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ABSTRACT

Betulinic acid (BA) and oleanolic acid (OA) are plant-derived conjugates found in various medicinal plants that have emerged as potential antitumor agents. Herein, a series of novel BA and OA derivatives were synthesized by conjugation with per-*O*-methylated-β-cyclodextrin (PM-β-CD), and their anticancer properties against a panel of three human cancer cell lines were evaluated. Two OA-PM-β-CD conjugates (**48** and **50)** were observed to be the most potent conjugates against the three cell lines (MCF-7, BGC-823, and HL-60), with a 15- to 20-fold decrease in the IC_{50} values (IC_{50}: 6.06–8.47 μM) compared with their parental conjugate (OA). Annexin V-FITC/propidium iodide staining and Western blot analysis revealed that both conjugates induced apoptosis in HL-60 cells. Additionally, in the representative conjugate **48**-treated HL-60 cells, a decrease in mitochondrial membrane potential and subsequent release of cytochrome *c* into the cytosol were observed, indicating the activation of the intrinsic apoptosis pathway. Furthermore, **48** dramatically induced the generation of reactive oxygen species (ROS) in HL-60 cells, and the corresponding effect could be reversed using the ROS scavenger *N*-acetylcysteine. Collectively, these results suggest that the novel pentacyclic triterpenoid derivatives trigger the intrinsic apoptotic pathways via the ROS-mediated activation of caspase-3 signaling, inducing cell death in human cancer cells.

KEYWORDS: pentacyclic triterpenoid, antitumor, apoptosis, reactive oxygen species, caspase-3

1. INTRODUCTION

Betulinic acid (BA, **1**, Figure 1) and oleanolic acid (OA, **2**) are two close structural isomers of naturally occurring pentacyclic triterpenoids that possess numerous biological activities.[1, 2] BA is a lupane-structured triterpenoid, generally isolated from birch bark extract. Since 1976, the remarkable anticancer activities of BA and its semisynthetic derivatives have attracted special attention.[3] As a novel and highly selective antimelanoma agent, BA has even been used in phase I/II clinical trials.[4] NVX-207 (**3**), a semisynthetic Tris ester of betulin, is well tolerated and has demonstrated potential anticancer activity in the treatment of drug-resistant malignancies in horses and dogs.[5, 6] Furthermore, numerous BA derivatives with improved water solubility and bioavailability, such as triphenylphosphonium cation ester **(4)**,[7] Boc-lysinated-betulonic acid (**5**),[8] and glucopyranoside derivative B10 (**6**)[9] and its analogs (**7**),[10] have been designed and synthesized to investigate their antiproliferative activity. OA has been widely used in clinics for ~30 years in China as an oral hepatoprotective drug.[11] Over the past several decades, the amount of interest in the scientific community toward the potential clinical application of OA derivatives as anticancer agents has substantially increased.[12] Three derivatives of OA—CDDO (RTA401, **8**), CDDO-Me (RTA402, **9**), and CDDO-Im (RTA403, **10**)—exhibited antitumor activity in phase I clinical trials.[13, 14] However, the subpar water solubility of pentacyclic triterpenoids can engender poor dissolution rates and slow absorption, thereby producing inadequate and variable bioavailability. The solubility of BA and OA in distilled water is ~0.021 μg/mL and 0.012 μg/mL, respectively.[15]

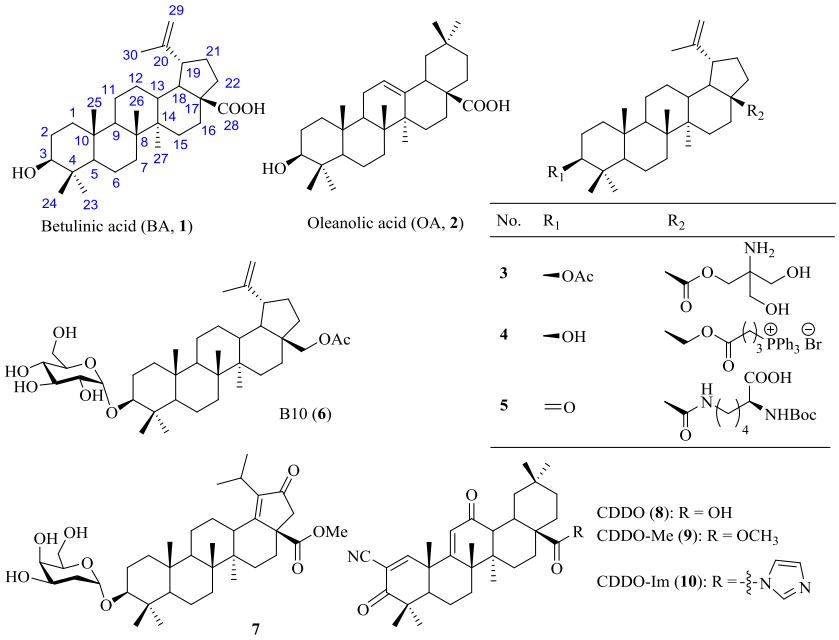


Figure 1. Chemical structures of BA (**1**), OA (**2**), and their anticancer derivatives **3**–**10**.

