

Full-length article

Levistolide A overcomes P-glycoprotein-mediated drug resistance in human breast carcinoma cells¹

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Key words

levistolide A; P-glycoprotein; drug resistance; apoptosis; breast carcinoma

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Abstract

Aim: The aim of the present study was to investigate the reversing effect of levistolide A (LA) on P-glycoprotein (P-gp)-mediated multidrug resistance (MDR) in human breast carcinoma Bcap37/MDR1 cells. **Methods:** After chemotherapeutic drugs (adriamycin or vincristine) used alone or in combination with LA, cell proliferation was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay and cell cycle distribution by flow cytometry. RT-PCR was used to detect MDR1 gene transcription and the Western blot assay was used to assess P-gp expression and the cleavages of poly(ADP-ribose) polymerase and caspase-3. Apoptosis was detected by terminal transferase-mediated dUTP nick end-labeling assay. Moreover, the P-gp function was evaluated by the intracellular accumulation of the P-gp substrate detected by flow cytometry. **Results:** We found the subcytotoxic doses of LA significantly enhanced adriamycin- or vincristine-induced G₂/M arrest and apoptosis. These effects were consistent with the ability of LA to inhibit P-gp function. Moreover, LA dramatically enhanced the verapamil (VER) ability to reverse drug resistance. **Conclusion:** LA has the potential to be developed as a novel P-gp modulator. Furthermore, the combination of LA and VER might represent a more sufficient but less toxic anti-MDR regimen.

Introduction

The multidrug resistance (MDR) phenotype is often associated with the overexpression of ATP-binding cassette (ABC) transporters, which can actively extrude a wide variety of structurally diverse anticancer agents, thereby reducing their intracellular drug concentrations^[1]. P-glycoprotein (P-gp) is a 170 kDa transmembrane glycoprotein encoded by the MDR1 gene^[2,3]. The overexpression of P-gp was shown to be resistant against various clinically relevant compounds, including anthracyclines and vinca alkaloids^[1]. In addition, several cytological dyes, such as 3-ethyl-2-(3-[3-ethyl-2-{3H}-benzoxazolylidene]-1-propenyl) benzoxazolium iodide [DiOC₂(3)] demonstrated decreased accumulation in P-gp overexpressing cells^[4]. P-gp overexpression appears to be closely correlated with poor prognosis for cancers, including breast carcinoma^[5]. Therefore, a successful inhibition of

the P-gp transporter function or its expression may overcome MDR by increasing the intracellular accumulation of anticancer drugs.

Although many agents have been identified as competitive antagonists of P-gp *in vitro*, most have little or no therapeutic potential because of high toxicity *in vivo* at the doses required to reverse MDR. For instance, verapamil (VER), a calcium channel blocker used in the treatment of ischemic heart disease, is a highly effective competitive antagonist of P-gp, but the doses required to overcome MDR result in unacceptable cardiovascular toxicity^[6].

Levistolide A (LA) is a naturally occurring compound extracted from the rhizomes of *Angelicae sinensis* (Oliv). In Chinese traditional medicine, the root of *Angelicae sinensis* is used for tonifying circulation and removing blood stasis. It has been applied in clinics for treating gynecological

symptoms, anemia, chronic bronchitis, asthma, rheumatism, and other diseases^[7]. It is also used as an essential component of antitumor formulas in China. The most important effect of *Angelicae sinensis* in antitumor formulas is to enhance the efficacy and reduce the toxicity of chemotherapy^[8–10]. Therefore, we hypothesized that some of the components in *Angelicae sinensis* may serve to enhance the efficacy of chemotherapy. We screened more than 50 fractions in the organic solution and found that LA had a potent capacity to sensitize MDR cells to a variety of chemotherapy drugs, including adriamycin (ADR) and vincristine (VCR). In this study, we investigated the reversing effect of LA on P-gp overexpressing Bcap37/MDR1 cells and compared its reversing efficiency with a typical P-gp modulator VER.

