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Discovery of FZU-03,010 as a self-assembling anticancer amphiphile for acute myeloid leukemia

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ABSTRACT

Recently various drug candidates with excellent anticancer potency have been demonstrated, whereas their clinical application largely suffers from several limitations especially poor solubility. Ursolic acid (UA) as one of ubiquitous pentacyclic triterpenes in plant kingdom exhibited versatile antiproliferative effects in various cancer cell lines. However, the unfavorable pharmaceutical properties became the main obstacle for its clinical development. With the aim of development of novel derivatives with enhanced potency, a series of diversified UA amphiphiles have been designed, synthesized, and pharmacologically evaluated. Amphiphile **10** (**FZU-03,010**) with significant improved antiproliferative effect can self-assemble into stable nanoparticles in water, which may serve as a promising candidate for further development.

Recently, natural products attract increasing attention in academic institutions and pharmaceutical industry due to their unparalleled resources, and diverse interesting biological characteristics.[1–3] Among them, pentacyclic triterpene compounds widely exist throughout nature, possessing considerable pharmacological activities, especially anticancer potency.[4,5] Therefore, they are expected to serve as a pivotal resource to develop novel promising drug candidates targeting diverse signaling pathways.[6,7] Ursolic acid (UA, Fig. 1) as one of the pentacyclic triterpenes is ubiquitous in plant kingdom.[8] UA is particularly valuable for its versatile bioactivities including anti-inflammatory, antioxidative, anticancer, antibacterial, and sedative activities.[9] Its significant anticancer and fascinating chemoprevention effects have been intensively studied. Recent studies revealed that UA is involved in a series of the activities of anticancer, such as induction of cancer cell apoptosis, prevention of tumorigenesis, and inhibition of cancer cell proliferation.[10] In addition, UA has been demonstrated to significantly enhance immune function of human body.[11] All these findings indicate that UA has great potential for clinical application. However, UA suffers from poor water solubility, rapid metabolism and low bioavailability, limiting its further clinical development.[12,13]

Despite these limitations, UA possesses the common structural characteristic of the plant-derived triterpenoids bearing two polar groups separated by a non-polar spacer of appropriate length.[14] This inherent amphiphilic feature renders it as one of the ideal building blocks for development of self-delivering amphiphiles with improved anticancer potency through self-assembly. Due to the diversity spacers and unique spatial structure arrangements, Bag, Ju and Hu, as well as Mezzenga studied the assembly behaviors of triterpenoids especially focusing on supramolecular recognition, assembly and diverse supramolecular architectures (including fibers, vesicles, flowers, helices, and spheres).[15–17] For example, Ju reported a novel uracil-appended glycyrrhetinic acid conjugate and fully investigated its gelation characteristics as well as the gel-to-sol phase transition process.[17]

Recently, construction of complex amphiphilic drug–drug conjugates (ADDCs) is considered as an emerging strategy for the enhancement of therapeutic efficacy *via* simple conjugation of a hydrophilic anticancer drug with a hydrophobic one through a biodegradable bond.[18] For example, Yan and Zhu synthesized Ir–Cb conjugate consisting of the hydrophilic anticancer drug irinotecan (Ir) and the hydrophobic anticancer drug chlorambucil (Cb) via a hydrolyzable ester linkage.[19] This complex conjugate could self-assemble into nanoparticles in water and facilitated the accumulation of drugs in tumor tissues, eventually resulting in significant improvement of therapeutic efficacy.[19]

Owing to the unique properties of self-assembled nanoparticles and higher accumulation in tumors *via* the enhanced permeation

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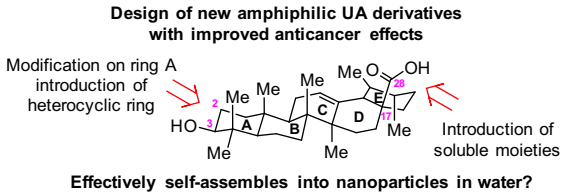


