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Paris saponin VII from *trillium tschonoskii* reverses multidrug resistance of adriamycin-resistant MCF-7/ADR cells via P-glycoprotein inhibition and apoptosis augmentation

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ABSTRACT

Ethnopharmacological relevance: Saponins of several herbs are known to induce apoptosis in some cancer cells and are proposed to be promising modulators of drug resistance. In the present study, we extracted Paris saponin VII (PS VII), a kind of saponin, from *Trillium tschonoskii* Maxim. and observed its effect on adriamycin-resistant breast cancer cells.
Materials and methods: An adriamycin-resistant human breast cancer cell line, MCF-7/ADR cells were exposed to different concentrations of PS VII (0–100 μmol/L). Then, flow cytometric assays and a human apoptosis array were used to detect apoptotic cells and apoptosis related protein expression. P-glycoprotein levels and intracellular rhodamine 123 (RH-123) accumulations were measured to evaluate the expression and activity of P-glycoprotein.
Results: PS VII dose dependently suppressed cell viability as well as triggered apoptosis and modulated drug resistance of MCF-7/ADR cells. Further results showed that PS VII treatment in MCF-7/ADR cells led to increased TNFR1, TRAIL R1/DR4, TRAIL R2/DR5, and FADD expression, and activation of PARP, caspase-8, and 3. In parallel to the alterations, P-glycoprotein expression and activity were also reduced.
Conclusion: These findings showed that PS VII might be an effective tumouristatic agent for the treatment of MDR breast cancer.

1. Introduction

Breast cancer is a major malignant disease affecting women worldwide, with 1, 050, 000 new cases and 3,72, 000 deaths annually (Wong et al., 2009). Chemotherapy plays an important role in the management of patients with breast carcinoma. Adriamycin (ADR), docetaxel, paclitaxel and other chemotherapeutic drugs, have been used as first- or second-line anti-cancer drugs in the treatment of breast cancer (Vanhoefer et al., 1997). However, development of a multidrug-resistant (MDR) phenotype, one of the most formidable challenges in the field of cancer therapy (Ross, 2000), is a main obstacle to the successful treatment of breast cancer (Hansen et al., 1999; Meyer zu Schwabedissen and Kroemer, 2011).

MDR is acquired via two major ways. One is the active export of drug from cells by the over-expression of P-glycoprotein (P-gp) (Roninson, 1992) and other ATP-binding cassette (ABC) transporters, which extrude the internalized drugs from the cancer cells. Another is direct suppression of apoptosis. Increased expression of anti-apoptotic proteins, such as Bcl-2, has been reported in MDR cells (Robert and Larsen, 1998). Patients with MDR tumor types are often left with few options but exceptionally high doses. Thus, development of new chemotheraputic drugs which will bypass this resistance is essential in treating MDR breast cancer.

Natural products have been shown to be excellent and reliable sources for pharmaceutical development. Paclitaxel, a natural product from the bark of the Pacific yew *Taxus brevifolia*, shows good curative effects on refractory breast cancer (Mann, 2002). Curcumin, a diferuloylmethane derived from the plant *Curcuma*

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longa, inhibits invasion and metastasis in K1 papillary thyroid cancer cells (Zhang et al., 2013).

Trillium tschonoskii Maxim., locally named "a pearl on head", is a perennial herb of the *Trilliaceae* found in mid-western China (Li et al., 2005; Zhang et al., 2013). It has been traditionally used for treatment of hypertension, neurasthenia, headache, cancer, and ameliorating pains (Fu, 1992) in folk medicine of China. The main bioactive constituents of genus *Trillium* are the steroidal saponins (Hayes et al., 2009; Nooter and Herweijer, 1991; Ong et al., 2008; Yokosuka and Mimaki, 2008). Phytochemical and pharmacological studies have further revealed a novel therapeutic role as an anticancer agent for these steroid saponins (Wang et al., 2013).

In the present study, we investigated the mechanism underlying the cytotoxic effects of a kind of steroidal saponins from *Trillium tschonoskii* Maxim., namely, Paris saponin VII (PS VII) and its putative antitumor properties on MCF-7/ADR, and the parental drug-sensitive MCF-7 cell lines. The results indicated that PS VII inhibited growth of these cells effectively. It not only upregulated TNFR1, TRAIL R1/DR4, TRAIL R2/DR5, and cleaved caspase-8 expression, but also targeted P-gp function and expression in MCF-7/ADR cells. These preclinical studies indicated that PS VII may have potentials in the treatment of MDR breast cancer.

