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Design, synthesis, and biofunctional evaluation of novel pentacyclic triterpenes bearing *O*-[4-(1-piperazinyl)-4-oxo-butyryl moiety as antiproliferative agents

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ABSTRACT

A series of pentacyclic triterpenoids derivatives bearing *O*-[4-(1-piperazinyl)-4-oxo-butyryl moiety has been synthesized and investigated for their potential antiproliferative activities. Pentacyclic triterpenoids derivative compounds were synthesized by a four or six step synthetic procedure. The activity studies were evaluated using Cell Counting Kit-8 method, and Western blotting analysis on A549 cells, MCF-7 cells and Hela cells. Compounds methyl 3-*O*-[4-(1-piperazinyl)-4-oxo-butyryl]olean-12-ene-28-oate (**OA-4**) and compound 2-*O*-[4-(1-piperazinyl)-4-oxo-butyryl]-3,23-dihydroxyurs-12-ene-28-oate (**AA-5**) were found to be promising antiproliferative agents. These compounds show potentiality for further optimization as antitumor drugs.

Modern medical science is constantly searching for new and more powerful agents to prevent, treat, or retard cancer. Therefore, a worldwide search for new anticancer drugs of natural origin is underway. An important part of this research field is the application of biosynthetic knowledge to synthesize interesting, natural products (i.e., biomimetic synthesis). To date, approximately 20,000 triterpenoids have been identified from various parts of medicinal plants.[1] The naturally occurring pentacyclic triterpenoids 18-β-glycyrrhetinic acid (GA), asiatic acid (AA), oleanolic acid (OA), and ursolic acid (UA) can inhibit tumor initiation and growth, and induce apoptosis in various cancer cells.[2–13] They all contain hydroxyl and carboxylic groups, as shown in Figure 1. Numerous attempts have been made to obtain potent synthetically feasible analogs of pentacyclic triterpenoids with a heteroatom incorporated in positions on the triterpenoid skeleton. Previous Letters have suggested that the activity of these pentacyclic triterpenoids is related to their basic triterpenoid skeletal structure, and the attached functional groups offer opportunities for chemical modification and improvement of activity.[13–16] Dicarboxylic acid hemiesters of pentacyclic triterpenoid such as UA, OA, and betulinic acid showed more potent inhibitory activity on the human immunodeficiency virus (HIV)-protease.[17]

Previous studies in our group on AA and related analogs showed that structurally modified compounds could inhibit the growth of human non-small cell lung cancer (NSCLC) A549 and PC9/G (acquired resistance to gefitinib) cell lines in vitro, when the carboxylic group at C-28 of AA was converted to methyl ester, and butanedioic acid was introduced into the hydroxyl group at the C-2 position.[18] These results encouraged us to select GA, AA, OA, and UA as templates, and to design a series of pentacyclic triterpenoids analogs.

Piperazine derivatives possess pharmacological properties. Many currently notable anticancer agents, including imatinib (STI571),[19] dasatinib (BMS-354825),[20] bosutinib (SKI-606),[21] danusertib (PHA-739358),[22] and VX-680,[23] contain a piperazine ring as part of their molecular structure.

In the present study, functional groups attached to pentacyclic triterpene molecules were modified to generate derivatives, while maintaining the pentacyclic triterpene skeletal structure. As a result of these modifications, the carboxylic group was converted to a methyl ester with retention of stereochemistry, which may significantly influence its physical properties or receptor interactions. Piperazine and butanedioic acid were introduced into the

