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# Synthesis and evaluation of ursolic acid derivatives as potent cytotoxic agents

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ABSTRACT

Structural modification was performed at the C-3 and C-28 positions of ursolic acid (UA). Ten UA derivatives with distinct electrical property were synthesized. They could be divided into two groups according to their charge under physiological conditions: (1) Group I negatively charged and (2) Group II positively charged. The anti-proliferative capability of the derivatives was evaluated against HepG2, AGS, HT-29 and PC-3 cells by the MTT assay. Flow cytometry and Annexin V/PI dual staining assay were carried out to explore the antitumor mechanism. The results showed the cytotoxic capacity of the compounds was: Group I < UA < Group II. The UA derivatives in Group II exhibited potent cytotoxicity and the enhancement of the lipophilicity could further strengthen the cytotoxicity. Triggering apoptosis and causing cell cycle arrest contributed to the anticancer mechanism. The UA derivative **UA-7** had the therapeutic potential in the treatment of gastric carcinoma since it showed potent cytotoxicity, reasonable oil/water partition, enhanced water solubility, and the ability to induce the apoptosis of AGS cells.



Ursolic acid (UA, 3β-hydroxy-urs-12-en-28-oic acid **1**) is a pentacyclic triterpene acid existing abundantly in the plant kingdom. It has been reported to possess a wide range of pharmacological properties, including anti-allergic, antiviral, anti-inflammatory, antibacterial, and antitumor activities.[1–3] The cytotoxicity of UA has attracted the attention of the pioneers who aim to develop novel antitumor agents.[1,3] As an effective natural anticancer compound, considerable structural modification has been performed on UA to obtain potential antitumor derivatives with enhanced physical/chemical and pharmacokinetic/pharmacodynamic properties.[2,4,5] It is expected that incorporation of polar moiety onto the C-3 or C-28 position might improve the water solubility and thus clinical utility.[1,6,7] Among these moieties, the ones possessing obvious electrical property are worthy of being taken into consideration in the case of their expected hydration-ability. The resultant charge of UA derivatives might play an important part in the structure–activity relationships of these derivatives. However, the work designed to explore the role of electrical property in the structure–activity relationships of UA derivatives, had not been systematically performed yet.

In this study, a series of novel UA derivatives with distinct electrical property were reported. These UA derivatives were prepared as showed in Scheme 1. Two compounds, **UA-4** and **UA-5** were derived from UA as described.[8] **UA-6**, a derivative converted from **UA-1**, was alkali hydrolyzed to prepare **UA-7**. **UA-6** and **UA-7** were conjugated with different amino acids such as L-glycine, L-methionine and L-phenylalanine, to obtain **UA-9a–c** and **UA-11a–c**, respectively. Structures of the UA derivatives and their high purity were confirmed by determination of FT-IR, ESI-MS, Elemental analysis and $^1$H NMR.[9] Based on their electrical property under physiological conditions, these UA derivatives could be divided into two groups: (1) Group I negatively charged including **UA-4** and **UA-5**; and (2) Group II positively charged containing **UA-6**, **UA-7**, **UA-9a–c** and **UA-11a–c**.

The in vitro cytotoxic activities of these derivatives were evaluated against human hepatoma cell HepG2, gastric carcinoma cell AGS, colorectal carcinoma cell HT-29 and prostatic carcinoma cell PC-3 by 3-(4,5-dimethylthiao-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) cell proliferation assay.[10] The antitumor drug Taxol was used as a positive control. The results were summarized in Table 1. To find out the effects of different kinds of electrical property borne by the derivatives on their anticancer activity, Figure 1 was plotted with $IC_{50}$ value as ordinate and type of the UA derivatives as the horizontal coordinate. Interestingly, a great difference of the anti-proliferative capability against the treated cell lines was observed: Group II > UA > Group I. The significant cytotoxicity of the UA derivatives with chemical group of positive charge at the C-28 position of UA might be explained by the electrostatic interaction between the derivatives and the assayed cells. The outer surface of cancer cells presented net negative charge in physiological environment,[11,12] which attach the positively charged compounds around cells, increasing in local drug concentration and higher inhibitory activity.[12]