Among the most common three natural cyclodextrins (CDs), *β*-CD is particularly important for drug delivery and pharmaceutical development because of its low production cost and moderate molecular void space.[16-19] Several chemically modified *β*-CDs, including hydroxypropyl-*β*-CD, sulfobutyl ether-*β*-CD, and branched-*β*-CD, have been successfully prepared to enhance inclusion capacity,[20] thereby improving certain physicochemical properties of poorly soluble drugs.[21] Recently, randomly *O*-methylated-*β*-CD (RM-*β*-CD), di-*O*-methylated-*β*-CD (DM-*β*-CD), and per-*O*-methylated-*β*-CD (PM-*β*-CD) have also attracted considerable attention from scientists owing to their potential application in many areas.[22, 23] More remarkably, it has also been reported that PM-*β*-CD can serve as a synthon for developing antitumor agents,[24] artificial O_2 carriers,[25] drug delivery systems,[26] and other interesting therapeutics.[27] Therefore, numerous chemically modified PM-*β*-CD derivatives possessing a few specifically located functionalized groups, such as 2^A-hydroxy-, 3^A-hydroxy- and 6^A-hydroxy-PM-*β*-CD,[28-30] 2^A,3^B-dihydroxy-PM-*β*-CD,[29] 3^A-amino-PM-*β*-CD,[31] and 6^A-amino- and $6^{A,D}$-diamino-PM-*β*-CD,[32] have been synthesized.

In our previous study, 12 water-soluble pentacyclic triterpenoid-*β*-CD derivatives were conveniently synthesized via click chemistry. Surprisingly, we found that only

the PM-*β*-CD–conjugated pentacyclic triterpenoid derivatives exhibited significant cytotoxicity against two cancer cell lines (HeLa, and HepG2) and two normal cell lines (MDCK, and 293T); however,[33] the exact mechanism of this cytotoxicity is yet to be elucidated. In the present study, a series of novel BA- and OA-PM-*β*-CD conjugates via direct amide bond was synthesized and their antitumor activities in a panel of cancer cells were evaluated. The apoptotic action of the conjugates was also investigated using flow cytometry and Western blot.

2. MATERIALS AND METHODS

2.1 Materials. Unless otherwise stated, all chemicals were purchased from J & K Scientific Co., Ltd. (Beijing, China) and used without further purification. BA and OA were purchased from Nanjing Zelang Medical Technology Co., Ltd. (Nanjing, China). *β*-CD was obtained from Shanghai Aladdin Bio-Chem Technology Co., Ltd. (Shanghai, China). NMR experiments were performed on Bruker DRX 400 MHz (100 MHz, ^{13}C) spectrometer at 298K. The spectra of samples in $CDCl_3$ were calibrated using the signal from residual solvent (7.26 ppm for ^{1}H and 77.00 ppm for ^{13}C). The HRMS spectra were recorded using Bruker APEX IV-FTMS (7.0T) mass spectrometer. Reactions were magnetically stirred and monitored via thin layer chromatography (TLC) conducted on a silica gel 60 F_{254} precoated plate (Merck, Germany). Column chromatography was performed on 200–300 mesh silica gel (Qingdao Haiyang Chemical Co., Ltd., Qingdao, China). Spots were stained with a yellow solution containing $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (24.0 g) and $Ce(NH_4)_2(NO_3)_6$ (0.5 g) in 6% H_2SO_4 (500 mL), followed by heating with a hot gun. Conjugates **1**, **12**–**34**, and **36**–**39** were synthesized according to previously described procedures.[32, 34, 35]

2.2. General Procedure for the Synthesis of Conjugates 40–51 by Amidation Reaction. Na_2CO_3 or EDC (0.045 mmol) was added to a solution of 6^{A}-amino-PM-*β*-CD (**39**, 0.03 mmol) in dried DMF (2 mL) followed by the addition of DMAP (0.03 mmol). After stirring the solution vigorously for 30 min at room temperature, conjugates **13**, **24**, **19–23** or **30–34** (0.02 mmol) were added. The progress of the reaction was monitored by TLC. After concentrating the reaction mixture *in vacuo*, the residue was dissolved in water (10 mL), and extraction was conducted using

dichloromethane (3 × 10 mL). The combined organic phase was washed with saturated $NaHCO_3$ (10 mL) and brine (10 mL) and dried over anhydrous sodium sulfate. Following filtration, the filtrate was concentrated *in vacuo*. The resulting crude product was purified using silica gel column chromatography to afford the final product.

The synthetic method of only compound **40** is described here, for the rest of compounds **41**–**51** readers are invited to refer to Supporting Information section.

2.3. Synthesis of *N*-(6^{A}-Deoxy-per-*O*-methylated-*β*-CD-6-yl)-3*β*-hydroxy-lup-20(29)-en-28-amide (40). Prepared from **13** and **39** according to general procedure, the residue was purified by flash chromatography (eluent: DCM/CH_3OH = 30:1) to afford **40** as a white foam in 85% yield. R_f = 0.26 (DCM/CH_3OH = 15:1). ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.05 (t, 1H, *J* = 5.6 Hz), 5.20 (d, 1H, *J* = 3.8 Hz), 5.15-5.17 (m, 2H), 5.06-5.09 (m, 4H), 4.71 (s), 4.58 (s), 3.33-3.95 (m, 93H), 3.91 (m, 1H), 3.37 (m, 1H), 3.10-3.24 (m, 6H), 3.16 (m, 1H), 3.13 (m, 1H), 3.05 (dd, 1H, *J* = 9.6, 3.1 Hz), 2.47-2.53 (m, 1H), 1.88-1.99 (m, 2H), 0.87-1.77 (m, other aliphatic ring protons), 1.67 (s, 3H), 0.96 (2 × s, 6H), 0.80 (s, 3H), 0.75 (s, 3H), 0.68 (d, 1H, *J* = 10.9 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): δ 176.14, 150.92, 109.24, 99.99, 99.15, 99.11, 99.07, 98.95, 98.75 [2C], 82.79, 82.18, 82.03, 81.84, 81.81, 81.74, 81.72, 81.66, 81.41, 81.38, 80.82, 80.75, 80.51, 80.42, 80.16, 80.14, 80.05, 78.93, 71.73, 71.47, 71.09, 70.88, 70.78, 70.07, 61.63, 61.61, 61.58, 61.53, 61.30, 61.19, 61.10, 59.36, 59.29, 59.01, 58.99, 58.90, 58.73, 58.70, 58.40, 58.33, 58.14, 58.09, 55.90, 55.33, 50.57, 50.15, 46.58, 42.59, 40.68, 38.82, 38.66, 38.33, 38.21, 37.44, 37.20, 34.55, 34.34, 30.89, 29.35, 27.96, 27.39, 25.54, 20.95, 19.50, 18.15, 16.30, 16.02, 15.31, 14.66; ESI-HRMS calcd for $C_{92}H_{161}N_2O_{36}$ $[M + NH_4]^+$: 1870.0829, Found 1870.0796.

