Bioorganic Chemistry 85 (2019) 179–190

Contents lists available at ScienceDirect

Bioorganic Chemistry

journal homepage: www.elsevier.com/locate/bioorg

Mitochondria-targeted triphenylphosphonium conjugated glycyrrhetinic acid derivatives as potent anticancer drugs

Le Jin[a,1], Lumei Dai[b,1], Min Ji[a,*], Hengshan Wang[c,*]

[a] School of Biological Science and Medical Engineering, Southeast University, Nanjing 210096, China
[b] Key Laboratory of Tropical Marine Bio-resources and Ecology, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou 510301, China
[c] State Key Laboratory for the Chemistry and Molecular Engineering of Medicinal Resources, School of Chemistry and Pharmaceutical Sciences of Guangxi Normal University, Guilin 541004, China

ARTICLE INFO

Keywords:
Glycyrrhetinic acid
Anti-tumor
Mitochondria
Apoptosis

ABSTRACT

Glycyrrhetinic acid has been usually studied for their anti-tumor activities. However, the low bioavailability and poor aqueous solubility as well as limited intracellular accumulation have limited their utility. In this present study, a series of new glycyrrhetinic acid conjugates with a triphenylphosphonium cation (TTP^+) moiety, meant to specifically target them to tumor cells mitochondria, have been designed and synthesized. Among them, compound **2f** possessed excellent antitumor activities against the tested human cancer cells, and simultaneously exhibited better cell selectivity between cancer cells and normal cells than glycyrrhetinic acid and HCPT. Moreover, **2f** significantly induced cell cycle arrest at the G2/M phase, and effectively inhibited cancer cells proliferation and migration. Mechanism studies revealed that **2f** triggered apoptosis through the mitochondrial pathway via the collapse of mitochondrial membrane potential, reactive oxygen species production and the activation of caspase-9 and caspase-3.

1. Introduction

One of the most challenging problems in developing cancer chemotherapeutics is to increase selectivity and to reduce side effects toward normal cells and tissues [1]. Since tumor cells easily obtain the ability to escape apoptosis by conferring insensitivity of mitochondria apoptotic signals for cell death, through disrupting the balance of pro-apoptotic and anti-apoptotic proteins [2–4]. An increasing number of studies have found that many human diseases have been linked with functional mitochondria, such as cancer, neurodegenerative, diabetes, etc [5–6]. Recent studies suggested that mitochondria are closely associated with both intrinsic and extrinsic apoptotic pathways, and recognized as one of the most important targets for new drug design in cancer [7–9]. In addition, mitochondria are vital subcellular organelles in eukaryotic cells, and mitochondrial dysfunctions have been linked to multiple aspects of tumorigenesis and tumor progression [10–11]. In recent years, precise delivery of chemotherapy agents at the subcellular level has acquired great attention because it can enhance therapy efficiency, reduce side effects, and overcome multi-drug resistance [12–14]. Moreover, increasing evidence indicated that the mitochondrial membrane potential difference between tumor and normal cells has offered further confidence for the strategy of mitochondria targeting in drug design [15,16]. The most common strategy to develop mitochondria targeting therapy agents is to conjugate a mitochondria targeting moiety such as triphenylphosphonium cation (TPP^+) with parent drugs or to direct drugs into surface-functionalized aggregates [13]. So far, some successful examples have been reported with improved therapy efficiency, reduced severe toxic side effects for normal cells and tissues and overcame multi-drug resistance such as doxorubicin, betulinic acid, ursolic acid, chlorambucil and among others [17–21].

Natural products and their analogues have pharmacological or biological activity that can be of therapeutic benefit in treating diseases, especially in the area of cancer therapy [22,23]. Recently, many studies reported that pentacyclic triterpene compounds including boswellic acid, oleanolic acid, betulinic acid, ursolic acid and glycyrrhetinic acid also exhibited a broad spectrum of biological activities, such as antioxidant, anti-bacterial, anti-inflammatory and anti-tumor activities [19–20,24–26]. Glycyrrhetinic acid (GA), also known as enoxolone, is a pentacyclic triterpenoid obtained from Glycyrrhiza glabra, and is effective in the treatment of peptic ulcer as well as expectorant properties [27,28]. More importantly, GA and their derivatives were also reported

* Corresponding authors.
E-mail addresses: jiminseu@163.com (M. Ji), whengshan@163.com (H. Wang).
[1] Co-first author: These authors contributed equally to this work.

https://doi.org/10.1016/j.bioorg.2018.12.036
Received 16 October 2018; Received in revised form 20 December 2018; Accepted 28 December 2018
Available online 03 January 2019
0045-2068/

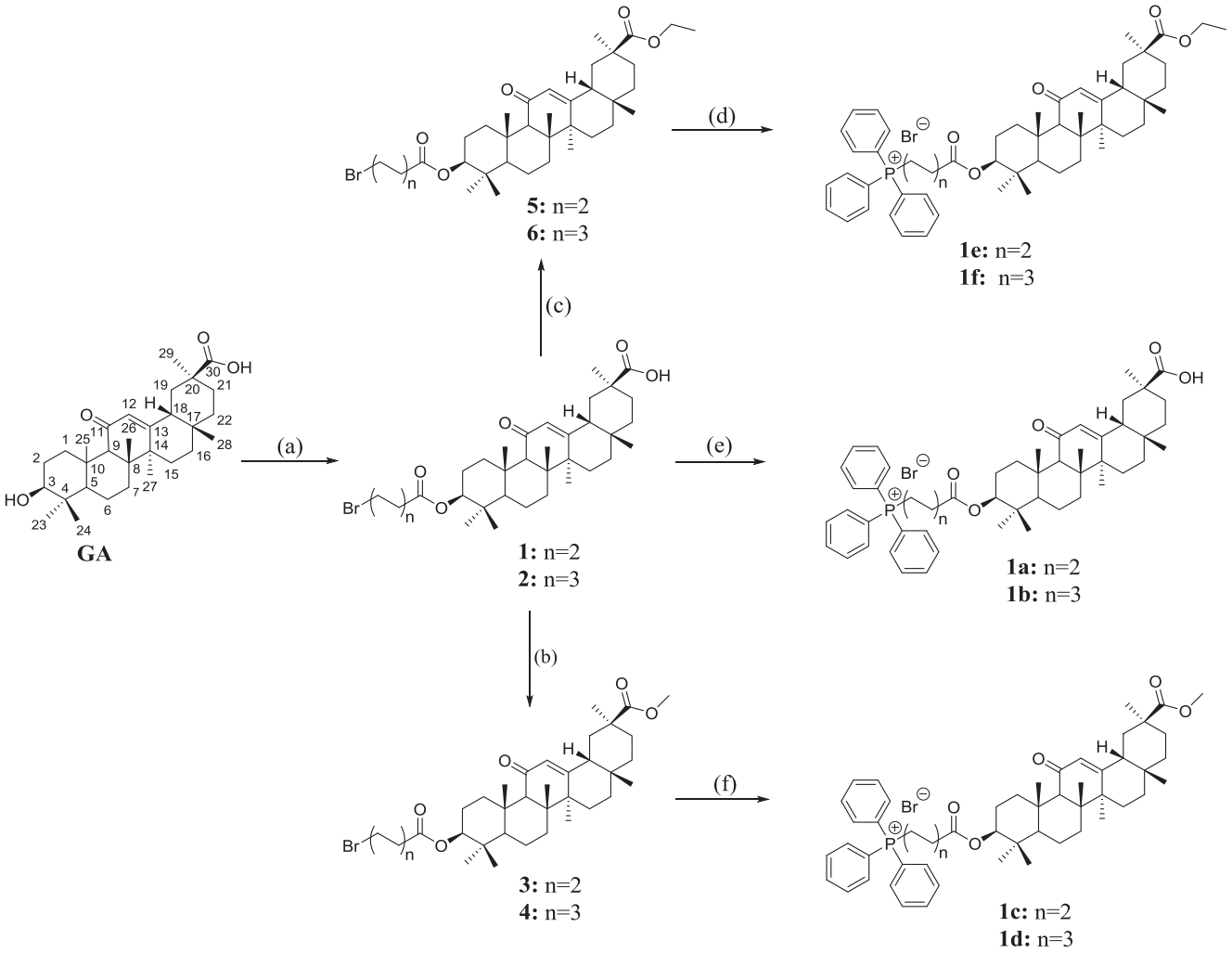
to exhibit moderate anti-proliferative activity toward various human cancer cell lines [28,29]. However, unfortunately, the high hydrophobicity and poor blood serum solubilization of these compounds limits their further development as cytotoxic drugs as well as used for the treatment of cancer. Up still now, a number of studies were attempted by structural modifications to enhance bioavailability, selectively and cytotoxicity [29–33]. Furthermore, mechanistic studies revealed that the antitumor activity of GA and its derivatives are associated with the mitochondrial apoptotic pathway by mitochondrial membrane potential depolarization [9,34–36]. This consequently results in activation of caspase-9, caspase-3 and stimulation of reactive oxygen species (ROS) production as well as affecting the balance between pro-proteins and anti-apoptotic proteins, and ultimately causing to apoptosis. Therefore, we hypothesized that specifically targeting GA to cancer cell mitochondria could significantly improve their anticancer activity and selectivity as well as reduce side effects.

In the light of above considerations, in this paper, we report the rational design and synthesis a series of triphenylphosphonium cation (TPP^+) group containing GA at the OH group in position of C-3 or C-30 These TPP^+-GA conjugates were expected to target mitochondria, and they *in vitro* cytotoxicity against a number of tumor cell lines and a human normal liver cell line were also evaluated. In vitro evaluation revealed that these analogs displayed more cytotoxicity to tumor cells than normal cells compared to parent compound GA, exhibiting selectivity to cancer cells. Moreover, our results clearly demonstrated that compound **2f** could markedly attenuate the migration of A549 cells and effectively induce apoptosis in A549 cells in a dose-dependent manner. Furthermore, the molecules mechanism of apoptotic pathway induced apoptosis in A549 cells by the representative of the target compound **2f** was also investigated.

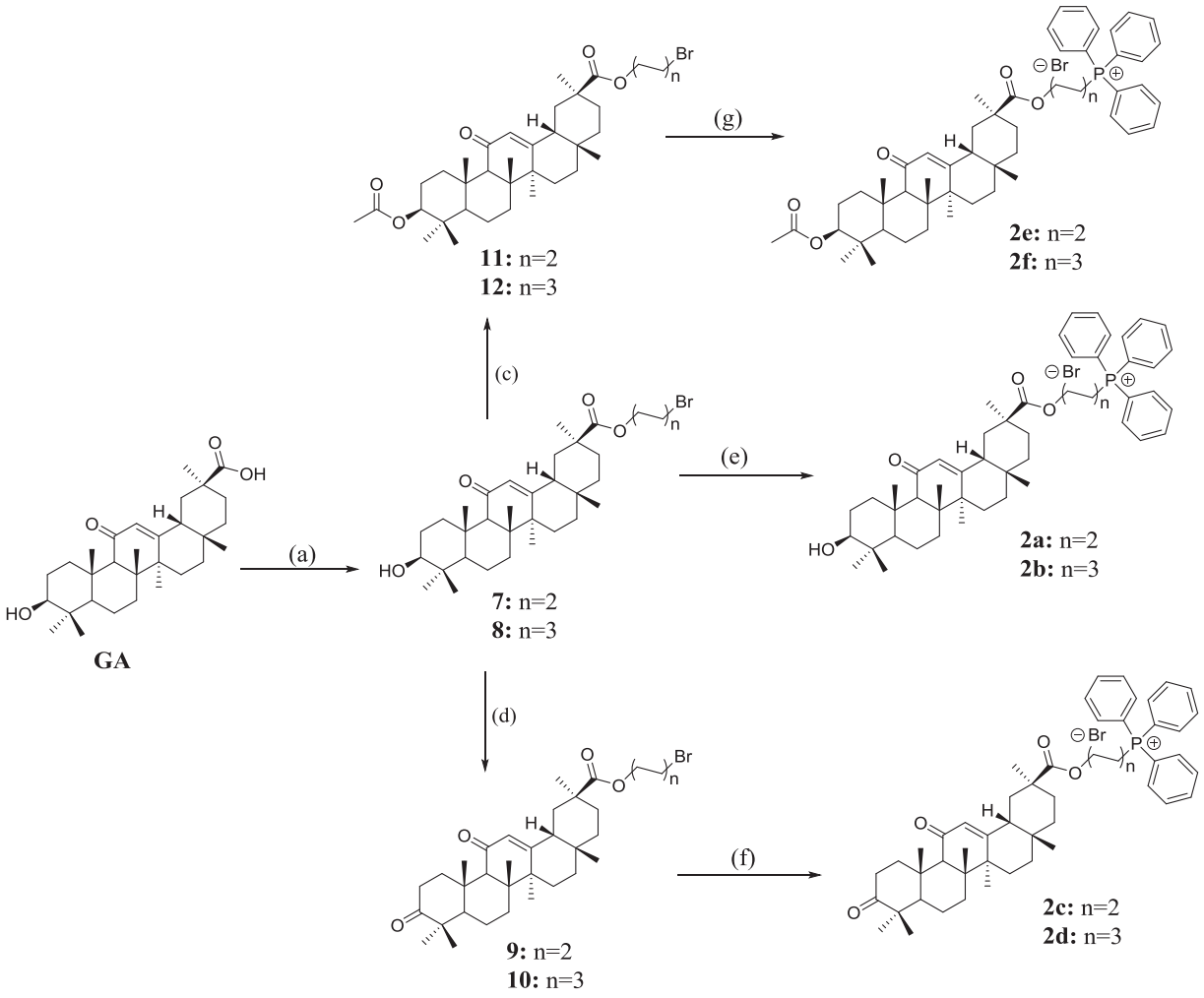
2. Results and discussion

2.1. Chemistry

The general procedures for the synthesis of glycyrrhetinic acid contained triphenylphosphine derivatives are shown in Scheme 1 and 2. The triphenylphosphonium cation (TPP^+) group was linked to a molecule of glycyrrhetinic acid at the C-3 or C-30 position of the triterpenoid skeleton through the hydrophobic alkyl spacer. The intermediates synthesis of these analogs is fairly straightforward by esterification of glycyrrhetinic acid with dibromo alkane or ω-bromoalkanoic acid following standard conditions. The final compounds were achieved by substitution of intermediates with triphenylphosphine. As these agents' anti-cancer activities can be influenced by the introduction of substituents, we further designed and synthesized analogs by modification of the remaining hydroxyl or carboxyl. The structures of target compounds were then confirmed by IR, ^{1}H NMR, ^{13}C NMR and high resolution mass spectrometry (HR-MS).



