Sesquiterpene lactone 6-O-angeloylplenolin reverses vincristine resistance by inhibiting YB-1 nuclear translocation in colon carcinoma cells

CHANGLONG LI[1,2], HEZHEN WU[3], YANFANG YANG[3], JIANWEN LIU[2] and ZHENWEN CHEN[1]

[1]School of Basic Medical Science, Capital Medical University, Beijing 100069; [2]School of Pharmacy, East China University of Science and Technology, Shanghai 200237; [3]Key Laboratory of Resources and Chemistry of Chinese Medicine of The Ministry of Education, Hubei University of Chinese Medicine, Wuhan, Hubei 430061, P.R. China

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Abstract. Multidrug resistance (MDR) is a major obstacle to cancer chemotherapy efficacy. In the present study, 6-O-angeloylplenolin repressed the overexpression of ATP binding cassette subfamily B member 1 (*MDR1*) and increasing the intracellular concentration of anticancer drugs. A reduction in P-glycoprotein expression (encoded by *MDR1*) was observed in parallel with a decline in mRNA expression in vincristine-resistant HCT (HCT-8/VCR) cells treated with 6-O-angeloylplenolin. In addition, 6-O-angeloylplenolin suppressed the activity of the *MDR1* gene promoter. Treatment with 6-O-angeloylplenolin also decreased the amount of the specific protein complex that interacted with the *MDR1* gene promoter in HCT-8/VCR cells, potentially leading to the suppression of *MDR1* expression. Treatment with 6-O-angeloylplenolin inhibited the nuclear translocation of Y-box binding protein-1 in HCT-8/VCR cells treated with 6-O-angeloylplenolin, contributing to the negative regulation of *MDR1*. Finally, 6-O-angeloylplenolin reversed VCR resistance in an HCT/VCR xenograft model. In conclusion, 6-O-angeloylplenolin exhibited a MDR-reversing effect by downregulating *MDR1* expression and could represent a novel adjuvant agent for chemotherapy.

Correspondence to: Dr Jianwen Liu, School of Pharmacy, East China University of Science and Technology, 130 Menlong Road, Xuhui, Shanghai 200237, P.R. China
E-mail: czwen@ccmu.edu.cn

Dr Zhenwen Chen, School of Basic Medical Science, Capital Medical University, 10 Youanmen Xitoutiao Road, Fengtai, Beijing 100069, P.R. China
E-mail: czwenteam@163.com

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Introduction

Multidrug resistance (MDR) is a major obstacle to successful cancer chemotherapy. MDR is often associated with the overexpression of the ATP binding cassette subfamily B member 1 (*ABCB1*, also known as *MDR1*) gene, which encodes P-glycoprotein (P-gp) (1,2). P-gp expression enhances drug efflux pump activity, depleting the intracellular concentration of anticancer drugs, driving drug resistance. Various environmental stimuli, including an inverted CCAAT box, can activate the *MDR1* promoter (3-5), which directly interacts with Y-box-binding protein (YB-1) (5-9). Assays into *MDR1* promoter activity using reporter genes demonstrated that the nuclear translocation of YB-1 was able to activate the *MDR1* promoter (5,7), contributing to the expression of *MDR1* and to drug resistance (5,9). In addition, the expression of *MDR1* was also observed in tumor specimens derived from cancer patients (9-11). Therefore, the nuclear localization of the YB-1 is a notable marker of disease progression (6). Moreover, multidrug resistance-associated protein 1 (MRP1) is a protein encoded by the ABCC1 gene in human, which may interacted with MDR1 to regulate the drug resistant as well (12). However, a previous study revealed that the amount of nuclear YB-1 was not associated with *MDR1* expression (13). The mechanism of YB-1 nuclear translocation is presently unclear, meaning that further research is required.

The sesquiterpene lactone 6-O-angeloylplenolin, a bioactive component of *Centipeda minima* (L.) A (14), has been reported to have various biological activities, including antiprotozoal and antibacterial activities (15). It was previously reported that 6-O-angeloylplenolin induced apoptosis through a mitochondrial/caspase pathway in acute promyelocytic leukemia HL-60 cells (16); it was also reported to trigger mitotic arrest and caspase-dependent apoptosis in human multiple myeloma cells (17). The result of proteome microarray analysis revealed that 6-O-angeloylplenolin inhibited S-phase kinase-associated protein 1 (Skp1) and signal transducer and activator of transcription 3 to repress Skp2, exhibiting inhibitory effects on lung cancer cell proliferation (18). In addition, it was reported that a number of sesquiterpene lactones could bind specifically to human P-gp and reverse cellular multidrug

resistance (19). However, the detailed mechanism behind the 6-O-angeloylplenolin-dependent rescue of drug resistance in cancer was not revealed. The present study demonstrated that 6-O-angeloylplenolin exhibited MDR-reversing activity in colon carcinoma cells and attempted to investigate the underlying mechanisms behind this reversal.

Materials and methods

Chemicals, cell lines and cell culture. 6-O-angeloylplenolin (also known as brevifolin or brevilin-A) (Fig. 1A) was isolated from *C. minima* in Capital Medical University (Beijing China), as described previously (17). The purity of 6-O-angeloylplenolin was >97% (HPLC). The human colon carcinoma HCT-8 cell line and its MDR variant HCT-8/VCR were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were maintained in RPMI 1640 (Life Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies; Thermo Fisher Scientific, Inc.), penicillin (100 ng/ml) and streptomycin (100 ng/ml) in a humidified atmosphere with 5% CO_2 at 37˚C. HCT-8/VCR cells were grown under the same conditions, but the medium contained 2 mg/l vincristine (Sigma Aldrich; Merck KGaA, Darmstadt, Germany), which was removed for 1 week prior to assay commencement. In terms of cell proliferation and cell cycle distribution, there was no significant difference between these two cell lines.

