Novel rotundic acid derivatives: Synthesis, structural characterization and *in vitro* antitumor activity

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Received September 25, 2012; Accepted November 16, 2012

DOI: 10.3892/ijmm.2012.1206

Abstract. Six novel rotundic acid (RA, 1) derivatives 4a-4f modified at the 28-COOH position were synthesized, and their structures were confirmed by IR, MS, ^{1}H NMR and ^{13}C NMR. The derivatives were evaluated for cytotoxic properties on the following three tumor cell lines: HeLa, HepG2 and SGC-7901. Compound 4f showed better cytotoxic activity compared with RA treatment and lower IC_{50} (4.16 μM) on HepG2 cells than on HeLa (8.54 μM) and SGC-7901 cells (11.32 μM). The anticancer mechanism of compound 4f was studied through cell cycle progression and apoptosis. Notably, compound 4f was able to induce apoptosis and G0/G1 cell cycle arrest of HepG2 at a concentration of 4.16 μM. In summary, RA was modified to obtain six novel derivatives. Compound 4f exhibited better cytotoxicity and may be developed as a potential agent against hepatocellular carcinoma.

Introduction

Hepatocellular carcinoma (HCC) is the fifth-most common malignant tumor in the world (1). The incidence of HCC has been on the rise in recent years (1,2), and more than 70% of all newly diagnosed cases of liver cancer occur in Asia (3). The treatment for HCC usually involves surgical resection or liver transplantation with curative options for the patients, when the disease is diagnosed at an early stage (4). However, only 30% of the patients are eligible for the curative treatment, and recurrence is a common concern affecting up to 70% of the patients after tumor ablation (5). Only a few non-curative treatment options exist for such patients. Some effective treatment modalities include: ethanol ablation, radiofrequency ablation, transarterial chemoembolization, and selective radiation of lesions (6). Therefore, new therapeutic agents for the treatment of hepatoma need to be explored.

Rotundic acid (RA, 1) (Fig. 1) is one of the pentacyclic triterpenoids, mainly found in *Ilex rotunda*, *Ilex purpurea* and other *Aquifoliaceae* plants (7-10). RA can also be isolated from *Mussaenda Pubescens*, *Olea europaea* and *Planchonella duclitan* (11-13). RA and its derivatives inhibit cell growth. Due to its unstable nature during the metabolic processes, RA induces serious gastrointestinal adverse effects. A considerable number of patents on RA and its derivatives have been applied by our research group in the recent years. Our studies explored these novel compounds extensively and focused on reducing these adverse effects (14-17). In our previous study, eight amino acid derivatives of RA at the 28-COOH position were synthesized, and their *in vitro* cytotoxic properties were evaluated on three tumor cell lines, A375 (human malignant melanoma cells), HepG2 (human hepatoma cells), and NCI-H446 (human small cell lung cancer cells) (18).

The present study aimed at synthesizing RA derivatives by making structural modifications at the 28-COOH position of RA. The synthesized compounds were characterized and their cytotoxic effects in the three cell lines, HeLa (human cervical cancer), HepG2 (human hepatoma cell) and SGC-7901 (human gastric carcinoma), were studied.

Materials and methods

Based on the natural structure of RA, six new derivatives 4a-4f were designed and synthesized in this study to improve its bioactivity. Their structures were elucidated on the basis of spectroscopic assays such as IR, MS, ^{1}H NMR and ^{13}C NMR. The cytotoxicity and antitumor effects of RA derivatives were assayed by MTT colorimetric assay with the HeLa, HepG2 and SGC-7901 cell lines. Cell cycle distribution and measurement of the percentage of apoptotic cells were performed by flow cytometry, following staining with propidium iodide (PI) and Annexin V-FITC.

Reagents and cell culture. Reagent-grade chemicals and solvents were obtained from Sigma Chemicals, Munich, Germany. All solvents were freshly distilled and dried prior to use, according to the standard procedures. The HeLa, HepG2 and SGC-7901 cell lines were purchased from the Tumor Center of the Chinese

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Key words: antitumor activity, apoptosis, characterization, cytotoxicity, derivative, rotundic acid, synthesis

Academy of Medical Sciences, Beijing, China. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37˚C in a humidified atmosphere of 5% CO_2.

Preparation of RA. The preparation of RA was reported in our previous study (18). The purity of RA used was ≥98% (HPLC assay) and the extraction yield of RA in our study was up to 100 mg/g, which made it suitable for industry production. Melting points were determined on a Fisher-Johns apparatus and were uncorrected. IR spectra were recorded on a Perkin-Elmer 983G spectrometer. NMR spectra were measured in C_5D_5N on a Bruker AM-400 spectrometer, using tetramethylsilane as an internal standard. Coupling constants (*J* values) were given in Hz, and an MS Agilent 1100 Series LC/MSD ion-trap mass spectrometer was used to record the HR-ESI-MS.

Structure modification of RA and synthesis of derivatives. The synthetic routes to the RA derivatives are outlined in Fig. 2. Firstly, RA (1) was converted to its 28-ethyl acetate (2), which was then treated with hydrazine hydrate to give the 28-acetohydrazide (3). It was then reacted with the appropriate aldehyde (benzaldehyde, p-methyl benzaldehyde, p-chloride benzaldehyde, m-nitryl benzaldehyde, p-nitryl benzaldehyde, p-methoxy benzaldehyde) in the presence of glacial acetic acid to give the benzaldehyde {2-[(3β,19α,23-trihydroxy-urs-12-en-28-oyl)-hydroxy]-acetic} hydrazone 4a-4f. The structures of these synthesized compounds were confirmed by IR, MS, ^{1}H NMR and ^{13}C NMR (25-28). All six compounds obtained were synthesized in high yields with purities of 98% or better and are reported for the first time.