2.4. Cell Culture. The HL-60, BGC-823, and MCF-7 cells were donated by Crown Bioscience, Inc. (Beijing, China). All the cell lines were cultured in RPMI 1640 medium (Invitrogen, A1049101) containing 10% fetal bovine serum (FBS) (Gibco, 10099141C) and maintained in a humidified incubator at 37°C under 5% CO_2.

2.5. Cell Viability Assay. The proliferative effect of each conjugate on the HL-60, BGC-823, and MCF-7 cells conjugate was measured using CellTiter-Glo luminescent

cell viability assay (Promega Corp Inc., USA) according to the original manufacturer's instructions. Briefly, the three cancer cell lines harvested in a logarithmic growth phase were seeded in 96-well plates at a density of 1×10^4 cells/well overnight and treated in triplicate with different conjugates (10 μM). After 48 h, CellTiter-Glo reagent was added to the cell culture medium present in each well in equal volumes. The emitted luminescence was measured using Infinite® M200 Pro multimode microplate reader (Tecan, Switzerland).

For determining IC_{50} values, the conjugates were diluted to different concentrations (0.078, 0.16, 0.31, 0.63, 1.25, 2.50, 5.00, 10.0, and 20.0 μM), and the final DMSO concentration was 0.05%. Then, the experiment proceeded as described above.

2.6. Cell Apoptosis Assay. HL-60 cells (1×10^6/well) were cultured in RPMI 1640 supplemented with 10% FBS in 12-well plates for 24 h and treated with 10 μM of OA, PM-β-CD, conjugate **48**, or conjugate **50** for 36 h. Cells treated with 0.05% DMSO were used as vehicle control. Then, the cells were harvested and washed with phosphate buffer saline (PBS), and the apoptotic status was analyzed using FITC-Annexin V/PI Kit (Solarbio, China), following the manufacturer's instructions. Briefly, resuspended cells were stained with FITC-Annexin-V in the dark for 5 min and then stained with PI for an additional 5 min. The percentages of apoptotic cells were analyzed using flow cytometry on CytoFLEX LX (Beckman Coulter, CA, USA).

2.7. Measure of Intracellular ROS. Intracellular ROS was further determined with conjugate **48** as a representative using ROS assay kit (Solarbio, China) based on the fluorescent probe DCFH-DA, according to the original manufacturer's instructions. Briefly, 1×10^6 HL-60 cells were seeded in 12-wells plates and pretreated with 2.5 mM NAC for 2 h or not. Then, the cells were treated with or without 10 μM conjugate **48** for 12 h. Treated cells were extensively washed with PBS and then incubated with 10 μM DCFH-DA at 37°C for 20 min. DCF fluorescence was determined using a flow cytometer at an emission wavelength of 535 nm and excitation wavelength of 488 nm.

2.8. Measure of $\Delta\psi_m$. JC-1 mitochondrial membrane potential detection kit (Solarbio, China) was used to assess the $\Delta\psi_m$ in cells. The treated cells were washed twice with ice-cold PBS and stained with 2 μM JC-1 for 20 min at 37°C. Then, the cells were washed, resuspended in PBS, and analyzed using flow cytometry on CytoFLEX LX (Beckman Coulter, CA, USA).