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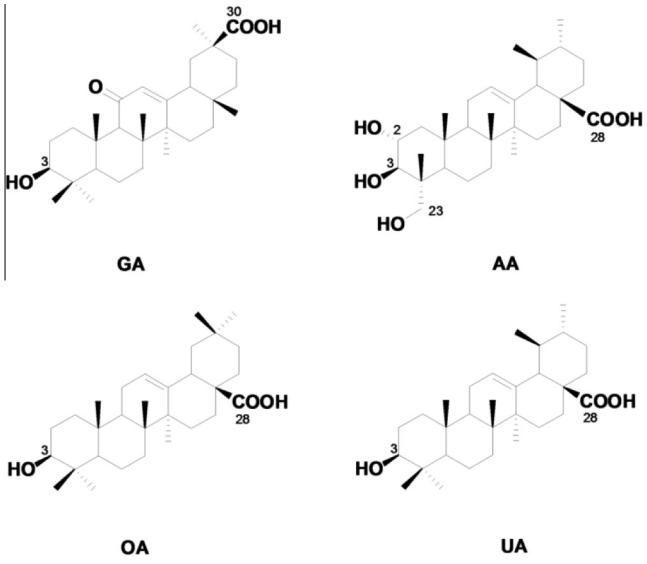


Figure 1. Chemical structures of pentacyclic triterpenoids.

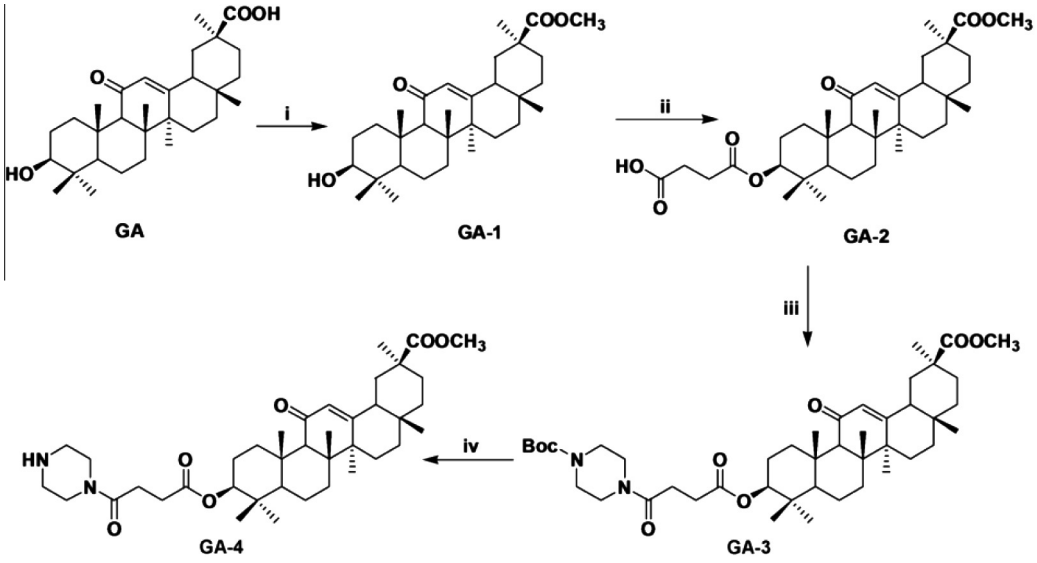
hydroxyl group at the C-2 position of AA, and at the C-3 positions of GA, OA, and UA. The dihydroxyl groups at C-3 and C-23 of AA were protected with acetonide using 2,2-dimethoxy propane. In addition, we described the synthesis and cytotoxicity evaluation of the derivatives. The effect of the novel compounds on cell cycle blockage was also analyzed.

