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 IC50 (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. The most effective of therapies used in cancer treatment continue to be traditional cytotoxic agents. 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. 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). Betulin has been shown to exert various pharmacological and biological activities, such as antibacterial, anti-HIV (human immunodeficiency virus) and anti-inflammatory properties. Recently, a plenty of studies 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.

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. 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. 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. 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.

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 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, Fujian, China) and were not corrected. 1H and 13C nuclear magnetic resonance (NMR) spectra were recorded with Bruker A V ANCE NEO600 spectrometer (tetramethylsilane (TMS) as internal standard) (Bruker, Berlin, Germany). Reaction progress was real-time monitored by TLC using F254 silica gel plates (Biohonor, Guangzhou, China). The intermediates and target compounds were purified by flash column chromatography using ethyl acetate/petroleum ether = 1/7) to give 2 (80%). White solid; mp 216.0-217.5 °C; 1H NMR (600 MHz, CDCl3) 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), 2.45 (d, 1H, J 10.9 Hz, CH–O), 3.85 (d, 1H, J 11.0 Hz, CH–O), 2.44 (td, 1H, J 11.1, 5.8 Hz, H3), 2.07 (s, 3H, CH3–COO), 2.04 (s, 3H, CH3–COO), 1.68 (s, 3H, CH3–C=C), 1.03 (s, 3H, –CH3), 0.97 (s, 3H, –CH3), 0.87-0.82 (m, 9H, –CH3 × 3), 0.78 (d, 1H, J 9.3 Hz); 13C NMR (150 MHz, CDCl3) 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 CH2Cl2 (50 mL) and water (50 mL). The filtration was washed with sat. NaHCO3. The combined solution was washed with brine twice and dried over anhydrous Na2SO4. The solvent was evaporated and purified by silica gel column chromatography (ethyl acetate/petroleum ether = 1/7) to give 2 (80%). White solid; mp 216.0-217.5 °C; 1H NMR (600 MHz, CDCl3) 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), 2.45 (d, 1H, J 10.9 Hz, CH–O), 3.85 (d, 1H, J 11.0 Hz, CH–O), 2.44 (td, 1H, J 11.1, 5.8 Hz, H3), 2.07 (s, 3H, CH3–COO), 2.04 (s, 3H, CH3–COO), 1.68 (s, 3H, CH3–C=C), 1.03 (s, 3H, –CH3), 0.97 (s, 3H, –CH3), 0.87-0.82 (m, 9H, –CH3 × 3), 0.78 (d, 1H, J 9.3 Hz); 13C NMR (150 MHz, CDCl3) 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 CH2Cl2 (50 mL) and water (50 mL). The filtration was washed with brine twice and dried over anhydrous Na2SO4. The solvent was evaporated and purified by silica gel column chromatography (ethyl acetate/petroleum ether = 1/7) to give 2 (80%). White solid; mp 256.1-257.8 °C; 1H NMR (600 MHz, CDCl3) 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, CH2–O), 3.33 (d, 1H, J 10.8 Hz, CH–O), 2.38 (td, 1H, J 11.0, 5.8 Hz), 2.04 (s, 3H, CH3–COO), 1.69 (s, 3H, CH3–C=C), 1.02 (s, 3H, –CH3), 0.97 (s, 3H, –CH3), 0.88-0.81 (m, 9H, –CH3 × 3); 13C NMR (150 MHz, CDCl3) 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-betulinaldehyde (4)

To a solution of 3-O-acetyl-betulin (3, 300 mg, 0.6 mmol) in CH2Cl2 (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; 1H NMR (600 MHz, CDCl3) 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), and then redissolved with CH2Cl2 (100 mL), washed with sat. NaHCO3. The combined solution was washed with brine twice and dried over anhydrous Na2SO4. The solvent was evaporated and purified by silica gel column chromatography (ethyl acetate/petroleum ether = 1/50) to give 2 (2.95 g, 80%).
2.86 (td, 1H, J 11.2, 5.9 Hz), 2.04 (s, 3H, CH3–COO), 1.70 (s, 3H, –CH3), 0.97 (s, 3H, –CH3), 0.92 (s, 3H, –CH3), 0.84 (d, 9H, J 8.5 Hz, –CH3 × 3); 13C NMR (150 MHz, CDCl3) 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: 483.4.

