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Original article

Design, synthesis and *in vitro* evaluation of novel ursolic acid derivatives as potential anticancer agents

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ABSTRACT

A series of novel ursolic acid (**UA**) derivatives modified at the C-3 and the C-28 positions were designed and synthesized in an attempt to develop potential antitumor agents. The *in vitro* cytotoxicity were evaluated against five cancer cell lines (MGC-803, HCT-116, T24, HepG2 and A549 cell lines) and a normal cell (HL-7702) by MTT assay. The screening results indicated that some of these target compounds displayed moderate to high levels of antiproliferative activities compared with ursolic acid and 5-fluorouracil (5-FU), and exhibited much lower cytotoxicity than 5-FU, indicating that the targeted compounds had selective and significant effect on the cell lines. The induction of apoptosis and affects on the cell cycle distribution of compound **6r** were investigated by acridine orange/ethidium bromide staining, Hoechst 33258 staining, JC-1 mitochondrial membrane potential staining and flow cytometry, which revealed that the antitumor activity of **6r** was possibly achieved through the induction of cell apoptosis by G1 cell-cycle arrest. Western blot and qRT-PCR (quantitative real-time PCR) experiments demonstrated that compound **6r** may induce apoptosis through both of intrinsic and extrinsic apoptosis pathway.

1. Introduction

Pentacyclic triterpenes represents a very powerful class of natural products due to their broad biological activity and amazing diversity of structures [1,2]. So over the ten decades, the development of novel pentacyclic triterpenes with potent medicinal virtue have greatly attracted bioorganic chemists' interest and become a hot research topic in bioorganic chemistry.

UA is a well-known pentacyclic triterpene that serves as one of the major effective elements of many traditional chinese medicines [3]. **UA** and its derivatives, has been reported to have anti-HIV [4], antidiabetic [5], antihepatodamage [6], antimalarial [7], antimicrobial, anti-inflammatory activity [8] and antitumor activities [9–14]. Moreover, previous research has shown that **UA** could act at various stages of tumor development to inhibit tumor initiation and promotion, as well as to induce tumor cell differentiation and apoptosis [15]. So many modification of **UA** have been developed to screen for novel potential antitumor drugs. Jing-Wei Shao et al. had shown that the modification in position 3 and/or 28 of **UA** was essential for their cytotoxic activity [16–18]. 3-Oacyl derivatives of **UA** exhibited potent cytotoxic activity [19,20], while the connection of amino acid and **UA** also led to significant cytotoxicity against some tumor cell lines [21,22]. Furthermore, Ming-Chuan Liu and his co-workers suggested that incorporation of an acyl piperazine moiety at C-28 of **UA** while retaining the polar group at C-3 could significantly improved their antitumor bioactivities [3]. However, to the best of our knowledge, **UA** derivatives had not been thoroughly developed for their antitumor activity.

Research had shown that the incorporation of an acyl piperazine moiety at C-28 could provide unexpected improvements to the antitumor activity of UA derivatives [3,16]. In addition, our previous studies had demonstrated that thiourea derivatives had potential

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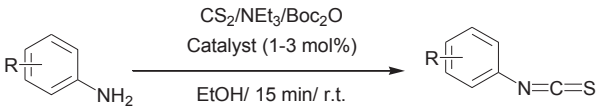
antitumor activity [23,24]. It is thus to expect that the combination of thiourea, piperazine and **UA** core may lead to important antitumour activity. Therefore, to search for novel **UA** derivatives with important antitumor bioactivities along with our previous work, the present study introduced a thiourea groups to the C-28 position based an acyl piperazine moiety of **UA**. Herein, a series of piperazine-thioureas **UA** derivatives were synthesized and their antitumor activities were evaluated against five cancer cell lines (MGC-803, HCT-116, T24, HepG2 and A549 cell lines) and a normal cell HL-7702. Further investigation of the mechanism on the mode of action of representative compound **6r** found that it could effectively induce cell apoptosis in HeG2 cells.

2. Results and discussion

2.1. Chemistry

A series of **UA** derivatives were synthesized as outlined in Scheme 1 and 2. As shown in Scheme 1, 3-acetyl ursolic acid **2** was synthesized by the treatment of ursolic acid **1** with acetic anhydride in dry pyridine in the presence of 4-dimethylaminopyridine (DMAP) [25]. Compound **2** was treated with oxalyl chloride to offer the intermediate 28-acyl chloride **3**, which was highly reactive and was then coupled with piperazine to produce compounds **4** [5]. Compound **4** was hydrolyzed in the presence of sodium hydroxide to give compound **5** [16]. Isothiocyanates were obtained according to the method of the literature [26] as shown in Scheme 2. **UA** derivatives (**6a**–**t** and **7a**–**r**) were obtained by the condensation of isothiocyanates with **4** (or **5**) in CH_2Cl_2 at room temperature. The structures of **UA** derivatives **6a**–**t** and **7a**–**r** were then confirmed by ^1H NMR, ^{13}C NMR and high resolution mass spectrum (HRMS).



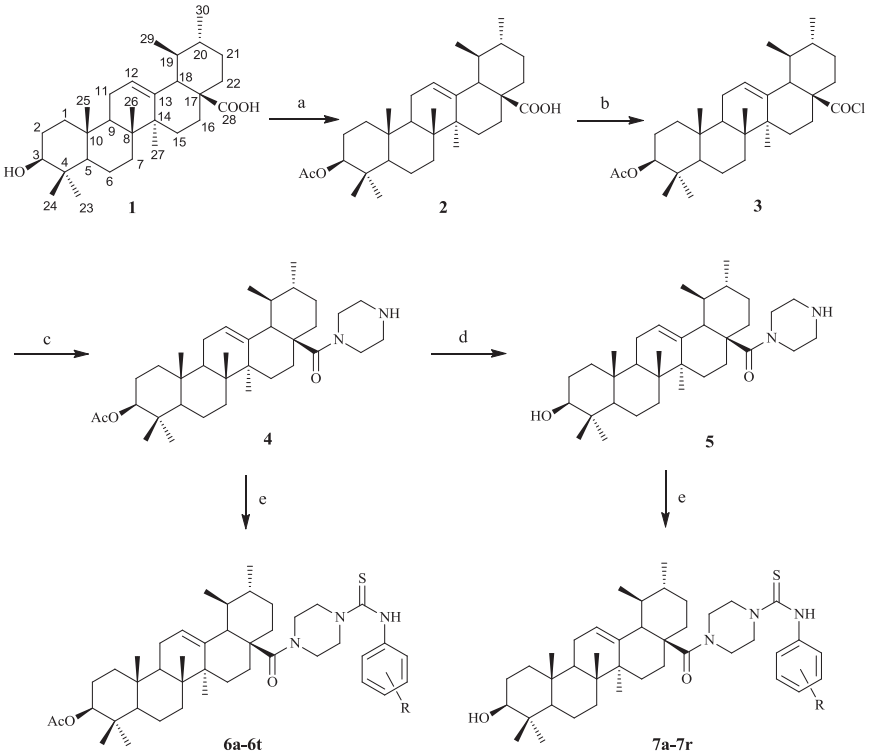
Scheme 2. Synthetic pathway to isothiocyanates.

2.2. Evaluation of antitumor activities

The *in vitro* cytotoxicity of **UA** derivatives **6a**–**t** and **7a**–**r** were evaluated by MTT assay against MGC-803, HCT-116, T24, HepG2 and A549 tumor cell lines, with **UA** and 5-FU as the positive control. All the tested target compounds were dissolved in DMSO and subsequently diluted in culture medium before treatment of cultured cells. The tested results were shown in Table 1.

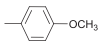
As shown in Table 1, **UA** suppressed proliferation of the above five cancer cell lines in different extents (from 27.08 to 38.78 μM), and most of the title compounds **6a**–**t** modified from **UA** displayed important proliferation activity. Table 1 also revealed that **UA** showed similar antiproliferative activity with 5-FU in these cell lines. Besides, it was important to note that compounds **6q** and **6r** showed better potent antiproliferative activity than **UA** (as well as 5-FU) against these five cancer lines. Evidently, compound **6r** displayed the best proliferation among compounds **6a**–**t**, with IC_{50} values of 9.8 μM, 18 μM, 13 μM, 5.4 μM and 11 μM against MGC-803, HCT-116, T24, HepG2 and A549 cancer cell lines, respectively. The result indicated that the synchronous introduction of piperazine-thiourea at C-28 and an acyl group at C-3 may improve the antitumor activity of **UA**, consistent with the previous study [3,16,27].

It also could be seen from Table 1 that the compounds **6a**–**t** showed more potent antiproliferative activities than compounds **7a**–**r** (with a free 3-OH group). It indicated that the hydrolyzing



Scheme 1. Synthetic pathway to target compounds **6a**–**t** and **7a**–**r**. Reagents and conditions: (a) Ac_2O/DMAP/pyridine, r.t; (b) ClCOCOCl/CH_2Cl_2, r.t; (c) Et_3N/CH_2Cl_2, piperazine; (d) 4 mol/L NaOH aq, THF/MeOH, r.t; (e) CH_2Cl_2, isothiocyanates, r.t.

Table 1
Effect of compounds **6a–t and 7a–r** against cell viability of different cell lines.

Compound	IC_{50} (μM)						
	R	MGC-803	HCT-116	T24	HepG2	A549	HL-7702
6a		38.73 ± 0.58	43.12 ± 0.83	37.94 ± 0.48	39.19 ± 0.93	45.52 ± 0.79	>100
6b		60.21 ± 0.26	46.18 ± 0.48	38.38 ± 0.56	45.21 ± 0.20	58.17 ± 0.74	>100
6c		34.54 ± 0.54	40.58 ± 0.17	39.50 ± 0.59	36.93 ± 0.38	40.32 ± 0.10	>100
6d		27.73 ± 0.76	37.79 ± 0.37	30.28 ± 0.64	28.29 ± 1.43	35.69 ± 1.20	>100
6e		36.71 ± 0.21	40.08 ± 0.56	34.54 ± 0.40	58.74 ± 0.76	44.03 ± 0.38	>100
6f		30.07 ± 0.18	37.58 ± 0.16	30.21 ± 0.20	44.54 ± 0.29	38.52 ± 0.46	>100
6g		35.73 ± 057	44.62 ± 0.45	39.78 ± 0.26	47.22 ± 0.76	42.76 ± 0.54	>100
6h		35.22 ± 0.46	40.43 ± 1.12	52.78 ± 0.76	54.14 ± 0.19	43.34 ± 1.32	>100
6i		30.54 ± 0.29	37.03 ± 2.01	37.43 ± 1.12	34.54 ± 0.59	36.13 ± 0.36	>100
6j		42.52 ± 0.38	42.88 ± 0.49	40.79 ± 0.31	38.69 ± 0.88	46.76 ± 0.67	>100
6k		48.28 ± 0.35	39.78 ± 0.96	48.55 ± 0.45	51.58 ± 0.95	58.34 ± 0.36	>100
6l		38.28 ± 1.05	30.73 ± 0.54	34.55 ± 0.75	41.44 ± 0.47	47.98 ± 0.86	>100
6m		37.50 ± 0.98	35.34 ± 0.89	30.78 ± 0.70	48.55 ± 0.65	38.38 ± 0.14	>100
6n		26.74 ± 0.62	27.08 ± 0.75	22.15 ± 0.67	32.36 ± 0.81	25.79 ± 0.53	>100
6o		38.62 ± 0.53	30.94 ± 0.43	29.84 ± 0.59	35.98 ± 0.85	39.11 ± 0.27	>100
6p		28.78 ± 1.70	37.18 ± 0.94	30.77 ± 0.24	26.11 ± 0.82	32.77 ± 0.51	>100
6q		16.34 ± 0.83	28.05 ± 0.35	19.60 ± 0.43	15.72 ± 0.84	20.79 ± 0.54	>100
6r		9.82 ± 0.29	18.97 ± 0.53	13.64 ± 0.43	5.40 ± 0.79	11.06 ± 0.37	>100
6s		56.12 ± 1.50	51.12 ± 1.09	45.58 ± 0.15	65.08 ± 0.98	>100	>100
6t		49.75 ± 0.29	47.30 ± 1.07	43.43 ± 0.73	37.44 ± 1.36	51.38 ± 0.70	>100
7a		64.35 ± 0.34	76.12 ± 1.02	60.09 ± 0.56	71.73 ± 0.10	54.08 ± 0.97	>100
7b		>100	64.74 ± 0.22	60.71 ± 0.40	67.26 ± 0.39	65.48 ± 0.76	>100
7c		>100	65.43 ± 0.82	>100	68.20 ± 0.72	>100	>100
7d		45.28 ± 0.21	50.46 ± 0.59	43.20 ± 0.43	41.41 ± 0.81	69.74 ± 0.92	>100
7e		55.26 ± 0.38	54.80 ± 0.75	60.90 ± 0.25	>100	53.54 ± 0.50	>100

(*continued on next page*)

Table 1 (*continued*)

Compound	IC_{50} (μM)						
	R	MGC-803	HCT-116	T24	HepG2	A549	HL-7702
7f		43.70 ± 0.14	50.40 ± 0.63	46.27 ± 0.52	78.54 ± 0.66	57.86 ± 0.85	>100
7g		>100	56.94 ± 0.46	45.32 ± 0.26	60.24 ± 0.16	52.85 ± 0.73	>100
7h		>100	60.41 ± 0.47	>100	70.76 ± 0.51	74.58 ± 0.96	>100
7i		43.82 ± 0.44	50.72 ± 0.36	46.33 ± 0.42	44.97 ± 0.10	57.73 ± 0.19	>100
7j		74.91 ± 0.15	68.47 ± 0.35	>100	>100	80.24 ± 0.78	>100
7k		43.36 ± 0.67	62.70 ± 0.88	52.59 ± 0.84	78.93 ± 0.93	54.10 ± 0.71	>100
7l		52.12 ± 0.97	62.32 ± 0.13	60.18 ± 0.27	57.90 ± 0.45	>100	>100
7m		45.79 ± 0.70	53.70 ± 1.08	41.03 ± 0.53	39.28 ± 0.64	54.82 ± 0.77	>100
7n		52.81 ± 0.47	59.23 ± 0.56	47.24 ± 0.90	44.14 ± 0.82	44.50 ± 0.73	>100
7o		41.78 ± 0.55	40.76 ± 0.72	35.80 ± 0.25	33.83 ± 0.24	39.84 ± 0.39	>100
7p		29.20 ± 0.12	39.19 ± 0.17	30.84 ± 0.83	23.88 ± 0.62	33.52 ± 0.33	>100
7q		58.67 ± 0.83	53.41 ± 0.65	64.44 ± 0.70	62.44 ± 0.31	50.73 ± 0.62	>100
7r		68.76 ± 0.11	59.72 ± 0.76	57.15 ± 0 0.95	59.06 ± 0.52	60.88 ± 0.41	>100
UA		27.08 ± 0.29	38.78 ± 0.16	29.29 ± 0.80	30.21 ± 0.58	35.79 ± 0.37	>100
5-FU		40.94 ± 0.95	29.58 ± 1.31	37.56 ± 0.49	30.79 ± 0.82	36.34 ± 0.57	58.74 ± 2.31

acyl group at C-3 may diminish the antitumor activity. In addition, by the comparison of compounds **7** with **1**, it could be concluded that soley introduction of an acyl piperazine thiourea at C-28 may decrease the antitumor activity. It was consistent with the previous study [3,16,27] that significant further improvement of cell growth inhibition was obtained when an acetyl group was introduced at the 3-OH position.

In addition, by the respective comparison of compounds **6c** with **6d** (**6e** with **6f**, **6h** with **6i**, **6k** with **6l**, **6m** with **6n** and **6p** with **6q**), it could be concluded that para-substituents in the phenyl ring were important than meta-substituents to their antiproliferative activities. Moreover, compounds **7a**–**r** also showed the similar effect.

The inhibition activities of compounds **6** and **7** against HL-7702 normal human river cell lines were also estimated. The data of MTT assay against HL-7702 cell lines were also listed in Table 1. The results indicated that most of compounds showed low cytotoxicity on HL-7702 cells (with IC_{50} greater than 100 μM) and high *in vitro* antiproliferative activity on the five cancer cell lines. It was worth noting that all the **UA** derivatives demonstrated lower cytotoxicity on HL-7702 than the commercial anticancer drug 5-FU. In addition, target compound **6r** displayed the highest antiproliferative activity against HepG2 cancer cells with IC_{50} values of 5.40 ± 0.79 μM and showed lower cytotoxicity against HL-7702 cells with IC_{50} values of greater than 100 μM, making it(them) good candidate as antitumor drugs. These results showed that the targeted compounds had selective and significant effect on the cell lines.

