

Phenolic alkaloids from *Menispermum dauricum* inhibits BxPC-3 pancreatic cancer cells by blocking of Hedgehog signaling pathway

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Submitted: 03-12-2014

Revised: 05-02-2015

Published: 16-09-2015

ABSTRACT

Background: The Hedgehog (Hh) signaling pathway plays an important role in pancreatic cancer (PC) cells. Phenolic alkaloids from *Menispermum dauricum* (PAMD), a traditional Chinese medicine used for the treatment of immune disorders, have been reported to have antitumor activity recently. **Objective:** To investigate the efficacy and mechanism of PAMD against PC cell BxPC-3. **Materials and Methods:** F assay was used to assess cell proliferation inhibition of PAMD; the apoptotic induction and cell cycle arrest was detected by flow cytometry; the BxPC-3 xenograft was established to evaluate the tumor growth inhibition of PAMD; hematoxylin-eosin staining was applied to analyze the pathological morphology of tumor tissues; immunohistochemistry (IHC) and Western blot was adopted to detect the protein levels; quantitative real-time polymerase chain reaction was used to determine the mRNA expressions. **Results:** PAMD shows time- and dose-dependent proliferation inhibition on the BxPC-3 cell, induced G0/G1 phase arrest and cell apoptosis *in vitro*. PAMD also showed better inhibition of tumor growth and a preferable safety profile compared with chemotherapeutic regimen 5-fluoro-2, 4 (1 H, 3 H) pyrimidinedione in BxPC-3 xenograft *in vivo*. Furthermore, PAMD directly decreases the protein and mRNA levels of Sonic Hedgehog (Shh) and its downstream transcription factor Gli-1 in the BxPC-3 tumor tissues. **Conclusion:** The treatment of PAMD displayed Hh signaling pathway blockade through decreasing the protein and mRNA levels of Shh and its downstream transcription factor Gli-1, suggesting a promising strategy in treating human PC.

Key words: BxPC-3, Gli-1, pancreatic cancer, phenolic alkaloids from *Menispermum dauricum*, Sonic Hedgehog

INTRODUCTION

Pancreatic cancer (PC) is one of the most aggressive malignancies among the human cancers, and its overall 5 years survival is <5%.^[1,2] The poor clinical prognosis may be due to the lack of early symptoms and reliable diagnostic markers for early detection.^[3] Furthermore, apart from chemotherapy and radiation therapy, there are no effective therapies for the treatment of refractory PC.^[4] Therefore, the development of novel therapeutic strategies is urgently required.

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The Hedgehog (Hh) is an essential protein during embryonic development and adult stem cell function.^[5-7] Its signaling is minimal in differentiated tissues of healthy adults,^[8] and aberrant activated Hh has been detected in about 70% of pancreatic ductal adenocarcinomas (PDAC),^[9] thus Hh has got a lot of attention as a critical target of PC. Sonic Hedgehog (Shh), a member of the Hh family, initiated the signaling pathway by binding to the Hh receptor Patched (Ptc) and removing the inhibition on the transmembrane protein Smothered,^[10] thereby leading to the activation of the Gli transcription factors that control cell growth, survival, and differentiation in various tissues.^[11-14] Blockade of Hh pathway has been shown to inhibit PC cancer cell growth, invasion, and metastases.^[6,15-17] Recent studies demonstrated that inhibition of the Hh pathway induces autophagy in PDAC cells,^[18] and combination with chemotherapy leads to tumor reduction in PC.^[19]

Access this article online

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DOI:

10.4103/0973-1296.165548

Quick Response Code:



Therefore, the inhibition of Hh signaling pathway have been considered to be a potent strategy in PC therapies.

Phenolic alkaloids from *Menispermum dauricum* (PAMD), a traditional Chinese herb prescription with main constituents of daurisolin, dauricine, daurinolone and dauricoline,^[20] has been widely used for the treatment of immune disorders such as cardiovascular and thrombosis disorders for thousands of years.^[21] Recent studies also show that PAMD has antitumor activity through inducing apoptosis and inhibiting the proliferation and invasion in colon and urinary cancer cells.^[22,23] The aim of the present work is to study the tumor suppressive efficacy of PAMD, which shows potent anti-PC ability through blockade of Hh signaling pathway.

MATERIALS AND METHODS

Cell lines and reagents

Human PC cell line BxPC-3, obtained from Shanghai Institute of Biochemistry and Cell Biology, was maintained in RPMI-1640 (Gibco) medium supplemented with 10% fetal calf serum in a humidified atmosphere containing 5% CO₂ at 37°C.

