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Synthesis and antitumor activity of fluorouracil – oleanolic acid/ursolic acid/glycyrrhetic acid conjugates†

Chun-Mei Liu,^a Jia-Yan Huang,^a Li-Xin Sheng,^a
Xiao-An Wen^b and Ke-Guang Cheng ^{*,a}

Due to the obvious adverse effects of 5-fluorouracil that limit its clinical usefulness and considering the diverse biological activities of pentacyclic triterpenes, twelve pentacyclic triterpene-5-fluorouracil conjugates were synthesized and their antitumor activities were evaluated. The results indicated that all the single substitution targeted hybrids (7a–12a) possessed much better antiproliferative activities than the double substitution targeted hybrids (7b–12b). Hybrid 12a exhibited good antiproliferative activities against all the tested MDR cell lines. Furthermore, it was revealed that 12a could induce intracellular calcium influx, the generation of ROS, arrest the cell proliferation at the G1 phase, and activate the apoptotic signaling caspase-8, which eventually activates the apoptotic effector caspase-3 and causes the later nuclear apoptosis.

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Introduction

5-Fluorouracil (5-FU, Fig. 1), as well as the oral pro-drug capecitabine, is a highly effective cell cycle-specific antitumor antimetabolite that interferes with DNA synthesis and inhibits RNA formation.^{1–4} It has been an essential part of the treatment of a wide range of solid tumors, such as esophageal adenocarcinoma, breast cancer, metastatic colorectal cancer and so on, since being introduced into clinical use in the 1950s.^{5–8} However, besides its broad antitumor spectrum, 5-FU also displays obvious adverse effects such as headache, depression, anxiety, myelosuppression, stomatitis, nausea and diarrhea,^{2,7,9} which limit its clinical usefulness. As such, structural modification of 5-FU is required to enhance its selectivity and minimize the toxic side effects.^{10–12}

Natural products, which have a long history in disease therapy, play a key role in anti-tumor drug investigations for their important pharmacological properties.^{13,14} Extensive research has revealed that pentacyclic triterpenes are the main active ingredients of many traditional Chinese medicines.^{15,16} Pentacyclic triterpenes, including oleanolic acid (OA, 1, Fig. 1), ursolic acid (UA, 2, Fig. 1) and glycyrrhetic acid (GA,

3, Fig. 1), which are the effective elements of many natural plants, have been widely researched for their diverse biological activities, such as anti-hepatodamage, anti-HIV, anti-inflammatory, anti-cancer, anti-diabetic, anti-bacterial and anti-proliferation.^{13,15,17–20} Particularly, the mechanism of the anti-tumour activity of natural pentacyclic triterpenes and their semisynthetic derivatives were summarized and analyzed.²¹ Regrettably, although there are many publications covering this research area, it is still not clear which proteins are the primary targets and which master regulators are switched on in response to these compounds.²¹

Nowadays, the pharmacophore hybridization principle is one of the important methods for designing and developing

Fig. 1 Chemical structures of OA, UA, GA and 5-FU.

^a State Key Laboratory for the Chemistry and Molecular Engineering of Medicinal Resources, School of Chemistry and Pharmacy of Guangxi Normal University, Guilin 541004, PR China. E-mail: kgcheng2008@gmail.com;
Fax: +86 0773 2120958; Tel: +86 0773 2120958

^b Jiangsu Key Laboratory of Drug Discovery for Metabolic Diseases and, State Key Laboratory of Natural Medicines, Center of Drug Discovery, China Pharmaceutical University, 24 Tongjia Xiang, Nanjing 210009, China

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new drug lead compounds. The principle mainly refers to combining two known potential pharmacophores by covalent bonds to generate a novel hybrid molecule that could optimize certain pharmacological activities to get over the adverse effects related to a single drug.^{22–24}

Alkyl chains are usually used as the linkers of two pharmacophores to increase the liposolubility. The different lengths of the alkyl chains could be used to obtain the targeted molecules with distinct physical and chemical properties for finding the optimal molecule.^{25,26} Therefore, modification through introducing active pharmacophores into natural products has become a research hot topic in drug development.²⁷ Based on pharmacophore hybridization, our group has already reported several series of pentacyclic triterpene conjugates and some of them possess much better inhibition of proliferation as well as the cytotoxicity selectivity as compared to monomer pentacyclic triterpenes and the commercial anticancer drug 5-FU.^{28,29} For example, the dimeric oleanolic acid linked at C-28 by 1,6-hexanediamine displayed moderate cytotoxicity towards Hep-G2, A549, BGC-823, MCF-7 and PC-3 tumor cell lines with the IC₅₀ value under 10.0 μ M, while low cytotoxicity against the normal human liver cell HL-7702.³⁰ Inspired by the evidence, in this study, 5-FU was respectively attached to the natural products OA/UA/GA to afford conjugates of pentacyclic triterpene-5-fluorouracil (Schemes 1 and 2, 7a/7b–12a/12b) and their anti-tumor activities were evaluated (Table 1). Finally, hybrid 12a was chosen for further preliminary investigation of its mechanism on A549 cell lines (Fig. 2–6).

Results and discussion

Synthesis

Pentacyclic triterpene-5-FU conjugates were synthesized as described in Schemes 1 and 2. Firstly, OA/UA/GA (1 equiv.)

was esterified with the corresponding α,ω -dibromoalkane (3 equiv.) in the presence of potassium carbonate (1 equiv.) to prepare compounds 4a/4b–6a/6b^{28,31} in moderate to good yield (62–89%). Compounds 4a/4b–6a/6b (1 equiv.) were treated with 5-FU (3 equiv.) under potassium carbonate (3 equiv.) at 50 °C by means of a nucleophilic substitution reaction to generate targeted products 7a/7b–12a/12b in the yields of 21–45%. Targeted compounds 7a/7b–12a/12b were fully characterized by ¹H-NMR, ¹³C-NMR spectroscopy and mass spectrometry.

