Decrease of ABCB1 protein expression and increase of G_1 phase arrest induced by oleanolic acid in human multidrug-resistant cancer cells

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Abstract. Oleanolic acid (OA) is a natural compound that can be found in a number of edible and medicinal plants and confers diverse biological actions. However, the direct target of OA in human tumor cells remains poorly understood, preventing its application in clinical and health settings. A previous study revealed that overexpression of caveolin-1 in human leukemia HL-60 cells can increase its sensitivity to OA. The present study aimed to investigate the effects of OA on the doxorubicin-resistant human breast cancer MCF-7 cell line (MCF-7/DOX), harringtonine-resistant human leukemia HL-60 cells (HL-60/HAR) and their corresponding parental cell lines. Western blotting was performed to measure protein expression levels, whilst Cell Counting Kit-8 (CCK-8) assays, cell cycle analysis (by flow cytometry) and apoptosis assays (with Annexin V/PI staining) were used to assess drug sensitivity. CCK-8 assay results suggested that MCF-7/DOX cells, which overexpress the caveolin-1 protein, have similar OA susceptibility to their parent line. In addition, sensitivity of MCF-7/DOX cells to OA was not augmented by knocking down caveolin-1 using RNA interference. HL-60/HAR cells exhibited a four-fold increased sensitivity to OA compared with that in their parental HL-60 cells according to CCK-8 assay. Both of the resistant cell lines exhibited higher numbers of cells at G_1 phase arrest compared with those in their parent lines, as measured via flow cytometry. Treatment of both MCF-7 cell lines with 100 μM OA for 48 h induced apoptosis, with increased effects observed in resistant cells. However, no PARP-1 or caspase-3 cleavage was observed, with some positive Annexin V staining found after HL-60/HAR cells were treated with OA, suggesting that cell death occurred via non-classical apoptosis or through other cell death pathways. It was found that OA was not a substrate of ATP-binding cassette subfamily B member 1 (ABCB1) in drug-resistant cells, as indicated by the accumulation of rhodamine 123 assessed using flow cytometry. However, protein expression of ABCB1 in both of the resistant cell lines was significantly decreased after treatment with OA in a concentration-dependent manner. Collectively, these results suggest that OA could reduce ABCB1 protein expression and induce G_1 phase arrest in multidrug-resistant cancer cells. These findings highlight the potential of OA for cancer therapy.

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Abbreviations: ABCB1, ATP-binding cassette subfamily B member 1; CASP-3, caspase-3; CAV-1, caveolin-1; CCK-8, cell counting kit-8; DOX, doxorubicin; HAR, harringtonine; MRP, multidrug resistance-associated protein; PARP-1, poly(ADP-ribose) polymerase 1; PI, propidium iodide; OA, oleanolic acid; Rho 123, rhodamine 123

Key words: oleanolic acid, multidrug resistance in tumor, cell cycle, ATP-binding cassette subfamily B member 1, caveolin-1

Introduction

Oleanolic acid (OA) is a bioactive triterpenoid that can exist in nature as a free acid in a number of edible and medicinal plants, including olives, sage *Lantana camara* and the privet *Lisgustrum lucidum* (1). OA has been reported to exert anti-oxidant, antibacterial and antitumor effects on human cells (such as hepatocellular carcinoma and breast cancer cells) and has been applied in China as a drug for treating liver diseases for >20 years (2). Several signaling pathways have been documented to be regulated by OA, including 5'adenosine monophosphate-activated protein kinase, NF-κB and mTOR pathways (3-5). In addition, OA can induce apoptosis and autophagy in numerous types of tumor cells, such as hepato-cellular carcinoma cells (1,6-9) and attenuate cisplatin-induced nephrotoxicity (10). Although previous studies have reported

that OA can arrest cell cycle progression at the G_1 phase, such as in gallbladder cancer cells (1,11), it also blocks liver cancer HepG2 cells at the G_2/M phase (7).

Multidrug resistance in tumors is one of the main causes of chemotherapy failure (12). Upregulation of transmembrane transporters in tumor cells, such as ATP-binding cassette subfamily B member 1 (ABCB1 or P-glycoprotein) and multidrug resistance-associated protein (MRP1 or ABCC1), mainly contribute to drug resistance, since these proteins pump antitumor agents out of the tumor cells (12-14). ABCB1 was primarily discovered as a mediator of multidrug resistance in breast cancer MCF-7 cells (12). It has been previously reported that OA can inhibit MRP1 function, but not ABCB1 function in drug-resistant sarcoma cells (15). However, a chemically modified OA derivative can target ABCB1 (16). To facilitate the clinical application of OA as a chemotherapeutic agent, it is necessary to characterize the actions of OA further in ABCB1-overexpressing tumor cells.

Molecules that directly mediate the actions of OA on tumor cells remain poorly understood. In a previous study, it was revealed that transfection of human acute myelogenous leukemia HL-60 cells with caveolin-1 (CAV-1) conferred increased susceptibility to OA (17), suggesting that CAV-1 may be a target for mediating the antitumor action of OA. In another study, which aimed to characterize the role of CAV-1 in the antitumor action of bleomycin (18), it was observed that CAV-1 was upregulated in the doxorubicin-resistant human breast cancer cell line MCF-7 (MCF-7/DOX). Therefore, the present study investigated whether OA can affect multidrug resistance in cancer cells via CAV-1. It also further determined the characteristics of drug resistance in cell lines after exposure to OA.

Materials and methods

Drugs and reagents. Harringtonine (HAR) and OA were purchased from the National Institute of Food and Drug Control (Beijing, China). Imatinib was obtained from Selleck Chemicals. Doxorubicin (DOX), vincristine (VCR), cis-diamminedichloroplatinum, dimethyl sulfoxide, propidium iodide (PI) and rhodamine 123 (Rho 123) were all acquired from Sigma-Aldrich, Merck KGaA. Cell Counting Kit-8 (CCK-8) was purchased from Bimake.com and the apoptotic Annexin V/PI kit was obtained from Beijing 4A Biotech Co., Ltd.