MTT assay. The MTT colorimetric assay was performed as described by Mosman *et al* (20). HCT-8 or HCT-8/VCR cells were seeded into 96-well plates at a density of $1x10^4$ cells/well. The cytotoxicity of 6-O-angeloylplenolin, vincristine, mitomycin (Sigma Aldrich; Merck KGaA), hydroxycamptothecin (Sigma Aldrich; Merck KGaA) or the compounds in combinations in HCT-8 and HCT-8/VCR cells was analyzed by MTT assay following incubation of cells with these compounds for 24 h. The concentrations of vincristine, mitomycin or hydroxycamptothecin were 1, 3, 10, 30, 100 and 300 μg/ml. The cytotoxicity was expressed as the half-maximal inhibitory concentration (IC_{50}), which was extrapolated from linear regression analysis of experimental data.

Detection of intracellular vincristine accumulation. High-performance liquid chromatography (HPLC) was used to measure the accumulation of drugs in cells and tissue, as described previously (21). HCT-8/VCR cells were incubated with 6-O-angeloylplenolin for 24 h and then the cells were treated with 80 μg/ml vincristine for 12 h. A total of $1x10^6$ cells were collected and centrifuged at 4˚C for 10 min at 15,996 x g. The supernatant was discarded. The cell pellet was resuspended and lysed (Cell Signaling Technology, Inc. Danvers, MA, USA) for 10 min at 4˚C. A total of 50 μl cell lysate samples were taken for HPLC evaluation. The concentrations of vincristine were determined by HPLC as described by Chinese pharmacopoeia (22). Briefly, separation was performed on a C_{18} column (Eclipse C_{18}, 150 by 4.6 mm, with a particle size of 5 μm; Agilent Technologies, Inc., Santa Clara, CA, USA) at 20˚C. The mobile phase was a 55:45 (v/v) mixture of methanol and 60 mM sodium phosphate buffer (pH 3.0). The UV detector was set at wavelength of 297 nm. Data were expressed as the ratio of the peak area to that of the internal standard.

Adriamycin and Rh123 accumulation assay. Accumulation of the fluorescent compounds adriamycin (Sigma-Aldrich; Merck KGaA) and Rh123 (0.5 mg/ml; Sigma-Aldrich; Merck KGaA) were used as a functional index of P-gp, as described previously (5,23). HCT-8/VCR cells ($1x10^4$ cells/well) were first treated with 0.35, 0.7 and 1.4 μM 6-O-angeloylplenolin for 24 h. Next, one group of cells were treated with 12 μM verapamil for 12 h, which was used as the positive control (24). Following this, the cells were incubated in medium containing 50 μM adriamycin and 10 μM Rh123 for 3 h. Finally, the cells were washed twice with PBS prior to measurement. The fluorescence intensity of were detected by fluorospectrophotometer (Synergy 2, BioTek Instruments, Inc., Winooski, VT, USA) for Rh123 at 530 nm (excitation wavelength, 500 nm) and adriamycin at 530 nm (excitation wavelength, 485 nm).

Determination of gene expression by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). HCT-8/VCR cells were treated with 1.4 μM 6-O-angeloylplenolin for 12 h. Total RNA was isolated by RNeasy Mini kit 250 (cat. no. 74106; Qiagen GmbH, Hilden, Germany) from HCT-8 or HCT-8 cells. QuantiTect Reverse Transcription kit 200 (cat. no. 205313; Qiagen GmbH) was used to synthesize complementary DNA (cDNA), according to manufacturer's protocol. The synthesized cDNA was subjected to RT-qPCR using QuantiTect SYBR® Green RT-PCR kit (Qiagen GmbH) on a Bio-Rad CFX Connect real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA) with conditions of 30 cycles of 40 sec at 95˚C, 40 sec at 60˚C and 30 sec at 72˚C. The primers used for PCR were shown below: *MDR1* forward, 5'-CGAAACCGTATCAGTCCTCG-3' and reverse, 5'-CTTGAGTCTGAGAGACCACC-3' (25); *MRP1* forward, 5'-GTGGAATTCCGGAACTAC-3' and reverse, 5'-CGGAGGTCGTGCAGGCCG-3' (24); *YBX1* forward, 5'-CGGAGGCAGCAAATGTTA-3' and reverse, 5'-CACCCTGGTTGTCAGCAC-3' (4); and *GAPDH* forward, 5'-GTCAACGGATTTGGTCGTATT-3' and reverse, 5'-AGTCTTCTGGGTGGCAGTGAT-3'. The PCR products were separated by electrophoresis on an 1.5% agarose gel and stained with 0.1 μg/ml ethidium bromide. Differential gene expression was quantified using the Image Analysis system, version 3 (FR-980A gel Image Analysis System, Shanghai Furi Science and Technology Co., Ltd., Shanghai, China).