Pentacyclic triterpenoids GA, AA, OA, and UA were used as lead compounds, and the structural modifications were made at positions C-3, C-30 of GA, C-2, C-3, C-23, C-28 of AA, and C-3 and C-28 of OA and UA, respectively. Similar procedures were used to synthesize derivatives of GA, OA, and UA, and the synthetic pathways are presented in Schemes 1–4. The carboxylic groups (C-30 of GA, and C-28 of OA and UA) were subjected to methyl iodide treatment in the presence of potassium carbonate in dimethyl formamide (DMF) to create methyl ester moieties with retention of stereochemistry (**GA-1**, **OA-1**, and **UA-1**). The hydroxyl groups at C-3 of these pentacyclic triterpenoids were treated with butanedioic anhydride in the presence of 4-dimethylaminopyridine (DMAP) in dichloromethane (CH_2Cl_2) to generate intermediates (**GA-2**, **OA-2**, and **UA-2**). Coupling reactions of the intermediates with *N-tert*-butoxycarbonylpiperazine and DMAP were performed in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) in CH_2Cl_2 to obtain compounds **GA-3**, **OA-3**, and **UA-3**. The protecting group was then removed by a treatment with a trifluoroacetic acid/dichloromethane (TFA/CH_2Cl_2) mixture to result in the deprotected analogs (**GA-4**, **OA-4**, and **UA-4**). AA preparation and synthesis of its derivatives (**AA-1**, **AA-2**, and **AA-3**) were performed as previously described.[18] As shown in Scheme 2, the dihydroxyl groups at C-3 and C-23 of AA were treated with 2, 2-dimethoxypropane ($(CH_3)_2C(OCH_3)_2$) in the presence of *p*-toluenesulfonic acid (*p*-TsOH) in DMF to generate acetonide. The carboxylic group at C-28 was converted to a methyl ester, and the hydroxyl group at C-2 was treated with succinic anhydride using the same synthetic pathways as used for other pentacyclic triterpenoids to obtain compounds **AA-4** and **AA-5**. All of the compounds were dissolved in dimethyl sulfoxide (DMSO) prior to use in biological activity assays.

We compared the inhibitory effects of the four pentacyclic triterpenoids (GA, AA, OA, and UA) and 17 analogs against three cancer cell lines: MCF-7 (human breast cancer cells), Hela (human cervical carcinoma cells), and A549 (human NSCLC cells). The screening procedure was performed using the Cell Counting Kit-8 (CCK-8) (Dojindo, Kumamoto, Japan), and the results are summarized in Table 1.

The pentacyclic triterpenoids derivatives with a piperazine group (**GA-4**, **AA-5**, **OA-4**, and **UA-4**) showed much stronger growth inhibitory effects than their corresponding analogs with IC_{50} values from 7.05 to 13.13 μM in the three cancer cells. However, **GA-2**, **AA-3**, **OA-2**, and **UA-2**, which contain a substituted butanedioic acid, displayed weak cytotoxicity against the cancer cell lines with IC_{50} values from 26.03 to more than 100 μM. The pronounced influence of the incorporation of piperazine on antiproliferative effects in the series of synthesized compounds could be explained on the capacity for the formation of hydrogen bonds, improvement of water-solubility, and adjustment of molecular physicochemical properties.

To evaluate the cytotoxic mechanism of **AA-5** and **OA-4** against MCF-7, Hela, and A549 cells and to gain further insight into the



Scheme 1. Synthesis of glycyrrhetinic acid derivatives. Reagent and conditions: (i) CH_3I, K_2CO_3, DMF, rt; (ii) butanedioic anhydride, DMAP, CH_2Cl_2, reflux; (iii) *N-tert*-butoxycarbonylpiperazine, EDCI, DMAP, CH_2Cl_2, rt; (iv) TFA, CH_2Cl_2, rt.