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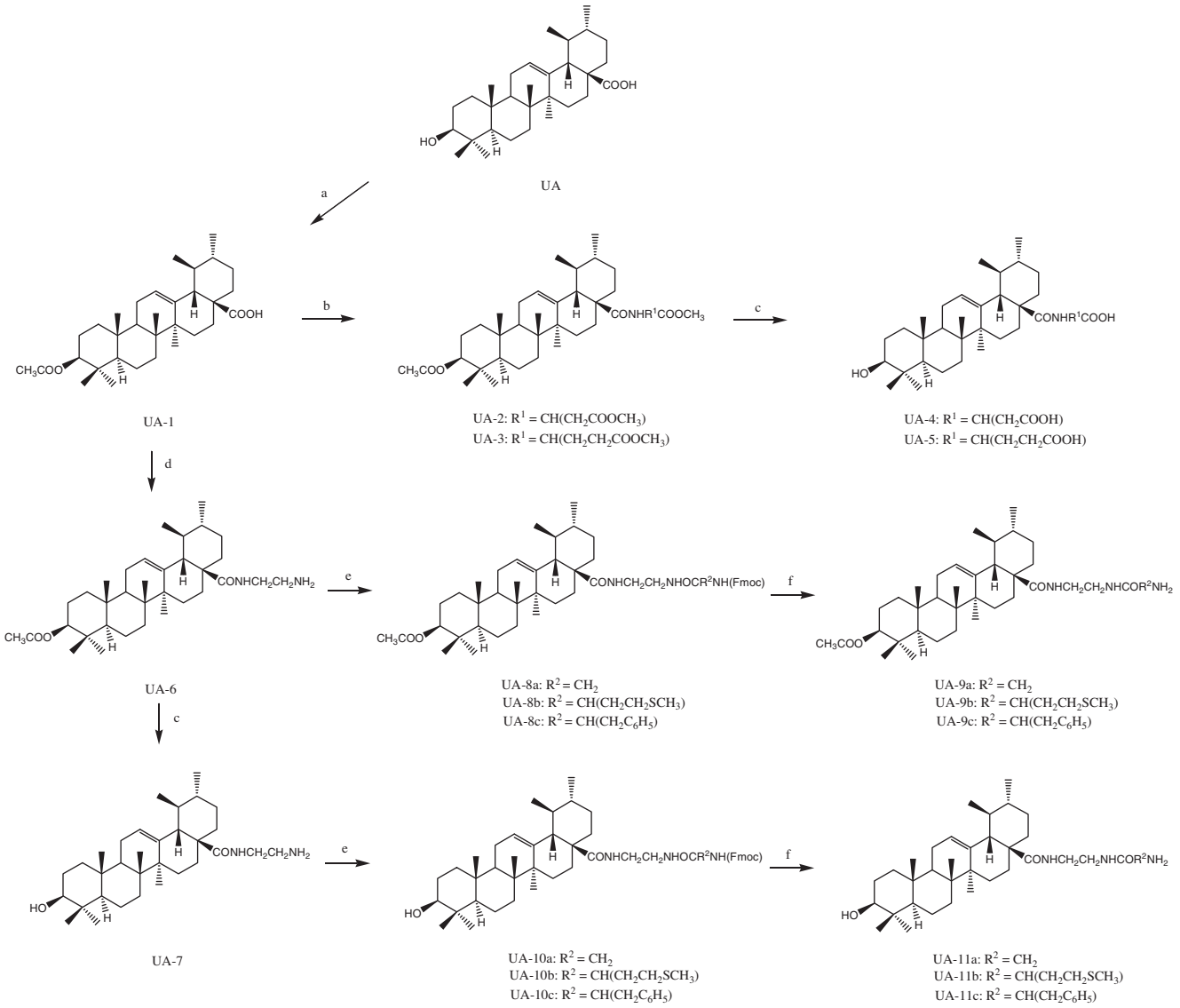


**Scheme 1.** Reagents and conditions: (a) $(CH_3CO)_2O$, DMAP, pyridine; (b) ①$(COCl)_2$, $CH_2Cl_2$ ②Asp(OMe)-OMe·HCl or Glu(OMe)-OMe·HCl, $Et_3N$; (c) NaOH, THF/$CH_3OH$; (d) ①$(COCl)_2$, $CH_2Cl_2$ ②1, 2-ethylenediamine (anhydrous); (e) (Fmoc)NH-$R^2$-COOH, EDC·HCl, dioxane; (f) $Et_3N/CH_2Cl_2$.

**Table 1**
The in vitro cytotoxicity of ursolic acid (UA) derivatives [expressed as $IC_{50}$ (μM)] against human cancer cell lines (48 h)

| Group | Compd | Polar moiety | AGS | HepG2 | HT-29 | PC-3 |
|---|---|---|---|---|---|---|
| Positive control | **Taxol** | — | <10 | 30.7 | <10 | 57.2 |
| Parent core | **UA** | 1 × OH, 1 × COOH | 20.6 | 53.4 | 25.3 | 22.3 |
| Group I (negatively charged) | **UA-4** | 1 × OH, 2 × COOH | >100 | >100 | >100 | >100 |
| | **UA-5** | 1 × OH, 2 × COOH | >100 | >100 | >100 | >100 |
| Group II (positively charged) | **UA-6** | 1 × $NH_2$ | <10 | 12.4 | <10 | <10 |
| | **UA-7** | 1 × $NH_2$, 1 × OH | 11.4 | 21.3 | 14.3 | <10 |
| | **UA-9a** | 1 × $NH_2$ | <10 | 15.5 | 20.2 | 14.3 |
| | **UA-9b** | 1 × $NH_2$ | <10 | <10 | <10 | <10 |
| | **UA-9c** | 1 × $NH_2$ | <10 | <10 | <10 | <10 |
| | **UA-11a** | 1 × $NH_2$, 1 × OH | 10.2 | nt | nt | 19.6 |
| | **UA-11b** | 1 × $NH_2$, 1 × OH | <10 | 16.0 | nt | <10 |
| | **UA-11c** | 1 × $NH_2$, 1 × OH | <10 | nt | nt | <10 |

nt: Not tested.