Materials and methods

Cell culture and reagents Bcap37 breast carcinomas cells and P-gp overexpressing subline Bcap37/MDR1 cells derived by transfecting the human MDR1 gene^[11], were generously provided by Dr Cao JIANG at Zhejiang University (Hangzhou, China). These cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) in 5% CO₂ at 37 °C. LA used in this study was purified from *Angelicae sinensis* according to the reported methods^[12]. Its structural identity was determined spectroscopically (¹H and ¹³C NMR, and MS) as described previously^[13,14]. ADR, VCR, VER, DiOC₂(3), and dimethyl thiazolyl-2,5-diphenyltetrazolium bromide (MTT) were from Sigma (St Louis, MO, USA).

MTT assay Chemosensitivity was measured by the MTT assay. Briefly, the cells were seeded into 96-well plates and allowed to attach for 24 h, then the drugs diluted with the medium at different concentrations were added into the wells. To determine the reversal effect, graded concentrations of ADR or VCR with or without P-gp modulators were added. Then the cells were exposed to the drugs for 68 h at 37 °C, after which 20 µL MTT (5 mg/mL diluted in phosphate-buffered saline [PBS]) was added to each well and incubated for an additional 4 h. The formed formazan was dissolved in DMSO after aspirating the culture medium. The plates were shaken mechanically and the optical density of each well was read on a microplate reader (Opsys MR, Dynex Technology, Chantilly, Virginia, USA) at 470 nm. The reversal activity of VER or LA on ADR and VCR resistance was expressed as the reversing factors (RF), which were calculated according to the following equation: RF=IC₅₀ antitumor drug alone/IC₅₀ antitumor drug+modulator.

Cell cycle analysis The cells were seeded into 6-well plates and allowed to attach for 24 h. Then various concen-

trations of drugs diluted with the medium were added into the wells and incubated for 24 h. After the treatment period, the cells were harvested and fixed in 70% ethanol. The cells were again washed with PBS and then incubated with propidium iodide (10 µg/mL) with simultaneous treatment of RNase at 37 °C for 30 min. The percentages of the cells in the different phases of the cell cycle were measured with a FACStar flow cytometer (Becton Dickinson, San Jose, CA, USA) and analyzed using ModFit software (Verity, Topsham, USA).

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling assay The terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay was performed using the commercial kit (Roche Molecular Biochemicals, Indianapolis, USA) according to the manufacturer's instructions^[15]. Briefly, the cells were collected and fixed in 1% formaldehyde in PBS for 15 min on ice, washed with PBS, resuspended in 70% ethanol, and stored overnight at -20 °C. After rehydration in PBS for 5 min, the cells were resuspended in 50 µL reaction buffer (5 U terminal transferase, 2.5 mmol/L cobalt chloride, 0.5 mmol/L biotin-dUTP, and TdT buffer containing 0.2 mol/L potassium cacodylate, 25 mmol/L Tris-HCl, and 0.25 mg/mL bovine serum albumin, pH 6.6) and incubated at 37 °C for 30 min. The cells were then washed with PBS containing 0.1% Triton X-100, resuspended in 100 µL staining buffer, and incubated for 30 min in the dark. The pellets were then washed twice with rinsing buffer, resuspended in PBS, and measured with a flow cytometer.

Western blot analysis The cell extracts were equally loaded onto 8%–12% SDS-PAGE and electrophoretically transferred to NC membranes (Bio-Rad, New Orleans, Louisiana, USA). The membranes were blocked with 5% skimmed milk in PBS containing 0.1% Tween 20 (PBST) and incubated overnight with each primary antibody against P-gp (Chemicon Tech, Hangzhou, China), poly(ADP-ribose) polymerase (PARP; Santa Cruz Biotechnology, Santa Cruz, CA, USA), caspase-3 (Cell Signaling Tech, Danvers, MA, USA), and β-actin (Sigma, St Louis, USA). The membranes were then washed with PBST and incubated for 1 h with a secondary antibody and visualized with an enhanced chemiluminescence detection kit (Pierce, Appleton, WI, USA).

