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Original Article

Triptolide reverses MCF-7/ADR cell resistance by down-regulating P-glycoprotein expression

Pu-Rong Zhang, Jian-Hui Zhang, Miao Yu, Xin Zhang

Department of Breast Surgery, Sichuan Cancer Hospital & Institute, Sichuan Cancer Center, School of Medicine, University of Electronic Science and Technology of China, Chengdu, China

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Abstract: Objective: To explore the reversal effects of triptolide (TP) on drug-resistance of adriamycin (Adriacin doxorubicin, ADR)-resistant human breast cancer cell lines (MCF-7/ADR) in vivo and in vitro. Methods: effects of triptolide and adriamycin on MCF-7 and MCF-7/ADR cell proliferation was measured by CCK-8, the reversal effect of triptolide on the drug-resistance of MCF-7/ADR cells was also measured by CCK-8; flow cytometer for testing the effect of triptolide and adriamycin on cell apoptosis was detected by flow cytometer; the expression level of P-glycoprotein was determined by Western blotting. Results: Different concentrations of adriamycin and triptolide at had inhibitory effects on the viability of MCF-7 and MCF-7/ADR cells respectively, with a concentration-dependent manner. To be specific, IC_{50} values of adriamycin on MCF-7 and MCF-7/ADR cells were 110.6±2.6 and 806.9±4.6 μg/ml, respectively, with an resistance index of 7.29. The IC_{50} values of triptolide on MCF-7 and MCF-7/ADR cells were 330.5±3.5 and 345.2±2.8 nmol/L, respectively, with a resistance index of 1.04. A combination of triptolide at non-toxic dosage levels with adriamycin reversed the drug resistance of MCF-7/ADR, with a reversal fold up to 3.65±0.21, induced cell apoptosis of MCF-7 and MCF-7/ADR, and could also significantly inhibit the growth of subcutaneous MCF-7/ADR tumor, as well as down-regulated the expression of P-glycoprotein in tumor cells *in vitro* and *in vivo*. Conclusions: Triptolide can significantly reverse the resistance of MCF-7/ADR cells, and P-glycoprotein is involved in regulating the drug-resistant effects of MCF-7/ADR cells.

Keywords: Drug resistance, P-glycoprotein, triptolide, drug-resistant human breast cancer cell

Introduction

Triptolide (TP), also known as tripterygium wilfordii lactone and tripterygium wilfordii lactone alcohol, is a diterpene lactone epoxide compound extracted from the root, leaf, flower and fruit of tripterygium wilfordii, a plant of *celastraceae* family. As an important active substance, triptolide has a special oxa-tricycle and an unsaturated five membered lactone ring in its structure [1, 2]. TP, which has potent pharmacological activities such as significant anti-inflammatory, anti-fertility and immune-modulatory effects, is widely used to treat autoimmune diseases, organ transplantation, etc. [3, 4]. In recent years, it was found that triptolide had certain anti-cancer effect. Wang Xiu *et al.* have revealed that triptolide induced apoptosis of nasopharyngeal carcinoma cells [5]; Ma Jia *et al.* have reported that triptolide could inhibit proliferation of liver cancer cells and induce apoptosis [6].

Drug resistance of tumorsrefers to the fact that desirable efficacy is detected in the initial stage of chemotherapy, but obviously decreased in the late stage of chemotherapy [7, 8]. The development of drug resistance is extremely unfavorable to the treatment of tumors. Therefore, it is an important subject in cancer research to reverse or overcome the drug resistance of tumor cells. In recent years, many domestic and foreign scholars have developed a large number of drug resistance reversal agents for tumors, but these reversal agents have many defects such as great side effects, unremarkable in-vivo reversal effects and high price and so on [9, 10]. Some researchers have found that the extract of traditional Chinese medicine can be used as a drug resistance reversal agent, and its characteristics such as high efficiency, low toxicity and multiple targets are very important for drug resistance reversal of chemotherapy [11]. Zhang Ying *et al.* have found out that astragalus polysaccharide for

injection has drug-resistance reversal effect on cisplatin-resistant human lung adenocarcinoma cells A549/DDP [12]; Tang Xiaoyong*et al.* have reported that peimine reverses the multidrug resistance of lung cancer A549/DDP cell lines [13].