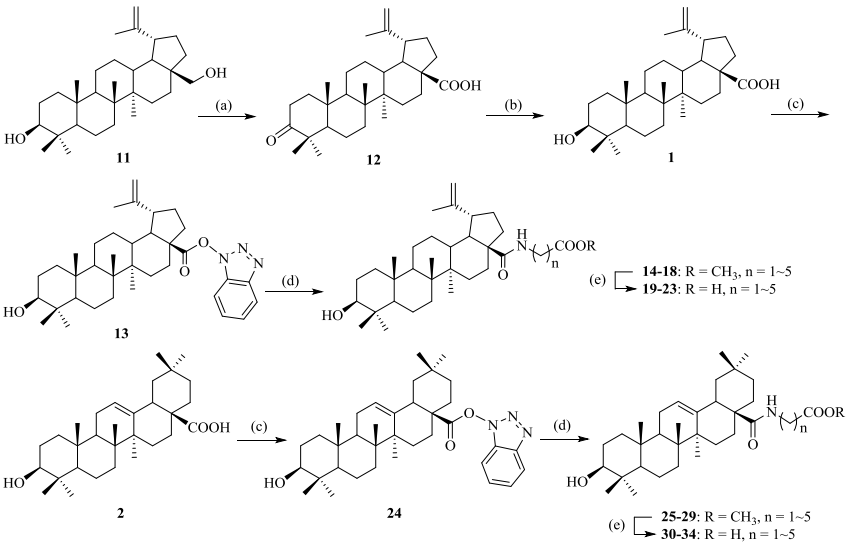
2.9. Western Blot Analysis. Western blots were used to evaluate apoptosis-related protein content in the cell extracts. HL-60 cells were seeded in 12-well plates at a density of 1×10^6 cells/well in RPMI 1640 supplemented with 10% FBS and treated with 10 μM OA, 10 μM PM-β-CD, and 1.25, 2.50, 5.00, or 10.0 μM of conjugate **48** or **50** for 12 h. Cells treated with 0.05% DMSO were used as control. Then, the cells were harvested and washed. The total proteins of HL-60 cells were extracted using RIPA lysis buffer (Thermo Fisher Scientific Inc. Illinois, USA) with protease and phosphatase inhibitor cocktails (Roche, Mannheim, Germany). The mitochondrial proteins were isolated according to the protocol of a commercial Mitochondria Isolation Kit (Yeasen, Shanghai, China). Proteins were quantified using BCA protein assay kit (Invitrogen, USA). After SDS-PAGE, the separated proteins were transferred from the gel onto a PVDF membrane. Antibodies used in this study included anti-ERK (Proteintech, cat. 16443-1-AP), anti-pERK (CST, cat. 4370, clone D13.14.4E), anti-STAT3 (Proteintech, cat. 10253-2-AP), anti-pSTAT3 (CST, cat. 9145, clone D3A7), anti-BAX (Proteintech, cat. 50599-2-Ig), anti-cytochrome *c* (Proteintech, cat. 10993-1-AP), anti-COX IV (Proteintech, cat. 11242-1-AP), and anti-caspase 3 (Proteintech, cat. 19677-1-AP), which were applied at the recommended dilution, following which an HRP-conjugated secondary antibody (CST, cat. 7074S) was applied. Immobilon Western Chemiluminescent HRP Substrate (Millipore, USA) was used to detect HRP. Images were captured by a chemiluminescent imaging system (Tanon, China). Signals from an anti-β-actin antibody (Proteintech, cat. 20536-1-AP) served as loading controls. Western blot strips were quantitatively analyzed using image analysis software Image J.

2.10. Statistical Analysis. Data were expressed as mean ± standard deviation. Assays were performed in triplicate, and the mean was used for statistical analysis. The IC_{50} values were calculated using GraphPad Prism 8.3.0 Software (GraphPad Inc., San Diego, CA, USA). The flow cytometric data were analyzed using CytExpert 2.3 Software (Beckman Coulter, Brea, CA, USA).

3. RESULTS AND DISCUSSION

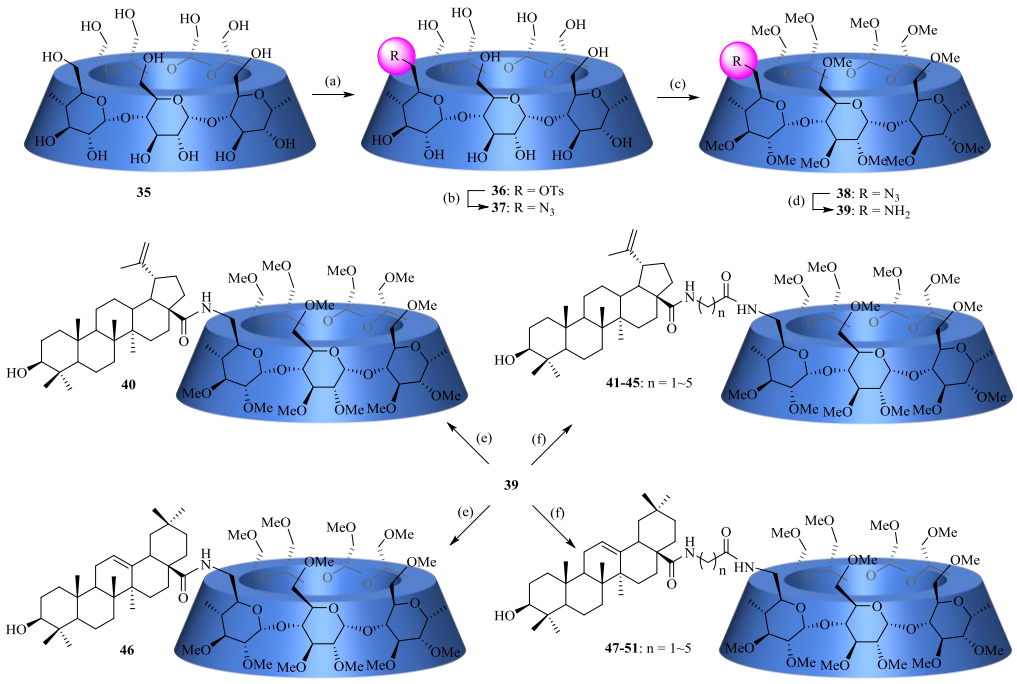
3.1. Synthesis of Conjugates 40–51. Scheme 1 outlines the preparation of ω-aminoalkanoic acid amide derivatives of two major pentacyclic triterpenoids, which were used as building blocks for synthesizing the target conjugates. BA (**1**) was synthesized via a convenient two-step conversion of betulin (**11**).[34] After the

activation of the carboxyl group at position 17 via 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate, a highly stable intermediate **13** reacted with *ω*-aminoalkanoic acid methyl ester [$NH_2(CH_2)_nCOOCH_3$] in the presence of Na_2CO_3, followed by the base-catalyzed hydrolysis of methyl ester in a methanol/THF mixture to afford known BA derivatives **19–23**.[35] Similarly, *ω*-aminoalkanoic acid–substituted OA derivatives **30–34** were also prepared.[36]



Scheme 1. Reagents and conditions: (a) chromic acid, H_2SO_4, acetone, 0°C→rt, 63%; (b) $NaBH_4$, THF, 79%; (c) TBTU, DIEA, THF, 91%; (d) *ω*-aminoalkanoic acid methyl ester, Na_2CO_3, DMF, 71-82% for **14–18**, 70-84% for **25–29**; (e) NaOH (5%, w/v), H_2O/THF (1:1, v/v), quantitative.

