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Synthesis of [3β-acetoxy-urs-12-en-28-oyl]-1-monoglyceride and investigation on its anti tumor effects against BGC-823

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ABSTRACT

Ursolic acid (UA) as the leader compound was designed to prepare a series of derivatives (three novel compounds **UA-1a**, **UA-1b** and **UA-2**) by modification at the C3 and C28 positions. Their chemical structures were confirmed by IR, ^{1}H NMR and MS. The cytotoxic activity of the derivatives was evaluated against HepG2, BGC-823 and HT-29 by the MTT assay. The novel derivative **UA-1a,** [3β-acetoxy-urs-12-en-28-oyl]-1-monoglyceride showed significant anti-growth ability against the assayed cancer cell lines, particularly against BGC-823, while low cytotoxicity to human normal gastric cell line GES-1. Further investigation revealed that **UA-1a** could induce apoptotic events of the treated BGC-823 cells, such as comet-like DNA bend, sub-G0/G1 phase accumulation and phosphatidylserine externalization. The activity of Caspase-3 was found to be up-regulated, while the expression of Bcl-2 and Survivin were down-regulated in **UA-1a** treated cells. **UA-1a** might trigger the death of BGC-823 cells by inducing apoptosis via the mitochondria pathway. **UA-1a** exerted stronger ability than Taxol to retard tumor growth in nude mice without leaving apparent toxicity to the hosts. The experimental data suggested that **UA-1a** would have a therapeutic potential in the treatment of gastric cancer.

1. Introduction

Current therapies for malignant epithelial tumor include chemotherapy, surgery and radiation. Chemotherapy is widely used for the cancer sufferers but always shows some undesirable side effects. In order to obtain novel anticancer compounds, more and more attention is paid to the plant kingdom. Some natural products have been successfully applied in clinic treatment of various cancers. Taxol which is extracted form *Taxus* chinensis, has been abroad utilized to cure malignant epithelial tumor.[1]

Ursolic acid (UA, 3β-hydroxy-urs-12-en-28-oic acid), a pentacyclic triterpene acid existing abundantly in the plant kingdom, has attracted the attention of the pioneers who aim to develop novel anti tumor agents.[2] Recent studies have shown that ursolic acid has marked anti-tumor activity toward various types of cancer cell lines in vitro and in vivo.[2,3] As a prospective anticancer drug, considerable structural modification has been performed on UA to obtain potential anticancer derivatives with enhanced pharmacokinetic/pharmacodynamic and physical/chemical properties. Meng[4] prepared fifteen UA derivatives, some of which exerted higher anticancer activity than UA against Hela cells. Structural modification was carried out by Ma[5] as well and some UA derivatives with enhanced cytotoxicity upon HL-60, Bel-7402, BGC-823 and Hela cell lines were obtained.

In order to hunt for the potential anti-tumor drug candidates and to study their structure-activity relationships, our group has prepared series of UA derivatives. In this study a series of novel UA derivatives were reported and their cytotoxic activities were evaluated against human hepatoma cell HepG2, gastric cancer cell BGC-823 and colorectal carcinoma cell HT-29. The anti-cancer capacity of the prepared **UA-1a** against BGC-823 was evaluated both in vitro and in vivo. The expression of Bcl-2 and Survivin and the activity of Caspase-3 were determined to explore the possible anticancer mechanism.

2. Experimental protocols

2.1. Materials

Human gastric adenocarcinoma cell line BGC-823, hepatoma cell line HepG2, colorectal carcinoma cell line HT-29 were obtained from Shanghai Cell Resource Center (Chinese Academy of Sciences, Shanghai, China). Taxol and UA were purchased from Sigma (St. Louis, MO, USA). Fetal calf serum (FCS), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Penicillin/Streptomycin, trypsin and RPMI-1640 medium, were provided by Gibco (Gaithersburg, MD, USA). Caspase-3 activity detection Kit was obtained from Beyond Time (Haimen, China). AnnexinV-FITC apoptosis detection Kit and Cell apoptosis PI cycle detection Kit were supplied by KeyGEN (Nanjing, China). Monoclonal anti-β-Actin,

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monoclonal anti-Survivin, monoclonal anti-Bcl-2 primary antibodies, and horseradish peroxidase (HRP) conjugated goat anti-rabbit/mouse IgG were from Santa Cruz Bio-technology (Inc., CA, USA). Low melting point (LMP) agarose was a product from Invitrogen (USA). Other reagents of an analytic grade were purchased from commercial suppliers.

2.2. The synthesis of UA derivatives

A series of ursolic acid derivatives were synthesized as shown in Figure 1.

2.2.1. Preparation of the intermediate product (UA-3)

UA was converted to its 3-*O*-acetate **UA-3** as previously described.[4–6] To be brief, UA (456 mg, 1 mmol) was dissolved in pyridine (5 mL), mixed with acetic anhydride (0.5 mL), and the solution was stirred at room temperature for 6 h. The reaction mixture was added water (40 mL) and partitioned with dichloromethane (60 mL × 3). The organic solution was dried over Na_2SO_4 and concentrated in vacuo to give crude product. The crude product was purified via crystallization and then recrystallization in ethanol to obtain **UA-3**.

2.2.2. General procedure for esterification at C-28 carboxylic acid of UA and the intermediate product

UA (182 mg, 0.4 mmol) or **UA-3** (200 mg, 0.4 mmol) was well distributed (or dissolved) in 20 mL acetone, to which was added K_2CO_3 (1 mmol) and KI (1 mmol). Stirred at room temperature for 6 h, the reactant was mixed with (DL)-3-Chloro-1,2-propanediol (100 μL, 1.2 mmol) or 2-chloroethanol (80 μL, 1.2 mmol) for another 24 h. The mixture was concentrated in vacuo, mixed with distilled water, neutralized with HCl, filtered to get crude solid. The crude was purified by chromatography on a silica gel column eluted with petroleumether/ethylacetate/triethylamine (V/V/V = 33:66:1) to get purified compounds **UA-1a**, **UA-1b** and **UA-2**.