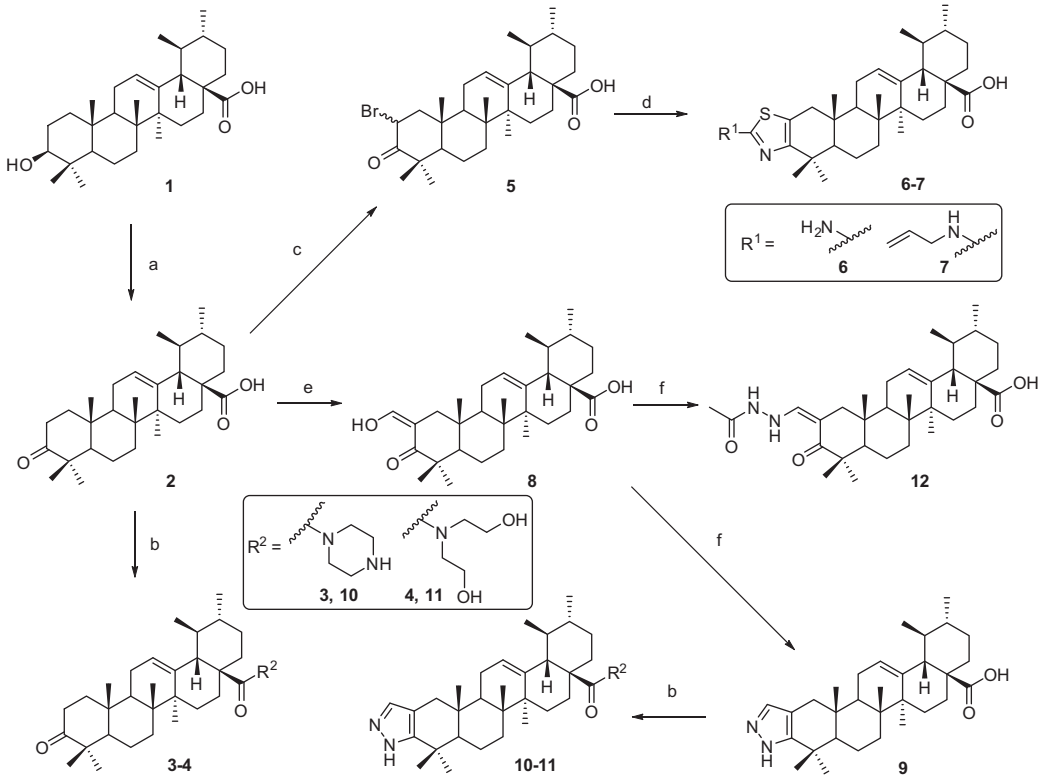
Fig. 1. Drug design self-delivery strategy for new amphiphilic UA derivatives with improved anticancer effects.

and retention (EPR) effect, amphiphiles with improved anticancer potency can enhance permeability and reduce side effects.[18] The skeleton of UA is lined with readily accessible A ring and carboxyl group at the position 28, which allows for the facile introduction of functional moieties to promote reversible and directional interactions and engender supramolecular assemblies. With these considerations in mind, we hypothesized that amphiphilic UA derivatives consisting of the hydrophilic privilege fragments around A-ring and hydrophobic tail at the 28-position might solve the limitation of poor solubility of UA.

In this work, we designed and synthesized a series of novel amphiphilic UA derivatives with favorable physicochemical properties which were associated with incorporating nitrogen into the core scaffold and evaluated their anticancer activities. Among them, UA derivative **10** (**FZU-03,010**) could be a promising anticancer agent for its highly inhibitory activity against human leukemia cells. To our knowledge, such a promising strategy through development of amphiphilic UA derivatives to self-assemble into its own nanostructures for the enhancement of anticancer efficacy has not been reported to date.