Scheme 1. Reagents and conditions: Synthetic route for the final compounds from 18β-glycyrrhetinic acid. (a) DMAP, EDCI, 5-Bromovaleric acid or 7-Bromoheptanoic acid, DCM, room temperature; (b) CH_3I, K_2CO_3, DMF, room temperature; (c) CH_3CH_2Br, K_2CO_3, acetone, 35 °C; (d) TPP (Triphenylphosphine), MeCN, reflux; (e) TPP, MeCN, reflux; (f) TPP, MeCN, reflux.



Scheme 2. Reagents and conditions: Synthetic route for the final compounds from 18β-glycyrrhetinic acid. (a) 1,4-dibromobutane or 1,6-dibromhexan, K_2CO_3, DMF, room temperature; (b) Jones reagent, acetone, 0 °C; (c) pyridine, 0 °C; (d) TPP, MeCN, reflux; (e) TPP, MeCN, reflux; (f) TPP, MeCN, reflux; (g) TPP, MeCN, reflux.

2.2. In vitro cytotoxicity assay

The *in vitro* cytotoxicity of compounds **1a–2f** were evaluated by MTT assays on five human cancer cell lines, HepG-2 (hepatocellular), A549 (lung), MCF-7 (breast), HT-29 (colorectal), A2780 (ovarian) and human normal liver HL-7702 cells, using 10-Hydroxycamptothecin (HCPT) and GA as the positive controls, respectively. The IC_{50} values (concentration required to reduce viability to 50%) were summarized in Table 1. As shown in Table 1, these GA-derivatives **1a–2f** showed a significantly higher cytotoxic potency against all five human cancer cells compared to the parent compound GA, respectively. It was noted that compounds **2e** and **2f**, the introduction of triphenylphosphonium cation (TPP^+) moiety at the C-30 position and acetylation of the 3-hydroxy group (C-3) in GA, possessed higher cytotoxicity against all test cancer cell lines compared to GA, with IC_{50} values in the range of 8.13 ~ 11.24 μM (**2e**) and 5.25 ~ 9.22 μM (**2f**), which showed 6.2 ~ 7.1 (**2e**) and 7.6 ~ 11.0 (**2f**) fold increase in activity than those of GA. Especially, **2e** and **2f** displayed better anti-tumor activities against all tested human cancer cell lines and exhibited lower cytotoxicity toward human normal liver HL-7702 cells compared to HCPT. Interestingly, **2f** showed significantly more effective anti-cancer activity than **2b** against the tested five human cancer cells, indicating that the acetylation at the C-3 position in GA could enhance the anti-tumor activity. However, after the oxidation of the hydroxyl group, the oxidized compound **2d** showed lower anti-proliferative activities against all human cancer cells compared to **2b**, suggesting the oxidation of the hydroxyl group at C-3 position resulted in a decreased anti-proliferative activity. Moreover, **1f** and **1d** were slightly more potent against all tested human cancer cell lines than **1b**, suggesting that the esterification at C-30 could improve the antitumor efficacy. More importantly, it was also noted that six sets of compounds (e.g., **1a** and **1b**; **1c** and **1d**; **1e** and **1f**; **2a** and **2b**; **2c** and **2d**; **2e** and **2f**) with different alkyl chains exhibited antitumor activities increased with the increasing carbon chain length.

2.3. Cellular uptake of *2f* in the mitochondria of A549 cells

In order to demonstrate that the increased cytotoxicity of the GA derivative **2f** was a result of mitochondria targeting, the uptake of **2f** to the mitochondria was investigated in human lung cancer A549 cells. As shown in Fig. 1, the mitochondrial uptake of **2f** was increased 2.5-fold after treatment of A549 cells for 4 h compared to parent compound GA. In shorts, these results indicated that the TPP^+ group significantly delivered GA to the mitochondria.

2.4. Compound *2f* induced apoptotic cell death

In order to investigate if the observed cytotoxicity of **2f** is due to induction of apoptosis, A549 cells were treated with various indicated concentrations of **2f** for 24 h, and then, the cells were harvested, stained with Annexin V-FITC and PI, and detected by flow cytometry. Q1, Q2, Q3, and Q4 represent four different cell states: necrotic cells, late apoptotic or necrotic cells, apoptotic cells and living cells, respectively.

Table 1
Cytotoxicity (IC_{50} values in μM) of **1a–2f** in a panel of various cancer cell lines including human normal liver HL-7702 cells.

Compd.	IC_{50} (μM)[a]					
	HepG-2	A549	MCF-7	HT-29	A2780	HL-7702
1a	21.03 ± 1.19[b]	18.51 ± 2.01	23.27 ± 2.08	19.05 ± 1.89	23.06 ± 2.23	71.37 ± 3.41
1b	18.54 ± 1.87	15.33 ± 2.04	16.35 ± 1.76	21.33 ± 1.63	20.25 ± 2.19	65.53 ± 3.01
1c	19.55 ± 2.21	16.79 ± 1.88	21.03 ± 2.28	17.08 ± 1.75	21.03 ± 1.51	63.87 ± 4.39
1d	15.24 ± 1.41	13.07 ± 1.36	18.21 ± 1.52	13.06 ± 1.17	18.01 ± 1.46	70.87 ± 4.21
1e	14.11 ± 1.32	15.08 ± 1.33	18.03 ± 1.68	12.05 ± 1.25	17.54 ± 1.63	67.36 ± 3.63
1f	9.36 ± 1.02	10.71 ± 1.53	12.03 ± 1.31	10.01 ± 1.41	10.27 ± 1.45	64.39 ± 4.15
2a	16.23 ± 1.33	15.21 ± 1.22	17.03 ± 2.13	15.25 ± 1.55	18.16 ± 2.28	70.21 ± 4.09
2b	13.61 ± 1.29	13.18 ± 1.44	15.83 ± 1.71	11.24 ± 1.50	16.04 ± 1.91	65.33 ± 3.91
2c	26.38 ± 2.34	20.13 ± 1.96	25.04 ± 2.70	24.18 ± 2.29	27.36 ± 2.61	67.51 ± 4.24
2d	22.36 ± 2.15	17.67 ± 2.01	22.80 ± 2.61	21.05 ± 2.11	23.65 ± 2.48	63.91 ± 4.18
2e	9.11 ± 0.96	8.13 ± 0.53	9.81 ± 1.02	10.31 ± 1.08	11.24 ± 0.93	66.14 ± 4.13
2f	7.58 ± 0.89	5.25 ± 0.84	7.23 ± 1.01	8.71 ± 1.06	9.22 ± 1.16	68.22 ± 3.87
GA	64.51 ± 3.75	57.85 ± 3.67	60.51 ± 3.49	66.35 ± 3.58	70.15 ± 4.35	64.31 ± 4.14
HCPT[c]	12.01 ± 1.14	11.27 ± 1.54	10.71 ± 1.26	13.13 ± 1.66	12.09 ± 1.28	15.03 ± 2.01

[a] IC_{50} values are presented as the mean ± SD (standard error of the mean) from five independent experiments.
[b] Significant difference from the viability of control ($p \leq 0.05$).
[c] 10-Hydroxy camptothecin.

As illustrated in Fig. 2, compound **2f** induced cell apoptosis in a dose-dependent manner. When the A549 cells were incubated with **2f** at 5, 10 and 20 μM for 24 h, the percentages of early and late apoptosis cells were 18.49%, 30.82%, and 45.0%, respectively. These results indicated that the observed cytotoxicity of **2f** in A549 cells is mainly through the apoptotic pathway.

2.5. Hoechst 33258 staining assay

In order to further validate the morphological changes of cell death, compound **2f** treated with A549 cells were stained with Hoechst 33258. As shown in Fig. 3, A549 cells with smaller nuclei and condensed chromatin were rarely observed in the control groups. When the A549

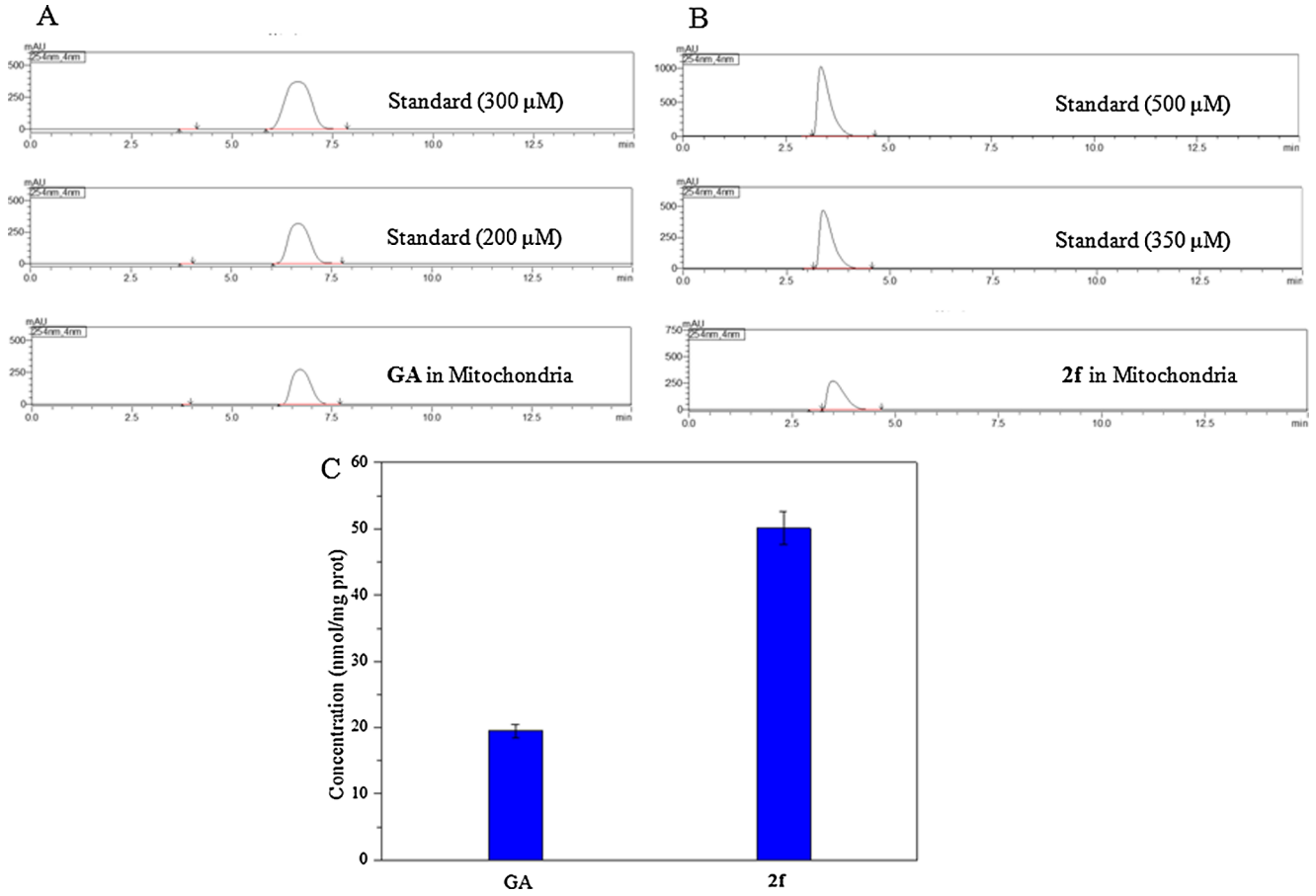


Fig. 1. Cellular uptake of **2f** to the mitochondria in human lung cancer A549 cells. A549 cells were treated with 20 μM **2f** or GA for 4 h, then mitochondria were isolated and **2f** and GA were extracted with organic solvent for analysis. (A) RP-HPLC chromatograms of GA standards and of extracts from the mitochondria of A549 cells. (B) RP-HPLC chromatograms of **2f** and of extracts from the mitochondria of A549 cells. (C) Quantitative data of **2f** and GA in mitochondria after normalization to the protein content. The experiments were performed three times, $p < 0.05$.