In addition, triptolide was also shown to have certain reversal effect on the multidrug-resistance of some cancer cells. Wang Zhonghua *et al.* have found out that triptolide reverses the resistance of cisplatin-resistant lung cancer A549/DDP cells [14]. However, no reports at present indicate the drug-resistance reversal effect of triptolide on drug-resistant breast cancer cell lines or the *in vivo* reversal effect. In this study, adriamycin-resistant breast cancer cell lines were induced by limiting dilution method, then, the drug-resistance reversal effect of triptolide on MCF-7/ADR cell lines and related mechanism were investigated *in vivo* and in *vitro*.

Materials and methods

Reagents

Triptolide (article No.: 38748-32-2, purity: > 98% by HPLC, Nanjing Qingze Medical Technology Development Co., Ltd.); adriamycin (article No.: 25316-40-9, purity: > 99% by HPLC, Beijing Nuoqiya Biotech. Co., Ltd.); cell viability testing kit (CCK-8, Shanghai Sangon Biotech Co., Ltd.); double staining kit for flow cytometry (Annexin V-FITC/PI, Beijing Chuanggenshengtai Technology Co., Ltd.); anti-P-glycoprotein monoclonal antibody (Merck Millipore China); RPMI-1640 medium (HYCLONE, U.S.); fetal calf serum (HYCLONE, U.S.); phosphate buffer (HYCLONE, U.S.); all the reagents associated with Western blotting were domestic reagents.

Cell lines and incubation

Human breast cancer MCF-7 cells purchased from Shanghai Cell Bank were incubated in RPMI-1640 complete culture fluids (containing 10% calf serum, penicillin 100 U/ml and streptomycin 100 mg/L) under the conditions of 37°C, 5% CO_2 in a constant temperature and humidity incubator.

Induction of drug-resistant MCF-7/ADR cell lines

Adriamycin-resistant human breast cancer MCF-7/ADR cell lines were induced by limiting dilution method. MCF-7 cells in log phase of growth were collected and cultured in media containing 10 μg/ml adriamycin. After incubation for 6 h, the mixture was replaced by complete culture fluids, and then the mixture was incubated until the cells resumed growth, and cultured in 10 μg/ml adriamycin media. The above process was repeated, until the cells became resistant. Then, cells were incubated in 0.1 μg/ml adriamycin media. Incubation with drug was stopped 15-30 days prior to the experiment.

Cytotoxicity and resistance was measured by CCK-8 method

MCF-7 and MCF-7/ADR cell lines in log phase of growth were digested, and seeded into a 96-well plate (100 μl/well, 10^5 cells/ml) and then incubated for 24 h. Fresh media containing different concentrations of adriamycin (10-200 μg/ml) and triptolide (10-320 nmol/L) as well as a combination of adriamycin and triptolide were added separately, after which the incubation was continued for 24 h. The media were then replaced by fresh culture fluids containing 10% CCK-8 reagent and was incubated at 37°C for 30 min. Optical density values (OD values) of the cells were measured at 450 nm in a plate reader, and the negative control group contained cells and CCK-8 reagent, while the blank control group contained none but cells, with 4 duplicate wells/group. Drug medium lethal concentration (IC_{50}) was calculated by the software SPSS 17.0.

$$\text{Inhibition of cell viability} = \left[1 - \frac{\text{OD Test group} - \text{OD Blank control group}}{\text{OD Negative control group} - \text{OD Blank control group}}\right] \times 100\% \quad (1)$$

$$\text{Resistance index} = IC_{50}\ (\text{MCF-7/ADR}) / IC_{50}\ (\text{MCF-7}) \quad (2)$$

$$\text{Drug resisitance reverse fold} = \text{TPpre-treatment } IC_{50} / \text{TPpost-treatment } IC_{50} \quad (3)$$

Apoptosis was detected by flow cytometer

MCF-7 and MCF-7/ADR cell lines in log phase of growth were digested, and seeded into a 6-well plate (1 ml/well, 10^5 cells/ml) and then incubated for 24 h. Fresh media containing adriamycin and triptolide as well as a combination of adriamycin and triptolide (the dosage of triptolide was non-toxic) were added separately, after which the incubation was continued for 24 h. Then, the media were discarded. The cells

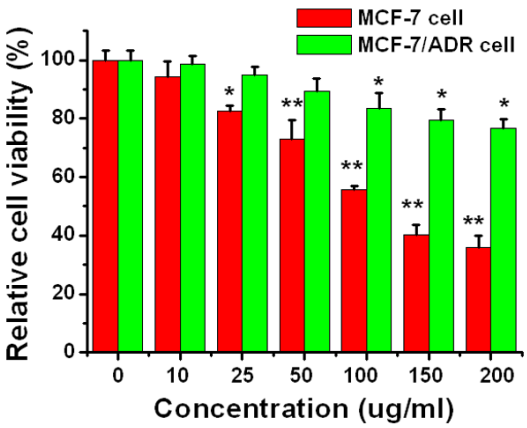

Figure 1. Adriamycin suppresses the viability of MCF-7 and MCF-7/ADR cells ($\bar{x} \pm s$, n=4). MCF-7 and MCF-7/ADR cell lines were digested, and seeded into a 96-well plate (100 μl/well, 10^5 cells/ml) and then incubated for 24 h. The incubation was continued for 24 h adding adriamycin at different concentrations.