Betulinaldehyde (5)

The solution of 3-O-acetyl-betulinaldehyde (4, 200 mg, 0.4 mmol) in 2% NaOH-MeOH (10 mL) was stirred for 2 h at 80°C. The solvent was evaporated and then redissolved with CH2Cl2 (100 mL), washed with brine twice and dried over anhydrous Na2SO4. 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; 1H NMR (600 MHz, CDCl3) 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 11.2, 5.9 Hz, C=C–CH–), 1.70 (s, 3H, CH3–C=C), 0.97 (s, 3H, –CH3), 0.92 (s, 3H, –CH3), 0.84 (d, 9H, J 8.5 Hz, –CH3 × 3); 13C NMR (150 MHz, CDCl3) δ 206.7, 171.0, 149.7, 110.2, 79.0, 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]+: 445.4.

General procedure for synthesis of compounds 7a-7o

To a solution of 28-hydrazonomethyl-betulin (6, 1 mmol) in 2% NaOH-MeOH (10 mL) was stirred for 2 h at 80°C. The solvent was evaporated and purified by silica gel column chromatography (ethyl acetate/petroleum ether = 1/10) to give 7a (116 mg, 65%). White solid; mp 285.3-287.2°C; 1H NMR (600 MHz, CDCl3) 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, CH3), 1.69-1.10 (m, 18H), 1.00 (s, 3H, CH3), 0.97 (m, 6H, CH3), 0.87 (m, 2H), 0.81 (s, 3H, CH3), 0.75 (m, 3H, CH3), 0.68 (d, 1H, J 11.2 Hz); 13C NMR (150 MHz, CDCl3) δ 196.8 (C=N), 150.0 (C-20), 143.1 (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 C37H54N2O [M + H]+: 543.4314, found: 543.4312.

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

Pale yellow solid; 75%; 1H NMR (600 MHz, CDCl3) 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
Pale yellow solid; 79%; ¹H NMR (600 MHz, CDCl₃) 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), 4.39 (d, 1H, J 11.5, 4.6 Hz), 2.73-2.61 (m, 1H), 2.12-2.04 (m, 1H), 0.97 (s, 1H, J 2.6 Hz), 0.75 (d, 3H, J 2.6 Hz), 0.71-0.64 (m, 1H); ¹C NMR (150 MHz, CDCl₃) δ 169.7 (C-28), 160.6 (C-20), 135.9 (C-Ar), 133.8 (C-Ar), 129.7 (C-Ar), 125.2 (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-31), 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 (s, 3H, CH₃), 0.76 (3H, CH₃), 0.75 (d, 3H, J 2.6 Hz), 0.71-0.64 (m, 1H); ¹C NMR (150 MHz, CDCl₃) δ 169.7 (C-28), 160.6 (C-20), 135.9 (C-Ar), 133.8 (C-Ar), 129.7 (C-Ar), 125.2 (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-31), 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 (s, 3H, CH₃), 0.76 (3H, CH₃), 0.75 (d, 3H, J 2.6 Hz), 0.71-0.64 (m, 1H); HRMS (ESI) m/z, calcd. for C₃₅H₅₇N₃O₃: 628.4240, found: 628.4162.
0.90 (s, 6H, CH₃), 0.83 (m, 2H), 0.74 (s, 3H, CH₃), 0.68 (s, 3H, CH₃), 0.62 (d, 1H, J 11.2 Hz); ¹³C NMR (150 MHz, CDCl₃) 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 (CF₃), 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, calcld. for C₃₈H₅₆N₂O₃ [M + H]⁺: 589.4188, found: 589.4185.