2.2.3. Determination of the physical/chemical properties of UA derivatives

The melting points were determined on an electrically heated X-4 digital visual melting point apparatus and are uncorrected. IR spectra were recorded on Thermo Nicolet Nexus 470 spectrometer. ^{1}H NMR spectra were recorded on a BRUKER AVANCE 600 MHz spectrometer at room temperature, and chemical shifts were measured in ppm downfield from TMS as internal standard. Mass spectra were recorded on Agilent 1100 LC/MSD Trap XCT equipment, in positive Electron Spray Ionization (ESI) mode and were reported as *m*/*z*. UV–vis absorbance spectra were recorded on a Shimadzu UV-1700 Pharma spectrometer.

2.3. Cell line and culture

The human cell lines including BGC-823, HepG2, HT-29 and GES-1 were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FCS, penicillin (100 U/mL) and streptomycin (100 μg/mL) in a humidified atmosphere supplied with 5% CO_2 at 37 °C. The cells in logarithmic phase were allowed for the following tests.

2.4. MTT assay for cell viability/proliferation

The cytotoxicity of the derivatives was determined by the MTT assay.[4–6] UA, Taxol and UA derivatives were dissolved in DMSO, then diluted with culture medium. The content of DMSO in each sample was 0.5%. Cells were seeded in 96-well plates at a density of 5×10^3 cells per well. After 24 h, the cells were treated with different concentrations of drugs for another setting period (24–72 h). The negative control cells were treated with culture medium containing 0.5% DMSO. Finally, excess MTT was added, and the culture was incubated for another 4 h. The resultant formazan formed by metabolically viable cells was well dissolved in 100 mL of DMSO. The absorbance was then measured on a microplate reader apparatus (DNA Expert, TECAN, Switzerland) at 570 nm. Each sample had six duplicates and the average absorbance was applied into the following formula to calculate the growth inhibition.[5] The concentration of the compound, which gives the 50% growth inhibition value, corresponds to the IC_{50}. Each test was repeated at least three times and the results were expressed as mean ± SD.

% inhibition = [1−(OD value for treated cells/OD value for untreated cells)] × 100%.

2.5. Cell cycle analysis

The cell cycle was analyzed by flow cytometry. Briefly, BGC-823 cells were treated with **UA-1a** (20 μM) for 24–72 h. After the incubation, approximately 10^6 treated cells were harvested, and the cell cycle phase distribution was assessed by propidium iodide (PI) staining, according to the manufacturer's instruction (Cell apoptosis PI cycle detection Kit) in a flow cytometer (Beckman Coulter, EPICS XL, USA). The percentage of cells in G0/G1, S, G2/M and sub-G0/G1 phase were analyzed by means of CellQuest software program.[7] Similar process was performed after the incubation with different concentration of **UA-1a** (10, 20, 40 μM) for 48 h to determine the effects of the dosage on the DNA content. All experiments were repeated at least three times.

2.6. Annexin-V/PI dual-staining assay

Tumor cells were treated with 0, 10, 20, 40 μM **UA-1a** for 48 h, washed and resuspended in PBS buffer. Apoptotic cells were identified by double staining with recombinant fluorescein isothiocyanate (FITC)-conjugated Annexin-V and PI[8], by using the AnnexinV-FITC apoptosis detection Kit following the manufacturer's instructions. Flow cytometric analysis was performed immediately after staining. Data acquisition and analysis were performed by using CellQuest software.[7]

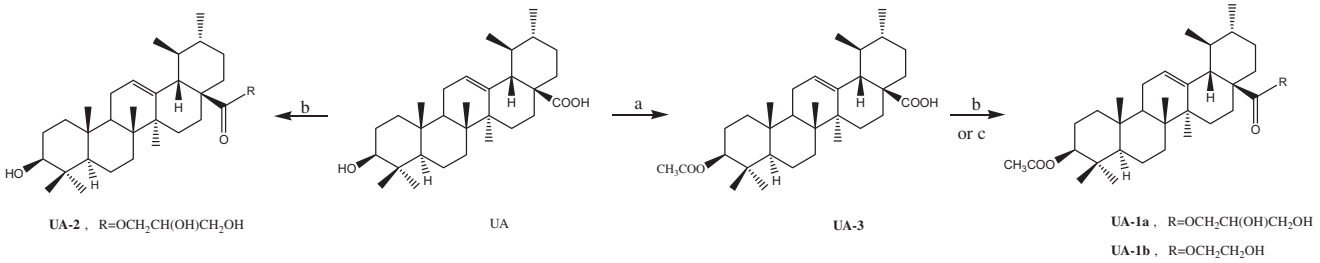

Figure 1. Route for synthesis of ursolic acid derivatives. Reagents and conditions: (a) $(CH_3CO)_2O$, pyridine, at room temperature; (b) (DL)-3-chloro-1,2-propanediol, K_2CO_3, acetone, reflux; (c) 2-chloroethanol, K_2CO_3, acetone, reflux.

2.7. Comet assay

BGC-823 cells were incubated for 48 h at 37 °C in the presence of **UA-1a** (10, 20, 40 μM). After the treatment, about 10^5 treated cells were harvested to perform the alkaline comet assay as described by Thierry Godard.[9] The comet bends were recorded by GelPro Documentation System (Biosens 810, Shanghai, China).

2.8. Determination of Total Caspase-3 enzyme activity

BGC-823 cells were treated with **UA-1a** (0, 10, 20 μM) for 48 h. After the treatments cells were detached with trypsin/EDTA and washed twice with cold PBS buffer. Then, about 10^6 cells were harvested and the total Caspase-3 activities were determined by using the Caspase-3 activity detection Kit following the manufacturer's instructions. Briefly, the collected cells were lysed and the lysis was incubated with sufficient Ac-DEVD-pNA (acetyl-Asp-Glu-Val-Asp p-nitroanilide, the corresponding fluorogenic substrate) for 1 h. Cleavage of substrate was monitored by DNA expert (TECAN,-CA) at 405 nm. Additionally, Bradford Protein Assay[10,11] was used to quantify the content of total protein. Results were expressed as the change in fluorescence units (per 1 g of protein) relative to control.