Based on the above observation, some interesting structure-activity relationships could be concluded: (1) the synchronous introduction of piperazine-thiourea at C-28 and an acyl group at C-3 was significant for improving their activity; (2) the acylation of OH group at C-3 was important for improving antitumor activity; (3) para-position of the phenyl ring favored better antitumor activity than meta-position.

2.3. Preliminary investigation of the apoptosis-inducing effect of target compound *6r*

Previous report had indicated that **UA** and its derivatives could potentially induce apoptosis in certain cancer cell lines [10–13]. In the present study, compound **6r** which showed good cytotoxic inhibition in these five cancer cell lines and could be used as a good representative of compounds **6** and **7** was selected and its mechanism of growth inhibition of HepG2 cells was evaluated.

2.3.1. Fluorescence staining

In order to investigate whether the growth inhibitory activity of compound **6r** was related to the induction of apoptosis, changes in the morphological character of HepG2 cells were determined using acridine orange (AO)/ethidium bromide (EB), Hoechst 33258 staining and JC-1 mitochondrial membrane potential staining under fluorescence microscopy.

2.3.1.1. AO/EB staining. AO is a vital dye which can stain nuclear DNA across an intact cell membrane, while EB can only stains cells that have lost their membrane integrity. Hence, after simultaneous treatment with AO and EB, live cells will be uniformly stained as green (in the web version) and early apoptotic cells will be densely stained as green yellow or show green yellow fragments (in the web version), while late apoptotic cells will be densely stained as orange or display orange fragments and necrotic cells will be stained as orange with no condensed chromatin. The cytotoxicity of compound **6r** at different concentrations for 24 h against HepG2 cells was detected by AO/EB staining, and HepG2 cells not treated with the **6r** were used as control for 24 h. The results were shown in Fig. 1.

Fig. 1 displayed that the HepG2 cells treated with **6r** at different concentrations had obviously changed. The nuclei stained as yellow green or orange, and the morphology showed pycnosis, membrane blebbing and cell budding. These phenomena were associated with cell apoptosis [23,24], indicating that compound **6r** was able to induce the apoptosis of HepG2 cells.

2.3.1.2. Hoechst 33258 staining. Hoechst 33258 is a membrane permeable dye with blue fluorescence. Live cells with uniformly light blue nuclei were obviously detected under fluorescence microscope after treatment with Hoechst 33258, whereas apoptotic cells had bright blue nuclei due to karyopyknosis and chromatin condensation. On the contrary, the nuclei of dead cells could not be stained. HepG2 cells which were treated with compound **6r** for 24 h at different concentrations were stained with Hoechst 33258. HepG2 cells not treated with compound **6r** were used as control at for 24 h. The results were given in Fig. 2.

As shown in Fig. 2, cells not treated with compound **6r** were normally blue. It was worth noting that, for **6r** treatment, the cells displayed strong blue fluorescence and indicated typical apoptotic morphology at 10 μM, 20 μM and 30 μM, respectively. The observation revealed that compound **6r** induced apoptosis against HepG2 cells in a dose-dependent manner [23,24]. These findings showed that compound **6r** induced apoptosis against HepG2 cell lines, consistent with the results for AO/EB double staining. Finally, the cells presented with apoptotic morphology. The complete absence of orange cells in compound **6r** revealed that it was associated with very low cytotoxicity. These findings indicated that compound **6r** could induce apoptosis in a dose-dependent manner with low cytotoxicity.

2.3.1.3. Mitochondrial membrane potential staining. Mitochondrial membrane potential changes were also designed and detected to further investigate the apoptosis inducing effect of target compound **6r**, using a fluorescent probe JC-1. JC-1, which was a lipophiliccationic dye, could easily pass through the plasma membrane into cells and accumulate in actively respiring mitochondria. HepG2 cells treated with **6r** at 10 μM, 20 μM, 30 μM for 24 h were stained with JC-1, whereas not treated with **6r** were used as control for 24 h. The results were given in Fig. 3.

The JC-1 monomer and J-aggregates were excited at 514 nm and 585 nm, respectively, and light emissions were collected at 515–545 nm (green) and 570–600 nm (red) [28,29]. For fluorescence microscopy, Fig. 3 showed that cells not treated with the compound were normally red, while for **6r** treatment, cells exhibited strong green fluorescence and indicated typical apoptotic morphology. Therefore, it could be concluded that compound **6r** induced apoptosis against HepG2 cell line in a concentration-dependent manner. These results were identical with that of previous experiment of AO/EB double staining and Hoechst 33258 staining.

2.3.2. Flow cytometry

Flow cytometry was used to investigate analog-induced apoptosis and cell death. The compound **6r** with cytotoxic effects

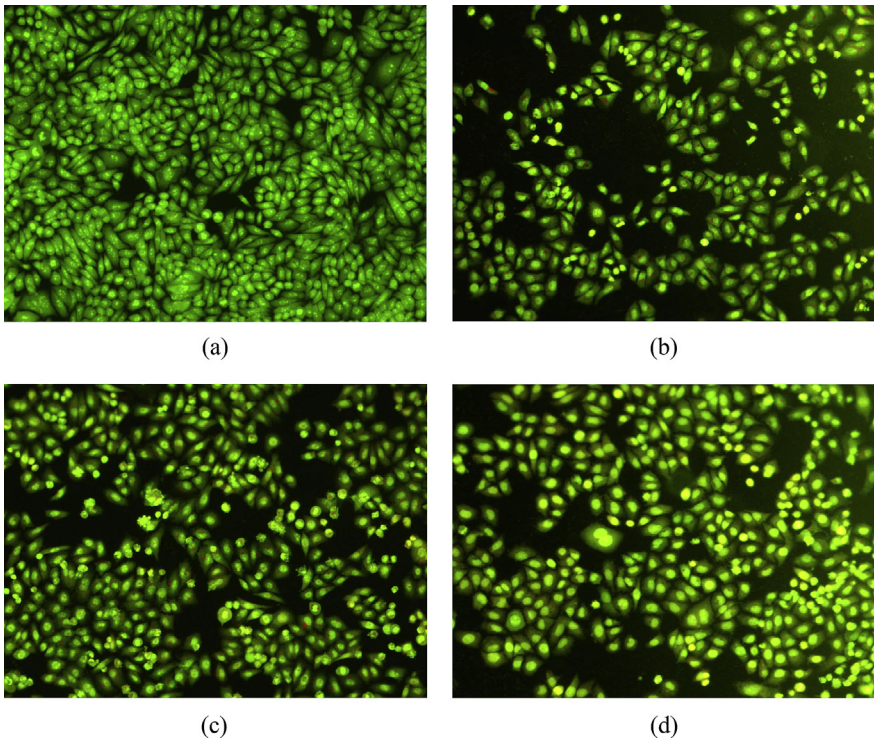


Fig. 1. AO/EB staining of compound **6r** in HepG2 cells. (a) Not treated with compound **6r** was used as control at for 24 h and (b, c, d) treatment with compound **6r** (10, 20, 30 μM) for 24 h, respectively.

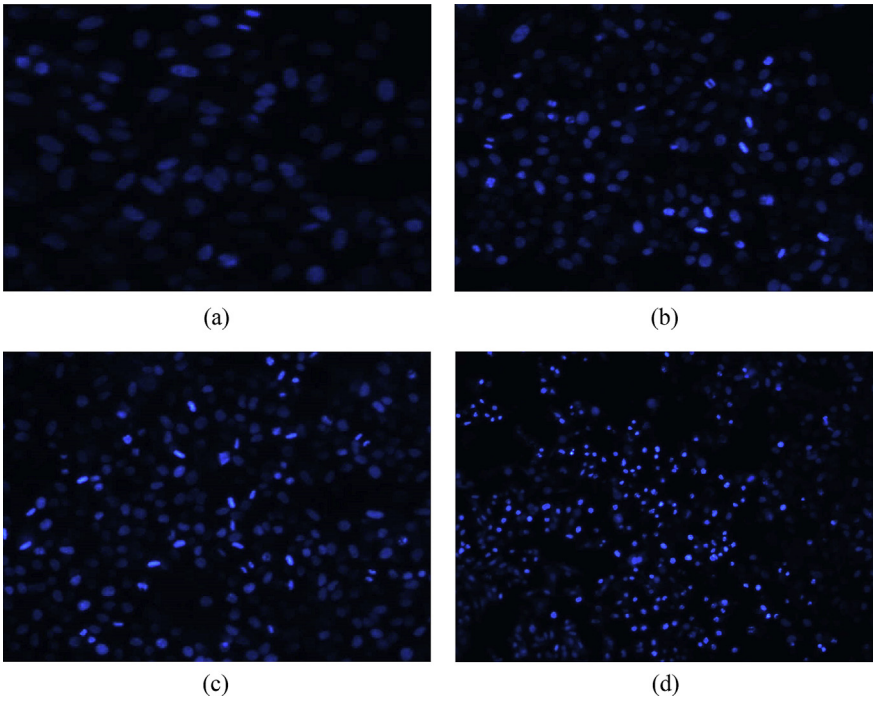


Fig. 2. Hoechst 33258 staining of compound **6r** in HepG2 cells. (a) Not treated with compound **6r** was used as control at for 24 h and (b, c, d) treatment with compound **6r** at 10 μM, 20 μM, 30 μM for 24 h, respectively.

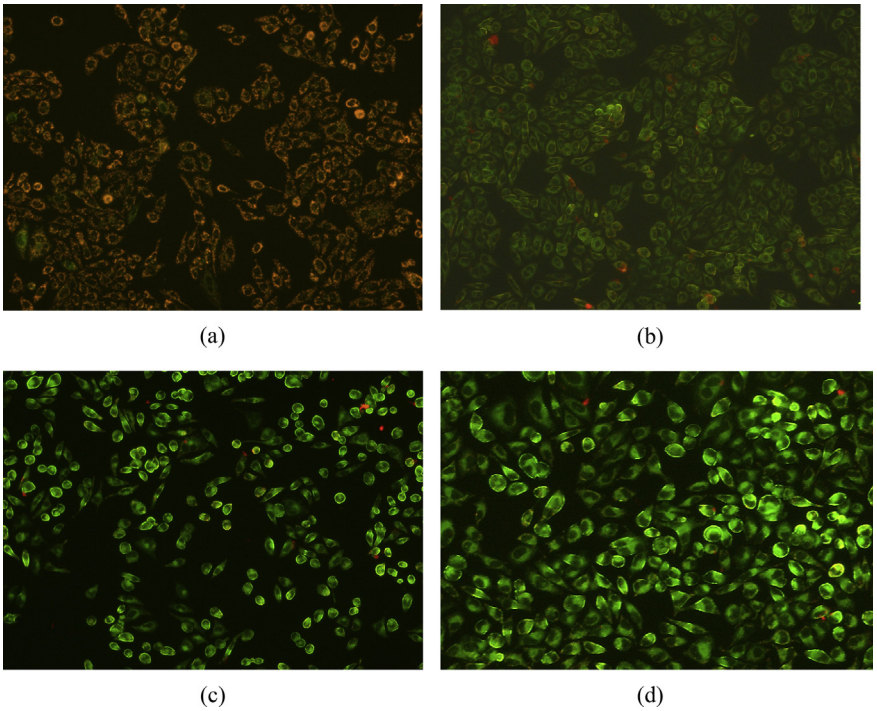


Fig. 3. JC-1 mitochondrial membrane potential staining of compound **6r** in HepG2 cells. (a) Not treated with compound **6r** was used as control at for 24 h and (b, c, d) treatment with compound **6r** at 10 μM, 20 μM, 30 μM for 24 h, respectively.

was investigated for the effects on the apoptosis of HepG2 cells. To illustrate the mechanism of cyclotriphosphazene-induced (compound **6r**) cell death in cells, we determined both early and late apoptosis using annexin V-FITC and PI (Propidium iodide) labeling of live cells. Annexin V binded to phosphatidylserine which was exposed on the cell membrane and it was one of the earliest indicators of cellular apoptosis. PI, which could be used to differentiate necrotic, apoptotic and normal cells, was used as a DNA stain for both flow cytometry to evaluate cell viability or DNA content in cell cycle analysis and microscopy to visualize the nucleus and

other DNA containing organelles.

2.3.2.1. Apoptosis analysis. In the present study, the apoptotic cell rates were determined for the HepG2 cells that were treated with compound **6r** at 20 μM for 6, 12, 24 h, respectively, and the results were shown in Fig. 4. It can be observed from Fig. 4 that compound **6r** significantly caused the early and late apoptosis. Specifically, compared with control (2.13%, 4.03%), the early and late apoptosis rates were gradually increased from (7.21%, 2.59%) to (14.4%, 7.36%) and (22.5%, 13.8%) after treatment for 6, 12, 24 h, respectively. The results showed that compound **6r** caused a markedly increased the cellular apoptosis in a time-dependent manner.

2.3.2.2. Cell cycle analysis. The cell cycle is a series of events that happen in a cell leading to its division and duplication (replication). The cell cycle contains four distinct phases: G1 phase, S phase

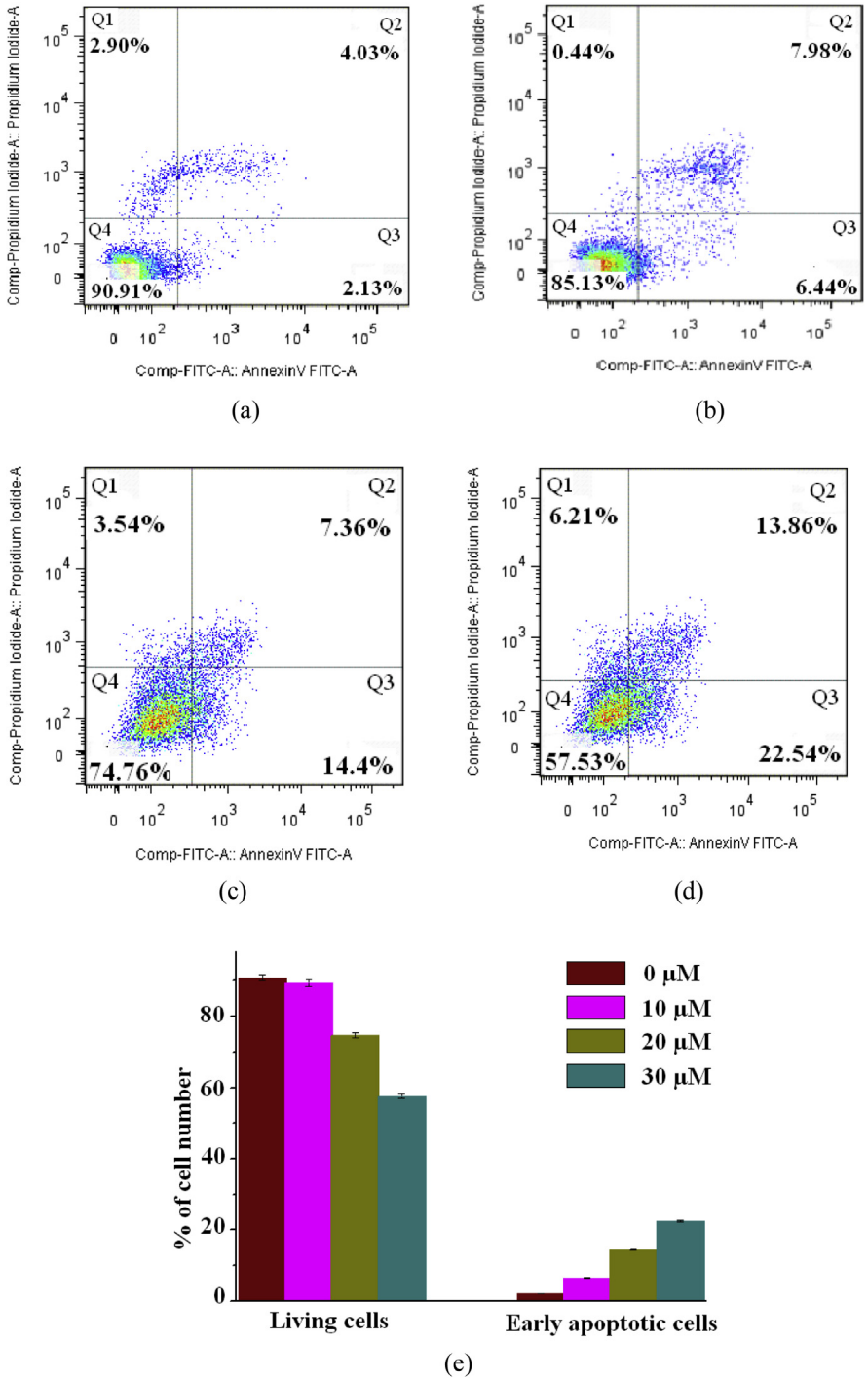


Fig. 4. (a–d) HepG2 Cells were treated with compound **6r** at 20 μM for 0, 6, 12, 24 h, respectively. The cells were trypsinized, loaded with PI and Annexin V, and then analyzed via flow cytometry. (e) The numbers of living cells and early apoptotic cells were expressed as a percentage of the total cell number. The values represent mean ± S.D of three independent experiments ($p < 0.01$).

