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An active molecule from *Pulsatilla chinensis*, *Pulsatilla saponin A*, induces apoptosis and inhibits tumor growth of human colon cancer cells without or with 5-FU

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Abstract. Colon cancer is one of the common types of digestive malignancy. The efficacy of the first-line chemotherapy drug for colon cancer, fluorouracil (5-FU), remains limited in clinical settings due to poor efficacy and significant side effects. In the present study, the anticancer activity of an active compound from *Pulsatilla chinensis* extracts, *Pulsatilla saponin A* (PsA), was isolated and examined *in vitro* and *in vivo*. It was demonstrated that PsA significantly inhibited the growth of human colon cancer HT-29 cells. This inhibitory activity was also observed when the compound was tested in a colon cancer xenograft mouse model. Additionally, the synergic antitumor effects of PsA and 5-FU on colon cancer cells were observed. Using annexin V and terminal deoxynucleotidyl transferase 2'-deoxyuridine 5'-triphosphate nick end labeling assays, it was demonstrated that levels of apoptosis induction in HT-29 cells treated with PsA or 5-FU were significantly increased compared with the untreated control cells ($P<0.05$). Western blot analyses were then performed, and the results revealed an increase in tumor protein 53 and cleaved caspase 9, and a decrease in B-cell lymphoma 2 protein expressions in PsA and PsA + 5-FU treated colon cancer cells compared with the vehicle-treated (PBS) cells. In summary, PsA exhibited anticancer activity in human colon cancer cells *in vitro* and *in vivo,* in isolation and synergistically with 5-FU, through apoptosis induction.

Introduction

Colon cancer is ranked as the third most common malignancy of the gastrointestinal system (1-3). At present, the comprehensive treatment based on surgery is the widely accepted treatment option in colon cancer therapeutics. Chemotherapy is an important part of colon cancer treatment, which has been demonstrated to be effective for metastasis control. However, the anticancer effects of these therapies, including fluorouracil (5-FU), which is regarded as the first-line drug for colon cancer chemotherapy, remains limited due to poor efficacy and significant side effects (1-3). Therefore, it is necessary to investigate novel bioactive molecules for the comprehensive treatment of colon cancer.

Pulsatilla chinensis regel is a traditional Chinese herb known to exhibit anti-inflammatory properties and may be used in various infectious diseases, such as malaria, intestinal amebiasis and bacterial infections (4,5). To date, at least 15 saponin derivatives had been found in *P chinensis* extracts (5), several of which have been reported to exhibit antitumor activities. For example, *Pulsatilla saponin A* (PsA), one of the *P chinensis* extracts, has been reported to demonstrate an antitumor effect by inducing DNA damage and apoptosis, and causing G2 arrest in hepatocellular carcinoma (HCC) cells (6).

In the present study, the anti-colon cancer activities of PsA or PsA combined with 5-FU were determined in cell culture and xenograft mouse models. The possible mechanisms of action were examined using western blot assays.

Materials and methods

Cell culture. The human colon cancer HT-29 cell line was purchased from the Cellular Biological Institute of the Science Academy of China in Shanghai (Shanghai, China), and cultured in McCoy's 5A medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum, 100 mg/ml streptomycin and 100 IU/ml penicillin. All cells were cultured in a 5% CO_2 humidified atmosphere at 37˚C.

Cell proliferation assay. Cell proliferation was measured in 96-well plates using the Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Kumamoto, Japan). Subsequent to incubation at 37˚C in corresponding drugs with a certain concentration (5 ng/µl PsA, 5 ng/µl 5-FU or 2.5 ng/µl 5-FU + 2.5 ng/µl PsA) for a 0, 1, 3 or 5 days all the cells were

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cultured with 10 ml CCK-8 solution at 37˚C for 2 h. Optical absorbance was monitored in a plate reader at a 450-nm wavelength. In these experiments, each data point was assayed in triplicate. Each experiment was performed at least three times as described previously (7).