BA- and OA-PM-*β*-CD conjugates **40–51** were synthesized from *β*-CD (Scheme 2). The key intermediate 6^A-amino-PM-*β*-CD (**39**) was synthesized from natural *β*-CD via a four-step procedure involving selective monotosylation, azide nucleophilic substitution, permethylation, and catalytic hydrogenation reactions.[32, 37] Then, an amide coupling reaction with **13** and **24** in the presence of Na_2CO_3 or with **19–23** and **30–34** in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was performed to finally afford **40–51**. All the synthesized conjugates **40–51** were purified using silica gel column chromatography and characterized using ^{1}H-NMR, ^{13}C-NMR, and ESI-HRMS spectra, which are presented in the supplementary data (Supporting Information).



Scheme 2. Reagents and conditions: (a) *p*-TsCl, NaOH, H_2O, 12%; (b) DMF, NaN_3, 60°C, 89%; (c) DMF, NaH, 1 h; then CH_3I, 77%; (d) Pd/C, H_2, CH_3OH, 82%; (e) **13** and **24**, Na_2CO_3, DMF, 85% for **40**, 81% for **46**; (f) **19–23** and **30–34**, EDC, DMAP, DMF, 68-75% for **41–45**, 65-79% for **47–51**.

3.2. Cytotoxic Activity of Pentacyclic Triterpenoid-PM-*β*-CD Derivatives. The cytotoxic activities of the two starting triterpenoids, BA (**1**) and OA (**2**), as well as their synthesized conjugates of PM-*β*-CD **40–51**, were evaluated in human acute promyelocytic leukemia (HL-60), human gastric carcinoma (BGC-823), and human breast adenocarcinoma (MCF-7) cells. Based on CellTiter-Glo luminescent cell viability assay, the half maximal inhibitory concentration (IC_{50}) of the cells was determined and summarized in Table 1. PM-*β*-CD was devoid of significant cytotoxicity (IC_{50} > 200 μM, Figure S1). BA (**1**) exhibited moderate cytotoxicity toward HL-60, MCF-7, and BGC-823 cells, with IC_{50} values of 19.2, 22.9, and 30.6 μM, respectively, which was in accordance with the literature (MCF-7, IC_{50} = 14.9 μM).[38] However, compared with BA (**1**), its analog OA (**2**) exhibited weak cytotoxicity (IC_{50}: 120~157 μM). Under the assayed conditions (0.078–20 μM), conjugates **41–44**, **48**, and **50** exhibited pronounced cytotoxic activities (lower micromolar range), with some preference for HL-60 cells (IC_{50} range: 5.76~7.04 μM).

The most effective conjugate was the PM-*β*-CD conjugate of OA linked by 5-aminopentanoic acid **44** (IC_{50} = 5.76 μM for HL-60 cells). Interestingly, two PM-*β*-CD conjugates **40** and **46**, wherein BA and OA were directly conjugated via amide bonds, respectively, showed inferior cytotoxicity toward the three cell lines with IC_{50} values all over 20 μM. Thus, due to the steric environment surrounding around the drug caused by the relative bulky *β*-CD group, the short linkage might have a detrimental effect by interrupting the binding of target protein with pharmacophore. Only two OA-PM-*β*-CD conjugates, **48** and **50**, showed significantly reduced cell viability in MCF-7 cells (IC_{50} = 8.18; 7.99 μM, respectively). These conjugates also showed cytotoxicity toward HL-60 and BGC-823 cells at the micromolar level. Therefore, they were used for further biological evaluation.

Table 1. Cytotoxic activity of OA- and BA-PM-*β*-CD conjugates **40–51** against HL-60, BGC-823, and MCF-7 cells.[a]

Conjugate	IC_{50} (μM) [b]		
	HL-60	BGC-823	MCF-7
PM-*β*-CD	>200	>200	>200
BA (**1**)	19.2 ± 0.63	30.6 ± 1.16	22.9 ± 0.94
OA (**2**)	120 ± 15.1	157 ± 5.78	128 ± 13.1
40	>20	>20	>20
41	7.04 ± 0.30	>20	>20
42	6.63 ± 0.66	>20	>20
43	6.27 ± 0.74	11.9 ± 0.47	>20
44	5.76 ± 0.90	9.95 ± 2.99	>20
45	16.4 ± 9.80	>20	>20
46	>20	>20	>20
47	12.9 ± 1.03	>20	>20
48	6.06 ± 0.65	6.64 ± 2.56	8.18 ± 3.65
49	12.4 ± 1.70	>20	>20
50	6.15 ± 0.65	8.47 ± 0.35	7.99 ± 3.33
51	>20	>20	>20

[a] Data are expressed as the means of three independent experiments with standard deviation.

[b] Half maximal inhibitory concentration.