Cell lines and culture. The human leukemia HL-60 cell line was acquired from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. The HAR-resistant HL-60 cell line (HL-60/HAR) was established by Professor Q. He, Institute of Medicinal Biotechnology, Peking Union Medical College and Chinese Academy of Medical Sciences (Beijing, China) (19). The human breast cancer MCF-7 and MCF-7/DOX cell lines were kindly provided by Dr Kenneth H Crown (National Institutes of Health, Baltimore, USA). The resistant cell lines were used in the experiments after the withdrawal of the resistance-inducing drugs for 4 weeks. Sensitive MCF-7 and MCF-7/DOX cells were cultured in RPMI-1640 medium (HyClone; Cytiva). Sensitive HL-60 and HL-60/HAR cells were maintained with Improved Minimum Essential Medium (HyClone; Cytiva). A total of 10% (v/v) FBS (PAN-Biotech GmbH) was supplemented to all media. All cell lines were cultured with 5% CO_2 in a humidified atmosphere at 37˚C.

Western blot analysis. Western blotting was performed as described previously (20). Cells (MCF-7, MCF-7/DOX, HL-60 and HL-60/HAR) were lysed with lysis buffer, which contained 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1% Triton X-100 and protease inhibitors [Roche Diagnostics (Shanghai) Co., Ltd.]. The protein concentrations of the samples were determined using the Quick Start Bradford 1X Dye Reagent (cat. no. 500-0205; Bio-Rad Laboratories Inc.), according to the manufacturer's protocol, and by measuring the absorbance at 590 nm with a microplate reader (Bio-Rad Laboratories, Inc.). The samples with 20 μg protein per lane were separated via SDS-PAGE on 7.5 or 12.5% gels and then transferred onto polyvinylidene fluoride membranes. The membrane was blocked with 5% BSA for 1 h at room temperature, incubated with primary antibodies for 12 h at 4˚C, washed and then incubated with secondary antibodies at room temperature. The immunoreactive bands were visualized with the ECL Plus Western Blotting Detection System (Bio-Rad Laboratories, Inc.) and detected with an Amersham Imager 600 (GE Healthcare). The bands were quantified with ImageJ 1.46 software (National Institutes of Health). Antibodies against ABCB1 (cat. no. 12273, 1:1,000), CAV-1 (cat. no. 3238, 1:1,000), poly(ADP-ribose) polymerase 1 (PARP-1; cat. no. 9532, 1:2,000), caspase-3 (CASP-3, cat. no. 9662, 1:1,000) and cleaved caspase-3 (CASP-3-C, cat. no. 9664, 1:500) were acquired from Cell Signaling Technology, Inc. β-actin (cat. no. sc-47778, 1:100,000) and p53 (cat. no. sc-126, 1:2,000) antibodies were obtained from Santa Cruz Biotechnology, Inc. Horseradish peroxidase-conjugated goat anti-rabbit (cat. no. hs101-01, 1:1,000) and anti-mouse IgG (cat. no. hs201-01, 1:1,000) antibodies were acquired from Transgen Biotech Co., Ltd.

Detection of Rho 123 accumulation. Since Rho 123 is the substrate of ABCB1, its fluorescence intensity can be used as an indicator of the relative levels of ABCB1 (15,21). A total of $2x10^5$ cells from the 4 cell lines per well at the logarithmic growth phase were seeded into a six-well plate. After 24 h incubation at 37˚C, the cells were exposed to different concentrations of OA (10, 20, 40, 80 and 100 μM) for 1 h at 37˚C. Rho 123 (5 μg/ml) was then added to the culture medium and the plate was incubated for 1.5 h at 37˚C. The total incubation time with OA was 2.5 h. The cells were then collected after centrifugation at 800 x g for 5 min at room temperature. The fluorescence intensity was detected using a BD FACSCalibur™ flow cytometer (BD Biosciences). The data were analyzed using CellQuest Pro software version 5.1 (BD Biosciences).

RNA interference. RNA interference was performed according to a previously described protocol (18). Small interfering (si)RNA against CAV-1 and scrambled siRNA were synthesized by Invitrogen (Thermo Fisher Scientific, Inc.). The sequence of CAV-1 siRNA was 5'-UUUCCCAACAGCUUC AAAGAGUGGG-3', and the sequence of scrambled siRNA was 5'-AAGUUGAAUGCGUCUGAAACGGUUC-3'. The

Table I. IC_{50} values of various drugs for MCF-7/DOX and sensitive MCF-7 cells.

Drug	IC_{50} for MCF-7 (μM)	IC_{50} for MCF-7/DOX (μM)	Resistant folds
DOX	0.23±0.02	108.00±2.94	469.6
Vincristine	0.03±0.01	5.92±1.63	196.6
cis-diamminedichloroplatinum	13.10±0.30	4.94±1.62	0.4
Oleanolic acid	83.40±5.61	79.13±3.71	0.9

The inhibitory rate of cell survival was determined with Cell Counting Kit-8 assay. The results are represented as the mean ± SD from three separate experiments. Resistant folds are obtained from IC_{50} values in MCF-7/DOX cells divided by those in MCF-7 cells. DOX, doxorubicin.

MCF-7/DOX cells were transfected with 100 pmol siRNA for 6 h using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The transfected cells were cultured in fresh culture medium for 18 h and then used for subsequent experiments.

CCK-8 assay. CCK-8 assay was performed by following a previously described protocol (20). A total of 3,000 cells from 4 the cell lines at the logarithmic growth phase was seeded into a 96-well plate and incubated for 24 h at 37˚C. Cells were exposed to the drugs for 72 h at 37˚C. The MCF-7 cells were treated with DOX (0.05, 0.1, 0.5 or 1, 5 μM), VCR (0.005, 0.01, 0.05, 0.1 or 0.2 μM), DDP (1, 5, 10, 20 or 50 μM) and OA (20, 40, 80, 100 or 120 μM) for 72 h at 37˚C. The MCF-7/DOX cells were treated with DOX (1, 5, 10, 20 or 50 μM), VCR (0.5, 1, 2, 5 or 10 μM), DDP (0.5, 1, 5, 10 or 20 μM) and OA (20, 40, 80, 100 or 120 μM) for 72 h at 37˚C. The HL-60 cells were treated with HAR (0.001, 0.005, 0.01, 0.05 or 0.1 μM), DOX (0.01, 0.05, 0.1, 0.2 or 0.5 μM), VCR (0.05, 0.1, 0.5, 1, 2 or 5 nM), imatinib (2, 5, 10, 20 or 50 μM) and OA (20, 40, 80, 100 or 120 μM) for 72 h at 37˚C. The HL-60/HAR cells were treated with HAR (0.1, 0.5, 1, 5 or 10 μM), DOX (0.1, 0.5, 1, 5 or 10 μM), VCR (100, 200, 500, 1,000 or 2,000 nM), imatinib (2, 5, 10, 20 or 50 μM) and OA (5, 10, 20, 40 or 80 μM) for 72 h at 37˚C. After co-incubation with the aforementioned drugs, 20 μl of CCK-8 per lane for 2 h at 37˚C, the optical density (OD) values at 450 nm were measured using a microplate reader (Bio-Rad Laboratories, Inc.). The viability of the control group that did not receive OA treatment was set as 100%. The cell survival rates of the experimental groups were calculated using the following equation: Viability (%)=(OD of the drug-treated groups-OD of background)/(OD of the control group-OD of background) x100%. The data were plotted with GraphPad Prism 5 (GraphPad Software, Inc.).