RNA extraction and RT-PCR The total RNA from Bcap37/MDR1 cells was extracted using TRIzol and isolated according to the procedure supplied by the manufacturer (Invitrogen, USA). Reverse transcription was carried out according to the manufacturer's instructions (GIBCO-BRL, USA). The first strand of the cDNA was generated from 5 µg of total RNA using the oligo(dT) primer and superscript

II reverse transcriptase (GIBCO-BRL, USA). The cDNA were amplified by PCR using the Supermix kit (Toyobo, Tokyo, Japan). The primer sets for P-gp were 5'-CCC ATC ATT GCA ATAGCA GG-3' and 5'-GTT CAAACTTCT GCT CCTGA-3'; and the primer sets for β -actin were 5'-GTG GGG CGC CCC AGG CAC CA-3' and 5'-CTC CCT AAT GTC ACG CAC GAT TTC-3'. Amplified DNA fragments were electrophoresed on 1% agarose gels.

DiOC₂(3) efflux assays The fluorescence intensity of intracellular DiOC₂(3) was determined by flow cytometry. Briefly, the Bcap37/MDR1 cells were harvested, incubated with 0.1 μ mol/L DiOC₂(3) at 37 °C for 1 h, washed with ice-cold PBS, and resuspended in fresh medium containing P-gp modulators for indicated time. Then the cells were washed with ice-cold PBS and the mean fluorescence intensity of intracellular DiOC₂(3) was detected using flow cytometry.

Statistical analysis All experiments were repeated at least 3 times and expressed as mean \pm SD. The statistical difference between the control and treatment groups was determined by *t*-test. A *P*-value of <0.05 was considered to be statistically significant.

Results

LA enhances ADR- or VCR-induced G₂/M arrest in Bcap37/MDR1 cells The chemosensitivity of MDR cells to the chemotherapy drugs was measured by IC₅₀ comparison expressed as RF. The IC₅₀ of LA were 89.31 and 93.14 μ mol/L, respectively, in Bcap37 and Bcap37/MDR1 cells (Table 1). At 39 μ mol/L (15 μ g/mL) or less, LA did not cause any cytotoxicity to Bcap37 or Bcap37/MDR1 cells. Therefore, 13, 26, and 39 μ mol/L were chosen as the working concentrations in the next experiments. As expected, the Bcap37/

MDR1 cells were more resistant to ADR and VCR. The IC₅₀ for ADR and VCR without a modulator in the Bcap37 cells were 0.10 and 0.11 μ mol/L, respectively (Table 1), while in the Bcap37/MDR1 cells, the IC₅₀ for ADR and VCR without a modulator were 1.84 and 4.57 μ mol/L, respectively (Table 1). LA dose dependently increased the sensitivity of Bcap37/MDR1 to ADR and VCR (Figure 2A). At a concentration of 26 μ mol/L, LA shifted the IC₅₀ for ADR and VCR to 0.17 and 0.2 μ mol/L with RF of 10.70- and 22.46-fold, respectively (Table 1). However, LA treatment did not significantly affect drug sensitivity in the Bcap37 cells (Table 1).

Usually, agents that induce cytotoxicity by damaging DNA (e.g. ADR) or inhibiting tubulin polymerization (e.g. VCR) arrest cell cycle at the G₂/M phase^[16]. As expected, we found ADR (2 μ mol/L and above) and VCR (5 μ mol/L and above) dose-dependently induced G₂/M arrest (data not shown). Neither ADR (1 μ mol/L) nor LA (26 μ mol/L) alone caused cell cycle arrest, whereas treatment with a combination of both induced a significant G₂/M arrest up to 60.94% (Figure 2B). However, higher doses of LA (>54 μ mol/L) induced

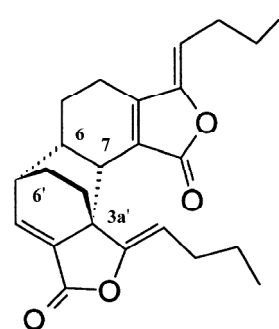


Figure 1. Chemical structure of LA (Molecular weight =380).