(synthesis), G2 phase (collectively known as inter phase) and M phase (mitosis). The G1 stage is the stage when preparation of energy and material for DNA replication occurs. The S stage is the stage when DNA replication occurs. The G2 stage is the stage when preparation for the M stage occurs. The M stage is "mitosis", and is when nuclear and cytoplasmic division occurs.

To determine whether the suppression of cancer cell growth by compound **6r** was caused by a cell cycle progression, a cell-cycle cytotoxicity assay was performed by treating HepG2 cells at different concentrations of compound **6r** (0, 5, 10, 20 μM) for 48 h (Fig. 5). As shown in Fig. 5, cells in the G1 phase increased from 45.19% in control to 54.64%, 60.03%, and 65.16%, and a decrease of population in S phase from 49.27% in control to 43.11%, 37.82%, and 28.37% was also found. Hence, compound **6r** caused a markedly G1 arrest in a concentration manner with a concomitant decrease in terms of the number of cells in the S phase of the cycle.

2.4. Compound **6r** induces caspase-dependent apoptosis in HepG2 cells

To further investigate the effect of ursolic acid derivatives on apoptosis in HepG2 cells, expression of three apoptosis-related genes (caspase-3, caspase-8, caspase-9) was detected by real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) after treatment of compound **6r** for 24 h at 0 μM, 10 μM, 20 μM and 30 μM, respectively. The levels of gene expression were

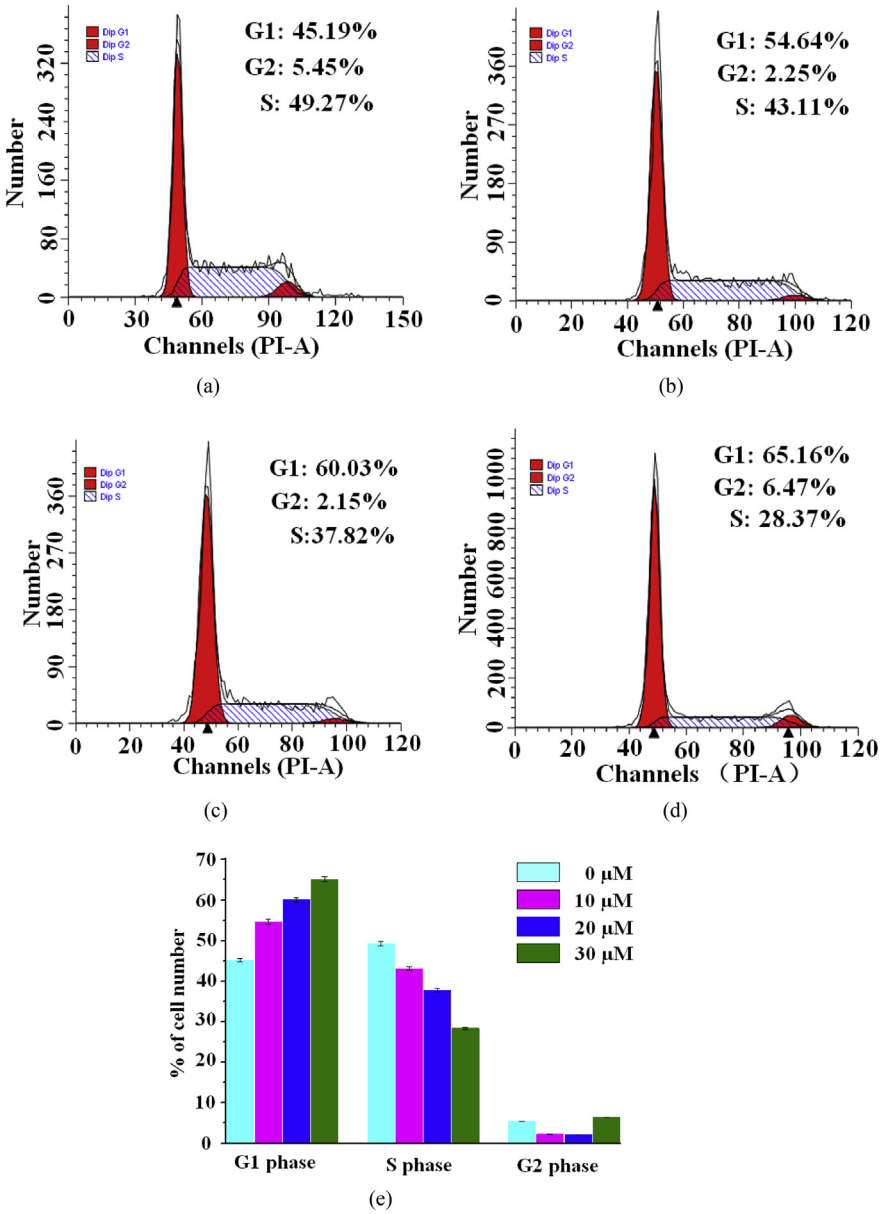


Fig. 5. Cell-cycle analysis of compound **6r** by flow cytometry in HepG2 cells. (a) Not treated with compound **6r** was used as control at for 48 h (b, c, d) treatment with compound **6r** at 10 μM, 20 μM and 30 μM for 48 h, respectively. (e) The numbers of G1, S, G2 phase cells were expressed as a percentage of the total cell number. The values represent mean ± S.D of three independent experiments ($p < 0.01$).

normalized to control cells and calibrated with reference to β-actin. Up-regulation of apoptosis-related genes was observed (Fig. 6). Caspase-3 was up-regulated 14.58-fold, and caspase-8 was up-regulated 4.46-fold, while caspase-9 was up-regulated 5.22-fold.

Caspase family of cysteinyl-proteases plays the critical role in the initiation and execution of programmed cell death. It has been reported natural pentacyclic triterpenoids induced apoptosis in different cell lines through extrinsic or intrinsic pathway by increasing the expression of caspase-3, caspase-8 and caspase-9 [30,31]. Thus, the over expression of compound **6r** upon caspase-3, caspase-8 and caspase-9 in HepG2 cells as we observed indicated that compound **6r** might possessed the ability to inhibit proliferation and migration of HepG2 cells via activating caspases through extrinsic or intrinsic pathway.

2.5. Effect of compound **6r** on intracellular reactive oxygen species (ROS)

Reactive oxygen species (ROS) plays an important role in apoptosis induction under physiologic and pathologic conditions. Interestingly, mitochondria are both source and target of ROS. Cytochrome *c* releases from mitochondria, that triggers caspase activation, appears to be largely mediated by direct or indirect ROS action. Furthermore, ROS have destructive actions on both DNA and proteins [32]. Natural pentacyclic triterpenoids have been shown to inhibit proliferation and induce apoptosis of some tumor cell lines [9–11]. Several studies have shown that Natural pentacyclic triterpenes trigger a rapid production of intracellular ROS, which might be responsible for their cytotoxic actions [33–35]. To determine whether ursolic acid derivatives could significantly increased the intracellular level of ROS in a concentration-dependent manner, we exposed HepG2 cells to compound **6r** (at the concentration of 0, 10, 20, 30 μM) for 24 h and examined them for evidence of ROS production using the oxidative stress-sensitive dyes DCFH-DA. Cellular fluorescence was quantified using fluorescence microplate reader (Nikon ECLIPSETE2000-S) at an excitation of 485 nm and an emission of 538 nm. The result was showed in Fig. 7. The results obtained revealed that cell treated with compound **6r** led to a remarkable increase of the DCFH-DA probe fluorescence emission that correlated well with increasing concentrations. Fig. 7 showed that cells not treated with compound **6r** displayed normally green (in the web version), while cells treated with **6r** displayed strong green fluorescence and the fluorescence intensity was gradually enhanced following the increasing concentrations. These findings demonstrated a significant increase in the intracellular levels of ROS.

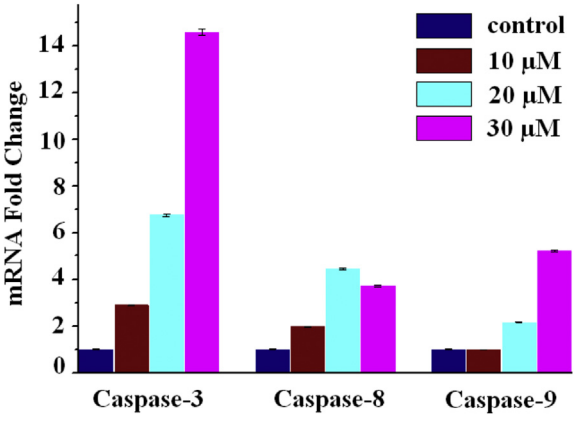


Fig. 6. Compound **6r** affected the activities of caspase-3, caspase-8 and caspase-9 of HepG2 cells.

2.6. Effect of compound **6r** induce apoptosis via intrinsic and extrinsic apoptosis pathway in HepG2 cells

Apoptosis is a cell suicide mechanism that enables metazoans to control cell number in tissues and to eliminate individual cells that threaten the animal's survival. There are two major apoptotic pathways known to date, initiated by either the mitochondria (the 'intrinsic' pathway) or the death receptor (the 'extrinsic' pathway) [36]. The mitochondria-dependent apoptotic pathway is regulated by the Bcl-2 family of pro- and anti-apoptotic proteins, which induce the permeabilization of the mitochondrial outer membrane and cytochrome *c* released into the cytosol, resulting in the activation of the caspase cascade and the induction of apoptotic cell death [37]. The extrinsic apoptosis pathway is mediated by plasma membrane death receptors and caspase-8 is a major initiator caspase in this pathway [38]. To evaluate the effects of compound **6r** on the intrinsic and extrinsic pathway, the cells were treated with ursolic acid derivatives at 20 μM for 0, 6, 12, 24 h and the total cell lysates were prepared for Western blot analysis.

Bax and Bcl-2 belong to the Bcl-2 family and serve as pro- and anti-apoptotic effectors, respectively. In addition, they also regulate mitochondrial outer membrane permeabilization (MOMP). The imbalance of anti- and pro-apoptotic protein expression is one of the major mechanisms underlying the ultimate fate of cells with respect to apoptosis. The effects of compound **6r** on the constitutive levels of Bax and Bcl-2 in HepG2 cells were shown in Fig. 8a. In comparison with the control cells, **6r** induced a significant increase in the levels of Bax and a reduction in the levels of Bcl-2, in a time-dependent fashion. The result indicated that treatment with compound **6r** (20 μM) could shift the ratio of Bax to Bcl-2 and lead to collapse of the mitochondrial membrane potential.

After loss of MOMP, cytochrome *c* is released into the cytosol leading to activation of the apoptosome complex and a caspase cascade. Therefore, we investigated cytochrome *c* release in the cytosolic fraction following compound **6r** treatment (Fig. 8a). The result showed that cytosolic cytochrome *c* expression was remarkably increased at a time-dependent manner, as compared to control cells, indicating that compound **6r** could regulate apoptosis of HepG2 cells through a mitochondria-mediated pathway.

The release of cytochrome *c* results in the activation of caspase-9 which then goes on to process and activate other downstream caspases including that of caspase-3 what leads to the cleavage of numerous down stream molecules including PARP leading to the final phase of the execution of compound induced cell death [39,40]. It has been reported that **UA** caused DNA-damage followed by the activation of a p53-, BAK-, and caspase-dependent cell-death pathway [41]. The effects of compound **6r** on the time course of Apaf-1, caspase-9, caspase-3 and PARP expression in HepG2 cells were shown in Fig. 8b. Exposure of HepG2 cells to this compound caused a dramatic increase in the levels of Apaf-1, caspase-9, caspase-3 and PARP, as compared with control cells. These observations suggested that compound **6r** might induce HepG2 cells apoptosis through a mitochondrial mediated pathway and caspase cascade.

Fas receptor and its ligand (FasL) are a pair of plasma membrane proteins whose interaction triggers one of the pathways for apoptosis. This intracellular cascade of events requires the Fas-associated death domain protein and the formation of death-inducing signaling complex, leading to caspase-8 activation and cell apoptosis. To determine whether compound **6r** altered the constitutive levels of Fas, caspase-8 and caspase-3, via western blot analysis, HepG2 cells were treated with 20 μM of compound

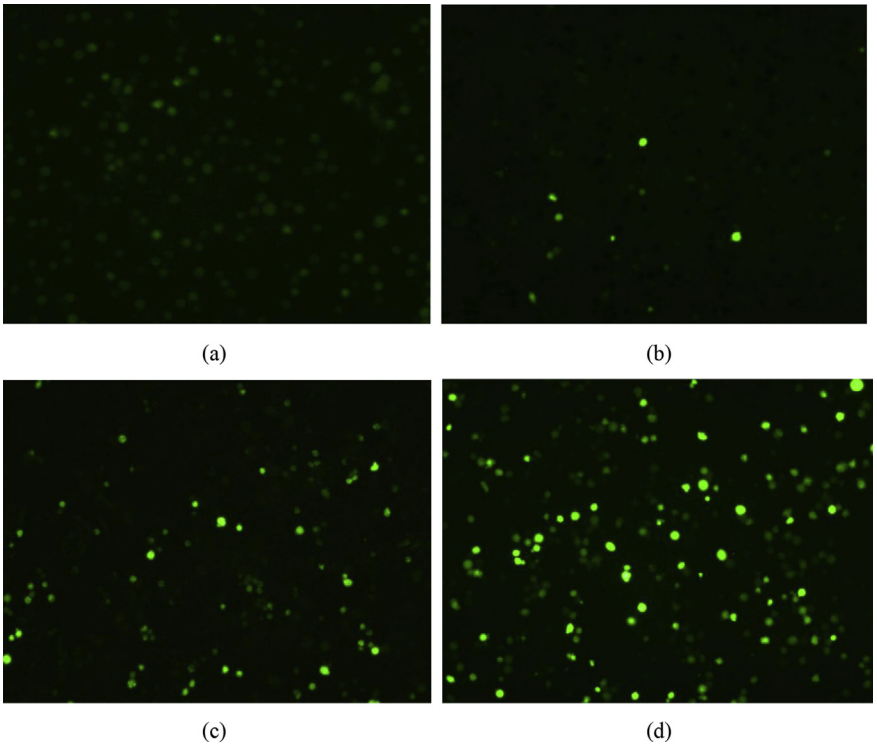


Fig. 7. ROS reactive oxygen species potential staining of compound **6r** in HepG2 cells. (a) Not treated with compound **6r** was used as control at for 24 h and (b, c, d) treatment with compound **6r** at 10 μM, 20 μM, 30 μM for 24 h, respectively.

6r for different time intervals (0–24 h) (Fig. 8c). As shown in Fig. 8c, compound **6r** stimulated levels of Fas, and the active form of caspase-8 and caspase-3. The results indicated that Fas activation contributed to apoptosis in compound **6r** treated cells, which may have been mediated by caspase-8 activation. Moreover, the activated caspase-8 may have directly activated caspase-3.

3. Conclusions

In summary, we designed and synthesized a set of ursolic acid derivatives (**6a**–**t** and **7a**–**r**) and the cytotoxicity of target compounds on five cancer cell lines (MGC-803, HCT-116, T24, HepG2 and A549) were determined. The *in vitro* antiproliferative activity screening indicated that most of target compounds exhibited a potent inhibitory activity. Especially, the compound **6r** exhibited excellent *in vitro* cytotoxicity against HepG2 cells (IC_{50} was 5.40 ± 0.79 μM). The incorporation of piperazine and thiourea at C-28 while retaining the polar group at C-3 significantly improved the antitumor bioactivities of the compounds. The apoptosis-inducing and affects on the cell cycle distribution of compound **6r** in HepG2 cells were investigated by AO/EB staining, Hoechst 33258 staining, JC-1 mitochondrial membrane potential staining, flow cytometry, which revealed that the antitumor activity of **6r** was achieved through the induction of cell apoptosis by G1 cell-cycle arrest. Western blot and qRT-PCR (quantitative real-time PCR) experiments demonstrated that compound **6r** may induce apoptosis through the both of intrinsic and extrinsic apoptosis pathway. Moreover, **6r** has shown a pronounced increase in Fas and caspase-8 activity. Therefore, these studies indicated that the target compound may induce apoptosis, arrest cell cycle progression at the S phase and increase the activity of caspase-3 to inhibit cell growth.