The lipophilicity brought by the conjugated moiety was also important to the anticancer activity of UA derivatives.[2,4] The introduction of acetyl group to the C-3 position of UA or UA derivatives might result in greater cytotoxicity (Table 1). Similar results had been reported by Meng[4] and Ma.[2] Additionally, we found that the cytotoxic capacity of the UA derivatives were **9a** < (**9b**, **9c**) and **11a** < (**11b**, **11c**), while the liposolubility of the concerning amino acids were: glycine < methionine and phenylalanine. The

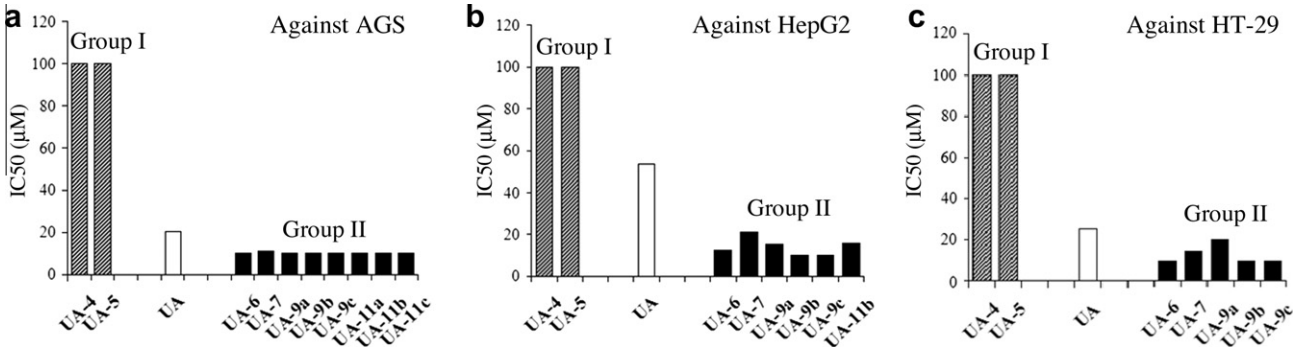


**Figure 1.** The effects of electro property on the cytotoxicity of UA derivatives against (a) AGS cells; (b) HepG2 cells; and (c) HT-29 cells.

reason was probably contributed to the better lipophilicity, which often lead to better membrane permeability, followed by better pharmaceutical effects.[13,14] The enhancement of lipophilicity possibly strengthened the cytotoxic abilities of the positively charged UA derivatives.

The structure–activity relationships of other pentacyclic triterpene acids such as Oleanolic acid (OA, 3β-hydroxy-olean-12-en-28-oic acid) and Betulinic acid (BA, 3β-hydroxy-lup-20(29)-en-28-oic acid), had been studied.[15,16] It was interesting that UA, OA and BA were different in their core structures while they shared similar observations of structure–activity relationships. The similar observations included (1) the positively charged derivatives seemed to be more potent than the mother nucleus; and (2) the enhancement of the derivatives' lipophilicity appeared to strengthen the anticancer capacity. The structure–activity relationships might be generally established among triterpenoid amides and amines analogous.

The derivatives in Group II presented much higher anti-proliferative activity than UA against all of the four cancer cell lines. These compounds might be developed as antitumor drugs. However, the research work to explore their antitumor mechanism was necessary. In this work, flow cytometry with propidium iodide (PI) dye[17] was applied and cell cycle analysis was performed to determine the impacts of the UA derivatives on the DNA content of AGS cells. The results were showed in Table 2. Fourty eight hours post treatment of AGS cells with UA (20.6 μM) resulted in 86.53% of the treated cells were accumulated at sub-G0/G1 phase. However **UA-6**, **UA-7**, **UA-9a–c** could achieve the same effects of similar level (75.45–92.64%) against AGS cells at a lower dose (10 μM). It indicated the apoptotic effects by these five UA derivatives were even more potent than that by Taxol at the same dose (10 μM). They might share the same anticancer mechanism as the leader compound UA.[4,18] In addition, marked cell cycle arrest was observed in Table 2. About 70% of the non-apoptotic AGS cells treated with 10 μM of **UA-11a** or **UA-11b** were arrested at G2/M phase, while both could induce the apoptosis of cells as well. UA derivatives of Group II might exert their antitumor activities by triggering apoptosis of cells and cell cycle arrest.

**UA-7** was most effective among the UA derivatives to induce the apoptosis of AGS cells. Its apoptotic effect was further evaluated by Annexin V-FITC/propidium iodide (AV/PI) dual staining experiment[19] to examine the occurrence of phosphatidylserine externalization onto the cell surface. The results were shown in Figure 2. When the cells were not treated with **UA-7**, as a control, 95.5% of cells were in the normal condition. When the dose of **UA-7** increased up to 10 μM, the population of normal cells was decreased to 59.9%, however 27.7% and 11.8% of treated cells entered into the early-apoptotic stage and the late one, respectively. Treating with higher dose of **UA-7** induced a significant shift of the cell population to the apoptotic stage with 68.6% of cells in the early-apoptotic status and 17.2% of cells late-apoptotic or necrotic (Fig. 2). It indicated that **UA-7** triggered the apoptosis of AGS cells in a dose-dependent manner.