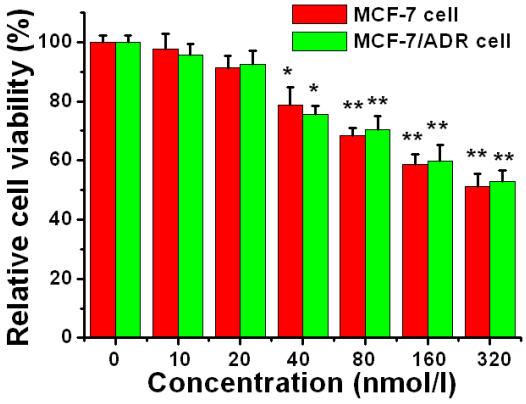

Figure 2. Triptolide inhibits the proliferation of MCF-7 and MCF-7/ADR ($\bar{x} \pm s$, n=4). MCF-7 and MCF-7/ADR cell lines were digested, and seeded into a 6-well plate (1 ml/well, 10^5 cells/ml) and then incubated for 24 h. The incubation was continued for 24 h adding adriamycin at different concentrations.

were digested, collected, and centrifuged at 1500 rpm for 3 min, and washed with PBS twice. Then 500 μl precooled cell resuspension were added, 5 μl FITC dye were added, the mixture was gently mixed and incubated at room temperature for 10 min in dark. Then 10 μl propidium iodide staining solution was added, the mixture was gently mixed and were allowed to interaction for 15 min in dark, then measured by flow cytometer.

Formation of subcutaneous mice tumor

BALB/c nude mice were female, 4-5 weeks old, and 18-22 g in weight. The mice were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. MCF-7 and MCF-7/ADR cell lines in log phase of growth were digested and counted, the number of cells was adjusted to 1×10^7/mL, and adjusted to single-cell suspension by PBS. The 150 μl single-cell suspension was seeded subcutaneously into right of the back of the healthy BALB/c mice. Subsequently, the subcutaneous injection sites were disinfected by ethanol, and then the animals were raised in animal rooms. 7-9 days later, growth status of subcutaneous mice tumor was observed, and the tumor was used in further experiment when their volumes reached 120 mm^3. Length and width of each tumor were measured by a vernier caliper, and the tumor volume was calculated according to the following equation:

$$\text{Tumor volume}=\text{tumor length}\times\text{tumor width}^2/2 \quad (4)$$

In vivo tumor treatment

MCF-7 and MCF-7/ADR tumor-bearing mice were divided into two groups randomly: blank control group (normal saline treatment group) and drug treatment group (adriamycin group, adriamycin + triptolide group), n=5. Normal saline and drug were injected intravenously via the tail vein of each mouse, and subcutaneous tumor volume and weight for each mouse were measured every 2 days. The relative tumor volume was calculated using the following equation:

$$V = \frac{V_1}{V_0} \quad (5)$$

In which, V represented relative tumor volume, V_1 the tumor volume after injection in mice, and V_0 the initial tumor volume before injection in mice.

The expression of P-glycoprotein in tumor cells was detected by western blotting

MCF-7 and MCF-7/ADR cells in log phase of growth were digested into single suspended cells (cell density: 1×10^5/ml), and seeded into a 25 ml flask, and incubated for 24 h, then different concentrations of drug were added into cells and treated for 24 h, with 4 bottles of cells per group. The cells were collected and the total proteins were extracted. The expression of P-glycoprotein in tumor tissue were measured as following: after tail intravenous injection of different drugs, the tumor-bearing mice were housed for 48 h, then the mice tumor were separated, cut into pieces with scissors, homoge-

