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The synthesis of ursolic acid derivatives with cytotoxic activity and the investigation of their preliminary mechanism of action

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ABSTRACT

Nineteen ursolic acid derivatives (15 novel compounds) modified at the C-3 and the C-28 positions were synthesized. The cytotoxic activity of the derivatives was evaluated against HeLa, BGC-823 and SKOV3 cells by MTT assay. Inducing apoptosis and affecting cell cycle distribution by the derivatives in HeLa cells were assessed by flow cytometry and DNA fragmentation. Compounds **10b and 11b** were particularly active to inhibit HeLa cells growth through inducing apoptosis and arresting cell cycle progression. The typical 'sub-G1 peak' and DNA ladder formation were checked and cell cycle was arrested at the S phase in a dose-dependent manner.

1. Introduction

Ursolic acid (UA, 3β-hydroxy-urs-12-en-28-oic acid, **1**), a pentacyclic triterpene acid, exists abundantly in the plant kingdom and as constituents of medicinal herbs. UA has been reported to display a remarkable spectrum of biochemical activities to influence processes that are dysregulated during cancer development. These include inhibition of tumorigenesis, tumor promotion, invasion, metastasis, angiogenesis and induction of tumor cell differentiation.[1–10] In addition, Ya-Ling Hsu et al. have reported that UA inhibits the cell proliferation of human lung cancer cell line A549 and provided a molecular understanding for this effect.[11] Mechanistic studies revealed that UA blocked the cell cycle progression in the G1 phase and UA treatment resulted in the triggering of apoptosis as determined by DNA fragmentation assay.[11]

The pleuripotent anti-tumor activities of UA have stimulated active research in this field. We think it is very important to probe novel ursane triterpenoids structures with a view to biological testing. Based on the reports that the acid moiety at C-17 and ester functionality at C-3 are essential for pharmacological activities of pentacyclic triterpenes[12,13], and a hydrogen donor group at either C-3 position and/or C-28 position of ursolic acid is essential for the cytotoxic activity[14], a series of UA derivatives have been synthesized and their cytotoxic activities have been evaluated against HeLa cells. The results showed that acetylation of C-3 alcohol together with coupling an amino alcohol or benzylamine at C-28 results in derivatives having stronger cell growth inhibitory than ursolic acid (IC_{50} values of compounds **10a**, **11a** and **12a**, 10.83 μM, 5.44 μM and 1.63 μM, respectively[15]).

Following on these previous findings, we now report the design and synthesis of UA-amino acid (or related amino alcohol compound) conjugates and preliminary structure-activity relationship (SAR) study of their cytotoxic activity. The present studies also explore preliminary mechanisms of action of UA–amino alcohol conjugates' antiproliferative effects: compounds **10b, 11b** may act through inducing apoptosis and blocking cell cycle in S phase.

2. Results and discussion

2.1. Chemistry

The synthetic routes of UA derivatives are outlined in Scheme 1. Ursolic acid **1** was first converted to its 3-*O*-acetate **2**, which was then treated with oxalyl chloride to give the 28-acyl chloride **3**.[14] This intermediate was then condensed with the appropriate amino compound (methyl ester of serine, threonine, tyrosine, glycine, isoleucine and phenylalanine; 2-aminoethanol, 2-amino-1-propanol; *o*-aminophenyl-carbinol) in the presence of triethylamine to give the compounds **4a**–**12a**. Saponification of compounds **4a**–**9a** gave the corresponding *N*-[3β-hydroxy-urs-12-en-28-oyl]-amino acids compounds **4b**–**9b**. Compounds of **10b**–**12b** were obtained by re-acetylating compounds **10a**–**12a**, respectively. The structures and high purity of the target compounds were confirmed by application of mp, Infrared (IR), mass spectra (MS), ^{1}H NMR and ^{13}C NMR.

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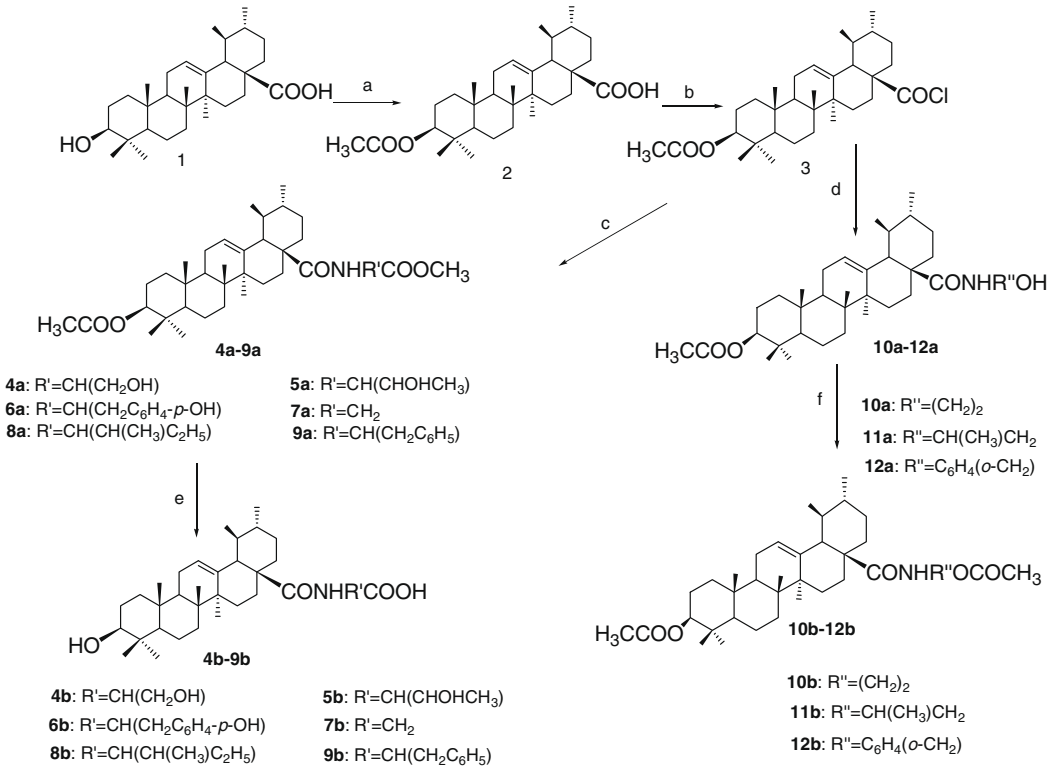

Scheme 1. Reagents and conditions: (a) and (f) $(CH_3CO)_2O$, DMAP, THF, rt; (b) $(COCl)_2$, CH_2Cl_2, rt; (c) NH_2-R′ CO_2CH_3, Et_3N, rt; (d) NH_2-R′′OH, Et_3N, rt; (e) NaOH, CH_3OH, THF, 40 °C.