Intensive researches demonstrated that various biological properties of pentacyclic triterpenes are related to the C-3 hydroxyl group.[20–23] In this respect, a variety of structure modifications of UA on C-3 position have been conducted to improve the biological activity.[24–26] The heterocyclic ring derivatives have been proved possess highly anticancer effects. To date, increasing studies have demonstrated that a variety of UA derivatives with structural modifications on A ring possess effective improvement of anticancer effects.[27,28] Accumulating investigation revealed that pentacyclic triterpenes with thiazole and pyrazole moiety at A-ring are expected to possess enhanced anticancer potency.[29,30] In addition, accumulating evidence indicated that the introduction of suitable moieties at the 28-position of UA would significantly enhance the anticancer activity.[31,32] Therefore, our efforts first focused on modification at C-2 and C-3 positions followed by introduction of the privileged fragments at the 28-position (Fig. 1) to synthesize amphiphilic UA derivatives.

The synthetic routes to new UA derivatives reported in this work are outlined in Scheme 1. Following the literature procedures, the key intermediate 3-keto UA (**2**) was prepared by the oxidation of UA (**1**) with Jones reagent in 90% yield. The compounds **3** and **4** were obtained in a two-step synthesis starting from 3-keto UA (**2**). The C-28 chloride of UA analogues was easily prepared from 3-keto UA (**2**) in oxalyl chloride in CH_2Cl_2, and then treated with piperazine or diethanolamine to give the corresponding analogues **3** and **4**, respectively. Notably, the moieties of piperazine and diethanolamine have been demonstrated to enhance potency and drug-like properties.[24,33] Bromination of **2** with $PyHBr_3$ in THF led to obtain the intermediate **5** in 88% yield. Hantzsch reaction of **5** with thiourea or allyl thiourea in the refluxing ethanol directly afforded corresponding derivatives **6** and **7** in the yield of 62% and 60%, respectively. Compound **8** was obtained by Claisen



Scheme 1. Reagents and conditions: (a) Jones reagent, acetone, rt, 2 h, 90%; (b) (i) oxalyl chloride, CH_2Cl_2, rt, 24 h; (ii) corresponding amines, EtOAc, rt, 3 h, 38–67%; (c) $PyHBr_3$, THF, rt, 16 h, 88%; (d) corresponding thioureas, EtOH, reflux, 5 h, 60–62%; (e) CH_3ONa, ethyl formate, benzene, rt, 12 h, 80%; (f) corresponding hydrazines, EtOH, reflux, 6 h, 80–85%.

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Table 1
Effects of newly synthesized compounds on proliferation of human promyelocytic leukemia (HL-60) cells.

No	cLogP[a]	TPSA[b]	Inhibitory effect (%)[c]							IC_{50} (μM)[c]
			0.63 μM	1.25 μM	2.5 μM	5 μM	10 μM	20 μM	40 μM	
1	6.48	57.5	N/A[d]	N/A	N/A	N/A	N/A	N/A	N/A	>40
2	6.37	54.4	N/A	N/A	N/A	N/A	N/A	N/A	N/A	>40
3	5.82	49.4	10.6[e]	22.5	43.7	82.2	92.7	92.5	94.8	2.64 ± 0.12
4	5.23	77.8	9.32	8.43	12.4	26.7	53.7	86.5	92.7	7.04 ± 1.37
6	6.73	76.2	N/A	N/A	N/A	N/A	N/A	12.9	59.2	~40
7	7.70	62.2	N/A	0.33	4.73	13.4	20.3	34.7	49.8	>40
8	5.98	74.6	9.03	18.5	33.9	50.3	67.0	72.4	89.8	5.44 ± 0.08
9	6.61	66.0	5.36	7.54	8.55	16.3	79.5	93.2	94.7	6.26 ± 1.61
10	6.07	61.0	14.7	66.4	86.0	91.6	95.0	93.5	94.0	0.91 ± 0.05
11	5.41	89.4	3.54	2.47	19.3	64.8	86.8	94.0	93.6	5.21 ± 1.41
12	6.63	72.2	9.96	20.7	28.8	41.7	61.7	69.7	93.1	5.64 ± 1.33
Doxorubicin (ADR)			80.8	82.5	85.6	95.4	>99	>99	>99	<0.63

[a] Average cLogP: http://146.107.217.178/lab/alogps/start.html.
[b] TPSA: http://www.molinspiration.com/cgi-bin/properties.
[c] The values are the mean ± SE of at least three independent experiments.
[d] N/A: no activity.
[e] The values are mean of at least three independent experiments.

condensation of **2** with ethyl formate in 80% yield. Treatment of **8** with hydrazine in EtOH under reflux for 6 h afforded pyrazole derivative **9**. To access compounds **10** and **11**, the pyrazole derivative **9** was submitted to the same reaction condition as the synthesis of **3** and **4**. Treatment of **8** with acetohydrazide provided compound **12** in the yield of 85%.