Flow cytometry assay. HCT-8/VCR cells were treated with 0.35, 0.7 or 1.4 μM 6-O-angeloylplenolin for 24 h, and flow cytometry was performed. A total of $1x10^5$ cells/ml cells were washed in PBS with 0.1% sodium azide (PBS-azide). The cells were firstly blocked with 3% Bovine Serum Albumin (BSA) at room temperature for 1 h. Then, HCT-8/VCR cells were incubated with 2 μg monoclonal antibodies against P-gp (Aviva Systems Biology, Corp., San Diego, CA, USA; cat. no. APR51326_P050) at a dilution of 1:100 for 2h at room temperature. Next, cells were washed twice with PBS and incubated with 1 μg goat anti-rabbit secondary antibodies

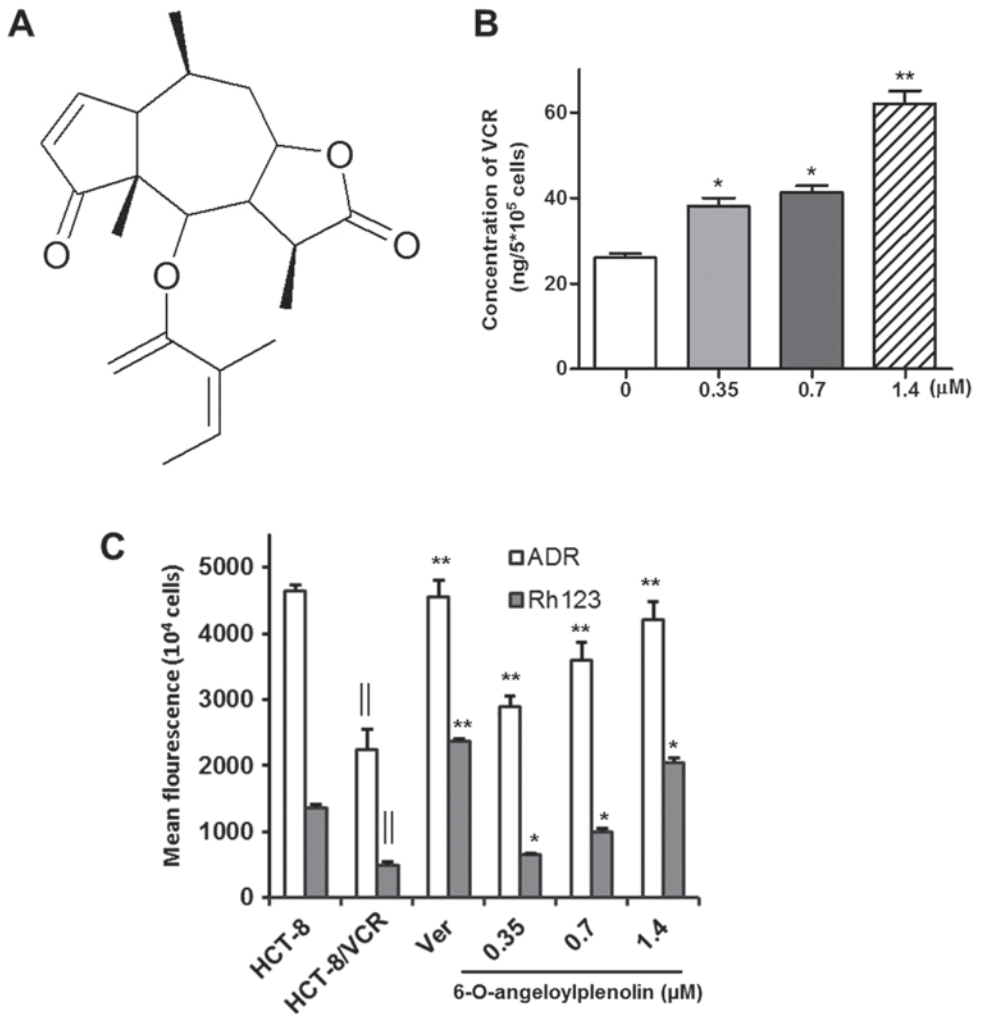

Figure 1. Chemical structure of 6-O-angeloylplenolin and effect of 6-O-angeloylplenolin on the intracellular accumulation of vincristine, adriamycin and Rh123. (A) The Chemical structure of 6-O-angeloylplenolin. (B) HCT-8/VCR cells were incubated with 80 μg/ml vincristine for 12 h following treatment with 6-O-angeloylplenolin for 24 h. The intracellular accumulation of vincristine was measured by HPLC in 1x10^6 cells (n=3). *P<0.05 and **P<0.01 vs. untreated HCT-8/VCR cells. (C) Cells were incubated with 50 μM adriamycin and 10 μM Rh123 for 3 h following treatment with 6-O-angeloylplenolin for 24 h. The fluorescence intensity was detected in 1x10^4 cells (n=4). *P<0.05 and **P<0.01 vs. untreated HCT-8/VCR cells; $^{\|}$P<0.01 vs. untreated HCT-8 cells.

(1:10,000) conjugated with fluorescein isothiocyanate (FITC) (Rockland Immunochemicals, Inc., Limerick, PA, USA; cat. no. 611-1102) at room temperature for 30 min in dark. Over 2x10^4 events were acquired and analyzed by a FACScan flow cytometer with CellQuest software (version 5.1; BD Biosciences, Franklin Lakes, NJ, USA).

