Levistolide A synergistically enhances doxorubicin-induced apoptosis of k562/dox cells by decreasing MDR1 expression through the ubiquitin pathway

YUANYUAN DING[1,2*], WEIBO NIU[3*], TAO ZHANG[2], JUE WANG[2], JIAO CAO[2],
HE CHEN[1], RUIQI WANG[1] and HONGLI AN[1,4]

[1]Center for Translational Medicine, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, Shaanxi 710061;
[2]Institute of Materia Medica, School of Pharmacy, Xi'an Jiaotong University, Xi'an, Shaanxi 710061;
[3]Department of General Surgery, Qilu Hospital of Shandong University, Jinan, Shandong 250012;
[4]Key Laboratory for Tumor Precision Medicine of Shaanxi Province,
The First Affiliated Hospital of Xi'an Jiaotong University,
Xi'an, Shaanxi 710061, P.R. China

Abstract. Multidrug resistance (MDR) is one of the main reasons underlying failure of cancer chemotherapy. Certain natural compounds may help prevent MDR, and may be used in combination with chemotherapeutic agents to enhance their efficacy. Levistolide A is a natural product that is extracted from the rhizome of *Angelicae sinensis* (Oliv.), which has been used as an essential component of antitumor formulas since ancient times in China. The present study conducted the following experiments: MTT assay, apoptosis analysis, cellular doxorubicin accumulation assay, immunoblotting and reverse transcription-quantitative polymerase chain reaction, to investigate whether levistolide A enhance doxorubicin-induced apoptosis of k562/dox cells and to determine the molecular mechanisms involved. When combined with doxorubicin, levistolide A exhibited a synergistic effect and induced cytotoxicity in k562/dox cells. Drug accumulation studies revealed that levistolide A increased the intracellular concentration of doxorubicin in a dose-dependent manner. Cell apoptosis experiments indicated that levistolide A increased the sensitivity of k562/dox cells to doxorubicin. Furthermore, detection of reactive oxygen species (ROS) revealed that levistolide A enhanced doxorubicin-induced cell death by increasing the levels of ROS. Mitochondrial potential detection with JC-1 staining also indicated that levistolide A synergistically enhanced doxorubicin-induced cell death. Immunoblotting demonstrated that levistolide A enhanced doxorubicin-induced cell death by decreasing the expression levels of B-cell lymphoma 2 and increasing caspase 3 expression. Furthermore, multidrug resistance protein 1 (MDR1) expression in k562/dox cells was downregulated by levistolide A in a dose-dependent manner, thus suggesting that levistolide A may modulate MDR1 during cancer therapy. Therefore, the combination of levistolide A with doxorubicin could result in more effective and less toxic anticancer regimens.

Introduction

Multidrug resistance (MDR) is a major impediment to effective cancer chemotherapy and the treatment of infectious diseases (1). The development of drug resistance remains a dominant obstacle toward curative cancer treatment. MDR can be induced by numerous mechanisms, including reduced drug uptake, altered cell cycle checkpoints and cell cycle arrest, altered drug targets, increased drug efflux by drug transporters, or sequestration of anticancer drugs in lysosomes, intracellular organelles and intercellular vesicles (2-12). Overexpression of various ATP-dependent efflux pumps, particularly ATP binding cassette (ABC) subfamily B member 1, which encodes multidrug resistance protein 1 (MDR1, also known as P-glycoprotein), is a well-known resistance mechanism against several chemotherapeutic agents; for example, taxanes, anthracyclines, vinca alkaloids and epipodopyllotoxins. This has been demonstrated in previous studies (13), both in tumor cell lines and in numerous tumor types, including solid tumors and hematological malignancies (13).

In tumor cells, MDR1 pumps out anticancer drugs, such as doxorubicin, taxanes, anthracyclines, vinca alkaloids and epipodopyllotoxins, resulting in decreased intracellular drug concentrations (14,15). MDR1-mediated drug resistance can be effectively overcome by either blocking the function of MDR1 or by inhibiting its expression (16). In addition,

Correspondence to: Dr Hongli An, Center for Translational Medicine, The First Affiliated Hospital of Xi'an Jiaotong University, 277 Yanta West Road, Xi'an, Shaanxi 710061, P.R. China
E-mail: anhongli@mail.xjtu.edu.cn

*Contributed equally

other mechanisms underlying drug resistance have been described, including activation of p53, deletion or inactivation of the pro-apoptotic gene caspase 3, increased expression of the proto-oncogene B-cell lymphoma 2 (Bcl-2), suppression of the apoptosis pathway and increased protein expression of ATP transporters, all of which can promote tumor cell resistance to chemotherapeutic drugs (17-21).

Apoptosis involves a series of morphological and biochemical events, including DNA degradation, cell shrinkage, protein cleavage, protein cross-linking, phosphatidylserine exposure, increased mitochondrial membrane permeability and cell membrane deformities (22). There are two major apoptotic pathways: The extracellular pathway, which is mediated by death receptors, and the intracellular pathway, which is also known as mitochondrial apoptosis. Although these two pathways function separately, they are interconnected and eventually converge on the same target (23). The process of apoptosis is closely regulated by cells through a series of mitochondrial pathway molecules, particularly the Bcl-2 family (24). Apoptosis is a normal form of cell death that is closely associated with biological development, organ formation and physical balance. The most prominent feature of tumor cells is the ability to escape control by the host and to avoid cell apoptosis.

The ubiquitin-proteasome pathway (UPP) is an important pathway that selectively degrades proteins *in vivo*; the majority of eukaryotic protein degradation is accomplished in this manner (25). This pathway also serves an important role in the regulation of apoptosis. It has previously been reported that proteasome inhibitors can induce cellular apoptosis in various tumor cells by blocking the ubiquitination pathway; these findings provide a direct and powerful means for studying the role of the UPP in apoptosis (25). In addition, the proteasome inhibitor, MG132, induces nuclear damage, reduces mitochondrial transmembrane potential, releases cytochrome *c*, induces the formation of reactive oxygen species (ROS), activates caspase-8, and affects Bcl-2 family proteins to affect MDR. Therefore, proteasome inhibitors are closely associated with drug resistance in cancer therapy (26-28).

Natural products are garnering interest in the field of cancer therapy. Some compounds extracted from fruits, vegetables, oilseeds and herbs have been reported to modulate the activity of MDR1 (29-31). Levistolide A is a natural compound extracted from the rhizome of *Angelicae sinensis* (Oliv.) (32). It has been used clinically in China to treat gynecological symptoms, anemia, chronic bronchitis, asthma, rheumatism and other diseases (33). With regards to antitumor therapies, the most important effects of *Angelicae sinensis* are that it enhances the efficacy and reduces the normal cell toxicity of chemotherapeutic drugs (34-36). Our previous study screened and identified levistolide A as an active compound capable of inhibiting tumor cell proliferation (37). The present study aimed to reveal the mechanisms underlying the synergistic effects of levistolide A towards doxorubicin-induced apoptosis of k562/dox cells.

Materials and methods

Reagents. Levistolide A (Fig. 1) was obtained from Shanghai Suo Laibao Biotechnology Co., Ltd. (Shanghai,

China). Doxorubicin was obtained from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). Antibodies against the apoptosis regulators Bcl-2 (cat. no. 4223) and Bcl-2-associated X protein (Bax; cat. no. 5023) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Primary antibodies against MDR1 (cat. no. sc-8313) were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA), against breast cancer resistance protein [BCRP (ERP2099); cat. no. ab108312) were purchased from Abcam (Cambridge, MA, USA) and GAPDH (cat. no. AB-P-R001) were obtained from Hangzhou Goodhere Biotechnology Co., Ltd. (Hangzhou, China) MG132, dimethyl sulfoxide (DMSO) and MTT were purchased from Sigma-Aldrich; Merck KGaA. RPMI-1640 medium was obtained from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA).

For all experiments, stock solutions of the drugs were dissolved in DMSO. The final concentrations of the drugs were obtained by diluting the stock solutions in RPMI-1640 culture medium. The final concentration of DMSO was always <0.2% in order to avoid cell toxicity.

Cell culture. Doxorubicin-induced MDR erythroleukemic k562/dox cells and parental k562 cells were obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). To maintain doxorubicin resistance, k562/dox cells were cultured with 1.0 μM doxorubicin. Cells were maintained in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) at 37°C under a humidified atmosphere containing 5% CO_2.

