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Inhibition of matrix metalloproteinases related to metastasis by diosgenyl and pennogenyl saponins

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ABSTRACT

Ethnopharmacological relevance: Diosgenyl and pennogenyl saponins isolated from *Rhizoma Paridis*, showed pro-apoptosis and immunoregulation with antitumor activity in cultured cells and animal systems.
Aim of the study: To evaluate their anti-metastatic mechanism on cancer cells and discuss their structure-activity relationship on anti-tumor effect.
Materials and methods : This research used the wound healing and migration assay to detect their anti-invasive effect on B16 melanoma cells. Through the gelatin zymography assay, immunofluorescence analysis and western blot, saponins exhibited different levels of protein expression inhibition of MMP-1, -2, -3, -9 and -14.
Results: Through the analysis, diosgenyl and pennogenyl saponins inhibited the metastasis of B16 melanoma cells. Diosgenyl saponins also showed strong suppression of enzyme activity of MMP-2 and -9. Different saponins exhibited different levels of inhibition on MMP expression.
Conclusions: 17-α OH increases the sensitivity of diosgenyl saponins to the membrane-bound protease which can stimulate proMMP-2 activation, but it also decreases the anti-metastatic activity of diosgenyl saponin. Furthermore, their combination might provide a potential therapeutic modality for metastasis.

1. Introduction

Paris polyphylla var. *yunnanensis* (Fr.) Hand.-Mazz. (Becker et al., 2005), mostly distributed in the southwest of China, is a traditional Chinese medicinal herb that has been used in treating cancer for many years (Su and Wei, 1983). In recent years, studies on the aqueous, ethanolic and methanolic extracts of *Paris polyphylla* showed anticancer activity on several types of cancer cell lines (Ji et al., 2001). More extensive phytochemical and pharmacological studies further identified *Rhizoma Paridis* saponins, which are steroid saponins, as the main antitumor active components in *Paris polyphylla* (Wu et al., 2004).

Polyphyllin D (PD) is a diosgenyl saponin originally found in PPY. With the ascertained chemical structure and the improved synthesis of Polyphyllin D, both *in vitro* and *in vivo* studies were performed on its effect. Recent research showed that PD is a potent apoptosis inducer through mitochondrial dysfunction in drug-resistant HepG2 cells (Cheung et al., 2005) and has inhibitory effects on the growth of human breast cancer cells and in xenograft (Lee et al., 2005). The proteomic and transcriptomic analyses revealed that PD induced the cytotoxic effect through a mechanism initiated by ER stress followed by mitochondrial apoptotic pathway (Siu et al., 2008). However, the anti-metastasis activity has not been discussed.

Paris H and Paris VII as pennogenyl saponins also displayed some cytotoxicity to mouse lung cancer LA795 cells (Wang et al., 2007b; Huang et al., 2010).

Our preliminary drug screening revealed that the components extracted from *Paris polyphylla* had strong anti-lung cancer activity (Wang et al., 2006, 2007a; Yan et al., 2008). The purified mixture isolated from RPS (PM), mainly composed of PD, Paris H and Paris VII (Fig. 1), showed a powerful anti-proliferation effect by inducing apoptosis and inhibiting metastasis (Man et al., 2011b). In this paper, we want to display the anti-tumor and anti-metastasis activity of Polyphyllin D, Paris H and Paris VII, respectively, and discuss their preliminary mechanism of antitumor effects on B16 melanoma cells.

Abbreviations: HE, histological examination; Mets, metastases; MMP, matrix metalloproteinase; PD, Polyphyllin D; PPY, *Paris polyphylla* var. *yunnanensis* (Fr.) Hand.-Mazz. (PPY); RPS, *Rhizoma Paridis* saponins.

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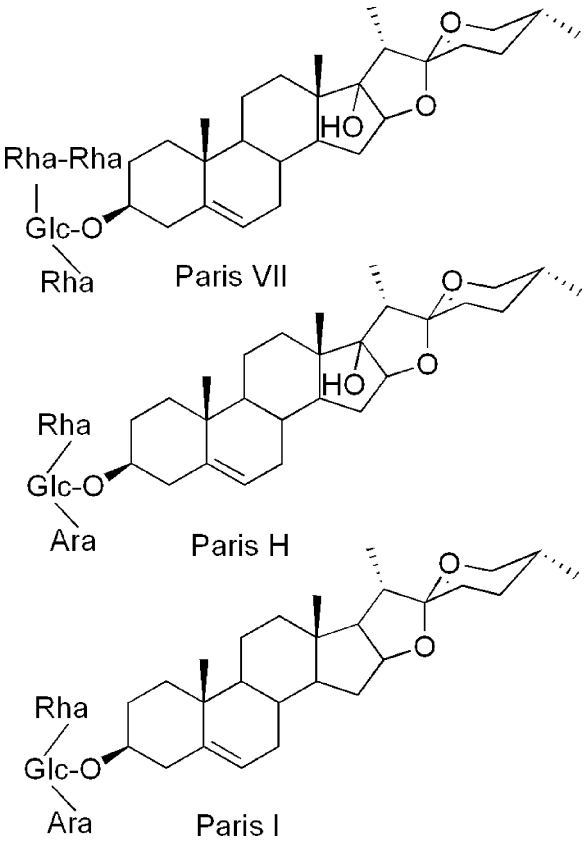

Fig. 1. Structures of paridis saponins.