2.2. Biological activity

2.2.1. Cytotoxic activity

In this experiment, HeLa cell lines were the model chosen to determine the cytotoxic activity of UA, its derivatives and paclitaxol (a positive control). Some compounds were also tested in two other tumorigenic human cell lines, SKOV3 and BGC-823 cells. Antiproliferative effects were identification with tetrazolium dye assay (MTT). Each experiment was repeated at least three times. The results are shown in Table 1.

As shown in Table 1, esterification of the 3-OH of ursolic acid to yield compound **2** resulted in increasing inhibitory activity against HeLa cell lines. Significant further improvement of the cell growth inhibition to HeLa cell lines was achieved when the selected amino acid methyl ester was coupled to the C-28. Compounds **4a**–**7a**, **9a** (IC_{50} values of **4a**–**7a**, 4.30, 6.86, 9.04, 4.86 μM) presented strong inhibition in the HeLa cell lines model at concentrations of 10 μM. (Compounds **4b**–**7b**, **9b** with the free 3-OH group, the saponification products of **4a–7a**, **9a** had lower anti-tumor effects than compounds **4a**–**7a**, **9a** but were still more active than the parent UA). Similar results had been seen in our previous study.[15] It is interesting to note that among other UA-amino acid conjugates tested, compounds **8a** and **8b** showed similar HeLa cell lines activity to that of UA. The reduced of **8a** and **8b** might be explained by a steric hindrance due to the presence of the *sec*-butyl on the C-28 amide side chain.

UA-amino alcohol conjugates **10a**–**12a** was acetylated to give compounds **10b–12b**. Compared to **10a** and **11a**, compounds **10b**, **11b** showed more potent anti-tumor activity in HeLa cell lines (IC_{50} values of **10b**, **11b**, 1.13, 2.62 μM). Additionally, compounds **10b** and **11b** also showed improved anti-tumor activity in SKOV3 (IC_{50} values of **10b** and **11b** 6.09 μM, 2.24 μM) and BGC-823 cell lines (IC_{50} value of **11b** 8.30 μM). Interestingly, compound **12b** is an exception, exhibiting less activity than compound **12a**, as seen in our previous study, incorporation of an anilino group at C-28 in UA-acetate to give N-[3β-acetoxy-urs-12-en-28-oyl]-aniline resulted in less activity than **12a**[15], while compound **12b** was slightly more active than *N*-[3β-acetoxy-urs-12-en-28-oyl]-aniline. These observations suggested that the electron-releasing group at benzene ring might be important factor on activity. In order to get a deeper understanding, many more derivatives containing a benzene ring with different substituent groups are being prepared in our laboratory.

We conclude that both the 3-*O*-acetyl group and a 28-amido group might be important for improving the tumor cell growth inhibitory activity. Compounds **4a**, **5a, 6a, 10a, 11a and 12a** with free hydroxyl group at the C-28 amide side chain showed similar inhibitory activity as compounds **7a**, **10b** and **11b** without the hydroxyl group on the amide side chain, suggesting that a hydroxyl group at the C-28 amide side chain could not influence the antiproliferative effect.

2.2.2. Investigation of apoptosis and cell cycle distribution

It has been reported that UA induces apoptosis and growth inhibition at the G1 phase of cell cycle in certain cancer cell systems.[16] Compounds **10b** and **11b** were selected for characterizing the mechanisms in inhibiting growth of UA derivatives using flow cytometric analysis of HeLa cells.

2.2.2.1. Apoptosis. The DNA fluorescence histograms of PI-stained cells showed low DNA stainability of compounds **10b** or **11b**-treated, apoptotic cells, which resulted in a distinct, quantifiable region below the G1 peak. In contrast, the G1 peak predominated in control cells (Fig. 1A). The apoptotic cells increased up to 35.3% and 32.5% (10 μM) after incubation in compounds **10b** or **11b** of concentrations higher than 2.5 μM for 24 h. Quantification of dose dependency was done by monitoring the amount of nuclei with sub-diploid DNA content with flow cytometry (Fig. 1B). Meanwhile,

Table 1
Inhibitory activity of UA and derivatives on the HeLa, SKOV3 and BGC-823 cells proliferation

Compound[a]	Inhibition rate%[b]			IC_{50}[c] (μM)		
	HeLa	SKOV3	BGC-823	HeLa	SKOV3	BGC-823
UA (1)	12.36 ± 3.01	9.06 ± 2.02	10.20 ± 1.02	>10	>10	>10
2	37.84 ± 6.28	nt[d]	nt	>10	nt	nt
4a	59.90 ± 8.79	nt	15.72 ± 2.81	4.30 ± 1.01	nt	>10
5a	55.92 ± 4.29	nt	nt	6.86 ± 0.98	nt	nt
6a	51.53 ± 5.09	nt	41.59 ± 7.08	9.04 ± 1.21	nt	>10
7a	61.69 ± 10.08	nt	17.85 ± 3.18	4.86 ± 0.81	nt	>10
8a	15.89 ± 6.79	nt	nt	>10	nt	nt
9a	32.86 ± 8.62	nt	nt	>10	nt	nt
10a	50.34 ± 5.71	nt	nt	10.82 ± 1.09	nt	nt
11a	58.38 ± 6.23	nt	nt	5.44 ± 0.68	nt	nt
12a	77.52 ± 12.09	nt	nt	1.63 ± 0.32	nt	nt
4b	16.84 ± 3.09	nt	nt	>10	nt	nt
5b	21.96 ± 4.15	nt	nt	>10	nt	nt
6b	23.58 ± 6.08	nt	nt	>10	nt	nt
7b	15.58 ± 2.59	nt	nt	>10	nt	nt
8b	16.79 ± 3.68	5.01 ± 1.28	nt	>10	>10	nt
9b	27.47 ± 5.36	nt	nt	>10	nt	nt
10b	73.50 ± 12.03	54.80 ± 10.12	42.05 ± 6.22	1.13 ± 0.09	6.09 ± 0.92	>10
11b	69.34 ± 9.35	70.54 ± 12.56	56.32 ± 8.58	2.62 ± 1.25	2.24 ± 0.56	8.30 ± 1.21
12b	30.53 ± 3.91	1.19 ± 1.02	13.0 ± 1.89	>10	>10	>10
Paclitaxol				0.001 ± 0.0005	0.016 ± 0.0037	0.0189 ± 0.00257