Reasonable oil/water partition property and good water solubility should be taken into account when developing clinic drugs with good delivery.[20–22] UA was poorly soluble in water.[1,6,7] No experimental data of its water solubility had been reported yet. We had assessed the water solubility and log*P*[22] value of UA and its derivatives. However their water solubility was too poor to be determined by HPLC equipped with UV detector. Likewise the log*P* value of the compounds could not be detected either. Theoretical arithmetic and computer-assistant calculations were then used to predict log*P* value, such as ACD\log*P*[23] and Xlog*P*3[24,25] and Molinspiration\log*P*.[26,27] With an observed melting point (MP) and calculated log*P*, a reasonable estimation of the aqueous solubility of

**Table 2**
Cell cycle distribution and sub-G0/G1 ratio (%) of AGS cells treated with UA and its derivatives ($n = 3$)

| Compd | Sub-G0/G1 (%) | Cell cycle distribution of non-apoptotic AGS (%) | | |
|---|---|---|---|---|
| | | G0/G1 | S | G2/M |
| Control | 1.56 ± 0.20 | 28.07 ± 3.52 | 36.56 ± 0.36 | 35.37 ± 3.73 |
| **Taxol** | 70.41 ± 1.10** | 5.92 ± 0.70** | 7.90 ± 1.58** | 86.18 ± 1.26** |
| **UA** | 86.53 ± 0.46** | 33.20 ± 6.62 | 14.64 ± 7.88** | 52.16 ± 2.98** |
| **UA-6** | 88.30 ± 1.85** | 51.86 ± 3.72** | 1.42 ± 1.36** | 46.72 ± 4.03* |
| **UA-7** | 92.64 ± 3.13** | 30.83 ± 10.96 | 66.66 ± 12.29* | 2.51 ± 0.35** |
| **UA-9a** | 75.45 ± 3.24** | 36.97 ± 4.17* | 25.37 ± 5.65* | 37.66 ± 1.51 |
| **UA-9b** | 82.38 ± 0.84** | 54.97 ± 3.49** | 0.00 ± 0.00** | 45.03 ± 3.49* |
| **UA-9c** | 87.64 ± 7.87** | 63.49 ± 2.26** | 2.06 ± 1.19** | 35.31 ± 3.59 |
| **UA-11a** | 44.69 ± 1.72** | 17.72 ± 3.33* | 11.23 ± 8.63** | 71.45 ± 5.57** |
| **UA-11b** | 50.03 ± 1.41** | 12.90 ± 1.66** | 18.46 ± 1.10** | 68.65 ± 2.66** |
| **UA-11c** | 47.73 ± 3.17** | 42.62 ± 2.37** | 11.79 ± 6.08** | 45.59 ± 5.76* |

* $p < 0.05$.
** $p < 0.01$, versus control.

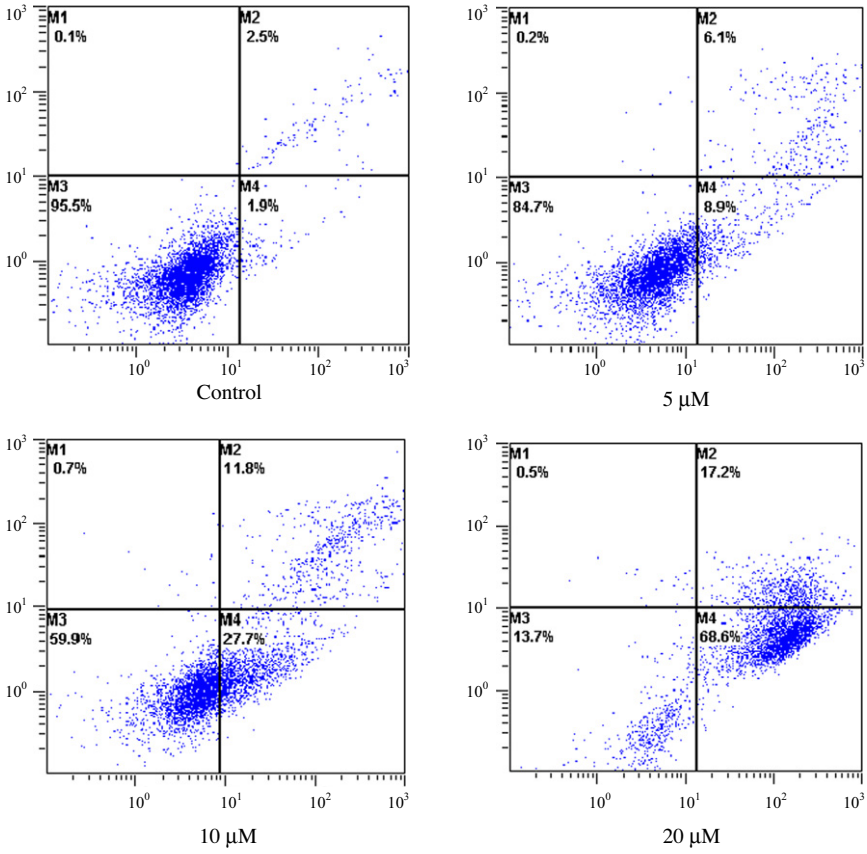


**Figure 2.** Annexin V/PI dual staining of AGS cells treated with **UA-7** (0, 5, 10 and 20 μM) for 24 h was carried out. The cells were harvested, stained and then analyzed by Flow cytometry. In all panels, cells in the lower left quadrant (M3: $AV^-/PI^-$) were alive, cells in the lower right quadrant (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.