2.9. Western blot analysis

After the incubation with **UA-1a** (10, 20, 40 μM) for 48 h, the BGC-823 cells were detached with trypsin/EDTA. The determination of Bcl-2 and Survivin proteins was performed as described.[12,13] To be brief, more than 10^6 cells were lysed and the total protein was harvested and quantified via Bradford Protein Assay.[10,11] For each sample, 150–200 μg of protein was loaded onto 12.5% SDS–polyacrylamide gel, electrophoresed and transferred to a PVDF membrane (0.45 μm). The primary antibodies were used at a concentration of 1:1000 for Survivin, 1:200 for Bcl-2 and 1:1000 for β-actin. The membranes were incubated for 1 h with HRP-labeled secondary antibodies, then developed by an ECL system following the manufacturer's instructions (Beyotime, Nanjing, China). Quantification of band intensity was performed by means of Gel-Pro Analyzer 3.1 software.[12]

2.10. Anti-tumor effect in vivo

The in vivo therapeutic effect of **UA-1a** was determined as described by Zheng.[8] Male nude mice (5 weeks old, 18–22 g body weight) were subcutaneously inoculated with 1×10^7 BGC-823 cells. After tumor volume reached about 200 mm^3, the tumor-bearing nude mice were randomized into four experimental groups, each with eight mice. The control mice were given with 0.2 mL 0.9% saline each. Two treatment groups were intravenously injected with 0.2 mL **UA-1a** solution at the doses of 6 mg/Kg and 30 mg/Kg, respectively. In addition, the positive control mice were given with 0.2 mL Taxol solution at the dose of 8 mg/Kg. The tumor volume were calculated once a day using the following formula: tumor volume = 0.5 × (length × width × width). After tail vein injections for five times (day 1st, 4th, 7th, 10th, and 13th), all the animals were sacrificed at day 16th and the tumors were dissected and weighted.

2.11. Statistic analysis

Results were expressed as the mean ± standard deviation (SD) of data obtained from at least triplicate experiments. A statistical analysis was performed with one-way analysis of variance (ANOVA) using SPSS 13.0 analytical software. Data were expressed as mean ± SD ($n \geqslant 3$, where n represents the number of independent experiments). Results with a P value less than 0.05 were considered to be statistically significant.

3. Results

3.1. Properties of UA derivatives

UA-3, UA-1a, **UA-1b** and **UA-2** with high purity were prepared as described in Section 2.2. Their physical/chemical properties were as followed:

3.1.1. 3β-Acetoxy-urs-12-en-28-oic acid (UA-3)

While crystalline powder; yield 70.5%; mp: 284–287 °C (lit.[14,15] 285 °C, 287–289 °C); IR (KBr): 3366, 2972, 2928, 1736, 1697, 1455, 1370, 1246 cm^{-1}; ESI-MS: m/z 499.6 $[M+H]^+$. UV–vis (methanol), λ_{max} = 210 nm; ^1H NMR (600 MHz, $CDCl_3$): 5.32 (t, 1H, J = 3.6 Hz, H-12), 4.42 (t-like, 1H, H-3), 2.22 (d, 1H, J = 11.2 Hz, H-18), 2.04 (s, 3H, CH_3CO), 0.92 (d, 3H, J = 6.4 Hz, CH_3), 0.85 (d, 3H, J = 6.6 Hz, CH_3), 1.09, 1.00, 0.91, 0.78, 0.74 (s, 15H, 5× CH_3).

3.1.2. [3β-Acetoxy-urs-12-en-28-oyl]-1-monoglyceride (UA-1a)

While crystalline powder; yield 85.8%; mp: 127–129 °C; IR (KBr): 3442, 2970, 2928, 2874, 1735, 1245 cm^{-1}; ESI-MS: m/z 595.5 $[M+Na]^+$. UV–vis (Methanol), λ_{max} = 209 nm; ^1H NMR (600 MHz, $CDCl_3$): 5.25 (t, 1H, J = 3.6 Hz, H-12), 4.51–4.48 (m, 1H, H-3), 4.12–4.06 (m, 1H, $COOCH_2a$), 4.20–4.17 (dd, 0.5H, J = 11.5,4.5 Hz, $COOCH_2b$), 4.04–4.01 (dd, 0.5H, J = 11.5,6.0 Hz, $COOCH_2b$), 3.95–3.91 (m, 0.5H, $CH(OH)CH_2OH$), 3.91–3.87 (m, 0.5H, $CH(OH)CH_2OH$), 3.70–3.67 (m, 1H, $HOCH_2a$), 3.60–3.57 (m, 1H, $HOCH_2b$), 2.22 (d, 1H, J = 11.2 Hz, H-18), 2.04 (s, 3H, CH_3CO), 0.95 (d, 3H, J = 6.2 Hz, CH_3), 0.87 (d, 3H, J = 6.6 Hz, CH_3), 1.08, 0.94, 0.86, 0.85, 0.75 (s, 15H, 5× CH_3).