4. Experimental

4.1. General

Compound **2** was synthesized according to the literature [25]. The isothiocyanate was synthesized according to the literature [26]. **UA** with more than 95% purity was purchased from wuhan shengtianyu Biotech Co. Ltd. All the chemical reagents and solvents used were of analytical grade. Silica gel (300–400 mesh) used in column chromatography was provided by Tsingtao Marine Chemistry Co. Ltd. ^{1}H NMR and ^{13}C NMR were recorded on a BRUKER AV-500 spectrometer with TMS as an internal standard in $CDCl_3$. Mass spectra were determined on an FTMS ESI spectrometer.

4.2. Synthesis: general procedure for compounds *6a–t*

3-O-acetylursolic acid **2** (4 mmol) added to dry CH_2Cl_2 (20 mL) was stirred at 0 °C and oxalyl chloride (12 mmol) was dripped into the mixture and stirred at room temperature for 6 h. The solvent was removed by evaporation under reduced pressure, and dry CH_2Cl_2 (5 mL × 3) was added to the residue, concentrated to dryness to give **3**. Compound **3** (4 mmol) was dissolved in dry CH_2Cl_2, after basified to pH 8–9 with triethylamine the piperazine (12 mmol) was added. The resultant mixture was stirred at room temperature for 3 h. After the solvent was removed by evaporation under reduced pressure, the resultant residue was dispersed in water, then acidified to pH 3–4 with 2N HCl, then filtered and extracted with CH_2Cl_2, dried over anhydrous Na_2SO_4, filtered, and the solvent was removed under reduced pressure. The crude material was purified on a silica gel column with the appropriate eluent to yield compounds to afford compound **4**. Compound **4** (0.26 mmol) was dissolved in CH_2Cl_2 (20 mL), isothiocyanate(0.31 mmol) and triethylamine (0.13 mmol) were added to the mixture and stirred at room temperature for 4 h. After

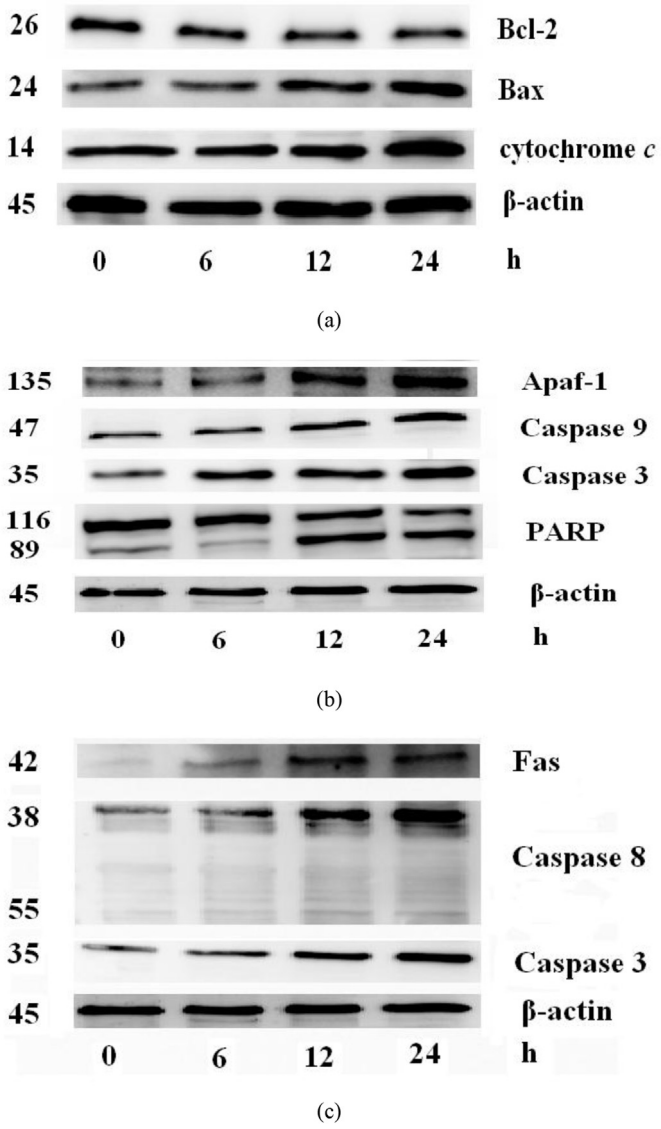


Fig. 8. Compound **6r** affected the apoptotic relative proteins expressions in HepG2 cells. A total of 5×10^5 HepG2 cells/mL cells were treated with 20 μM **6r** for 0, 6, 12, 24 h. Cells were harvested from each sample, and associated proteins were determined by Western blotting. (a) Cytochrome *c*, Bax, Bcl-2 (b) Apaf-1, caspase-9, and caspase-3, PARP (c) Fas, caspase-8 and caspase-3 expressions were examined using SDS-PAGE and Western blotting as described under Materials and Methods.

the reaction, the solvent was evaporated under reduced pressure, and the crude product was purified by chromatography on silica gel eluted with petroleum ether/ethyl acetate (V:V = 3:1) to offer compounds **6a**–**t**. The structures were confirmed by ^{1}H NMR, ^{13}C NMR and HR-MS (see supporting information).

4.2.1. *N-[3β-acetoxy-urs-12-en-28-oyl]-amino-N-phenylpiperazine-1-carbothioamide (**6a**)*

According to the general procedure, 3β-acetoxy-urs-12-en-28-acyl piperazine was treated with isothiocyanate, and then purified on silica gel eluted with petroleum ether/ethyl acetate (V:V = 3:1) as eluent to give compound **6a**.

Yields 90.43%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.59 (s, 1H, NH), 7.31 (t, *J* = 7.8 Hz, 2H), 7.17–7.14 (m, 3H), 5.18 (s, 1H, H-12), 4.48–4.44 (m, 1H, H-3), 3.91–3.64 (m, 8H, H in piperazine), 2.02 (s, 3H, $-OCOCH_3$), 1.06 (s, 3H, CH_3), 0.93 (d, *J* = 5.2 Hz, 3H, CH_3), 0.91 (s, 3H, CH_3), 0.86–0.83 (m, 9H, $3CH_3$), 0.69 (s, 3H, CH_3); ^{13}C NMR (500 MHz, $CDCl_3$): δ 183.2 (C═S), 175.9 (–CON–), 171.1 (–COO–), 139.8, 129.11, 125.6, 125.3, 123.9, 80.9, 55.30, 48.5, 47.5, 44.5, 42.2, 39.4, 38.7, 38.2, 37.7, 36.9, 34.3, 33.1, 31.5, 30.4, 29.7, 28.1, 23.5, 23.3, 21.4, 21.2, 18.2, 17.4, 16.8, 15.5; HR-MS (*m*/*z*) (ESI): calcd for $C_{43}H_{63}N_3O_3S$ [M– H^+]: 700.45902; found: 700.45123.

4.2.2. *N-[3β-acetoxy-urs-12-en-28-oyl]-amino-N-(1-naphthalene) piperazine-1-carbothioamide (**6b**)*

According to the general procedure, 3β-acetoxy-urs-12-en-28-acyl piperazine was treated with isothiocyanate, and then purified on silica gel eluted with petroleum ether/ethyl acetate (V:V = 3:1) as eluent to give compound **6b**.

Yields 80.53%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.01 (s, 1H, NH), 7.91 (d, *J* = 7.0 Hz, 1H), 7.84 (d, *J* = 7.4 Hz, 1H), 7.72 (d, *J* = 8.2 Hz, 1H), 7.49 (d, *J* = 2.9 Hz, 2H), 7.41 (t, *J* = 7.7 Hz, 1H), 5.16 (s, 1H, H-12), 4.46 (t, *J* = 7.7 Hz, 1H, H-3), 3.92–3.55 (m, 8H, H in piperazine), 2.03 (s, 3H, $-OCOCH_3$), 1.06 (s, 3H, CH_3), 0.91 (s, 6H, $2CH_3$), 0.85–0.82 (m, 9H, $3CH_3$), 0.66 (s, 3H, CH_3); ^{13}C NMR (500 MHz, $CDCl_3$): δ 184.4 (–C═S), 175.8 (–CON–), 171.1 (–COO–), 136.1, 134.3, 129.2, 128.4, 127.0, 126.6, 126.5, 125.5, 125.2, 122.5, 80.9, 55.2, 48.5, 48.0, 47.5, 45.5, 42.1, 39.4, 38.6, 38.2, 37.6, 36.8, 34.2, 31.4, 30.3, 29.7, 28.1, 23.5, 23.2, 21.4, 21.2, 18.2, 17.4, 16.7, 15.5; HR-MS (*m*/*z*) (ESI): calcd for $C_{47}H_{65}N_3O_3S$ [M– H^+]: 750.47472; found: 750.46600.

4.2.3. *N-[3β-acetoxy-urs-12-en-28-oyl]-amino-N-(3-fluoro phenyl) piperazine-1-carbothioamide (**6c**)*

According to the general procedure, 3β-acetoxy-urs-12-en-28-acyl piperazine was treated with isothiocyanate, and then purified on silica gel eluted with petroleum ether/ethyl acetate (V:V = 3:1) as eluent to give compound **6c**.

Yields 86.87%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.47 (s, 1H, NH), 7.29 (dd, *J* = 11.1, 5.2 Hz, 1H), 6.97–6.91 (m, 2H), 6.86 (t, *J* = 8.3 Hz, 1H), 5.20 (s, 1H, H-12), 4.47 (t, *J* = 7.5 Hz, 1H, H-3), 3.95–3.68 (m, 8H, H in piperazine), 2.04 (s, 3H, $-OCOCH_3$), 1.07 (s, 3H, CH_3), 0.94 (d, *J* = 5.5 Hz, 3H, CH_3), 0.92 (s, 3H, CH_3), 0.87–0.84 (m, 9H, $3CH_3$), 0.70 (s, 3H, CH_3); ^{13}C NMR (500 MHz, $CDCl_3$): δ 183.0 (–C═S), 177.1 (–CON–), 171.1 (–COO–), 163.8, 163.6, 161.9, 142.1, 141.2, 130.2, 118.8, 115.9, 112.3, 112.2, 110.6, 80.9, 80.4, 55.3, 48.6, 47.4, 44.5, 42.1, 40.8, 39.4, 38.2, 37.7, 36.8, 36.3, 34.3, 30.6, 30.4, 29.7, 28.1, 23.5, 23.2, 21.3, 21.2, 18.1, 17.4, 16.7, 15.5; HR-MS (*m*/*z*) (ESI): calcd for $C_{43}H_{62}FN_3O_3S$ [M–H^+]: 718.44964; found: 718.44174.

4.2.4. *N-[3β-acetoxy-urs-12-en-28-oyl]-amino-N-(4-fluorophenyl) piperazine-1-carbothioamide (**6d**)*

According to the general procedure, 3β-acetoxy-urs-12-en-28-acyl piperazine was treated with isothiocyanate, and then purified on silica gel eluted with petroleum ether/ethyl acetate (V:V = 3:1) as eluent to give compound **6d**.

Yields 88.92%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.47 (s, 1H, NH), 7.19–7.15 (m, 2H), 7.02 (t, *J* = 8.5 Hz, 2H), 5.19 (s, 1H, H-12), 4.49–4.43 (m, 1H, H-3), 3.99–3.69 (m, 8H, H in piperazine), 2.03 (s, 3H, $-OCOCH_3$), 1.07 (s, 3H, CH_3), 0.94 (s, 3H, CH_3), 0.91 (s, 3H, CH_3), 0.86–0.83 (m, 9H, $3CH_3$), 0.70 (s, 3H, CH_3); ^{13}C NMR (500 MHz, $CDCl_3$): δ 183.1 (–C═S), 175.9 (–CON–), 171.1 (–C═O), 161.4, 159.5, 135.8, 126.9, 126.9, 125.2, 115.7, 115.6, 80.9, 55.3, 48.7, 47.9, 47.4, 44.5, 42.1, 39.4, 38.7, 38.2, 37.6, 36.8, 34.3, 32.9, 31.4, 30.3, 29.6, 28.0, 23.5, 23.2, 21.3, 21.1, 18.1, 17.4, 16.8, 16.7, 15.4; HR-MS (*m*/*z*) (ESI): calcd for $C_{43}H_{62}FN_3O_3S$ [M– H^+]: 718.44962; found: 718.44138.

4.2.5. *N-[3β-acetoxy-urs-12-en-28-oyl]-amino-N-(3-bromophenyl) piperazine-1-carbothioamide (**6e**)*

According to the general procedure, 3β-acetoxy-urs-12-en-28-acyl piperazine was treated with isothiocyanate, and then purified on silica gel eluted with petroleum ether/ethyl acetate

(V:V = 3:1) as eluent to give compound **6e**.

Yields 85.41%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.59–7.42 (m, 1H, NH), 7.33 (s, 1H), 7.29 (d, J = 7.5 Hz, 1H), 7.22–7.13 (m, 2H), 5.19 (s, 1H, H-12), 4.47 (t, J = 7.5 Hz, 1H, H-3), 4.00–3.68 (m, 8H, H in piperazine), 2.04 (s, 3H, $-OCOCH_3$), 1.07 (s, 3H, CH_3), 0.94 (d, J = 5.1 Hz, 3H, CH_3), 0.92 (s, 3H, CH_3), 0.85 (t, J = 5.4 Hz, 9H, $3CH_3$), 0.70 (s, 3H, CH_3); ^{13}C NMR (500 MHz, $CDCl_3$): δ 182.8 (−C═S), 175.9 (−CON−), 171.0 (−COO−), 141.1, 130.1, 128.3, 126.8, 125.2, 122.3, 80.9, 55.3, 48.7, 47.4, 44.5, 42.1, 39.4, 38.7, 38.2, 37.7, 36.9, 34.3, 33.8, 31.4, 30.3, 29.6, 28.0, 23.5, 23.3, 21.3, 21.2, 19.1, 18.1, 17.3, 16.8, 16.7, 15.5; HR-MS (m/z) (ESI): calcd for $C_{43}H_{62}BrN_3O_3S$ [M− H^+]: 778.36955; found: 778.36169.

4.2.6. N-[3β-acetoxy-urs-12-en-28-oyl]-amino-N-(4-bromophenyl) piperazine-1-carbothioamide (**6f**)

According to the general procedure, 3β-acetoxy-urs-12-en-28-acyl piperazine was treated with isothiocyanate, and then purified on silica gel eluted with petroleum ether/ethyl acetate (V:V = 3:1) as eluent to give compound **6f**.

Yields 83.65%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.67 (s, 1H, NH), 7.41 (d, J = 8.5 Hz, 2H), 7.08 (d, J = 8.5 Hz, 2H), 5.17 (s, 1H, H-12), 4.44 (t, J = 7.8 Hz, 1H, H-3), 4.00–3.64 (m, 8H, H in piperazine), 2.02 (s, 3H, $-OCOCH_3$), 1.06 (s, 3H, CH_3), 0.93 (s, 3H, CH_3), 0.90 (s, 3H, CH_3), 0.85–0.81 (m, 9H, $3CH_3$), 0.68 (s, 3H, CH_3); ^{13}C NMR (500 MHz, $CDCl_3$): δ 182.6 (−C═S), 175.9 (−CON−), 171.1 (−COO−), 138.8, 131.9, 126.0, 118.7, 80.9, 55.3, 48.7, 48.1, 47.4, 44.5, 42.1, 39.4, 38.7, 38.1, 37.6, 36.8, 34.3, 32.9, 31.4, 30.3, 29.7, 28.0, 23.5, 23.2, 21.3, 21.2, 18.1, 17.4, 16.7, 15.5; HR-MS (m/z) (ESI): calcd for $C_{43}H_{62}BrN_3O_3S$ [M− H^+]: 778.36955; found: 778.36210.

4.2.7. N-[3β-acetoxy-urs-12-en-28-oyl]-amino-N-(2,4-DiBromophenyl)piperazine-1-carbothioamide (**6g**)

According to the general procedure, 3β-acetoxy-urs-12-en-28-acyl piperazine was treated with isothiocyanate, and then purified on silica gel eluted with petroleum ether/ethyl acetate (V:V = 3:1) as eluent to give compound **6g**.

Yields 84.62%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.72 (d, J = 2.2 Hz, 1H, NH), 7.67 (dd, J = 8.7, 2.7 Hz, 1H), 7.43 (dd, J = 8.7, 2.2 Hz, 1H), 7.23 (s, 1H), 5.21 (s, 1H, H-12), 4.50–4.45 (m, 1H, H-3), 4.08–3.74 (m, 8H, H in piperazine), 2.04 (s, 3H, $-OCOCH_3$), 1.08 (s, 3H, CH_3), 0.95 (d, J = 5.9 Hz, 3H, CH_3), 0.92 (s, 3H, CH_3), 0.86 (dd, J = 12.0, 5.9 Hz, 9H, $3CH_3$), 0.71 (s, 3H, CH_3); ^{13}C NMR (500 MHz, $CDCl_3$): δ 181.7 (−C═S), 176.0 (−CON−), 171.1 (−COO−), 136.9, 134.9, 130.9, 127.8, 125.2, 118.9, 118.8, 80.9, 55.3, 48.7, 47.9, 47.4, 44.3, 42.1, 39.4, 38.7, 38.1, 37.6, 36.8, 34.3, 32.9, 31.4, 30.3, 29.7, 28.0, 23.5, 23.3, 21.3, 21.2, 18.1, 17.4, 16.7, 15.5; HR-MS (m/z) (ESI): calcd for $C_{43}H_{61}Br_2N_3O_3S$ [M− H^+]: 856.28002; found: 856.27097.