Tumor xenograft study. To establish the human colon cancer mouse xenograft models, 20 six-week-old male athymic *BALB/c* mice (18-20 g/each) were fed in center animals rooms of the Second Affiliated Hospital of Soochow University (temperature, 18-22˚C; humidity 50-60%) and injected subcutaneously with $5x10^7$ HT-29 cells on the right dorsal flank to initiate tumor growth. Subsequent to tumor volumes reaching 50-100 mm^3 at ~2 weeks, the mice were randomly divided into four groups for additional treatment. The control group mice were administered purified saline, PsA group were injected with 10 mg/kg PsA, the 5-FU group was provided 20 mg/kg 5-FU and a combination of 5-FU and PsA was administered to the 5-FU + PsA group. The injections were performed intraperitoneally 3 times/week for 3 weeks. Body weight and tumor size in the mice were recorded every 3 days. Tumor size (in the living animal) was measured using a slide gauge. Tumor volume was calculated using the following equation: Volume (v)=tumor mass length x $width^2/2$ (6). The present study was approved by the ethic committees at the Second Affiliated Hospital of Soochow University hospital and was conducted in accordance with the Declaration of Helsinki. All experiments were conducted in accordance with Animal Ethical Care (3).

Assessment of apoptosis by annexin V staining and terminal deoxynucleotidyl transferase 2'-deoxyuridine 5'-triphosphate nick end labeling (TUNEL) assay. Subsequent to drug treatment as aforementioned, for 3 h, the cells were washed with 1X PBS, harvested and resuspended in 100 ml staining solution containing annexin V fluorescein and propidium iodine in a N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer; Annexin-V FLUOS staining kit (Boehringer-Mannheim, China). Subsequent to incubation at room temperature for 15 min, cells were analyzed by flow cytometry (7). The cells were treated for 8-12 h, then the apoptotic tumor cells were observed by fluorescence microscope using the TUNEL assay kits (Shanghai XiTang Biotechnology Corp., Shanghai, China). Apoptotic cells were stained with green fluorescence (3).

Western blot assay. Subsequent to treatment at 37˚C for 24-48 h, the cells were collected and lysed in a buffer containing 50 mmol/l pH 8.0 Tris-HCl, 150 mmol/l NaCl, 1% (v/v) Triton X-100, and a protease inhibitor mixture [4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, aprotinin, bestatin, EDTA, E-64 and leupeptin; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany; dilution, 1:100]. Protein samples were analyzed by 10 or 12% SDS-PAGE and western blotting using antibodies against B-cell lymphoma 2 (catalog no. o.3033; dilution 1:1,500; BioVision, Inc., Milpitas, CA, USA), tumor protein p53 (catalog no., 2524S; dilution, 1:1,000) or cleaved caspase 9 (grant no. 9505T; dilution, 1:1,500; Cell Signaling Technology, Inc., Danvers, MA, USA). Nitrocellulose membranes were developed using electrochemiluminescent reagents (Denville Scientific, South Plainfield, NJ, USA) and exposed to X-ray films as described previously (8).

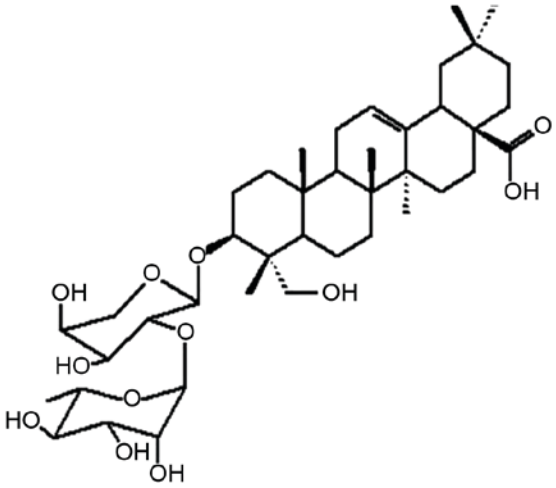

Figure 1. Chemical structure of *Pulsatilla saponin A*.