Cell cycle analysis via flow cytometry. The cells at the logarithmic growth phase were seeded into a six-well plate with a density of $2x10^5$ cells/well and incubated for 24 h at 37˚C. After exposure to OA for 24 h at 37˚C, the MCF-7 and MCF-7/DOX cells were digested and collected after centrifugation at 800 x g for 5 min at room temperature. HL-60 and HL-60/HAR cells were collected for centrifugation at 800 x g for 5 min at room temperature. The cells were then fixed with 70% (v/v) ethanol overnight at 4˚C. Before the assay, the cells were washed twice with cold PBS and incubated with 100 μg/ml RNase A (Beijing Solarbio Science & Technology Co., Ltd.) for 30 min at 37˚C. The cells from the 4 cell lines were stained with 50 μg/ml PI for 1 h in the dark at room temperature. Finally, the fluorescence intensities of the groups were measured using a BD FACSCalibur™ flow cytometer (BD Biosciences). The data were analyzed using ModFit LT software version 5.1 (Verity Software House, Inc.).

Measurement of apoptotic cells using Annexin V/PI staining. Cells ($3x10^5$) at the logarithmic growth phase were seeded into a six-well plate and maintained for 24 h at 37˚C. After exposure to various concentrations of OA (20, 40, 80 and 100 μM) for 48 h at 37˚C, the cells were collected and washed twice with cold PBS. The cells (MCF-7, MCF-7/DOX, HL-60 and HL-60/HAR) were resuspended with binding buffer and stained with 5 μl Annexin V-FITC for 5 min at room temperature in the dark. After the addition of 2 μg/ml PI to the cell suspension for 15 min at room temperature, the fluorescence intensities were analyzed using a BD FACSCalibur™ flow cytometer (BD Biosciences). The data were processed with CellQuest Pro software version 5.1 (BD Biosciences).

Statistical analysis. Data are presented as the mean ± SD from three independent experiments. Statistical analysis was conducted using one-way ANOVA followed by Tukey's test using SPSS 19.0 (IBM Corp.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Characterization of drug-resistant MCF-7/DOX and HL-60/HAR cells. The viability of cells treated with the indicated antitumor drugs was determined using CCK-8 assay. The IC_{50} values are summarized in Tables I and II. Compared with their respective parental cell lines, the increase of resistance against DOX in MCF-7/DOX cells was ~469-fold, whilst resistance against HAR in HL-60/HAR cells was 240-fold (Tables I and II), suggesting increased resistance to both antitumor agents. Upregulation in ABCB1 expression was observed in both drug-resistant cells compared with that in their respective parental cell lines (Fig. 1A), indicating that the drug resistance was associated with ABCB1 protein expression. This is consistent with findings from previous studies on these two drug-resistant cell lines (19,22). The protein expression levels of p53 and CAV-1 were also increased in MCF-7/DOX cells compared those in their parental MCF-7 cells. As presented in Fig. 1B and C, Rho 123 was markedly

Table II. IC_{50} values of various drugs for the inhibitions of HL-60/HAR and sensitive HL-60 cells.

Drug	IC_{50} for HL-60 (μM)	IC_{50} for HL-60/HAR (μM)	Resistant folds
HAR	0.01±0.01	2.40±0.40	240
Doxorubicin	0.06±0.01	0.92±0.23	15
Vincristine	0.008±0.001	0.62±0.05	750
Imatinib	21.84±2.62	18.31±0.73	0.8
Oleanolic acid	82.24±2.22	18.50±6.34	0.2

The inhibitory rate of cell survival was determined using a Cell Counting Kit-8 assay. The results are represented as the mean ± SD from three separate experiments. Resistant folds are obtained from IC_{50} values in HL-60/HAR cells divided by those in HL-60 cells. HAR, harringtonine.

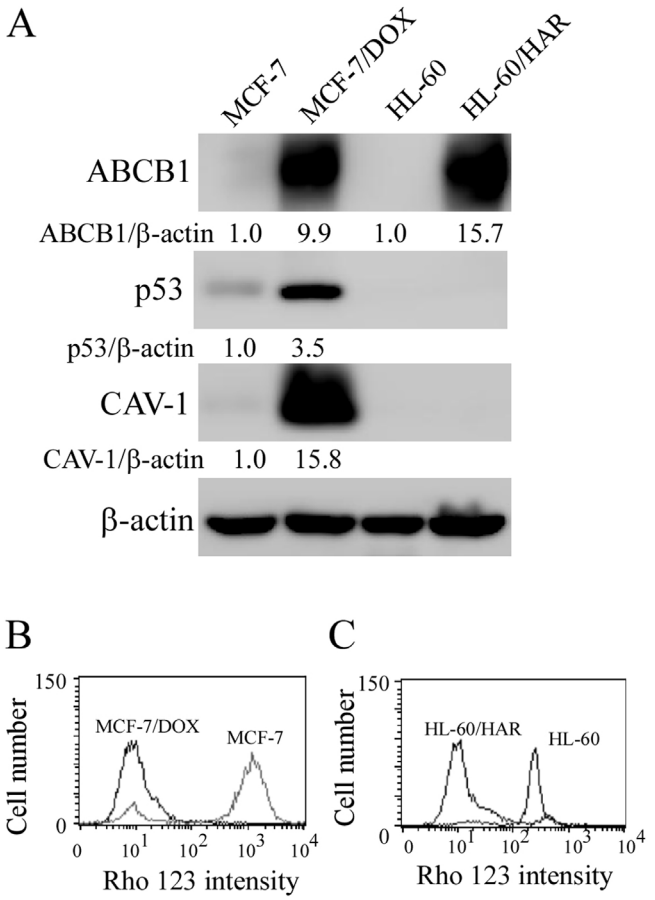

Figure 1. Characterization of drug-resistant MCF/DOX and HL-60/HAR cells. (A) ABCB1, p53 and CAV-1 expression levels in both cell lines were detected via western blotting. Accumulations of rhodamine 123 in (B) MCF-7/DOX, MCF-7, (C) HL-60 and HL-60/HAR both cell lines as determined using flow cytometry. One of representative results from three independent experiments is presented. ABCB1, ATP-binding cassette subfamily B member 1; CAV-1, caveolin-1; Rho 123, rhodamine 123; DOX, doxorubicin; HAR, harringtonine.