Procedure for the preparation of ethyl 2-[(3β,19α,23-trihydroxy-urs-12-en-28-oyl)-hydroxy]-acetate (2). RA (10 mmol) was dissolved in acetone (500 ml) and heated to clarity. Potassium carbonate (100 mmol), potassium iodide (0.6 mmol), and triethylamine (6 ml) were added to it. Ethyl chloroacetate (5 ml) was then added drop-wise and refluxed for 24 h. The refluxed content was filtered, and the filtrate was concentrated to 100 ml. Saturated salt water (100 ml) was added and was extracted with ethyl acetate five times (30 ml each time). It was then washed with saturated sodium carbonate and water four times (25 ml each time). Anhydrous sodium sulfate was added and filtered. The filtrate was evaporated to dryness. The crude was purified by column chromatography on a silica gel column with petroleum ether, ethyl acetate, and formic acid as eluents to give compound 2 as a white powder. The yield was 83.9%, and its melting point was between 133 and 135˚C. IR(KBr) cm^{-1}: 3545, 3404, 3250, 2929, 2882, 1765, 1715, 1634, 1469, 1455, 1381, 1207, 1148, 1055 and 1005; ^{1}H NMR (400 MHz, C_5D_5N) δ: 5.40 (1H, m, H-12), 5.06 (1H, m, H-3), 4.78 (2H, dd, *J*=15.6, 27.6 Hz, H-2'), 4.07 (2H, q, *J*=7.2Hz, H-1''), 4.06 (1H, d, *J*=10.4 Hz, H-23a), 3.61 (1H, d, *J*=10.4 Hz, H-23b), 2.75 (1H, br s, H-18), 1.51 (3H, s, CH_3-25), 1.26 (3H, s, CH_3-27), 1.03 (3H, t, *J*=7.2 Hz, CH_3-2''), 0.97 (3H, d, *J*=6.64 Hz, CH_3-30), 0.96 (3H, s, CH_3-29), 0.93 (3H, s, CH_3-26), 0.82 (3H, s, CH_3-24); ^{13}C NMR (100 MHz, C_5D_5N) δ: 177.7 (C-28), 139.4 (C-13), 128.6 (C-12), 73.7 (C-3), 72.7 (C-19), 68.1 (C-23), 54.5 (C-18), 48.8 (C-5), 48.8 (C-9), 47.8 (C-17), 43.0 (C-20), 42.2 (C-14), 42.1 (C-8), 40.5 (C-1), 39.0 (C-4), 38.0 (C-22), 37.3 (C-10), 33.3 (C-7), 29.1 (C-15), 27.8 (C-21), 27.0 (C-29), 26.8 (C-2), 26.3 (C-16), 24.7 (C-27), 24.2 (C-11), 18.8 (C-6), 17.2 (C-25), 16.8 (C-26), 16.2 (C-30), 13.2 (C-24), 61.3 (C-2'), 168.7 (C-1'), 61.0 (C-1''), 14.3 (C-2''). HR-ESI-MS found 575.3953. Calculated 575.3948 for $C_{34}H_{55}O_7$ [(M+H)+].

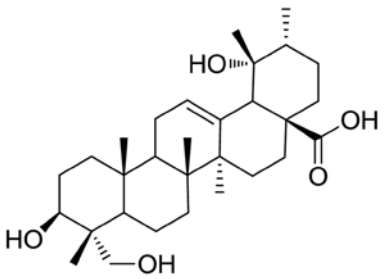

Figure 1. Chemical structure of rotundic acid.

Procedure for the preparation of 2-[(3β,19α,23-trihydroxy-urs-12-en-28-oyl)-hydroxy]-acetic hydrazide (3). Compound 2 (15 mmol) was dissolved in absolute ethyl alcohol (EtOH) (90 ml) and 80% hydrazine hydrate (7.5 ml) was added. The mixture was refluxed for 11 h and the solvent was removed under reduced pressure using a rotary evaporator to 50 ml. Water (250 ml) was added and extracted with chloroform five times (50 ml each time). It was then washed with saturated salt water four times (50 ml each time). Anhydrous sodium sulfate was added and filtered. The filtrate was evaporated to dryness to yield crude, which was then purified by column chromatography on a silica gel column with petroleum ether and ethyl acetate as eluents to give compound 3 as colorless needles. The yield was 97.7% and its melting point was between 225 and 226˚C. IR(KBr) cm^{-1}: 3624, 3398, 3335, 2930, 2876, 1731, 1683, 1450, 1387, 1206, 1147, 1046 and 756; ^{1}H NMR (400 MHz, C_5D_5N) δ: 9.76 (2H, s, -NH2), 7.45 (1H, s, -NH), 5.43 (1H, m, H-12), 5.04 (1H, m, H-3), 4.85 (2H, dd, *J*=14.2, 33.2 Hz, H-1'), 4.06 (1H, d, *J*=10.4 Hz, H-23a), 3.60 (1H, d, *J*=10.4 Hz, H-23b), 2.71 (1H, br s, H-18), 1.50 (3H, s, CH_3-25), 1.23 (3H, s, CH_3-27), 0.96 (3H, s, CH_3-29), 0.94 (3H, d, *J*=6.64 Hz, CH_3-30), 0.90 (3H, s, CH_3-26), 0.79 (3H, s, CH_3-24); ^{13}C NMR (100 MHz, C_5D_5N) δ: 177.5 (C-28), 139.7 (C-13), 128.5 (C-12), 73.6 (C-3), 72.7 (C-19), 68.1 (C-23), 54.5 (C-18), 48.8 (C-5), 48.7 (C-9), 47.8 (C-17), 43.0 (C-20), 42.1 (C-14), 42.1 (C-8), 40.4 (C-1), 39.0 (C-4), 38.0 (C-22), 37.3 (C-10), 33.2 (C-7), 29.1 (C-15), 27.8 (C-21), 27.0 (C-29), 26.8 (C-2), 26.2 (C-16), 24.7 (C-27), 24.1 (C-11), 18.8 (C-6), 17.2 (C-25), 16.7 (C-26), 16.1 (C-30), 13.2 (C-24), 62.8 (C-1'), 168.2 (C-1''). HR-ESI-MS found 561.3887. Calculated 561.3898 for $C_{32}H_{53}N_2O_6$ [(M+H)+].

General procedure for the preparation of benzaldehyde {2-[(3β,19α,23-trihydroxy-urs-12-en-28-oyl)-hydroxy]-acetic} hydrazone 4a-4f.

