (m, 1H), 1.65 (s, 3H, CH_3), 1.62-1.01 (m, 17H), 0.94 (s, 3H, CH_3),

0.90 (s, 6H, CH_3), 0.83 (m, 2H), 0.74 (s, 3H, CH_3), 0.68 (s, 3H, CH_3), 0.62 (d, 1H, *J* 11.2 Hz); ^{13}C NMR (150 MHz, $CDCl_3$) δ 169.9 (C-28), 157.7 (C=N), 148.9 (C-20), 133.9 (C-Ar), 130.6 (C-Ar), 128.2 (C-Ar), 126.3 (C-Ar), 126.2 (C-Ar), 123.8 (CF_3), 123.7 (C-Ar), 109.0 (C-29), 77.9 (C-3), 54.3 (C-5), 50.3 (C-9), 49.4 (C-19), 48.4 (C-17), 41.8 (C-14), 39.9 (C-8), 37.8 (C-4), 37.6 (C-1), 36.2 (C-13), 36.1 (C-10), 34.8 (C-7), 33.3 (C-22), 31.1 (C-21), 29.9 (C-16), 28.9 (C-23), 27.1 (C-2), 26.9 (C-15), 24.3 (C-12), 20.8 (C-11), 19.7 (C-30), 18.2 (C-6), 17.2 (C-25), 15.1 (C-26), 15.0 (C-24), 14.3 (C-27); HRMS (ESI) *m/z*, calcd. for $C_{38}H_{53}F_3N_2O$ $[M + H]^+$: 611.4188, found: 611.4185.

Betulin-28-(3-methoxy-4-hydroxy-benzylidene)hydrazone (**7i**)

Pale yellow solid; 84%; ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.42 (s, 1H), 8.11 (s, 1H), 7.49 (s, 1H), 7.17 (dd, 1H, *J* 8.1, 1.7 Hz), 6.95 (d, 1H, *J* 8.1 Hz), 6.08 (s, 1H, Ar-OH), 4.73 (d, 1H, *J* 1.6 Hz), 4.62 (s, 1H), 3.97 (d, 3H, *J* 3.4 Hz, OCH_3), 3.19 (dd, 1H, *J* 11.5, 4.7 Hz, OH), 2.66 (m, 1H), 2.20-2.11 (m, 1H), 2.01 (m, 1H), 1.97-1.92 (m, 2H), 1.88 (m, 1H), 1.74 (s, 1H), 1.72 (s, 3H, CH_3), 1.69-1.06 (m, 17H), 1.01 (s, 3H, CH_3), 0.97 (s, 6H, CH_3), 0.94-0.86 (m, 2H), 0.81 (s, 3H, CH_3), 0.75 (s, 3H, CH_3), 0.68 (d, 1H, *J* 11.2 Hz); ^{13}C NMR (150 MHz, $CDCl_3$) δ 169.0 (C-28), 160.5 (C=N), 150.1 (C-20), 148.6 (C-Ar), 146.9 (C-Ar), 124.5 (C-Ar), 114.4 (C-Ar), 109.9 (C-29), 108.5 (C-Ar), 79.0 (C-3), 56.1 (OCH_3), 55.3 (C-5), 50.4 (C-9), 49.5, (C-19) 47.9 (C-17), 42.9 (C-14), 38.8 (C-8), 38.7 (C-4), 37.3 (C-1), 37.1 (C-13), 34.3 (C-10), 34.8 (C-7), 32.2 (C-22), 29.9 (C-21), 29.8 (C-16), 28.1 (C-23), 27.4 (C-2), 25.3 (C-15), 20.8 (C-12), 20.7 (C-11), 19.2 (C-30), 18.3 (C-6), 17.1 (C-25), 16.1 (C-26), 15.4 (C-24), 14.7 (C-27); HRMS (ESI) *m/z*, calcd. for $C_{38}H_{56}N_2O_3$ $[M + H]^+$: 589.4369, found: 589.4366.

Betulin-28-(2,4-dichloro-4-hydroxy-benzylidene)hydrazone (**7j**)