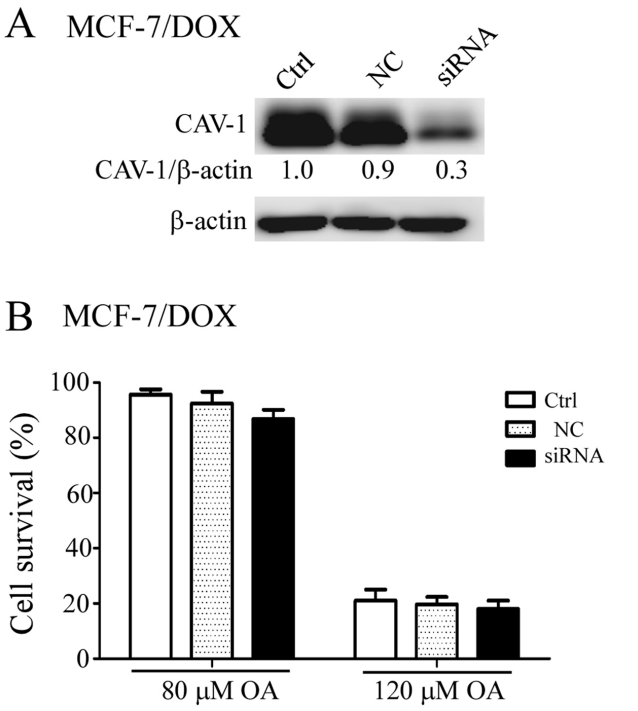

Figure 2. Effect of caveolin-1 knockdown on the actions of OA in MCF-7/DOX cells. (A) CAV-1 protein expression after CAV-1 knockdown using RNA interference in MCF-7/DOX cells. (B) Cell survival rates in OA treatment group after knocking down CAV-1 expression. Data are present as the mean ± SD from three separate experiments. Ctrl, control; OA, oleanolic acid; NC, negative control; siRNA, small interfering RNA; DOX, doxorubicin.

accumulated in sensitive MCF-7 and HL-60 cells, more than that observed in MCF-7/DOX and HL-60/HAR cells.

There was a notably different result in the cell survival rates of OA treatment groups between MCF-7/DOX and HL-60/HAR cells. MCF-7/DOX cells exhibited little difference in sensitivity to OA compared with that in their parental MCF-7 cells (Table I). However, HL-60/HAR cells were four-fold more sensitive to OA compared with that in their parental HL-60 cells (Table II). Based on this finding, HL-60/HAR cells were treated with lower concentrations of OA (2, 5, 10, 20 and 40 μM) for subsequent experiments. It should be noted that additional cell proliferation methods, such as BrdU, should also be used for precisely measuring proliferation of drug resistant cells.

Effect of CAV-1 knockdown on OA sensitivity in MCF-7/DOX cells. Overexpression of CAV-1 in HL-60 cells increases their sensitivity to OA, as demonstrated in a previous study (17). In the present study, upregulation of CAV-1 was found in MCF-7/DOX cells compared with that in their parental MCF-7 cells (Fig. 1A). Therefore, reduction in CAV-1 expression in MCF-7/DOX cells may enhance their sensitivity to OA. Therefore, CAV-1 expression was successfully downregulated in MCF-7/DOX cells using RNA interference (Fig. 2A). However, there was no significant difference in the sensitivity to OA between the scrambled siRNA-transfected and the

