Pien Tze Huang inhibits tumor angiogenesis in a mouse model of colorectal cancer via suppression of multiple cellular pathways

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Abstract. Angiogenesis plays an essential role in cancer progression, which therefore has become an attractive target for anticancer treatment. Tumor angiogenesis is tightly regulated by multiple signaling pathways that usually function redundantly; in addition, crosstalk between these pathways forms a complicated network that is regulated by compensatory mechanisms. Given the complexity of pathogenic mechanisms underlying tumor angiogenesis, most currently used angiogenesis inhibitors that only target single pathways may be insufficient and probably generate drug resistance, thus, increasing the necessity for development of novel anticancer agents. Traditional Chinese medicines (TCM) are receiving great interest since they have relatively fewer side-effects and have been used for thousands of years to clinically treat various types of diseases including cancer. Pien Tze Huang (PZH), a well-known traditional Chinese formulation that was first prescribed 450 years ago, has long been used as an alternative remedy for cancers. However, the precise mechanism of PZH's anticancer activity remains to be further elucidated. Using a colorectal cancer mouse xenograft model, in the present study, we evaluated the effect of PZH on tumor angiogenesis and investigated the underlying molecular mechanisms. We found that PZH inhibited tumor growth since PZH treatment resulted in decrease in both tumor volume and tumor weight in CRC mice. In addition, PZH suppressed the activation of several signaling pathways such as STAT3, Akt and MAPKs. Consequently, the inhibitory effect of PZH on these pathways resulted in the inhibition of tumor angiogenesis as demonstrated by the decrease of microvessel density in tumor tissues. Moreover, PZH treatment reduced the expression of angiogenic factors including iNOS, eNOS, VEGF-A, bFGF as well as their specific receptors VEGFR2 and bFGFR. Altogether, our findings suggest that inhibition of tumor angiogenesis via suppression of multiple signaling pathways might be one of the mechanisms whereby PZH affects cancers.

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Abbreviations: PZH, Pien Tze Huang; TCM, traditional Chinese medicine; STAT3, signal transducer and activator of transcription 3; MAPK, mitogen-activated protein kinase; CRC, colorectal cancer; IHS, immunohistochemical staining; MVD, microvessel density; VEGF-A, vascular endothelial growth factor A; bFGF, basic fibroblast growth factor; NOS, nitric oxide synthase

Key words: Pien Tze Huang, traditional Chinese medicine, anticancer treatment, tumor angiogenesis, signaling pathway

Introduction

Angiogenesis, the formation of new blood vessels from the pre-existing vasculatures, plays an important role in a wide range of biological processes including wound healing, reproduction and embryonic development. However, deregulation of this vital process is also essential for cancer progression (1-4). In the initial stage, tumor cells obtain oxygen and nutrients from nearby blood vessels by simple passive diffusion. However, when tumor grows to reach a size larger than 2 mm^3, oxygen delivery by diffusion is no longer sufficient, which causes tumor cells to induce the sprouting of new blood vessels to support the continued growth of tumor and provide an avenue for hematogenous metastasis (5-9).

The process of angiogenesis is highly regulated by multiple cellular signaling transduction pathways including signal transducer and activator of transcription 3 (STAT3), serine-threonine kinase Akt and mitogen-activated protein kinases

(MAPKs) (10-14). Aberrant activation of these pathways promotes tumor angiogenesis by inducing the expression of numerous critical angiogenic stimulators (15-17), including vascular endothelial growth factor A (VEGF-A), basic fibroblast growth factor (bFGF) and nitric oxide (NO) (18-22).

Due to the essential role of angiogenesis in cancer progression and metastasis, inhibition of tumor angiogenesis has become a promising strategy for anticancer chemotherapy. A variety of anti-angiogenic agents is currently in preclinical development, with some of them now entering clinical trials. However, the angiogenesis-related signaling pathways are usually redundant; and crosstalk between these pathways form a complicated and robust network that is regulated by compensatory mechanisms. Therefore, most currently used angiogenesis inhibitors that target only a single pathway may be insufficient and probably generate drug resistance (23). These problems highlight the need for the development of novel anticancer agents. Natural products, such as traditional Chinese medicine (TCM), have been used clinically to treat various kinds of diseases including cancer for thousands of years (24-26). TCM formula is a complex combination of many natural products, each of which contains numerous chemical compounds. TCM formulas, therefore, are considered to be multi-component and multi-target agents exerting their therapeutic function in a more holistic way. Pien Tze Huang (PZH) is a well-known traditional Chinese formulation that was first prescribed 450 years ago by a royal physician in the Ming Dynasty. The main ingredients of PZH include *Moschus*, *Calculus Bovis*, *Snake Gall* and *Radix Notoginseng*. These products together confer PZH properties of heat-clearing, detoxification, promotion of blood circulation and removal of blood stasis (27). Since in the Chinese medicine system accumulation of toxic dampness and heat is one of the major causative factors in the pathogenesis of cancers, PZH is believed to be an effective anticancer agent. In fact, PZH has long been used as an alternative remedy for cancers in China and Southeast Asia. Recently, we reported that PZH can inhibit colorectal cancer growth *in vivo* and *in vitro* via promotion of cancer cell apoptosis and inhibition of cell proliferation, which is probably mediated by its inhibitory effect on activation of STAT3 pathway in tumor tissues (28-32). To further elucidate the mechanism of the tumoricidal activity of PZH, in the present we used a colorectal cancer mouse xenograft model to evaluate the effect of PZH on tumor angiogenesis and investigated the underlying molecular mechanisms.