4.2.8. N-[3β-acetoxy-urs-12-en-28-oyl]-amino-N-(3-chlorophenyl) piperazine-1-carbothioamide (**6h**)

According to the general procedure, 3β-acetoxy-urs-12-en-28-acyl piperazine was treated with isothiocyanate, and then purified on silica gel eluted with petroleum ether/ethyl acetate (V:V = 3:1) as eluent to give compound **6h**.

Yields 81.79%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.69 (s, 1H, NH), 7.23 (t, J = 8.0 Hz, 1H), 7.18 (d, J = 1.4 Hz, 1H), 7.11 (t, J = 7.6 Hz, 2H), 5.18 (s, 1H, H-12), 4.46 (t, J = 7.2 Hz, 1H, H-3), 3.97–3.65 (m, 8H, H in piperazine), 2.03 (s, 3H, $-OCOCH_3$), 1.06 (s, 3H, CH_3), 0.93 (s, 3H, CH_3), 0.91 (s, 3H, CH_3), 0.86–0.82 (m, 9H, $3CH_3$), 0.69 (s, 3H, CH_3); ^{13}C NMR (500 MHz, $CDCl_3$): δ 182.7 (−C═S), 176.0 (−CON−), 171.1 (−COO−), 140.9, 134.4, 129.9, 125.5, 125.2, 123.9, 122.2, 80.9, 55.3, 48.3, 47.9, 47.4, 42.1, 39.4, 38.2, 38.1, 37.6, 36.8, 34.3, 32.9, 31.4, 30.3, 29.7, 28.0, 23.5, 23.2, 21.3, 21.2, 18.1, 17.4, 16.7, 15.5; HR-MS (m/z) (ESI): calcd for $C_{43}H_{62}ClN_3O_3S$ [M− H^+]: 734.42005; found: 734.41115.

4.2.9. N-[3β-acetoxy-urs-12-en-28-oyl]-amino-N-(4-chlorophenyl) piperazine-1-carbothioamide (**6i**)

According to the general procedure, 3β-acetoxy-urs-12-en-28-acyl piperazine was treated with isothiocyanate, and then purified on silica gel eluted with petroleum ether/ethyl acetate (V:V = 3:1) as eluent to give compound **6i**.

Yields 84.14%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.46 (d, J = 4.4 Hz, 1H), 7.31–7.27 (m, 2H), 7.17–7.12 (m, 2H), 5.19 (s, 1H, H-12), 4.47 (t, J = 7.8 Hz, 1H, H-3), 4.00–3.69 (m, 8H, H in piperazine), 2.03 (s, 3H, $-OCOCH_3$), 1.07 (s, 3H, CH_3), 0.94 (d, J = 6.1 Hz, 3H, CH_3), 0.92 (s, 3H, CH_3), 0.87–0.84 (m, 9H, $3CH_3$), 0.70 (s, 3H, CH_3); ^{13}C NMR (500 MHz, $CDCl_3$): δ 182.9 (−C═S), 176.9 (−CON−), 171.1 (−COO−), 138.2, 131.0, 129.1, 126.9, 125.5, 80.9, 55.3, 48.2, 47.4, 39.4, 38.1, 37.6, 36.8, 34.3, 31.9, 31.4, 30.5, 30.4, 30.3, 30.1, 29.7, 28.0, 23.5, 23.3, 22.7, 21.3, 21.2, 19.1, 18.1, 17.4, 16.7, 15.5; HR-MS (m/z) (ESI): calcd for $C_{43}H_{62}ClN_3O_3S$ [M− H^+]: 734.42005; found: 734.41178.

4.2.10. N-[3β-acetoxy-urs-12-en-28-oyl]-amino-N-(3,4-dichlorophenyl)piperazine-1-carbothioamide (**6j**)

According to the general procedure, 3β-acetoxy-urs-12-en-28-acyl piperazine was treated with isothiocyanate, and then purified on silica gel eluted with petroleum ether/ethyl acetate (V:V = 3:1) as eluent to give compound **6j**.

Yields 82.73%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.85 (s, 1H, NH), 7.37–7.29 (m, 2H), 7.13 (dd, J = 8.7, 2.4 Hz, 1H), 5.15 (s, 1H, H-12), 4.44 (t, J = 7.7 Hz, 1H, H-3), 4.01–3.67 (m, 8H, H in piperazine), 2.02 (s, 3H, $-OCOCH_3$), 1.06 (s, 3H, CH_3), 0.93 (s, 3H, CH_3), 0.90 (s, 3H, CH_3), 0.83 (s, 9H, $3CH_3$), 0.68 (s, 3H, CH_3); ^{13}C NMR (500 MHz, $CDCl_3$): δ 182.4 (−C═S), 176.0 (−CON), 171.2 (−COO−), 139.3, 132.4, 130.3, 129.0, 126.1, 125.2, 124.2, 80.9, 55.2, 48.7, 48.0, 47.4, 42.2, 39.4, 38.7, 38.1, 37.6, 36.8, 34.3, 32.8, 31.4, 30.3, 29.7, 28.0, 26.9, 23.5, 23.2, 21.3, 21.2, 18.1, 17.4, 16.7, 15.5; HR-MS (m/z) (ESI): calcd for $C_{43}H_{61}Cl_2N_3O_3S$ [M− H^+]: 768.38113; found: 768.37199.

4.2.11. N-[3β-acetoxy-urs-12-en-28-oyl]-amino-N-(3-trifluoromethylphenyl)piperazine-1-carbothioamide (**6k**)

According to the general procedure, 3β-acetoxy-urs-12-en-28-acyl piperazine was treated with isothiocyanate, and then purified on silica gel eluted with petroleum ether/ethyl acetate (V:V = 3:1) as eluent to give compound **6k**.

Yields 80.91%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.67 (s, 1H, NH), 7.42 (dt, J = 8.4, 7.0 Hz, 4H), 5.19 (s, 1H, H-12), 4.50–4.43 (m, 1H, H-3), 4.00–3.69 (m, 8H, H in piperazine), 2.03 (s, 3H, $-OCOCH_3$), 1.07 (s, 3H, CH_3), 0.94 (s, 3H, CH_3), 0.91 (s, 3H, CH_3), 0.85 (t, J = 5.1 Hz, 9H, $3CH_3$), 0.69 (s, 3H, CH_3); ^{13}C NMR (500 MHz, $CDCl_3$): δ 182.7 (−C═S), 176.0 (−CON−), 171.1 (−COO−), 140.2, 131.4, 131.1, 129.4, 127.6, 124.8, 122.6, 122.0, 120.7, 80.9, 55.2, 48.2, 47.4, 44.3, 42.2, 39.4, 38.7,38.1, 37.6, 36.8, 34.3, 33.1, 32.1, 30.3, 29.6, 28.0, 23.5, 23.2, 21.3, 21.2, 18.1, 17.4, 16.7, 15.5; HR-MS (m/z) (ESI): calcd for $C_{44}H_{62}F_3N_3O_3S$ [M− H^+]: 768.44643; found: 768.43730.

4.2.12. N-[3β-acetoxy-urs-12-en-28-oyl]-amino-N-(4-trifluoromethylphenyl)piperazine-1-carbothioamide (**6l**)

According to the general procedure, 3β-acetoxy-urs-12-en-28-acyl piperazine was treated with isothiocyanate, and then purified on silica gel eluted with petroleum ether/ethyl acetate (V:V = 3:1) as eluent to give compound **6l**.

Yields 82.76%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.90 (s, 1H, NH), 7.53 (d, J = 8.1 Hz, 2H), 7.30 (d, J = 8.4 Hz, 2H), 5.16 (s, 1H, H-12), 4.44 (t, J = 7.7 Hz, 1H, H-3), 4.02–3.67 (m, 8H, H in piperazine), 2.02 (s, 3H, $-OCOCH_3$), 1.05 (s, 3H, CH_3), 0.92 (s, 3H, CH_3), 0.90 (s, 3H, CH_3), 0.83 (s, 9H, $3CH_3$), 0.68 (s, 3H, CH_3); ^{13}C NMR (500 MHz, $CDCl_3$): δ 182.5 (−C═S), 176.1 (−CON−), 171.2 (−COO−), 143.1, 126.8, 126.5, 125.9, 125.8, 125.0, 123.6, 122.9, 120.7, 81.0, 55.2, 48.7, 48.3, 47.4, 44.4, 42.1, 39.4, 38.7, 38.1, 37.6, 36.8, 34.3, 32.9, 31.4, 30.3, 29.6, 28.0, 23.5, 23.2,

21.3, 21.1, 18.1, 17.3, 16.8, 16.6, 15.4; HR-MS (*m*/*z*) (ESI): calcd for $C_{44}H_{62}F_3N_3O_3S$ [M− H^+]: 768.44643; found: 768.43782.

4.2.13. N-[3β-acetoxy-urs-12-en-28-oyl]-amino-N-(3-methylphenyl) piperazine-1-carbothioamide (**6m**)

According to the general procedure, 3β-acetoxy-urs-12-en-28-acyl piperazine was treated with isothiocyanate, and then purified on silica gel eluted with petroleum ether/ethyl acetate (V:V = 3:1) as eluent to give compound **6m**.

Yields 88.43%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.32 (s, 1H, NH), 7.22 (t, *J* = 8.0 Hz, 1H), 7.00–6.93 (m, 3H), 5.20 (s, 1H, H-12), 4.51–4.45 (m, 1H, H-3), 3.80 (d, *J* = 90.2 Hz, 8H, H in piperazine), 2.33 (s, 3H, Ph-CH_3), 2.04 (s, 3H, −$OCOCH_3$), 1.07 (s, 3H, CH_3), 0.94 (d, *J* = 5.9 Hz, 3H, CH_3), 0.92 (s,3H, CH_3), 0.86 (t, *J* = 6.5 Hz, 9H, $3CH_3$), 0.70 (s, 3H, CH_3); ^{13}C NMR (500 MHz, $CDCl_3$): δ 183.3 (−C═S), 175.8 (−CON−), 171.0 (−COO−), 139.7, 139.0, 128.8, 126.3, 125.2, 124.4, 120.9, 119.0, 80.9, 55.3, 48.5, 47.4, 44.4, 42.1, 39.4, 38.7, 38.2, 37.6, 36.8, 34.3, 32.9, 31.4, 30.3, 29.6, 28.0, 23.5, 23.2, 21.3, 21.2, 18.1, 17.3, 16.8, 16.7, 15.5; HR-MS (*m*/*z*) (ESI): calcd for $C_{44}H_{65}N_3O_3S$ [M− H^+]: 714.47475; found: 714.46634.

4.2.14. N-[3β-acetoxy-urs-12-en-28-oyl]-amino-N-(4-methylphenyl) piperazine-1-carbothioamide (**6n**)

According to the general procedure, 3β-acetoxy-urs-12-en-28-acyl piperazine was treated with isothiocyanate, and then purified on silica gel eluted with petroleum ether/ethyl acetate (V:V = 3:1) as eluent to give compound **6n**.

Yields 85.62%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.44 (s, 1H, NH), 7.12–7.09 (m, 2H), 6.86–6.83 (m, 2H), 5.19 (s, 1H, H-12), 4.48–4.44 (m, 1H, H-3), 3.92–3.65 (m, 11H, H in piperazine/Ph-CH_3), 2.02 (s, 3H, −$OCOCH_3$), 1.06 (s, 3H, CH_3), 0.93 (d, *J* = 5.3 Hz, 3H, CH_3), 0.91 (s, 3H, CH_3), 0.86–0.83 (m, 9H, $3CH_3$), 0.69 (s, 3H, CH_3); ^{13}C NMR (500 MHz, $CDCl_3$): δ 183.3 (−C═S), 175.9 (−CON−), 171.1 (−COO−), 157.7, 132.6, 126.7, 114.1, 80.9, 55.4, 55.3, 48.6, 47.9, 47.4, 44.3, 42.1, 39.4, 38.1, 37.6, 36.8, 34.3, 31.5, 30.3, 30.1, 29.7, 28.0, 23.5, 23.2, 22.7, 21.3, 21.2, 18.1, 17.4, 16.7, 15.5; HR-MS (*m*/*z*) (ESI): calcd for $C_{44}H_{65}N_3O_3S$ [M− H^+]: 714.47475; found: 714.46614.

4.2.15. N-[3β-acetoxy-urs-12-en-28-oyl]-amino-N-(3,4-dimethylphenyl)piperazine-1-carbothioamide (**6o**)

According to the general procedure, 3β-acetoxy-urs-12-en-28-acyl piperazine was treated with isothiocyanate, and then purified on silica gel eluted with petroleum ether/ethylacetate (V:V = 3:1) as eluent to give compound **6o**.

Yields 89.20%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.44 (s, 1H, NH), 7.07 (d, J = 7.9 Hz, 1H), 6.92–6.86 (m, 2H), 5.19 (s, 1H, H-12), 4.50–4.43 (m, 1H, H-3), 3.79 (d, J = 107.9 Hz, 8H, H in piperazine), 2.21 (s, 6H, 2Ph-CH_3), 2.03 (s, 3H, −$OCOCH_3$), 1.06 (s, 3H, CH_3), 0.93 (s, 3H, CH_3), 0.91 (s, 3H, CH_3), 0.86–0.83 (m, 9H, $3CH_3$), 0.69 (s, 3H, CH_3); ^{13}C NMR (500 MHz, $CDCl_3$): δ 183.3 (−C═S), 175.8 (−CON−), 171.1 (−COO−), 137.4, 134.3, 130.1, 125.5, 121.8, 80.9, 55.3, 48.3, 47.4, 44.3, 42.1, 39.4, 38.6, 38.1, 37.6, 36.8, 34.3, 33.0, 30.3, 29.7, 28.0, 23.5, 23.2, 21.3, 21.2, 19.9, 19.3, 18.1, 17.3, 16.7, 15.5; HR-MS (*m*/*z*) (ESI): calcd for $C_{45}H_{67}N_3O_3S$ [M− H^+]: 728.49032; found: 728.48088.

4.2.16. N-[3β-acetoxy-urs-12-en-28-oyl]-amino-N-(3-methoxyphenyl)piperazine-1-carbothioamide (**6p**)

According to the general procedure, 3β-acetoxy-urs-12-en-28-acyl piperazine was treated with isothiocyanate, and then purified on silica gel eluted with petroleum ether/ethyl acetate (V:V = 3:1) as eluent to give compound **6p**.

Yields 82.94%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.57 (s, 1H, NH), 7.20 (t, *J* = 8.0 Hz, 1H), 6.73–6.67 (m, 3H), 5.18 (s, 1H, H-12), 4.46 (t, *J* = 7.9 Hz, 1H, H-3), 3.88–3.65 (m, 11H, H in piperazine/Ph-OCH_3), 2.02 (s, 3H, −$OCOCH_3$), 1.05 (s, 3H, CH_3), 0.93 (s, 3H, CH_3), 0.90 (s, 3H, CH_3), 0.84 (t, *J* = 5.2 Hz, 9H, $3CH_3$), 0.68 (s, 3H, CH_3); ^{13}C NMR (500 MHz, $CDCl_3$): δ 183.2 (−C═S), 175.9 (−CON−), 171.1 (−COO−), 160.1, 140.9, 129.8, 125.2, 115.6, 110.7, 109.4, 80.9, 55.3, 55.3, 48.7, 47.4, 42.1, 39.4, 38.7, 38.1, 37.6, 36.8, 34.3, 32.9, 31.4, 30.3, 29.7, 28.0, 23.5, 23.2, 21.3, 21.2, 19.1, 18.1, 17.4, 16.7, 15.5; HR-MS (*m*/*z*) (ESI): calcd for $C_{44}H_{65}N_3O_4S$ [M−H^+]: 730.46964; found: 730.46067.

4.2.17. N-[3β-acetoxy-urs-12-en-28-oyl]-amino-N-(4-methoxyphenyl)piperazine-1-carbothioamide (**6q**)

According to the general procedure, 3β-acetoxy-urs-12-en-28-acyl piperazine was treated with isothiocyanate, and then purified on silica gel eluted with petroleum ether/ethyl acetate (V:V = 3:1) as eluent to give compound **6q**.