White solid; 80%; ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.82 (s, 1H), 8.09 (s, 1H), 8.07 (s, 1H), 7.44 (d, 1H, *J* 2.0 Hz), 7.30 (dd, 1H, *J* 8.4, 1.9 Hz), 4.74 (d, 1H, *J* 1.3 Hz), 4.63 (s, 1H), 3.19 (dd, 1H, *J* 11.5, 4.6 Hz), 2.69-2.59 (m, 1H), 2.24-2.15 (m, 1H), 2.04-1.98 (m, 1H), 1.96-1.89 (m, 2H), 1.89-1.82 (m, 1H), 1.75 (m, 1H), 1.71 (s, 3H, CH_3), 1.69-1.07 (m, 17H), 1.01 (s, 3H, CH_3), 0.97 (s, 6H, CH_3), 0.93-0.86 (m, 2H), 0.81 (s, 3H, CH_3), 0.76 (s, 3H, CH_3), 0.69 (d, 1H, *J* 11.1 Hz); ^{13}C NMR (150 MHz, $CDCl_3$) δ 170.7 (C-28), 155.7 (C=N), 149.9 (C-20), 137.2 (C-Ar), 136.0 (C-Ar), 130.2 (C-Ar), 129.1 (C-Ar), 128.9 (C-Ar), 127.5 (C-Ar), 110.1 (C-29), 79.0 (C-3), 55.3 (C-5), 50.4 (C-9), 49.5 (C-19), 47.9 (C-17), 42.9 (C-14), 40.9 (C-8), 38.9 (C-4), 38.8 (C-1), 37.2 (C-13), 37.1 (C-10), 34.8 (C-7), 34.3 (C-22), 32.1 (C-21), 29.8 (C-16), 28.1 (C-23), 27.4 (C-2), 25.4 (C-15), 25.3 (C-12), 20.8 (C-11), 20.6 (C-30), 18.3 (C-6), 16.1 (C-25), 16.0 (C-26), 15.4 (C-24), 14.7 (C-27); HRMS (ESI) *m/z*, calcd. for $C_{37}H_{52}Cl_2N_2O$ $[M + H]^+$: 611.3535, found: 611.3532.

Betulin-28-(pyridin-4-ylmethylene)hydrazone (**7k**)

White solid; 81%; ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.75 (s, 2H), 8.43 (s, 1H), 8.11 (s, 1H), 7.68 (s, 2H), 4.75 (s, 1H), 4.63 (s, 1H), 3.19 (dd, 1H, *J* 11.5, 4.6 Hz), 2.72-2.62 (m, 1H), 2.16 (d, 1H, *J* 12.1 Hz), 2.00 (s, 1H), 1.92 (m, 2H), 1.84 (m, 1H), 1.76 (m, 1H), 1.72 (s, 3H, CH_3), 1.69-1.09 (m, 18H), 1.02 (s, 3H, CH_3), 0.97 (s, 6H, CH_3), 0.90 (m, 1H), 0.81 (s, 3H, CH_3), 0.75 (s, 3H, CH_3), 0.69 (d, 1H, *J* 11.1 Hz); ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.5 (C-28), 157.8 (C=N), 149.8 (C-20), 149.4 (Py), 114.3 (Py), 120.4 (Py), 110.1 (C-29), 78.9 (C-3), 55.3 (C-5), 50.4 (C-9), 49.4 (C-19), 47.9 (C-17), 42.8 (C-14), 40.9 (C-8), 38.9 (C-4), 38.8 (C-1), 37.2 (C-13), 37.1 (C-10), 34.8 (C-7), 34.3 (C-22), 32.1 (C-21), 29.8 (C-16), 28.1 (C-23), 27.3 (C-2), 25.4 (C-15), 25.3 (C-12), 20.8 (C-11), 20.7 (C-30), 18.3 (C-6), 16.1 (C-25), 16.0 (C-26), 15.4 (C-24), 14.7 (C-27); HRMS (ESI) *m/z*, calcd. for $C_{36}H_{53}N_3O$ $[M + H]^+$: 544.4268, found: 544.4264.

Betulin-28-(indole-4-ylmethylene)hydrazone (**7l**)

Pale yellow solid; 77%; ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.84 (s, 1H), 8.46 (s, 1H), 8.21 (s, 1H), 7.49 (dd, 2H, *J* 7.5, 4.1 Hz), 7.35 (s, 1H), 7.31 (s, 1H), 7.27 (s, 1H), 4.76 (s, 1H), 4.63 (s, 1H), 3.19 (dd, 1H, *J* 11.5, 4.5 Hz), 2.74-2.66 (m, 1H), 2.22 (d, 1H, *J* 12.9 Hz), 2.09-2.02 (m, 1H), 2.01-1.95 (m, 2H), 1.93 (s, 1H), 1.76 (s, 1H), 1.74 (s, 3H) , CH_3, 1.70-1.08 (m, 18H), 1.02 (s, 3H, CH_3), 0.97 (s, 6H, CH_3), 0.93-0.84 (m, 2H), 0.80 (s, 3H, CH_3), 0.75 (s, 3H, CH_3), 0.69 (d, 1H, *J* 11.2 Hz); ^{13}C NMR (150 MHz, $CDCl_3$) δ 169.2 (C-28), 161.1 (C=N), 150.1 (C-20), 136.3 (C-Ar), 126.0 (C-Ar), 125.8 (C-Ar), 125.6 (C-Ar), 123.1 (C-Ar), 121.7 (C-Ar), 113.9 (C-Ar), 109.9 (C-29), 103.4 (C-Ar), 79.0 (C-3), 55.3 (C-5), 50.4 (C-9), 49.5 (C-19), 47.9 (C-17), 42.8 (C-14), 40.9 (C-8), 38.8 (C-4), 38.7 (C-1), 37.4 (C-13), 37.1 (C-10), 34.8 (C-7), 34.3 (C-22), 32.2 (C-21), 29.8 (C-16), 28.2 (C-23), 27.4 (C-2), 25.4 (C-15), 25.3 (C-12), 20.8 (C-11), 20.7 (C-30), 18.3 (C-6), 16.1 (C-25), 16.0 (C-26), 15.3 (C-24), 14.7 (C-27); HRMS (ESI) *m/z*, calcd. for $C_{39}H_{55}N_3O$ $[M + H]^+$: 582.4421, found: 582.4420.

