Puerarin 6''-O-xyloside possesses significant antitumor activities on colon cancer through inducing apoptosis

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Abstract. Puerarin 6''-O-xyloside (PRX) is a major compound found in the root of the Pueraria lobata (Willd.) Ohwi. The present study aimed to investigate the antitumor activity of PRX against colon cancer and examine its possible mechanism. In the present study, the anti-proliferative effects of PRX against colon cell lines (SW480, LoVo and HCT-116) were evaluated using a Cell Counting Kit-8 assay, and the half maximal inhibitory concentration values of the SW480, LoVo and HCT-11 cells were 37.114, 49.213 and 43.022 µg/ml, respectively. Furthermore, the apoptosis of SW480 cells was detected using flow cytometry with Annexin V-fluorescein isothiocyanate/propidium iodide staining. Subsequently, western blot analysis was performed to examine the expression of proteins associated with apoptosis, invasion and metastasis of tumors. The results showed that PRX possessed antitumor activity against colon cancer cell lines in a dose-dependent and time-dependent manner. In addition, PRX significantly upregulated the expression levels of cleaved (c)-caspase-3, c-caspase-9, B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X protein and phosphorylated c-Jun terminal kinase, and downregulated the expression levels of Bcl-2, matrix metalloproteinase (MMP)-3, MMP-9 and vascular endothelial growth factor (Bcl -2), Bcl -2-associated X protein and phosphorylated c-Jun terminal kinase, and downregulated the expression levels of Bcl-2, matrix metalloproteinase (MMP)-3, MMP-9 and vascular endothelial growth factor (P<0.01). Therefore, the present study demonstrated the PRX exerted antitumor activity against colon cancer cell lines and that the anticancer mechanisms of PRX may be associated with the induction of mitochondria-mediated intrinsic apoptosis, and inhibition of tumor invasion and metastasis. The present study provides a scientific basis for the clinical use of PRX for the treatment of colon cancer.

Introduction

Cancer is the second major cause of mortality around the world, second to cardiovascular diseases (1). The causes of cancer are mainly associated with poor lifestyle behaviors, including smoking, obesity, physical inactivity and poor diet, and changing reproductive patterns (2). In addition, colon cancer is considered the third most commonly diagnosed cancer clinically in the world (3). Currently, the treatment of colon cancer comprises mainly conventional chemotherapy and radiotherapy; however, these therapeutic methods are often accompanied with serious side effects (4). Therefore, it is essential to identify novel effective and safe therapeutic strategies for treating colon cancer. At present, phytotherapy, which uses active anticancer agents isolated from plants to treat cancer, has gained increased attention and has become a widely accepted alternative drug for treating cancer (4). It is reported that etoposide and teniposide, which are extracted from the roots and rhizomes of the Mayapple tree, are used for treating lymphoma, bronchial and testicular cancer (5).

Puerarin, a type of flavonoid, is the major compound in the root of Pueraria lobata (Willd.) Ohwi (P. lobata) (6). It has been widely used in the treatment of cancer, cardiovascular diseases, Parkinson’s disease, Alzheimer’s disease and diabetes (7). It also exerts protective effects against fever, inflammation, hyperlipidemia and oxidative damage (7). Puerarin injection and other forms (tablet and capsule) of puerarin have been used in clinics extensively in China (8). The hydroxyl group at C-6’ of puerarin is often substituted with xylose residues to form puerarin 6''-O-xyloside (PRX; chemical structure shown in Fig. 1) with a high content in the root of P. lobata (9). PRX is one of the major isoflavones of P. lobata (10). Currently, there are numerous investigations on the activities of puerarin. It is reported that PRX has significant antitumor activities against A549 human lung cancer cells (10). However, to the best of our knowledge, there is no relevant report on the effect of PRX against colon cancer.

Therefore, the present study was designed to systemically investigate the antitumor effects of PRX on colon cancer in vitro and examine its possible molecular mechanism. This may be of significant value for the further identification of useful therapeutic agents from this plant to treat diseases clinically.
Materials and methods