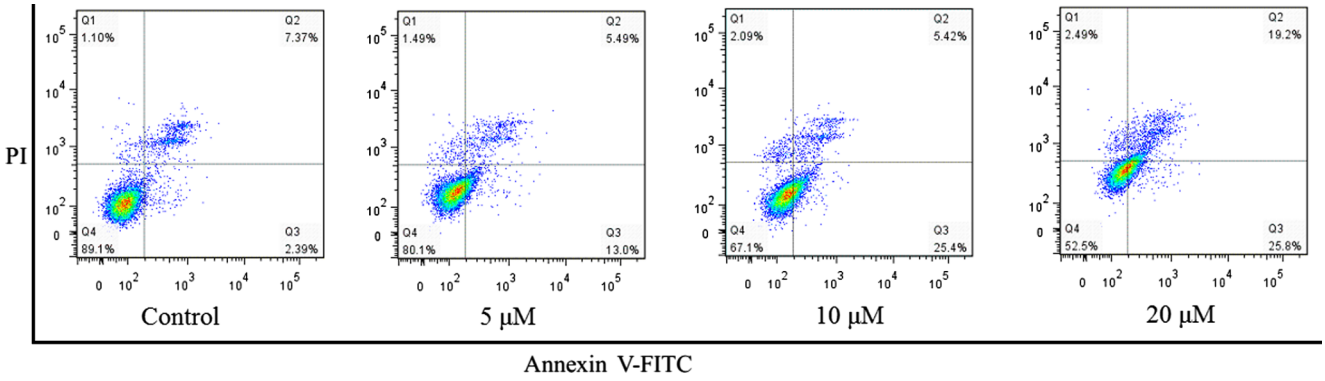


Fig. 2. Compound **2f** induced the apoptosis of A549 cells. Cells were treated with **2f** at the tested concentrations (5, 10 and 20 μM) for 24. Then, the cells were harvested, stained with Annexin V-FITC and PI, and analyzed by flow cytometry, respectively. The experiments were performed three times, and the results of the representative experiments are shown.

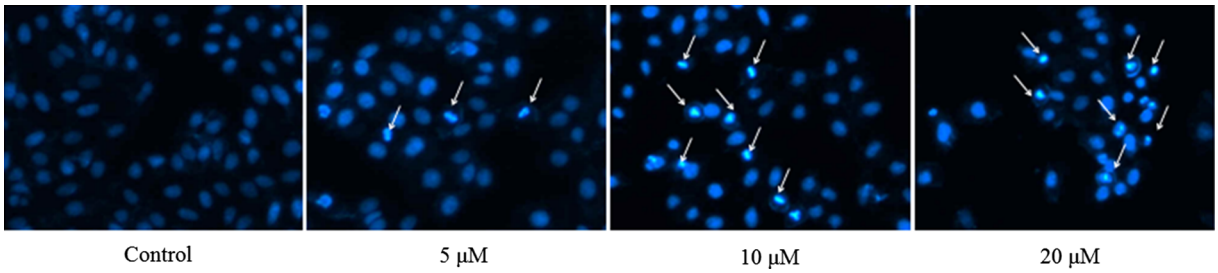


Fig. 3. Morphological changes in the nuclei (typical of apoptosis) of A549 cells treated with **2f** at the tested concentrations (5, 10 and 20 μM) for 24 h, respectively. Then, the cells were harvested, and stained by Hoechst 33258. The experiments were performed three times, and the results of the representative experiments are shown.

cells were incubated with **2f** at 5, 10 and 20 μM for 24 h, nuclei morphology of the cells slightly changed; most of A549 cells emitted brilliant blue fluorescence and revealed typical apoptotic morphology (in the web version), respectively. These results revealed that **2f** could induce apoptosis toward A549 cells.

2.6. Cell cycle effects.

In order to explore if the cytotoxicity of **2f** was due to the cell cycle arrest, we further examined the effect on cell cycle progression using propidium iodide (PI) staining by flow cytometry analysis in A549 cells. As illustrated in Fig. 4, compound **2f** induced cell cycle arrest at the G2 phase in a dose-dependent manner. When A549 cells were treated with **2f** from 5 to 20 μM for 24 h, the percentage of cells at the G2 phase increased sharply. The percentage of G2 peak increased from 10.66% (control group) to 13.21% at 5 μM of compound **2f**, while at higher concentrations, more than 53.33% of the A549 cells were arrested in G2 stage after 24 h treatment. In short, these results revealed that **2f** arrested the cell cycle of A549 cells at the G2 stage.

2.7. Migration assay.

Recently, many studies demonstrated that the metastatic cancer cells exhibited great capability of migration and invasion [37,38]. Moreover, migration is a pivotal step during tumor metastasis. Thus, a well-established wound-healing assay was conducted to examine if **2f** could prevent the migration of human lung cancer A549 cells. As illustrated in Fig. 5, 24 h after wounding, untreated A549 cells could be seen to have migrated into the denuded area. As expected, compound **2f** was able to inhibit the basal migration of A549 cells at the indicated concentrations (5, 10 and 20 μM) after 24 h of treatment.

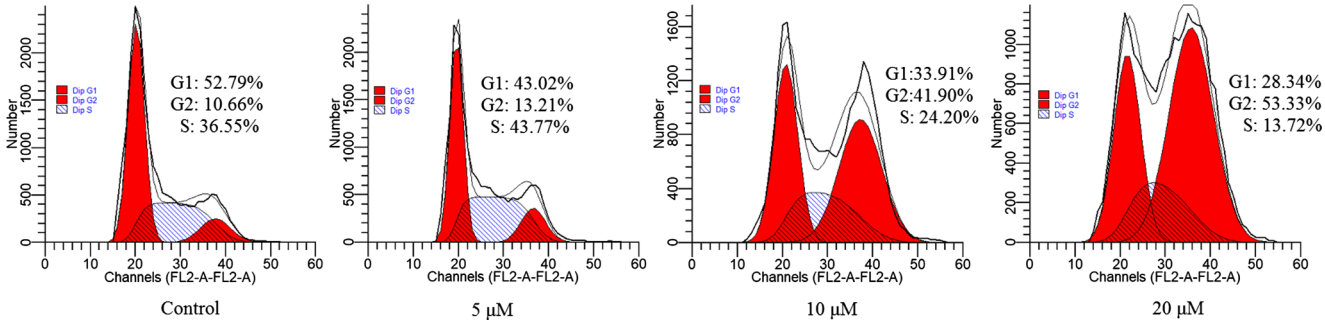


Fig. 4. Cell cycle arrest effect of compound **2f**. A549 cells treated with **2f** at the tested concentrations (5, 10 and 20 μM) for 24 h. The cells were trypsinized, harvested and washed three times with ice-PBS for PI-stained DNA content detected by flow cytometry. The experiments were performed three times, and the results of the representative experiments are shown.

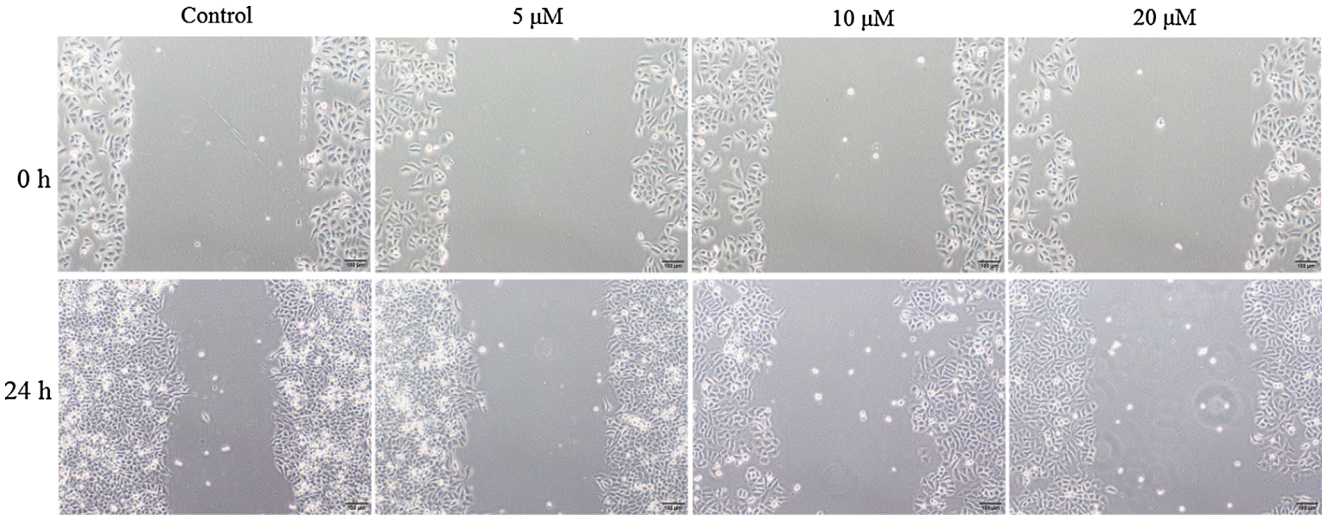


Fig. 5. Compound **2f** inhibited the migration of A549 cells *in vitro*. At the different concentrations of **2f** suppressed A549 cells migration. The experiments were performed three times, and the results of the representative experiments were shown.

*2.8. Effects on the mitochondrial membrane potential by **2f***

Increasing evidence has demonstrated that mitochondria play an important role in both extrinsic and intrinsic apoptosis [39,40]. Decreased mitochondrial membrane potential (MMP) has been implicated as an early event in apoptotic cells [40]. Since the new analogs were designed to target the mitochondria, we therefore decided to investigate the influence of **2f** on the MMP of A549 cells using JC-1 staining assay. As illustrated in Fig. 6, compared to control cells, treatment of A549 cells with **2f** at the indicated concentrations (5, 10 and 20 μM) induced significant loss of MMP as reflected by the increase of J-monomers (exhibiting green fluorescence and suggesting depolarized mitochondria) and concurrent decrease of Jaggregates (exhibiting red fluorescence and suggesting hyperpolarized mitochondria). In shorts, these results further suggested that **2f** significantly induced MMP collapse and mitochondrial dysfunction, and eventually triggered apoptotic cell death.

2.9. Role of reactive oxygen species (ROS) in apoptosis

Recent studies indicated that mitochondria are the main source of ROS in mammalian cells, and ROS has been indicated to have a double sword role in the cytotoxicity in cancer cells [40,41]. Hence, intracellular ROS generation was thus investigated via 2′, 7′-dichlorodihydrofluorescein diacetate (DCFH-DA) staining. As illustrated in Fig. 7, no obvious green fluorescence was observed in the control cells, while **2f** effectively caused ROS production at the indicated concentrations (5, 10 and 20 μM) as by the presented increase green fluorescence in cytoplasm.

*2.10. Mechanistic studies of the apoptotic effects triggered by **2f***

In order to further confirm that the observed apoptotic effects in A549 cells by the **2f** was exerted through the mitochondrial pathway, we therefore examined the expression levels of Bcl-2, Bax, Cleaved-caspase-3, and Cleaved-caspase-9 using western blotting method. As illustrated in Fig. 8, compared to control cells, **2f** dramatically up-regulated the expression of pro-apoptotic protein Bax and correspondingly down-regulated the expression of anti-apoptotic protein Bcl-2 in a dose-dependent manner. Moreover, treatment with **2f** at 5, 10 and 20 μM for 24 h significantly increased in the expression of Cleaved-caspase-3 and Cleaved-caspase-9, respectively, compared to control cells (Fig. 8). Also, we detected the effect of **2f** on dihydrofolate reductase (DHFR) by Western blotting. Western blot analyses revealed that **2f** had litter effect on the protein levels of DHFR (Fig. S1). These results further demonstrated that **2f** caused MMP collapse and mitochondrial dysfunction, altered the expression of apoptosis related proteins, and ultimately triggered apoptotic cell death.