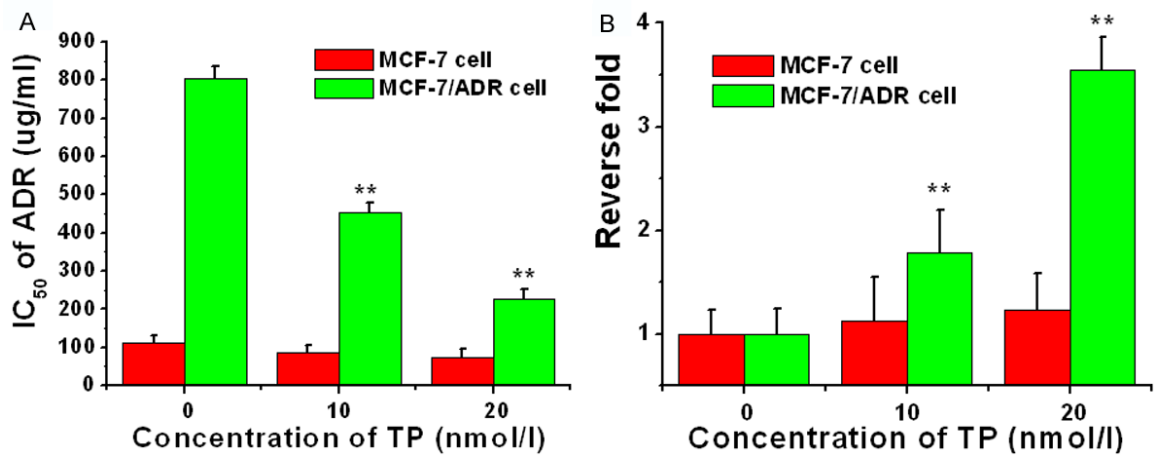

Figure 3. The drug resistance reversal effect of TP on MCF-7 and MCF-7/ADR cells ($\bar{x} \pm s$, n=4).