Benzaldehyde {2-[(3β,19α,23-trihydroxy-urs-12-en-28-oyl)-hydroxy]-acetic} hydrazone (4a, $C_{39}H_{56}N_2O_6$, R_1=C_6H_5, R_2=H). Compound 3 (2 mmol) was dissolved in absolute EtOH (30 ml) and benzaldehyde (0.3 ml) was added. Glacial acetic acid (0.2 ml) was added drop-wise and the mixture was refluxed for 8 h. Water (100 ml) was then added, stirred

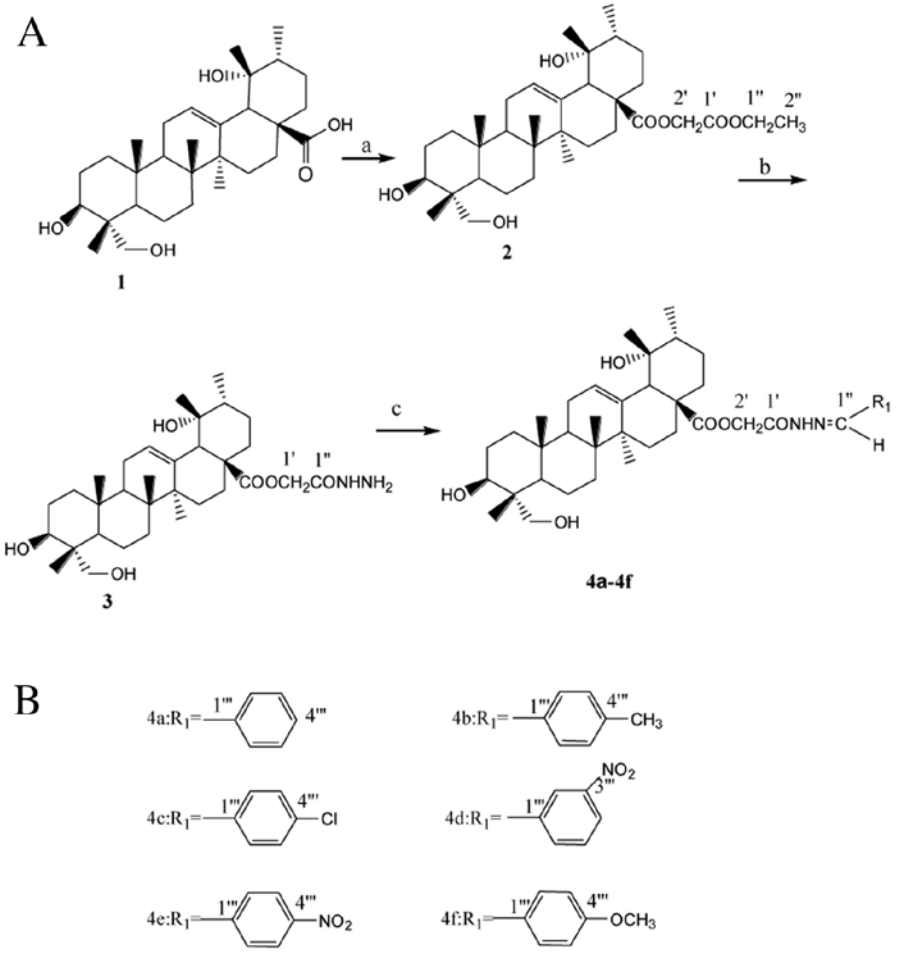

Figure 2. (A) Synthesis of RA derivatives. Reagents and conditions: (a) acetone/potassium carbonate/potassium iodide/triethylamine/ethyl chloroacetate/ refluxed/24 h; (b) hydrazine hydrate/ethanol/refluxed/11 h; (c) R_1COH/glacial acetic acid/refluxed/8 h. (B) Six novel compounds 4a-4f.

and filtered. The crude obtained was then purified on a silica gel column with petroleum ether and ethyl acetate as eluents to yield white needles. The yield was 80.3%, and its melting point was between 169 and 171˚C. IR(KBr) cm^{-1}: 3619, 3425, 2929, 2876, 1693, 1449, 1388, 1204, 1140, 1047, 755 and 695; ^{1}H NMR (400 MHz, C_5D_5N) δ: 12.50 (1H, s, -NH), 8.05 (1H, s, H-1''), 7.64 (2H, d, *J*=8.0 Hz, H-2''', 6'''), 7.25 (2H, t, *J*=8.0 Hz, H-3''', 5'''), 7.21 (1H, t, *J*=8.0 Hz, H-4'''), 5.51 (2H, dd, *J*=15.9, 26.9 Hz, H-2'), 5.47 (1H, m, H-12), 4.98 (1H, m, H-3), 4.06 (1H, d, *J*=10.4 Hz, H-23a), 3.61 (1H, d, *J*=10.4 Hz, H-23b), 2.90 (1H, br s, H-18), 1.55 (3H, s, CH_3-25), 1.28 (3H, s, CH_3-27), 0.97 (3H, s, CH_3-29), 0.96 (3H, d, *J*=6.64 Hz, CH_3-30), 0.94 (3H, s, CH_3-26), 0.90 (3H, s, CH_3-24); ^{13}C NMR (100 MHz, C_5D_5N) δ: 177.9 (C-28), 139.6 (C-13), 128.5 (C-12), 73.7 (C-3), 72.8 (C-19), 68.2 (C-23), 54.5 (C-18), 49.0 (C-5), 48.8 (C-9), 47.9 (C-17), 43.0 (C-20), 42.3 (C-14), 42.2 (C-8), 40.5 (C-1), 39.0 (C-4), 38.3 (C-22), 37.3 (C-10), 33.3 (C-7), 30.1 (C-15), 29.3 (C-21), 27.8 (C-29), 27.0 (C-2), 26.4 (C-16), 24.7 (C-27), 24.2 (C-11), 18.8 (C-6), 17.4 (C-25), 16.9 (C-26), 16.2 (C-30), 13.2 (C-24), 61.8 (C-2'), 169.5 (C-1'), 144.0 (C-1''), 135.0 (C-1'''), 129.2 (C-3''', 5'''), 127.6 (C-2''', 6'''), 130.3 (C-4'''). HR-ESI-MS found 649.4211. Calculated 649.4212 for $C_{39}H_{57}N_2O_6$ [(M+H)+].