Cytotoxicity assays. Cell viability was determined using the MTT assay. k562 and k562/dox cells were seeded into 96-well plates ($5x10^3$ cells/well) and were then treated with various concentrations of doxorubicin (with or without levistolide A) for 48 h at 37°C. In addition, k562/dox cells were incubated with 0, 2, 4, 8 and 10 μM MG132 for 24 h at 37°C. After discarding the medium, the MTT solution (500 μg/ml) was added and cells were incubated for 4 h at 37°C. The formazan was solubilized with DMSO and relative cell viability was measured at 490 nm with a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Growth inhibition in response to various concentrations of doxorubicin and levistolide A was calculated using GraphPad Prism 7.0 (GraphPad Software, Inc., La Jolla, CA, USA).

The nature of the interaction (whether it was synergistic, additive or antagonistic) between doxorubicin and levistolide A, as a function of their concentrations and cell growth inhibition, was assessed using the combination index (CI) method (38). For the combination of two compounds: $CI=D1/D_{x1} + D2/D_{x2} + D1xD2/D_{x1}xD_{x2}$; where D1 and D2 are the concentrations required to observe the x % effect when K562/dox cells were cotreated with doxorubicin and levistolide A; D_{x1} and D_{x2} are the concentrations required to observe the x effect with each of the two drugs alone. The CI helps to identify synergistic (CI<1), additive (CI=1) or antagonistic (CI>1) interactions.

Cellular doxorubicin accumulation assay. The k562/dox and k562 cells were cultured in 6-well plates for 24 h to allow
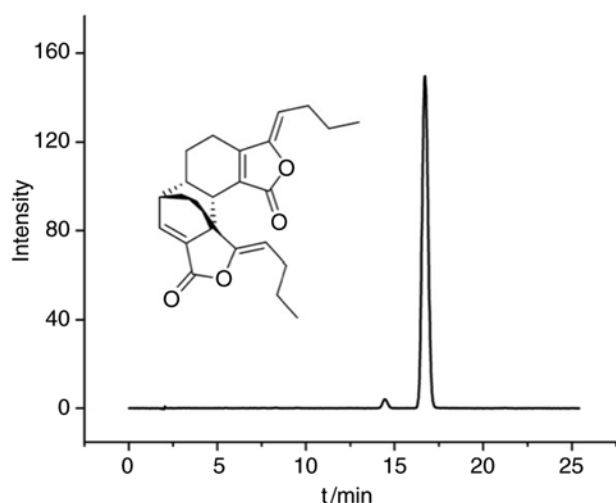
Figure 1. High-performance liquid chromatography chromatogram and structural diagram of levistolide A (purity, >99%).

attachment. Subsequently, the cells were incubated with DMSO alone (control), with 1.2 μM doxorubicin, or with doxorubicin plus 50 or 100 μM levistolide A for 24 h. After washing twice with PBS, the cells were treated with 400 μl PBS and were gently mixed before being analyzed using a FACSort flow cytometer with CellQuest Pro Ver. 6.0 software (both BD Biosciences, Franklin Lakes, NJ, USA).

Cell apoptosis. The k562/dox cells (1×10^5 cells/ml) were seeded into 6-well plates. Once they reached 80% confluence, cells were treated with 1.2 μM doxorubicin; 0, 10 or 100 μM levistolide A; or with both drugs for 24 h. Apoptosis was detected using the Annexin V-fluorescein isothiocyanate/propidium iodide (PI) double staining method. The cells were washed twice with ice-cold PBS, resuspended in PBS (100 μl) and were incubated with Annexin V and PI labeling solution (5 μl) at room temperature for 15 min. Subsequently, the cells were gently vortexed and incubated for 15 min at room temperature in the dark. Following the addition of 400 μl 1X binding buffer, the percentage of apoptotic cells was measured within 1 h using the BD Accuri™ C6 Plus Flow Cytometer (BD Biosciences).

ROS assay. The k562/dox cells (1×10^5 cells/ml) were seeded into 6-well plates. Once they reached 80% confluence, cells were treated with 1.2 μM doxorubicin; 0, 10 or 100 μM levistolide A; or with both drugs for 12 h. ROS were detected using the ROS Assay kit (cat. no. 50101ES01; Shanghai Yi San Biotechnology Co., Ltd., Shanghai, China), according to the manufacturer's protocol.

JC-1 mitochondrial membrane potential assay. The k562/dox cells (1×10^5 cells/ml) were seeded into 6-well plates. Once they reached 80% confluence, cells were treated with 1.2 μM doxorubicin; 0, 10 or 100 μM levistolide A; or with both drugs for 24 h. The mitochondrial membrane potential was analyzed using the JC-1 Mitochondrial Membrane Potential kit (cat. no. 40706ES60; Shanghai Yi San Biotechnology Co., Ltd.), according to the manufacturer's protocol.

Caspase 3 colorimetric assay. The k562/dox cells (1×10^5 cells/ml) were seeded into 6-well plates. Once they reached 80% confluence, cells were treated with 1.2 μM doxorubicin; 0, 50 or 100 μM levistolide A; or with both drugs for 24 h. Caspase 3 levels were detected using the caspase 3 colorimetric assay kit (cat. no. BC3830; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China), according to the manufacturer's protocol.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RT-qPCR was used to evaluate MDR1 mRNA expression following treatment with levistolide A (0, 0.01, 0.1, 1, 10 and 100 μM) for 24 h. Total RNA was extracted from k562/dox cells using the E.Z.N.A.® Total RNA kit I (Omega Bio-tek, Inc., Norcross, GA, USA), according to the manufacturer's protocol, and cDNA was synthesized using reverse transcriptase (PrimeScript™ RT reagent kit; cat. no. RR047A; Takara Bio, Inc., Otsu, Japan), according to the manufacturer's protocol. RT-qPCR (SYBR®-Green JumpStart™ *Taq* ReadyMix™; cat. no. S5193; Sigma-Aldrich; Merck KGaA) amplification was performed using a TP-810 Thermal Cycler system (Takara Bio, Inc.). The primer sequences were as follows: MDR1, forward 5'-GCA GCTGGAAGACAAATACACAAA-3', and reverse 5'-CCC CAACATCGT-GCACATC-3'; and GAPDH, forward 5'-TCT GCAGGAGACAAGACCTG-3' and reverse 5'-GACTTGAGG AGCTGGAGAGG-3'. The PCR protocol was as follows: One cycle of denaturation at 95°C for 2 min; 40 cycles of denaturation at 95°C for 15 sec, annealing at 60°C for 30 sec and extension at 72°C for 30 sec, followed by a final extension step at 4°C for 1 min. Relative quantification (ΔCq) (39) was used to determine relative mRNA expression levels, with GAPDH as the internal reference.