Phenolic alkaloids from *Menispermum dauricum* was prepared as mentioned in,^[20] by Prof. Wang Dong from Heilongjiang University of Chinese Medicine. Primary antibodies against Ki67, Shh and Gli-1 were purchased from Santa Cruz. 5-fluoro-2,4-dihydro-2,4-dimethylthiazol-5(4H)-pyrimidin-6(1H)-one (5-Fu), 1-(4, 5-Dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) and trypsin were obtained from Sigma. SYBR Premix Ex Taq and PrimeScript RT kits were brought from Takara Company.

1-(4, 5-Dimethylthiazol-2-yl)-3,5-diphenylformazan assay

Cells (5×10^3 /well) were seeded into 96-well plates and treated with PAMD, 5-Fu or saline control. At the end of the incubation period, cell viability was assessed by MTT assay. Formazan crystals formed were solubilized in dimethyl sulfoxide, and the optical density of the resulting solution was recorded at 570 nm on a microplate reader (Thermo, USA).

Cell cycle analysis

Cells were seeded in 6-well plates and treated with PAMD, 5-Fu or saline for 24 h. The cells were harvested, washed with phosphate-buffered saline (PBS), and fixed with 70% ethanol at -20°C overnight. After an additional washing, cells were incubated with RNase A (20 µg/mL) at 37°C for 30 min, stained with propidium iodide (100 µg/mL; Sigma-Aldrich) for 10 min, and analyzed with flow cytometry (BD FACSC autoTM II).

Apoptosis assay

Cells were seeded in 6-well plates and treated with PAMD, 5-Fu or saline for 24 h. Apoptosis was determined with the Annexin-V: FITC Apoptosis Detection Kit I (BD Biosciences) according to the manufacturer's protocol. Briefly, the saline control and drug-treated tumor cells were collected via centrifugation and washed once with PBS. The cells were subsequently stained with fluorescein and propidium iodide for 15 min at room temperature and analyzed with flow cytometry (BD FACSC autoTM II).

Tumor xenografts

BALB/C nu/nu female mice, aged 4–6 weeks, weighing about 20 g, were obtained from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. BxPC-3 cells (1×10^7 , suspended in 200 µL of PBS) were implanted s. c. in the hind flank of each mouse. Once palpable tumors were established, animals were randomly divided into four groups so that all groups had similar starting mean tumor volumes of 100–150 mm³. The six mice in each group were injected intraperitoneally with the saline control, 5-Fu or PAMD. Tumor volumes were calculated using the following ellipsoid formula: $(D \times [d^2])/2$, in which D is the large diameter of the tumor, and d is the small diameter. Tumor growth inhibition was determined using the following formula: $100\% \times ([WC-WT]/WC)$, in which WC represents mean tumor weight of the saline group, and WT represents mean tumor weight of a treated group. Spleen index = mass of spleen (mg)/body weight of mouse (g). All animal experiments were approved by Animal Care and Use Committee, Heilongjiang University of Chinese Medicine, China.

Hematoxylin-eosin staining

Tumor tissues were taken out before fixed in 4% paraformaldehyde and embedded in paraffin. Paraffin sections were treated by dewaxing, dehydration, hematoxylin staining, hydrochloric acid ethanol differentiation, ammonia treatment, eosin staining, ethanol dehydration, transparentization, drying and gum mounting processes before optical microscope observation.

Immunohistochemistry

After fixation with formalin, tissues were embedded with paraffin, cut, and mounted on slides. Then, slides were washed with xylene to deparaffinize, with graded ethanol to rehydrate, incubated with citrate buffer to retrieve antigen and blocked with 3% H₂O₂ to inactivate endogenous peroxidase. Slides were incubated with primary antibody at 4°C overnight, followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody for 30 min at room temperature. Complex visualization was done with a 2-solution DAB

Kit (Invitrogen). Contrast was applied with hematoxylin, and the slides were mounted in Canadian balsam and observed with a light microscope.

Western blot

Tumor tissue was homogenated with NP-40 lysis buffer (50 mmol/L Tris pH 8.0, 150 mmol/L NaCl, and 1% NP-40) at the proportion of 1 g/mL supplemented with phenylmethanesulfonyl fluoride (Sigma Aldrich) and PhosSTOP (Roche Diagnostics) at 4°C, and centrifuged at $12,000 \times g$ for 10 min. The supernatant was collected as the total protein extract. Protein concentration was estimated using a Pierce BCA Protein Assay Kit (Thermo Scientific) according to the manufacturer's protocol. Equal amounts of protein were analyzed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Thereafter, proteins were transferred to nitrocellulose membranes and blotted with specific primary antibodies. Proteins were detected via incubation with HRP-conjugated secondary antibodies. Results were recorded by Alpha image 2000 and analyzed by Image-Pro Plus.