Materials and methods

Materials and reagents. Pien Tze Huang (PZH) was obtained from and authenticated by the sole manufacturer Zhangzhou Pien Tze Huang Pharmaceutical Co., Ltd., China (Chinese FDA approval No: Z35020242). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, Trypsin-EDTA, TRIzol reagent, were purchased from Invitrogen (Grand Island, NY, USA). All antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Bio-Plex phosphoprotein assay kits were purchased from Bio-Rad Laboratories (Hercules, CA, USA). All the other chemicals, unless otherwise stated, were obtained from Sigma Chemicals (St. Louis, MO, USA).

Cell culture. Human colon carcinoma HT-29 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were grown in DMEM containing 10% (v/v) FBS, 100 Units/ml penicillin and 100 μg/ml streptomycin in a 37˚C humidified incubator with 5% CO_2. The cells were subcultured at 80-90% confluency.

Animals. Athymic male nude mice were obtained from the Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and housed in specific pathogen-free rooms in an environment with controlled temperature (22˚C), humidity, and a 12-h light/dark cycle. Food and water were given *ad libitum* throughout the experiment. All animal treatments were strictly in accordance with the international ethics guidelines and the National Institutes of Health Guide concerning the Care and Use of Laboratory Animals, and the experiments were approved by the Institutional Animal Care and Use Committee of the Fujian University of Traditional Chinese Medicine.

In vivo nude mice xenograft study. Cells ($1.5x10^6$) mixed with Matrigel (1:1) were subcutaneously injected in the right flank area of athymic nude mice to initiate tumor growth. After 3 days of xenograft implantation, mice were randomized into two groups (n=10) and given intragastric administration of 234 mg/kg/day dose of PZH or saline daily, 5 days a week for 16 days. Tumor growth were measured every two days. Tumor growth was determined by measuring the major (L) and minor (W) diameter with a caliper. The tumor volume was calculated according to the following formula: tumor volume = $\pi/6$ x L x W^2. At the end of the experiment, the animals were anaesthetized and the tumor tissue was removed and weighed.

Immunohistochemstry analysis. Tumor samples were fixed with 10% formaldehyde for 12 h and subsequently processed conventionally for paraffin-embedded tumor slides. The slides were subjected to antigen retrieval and the endogenous peroxidase activity was blocked with 3% hydrogen peroxide in water. For immunohistochemical staining, slides were incubated with rabbit polyclonal antibodies against CD31, VEGF-A, VEGFR2, bFGF, bFGFR, iNOS or eNOS (all in 1:200 dilution; Santa Cruz Biotechnology). After washing with PBS, slides were incubated with biotinylated secondary antibody followed by conjugated horseradish peroxidase (HRP)-labelled streptavidin (Dako) and then washed with PBS. The slides were then incubated with diamino-benzidine (DAB, Sigma) as the chromogen, followed by counterstaining with diluted Harris hematoxylin (Sigma). After staining, five high-power fields (x400) were randomly selected in each slide, and the average proportion of positive cells in each field were counted using the true color multi-functional cell image analysis management system (Image-Pro Plus; Media Cybernetics, Silver Spring, MD, USA). To rule out any non-specific staining, PBS was used to replace the primary antibody as a negative control.

Bio-Plex phosphoprotein assay. Tumors were homogenized and then lysed using a commercially available lysis kit (Bio-Rad Laboratories), followed by centrifugation at 14,000 x g for 15 min. Protein concentrations of the clarified supernatants were determined by BCA protein assay. The presence of p-STAT3, p-Akt, p-Erk1/2, p-JNK and p-p38 was detected

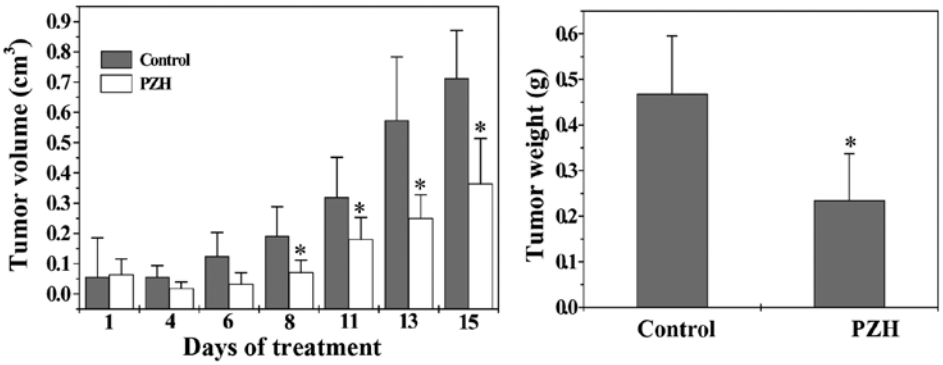

Figure 1. Effect of PZH on tumor growth in CRC xenograft mice. After tumor development, the mice were given intragastric administration of 234 mg/kg of PZH or saline daily, 5 days a week for 15 days. Tumor volume and tumor weight were measured. Data shown are the averages with SD (error bars) from 10 mice in each group. *P<0.05, vs. controls