Western blot analysis. Following incubation with various concentrations of levistolide A, doxorubicin, or both drugs for 24 h; or incubation with various concentrations of levistolide A combined with MG132 (2 μM), k562/dox cells or k562 cells were washed twice with cold PBS and lysed with lysis buffer (Xi'an Hat Biotechnology Co., Ltd., Xi'an, China). Proteins were extracted using the total protein extraction kit (Xi'an Hat Biotechnology Co., Ltd.); the cells were treated with lysis buffer and proteins were extracted from the lysates using the protein extraction kit. Lysates were centrifuged at 3,600 x g for 20 min at 4°C. The bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc.) was used to quantify protein concentrations. Proteins were denatured by mixing with an equal volume of loading buffer (100 μl loading buffer and 4 μl mercaptoethanol) and boiling at 100°C for 5 min. An aliquot (30 μg protein) of the supernatants was run on a 10% SDS polyacrylamide gel. Proteins were then transferred onto polyvinylidene fluoride membranes, which were blocked with 5% non-fat milk in Tris-buffered saline plus 0.1% Tween-20 for 2 h at 25°C. Proteins were detected using the following antibodies at 4°C for 24 h: Anti-GAPDH (1:1,000), anti-MDR1 (1:1,000), anti-Bcl-2 (1:1,000), anti-Bax (1:1,000) and anti-BCRP (1:1,000). The blots were washed and were then incubated with their respective secondary antibodies (1:20,000; cat. no. ANM02-4; Zhuangzhi Biotechnology Co., Ltd., Xi'an, China) at 25°C for 2 h. Proteins were visualized using an
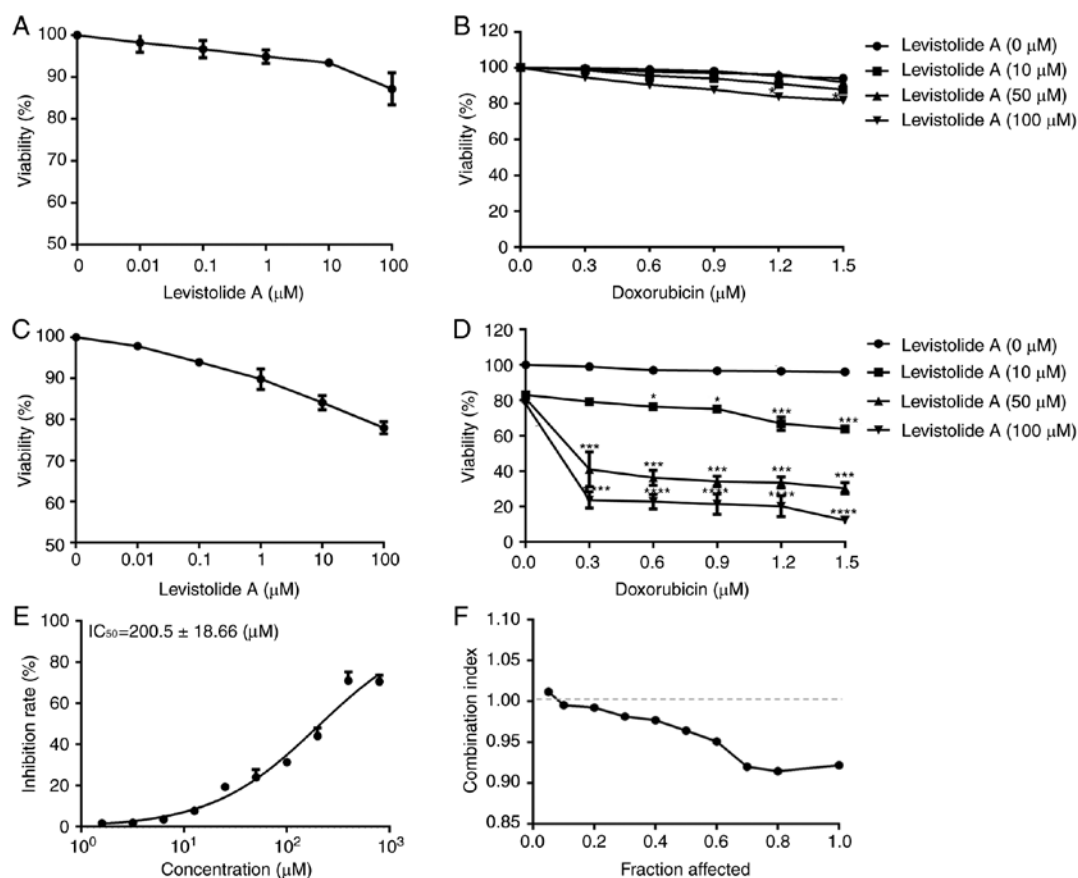
Figure 2. Effects of levistolide A on k562 and k562/dox cell viability. (A) Following treatment with various concentrations of levistolide A for 48 h, the viability of k562 cells was evaluated with MTT assays. (B) After k562 cells were treated with 0, 0.3, 0.6, 0.9, 1.2 or 1.5 μM doxorubicin together with 0, 10, 50 or 100 μM levistolide A for 48 h, cell viability was determined with MTT assays. (C) Following treatment with various concentrations of levistolide A in for 48 h, the viability of k562/dox cells was evaluated with MTT assays. (D) After k562/dox cells were treated with 0, 0.3, 0.6, 0.9, 1.2 or 1.5 μM doxorubicin together with 0, 10, 50, or 100 μM levistolide A for 48 h, cell viability was determined with MTT assays. (E) Following treatment with various concentrations of levistolide A for 48 h, the IC$_{50}$ in k562/dox cells was evaluated with MTT assays. (F) Combination index of levistolide A combined with doxorubicin in k562/dox cells. One-way analysis of variance (Bonferroni's multiple comparisons test) was used to determine statistical significance. Data are presented as the means ± standard deviation, n=3. *P<0.05 vs. doxorubicin (0 μM); ***P<0.001 vs. doxorubicin (0 μM); ****P<0.0001 vs. doxorubicin (0 μM).

enhanced chemiluminescence detection reagent and GAPDH was used as a reference to estimate relative protein levels. ImageJ k1.45 (National Institutes of Health, Bethesda, MD, USA) was used to semi-quantify protein expression.

Statistical analysis. Data are expressed as the means ± standard deviation. Differences between control and experimental values were assessed using one-way analysis of variance followed by Bonferroni's multiple comparisons test (GraphPad 7.0 Software; GraphPad Software, Inc., La Jolla, CA, USA.). P<0.05 was considered to indicate a statistically significant difference.

Results

Antiproliferative effects of levistolide A on k562 and k562/dox cells. The effects of levistolide A and doxorubicin on k562 and k562/dox proliferation after 48 h were assessed using the MTT assay (Fig. 2A-D). Levistolide A exhibited a mild, dose-dependent inhibitory effect on the survival of k562 (Fig. 2A) and k562/dox (Fig. 2C) cells at 100 μM; however, cell viability was still >80% at this concentration. The half maximal inhibitory concentration (IC$_{50}$) of doxorubicin in k562 and k562/dox cells was 2.86±0.64 and 102.56±2.89 μM,

Table I. Determination of multidrug resistance according to the sensitivity of k562 and k562/dox cells to doxorubicin.

Treatment	IC$_{50}$ (μM)		Fold increase in resistance
	k562	k562/dox	
Doxorubicin	2.86±0.64	102.56±2.89[a]	35.86

[a]P<0.05 vs. k562 cells. Two-tailed unpaired Student's t-test was used to determine statistical significance. Values are presented as the means ± standard deviation of triplicate experiments. IC$_{50}$, half maximal inhibitory concentration.