3.1.3. [3β-Hydroxy-urs-12-en-28-oyl]-1-monoglyceride (UA-2)

While crystalline powder; yield 86.0%; mp: 100–102 °C; IR (KBr): 3351, 2948, 2925, 2869, 1788, 1718, 1456, 1184,1044 cm^{-1}; UV–vis (methanol), λ_{max} = 207 nm; ESI-MS: m/z 553.5 $[M+Na]^+$; ^1H NMR (600 MHz, $CDCl_3$): 5.26 (t, 1H, J = 3.6 Hz, H-12), 4.12–4.06 (m, 1H, $COOCH_2a$), 4.20–4.17 (dd, 0.5H, J = 11.5,4.5 Hz, $COOCH_2b$), 4.04–4.01 (dd, 0.5H, J = 11.5,6.0 Hz, $COOCH_2b$), 3.94–3.91 (m, 0.5H, $CH(OH)CH_2OH$), 3.90–3.87 (m, 0.5H, $CH(OH)CH_2OH$), 3.70–3.67 (m, 1H, $HOCH_2a$), 3.60–3.57 (m, 1H, $HOCH_2b$), 3.22–3.20 (dd, 1H, J = 11.3,4.5 Hz, H-3), 2.22 (d, 1H, J = 11.2 Hz, H-18), 0.95 (d, 3H, J = 6.2 Hz, CH_3), 0.85 (d, 3H, J = 6.4 Hz, CH_3), 1.09, 0.99, 0.91, 0.78, 0.75 (s, 15H, 5× CH_3). .

3.1.4. [3β-Acetoxy-urs-12-en-28-oyl]-1-ethylene gylcol monoester (UA-1b)

While crystalline powder; yield 90.1%; mp: 159–161 °C; IR (KBr): 3511, 2970, 2948, 2930, 2878, 1731, 1694, 1455, 1371, 1243 cm^{-1}; UV–vis (methanol), λ_{max} = 212 nm; ESI-MS: m/z 565.6 $[M+Na]^+$; ^1H NMR(600 MHz, $CDCl_3$): 5.26 (t, 1H, J = 3.6 Hz, H-12), 4.51–4.48 (m, 1H, H-3), 4.22–4.18 (t, 1H, $COOCH_2a$), 4.10–4.06 (m, 1H, $COOCH_2b$), 3.82–3.75 (m, 2H, $HOCH_2$), 2.25 (d, 1H, J = 11.2 Hz, H-18), 2.04 (s, 3H, CH_3CO), 0.95 (d, 3H, J = 6.2 Hz, CH_3), 0.87 (d, 3H, J = 6.6 Hz, CH_3), 1.09, 0.94, 0.86, 0.85, 0.77 (s, 15H, 5× CH_3);

3.2. Cytotoxicity of the UA derivatives against different cell lines

The in vitro cytotoxic activity was evaluated for UA and its derivatives against three tumor cell lines (HepG2, HT-29, BGC-823) and a human normal gastric cell line (GES-1). Taxol was used as a positive control. The results were shown in Table 1. It can be seen from Table 1 that UA suppressed proliferation of the three assayed cancer

cells in different extents (IC_{50} values of 23.0–53.4 μM) and exhibited some toxic effect on GES-1 (IC_{50} value of 93.8 μM). The prepared UA derivatives exhibited more effective anti cancer activity. Introduction of a monoglyceride at the C-28 position, giving birth to **UA-2**, might obviously reduce the toxicity for GES-1 cells. **UA-3**, the 3-O-acetate of UA, demonstrated higher cytotoxicity than UA against the three cancer cell lines. **UA-1a** and **UA-1b** were two compounds resulting from the esterification of the carboxyl group at the C-28 position of **UA-3** with glycerol and ethylene glycol, respectively. Compared with UA, both **UA-1a** and **UA-1b** showed the higher anti cancer activity and the lower cytotoxicity for the normal GES-1cells.

UA-1a showed the most significant antiproliferative ability among the synthesized UA derivatives. It possessed stronger anti-tumor activity against BGC-823 cells than Taxol. The IC_{50} value of **UA-1a** was 15.66 μM after treating BGC-823 cells for 48 h, which was 50% lower than that of Taxol (Table 1). This UA derivative inhibited the growth of BGC-823 cells in a dose- and time-dependent manner (Fig. 2). The suppression of cells growth was rapidly heightened when treated with **UA-1a** at the concentrations ranged from 10 μM to 30 μM. Cell shrinkages and other obvious morphological changes of BGC-823 cells were observed after the treatment with **UA-1a** for 48 h at 30 μM (data not shown). In addition, **UA-1a** had lower toxicity than Taxol against GES-1 cells (Table 1).

The novel UA derivative **UA-1a** might have a therapeutic potential in the treatment of gastric tumor since it showed more significant anti-proliferation activity against BGC-823 cells and greater safety for the normal cells than UA and Taxol. The anti-cancer mechanism and the in vivo anti-tumor effects needed further research.

3.3. Cell cycle distribution of BGC-823 cells treated by UA-1a

To characterize the cell death triggered by **UA-1a**, cell cycle analysis was performed by staining the cells with PI. The results were shown in Figure 3. Compared with the negative control, a dose-dependent increasing in the percentage of cells in sub-G0/G1 phase was observed (from 0.66% in control cells to 96.13% when treated by 40 μM **UA-1a**) (Fig. 3A). Meanwhile, the percentage of cells in sub-G0/G1 phase was increased in a time-dependent manner, with 3.71%, 7.91% and 13.63% of sub-G0/G1 cells for 24 h, 48 h, and 72 h, respectively when treated by 20 μM **UA-1a** (Fig. 3B). Treatment of BGC-823 cells with **UA-1a** distinctly resulted in a dose- and time-dependent apoptosis. The cell cycle distribution (such as G0/G1, S, G2/M) was slightly modified under different treating conditions and no marked arrest at any phase of the cell cycle was found.