4-methyl-benzaldehyde {2-[(3β,19α,23-trihydroxy-urs-12-en-28-oyl)-hydroxy]-acetic} hydrazone (4b, $C_{40}H_{58}N_2O_6$, R_1=$CH_3C_6H_4$, R_2=H). Compound 3 was reacted with p-methyl benzaldehyde using the general procedure to give compound 4b, which was eluted with petroleum ether/ethyl acetate (V/V)=1:5 to give white powder. The yield was 87.2% and its melting point was between 210 and 211˚C. IR(KBr) cm^{-1}: 3620, 3275, 2928, 2874, 1739, 1688, 1468, 1388, 1229, 1149, 1046, 813 and 514; ^{1}H NMR (400 MHz, C_5D_5N) δ: 12.43 (1H, s, -NH), 8.04 (1H, s, H-1''), 7.57 (2H, d, *J*=8.0 Hz, H-2''',6'''), 7.08 (2H, d, *J*=8.0 Hz, H-3''', 5'''), 5.54 (2H, dd, *J*=15.8, 27.0 Hz, H-2'), 5.46 (1H, m, H-12), 4.97 (1H, m, H-3), 4.07 (1H, d, *J*=10.4 Hz, H-23a), 3.61 (1H, d, *J*=10.4 Hz, H-23b), 2.90 (1H, br s, H-18), 2.10 (3H, s, 4'''-CH_3), 1.55 (3H, s, CH_3-25), 1.28 (3H, s, CH_3-27), 0.97 (3H, s, CH_3-29), 0.96 (3H, d, *J*=6.64 Hz, CH_3-30), 0.91 (3H, s, CH_3-26), 0.89 (3H, s, CH_3-24); ^{13}C NMR (100 MHz, C_5D_5N) δ: 177.9 (C-28), 139.6 (C-13), 128.5 (C-12), 73.7 (C-3), 72.8 (C-19), 68.2 (C-23), 54.5 (C-18), 49.0 (C-5), 48.8 (C-9), 47.9 (C-17), 43.0 (C-20), 42.3 (C-14), 42.2 (C-8), 40.5 (C-1), 39.0 (C-4), 38.3 (C-22), 37.3 (C-10), 33.3 (C-7), 30.1 (C-15), 29.3 (C-21), 27.8 (C-29), 27.1 (C-2), 26.4 (C-16), 24.7 (C-27), 24.2 (C-11), 18.9 (C-6), 17.4 (C-25), 16.8 (C-26), 16.2 (C-30), 13.2 (C-24), 61.8 (C-2'), 169.4 (C-1'), 144.2 (C-1''), 132.3 (C-1'''), 129.9 (C-3''', 5'''), 127.6 (C-2''', 6'''), 140.3 (C-4'''), 21.4 (4'''-CH_3). HR-ESI-MS found 663.4388. Calculated 663.4368 for $C_{40}H_{59}N_2O_6$ [(M+H)+].

4-chloride-benzaldehyde {2-[(3β,19α,23-trihydroxy-urs-12-en-28-oyl)-hydroxy]-acetic} hydrazone (4c, $C_{39}H_{55}ClN_2O_6$, R_1=p-ClC_6H_4, R_2=H). Compound 3 was reacted with p-chlorine benzaldehyde using the general procedure to give compound 4c, which was eluted with petroleum ether/ethyl acetate (V/V)=1:5 to give white needles. The yield was 91.0% and its melting point was between 205 and 207˚C. IR(KBr) cm^{-1}: 3620, 3440, 2930, 2876, 1699, 1459, 1388, 1259, 1140, 1046, 826 and 515; ^{1}H NMR (400 MHz, C_5D_5N) δ: 12.60 (1H, s, -NH), 7.98 (1H, s, H-1''), 7.57 (2H, d, *J*=8.8 Hz, H-2''', 6'''), 7.31 (2H, d, *J*=8.8 Hz, H-3''', 5'''), 5.49 (2H, dd, *J*=15.8, 28.4 Hz, H-2'), 5.43 (1H, m, H-12), 5.00 (1H, m, H-3), 4.08 (1H, d, *J*=10.4 Hz, H-23a), 3.61 (1H, d, *J*=10.4 Hz, H-23b), 2.90 (1H, br s, H-18), 1.55 (3H, s, CH_3-25), 1.28 (3H, s, CH_3-27), 0.97 (3H, s, CH_3-29), 0.96 (3H, d, *J*=6.64 Hz, CH_3-30), 0.94 (3H, s, CH_3-26), 0.91 (3H, s, CH_3-24); ^{13}C NMR (100 MHz, C_5D_5N) δ: 177.9 (C-28), 139.6 (C-13), 128.5 (C-12), 73.7 (C-3), 72.8 (C-19), 68.2 (C-23), 54.5 (C-18), 49.0 (C-5), 48.8 (C-9), 47.9 (C-17), 43.0 (C-20), 42.3 (C-14), 42.2 (C-8), 40.5 (C-1), 39.0 (C-4), 38.3 (C-22), 37.3 (C-10), 33.3 (C-7), 30.1 (C-15), 29.3 (C-21), 27.8 (C-29), 27.1 (C-2), 26.4 (C-16), 24.7 (C-27), 24.2 (C-11), 18.9 (C-6), 17.4 (C-25), 16.8 (C-26), 16.2 (C-30), 13.2 (C-24), 61.7 (C-2'), 169.6 (C-1'), 142.6 (C-1''), 129.2 (C-1'''), 129.4 (C-3''', 5'''), 128.9 (C-2''', 6'''), 133.7 (C-4'''). HR-ESI-MS found 683.3821. Calculated 683.3821 for $C_{39}H_{56}ClN_2O_6$ [(M+H)+].