respectively (Table I). The IC$_{50}$ of levistolide A in k562/dox cells was 200.5±18.66 μM (Fig. 2E). Subsequently, the present study examined whether levistolide A modified the sensitivity of k562 and k562/dox cells to doxorubicin. Co-incubation with ≥10 μM levistolide A markedly increased the response of k562/dox cells to doxorubicin (Fig. 2D). Conversely, the synergistic effect of levistolide A plus doxorubicin was absent in k562 cells (Fig. 2B). The interaction between levistolide A and
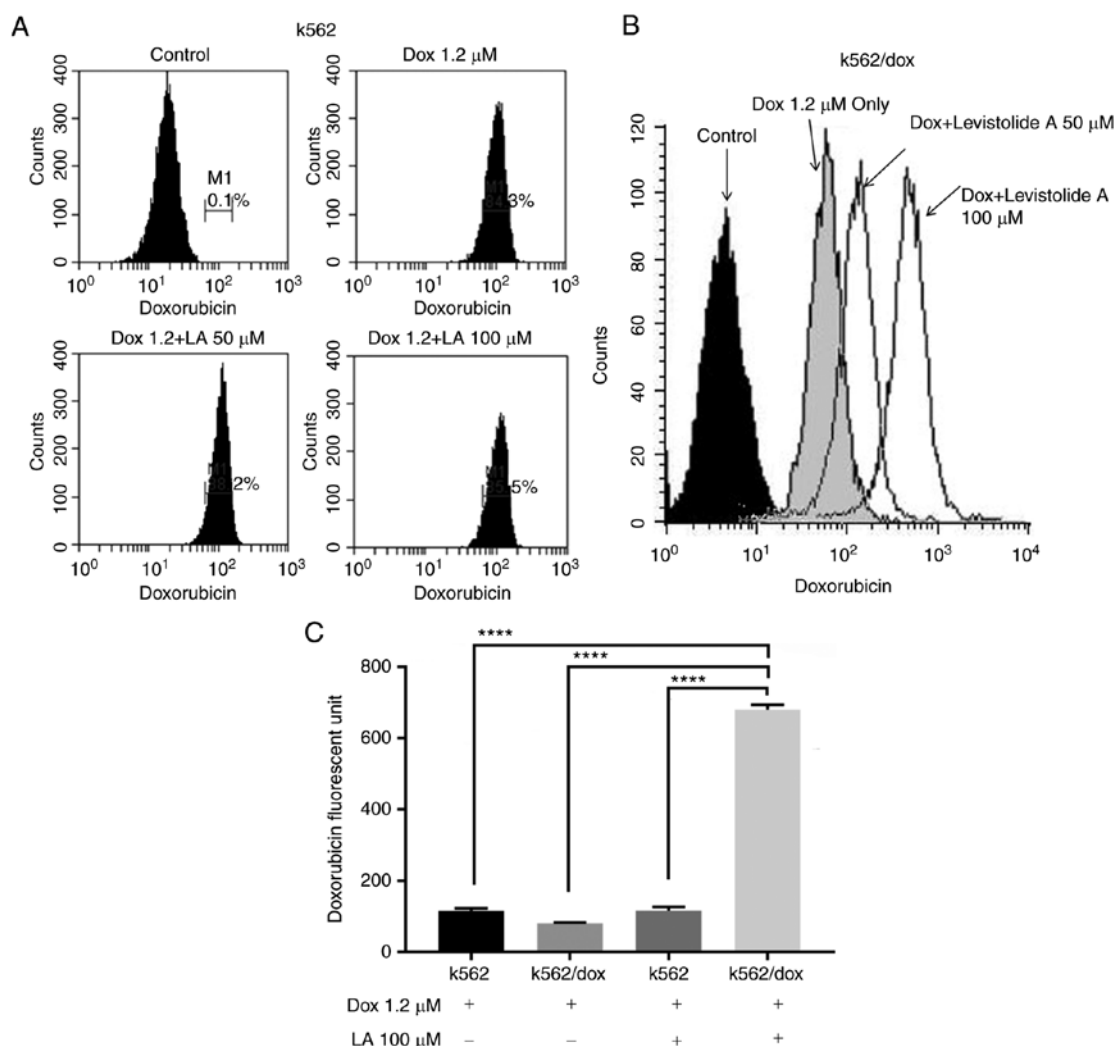
Figure 3. Levistolide A enhances doxorubicin accumulation in k562/dox cells. (A) Cellular doxorubicin accumulation levels were measured in k562 cells using flow cytometry. The cells were incubated with 1.2 μM doxorubicin alone or together with 50 or 100 μM levistolide A for 24 h. (B) Cellular doxorubicin accumulation levels of k562/dox cells were measured using flow cytometry. The cells were incubated with 1.2 μM doxorubicin, alone or together with 50 or 100 μM levistolide A for 24 h. (C) Percentage of doxorubicin accumulation. One-way analysis of variance (Bonferroni's multiple comparisons test) was used to determine statistical significance. Data are presented as the means $\pm$ standard deviation, n=3. ****P<0.01 vs. K562/dox cells.

doxorubicin was analyzed using the CI method, as shown in Fig. 2F. In k562/dox cells, levistolide A and doxorubicin exhibited a synergistic effect when the doxorubicin concentration was >0.3 μM, the levistolide A concentration was >10 μM, and the combined inhibition was >5% (Fig. 2D). Conversely, treatment of k562/dox (Fig. 2A) and k562 (Fig. 2C) cells with each of these two compounds individually produced lower antiproliferative effects. These results indicated that levistolide A could increase the antiproliferative effects of doxorubicin in k562/dox cells (Fig. 2D), but not in k562 cells (Fig. 2B).

Levistolide A promotes doxorubicin-induced apoptosis by increasing its intracellular concentration. The present study conducted fluorescence-activated cell sorting analyses to investigate whether levistolide A increased the sensitivity of k562/dox cells to doxorubicin by increasing its intracellular accumulation (Fig. 3). The experiments revealed that the fluorescence intensity of intracellular doxorubicin was increased in a dose-dependent manner in k562/dox cells, indicating that levistolide A promoted the intracellular accumulation of

doxorubicin (Fig. 3B and C). Conversely, levistolide A had little effect on the intracellular accumulation of doxorubicin in k562 cells (Fig. 3A and C). In addition, levistolide A increased the doxorubicin effective concentration in k562/dox cells (Fig. 2D). Therefore, combined treatment of k562/dox cells with levistolide A and doxorubicin significantly increased their response to doxorubicin.

K562/dox cells were treated with various concentrations of levistolide A alone or combined with 1.2 μM doxorubicin, and apoptosis was analyzed by flow cytometry (Fig. 4). The percentage of apoptotic cells was significantly increased in the combined treatment group (Fig. 4B). The results revealed that levistolide A combined with doxorubicin increased the percentage of apoptotic cells in a dose-dependent manner (Fig. 4).

The present study also investigated whether increased expression of ROS could explain the synergistic effects of levistolide A and doxorubicin (Fig. 5). The k562/dox cells were treated with 0, 10 or 100 μM levistolide A, either alone or combined with 1.2 μM doxorubicin for 12 h.
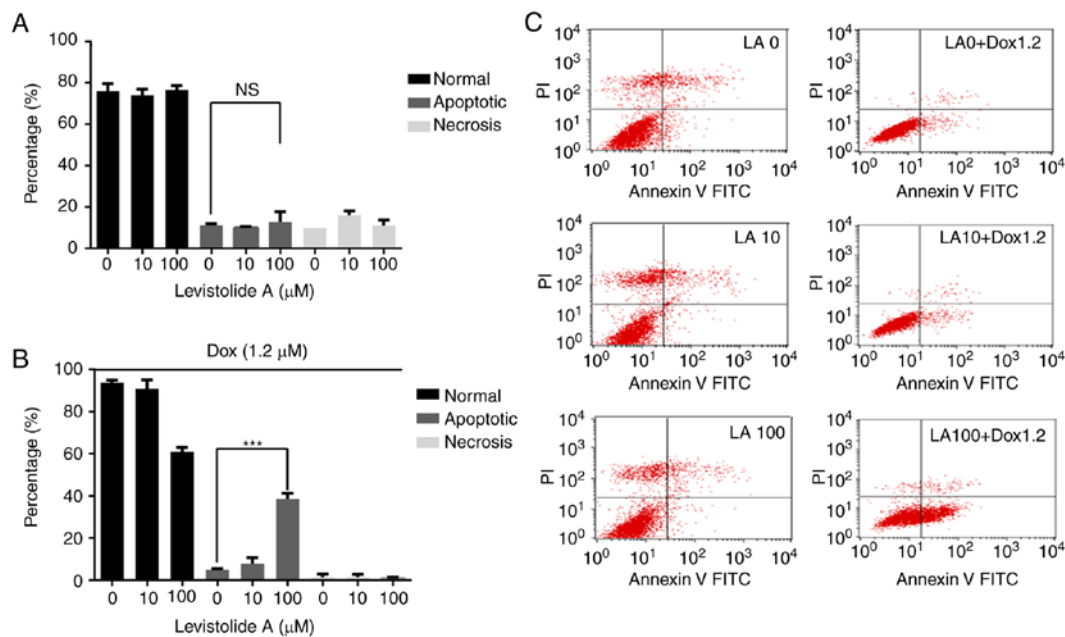
Figure 4. Levistolide A enhances k562/dox cell sensitivity to doxorubicin treatment. (A) The k562/dox cells were incubated with the indicated concentrations of levistolide A (0, 10 and 100 μM) for 24 h. Apoptosis was determined using Annexin V/PI double staining. (B) The k562/dox cells were incubated with the indicated concentrations of levistolide A (0, 10 and 100 μM) combined with 1.2 μM doxorubicin for 24 h. Apoptosis was determined using Annexin V/PI double staining. (C) Flow cytometry plots of apoptotic cells are presented. One-way analysis of variance (Bonferroni's multiple comparisons test) was used to determine statistical significance. Data are presented as the means ± standard deviation, n=3. ***P<0.001 vs. apoptotic levistolide A (0 μM)-treated cells. FITC, fluorescein isothiocyanate; PI, propidium iodide.
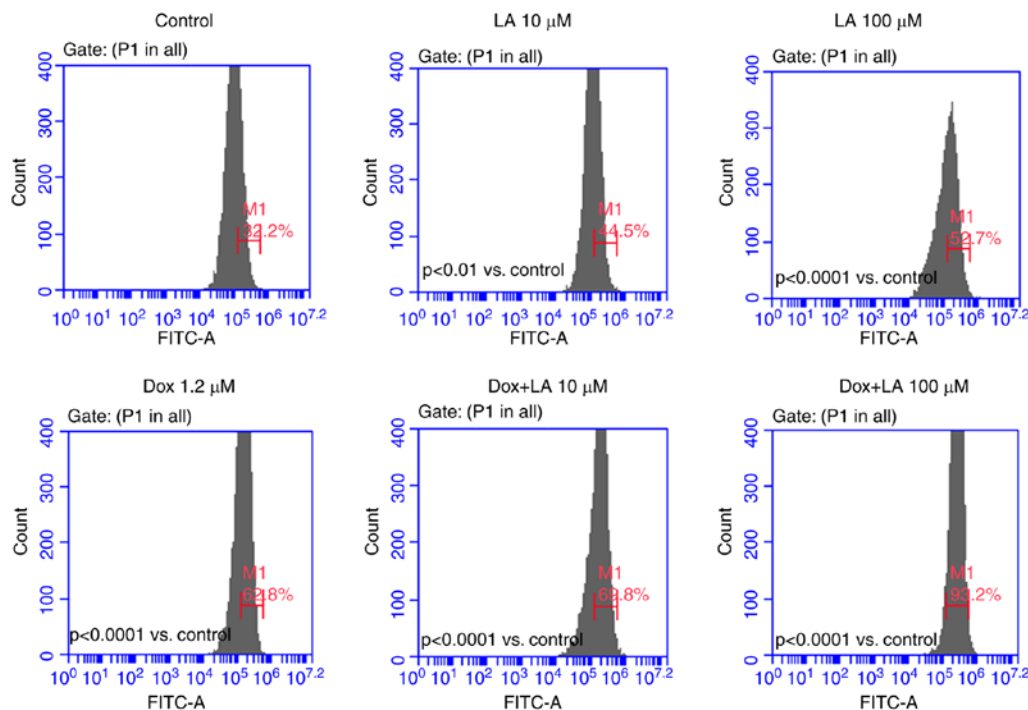