3.4. Comet assay of BGC-823 cells treated by UA-1a

The comet assay is a sensitive method for detection of DNA fragmentation occurring during apoptosis.[9] The comet effect of **UA-1a** on BGC-823 cells was shown in Figure 4. Cells with larger,

Table 1
The in vitro cytotoxicity of ursolic acid (UA) derivatives [expressed as IC_{50} (μM)] against HT-29, HepG2 and BGC-823 human cancer cell lines, as well as the normal human gastric epithelial cell line GES-1 (48 h)

Compound	HT-29	HepG2	BGC-823	GES-1
Taxol	<10	30.74 ± 4.59	33.09 ± 5.91	106.2 ± 13.1
UA	26.31 ± 2.74	53.42 ± 4.07	22.96 ± 0.99	93.83 ± 8.51
UA-2a	nt[b]	44.23 ± 2.81**	20.51 ± 4.26	154.3 ± 19.2**
UA-3	23.89 ± 1.52*	37.67 ± 2.92**	19.36 ± 1.70*	92.94 ± 6.34
UA-1a	18.43 ± 1.83**	27.46 ± 1.78**	15.66 ± 1.38**,a	155.4 ± 11.4**,a
UA-1b	23.56 ± 1.69*	29.83 ± 3.34**	17.22 ± 2.50**	137.2 ± 10.9**

* $P < 0.05$ versus UA.
** $P < 0.01$ versus UA.
[a] $P < 0.01$ versus Taxol.
[b] Not tested.

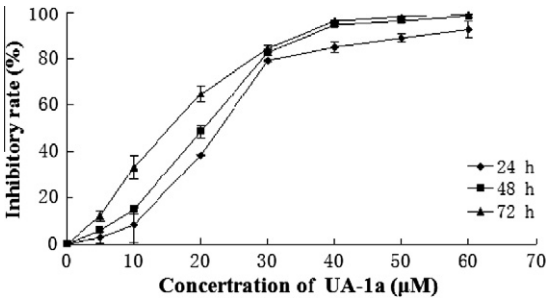

Figure 2. Cytotoxicity of **UA-1a** against human gastric carcinoma cell line BGC-823. Growth inhibition was determined by MTT assay. Each value represented the mean ± SD of three independent experiments, each of which had six replicates.

more defined heads were scored as normal cells with minimal DNA diffusion, whereas cells with diffuse fan-like tails and small heads were considered as apoptotic cells. It was clearly demonstrated that the capacity of **UA-1a** to trigger apoptosis of BGC-823 cells was dependent on the dose.

3.5. Annexin-V/PI dual-staining assay of BGC-823 cells treated by UA-1a

The apoptotic effect of **UA-1a** was further evaluated by AnnexinV-FITC/PI (AV/PI) dual staining experiment to examine the occurrence of phosphatidylserine externalization onto the cell surface. This experiment allowed the differentiation among normal alive cells (AV^-/PI^-), early-phase apoptotic cells (AV^+/PI^-), and late-phase apoptotic or necrotic cells (AV^+/PI^+). As shown in Figure 5, when the cells were not treated with **UA-1a**, as a control, 95.5% of cells were in the normal condition. When the dose of **UA-1a** increased up to 20 μM, the population of normal cells was decreased to 72.2%, however 19.4% of treated cells entered into the early apoptotic stage. A substantial amount of apoptotic cells, 78.8% both in the early apoptotic stage and in the late apoptotic stage, were detected after being treated by **UA-1a** at 30 μM. Treating with higher concentration of **UA-1a** induced a shift of the cell population to the late apoptotic stage. 89.5% of cells were observed in the late apoptotic/necrotic phase when treated with 40 μM of **UA-1a** for 48 h. The data indicated that the population of apoptotic cells increased in a dose-dependent manner and the critical concentration for the induction of apoptosis of BGC-823 by **UA-1a** was about 30 μM.

3.6. Determination of Caspase-3 activity in BGC-823 cells treated by UA-1a

The proteolytic activity of the executioner Caspase-3 was determined to explore the role of caspases in the apoptosis induced by **UA-1a**. As shown in Figure 6, a significant increase of Caspase-3 activity in the BGC-823 cells was observed after 48 h exposure to **UA-1a**. Compared with the control, the total Caspase-3 activity was increased up to 2.54-fold and 3.65-fold, respectively after BGC-823 cells were separately incubated with 10 μM and 20 μM of **UA-1a**. It was probable that **UA-1a** induced the apoptosis of BGC-823 cells via the caspase-dependent pathways mediated by the activation of Caspase-3.

3.7. Effect of UA-1a on the expression of Survivin and Bcl-2

Some special proteins have been reported to be associated with the apoptosis pathway. In this work the effect of **UA-1a** on the expression of Survivin and Bcl-2 proteins was examined (Fig. 7A). The Survivin expression in cells treated with **UA-1a** (10, 20, 40 μM) were decreased by 37.6%, 43.1% and 53.1%, respectively

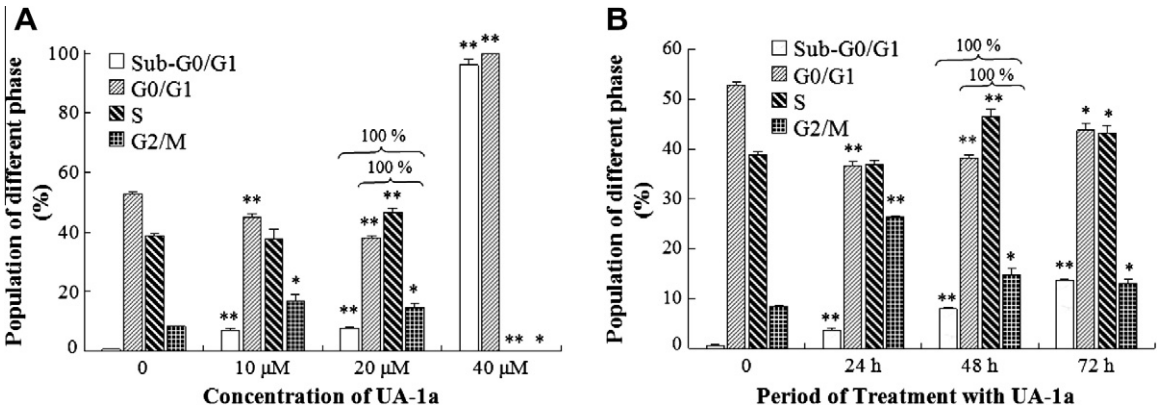

Figure 3. The cell cycle distribution of BGC-823 cells treated with (A) 0, 10, 20, 40 μM of **UA-1a** for 48 h and with (B) 20 μM of **UA-1a** for 0, 24, 48, 72 h was shown. The percentage of cells residing in sub-G0/G1phase was counted. Simultaneously, the cell population of G0/G1, S and G2/M phases that constituted the DNA-integrity cells (taken as 100%) were analysed. Results were expressed as mean ± SD. $^{*}P < 0.05$; $^{**}P < 0.01$, versus control (0 μM)