Betulin-28-(quinolin-4-ylmethylene)hydrazone (**7m**)

Pale yellow solid; 78%; ^{1}H NMR (600 MHz, $CDCl_3$) δ 9.14 (s, 1H), 9.02 (d, 1H, *J* 4.5 Hz), 8.66 (d, 1H, *J* 8.4 Hz), 8.22 (d, 2H, *J* 6.2 Hz), 7.89 (d, 1H, *J* 4.4 Hz), 7.80 (dd, 1H, *J* 11.2, 3.9 Hz), 7.68 (t, 1H, *J* 7.2 Hz), 4.77 (s, 1H), 4.65 (s,

1H), 3.19 (dd, 1H, *J* 11.5, 4.6 Hz), 2.71 (d, 1H, *J* 5.0 Hz), 2.28-2.20 (m, 1H), 2.08-2.04 (m, 2H), 1.99-1.93 (m, 2H), 1.89 (m, 1H), 1.79 (m, 1H), 1.74 (s, 3H, CH_3), 1.71-1.10 (m, 18H), 1.04 (s, 3H, CH_3), 0.99 (s, 3H, CH_3), 0.97 (s, 3H, CH_3), 0.95-0.86 (m, 2H), 0.81 (s, 3H, CH_3), 0.76 (s, 3H, CH_3), 0.69 (d, 1H, *J* 11.5 Hz); ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.9 (C-28), 157.2 (C=N), 149.9 (C-20), 149.8 (C-Ar), 130.0 (C-Ar), 129.8 (C-Ar), 127.7 (C-Ar), 125.8 (C-Ar), 124.0 (C-Ar), 120.4 (C-Ar), 110.1 (C-29), 78.9 (C-3), 55.3 (C-5), 50.4 (C-9), 49.5 (C-19), 47.9 (C-17), 42.9 (C-14), 40.9 (C-8), 38.8 (C-4), 38.7 (C-1), 37.3 (C-13), 37.2 (C-10), 34.8 (C-7), 34.3 (C-22), 32.1 (C-21), 29.8 (C-16), 28.2 (C-23), 27.4 (C-2), 25.4 (C-15), 25.3 (C-12), 20.8 (C-11), 20.7 (C-30), 18.3 (C-6), 16.1 (C-25), 16.0 (C-26), 15.3 (C-24), 14.7 (C-27); HRMS (ESI) *m/z*, calcd. for $C_{40}H_{55}N_3O$ $[M + H]^+$: 594.4425, found: 594.4420.

Betulin-28-(thiophene-2-ylmethylene)hydrazone (**7n**)