Quantitative real-time polymerase chain reaction

Total RNA extraction of tumor tissue was performed with TRIzol reagent (Life Technologies Corporation), and first-strand cDNA was synthesized using 1 µg of total RNA (concentrations measured by NanoDrop 2000, Thermo Scientific) according to the manufacturer's instructions. Quantitative RT-PCR analysis was performed in triplicate with StepOnePlus RT-PCR system (Life Technologies).

The $\Delta\Delta CT$, two triangles+CT method was used to calculate relative expression. Primer sequences (synthesized by Shanghai Generay Biotech Co., Ltd.) used in RT-PCR for Shh (forward 5'-GCTCGGTGAAAGCAGAGAACT-3' and reverse 5'-CCCAGGAAAGTGAGGAAGTCG-3'), and Gli-1 (forward 5'-ATCCTTACCTCCCAACCTCTGT-3' and reverse 5'-CCAACCTTCTGGCTCTTTCCTGTA-3'), using GAPDH (forward 5'-AGAAGGCTGGGGCTCAITTG-3' and reverse 5'-AGGGGCCATCCACAGTCTTC-3') as control.

Statistical analysis

Analysis of variance was used for comparisons across multiple groups. The data are reported as means \pm standard deviation. Statistical analysis was performed using SPSS Statistics 19.0; ($P < 0.05$) was considered statistically significant.

RESULTS

Phenolic alkaloids from *Menispermum dauricum* inhibits the proliferation of BxPC-3 pancreatic cancer cells

The effects of PAMD on cell proliferation were assessed with an MTT assay. The proliferation of BxPC-3 cells was markedly inhibited by PAMD and showed a time and dose-dependent manner, with IC_{50} values of 2.37, 1.78 and 1.25 µg/mL for 24, 48 and 72 h, respectively [Figure 1].

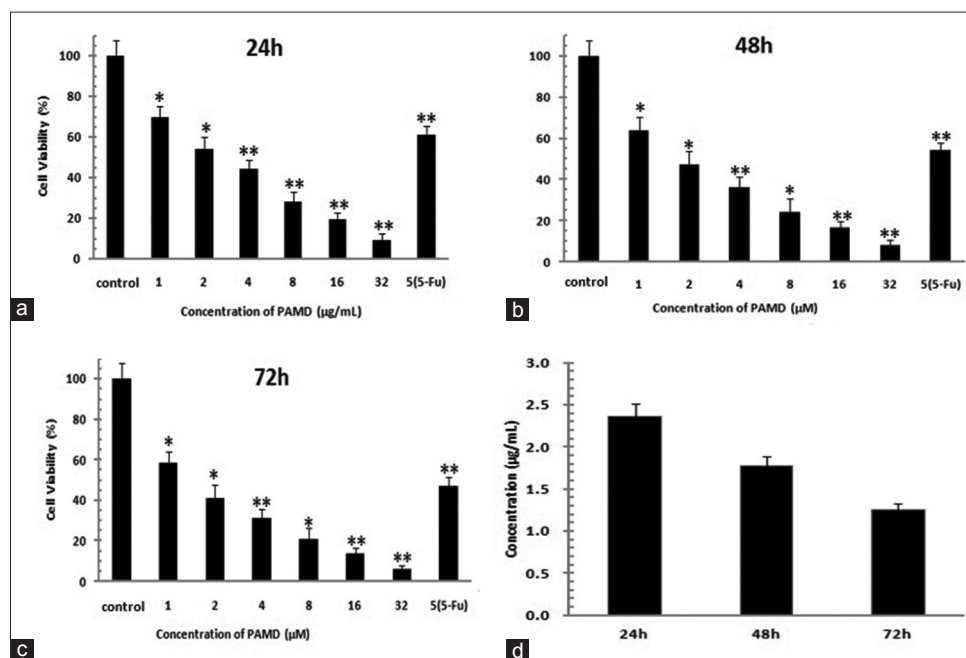


Figure 1: Phenolic alkaloids from *Menispermum dauricum* (PAMD) inhibits the proliferation of BxPC-3 pancreatic cancer cells. BxPC-3 pancreatic cancer cells were treated for (a) 24 h, (b) 48 h and (c) 72 h in the absence or presence of PAMD ranging from 1 to 32 µg/mL. Cell viability was measured with 1-(4, 5-Dimethylthiazol-2-yl)-3,5-diphenylformazan and expressed as a percentage of saline control. Results are presented as means \pm standard deviation of three independent experiments. * $P < 0.05$: Significant difference; ** $P < 0.01$: Very significant difference from control by analysis of variance. (d) The half maximal inhibitory concentrations (IC_{50}) of PAMD against BxPC-3 for 24, 48 and 72 h, respectively

Phenolic alkaloids from *Menispermum dauricum* induces BxPC-3 pancreatic cancer cells G0/G1 phase arrest and induces apoptosis

Compared to the control cells, PAMD-treated cells displayed obvious arrest of cell cycle in the G0/G1 phase after 24 h. The increase in the G0/G1 cell population was accompanied by a concomitant decrease in the population in the S and G2/M phases of the cell cycle, while the 5-Fu-treated groups showed an enhanced cell population in S phase, indicating the difference of anti-tumor mechanism [Figure 2a and b], ($P < 0.05$).