2. Materials and methods

2.1. Reagents

Fluorescein (FITC)-conjugated AffiniPure Rabbit Anti-Goat IgG (ZSGB-bio, Beijing, China, ZF-0314), Fluorescein (FITC)-conjugated AffiniPure Goat Anti-Mouse IgG (ZSGB-bio, Beijing, China, ZF-0312), Fluorescein (FITC)-conjugated AffiniPure Goat Anti-Rabbit IgG (ZSGB-bio, Beijing, China, ZF-0311).

Mouse monoclonal antibody specific for MMP-2 (sc-13595) and Goat monoclonal antibody specific for MMP-9 (sc-6840) were purchased from Santa Cruz Biotechnology, INC. (USA). Rabbit monoclonal antibody specific for MMP-1 (BA-1270), -3 (BA-1531) and -14 (BA-1278) were purchased from Boster Biological Technology (Boster, Wuhan, China). Electrophoretic reagents were bought from BioRad (Richmond, CA.). Other chemicals were of the highest grade available.

2.2. Isolation of diosgenyl and pennogenyl saponins

Paris polyphylla Smith var. *yunnanensis* (Becker et al.) and *Paris polyphylla* Smith var. *Chinensis* (Franch) (PPC), commonly known as *Rhizoma Paridis* in China, grow primarily in the temperate zone and tropical regions of Europe and Asia continent, especially in Guangxi, Yunnan and Guizhou province of China. The structure of Polyphyllin D, Paris H and Paris VII were identified before (Huang et al., 2010). The purity of these monomers was determined to be more than 98% by normalization of the peak areas detected by HPLC, and was stable in methanol solution.

2.3. Cells

Mouse B16 melanoma cell line obtained from Peking Union Medical College (Beijing, China), was maintained in RPMI-1640 Medium (Gibco, BRL) supplemented with 10% heat-inactivated (56 °C, 30 min) fetal calf serum (FCS) (Gibco, BRL), penicillin (100 U/mL) and streptomycin (100 μg/mL). The cell culture was maintained at 37 °C in a humidified atmosphere of 5% CO_2. Cells were passaged each 3–4 days.

2.4. In vitro proliferation assay by MTT

Cell proliferation was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay (Sigma, USA). The B16 melanoma cells were seeded at a density of 1×10^5 cells per well into 96-well plates in a culture medium containing 10% FCS. After 24 h, the cultures were washed and added with 5 different kinds of concentration of the drug. MTT (0.5 mg/mL) was added for an additional 4 h after being cultured for 3 days. Absorbance at 570 nm was determined for each well using ELISA reader. The experiments were repeated in triplicate wells.

2.5. Wound healing assay

For the wounding healing experiment, B16 melanoma cells were seeded in 6 well plates and allowed to grow to complete confluence. Subsequently, a plastic pipette tip was used to scratch the cell monolayer to create a cleared area, and the wounded B16 melanoma cell layer was washed with fresh medium to remove loose cells. The cells were then re-fed with fresh medium containing 0.5% FBS and were treated with different non-cytotoxic concentration of drugs for 24 h. Immediately following scratch wounding (0 h) and after incubation of cells at 37 °C for 24 h, phase-contrast images (10× fields) of the wound healing process were photographed digitally with an inverted microscope (Olympus IX50). The distance of the wound areas were measured on the images, and the mean percentage of the total distances of the wound areas were calculated. The inhibition of the migration = (1 – the width of the wound area at 0 h/the width of the wound area after 24 h) × 100%.

2.6. In vitro migration assay

Cell migration was assayed using 18-mm diameter chambers with 8-μm pore filters (Transwell, 12-well cell culture). The B16 melanoma cells were removed from the culture flasks and resuspended at 1.5×10^5 cells/mL in serum-free medium, and then 0.4 mL cell suspension was added to the upper chambers. Afterwards, the lower chambers were added with a culture medium containing 2.5% FBS (2 mL). The chambers were incubated for 24 h at 37 °C in a humid atmosphere of 5% CO_2/95% air. And then the filters were dried in the air. The upper surfaces of the filters were scraped twice with cotton swabs to remove non-migrated cells. The undersides of the filters were stained with Crystal violet and then photographed digitally with an inverted microscope (Olymus IX50). In the end, the chambers washed with 33% acetic acid. Absorbance at 570 nm was determined for each well using UV detector. The experiments were repeated in triplicate wells.

2.7. Gelatin zymography

The gelatinolytic activity of MMP-2 and -9 secreted in conditioned media was assayed by means of gelatin-substrate gel electrophoresis to identify the levels of metalloproteinase activity. Conditioned media were harvested and the protein concentration measured, followed by concentrating by precipitation with 2-volume of absolute ethanol. Twenty micrograms of protein was resuspended with 2× sample buffer without reducing agent (0.5 M Tris–HCl, pH 6.8, 10% SDS, 0.1% bromophenol blue, 10% glycerol) and then subjected to 10% SDS-PAGE gel containing 0.1% (w/v)