[a] Compounds **2**, **10a**, **11a** and **12a** are known compounds.
[b] Inhibitory percentage of cells treated with each compound at a concentration of 10 μM for 96 h.
[c] The agent concentration that inhibited HeLa cells growth by 50%.
[d] Not tested.

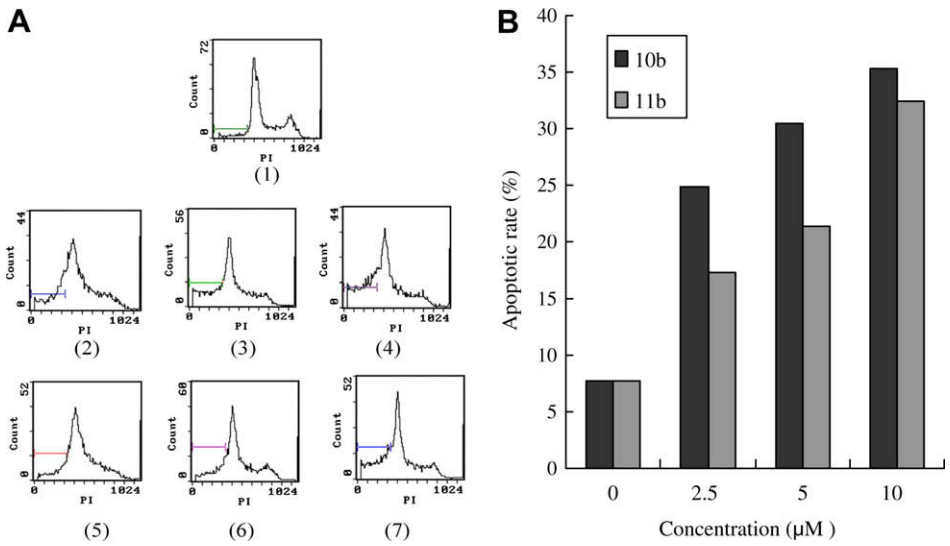

Figure 1. (A) Demonstration of apoptosis by flow cytometric analysis. (1) Untreated Hela cells. (2)–(4) and (5)–(7) Appearance of cells with sub-diploid DNA content after exposed to increasing concentrations of compounds **10b** and **11b** (2.5, 5.0, 10.0 μM). (B) Dose-dependent induction of apoptosis by **10b** and **11b**.

agarose gel electrophoresis showed typical DNA fragmentation pattern of apoptosis (Fig. 2). DNA fragmentation caused by **10b** or **11b** was also dose dependent, the intensity of DNA fragments increased when the amounts of **10b** or **11b** (2.5–5.0 μM) were increased.

2.2.2.2. Cell cycle distribution. To evaluate the possible role of cell cycle arrest in UA derivatives-caused growth inhibition, HeLa cell lines were treated with compounds **10b** and **11b**. Cell cycle distribution was evaluated by flow cytometric analysis after staining of cellular DNA with propidium iodide at the different concentrations. This indicated that **10b** and **11b** induced an accumulation in S phase of cell cycle. After treatment with 2.5–10 μM of **10b** and **11b** for 24 h, the number of cells in S phase was higher than that in untreated cells (Fig. 3A).

The percentage of cells arrested in S phase increased with the increase of concentration. The curve of S arrest following treatment for 24 h with increasing doses of **10b** is shown in Figure 3B. The percentage of cells in S peaked when cells were exposed to 2.5 μM of **10b**. The dose-dependent curve of S arrest following treatment for 24 h with increasing doses of **11b** is shown in Figure 3C. The percentage of cells in S peaked when cells were exposed to10 μM of **11b**.

3. Conclusion

In summary, our data suggest that (1)Acetylation of C-3 alcohol together with coupling an amino acid methyl ester or amino alcohol acetate at C-28 results in derivatives having stronger antiproliferative ability. (2) Six compounds (**4a, 5a, 6a, 7a, 10b, 11b**) showed

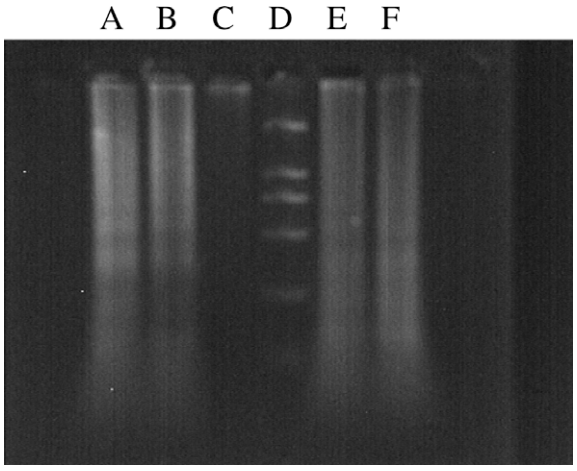

Figure 2. Electrophoresis analysis of apoptosis in Hela cells treated with **10b** and **11b**: (A) 5.0 μM **10b**, (B) 2.5 μM **10b**, (C) untreated Hela cells, (D) marker, (E) 2.5 μM **11b**, (F) 5.0 μM **11b**.

significant anti-tumor activity against the HeLa cell lines in the preliminary pharmacological test, and compounds **10b, 11b** also showed improved anti-tumor activity in SKOV3 and BGC-823 cells. (3) The C-3 free hydroxyl might decrease the inhibitory activity, while the free hydroxyl at amide side chain might not influence the activity. (4) Too many alkyl side chains at C-28 amide chain can decrease the inhibitory activity, such as compounds **8a** and **8b**. (5) Preliminary mechanisms study demonstrated that compounds **10b, 11b** may inhibit cell growth through inducing apoptosis and arresting cell cycle progression at the S phase. These results indicated that UA–amino alcohol conjugates may share some similarities in their mechanism of action with HeLa cell lines, but this should be more deeply investigated. The finding of these new active leads provides a powerful incentive for further research on the chemical modifications and structure-activity relationships of ursolic acid and other triterpenoid acids.