The calculated lipophilicity (cLogP) and topological polar surface area (TPSA) values of synthesized UA derivatives are listed in Table 1. The result suggests that most of these new compounds are conformed to the criteria of Lipinski's "Rule of Five" and may have good physicochemical properties. In the present study, to explore a meaningful SAR and examine how the substitutions on the key moieties affect biological activities of newly UA derivatives, the antiproliferative potency of these analogues was evaluated on the proliferation of human promyelocytic leukemia (HL-60) cells.[34,35] The capability of novel synthesized analogues to inhibit the growth of HL-60 cells is summarized in Table 1.

The key intermediate **2** did not show any antiproliferative effect even at 40 μM. After introduction of soluble moieties into the derivative **2** at position 28, compound **3** with piperazine moiety exhibited significantly improved antiproliferative effect with an IC_{50} value of 2.64 μM and compound **4** showed a slightly enhanced potency, indicating that appropriate modifications on 28-position may improve the antiproliferative activity. It was disappointed to find that the thiazole derivatives **6** and **7** were inactive with a dramatic loss of antiproliferative activity. Therefore, more extensive SAR study on thiazole ring was not pursued. Noteworthy, the pyrazole derivative **9** displayed significantly enhanced potency. Thus we turned our extensive SAR efforts on the scaffold of compound **9**. As expected, amphiphilic UA derivative **10** (**FZU-03,010**) with piperazine moiety displayed an IC_{50} value of 0.91 μM against HL-60 cells, while derivative **11** showed a slight enhanced potency with an IC_{50} value of 5.21 μM. Notably, compound **12** also exhibited significant improved antiproliferative effect, suggesting further structural modifications are needed.

After adding deionized water into the DMSO solution of compound **10**, DMSO was removed by dialysis and a stable and bluish nanoparticle solution with the final concentration of 1.5 mg/mL was obtained (Fig. 2A). To confirm that UA derivative **10** can form nanoparticles, the dynamic light scattering (DLS) measurement was used to study the size of nanoparticles. The DLS results in Fig. 2B showed that compound **10** in aqueous solution forms aggregates and the average hydrodynamic diameter aggregates was about 179.9 nm with a narrow unimodal size distribution