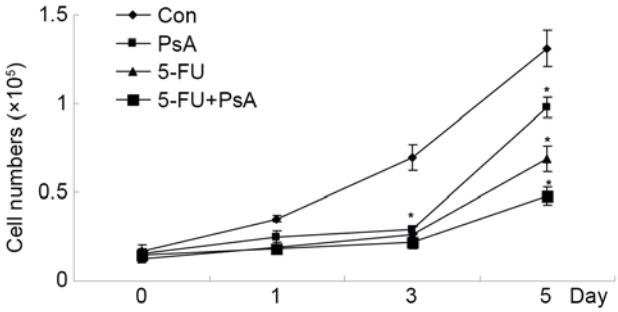

Figure 2. PsA inhibited colon cancer cell proliferation. Subsequent to incubation with 5 ng/μl PsA, 5 ng/μl 5-FU or 2.5 ng/μl 5-FU + 2.5 ng/μl PsA, the viability of HT-29 cells was measured by CCK-8 assay. Data were obtained from 3 independent experiments. *P<0.05 vs. vehicle control at the same time point. PsA, *Pulsatilla saponin A*; 5-FU, fluorouracil; Con, control.

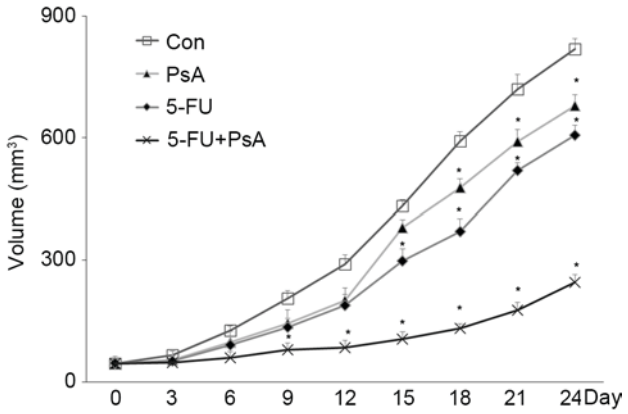

Figure 3. PsA inhibited tumor growth in human colon cancer xenograft mouse models. Tumor growth in a HT-29 cell-based mouse xenograft model treated with PsA, 5-FU, 5-FU+PsA or vehicle control. *P<0.05 PsA, 5-FU or 5-FU+PsA vs. vehicle control at the same time point. PsA, *Pulsatilla saponin A*; 5-FU, fluorouracil; Con, control.

Statistical analysis. All data were presented as the mean ± standard deviation, and all experiments were repeated at least three times. Statistical analysis was conducted with an unpaired Student t-test using SPSS 15 software (SPSS, Inc., Chicago,