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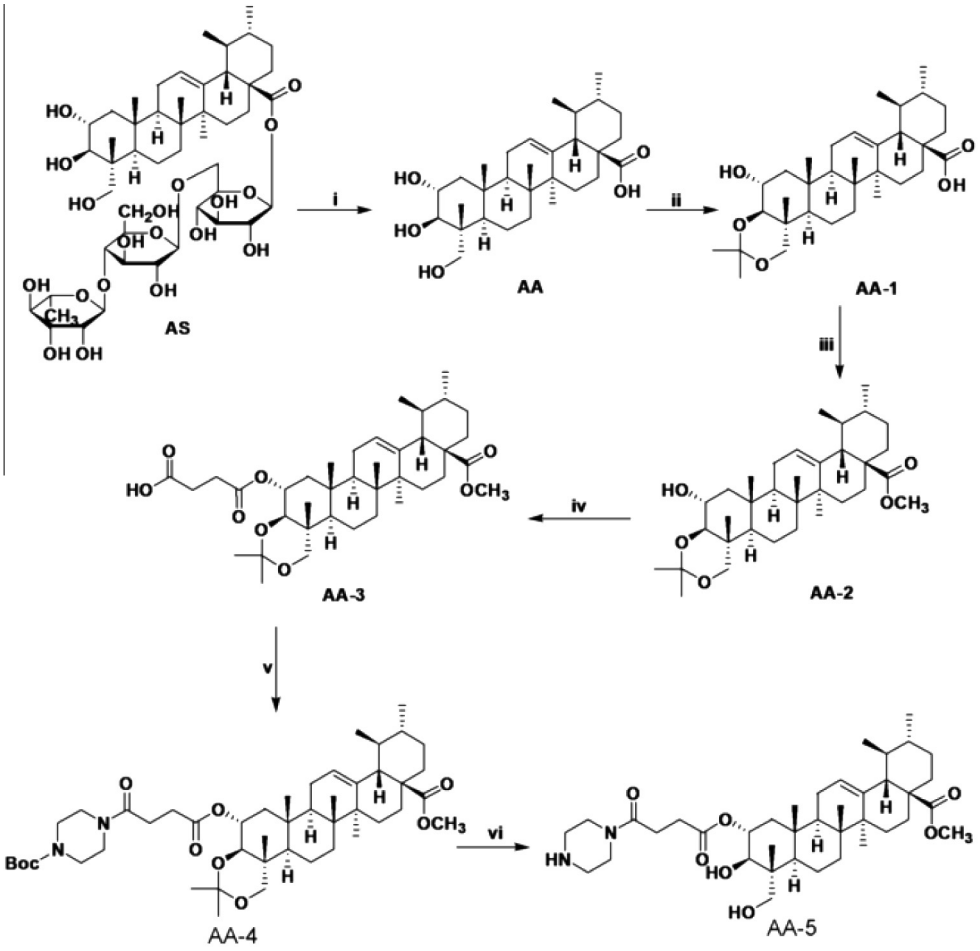
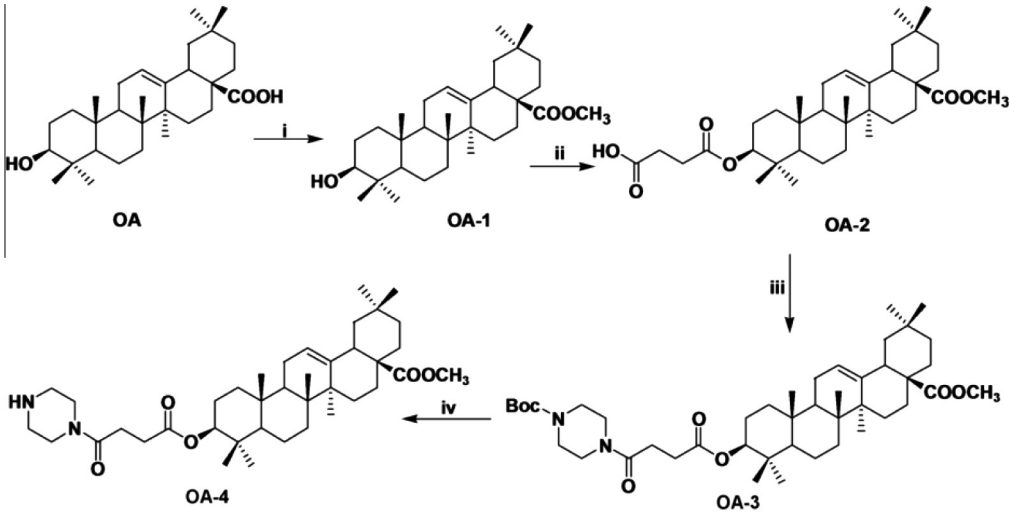


Scheme 2. Synthesis of asiatic acid derivatives. Reagent and conditions: (i) 5*N*-NaOH, MeOH, reflux; (ii) $(CH_3)_2C(OCH_3)_2$, *p*-TsOH, DMF, rt; (iii) CH_3I, K_2CO_3, DMF, rt; (iv) butanedioic anhydride, DMAP, CH_2Cl_2, reflux; (v) *N-tert*-butoxycarbonylpiperazine, EDCI, DMAP, CH_2Cl_2, rt; (vi) TFA, CH_2Cl_2, 1 N HCl, CH_3OH, rt.