Antitumor activities evaluation

The cytotoxicities of the synthesized targeted pentacyclic triterpene-5-FU hybrids (7a/7b–12a/12b) were evaluated by MTT assay,^{32,33} firstly against the A549, MCF-7, Bel-7402 and K562 tumor cell lines as well as human normal liver cells L-O2 with 5-FU as positive controls (Table 1). However, the results showed that with the exception of 8a, all the targeted hybrids did not display selectivity against the tumor cell lines and liver cell L-O2 cell line. The selectivity of 8a was consistent with the proliferation activity of 5-FU on the tested cell lines. It was found that all the double substitution targeted hybrids (7b–12b) did not possess any anti-tumor activity, while the single substitution targeted hybrids (7a–12a), with the exception of 10a, exhibited moderate to good anti-tumor activity against tested cell lines (A549, MCF-7, Bel-7402 and K562).

Among the single substitution targeted hybrids, though hybrids 7a/9a/12a showed good antiproliferative activities, only hybrid 8a showed potential selectivity against tumor cells. 8a and 12a were tested against multidrug resistant (MDR) cell lines (A549/T, MCF-7/ADR, Bel-7402/FU and K562/ADR). The results showed that 8a exhibited moderate anti-proliferative activity against A549/T and Bel-7402/FU cell

Scheme 1 Synthesis of OA/UA/-5-FU derivatives, where *n* is the number of methylene groups.

Scheme 2 Synthesis of GA-5-FU derivatives, where n is the number of methylene groups.

Table 1 *In vitro* cytotoxicity of the target compounds after 48 h of treatment

Compd	IC_{50}^a (μM)								
	A549	MCF-7	Bel-7402	K562	L-O2	A549/T	MCF-7/ADR	Bel-7402/FU	K562/ADR
5-FU	50.43	52.07	53.33	41.66	>200	84.28	54.72	38.24	31.90
OA	41.65	>200	59.94	>200	>200	>200	>200	>200	>200
UA	77.65	70.12	64.64	41.01	58.37	— ^b	—	—	38.06
GA	>200	>200	>200	>200	>200	99.85	74.49	69.77	86.46
7a	23.44	24.33	25.22	14.92	46.64	—	—	—	—
7b	>200	>200	>200	>200	>200	—	—	—	—
8a	50.54	53.63	43.82	22.99	>200	43.07	166.2	31.42	>200
8b	>200	>200	>200	>200	>200	—	—	—	—
9a	17.35	18.86	32.50	14.89	22.25	—	—	—	—
9b	>200	>200	>200	>200	>200	—	—	—	—
10a	>200	>200	>200	>200	>200	—	—	—	—
10b	>200	>200	>200	>200	>200	—	—	—	—
11a	49.35	50.31	40.92	35.05	73.24	—	—	—	—
11b	>200	>200	>200	>200	>200	—	—	—	—
12a	15.68	12.89	20.98	9.80	20.90	20.73	23.42	19.77	26.98
12b	>200	>200	>200	>200	>200	—	—	—	—

^a The IC_{50} values are the concentrations of the compounds that inhibit tumor cell growth by 50%, and are presented as the mean from three separate experiments. ^b Inhibition effects have not been detected.

lines, which was the same level as against A549 and Bel-7402 cell lines; **12a** exhibited good antiproliferative activities against all the tested MDR cell lines.

Flow cytometry analysis

The detection of intracellular Ca^{2+} production. As the important second messenger in the cell, calcium ions regulate most of the signaling pathways in cells and play an important role in the physiological activities of cells.³⁴ The change in the free calcium ion concentration is closely related to the cell function, signal transmission, damage and apoptosis.³⁵

In this study, the Fluo-3 AM (Calcium Indicator, CAS 121714-22-5) was used to detect the variety of calcium ion to determine whether the apoptosis was related to the intracellular calcium influx. As shown in Fig. 2, the A549 cells treated with hybrid **12a** showed a right-shift fluorescence peak as compared with the control group, which indicated that the hybrid **12a** could induce intracellular calcium influx.

Evaluation of ROS levels. It was reported that ROS (reactive oxygen species) could induce apoptosis through cellular oxidative stress levels and affect cell signal transduction pathways to regulate tumor cell apoptosis and proliferation.³⁶ Thus, in order to investigate whether the hybrid **12a** could

Fig. 2 Analysis of Ca^{2+} production by flow cytometry after A549 cells were treated with hybrid **12a** for 48 h.

influence the ROS production in A549 cells, the fluorescent probe DCFH-DA (Reactive Oxygen Species Assay Kit) combined with flow cytometry was used to detect the level of ROS. As shown in Fig. 3, the A549 cells treated with hybrid **12a** demonstrated a right-shift fluorescence peak as compared with the control group, which indicated that hybrid **12a** could induce the generation of ROS.

Cell cycle analysis. The cell cycle regulates cell differentiation and proliferation, and also regulates cell function decline and cell senescence. The regulation mechanism of the cell cycle could reveal the nature of tumor development and explain the mechanism of cancer. The cell cycle can be divided into four phases: G1 phase (synthesis of RNA and ribosomes), G2 phase (mass synthesis of RNA and protein), S phase (synthesis of histones, DNA and corresponding enzymes) and M phase (average distribution of genetic material). The influence of **12a** on the cell cycle of A549 cells was detected by propidium iodide (PI) through flow cytometry (Fig. 4). The results revealed that the A549 cell ratio in the G1 phase rose from 70.63% to 92.18%, indicating that the hybrid **12a** could block the cell cycle in the G1 phase.