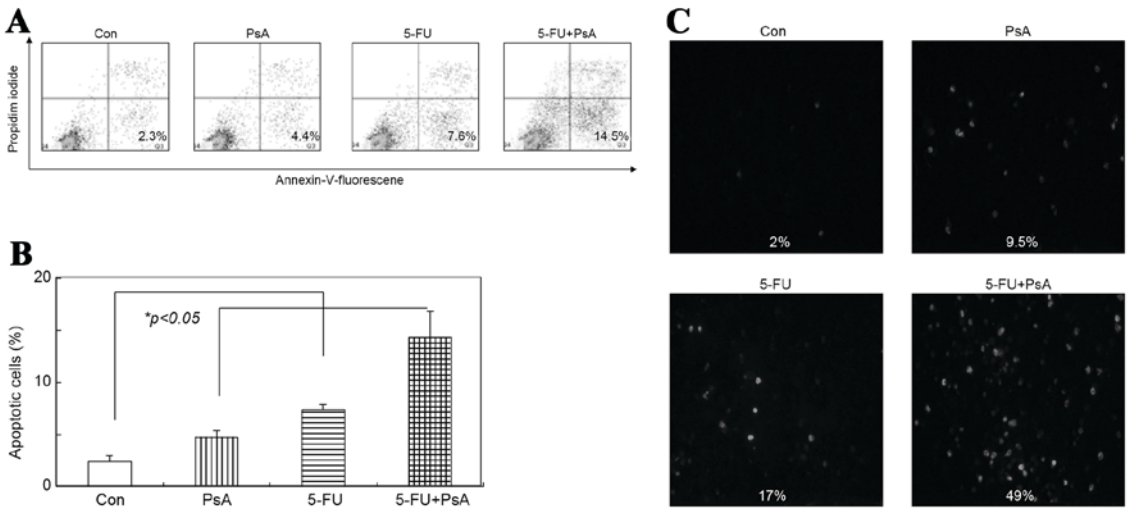

Figure 4. PsA induced apoptosis in HT-29 cells. (A) HT-29 cells were treated with a vehicle control or PsA, 5-FU, 5-FU+PsA for 3 h. The cells were incubated with an anti-annexin-V antibody and analyzed by flow cytometry. (B) The percentage of Annexin V positive cells was calculated. The data presented in the bar graph were obtained from three independent experiments. (C) HT-29 cells were treated for 6-8 h. Percentage of apoptotic tumor cells were observed by fluorescence microscopy. Magnification, x40. PsA, *Pulsatilla saponin A*; 5-FU, fluorouracil; Con, control.

IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

PsA inhibited cancer cell proliferation. The molecule structure of PsA is illustrated in Fig. 1. Subsequent to incubation with 5 ng/μl PsA, 5 ng/μl 5-FU or 2.5 ng/μl 5-FU + 2.5 ng/μl PsA, the viability of the HT-29 cells was measured using a CCK-8 assay. In the presence of PsA, 5-FU, or PsA + 5-FU, the proliferation rates were inhibited significantly compared with the PBS-treated control cells (P<0.05). The combination of PsA and 5-FU demonstrated a synergic inhibition on HT-29 cells growth (P<0.05). The results indicate that PsA exhibited an antiproliferative effect on cultured colon cancer cells, in isolation or combined with 5-FU (Fig. 2).

PsA inhibited tumor growth in mouse xenograft models. To examine whether PsA exhibited antitumor growth activity *in vivo*, xenograft tumor models were established in athymic BALB/c mice using HT-29 cells. The mice were then treated with PsA, 5-FU, PsA + 5-FU or a vehicle. As demonstrated in Fig. 3, PsA and 5-FU markedly inhibited HT-29 derived tumor growth in mice compared with the vehicle treated control, as indicated by smaller tumor volumes (P<0.05). Additionally, the tumors grew more slowly subsequent to the combined application of PsA and 5-FU, compared with each treatment alone (P<0.05). These data indicate that PsA exhibited inhibitory activities against human colon tumor *in vivo*, and that PsA and 5-FU possessed synergic antitumor effects *in vivo*.

PsA and/or 5-FU induced apoptosis in colon cancer cells. To examine whether PsA induces apoptosis in cancer cells, the annexin V expression in HT-29 cells was analyzed. The cells were incubated with 5 ng/μl PsA, 5 ng/μl 5-FU or 2.5 ng/μl 5-FU + 2.5 ng/μl PsA for 3 h and analyzed by flow cytometry using an anti-annexin V antibody. As illustrated in Fig. 4A and B, 4.4, 7.6 and 14.5% of the cells were positive for annexin V subsequent to treatment with PsA, 5-FU and 5-FU+PsA, respectively, however only 2.3% of the cells were annexin V positive in the vehicle-treated group (P<0.05).

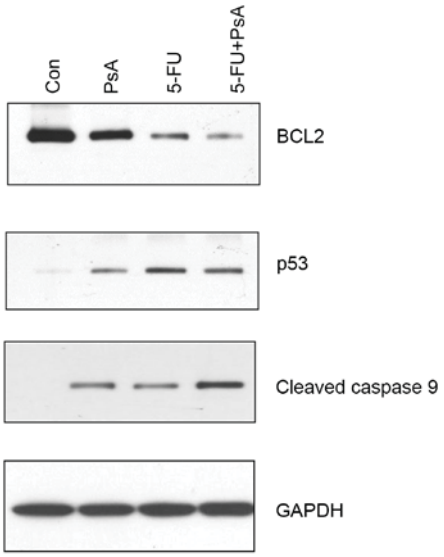

Figure 5. PsA affected the p53, BCL-2, and caspase 9 protein expression levels. HT-29 cells were treated with PsA, 5-FU, 5-FU+PsA at 37˚C for 24 h. The cells were lysed, and protein samples were analyzed by western blot assay for BCL-2 (top panel), p53 (second panel), and cleaved caspase 9 (third panel) protein expression levels. The level of GAPDH protein expression was used as a sample loading control (bottom panel). PsA, *Pulsatilla saponin A*; 5-FU, fluorouracil; Con, control; BCL-2, B-cell lymphoma 2, p53, tumor protein 53.