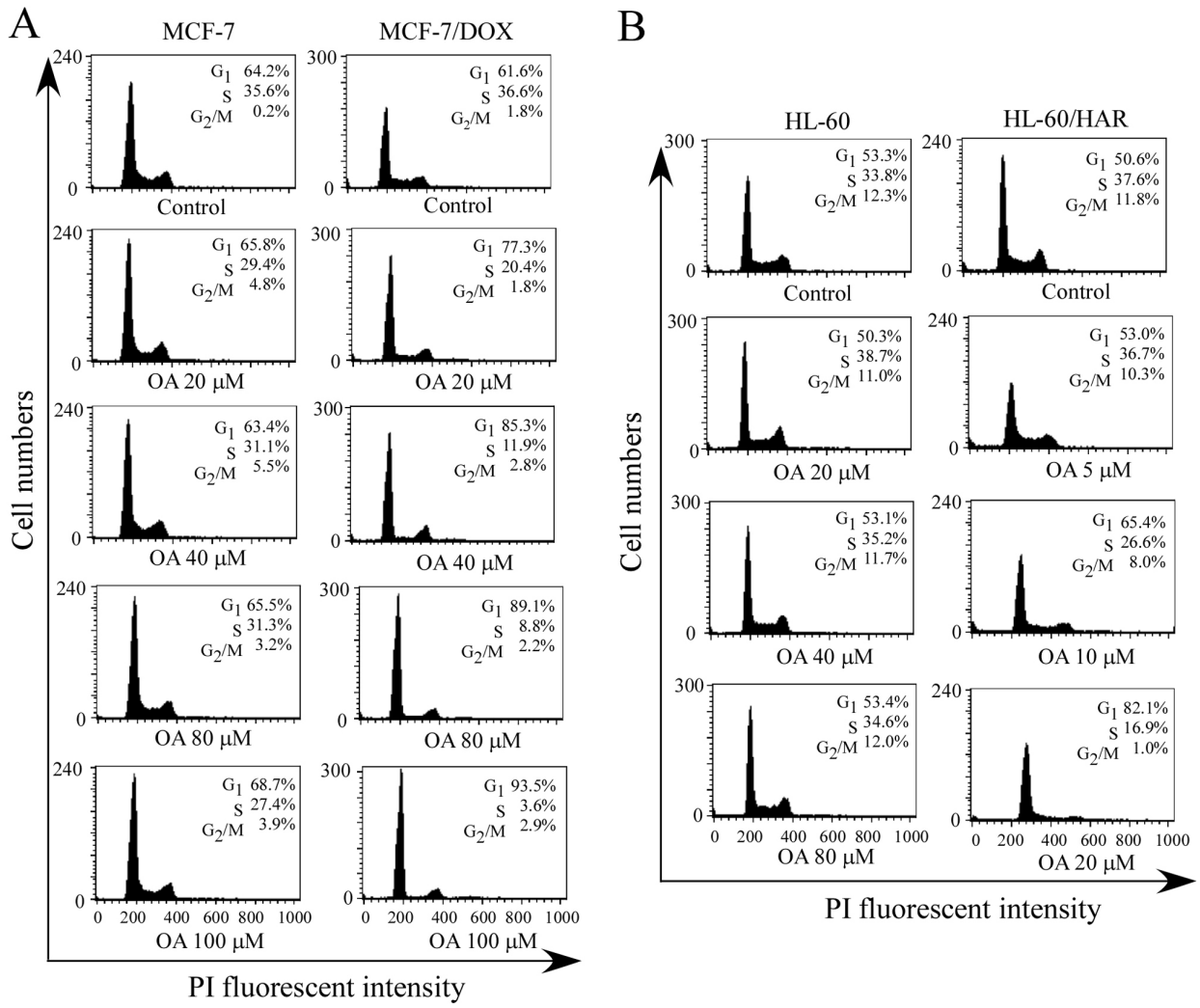

Figure 3. Increase of G_1 phase arrest by OA in both drug-resistant cell lines. (A) Cell cycle analysis of MCF-7/DOX and sensitive MCF-7 cell lines after exposure to OA for 24 h, as determined using flow cytometry. (B) Distribution of the cell cycle after treatment with OA for 24 h in HL-60/HAR and sensitive HL-60 cell lines. One representative result from three independent experiments is presented. OA, oleanolic acid; DOX, doxorubicin; HAR, harringtonine.

CAV-1 siRNA-transfected MCF-7/DOX cells after the cells were treated with 80 and 120 μM OA for 48 h (Fig. 2B).

G_1 phase cell cycle arrest after OA treatment in both drug resistant cell lines. Cell cycle distribution was analyzed using flow cytometry after the cells were treated with different concentrations of OA for 24 h. The results demonstrated that the percentage of cells at G_1 phase increased a small amount with increasing concentrations of OA in sensitive MCF-7 cells (Fig. 2A). For example, the G_1 percentage was 64.2% in the control group and was 68.7% in the 100 μM OA-treatment group. However, the G_1 percentage in MCF-7/DOX cells treated with the same concentration of OA was 93.5%, indicating an increased percentage of G_1 phase cells compared with that in the sensitive cells (Fig. 3A). In addition, the percentage of G_1 phase cells was 53.3% in the control group and 53.4% after sensitive HL-60 cells were treated with 80 μM OA (Fig. 3B). OA concentrations of 5, 10 and 20 μM were used for cell cycle analysis as HL-60/HAR cells tended to be more sensitive to OA, compared with sensitive HL-60 cells, as shown in a preliminary cell survival experiment (Table II). The percentage of G_1 phase cells was 82.1% in the 20 μM OA-treatment group, whilst no notable changes in the percentage of G_1 phase cells were detected with high concentrations of OA treatment (80 μM) in sensitive HL-60 cells compared with that in the control cells (Fig. 3B). These results suggested that HL-60/HAR cells were more sensitive to OA in comparison with their corresponding sensitive parental HL-60 cells.

Induction of apoptosis by OA in MCF-7 cells. The apoptotic rate of cells was next detected using Annexin V/PI-staining after MCF-7 cells were treated with different concentrations of OA for 48 h. The apoptotic rates of MCF-7 cells treated with OA were gradually increased in a concentration-dependent manner (Fig. 4A and B). The apoptosis rate was 13.4% in sensitive MCF-7 cells after exposure to 100 μM OA. However, the apoptotic rate in MCF-7/DOX cells was 22.7% after OA treatment at the same concentration, indicating enhanced apoptosis compared with that observed in their corresponding parental sensitive cells. Changes in apoptosis-related protein expression were detected by western blotting. The cleavage of PARP-1 is a biomarker of apoptosis that is used as an indicator of apoptotic occurrence (23). The cleavage fragments of PARP-1 and activated CASP-3 were detected after sensitive MCF-7 cells and MCF-7/DOX cells were treated with increasing concentrations of OA up to 100 μM for 48 h (Fig. 4C),