4. Experimental

4.1. Chemicals

UA was purchased from China Chengdu Scholar Bio-Tech. Co. Ltd., with a over 98% purity. Other reagents were bought from commercial suppliers in an analytic grade without further purification unless otherwise noted. The melting points were determined on an electrically heated X-4 digital visual melting point apparatus and were uncorrected. IR spectra were recorded on Thermo Nicolet 470FT spectrometer. ^{1}H NMR, ^{13}C NMR spectra were recorded on a BRUKER ARX-300MHz spectrometer at room temperature, and chemical shifts were measured in ppm downfield from TMS as internal standard. Mass spectra were recorded on Thermo-Finnigan LCQ equipment, in positive Electron Spray Ionization (ESI) mode and are reported as *m/z*.

4.2. General procedure for the synthesis of *N*-[3β-acetoxy-urs-12-en-28-oyl]-amino acid methyl-esters (4a–9a)

To a solution of compound **2** (0.20 mmol) in 3 mL CH_2Cl_2 was added oxalyl chloride (0.07 mL) and stirred at room temperature for 24 h. The mixture was concentrated to dryness under reduced pressure. Cyclohexane (2× 2 mL) was added to the residue, then the concentrated to dryness to yield crude 3-*O*-acetylursolyl chloride **3**. To a CH_2Cl_2 (4 mL) solution of **3** was added glycine methyl ester (0.80 mmol). The reaction mixture was stirred in presence of triethylamine at room temperature for 6 h, the reactant was partitioned in 3 mL water, then treated with 2 N HCl to pH 3. CH_2Cl_2 was removed under vacuum to precipitate white solid, then filtered. The filter was washed with water to pH 6, and dried. The crude was purified on a silica gel column with petroleum ether and ethyl acetate to yield a white powder.

4.2.1. Methyl *N*-[3β-acetoxy-urs-12-en-28-oyl]-2-amino-3-hydroxypropionate (4a)

Compound **2** was reacted by using general procedure with serine methyl ester to get compound 4a. Eluted by petroleum ether/ethyl acetate (V/V) = 3:1. Yield 55.4%; mp 182–184 °C; IR (KBr): 3454, 1736, 1639, 1511, 1247 cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6):δ 7.17 (1H, d, J = 7.2 Hz, NH), 5.19 (s, 1H, H-12), 4.97 (1H, t, OH), 4.39 (t, 1H, J = 6.3 Hz, H-3), 4.29–4.26 (m, 1H, NHC*H*), 3.59 (m, 2H, CH_2OH) 3.59 (m, 3H, OCH_3), 1.99 (s, 3H, CH_3CO), 1.04 (s, 3H, CH_3), 0.92 (s, 3H, CH_3), 0.89 (d, 3H, J = 6.2 Hz, CH_3), 0.84 (s, 3H, CH_3),0.83(d, 3H, J = 6.3 Hz, CH_3) 0.81 (s, 3H, CH_3), 0.65 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 178.1 (C-28), 173.8 (–COOCH$_3$), 172.0 (CH_3CO), 138.8 (C-13), 126.8 (C-12), 81.9 (C-3), 63.4 (CH_2OH); ESI-MS *m/z*: 600.3 (M+H)$^+$.

4.2.2. Methyl *N*-[3β-acetoxy-urs-12-en-28-oyl]-2-amino-3-hydroxybutyrate (5a)

Compound **2** was reacted by using general procedure with threonine methyl ester to get compound **5a**. Eluted by petroleum ether/ethyl acetate (V/V) = 6:1; Yield: 41.2%; mp 123–125 °C; IR (KBr): 3424, 2949, 1737, 1655, 1508, 1456, 1373, 1246 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 6.61 (br, 1H, NH), 5.39 (s, 1H, H-12), 4.52 (br, 1H, H-3), 4.52 (br, 1H, NHC*H*), 4.18 (br, 1H, C*H*OH), 3.74 (s, 3H, OCH$_3$), 2.04 (s, 3H, CH_3CO), 1.09 (s, 3H, CH_3), 1.00 (s, 3H, CH_3), 0.96 (d, 3H, J = 6.5 Hz, CH_3), 0.93 (s, 3H, CH_3),0.89 (d, 3H, J = 6.6 Hz, CH_3), 0.85 (s, 3H, CH_3), 0.69 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 177.1 (C-28), 172.6 (–COOCH$_3$), 171.0 (CH_3CO), 138.5 (C-13), 126.1 (C-12), 81.4 (C-3), 67.4 (CH_2OH), 59.0 (NHCH); ESI-MS *m/z*: 614.4 (M+H)$^+$.