Figure 5. Levistolide A enhances doxorubicin-induced k562/dox cell apoptosis by increasing the levels of ROS. ROS levels in k562/dox cells were detected using the ROS Assay kit. Cells were treated with various concentrations of levistolide A (0, 50 and 100 μM) alone or combined with 1.2 μM doxorubicin for 12 h. One-way analysis of variance (Bonferroni's multiple comparisons test) was used to determine statistical significance. Data are presented as the means ± standard deviation, n=3. FITC, fluorescein isothiocyanate; ROS, reactive oxygen species.

The results revealed that levistolide A dose-dependently promoted doxorubicin-induced apoptosis of k562/dox cells via increasing ROS levels. Furthermore, analysis of the mitochondrial potential using JC-1 staining indicated that

levistolide A synergistically enhanced doxorubicin-induced cell death (Fig. 6). The k562/dox cells were treated with 0, 10 or 100 μM levistolide A, either alone or combined with 1.2 μM doxorubicin for 24 h. The present study revealed that
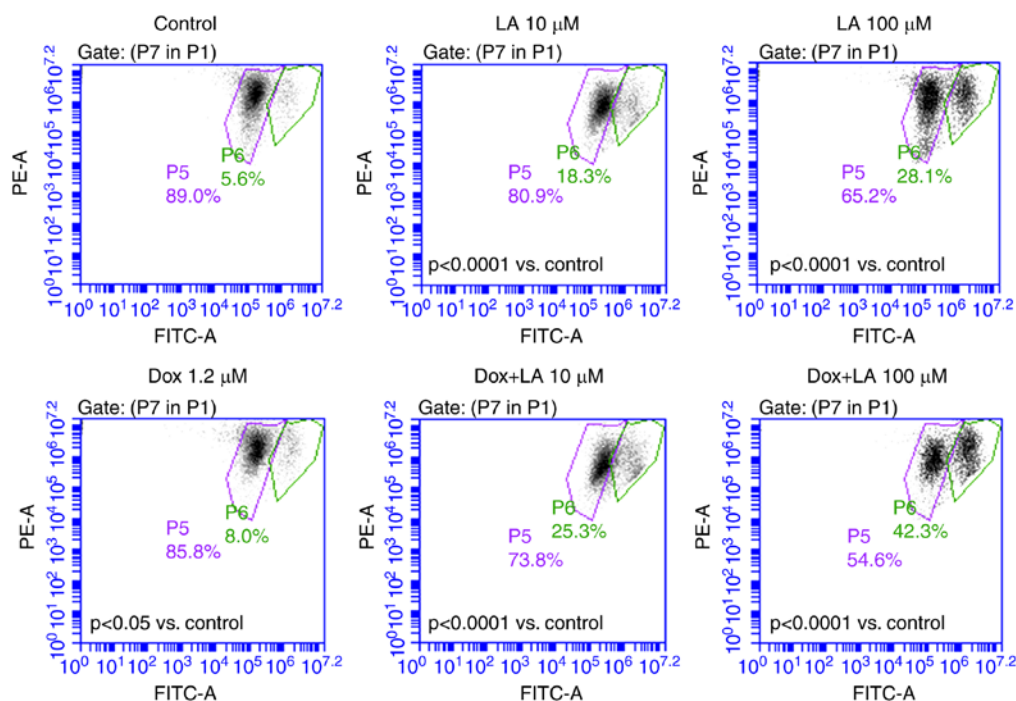
Figure 6. Levistolide A enhances doxorubicin-induced k562/dox cell apoptosis by disrupting the mitochondrial system. The levels of JC-1 in k562/dox cells were determined using the JC-1 Mitochondrial Membrane Potential kit. Cells were treated with various concentrations of levistolide A (0, 50 and 100 μM) alone or combined with 1.2 μM doxorubicin for 24 h. One-way analysis of variance (Bonferroni's multiple comparisons test) was used to determine statistical significance. Data are presented as the means ± standard deviation, n=3.
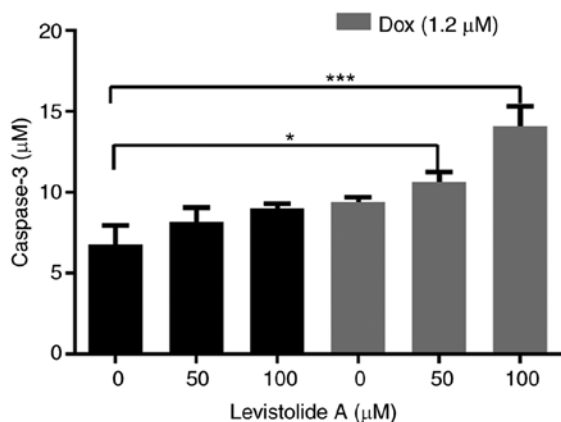

Figure 7. Levistolide A enhances doxorubicin-induced k562/dox cell apoptosis by increasing the levels of caspase 3. The levels of caspase 3 in k562/dox cells were determined using a caspase 3 colorimetric assay kit. Cells were treated with various concentrations of levistolide A (0, 50 and 100 μM) alone or combined with 1.2 μM doxorubicin for 24 h. One-way analysis of variance (Bonferroni's multiple comparisons test) was used to determine statistical significance. Data are presented as the means ± standard deviation, n=3. *P<0.05, ***P<0.001 vs. levistolide A (0 μM)-treated cells.

levistolide A dose-dependently promoted doxorubicin-induced apoptosis of k562/dox cells by reducing mitochondrial membrane potential. In addition, increased expression levels of caspase 3 were detected in the combined treatment group (Fig. 7). Western blotting was also performed to analyze the expression levels of apoptotic markers, including Bax and Bcl-2, in k562/dox cells treated with levistolide A alone or combined with doxorubicin for 24 h (Fig. 8A). Levistolide A alone (50 and 100 μM) induced a slight dose-dependent

decrease in Bcl-2 (Fig. 8Ac) compared with in control, untreated cells. However, levistolide A (50 and 100 μM) combined with doxorubicin (1.2 μM) resulted in a significant (P<0.001) dose-dependent reduction in Bcl-2 expression (Fig. 8Ac), when compared with untreated, control cells. Treatment of k562 cells with levistolide A alone or in combination with doxorubicin for 24 h revealed that levistolide A alone induced a slight dose-dependent decrease in Bcl-2 when compared with control, untreated cells; levistolide A (50 and 100 μM) combined with doxorubicin (1.2 μM) resulted in a similar downregulation in Bcl-2 (Fig. 8Bc), thus indicating that levistolide A does not enhance doxorubicin-induced apoptosis of k562 cells. On the basis of these results, it may be suggested that levistolide A markedly enhanced the doxorubicin-induced mitochondrial apoptotic cascade in k562/dox cells.