BxPC-3 cells were treated with PAMD for 24 h and analyzed for apoptotic cell death using an Annexin-V: FITC Apoptosis Detection Kit. The results revealed a dose-dependent induction of apoptotic cell death by PAMD [Figure 2c and d].

Phenolic alkaloids from *Menispermum dauricum* inhibits the tumor growth of BxPC-3 xenografts

BxPC-3 human PC xenografts were established to assess the chemotherapeutic potential of PAMD. The

antitumor activity of PAMD were determined with the positive control of 20 mg/kg/day 5-Fu [Figure 3a and b]. The results showed that PAMD exhibits better PC tumor suppression than 5-Fu, with tumor inhibition rates of 45.17% ($P < 0.05$) and 57.35% ($P < 0.01$) for 10 and 20 mg/kg¹/day PAMD, while 41.38% ($P < 0.05$) for 20 mg/kg¹/day 5-Fu. All treatment groups exhibited weight loss with significant tumor suppression compared with the saline group ($P < 0.01$). However, PAMD also showed the better safety profile than 5-Fu with respect to variation in body weight ($P < 0.05$), while no significant changes between the body weight of different PAMD dose groups ($P > 0.05$) [Figure 3c]. Importantly, the spleen indexes indicates better mice immunity after treated with PAMD than that of 5-Fu ($P < 0.01$), and no significant difference between PAMD groups ($P > 0.05$) [Figure 3d].

Pathological analysis of the BxPC-3 tumor tissues

Hematoxylin and eosin stained tissue sections from each group were distinct with each other [Figure 4A]. Samples from the saline group showed imbalanced nuclear/plasma