3.3. Conjugates 48 and 50 Induced Significant Apoptosis in HL-60 Cells. Over the last decade, numerous studies have aimed to elucidate the molecular mechanisms of pentacyclic triterpenoid–mediated antitumor activity.[7, 38] As a potent human melanoma-specific inhibitor, the ability of BA to induce apoptosis forms the basis of its antitumor activity.[39] To test whether the OA-PM-β-CD conjugates described herein also act via this mechanism, the apoptosis rate of HL-60 cells, which responded best to the conjugates, was detected using Annexin V and propidium iodide (PI) staining. The typical dot plots of the live/dead assay are shown in Figure 2A. The results revealed that only 2.45% and 3.37% of apoptotic cells were detected after 36 h of treatment with 10 μM PM-β-CD and BA, respectively. However, majority of the cells treated with the same concentration of conjugates **48** and **50** were found to be either in early (77.53% and 72.30%, respectively) or in later (21.46% and 27.17%, respectively) apoptosis. These results demonstrate that conjugates **48** and **50** exert antitumor activity by inducing apoptosis in cancer cells within 36 h after treatment.

Subsequently, the expression of apoptosis-related proteins was investigated using Western blot. The expression levels of cleaved caspase-3 (pro-caspase-3), an apoptosis-associated protein, in HL-60 cells exhibited dramatic decrease after a 12-h incubation with conjugates **48** and **50** (2.5, 5.0, and 10.0 μM) in a dose-dependent manner (Figure 2B). In addition, exposing the cells to 10.0 μM of conjugates **48** and **50** induced remarkable proteolysis of pro-caspase-3conjugate, indicating that activation of caspase-3 and promotion of cell apoptosis. Although no visible changes could be observed for the proapoptotic protein BAX, a minor increase in the levels of the cleaved form of BAX (p18 BAX) protein, which is known to be more potent in disrupting mitochondrial membrane integrity and inducing apoptosis, was detected at a high concentration (10.0 μM). ERK, pERK, STAT3, and pSTAT3 protein levels in the conjugate-treated HL-60 cells were evaluated to explore the signaling pathways associated with the induction of apoptosis. Treating the cells with PM-β-CD, **2**, **48**, **50**,

or 0.05% DMSO for 12 h did not significantly affect ERK and STAT3 protein expression. However, treatment with 10 μM of conjugates **48** and **50** significantly increased pERK1/2 protein expression. Notably, incubating HL-60 cells with conjugates **48** and **50** (2.5, 5.0, and 10.0 μM) induced a dose-dependent reduction in pSTAT3 protein levels. These findings suggest that the antitumor effect of conjugates **48** and **50** is related to caspase-3-dependent apoptosis, and pERK1/2 and pSTAT3 play a crucial role in this process. In addition, conjugate **48** is more potent than **50**, which is consistent with the antitumor effect these conjugates exhibited toward HL-60 cells. Therefore, conjugate **48** was selected as a representative for further study.

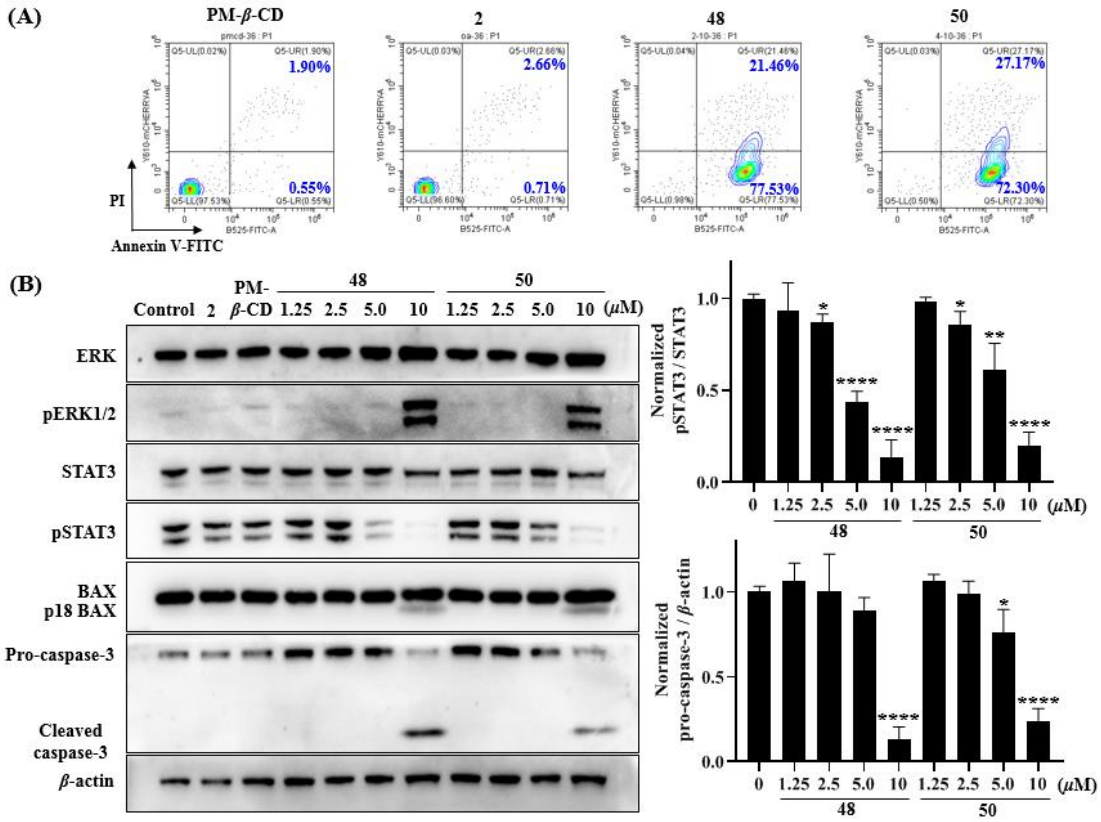


Figure 2. Conjugates **48** and **50** induced apoptosis in HL-60 cells. (**A**) Apoptosis-inducing ability of OA-PM-β-CD conjugates **48** and **50** in HL-60 cells was assessed via the flow cytometric analysis of Annexin V-FITC and PI staining. (**B**) Western blot analysis of ERK, pERK, STAT3, pSTAT3, BAX, caspase-3, and β-actin protein levels was performed using extracts of HL-60 cells treated with 0.05% DMSO (control), conjugate **2** (10 μM), PM-β-CD (10 μM), and the indicated amounts of conjugates **48** and **50** for 12 h. Commercially provided antibodies were used. Data are expressed as

the mean of three separate experiments with standard deviation. ***P* < 0.05, ****P* < 0.01, and *****P* < 0.0001 *vs*. the control group.