Annexin V/propidium iodide assay. Apoptosis is a mode of programmed cell death, it occurs through the regulation of intracellular genes and their products during development or under certain factors. It involves the activation, expression, and regulation of a range of genes, and it is a gradual process

Fig. 4 Cell cycle progress of A549 cells treated with hybrid **12a** at the specified concentration for 48 h, which were analyzed by flow cytometry assay after PI staining.

that can be divided into four periods. At different stages of apoptosis, the cells undergo the corresponding apoptotic molecular signals, produce different cellular stress responses, and exhibit different morphological features such as cell morphology. Therefore, to investigate cell apoptosis in the presence of **12a**, FITC-Annexin V/PI dual staining and flow cytometric analysis were performed on A549 cells (Fig. 5). The results showed that as the concentration of **12a** increased, the proportion of the A549 cells in the Q2 area increased from 4.19% to 5.43%, 31.90% and 46.40% in a dose-dependent manner. Hence, flow cytometric analysis indicated that **12a** could induce later apoptosis in A549 cells in a concentration-dependent way.

Caspase-3/8 activation assay. Caspase-8 plays a key role in triggering apoptosis upon endoplasmic reticulum stress.³⁷ Caspase-3, which can promote cell apoptosis through intracellular or extracellular pathways, is the main effector in the process of apoptosis.³⁸ The activation of caspase-3 is a sign that apoptosis has entered an irreversible phase. In this study, the activation of caspase-3/8 was measured by flow cytometry in active caspase-3/8 staining kits. As shown in

Fig. 3 Analysis of ROS production by flow cytometry after A549 cells were treated with hybrid **12a** for 48 h.

Fig. 5 Apoptosis effects on A549 cells treated with hybrid 12a at the specified concentration for 48 h, which were analyzed by flow cytometry after Annexin V-FITC/PI staining.

Fig. 6, activated caspase-8 levels increased to 12.70% and 32.40% after treatment with hybrid 12a (15 μ M, 20 μ M, respectively) as compared to the control. Similarly, activated caspase-3 levels increased significantly from 5.99% to 24.50%, 50.90% and 64.70% after treatment 12a (0 μ M, 10 μ M, 15 μ M, 20 μ M, respectively). These results indicate that 12a could induce apoptosis *via* activating caspase-3/8 in A549 cells.

Experimental

General

All the chemical reagents and solvents were of analytical grade and were used without further purification unless specified. All commercial reagents were purchased from Aladdin (Shanghai) Industrial Corporation. Melting points were measured on a RY-1 melting point apparatus. ^1H and ^{13}C NMR-

spectra were recorded on a Bruker AV-500 or AV-300 spectrometer. Chemical shifts are reported as values from an internal tetramethylsilane standard. Mass spectra were recorded on an Agilent 6500 Series Q-TOF LC/MS System, or on a Thermo Scientific Accela – Exactive High-Resolution Accurate Mass spectrometer. The item numbers of the cell lines from Nanjing Kaiji Biotechnology Development Co. Ltd. (Nanjing, China) are as follows: Hep-G2 (KG020), A549 (KG007), Bel-7402 (KG022), BGC-823 (KG025), MCF-7 (KG031), MDA-MB-231 (KG033), PC-3 (KG044), L-O2 (HL-7702, KG063), A549/T (KG124), MCF-7/ADR (KG0311), Bel-7402/FU (KG125) and K562/ADR (KG301).

Synthesis

General procedure for *N*-alkylation reaction of 5-FU to afford 7a/7b to 12a/12b. To a solution of ω -bromoalkyl oleanate/ursolate/glycyrrhetinate^{28,31} (0.404 mmol) in DMF (5 mL), K_2CO_3 (167.2 mg, 1.21 mmol) and 5-fluorouracil (167.2 mg, 1.29 mmol) were added. After stirring at 50 $^\circ\text{C}$ for 4 hours, the mixture was diluted with EtOAc (50 mL). Then, the organic layer was washed successively with 1 N HCl, saturated NaHCO_3 and saturated brine, dried over anhydrous Na_2SO_4 , filtered and concentrated to obtain the residue, which was purified by flash column chromatography.

Compound 7a. Prepared from compound 4a^{28,31} (250 mg, 0.404 mmol) and 5-fluorouracil (167.2 mg, 1.29 mmol) according to the general procedure, compound 7a was obtained by flash column chromatography. Yield 63 mg, 24%, white solid, mp 93–95 $^\circ\text{C}$; R_f = 0.28 (petroleum ether: EtOAc = 1 : 1). ^1H NMR (500 MHz, CDCl_3) δ (ppm): 0.88 (s, 6 H, 2 $\times$ CH_3), 0.70 (s, 3 H, CH_3), 0.76 (s, 3 H, CH_3), 0.90 (s, 3 H, CH_3), 0.97 (s, 3 H, CH_3), 1.11 (s, 3 H, CH_3), 0.67–2.05 (m, 30 H), 2.84 (dd, J = 4.0, 13.9 Hz, 1 H, 18-H), 3.20 (dd, J = 4.6, 11.2 Hz, 1 H, 3-H), 3.69 (t, 1 H, J = 7.4 Hz, NCH_a), 3.86–4.00 (m, 3 H, COOCH_2 and NCH_b), 5.25 (t, 1 H, J = 3.5 Hz, 12-H), 7.24 (d, J = 5.4 Hz, 1 H, 6- CH^{flu}), 9.72 (s, 1 H, 3- NH^{flu}).

Fig. 6 Activation of caspase-3/8 on A549 cells treated with hybrid 12a was measured by flow cytometry in active caspase-3/8 staining kits.

^{13}C NMR (125 MHz, CDCl_3) δ (ppm): 15.4, 15.7, 17.2, 18.4, 23.1, 23.5, 23.7, 25.8, 26.0, 26.1, 27.2, 27.8, 28.2, 28.5, 28.9, 30.8, 32.6, 32.9, 33.2, 34.0, 37.1, 38.5, 38.8, 39.4, 41.4, 41.8, 46.0, 46.8, 47.7, 49.1, 55.3, 64.0, 79.1, 122.5, 128.6, 139.6, 143.9, 149.6, 157.5, 177.9. HRMS (ESI) m/z : $[\text{M} + \text{Cl}]^+$ calcd for $\text{C}_{40}\text{H}_{61}\text{ClFN}_2\text{O}_5$ 703.4253; found 703.4279.