**Table 3**
Predicted log*P* value and water solubility of UA and its derivative

| Type | Compd | Log*P* value predicted by | | | Predicted log$S_0$* | Relative water solubility (vs UA) |
|---|---|---|---|---|---|---|
| | | ACD/log*P* | Molinspiration/log*p* | Xlog*P*3 | | |
| Parental | **UA** | 9.01 ± 0.37 | 6.789 | 7.34 | −9.34 ± 0.38 | 1 |
| Group II | **UA-7** | 7.67 ± 0.49 | 5.783 | 6.15 | −6.88 ± 0.38 | 288 |

* $S_0$, water solubility in pure $H_2O$, mol/L.

any organic non-electrolyte could be obtained via the equation (1) that described the general solubility equation (GSE).[28] The aqueous solubility of UA or its derivative was expressed as the logarithm of water solubility (log$S_0$,). The average absolute error and the root-mean-square error in the solubility estimates were 0.38 and 0.53 logarithm units, respectively.[28] Moreover, the equation (2) was designed to evaluate the improvement of solubility of **UA-7** in water. The results shown in Table 3 indicated the estimated log*P* of **UA-7** was much lower than that of UA (by 1–2 logarithm units). More excitedly, the predicted water solubility of **UA-7** was 288-fold of that of UA. This UA derivative had more reasonable log*P* value and better aqueous solubility than the mother nucleus. It suggested that **UA-7** has appropriate properties for good absorption, distribution, and delivery in living body.[13,29]

$$logS_0 = 0.3814 - 0.00961(MP - 25) - 1.0223\ logP \quad (1)$$

Relative water solubility (compared with UA)

$$= \frac{S_{0(UADerivative)}}{S_{0(UA)}} = 10^{[logS_0(UADerivative) - logS_0(UA)]} \quad (2)$$

In conclusion, a series of UA derivatives with distinct electrical property were reported in this work. The structure–activity relationships were established. The positively charged UA derivatives exerted more potent cytotoxicity than UA against tumor cell lines. The anticancer capacity of these UA derivatives could be further strengthened by the enhancement of their lipophilicity. Triggering apoptosis and inducing cell cycle arrest contributed to the antitumor mechanism of these UA derivatives. **UA-7**, a UA derivative capable of inducing AGS cells to apoptosis, possessed potent cytotoxic activity, enhanced water solubility and logical log*P*. It might have a therapeutic potential in the treatment of gastric cancer.

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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2012.02.009. These data include MOL files and InChiKeys of the most important compounds described in this article.