Levistolide A downregulates MDR1 expression through the UPP. MDR1 has been reported to be overexpressed in k562/dox cells (40) and absent in k562 cells; this finding was confirmed in the present study (Fig. 9A and B). Notably, levistolide A could downregulate MDR1 expression in k562/dox cells (Fig. 9A and B) without affecting MDR1 mRNA expression (Fig. 9C), thus suggesting that it acted via a post-translational pathway. Therefore, k562/dox cells were treated with MG132, a potent and cell-permeable proteasome inhibitor, at a low, non-toxic concentration (2 μM); the results revealed that MG132 exhibited a dose-dependent inhibitory effect on the survival of k562/dox cells (Fig. 10A). The results indicated that MG132 may attenuate levistolide A-induced downregulation (Fig. 9A) of MDR1 protein levels (Fig. 10B). Based on these results, it may be hypothesized that levistolide A induces MDR1 degradation via the UPP.
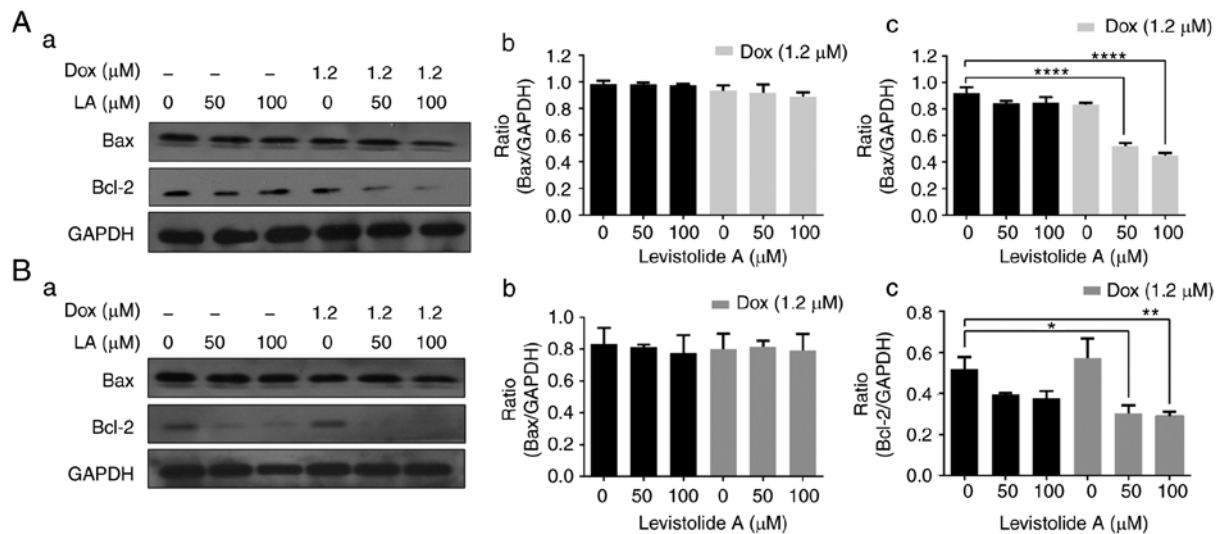
Figure 8. Levistolide A enhances doxorubicin-induced k562/dox cell apoptosis by decreasing the levels of Bcl-2. (Aa) Protein expression levels of Bcl-2 and Bax were detected in k562/dox cells by western blotting. Cells were treated with various concentrations of levistolide A (0, 50 and 100 μM) alone or combined with 1.2 μM doxorubicin for 24 h. (Ab) Bax protein expression in k562/dox cells was semi-quantified by densitometric analysis, and was normalized to GAPDH protein. (Ac) Bcl-2 protein expression in k562/dox cells was semi-quantified by densitometric analysis, and was normalized to GAPDH protein. (Ba) Protein expression levels of Bcl-2 and Bax were detected in k562 cells by western blotting. Cells were treated with various concentrations of levistolide A (0, 50 and 100 μM) alone or combined with 1.2 μM doxorubicin for 24 h. (Bb) Bax protein expression in k562 cells was semi-quantified by densitometric analysis, and was normalized to GAPDH protein. (Bc) Bcl-2 protein expression in k562 cells was semi-quantified by densitometric analysis, and was normalized to GAPDH protein. One-way analysis of variance (Bonferroni's multiple comparisons test) was used to determine statistical significance. Data are presented as the means ± standard deviation, n=3. *P<0.05, **P<0.01, ****P<0.0001 vs. levistolide A (0 μM)-treated cells. Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2.
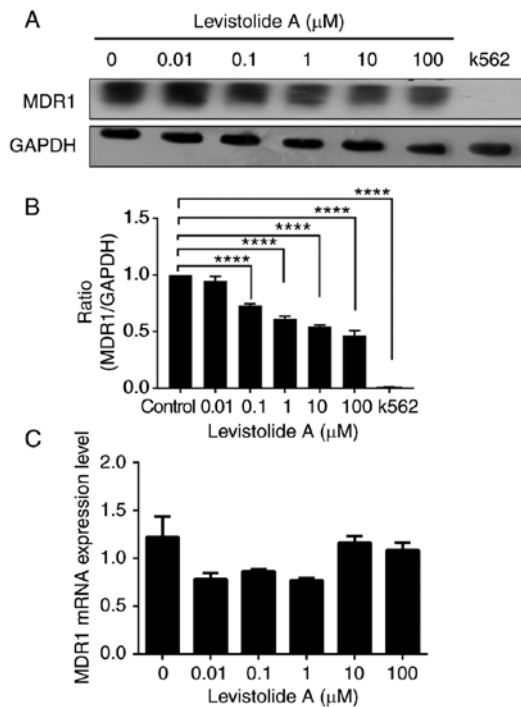

Figure 9. Levistolide A inhibits the protein expression levels of MDR1 in k562/dox cells. (A) Protein expression levels of MDR1 in k562/dox and k562 cells, as determined by western blotting. Cells were treated with various concentrations of levistolide A (0, 0.01, 0.1, 1, 10 or 100 μM) for 24 h. (B) MDR1 expression was semi-quantified by densitometric analysis, and was normalized to GAPDH protein. (C) mRNA expression levels of MDR1 in k562/dox cells, as determined by reverse transcription-quantitative polymerase chain reaction. Cells were treated with levistolide A for 24 h. The mRNA expression levels of MDR1 were normalized to GAPDH. One-way analysis of variance (Bonferroni's multiple comparisons test) was used to determine statistical significance. Data are presented as the means ± standard deviation of three independent experiments. ****P<0.0001 vs. control cells. MDR, multidrug resistance protein 1.
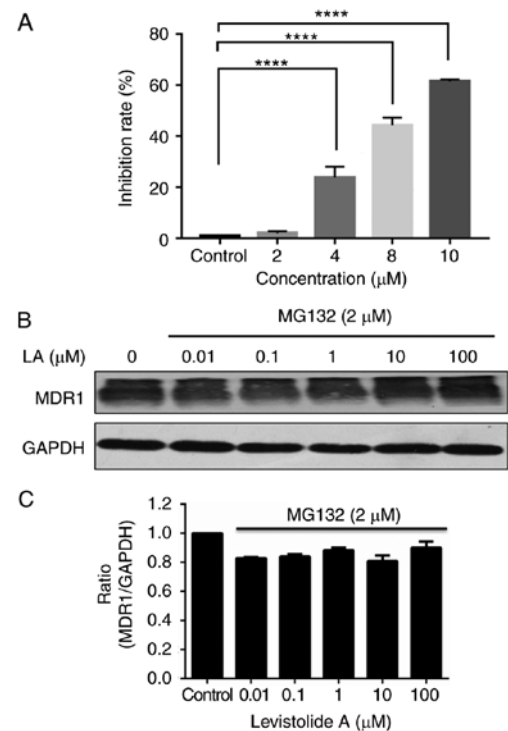
Figure 10. Levistolide A inhibits the protein expression levels of MDR1 in k562/dox cells via the ubiquitin-proteasome pathway. (A) MG132 exhibited a dose-dependent inhibitory effect on the survival of k562/dox cells; the k562/dox cells were incubated with 0, 2, 4, 8 and 10 μM MG132 for 24 h. (B) The k562/dox cells were incubated with 2 μM MG132 together with 0, 0.01, 0.1, 1, 10 or 100 μM levistolide A for 24 h, and MDR1 protein expression was detected by western blotting. (C) MDR1 protein expression in k562/dox cells was semi-quantified by densitometric analysis, and was normalized to GAPDH protein. One-way analysis of variance (Bonferroni's multiple comparisons test) was used to determine statistical significance. Data are presented as the means ± standard deviation of three independent experiments. ****P<0.0001 vs. control cells. MDR, multidrug resistance protein 1.
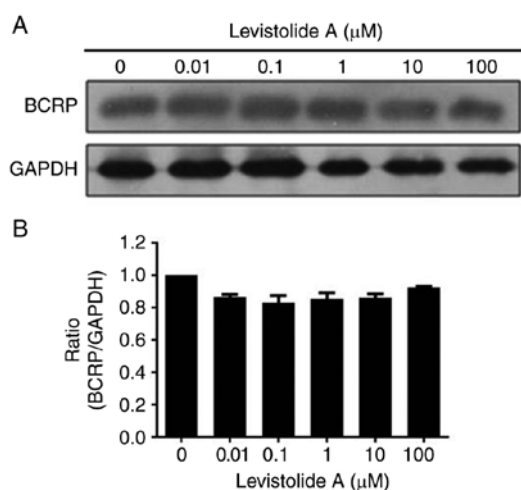
A