Western blot analysis. Following treatment with 0.35, 0.7 or 1.4 μM 6-O-angeloylplenolin for 12 h, the cytosolic and nuclear proteins in HCT-8/VCR cells were extracted described previously (26). A total of 20 μg total cytosolic and nuclear proteins were separated by 12% SDS-PAGE. Following electrophoresis, the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane, which were blocked with 3% BSA at room temperature for 1 h. The PVDF membranes were incubated with anti-YB-1 (monoclonal rabbit IgG; Cell Signaling Technology, Inc.) or rabbit anti-GAPDH antibody (dilution, 1:5,000; Sigma-Aldrich, Merck KGaA; cat. no. PLA0125) antibodies at 4˚C for overnight. The primary antibodies were detected with anti-rabbit IgG conjugated to alkaline phosphatase (1:3,000 dilutions, Bio-Rad Laboratories, Inc., Hercules, CA, USA; cat. no. 170-6518) to generate a BCIP/NBT (Promega Corporation, Madison, WI, USA; cat. no. S3771) signal. Gel analysis was performed with Image Lab 3.0 software (Bio-Rad Laboratories, Inc.).

Transient transfection and luciferase assay. Plasmid construction was performed as described previously (7,27). HCT-8/VCR cells were seeded into six-well plates (3x10^5 cells/well) and incubated for 24 h before transfection. Using Lipofect Transfection reagent (Beijing Tiangen Biotech Co., Ltd., Beijing, China), the cells were co-transfected with 4 μg/well plasmid pGL3-MDR1 (Promega Corporation, Madison, WI, USA). The sequence for MDR1 promoter was: Forward, 5'-CTAGAGAGGTGCAACGGA-3' (-198 to -181) and reverse, 5'-GCGGCCTCTGCTTCTTTGA-3' (+25 to +43). Next, the cells were treated with 0.35, 0.7 or 1.4 μM 6-O-angeloylplenolin for 24 h. Firefly and *Renilla* luciferase activities in 1x10^4 cells/well were measured using the Dual-Luciferase Assay system (Promega Corporation) using a Microplate Luminometer (Berthold Technologies GmbH, Bad Wildbad, Germany).

Table I. Cytotoxicity of drugs in HCT-8 and HCT-8/VCR cells by MTT assay.

	IC_{50}			
Cell line	VCR, μg/ml	HEPT, μg/ml	MIT, μg/ml	6-O-angeloylplenolin, μM
HCT-8	12.7±0.59	23.3±0.47	1.1±0.11	5.97±0.47
HCT-8/VCR	250±8.45[a]	40±0.35[a]	3.9±0.15[a]	24.21±0.64[a]
HCT-8/VCR + 6-O-angeloylplenolin	13.8±0.78[b]	18.5±0.15[b]	0.48±0.02[b]	-

Each value represents the mean ± standard deviation (n=3). [a]P<0.01 vs. untreated HCT-8 cells; [b]P<0.01 vs. untreated HCT-8/VCR cells. IC_{50}, half-maximal inhibitory concentration; VCR, vincristine; MIT, mitomycin; HEPT, hydroxycamptothecin.

Electrophoretic mobility shift assay (EMSA). HCT-8/VCR ($3x10^5$ cells/well) cells were treated with 0.35, 0.7 or 1.4 μM 6-O-angeloylplenolin for 12 h and the EMSA was performed as described by Han *et al* (24). The DNA sequence of the ds-oligomer used was a CAAT-like motif (5'-ATCAGCATT CAGTCAATCCGGGCC-3') (5,7). The gels were stained using the EMSA kit (Invitrogen; Thermo Fisher Scientific, Inc.) as described by the manufacturer and images were captured using an Olympus Standard fluorescence microscope (Olympus Corporation, Tokyo, Japan). The magnification used was x100.

Animal study. Nude mice (BALB/c; female; age, 6-8 week; 20-22 g; n=5) were provided by Vital River Laboratories Co., Ltd. (Beijing, China), which were housed in barrier facilities on a 12-h light/dark cycle at 23-25˚C with free access to food and water. Each mouse was subcutaneously injected with $5x10^6$ HCT-8/VCR cells in the left dorsal flank. When the tumor reached about 150 mm^3, the mice were randomly assigned to 5 groups and received the following treatments for 12 days: Control (PBS), 6-O-angeloylplenolin (7 mg/kg per day, oral), VCR (2.5 mg/kg per day, oral), VCR + 6-O-angeloylplenolin (3.5 mg/kg per day, oral), VCR + 6-O-angeloylplenolin (7 mg/kg per day, oral). Following 12 days, the tumors were isolated and weighed. The tumor growth inhibitory rate (IR) was calculated as follows: $IR\ (\%)=1-(TW_{treated}/TW_{control})x100$. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Capital Medical University.

Statistical analysis. Statistical analysis was performed using one-way analysis of variance followed by Dunnett's test (version 5.0; GraphPad Software, Inc., La Jolla, CA, USA). All results are presented as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.

Results

MDR-reversing effect of 6-O-angeloylplenolin. HCT-8 and HCT-8/VCR cells were incubated with vincristine, mitomycin and hydroxycamptothecin for 24 h and subjected to MTT assays to assess cell viability. HCT-8/VCR cells were significantly more resistant to vincristine, 6-O-angeloylplenolin, mitomycin and hydroxycamptothecin than HCT-8 cells (Table I). Treatment with 1.4 μM 6-O-angeloylplenolin had no significant effect on the viability of HCT-8/VCR cells. Therefore, the concentration of 6-O-angeloylplenolin was applied to indicate a reversal of resistance. HCT-8/VCR cells were co-incubated with 1.4 μM 6-O-angeloylplenolin and one of three anti-cancer drugs vincristine, mitomycin or hydroxycamptothecin for 24 h. The concentrations of vincristine, mitomycin or hydroxycamptothecin were 1, 3, 10, 30, 100 and 300 μg/ml. The results of the MTT assay revealed that 1.4 μM 6-O-angeloylplenolin significantly increased the cytotoxicity of each of the three anticancer drugs on HCT-8/VCR cells (Table I).