4.2.3. Methyl *N*-[3β-acetoxy-urs-12-en-28-oyl]-2-amino-3-(*p*-hydroxy)-phenyl propionate (6a)

Compound **2** was reacted by using general procedure with tyrosine methyl ester to get compound **6a**. Eluted by petroleum ether/ethyl acetate (V/V) = 3:1; Yield: 44.6%; mp > 300 °C; IR(KBr): 3410, 2949, 1736, 1635, 1616, 1516, 1451, 1369, 1246, 830 cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.14 (s, 1H, Ar-O*H*), 7.39 (d, 1H, J = 7.4 Hz, NH), 7.01 (d, 2H, J = 8.3 Hz, Ar-H), 6.62 (d, 2H, J = 8.28 Hz, Ar-H), 5.02 (s, 1H, H-12), 4.39–4.38 (m, 1H, H-3), 4.22 (br, 1H, NHC*H*), 3.58 (s, 3H, OCH_3), 2.85–2.82 (m, 2H, Ar-CH_2), 1.99 (s, 3H, CH_3CO), 0.95 (s, 3H, CH_3), 0.90 (s, 3H, CH_3), 0.83 (d, 3H, J = 6.0 Hz, CH_3), 0.81(s, 3H, CH_3), 0.78 (d, 3H, J = 6.1, CH_3), 0.75 (s, 3H, CH_3), 0.70 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 176.8(C-28), 171.7 (–COOCH$_3$), 170.1 (CH_3CO), 158.7 (Ar-C 4′), 138.2 (C-13), 131.8, 130.9 (Ar-C2′, 6′), 129.4 (Ar-C1′), 125.8 (C-12), 118.2, 117.8 (Ar-C 3′, 5′), 81.0(C-3); ESI-MS *m/z*: 676.3 (M+H)$^+$.

4.2.4. Methyl *N*-[3β-acetoxy-urs-12-en-28-oyl]-2-amino acetate (7a)

Yield: 51.6%; mp 82–84 °C ;IR (KBr): 3421, 1736, 1656, 1519, 1460, 1384, 1246 cm^{-1}; ^{1}H NMR(300 MHz, $CDCl_3$): δ 6.52 (br, 1H, NH), 5.40 (t-like, 1H, H-12), 4.49 (t, 1H, J = 7.9 Hz, H-3), 4.11 (dd, 1H, J^2 = 18.7 Hz, J^3 = 5.5 Hz, NHC*Ha*CO), 3.84 (dd, 1H, J^2 = 18.6 Hz, J^3 = 3.5 Hz, 1H, NHC*Hb*CO), 3.76 (s, 3H, OCH$_3$), 2.04 (s, 3H, CH_3CO), 1.09 (s, 3H, CH_3), 0.98 (s, 3H, CH_3), 0.96 (d, J = 6.3 Hz, 3H, CH_3), 0.93 (s,3H, CH_3), 0.89 (d, 3H, J = 6.2 Hz CH_3), 0.85 (s, 3H, CH_3), 0.71 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 176.5 (C-28), 170.7 (CH_3CO), 169.8 (–COOCH$_3$), 138.4 (C-13), 126.0 (C-12), 80.5 (C-3); ESI-MS *m/z*: 570.3 (M+H)$^+$.

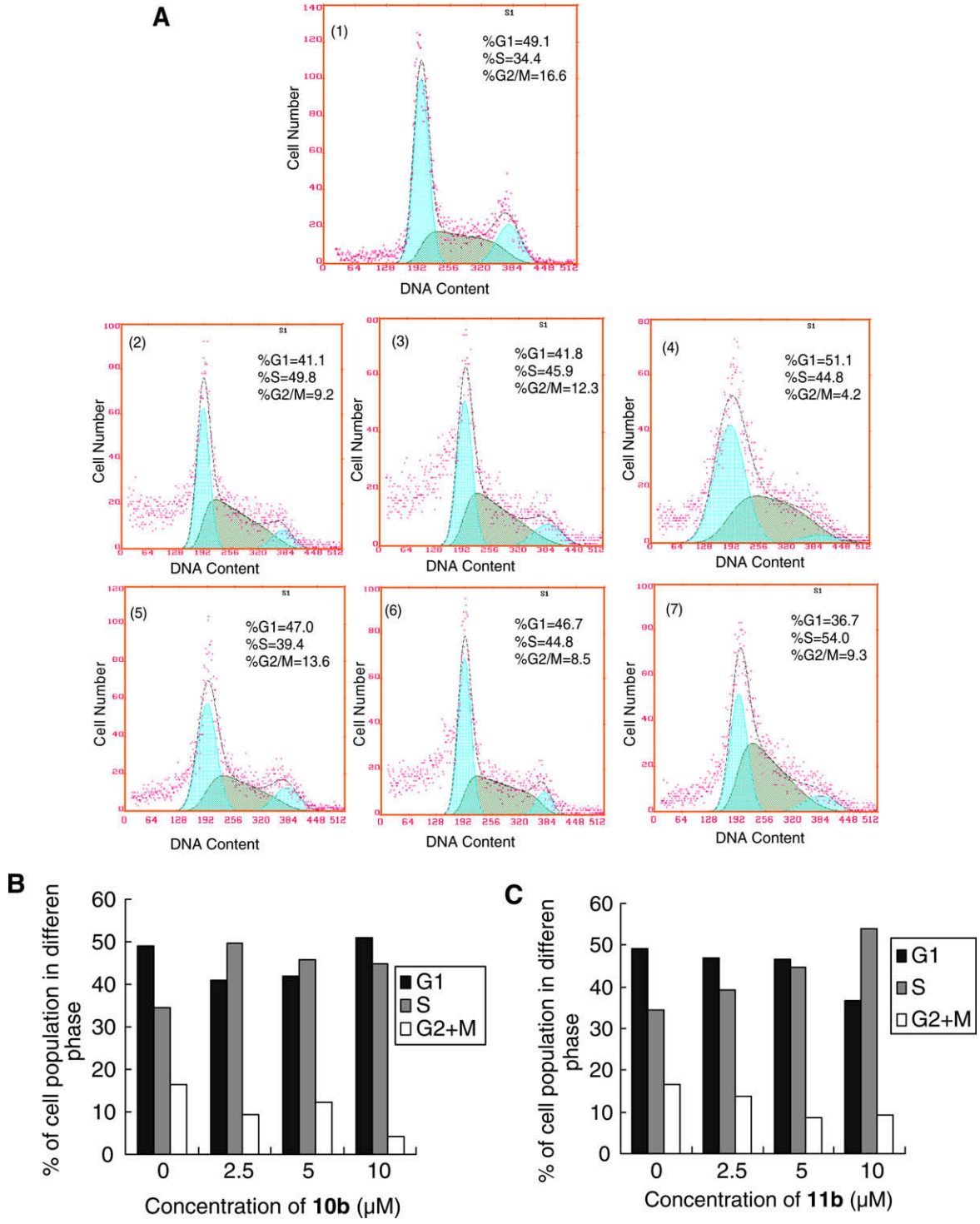

Figure 3. Effect of compounds **10b** and **11b** on cell cycle distribution. (A) Cell cycle fraction of HeLa cells grown exposed to 0.1% DMSO (1) and 2.5, 5.0, 10.0 μM of **10b** and **11b** (2–4 and 5–7). (B) and (C) effect results of Hela cells exposed for 24 h to concentration of compounds **10b** and **11b** varying from 2.5 to 10.0 μM.