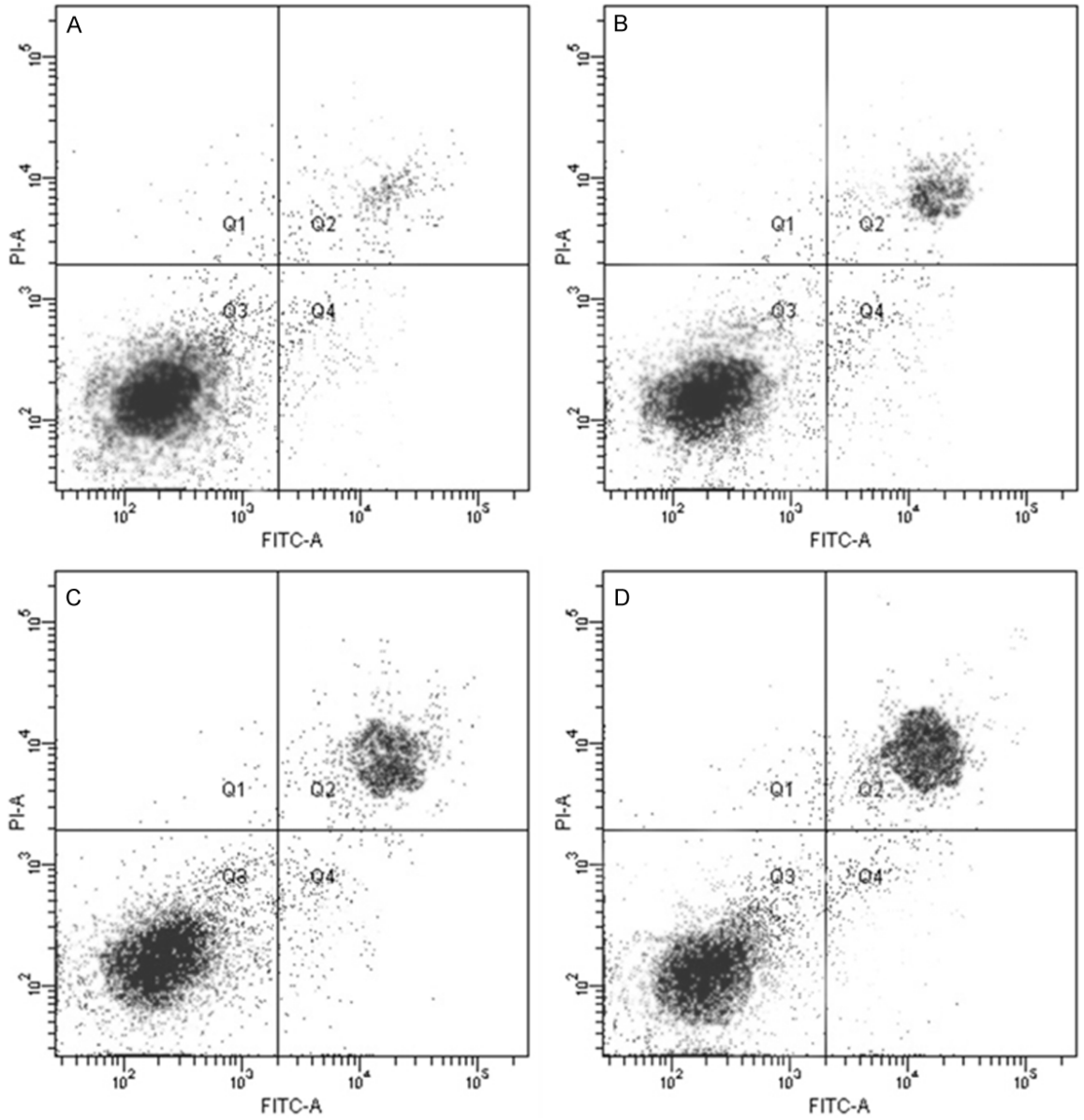

Figure 4. Triptolide induced MCF-7/ADR cell apoptosis. A: MCF-7/ADR cells; B: MCF-7/ADR cells +50 μg/ml ADR; C: MCF-7/ADR cells +50 μg/ml ADR+10 nmol/l TP; D: MCF-7/ADR cells +50 μg/ml ADR+20 nmol/l TP.

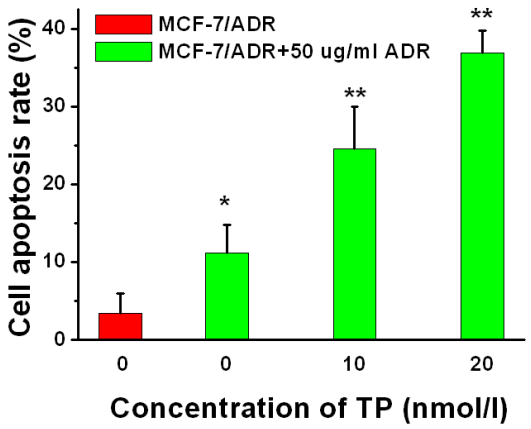

Figure 5. Triptolide induced MCF-7/ADR cell apoptosis ($\bar{x} \pm s$, n=4).

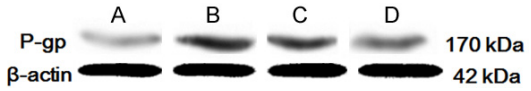

Figure 6. Triptolide decreases the expression level of P-glycoprotein. A: MCF-7 cells; B: MCF-7/ADR cells; C: MCF-7/ADR cells +10 nmol/l TP; D: MCF-7/ADR cells +20 nmol/l TP.

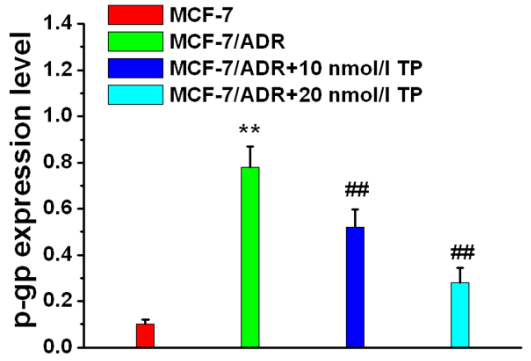

Figure 7. Triptolide decreases the expression level of P-glycoprotein ($\bar{x} \pm s$, n=4). $^{**}P < 0.01$ versus MCF-7 group; $^{\#\#}P < 0.01$ versus MCF-7/ADR group.

nized in a homogenizer until no macroscopic blocks could be observed. The homogenate was then centrifuged at 10,000 rpm for 10 min, then the pellet was discarded and the supernatant was taken. The above procedures were carried out at about 4°C. Total proteins were extracted from the supernatant. Protein concentrations of the above samples were tested by BCA method, and the protein samples were loaded on polyacrylamide gel and electrophoresed (voltage: 120 V). Subsequently, the polyacrylamide gel was transferred at 150 mA for about 1 to 1.5 h, so that the proteins on the gel were transferred to a cellulose nitrate membrane (NC), and the membrane was blocked by 5% skim milk powder. Then, P-glycoprotein monoclonal antibody (1:500 dilution) and β-actin primary antibody (1:500 dilution) were added and lethal concincubated at 4°C overnight. The resultant was incubated at room temperature for 2 h with horseradish peroxidase conjugated secondary antibody, and finally the protein expression results were tested by enhanced chemiluminescence (ECL) assay.