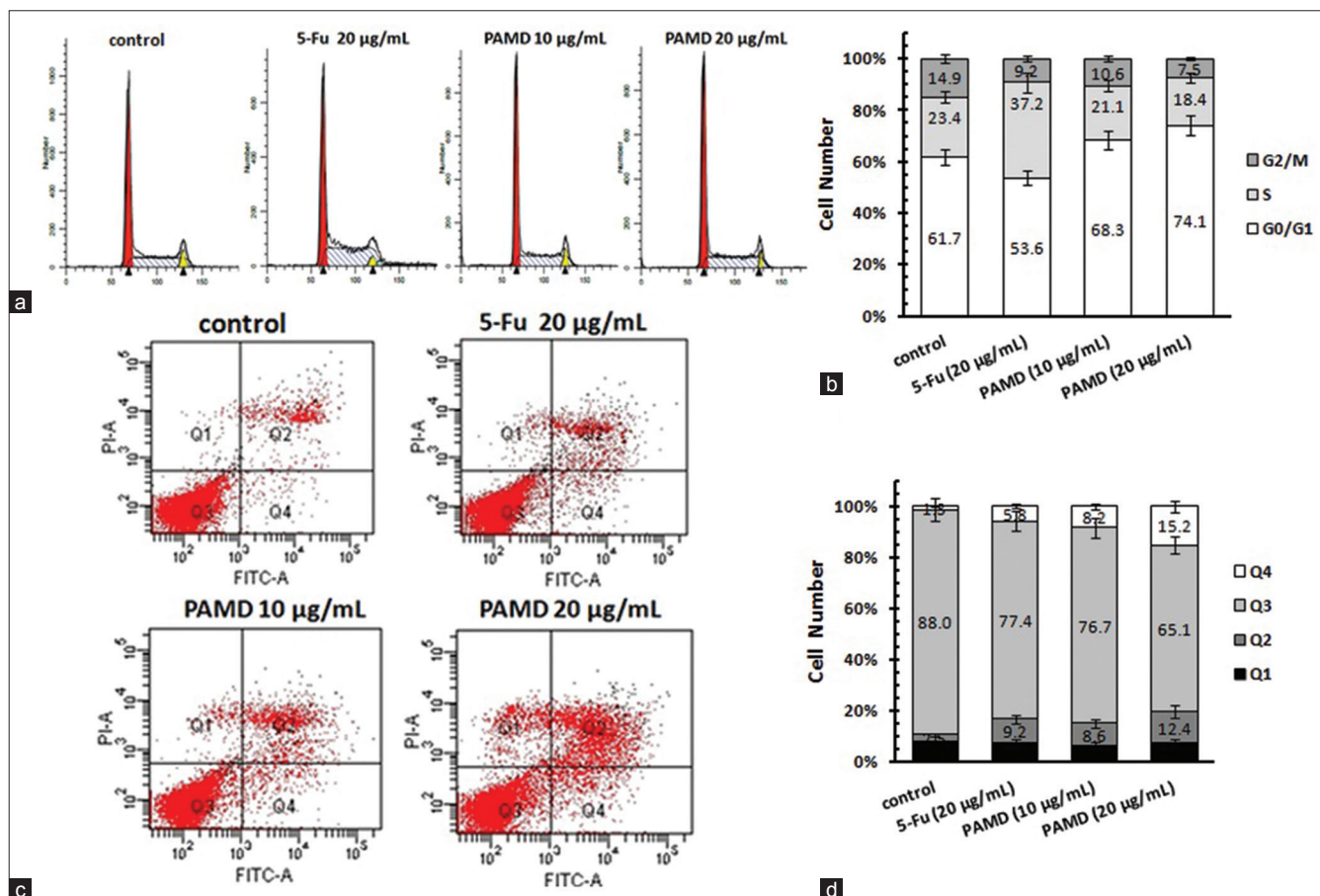


Figure 2: Phenolic alkaloids from *Menispermum dauricum* (PAMD) induces BxPC-3 pancreatic cancer cells G0/G1 phase arrest and induces apoptosis. (a) and (b) BxPC-3 cells were treated with PAMD, 5-fluoro-2, 4 (1 H, 3 H) pyrimidinedione (5-Fu) or saline for 24 h. The cells were then fixed with 70% ethanol at -20°C overnight, incubated with RNase A at 37°C for 30 min, stained with propidium iodide for 10 min, and analyzed with flow cytometry. (c) and (d) BxPC-3 cells were treated with PAMD, 5-Fu or saline for 24 h, and apoptotic cell death was detected by staining cells with an Annexin-V: FITC Apoptosis Detection Kit for analysis with flow cytometry

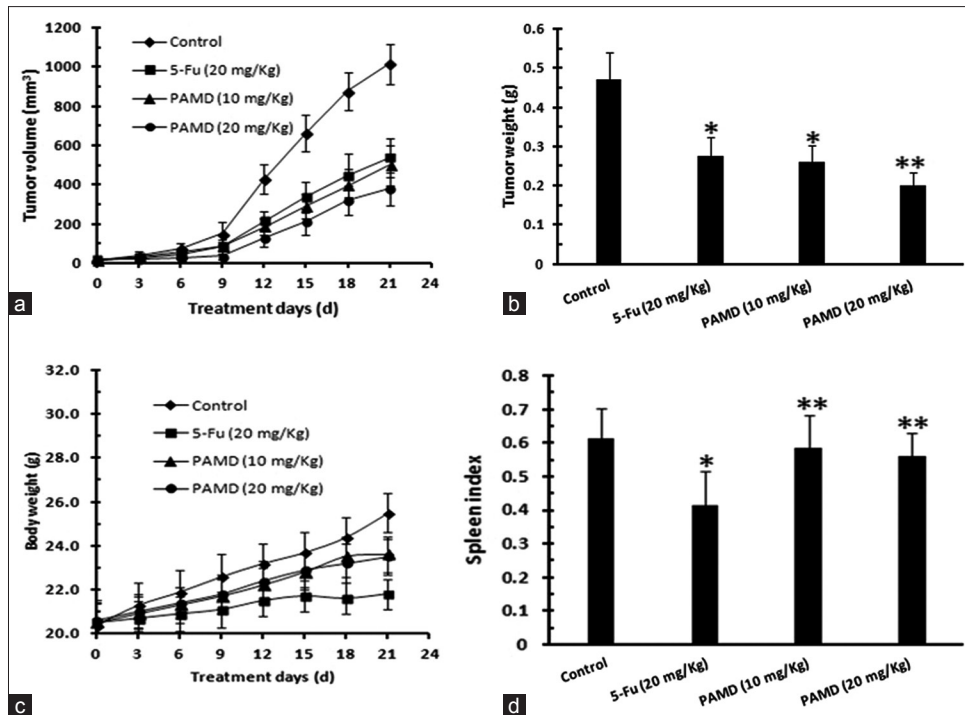


Figure 3: Phenolic alkaloids from *Menispermum dauricum* (PAMD) inhibits the tumor growth of BxPC-3 xenografts. Randomly grouped nude mice bearing BxPC-3 tumors were treated with saline control (control, C), 5-Fu (20 mg/kg), PAMD (10 mg/kg) or (20 mg/Kg) per day. Tumor volume (a) and body weight (c) were measured every 3 days. The mice were sacrificed until the tumor sizes of the saline control groups reached approximately 1000 mm³. Tumors weight (b) and spleen weights were recorded. (d) The spleen index were determined by the ratio of spleen/body weight