Scheme 3. Synthesis of oleanolic acid derivatives. Reagent and conditions: (i) CH_3I, K_2CO_3, DMF, rt; (ii) butanedioic anhydride, DMAP, CH_2Cl_2, reflux; (iii) *N-tert*-butoxycarbonylpiperazine, EDCI, DMAP, CH_2Cl_2, rt; (iv) TFA, CH_2Cl_2, rt.

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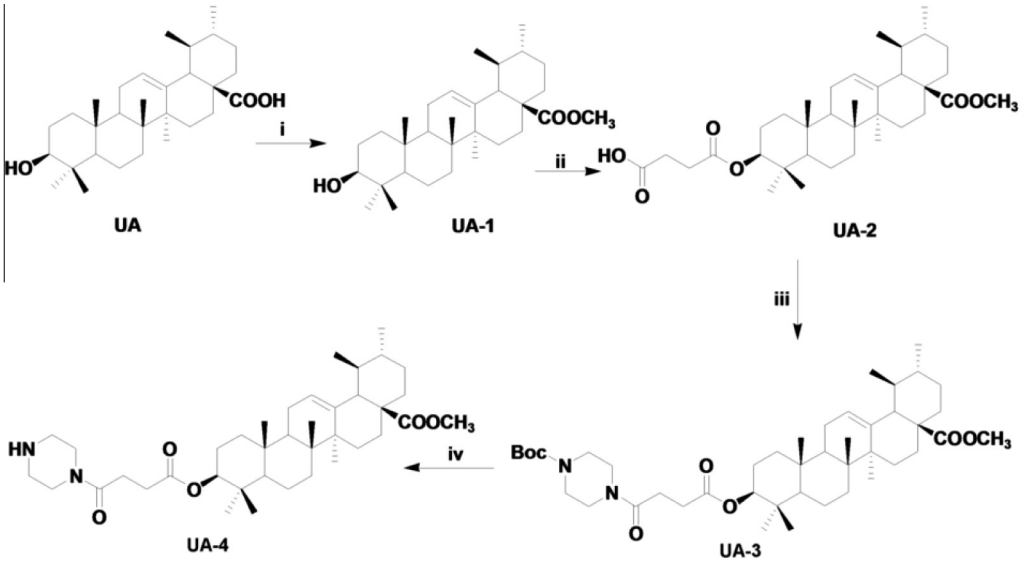

Scheme 4. Synthesis of ursolic acid derivatives. Reagent and conditions: (i) CH_3I, K_2CO_3, DMF, rt; (ii) butanedioic anhydride, DMAP, CH_2Cl_2, reflux; (iii) *N-tert*-butoxycarbonylpiperazine, EDCI, DMAP, CH_2Cl_2, rt; (iv) TFA, CH_2Cl_2, rt.

Table 1
Cytotoxicity of compounds

Compounds	IC_{50}[a] (μM)		
	MCF-7	Hela	A549
GA	>100	>100	>100
GA-1	>100	>100	>100
GA-2	>100	>100	>100
GA-3	>100	>100	>100
GA-4	17.74 ± 2.33	18.72 ± 2.54	15.14 ± 3.02
AA	67.58 ± 6.21	91.07 ± 10.14	>100
AA-1	59.85 ± 7.84	60.04 ± 5.87	34.73 ± 1.74[b]
AA-2	>100	>100	48.86 ± 2.93[b]
AA-3	45.55 ± 5.97	55.63 ± 7.63	26.03 ± 2.47[b]
AA-4	21.12 ± 3.29	23.75 ± 2.87	33.61 ± 4.12
AA-5	7.58 ± 1.25	8.13 ± 1.69	13.13 ± 4.37
OA	>100	>100	>100
OA-1	>100	>100	>100
OA-2	55.71 ± 4.81	57.81 ± 6.62	51.84 ± 4.94
OA-3	>100	>100	>100
OA-4	7.05 ± 1.45	7.75 ± 1.09	9.91 ± 2.11
UA	44.84 ± 5.41	64.48 ± 6.75	74.65 ± 9.42
UA-1	>100	>100	>100
UA-2	36.04 ± 3.25	46.68 ± 3.68	74.24 ± 8.77
UA-3	50.16 ± 4.39	>100	>100
UA-4	10.72 ± 2.24	13.07 ± 1.98	17.63 ± 2.54
Gefitinib	17.83 ± 7.85	15.40 ± 4.63	11.02 ± 3.27