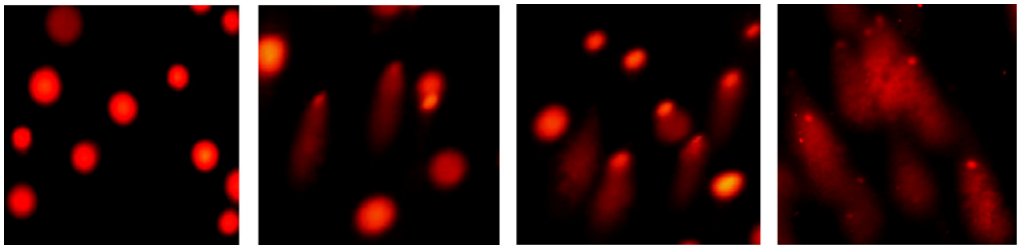

Figure 4. Comet-like DNA electrophoresis bends in BGC-823 cells treated with **UA-1a** at different concentrations (0, 10, 20 and 40 μM) for 48 h.

when compared to that of the control (Fig. 7B). In addition, **UA-1a** significantly down-regulated the expression of Bcl-2 (Fig. 7B). It was evident that **UA-1a** suppressed the expression of Survivin and Bcl-2 in a dose-dependent manner. The results indicated that the inhibition of expression of Survivin and *bcl-2* genes was involved in the anti-tumor activity of **UA-1a**.

3.8. In vivo anti-tumor effect of UA-1a

The above experiments indicated **UA-1a** exhibited an anti-proliferation effect on the BGC-823 cells in vitro. Subsequently, a kind of animal model, nude mice subcutaneously injected with BGC-823 cells was employed to explore if **UA-1a** was able to suppress the tumor growth in vivo. It was found that the tumor sizes in the mice treated by **UA-1a** were significantly smaller than that of the mice in the control group. After five times of tail vein injection, the tumor volume were diminished by 29.3% (at low dose, 6 mg/Kg) and 41.9% (at high dose, 30 mg/Kg), when compared with the control (Fig. 8A). The solid tumors of each group were weighted and the data showed that the tumors treated with **UA-1a** were markedly lighter than that without any treatment (Fig. 8B).

Taxol was used as a positive control. The determined tumor volume was decreased by 15.2% when treated with Taxol at the dose of 8 mg/Kg (Fig. 8 A&B). We concluded **UA-1a** exerted stronger ability to inhibit tumor growth than Taxol. Moreover, no significant difference was observed on the body weight of nude mice treated with **UA-1a** when compared with the control group (Table 2), which demonstrated that **UA-1a** retarded tumor growth in nude mice without leaving apparent toxicity to the hosts.

4. Discussion

Ursolic acid (UA) has been shown to possess anti-proliferative ability against various types of cancer cells.[2–6] More and more structural modification has been performed at the C-3 or C-28 position of UA to enhance its pharmacokinetic /pharmacodynamic properties. The results of Ma[5] revealed that the triterpenes possessing two hydrogen-bond forming groups (an H-donor and a carbonyl group) at positions C-3 and C-28 exhibit cytotoxic activity. Introduction of an amino group at these two positions might increase the cytotoxicity. Meng[4] concluded that both the 3-O-acetyl group and a 28-amido group might be important for improving the anti tumor activity, however a hydroxyl group at the C-28 amide side chain could not influence the antiproliferative effect.

In this study, some UA derivatives were prepared by esterification at C-3 or/and C-28 positions of the parent ursolic acid. The structure-activity relationships (SAR) of these UA derivatives suggested the 3-O-acetyl group might be important for improving the anti-tumor activity. The introduction of a monoglyceride or a ethylene gylcol monoester at the C-28 position could reduce the toxicity of ursolic acid or its 3-O-acetate toward the normal gastric cells.

UA-1a was synthesized via condensation of acetic acid with the hydroxyl group at the C-3 position of ursolic acid and then by esterification of the carboxyl group at C-28 position with glycerol. This novel compound showed strengthened anti-cancer effect. The reason was probably contributed to the obtained better lipophilicity, which often resulted in better membrane permeability, followed with better pharmaceutical effects.[16] The experimental data also revealed the esterification of the carbonyl group of the mother nucleus might be important for the cytotoxicity. The introduction of polylol to the C-28 position could benefit both of the anti-tumor activity and the safety toward normal cells.

Apoptosis, or programmed cell death, plays an essential role in developing and maintaining tissue homeostasis through eliminating genetically damaged cells and excess cells.[12] It is a fundamental process involving the degradation of DNA in a scheduled manner.[17] After **UA-1a** treatment, the apoptosis events of the treated BGC-823 cells,