White solid; 73%; ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.67 (s, 1H), 8.10 (s, 1H), 7.45 (d, 1H, *J* 5.0 Hz), 7.40 (d, 1H, *J* 3.5 Hz), 7.13-7.07 (m, 1H), 4.73 (d, 1H, *J* 1.5 Hz), 4.61 (s, 1H), 3.18 (dd, 1H, *J* 11.5, 4.6 Hz), 2.73-2.63 (m, 1H), 2.15 (m, 1H), 2.04-1.97 (m, 1H), 1.95-1.86 (m, 3H), 1.74 (s, 1H), 1.72 (s, 3H, CH_3), 1.69-1.06 (m, 17H), 1.01 (s, 3H, CH_3), 0.96 (s, 6H, CH_3), 0.90 (m, 2H), 0.80 (s, 3H, CH_3), 0.74 (s, 3H, CH_3), 0.68 (d, 1H, *J* 11.0 Hz); ^{13}C NMR (150 MHz, $CDCl_3$) δ 170.1 (C-28), 154.7 (C=N), 150.0 (C-20), 138.8 (C-Ar), 132.0 (C-Ar), 129.5 (C-Ar), 127.7 (C-Ar), 110.1 (C-Ar), 109.9 (C-29), 78.9 (C-3), 55.3 (C-5), 50.4 (C-9), 49.5 (C-19), 47.8 (C-17), 42.8 (C-14), 40.9 (C-8), 38.9 (C-4), 38.7 (C-1), 37.3 (C-13), 37.1 (C-10), 34.8 (C-7), 34.3 (C-22), 29.8 (C-16), 28.1 (C-23), 27.4 (C-2), 25.4 (C-15), 25.3 (C-12), 20.8 (C-11), 20.7 (C-30), 18.8 (C-6), 16.1 (C-25), 16.0 (C-26), 15.4 (C-24), 14.7 (C-27); HRMS (ESI) *m/z*, calcd. for $C_{35}H_{52}N_2OS$ $[M + H]^+$: 548.3878, found: 548.3875.

Betulin-28-(furan-2-ylmethylene)hydrazone (**7o**)

Pale yellow solid; 72%; ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.39 (s, 1H), 8.20 (s, 1H), 7.58 (d, 1H, *J* 1.3 Hz), 6.85 (d, 1H, *J* 3.4 Hz), 6.53 (dd, 1H, *J* 3.4, 1.7 Hz), 4.72 (d, 1H, *J* 1.4 Hz), 4.61 (s, 1H), 3.18 (dd, 1H, *J* 11.5, 4.7 Hz), 2.71-2.62 (m, 1H), 2.22-2.12 (m, 1H), 2.02-1.96 (m, 1H), 1.94-1.87 (m, 2H), 1.83 (m, 1H), 1.73 (m, 1H), 1.70 (s, 3H, CH_3), 1.68-1.05 (m, 18H), 1.00 (s, 3H, CH_3), 0.97 (s, 3H, CH_3), 0.93 (s, 3H, CH_3), 0.90 (m, 2H), 0.80 (s, 3H, CH_3), 0.75 (s, 3H, CH_3), 0.68 (d, *J* 11.0 Hz, 1H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 171.3 (C-28), 154.6 (C=N), 149.9 (C-20), 149.5 (C-20), 145.4 (C-Ar), 128.8 (C-Ar), 116.2 (C-Ar), 112.1 (C-Ar), 109.9 (C-29), 78.9 (C-3), 55.3 (C-5), 50.4 (C-9), 49.5 (C-19), 47.9 (C-17), 42.8 (C-14), 40.9 (C-8), 38.9 (C-4), 38.7 (C-1), 37.3 (C-13), 37.1 (C-10), 34.8 (C-7), 34.3 (C-22), 29.8 (C-16), 28.1 (C-23), 27.4 (C-2), 27.2 (C-15), 25.3 (C-12), 20.8 (C-11), 20.7 (C-30), 19.1 (C-6), 16.1 (C-25), 16.0 (C-26), 15.4 (C-24), 14.7 (C-27); HRMS (ESI) *m/z*, calcd. for $C_{35}H_{52}N_2O_2$ $[M + H]^+$: 533.4110, found: 533.4104.

Cells culture and MTT assays

All cell lines were obtained from the Shanghai cell Bank of the Chinese Academy of Science. Cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 units mL^{-1} of penicillin and 100 μg mL^{-1} streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. Cytotoxic activities of all tested compounds against four cancer cell lines were evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded into 96-well plates (1×10^4 cells *per* well) for 24 h. Then the cells were treated with compounds at gradient concentrations from 1 to 60 μM for 48 h and then 10 μL MTT (Sigma Chemical Co., Ltd., Milwaukee, USA) solution (5 mg mL^{-1} in phosphate buffered saline (PBS)) were added for 2 h. The solution was replaced by 100 μL dimethyl sulfoxide (DMSO), and the absorbance was measured at 490 nm on a Spectra Max 340 microplate reader. The IC_{50} (concentration of the tested compound that inhibits 50% of cell growth) values were derived by SPSS[38] nonlinear regression analysis.