4.2.5. Methyl *N*-[3β-acetoxy-urs-12-en-28-oyl]-2-amino-3-methyl-valerate (8a)

Compound **2** was reacted by using general procedure with isoleucine methyl ester to get compound **8a**. Eluted by petroleum ether/ethyl acetate (V/V) = 10:1, Yield: 64.9%; mp 196–198 °C; IR(KBr): 3419, 2927, 1735, 1652, 1511, 1457, 1370, 1251 cm^{-1}; ^1H NMR (300 MH_Z, $CDCl_3$): δ 6.46 (br, 1H, NH), 5.38 (s, 1H, H-12), 4.50 (t-like, 1H, H-3), 4.42 (m, 1H, NH*CH*), 3.70 (s, 3H, OCH_3), 2.05 (s, 3H, CH_3COO), 1.08 (s, 3H, CH_3), 0.95 (s, 3H, CH_3), 0.93 (d, 3H, J = 6.4 Hz, CH_3), 0.90 (s, 3H, CH_3), 0.88 (d, 3H, J = 6.3 Hz, CH_3), 0.85 (s, 3H, CH_3), 0.66 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 177.5 (C-28), 172.5 (–$COOCH_3$), 171.0 (CH_3CO), 138.4 (C-13), 126.4 (C-12), 80.9 (C-3), 56.2 (C-5), 55.3 (C-18), 53.9 (NHCH), 51.9 (OCH_3); ESI-MS m/z: 626.5 $(M+H)^+$.

4.2.6. Methyl *N*-[3β-acetoxy-urs-12-en-28-oyl]-2-amino-3-phenylpropionate (9a)

Compound **2** was reacted by using general procedure with phenylalanine methyl ester to get compound **9a**. Eluted by petroleum ether/ethyl acetate (V/V) = 10:1; Yield: 43.4%; mp > 300 °C;

IR (KBr): 3414, 2948, 1736, 1666, 1497, 1456, 1369, 1246, 701 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 7.26–7.28 (m, 3H, Ar-H), 7.11–7.08 (m, 2H, Ar-H), 6.39 (br, 1H, NH), 5.24 (s, 1H, H-12), 4.47 (t-like, 1H, H-3), 4.13–4.11 (m, 1H, NHC*H*), 3.67 (s, 3H, OCH_3), 3.12–3.05 (m, 2H, Ar-CH_2), 2.04 (s, 3H, CH_3CO), 1.05 (s, 3H, CH_3), 0.98 (s, 3H, CH_3), 0.94 (d, 3H, J = 6.2 Hz, CH_3), 0.89 (s, 3H, CH_3), 0.85 (d, 3H, J = 6.3 Hz, CH_3), 0.84 (s, 3H, CH_3), 0.60 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 175.8 (C-28), 171.8 (–COOCH_3), 170.2 (CH_3CO), 138.5 (C-13), 136.8 (Ar-C1′), 129.1 (Ar-C3′, 5′), 128.5 (Ar-C2′, 6′), 126.6 (Ar-C4′), 125.8(C-12), 80.6 (C-3). ESI-MS *m/z*: 660.4 $(M+H)^+$.

4.3. General procedure for the synthesis of *N*-[3β-Hydroxy-urs-12-en-28-oyl]-amino acids (4b–9b)

A solution of **4a (or 5a–9a)** with aqueous NaOH (4 N) in CH_3OH/THF was stirred for 3.5 h at 40 °C and concentrated in vacuo. The residue was suspended in water treated with 2 N HCl to pH 3. Organic solvent was removed under vacuum to precipitate white solid, filtered. The filter was washed with water to pH 6, and dried to give a white powder.

4.3.1. *N*-[3β-Hydroxy-urs-12-en-28-oyl]-2-amino-3-hydroxy propionic acid (4b)

Yield: 88.0%; mp 190–192 °C; IR(KBr): 3407, 2927, 1736, 1633, 1512, 1457, 1388 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6): δ 12.62 (br, 1H, CO*OH*), 6.98 (d, 1H, J = 6.6 Hz, NH), 5.29 (s, 1H, H-12), 4.88 (br, 1H, NH*CH*), 4.29 (br, 1H, *CHa*OH), 4.17 (br, 1H, *CHb*OH), 2.99 (s, 1H, H-3), 1.04 (s, 3H, CH_3), 0.97 (s, 3H, CH_3), 0.92 (d, 3H, J = 6.4 Hz, CH_3), 0.88(s, 3H, CH_3), 0.84 (d, 3H, J = 6.4 Hz, CH_3), 0.67 (s, H, CH_3), 0.64 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 178.1 (C-28), 174.8 (–*C*OOH), 138.7 (C-13), 126.8 (C-12), 79.2 (C-3), 63.4 (CH_2OH). ESI-MS *m/z*: 544.3 $(M+H)^+$.

4.3.2. *N*-[3β-Hydroxy-urs-12-en-28-oyl]-2-amino-3-hydroxy-butyric acid (5b)

Yield: 74.5%; mp 207–210 °C; IR(KBr): 3417, 2927, 1715, 1609, 1513, 1459, 1391 cm^{-1}; ^1H NMR (600 MHz, $CDCl_3$): δ 7.04 (br, 1H, NH), 5.39 (s, 1H, H-12), 4.28 (br, 1H, *CH*OH), 4.28 (br, 1H, NH*CH*), 3.22–3.20 (m, 1H, H-3), 1.09 (s, 3H, CH_3), 0.98 (s, 3H, CH_3), 0.95 (d, 3H, J = 6.2 Hz, CH_3), 0.88 (s, 3H, CH_3), 0.86 (d, 3H, J = 6.2 Hz, CH_3), 0.76 (s, 3H, CH_3), 0.71 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 177.2 (C-28), 176.5 (–*C*OOH), 138.5 (C-13), 126.2 (C-12), 79.5 (C-3), 67.4 (CH_2OH), 60.0 (NH*C*H); ESI-MS: 558.4 $(M+H)^+$.