Table 1. Effect of LA on the cytotoxicity of ADR and VCR in Bcap37 and Bcap37/MDR1 cells. *n*=4. Mean \pm SD.

	IC ₅₀		RF	
	Bcap37	Bcap37/MDR	Bcap37	Bcap37/MDR
LA	89.31 \pm 6.221	93.14 \pm 5.262		
ADR	0.101 \pm 0.027	1.84 \pm 0.021		
+LA (13 μ mol/L)	0.088 \pm 0.002	0.899 \pm 0.008	1.148	2.049
+LA (26 μ mol/L)	0.083 \pm 0.011	0.171 \pm 0.034	1.217	10.77
+VER (5 μ mol/L)	0.064 \pm 0.006	0.151 \pm 0.014	1.578	12.20
VCR	0.122 \pm 0.081	4.567 \pm 0.271		
+LA (13 μ mol/L)	0.134 \pm 0.009	0.847 \pm 0.021	0.911	5.392
+LA (26 μ mol/L)	0.109 \pm 0.035	0.202 \pm 0.029	1.119	22.61
+VER (5 μ mol/L)	0.112 \pm 0.007	0.117 \pm 0.002	1.089	39.03

Reversing factors (RFs) were calculated according to the following equation: RFs=IC₅₀ antitumor drug alone/IC₅₀ antitumor drug+modulator

cell cycle arrest at the G₀/G₁ phase (data not shown), suggesting that LA enhanced the ADR-induced G₂/M arrest. Similarly, we found that the VCR-induced G₂/M arrest was also increased by LA by 82.04% (Figure 2B). The potentiated effects of LA (26 μmol/L) on G₂/M arrest were a little less than those of VER at 5 μmol/L. These results suggest that like VER, LA enhanced ADR- or VCR-induced G₂/M arrest, which may attribute its sensitizing effect to drug resistance.

LA treatment potentiates ADR-induced apoptosis in Bcap37/MDR1 cells Inducing apoptosis is the main mechanism for ADR and VCR to treat carcinomas. We therefore investigated whether LA increased the cytotoxicity of ADR or VCR by apoptosis. Apoptosis was determined by TUNEL

assay. Treatment with ADR (1 μmol/L), VER (5 μmol/L), or LA (26 μmol/L) alone did not increase TUNEL-positive cells compared to the control, while the cotreatment of ADR with LA or VER significantly increased TUNEL staining up to 53.6% and 59.8%, respectively (Figure 3A). Similarly, LA (26 μmol/L) cotreatment with VCR (0.5 μmol/L) significantly enhanced VCR-induced TUNEL staining (data not shown). The effect of LA (13 and 26 μmol/L) on apoptosis potentiation was further evidenced by caspase-3 activation and PARP cleavage. As illustrated in Figure 3B, caspase-3 was activated and PARP was cleaved in VER and ADR (or VCR)-cotreated MDR cells, but not in ADR (or VCR) alone-treated cells. Similar to VER, cotreatment with LA also caused a notable increase of caspase-3 activation and PARP cleavage (Figure 3B). These