3. Conclusion

Many studies indicated that the trans-membrane potential of mitochondria of cancer cells is higher than the trans-membrane potential of normal cells, which contributed to the selective cytotoxicity of anticancer substances [42,43]. Thus, conjugation with a lipophilic mitochondriotropic triphenylphosphonium cation (TPP^+) has been widely used to improve selectivity and reduce side effects of mitochondria targeted anti-tumor agents. In this paper, based on the hypothesis that the cytotoxicity of a given compound to cancer cells is closely associated with its accumulation in the mitochondria, we hence designed

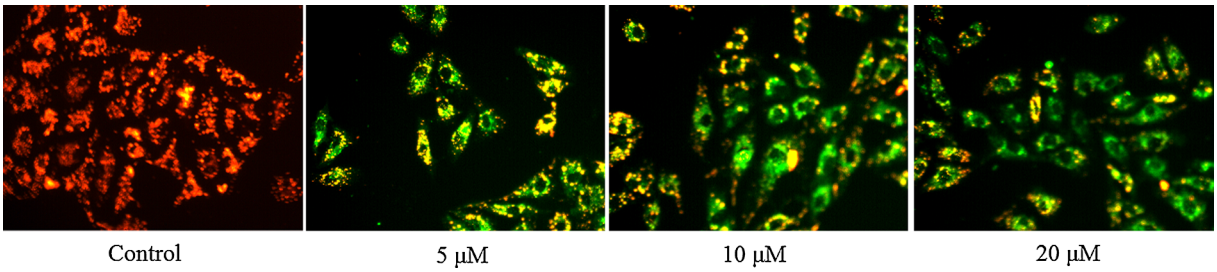


Fig. 6. Compound **2f** decreased the MMP of A549 cells. The A549 cells were treated with **2f** at the tested concentrations (5, 10 and 20 μM) for 24 h followed by incubation with the fluorescence probe JC-1 for 30 min. Then, the cells were visualized by fluorescence microscope. The experiments were performed three times, and the results of the representative experiments are shown.

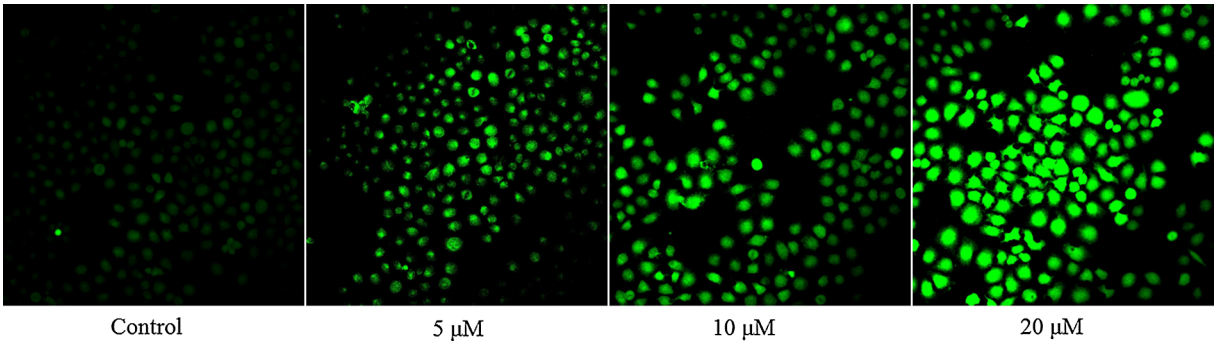


Fig. 7. Intracellular production of ROS by **2f** following a 24 h incubation visualized by fluorescence microscope. The experiments were performed three times, and the results of the representative experiments are shown.

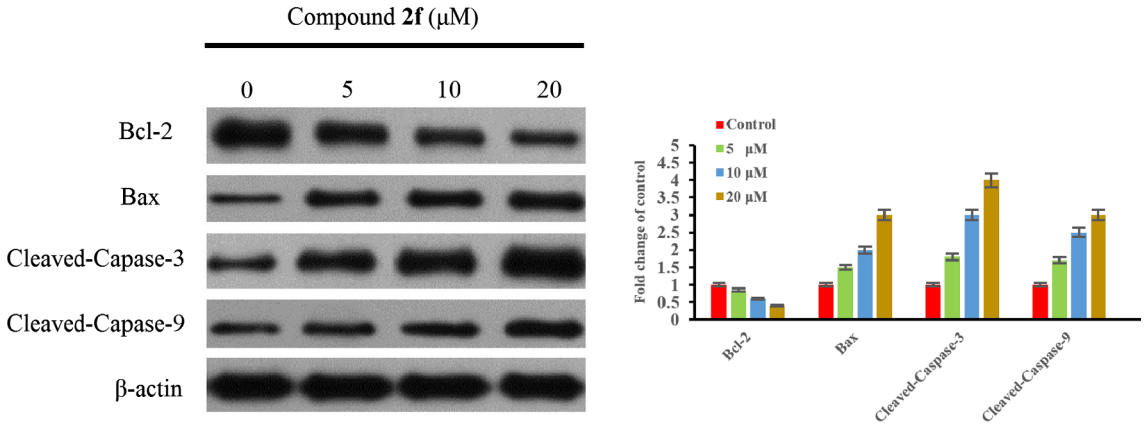


Fig. 8. A549 cells were incubated with **2f** at the tested concentrations (5, 10 and 20 μM) for 24 h. The expressions of Bax, Bcl-2, Cleaved-Caspase-3 and Cleaved-Caspase-9 were determined by western blotting assay. β-Actin antibody was used as reference control. Data are expressed as the mean ± S.D. of three independent experiments, $P < 0.05$.

and synthesized twelve TPP-linked derivatives of GA and compared their biological activity with GA. Our results suggested that the anti-proliferative effects of these analogues are evidently compared with the parent compound GA against the tested human cancer cells. Interestingly, the lower toxicities of these analogues to the human normal liver HL-7702 cells compared to human hepatoma carcinoma HepG-2 cells were also observed. Especially, **2f** displayed excellent anti-tumor activities against the tested human cancer cells and exhibited lower cytotoxicity toward human normal liver HL-7702 cells compared with HCPT. Preliminary mode of action studies indicated that **2f** caused accumulation of cells in the G2/M phase of the cell cycle and induced apoptosis in A549 cells. More importantly, **2f** obviously caused loss of MMP and effectively induced ROS production, altered the expression of mitochondrial-apoptosis related proteins, and eventually triggered apoptotic cell death. In shorts, these results of our studies provide further evidence that natural pentacyclic triterpene compounds can be optimized by the mitochondria-targeting strategy to increase potency and selectivity.

4. Experimental section

4.1. General information.

All chemicals (reagent grade) used were purchased from Aldrich (U.S.A) and Sinopharm Chemical Reagent Co., Ltd (China). Separation of the compounds by column chromatography was carried out with silica gel 60 (200–300 mesh ASTM, E. Merck). The quantity of silica gel used was 30–70 times the weight charged on the column. Then, the eluates were monitored using TLC (thin-layer chromatography). Melting points were uncorrected and were taken in an open capillary tube on a Stuart melting point apparatus (Stuart Scientific, Redhill, UK). The IR spectra of the compounds were recorded on BB Bomem FT-IR spectrometer MB 104 with KBr pellets. ESI mass spectra were obtained on a Mariner System 5304 mass spectrometer, and ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker AV-300 or 500 spectrometer with chemical shifts reported as ppm (in $CDCl_3$, TMS as the internal standard).

4.2. Experimental section.

4.2.1. General procedure for preparation of compound *1* and *2*

A solution of glycyrrhetinic acid (470 mg, 1 mmol) in DCM (20 mL) was stirred. EDCI (482 mg, 2.5 mmol), DMAP (49 mg, 0.4 mmol) and 5-bromovaleric acid (750 mg, 4 mmol) or 7-bromoheptanoic acid (836 mg, 4 mmol) were added successively, and the mixture was stirred at 25 °C until the reaction was complete based on TLC detection. The solvent was removed under diminished pressure, and the residue was purified by column chromatography using 10:1:0.1 petroleum ether/ethyl acetate/formic acid to obtain the target product of compound **1** (518.2 mg, 82%) and **2** (514.8 mg, 78%) as a white powder.

18β-3-O-(5′-Bromovaleryl)-11-oxo-olean-12-en-30-oic acid (1). ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.71 (s, 1H), 4.53 (dd, J = 11.3, 4.8 Hz, 1H), 3.42 (t, J = 6.5 Hz, 2H), 2.80 (d, J = 13.5 Hz, 1H), 2.36 (d, J = 7.1 Hz, 2H), 2.33 (s, 1H), 2.19 (d, J = 9.6 Hz, 1H), 2.07 – 1.92 (m, 4H), 1.88 – 1.67 (m, 6H), 1.65 – 1.40 (m, 8H), 1.37 (s, 3H, CH_3), 1.26 (s, 2H), 1.23 (s, 3H, CH_3), 1.15 (d, J = 10.7 Hz, 6H, 2 × CH_3), 1.03 (d, J = 13.0 Hz, 2H), 0.88 (s, 6H, 2 × CH_3), 0.84 (s, 3H, CH_3).

18β-3-O-(7′-Bromoheptanoyl)-11-oxo-olean-12-en-30-oic acid (2). ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.71 (s, 1H), 4.53 (dd, J = 11.3, 4.8 Hz, 1H), 3.40 (t, J = 6.7 Hz, 2H), 2.80 (d, J = 13.6 Hz, 1H), 2.37 (s, 1H), 2.31 (t,

J = 7.5 Hz, 2H), 2.19 (d, J = 11.3 Hz, 1H), 2.09 – 1.82 (m, 8H), 1.71 – 1.58 (m, 8H), 1.50 – 1.42 (m, 6H), 1.37 (s, 3H, CH_3), 1.36 – 1.30 (m, 2H), 1.22 (s, 3H, CH_3), 1.15 (d, J = 10.8 Hz, 6H, 2 × CH_3), 1.03 (d, J = 12.0 Hz, 2H), 0.88 (s, 6H, 2 × CH_3), 0.84 (s, 3H, CH_3).

*4.2.2. General procedure for preparation of compound **3** and **4***

A solution of compound **1** (303 mg, 0.48 mmol) or compound **2** (317 mg, 0.48 mmol) in DMF (20 mL) was stirred. Then, CH_3I (127.5 μL, 2.04 mmol) and K_2CO_3 (284.5 mg, 2.04 mmol) were added, and the mixture was stirred at 25 °C until the reaction was complete based on TLC detection. The reaction solution was extracted by DCM (3 × 5 mL) and ice cold water to remove DMF and K_2CO_3. The combined organic phases were dried (Na_2SO_4) and concentrated. The residue was purified by column chromatography using 10:1 petroleum ether/ethyl acetate to obtain the target product of compound **3** (263.6 mg, 85%) and **4** (252.3, 78%) as a white solid.

*18β-3-O-(5′-Bromovaleryl)-11-oxo-olean-12-en-30-oicacidmethyl ester (**3**)*. ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.60 (s, 1H), 4.46 (dd, J = 11.4, 5.0 Hz, 1H), 3.62 (s, 3H, CH_3), 3.33 (t, J = 6.8 Hz, 2H), 2.73 (d, J = 13.9 Hz, 1H), 2.30 (s, 1H), 2.26 (d, J = 7.4 Hz, 2H), 2.04 – 1.70 (m, 9H), 1.61 – 1.53 (m, 6H), 1.44 – 1.37 (m, 4H), 1.30 (s, 3H, CH_3), 1.26 – 1.22 (m, 2H), 1.09 (d, J = 4.9 Hz, 6H, 2 × CH_3), 1.06 (s, 3H, CH_3), 0.96 (d, J = 13.6 Hz, 2H), 0.81 (d, J = 2.6 Hz, 6H, 2 × CH_3), 0.74 (s, 3H, CH_3).

*18β-3-O-(7′-Bromoheptanoyl)-11-oxo-olean-12-en-30-oicacidmethyl ester (**4**)*. ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.67 (s, 1H), 4.53 (dd, J = 11.4, 5.0 Hz, 1H), 3.69 (s, 3H, CH_3), 3.42 (t, J = 6.5 Hz, 2H), 2.81 (d, J = 13.6 Hz, 1H), 2.36 (s, 2H), 2.33 (s, 1H), 2.28 – 1.83 (m, 9H), 1.82 – 1.48 (m, 10*H*), 1.47 – 1.37 (m, 4H), 1.37 (s, 3H, CH_3), 1.33 – 1.24 (m, 2H), 1.16 (d, J = 4.7 Hz, 6H, 2 × CH_3), 1.13 (s, 3H, CH_3), 1.03 (d, J = 13.5 Hz, 2H), 0.89 (s, 6H, 2 × CH_3), 0.81 (s, 3H, CH_3).

*4.2.3. General procedure for preparation of compound **5** and **6***

Anhydrous K_2CO_3 (69.1 mg, 0.5 mmol) and CH_3CH_2Br (149 μL, 2 mmol) were added to a stirred solution of compound **1** (315.6 mg, 0.5 mmol) or compound **2** (330.2 mg, 0.5 mmol) in acetone (10 mL) at room temperature. The resulting solution was stirred at 35 °C for 48 h. Progress of the reaction was monitored by TLC. After completion of the reaction, the acetone was distilled off. The resulting mixture was diluted with water (15 mL) and extracted with dichloromethane (30 mL × 2). The combined organic layers were washed with brine (10 mL) and dried over sodium sulfate. After evaporation of the solvent, the crude residue was purified by column chromatography using 10:1 petroleum ether/ethyl acetate to obtain the target product of compound **5** (273.9 mg, 83%) and compound **6** (271.8 mg, 79%) as a white solid.