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9. Properties of the novel UA derivatives: *N*-[3β-Hydroxy-urs-12-en-28-oyl]-2-amino-1,5-pentanedioic acid (**UA-4**): white crystalline powder. Yield 95.3%; mp 317–320 °C; ESI-MS: *m*/*z* 610.9 $[M+K]^+$; UV–Vis (Methanol), $\lambda_{max}$ = 210 nm; IR (KBr): 3384, 2976, 2936, 2872, 1712, 1635 $cm^{-1}$; $^1H$ NMR (600 MHz, DMSO-$d_6$): 7.22 (d, 1H, *J* = 7.3 Hz, NH), 5.19 (t, 1H, *J* = 3.6 Hz, H-12), 4.24 (s, 1H, OH-3), 4.13–4.09 (m, 1H, NH*CH*COOH), 2.99 (s, 1H, H-3), 2.22 (t, 2H, *J* = 7.3 Hz, $CH_2$COOH), 0.83 (d, 3H, *J* = 6.4 Hz, $CH_3$), 1.03, 0.91, 0.89, 0.85, 0.67, 0.65 (s, 18H, 6×$CH_3$); Anal. Calcd for $C_{34}H_{53}NO_6$: C 71.42, H 9.34, N 2.45; Found: C 71.60, H 9.26, N 2.27.
   *N*-[3β-Hydroxy-urs-12-en-28-oyl]-2-amino-1,4-butanedioic acid (**UA-5**): white crystalline powder. Yield 93.6%; mp 302–305 °C; ESI-MS: *m*/*z* 624.8 $[M+K]^+$, 1194.4 [2 M+Na]$^+$; UV–Vis (Methanol), $\lambda_{max}$ = 204 nm; IR (KBr): 3417, 2971, 2928, 2871, 1731, 1636 $cm^{-1}$; $^1H$ NMR (600 MHz, DMSO-$d_6$): 7.27 (d, 1H, *J* = 7.2 Hz, NH), 5.20 (t, 1H, *J* = 3.6 Hz, H-12), 4.33–4.29 (m, 1H, NH*CH*COOH), 4.24 (s, 1H, OH-3), 2.99 (s, 1H, H-3), 2.22 (t, 2H, *J* = 7.3 Hz, $CH_2$COOH), 0.83 (d, 3H, *J* = 6.4 Hz, $CH_3$), 1.02, 0.90, 0.89, 0.84, 0.67, 0.65 (s, 18H, 6×$CH_3$); Anal. Calcd for $C_{35}H_{55}NO_6$: C 71.76, H 9.46, N 2.39; Found: C 71.77, H 9.34, N 2.07.
   *N*-[3β-acetoxy-urs-12-en-28-oyl]-2-aminoethylamine (**UA-6**): white crystalline powder. Yield 75.5%; mp 140–142 °C; ESI-MS: *m*/*z* 541.5 $[M+H]^+$; UV–Vis (Methanol), $\lambda_{max}$ = 208 nm; IR (KBr): 3365, 2950, 2853, 1728, 1631, 1252, 1026 $cm^{-1}$; $^1H$ NMR (600 MHz, $CDCl_3$): 6.32 (s, 1H, CO*NH*$CH_2$), 5.33 (s, 1H, H-12), 4.49 (t, 1H, *J* = 7.6 Hz, H-3), 3.41–3.38 (m, 1H, CONH$CH_2CH_2NH_2$), 3.06–3.04 (m, 1H, CONH$CH_2CH_2NH_2$), 2.79 (s, 2H, $CH_2NH_2$), 2.05 (s, 3H, $CH_3$CO), 1.09, 0.95, 0.94, 0.86, 0.85, 0.84, 0.79 (s, 21H, 7×$CH_3$); Anal. Calcd for $C_{34}H_{56}N_2O_3$: C 75.51, H 10.44, N 5.18; Found: C 75.76, H 10.71, N 4.94.
   *N*-[3β-hydroxy-urs-12-en-28-oyl]-2-aminoethylamine(**UA-7**): white crystalline powder. Yield 86.7%; mp 145–147 °C; ESI-MS: *m*/*z* 499.5 $[M+H]^+$; UV–Vis (Methanol), $\lambda_{max}$ = 206 nm; IR (KBr): 3362, 2925, 2868, 1640, 1043, 999 $cm^{-1}$; $^1H$ NMR (600 MHz, DMSO-$d_6$): 7.13 (s, 1H, CO*NH*$CH_2$), 5.20 (s, 1H, H-12), 3.5–3.1 (br s, OH), 3.02∼2.98 (m, 2H, CONH$CH_2CH_2NH_2$), 2.97–2.92 (m, 2H, CONHCH$_2CH_2$NH$_2$), 2.15(d, 1H, H-18), 0.82 (d, 3H, *J* = 5.2 Hz , $CH_3$), 1.03, 0.91, 0.89, 0.85, 0.69, 0.67 (s, 18H, 6×$CH_3$); Anal. Calcd for $C_{32}H_{54}N_2O_2$: C 77.06, H 10.91, N 5.63; Found: C 76.56, H 10.54, N 5.27.
   *N*-(3β-acetoxy-urs-12-en-28-oyl)-1-amino, *N*-glycyl-2-aminoethane (**UA-9a**): white powder. Yield 88.5%; mp 147–149 °C; ESI-MS: *m*/*z* 498.5 $[M+H]^+$; UV–Vis (Methanol), $\lambda_{max}$ = 210 nm; IR (KBr): 3397, 2925, 2854, 1736, 1681, 1640, 1246, 1027, 805 $cm^{-1}$; $^1H$ NMR (600 MHz, $CDCl_3$): 7.62 (s, 1H, CO*NH*$CH_2$), 6.31 (s, 1H, CO*NH*$CH_2$), 5.29 (s, 1H, H-12), 4.42 (d, 1H, *J* = 7.4 Hz, H-3), 3.54–3.52 (m, 1H, HN*CH*CON), 3.10–3.08 (m, 1H, HN*CH*CON), 3.36, 3.30 (s, 1H, each, COHN$CH_2CH_2$NHCO), 3.27 (s, 1H, COHNCH$_2$*CH$_2$*NHCO), 3.22 (d, 1H, *J* = 13.4 Hz, COHNCH$_2$*CH$_2$*NHCO), 1.97 (s, 3H, $CH_3$COO), 1.01, 0.86, 0.86, 0.80, 0.78, 0.78, 0.69 (s, 21H, 7×$CH_3$); Calcd for $C_{36}H_{59}N_3O_4$: C 72.32, H 9.95, N 7.03; Found: C 71.95, H 9.94, N 6.87.