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

Pale yellow solid; 84%; ¹H NMR (600 MHz, CDCl₃) 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-CH), 4.73 (d, 1H, J 1.6 Hz), 4.62 (s, 1H), 3.97 (d, 3H, J 3.4 Hz, OCH₃), 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, CH₃), 1.69-1.06 (m, 17H), 1.01 (s, 3H, CH₃), 0.97 (s, 6H, CH₃), 0.94-0.86 (m, 2H), 0.81 (s, 3H, CH₃), 0.75 (s, 3H, CH₃), 0.68 (d, 1H, J 11.2 Hz); ¹³C NMR (150 MHz, CDCl₃) 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 (OCH₃), 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, calcld. for C₃₉H₅₅F₃N₂O [M + H]⁺: 544.4268, found: 544.4264.

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

White solid; 80%; ¹H NMR (600 MHz, CDCl₃) 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, CH₃), 1.69-1.07 (m, 17H), 1.01 (s, 3H, CH₃), 0.97 (s, 6H, CH₃), 0.93-0.86 (m, 2H), 0.81 (s, 3H, CH₃), 0.76 (s, 3H, CH₃), 0.69 (d, 1H, J 11.1 Hz); ¹³C NMR (150 MHz, CDCl₃) 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), 32.5 (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, calcld. for C₃₉H₅₅F₃N₂O [M + H]⁺: 582.4421, found: 582.4420.

Betulin-28-(quinalino-4-yldimethylene)hydrazone (7m)

White solid; 78%; ¹H NMR (600 MHz, CDCl₃) 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,
Betulin-28-(thiophene-2-ylmethylene)hydrazone (7n)

White solid; 73%; 1H NMR (600 MHz, CDCl3) 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, CH3), 1.69-1.06 (m, 17H), 1.01 (s, 3H, CH3), 0.96 (s, 6H, CH3), 0.90 (m, 2H), 0.80 (s, 3H, CH3), 0.74 (s, 3H, CH3), 0.68 (d, 1H, J 11.0 Hz); 13C NMR (150 MHz, CDCl3) δ 171.0 (C-28), 154.6 (C=N), 150.0 (C-20), 138.8 (C-Ar), 132.0 (C-Ar), 127.7 (C-Ar), 124.0 (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 C35H52N2O2 [M+H]+: 594.4420, found: 594.4420.

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

Pale yellow solid; 72%; 1H NMR (600 MHz, CDCl3) 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 (d, 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, CH3), 1.68-1.05 (m, 18H), 1.00 (s, 3H, CH3), 0.97 (s, 3H, CH3), 0.93 (s, 3H, CH3), 0.90 (m, 2H), 0.80 (s, 3H, CH3), 0.75 (s, 3H, CH3), 0.68 (d, J 11.0 Hz, 1H); 13C NMR (150 MHz, CDCl3) 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 C35H52N2OS [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% CO2. Cytotoxic activities of all tested compounds against four cancer cell lines were evaluated by 3-(4,5-dimethylthiazol-2-y)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded into 96-well plates (1 × 10⁴ 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 IC₅₀ (concentration of the tested compound that inhibits 50% of cell growth) values were derived by SPSS™ 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 × 10⁴ cells per mL. The cells were incubated overnight at 37 °C in a humidified atmosphere of 5% CO₂. 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/EB stain (100 μg mL⁻¹). The fluorescence was read using an IX71SIF-3 fluorescence microscope.

Flow cytometry analysis

The MCF-7 cells were seeded into six-well plates at a concentration of 6 × 10⁴ cells per mL. The cells were incubated overnight at 37 °C in a humidified atmosphere of 5% CO₂. 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).  

**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){sub 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.

![Scheme 1](image)

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){sub 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%.

The structures of all new compounds were characterized by HRMS, 1H NMR and 13C NMR spectrum methods. Taking compound 7a as a typical example, in the 1H 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 13C 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...
group displayed significant cytotoxic activity with IC\textsubscript{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\textsubscript{50} values lower than 15 μM. Among them, compound 7l (IC\textsubscript{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\textsubscript{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.

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

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

\textsuperscript{4}IC\textsubscript{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.

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. 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%.
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 hydrazine 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 71 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.

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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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