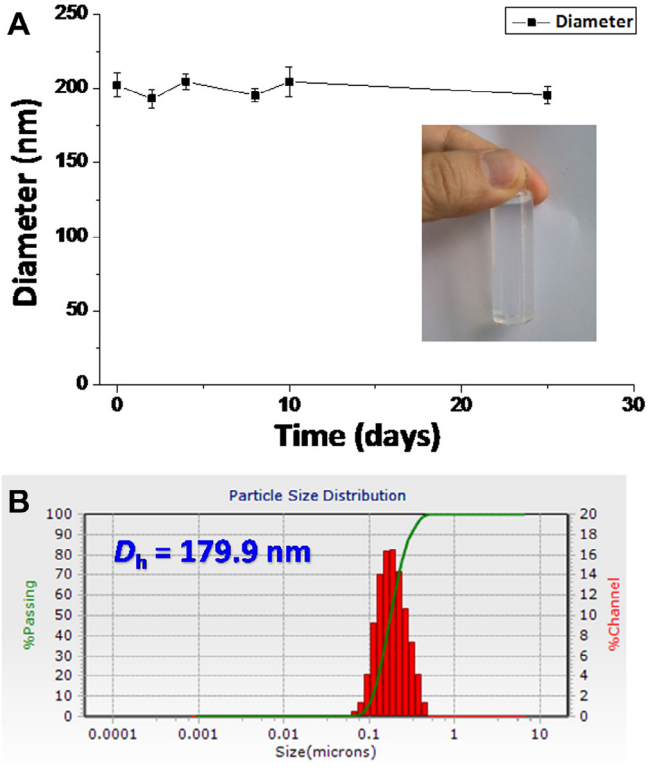

Fig. 2. (A) Relationship between the storage on diameter and time of FZU-03,010 (**10**) nanoparticles; inset: a digital photograph of FZU-03,010 (**10**) nanoparticle solution, exhibiting a stable, transparent bluish solution. (B) DLS plot of FZU-03,010 (**10**) nanoparticles (placed at room temperature for 25 days), which shows the average size (D_h = 179.9 nm).

(data derived from the sample placing at room temperature for 25 days). Besides, the DLS measurements were performed at different time intervals. The results demonstrated that nanoparticles of UA derivative **10** were stable enough for more than three-week storage. All results demonstrated that compound **10** can self-assemble into stable and well-defined nanoparticles. It also noted that UA is a little different to effectively form nanoparticles at the same condition for a large amount of solid was precipitated. One potential reason is that the water solubility of UA is extraordinarily poor, while compound **10** displayed favorable water

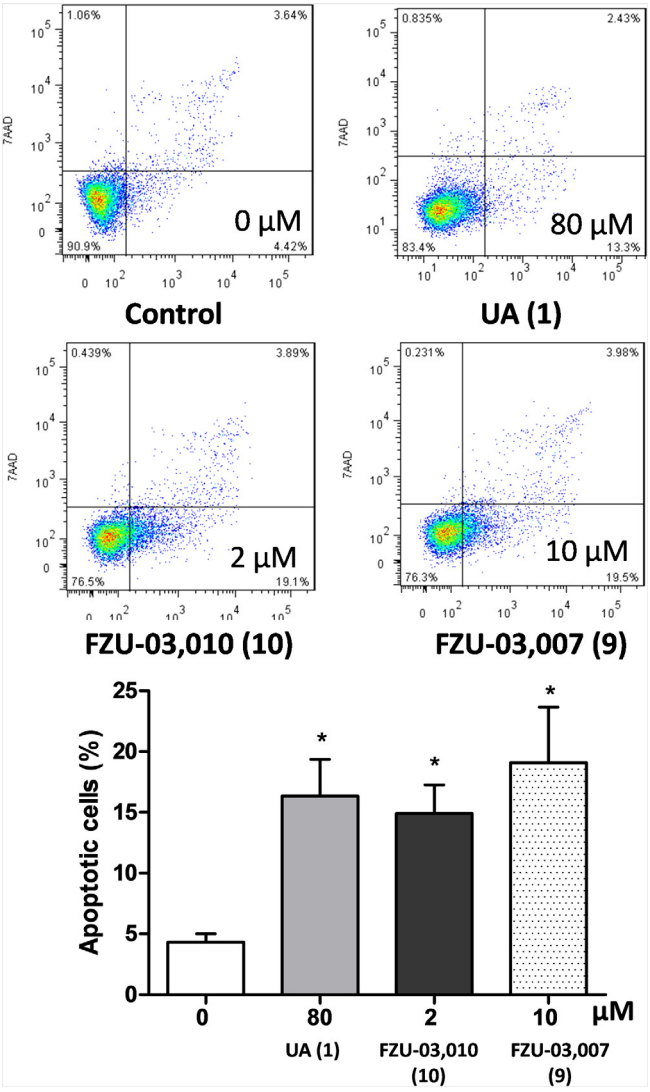

Fig. 3. The apoptotic cell percentages in HL-60 cells after incubation with 80 μM of UA (**1**), 2 μM of FZU-03,010 (**10**), and 10 μM of FZU-03,007 (**9**) for 24 h, respectively.