   *N*-(3β-acetoxy-urs-12-en-28-oyl)-1-amino, *N*-(2-amino-4-methylthio-1-butyl)-2-aminoethane (**UA-9b**): white powder. Yield 89.2%; mp 96∼98 °C; ESI-MS: *m*/*z* 672.6 $[M+H]^+$; UV–Vis (Methanol), $\lambda_{max}$ = 208 nm; IR (KBr): 3355, 2925, 2871, 1736, 1650, 1246, 1027, 804 $cm^{-1}$; $^1H$ NMR (600 MHz, $CDCl_3$): 7.67 (s, 1H, CO*NH*$CH_2$), 6.34 (s, 1H, CO*NH*$CH_2$), 5.28 (s, 1H, H-12), 4.42 (d, 1H, *J* = 7.4 Hz, H-3), 3.43,3.39 (s, 2H, each, COHN$CH_2CH_2$NHCO), 3.31,3.27 (s, 2H, each, COHN$CH_2CH_2$NHCO), 3.11∼3.10 (m, 1H, HN*CH*CON), 2.54 (m, 2H, $CH_3SCH_2$), 2.04 (s, 3H, $CH_3$CO), 1.97 (s, 3H, $SCH_3$), 1.65 (t, 2H, *J* = 12.4 Hz, $CH_2CH_2SCH_3$), 1.01, 0.88, 0.87, 0.80, 0.79, 0.78, 0.69 (s, 21H, 7×$CH_3$); Calcd for $C_{39}H_{65}N_3O_4S$: C 69.70, H 9.75, N 6.25, S 4.77; Found: C 69.29, H 9.66, N 6.24, S 4.27.
   *N*-(3β-acetoxy-urs-12-en-28-oyl)-1-amino, *N*-(2-amino-3-phenyl-1-propionyl-)-2-aminoethane (**UA-9c**): white powder. Yield 96.8%; mp 82∼84 °C, ESI-MS: *m*/*z* 688.6 $[M+H]^+$; UV–Vis (Methanol), $\lambda_{max}$ = 205, 241 nm; IR (KBr): 3363, 2924, 2853, 1735, 1648, 1246, 1095, 1027, 803, 746, 701, 665 $cm^{-1}$; $^1H$ NMR (600 MHz, $CDCl_3$): 8.63 (s, 1H, CO*NH*$CH_2$), 6.86 (s, 1H, CO*NH*$CH_2$), 5.34 (s, 1H, H-12), 4.49 (s, 1H, H-3), 3.88 (s, 1H, HN*CH*CON), 3.40 (s, 1H, Ar-$CH_2$), 3.30 (s, 2H, $NH_2$), 3.16 (s, 1H,Ar-$CH_2$), 3.12 (t, 2H, COHN$CH_2$CH$_2$NHCO), 3.10 (t, 2H, COHNCH$_2$*CH$_2$*NHCO), 2.05 (s, 3H, $CH_3$COO), 1.07, 0.94, 0.93, 0.87, 0.86, 0.85, 0.74 (s, 21H, 7×$CH_3$); Calcd for $C_{43}H_{65}N_3O_4$: C 75.07, H 9.52, N 6.11; Found: C 74.52, H 10.03, N 6.12.
   *N*-(3β-hydroxy-urs-12-en-28-oyl)-1-amino, *N*-glycyl-2-aminoethane (**UA-11a**): white crystalline powder. Yield 87.1%; mp 205–208 °C; ESI-MS: *m*/*z* 556.5 $[M+H]^+$; UV–Vis (Methanol), $\lambda_{max}$ = 208 nm; IR (KBr): 3412, 3087, 2926, 1683, 1641, 1251, 1044, 997 $cm^{-1}$; $^1H$ NMR (600 MHz, DMSO-$d_6$): 8.56 (s, 1H, CO*NH*$CH_2$), 7.39 (s, 1H, CO*NH*$CH_2$), 5.21 (s, 1H, H-12), 4.31 (s, 1H, HO-3), 4.03–4.02 (d, 1H, *J* = 6.3 Hz, $H_2NCH_2$CON), 3.46 (s, 1H, $H_2NCH_2$CON), 3.35 (br s, 2H, $NH_2$), 3.10 (s, 1H, CONH$CH_2H_2$NHCO), 3.03–2.99 (m, 1H, CONH$CH_2CH_2$NHCO), 2.18 (d, 1H, *J* = 10.1 Hz, H-18), 1.02, 0.91, 0.88, 0.84, 0.82, 0.67, 0.67 (s, 21H, 7×$CH_3$); Calcd for $C_{34}H_{57}N_3O_3$: C 73.47, H 10.34, N 7.56; Found: C 73.29, H 9.91, N 7.53.
   *N*-(3β-hydroxy-urs-12-en-28-oyl)-1-amino, *N*-(2-amino-4-methylthio-1-butyl-)-2-aminoethane (**UA-11b**): white powder. Yield 89.2%; mp 99–101 °C; ESI-MS: *m*/*z* 630.6 $[M+H]^+$; UV–Vis (Methanol), $\lambda_{max}$ = 210 nm; IR (KBr): 3378, 2924, 2854, 1641, 1260, 1093, 1028, 802 $cm^{-1}$; $^1H$ NMR (600 MHz, DMSO-$d_6$): 8.01 (s, 1H, CO*NH*$CH_2$), 7.28 (s, 1H, CO*NH*$CH_2$), 5.28 (s, 1H, H-12), 4.34 (s, 1H, HO-3), 3.38 (br s, 2H, $NH_2$), 3.25 (s, 1H, HN*CH*CON), 3.15 (s, 1H, CONH$CH_2CH_2$NHCO), 3.04 (s, 1H, CONH$CH_2CH_2$NHCO), 2.17 (d, 1H, *J* = 10.1 Hz, H-18), 2.08 (s, 3H, $SCH_3$), 1.63 (t, 2H, *J* = 12.4 Hz, $CH_2CH_2SCH_3$), 1.08, 0.96, 0.94, 0.90, 0.87, 0.72, 0.72 (s, 21H, 7×$CH_3$); Calcd for $C_{37}H_{63}N_3O_3S$: C 70.54, H 10.08, N 6.67, S 5.09; Found: C 70.05, H 9.92, N 6.26, S 4.62.