Acridine orange (AO)/ethidium bromide (EB) staining

The MCF-7 cells were seeded into six-well plates at a concentration of 5×10^4 cells *per* mL. The cells were incubated overnight at 37 °C in a humidified atmosphere of 5% CO_2. Then the MCF-7 cells were treated with compound **7l** (0, 4, 8 and 16 μM) for 24 h. The cover slip with monolayer cells was inverted on the glass slide with 20 μL of AO/BE stain (100 μg mL^{-1}). The fluorescence was read using an IX71SIF-3 fluorescence microscope.[39]

Flow cytometry analysis

The MCF-7 cells were seeded into six-well plates at a concentration of 6×10^4 cells *per* mL. The cells were incubated overnight at 37 °C in a humidified atmosphere of 5% CO_2. Then the MCF-7 cells were treated with compound **7l** (0, 4, 8 and 16 μM) for 24 h. The cells were collected, washed twice in PBS, and resuspended in 120 μL of binding buffer. Then the cells were incubated with 5 μL of annexin V-FITC and 5 μL of propidium iodide (PI) staining

solution for 15 min at 4 °C in the dark (annexin V-FITC/PI apoptosis detection kit, Beyotime, Shanghai, China). The cells apoptosis analysis was examined by flow cytometry and system software (BD Biosciences, San Jose, CA, USA).[40]