*18β-3-O-(5′-Bromovaleryl)-11-oxo-olean-12-en-30-oic acidethyl ester (**5**)*. ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.65 (s, 1H), 4.53 (dd, J = 11.4, 5.0 Hz, 1H), 4.16 (dd, J = 9.3, 7.2 Hz, 2H), 3.42 (t, J = 6.5 Hz, 2H), 2.81 (d, J = 13.7 Hz, 1H), 2.36 (d, J = 5.7 Hz, 2H), 2.32 (s, 1H), 2.10 (d, J = 13.4 Hz, 1H), 2.06 – 1.85 (m, 6H), 1.84 – 1.58 (m, 8H), 1.56 – 1.38 (m, 4H), 1.37 (s, 3H, CH_3), 1.34 – 1.29 (m, 2H), 1.26 (t, J = 7.1 Hz, 3H, CH_3), 1.17 (s, 3H, CH_3), 1.14 (d, J = 4.3 Hz, 6H, 2 × CH_3), 1.02 (d, J = 15.9 Hz, 2H), 0.88 (d, J = 2.6 Hz, 6H, 2 × CH_3), 0.81 (s, 3H, CH_3).

*18β-3-O-(7′-Bromoheptanoyl)-11-oxo-olean-12-en-30- oic acidethyl ester (**6**)*. ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.65 (s, 1H), 4.53 (dd, J = 11.3, 4.9 Hz, 1H), 4.22 – 4.11 (m, 2H), 3.40 (t, J = 6.8 Hz, 2H), 2.80 (d, J = 13.6 Hz, 1H), 2.37 (s, 1H), 2.31 (t, J = 7.4 Hz, 2H), 2.10 (d, J = 13.4 Hz, 1H), 2.06 – 1.70 (m, 8H), 1.69 – 1.56 (m, 8H), 1.53 – 1.39 (m, 6H), 1.37 (s, 3H, CH_3), 1.30 (s, 2H), 1.27 (t, J = 7.1 Hz, 3H, CH_3), 1.17 (s, 3H, CH_3), 1.14 (d, J = 4.4 Hz, 6H, 2 × CH_3), 1.03 (d, J = 15.7 Hz, 2H), 0.88 (d, J = 2.4 Hz, 6H, 2 × CH_3), 0.81 (s, 3H, CH_3).

*4.2.4. General procedure for preparation of compound **7** and **8***

A solution of glycyrrhetinic acid (470 mg, 1 mmol) in DMF (10 mL) was stirred. K_2CO_3 (552 mg, 4 mmol), 1, 4-dibromobutane (864 mg, 4 mmol) or 1, 6-dibromhexan (976 mg, 4 mmol), were added successively, and the mixture was stirred at 25 °C until the reaction was complete based on TLC detection. The reaction solution was extracted by DCM (3 × 10 mL) and ice cold water to remove DMF and K_2CO_3. The combined organic phases were dried (Na_2SO_4) and concentrated. The residue was purified by column chromatography using 8:1 petroleum ether/ethyl acetate to obtain of the target product of compound **7** (501.3 mg, 83%) and **8** (505.6 mg, 80%) as a white powder.

*18β-11-Oxo-olean-12-en-30-oic acid- (4′-bromobutyl) ester (**7**)*. ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.67 (s, 1H), 4.17 (t, J = 6.4 Hz, 2H), 3.48 (t, J = 6.6 Hz, 2H), 3.26 (d, J = 6.3 Hz, 1H), 2.83 (d, J = 13.5 Hz, 1H), 2.38 (s, 1H), 2.14 (dd, J = 13.5, 3.3 Hz, 1H), 2.10 – 1.82 (m, 9H), 1.72 – 1.61 (m, 6H), 1.53 – 1.43 (m, 3H), 1.41 (s, 3H, CH_3), 1.38 – 1.35 (m, 2H), 1.20 – 1.16 (m, 9H, 3 × CH_3), 1.05 (s, 3H, CH_3), 1.03 – 0.90 (m, 1H), 0.85 (d, J = 2.1 Hz, 6H, 2 × CH_3), 0.74 (d, J = 10.9 Hz, 1H).

*18β-11-Oxo-olean-12-en-30-oic acid- (6′-Bromohexyl) ester (**8**)*. ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.63 (s, 1H), 4.10 (t, J = 6.5 Hz, 2H), 3.41 (t, J = 6.7 Hz, 2H), 3.22 (d, J = 9.2 Hz, 1H), 2.84 – 2.76 (m, 1H), 2.34 (s, 1H), 2.08 (d, J = 3.6 Hz, 1H), 2.05 – 1.75 (m, 7H), 1.73 – 1.58 (m, 8H), 1.57 – 1.40 (m, 7H), 1.37 (s, 3H, CH_3), 1.32 – 1.21 (m, 2H), 1.15 – 1.12 (m, 9H, 3 × CH_3), 1.01 (s, 3H, CH_3), 0.98 – 0.87 (m, 1H), 0.81 (s, 6H, 2 × CH_3), 0.70 (d, J = 11.4 Hz, 1H).

*4.2.5. General procedure for preparation of compound **9** and **10***

Jones reagent (prepared from 107.9 mg of CrO_3) was added to a solution of **7** (604 mg, 1 mmol) or **8** (632 mg, 1 mmol) in acetone (10 mL) at 0 °C over a period of 30 min till the brown color persisted. The resulting solution was stirred for further 30 min. Progress of the reaction was monitored by TLC. After completion of the reaction, isopropanol (0.5 mL) was added. After evaporation of the solvent, the crude residue was diluted with dichloromethane (100 mL). The organic layer was washed with water (25 mL × 2) and brine (25 mL), and dried over sodium sulfate. The residue was purified by column chromatography using 10:1 petroleum ether/ethyl acetate to obtain of the target product of compounds **9** (523.7 mg, 87%) and **10** (535.5 mg, 85%) as a white powder.

*18β-3, 11-Dioxo-olean-12-en-30-oic acid- (4′-bromobutyl) ester (**9**)*. ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.61 (s, 1H), 4.07 (t, J = 6.2 Hz, 2H), 3.38 (t, J = 6.4 Hz, 2H), 2.93 – 2.84 (m, 1H), 2.62 – 2.50 (m, 1H), 2.37 (s, 1H), 2.34 – 2.24 (m, 1H), 2.05 (d, J = 14.0 Hz, 1H), 1.99 – 1.75 (m, 8H), 1.73 – 1.50 (m, 4H), 1.48 – 1.33 (m, 4H), 1.31 (s, 3H, CH_3), 1.28 – 1.24 (m, 2H), 1.20 (s, 3H, CH_3), 1.17 – 1.13 (m, 1H), 1.09 (d, J = 4.0 Hz, 6H, 2 × CH_3), 1.02 (d, J = 10.7 Hz, 6H, 2 × CH_3), 0.95 (s, 1H), 0.76 (s, 3H, CH_3).

*18β-3, 11-Dioxo-olean-12-en-30-oic acid- (6′-Bromohexyl) ester (**10**)*. ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.61 (s, 1H), 4.04 (t, J = 6.5 Hz, 2H), 3.35 (t, J = 6.7 Hz, 2H), 2.95 – 2.85 (m, 1H), 2.64 – 2.51 (m, 1H), 2.37 (s, 1H), 2.34 – 2.25 (m, 1H), 2.06 (d, J = 13.8 Hz, 1H), 2.00 – 1.78 (m, 6H), 1.75 – 1.51 (m, 6H), 1.49 – 1.33 (m, 8H), 1.31 (s, 3H, CH_3), 1.28 – 1.24 (m, 2H), 1.20 (s, 3H, CH_3), 1.14 (s, 1H), 1.09 (d, J = 6.2 Hz, 6H, 2 × CH_3), 1.02 (d, J = 10.7 Hz, 6H, 2 × CH_3), 0.98 – 0.89 (m, 1H), 0.76 (s, 3H, CH_3).

*4.2.6. General procedure for preparation of compound **11** and **12***

Acetic anhydride (5.0 mL) was added dropwise to a stirred solution of compound **7** (604 mg, 1 mmol) or compound **8** (632 mg, 1 mmol) in pyridine (7.5 mL) at 0 °C. The resulting solution was stirred at room temperature for 24 h and then poured into ice water (75 mL), resulting in the compound **11** (497.4 mg, 77%) and **12** (498.8 mg, 74%) as the white solid precipitate.

*18β-3-O-Acetyl-11-Oxo-olean-12-en-30-oic acid- (4′-bromobutyl) ester (**11**)*. ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.64 (s, 1H), 4.52 (dd, J = 11.4, 5.0 Hz, 1H), 4.14 (t, J = 6.3 Hz, 2H), 3.45 (t, J = 6.5 Hz, 2H), 2.80 (d, J = 13.6 Hz, 1H), 2.36 (s, 1H), 2.14 – 2.07 (m, 1H), 2.05 (s, 3H, CH_3), 2.03 – 1.93 (m, 4H), 1.93 – 1.68 (m, 6H), 1.67 – 1.38 (m, 8H), 1.37 (s, 3H, CH_3), 1.33 – 1.21 (m, 2H), 1.16 (d, J = 2.7 Hz, 6H, 2 × CH_3), 1.13

(s, 3H, CH_3), 1.03 (d, J = 13.3 Hz, 2H), 0.88 (s, 6H, 2 × CH_3), 0.81 (s, 3H, CH_3).

*18β-3-O-Acetyl-11-Oxo-olean-12-en-30-oic acid- (6′-Bromohexyl) ester (**12**)*. ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.64 (s, 1H), 4.52 (dd, J = 11.4, 5.0 Hz, 1H), 4.13 – 4.07 (m, 2H), 3.41 (t, J = 6.7 Hz, 2H), 2.80 (dd, J = 10.2, 3.4 Hz, 1H), 2.36 (s, 1H), 2.09 (s, 1H), 2.05 (s, 3H, CH_3), 2.03 – 1.74 (m, 7H), 1.73 – 1.58 (m, 8H), 1.54 – 1.39 (m, 7H), 1.37 (s, 3H, CH_3), 1.33 – 1.22 (m, 2H), 1.17 – 1.13 (m, 9H, 3 × CH_3), 1.03 (d, J = 13.7 Hz, 2H), 0.88 (s, 6H, 2 × CH_3), 0.81 (s, 3H, CH_3).

4.2.7. General procedure for preparation of compound *1a* and *1b*

Triphenylphosphine (262 mg, 1 mmol) was added to a solution of compound **1** (151.5 mg, 0.24 mmol) or compound **2** (158.5 mg, 0.24 mmol) in CH_3CN (10 mL). The mixture was then stirred at 80 °C until the reaction was complete according to TLC detection. The solvent was subsequently removed under diminished pressure, and the residue was purified by column chromatography using 25:1 CH_2Cl_2/CH_3OH to obtain of the target product of compound **1a** and **1b**.

*18β-3-O-(5′-Triphenylphosphonio-pentanoate bromide)-11-oxo-olean-12-en-30-oic acid (**1a**)*. Light yellow solid; Yield: (79.4 mg, 37%); mp: 242–245 °C; [α] = +92.1° (c = 0.5, $CHCl_3$); IR ν_{max} 2928, 2867, 1716, 1647, 1544, 1437, 1386, 1259, 1209, 1111, 996, 722, 688, 529, 506 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.82 – 7.77 (m, 7H), 7.74 – 7.68 (m, 8H), 5.64 (s, 1H), 4.41 (dd, J = 11.5, 4.5 Hz, 1H), 3.60 (s, 2H), 2.72 (d, J = 13.3 Hz, 1H), 2.38 (t, J = 6.5 Hz, 2H), 2.33 (s, 1H), 2.21 (d, J = 14.4 Hz, 1H), 2.14 – 1.96 (m, 4H), 1.95 – 1.69 (m, 6H), 1.68 – 1.45 (m, 8H), 1.36 (s, 3H, CH_3), 1.28 – 1.25 (m, 2H), 1.18 (s, 3H, CH_3), 1.11 (s, 6H, 2 × CH_3), 0.99 – 0.96 (m, 1H), 0.82 – 0.78 (m, 9H, 3 × CH_3), 0.73 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 200.2, 180.2, 172.8, 169.9, 135.2, 135.1, 133.6, 133.5, 130.6, 130.4, 128.3, 118.7, 117.6, 80.7, 61.6, 55.0, 48.2, 45.4, 43.7, 43.2, 41.0, 38.7, 38.0, 36.9, 33.6, 32.6, 31.8, 31.0, 28.5, 28.1, 26.5, 26.4, 25.7, 25.5, 23.5, 23.3, 22.5, 21.8, 18.7, 18.5, 17.3, 16.7, 16.4. HR-MS (*m/z*) (ESI): calcd for $C_{53}H_{68}O_5P^+$ [M-Br] $^+$: 815.47989; found: 815.47910.