Reagents and cell lines. PRX (cat. no. JD-24146) was purchased from Shanghai Jindow Biological Technology Co., Ltd. (Shanghai, China). The SW480, LoVo and HCT-116 colon cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). The DMEM and fetal bovine serum (FBS) were obtained from Invitrogen; Thermo Fisher Scientific, Inc. (Waltham, MA, USA); the Cell Counting Kit-8 (CCK-8) was purchased from Beyotime Institute of Biotechnology (Shanghai, China). Cleaved (c)-caspase-3 (cat. no. ab136812) and c-caspase-9 (cat. no. ab2324) antibodies were purchased from Abcam (Cambridge, MA, USA). B-cell lymphoma 2 (Bcl-2)-associated X protein (Bax; cat. no. ab32503), Bcl-2 (cat. no. ab32124), Bcl-2-associated death promoter (Bad; cat. no. ab32445), c-Jun N-terminal kinase (JNK; cat. no. ab76125), phosphorylated (p)-JNK (cat. no. ab4821), p-Akt (cat. no. ab38449), Akt (cat. no. ab8805), matrix metalloproteinase (MMP)-3 (cat. no. ab38907), MMP-9 (cat. no. ab73734) and vascular endothelial growth factor (VEGF; cat. no. ab1939) antibodies were products of Abcam. Bicinchoninic acid (BCA) protein assay reagent was purchased from Beyotime Institute of Biotechnology (cat. no. P0012S). Silica-gel (100-200 mesh) was purchased from Qingdao Haiyang Chemical Co., Ltd. (Qingdao, China). The Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) kit was purchased from BD Biosciences (San Jose, CA, USA). All other chemicals used in the present study were of analytical reagent grade.

Cell culture. The SW480, LoVo and HCT-116 colon cancer cell lines were maintained in DMEM supplemented with 10% FBS and antibiotics (1% penicillin and 100 µg/ml streptomycin; Beyotime Institute of Biotechnology). The cell lines were cultured in an atmosphere containing 5% CO₂/95% air at 37°C.

Determination of cytotoxicity. The cytotoxicity was evaluated using the CCK-8 assay. A 100-µl cell suspension (5x10⁴ cells/ml) was seeded in 96-well plates and incubated in an atmosphere containing 5% CO₂/95% air at 37°C for 24 h. The cells were then administrated PRX at a series of concentrations (4, 8, 16, 32, 64, 128 and 256 µg/ml) and maintained for 24 h at 37°C. The control cells were treated with 10 µl DMEM for 24 h at 37°C. Subsequently, the CCK-8 assay was performed to determine the percentage of cell proliferation inhibition (n=4) by detecting the optical density (OD) at 450 nm using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The half maximal inhibitory concentration \((IC₅₀)\) values of PRX on the SW480, LoVo and HCT-116 cells were calculated. Additionally, SW480 cells were treated with PRX (16, 32 and 64 µg/ml) for 12, 24, 36 and 48 h to determine the dose-dependent and time-dependent effects of PRX. The inhibitory rate was calculated according to the following formula: \(\frac{(OD_{\text{control}}-OD_{\text{treatment}})}{OD_{\text{control}}}\times100\%\). washed with PBS, centrifuged for 5 min at 500 x g at room temperature and stained using the Annexin V-FITC/PI kit (200 µl Annexin V-FITC and 10 ml PI for every 1x10⁶ cells) for 5 min at room temperature in the dark, according to the manufacturer's protocol.

Western blot analysis. The cells were treated with PRX (15, 30 and 60 µg/ml) for 24 h at 37°C. Total protein was extracted from the cells using the cell lysis buffer for western blot analysis and immunoprecipitation (Beyotime Institute of Biotechnology; cat. no. P0013), and the protein concentration was determined using the BCA protein assay reagent. Subsequently, 35 µg of protein was separated by 12% SDS-PAGE and running buffer [0.3% Tris Base, 1.4% glycine and 20% SDS (pH 8.3)]. The proteins were then transferred onto polyvinylidene fluoride membranes. The membranes were blocked with 5% fat-free dry milk in 1X TBS (containing 0.1% Tween-20) for 2 h at room temperature. The membranes were then incubated at 4°C overnight with the following primary antibodies: Bax (dilution 1:1,000), Bcl-2 (dilution 1:1,000), Bad (dilution 1:1,000), c-caspase-3 (dilution 1:1,000), c-caspase-9 (dilution 1:1,000), JNK (dilution 1:1,000), p-JNK (dilution 1:1,000), p-Akt (dilution 1:1,000), Akt (dilution 1:1,000), MMP-9 (dilution 1:1,000), VEGF (dilution 1:1,000), MMP-3 (dilution 1:1,000) and GAPDH (dilution 1:2,000). The membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:2,000; cat. no. A0286; Beyotime Institute of Biotechnology) at room temperature for 1 h. The proteins were detected using the chemiluminescence ECL kit (Abcam). The signals were quantitated using ImageLab software (version 4.0; Bio-Rad Laboratories, Inc.) on a Chemidoc Imaging instrument. To normalize for protein loading, antibodies directed against GAPDH were used, with protein expression levels expressed relative to GAPDH.