Treatment with 6-O-angeloylplenolin increases the intracellular accumulation of vincristine, adriamycin and Rh123. Inhibiting drug efflux and increasing the intracellular accumulation of drugs is an effective way of overcoming drug resistance (24). To investigate whether 6-O-angeloylplenolin increased the intracellular accumulation of vincristine, adriamycin and Rh123 in HCT-8/VCR cells, HPLC and fluorescence intensity assays were performed. The results indicated that 6-O-angeloylplenolin significantly increased the intracellular accumulation of vincristine, adriamycin and Rh123 in HCT-8/VCR cells following treatment with 6-O-angeloylplenolin for 24 h (Fig. 1B and C; P<0.01).

Treatment with 6-O-angeloylplenolin does not alter the expression of MRP1 and YB-1. It is reported that vincristine is susceptible to common mechanisms of multidrug resistance, including the overexpression of P-gp (28). Here, 6-O-angeloylplenolin did not affect the expression of *MRP1* in colon cancer cells. This result indicated that 6-O-angeloylplenolin reversed vincristine resistance by suppressing *MDR1* expression. Moreover, 6-O-angeloylplenolin had no effect on the expression of *YBX1*, which encodes YB-1 (Fig. 2A and B). These results indicated that 6-O-angeloylplenolin suppressed YB-1 nuclear translocation, but not YB-1 expression.

Treatment with 6-O-angeloylplenolin downregulates expression of MDR1 mRNA and protein. The overexpression of *MDR1* and its protein product have been associated with the MDR phenotype (1). Therefore, the expression of *MDR1* mRNA was assessed by RT-PCR and flow cytometry analysis. Whether 6-O-angeloylplenolin could downregulate the expression of *MDR1* in HCT-8/VCR cells was examined. The results of this analysis revealed that 6-O-angeloylplenolin decreased *MDR1* expression in HCT-8/VCR cells (Fig. 2),

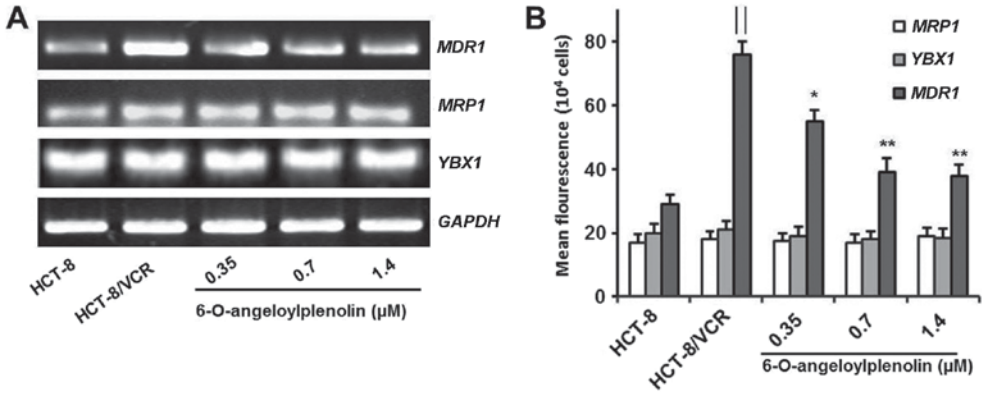

Figure 2. Effect of 6-O-angeloylplenolin on *MDR1*, *MRP1* and *YBX1* mRNA level. Cells were treated with 6-O-angeloylplenolin for 12 h. RT-PCR analysis was applied to expression of *MDR1, MRP1*, and *YBX1*. (A) Electrophoresis gel image of RT-PCR products. (B) The ratio (to GAPDH) of in the cells treated with different concentrations of 6-O-angeloylplenolin by scanning densitometry (n=4). *P<0.05 and **P<0.01 vs. HCT-8/VCR cells; $^{||}$P<0.01 vs. untreated HCT-8 cells. RT-PCR, reverse transcription-polymerase chain reaction; *MDR1*, ATP-binding cassette sub-family B member 1; *MRP1*, ATP binding cassette subfamily C member 1; *YBX1*, Y-box binding protein 1.