3-nitryl-benzaldehyde {2-[(3β,19α,23-trihydroxy-urs-12-en-28-oyl)-hydroxy]-acetic} hydrazone (4d, $C_{39}H_{55}N_3O_8$, R_1=m-$NO_2C_6H_4$, R_2=H). Compound 3 was reacted with m-nitryl benzaldehyde using the general procedure to give compound 4d, which was eluted with petroleum ether/ethyl acetate (V/V)=1:5 to give white needles. The yield was 93.0% and its melting point was between 175 and 177˚C. IR(KBr) cm^{-1}: 3423, 2929, 2876, 1702, 1534, 1450, 1388, 1352, 1228, 1140, 1045, 736 and 678; ^{1}H NMR (400 MHz, C_5D_5N) δ: 12.88 (1H, s, -NH), 8.46 (1H, s, H-1''), 8.11 (1H, brs, H-2'''), 8.06 (1H, d, *J*=8.0 Hz, H-4'''), 8.06 (1H, d, *J*=8.0 Hz, H-5'''), 7.37 (1H, t, *J*=8.0 Hz, H-6'''), 5.52 (2H, dd, *J*=15.8, 24.3 Hz, H-2'), 5.46 (1H, m, H-12), 4.97 (1H, m, H-3), 4.08 (1H, d, *J*=10.4 Hz, H-23a), 3.61 (1H, d, *J*=10.4 Hz, H-23b), 2.90 (1H, br s, H-18), 1.55 (3H, s, CH_3-25), 1.29 (3H, s, CH_3-27), 0.97 (3H, s, CH_3-29), 0.96 (3H, d, *J*=6.64 Hz, CH_3-30), 0.96 (3H, s, CH_3-26), 0.92 (3H, s, CH_3-24); ^{13}C NMR (100 MHz, C_5D_5N) δ: 180.0 (C-28), 139.6 (C-13), 128.5 (C-12), 73.7 (C-3), 72.8 (C-19), 68.2 (C-23), 54.6 (C-18), 49.0 (C-5), 48.8 (C-9), 47.9 (C-17), 43.0 (C-20), 42.3 (C-14), 42.2 (C-8), 40.5 (C-1), 39.0 (C-4), 38.3 (C-22), 37.3 (C-10), 33.3 (C-7), 30.1 (C-15), 29.3 (C-21), 27.8 (C-29), 27.1 (C-2), 26.5 (C-16), 24.7 (C-27), 24.2 (C-11), 18.9 (C-6), 17.4 (C-25), 16.8 (C-26), 16.2 (C-30), 13.2 (C-24), 61.7 (C-2'), 169.8 (C-1'), 141.3 (C-1''), 149.1 (C-3'''), 136.9 (C-1'''), 132.6 (C-6'''), 130.2 (C-5'''), 124.5 (C-4'''), 122.3 (C-2'''). HR-ESI-MS found 694.4062. Calculated 694.4062 for $C_{39}H_{56}N_3O_8$ [(M+H)+].

4-nitryl-benzaldehyde {2-[(3β,19α,23-trihydroxy-urs-12-en-28-oyl)-hydroxy]-acetic} hydrazone (4e, $C_{39}H_{55}N_3O_8$, R_1=p-$NO_2C_6H_4$, R_2=H). Compound 3 was reacted with p-nitryl benzaldehyde using the general procedure to give compound 4e, which was eluted with petroleum ether/ethyl acetate (V/V)=1:5 to give pale yellow needles. The yield was 85.1% and its melting point was between 230 and 231˚C. IR(KBr) cm^{-1}: 3606, 3499, 2925, 2879, 1713, 1699, 1517, 1407, 1341, 1234, 1151, 1043, 835 and 752; ^{1}H NMR (400 MHz, C_5D_5N) δ: 12.96 (1H, s, -NH), 8.13 (2H, d, *J*=8.8 Hz, H-2''',6'''), 8.06 (1H, s, H-1''), 7.75 (2H, d, *J*=8.8 Hz, H-3''', 5'''), 5.52 (2H, dd, *J*=15.9, 26.8 Hz, H-2'), 5.47 (1H, m, H-12), 5.10 (1H, m, H-3), 4.08 (1H, d, *J*=10.4 Hz, H-23a), 3.61 (1H, d, *J*=10.4 Hz, H-23b), 2.90 (1H, br s, H-18), 1.55 (3H, s, CH_3-25), 1.29 (3H, s, CH_3-27), 0.97 (3H, s, CH_3-29), 0.96 (3H, d, *J*=6.64 Hz, CH_3-30), 0.96 (3H, s, CH_3-26), 0.91 (3H, s, CH_3-24); ^{13}C NMR (100 MHz, C_5D_5N) δ: 180.0 (C-28), 139.6 (C-13), 128.5 (C-12), 73.7 (C-3), 72.8 (C-19), 68.1 (C-23), 54.5 (C-18), 49.0 (C-5), 48.8 (C-9), 47.9 (C-17), 43.0 (C-20), 42.3 (C-14), 42.2 (C-8), 40.5 (C-1), 39.0 (C-4), 38.3 (C-22), 37.3 (C-10), 33.3 (C-7), 30.1 (C-15), 29.3 (C-21), 27.8 (C-29), 27.1 (C-2), 26.4 (C-16), 24.7 (C-27), 24.2 (C-11), 18.9 (C-6), 17.4 (C-25), 16.8 (C-26), 16.2 (C-30), 13.2 (C-24), 61.7 (C-2'), 169.6 (C-1'), 141.3 (C-1''), 140.9 (C-1''''), 128.0 (C-3''', 5'''), 124.4 (C-2''', 6'''), 148.6 (C-4'''). HR-ESI-MS found 694.4061. Calculated 694.4062 for $C_{39}H_{56}N_3O_8$ [(M+H)+].