Results and Discussion

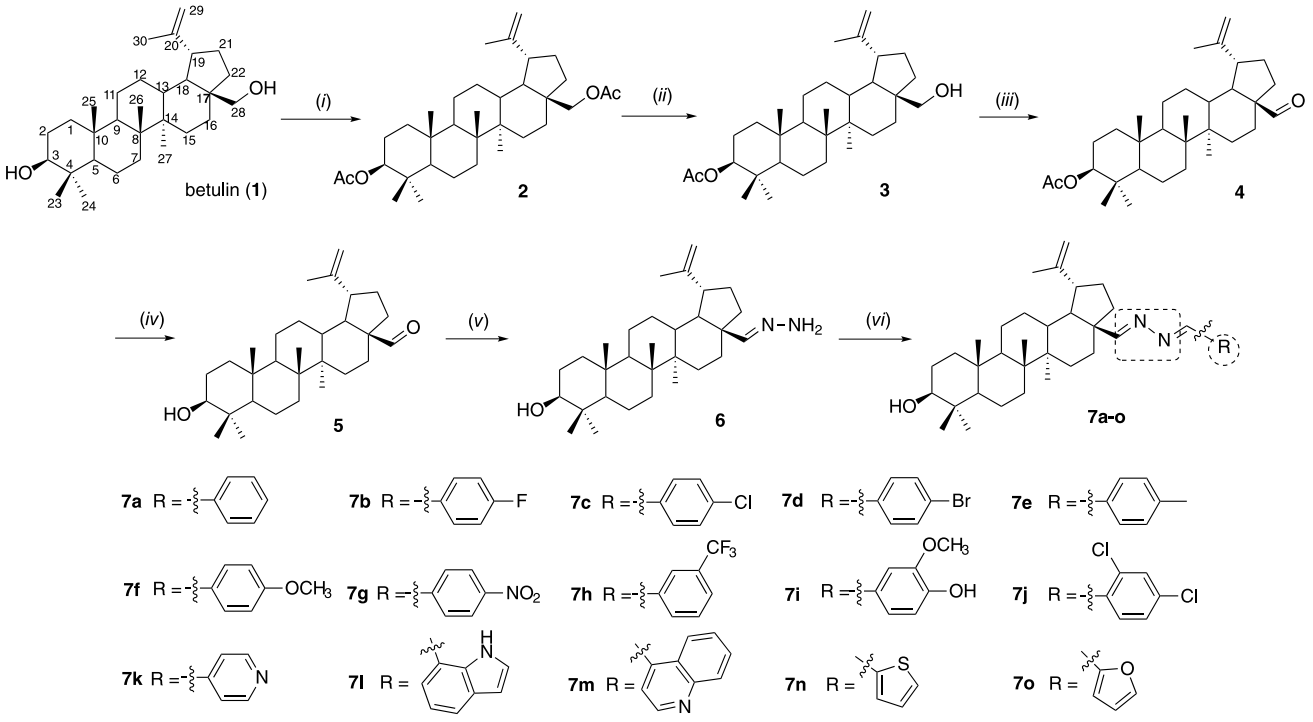
Chemistry

The general procedure for the synthesis of betulin derivatives is shown in Scheme 1. The 3-OH and 28-OH of betulin (**1**) were acetylated with acetic anhydride in the presence of DMAP (4-dimethylaminopyridine) in dry pyridine at room temperature to give compound **2**. Compound **2** further reacted with titanium propoxide (Ti(*i*-PrOH)$_4$) in dry isopropyl alcohol (*i*-PrOH) for selective deacetylation at C-28 to give compound **3**. Then the 28-OH of betulin was oxidized to a carbonyl group in the presence of pyridinium chlorochromate (PCC) in dry dichloromethane to give compound **4**. Subsequently, compound **4** was reacted with sodium hydroxide for deacetylation at C-3 to give compound **5**. Then, compound **5** was reacted with hydrazine hydrate in ethanol to obtain the corresponding hydrazine **6**. At last, the resulting hydrazine **6** was reacted with different aldehyde substituents in the presence of acetic acid in ethanol to obtain target novel betulin derivatives **7a**-**7o**. The structures of all new compounds were characterized by HRMS, ^{1}H NMR and ^{13}C NMR spectrum methods. Taking compound **7a** as a typical example, in the ^{1}H NMR spectrum, the chemical shifts of 8.50 (s, 1H) ppm and 8.12 (s, 1H) ppm demonstrate the formation of –CH=N–N=CH– group at side chain. In addition, the chemical shifts of two C=N bonds at 169.8 and 160.5 ppm in the ^{13}C NMR spectrum also demonstrate the formation of –CH=N–N=CH– group in compound **7a**.

Evaluation of antitumor activities

The *in vitro* cytotoxicities of all newly synthesized betulin derivatives **7a**-**7o** were evaluated using MTT assays against human hepatocellular carcinoma cells (HepG2), human breast carcinoma cells (MCF-7), human colorectal cells (HCT-116) and human lung carcinoma cells (A549). Mitomycin C was used as the positive drug control. The cytotoxicities of all compounds were summarized as IC_{50} values in Table 1. The results showed that some of the synthesized compounds displayed significant cytotoxicities toward all four tested human tumor cell lines. The results suggested the following rough structure-activity relationships considerations.

For HepG2 cell line, compounds **7g**, **7h**, **7j**, **7k** and **7l** displayed greater cytotoxic activities than betulin (IC_{50} = 20.60 μM). Compound **7l** possessing indole



Scheme 1. Synthesis of betulin derivatives **7a**-**7o**. Reagents and conditions: (*i*) acetic anhydride, DMAP, pyridine, r.t., 6 h, 80%; (*ii*) Ti(*i*-PrOH)$_4$, *i*-PrOH, 85 °C, 5 h; 76%; (*iii*) PCC, dichloromethane, 35 °C, 1 h, 80%; (*iv*) NaOH, methanol, 80 °C, 2 h, 65%; (*v*) hydrazine hydrate, ethanol, 40 °C, 5 h, 70%; (*vi*) ethanol, acetic acid, aldehyde, r.t., 2-5 h, 72-88%.

Table 1. Cytotoxicities of compounds **7a**-**7o** in human cancer cells

Compound	IC_{50}[a] / μM			
	HepG2	MCF-7	HCT-116	A549
7a	> 60	47.34 ± 4.34	> 60	> 60
7b	27.42 ± 0.85	27.09 ± 1.12	32.14 ± 2.13	36.84 ± 5.13
7c	21.42 ± 2.20	25.37 ± 1.33	31.15 ± 2.65	29.40 ± 1.92
7d	25.87 ± 1.10	12.24 ± 0.47	19.53 ± 0.64	32.26 ± 2.11
7e	34.05 ± 2.33	> 60	> 60	> 60
7f	> 60	> 60	> 60	> 60
7g	11.35 ± 0.88	10.64 ± 0.53	17.52 ± 1.13	26.77 ± 1.40
7h	14.50 ± 1.17	17.65 ± 0.92	12.55 ± 0.87	24.14 ± 1.13
7i	39.52 ± 1.44	23.80 ± 1.30	29.55 ± 1.62	40.27 ± 1.38
7j	12.93 ± 0.67	10.48 ± 0.61	16.63 ± 0.53	22.72 ± 2.02
7k	9.32 ± 0.47	8.76 ± 0.44	18.43 ± 1.22	28.55 ± 1.33
7l	8.60 ± 0.84	7.37 ± 0.38	14.24 ± 1.31	27.54 ± 1.55
7m	18.27 ± 1.23	14.88 ± 1.36	28.58 ± 0.70	> 60
7n	31.06 ± 3.14	28.17 ± 1.58	> 60	> 60
7o	34.25 ± 1.35	27.66 ± 1.91	31.54 ± 2.46	> 60
1	20.60 ± 1.14	19.67 ± 0.93	27.46 ± 1.33	31.12 ± 2.05
Mitomycin C	26.60 ± 1.30	13.03 ± 1.10	11.09 ± 0.78	12.36 ± 0.99

[a]IC_{50}: concentration of the tested compound that inhibits 50% of cell growth. All data are presented as means ± standard deviation (SD) of three independent experiments. HepG2: human hepatocellular carcinoma cells; MCF-7: human breast carcinoma cells; HCT-116: human colorectal cells; A549: human lung carcinoma cells.