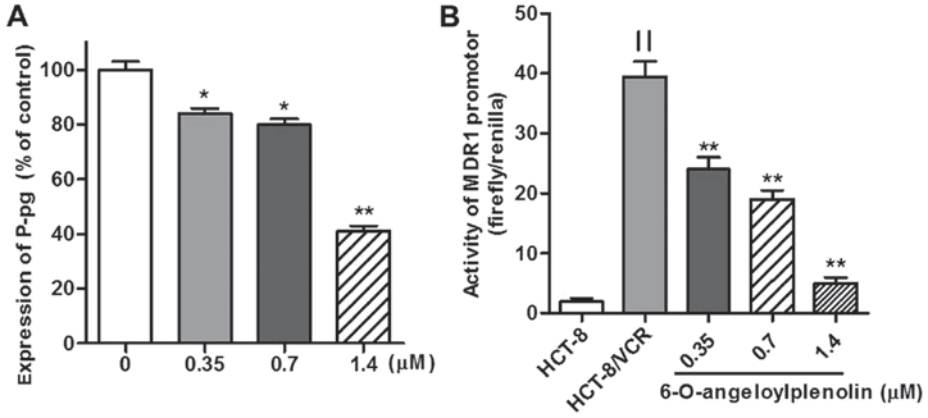

Figure 3. Effect of 6-O-angeloylplenolin on protein expression of MDR1 and activity of the *MDR1* promoter. (A) HCT-8/VCR cells were treated with 6-O-angeloylplenolin for 24 h, and flow cytometry assay was performed (n=4). *P<0.05 and **P<0.01 vs. untreated HCT-8/VCR cells. (B) Cells were treated with 6-O-angeloylplenolin for 2 days following co-transfection with pGL3-MDR and pRL-TK. The activity of *MDR1* promoter was measured using a dual-luciferase assay. Data are represented as mean ± standard deviation (n=4). *P<0.05 and **P<0.01 vs. untreated HCT-8/VCR cells; $^{||}$P<0.01 was compared with untreated HCT-8 cells. *MDR1*, ATP-binding cassette sub-family B member 1.

in parallel with a reduction of the protein expression of *MDR1* (Fig. 3A).

Treatment with 6-O-angeloylplenolin suppresses MDR1 promoter activity. To determine whether the CAAT segment of the *MDR1* promoter was regulated by 6-O-angeloylplenolin or not, the wild-type *MDR1* promoter (residues -198 to +43,241 bp) DNA fragment was cloned into a luciferase-expressing pGL3-basic vector to construct pGL3-MDR, as described previously (27). The data revealed that there was an 18-fold increase in *MDR1* promoter activity in HCT-8/VCR cells transiently transfected with dual-reporter gene vectors, compared with the level in HCT-8 (Fig. 3B). *MDR1* promoter activity was significantly decreased in HCT-8/VCR cells following incubation with 6-O-angeloylplenolin for 24 h (Fig. 3B).

6-O-angeloylplenolin suppresses YB-1 nuclear translocation. Whether the expression and localization of YB-1 is associated with the expression of *MDR1* gene is key to understanding the mechanism of P-gp action. To confirm the effect of 6-O-angeloylplenolin on YB-1 nuclear translocation, western blot analysis was performed. Compared with HCT-8 cells, nuclear translocation of YB-1 was enhanced in HCT-8/VCR cells. The data revealed that 6-O-angeloylplenolin effectively inhibited the nuclear translocation of YB-1 in HCT-8/VCR cells (Fig. 4A-C).

6-O-angeloylplenolin downregulates MDR1 expression by decreasing binding of MDR1 promoter with nuclear transcription factors. A number of studies (29,30) have provided evidence implicating complex mechanisms for the transcriptional regulation of *MDR1* in human cancer cells. Since the present study demonstrated that 6-O-angeloylplenolin could inhibit the expression of *MDR1* mRNA, it was necessary to examine whether 6-O-angeloylplenolin had any effect on *MDR1* promoter. An EMSA was performed using a probe from the *MDR1* promoter sequence, -86 to -67 bp. The amount of specific protein complex interacting with the probe was lower in the HCT-8 cells than in HCT-8/VCR cells (Fig. 4D; lanes 2-3).