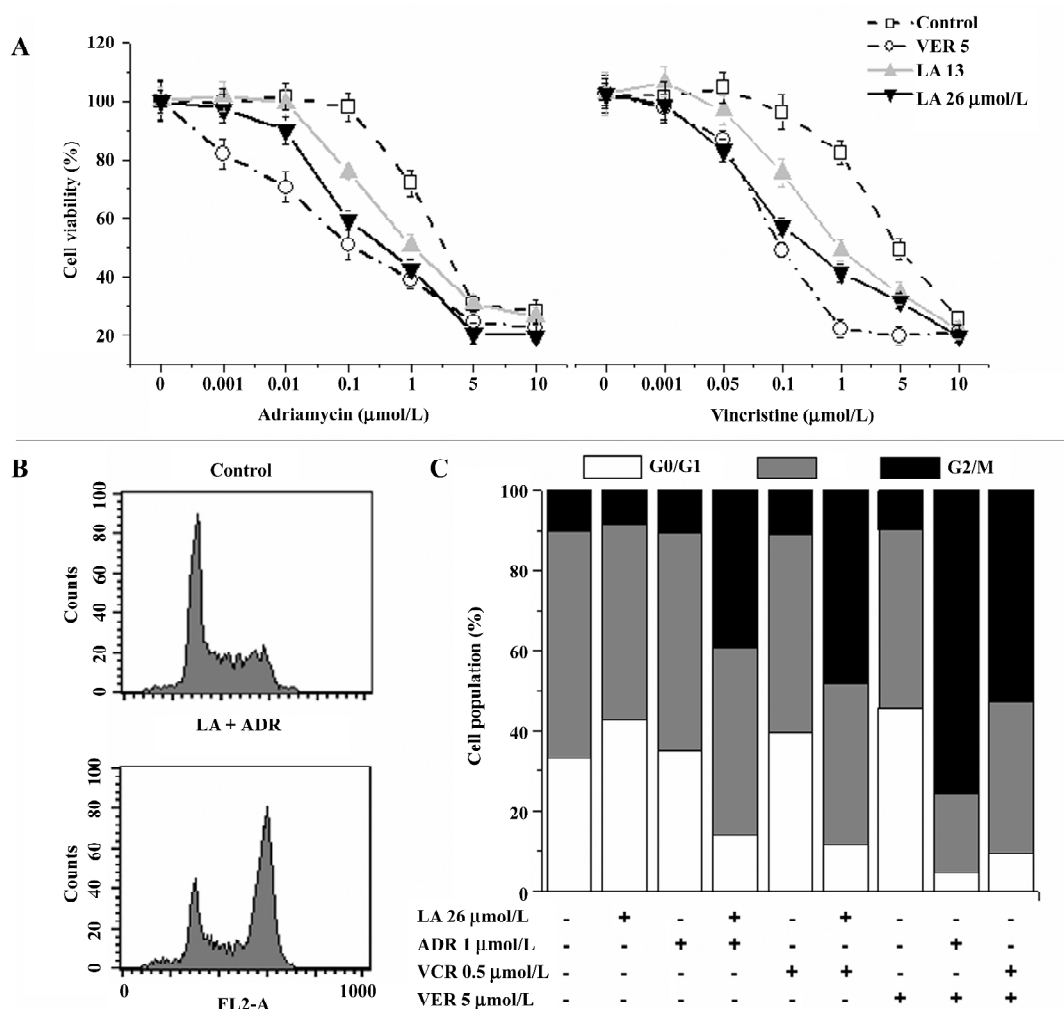


Figure 2. LA potentiates ADR- or VCR-induced cell growth inhibition in Bcap37/MDR1 cells. (A) cells were incubated with various concentrations of ADR or VCR with or without VER (5 μmol/L) or LA (13 and 26 μmol/L) for 68 h. Cell viability was determined by MTT assay. (B, C) Cells were cultured with ADR (1 μmol/L) and VCR (0.5 μmol/L) with or without VER (5 μmol/L) or LA (26 μmol/L) for 48 h and the cells were fixed and stained with PI. DNA content was analyzed by flow cytometry. G₀/G₁, G₂/M and S indicate the cell phases. Each phase was calculated using the Modifit program. Three duplicate experiments were performed with similar results.