3.4. Conjugate 48 Caused Mitochondrial Dysfunction in HL-60 Cells. Apoptosis is essential for physiology and pathology and has two major pathways:[40] death receptor-mediated extrinsic pathway and mitochondrial-mediated intrinsic pathway. The convergence of the two pathways at the outer mitochondrial membrane leads to its permeabilization. Growing evidence suggests that the oligomerization of BAX in the outer membrane of mitochondria creates pores to allow the release of cytochrome *c*, thereby inducing apoptosis.[41] In the present study, mitochondrial cytochrome *c* and BAX protein expression levels were examined using Western blot analysis to determine whether conjugate **48** uses a similar mechanism to induce apoptosis (Figure 3A). The mitochondrial BAX levels considerably increased following the 12-h treatment with conjugate **48**. Concurrently, a significant loss of cytochrome *c* was detected in conjugate **48**-treated HL-60 cells, indicating its release into the cytosol due to the permeabilization of the outer mitochondrial membrane.

This permeabilization also causes the loss of mitochondrial membrane potential ($\Delta\psi_m$). To investigate whether conjugate **48** altered the $\Delta\psi_m$, HL-60 cells were treated with 10-μM conjugate **48** for 12 h, and the $\Delta\psi_m$ was then investigated with a JC-1 probe using a flow cytometer assay. As illustrated in Figure 3B, a substantial decrease was detected in the red fluorescence, indicating a drop in the $\Delta\psi_m$. These results suggest that the mitochondrial-mediated apoptosis pathway is involved in conjugate **48**-induced apoptosis of HL-60 cells.

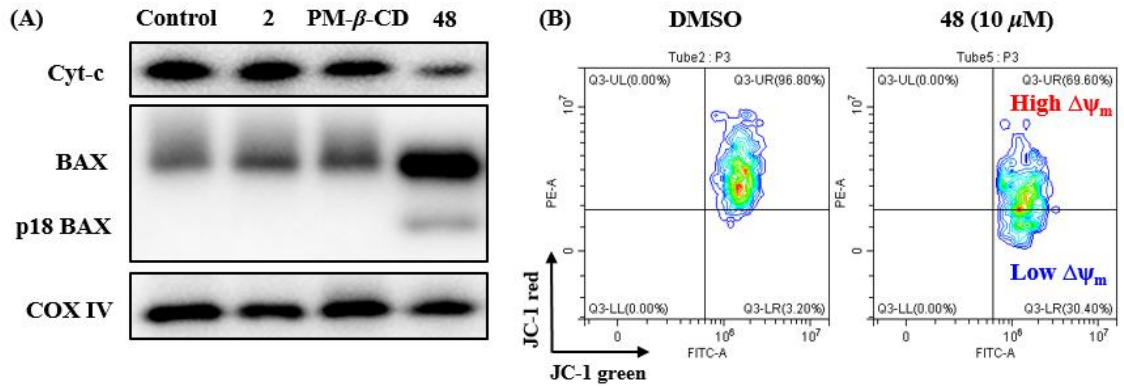


Figure 3. Effect of **48** on mitochondrial membrane potential and related protein

expression. (**A**) Western blot analysis of the mitochondrial cytochrome *c* and BAX protein levels was performed in mitochondrial extracts from HL-60 cells treated with 0.05% DMSO (control), conjugate **2** (10 μM), PM-β-CD (10 μM), and conjugate **48** (10 μM) for 12 h. COX IV was used as a loading control. Commercially provided antibodies were used. (**B**) Flow cytometric analysis of HL-60 cells labeled with JC-1 (2 μM), untreated (left), or conjugate **48** treated (10 μM) (right).

3.5. Conjugate 48 Promoted ROS-mediated Apoptosis in HL-60 Cells. Mitochondrial dysfunction is often associated with increased levels of reactive oxygen species (ROS), which are pivotal in the regulation of cellular apoptosis.[42] The change in intracellular ROS levels is determined based on a flow cytometric assay using a cell-penetrable ROS sensor dye; 2′-7′-dichlorodihydrofluorescein diacetate (DCFH-DA).[43] In the current study, the intracellular ROS level increased to 67.68% after HL-60 cells were treated with 10 μM of conjugate **48** (Figure 4A).

To address whether conjugate **48** induces apoptosis via the generation of ROS, the protection offered by *N*-acetyl-*L*-cysteine (NAC), a well-known free radical scavenger, against conjugate **48**-induced toxicity was investigated. Notably, pretreatment with 2.5 mM NAC significantly scavenged conjugate **48**-induced ROS levels in HL-60 cells. The fluorescence intensity of DCF decreased from 67.68% to 13.46% (Figure 4A). Furthermore, NAC treatment significantly decreased apoptosis in HL-60 cells, from 36.54% to 16.72% (Figure 4B). NAC pretreatment also reversed, at least partially, the effect of conjugate **48** on BAX cleavage and pro-caspase-3 activation (Figure 4C). These results clearly indicate that the new pentacyclic triterpenoid derivatives induce ROS-mediated apoptosis in HL-60 cells.