Statistical analysis. The significance of differences between groups was determined by one-way analysis of variance followed by Dunnett's multiple comparisons post hoc test using SPSS software (SPSS for Windows 19.0; IBM SPSS, Armonk, NY, USA). The results are presented as the mean ± standard deviation. \(P<0.05\) was considered to indicate a statistically significant difference.

![Figure 1. Chemical structure of puerarin 6''-O-xyloside.](image-url)
Results

Inhibitory effects of PRX on colon cancer. As shown in Fig. 2, PRX exerted marked inhibitory effects on the three colon cell lines (SW480, LoVo and HCT-116). The IC$_{50}$ values of the SW480, LoVo and HCT-116 cells were 37.114, 49.231 and 43.022 µg/ml, respectively. It was shown that PRX exerted the highest anti-proliferative effect on the SW480 cells, therefore, the SW480 cell line was selected from the three cell lines for further experiments. In addition, as shown in Fig. 3, PRX (16, 32 and 64 µg/ml) exhibited dose-dependent and time-dependent cytotoxic effects against the SW480 cells.

Pro-apoptotic effect of PRX on SW480 cells. The results of the cell viability experiment indicated that PRX exerted notable antitumor activity against the colon cancer cells. To determine whether the anticancer activity of PRX was associated with apoptosis, the apoptosis induced by PRX was determined by staining with Annexin V-FITC/PI followed by flow cytometric analysis. It was found, as shown in Fig. 4A-E, that the number of apoptotic cells was increased gradually by treating the cells with increased concentrations of PRX (15, 30 and 60 µg/ml). These results showed that PRX induced the death of colon cancer cells due to the induction of apoptosis.

Effects of PRX on the protein expression of caspase-3, caspase-9, Bad, Bax and Bcl-2 in SW480 cells. As shown in Fig. 5, the expression levels of caspase-3 and caspase-9 were upregulated gradually following treatment with increased concentrations of PRX (15, 30 and 60 µg/ml; P<0.01). In addition, as shown in Fig. 6, the expression levels of Bad and Bax were significantly increased in a concentration-dependent manner, whereas that of Bcl-2 was significantly decreased (P<0.01). These results demonstrated that PRX-induced apoptosis of SW480 cells may be associated with the mitochondria-mediated apoptotic pathway.

Effects of PRX on the protein levels of PRX on JNK, p-JNK, p-Akt and Akt in SW480 cells. To examine other potential
mechanisms underlying the effect of PRX on the SW480 cells, the expression levels of proteins associated with the JNK/Akt signal pathway were detected, including JNK, p-JNK, p-Akt and Akt. As shown in Fig. 7, PRX had no significant effect on JNK, p-Akt or Akt (P>0.05). However, PRX (15, 30 and 60 µg/ml) significantly upregulated the expression of p-JNK (P<0.01) in a dose-dependent manner, compared with that in the control group.

Effects of PRX on the protein expression of MMP-9, VEGF and MMP-3 in SW480 cells. To examine other possible mechanisms underlying the antitumor activities of PRX on SW480 cells, the expression of proteins associated with tumor invasion and metastasis were detected in the present study. The effects of PRX on the expression levels of MMP-3, MMP-9 and VEGF are shown in Fig. 8. It was found that PRX significantly decreased the expression levels of MMP-9 and VEGF in a concentration-dependent manner (P<0.01). Additionally, the expression of MMP-3 was downregulated following treatment with PRX (30 and 60 µg/ml, P<0.01).

Discussion

The present study is the first, to the best of our knowledge, to systemically investigate the antitumor effect of PRX on colon cancer cell lines in vitro. The results showed that PRX exerted significant anticancer effects against colon cancer cells in vitro through the induction of mitochondria-mediated intrinsic apoptosis and through inhibiting the invasion and metastasis of tumor cells.

It has been reported that plant-derived medicines are safer than synthetic drugs (11), and they have also been demonstrated to be effective in the treatment of various diseases, particularly those that cannot be treated by modern synthetic drugs (12). Therefore, the aim of the present study was to investigate the antitumor activities of PRX isolated from the root of the P. lobata.