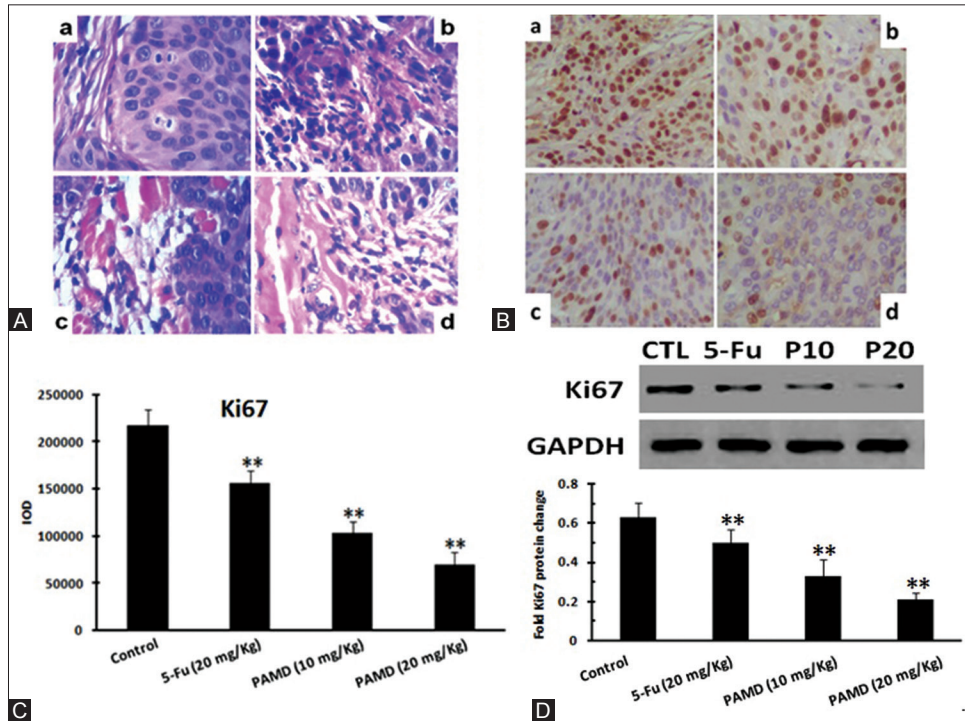


Figure 4: Pathological analysis of the BxPC-3 tumor tissues. (A) HE staining and (B) immunohistochemistry showed Ki67 expressions in a-d. (a) Control group; (b) 5-fluoro-2, 4 (1 H, 3 H) pyrimidinedione (20 mg/Kg) treated group; (C) Phenolic alkaloids from *Menispermum dauricum* (PAMD) (10 mg/Kg) treated group; (d) PAMD (20 mg/Kg) treated group. (C) The integrated option density of immunohistochemistry and (D) Western blot pictures showed Ki67 levels in the BxPC-3 tumor tissues and were analyzed by ImagePro Plus. Results are presented as means ± standard deviation of three independent experiments. **P* < 0.05: Significant difference; ***P* < 0.01: Very significant difference

ratio and multiple pathologic mitoses. The cancer cells were infiltrative when small glands invaded in a diffuse manner, and the tumor border was not distinct. The 5-Fu treated tumor tissues displayed less pathologic mitoses and invasive cancer cells with slight lymphocytes around the tumor. The tumor tissue was significantly reduced in PAMD treated groups. Minimal necrosis existed in the tumor tissues of low dose group while large areas of necrosis and white cell debris could be noticed in that of the high dose group.

We next examined the effects of PAMD on cell proliferation in tumor tissues derived from control and drug-treated mice using anti-Ki67 antibody [Figure 4B-D]. IHC and the Western blot analysis confirmed greater decreases of Ki67 expression in the both PAMD-treated groups compared with the control ($P < 0.01$) or 5-Fu-treated groups ($P < 0.01$), and the high dose group downregulated Ki67 expression more effectively than the low-dose group ($P < 0.05$) tested by both IHC and Western blot, indicating the cell proliferative inhibition of PAMD in BxPC-3 tumor tissues.