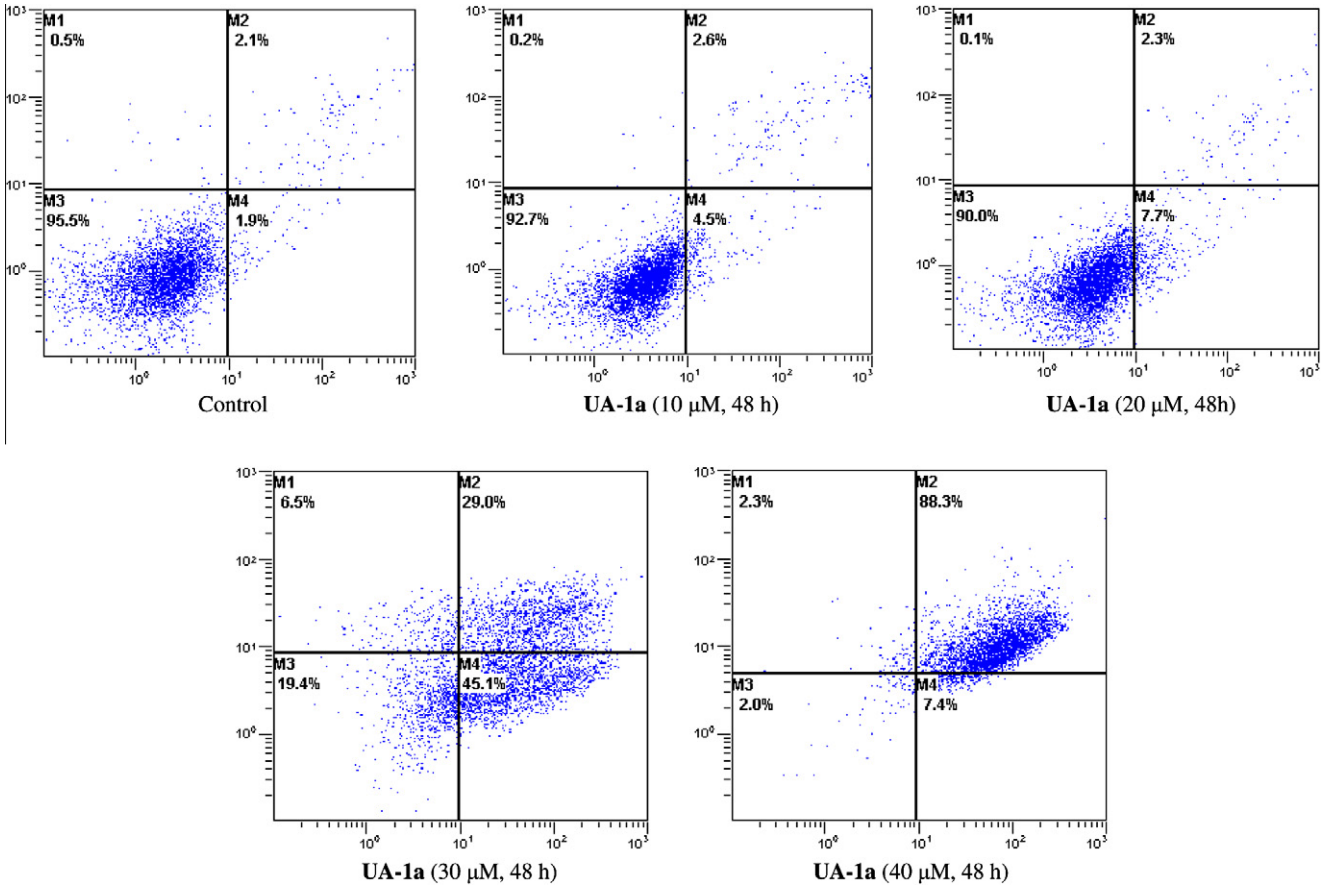

Figure 5. Annexin V/PI dual staining of BGC-823 cells treated with **UA-1a** (0, 10, 20, 30 and 40 μM) for 48 h was carried out. The cells were harvested, stained and then analyzed by Flow cytometry. In all panels, cells in the lower left quadrant (M3: AV^-/PI^-) were alive, cells in the lower right quadrant (M4: AV^+/PI^-) were in early apoptosis, cells in the upper right quadrant (M2: AV^+/PI^+) were in late apoptosis/necrosis, and cells in the upper left quadrant (M1: AV^-/PI^+) were damaged appearing in the process of cell collection. Percentage of total signal within the quadrant was indicated.

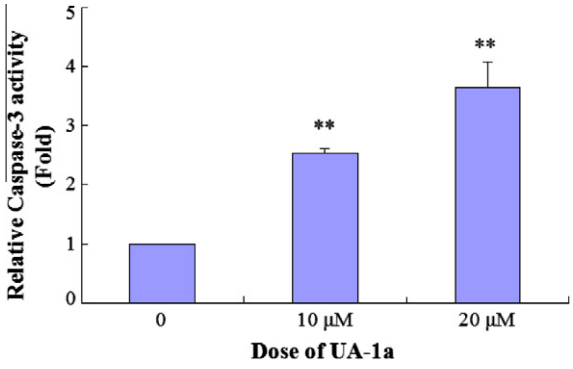

Figure 6. The relative Caspase-3 activity in BGC-823 cells treated by **UA-1a** (0, 10, 20 μM). Enzymatic activities of Caspase-3 were defined as the fluorescence per one gram of protein in the treated sample. Relative Caspase-3 activity was shown as the ratio of enzymatic activities of **UA-1a** treated cells in respect to that of the control (0 μM). Results were express as mean ± SD of three independent experiments. $^*P<0.05$; $^{**}P<0.01$ versus control.

including comet-like DNA bend and sub-G0/G1 phase were observed. Annexin V/PI dual staining assay further provided an explicit evidence for the induction-effect of **UA-1a** on the apoptosis of BGC-823 cells. The experimental data suggested that apoptosis play an important role in the proliferation inhibition of BGC-823 cells by **UA-1a**.

Caspase-3 is a key apoptotic executive caspase, known to mainly mediate the caspase activated DNase (CAD) which is an endonulease responsible for DNA degradation as well as forming of comet-like DNA bend.[18,19] It was found in our experiments that the activity of caspase-3 could be enhanced more than 2 times by **UA-1a**. These results suggested that **UA-1a** trigger the cancer cell death by caspases-dependent apoptotic mechanism involving the activation of caspase-3.