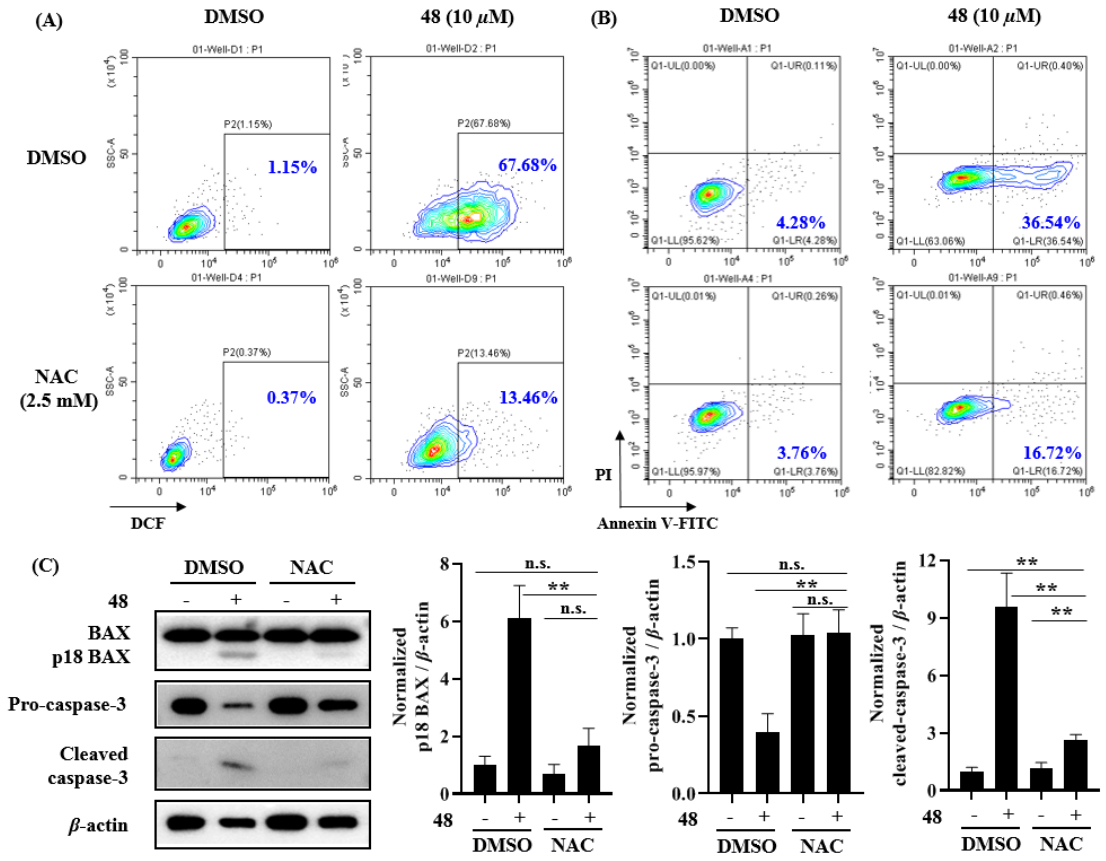


Figure 4. ROS-mediated apoptosis induction by conjugate **48**. HL-60 cells were preincubated with or without pretreatment with 2.5 mM *N*-acetyl-*L*-cysteine (NAC) for 2 h followed by the addition of 10 μM of **48** for another 12 h. (**A**) Intracellular ROS level assay via flow cytometry evaluated at a wavelength of 535 nm with excitation at 488 nm. (**B**) Apoptosis assay assessed using flow cytometric analysis via Annexin V-FITC and PI staining. (**C**) Western blot and the corresponding quantification data analysis of BAX, caspase-3, and β-actin protein levels. Data are expressed as the mean of three separate experiments with standard deviation. $^{**}P < 0.01$ *vs*. the control group.

In conclusion, this study shed light on the antitumor activity and mechanism of a series of novel and water-soluble BA/OA derivatives **40**–**51**, wherein BA or OA triterpenoid was tethered to the primary face of PM-β-CD via various alkyl linkers. The antitumor activities of the synthesized conjugates were evaluated, and the structure-activity relationships (SARs) were discussed. Among them, conjugates **48** and **50** exhibited optimal and broad antitumor activities against three different cancer cell lines, with IC_{50} values between 6.06 and 8.47 μM. Further antitumor-mechanism

studies revealed that the two conjugates induced the apoptosis of HL-60 cells via the ROS-dependent, mitochondrial-mediated pathway by downregulating pSTAT3 and upregulating pERK1/2 and cleaved caspase-3 expressions. In addition, conjugate **48** caused the collapse of the mitochondrial membrane potential and spontaneous release of cytochrome *c* into the cytosol, which further confirmed that **48**-induced apoptosis occurred via the mitochondrial-mediated pathway. These results indicate that modifying pentacyclic triterpenoids with a PM-*β*-CD group can generate novel 28-substituted triterpenoid derivatives with enhanced antitumor activities.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at http://pubs.acs.org/doi/.

Synthesis procedures of conjugates **41-51**; IC_{50} determination curves for compounds **1**, **2**, **35**, **48**, and **50**; and ^{1}H NMR, ^{13}C NMR, and ESI-HRMS spectra of conjugates **41-51**.

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Notes

The authors declare no competing financial interest.

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