Yields 83.53%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.47 (s, 1H, NH), 7.10 (d, *J* = 8.8 Hz, 2H), 6.85 (d, *J* = 8.9 Hz, 2H), 5.18 (s, 1H, H-12), 4.50–4.43 (m, 1H, H-3), 3.97–3.66 (m, 11H, H in piperazine/Ph-OCH_3), 2.02 (s, 3H, −$OCOCH_3$), 1.06 (s, 3H, CH_3), 0.93 (d, *J* = 5.2 Hz, 3H, CH_3), 0.91 (s, 3H, CH_3), 0.84 (t, *J* = 6.1 Hz, 9H, CH_3), 0.69 (s, 3H, CH_3); ^{13}C NMR (500 MHz, $CDCl_3$): δ 183.3 (−C═S), 175.9 (−CON−), 171.1 (−COO−), 157.7, 132.6, 126.6, 114.2, 80.9, 55.4, 55.3, 48.6, 48.0, 47.4, 42.1, 39.4, 38.7, 38.1, 37.6, 36.8, 34.3, 31.4, 30.38, 30.1, 29.7, 28.0, 23.5, 23.2, 21.3, 21.2, 18.1, 17.4, 16.7, 15.5; HR-MS (*m*/*z*) (ESI): calcd for $C_{44}H_{65}N_3O_4S$ [M− H^+]: 730.46964; found: 730.45965.

4.2.18. N-[3β-acetoxy-urs-12-en-28-oyl]-amino-N-(3,4,5-trimethoxyphenyl)piperazine-1-carbothioamide (**6r**)

According to the general procedure, 3β-acetoxy-urs-12-en-28-acyl piperazine was treated with isothiocyanate, and then purified on silica gel eluted with petroleum ether/ethyl acetate (V:V = 3:1) as eluent to give compound **6r**.

Yields 88.52%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.47 (s, 1H, NH), 6.44 (s, 2H), 5.18 (s, 1H, H-12), 4.49–4.43 (m, 1H, H-3), 3.94–3.67 (m, 17H, H in piperazine/3Ph-OCH_3), 2.02 (s, 3H, −$OCOCH_3$), 1.05 (s, 3H, CH_3), 0.92 (d, *J* = 5.8 Hz, 3H, CH_3), 0.90 (s, 3H, CH_3), 0.84 (t, *J* = 6.7 Hz, 9H, $3CH_3$), 0.69 (s, 3H, CH_3); ^{13}C NMR (500 MHz, $CDCl_3$): δ 182.9 (−C═S), 175.9 (−CON−), 171.1 (−COO−), 138.5, 134.3, 133.4, 130.9, 124.9, 123.1, 80.9, 55.2, 48.7, 48.2, 47.4, 42.1, 39.4, 38.7, 38.1, 37.6, 36.8, 34.3, 33.0, 31.5, 30.3, 29.7, 28.0, 23.5, 23.2, 21.3, 21.2, 19.6, 18.1, 17.3, 16.7, 15.5; HR-MS (*m*/*z*) (ESI): calcd for $C_{46}H_{69}N_3O_6S$ [M-H^+]: 790.50603; found: 790.48297.

4.2.19. N-[3β-acetoxy-urs-12-en-28-oyl]-amino-N-(3-Chloro-4-fluorophenyl)piperazine-1-carbothioamide (**6s**)

According to the general procedure, 3β-acetoxy-urs-12-en-28-acyl piperazine was treated with isothiocyanate, and then purified on silica gel eluted with petroleum ether/ethyl acetate (V:V = 3:1) as eluent to give compound **6s**.

Yields 84.72%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.74 (s, 1H, NH), 7.27 (d, *J* = 2.1 Hz, 1H), 7.26 (s, 1H), 7.13 (ddd, *J* = 8.7, 4.1, 2.6 Hz, 1H), 7.07 (td, *J* = 8.6, 1.9 Hz, 1H), 5.16 (s, 1H, H-12), 4.44 (t, *J* = 7.5 Hz, 1H, H-3), 4.08–3.66 (m, 8H, H in piperazine), 2.02 (s, 3H, −$OCOCH_3$), 1.06 (s, 3H, CH_3), 0.93 (s, 3H, CH_3), 0.90 (s, 3H, CH_3), 0.83 (s, 9H, $3CH_3$), 0.68 (s, 3H, CH_3); ^{13}C NMR (500 MHz, $CDCl_3$): δ 182.74 (−C═S), 176.0 (−CON−), 171.2 (−COO−), 156.9, 154.9, 136.3, 127.4, 125.4, 120.9, 120.8, 116.5, 116.4, 80.9, 55.2, 48.7, 47.8, 47.4, 42.2, 39.4, 38.7, 38.1, 37.6, 36.8, 34.3, 31.5, 31.4, 30.3, 30.1, 29.7, 28.0, 23.5, 23.2, 21.3, 21.2, 18.1, 17.4, 16.7, 15.5; HR-MS (*m*/*z*) (ESI): calcd for $C_{43}H_{61}ClFN_3O_3S$ [M− H^+]: 752.41062; found: 752.40183.

4.2.20. N-[3β-acetoxy-urs-12-en-28-oyl]-amino-N-(3-Chloro-4-methylphenyl)piperazine-1-carbothioamide (**6t**)

According to the general procedure, 3β-acetoxy-urs-12-en-28-acyl piperazine was treated with isothiocyanate, and then purified on silica gel eluted with petroleum ether/ethyl acetate (V:V = 3:1) as eluent to give compound **6t**.

Yields 85.41%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.69 (s, 1H, NH), 7.16–7.13 (m, 2H), 7.02 (dd, J = 8.1, 2.1 Hz, 1H), 5.16 (s, 1H, H-12), 4.45 (t, J = 7.9 Hz, 1H, H-3), 3.98–3.66 (m, 8H, H in piperazine), 2.31 (s, 3H, Ph-CH_3), 2.02 (s, 3H, −OCOCH_3), 1.05 (s, 3H, CH_3), 0.93 (s, 3H, CH_3), 0.90 (s, 3H, CH_3), 0.83 (d, J = 4.0 Hz, 9H, 3CH_3), 0.68 (s, 3H, CH_3); ^{13}C NMR (500 MHz, $CDCl_3$): δ 182.8 (−C═S), 175.9 (−CON−), 171.1 (−COO−), 138.5, 134.2, 133.4, 130.9, 124.9, 123.1, 80.9, 55.2, 48.7, 48.1, 47.4, 42.2, 39.4, 38.6, 38.1, 37.6, 36.8, 34.3, 33.0, 30.3, 29.7, 28.0, 23.5, 23.2, 21.3, 21.2, 19.6, 18.1, 17.3, 16.7, 15.5; HR-MS (m/z) (ESI): calcd for $C_{44}H_{64}ClN_3O_3S$ [M− H^+]: 748.43573; found: 748.42779.

4.3. General procedure for compounds **7a**–**r**

A solution of compound **4** in aqueous NaOH (4 N) in CH_3OH: THF (1:1.5, v:v) was stirred for 4 h reflux at 60 °C and concentrated under reduced pressure. The residue was suspended in water, adjusted with 2 N HCl to pH 3–4 and then partitioned with ethyl acetate, The organic layer was washed with saturated sodium chloride, dried over Na_2SO_4, and purified via silica gel column chromatography with petroleum ether/ethyl acetate to offer compound 5 [16]. Compound **5** (0.26 mmol) was dissolved in CH_2Cl_2 (20 mL), isothiocyanate (0.31 mmol) and triethylamine (0.13 mmol) were added to the mixture and stirred at room temperature for 4 h. After the reaction, the solvent was evaporated under reduced pressure, and the crude product was purified by chromatography on silica gel eluted with petroleum ether/ethyl acetate (V:V = 4: 1) to offer compounds **7a**–**r**. The structures were confirmed by ^{1}H NMR, ^{13}C NMR and HR-MS (see supporting information).

4.3.1. N-[3β-hydroxy-urs-12-en-28-oyl]-amino-N-phenylpiperazine-1-carbothioamide (**7a**)

According to the general procedure, 3β-hydroxy-urs-12-en-28-acyl piperazine was treated with isothiocyanate, and then purified on silica gel eluted with petroleum ether/ethyl acetate (V:V = 4:1) as eluent to give compound **7a**.

Yields 89.37%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.75 (d, J = 10.4 Hz, 1H, NH), 7.31–7.26 (m, 2H), 7.14 (d, J = 8.0 Hz, 3H), 5.16 (s, 1H, H-12), 3.88–3.60 (m, 8H, H in piperazine), 3.17 (d, J = 5.6 Hz, 1H, H-3), 1.05 (s, 3H, CH_3), 0.95 (s, 3H, CH_3), 0.92 (s, 3H, CH_3), 0.87 (s, 3H, CH_3), 0.83 (d, J = 5.8 Hz, 3H, CH_3), 0.74 (s, 3H, CH_3), 0.67 (s, 3H, CH_3); ^{13}C NMR (500 MHz, $CDCl_3$): δ 183.1 (−C═S), 175.9 (−CON−), 139.8, 129.5, 129.0, 125.5, 125.3, 124.1, 78.9, 55.2, 48.6, 48.4, 47.5, 44.4, 42.1, 39.4, 38.7, 38.5, 36.9, 34.3, 33.0, 31.9, 31.5, 30.3, 30.1, 29.6, 28.1, 27.1, 23.3, 22.6, 21.2, 18.2, 17.3, 16.8, 15.7, 15.4; HR-MS (m/z) (ESI): calcd for $C_{41}H_{62}N_3O_2S$ [M− H^+]: 658.44843; found: 658.44090.

4.3.2. N-[3β-hydroxy-urs-12-en-28-oyl]-amino-N-(1-naphthalene) piperazine-1-carbothioamide (**7b**)

According to the general procedure, 3β-hydroxy-urs-12-en-28-acyl piperazine was treated with isothiocyanate, and then purified on silica gel eluted with petroleum ether/ethyl acetate (V:V = 4:1) as eluent to give compound **7b**.

Yields 81.63%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.99–7.93 (m, 1H), 7.88 (dd, J = 6.6, 2.7 Hz, 1H), 7.75 (d, J = 8.2 Hz, 1H, NH), 7.57–7.52 (m, 2H), 7.50 (s, 1H), 7.47–7.42 (m, 1H), 7.27 (d, J = 7.5 Hz, 1H), 5.19 (s, 1H, H-12), 3.71 (d, J = 47.0 Hz, 8H, H in piperazine), 3.21 (dd, J = 11.2, 4.6 Hz, 1H, H-3), 1.07 (s, 3H, CH_3), 0.99 (s, 3H, CH_3), 0.93 (d, J = 6.1 Hz, 3H, CH_3), 0.90 (s, 3H, CH_3), 0.85 (d, J = 6.4 Hz, 3H, CH_3), 0.78 (s, 3H, CH_3), 0.68 (s, 3H, CH_3); ^{13}C NMR (500 MHz, $CDCl_3$): δ 184.5 (−C═S), 175.8 (−CON−), 136.1, 134.4, 129.1, 128.5, 126.9, 126.7, 126.5, 125.5, 125.2, 123.4, 123.0, 122.4, 78.9, 55.2, 48.6, 47.5, 44.4, 42.1, 39.4, 38.7, 38.5, 36.9, 34.2, 33.0, 31.9, 31.4, 30.3, 30.2, 29.7, 28.1, 27.2, 23.7, 23.2, 22.7, 21.2, 18.2, 17.3, 16.8, 15.6, 15.4; HR-MS (m/z) (ESI): calcd for $C_{45}H_{63}N_3O_2S$ [M−H^+]: 708.46412; found: 708.45232.

4.3.3. N-[3β-hydroxy-urs-12-en-28-oyl]-amino-N-(3-fluoro phenyl) piperazine-1-carbothioamide (**7c**)

According to the general procedure, 3β-hydroxy-urs-12-en-28-acyl piperazine was treated with isothiocyanate, and then purified on silica gel eluted with petroleum ether/ethyl acetate (V:V = 4:1) as eluent to give compound **7c**.

Yields 80.79%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.70 (s, 1H, NH), 7.32–7.25 (m, 1H), 6.98 (d, J = 6.9 Hz, 2H), 6.87 (t, J = 8.2 Hz, 1H), 5.21 (s, 1H, H-12), 3.98–3.66 (m, 8H, H in piperazine), 3.22 (d, J = 10.5 Hz, 1H, H-3), 1.09 (s, 3H, CH_3), 0.99 (s, 3H, CH_3), 0.96 (s, 3H, CH_3), 0.91 (s, 3H, CH_3), 0.87 (d, J = 5.7 Hz, 3H, CH_3), 0.78 (s, 3H, CH_3), 0.72 (3H, CH_3); ^{13}C NMR (500 MHz, $CDCl_3$): δ 182.9 (−C═S), 176.0 (−CON−), 163.8, 161.8, 141.4, 130.1, 125.3, 119.1, 112.2, 111.0, 79.0, 55.2, 48.7, 48.5, 47.5, 44.3, 42.1, 39.4, 38.7, 38.5, 36.9, 34.3, 33.0, 31.4, 30.3, 30.1, 29.7, 28.1, 28.0, 27.2, 23.8, 23.2, 21.2, 18.2, 17.3, 16.8, 15.6, 15.4; HR-MS (m/z) (ESI): calcd for $C_{41}H_{60}FN_3O_2S$ [M− H^+]: 676.43903; found: 676.42848.

4.3.4. N-[3β-hydroxy-urs-12-en-28-oyl]-amino-N-(4-fluorophenyl) piperazine-1-carbothioamide (**7d**)

According to the general procedure, 3β-hydroxy-urs-12-en-28-acyl piperazine was treated with isothiocyanate, and then purified on silica gel eluted with petroleum ether/ethyl acetate (V:V = 4:1) as eluent to give compound **7d**.

Yields 88.23%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.47 (s, 1H, NH), 7.17 (dd, J = 8.8, 4.7 Hz, 2H), 7.02 (t, J = 8.2 Hz, 2H), 5.19 (s, 1H, H-12), 4.02–3.67 (m, 8H, H in piperazine), 3.19 (dd, J = 10.9, 4.7 Hz, 1H, H-3), 1.07 (s, 3H, CH_3), 0.97 (s, 3H, CH_3), 0.94 (s, 3H, CH_3), 0.89 (s, 3H, CH_3), 0.85 (d, J = 6.3 Hz, 3H, CH_3), 0.76 (s, 3H, CH_3), 0.70 (d, J = 8.5 Hz, 3H, CH_3); ^{13}C NMR (500 MHz, $CDCl_3$): δ 183.2 (−C═S), 176.0 (−CON−), 161.5, 159.5, 135.6, 126.8, 126.7, 125.3, 115.9, 115.7, 79.0, 55.2, 48.7, 48.0, 47.5, 44.3, 42.1, 39.4, 38.7, 38.5, 37.0, 34.3, 33.0, 31.4, 30.3, 30.1, 29.7, 28.1, 27.2, 23.3, 22.7, 21.2, 18.2, 17.3, 16.8, 15.6, 15.4; HR-MS (m/z) (ESI): calcd for $C_{41}H_{60}FN_3O_2S$ [M− H^+]: 676.43903; found: 676.42988.

4.3.5. N-[3β-hydroxy-urs-12-en-28-oyl]-amino-N-(3-bromophenyl) piperazine-1-carbothioamide (**7e**)

According to the general procedure, 3β-hydroxy-urs-12-en-28-acyl piperazine was treated with isothiocyanate, and then purified on silica gel eluted with petroleum ether/ethyl acetate (V:V = 4:1) as eluent to give compound **7e**.

Yields 85.01%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.63 (s, 1H, NH), 7.34 (s, 1H), 7.28 (d, J = 7.6 Hz, 1H), 7.21–7.13 (m, 2H), 5.19 (s, 1H, H-12), 3.95–3.65 (m, 8H, H in piperazine), 3.20 (dd, J = 10.9, 4.6 Hz, 1H, H-3), 1.07 (s, 3H, CH_3), 0.97 (s, 3H, CH_3), 0.94 (d, J = 4.6 Hz, 3H, CH_3), 0.89 (s, 3H, CH_3), 0.85 (d, J = 6.3 Hz, 3H, CH_3), 0.77 (s, 3H, CH_3), 0.71 (d, J = 6.7 Hz, 3H, CH_3); ^{13}C NMR (500 MHz, $CDCl_3$): δ 182.9 (−C═S), 175.9 (−CON−), 156.8, 141.1, 130.1, 128.3, 126.7, 125.3, 122.6, 122.3, 79.0, 55.2, 49.1, 48.4, 47.5, 42.1, 39.4, 38.7, 38.5, 37.0, 34.3, 33.9, 31.4, 30.4, 29.7, 28.1, 27.2, 25.6, 24.9, 23.3, 22.6, 21.2, 18.2, 17.3, 16.8, 15.6, 15.4; HR-MS (m/z) (ESI): calcd for $C_{41}H_{60}BrN_3O_2S$ [M− H^+]: 736.35904; found: 736.35149.