Compound 7b. Prepared from compound **4a**^{28,31} (500 mg, 0.807 mmol) and 5-fluorouracil (314.8 mg, 2.42 mmol) according to the general procedure, compound **7b** was obtained by flash column chromatography. Yield 242 mg, 25%, white solid, mp 135–137 °C; R_f = 0.288 (petroleum ether:EtOAc = 2:1). ^1H NMR (300 MHz, CDCl_3) δ (ppm): 0.89 (s, 12 H, 4 $\times$ CH_3), 0.72 (s, 6 H, 2 $\times$ CH_3), 0.77 (s, 6 H, 2 $\times$ CH_3), 0.91 (s, 6 H, 2 $\times$ CH_3), 0.98 (s, 6 H, 2 $\times$ CH_3), 1.12 (s, 6 H, 2 $\times$ CH_3), 0.67–2.19 (m, 60 H), 2.85 (dd, J = 4.6, 14.9 Hz, 2 H, 18-H and 18'-H), 3.22 (dd, J = 4.8, 10.4 Hz, 2 H, 3-H and 3'-H), 3.71 (t, J = 7.4 Hz, 2 H, NCH_a and NCH_a), 3.81–4.31 (m, 6 H, 2 $\times$ OCH_2 , NCH_b and NCH_b), 5.27 (brs, 2 H, 12-H, 12'-H), 7.20 (d, J = 5.1 Hz, 1 H, 6- CH^{flu}). ^{13}C NMR (125 MHz, CDCl_3) δ (ppm): 15.4, 15.7, 17.1, 17.2, 18.5, 23.2, 23.6, 23.8, 25.8, 25.9, 26.0, 26.2, 26.7, 27.3, 27.5, 27.8, 28.2, 28.57, 28.63, 29.0, 30.8, 32.60, 32.63, 32.9, 33.20, 33.23, 34.0, 37.2, 38.6, 38.9, 39.5, 41.5, 41.8, 42.1, 46.00, 46.01, 46.79, 46.81, 47.72, 47.76, 50.0, 55.34, 55.37, 64.0, 64.2, 79.08, 79.13, 122.5, 126.3, 126.6, 139.2, 141.1, 143.9, 149.9, 157.4, 177.8. HRMS (ESI) m/z : $[\text{M} + \text{Cl}]^+$ calcd for $\text{C}_{76}\text{H}_{119}\text{ClFN}_2\text{O}_8$, 1241.8639; found 1241.8646.

Compound 8a. Prepared from compound **4b**^{28,31} (250 mg, 0.386 mmol) and 5-fluorouracil (150.9 mg, 1.16 mmol) according to the general procedure, compound **8a** was obtained by flash column chromatography. Yield 61 mg, 23%, white solid, mp 92–94 °C; R_f = 0.281 (petroleum ether:EtOAc = 1:1). ^1H NMR (500 MHz, CDCl_3) δ (ppm): 0.88 (s, 6 H, 2 $\times$ CH_3), 0.71 (s, 3 H, CH_3), 0.76 (s, 3 H, CH_3), 0.90 (s, 3 H, CH_3), 0.97 (s, 3 H, CH_3), 1.11 (s, 3 H, CH_3), 0.67–2.08 (m, 34 H), 2.84 (dd, J = 4.0, 13.8 Hz, 1 H, 18-H), 3.21 (dd, J = 4.6, 11.1 Hz, 1 H, 3-H), 3.68 (t, J = 7.4 Hz, 1 H, NCH_a), 3.85–4.00 (m, 3 H, COCH_2 and NCH_b), 5.25 (t, J = 3.3 Hz, 1 H, 12-H), 7.24 (d, J = 5.4 Hz, 1H, 6- CH^{flu}), 9.81 (s, 1 H, 3- NH^{flu}). ^{13}C NMR (125 MHz, CDCl_3) δ (ppm): 15.4, 15.7, 17.1, 18.4, 23.1, 23.5, 23.7, 25.97, 26.03, 26.4, 27.2, 27.7, 28.2, 28.6, 29.0, 29.1, 29.2, 30.8, 32.6, 32.9, 33.2, 34.0, 37.1, 38.5, 38.8, 39.4, 41.4, 41.8, 46.0, 46.8, 47.7, 49.2, 55.3, 64.2, 79.1, 122.4, 128.7, 139.6, 143.9, 149.6, 157.5, 178.0. HRMS (ESI) m/z : $[\text{M} - \text{H}]^+$ calcd for $\text{C}_{42}\text{H}_{64}\text{FN}_2\text{O}_5$, 695.4799; found 695.4811.

Compound 8b. Prepared from compound **4b**^{28,31} (500 mg, 0.772 mmol) and 5-fluorouracil (301.8 mg, 2.32 mmol) according to the general procedure, compound **8b** was obtained by flash column chromatography. Yield 202 mg, 21%, white solid, mp 118–120 °C; R_f = 0.356 (petroleum ether:EtOAc = 2:1). ^1H NMR (300 MHz, CDCl_3) δ (ppm): 0.72 (s, 6 H, 2 $\times$ CH_3), 0.77 (s, 6 H, 2 $\times$ CH_3), 0.89 (s, 12 H, 4 $\times$ CH_3), 0.92 (s, 6 H, 2 $\times$ CH_3), 0.98 (s, 6 H, 2 $\times$ CH_3), 1.12 (s, 6 H, 2 $\times$ CH_3), 0.65–2.12 (m, 68 H), 2.86 (dd, J = 3.75, 13.9 Hz, 2 H, 18-H and 18'-H), 3.22 (dd, J = 4.8, 10.5 Hz, 2 H, 3-H and 3'-H), 3.68–3.73 (t, J = 7.4 Hz, 2 H, NCH_a and NCH_a), 3.91–