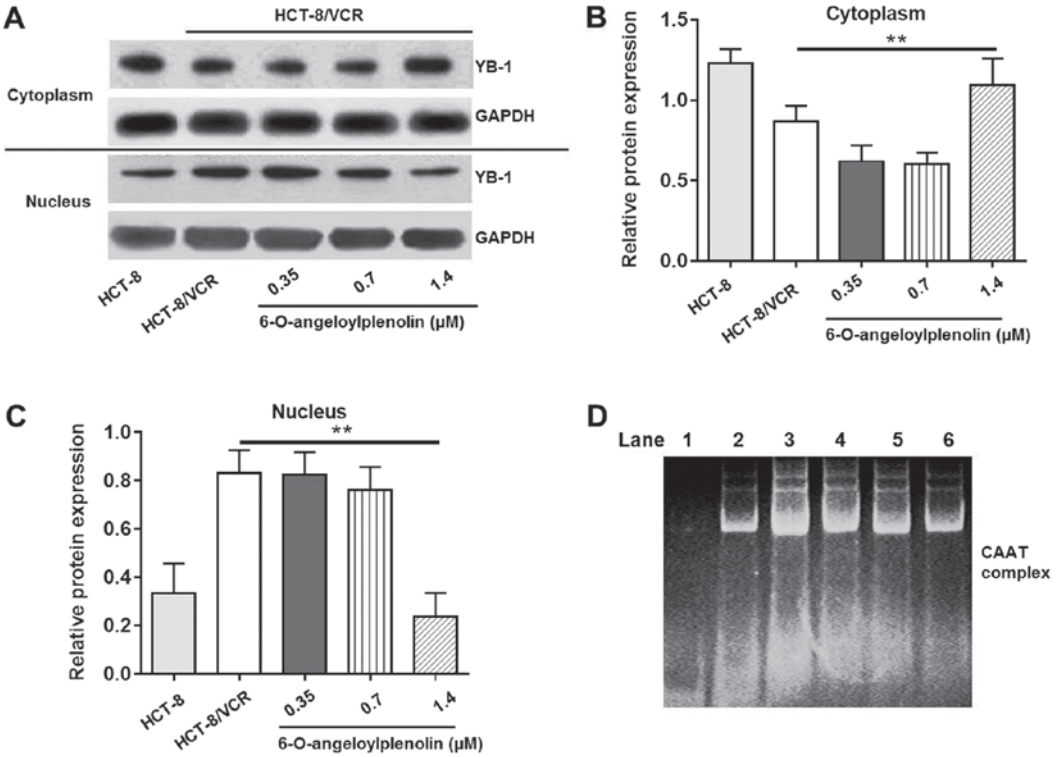

Figure 4. Effect of 6-O-angeloylplenolin on YB-1 nuclear translocation and interaction between the nuclear protein and the CAAT region of the *MDR1* promoter. (A) The cytoplasm and nucleolus protein were extracted following incubation with 6-O-angeloylplenolin for 12 h, resolved using SDS-PAGE, and western blot analysis was performed with the indicated antibodies. GAPDH was used as the loading control. (B) The quantification of the YB-1 protein expression in the cytoplasm. (C) Quantification of the YB-1 protein expression in cytoplasm. **P<0.01 vs. untreated HCT-8/VCR cells. (D) The nucleolar protein was extracted following incubation with 6-O-angeloylplenolin for 12 h, and the amount of complexed nuclear protein and DNA were measured using an electrophoretic mobility shift assay. Lane 1, only nuclear extracts; lane 2, probe incubated with nuclear extracts of HCT-8 cells; lane 3, probe incubated with nuclear extracts of HCT-8/VCR cells; lane 4, probe incubated with nuclear extracts of HCT-8/VCR cells following treatment with 0.35 μM 6-O-angeloylplenolin; lane 5, probe incubated with nuclear extracts of HCT-8/VCR cells following treatment with 0.7 μM 6-O-angeloylplenolin; lane 6, probe incubated with nuclear extracts of HCT-8/VCR cells following treatment with 1.4 μM 6-O-angeloylplenolin. YB-1, Y-box binding protein 1.

Treatment with 6-O-angeloylplenolin decreased the number of protein/DNA complexes in HCT-8/VCR cells (Fig. 4B, lanes 3-6). This result indicated that 6-O-angeloylplenolin inhibited the binding of nuclear proteins to the CAAT region of the *MDR1* promoter in HCT-8/VCR cells, which could result in the inhibition of *MDR1* expression.

6-O-angeloylplenolin reverses VCR resistance in HCT/VCR xenograft model. To evaluate whether 6-O-angeloylplenolin could reverse vincristine resistance *in vivo*, a HCT/VCR xenograft model was established by injecting HCT/VCR cells into the left dorsal flanks of nude mice. The data demonstrated that 6-O-angeloylplenolin or vincristine treatment alone had no effect on tumor growth. However, the combination of 6-O-angeloylplenolin with vincristine significantly inhibited tumor growth (Fig. 5A and B). In addition, the body weights of mice were stable, indicating the combination treatments were tolerable (Fig. 5C).

Discussion

Resistance to chemotherapeutics remains a major cause of cancer treatment failure. Thus, in addition to investigating more efficient therapeutic drugs, there is a requirement to develop compounds to inhibit MDR activity or to synergize with existing treatments. The current study revealed that 6-O-angeloylplenolin treatment reversed vincristine resistance in HCT-8/VCR cells, increasing the intracellular accumulation. MDR is often associated with the overexpression of *MDR1*, which causes the enhancement of drug efflux pump activity and drug resistance (29). Treatment with 6-O-angeloylplenolin inhibited the expression or function of P-gp, data that were further supported by RT-PCR, flow cytometry and promoter activity analysis.

Several approaches to overcoming MDR have been proposed (12,28,29); of them, inhibition of MDR-associated genes has promise. P-gp is encoded by *MDR1*, which was investigated in the present study. The expression of *MDR1* was depleted following 6-O-angeloylplenolin administration. Besides P-gp, MRP1 is another protein that is important to MDR; it is encoded by *MRP1*. The results of the present study indicated that the mRNA level of *MRP1* was unchanged following 6-O-angeloylplenolin treatment (Fig. 2) and that 6-O-angeloylplenolin exerts its main effect via regulating the expression of *MDR1 in vitro*.

Previous reports have demonstrated that YB-1 activity is closely associated with the expression of *MDR1 in vivo* and *in vitro* (5-9). Treatment with 6-O-angeloylplenolin decreased the level of YB-1 in nucleus and complexes of this nuclear protein with *MDR1* promoter in a dose-dependent manner. However, 6-O-angeloylplenolin regulating the expression of *YBX1* on mRNA level was not observed. Therefore, 6-O-angeloylplenolin