4.3.3. *N*-[3β-Hydroxy-urs-12-en-28-oyl]-2-amino-3-(*p*-hydroxy)-phenylpropionic acid (6b)

Yield: 78.3%; mp > 300 °C; IR(KBr): 3401, 2927, 1733, 1633, 1616, 1516, 1454, 1377, 830 cm^{-1}; ^1H NMR(300 MHz, DMSO-d_6): δ 9.13 (s, 1H, Ar-O*H*), 7.14 (s, 1H, NH), 6.98 (s, 2H, Ar-*H*), 6.61 (s, 2H, Ar-*H*), 5.04 (s, 1H, H-12), 4.27 (s, 1H, NH*CH*), 4.15 (s, 1H, H-3), 2.94 (br, 2H, Ar-CH_2), 1.09 (s, 3H, CH_3), 0.99 (s, 3H, CH_3), 0.95 (d, 3H, J = 6.5 Hz, CH_3), 0.89 (s, 3H, CH_3), 0.79 (d, 3H, J = 6.4 Hz, CH_3), 0.72 (s, 3H, CH_3), 0.67 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 175.8(C-28), 174.7(–*C*OOH), 157.7(Ar-C 4′), 138.2(C-13), 131.5, 130.4(Ar-C 2′, 6′), 129.4(Ar-C1′), 125.8(C-12), 118.0, 117.4(Ar-C 3′, 5′), 78.6(C-3); ESI-MS: 620.4$(M+H)^+$.

4.3.4. *N*-[3β-Hydroxy-urs-12-en-28-oyl]-2-amino acetic acid (7b)

Yield: 85.6%; mp 162–164 °C; IR(KBr): 3441, 2926, 1750, 1639, 1527, 1456, 1413 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6): δ 7.26 (s, 1H, NH), 5.21 (s, 1H, H-12), 3.43 (br, 1H, *CHa*COOH), 3.00 (br, 1H, H-3), 3.00 (br, 1H, *CHb*COOH), 1.04 (s, 3H, CH_3), 0.96 (s, 3H, CH_3), 0.91 (d, 3H, J = 6.4 Hz, CH_3), 0.89 (s, 3H, CH_3), 0.84 (d, 3H, J = 6.3 Hz, CH_3), 0.71 (s, 3H, CH_3), 0.66 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 175.8 (C-28), 173.2 (–*C*OOH), 138.4 (C-13), 126.0 (C-12), 78.5 (C-3); ESI-MS *m/z*: 514.3 $(M+H)^+$.

4.3.5. *N*-[3β-Hydroxy-urs-12-en-28-oyl]-2-amino-3-methyl-valeric acid (8b)

Yield: 60.1%; mp 257–259 °C; IR(KBr): 3417, 2927, 1726, 1636, 1510, 1458, 1383 cm^{-1}; ^1H NMR (300 MHZ, $CDCl_3$): δ 6.56 (br, 1H, NH), 5.37 (s, 1H, H-12), 4.45 (m, 1H, NH*CH*), 3.23 (m, 1H, H-3), 1.09 (m, 3H, CH_3), 0.98 (m, 3H, CH_3), 0.95 (d, 3H, J = 6.4 Hz, CH_3),0.92 (s, 3H, CH_3), 0.88 (d, 3H, J = 6.4 Hz, CH_3), 0.77 (s, 3H, CH_3), 0.71 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 178.9 (C-28), 173.8 (–*C*OOH), 138.4 (C-13), 126.3 (C-12), 78.7 (C-3), 56.3 (C-5), 54.8 (C-18), 53.7 (NH*C*H);ESI-MS *m/z*: 626.5 $(M+H)^+$.

4.3.6. *N*-[3β-Hydroxy-urs-12-en-28-oyl]-2-amino-3-phenylpropionic acid (9b)

Yield:73.1%; mp > 300 °C; IR(KBr): 3419, 2926, 1636, 1604, 1509, 1455, 1391, 700 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 7.26–7.23 (m, 5H, Ar-*H*), 6.44 (br, 1H, NH), 5.07 (1H, t-like, H-12), 4.36 (m, 1H, NH*CH*), 3.18 (m,1H, H-3), 3.18 (m, 1H, OH), 2.98–2.96 (m, 2H, Ar-CH_2), 0.98 (s, 3H, CH_3), 0.95 (s, 3H, CH_3), 0.90(d, 3H, J = 6.2 Hz, CH_3) 0.87 (s, 3H, CH_3), 0.84 (d, 3H, J = 6.1 Hz, CH_3), 0.79 (s, 3H, CH_3), 0.76 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 176.9 (C-28), 173.6 (–*C*OOH), 138.5 (C-13), 136.8 (Ar-C1′), 130.1 (Ar-C3′, 5′), 129.3 (Ar-C2′, 6′), 126.2 (Ar-C4′), 125.6(C-12), 78.5(C-3); ESI-MS *m/z*: 604.5 $(M+H)^+$.

4.4. General procedure for the synthesis of *N*-[3β-acetoxy-urs-12-en-28-oyl]-amino alcohol acetates (10b–12b)

A solution containing compound **3** and ethanolamine (or 2-amino-1-propanol, 3-amino-1-propanol, 2-aminophenylcarbinol,) was stirred in the presence of tri-ethylamine for 1.5 h at room temperature. The reactant was partitioned in 3 mL water, then treated with 2 N HCl to pH 3. CH_2Cl_2 was removed under vacuum to precipitate white solid, then filtered. The filter was washed with water to pH 6, and dried. The crude was purified on a silica gel column with petroleum ether and ethyl acetate to yield a white powder **10a**–**12a**. Compounds **10a**–**12a** was acetylated with two equivalence of acetic anhydride in anhydrous THF in presence of 4-(dimethylamino)-pyridine to give compounds **10b–12b**.