Statistical analysis

Data were expressed in $\bar{x} \pm s$, the statistical analysis was performed with the SPSS17.0 software, and independent sample t-test was used for intergroup pairwise comparison. $P < 0.05$ indicated statistical significance.

Results

Adriamycin suppresses the viability of MCF-7 and MCF-7/ADR cells

As shown in **Figure 1**, 10-200 μg/ml adriamycin had inhibitory effects on the viability of both MCF-7 and MCF-7/ADR cells, with a concentration-dependent manner. Medium lethal concentration of adriamycin on MCF-7 cells was: IC_{50}=(110.6±2.6) μg/ml, and the medium lethal concentration of MCF-7/ADR was: IC_{50}=(806.9±4.6) μg/ml, with a resistance index of 7.29 folds, indicating that MCF-7/ADR cells in this experiment exhibited significant resistance to adriamycin.

Triptolide inhibits the proliferation of MCF-7 and MCF-7/ADR cells

As shown in **Figure 2**, 10-320 nmol/l triptolide had inhibitory effects on the viability of MCF-7 and MCF-7/ADR cells, with a concentration-dependent manner. The medium lethal concentration of triptolide on MCF-7 cells was: IC_{50}=(330.5±3.5) nmol/l, and the medium lethal concentration of MCF-7/ADR was: IC_{50}=(345.2±2.8) μg/ml, with a resistance index of 1.04 folds, indicating that the MCF-7/ADR cells in this experiment had no significant resistance

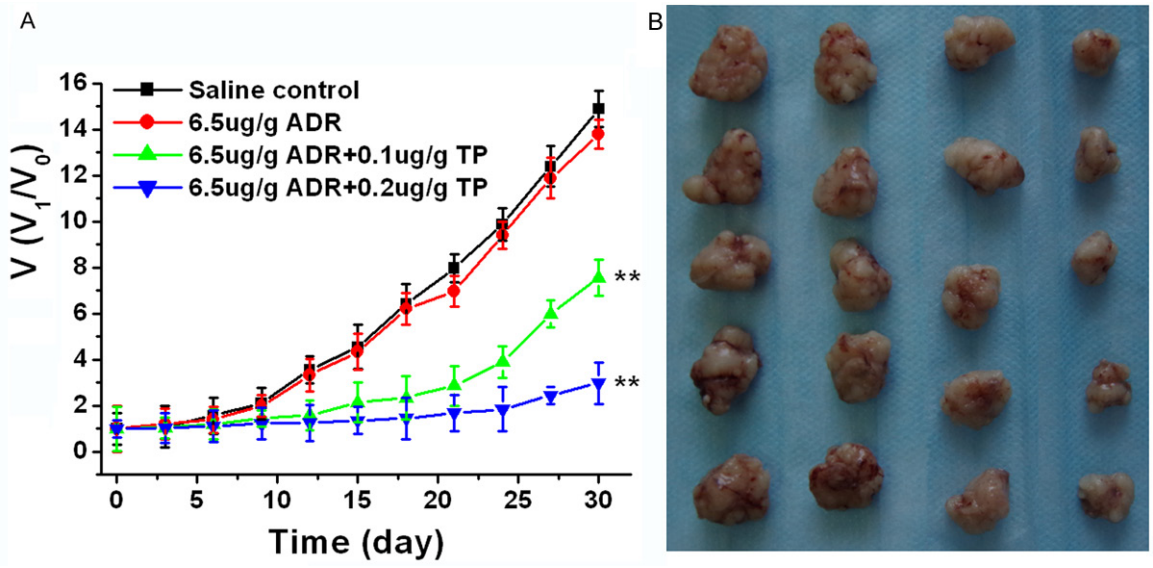

Figure 8. Triptolide inhibits tumor growthin MCF-7/ADR tumor bearing mice ($\bar{x} \pm s$, n=5).

to triptolide. As reported in [15], the dosage less than IC_5 value was non-toxic. The calculated IC_5 of triptolide was (24.6±2.1) nmol/l, therefore in this experiment, the non-toxic dosages of triptolide were 10 and 20 nmol/l, which were used in further experiment.