*18β-3-O-(7′-Triphenylphosphonio-heptanoate bromide)-11-oxo-olean-12-en-30-oic acid (**1b**)*. Light yellow solid; Yield: (77.5 mg, 35%); mp: 158–161 °C; [α] = +81.7° (c = 0.5, $CHCl_3$); IR ν_{max} 2928, 2868, 1727, 1644, 1437, 1383, 1251, 1209, 1143, 1112, 984, 722, 689, 533, 507 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.90 – 7.82 (m, 6H), 7.81 – 7.78 (m, 3H), 7.74 – 7.68 (m, 6H), 5.67 (s, 1H), 4.46 (dd, J = 11.3, 4.8 Hz, 1H), 3.78 (s, 2H), 2.76 (d, J = 13.2 Hz, 1H), 2.35 (s, 1H), 2.24 (d, J = 7.4 Hz, 2H), 2.16 (s, 1H), 2.08 – 1.67 (m, 8H), 1.66 – 1.52 (m, 10*H*), 1.49 – 1.39 (m, 4H), 1.36 (s, 3H, CH_3), 1.32 – 1.28 (m, 2H), 1.20 (s, 3H, CH_3), 1.13 (d, J = 5.5 Hz, 6H, 2 × CH_3), 1.04 (d, J = 7.8 Hz, 2H), 0.83 (d, J = 5.6 Hz, 9H, 3 × CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 200.2, 180.7, 173.4, 169.6, 135.0, 134.9, 133.7, 133.6, 130.5, 130.4, 128.3, 118.9, 117.8, 80.3, 61.6, 55.0, 48.1, 45.4, 43.7, 43.2, 40.9, 38.7, 38.0, 37.7, 36.9, 34.4, 32.6, 31.8, 30.9, 30.0, 29.8, 28.4, 28.4, 28.0, 26.3, 24.5, 23.5, 23.3, 23.0, 22.4, 22.3, 18.6, 17.3, 16.7, 16.3. HR-MS (*m/z*) (ESI): calcd for $C_{55}H_{72}O_5P^+$ [M-Br] $^+$: 843.5112; found: 843.5108.

4.2.8. General procedure for preparation of compound *1c* and *1d*

Compound **1c** and **1d** were obtained from compound **3** and **4**, according to the procedure of compound **1a** and **1b**.

*18β-3-O-(5′-Triphenylphosphonio-pentanoate bromide)-11-oxo-olean-12-en-30-oicacidmethyl ester (**1c**)*. Light yellow solid; Yield: (93.7 mg, 43%); mp: 153–155 °C; [α] = +95.1° (c = 0.5, $CHCl_3$); IR ν_{max} 2945, 2868, 1721, 1651, 1437, 1386, 1257, 1213, 1155, 1111, 995, 722, 689, 530, 507 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.92 – 7.84 (m, 6H), 7.84 – 7.80 (m, 3H), 7.76 – 7.70 (m, 6H), 5.69 (s, 1H), 4.42 (dd, J = 11.5, 4.4 Hz, 1H), 3.96 – 3.86 (m, 2H), 3.72 (s, 3H, CH_3), 2.76 (d, J = 13.7 Hz, 1H), 2.42 (t, J = 6.5 Hz, 2H), 2.36 (s, 1H), 2.15 – 1.93 (m, 9H), 1.86 – 1.61 (m, 6H), 1.56 – 1.44 (m, 4H), 1.38 (s, 3H, CH_3), 1.32 (s, 2H), 1.17 (s, 3H, CH_3), 1.14 (s, 6H, 2 × CH_3), 1.05 (d, J = 13.5 Hz, 2H), 0.82 (d, J = 2.6 Hz, 9H, 3 × CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 200.1, 176.9, 172.8, 169.4, 135.0, 134.9, 133.7, 133.6, 130.5, 130.4, 128.4, 118.9, 117.7, 80.6, 61.7, 55.0, 51.7, 48.4, 45.3, 44.0, 43.2, 41.1, 38.7, 37.9, 37.7, 36.9, 33.7, 32.6, 31.8, 31.1, 28.5, 28.3, 28.0, 26.4, 25.7, 23.5, 23.3, 22.8, 22.2, 21.9, 18.6, 17.3, 16.7, 16.3. HR-MS (*m/z*) (ESI): calcd for $C_{54}H_{70}O_5P^+$ [M-Br] $^+$: 829.49554; found: 829.49308.

*18β-3-O-(7′-Triphenylphosphonio-heptanoate bromide)-11-oxo-olean-12-en-30-oicacidmethyl ester (**1d**)*. Light yellow solid; Yield: (89.9 mg, 40%); mp: 128–131 °C; [α] = +81.1° (c = 0.5, $CHCl_3$); IR ν_{max} 2929, 2859, 1724, 1651, 1436, 1387, 1250, 1212, 1189, 1112, 985, 721, 690, 533, 508 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.91 – 7.84 (m, 6H), 7.83 – 7.80 (m, 3H), 7.76 – 7.72 (m, 6H), 5.69 (s, 1H), 4.52 – 4.46 (m, 1H), 3.80 (s, 2H), 3.71 (s, 3H, CH_3), 2.80 (d, J = 13.6 Hz, 1H), 2.37 (s, 1H), 2.29 (d, J = 7.2 Hz, 2H), 2.19 – 1.75 (m, 10*H*), 1.69 – 1.59 (m, 9H), 1.54 – 1.42 (m, 4H), 1.38 (s, 3H, CH_3), 1.34 – 1.32 (m, 2H), 1.17 (s, 6H, 2 × CH_3), 1.15 (s, 3H, CH_3), 1.08 – 1.03 (m, 2H), 0.87 (s, 6H, 2 × CH_3), 0.83 (s, 3H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 200.0, 176.9, 173.4, 169.3, 135.0, 134.9, 133.7, 133.6, 130.5, 130.4, 128.5, 119.0, 117.8, 80.4, 61.7, 55.0, 51.7, 48.4, 45.4, 44.0, 43.2, 41.1, 38.8, 38.0, 37.7, 36.9, 34.4, 32.7, 31.8, 31.1, 30.0, 29.8, 28.5, 28.3, 28.1, 26.5, 24.5, 23.6, 23.3, 22.9, 22.5, 22.3, 18.7, 17.4, 16.7, 16.4. HR-MS (*m/z*) (ESI): calcd for $C_{56}H_{74}O_5P^+$ [M-Br] $^+$: 857.5268; found: 857.5268.

4.2.9. General procedure for preparation of compound *1e* and *1f*

Compound **1e** and **1f** were obtained from compound **5** and **6**, according to the procedure of compound **1a** and **1b**.

*18β-3-O-(5′-Triphenylphosphonio-pentanoate bromide)-11-oxo-olean-12-en-30-oic acidethyl ester (**1e**)*. Light yellow solid; Yield: (97.4 mg, 44%); mp: 130–133 °C; [α] = +93.3° (c = 0.5, $CHCl_3$); IR ν_{max} 2929, 2868, 1718, 1654, 1437,1385, 1323, 1256, 1212, 1173, 1111, 995, 921, 722, 688, 530, 507 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.83 – 7.75 (m, 6H), 7.74 – 7.69 (m, 3H), 7.67 – 7.60 (m, 6H), 5.58 (s, 1H), 4.33 (dd, J = 11.5, 4.5 Hz, 1H), 4.14 – 4.04 (m, 2H), 3.78 (d, J = 13.2 Hz, 2H), 2.66 (d, J = 13.8 Hz, 1H), 2.32 (t, J = 6.5 Hz, 2H), 2.27 (s, 1H), 2.06 (s, 1H), 2.01 – 1.70 (m, 8H), 1.68 – 1.46 (m, 6H), 1.43 – 1.32 (m, 4H), 1.29 (s, 3H, CH_3), 1.27 – 1.23 (m, 2H), 1.20 (s, 3H, CH_3), 1.08 (s, 3H, CH_3), 1.05 (s, 6H, 2 × CH_3), 0.93 (s, 2H), 0.73 (s, 9H, 3 × CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 200.0, 176.3, 172.8, 169.5, 135.0, 134.9, 133.7, 133.6, 130.5, 130.4, 128.4, 118.9, 117.8, 80.6, 61.7, 60.3, 55.0, 48.4, 45.4, 43.8, 43.2, 41.1, 38.7, 37.9, 37.7, 36.9, 33.7, 32.6, 31.8, 31.1, 28.5, 28.3, 28.0, 26.5, 25.7, 25.5, 23.5, 23.3, 22.8, 21.9, 18.7, 17.3, 16.7, 16.3, 14.3. HR-MS (*m/z*) (ESI): calcd for $C_{55}H_{72}O_5P^+$ [M-Br] $^+$: 843.5112; found: 843.5115.

*18β-3-O-(7′-Triphenylphosphonio-heptanoate bromide)-11-oxo-olean-12-en-30- oic acidethyl ester (**1f**)*. Light yellow solid; Yield: (111.8 mg, 49%); mp: 115–118 °C; [α] = +84.1° (c = 0.5, $CHCl_3$); IR ν_{max} 2929, 2864, 1719, 1654, 1437, 1385, 1323, 1248, 1210, 1173, 1111, 984, 921, 722, 688, 532, 507 cm^{-1}. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.83 – 7.75 (m, 6H), 7.74 – 7.69 (m, 3H), 7.68 – 7.62 (m, 6H), 5.57 (s, 1H), 4.39 (dd, J = 11.3, 4.7 Hz, 1H), 4.13 – 4.02 (m, 2H), 3.73 (s, 2H), 2.71 (d, J = 13.6 Hz, 1H), 2.28 (s, 1H), 2.18 (s, 2H), 2.02 (s, 1H), 1.99 – 1.60 (m, 8H), 1.59 – 1.49 (m, 8H), 1.48 – 1.31 (m, 6H), 1.29 (s, 3H, CH_3), 1.27 – 1.24 (m, 2H), 1.19 (s, 3H, CH_3), 1.07 (d, J = 5.8 Hz, 9H, 3 × CH_3), 0.97 – 0.88 (m, 2H), 0.78 (s, 6H, 2 × CH_3), 0.74 (s, 3H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 200.0, 176.3, 173.6, 169.4, 135.0, 134.9, 133.7, 133.6, 130.5, 130.4, 128.4, 119.0, 117.9, 80.4, 61.7, 60.2, 55.0, 48.4, 45.4, 43.8, 43.2, 41.1, 38.7, 38.0, 37.7, 36.9, 34.4, 32.7, 31.8, 31.1, 30.0, 29.8, 28.5, 28.3, 28.1, 26.5, 24.5, 23.6, 23.3, 22.9, 22.5, 22.3, 18.7, 17.4, 16.7, 16.3, 14.3. HR-MS (*m/z*) (ESI): calcd for $C_{57}H_{76}O_5P^+$ [M-Br] $^+$: 871.5425; found: 871.5421.

4.2.10. General procedure for preparation of compound *2a* and *2b*

Compound **2a** and **2b** were obtained from compound **7** and **8**, according to the procedure of compound **1a** and **1b**.

*18β-11-Oxo-olean-12-en-30-oic acid- (4′-Triphenylphosphonio-butyrate bromide) ester (**2a**)*. Light yellow solid; Yield: (81.1 mg, 39%); mp: 157–161 °C; [α] = +107.0° (c = 0.5, $CHCl_3$); IR ν_{max} 2925, 2864,

1716, 1651, 1437, 1385, 1313, 1257, 1208, 1153, 1111, 994, 722, 688, 530, 506 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ 7.89 – 7.85 (m, 6H), 7.81 – 7.78 (m, 3H), 7.72 – 7.68 (m, 6H), 5.49 (s, 1H), 4.18 – 4.11 (m, 2H), 4.02 – 3.84 (m, 2H), 3.24 (dd, J = 11.0, 4.9 Hz, 1H), 2.75 (d, J = 13.4 Hz, 1H), 2.30 (s, 1H), 2.10 (s, 1H), 2.04 – 1.94 (m, 4H), 1.89 – 1.67 (m, 6H), 1.63 – 1.39 (m, 8H), 1.34 (s, 3H, CH_3), 1.26 (s, 2H), 1.11 (d, J = 10.9 Hz, 6H, 2 × CH_3), 1.00 (d, J = 6.0 Hz, 6H, 2 × CH_3), 0.96 – 0.87 (m, 1H), 0.80 (s, 3H, CH_3), 0.74 (s, 3H, CH_3), 0.70 (d, J = 11.2 Hz, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 199.9, 176.2, 169.2, 135.0, 134.9, 133.7, 133.6, 130.5, 130.4, 128.3, 118.5, 117.8, 78.5, 63.0, 61.8, 54.9, 48.2, 45.3, 43.9, 43.1, 41.0, 39.1, 39.1, 37.6, 37.0, 32.7, 31.7, 30.9, 29.5, 29.4, 28.5, 28.2, 28.0, 27.2, 26.4, 26.3, 23.3, 19.3, 18.6, 17.4, 16.3, 15.5. HR-MS (m/z) (ESI): calcd for $C_{52}H_{68}O_4P^+$ [M-Br] $^+$: 787.4850; found: 787.5234.