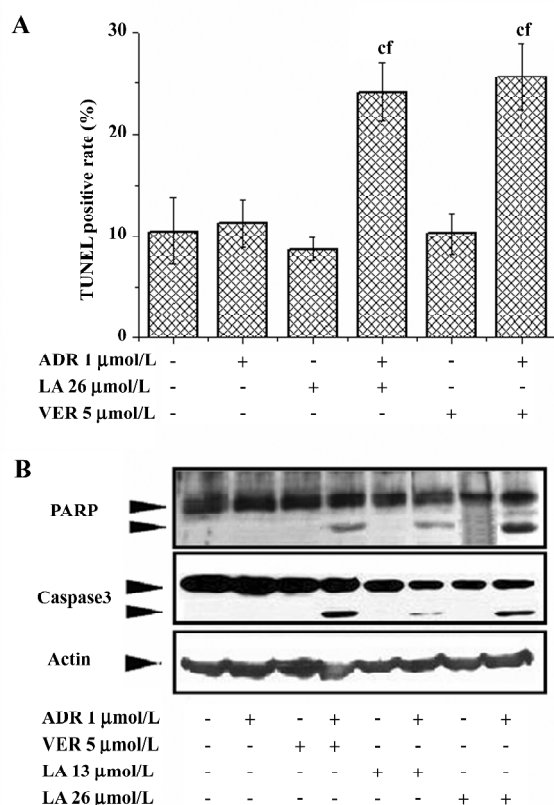


Figure 3. LA enhanced ADR-induced apoptosis in Bcap37/MDR1 cells. (A) cells were treated with ADR (1 μmol/L) with or without VER (5 μmol/L) or LA (26 μmol/L) for 48 h, then fixed and incubated with TUNEL reaction buffer and measured by flow cytometry. Data are represented as the TUNEL-positive rates. ^c $P < 0.01$ vs control; ^f $P < 0.01$ vs ADR-treated group. (B) cells were treated with ADR (1 μmol/L) with or without VER (5 μmol/L) or LA (13, 26 μmol/L) for 48 h, then the cell lysates were examined for the cleavage of PARP and caspase-3 activation by Western blotting.

results suggest that LA treatment might inhibit P-gp function and subsequently lead to an increase in its apoptotic potential resulting from the intracellular accumulation of anticancer drugs.

LA inhibits DiOC₂(3) efflux out of Bcap37/MDR1 cells without affecting P-gp expression In order to determine whether LA reversed MDR by inhibiting P-gp function, we investigated the effect of LA on the intracellular accumulation of a specific P-gp substrate DiOC₂(3). As illustrated in Figure 4A, the inhibition of P-gp with LA dose dependently caused an increase in the accumulation of DiOC₂(3) in MDR cells. LA at 39 μmol/L increased drug accumulation by 6.5-fold, with the efficiency similar to that of VER at 10 μmol/L (Figure 4A). P-gp is a drug transporter and functions by pumping its substrates out of

MDR cells. By increasing the intracellular DiOC₂(3) accumulation of LA suggests that LA has a capacity to suppress P-gp function. To determine whether LA also affects P-gp expression, we exposed the MDR cells to LA for indicated times, then detected MDR1 gene transcription by RT-PCR and P-gp protein expression by Western blotting. As shown in Figure 4B, LA at 26 μmol/L did not affect MDR1 mRNA or P-gp expression during 48 h exposure. These results indicate that LA overcame P-gp-mediated MDR by inhibiting P-gp function without affecting the P-gp expression.

LA inhibits LA-enhanced VER ability to reverse drug resistance To investigate whether LA targets the same site of VER or other modulators, the MDR cells were incubated with paired modulators and then their effects on DiOC₂(3) accumulation were analyzed. Cotreatment with LA (13 μmol/L) and VER (5 μmol/L) for 2 h dramatically increased DiOC₂(3) accumulation much more than LA (13 μmol/L) or VER (5 μmol/L) alone (Figure 4C). After incubation for 2 h, the drug accumulation peaks became more flat than those of incubation at 1 h (Figure 4A) in the control, VER, or LA alone-treated groups, suggesting the drug efflux was over influx in these groups (Figure 4C). However, in the LA and VER cotreated group, the peak remained sharp (Figure 4C), suggesting that cotreatment significantly potentiated the efflux pump inhibitory effect of LA or VER. However, we did not show similar results in the pair of LA and cyclosporin A (CsA), or of VER and CsA (data not shown). It is therefore reasonable to hypothesize that by using relatively low concentrations of LA and VER, it may be possible to develop a less toxic regimen to reverse MDR. As illustrated in Figure 4D, a combination of 13 μmol/L LA and 5 μmol/L VER potentiated ADR-induced cytotoxicity in Bcap37/MDR1 cells, with inhibitory rates up to 35%–50% compared to LA or VER alone treatment.