   *N*-(3β-hydroxy-urs-12-en-28-oyl)-1-amino, *N*-(2-amino-3-phenyl-1-propionyl-)-2-aminoethane (**UA-11c**): white powder. Yield 96.8%, mp 154–147 °C; ESI-MS: *m*/*z* 668.6, [M+Na]$^+$; UV–Vis (Methanol), $\lambda_{max}$ = 205, 242 nm; IR (KBr): 3355, 2926, 2869, 1673, 1638, 1538, 1455, 1251, 1042, 746, 700, 663 $cm^{-1}$; $^1H$ NMR (600 MHz, DMSO-$d_6$): 8.49 (s, 1H, CO*NH*$CH_2$) , 7.35 (s, 1H, CO*NH*$CH_2$), 7.30–7.24 (5H, Ar-H), 5.20 (s, 1H, H-12), 4.31 (s, 1H, HO-3), 3.79 (s, 1H, HN*CH*CON), 3.38 (s, 1H, Ar-$CH_2$), 3.06 (s, 1H, Ar-$CH_2$), 3.34 (br s, 2H, $NH_2$), 3.03–3.00 (m, 1H, CONH$CH_2CH_2$NHCO), 2.91–2.88 (m, 1H, CONH$CH_2CH_2$NHCO), 2.16 (d, 1H, *J* = 10.2 Hz, H-18), 1.02, 0.90, 0.89, 0.85, 0.81, 0.67, 0.67 (s, 21H, 7×$CH_3$); Calcd for $C_{41}H_{63}N_3O_3$: C 76.23, H 9.83, N 6.51; Found: C 75.59, H 9.37, N 6.47.
10. *MTT assay*: The anti-proliferative activities of the molecules were determined by the MTT assay. UA, Taxol and UA derivatives were initially dissolved in DMSO at 20 mM and serially diluted with culture medium to various concentrations. Cells were seeded in 96-well plates at a density of $1 \times 10^4$ cells per well. After 24 h, the cells were treated with different concentrations of drugs for another 48 h. Each compound was tested at six different concentrations, 10, 20, 40, 60, 80 and 100 μM, and each concentration was replicated in six wells. The negative control (0 μM of test compound) contained the same amount of DMSO as that of the highest test concentration. Next, excess MTT was added, and the culture was incubated for additional 4 h. The resultant formazan formed by metabolically viable cells was well dissolved in 100 μL of DMSO. The absorbance was then measured on a microplate reader apparatus (TECAN DNA Expert, Switzerland) at 570 nm. The average absorbance of the six duplicates was applied to calculate the inhibitory effects by subtracting the absorbance measured at the same wavelength from DMSO treated cells. The concentration of the compound, which gives the 50% growth inhibition value, corresponds to the $IC_{50}$.
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17. *Cell cycle analysis*: AGS cells were used to analyze the cell cycle effects of the compounds. Cells were seeded at $1 \times 10^5$ cells per well in six-well culture plates. After 24 h, the cells were treated with tested compounds at 10 μM or at concentrations equivalent to their $IC_{50}$ values, and incubated for an additional 48 h. The cells were harvested and fixed with 70% ethanol at 4 °C overnight, then treated with RNAse (100 μg/mL) for 20 min, stained with propidium iodide (Sigma, USA) for 10 min, and finally analyzed using a flow cytometer (Beckman Coulter, EPICS XL, USA). The percentages of cells in G0/G1, S, and G2/M phases were determined by CellQuest software (Becton, Dickinson and Company). All experiments were performed in triplicate and gave the similar results.
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19. *Annexin V/propidium iodide (AV/PI) dual staining*: AGS cells were treated with 0, 5, 10, 20 μM **UA-7** for 24 h, washed and resuspended in PBS buffer. Apoptotic cells were identified by double staining with recombinant fluorescein isothiocyanate (FITC)-conjugated Annexin V and propidium iodide, by using the Annexin V-FITC apoptosis detection Kit (KeyGEN, China) following the manufacturer's instructions. Flow cytometric analysis was performed immediately after staining. Data acquisition and analysis were performed by using CellQuest software.
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