*18β-11-Oxo-olean-12-en-30-oic acid- (6′-Triphenylphosphoniocaproate bromide) ester (**2b**)*. Light yellow solid; Yield: (96.6 mg, 45%); mp: 130–132 °C; [α] = +83.1° (c = 0.5, $CHCl_3$); IR ν_{max} 2927, 2862, 1716, 1651, 1437, 1385, 1314, 1208, 1154, 1112, 994, 722, 688, 533, 507 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$) δ 7.90 – 7.82 (m, 6H), 7.80 (d, J = 7.7 Hz, 3H), 7.75 – 7.69 (m, 6H), 5.54 (s, 1H), 4.09 – 3.99 (m, 2H), 3.82 – 3.73 (m, 2H), 3.27 – 3.19 (m, 1H), 2.63 (d, J = 13.4 Hz, 1H), 2.31 (s, 1H), 2.16 (s, 1H), 2.05 – 1.73 (m, 7H), 1.72 – 1.60 (m, 8H), 1.59 – 1.39 (m, 7H), 1.36 (s, 3H, CH_3), 1.34 – 1.28 (m, 2H), 1.12 (s, 3H, CH_3), 1.09 (d, J = 4.7 Hz, 6H, 2 × CH_3), 1.00 (s, 3H, CH_3), 0.94 – 0.87 (m, 1H), 0.79 (d, J = 4.9 Hz, 6H, 2 × CH_3), 0.69 (d, J = 11.4 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 199.9, 176.2, 169.5, 134.9, 134.9, 133.6, 133.5, 130.4, 130.3, 128.1, 118.8, 117.6, 78.3, 64.1, 61.7, 54.7, 48.4, 45.2, 43.8, 43.1, 41.0, 39.0, 37.5, 36.9, 32.6, 31.7, 30.9, 30.2, 30.0, 28.4, 28.2, 28.0, 27.1, 26.3, 26.2, 25.8, 23.2, 22.9, 22.5, 22.3, 18.6, 17.3, 16.2, 15.5. HR-MS (m/z) (ESI): calcd for $C_{54}H_{72}O_4P^+$ [M-Br] $^+$: 815.5163; found: 815.5160.

4.2.11. General procedure for preparation of compound **2c** and **2d**

Compound **2c** and **2d** were obtained from compound **9** and **10**, according to the procedure of compound **1a** and **1b**.

*18β-3, 11-Dioxo-olean-12-en-30-oic acid- (4′-Triphenylphosphoniobutyrate bromide) ester (**2c**)*. Light yellow solid; Yield: (105.8 mg, 51%); mp: 155–158 °C; [α] = +105.5° (c = 0.5, $CHCl_3$); IR ν_{max} 2927, 2866, 1700, 1651, 1458, 1437, 1384, 1313, 1209, 1153, 1111, 996, 748, 722, 688, 529, 507 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$) δ 7.90 – 7.82 (m, 6H), 7.78 (d, J = 6.9 Hz, 3H), 7.73 – 7.67 (m, 6H), 5.56 (s, 1H), 4.15 (s, 2H), 3.91 (s, 2H), 2.99 – 2.87 (m, 1H), 2.67 – 2.57 (m, 1H), 2.41 (s, 1H), 2.34 (s, 1H), 2.21 (s, 1H), 2.16 – 1.89 (m, 6H), 1.87 – 1.64 (m, 6H), 1.56 – 1.44 (m, 4H), 1.35 (s, 3H, CH_3), 1.30 (s, 2H), 1.25 (s, 3H, CH_3), 1.21 – 1.19 (m, 1H), 1.15 (s, 3H, CH_3), 1.09 (d, J = 11.6 Hz, 6H, 2 × CH_3), 0.99 (s, 3H, CH_3), 0.91 – 0.82 (m, 1H), 0.76 (s, 3H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 217.0, 199.2, 176.2, 169.8, 135.0, 134.9, 133.7, 133.6, 130.5, 130.4, 128.2, 118.7, 117.6, 63.0, 61.0, 55.3, 48.2, 47.7, 45.2, 43.9, 41.1, 39.8, 37.7, 36.7, 34.1, 32.0, 31.7, 30.9, 29.5, 29.3, 28.6, 28.2, 26.4, 26.3, 23.3, 22.4, 21.7, 21.3, 19.3, 18.7, 18.5, 15.6. HR-MS (m/z) (ESI): calcd for $C_{52}H_{66}O_4P^+$ [M-Br] $^+$: 785.4693; found: 785.4689.

*18β-3, 11-Dioxo-olean-12-en-30-oic acid- (6′-Triphenylphosphoniocaproate bromide) ester (**2d**)*. Light yellow solid; Yield: (102.8 mg, 48%); mp: 128–130 °C; [α] = +134.8° (c = 0.5, $CHCl_3$); IR ν_{max} 2929, 2863, 1701, 1651, 1458, 1437, 1384, 1313, 1207, 1154, 1111, 996, 746, 721, 689, 532, 507 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$) δ 7.88 – 7.81 (m, 6H), 7.79 (d, J = 7.7 Hz, 3H), 7.74 – 7.69 (m, 6H), 5.61 (s, 1H), 4.04 (t, J = 5.8 Hz, 2H), 3.80 (s, 2H), 2.85 – 2.77 (m, 1H), 2.57 (dd, J = 15.9, 10.5 Hz, 1H), 2.42 (s, 1H), 2.39 – 2.32 (m, 1H), 2.11 – 1.82 (m, 8H), 1.71 – 1.56 (m, 9H), 1.51 – 1.40 (m, 4H), 1.37 (s, 3H, CH_3), 1.33 – 1.30 (m, 2H), 1.21 (s, 3H, CH_3), 1.16 (s, 1H), 1.14 (s, 3H, CH_3), 1.11 (d, J = 4.7 Hz, 6H, 2 × CH_3), 1.06 (s, 3H, CH_3), 0.99 (d, J = 10.8 Hz, 1H), 0.80 (s, 3H, CH_3). ^{13}C NMR (75 MHz, CDCl3) δ 217.1, 199.3, 176.3, 170.1, 134.9, 134.9, 133.8, 133.6, 130.5, 130.3, 128.2, 119.0, 117.9, 64.3, 61.0, 55.2, 48.5, 47.7, 45.2, 43.9, 43.3, 41.2, 39.7, 37.6, 36.6, 34.1, 32.0, 31.8, 31.0, 30.3, 30.0, 28.6, 28.5, 28.3, 26.5, 25.8, 23.3, 22.9, 22.6, 22.2, 21.3, 18.8, 18.5, 15.7. HR-MS (m/z) (ESI): calcd for $C_{54}H_{70}O_4P^+$ [M-Br] $^+$: 813.50062; found: 813.50342.

4.2.12. General procedure for preparation of compound **2e** and **2f**

Compound **2e** and **2f** were obtained from compound **11** and **12**, according to the procedure of compound **1a** and **1b**.

*18β-3-O-Acetyl-11-Oxo-olean-12-en-30-oic acid- (4′-Triphenylphosphoniobutyrate bromide) ester (**2e**)*. Light yellow solid; Yield: (85.0 mg, 39%); mp: 159–163 °C; [α] = +90.5° (c = 0.5, $CHCl_3$); IR ν_{max} 2925, 2858, 1717, 1650, 1437, 1364, 1319, 1247, 1212, 1155, 1112, 1026, 985, 748, 721, 689, 531, 509 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$) δ 7.92 – 7.84 (m, 6H), 7.81 – 7.76 (m, 3H), 7.73 – 7.67 (m, 6H), 5.49 (s, 1H), 4.51 (dd, J = 11.3, 4.9 Hz, 1H), 4.21 – 4.12 (m, 2H), 4.05 – 3.83 (m, 2H), 2.77 (d, J = 13.5 Hz, 1H), 2.32 (s, 1H), 2.11 (s, 1H), 2.05 (s, 3H, CH_3), 2.04 – 1.91 (m, 4H), 1.90 – 1.68 (m, 6H), 1.67 – 1.37 (m, 8H), 1.33 (s, 3H, CH_3), 1.27 – 1.22 (m, 2H), 1.13 (d, J = 14.4 Hz, 6H, 2 × CH_3), 1.03 (s, 1H), 0.99 (s, 3H, CH_3), 0.89 (s, 6H, 2 × CH_3), 0.80 (d, J = 11.2 Hz, 1H), 0.74 (s, 3H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 199.7, 176.1, 170.9, 169.3, 134.9, 134.9, 133.8, 133.6, 130.5, 130.3, 128.2, 118.7, 117.6, 80.5, 63.0, 61.7, 55.0, 48.2, 45.3, 43.8, 43.1, 41.0, 38.8, 38.0, 37.6, 36.9, 32.6, 31.7, 30.9, 29.5, 29.3, 28.5, 28.2, 28.0, 26.3, 26.3, 23.5, 23.3, 21.2, 19.3, 18.6, 17.3, 16.6, 16.3. HR-MS (m/z) (ESI): calcd for $C_{54}H_{70}O_5P^+$ [M-Br] $^+$: 829.4955; found: 829.4958.

*18β-3-O-Acetyl-11-Oxo-olean-12-en-30-oic acid- (6′-Triphenylphosphoniocaproate bromide) ester (**2f**)*. Light yellow solid; Yield: (83.2 mg, 37%); mp: 142–145 °C; [α] = +91.9° (c = 0.5, $CHCl_3$); IR ν_{max} 2927, 2856, 1720, 1651, 1437, 1365, 1319, 1247, 1213, 1154, 1112, 1027, 985, 744, 722, 689, 532, 507 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$) δ 7.90 – 7.82 (m, 6H), 7.80 – 7.75 (m, 3H), 7.74 – 7.68 (m, 6H), 5.56 (s, 1H), 4.50 (dd, J = 11.3, 5.0 Hz, 1H), 4.07 – 4.00 (m, 2H), 3.87 – 3.77 (m, 2H), 2.67 (d, J = 13.5 Hz, 1H), 2.32 (s, 1H), 2.05 (s, 3H, CH_3), 2.02 (d, J = 3.7 Hz, 1H), 1.99 – 1.72 (m, 7H), 1.71 – 1.59 (m, 8H), 1.58 – 1.38 (m, 7H), 1.36 (s, 3H, CH_3), 1.29 – 1.22 (m, 2H), 1.11 (d, J = 4.4 Hz, 9H, 3 × CH_3), 1.01 (d, J = 13.4 Hz, 2H), 0.88 (s, 6H, 2 × CH_3), 0.78 (s, 3H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 199.8, 176.3, 170.9, 169.6, 134.9, 134.9, 133.7, 133.6, 130.5, 130.3, 128.2, 118.9, 117.8, 80.5, 64.2, 61.6, 54.9, 48.5, 45.3, 43.9, 43.2, 41.1, 38.7, 38.0, 37.6, 36.9, 32.6, 31.8, 31.0, 30.2, 30.0, 28.5, 28.3, 28.0, 26.4, 26.3, 25.8, 23.5, 23.3, 22.6, 22.4, 21.2, 18.6, 17.3, 16.6, 16.3. HR-MS (m/z) (ESI): calcd for $C_{56}H_{74}O_5P^+$ [M-Br] $^+$: 857.5268; found: 857.5266.

4.3. Cell culture and maintenance

All human cancer cell lines including human normal liver HL-7702 cells in this study were purchased from China Life Science Collage (Shanghai, PRC). Culture medium Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), phosphate buffered saline (PBS, pH = 7.2), and Antibiotice-Antimycotic came from KeyGen Biotech Company (China). Cell lines were grown in the supplemented with 10% FBS, 100 units/ml of penicillin and 100 g/ml of streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C.

4.4. Cytotoxicity assay

The anticancer activity of the title compounds were dissolved in DMSO and evaluated in five human cancer cells (HepG-2, A549, MCF-7, HT-29 and A2780) and human normal cells (HL-7702), respectively. About 1.0 × 10^5 cells/mL cells, which were in the logarithmic phase, were grown in each well of 96-well plates and incubated for 12 h at 37 °C in 5% CO_2. Compounds at six different concentrations (2.5, 5, 10, 20, 40 and 80 μM) were also added to the test well and then the cells were incubated at 37 °C in a 5% CO_2 atmosphere for 72 h. An enzyme labeling instrument was used to read absorbance with 570/630 nm double wavelength measurement. Cytotoxicity was detected on the percentage of cell survival compared with the negative control. The final IC_{50} values were calculated by the Bliss method (n = 5). All of the tests were repeated in triplicate.

4.5. Measurement of mitochondrial uptake of compounds **2f** and GA.

HPLC was used to detect and quantify **2f** and GA. For this purpose, A549 cells were grown in 100 mm dishes and then treated with **2f** or GA at 10 μM for 4 h. After the treatment, the cells were washed twice with ice-cold PBS. Mitochondrial isolation was then performed using the cell mitochondrial isolation kit (Beyotime) according to themanufacturer's recommended protocol. The mitochondria pellet was diluted in PBS and extracted twice with a dichloromethane/methanol (4:1) mixture. After the organic layers were combined and dried, the dry residue was dissolved in methanol containing 0.1% of formic acid and was then used for HPLC analysis. The extract of GA-treated cells was dissolved in methanol containing 0.1% of formic acid, and 20 μL of the solution was injected into the HPLC system (Agilent Technologies, CA, USA). Analyses were performed on a ZORBAX SB-C18 column (5 μm, 4.6 × 250 mm) using methanol: 1.0% formic acid solution (90:10) as the mobile phase, and peaks were detected at 254 nm. Meanwhile, the extract of **2f**-treated cells was dissolved in methanol containing 0.1% formic acid, and the mobile phase was methanol: 1.0% formic acid solution (90:10). The wavelength for the detection of **2f** was 254 nm.