3.99 (m, 6 H, 2 $\times$ OCH_2 , NCH_b and NCH_b), 5.27 (t, J = 3.0 Hz, 2 H, 12-H and 12'-H), 7.19 (d, J = 5.1 Hz, 1 H, 6- CH^{flu}). ^{13}C NMR (125 MHz, CDCl_3) δ (ppm): 15.4, 15.7, 17.2, 18.5, 23.2, 23.6, 23.7, 25.99, 26.04, 26.1, 26.5, 26.9, 27.3, 27.6, 27.8, 28.2, 28.66, 28.71, 29.0, 29.1, 29.20, 29.24, 29.3, 30.8, 32.6, 32.9, 33.2, 34.0, 37.2, 38.6, 38.9, 39.5, 41.5, 41.8, 42.2, 46.0, 46.8, 47.8, 50.0, 55.4, 64.3, 79.1, 122.5, 126.5, 139.2, 144.0, 149.9, 157.3, 177.9. HRMS (ESI) m/z : $[\text{M} + \text{Cl}]^+$ calcd for $\text{C}_{80}\text{H}_{127}\text{ClFN}_2\text{O}_8$, 1297.9265; found 1297.9274.

Compound 9a. Prepared from compound **5a**³¹ (250 mg, 0.403 mmol) and 5-fluorouracil (157.42 mg, 1.21 mmol) according to the general procedure, compound **9a** was obtained by flash column chromatography. Yield 70 mg, 26%, white solid, mp 85–87 °C; R_f = 0.433 (petroleum ether:EtOAc = 1:1). ^1H NMR (500 MHz, CDCl_3) δ (ppm): 0.72 (s, 3 H, CH_3), 0.76 (s, 3 H, CH_3), 0.84 (s, 3 H, CH_3), 0.89 (s, 3 H, CH_3), 0.92 (s, 3 H, CH_3), 0.97 (s, 3 H, CH_3), 1.06 (s, 3 H, CH_3), 0.67–2.08 (m, 30 H), 2.19 (d, J = 11.2 Hz, 1 H, 18-H), 3.20 (dd, J = 4.6, 11.1 Hz, 1 H, 3-H), 3.69 (t, J = 7.4 Hz, 2 H, OCH_2), 3.71–4.22 (m, 3 H, OH and NCH_2), 5.21 (t, J = 3.5 Hz, 1 H, 12-H), 7.24 (d, J = 5.4 Hz, 1 H), 9.89 (s, 1 H, 3- H^{flu}). ^{13}C NMR (125 MHz, CDCl_3) δ (ppm): 15.5, 15.7, 17.0, 17.1, 18.3, 21.2, 23.3, 23.5, 24.2, 25.7, 26.0, 27.2, 28.0, 28.1, 28.4, 28.8, 30.7, 33.1, 36.8, 37.0, 38.6, 38.7, 38.9, 39.1, 39.6, 42.1, 47.5, 48.1, 49.0, 52.9, 55.2, 63.9, 79.0, 125.5, 128.5, 138.2, 139.5, 149.5, 157.3, 177.6. HRMS (ESI) m/z : $[\text{M} - \text{H}]^+$ calcd for $\text{C}_{40}\text{H}_{60}\text{FN}_2\text{O}_5$, 667.4486; found 667.4494.

Compound 9b. Prepared from compound **5a**³¹ (500 mg, 0.807 mmol) and 5-fluorouracil (314.83 mg, 2.42 mmol) according to the general procedure, compound **9b** was obtained by flash column chromatography. Yield 316 mg, 32%, white solid, mp 71–73 °C; R_f = 0.156 (petroleum ether:EtOAc = 2:1). ^1H NMR (300 MHz, CDCl_3) δ (ppm): 0.74 (s, 6 H, 2 $\times$ CH_3), 0.77 (s, 6 H, 2 $\times$ CH_3), 0.85 (d, J = 6.2 Hz, 6 H, 2 $\times$ CH_3), 0.90 (s, 6 H, 2 $\times$ CH_3), 0.94 (d, J = 6.2 Hz, 6 H, 2 $\times$ CH_3), 0.98 (s, 6 H, 2 $\times$ CH_3), 1.07 (s, 6 H, 2 $\times$ CH_3), 0.53–2.19 (m, 60 H), 2.21 (d, J = 11.2 Hz, 2 H, 18-H and 18'-H), 3.21 (dd, J = 4.6, 10.8 Hz, 2 H, 3-H and 3'-H), 3.71 (t, J = 7.3 Hz, 2 H, NCH_a and NCH_a), 3.93–3.99 (m, 6 H, 2 $\times$ COOCH_2 , NCH_b and NCH_b), 5.23 (brs, 2 H, 12-H and 12'-H), 7.19 (d, J = 5.1 Hz, 1 H, 6- CH^{flu}). ^{13}C NMR (125 MHz, CDCl_3) δ (ppm): 14.2, 15.6, 15.8, 17.1, 17.3, 18.4, 21.5, 22.8, 23.4, 23.7, 24.3, 25.8, 25.9, 26.2, 26.7, 27.3, 27.5, 28.1, 28.3, 28.56, 28.62, 29.0, 29.5, 29.77, 29.82, 30.8, 32.0, 33.2, 36.9, 37.1, 38.8, 38.9, 39.0, 39.2, 39.7, 42.2, 47.7, 48.2, 50.0, 53.0, 55.3, 64.0, 64.2, 79.1, 125.6, 126.5, 138.3, 141.1, 149.9, 157.3, 177.7. HRMS (ESI) m/z : $[\text{M} + \text{Cl}]^+$ calcd for $\text{C}_{76}\text{H}_{119}\text{ClFN}_2\text{O}_8$, 1241.8639; found 1241.8586.