The TUNEL assays demonstrated that the number of apoptotic cells markedly increased in the treated cells compared with the control group; the apoptotic rate was 9.5, 17 and 49% in the PsA group, 5-FU group and 5-FU+PsA group, respectively, while only 2% in the control group (P<0.05), as demonstrated in Fig. 4C. These data suggest that PsA may

induce apoptosis in HT-29 cells, and the combined treatment with PsA and 5-FU increased the rate of apoptosis in HT-29 cells compared with these drugs in isolation.

PsA affected the p53, Bcl-2, and caspase 9 protein expression in HT-29 cells. The incidence and mechanisms of action of PsA on the expression of proteins involved in apoptosis were examined. HT-29 cells were treated with 5 ng/μl PsA, 5 ng/μl 5-FU or 2.5 ng/μl 5-FU + 2.5 ng/μl PsA at 37˚C for between 24 and 48 h. Subsequent to cell lysis, the proteins in the cell lysate were analyzed by SDS-PAGE and western blotting. As demonstrated in Fig. 5, BCL-2 protein expression levels were reduced in the PsA-treated cells, whilst the cleaved caspase 9 and p53 protein expression levels were increased in these cells.

Discussion

As one of the most common clinical chemotherapy drugs for gastrointestinal tumors, 5-FU exerts antitumor effects through transforming into corresponding nucleotides (3), and by inducing cancer cell apoptosis. Hypothetically, 5-FU may cause DNA deterioration, thus accelerating the rates of apoptosis in cancer cells (9,10). However, the effective rate of inducing cancer cell apoptosis for 5-FU was only ~30% with respect to colon cancer clinical chemotherapy. The severe levels of toxicity and side-effects, and emerging drug resistance, have limited the dosage and therapeutic applications of 5-FU (1-3). Therefore, the investigation of novel therapeutic strategies, which may improve the effect and reduce the possibility of adverse reactions of 5-FU, is required.

PsA is an active compound extracted from *P chinensis*, similar to several saponin derivatives, which also exhibits antitumor activities via inducing DNA damage, G2 arrest and apoptosis among HCC and pancreatic cancer cells. However, the effects of PsA on colon cancer cells has not been fully characterized, particularly when administered in combination with 5-FU.

In cell line experiments, PsA in isolation significantly inhibited the proliferation of human colon cancer HT-29 cell line, similar to 5-FU. In addition, the combined treatment with 5-FU and PsA enhanced the inhibitory effects on HT-29 cells growth. The antitumor activity of PsA was also confirmed *in vivo*, as in a human colon cancer xenograft mouse model, PsA elicited inhibitory effects on tumor growth in isolation and PsA+5-FU treatment exhibited marked synergic antitumor effects *in vivo*. Using a TUNEL assay and flow cytometric analysis, apoptotic cells were detected in the PsA and PsA+5-FU-treated HT-29 cells. Notably, a larger number of apoptotic cells were identified the PsA+5-FU-treated group. These data indicate one of the possible mechanisms through which PsA and 5-FU induce the synergic anticancer effects.

To elucidate the possible mechanisms underlying PsA-mediated tumor inhibition, western blot analyses were performed. It was revealed that p53 and cyclin B protein expression levels were increased in PsA-treated cells, whereas BCL-2 protein expression levels were decreased. All these data were in agreement with the previous findings that PsA may induce apoptosis. Unlike PsA, which induces DNA damage and cell apoptosis, 5-FU may elicit antitumor activities through serving as a thymidylate synthase inhibitor, thus blocking the pyrimidine-thymidine synthesis and DNA replication of cancer cells (2,3). Therefore, evidence was provided for the mechanisms through which PsA and 5-FU may exhibit synergic antitumor effects.

In summary, PsA may inhibit human colon cancer cell growth *in vitro* and *in vivo*, in isolation or synergistically with 5-FU, through apoptosis induction. The data from the present study suggest that PsA and related compounds may be a novel class of anti-colon cancer drugs.

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