2. Materials and methods

2.1. Chemicals and reagents

The cytotoxic drugs adriamycin (ADR) was purchased from Sigma (St. Louis, MO, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), and rhodamine 123 (Rh123) were purchased from Sigma (St Louis, MO, USA). Anti-poly (ADP-ribose) polymerase (PARP), cleaved caspase-3, cleaved caspase-8, TNFR1, TRAIL R1/DR4, TRAIL R2/DR5, and FADD antibodies were obtained from Cell Signaling (Beverly, MA, USA). Anti-P-glycoprotein antibody was purchased from Abcam (Cambridge, UK). Anti-β-actin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Preparation of PSVII

The crude drugs were collected from Taibaishan of Qinling Moutains and identified morphologically, histologically, and chemically by Guo yaowu, a pharmacist in laboratory for the control of drugs of Shaanxi Province. A voucher specimen (No. 20101015) including the identification and classification of the plant material was preserved in the herbarium of Collaborative Innovation Center for Chinese Medicine in Qinba Mountains. PSVII, with a purity of $>99\%$, was isolated from the root and rhizome of *Trillium tschonoskii* Maxim. in our lab (Li et al., 2013). Briefly, the dried root and rhizome of Trillium (20.0 Kg) were extracted with 70% ethanol for three times. After removal of the solvent under reduced pressure, the ethanol extract was suspended in H_2O, and subjected to macroporous resin (D 101) column chromatography and eluted with increasing amounts of ethanol (i.e. 0% ethanol, 20% ethanol, 60% ethanol, and 95% ethanol). The 60%-ethanol fraction was separated by a silica gel column, Sephadex LH-20, reverse phase C18 chromatography, and MHPLC chromatography, final purification was achieved by HPLC to get compound PS VII. And its chemical structure is presented in Fig. 1.

2.3. Cell culture

MCF-7, a human breast adenocarcinoma cell line, was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in DMEM medium. Its ADR resistant cell line MCF-7/ADR cells, derived from the parental drug-sensitive MCF-7 cells by stepwise selection with ADR (Cohen et al., 1986), was obtained from Nanjing Keygen Biotech. Co., Ltd. (Nanjing, China). To keep the drug resistance phenotype, this cell was cultured in the presence of 1 μg/ml ADR in RPMI 1640 culture medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 U/ml streptomycin. The cells were maintained in a humidified atmosphere at 37 °C in 5% CO_2.

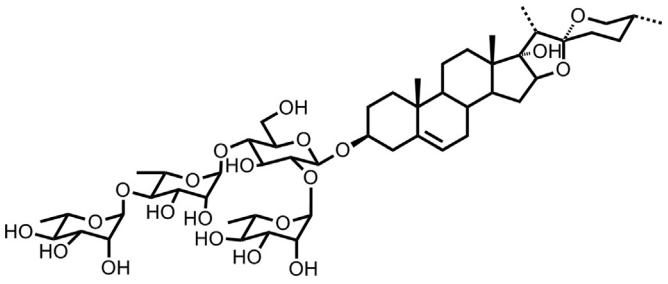

Fig. 1. Chemical structure of Paris saponin VII (PS VII).

2.4. Cell proliferation assays

MCF-7 and MCF-7/ADR cells were plated in 96-well plates at a density of 2×10^4 cells/well and allowed to grow for 24 h. The cells were then treated with PS VII for 24 h in complete medium. After drug treatment, 20 μL of MTT solution (0.5 mg/mL) was added to each well. The cells were then washed thrice with PBS after 4 h incubation and the formazan was resuspended in 150 μL DMSO. Absorbance was measured at 490 nm by using a Bio-Rad ELISA reader. Half maximal inhibitory concentrations (IC_{50}) of PS VII were determined by curvefitting analyses using Prism software (GraphPad Software, San Diego, CA). The experiment was repeated independently three times.

2.5. Flow cytometric assays for annexin-V

MCF-7/ADR Cells were plated at a density of 5×10^6 per 10 cm^2 dish and cultured with different concentrations of PS VII for 24 h or 48 h. A total of 1×10^6 to 3×10^6 cells were washed with ice-cold PBS and resuspended in 1 × binding buffer [10 mM Hepes/NaOH (pH 7.4), 140 mM NaCl, 2.5 mM $CaCl_2$] at a concentration of 1×10^6 cells/ml. 5 μl of annexin V-FITC solution (25 μg/ml) and 5 μl of dissolved PI (250 μg/ml) were added to 100 μl of the cell suspensions. Cells were then gently vortexed and incubated at room temperature in the dark for 15 min. Then 400 μl of ice-cold binding buffer was added and mixed gently before the cell preparations were examined by flow cytometry (FACSCalibur, Becton Dickinson, San Jose, CA, USA).

2.6. Human apoptosis array

A human apoptosis array kit (ARY009) was purchased from R&D Systems, Inc. (MN, USA). MCF-7/ADR Cells were treated with or without PS VII at the concentration of 3 μM for 24 h. Then, total proteins were extracted by using the lysis buffer in the kit and the concentrations were determined using bicinchoninic acid reagent (Thermo Fisher Scientific Inc., IL, USA) with bovine serum albumin as a standard. Equal amounts of proteins (300 μg/array) were added and all the procedures were carried out according to the manufacturer's instructions.

2.7. Western blot analysis

MCF-7/ADR cells were incubated with 1.5 or 3 μM of PS VII for 24 h or 48 h. Cells were then harvested and resuspended in lysis buffer [(pH 7.9) 10 mM Hepes, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, plus the protease inhibitors leupeptin 10 μg/ml, aprotinin