Compound 10a. Prepared from compound **5b**³¹ (250 mg, 0.386 mmol) and 5-fluorouracil (150.6 mg, 1.16 mmol) according to the general procedure, compound **10a** was obtained by flash column chromatography. Yield 71 mg, 26%, white solid, mp 83–85 °C; R_f = 0.393 (petroleum ether:EtOAc = 1:1). ^1H NMR (500 MHz, CDCl_3) δ (ppm): 0.73 (s, 3 H, CH_3), 0.76 (s, 3 H, CH_3), 0.84 (d, J = 6.3 Hz, 3 H, CH_3), 0.89 (s, 3 H, CH_3), 0.93 (d, J = 6.3 Hz, 3 H, CH_3), 0.97 (s, 3 H, CH_3), 1.06 (s, 3 H, CH_3), 0.67–2.11 (m, 34 H), 2.20 (d, J = 11.4

Hz, 1 H, 18-H), 3.21 (dd, $J = 4.7, 11.1$ Hz, 1 H, 3-H), 3.67 (t, $J = 7.4$ Hz, 1 H, NCH_a), 3.96 (t, $J = 6.6$ Hz, 1 H, NCH_b), 3.91–4.01 (m, 2 H, COOCH₂), 5.21 (t, $J = 3.6$ Hz, 1 H, 12-H), 7.24 (d, $J = 5.4$ Hz, 1H, 6-CH^{flu}), 9.72 (s, 1 H, 3-NH^{flu}). ¹³C NMR (125 MHz, CDCl₃) δ (ppm): 15.6, 15.8, 17.1, 17.2, 18.4, 21.3, 23.4, 23.7, 24.5, 26.0, 26.4, 27.3, 28.4, 28.6, 29.0, 29.1, 29.2, 30.8, 33.2, 36.9, 37.1, 38.7, 38.8, 39.0, 39.2, 39.7, 42.2, 47.6, 48.2, 49.2, 53.0, 55.3, 64.2, 79.1, 125.6, 128.6, 138.3, 139.6, 149.6, 157.4, 177.8. HRMS (ESI) m/z : [M – H]⁺ calcd for C₄₂H₆₄FN₂O₅, 695.4799; found 695.4810.

Compound 10b. Prepared from compound 5b³¹ (500 mg, 0.772 mmol) and 5-fluorouracil (301.2 mg, 2.32 mmol) according to the general procedure, compound 10b was obtained by flash column chromatography. Yield 294 mg, 30%, white solid, mp 59–61 °C; $R_f = 0.213$ (petroleum ether: EtOAc = 2 : 1). ¹H NMR (300 MHz, CDCl₃) δ (ppm): 0.74 (s, 6 H, 2 × CH₃), 0.77 (s, 6 H, 2 × CH₃), 0.85 (d, $J = 6.2$ Hz, 6 H, 2 × CH₃), 0.90 (s, 6 H, 2 × CH₃), 0.94 (d, $J = 6.2$ Hz, 6 H, 2 × CH₃), 0.98 (s, 6 H, 2 × CH₃), 1.07 (s, 6 H, 2 × CH₃), 0.65–2.02 (m, 68 H), 2.22 (d, $J = 11.3$ Hz, 2 H, 18-H and 18'-H), 3.21 (dd, $J = 4.5, 10.5$ Hz, 2 H, 3-H and 3'-H), 3.67 (t, $J = 7.4$ Hz, 2 H, NCH_a and NCH_{a'}), 3.82–4.03 (m, 6 H, 2 × OCH₂, NCH_b and NCH_{b'}), 5.23 (t, $J = 3.5$ Hz, 2 H, 12-H and 12'-H), 7.19 (d, $J = 5.1$ Hz, 1 H, 6-CH^{flu}). ¹³C NMR (125 MHz, CDCl₃) δ (ppm): 14.1, 15.5, 15.6, 17.0, 17.1, 18.3, 21.2, 22.7, 23.3, 23.6, 24.2, 25.9, 26.0, 26.4, 26.8, 27.2, 27.4, 28.0, 28.3, 28.5, 28.6, 28.9, 29.1, 29.2, 29.4, 29.7, 30.7, 31.9, 33.1, 36.7, 37.0, 38.7, 38.8, 38.9, 39.1, 39.6, 42.1, 47.6, 48.1, 49.9, 52.9, 55.2, 64.2, 79.0, 125.5, 126.3, 138.2, 141.0, 149.8, 157.2, 177.6. HRMS (ESI) m/z : [M + Cl]⁺ calcd for C₈₀H₁₂₇ClFN₂O₈, 1297.9265; found 1297.9225.

Compound 11a. Prepared from compound 6a³¹ (250 mg, 0.395 mmol) and 5-fluorouracil (153.95 mg, 1.18 mmol) according to the general procedure, compound 11a was obtained by flash column chromatography. Yield 66 mg, 25%, white solid, mp 111–113 °C; $R_f = 0.283$ (petroleum ether: EtOAc = 1 : 1). ¹H NMR (500 MHz, CDCl₃) δ (ppm): 0.77 (s, 3 H, CH₃), 0.78 (s, 3 H, CH₃), 0.98 (s, 3 H, CH₃), 1.12 (s, 3 H, CH₃), 1.10 (s, 9 H, 3 × CH₃), 0.62–2.33 (m, 27 H), 2.32 (s, 1 H, 9-H), 2.73 (m, 1 H, 18-H), 3.20 (dd, $J = 5.1, 11.1$ Hz, 1 H, 3-H), 3.72 (m, 2 H, NCH₂), 3.89–4.19 (m, 3 H, COOCH₂ and 3-OH), 5.57 (s, 1 H, 12-H), 7.54 (d, $J = 5.6$ Hz, 1H, 6-CH^{flu}), 9.87 (s, 1 H, 3-NH^{flu}). ¹³C NMR (125 MHz, CDCl₃) δ (ppm): 15.7, 16.4, 17.6, 18.8, 23.5, 26.2, 26.3, 26.5, 26.6, 27.3, 28.2, 28.6, 28.7, 28.8, 29.0, 31.2, 32.0, 32.8, 37.2, 37.7, 39.2, 41.2, 43.4, 44.1, 45.6, 48.8, 48.9, 55.0, 62.0, 64.3, 78.8, 128.3, 129.2, 139.6, 149.7, 169.9, 176.6, 200.6. HRMS (ESI) m/z : [M – H]⁺ calcd for C₄₀H₅₈FN₂O₆, 681.4279; found 681.4282.