4-methoxy-benzaldehyde {2-[(3β,19α,23-trihydroxy-urs-12-en-28-oyl)-hydroxy]-acetic} hydrazone (4f, $C_{40}H_{58}N_2O_7$, R_1=p-$CH_3OC_6H_4$, R_2=H). Compound 3 was reacted with p-methoxy benzaldehyde using the general procedure to give compound 4f, which was eluted with petroleum ether/ethyl acetate (V/V)=1:3 to give white needles. The yield was 89.2% and its melting point was between 173 and 175˚C. IR(KBr) cm^{-1}: 3622, 3483, 2932, 2875, 1688, 1607, 1516, 1462, 1377, 1252, 1170, 1034, 831 and 530; ^{1}H NMR (400 MHz, C_5D_5N) δ: 12.40 (1H, s, -NH), 8.04 (1H, s, H-1''), 7.63 (2H, d, *J*=8.8 Hz, H-2''', 6'''), 6.87 (2H, d, *J*=8.8 Hz, H-3''', 5'''), 5.49 (2H, dd, *J*=15.9, 28.2 Hz, H-2'), 5.43 (1H, m, H-12), 4.99 (1H, m, H-3), 4.08 (1H, d, *J*=10.4 Hz, H-23a), 3.61 (1H, d, *J*=10.4 Hz, H-23b), 3.56 (3H, s, -OCH_3), 2.90 (1H, br s, H-18), 1.55 (3H, s, CH_3-25), 1.28 (3H, s, CH_3-27), 0.97 (3H, s, CH_3-29), 0.96 (3H, d, *J*=6.64 Hz, CH_3-30), 0.94 (3H, s, CH_3-26), 0.90 (3H, s, CH_3-24); ^{13}C NMR (100 MHz, C_5D_5N) δ: 177.9 (C-28), 139.6 (C-13), 128.5 (C-12), 73.7 (C-3), 72.8 (C-19), 68.2 (C-23), 54.5 (C-18), 49.0 (C-5), 48.8 (C-9), 47.9 (C-17), 43.0 (C-20), 42.3 (C-14), 42.2 (C-8), 40.5 (C-1), 39.0 (C-4), 38.3 (C-22), 37.3 (C-10), 33.3 (C-7), 30.1 (C-15), 29.3 (C-21), 27.8 (C-29), 27.1 (C-2), 26.4 (C-16), 24.7 (C-27), 24.2 (C-11), 18.9 (C-6), 17.4 (C-25), 16.8 (C-26), 16.2 (C-30), 13.2 (C-24), 62.8 (C-2'), 169.3 (C-1'), 143.9 (C-1''), 127.7 (C-1'''), 129.2 (C-3''', 5'''), 114.8 (C-2''', 6'''), 161.7 (C-4'''), 55.4 (C-OCH_3). HR-ESI-MS found 679.4332. Calculated 679.4317 for $C_{40}H_{59}N_2O_7$ [(M+H)+].

Cell cytotoxicity assays. The cell survival rate was measured by an MTT assay (29). Aliquots (200 μl) of $5x10^3$ cells/ml of HeLa, HepG2 and SGC-7901 cells were seeded in 96-well plates in DMEM medium containing 10% FBS at 37˚C in a humidified atmosphere of 5% CO_2. The test drugs were dissolved in dimethyl sulfoxide (DMSO). The incubation medium was replaced with each test medium, making a final concentration of 1.25 to 100 μM of RA and compounds 4a-4f for 48 h and DMSO at 0.1% in media as a vehicle control. After adding 5 μl of MTT labeling reagent (MTT Cell Proliferation Assay kit; Trevigen, USA) to each well, the plates were incubated for 4 h, before each well was supplemented with 100 μl solubilization solution. The absorbance at 570 nm was then measured in a microtiter plate reader (Bio-Tek, Winooski, VT, USA). The drug treatments were performed separately three times.

Table I. The IC_{50} values of RA and its derivatives 4a-4f on human cancer cell lines.

Compound	IC_{50} (μM)		
	HeLa	HepG2	SGC-7901
RA	18.70±1.61	8.26±1.24	16.48±2.32
4a	86.67±3.86	>100[a]	44.29±4.27
4b	20.58±0.79	34.60±3.55	41.22±2.98
4c	>100[a]	>100[a]	>100[a]
4d	9.58±1.14[b]	45.36±3.36	>100[a]
4e	18.83±2.26	8.74±1.28	15.38±1.58
4f	8.54±0.97[b]	4.16±0.73[b]	11.32±1.20[b]

Data are represented as means ± SD; n=3. [a]IC_{50} values >100 μM are indicated as >100; [b]P<0.05 vs. RA.

Apoptosis determination by DAPI staining. Apoptotic morphological changes were determined by DAPI staining. The harvested cells were exposed with compound 4f for 48 h. The medium was then removed from the culture, and the cells were washed twice with cold PBS and fixed with 100% EtOH for 20 min at room temperature. It was washed twice again with PBS and incubated with DAPI solution (0.5 μg/ml) for 30 min. Then, cell morphology was evaluated by fluorescence microscopy (IX70-SIF2; Olympus).

Annexin V-FITC and PI double staining analysis by flow cytometry. The cells were plated at appropriate densities (~2.5x10^4 cells/well) in 3 ml of medium in 6-well plates. The induction of apoptosis was evaluated in HepG2 cells plated on 6-well plates overnight with compound 4f dissolved in DMEM medium. After 48 h in culture, the adherent cells were harvested, washed twice with cold PBS, and then assayed for apoptosis by double staining with Annexin V-FITC and PI (Annexin V-FITC Apoptosis Detection kit; KeyGEN Biotech, Nanking, China). Briefly, 5x10^5 cells were re-suspended in 1x binding buffer and stained with 5 μl of FITC-Annexin V. After 10 min of incubation, 5 μl of PI was added, and the samples were incubated again for 15 min. The samples were then immediately analyzed using a flow cytometer (FACScan; BD Biosciences, Milan, Italy) with a dedicated software. The cells in the upper right portion were late-apoptotic cells. The cells in the lower left portion were viable cells. The cells in the lower right portion were early apoptotic cells.

Cell cycle analysis. HepG2 cells (2x10^5/well) were placed in 6-well plates, and then compound 4f was added to the wells and cultured for 48 h. The vehicle control in the media was DMSO at 0.1%. The cells from each well were harvested individually by centrifugation. The isolated cells were fixed by 70% EtOH at 4˚C overnight, and then re-suspended in PBS containing 40 μg/ml PI, 0.1 mg/ml RNase A, and 0.1% Triton X-100 in a dark room for 30 min at 37˚C. The DNA content of 20,000 cells for each determination was measured using EPICS XL-MCL flow cytometer (Beckman Coulter, Inc., Fullerton, CA, USA), in which an argon laser (488 nm) was used to excite PI. An emission above 550 nm was collected.

Statistical analysis. The data were analyzed for mean values and standard deviation for all the experiments. Statistically significant differences were determined between the control and treated groups using Student's t-test. P<0.05 was considered to indicate a statistically significant difference. Statistical package for the Social Sciences (SPSS, version 13.0) was used for statistical analyses.

Results

Preparation of RA. The barks of *I. rotunda* were shade-dried, grounded, and extracted with refluxing 80% EtOH. The air-dried and powdered total saponins from EtOH extract were hydrolyzed by 4% sodium hydroxide in 30% EtOH and purified by recrystallization to prepare RA. The purity of RA used was found to be ≥98% in a high performance liquid chromatography assay.