Triptolide reverses drug-resistance

As shown in **Figure 3**, for MCF-7 cells, the IC_{50} value for adriamycin treated alone was: 100.3±2.6 nmol/l; the IC_{50} values for adriamycin + triptolide (10, 20 nmol/l) were: 98.4±3.6 μg/ml and 95.1±2.5 μg/ml, respectively. No significant difference of IC_{50} values was detected in MCF-7 cells in each group. However, for MCF-7/ADR cells, the IC_{50} value for adriamycin treated alone was: 803.4±2.8 μg/ml; the IC_{50} values for adriamycin + triptolide (10, 20 nmol/l) were: 431.6±3.9 μg/ml and 235.1±4.5 μg/ml, respectively, which had significant difference compared to the IC_{50} values in adriamycin treated alone group ($P < 0.01$). When combined with 10 nmol/l triptolide, drug-resistance reversal fold for the MCF-7/ADR cells was 1.84±0.24 ($P < 0.01$), and when combined with 20 nmol/L triptolide, the drug-resistance reversal fold for MCF-7/ADR cells was 3.65±0.21 ($P < 0.01$). The results suggested that combination adriamycin with triptolide (10, 20 nmol/l) could reverse drug-resistance in MCF-7/ADR cells.

Triptolide induced MCF-7/ADR cell apoptosis

As shown in **Figure 4**, a combination of triptolide at non-toxic dosage levels and adriamycin at moderate concentration (50 μg/ml) induced MCF-7/ADR apoptosis. The statistical results for apoptosis was shown in **Figure 5**, the apoptosis rate was (13.2±2.6)% in MCF-7/ADR cells induced by 50 μg/ml adriamycin alone, the apoptosis rate was (25.6±3.7)% in MCF-7/ADR cells induced by a combination of 50 μg/ml adriamycin and 10 nmol/l triptolide, and the apoptosis rate induced by a combination of 50 μg/ml adriamycin and 20 nmol/l triptolidewas (37.8±2.5)%. The above results had statistical difference compared with the blank control group ($P < 0.01$).

Triptolide decreases the expression level of P-glycoprotein

As shown in **Figure 6**, P-glycoprotein expression level in MCF-7 cells was very low, and P-glycoprotein expression level in MCF-7/ADR cells was significantly higher than that in MCF-7 cells ($P < 0.01$). However, after treatment with triptolide at non-toxic dosage levels, the expression level of P-glycoprotein decreased gradually, showing significant difference compared with the result before treatment ($P < 0.01$). The statistical results were as shown in **Figure 7**. The results indicated that the expression of P-glycoprotein might be involved in the drug-resistance reversal effect of triptolide on MCF-7/ADR cells.

Triptolide inhibits tumor growth

As shown in **Figure 8**, the MCF-7/ADR tumor-bearing mice displayed different tumor inhibitory effects after treatment with different

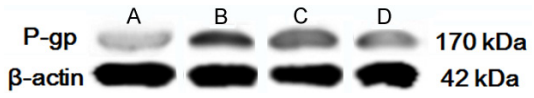

Figure 9. Triptolide decreases the level of P-glycoprotein in MCF-7/ADR tumor bearing mice; A: MCF-7 tumor; B: MCF-7/ADR tumor; C: MCF-7/ADR tumor +0.1 μg/g TP; D: MCF-7/ADR tumor +0.2 μg/g TP.

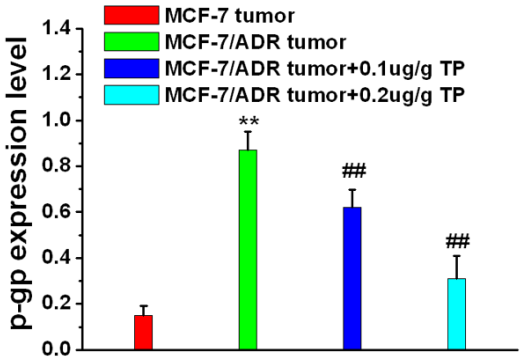

Figure 10. Triptolide decreases the level of P-glycoprotein in tumor tissue cells ($\bar{x} \pm s$, n=4); $^{**}P < 0.01$ versus MCF-7 group; $^{\#\#}P < 0.01$ versus MCF-7/ADR group.

drugs. When the mice were treated with adriamycin alone, the tumor growth speed was similar to that of the normal saline treatment group, indicating that MCF-7/ADR tumor-bearing mice were adriamycin-resistant. However, after combination with triptolide, the tumor growth was significantly inhibited. The tumor inhibitory effect would be more significant with increase in triptolide dosage, and the inhibitory effect had significant statistical difference compared with normal saline group ($P < 0.01$). The results indicated that triptolide could reverse adriamycin-resistance of MCF-7/ADR tumor cells *in vivo*.