Figure 11. BCRP expression in k562/dox cells. (A) Protein expression levels of BCRP in k562/dox cells, as determined by western blotting. Cells were treated with various concentrations of levistolide A (0, 0.01, 0.1, 1, 10 or 100 μM) for 24 h. (B) BCRP protein expression in k562/dox cells was semi-quantified by densitometric analysis, and was normalized to GAPDH protein. One-way analysis of variance (Bonferroni's multiple comparisons test) was used to determine statistical significance. Data are presented as the means ± standard deviation of three independent experiments. BCRP, breast cancer resistance protein.

Western blot analysis revealed that BCRP protein expression was not significantly altered following treatment with levistolide A (Fig. 11). Therefore, it may be hypothesized that the effects of levistolide A on k562/dox cells specifically involves attenuation of MDR via the UPP, rather than BCRP.

Discussion

Levistolide A is a natural compound extracted from the rhizome of *Angelicae sinensis* (Oliv.). According to Traditional Chinese Medicine, the roots of *Angelicae sinensis* are used to tonify circulation and improve blood stasis. It has also been used clinically for the treatment of gynecological symptoms, anemia, chronic bronchitis, asthma, rheumatism and other diseases (33). *Angelicae* is often included in vegetable-rich diets due to its beneficial chemopreventive, antitumor, antioxidant and antimutagenic effects (41).

The IC$_{50}$ of levistolide A in k562/dox cells was 200.5±18.66 μM, whereas the IC$_{50}$ of doxorubicin in k562 and k562/dox cells was 2.86±0.64 and 102.56±2.89 μM, respectively. Individually, and at low concentrations ($\leq$100 μM), levistolide A and doxorubicin exhibited little effects on k562 and k562/dox cell proliferation and apoptosis. However, levistolide A increased the cytotoxic effects of doxorubicin against k562/dox cells compared with its effect on k562 cells. In addition, levistolide A promoted the intracellular accumulation of doxorubicin in k562/dox cells when compared with k562 cells, thus suggesting that the MDR1 pump was inhibited by levistolide A. Compared with in k562/dox cells, k562 cells exhibited lower sensitivity to the combination of levistolide A and doxorubicin. The process of apoptosis is closely regulated by a series of molecules associated with the Bcl-2 family, which are particularly linked to mitochondrial pathways (24).

The present study revealed that levistolide A promoted doxorubicin-induced apoptosis of k562/dox cells via increased ROS, reduced mitochondrial membrane potential, decreased Bcl-2 levels and increased caspase 3 expression. Treatment with levistolide A alone had only a mild effect on ROS, JC-1 mitochondrial membrane potential, Bcl-2 and caspase 3 levels when compared with control, untreated cells. The combination of the two drugs had little effect on Bax, thus suggesting that apoptosis was Bax-independent. In k562 cells, levistolide A alone or combined with doxorubicin induced a significant dose-dependent decrease in Bcl-2 expression, indicating that levistolide A induced apoptosis of k562 cells, whereas the pro-apoptotic effect of doxorubicin was weaker in k562/dox cells. These findings indicated that levistolide A promoted doxorubicin-induced cell apoptosis via mitochondrial pathways and by increasing the intracellular concentration of doxorubicin.

MDR1 is a member of the ABC transporter superfamily. Various ABC transporters, such as MDR1 and BCRP, have overlapping substrates (42). MDR1 has been reported to be overexpressed in k562/dox cells (40), and this was confirmed in the present study. Notably, this study revealed that levistolide A effectively suppressed MDR1 protein expression; however, MDR1 mRNA expression was not significantly altered in cells treated with levistolide A. Therefore, it may be hypothesized that the decrease in MDR1 levels is a result of post-transcriptional regulation. The majority of eukaryotic protein degradation is accomplished by the UPP, and MG132 is an effective and reversible aldehyde peptide-specific proteasome inhibitor. Therefore, this study used MG132 to validate the hypothesis that levistolide A downregulates the expression of MDR1 through the UPP pathway. It was revealed that MG132 (2 μM) could reverse the levistolide A-induced reduction in MDR1 expression, thus indicating that levistolide A may negatively regulate MDR1 expression via the UPP. In addition, the protein expression levels of BCRP were not significantly altered following treatment with levistolide A. Therefore, the effects of levistolide A may be mediated by downregulation of MDR1 expression, and not BCRP.

Fei *et al* reported that levistolide A modulates MDR1 function and can overcome MDR1-mediated MDR in Bcap37/MDR1 cells. In addition, levistolide A inhibits 3,3'-diethyloxacarbocyanine iodide efflux from Bcap37/MDR1 cells without affecting MDR1 expression (32). In the present study, the levistolide A-mediated reversal of doxorubicin resistance in k562/dox cells occurred via modulation of MDR1 expression.

In conclusion, the present findings suggested that levistolide A synergistically increased the cytotoxicity of doxorubicin and promoted doxorubicin-induced apoptosis of k562/dox cells. This effect involved the apoptotic cascade, which was regulated by the mitochondrial system, and downregulation of MDR1 expression. Therefore, levistolide A may be a promising drug candidate for the treatment of doxorubicin-resistant cancer by targeting MDR1.

Funding

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

YD performed the research, analyzed the data and wrote the paper. WN and TZ performed the research and wrote the paper. JW performed statistical analysis. JC, HC and RW performed cell culture. HA designed the study, performed the research, analyzed the data and wrote the paper. All authors read and approved the final manuscript.