Compound 11b. Prepared from compound 6a³¹ (500 mg, 0.789 mmol) and 5-fluorouracil (307.89 mg, 2.36 mmol) according to the general procedure, compound 11b was obtained by flash column chromatography. Yield 204 mg, 21%, white solid, mp 108–109 °C; $R_f = 0.203$ (petroleum ether: EtOAc = 2 : 1). ¹H NMR (300 MHz, CDCl₃) δ (ppm): 0.80 (s, 12 H, 4 × CH₃), 1.00 (s, 12 H, 4 × CH₃), 1.13 (s, 18 H, 6 × CH₃), 0.49–2.19 (m, 54 H), 2.34 (s, 2 H, 9-H and 9'-H), 2.75

(d, $J = 4.3$ Hz, 2 H, 18-H and 18'-H), 3.23 (brs, 2 H, 3-H and 3'-H), 3.76 (brs, 2 H, NCH₂), 3.96–4.15 (m, 6 H, 2 × OCH₂, 2 × OH), 5.62 (s, 2 H, 12-H and 12'-H), 7.48 (d, 1 H, $J = 5.1$ Hz, 6-CH^{flu}). ¹³C NMR (125 MHz, CDCl₃) δ (ppm): 14.2, 15.7, 16.5, 17.6, 18.8, 22.8, 23.5, 25.8, 26.2, 26.4, 26.6, 27.5, 28.2, 28.6, 28.8, 28.9, 29.1, 29.5, 29.8, 31.2, 32.0, 32.9, 37.2, 37.8, 37.9, 39.3, 41.2, 41.3, 42.0, 43.3, 43.4, 44.10, 44.13, 45.5, 45.6, 48.5, 48.8, 49.8, 55.1, 62.0, 64.4, 78.8, 126.9, 128.4, 139.2, 150.0, 157.4, 169.5, 176.5, 200.3. HRMS (ESI) m/z : [M + Cl]⁺ calcd for C₇₆H₁₁₅ClFN₂O₁₀, 1269.8224; found 1269.8228.

Compound 12a. Prepared from compound 6b³¹ (300 mg, 0.453 mmol) and 5-fluorouracil (176.9 mg, 1.36 mmol) according to the general procedure, compound 12a was obtained by flash column chromatography. Yield 75 mg, 23%, white solid, mp 65–67 °C; $R_f = 0.295$ (petroleum ether: EtOAc = 1 : 1). ¹H NMR (500 MHz, CDCl₃) δ (ppm): 0.76 (s, 3 H, CH₃), 0.77 (s, 3 H, CH₃), 0.97 (s, 6 H, 2 × CH₃), 1.08 (s, 3 H, CH₃), 1.09 (s, 3 H, CH₃), 1.11 (s, 3 H, CH₃), 0.61–2.35 (m, 31 H), 2.31 (s, 1 H, 9-H), 2.71 (m, 1H, 18-H), 3.20 (dd, $J = 4.9, 11.3$ Hz, 1 H, 3-H), 3.69 (t, $J = 7.5$ Hz, 1 H, NCH_a), 4.00–4.15 (m, 3 H, OCH₂ and NCH_b), 5.58 (s, 1 H, 12-H), 7.37 (d, $J = 5.5$ Hz, 1 H, 6-CH^{flu}), 10.02 (s, 1 H, 3-NH^{flu}). ¹³C NMR (125 MHz, CDCl₃) δ (ppm): 14.2, 15.7, 16.4, 17.5, 18.6, 23.4, 25.9, 26.0, 26.3, 26.45, 26.53, 26.7, 27.2, 28.2, 28.5, 28.6, 28.7, 28.8, 29.0, 29.1, 31.2, 31.9, 32.8, 37.1, 37.8, 39.2, 41.1, 43.3, 44.1, 45.5, 48.6, 49.1, 55.0, 61.9, 64.5, 78.8, 128.4, 129.0, 139.5, 149.2, 169.7, 176.6, 200.4. HRMS (ESI) m/z : [M + Na]⁺ calcd for C₄₂H₆₃FN₂O₆Na, 733.4568; found 733.4575.

Compound 12b. Prepared from compound 6b³¹ (900 mg, 1.36 mmol) and 5-fluorouracil (530.71 mg, 4.08 mmol) according to the general procedure, compound 12b was obtained by flash column chromatography. Yield 793 mg, 45%, white solid, mp 126–128 °C; $R_f = 0.164$ (petroleum ether: EtOAc = 2 : 1). ¹H NMR (300 MHz, CDCl₃) δ (ppm): 0.80 (s, 18 H, 6 × CH₃), 1.00 (s, 6 H, 2 × CH₃), 1.12 (s, 12 H, 4 × CH₃), 1.13 (2 s, 6 H, 2 × CH₃), 0.60–2.43 (m, 62 H), 2.34 (s, 2 H, 9-H and 9'-H), 2.77 (m, 2 H, 18-H and 18'-H), 3.23 (m, 2 H, 3-H and 3'-H), 3.73 (t, $J = 7.5$ Hz, 2 H, NCH_a and NCH_{a'}), 3.94 (t, $J = 7.5$ Hz, 2 H, NCH_b and NCH_{b'}), 4.00–4.09 (m, 4 H, 2 × OCH₂), 5.62 and 5.63 (2 s, each 1 H, 12-H and 12'-H), 7.30 (d, 1 H, $J = 5.1$ Hz, 6-CH^{flu}). ¹³C NMR (125 MHz, CDCl₃) δ (ppm): 15.7, 16.5, 17.6, 18.8, 23.5, 26.0, 26.1, 26.4, 26.57, 26.62, 26.9, 27.40, 27.44, 27.5, 28.2, 28.5, 28.6, 28.7, 28.8, 28.9, 29.2, 31.3, 32.0, 32.9, 37.2, 37.9, 39.3, 41.2, 42.1, 43.4, 44.1, 45.5, 48.4, 48.6, 50.0, 55.1, 62.0, 64.6, 78.9, 126.7, 128.6, 139.2, 149.9, 157.4, 169.4, 176.6, 200.3. HRMS (ESI) m/z : [M + H]⁺ calcd for C₈₀H₁₂₄FN₂O₁₀, 1291.9240; found 1291.9290.