Triptolide decreases the level of P-glycoprotein in tumor tissues

As shown in **Figure 9**, expression level of P-glycoprotein in in-vivo tumor cell was detected by Western blotting, the results were similar to the findings in vitro cells. P-glycoprotein expression level in MCF-7/ADR tumor was higher than that in MCF-7 tumor, and after treatment with triptolide, the expression level decreased with increase in dosage. The statistical results were shown in **Figure 10**.

Discussion

Breast cancer is one of the most common malignant tumors, and may have great impact on physical and psychological health of women and may even be life-threatening [16]. At present, there are various clinical treatments for breast cancer in China and abroad, including preoperative neoadjuvant chemotherapy, local surgical excision, postoperative adjuvant chemotherapy, etc. [17]. Chemotherapy plays a very important role in prognosis of the patients. However, not all chemotherapy could result in good efficacy. There are many reasons for the failure of chemotherapy, the most important reason is the acquired resistance of tumor cells [13]. Once the tumor cells acquire resistance to chemotherapy drugs, they would be resistant to anti-cancer drugs, leading to failure of chemotherapy. Therefore, it is of particular importance to develop or screen new drug-resistant reversal agents for improving the prognosis of chemotherapy for breast cancer.

In this study, adriamycin-resistant breast cancer cell lines were induced by limiting dilution method, and results suggested that adriamycin had certain inhibitory effect on the viability of normal MCF-7 cells and adriamycin-resistant MCF-7/ADR cells. However, IC_{50} value of adriamycin on MCF-7/ADR cells was 7.29 times than the effect of adriamycin on MCF-7 cells, indicating that MCF-7/ADR cells induced in this experiment exhibited significant resistance to adriamycin. That is, decrease of IC_{50} values of adriamycin on MCF-7/ADR cells means reversal of resistance of MCF-7/ADR cells to adriamycin. In this experiment, triptolide at non-toxic dosage levels (10, 20 nmol/l) combined with adriamycin were used to treat MCF-7/ADR cells. It was revealed that triptolide could significantly reduce the IC_{50} value of adriamycin for the MCF-7/ADR cells, which clearly indicated that triptolide could effectively reverse adriamycin-resistance of MCF-7/ADR cells. The results of flow cytometry suggested that a combination of adriamycin and triptolide induced MCF-7/ADR apoptosis.

In order to further investigate the drug-resistant mechanism of MCF-7/ADR cells, the expression level of P-glycoprotein, a typical drug-resistant protein, was detected, and the expression level of P-glycoprotein in MCF-7/

ADR cells were found to be significantly higher than that in normal MCF-7 cells, indicating that P-glycoprotein might be involved in the resistance of MCF-7/ADR cells to adriamycin. After treatment with triptolide, the expression level of P-glycoprotein in MCF-7/ADR cells was decreased, indicating that drug-resistance reversal effect of triptolide might due to the inhibition of P-glycoprotein expression.

P-glycoprotein, a transmembrane protein mediated by ATP, is a broad-spectrum multidrug efflux pump consisted of 1280 amino acids. P-glycoprotein was discovered by Juliano 40 years ago, and high expression of P-glycoprotein was demonstrated to be one of the mechanisms in tumor cell drug resistance [18]. Some investigators have developed reversal agents specific for P-glycoprotein, i.e., reversal agents, which inhibit expression of P-glycoprotein in cells. The reversal agents mainly include calcium channel blockers, immunosuppressants, protein kinase C inhibitors, etc. [19, 20]. These reversal agents, such as verapamil and cydosporine have certain drug-resistance reversal effect. However, most of the reversal agents are dose dependent. Therefore, high doses of drugs are required to achieve significant drug-resistance reversal effect, leading to high toxicity and side effects which may limit the clinical application of the reversal agents [21, 22].

In addition, subcutaneous animal tumor models were also established in the experiment to further investigate the drug-resistance reversal effect of triptolide on MCF-7/ADR cells. Meanwhile, the *in-vivo* assay also indicated that P-glycoprotein was involved in the drug-resistance reversal effect of triptolide. In summary, triptolide can significantly reverse resistance of the MCF-7/ADR cells to adriamycin, due to the inhibitory effect of triptolide on the expression of P-glycoprotein in MCF-7/ADR cells.

Disclosure of conflict of interest

None.

Address correspondence to: Jian-Hui Zhang, Department of Breast Surgery, Sichuan Cancer Hospital & Institute, Sichuan Cancer Center, School of Medicine, University of Electronic Science and Technology of China, Chengdu, China. E-mail: zhangjhscth@sohu.com

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