4.4.1. *N*-[3β-Acetoxy urs-12-en-28-oyl]-2-aminoethanol acetate (10b)

Yield: 29.7%; mp 83–85 °C; IR(KBr): 3418, 2947, 1736, 1652, 1521, 1458, 1384, 1245; ^1H NMR (300 MHz, $CDCl_3$): δ 6.17 (br, 1H, NH), 5.31(s, 1H, H-12), 4.49 (t-like, 1H, H-3), 4.15–4.08 (m, 2H, CH_2OCOCH_3), 3.58 (br, 1H, NH*CH*a), 3.34 (br, 1H, NH*CH*b), 2.08 (s, 3H, OCO*CH*$_3$), 2.04 (s, 3H, CH_3COO), 1.09 (s, 3H, CH_3), 0.94 (s, 6H, CH_3×2), 0.89–0.87 (m, 9H, CH_3×3), 0.77 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$): δ 179.5(C-28), 171.7(CH_3COO), 138.7(C-13), 125.6 (C-12), 80.9 (C-3), 64.8 (CH_2OCOCH_3); ESI-MS: 584.4 $(M+H)^+$.

4.4.2. *N*-[3β-Acetoxy-urs-12-en-28-oyl]- 2-amino-1-propanol acetate (11b)

Yield: 44.3%; mp 77–80 °C; IR(KBr): 3263, 2925, 1725, 1693, 1459, 1378, 1223, 1189; ^1H NMR (300 MHz, $CDCl_3$): δ 5.91 (d, J = 7.7 Hz, 1H, NH), 5.27 (s, 1H, H-12), 4.49 (t, 1H, J = 7.8 Hz, H-3), 4.24 (br, 1H, NH*CH*), 4.10–3.97 (m, 2H, CH_2OCOCH_3), 2.09 (s, 3H, OCO*CH*$_3$), 2.04 (s, 3H, CH_3COO), 1.14 (d, 3H, J = 6.6 Hz, NHCH(CH_3)CH_2OCOCH_3), 1.09 (s, 3H, CH_3), 0.94 (s, 6H, CH_3×2), 0.86–0.85 (m, 9H, CH_3×3), 0.80 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$): δ 179.1 (C-28), 171.3 (CH_3COO), 138.6 (C-13), 125.8 (C-12), 81.49 (C-3), 71.7 (CH_2OCOCH_3); ESI-MS:598.4 $(M+H)^+$.

4.4.3. *N*-[3β-Acetoxyurs-12-en-28-oyl]-2-aminophenyl-carbinol acetate (12b)

Yield: 30.3%; mp 238–240 °C; ^{1}H NMR(300 MHz, $CDCl_3$): δ 8.35 (s, 1H, NH), 7.86 (d, 1H, J = 8.3 Hz, Ar-H), 7.34–7.33 (m, 2H, Ar-H), 7.11 (t, 1H, J = 7.4 Hz, Ar-H), 5.36 (s, 1H, H-12), 5.05 (s, 2H, CH_2OH), 4.48 (t-like, 1H, H-3), 2.10 (s, 3H, OCOCH_3), 2.04 (s, 3H, CH_3COO),1.13 (s, 3H, CH_3), 0.99 (s, 3H, CH_3), 0.94–0.91 (m, 6H, $CH_3\times2$), 0.86–0.84 (m, 6H, $CH_3\times2$), 0.80 (s, 3H, CH_3); ^{13}C NMR ($CDCl_3$): δ 178.5 (C-28), 170.7 (CH_3COO), 140.1 (C-1′-Ar), 138.5 (C-13), 132.5 (C-2′-Ar), 128.8 (C-3′, 5′-Ar), 126.1 (C-4′-Ar), 125.4 (C-12), 121.2 (C-6′-Ar), 81.0 (C-3), 65.8 (CH_2 OCOCH_3); ESI-MS: 646.4 $(M+H)^+$.

4.5. Biological activity assays

4.5.1. Cell culture

All human tumor cells were cultured in RPMI 1640 medium supplemented with 10% foetal bovine serum and penicillin/streptomycin. A humidified incubator was set at 37.8 °C; the air contained 5% CO_2.

4.5.2. Antiproliferative effects

Antiproliferative effects were identification with tetrazolium dye assay (MTT). Cells were detached by trypsinisation, seeded at 1.0–2.0 $\times$ 10^3 cells/well in a 96-well microtitre plate overnight, and treated with different concentrations of drugs in RPMI 1640with 10% foetal bovine serum. Finally, 50 mL of MTT solution (1mg/mL in RPMI 1640) was added to each well and incubated at 37.8 °C for 4 h. The MTT-formazan formed by metabolically viable cells was dissolved in 150 mL of DMSO, and monitored by a microplate reader at dual-wavelength of 490 nm; IC_{50} was defined as the drug concentrations that inhibited the cell number to 50% after 96 h. Each experiment was repeated at least three times.

4.5.3. Assessment of apoptosis and cell cycle distribution by flow cytometry

Assessment of apoptosis and cell cycle kinetic by flow cytometry: HeLa cells were washed with PBS and then fixed with 70% ethanol. The fixed cells were kept at least overnight at −20 °C. The cells were washed with PBS before analysis, and then the fluorochrome solution (50 μg/mL propidium iodide in PBS, plus RNase, 50 μg/mL) was added. Distribution of the cell cycle phases was determined by analytical flow cytometry using a Coulter Epics XL (Coultronics, France SA) with an excitation/emission of 488/525 nm. All experiments were performed three times.

4.5.4. Assessment of apoptosis by DNA fragmentation

Assessment of apoptosis by DNA fragmentation: HeLa cells incubated with **10b** or **11b** for 24 h were collected and washed two times with PBS, then resuspended in 0.5 ml of extraction buffer (100 mmol/L NaCl, 10 mmol/L Tris–HCl pH 8.0, 25 mmol/L EDTA pH 8.0, 0.1 mg/ml Proteinase K) at 50 °C for 12 h. DNA was extracted with an equal volume of phenol saturated/chloroform/isoamyl alcohol (25:24:1) and extracted again with a combination of chloroform/isoamyl alcohol (24:1), then centrifuged at 12,000g for 30 min. Precipitated DNA was analysed on a 2.0% agarose gel.

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Supplementary data

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