Ethical approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Wang YL: Tiny mutation triggers drug resistance for patients with one type of leukemia. The University of Chicago Medicine Communications. http://www.uchospitals.edu/news/2014/20140528-leukemia.html.
2. Chai S, To KK and Lin G: Circumvention of mult-drug resistance of cancer cells by Chinese herbal medicines. Chin Med 5: 26, 2010.
3. Goler-Baron V and Assaraf YG: Structure and function of ABCG2-rich extracellular vesicles mediating multidrug resistance. PLoS One 6: e16007, 2011.
4. Goler-Baron V, Sladkevich I and Assaraf YG: Inhibition of the PI3K-Akt signaling pathway disrupts ABCG2-rich extracellular vesicles and overcomes multidrug resistance in breast cancer cells. Biochem Pharmacol 83: 1340-1348, 2012.
5. Gonen N and Assaraf YG: Antifolates in cancer therapy: Structure, activity and mechanisms of drug resistance. Drug Resist Updat 15: 183-210, 2012.
6. Ifergan I, Scheffer GL and Assaraf YG: Novel extracellular vesicles mediate an ABCG2-dependent anticancer drug sequestration and resistance. Cancer Res 65: 10952-10958, 2005.
7. Lebedeva IV, Goler-Baron V and Assaraf YG: Overcoming multidrug resistance via photo destruction of ABCG2-rich extracellular vesicles sequestering photosensitive chemotherapeutics. PLoS One 7: e35487, 2012.
8. Livney YD and Assaraf YG: Rationally designed nanovehicles to overcome cancer chemoresistance. Adv Drug Deliv Rev 65: 1716-1730, 2013.
9. Raz S, Sheban D, Gonen N, Stark M, Berman B and Assaraf YG: Severe hypoxia induces complete antifolate resistance in carcinoma cells due to cell cycle arrest. Cell Death Dis 5: e1067, 2014.
10. Zhitomirsky B and Assaraf YG: Lysosomal sequestration of hydrophobic weak base chemotherapeutics triggers lysosomal biogenesis and lysosome-dependent cancer multidrug resistance. Oncotarget 6: 1143-1156, 2015.
11. Zhitomirsky B and Assaraf YG: Lysosomes as mediators of drug resistance in cancer. Drug Resist Updat 24: 23-33, 2016.
12. Zhou X, Li D, Wang X, Zhang B, Zhu H and Zhao J: Galectin-1 is overexpressed CD133+ human lung adenocarcinoma cells and promotes their growth and invasiveness. Oncotarget 6: 3111-3122, 2015.
13. Genovese I, Ilari A, Assaraf YG, Fazi F and Colotti G: Not only P-glycoprotein: Amplification of the ABCB1-containing chromosome region 7q21 confers multidrug resistance upon cancer cells by coordinated overexpression of an assortment of resistance related proteins. Drug Resist Updat 32: 23-46, 2017.
14. Glavinas H, Krajcsi P, Cserepes J and Sarkadi B: The role of ABC transporters in drug resistance, metabolism and toxicity. Curr Drug Deliv 1: 27-42, 2004.
15. Varma MV, Ashokraj Y, Dey CS and Panchagnula R: P-glycoprotein inhibitors and their screening: A perspective from bioavailability enhancement. Pharmacol Res 48: 347-359, 2003.
16. Gottesman MM, Fojo T and Bates SE: Multidrug resistance in cancer: Role of ATP-dependent transporters. Nat Rev Cancer 2: 48-58, 2002.
17. Hipfner DR, Deeley RG and Cole SP: Structural, mechanistic and clinical aspects of MRP1. Biochim Biophys Acta 146: 1359-1376, 1999.
18. Ozvegy C, Litman T, Szakács G, Nagy Z, Bates S, Váradi A and Sarkadi B: Functional characterization of the human multidrug transporter, ABCG2, expressed in insect cells. Biochem Biophys Res Commun 285: 111-117, 2001.
19. Roy S, Kenny E, Kennedy S, Larkin A, Ballot J, Perez De Villarreal M, Grown J and O'Driscoll L: MDR1/P-glycoprotein and MRP-1mRNA and protein expression in non-small cell lung cancer. Anticancer Res 27: 1325-1330, 2007.
20. Sparrebppm A, Danesi R, Ando Y, Chan J and Figg WD: Phamacogenomics of ABC transporters and its role in cancer chemotherapy. Drug Resist Updat 6: 71-84, 2003.
21. Sanchez C, Mendoza P, Contreras HR, Vergara J, McCubrey JA, Huidobro C and Castellón EA: Expression of multidrug resistance proteins in prostate cancer is related with cell sensitivity to chemotherapeutic drugs. Prostate 69: 1448-1459, 2009.
22. Hengartner MO: The biochemistry of apoptosis. Nature 407: 770-776, 2000.
23. Igney FH and Krammer PH: Death and anti-death: Tumour resistance to apoptosis. Nat Rev Cancer 2: 277-288, 2002.
24. Shore GC and Nguyen M: Bcl-2 proteins and apoptosis: Choose your partner. Cell 135: 1004-1006, 2008.
25. Glickman MH and Ciechanover A: The ubiquitin-proteasome protcolytic pathway: Destruction for the sake of construction. Physiol Rev 82: 373-428, 2002.
26. Lee CS, Han ES, Ha YS and Bang H: Differential effect of calmodulin antagonists on MG132-induced mitochondrial dysfunction and cell death in PC12 cells. Brain Res Bull 67: 225-234, 2005.
27. Bang JH, Han ES, Lim L and Lee CS: Differential response of MG132 cytotoxicity against small cell lung cancer cells to change in cellular GSH contents. Biochem Pharmacol 68: 659-666, 2004.
28. Yan XB, Yang DS, Gao X, Feng J, Shi ZL and Ye Z: Caspase-8 dependent osteosarcoma cell apoptosis induced by proteasome inhibitor MG132. Cell Biol Int 31: 1136-1143, 2007.
29. Chung SY, Sung MK, Kim NH, Jang JO, Go EJ and Lee HJ: Inhibition of Pglycoprotein by natural products in human breast cancer cells. Arch Pharm Res 28: 823-828, 2005.
30. Mothana RA, Lindequist U, Gruenert R and Bednarski PJ: Studies of the in vitro anticancer, antimicrobial and antioxidant potentials of selected Yemeni medicinal plants from the island Soqotra. BMC Complement Altern Med 9: 7, 2009.
31. Patanasethanont D, Nagai J, Matsuura C, Fukui K, Sutthanut K, Sripanidkulchai BO, Yumoto R and Takano M: Modulation of function of multidrug resistance associated-proteins by Kaempferia parviflora extracts and their components. Eur J Pharmacol 566: 67-74, 2007.
32. Chen F, Wang T, Wang J, Wang ZQ and Qian M: Levistolide A overcomes P-glycoprotein-mediated drug resistance in human breast carcinoma cells. Acta Pharmacol Sin 29: 458-464, 2008.
33. Tang JC, Zhang JN, Wu YT and Li ZX: Effect of the water extract and ethanol extract from traditional Chinese medicines Angelicae sinensis (Oliv.) Diels, Ligusticum chuanxiong Hort. and Rheum palmatum L on rat liver cytochrome P450 activity. Phytother Res 20: 1046-1051, 2006.

34. Lian N, Liu JB and Yan QM: Effect of jiawei donggui buxue decoction on the survival time of mice with cancer. Chin J Clin Rehabil 8: 5736-5737, 2004.
35. Lian N, Liu YM and Liu Y: Effect on modified Danggui Buxue decoction to curative effect of tumor treated by chemotherapy. J Chengdu Univ TCM 26: 18-19, 2003. http://kns.cnki.net/KCMS/detail/detail.aspx?dbcode=CJFQ&dbname=CJFD2003&filename=CDZY200303006&v=MzI0MTNJOUZZb1I4ZVgxTHV4WVM3RGgxVDNxVHJXTTFGckNVUkxLZVp1ZG1GeURrVjc3SkppblJkN0c0SHRMTXI=.
36. Li BH and Lian N: Clinical observation on synergia and attenuation of Jiawei Danggui buxue decoction to radiotherapy and chemotherapy of tumor: Attachment report of 392 cases. J Cheng Univ TCM 28: 7-9, 2005. http://kns.cnki.net/KCMS/detail/detail.aspx?dbcode=CJFQ&dbname=CJFD2005&filename=CDZY200502002&v=MjE2MzZyV00xRnJDVVJMS2VadWRtRnlEbFZZMM0pKaW5SZDdHNEh0VE1yWTlGWm9SOGVYMUx1eFlTN0RoMVQzcVQ=.
37. Zhang T, Ding Y, An H, Feng L and Wang S: Screening anti-tumor compounds from *Ligusticum wallichii* using cell membrane chromatography combined with high performance liquid chromatography and mass spectrometry. J Sep Sci 38: 3247-3253, 2015.
38. Jin ZJ: Addition in drug combination (author's transl). Zhongguo Yao Li Xue Bao 1: 70-76, 1980 (In Chinese). https://www.ncbi.nlm.nih.gov/pubmed/6461187.
39. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. Methods 25: 402-408, 2001.
40. Zhu F, Wang Y, Zeng S, Fu X, Wang L and Cao J: Involvement of annexin A1 in multidrug resistance of K562/ADR cells identified by the proteomic study. OMICS 13: 467-476, 2009.
41. Chen D, Shi Y and Tian G: Chuanxiong and aspirin treatment of transient ischemic attack of clinical research. J Int Tra Chi West Med 12: 672-674, 1992.
42. Ling V, Kartner N, Sudo T, Siminovitch L and Riordan JR: Multidrug resistance phenotype in Chinese hamster ovary cells. Cancer Treat Rep 67: 869-874, 1983.