Inhibition of Hedgehog signaling pathway by Phenolic alkaloids from *Menispermum dauricum* downregulates the protein and mRNA levels of Sonic Hedgehog and Gli

As Hh signaling is an essential pathway during embryonic pancreatic development, we also tested the expression of Shh and its downstream transcription factor Gli-1 through IHC [Figure 5A-D].^[24-26] The immunostained tissue samples revealed that greater decreases in Shh and Gli-1 expressions in the PAMD-treated group than that of the 5-Fu-treated group compared with the control group in BxPC-3 tumor, indicating that the anti-PC mechanism of PAMD probably act through the blockade of Hh pathway.

To get further insight into the anti-PC mechanism of PAMD, we tested the BxPC-3 tumor tissues with western blot and quantitative RT-PCR. Our data showed that treatment with PAMD results in decreases in Shh and Gli-1 protein levels [Figure 5E and F]. Similar results were obtained for mRNA expressions. Quantitative RT-PCR showed that the mRNA levels of Shh and Gli-1 was sharply downregulated by 10 and 20 mg/Kg PAMD, while 5-Fu only merely affect the expression of mRNA [Figure 5G and H].

DISCUSSION

Hedgehog directed therapeutics offer new hope in the treatment of PC, as it potentially causes tumor regression by intrinsic inhibition of the Hh pathway, decreases

the cancer stem population, has a paracrine effect on the tumor stromal cells and improves the therapeutic efficacy of cytotoxic drugs by affecting the desmoplastic fibrotic response and improving chemotherapeutic drug bioavailability.^[27] In the present study, we demonstrated both the *in vitro* and *in vivo* antitumor effects of the traditional Chinese medicine PAMD on BxPC-3 PC cells.

The antagonistic efficacy against human PC cells has been demonstrated that PAMD significantly inhibits the proliferation of BxPC-3 cells in a time and dose-dependent manner, with the IC_{50} of 2.37, 1.78 and 1.25 $\mu\text{g}/\text{mL}$ for 24, 48 and 72 h, respectively [Figure 1]. It also arrests G0/G1 cell cycle progression and induces cell apoptosis detected by flow cytometry [Figure 2]. Furthermore, it has been shown that PAMD displays anti-PC effects in the *in vivo* animal model [Figures 3A and B]. The administration of PAMD displayed an inhibitory effect on BxPC-3-derived tumors was more preferable in both the antitumor activity and mouse body weight than that of 5-Fu, one of the most widely used chemotherapy drugs. The pathological analysis showed that PAMD reduces the tumor tissue size, inhibits tumor cell proliferation, induces cancer cell necrosis and reduced the side and toxic effect by protecting mice immune function [Figures 3C, 3D and 4]. We have showed that PAMD directly decreases the protein and mRNA levels of Shh and its downstream transcription factor Gli-1 in the BxPC-3 tumor tissues [Figure 5], leading to the blockade of Hh pathway, which may be the primary mechanism mediating the anti-PC activity of PAMD.

Traditional Chinese medicine has been studied as a treatment to the malignant tumor for a long time, with the advantage of synergic effect on multiple oncogenes and overall coordination with preferable safety profile.^[28] In the present study, we confirmed the outstanding anti-PC activity and safety profile of PAMD compared with the traditional chemotherapy drug 5-Fu. These data suggest that the administration of PAMD may be a novel approach to PC treatment, and further investigation should be conducted for the detailed antitumor mechanism of PAMD.

CONCLUSION

The traditional Chinese medicine PAMD shows potent anti-tumor effects in BxPC-3 human PC cell both *in vitro* and *in vivo*. The treatment of PAMD displayed Hh signaling pathway blockade through decreasing the protein and mRNA levels of Sonic (Hh) and its downstream transcription factor Gli-1, suggesting a promising strategy in treating human PC.