In vitro cytotoxicity

The MTT assay^{32,33} was carried out as follows. Cells were seeded in 96-well plates and incubated in the CO₂ (5%) incubator at 37 °C. When the cells adhered to the plate, compounds at different concentrations (20, 10, 5, 2.5, 1.25 μ M) were added to each well. After incubation for another 48 h, 20 μ L MTT (5%) was added to each well and incubated for an

additional 4 h. The viable cells were stained with MTT and scanned with a spectrophotometer at 570 nm. Each treatment was done in triplicate.

Intracellular Ca^{2+} generation

The intracellular Ca^{2+} production was measured by a Fluo-3 AM probe. The cells were seeded in six-well plates for 24 h, and then with the selected compound (0 μM , 10 μM , 15 μM) for 48 h. After that, the cells were harvested and washed three times with serum-free medium (RPMI medium 1640 basic (1 $\times$)). The Fluo-3 AM (0.5 μM , 500 μL) was added to each sample and then incubated in an incubator for 60 min. The mixture was washed twice with a serum-free medium (RPMI medium 1640 basic (1 $\times$)) and suspended with 500 μL of corresponding serum-free medium. The generation of Ca^{2+} was immediately measured by flow cytometry.

Detection of intracellular reactive oxygen species

Intracellular reactive oxygen species (ROS) production was detected with a DCFH-DA probe. Appropriate A549 cells were incubated with the selected compound (0 μM , 20 μM , 25 μM) in six-well plates for 48 h. The cells were then harvested and washed twice with PBS, followed by suspension in 500 μL of the serum-free medium (RPMI medium 1640 basic (1 $\times$)) containing 10 $\mu\text{M L}^{-1}$ DCFH-DA probe. After 30 min of incubation at 37 $^{\circ}\text{C}$ in the dark, the cells were centrifuged and washed three times with serum-free medium. The generation of ROS in the A549 cells was detected by flow cytometry.

Annexin V/propidium iodide assay

In the apoptosis experiments, the Annexin V-FITC kit was used. A549 cells were seeded in six-well plates and incubated with the selected compound at different concentrations (0 μM , 10 μM , 15 μM , 20 μM) for 48 h. The A549 cells were then collected and suspended in annexin binding buffer (1 $\times$, 200 μL). In the next step, 5 μL Annexin V and 5 μL propidium iodide were added to the corresponding sample, and the mixture was incubated on the ice in the dark for 30 min. Subsequently, the extra binding buffer (300 μL) was added to the mixture, and the cell apoptosis was analyzed by flow cytometry.

Cell cycle assay

To investigate the influence of the selected compound on the cell cycle of tumor cells, flow cytometry was used to measure the changes in the cell cycle distribution in A549 cells at different concentrations. The A549 cells were seeded in cell and tissue culture dishes (70 mm) for 24 h, then treated with the selected compound (0 μM , 10 μM , 15 μM , 20 μM) for 48 h. The cells were then collected and washed with cool phosphate buffer saline (PBS) solution. The cells were fixed with cold 75% ethanol and stored at -20°C for 36 h. Later, the cells were centrifuged and washed with cold PBS. RNase A (100 $\mu\text{g mL}^{-1}$, 500 μL) was added to each sample and incubated in an incubator for 30 min.

Propidium iodide (1 mg mL^{-1} , 25 μL) was added to each sample for 10 min in the dark, and then detected by flow cytometry.

Detection of caspase-3/8

The activation of caspase-3/8 was detected by flow cytometry *via* the Caspase-3/8 Staining Kit. Appropriate A549 cells in six-well plates were exposed to the selected compounds (0 μM , 10 μM , 15 μM , 20 μM) for 48 h. Cells were collected and washed with PBS solution, suspended with PBS 300 μL containing 0.5 μL FITC-DEVD-FMK, and incubated for 50 minutes. The mixture was centrifuged and washed with wash buffer twice, and suspended in 500 μL wash buffer. A flow cytometer was immediately employed to detect caspase-3/8 activity.

Statistics

Data processing included the Student's *t*-test with $P \leq 0.05$ taken as the significance level, using SPSS 17.0.

Conclusions

Twelve pentacyclic triterpene-5-FU conjugates were synthesized and their antitumor activities were evaluated. The results outlined that all the single substitution targeted hybrids (7a–12a) possessed much better antiproliferative activities than the double substitution targeted hybrids (7b–12b). Hybrid 8a showed potential selectivity against tumor cells, and also possessed moderate antiproliferative activities against MDR cell lines A549/T and Bel-7402/FU, which were at the same levels as against A549 and Bel-7402 cell lines. However, only 12a exhibited good antiproliferative activities against all the tested MDR cell lines and possessed much better antiproliferative activities than 8a. Hybrid 12a was chosen for further preliminary investigation of its mechanism on A549 cell lines. It was revealed that after the treatment of A549 cells with 12a, the changes in cellular morphology could induce intracellular calcium influx and the generation of ROS as a result of significant apoptosis induction. Subsequently, cell proliferation in the G1 phase was arrested and apoptotic signaling of caspase-8 was activated. Furthermore, as a protease, caspase-8 could activate the apoptotic effector caspase-3, which eventually caused the later nuclear apoptosis. The other specific mechanisms of 12a are currently underway.

Conflicts of interest

There are no conflicts of interest to declare.

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