Structure modification of RA. The 28-COOH position of RA was modified to obtain six new compounds (Fig. 2). The cytotoxic activity of RA and its six derivatives were studied.

Cell cytotoxicity assays. The effects of RA and its derivatives 4a-4f on cell cytotoxicity were measured by MTT assay. The three types of human cancer cell lines were exposed to 1.25 to 100 μM of RA derivatives for 48 h. RA showed significant IC_{50} values of 18.70, 8.26 and 16.48 μM on the HeLa, HepG2 and SGC-7901 cell lines, respectively (Table I).

Apoptosis determination by 4'-6-diamidino-2-phenylindole (DAPI) staining. HepG2 cells were exposed to compound 4f in its IC_{50} (4.16 μM) for 48 h. The occurrence of nuclear condensation upon treatment with compound 4f could be revealed by DAPI staining. Apoptotic bodies, one of the stringent morphological criteria of apoptosis, were present in the compound 4f-treated HepG2 cells stained with DAPI (Fig. 3).

Annexin V-FITC assay. HepG2 cells were treated with compound 4f at a concentration 4.16 μM for 48 h and were analyzed by flow cytometry. As shown in Fig. 4A, the numbers of early and late apoptotic cells were significantly increased compared to the control group. The proportion of early and late

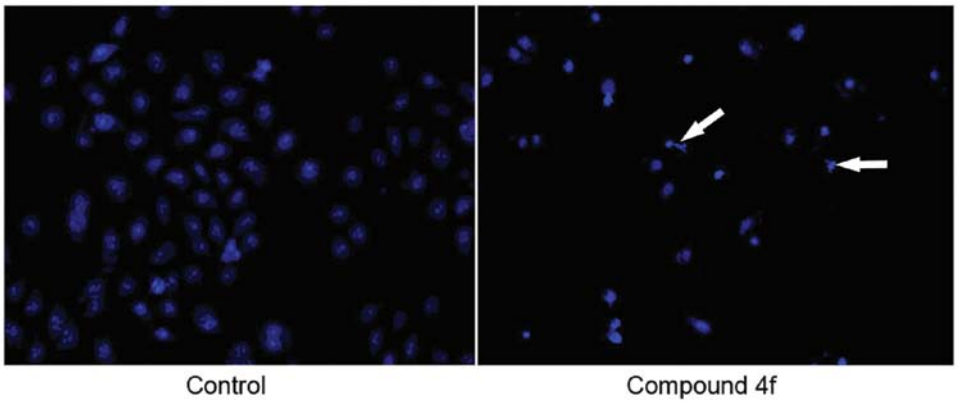

Figure 3. DAPI staining of compound 4f-treated HepG2 cells. Cells were exposed to either control (0.1% DMSO in medium) or compound 4f at 4.16 μM and incubated for 48 h. (Magnification, x200).

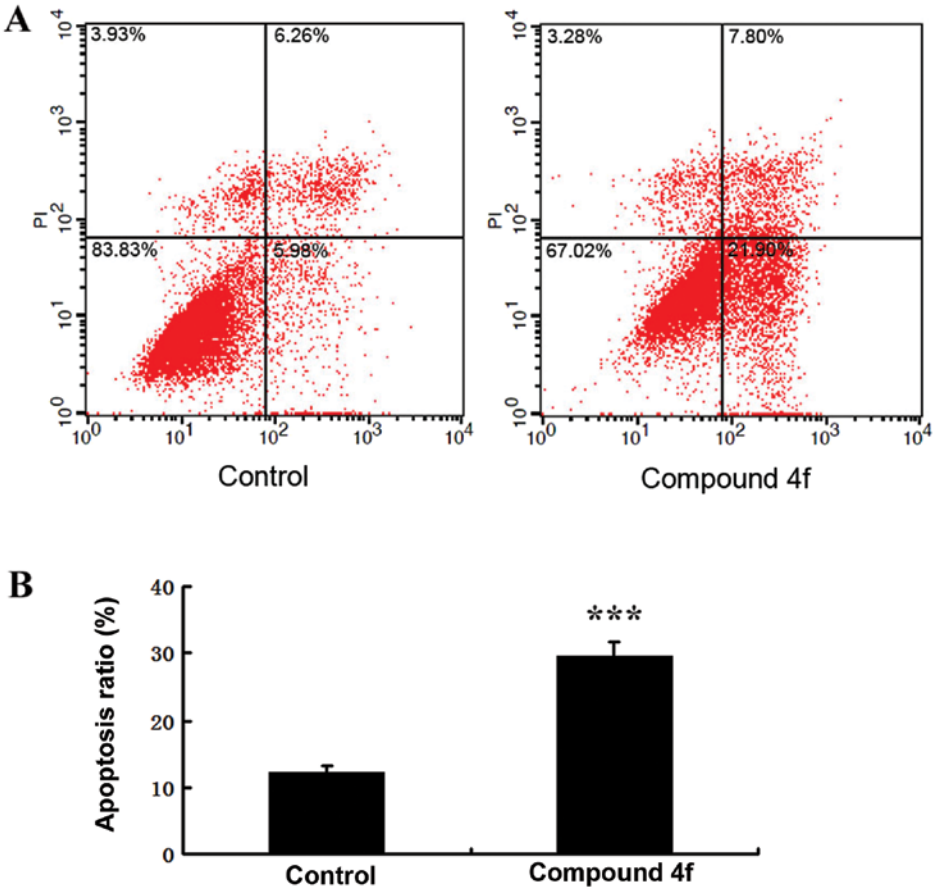

Figure 4. Apoptosis detection, by flow cytometry, of HepG2 cells treated with compound 4f. (A) Flow cytometric analysis of apoptosis in HepG2 cells treated with compound 4f. Cells were exposed to either control (0.1% DMSO in medium) or compound 4f at 4.16 μM and incubated for 48 h. The experiments were repeated at least three times. (B) Percentage of apoptotic cells. The apoptotic cells were calculated as the percentage of apoptotic cells in the upper right portion and lower right portion to the total number of the cells. Data are expressed as the means ± SD of three experiments (***P<0.001 vs. control).

apoptotic cells in the compound 4f treatment group reached 30.38%, which was higher than the control group (12.5%) (P<0.001) (Fig. 4B). This finding suggested that compound 4f suppressed cell proliferation possibly by inducing apoptosis.