Discussion

The treatment of human cancer has been seriously hampered for decades by resistance to chemotherapeutic drugs^[17]. Mechanisms underlying this resistance are far from being entirely known. The major hypothesis involves the existence of some cellular activities that tumor cells hijack to overcome the effects of cytotoxic drugs. Both biochemical and microenvironmental factors are involved in the mechanisms responsible for drug resistance. Between the cellular mechanisms, those mediated by proteins belonging to the family of ABC transporters are best known. These proteins are P-gp, the multi-drug resistance associated protein-1, and the breast cancer resistance protein. The ABC

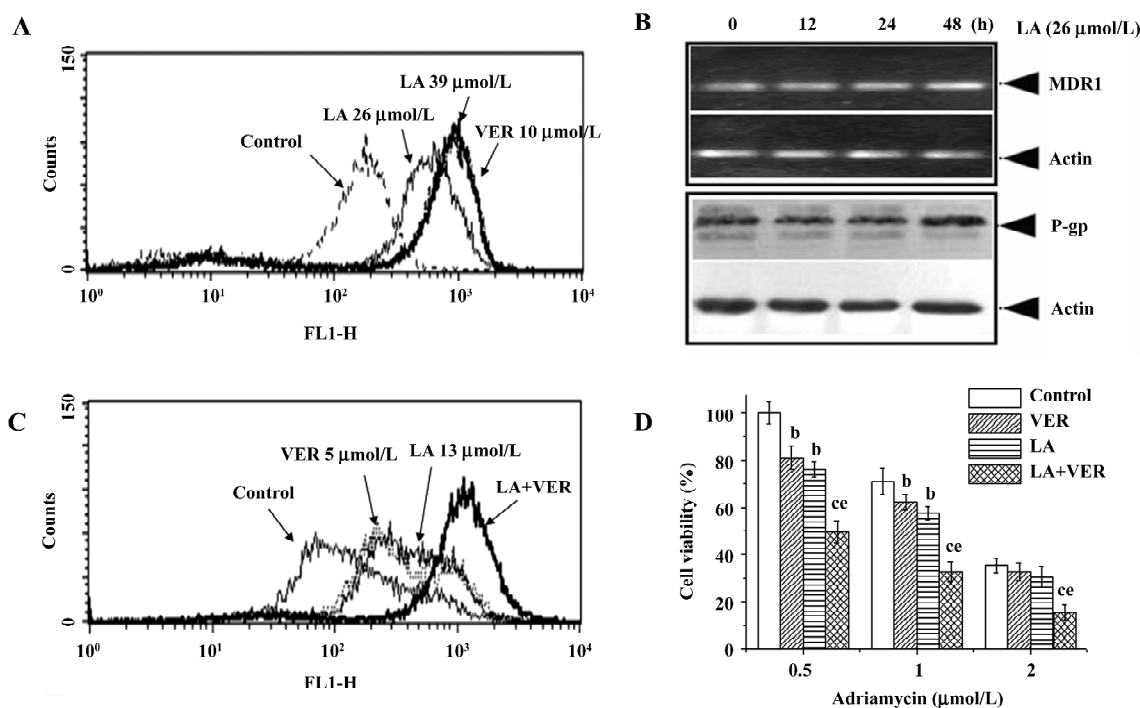


Figure 4. LA inhibits DiOC₂(3) efflux without affecting the P-gp expression. (A,C) Bcap37/MDR1 cells were incubated with DiOC₂(3) for 1 h, washed with ice-cold PBS, then incubated with LA (26 and 39 μmol/L) or VER (10 μmol/L) for another 1 h (A), or LA (13 μmol/L) and/or VER (5 μmol/L) for 2 h (C). Intracellular DiOC₂(3) was measured by flow cytometry at FL1. (B) cells were treated with 26 μmol/L LA for the indicated times. Expression of MDR1 transcription (upper) and P-gp protein expression (lower) levels were analyzed by RT-PCR and Western blotting, respectively. (D) cells were incubated for 48 h with ADR (0.5–2 μmol/L) with or without VER (5 μmol/L) or LA (13 μmol/L), or with a combination of VER (5 μmol/L) and LA (13 μmol/L). Cell viability was determined by the MTT assay. ^bP<0.05, ^cP<0.01 vs control group; ^{cc}P<0.05 vs VER- or LA-treated group.

transporters are associated with drug efflux pumps and have been associated with MDR. Their treatment causes MDR by decreasing intracellular drug retention or by altering the target distribution of the drugs. New data suggest that the linkage of some of these proteins (e.g. P-gp) with the actin cytoskeleton may be of importance for their activity^[18]. Thus, it seems that the function of these proteins may depend on a more complex cellular mechanism that involves more than a cellular structure. Although these proteins need to be characterized further, their significance as membrane efflux pump-related proteins in the resistance of human tumors to cytotoxic drugs has yet to be clarified.