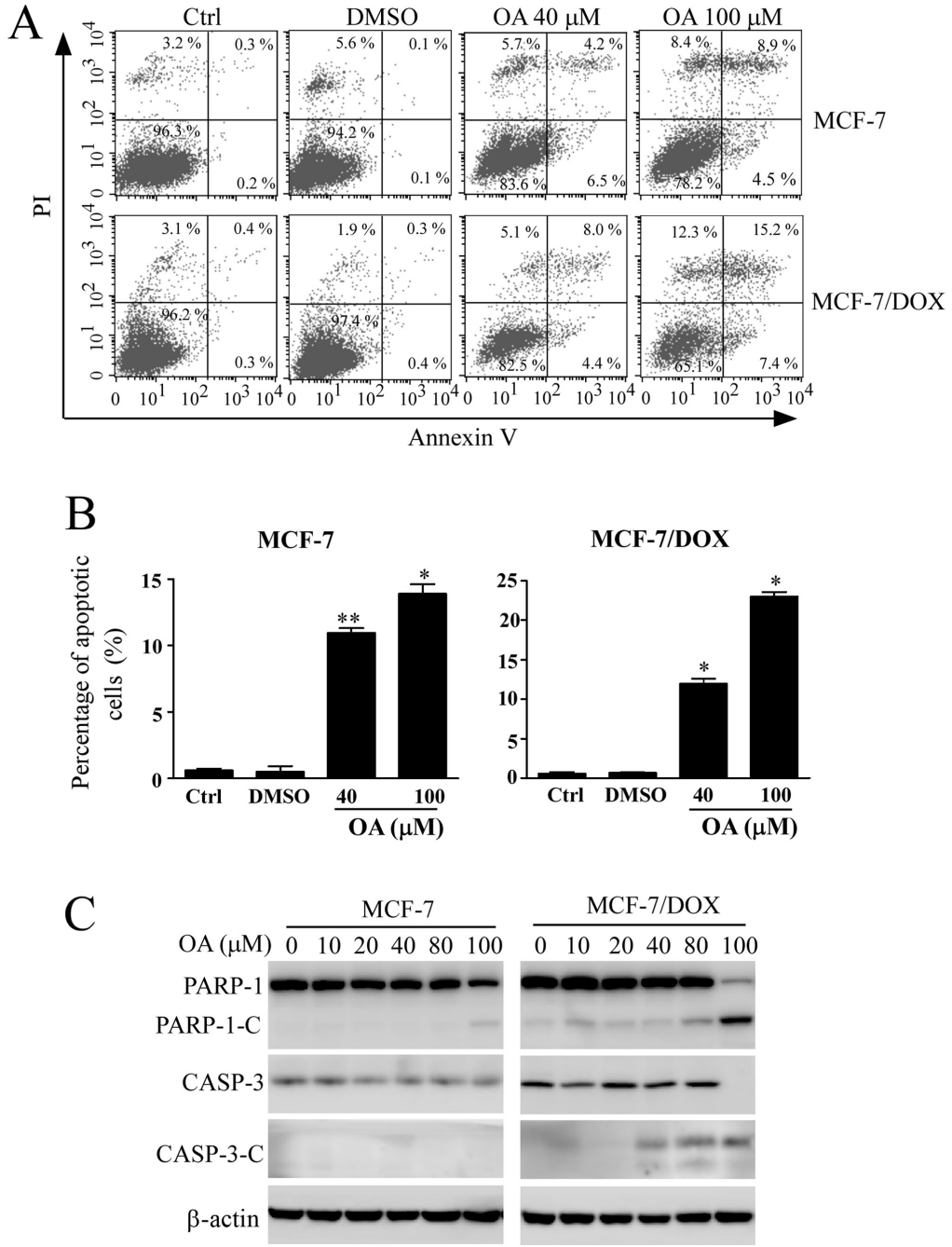

Figure 4. Induction of cell apoptosis by OA in MCF-7 cell lines. (A) Cell apoptosis was determined with Annexin V/PI staining after exposure to OA for 48 h. (B) Quantification of apoptotic cell number in (A). *P<0.05 and **P<0.01 vs. Ctrl. (C) Changes in the levels of apoptosis-related proteins were detected by western blotting after treatment with OA for 48 h. One representative result from three independent experiments is presented. Ctrl, control; CASP-3, caspase-3; CASP3-C, cleaved caspase-3; PARP-1, poly(ADP-ribose) polymerase 1; PARP-1-C, cleaved fragment of poly(ADP-ribose) polymerase; OA, oleanolic acid; DOX, doxorubicin.

where an increased number of cleaved fragments of PARP-1 and CASP-3 were observed in MCF-7/DOX cells treated with the same concentration (100 μM), compared with sensitive MCF-7 cells. The cleaved fragments were also observed at lower concentrations of OA treatment in MCF-7/DOX cells (Fig. 4C). The results from the apoptosis assay further indicated that MCF-7/DOX cells were more sensitive to OA.

Effect of OA treatment on apoptosis in HL-60/HAR cells. As detected using an Annexin V/PI staining assay, the apoptotic rate of sensitive HL-60 cells was 35.8% after exposure to 80 μM OA, whilst the apoptotic rate was 30.4% in 40 μM OA-treated HL-60/HAR cells (Fig. 5A and B), suggesting that HL-60/HAR cells were more sensitive to OA compared with their parental HL-60 cells. The pattern of staining of the apoptotic markers differed between the resistant and sensitive cell lines. Cleaved fragments from CASP-3 and PARP-1 were observed in sensitive cells after exposure to 80 μM OA. However, no cleavage fragments of PARP-1 were detected in HL-60/HAR cells (Fig. 5C). In addition, lower expression levels of CASP-3 were observed after treatment with the highest concentration of OA (40 μM), but no cleavage of CASP-3 was identified. These results suggested that OA induces a non-classical apoptosis pathway or other cell death pathways in resistant HL-60 cells.