group displayed significant cytotoxic activity with IC_{50} value of 8.60 μM. It is about 2.4-fold higher than betulin. Compounds **7g** and **7k** also displayed significant cytotoxic activities. The data showed that the incorporation of electron-donating group at the C28 of betulin led to significant improvement in cytotoxic activity than methyl or methoxy group. For MCF-7 cell line, compounds **7d**, **7g**, **7j**, **7k**, **7l** and **7m** also possessed stronger cytotoxicity than that of betulin, all IC_{50} values lower than 15 μM. Among them, compound **7l** (IC_{50} = 7.37 μM*)* was the most active, which was 2.7-fold more potent than betulin. The results suggested that the electron-donating substitution with aromatic hydrazone side chain at the C28 of betulin was beneficial for compounds which displayed significant cytotoxicity against MCF-7 cells. For HCT-116 cells, only compounds **7h** and **7l** exhibited moderate cytotoxic activities. For A549 cells, none of the compounds showed ideal inhibitory activity.

Among the compounds under biological study, compound **7l** was the most potent compound against HepG2 and MCF-7 cell lines, with IC_{50} values of 8.60 and 7.37 μM*,* respectively. One of the major indexes of a potent effective anti-cancer drug lies in that it can inhibit cancer cell growth, and subsequently induces apoptosis. To further investigate the cellular mechanism of this kind of new compounds, compound **7l** was chosen for subsequent biological functions experiments in MCF-7 cells.

Preliminary investigation of the apoptosis-inducing effect of compound **7l**

Firstly, the AO/EB staining of MCF-7 cells treated with compound **7l** was observed under a fluorescence microscope. A large number of normal cells in the control group were stained green and their nuclei were intact. As the concentration of compound **7l** increased (0, 4, 8 and 16 μM), some cells showed apoptotic characteristics such as chromosome pyknosis, fragmentation and sparse cytoplasm, and the number of cells gradually increased. Furthermore, the number of early apoptotic cells and late apoptotic cells also increased, the latter was characterized by the nucleus with EB staining, orange red, concentration or bias. The necrotic cells showed uneven orange-red fluorescence and were not clearly defined and disintegrated or nearly disintegrated. The results are shown in Figure 3.

In order to confirm whether apoptosis was induced by compound **7l** in tumor cells, the MCF-7 cells were doubly stained with annexin V-FITC and propidium iodide (PI). The MCF-7 cells were treated with compound **7l** at the indicated concentrations (0, 4, 8 and 16 μM) for 24 h, and the rates of apoptotic cells were detected by flow cytometry. As shown in Figure 4, after treatment with 4, 8 and 16 μM of **7l** for 24 h, the percentage of apoptosis cells was increased from 17.06 to 31.96%, while the control group was only 14.90%.

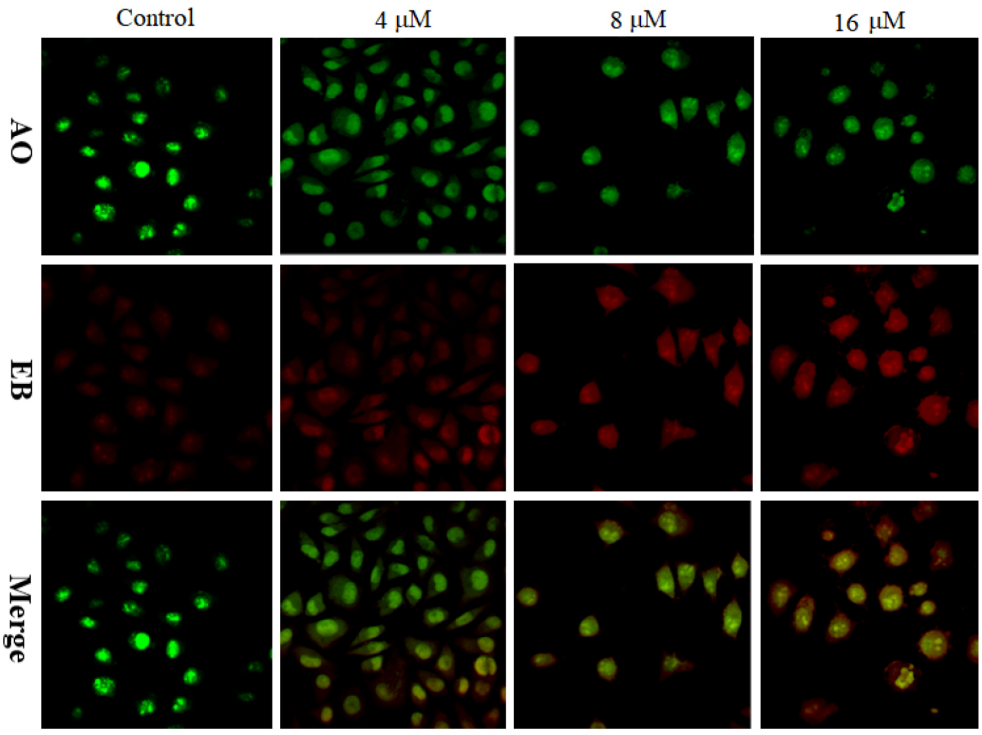


Figure 3. Compound **7l** induced apoptosis in MCF-7 cells. MCF-7 cells were treated with compound **7l** (0, 4, 8 and 16 μM) for 24 h and then stained with AO/EB.