Compound 4f causes G0/G1 cell cycle arrest in HepG2 cells. As shown in Fig. 5A, compared with the control group, compound 4f resulted in a significant accumulation of HepG2 cells in the G0/G1 phase and a decrease in the number of cells in the G2/M phase. Markedly, more HepG2 cells treated by compound 4f were in the G1 phase compared to the control (78.97 vs. 65.49%; P<0.05). These results indicated that compound 4f caused cell cycle arrest in the G0/G1 phase (Fig. 5B).

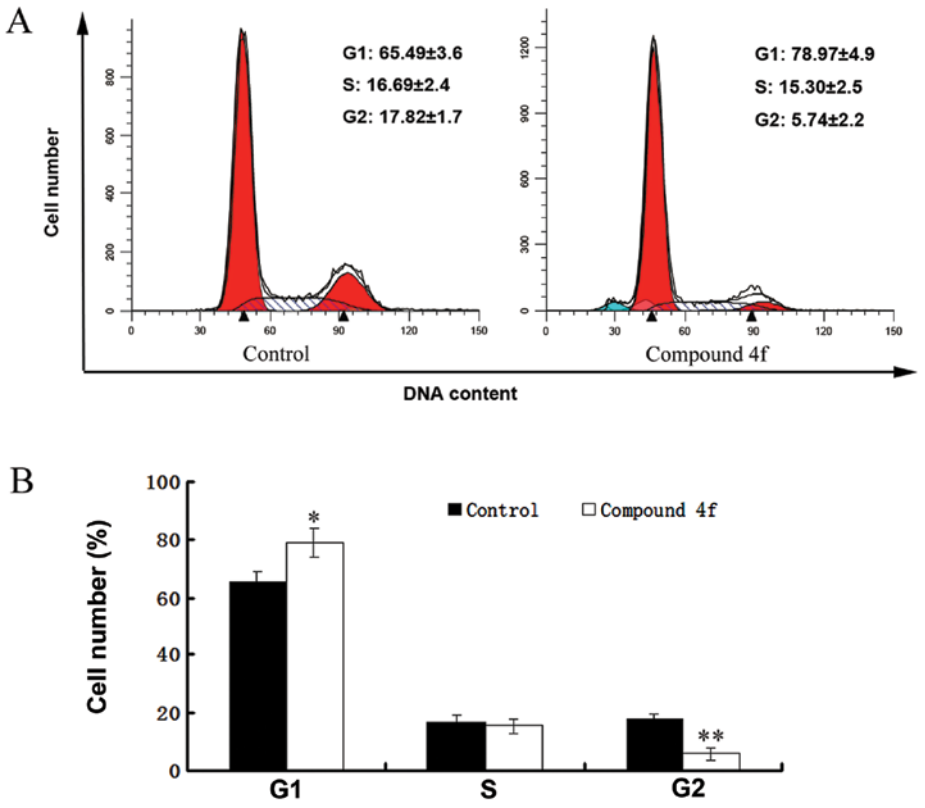

Figure 5. Compound 4f induces cell cycle arrest of HepG2 cells in the G0/G1 phase. (A) Effects of compound 4f on cell cycle distribution of HepG2 cells after staining with PI. Cells were exposed to either control (0.1% DMSO in medium) or compound 4f at 4.16 μM and incubated for 48 h. (B) The percentage of cells in each phase of the cell cycle. The columns or points represent the means ± SD of three independent experiments. *P<0.05, **P<0.01 vs. control.

Discussion

It has been reported that structural modifications could enhance the anticancer activities of parent compounds (19,20). In the present study, the 28-COOH position of RA was modified, and six new compounds were obtained. The synthesized derivatives were tested for antitumor activities in order to test previous evidence that the amino acid modification may enhance the antitumor activities of the parent (21).

The cytotoxicity results of the RA derivatives, compounds 4a-4f, demonstrated that the IC_{50} of compound 4f was significantly less than the groups treated with compounds 4c and 4e. The results may be explained by the difference of substituent group in para-position of benzene of R_1 group. Antitumor activities of these compounds were enhanced when they were substituted with electron-donating groups on para-position of benzene. In addition, the antitumor activity of compound 4f was found to be better than 4b. Hence, compound 4f was substituted with a methoxy group (a stronger electron-donation group) on para-position of benzene of R_1 group. On the other hand, since the compounds 4c, 4d and 4e were substituted with electron-attracting groups, their antitumor activities were weaker than RA. Based on the results, the IC_{50} of compound 4f was 8.54, 4.16 and 11.32 μM on the HeLa, HepG2 and SGC-7901 cell lines, respectively. This indicated that compound 4f could be further studied as a novel antitumor agent.

Evasion of apoptosis is one of the major hallmarks of cancer and a target for cancer treatment (22). Previous data suggested apoptosis as an underlying mechanism, by which various anticancer and chemopreventive agents exert their antitumor effects (23,24). The current study included the preliminary research on apoptosis and cell cycle. In the early stages of apoptosis, phosphatidylserine in the cell membrane was translocated outside. Compound 4f was able to induce apoptosis of HepG2 cells, and the apoptosis ratio in the early stage was about three times higher than in the control group.

The results of fluorescence-activated cell sorting of HepG2 cells treated with compound 4f at its IC_{50} showed significant cell cycle arrest at the G0/G1 interface, suggesting this as a mechanism for the antiproliferative effect of compound 4f. However, further mechanistic investigation of compound 4f remains to be conducted. These findings will provide new insight into the cancer chemotherapeutic properties of RA and its derivatives.

In conclusion, six novel RA derivatives were synthesized and evaluated for their cytotoxic properties on three tumor cell lines. Compound 4f had a substantially better antitumor effect than RA *in vitro* on HepG2 cells. Compound 4f induced apoptosis in HepG2 cells and G0/G1 cell cycle arrest. These results provide further insight into compound 4f-induced apoptosis and deepen our previous knowledge of the cytotoxicity and anticancer ability of RA derivatives.

Acknowledgements

The authors thank Professor Richard Nicholson at the University of Newcastle, Australia, for editing this manuscript and providing valuable suggestions.

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