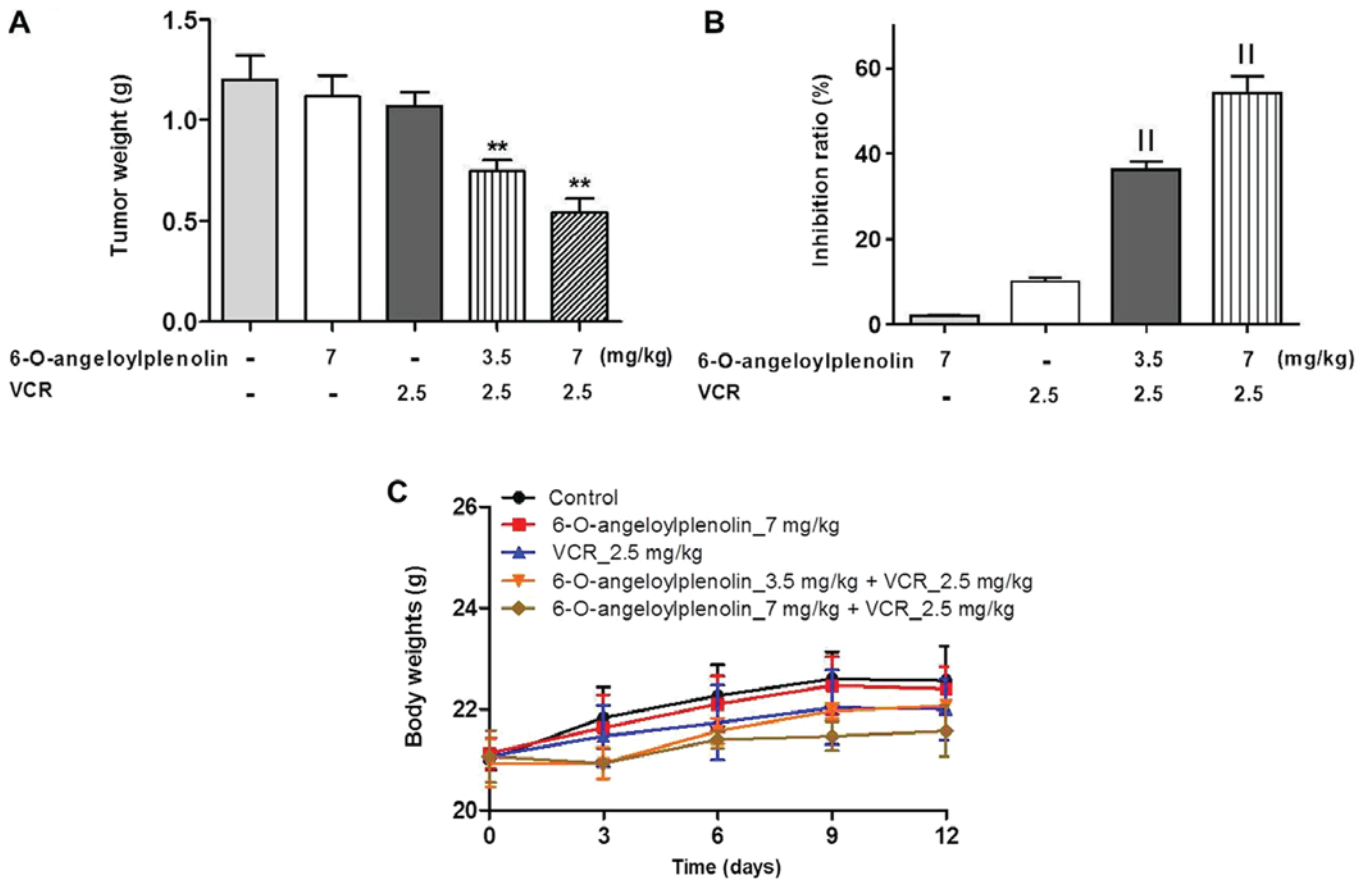

Figure 5. Treatment with 6-O-angeloylplenolin reversed vincristine resistance in the HCT/VCR xenograft model. (A) The tumors were isolated and weighted after 12 days of treatment. **P<0.01 were compared with control group. (B) The tumor growth inhibition ratio was calculated (n=5). IIP<0.01 was compared with vincristine treatment alone. (C) The body weights of mice were monitored on the indicated day (n=5). VCR, vincristine.

regulated the expression of MDR1 by inhibiting YB-1 nuclear translocation, not by depressing its expression. These findings differ from previous reports (9,12), which might be due to the different tumor type. Therefore, these novel results shed light on the mechanism of chemotherapy resistance in colon cancer. These results provide evidence for the combination use of 6-O-angeloylplenolin with vincristine in patients with refractory colon cancer. However, there were certain limitations to the current study. The detailed interaction between YB1 and MDR1 following 6-O-angeloylplenolin treatment in colon cancer was not clarified. Future studies should focus on the protein kinase B-nuclear factor-κB-YB-1-MDR1 and tumor protein p53-YB-1-MDR1 signaling pathways in the future.

Taken together, the results of the present study indicated that 6-O-angeloylplenolin displayed a significant antitumor activity by reserving drug resistance. The effect of 6-O-angeloylplenolin was exerted via inhibition of the intracellular accumulation of YB-1 and the expression of *MDR1*, resulting in a decrease in efflux pump activity. These results demonstrated that 6-O-angeloylplenolin may represent a potential anticancer drug adjuvant with the potential to reverse drug resistance.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

CL was mainly responsible for study design, data analysis, manuscript development and conduction of experiments, including cell culture, MTT assay, RT-PCR, and the accumulation assay of vincristine, adriamycin and Rh123. HW performed flow cytometry assay of HCT-8/VCR cells, western blot analysis, and assisted with statistical analysis. YY helped with transient transfection and luciferase assay. JL was involved in electrophoretic mobility shift assay and animal study. ZC performed experiments and reviewed and revised the manuscript.

Ethics approval and consent to publish

Ethics approval for animal study was given by the local research ethics committee at East China University of Science and Technology.

Consent for publication

There are no human participants, human data or human tissue involved in this manuscript.

Competing interests

The authors declare no competing financial interests.

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