Uncontrolled cell proliferation and insufficient apoptosis may be considered primary causes of cancer (13). Apoptosis is a physiological cell suicide process and is regulated by a series of biochemical events that eventually result in cell death (14,15). In addition, apoptosis is considered to be an ideal...
Figure 5. Regulatory effects of PRX on c-caspase-3 and c-caspase-9. Cells were treated with PRX for 24 h, following which total proteins were extracted and subjected to western blot assays. GAPDH was used as an internal control. Data are expressed as the mean ± standard deviation (n=4). **P<0.01 vs. Control. PRX, puerarin 6''-O-xyloside; c-caspase-3, cleaved caspase-3; c-caspase-9, cleaved caspase-9.

Figure 6. Regulatory effects of PRX on Bax, Bad and Bcl-2. Cells were treated with PRX for 24 h, following which total proteins were extracted and subjected to western blot assays. GAPDH was used as an internal control. Data are expressed as the mean ± standard deviation (n=4). **P<0.01 vs. Control. PRX, puerarin 6''-O-xyloside; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; Bad, Bcl-2-associated death promoter.
target for cancer therapy (16). In the present study, PRX exhibited significantly pro-apoptotic effects against SW480 cells. Mitochondria-mediated apoptosis is considered to be a major apoptotic pathway (17). Previous investigations have demonstrated that caspase family proteins are important in apoptosis and inflammatory responses (18). Caspase-9 is considered to be the initiating caspase in the caspase cascade reaction, and activated by cytochrome c (19). Caspase-3 activated by caspase-9 is a crucial death protease and is considered to be a bio-marker for identifying whether cells are undergoing apoptosis (20,21). Additionally, the Bcl-2 family proteins are crucial in mitochondria-mediated apoptosis, and are considered the initial regulatory step in the induction of mitochondrial apoptosis (22). In the Bcl-2 family, Bcl-2, Bax and Bad are considered to be apoptosis-associated proteins. The function of Bcl-2 is to bind and suppress the other pro-apoptotic relevant proteins of the Bcl-2 family; however, Bax and Bad directly promote the release of cytochrome c into the cytoplasm and inhibit anti-apoptotic Bcl-2 proteins (23). The results of the present study demonstrated that treatment of SW480 cells with PRX resulted in a significant upregulation in the expression levels of c-caspase-3, c-caspase-9, Bad and Bax, and the downregulation of Bcl-2. These findings showed that PRX induced mitochondria-mediated apoptosis in the SW480 colon cancer cell line.

JNKs can bind to and phosphorylate c-Jun on Ser-63 and Ser-73 within its transcriptional activation domain. p-JNK can modify the activity of proteins that reside in the mitochondria or act in the nucleus (24). In addition, JNK can lead to cell apoptosis via triggering the release of cytochrome c from the mitochondria into the cytoplasm and triggering the caspase cascade reactions (24,25). Akt is a serine/threonine-specific protein kinase, which is important in apoptosis and cell proliferation. Activated Akt inhibits several pro-apoptotic factors, including Bad and procaspase-9 (23). The results of the present study indicated that PRX significantly upregulated the expression levels of p-JNK, which indicated that the mitochondria-mediated apoptosis induced by PRX may be associated with the Akt/JNK signaling pathway.

MMPs are important in the degradation of extracellular matrix (ECM) components, which is important in tumor invasion and metastasis (26,27). MMP-3 and MMP-9 are the important proteases involved in cancer invasion and
metastasis. MMP-9, activated by MMP-3, can destroy and degrade ECM on the surface of the tumor, and eventually accelerate the invasion and metastasis of the tumor (28). In addition, MMP-9 promotes the growth and diffusion of tumors (28). It has been reported that VEGF is one of the key components of wound healing by promoting angiogenesis (29). VEGF can promote the formation of MMPs and the growth and metastasis of tumor via accelerating the formation of new vessels (30). Therefore, the expression of MMP-3, MMP-9 and VEGF can be used to determine the possibility of growth and metastasis of tumors. In the present study, the results showed the downregulation of MMP-3, MMP-9 and VEGF, indicating that PRX inhibited SW480 colon cancer cell invasion and metastasis.

In conclusion, the present study demonstrated that PRX exerted notable antitumor effects against colon cancer cell lines through the induction of mitochondria-mediated intrinsic apoptosis, and inhibition of tumor invasion and metastasis.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

BBW conceived and designed the study; XLZ and JSM performed the experiments; XLZ and BBW analyzed the data; BBW and XLZ wrote the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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