4.3.6. N-[3β-hydroxy-urs-12-en-28-oyl]-amino-N-(4-bromophenyl) piperazine-1-carbothioamide (**7f**)

According to the general procedure, 3β-hydroxy-urs-12-en-28-acyl piperazine was treated with isothiocyanate, and then purified on silica gel eluted with petroleum ether/ethyl acetate (V:V = 4:1) as eluent to give compound **7f**.

Yields 84.93%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.70 (s, 1H, NH), 7.43–7.39 (m, 2H), 7.10–7.06 (m, 2H), 5.17 (t, J = 3.3 Hz, 1H, H-12), 4.00–3.61 (m, 8H, H in piperazine), 3.18 (dd, J = 10.8, 5.0 Hz, 1H, H-

3), 1.06 (s, 3H, CH_3), 0.96 (s, 3H, CH_3), 0.93 (d, J = 1.8 Hz, 3H, CH_3), 0.88 (s, 3H, CH_3), 0.84 (d, J = 6.3 Hz, 3H, CH_3), 0.75 (s, 3H, CH_3), 0.68 (s, 3H, CH_3); ^{13}C NMR (500 MHz, $CDCl_3$): δ 182.8 (−C═S), 176.0 (−CON−), 138.8, 132.0.125.9, 118.7, 79.0, 55.2, 48.7, 48.4, 48.2, 47.5, 42.1, 39.4, 38.7, 38.5, 37.0, 34.3, 33.8, 31.4, 30.5, 30.3, 30.1, 29.6, 28.1, 27.2, 23.3, 22.6, 21.2, 19.1, 18.2, 17.3, 16.8, 15.6, 15.4; HR-MS (m/z) (ESI): calcd for $C_{41}H_{60}BrN_3O_2S$ [M− H^+]: 736.35904; found: 736.34776.

4.3.7. N-[3β-hydroxy-urs-12-en-28-oyl]-amino-N-(2,4-DiBromophenyl)piperazine-1-carbothioamide (**7g**)

According to the general procedure, 3β-hydroxy-urs-12-en-28-acyl piperazine was treated with isothiocyanate, and then purified on silica gel eluted with petroleum ether/ethyl acetate (V:V = 4:1) as eluent to give compound **7g**.

Yields 89.10%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.72 (d, J = 1.9 Hz, 1H, NH), 7.65 (d, J = 8.7 Hz, 1H), 7.42 (dd, J = 8.7, 1.9 Hz, 1H), 7.30 (s, 1H), 5.21 (s, 1H, H-12), 4.04−3.74 (m, 8H, H in piperazine), 3.19 (dd, J = 10.8, 4.3 Hz, 1H, H-3), 1.08 (s, 3H, CH3), 0.97 (s, 3H, CH3), 0.94 (d, J = 5.1 Hz, 3H, CH3), 0.89 (s, 3H, CH3), 0.86 (d, J = 6.2 Hz, 3H, CH3), 0.76 (s, 3H, CH3), 0.71 (s, 3H, CH3); ^{13}C NMR (500 MHz, $CDCl_3$): δ 181.8 (−C═S), 176.0 (−CON−), 136.9, 134.9, 130.9, 127.7, 125.4, 118.8, 118.7, 79.0, 55.2, 48.7, 47.9, 47.5, 44.2, 42.1, 39.4, 38.7, 38.5, 37.0, 34.3, 33.0, 31.4, 30.5, 30.3, 30.2, 29.7, 28.1, 27.2, 23.8, 23.3, 21.2, 19.1, 18.2, 17.3, 16.8, 15.6, 15.4: HR-MS (m/z) (ESI): calcd for $C_{41}H_{59}Br_2N_3O_2S$ [M + H^+]: 816.26902; found: 816.27361.

4.3.8. N-[3β-hydroxy-urs-12-en-28-oyl]-amino-N-(3-chlorophenyl) piperazine-1-carbothioamide (**7h**)

According to the general procedure, 3β-hydroxy-urs-12-en-28-acyl piperazine was treated with isothiocyanate, and then purified on silica gel eluted with petroleum ether/ethyl acetate (V:V = 4:1) as eluent to give compound **7h**.

Yields 86.34%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.51 (s, 1H, NH), 7.24 (d, J = 8.0 Hz, 1H), 7.19 (d, J = 1.8 Hz, 1H), 7.13 (d, J = 7.9 Hz, 1H), 7.09 (d, J = 8.0 Hz, 1H), 5.19 (s, 1H, H-12), 3.97−3.66 (m, 8H, H in piperazine), 3.20 (dd, J = 10.9, 4.5 Hz, 1H, H-3), 1.07 (s, 3H, CH_3), 0.98 (s, 3H, CH_3), 0.94 (d, J = 5.4 Hz, 3H, CH_3), 0.89 (s, 3H, CH_3), 0.85 (d, J = 6.3 Hz, 3H, CH_3), 0.77 (s, 3H, CH_3), 0.70 (s, 3H, CH_3); ^{13}C NMR (500 MHz, $CDCl_3$): δ 182.9 (−C═S), 175.9 (−CON−), 140.9, 134.5, 129.9, 125.4, 123.7, 121.9, 79.0, 55.2, 48.7, 48.5, 47.5, 44.4, 42.1, 39.4, 38.7, 38.5, 37.0, 34.3, 33.0, 31.4, 30.3, 30.2, 29.7, 28.1, 27.2, 23.3, 22.7, 21.2, 18.2, 17.3, 16.8, 15.6, 15.4; HR-MS (m/z) (ESI): calcd for $C_{41}H_{60}ClN_3O_2S$ [M−H^+]: 692.40953; found: 692.39804.

4.3.9. N-[3β-hydroxy-urs-12-en-28-oyl]-amino-N-(4-chlorophenyl) piperazine-1-carbothioamide (**7i**)

According to the general procedure, 3β-hydroxy-urs-12-en-28-acyl piperazine was treated with isothiocyanate, and then purified on silica gel eluted with petroleum ether/ethyl acetate (V:V = 4:1) as eluent to give compound **7i**.

Yields 82.47%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.74 (s, 1H, NH), 7.25 (d, J = 7.6 Hz, 2H), 7.13 (d, J = 8.7 Hz, 2H), 5.16 (s, 1H, H-12), 3.96−3.60 (m, 8H, H in piperazine), 3.17 (dd, J = 10.6, 5.0 Hz, 1H, H-3), 1.06 (s, 3H, CH_3), 0.95 (s, 3H, CH_3), 0.93 (s, 3H, CH_3), 0.87 (s, 3H, CH_3), 0.83 (d, J = 6.3 Hz, 3H, CH_3), 0.75 (s, 3H, CH_3), 0.68 (s, 3H, CH_3); ^{13}C NMR (500 MHz, $CDCl_3$): δ 182.7 (−C═S), 176.0 (−CON−), 138.4, 130.9, 128.9, 125.8, 78.9, 55.2, 48.7, 48.1, 47.5, 42.1, 39.4, 38.7, 38.5, 36.9, 34.3, 31.9, 31.4, 30.5, 30.3, 29.6, 28.1, 27.1, 23.3, 22.6, 21.2, 19.1, 18.2, 17.3, 16.8, 15.7, 15.4; HR-MS (m/z) (ESI): calcd for $C_{41}H_{60}ClN_3O_2S$ [M− H^+]: 692.40953; found: 692.39862.

4.3.10. N-[3β-hydroxy-urs-12-en-28-oyl]-amino-N-(3-trifluoromethylphenyl) piperazine-1- carbothioamide (**7j**)

According to the general procedure, 3β-hydroxy-urs-12-en-28-acyl piperazine was treated with isothiocyanate, and then purified on silica gel eluted with petroleum ether/ethyl acetate (V:V = 4:1) as eluent to give compound **7j**.

Yields 80.71%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.71 (s, 1H, NH), 7.46−7.39 (m, 4H), 5.18 (s, 1H, H-12), 4.04−3.67 (m, 8H, H in piperazine), 3.19 (dd, J = 11.0, 4.5 Hz, 1H, H-3), 1.07 (s, 3H, CH_3), 0.97 (s, 3H, CH_3), 0.94 (s, 3H, CH_3), 0.88 (s, 3H, CH_3), 0.84 (d, J = 6.3 Hz, 3H, CH_3), 0.76 (s, 3H, CH_3), 0.69 (s, 3H, CH_3); ^{13}C NMR (500 MHz, $CDCl_3$): δ 182.8 (−C═S), 176.0(−CON−), 140.3, 131.4, 131.1, 129.4, 127.5, 125.3, 124.8, 122.6, 122.0, 120.7, 79.0, 55.2, 48.7, 48.2, 47.5, 44.4, 42.1, 39.4, 38.7, 38.5, 36.9, 34.3, 33.0, 31.9, 31.4, 30.3, 30.1, 29.7, 28.1, 28.0, 27.2, 23.2, 21.1, 18.2, 17.3, 16.8, 15.6, 15.4; HR-MS (m/z) (ESI): calcd for $C_{42}H_{60}F_3N_3O_2S$ [M−H^+]: 726.43581; found: 726.42491.

4.3.11. N-[3β-hydroxy-urs-12-en-28-oyl]-amino-N-(4-trifluoromethylphenyl) piperazine-1- carbothioamide (**7k**)

According to the general procedure, 3β-hydroxy-urs-12-en-28-acyl piperazine was treated with isothiocyanate, and then purified on silica gel eluted with petroleum ether/ethyl acetate (V:V = 4:1) as eluent to give compound **7k**.

Yields 85.20%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.70 (s, 1H, NH), 7.56 (d, J = 8.4 Hz, 2H), 7.30 (d, J = 8.1 Hz, 2H), 5.19 (s, 1H, H-12), 3.99−3.69 (m, 8H, H in piperazine), 3.20 (dd, J = 10.8, 4.5 Hz, 1H, H-3), 1.07 (s, 3H, CH_3), 0.97 (s, 4H, CH_3), 0.94 (s, 3H, CH_3), 0.89 (s, 3H, CH_3), 0.85 (d, J = 6.3 Hz, 3H, CH_3), 0.76 (s, 3H, CH_3), 0.70 (s, 3H, CH_3); ^{13}C NMR (500 MHz, $CDCl_3$): δ 182.7 (−C═S), 176.0 (−CON−), 142.8, 127.0, 126.7, 126.2, 126.1, 125.3, 125.0, 123.0, 122.8, 79.0, 55.2, 48.7, 48.5, 47.5, 44.5, 42.1, 39.4, 38.7, 38.5, 36.9, 34.3, 33.0, 31.4, 30.3, 29.7, 29.3, 28.1, 27.2, 23.3, 22.7, 21.1, 21.0, 18.2, 17.3, 16.8, 15.6, 15.4; HR-MS (m/z) (ESI): calcd for $C_{42}H_{60}F_3N_3O_2S$ [M− H^+]: 726.43581; found: 726.42816.

4.3.12. N-[3β-hydroxy-urs-12-en-28-oyl]-amino-N-(3-methylphenyl) piperazine-1-carbothioamide (**7l**)

According to the general procedure, 3β-hydroxy-urs-12-en-28-acyl piperazine was treated with isothiocyanate, and then purified on silica gel eluted with petroleum ether/ethyl acetate (V:V = 4:1) as eluent to give compound **7l**.

Yields 83.09%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.48 (s, 1H, NH), 7.20 (t, J = 8.0 Hz, 1H), 6.95 (dd, J = 16.2, 4.8 Hz, 3H), 5.19 (s, 1H, H-12), 3.91−3.63 (m, 8H, H in piperazine), 3.19 (dd, J = 10.8, 4.5 Hz, 1H, H-3), 2.32 (s, 3H, CH_3), 1.06 (s, 3H, CH_3), 0.97 (s, 3H, CH_3), 0.93 (d, J = 5.4 Hz, 3H, CH_3), 0.89 (s, 3H, CH_3), 0.85 (d, J = 6.2 Hz, 3H, CH_3), 0.76 (s, 3H, CH_3), 0.69 (s, 3H, CH_3); ^{13}C NMR (500 MHz, $CDCl_3$): δ 183.3 (−C═S), 175.9 (−CON−), 139.6, 139.1, 128.9, 126.4, 125.3, 124.2, 120.7, 79.0, 55.2, 48.6, 48.0, 47.5, 44.3, 42.1, 39.4, 38.7, 38.5, 37.0, 34.3, 32.9, 31.4, 30.4, 30.0, 29.7, 28.1, 27.2, 23.3, 21.4, 21.2, 18.2, 17.3, 16.8, 15.6, 15.4; HR-MS (m/z) (ESI): calcd for $C_{42}H_{63}N_3O_2S$ [M− H^+]: 672.46412; found: 672.45280.

4.3.13. N-[3β-hydroxy-urs-12-en-28-oyl]-amino-N-(3,4-dimethylphenyl) piperazine-1- carbothioamide (**7m**)

According to the general procedure, 3β-hydroxy-urs-12-en-28-acyl piperazine was treated with isothiocyanate, and then purified on silica gel eluted with petroleum ether/ethyl acetate (V:V = 4:1) as eluent to give compound **7m**.

Yields 88.39%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.55 (s, 1H, NH), 7.06 (d, J = 8.0 Hz, 1H), 6.92−6.85 (m, 2H), 5.18 (s, 1H, H-12), 3.93−3.63 (m, 8H, H in piperazine), 3.18 (dd, J = 10.8, 4.8 Hz, 1H, H-3), 2.20 (s, 6H, $2CH_3$), 1.06 (s, 3H, CH_3), 0.96 (s, 3H, CH_3), 0.92 (s, 3H, CH_3), 0.88 (s, 3H, CH_3), 0.83 (d, J = 6.3 Hz, 3H, CH_3), 0.75 (s, 3H, CH_3), 0.68 (s, 3H, CH_3); ^{13}C NMR (500 MHz, $CDCl_3$): δ 182.9 (−C═S), 176.0 (−CON−), 163.8, 161.8, 141.4, 141.3, 130.1, 130.0, 125.3, 119.1, 112.2, 112.1, 111.0, 110.8, 79.0, 55.2, 48.7, 48.5, 47.5, 44.3, 42.1, 39.4, 38.7,

38.5, 36.9, 34.3, 33.0, 31.4, 30.3, 30.1, 29.7, 28.1, 28.0, 27.2, 23.8, 23.2, 21.2, 18.2, 17.3, 16.8, 15.6, 15.4; HR-MS (*m*/*z*) (ESI): calcd for $C_{43}H_{65}N_3O_2S$ [M−H^+]: 686.47968; found:686.46913.

4.3.14. *N-[3β-hydroxy-urs-12-en-28-oyl]-amino-N-(3-methoxyphenyl) piperazine-1- carbothioamide (**7n**)*

According to the general procedure, 3β-hydroxy-urs-12-en-28-acyl piperazine was treated with isothiocyanate, and then purified on silica gel eluted with petroleum ether/ethyl acetate (V:V = 4:1) as eluent to give compound **7n**.

Yields 85.21%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.49 (s, 1H, NH), 7.21 (td, *J* = 8.1, 2.0 Hz, 1H), 6.73–6.68 (m, 3H), 5.19 (s, 1H, H-12), 3.91–3.66 (m, 11H, H in piperazine/Ph-OCH3), 3.19 (dd, *J* = 10.7, 4.1 Hz, 1H, H-3), 1.07 (s, 3H, CH_3), 0.97 (s, 3H, CH_3), 0.93 (d, *J* = 5.6 Hz, 3H, CH_3), 0.89 (s, 3H, CH_3), 0.85 (d, *J* = 6.0 Hz, 3H, CH_3), 0.76 (s, 3H, CH_3), 0.69 (s, 3H, CH_3); ^{13}C NMR (500 MHz, $CDCl_3$): δ 183.3 (−C═S), 175.9(−CON−), 160.2, 140.9, 129.8, 125.3, 115.4, 110.8, 109.2, 79.0, 55.3, 55.2, 48.8, 47.5, 44.4, 42.1, 39.4, 38.7, 38.5, 37.0, 34.8, 34.3, 32.9, 31.4, 30.5, 30.4, 30.1, 29.7, 28.1, 28.0, 27.2, 23.2, 22.6, 21.2, 19.1, 18.2, 17.3, 16.8, 15.6, 15.4; HR-MS (*m*/*z*) (ESI): calcd for $C_{42}H_{63}N_3O_3S$ [M− H^+]: 688.45903; found: 688.44804.