4.6. Apoptosis analysis

Apoptosis was also evaluated by flow cytometric analysis of annexin V/PI staining. A549 cells were grown in each well of six-well plates at the density of 5.0×10^4 cells/mL of the DMEM medium with 10% FBS to the final volume of 2 mL. The plates were incubated for overnight and treated with **2f** at the indicated concentration (5, 10 and 20 μM) for 24 h. Briefly, cells were harvested and washed with twice ice-cold PBS, and then suspended cells in the annexin-binding buffer at a concentration of 5×10^5 cells/ml. cells were then incubated with 5 μL of annexin V-FITC and 5 μL of PIin the dark at 4 °C for 30 min. The cells were detected by system software (Cell Quest; BD Biosciences).

4.7. Hoechst 33258 staining

A549 cells were grown in each well of six-well plates at the density of 5.0×10^4 cells/mL of the DMEM medium with 10% FBS to the final volume of 2 mL. The plates were incubated for overnight and treated with **2f** at the indicated concentration (5, 10 and 20 μM) for 24 h. After the treatment period, the cells were then washed twice with ice-PBS and incubated with 0.5 mL of Hoechst 33,258 at dark for 30 min. After 30 min incubation, the cells were washed twice with cold PBS and the results were analysis by fluorescence microscope using 350 nm excitation and 460 nm emissions.

4.8. Flow cytometry analysis of cell cycle distribution

A549 cells were grown on 6-well plates and treated with **2f** at the indicated concentration (5, 10 and 20 μM) and maintained with of the proper culture medium in 5% CO_2 at 37 °C for 24 h. After completion of incubation, cells were harvested and washed three times with ice-cold PBS, fixed with ice-cold 70% ethanol at −20 °C for overnight. The cells were treated with 100 μg/mL RNase A for 30 min at 37 °C after washed with twice 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. Migration assay

The migration effects of **2f** on A549 cells were detected by wound-heal assay. A549 cells were grown on 6-well plates and treated with **2f** at the indicated concentration (5, 10 and 20 μM) and maintained with of the proper culture medium in 5% CO_2 at 37 °C for 24 h. After completion of incubation, the extent of wound heal was observed by imaging with fluorescence microscope.

4.10. Mitochondrial membrane potential assay

A549 cells were grown on 6-well plates and treated with **2f** at the indicated concentration (5, 10 and 20 μM) and maintained with of the proper culture medium in 5% CO_2 at 37 °C for 24 h. After for 24 h, the JC-1 fluorescent probe was added 20 min after replacing with fresh medium. Cells were harvested at 2000 rpm and washed twice with ice-cold PBS and the lost of mitochondrial membrane potential were investigated by fluorescent microscope. Mitochondrial depolarization is indicated by an increase in the green/red fluorescence intensity ratio. The emission fluorescence for JC-1 was monitored at 530 and 590 nm, under the excitation wavelength at 488 nm, respectively.

4.11. ROS assay

A549 cells were grown on 6-well plates and treated with **2f** at the indicated concentration (5, 10 and 20 μM) and maintained with of the proper culture medium in 5% CO_2 at 37 °C for 24 h. On the following treatment, cells were collected at 2000 rpm and washed twice with ice-cold PBS, and then resuspend cells in 10 mM DCFH-DA dissolved in cell free medium at 37 °C for 20 min in dark, and then washed twice with ice-cold PBS. Cellular fluorescence was analyzed by fluorescent microscope at an excitation of 485 nm and an emission of 538 nm.

4.12. Western blot analysis

A549 cells were grown on 6-well plates and treated with **2f** at the indicated concentration (5, 10 and 20 μM) and maintained with of the proper culture medium in 5% CO_2 at 37 °C for 24 h, respectively. After incubation for 24 h, cells were collected, centrifuged, and washed twice with ice-cold PBS. The pellet was then resuspended in lysis buffer. After the cells were lysed on ice for 30 min, lysates were centrifuged at 20000g at 4 °C for 5 min. The protein concentration in the supernatant was examined using the BCA protein assay reagents (Imgenex, USA). Equal amounts of protein per line were was separated on 12% SDS polyacrylamide gel electrophoresis and transferred to PVDF Hybond-P membrane (GE Healthcare). Membranes were incubated with 5% skim milk in Tris-buffered saline with Tween 20 (TBST) buffer for 1 h and then the membranes being gently rotated overnight at 4 °C. Membranes were then incubated with primary antibodies against Bcl-2, Bax, Cleaved-caspase-9, Cleaved-caspase-3 and DHFR for overnight at 4 °C. Membranes were next incubated with peroxidase labeled secondary anti-bodies for 2 h, and then all membranes were washed with TBST three times for 15 min and the protein blots were detected with chemiluminescence reagent (Thermo Fischer Scientifics Ltd.). The X-ray films were developed with developer and fixed with fixer solution.

4.13. Statistical analysis

All statistical analysis was performed with SPSS Version 10. Data was analyzed by one-way ANOVA. Mean separations were performed using the least significant difference method. Each experiment was replicated thrice, and all experiments yielded similar results. Measurements from all the replicates were combined, and treatment effects were analyzed.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This research was financially supported by National Key Basic Research Program of China (grant No. 2013CB933904), by Natural Science Foundation of China (Grant Nos. 81671745, 21431001 and 81760626), by Natural Science Foundation of Guangxi Province

(AB17292075) and Guangxi Funds for Distinguished Experts, by Industry Project of Jiangsu Science-technology Support Plan (grant No. BE2013840), by Science and Technology Development Program of Suzhou (grant No. ZXY201412).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2018.12.036.

References

[1] H.E. Wheeler, M.L. Maitland, M.E. Dolan, N.J. Cox, M.J. Ratainet, Nat. Rev. Genet. 14 (2013) 23.
[2] S.W. Tait, D.R. Green, J. Cell Sci. 125 (2012) 807.
[3] T. Moldoveanu, A.V. Follis, R.W. Kriwacki, D.R. Green, Trends. Biochem. Sci. 39 (2014) 101.
[4] S. Fulda, L. Galluzzi, G. Kroemer, Nat. Rev. Drug. Discov. 9 (2010) 447.
[5] X. Ma, N. Gong, L. Zhong, J. Sun, X.J. Liang, Biomaterials 97 (2016) 10.
[6] Z. Wang, Y.Z. Chen, H. Zhang, Y.W. Li, Y.F. Ma, J. Huang, X.L. Liu, F. Liu, T.X. Wang, X. Zhang, Bioconjugate Chem. 29 (2018) 2415.
[7] D.C. Wallace, Nat. Rev. Cancer 12 (2012) 685.
[8] S.T. Xu, H. Yao, S.S. Luo, Y.K. Zhang, D.H. Yang, D.H. Li, G.Y. Wang, M. Hu, Y.Y. Qiu, X.M. Wu, H.Q. Yao, W.J. Xie, Z.S. Chen, J.Y. Xu, J. Med. Chem. 60 (2017) 1449.
[9] Z. Gao, X. Kang, Y. Ju, C.L. Xu, Cytotechnology 64 (2012) 421.
[10] D. Trachootham, J. Alexandre, P. Huang, Nat. Rev. Drug Discov. 8 (2009) 579.
[11] V. Gogvadze, Curr. Pharm. Des. 17 (2011) 4034.
[12] N.M. Sakhrani, H. Padh, Drug Des. Devel. Ther. 7 (2013) 585.
[13] J. Zielonka, J. Joseph, A. Sikora, M. Hardy, O. Ouari, J. Vasquez-Vivar, G. Cheng, M. Lopez, B. Kalyanaraman, Chem. Rev. 117 (2017) 10043.
[14] L. Rajendran, H.J. Knölker, K. Simons, Nat. Rev. Drug Discov. 9 (2010) 29.
[15] G.G.M. D'Souza, M.A. Wagle, V. Saxena, S. Vaibhav, S. Anee, Biochim. Biophys. Acta, Bioenerg. 1807 (2011) 689.
[16] V. Gogvadze, S. Orrenius, B. Zhivotovsky, Trends Cell Biol. 18 (2008) 165.
[17] K.Y. Kim, H. Jin, J. Park, S.H. Jung, J.H. Lee, H. Park, S.K. Kim, J. Bae, J.H. Jung, Nano Res. 11 (2018) 1082.
[18] M. Millard, J.D. Gallagher, B.Z. Olenyuk, N. Neamati, J. Med. Chem. 56 (2013) 9170.
[19] D.A. Nedopekina, R.R. Gubaidullin, V.N. Odinokov, P.V. Maximchik, B. Zhivotovsky, Y.P. Bel'skii, V.A. Khazanov, A.V. Manuylova, V. Gogvadze, A.Y. Spivak, Med. Chem. Commun. 8 (2017) 1934.
[20] J.A. Jara, V. Castro-Castillo, J. Saavedra-Olavarria, L. Peredo, M. Pavanni, F. Jaña, M.E. Letelier, E. Parra, M.I. Becker, A. Morello, U. Kemmerling, J.D. Maya, J. Ferreira, J. Med. Chem. 57 (2014) 2440.
[21] Y. Ye, T. Zhang, H. Yuan, D.F. Li, H.X. Lou, P.H. Fan, J. Med. Chem. 60 (2017) 6353.
[22] G.M. Cragg, D.J. Newman, Biochim. Biophys. Acta 1830 (2013) 3670.
[23] D.J. Newman, G.M. Cragg, K.M. Snader, J. Nat. Prod. 66 (2003) 1022.
[24] M. Chudzik, I. Korzonek-Szlacheta, W. Krol, Molecules 20 (2015) 1610.
[25] M.K. Shanmugam, A.H. Nguyen, A.P. Kumar, B.K.H. Tan, G. Sethi, Cancer Lett. 320 (2012) 158.
[26] C.Y. Wang, T.C. Kao, W.H. Lo, G.C. Yen, J. Agric. Food Chem. 59 (2011) 7726.
[27] L. Badam, J Commun Dis. 29 (1997) 91.
[28] B. Xu, G.R. Wu, X.Y. Zhang, M.M. Yan, R. Zhao, N.N. Xue, K. Fang, H. Wang, M. Chen, W.B. Guo, P.L. Wang, H.M. Lei, Molecules. 22 (2017) 924.
[29] R. Csuk, S. Schwarz, R. Kluge, D. Ströhl, Eur. J. Med. Chem. 45 (2010) 5718.
[30] D. Liu, D. Song, G. Guo, R. Wang, J.L. Lv, Y.K. Jing, L.X. Zhao, Bioorg. Med. Chem. 15 (2007) 5432.
[31] S. Schwarz, R. Csuk, Bioorg. Med. Chem. 18 (2010) 7458.
[32] C. Gauthier, J. Legault, M. Lebrun, P. Dufour, A. Pichette, Bioorg. Med. Chem. 14 (2006) 6713.
[33] J. Atsuzaki, M. Taniguchi, K.F. Bastow, Bioorg. Med. Chem. 15 (2007) 6193.
[34] Y.C. Huang, C.L. Kuo, K.W. Lu, J.J. Lin, J.L. Yang, R.S.C. Wu, P.P. Wu, J.G. Chung, Molecules 21 (2016) 872.
[35] L. Jin, R.Z. Huang, X.C. Huang, B. Zhang, M. Ji, H.S. Wang, Bioorg. Med. Chem. 26 (2018) 1759.
[36] G. Sharma, S. Kar, S. Palit, P.K. Das, J. Cell. Physiol. 227 (2012) 1923.
[37] J.J. Chen, J.X. Ding, W.G. Xu, T.M. Sun, H.H. Xiao, X.L. Zhuang, X.S. Chen, Nano Lett. 17 (2017) 4526.
[38] L.L. Ma, R. Ma, Z.G. Wang, S.M. Yiu, G.Y. Zhu, Chem. Commun. 52 (2016) 10735.
[39] D.R. Green, G. Kroemer, Science 305 (2005) 626.
[40] K. Sinha, J. Das, P.B. Pal, P.C. Sil, Arch. Toxicol. 87 (2013) 1157.
[41] A. Zheng, H. Li, X. Wang, Z.H. Feng, J. Xu, K. Cao, B. Zhou, J. Wu, J.K. Liu, Curr. Cancer Drug Targets 14 (2014) 156.
[42] J.S. Modica-Napolitano, V. Weissig, Int. J. Mol. Sci. 16 (2015) 17394.
[43] J.S. Modica-Napolitano, M. Kulawiec, K.K. Singh, Curr. Mol. Med. 7 (2007) 121.