Gastric carcinoma exhibits high level of Bcl-2 protein expression. Bcl-2 overexpression protects cancer cells from apoptosis induced by different stimuli.[20] In this work, down-regulation of Bcl-2 expression by **UA-1a** initiated apoptosis of BGC-823 cells in a caspases-dependent apoptotic pathway mediated by Bcl-2 protein. Moreover, Bcl-2 is a member of the Bcl-2 family proteins which provided the link between the caspase cascade and the mitochondria.[12,21] It was probable that the programmed cell death of BGC-823 cells treated by **UA-1a** proceeded in the mitochondria pathway.

Survivin is a member of the inhibitors of apoptosis protein family (IAP).[13] A sustained overexpression of Survivin is a characteristic feature of gastric cancer, and plays an important role in tumor progression by inhibiting apoptosis and facilitating mitosis, giving cancer cells a survival and growth advantage.[22] In this work, the expression of Survivin was greatly inhibited by **UA-1a** in a dose-dependent manner. The down-regulation of Survivin expression resulted in the increase in the population of apoptotic cells.

In conclusion, our experimental data exhibited a satisfactory anti-cancer activity of **UA-1a** against BGC-823 cells and suggested a clear anti-cancer mechanism. It was notable that **UA-1a** exerted stronger ability than Taxol to retard tumor growth in nude mice

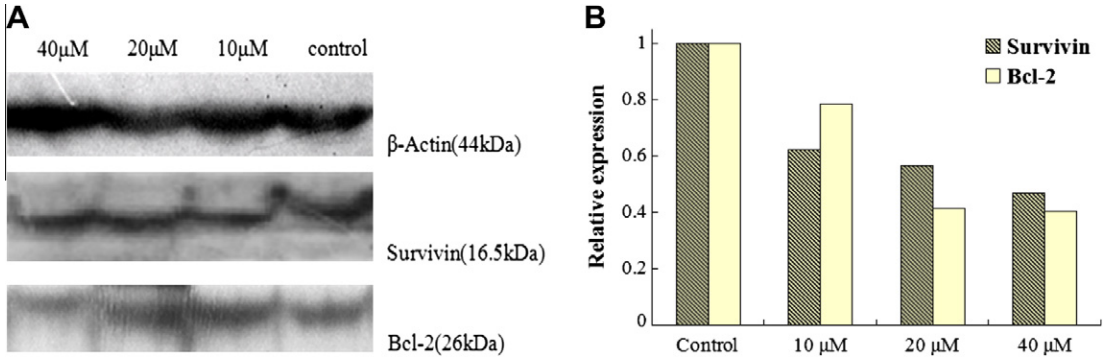

Figure 7. Effects of various concentrations of **UA-1a** on the expression of Survivin and Bcl-2 proteins in BGC-823 cells. (A) Western blot analysis of Survivin and Bcl-2; (B) Quantification of relative expression of Survivin and Bcl-2 when comparing with the control.

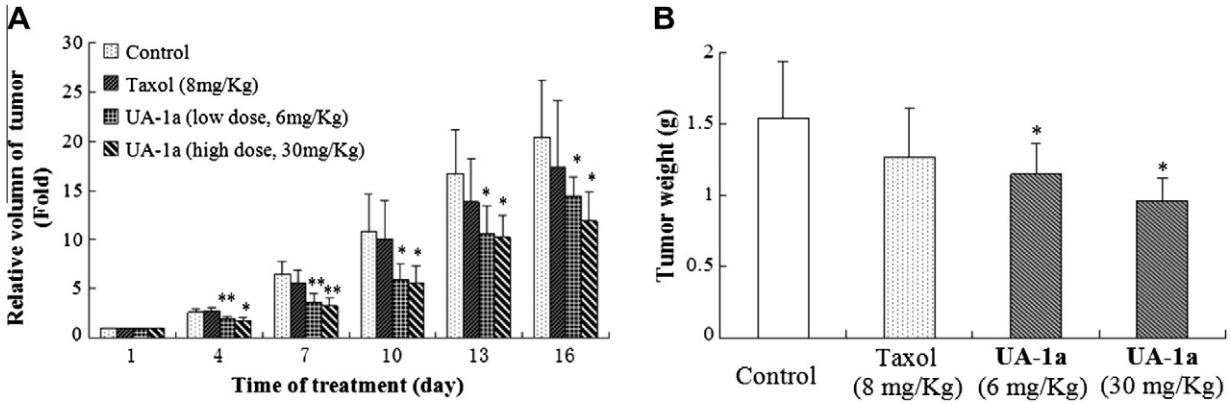

Figure 8. Anti-tumor effects of **UA-1a** on nude mice bearing BGC-823 cells. (A) The relative tumor volume and (B) the tumor weight were shown as mean ± SD of eight replicates. $^{*}P<0.05$; $^{**}P<0.01$ versus control.

Table 2
Effects of **UA-1a** on the body weight (gramme) of BGC-823 cells xenograft nude mice ($n = 8$)

Days of treatment	Control	Taxol (8 mg/Kg)	**UA-1a** (6 mg/Kg)	**UA-1a** (30 mg/Kg)
1	19.69 ± 1.21	18.76 ± 1.49	19.21 ± 0.82	19.26 ± 1.16
4	20.58 ± 1.35	19.72 ± 0.92	20.66 ± 1.16	19.61 ± 1.04
7	20.79 ± 1.29	19.70 ± 1.10	20.13 ± 1.20	19.29 ± 0.92
10	20.54 ± 1.45	20.13 ± 1.37	20.03 ± 1.18	19.16 ± 2.23
13	20.21 ± 1.83	20.17 ± 1.61	20.07 ± 1.14	19.69 ± 2.02
16	20.12 ± 1.52	20.25 ± 1.43	20.09 ± 1.25	19.57 ± 1.78

without leaving apparent toxicity to the hosts. **UA-1a** might have a therapeutic potential in the treatment of gastric cancer.

Acknowledgments

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.05.017.

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