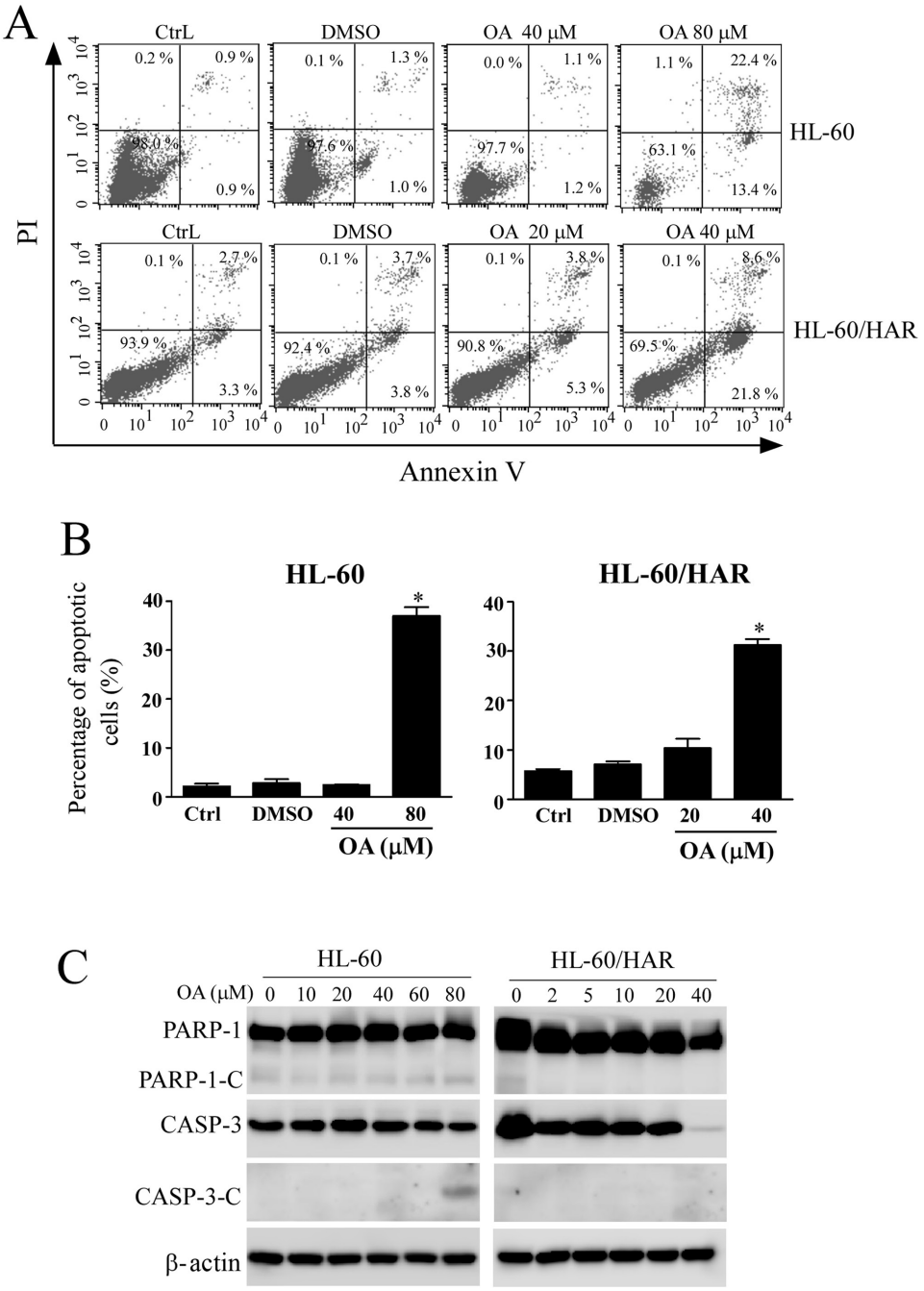

Figure 5. Effect of OA on cell death in HL-60 cell lines. (A) Apoptotic rates were determined with Annexin V/PI staining after exposure to OA for 48 h. (B) Quantifying the apoptotic cell number in (A). *P<0.05 vs. Ctrl. (C) Changes in the levels of apoptosis-related proteins were detected by western blotting after treatment with OA for 48 h. One representative result from three independent experiments is presented. Ctrl, control; CASP-3, caspase-3; CASP3-C, cleaved caspase-3; PARP-1, poly(ADP-ribose) polymerase 1; PARP-1-C, cleaved fragment of poly(ADP-ribose) polymerase; OA, oleanolic acid; HAR, harringtonine.

Rho 123 accumulation after exposure to OA in both resistant cell lines. A slight accumulation of Rho 123 was observed after the MCF-7/DOX cells were treated with OA for 1 h, but this was not concentration-dependent (Fig. 6A). Similarly, a slight Rho 123 accumulation was detected in HL-60/HAR cells after OA treatment for 1 h (Fig. 6B). These results indicated that OA does not directly bind with ABCB1.

Reduction in ABCB1 protein expression by OA in both resistant cells. ABCB1 mainly mediates multidrug resistance in tumor cells (12). Therefore, it was examined whether OA influences the protein expression levels of ABCB1 in the resistant cell lines. ABCB1 protein expression levels in both resistant cell lines were significantly decreased with increasing OA concentrations (Fig. 7). These effects occurred at lower concentrations of OA treatment in HL-60/HAR cells, when compared with those in MCF-7/DOX cells (Fig. 7A and C). These findings were consistent with aforementioned results, suggesting that HL-60/HAR cells were more sensitive to OA compared with MCF-7/DOX cells.

Discussion

The present study characterized the effects of OA on the cell viability and apoptosis of multidrug-resistant tumor cell lines.

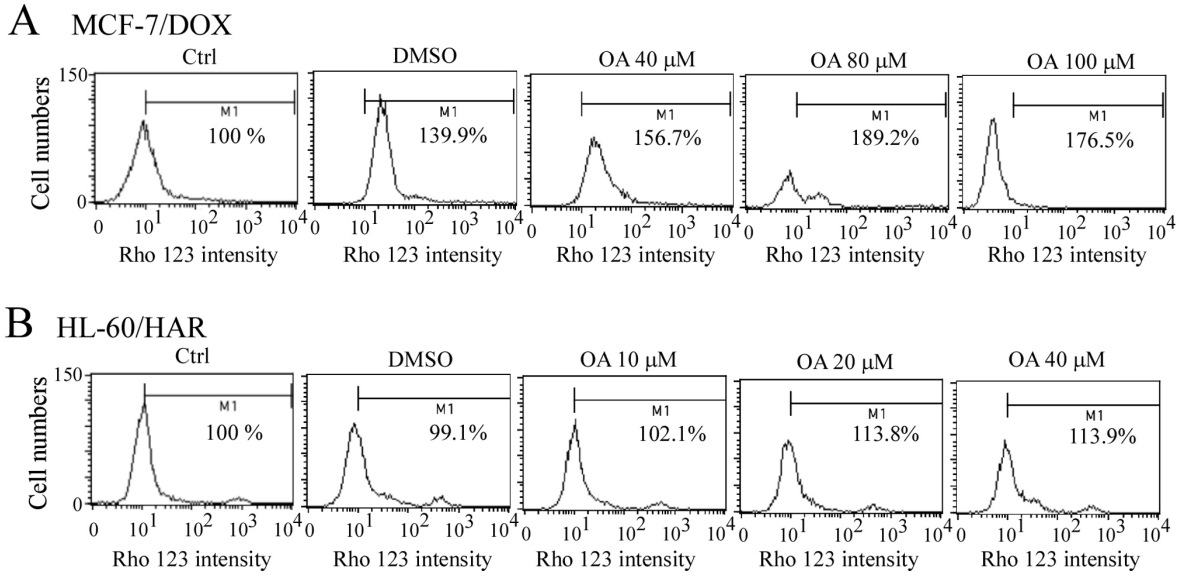

Figure 6. Effects of OA on the accumulation of Rho 123 in both drug-resistant cell lines. (A) OA slightly increases the accumulation of Rho 123 in MCF-7/DOX cells, as determined via flow cytometry. (B) Accumulation of Rho 123 after exposure to OA in HL-60/HAR cells. One representative result from three independent experiments is presented. Ctrl, control; Rho 123, rhodamine 123; OA, oleanolic acid; DOX, doxorubicin; HAR, harringtonine.