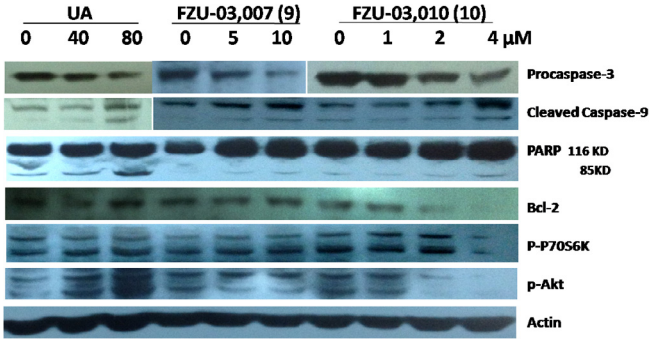

Fig. 4. Western blot analysis of biochemical markers for apoptosis induction and inhibition by UA, FZU-03,007 (**9**), and FZU-03,010 (**10**) in the HL-60 cell line. Cells were treated with UA, FZU-03,007 (**9**), and FZU-03,010 (**10**) at different concentrations, respectively. Levels of procaspase-3, cleaved caspase-9, PARP, Bcl-2, P-P70S6K, and p-Akt were probed by specific antibodies. Actin was used as the loading control.

solubility (as HCl salt, aqueous solubility >0.15 mg/mL, measured by an HPLC method[36]). Through our preliminary studies, compound **10** with favorable nanoscale characteristics was subjected to further biological characterization.

To determine whether amphiphilic UA derivative **10** induces cell apoptosis in HL-60 cells, we next used Annexin V-PE/7-AAD staining assay to investigate the role of UA derivatives on the induction of apoptosis. As shown in Fig. 3, compound **10** significantly induced apoptosis at 2 μM, while UA and compound **9** showed the similar effects at 80 μM and 10 μM, respectively. Despite recent advances in the development of effective molecularly targeted agents, acute myeloid leukemia (AML) still remains a lethal and incurable disease due to drug resistance in the prolonged chemotherapy.[37–39] Therefore, innovative single agent targeting multiple aberrant signaling pathways in AML might provide an additional therapeutic approach for AML.[40,41] To further investigate the potential mechanism of compound **10** induced apoptosis, we next examined the apoptotic signaling pathways involved in HL-60 cells. As shown in Fig. 4, the treatment with UA or UA derivatives (**9** and **10**) reduced the expression level of procaspase-3 and induced the cleavage of caspase-9 in a dose-dependent manner. However, the cleavage of PARP, one of the important caspase-3 substrates, was only slightly induced. Notably, the changes of expression levels of Bcl-2 after the treatment with UA and UA derivatives were different. Treatment with UA increased Bcl-2 expression, while, pyrazole derivatives **9** and **10** markedly decreased the expression of Bcl-2. We next evaluated whether UA derivatives (**9** and **10**) modulates PI3K/Akt signaling pathway for constitutive PI3K/Akt activation is frequently observed in AML.[42–46] Compared with UA treatment group, UA derivatives **9** and **10** markedly reduced phosphorylation of Akt at Ser473 and inhibited mTOR downstream targeted protein p-p70S6K. Our observation indicated that pyrazole derivatives **9** and **10** down-regulated the activation of crucial molecules in PI3K/Akt pathway.

In summary, an appropriate chemical modification of UA scaffold enabled us to identify several potent UA derivatives with improved anticancer potencies as well as enhanced druglike properties. A series of novel diversified analogues displayed improved antiproliferative effects. Among them, amphiphile **10** (**FZU-03,010**) exhibited remarkably growth inhibitory effect against HL-60 cells with an IC_{50} value of 0.91 μM, which is approximately 100-fold more potent than UA. This most potent pyrazole derivative also has demonstrated to modulate the apoptotic signaling pathway as well as PI3K/Akt pathway. Particularly, the data in this study clearly showed that amphiphile **10** with favorable nanoscale characteristics can self-assemble into nanoparticles in an aqueous solution. Design of self-assembled derivatives based on the amphiphilic building block is a promising strategy that can be both practical and inspiring, affording nanostructures with favorable pharmaceutical properties that the initial skeleton structure does not effectively possess.

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A. Supplementary material

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