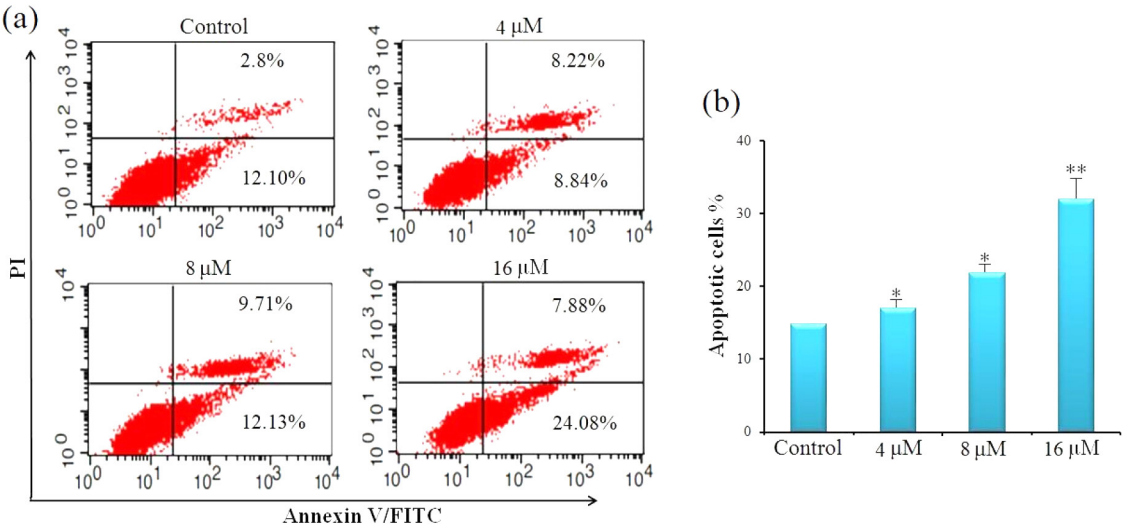


Figure 4. Compound **7l** induced MCF-7 cells apoptosis. (a) MCF-7 cells were treated with compound **7l** (4, 8 and 16 μM) for 24 h and then stained with annexin V-FITC/PI; (b) data were expressed as the mean ± SEM for three independent experiments. *$P < 0.05$, **$P < 0.01$ *versus* control (0 μM).

Notably, the apoptosis rate of MCF-7 cells treatment with compound **7l** increased in a dose-dependent manner. The above results suggested that compound **7l** could induce apoptosis in MCF-7 cells significantly.

Conclusions

In summary, according to the special structural features of betulin and hydrazone group, fifteen newly betulin derivatives with aromatic hydrazone side chain on the C-28 position were designed and synthesized. All compounds were evaluated for their *in vitro* cytotoxicities in four human carcinoma cells (HepG2, MCF-7, HCT-116 and A549). Among them, compound **7l** displayed the most potent antiproliferative with an IC_{50} value of 7.37 ± 0.38 μM against MCF-7 cells. Furthermore, the preliminary cellular mechanism studies indicated that compound **7l** could induce MCF-7 cells apoptosis. The above findings indicated

that compound **7l** may be used as a promising skeleton for antitumor agents with improved efficacy.

Supplementary Information

Supplementary file (containing the NMR and HRMS spectra for the synthesized compounds) is available free of charge at https://jbcs.sbq.org.br as PDF file.

Acknowledgments

We gratefully acknowledge the financial support by Central Government Support Fund for the Reform and Development of Local Universities-Talent Training Support Program Project (ZYZX2019) and Qiqihar Academy of Medical Sciences Project (QY-2016B-34).

Author Contributions

M. B. was responsible for conceptualization; J. F. W., J. L. W. and J. Z. for methodology and chemistry experiments; Y. L. H., H. J. W. and Y. L. for biology experiments; J. F. W. and J. W. for writing original draft; M. B. for writing-review and editing.

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Submitted: August 13, 2021
Published online: October 13, 2021