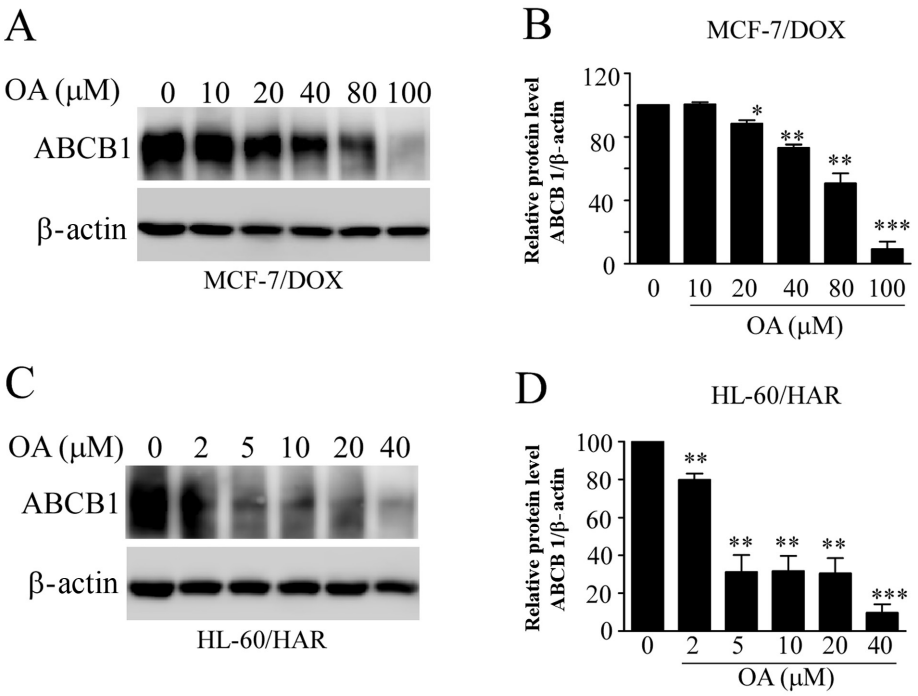

Figure 7. Reduction in ABCB1 protein expression induced by OA treatment in both drug-resistant cell lines. (A) ABCB1 protein expression was decreased by OA treatment in MCF-7/DOX cells. One representative result from three independent experiments is presented. (B) Semi-quantification of ABCB1 protein expression. (C) OA treatment reduced ABCB1 protein expression in HL-60/HAR cells. (D) Semi-quantification of ABCB1 protein expression. *P<0.05, **P<0.01 and ***P<0.001 vs. 0. ABCB1, ATP-binding cassette subfamily B member 1; OA, oleanolic acid; DOX, doxorubicin; HAR, harringtonine.

The MCF-7/DOX and HL-60/HAR cell lines have multiple drug resistance (Tables I and II). This is consistent with findings from previous studies on these two drug-resistant cell lines (19,22). To the best of our knowledge, the present study was the first to demonstrate that OA can arrest the cell cycle at the G_1 phase in drug-resistant cells. Although OA cannot enhance Rho 123 accumulation in drug-resistant cells, it can decrease ABCB1 protein expression in a concentration-dependent manner. These findings could be valuable for the clinic application of OA in tumor therapy. Moreover, some types of drug-resistant tumor cells in particular may be more suitable for OA treatment, such as leukemia cells.

Accumulating evidence has revealed that OA can arrest the cell cycle at the G_1 phase in a variety of tumor cell types, including prostate cancer PC-3 cells (3) and gallbladder cancer cells (11). In the present study, G_1 arrest was induced by OA treatment in both drug-resistant cell lines (Fig. 3). Furthermore, the percentage of cells in G_1 phase was increased after exposure

to low concentrations of OA in resistant HL-60/HAR cells, suggesting that there is a novel mechanism of the regulation of the G_1 phase of the cell cycle in drug-resistant cells. Induction of autophagy by OA in lung cancer A549 cells and breast cancer MCF-7 cells has been reported in previous studies (5,24), where G_1 phase cell cycle arrest frequently occurs during autophagy (25). Therefore, it was hypothesized that the autophagic process may differ in the drug-resistant cells compared with their parent cell lines. However, further investigation is required to test this hypothesis.

The present results suggested that OA was not a substrate of ABCB1, which is consistent with previous studies (15,26), which observed that OA affected MRP1 function but not ABCB1. The OA derivative, methyl 3,11-dioxoolean-12-en-28-olate, can target ABCB1 (16), suggesting that the chemical structure of OA prevents its interaction with ABCB1 protein. Since the present study only determined ABCB1 function after OA treatment for 2.5 h and detected Rho 123 accumulation by flow cytometry, limitations exist of regarding this method, including the specificity of Rho 123, as Rho 123 activity may not be representative of ABCB1 levels. Attention should be paid to the difference between Rho 123 accumulation and OA-induced reductions in ABCB1 protein expression.

To the best of our knowledge, the present study was the first to demonstrate that OA can decrease ABCB1 protein levels (Fig. 7). However, the signaling pathway mechanism for this reduction in ABCB1 was not investigated in the present study. It may possibly involve the mTOR pathway since OA has been previously reported to inhibit it's signaling (8,17). Further investigations currently underway to test this hypothesis.

The direct targets of OA in tumor cells remain unknown. OA can reportedly promote the dimerization of inducible nitric oxide synthase, thereby inhibiting hepatocellular carcinoma growth (27). It also been reported that transfection of CAV-1 into HL-60 cells can enhance their sensitivity to OA (17). However, the present results indicated that CAV-1 knockdown did not influence the survival of MCF-7/DOX cells after OA treatment, suggesting that CAV-1 serves an indirect role in the mechanism of OA action. In the present study, it has not determined whether OA treatment can increase CAV-1 expression. OA derivative, 2-Cyano-3,12-dioxoolean-1,9-dien-28-oic acid, can increase CAV-1 expression in colon cancer cells (28). Further studies are needed to demonstrate if overexpression of CAV-1 serves a role in MCF-7/DOX cell lines. The human leukemia HL-60 cell line lacks the *TP53* gene (29), where introduction of the wild-type *TP53* gene potentiates its susceptibility to chemotherapy (30). It was speculated that CAV-1 may function similarly during OA-induced apoptosis in sensitive HL-60 cells (17). OA has a selective action on melanoma cells, but mediated no cytotoxicity towards normal stem cells (31). In addition, the present study demonstrated that HL-60/HAR cells are more sensitive to OA compared with parental HL-60. These findings suggest that the molecular target of OA should be further investigated using drug-resistant HL-60 cells.

In conclusion, OA treatment can arrest the cell cycle at the G_1 phase in drug-resistant tumor cells and efficiently reduce ABCB1 protein expression, indicating its potential in reversing drug resistance. The present findings suggested that it may be possible to effectively target certain types of cancer using OA, such as leukemia, in the clinical setting. In recent years, numerous dietary components have been demonstrated to enhance the efficacy of cancer therapy, such as in the treatment of lymphoma (32). The present findings indicated the potential applicability of OA, which has low toxicity and few adverse effects, for tumor prevention and therapy.

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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

QH, PZ and JiaW conceived and designed the study. DW, JinW, JZ, XY, JP and LL performed the experiments and analyzed the data. DW and QH authenticated the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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