4.3.15. *N-[3β-hydroxy-urs-12-en-28-oyl]-amino-N-(4-methoxyphenyl) piperazine-1- carbothioamide (**7o**)*

According to the general procedure, 3β-hydroxy-urs-12-en-28-acyl piperazine was treated with isothiocyanate, and then purified on silica gel eluted with petroleum ether/ethyl acetate (V:V = 4:1) as eluent to give compound **7o**.

Yields 89.05%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.31 (s, 1H, NH), 7.12–7.09 (m, 2H), 6.88–6.85 (m, 2H), 5.20 (s, 1H, H-12), 3.96–3.67 (m, 11H, H in piperazine/Ph-OCH3), 3.20 (dd, *J* = 11.0, 4.6 Hz, 1H, H-3), 1.07 (s, 3H, CH_3), 0.98 (s, 3H, CH_3), 0.94 (d, *J* = 5.9 Hz, 3H, CH_3), 0.89 (s, 3H, CH_3), 0.86 (d, *J* = 6.3 Hz, 3H, CH_3), 0.77 (s, 3H, CH_3), 0.70 (s, 3H, CH_3); ^{13}C NMR (500 MHz, $CDCl_3$): δ 183.5 (−C═S), 175.9(−CON−), 157.7, 132.5, 126.4, 114.3, 79.0, 55.4, 55.3, 55.2, 48.7, 48.0, 47.5, 44.4, 42.1, 39.4, 38.7, 38.5, 37.0, 34.3, 33.0, 31.4, 30.4, 30.1, 29.7, 28.1, 27.2, 23.3, 22.7, 21.2, 19.1, 18.2, 17.3, 16.8, 15.6, 15.5, 15.4; HR-MS (*m*/*z*) (ESI): calcd for $C_{42}H_{63}N_3O_3S$ [M− H^+]: 688.45903; found: 688.44970.

4.3.16. *N-[3β-hydroxy-urs-12-en-28-oyl]-amino-N-(3,4,5-trimethoxyphenyl) piperazine-1-carbothioamide(**7P**)*

According to the general procedure, 3β-hydroxy-urs-12-en-28-acyl piperazine was treated with isothiocyanate, and then purified on silica gel eluted with petroleum ether/ethyl acetate (V:V = 4:1) as eluent to give compound **7P**.

Yields 87.29%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.44 (s, 1H, NH), 6.45 (s, 2H), 5.19 (s, 1H, H-12), 3.96–3.68 (m, 17H, H in piperazine/2Ph-CH_3), 3.18 (dd, *J* = 10.9, 4.6 Hz, 1H, H-3), 1.06 (s, 3H, CH_3), 0.96 (s, 3H, CH_3), 0.93 (d, *J* = 5.8 Hz, 3H, CH_3), 0.88 (s, 3H, CH_3), 0.84 (d, *J* = 6.3 Hz, 3H, CH_3), 0.75 (s, 3H, CH_3), 0.70 (s, 3H, CH_3); ^{13}C NMR (500 MHz, $CDCl_3$): δ 182.9 (−C═S), 175.9(−CON−), 153.3, 135.8, 135.5, 102.0, 78.9, 60.9, 60.9, 56.2, 56.1, 55.2, 48.7, 48.2, 47.5, 44.4, 42.1, 39.4, 38.7, 38.7, 38.5, 36.9, 34.3, 31.4, 30.3, 30.1, 29.6, 28.1, 27.1, 23.2, 22.7, 21.2, 18.2, 17.3, 16.8, 15.6, 15.4; HR-MS (*m*/*z*) (ESI): calcd for $C_{44}H_{67}N_3O_5S$ [M−H^+]: 748.49543; found: 748.47159.

4.3.17. *N-[3β-hydroxy-urs-12-en-28-oyl]-amino-N-(3-Chloro-4-fluorophenyl) piperazine-1- carbothioamide (**7q**)*

According to the general procedure, 3β-hydroxy-urs-12-en-28-acyl piperazine was treated with isothiocyanate, and then purified on silica gel eluted with petroleum ether/ethyl acetate (V:V = 4:1) as eluent to give compound **7q**.

Yields 85.73%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.44 (s, 1H, NH), 7.29 (dd, *J* = 6.4, 2.4 Hz, 1H), 7.14–7.08 (m, 2H), 5.19 (t, *J* = 3.5 Hz, 1H, H-12), 4.07–3.68 (m, 8H, H in piperazine), 3.20 (dd, *J* = 11.1, 4.4 Hz, 1H, H-3), 1.07 (s, 3H, CH_3), 0.97 (s, 3H, CH_3), 0.94 (d, *J* = 5.4 Hz, 3H, CH_3), 0.89 (s, 3H, CH_3), 0.85 (d, *J* = 6.4 Hz, 3H, CH_3), 0.77 (s, 3H, CH_3), 0.70 (s, 3H, CH_3); ^{13}C NMR (500 MHz, $CDCl_3$): δ 182.8 (−C═S), 176.1 (−CON−), 156.9, 154.9, 136.2, 136.2, 127.2, 125.1, 121.1, 120.9, 116.7, 116.5, 79.0, 55.2, 48.7, 47.9, 47.5, 44.3, 42.2, 39.4, 38.7, 38.5, 37.0, 34.3, 33.0, 31.4, 30.3, 30.1, 29.7, 28.1, 27.2, 23.8, 23.30 21.2, 18.2, 17.3, 16.8, 15.67, 15.4; HR-MS (*m*/*z*) (ESI): calcd for $C_{41}H_{59}ClFN_3O_2S$ [M−H^+]: 710.40014; found: 710.38810.

4.3.18. *N-[3β-hydroxy-urs-12-en-28-oyl]-amino-N-(3-chloro-4-methylphenyl) piperazine-1-carbothioamide (**7r**)*

According to the general procedure, 3β-hydroxy-urs-12-en-28-acyl piperazine was treated with isothiocyanate, and then purified on silica gel eluted with petroleum ether/ethyl acetate (V:V = 4:1) as eluent to give compound **7r**.

Yields 88.43%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.90 (s, 1H, NH), 7.14 (dd, *J* = 9.8, 4.9 Hz, 2H), 7.02 (d, *J* = 8.0 Hz, 1H), 5.14 (s, 1H, H-12), 3.98–3.60 (m, 8H, H in piperazine), 3.16 (dd, *J* = 10.5, 4.8 Hz, 1H, H-3), 2.29 (s, 3H, CH_3), 1.04 (s, 3H, CH_3), 0.94 (s, 3H, CH_3), 0.91 (s, 3H, CH_3), 0.86 (s, 3H, CH_3), 0.81 (d, *J* = 6.1 Hz, 3H, CH_3), 0.73 (s, 3H, CH_3), 0.66 (s, 3H, CH_3); ^{13}C NMR (500 MHz, $CDCl_3$): δ 182.7 (−C═S), 176.0(−CON−), 138.7, 134.1, 133.3, 130.8, 125.3, 125.1, 123.4, 78.9, 55.2, 48.7, 48.1, 47.5, 44.4, 42.1, 39.4, 38.7, 38.5, 36.9, 34.2, 33.0, 31.9, 31.4, 30.3, 29.6, 28.1, 27.1, 23.2, 22.6, 21.2, 19.5, 18.2, 17.3, 16.8, 15.7, 15.4; HR-MS (*m*/*z*) (ESI): calcd for $C_{42}H_{62}ClN_3O_2S$ [M− H^+]: 706.42513; found: 706.41463.

4.4. *In vitro cytotoxicity*

4.4.1. *Cell lines*

The procedure was performed as described previously [23,24]. The following *in vitro* human cancer cell lines were used: T24 (human bladder carcinoma cells), MGC-803 (human gastric cancer cells), HepG2 (human epidermoid larynx carcinoma cells), Hct-116 (human colorectal cells), A549 (human lung adenocarcinoma cells), HUVEC (human umbilical vein endothelial cells). The cell lines (T24, MGC-803, HepG2, Hct-116, A549, HUVEC) were purchased from the Cell Resource Center of Shanghai Institutes for Biological Sciences, the Academy of Sciences of China.

4.4.2. *Cell culture*

The procedure was performed as described previously [23,24]. T24, MGC-803, HepG-2, Hct-116, A549, HUVEC cells were cultured in Dulbecco Modified Eagle Medium (DMEM) (HyClone, USA), containing 4.0 mM L-Glutamine and 4500 mg/L Glucose, supplemented with 10% (*v*/*v*) foetalbovine serum (FBS; HyClone, USA). T24 cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) (HyClone, USA), medium, containing 2.05 mM L-Glutamine without Calcium nitrate, supplemented with 10% (*v*/*v*) foetalbovine serum. The cell culture media was supplemented with penicillin/streptomycin at 100 Units/mL as adherent monolayers. Cell cultures were kept in a humidified incubator with 5% CO_2 at 37 °C. Stock solutions were prepared in dimethyl sulfoxide (DMSO) and further dilutions were made with fresh culture medium. The concentration of DMSO in the final culture medium was 1%, which had no effect on the cell viability.

4.4.3. *MTT assay*

The procedure was performed as described previously [23,24]. Chemosensitivity was assessed using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay. Briefly, exponentially growing HepG-2 (2000–3000 cells/well), MGC-803 (2000–3000 cells/well), T24 (2000–3000 cells/well), Hct-116 (3000–4000 cells/well), HUVEC(2000–3000 cells/well) and A549

(2000–3000 cells/well), were seeded into 96-well plates and treated with indicated concentrations of samples for 48 h, and then 10 μL of MTT (10 mg/mL) (Sigma, USA) was added. After incubation for 4 h at 37 °C, the purple formazan crystals (i.e. a reduced form of MTT) generated from viable cells were dissolved by adding 100 μL DMSO in each well. The plates were swirled gently for 10 min to dissolve the precipitate, and quantified by measuring the optical density (OD) of the plates at a wavelength of 490 nm on plate reader (TECAN, infinite, M1000). Each concentration was repeated in three wells and the same experimental conditions were provided for all compounds and MTT analysis was repeated three times for each cell line.

4.5. AO/EB staining

The test was performed as described previously [23,24]. Cells were seeded at a concentration of 5 × 104 cell/mL in a volume of 2 mL on a sterile cover slip in six-well tissue culture plates. Following incubation, the medium was removed and replaced with fresh medium plus 10% fetal bovine serum and supplemented with compound **6r**. After the treatment period, the cover slip with monolayer cells was inverted on a glass slide with 20 μL of AO/EB stain (100 mg/mL). Fluorescence was read on a Nikon ECLIPSETE2000-S fluorescence microscope (OLYMPUS Co, Japan).

4.6. Hoechst 333258 staining

The procedure was performed as described previously [23,24]. Cells grown on a sterile cover slip in six-well plates were treated with compounds for a certain range of time. The culture medium containing compounds was removed, and the cells were fixed in 4% paraformaldehyde for 10 min. After being washed twice with PBS, the cells were stained with 0.5 mL of Hoechst 33258 (Beyotime, Haimen, China) for 5 min and then again washed twice with PBS. The stained nuclei were observed under a Nikon ECLIPSETE2000-S fluorescence microscope using 350 nm excitation and 460 nm emission.

4.7. Mitochondrial membrane potential staining

The procedure was performed as described previously [23,24]. JC-1 (Beyotime, Haimen, China) probe was employed to measure mitochondrial depolarization in HepG2 cells. Briefly, cells cultured in six-well plates after indicated treatments were incubated with an equal volume of JC-1 staining solution (5 μg/ml) at 37 °C for 20 min and rinsed twice with PBS. Mitochondrial membrane potentials were monitored by determining the relative amounts of dual emissions from mitochondrial JC-1 monomers or aggregates using a Nikon ECLIPSETE2000-S fluorescent microscope. Mitochondrial depolarization is indicated by an increase in the green/red fluorescence intensity ratio.

4.8. Flow cytometry

4.8.1. Apoptosis analysis

The approach was performed as described previously [23,24]. Apoptosis was discriminated with the annexin V-FITC/propidium iodide test. Cells were seeded at 2 × 10^6/well in 10% FBS-DMEM into 6-well plates, and treated with compounds various time intervals. The cells were washed twice with cold Phosphate Buffered Saline (PBS) and then resuspend cells in 1 × Binding Buffer (0.1 M Hepes/NaOH (pH 7.4), 1.4 M NaCl, 25 mM $CaCl_2$) at a concentration of 1 × 10^6 cells/mL. Transfer 100 μL of the solution (1 × 10^5 cells) to a 5 mL culture tube, and add 5 μL of FITC Annexin V (BD, Pharmingen) and 5 μL propidium iodide (PI) to each tube. Gently vortex the cells and incubate for 30 min at RT (25 °C) in the dark. Add 200 μl PBS to each tube. Analysis was performed with the system software (Cell Quest; BD Biosciences). Lower left quadrant, viable cells (annexin V-/PI-); lower right quadrant, early apoptotic cells (annexin V+/PI-); upper right quadrant, late apoptotic cells (annexin V+/PI+); upper left quadrant, necrotic cells (annexin V-/PI+). The percentage of cells positive for PI and/or Annexin V-FITC was reported inside the quadrants.

4.8.2. Cell cycle analysis

The step was performed as described previously [23,24]. The cells lines were treated with indicated concentrations of compounds. After incubated for 48 h, cells were washed twice with ice-cold PBS, fixed and permeabilized with ice-cold 70% ethanol at −20 °C overnight. The cells were treated with 100 μg/ml RNase A at 37 °C for 30 min after washed with ice-cold PBS, and finally stained with 1 mg/ml propidium iodide (PI) in the dark at 4 °C for 30 min. Analysis was performed with the system software (Cell Quest; BD Biosciences).

4.9. Immunoblotting and real-time quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted from the HepG2 cells after treatment with 10 μM, 20 μM, 30 μM ursolic acid derivatives for 24 h using the RNApure Kit (Aidlab, RN0302, China) as described previously [42]. RNA samples were reverse-transcribed for 30 min at 42 °C with the High Capacity cDNA Reverse Transcription Kit (TaKaRa, Biotechnology, Dalian). The SYBR® Green PCR Master Mix (Fermentas, K0251, Lithuania) and specific primer pairs were used for selected genes, and the primer pair for actin was used as the reference gene. RT-PCR was performed according to the following conditions: 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 15 s at 95 °C and 1 min at 60 °C using 0.5 μl of complementary (c) DNA, 2 × SYBR Green PCR Master Mix, and 500 nM of the forward and reverse primers on a 7500 real-time PCR System (Applied Biosystems). The threshold cycle number (Ct) was calculated with the 7500 ABI software. Relative transcript quantities were calculated using the △Ct method with actin as the reference gene amplified from the same samples. Ct is the difference in the threshold cycles of messenger (m)RNA for selected genes relative to those of actin mRNA. The real-time RT-PCR was performed in triplicate for each experimental group. The PCR primers used here are given in Table 2S, ESI.

4.10. ROS assay

The procedure was performed as described previously [23,24]. HepG2 cells were seeded into six-well plates and subjected to various treatments. Following treatment, cells were incubated with 10 mM DCFH-DA (Beyotime, Haimen, China) dissolved in cell-free medium at 37 °C for 30 min in dark, and then washed three times with PBS. Cellular fluorescence was quantified using Nikon ECLIPSETE2000-S fluorescence microscope at an excitation of 485 nm and an emission of 538 nm.

4.11. Western blotting analysis

The western blot procedure was performed as described previously [43]. HepG2 cells were collected after treatment with compound **6r** (20 μM) for 0, 6, 12, 24 h and then lysed in ice-cold lysis buffer (1% SDS in 25 mM TriseHCl, pH 7.5, 4 mM EDTA, 100 mM NaCl, 1 mM PMSF, 10 mg/mL^{-1}leupeptin and 10 mg/mL^{-1} soybean trypsin inhibitor). Whole cell lysates were centrifuged at 12,000 × g for 5 min. Thereafter, the protein concentration was

determined with bicinchoninic acid (BCA) protein assay kit (Beyotime Co, China). An aliquot of cell lysate (40–50 μg) was fractionated by SDS-PAGE on a 12% polyacrylamide gel for 2 h and transferred to PVDF membrane. After blocked with 5% nonfat dry milk in PBS-t for 1 h at room temperature, the membranes was incubated with β-actin, cytochrome *c*, Apaf-1, caspase-9, caspase-3, caspase-8, Fas, PARP, Bax, Bcl-2 antibodies (Bioworld Technology Inc, USA) overnight at 4 °C, washing with TBST, and then incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. Proteins were detected by ECL procedures (Thermo Fisher Scientific, USA) and analyzed by Image J software.

4.12. Statistical analysis

Data are expressed as mean ± SD for three different determinations. Statistical significance was analyzed by one-way ANOVA. Mean separations were performed using the least significant difference method. $P < 0.05$ was defined as statistically significant.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2015.03.051.

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