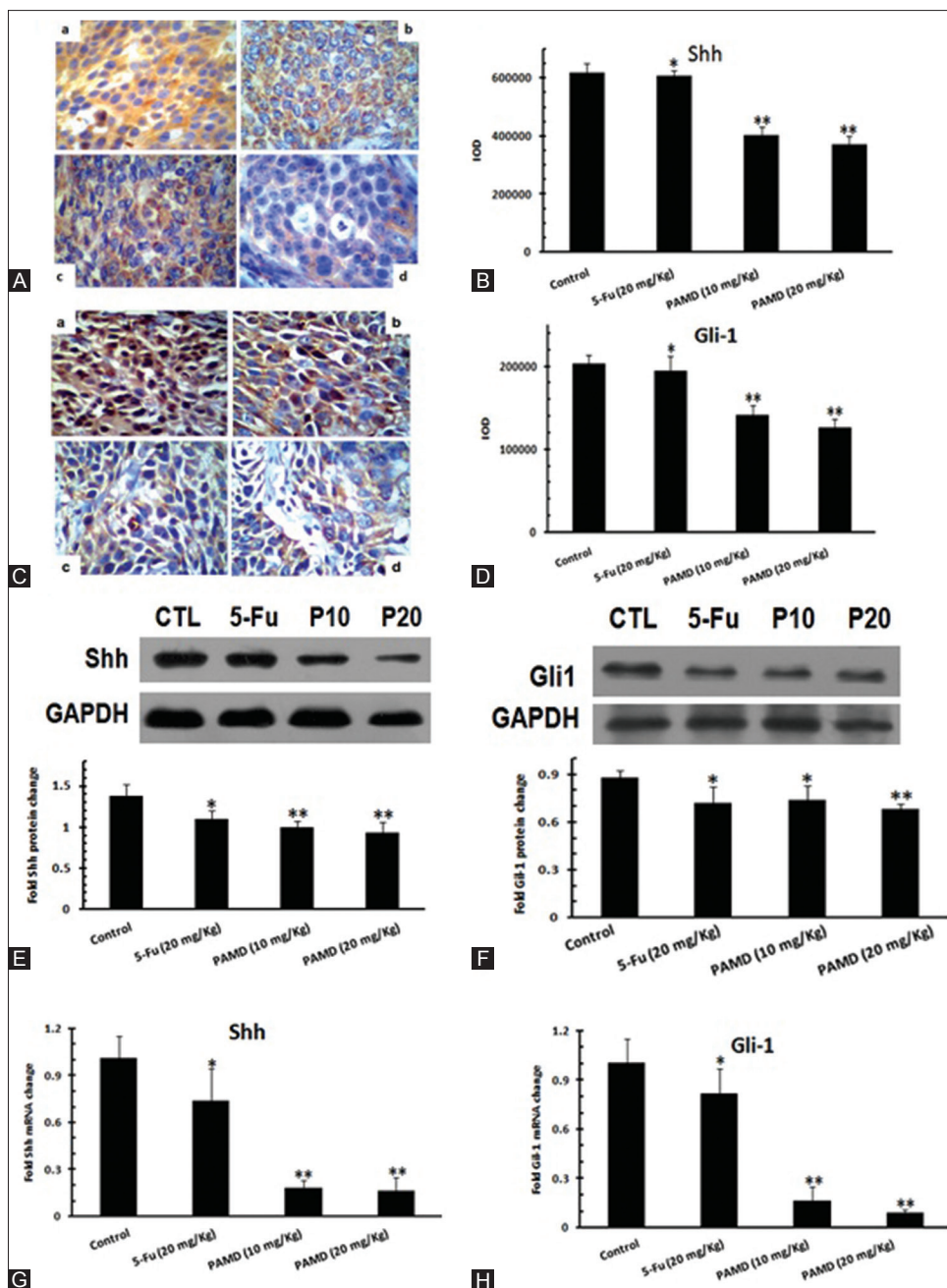


Figure 5: Inhibition of Hedgehog signaling pathway by Phenolic alkaloids from *Menispermum dauricum* (PAMD). (A) and (C) immunohistochemistry showed Shh and Gli-1 levels in the BxPC-3 tumor tissues. (a) Control group; (b) 5-Fu (20 mg/Kg) treated group; (c) PAMD (10 mg/Kg) treated group; (d) PAMD (20 mg/Kg) treated group. (B) and (D) integrated optical density of immunohistochemistry analyzed by ImagePro Plus. (E) and (F) Western blot showed Sonic Hedgehog (Shh) and Gli-1 protein levels. (G) and (H) qRT PCR of Shh and Gli-1. Results are presented as means \pm standard deviation of three independent experiments. * $P < 0.05$: Significant difference; ** $P < 0.01$: Very significant difference

ACKNOWLEDGMENTS

The work is funded by the Natural Science Foundation of China (81373777 and 81173599), Chinese Education Department Foundation for high school doctoral program (20112327120004), Heilongjiang province postdoctoral scientific research program (LBH-Z14196), and Heilongjiang University of Chinese medicine outstanding innovative talents support program (2012 red18).

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Cite this article as: Zhou Zg, Zhang Cy, Fei Hx, Zhong Li, Bai Y. Phenolic alkaloids from *Menispermum dauricum* inhibits BxPC-3 pancreatic cancer cells by blocking of Hedgehog signaling pathway. *Phcog Mag* 2015;11:690-7.

Source of Support: Nil, **Conflict of Interest:** None declared.