Human tumor cells were treated with different concentrations of samples for 48 h ($n = 3$ independent experiments).
[a] Data are presented as IC_{50} (μM, the concentration of 50% proliferation-inhibitory effect).
[b] Cited from Ref. 18.

mode of action, we systematically assessed cell cycle signal checkpoint proteins, including p16, p21, CDK1, Cyclin D1, and Cyclin B in MCF-7, Hela, and A549 cells after treating the cells with or without the analogs. p16 and p21 play an important role in cell cycle regulation by decelerating the progression of cells from G1 to S phase, and therefore act as a tumor suppressor that is implicated in the prevention of cancers. As shown in Figure 2, **AA-5** and **OA-4** at 15 μM induced the significant expression of p16 and p21 compared with the control in the three cell lines. In contrast, **AA-5** and **OA-4** inhibited the expression of Cyclin D1, Cyclin B, and CDK1.

In conclusion, a series of pentacyclic triterpenoid derivatives bearing the *O*-[4-(1-piperazinyl)-4-oxo-butyryl moiety were synthesized and investigated for their cytotoxic potential against three human cancer cell lines. Most of these compounds showed significant antiproliferative effects compared to their original compounds when tested in MCF-7, Hela, and A549 cells using the CCK-8 assay. In particular, compounds methyl 3-*O*-[4-(1-piperazinyl)-4-oxo-butyryl]olean-12-ene-28-oate (**OA-4**) and 2-*O*-[4-(1-piperazinyl)-4-oxo-butyryl]-3,23-dihydroxyurs-12-ene-28-oate (**AA-5**) showed the highest inhibitory activity against three human cancer cell lines. Furthermore, **OA-4** and **AA-5** can induce cell cycle arrest in all of the three cells. Therefore, as indicated by our results, compounds **OA-4** and **AA-5** were identified as promising candidate drugs.

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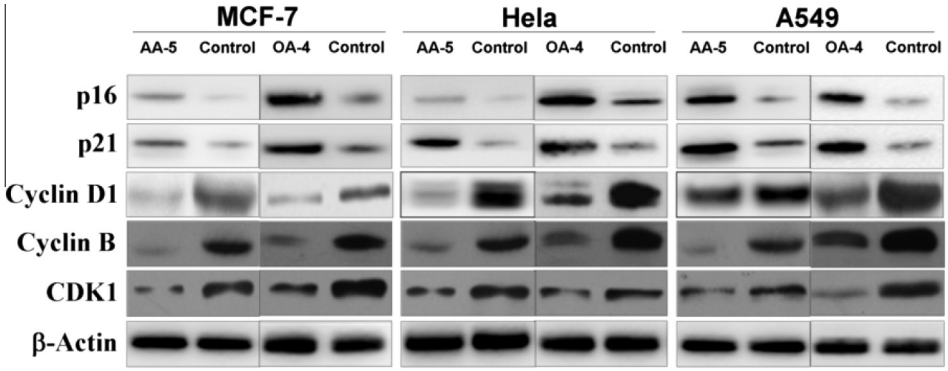

Figure 2. Effects of **AA-5** and **OA-4** on cell cycle related proteins in MCF-7 (human breast cancer cells), Hela (human cervical carcinoma cells), and A549 (human NSCLC cells). Cells were treated for 48 h with 15 μM of **AA-5** and **OA-4** and the expressions of cell cycle signal checkpoint proteins, including p16, p21, Cyclin D1, Cyclin B, and CDK1 were analyzed by Western blot. DMSO (0.1%) was employed as negative control.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2015.08.076.

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