In this study, the problem of MDR of human tumors has been faced by the use of an extract from *Angelicae sinensis*, an important herb in Chinese traditional medicine. Some formulas containing *Angelicae sinensis*, such as Siwu mixture, have been used in clinics as an auxiliary chemotherapy and have demonstrated reversing multidrug resistance in P-gp overexpressing human leukemia cells^[19]. Based on the extraction of the mixture formula, it suggested

the activation component might be a polysaccharide^[20]. In our previous study, however, we found that the chloroform extracts of *Angelicae sinensis* had a more potent reversing effect than that of the polysaccharide fraction. Through step-by-step extracting and screening, we finally found that the dimmer of (*Z*)-ligustilide (LA) had the potential to reverse multidrug resistance in a variety of P-gp overexpressing MDR cells which were selected by increased doses of ADR (e.g. K562/ADR and MCF-7/ADR) or vincristine (e.g. KBv200). In order to further investigate the reversing effect of LA on P-gp-mediated drug resistance, we used Bcap37/MDR1 cells which were derived from the Bcap37 cells transfected with the human MDR1 gene^[11]. With this model, we demonstrated that LA at subcytotoxic doses significantly reversed MDR by enhancing ADR- or VCR-induced G₂/M arrest and apoptosis (Figures 2,3). Its reversing capacity was associated with its inhibitory effect on P-gp function (Figure 4). Both ADR and VCR are substrates of P-gp^[6]. Typical P-gp modulators, such as VER and CsA, reverse MDR by competitively binding to P-gp. However,

the effective doses of these drugs have been proven to induce severe side-effects in the treatment of cancer patients^[6]. The results of our study have shown that LA behaves differently to the typical P-gp substrates. First, unlike VER, LA shows a similar IC₅₀ between P-gp-negative and -overexpressing cells (Table 1), suggesting it is not a P-gp substrate. However, cotreatment with LA and VER synergistically enhances either ADR- or VCR-induced cytotoxicity (Figure 4).

In conclusion, our data demonstrate for the first time that LA, a component of *Angelicae sinensis*, can modulate P-gp function and overcome P-gp-mediated MDR in Bcap37/MDR1 cells, indicating that LA could be a highly feasible candidate for the development of a new P-gp modulator. Moreover, LA and VER cotreatment can synergistically inhibit P-gp function and enhance ADR-induced cytotoxicity, suggesting that a combination of LA and VER may be possible to develop a more sufficient with less toxic anti-MDR method.

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