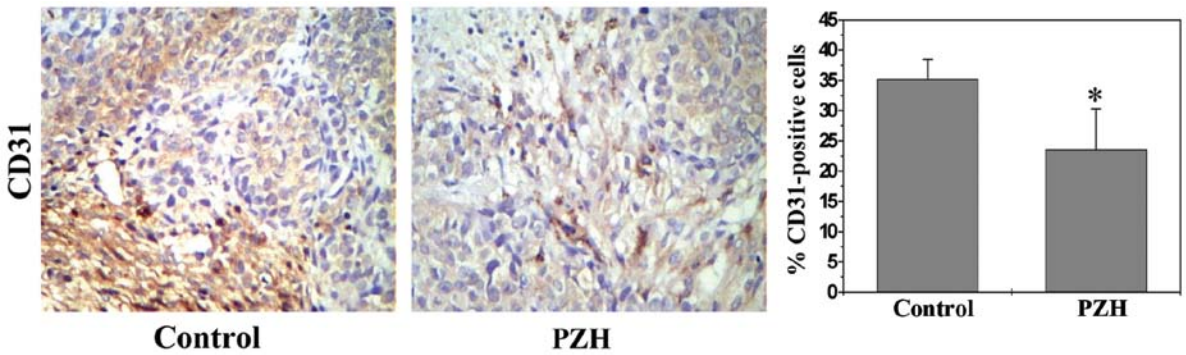

Figure 2. Effect of PZH on microvessel density (MVD) in CRC xenograft mice. At the end of the study, tumor tissues were processed for IHS for CD31. The photographs are representative images taken at a magnification of x400. Quantification of IHS assay is presented as percentage of positively-stained cells. Data shown are the average with SD (error bars) from 10 mice in each group. *P<0.05, vs. controls.

using a bead-based multiplex assay for phosphoproteins (Bio-Plex phosphoprotein assay; Bio-Rad Laboratories) according to the manufacturer's protocol. Data were collected and analyzed using the Bio-Plex 200 suspension array system (Bio-Rad Laboratories).

Statistical analysis. Data are presented as mean ± SD for the indicated number of independently performed experiments. Statistical analysis was carried out with the Student's t-test. Differences with P<0.05 were considered to be statistically significant.

Results and Discussion

PZH suppresses colorectal cancer growth via inhibition of tumor angiogenesis. The therapeutic efficacy of PZH against tumor growth was evaluated through comparison of tumor weight and volume in treated and control CRC xenograft mice. As shown in Fig. 1, tumor growth was significantly suppressed by PZH treatment throughout the experiment. The final tumor volume or tumor weight per mouse in control group was 0.71±0.16 cm^3 or 0.47±0.13 g; while that in PZH-treated group was 0.36±0.15 cm^3 or 0.23±0.10 g (P<0.05), demonstrating that PZH is effective in suppressing colorectal tumor growth.

Angiogenesis plays an essential role in cancer development; we therefore investigated the effect of PZH on intratumoral microvessel density (MVD) that is an indicator of new blood vessel growth. Tumors from CRC xenograft mice were evaluated by immunohistochemical staining (IHS) for the expression of an endothelial cell-specific marker CD31; and data in Fig. 2 show that the percentage of CD31-positive cells in control or PZH-treated mice was 35.22±3.26 or 23.60±6.73%, respectively (P<0.05), suggesting that PZH-caused inhibition of tumor growth is associated with its anti-angiogenic activity.

PZH inhibits the expression of VEGF-A, VEGFR2, bFGF and bFGFR. As most potent angiogenic stimulators, VEGF-A and bFGF are commonly overexpressed in many kinds of human cancer, which is correlated with tumor progression, invasion and metastasis, and poorer survival and prognosis in patients (33-36). VEGF-A and bFGF exert their biological function primarily through interaction with their specific receptors located on the surface of vascular endothelial cells, such as VEGFR-2 and bFGFR. Binding of VEGF-A and bFGF and their receptors leads to receptor dimerization, which in turn activates downstream signaling cascades such as the Akt and Erk pathways, leading to the proliferation, migration, survival, sprouting and eventually tube formation of endothelial cells (35-37).

By performing IHS analysis we found that PZH treatment profoundly inhibited the expression of VEGF-A and bFGF as well as their receptors in tumor tissues. The percentage of VEGF-A, VEGFR2, bFGF or bFGFR-positive cells in control group was, respectively, 38.77±7.76, 31.38±3.36, 23.80±6.73

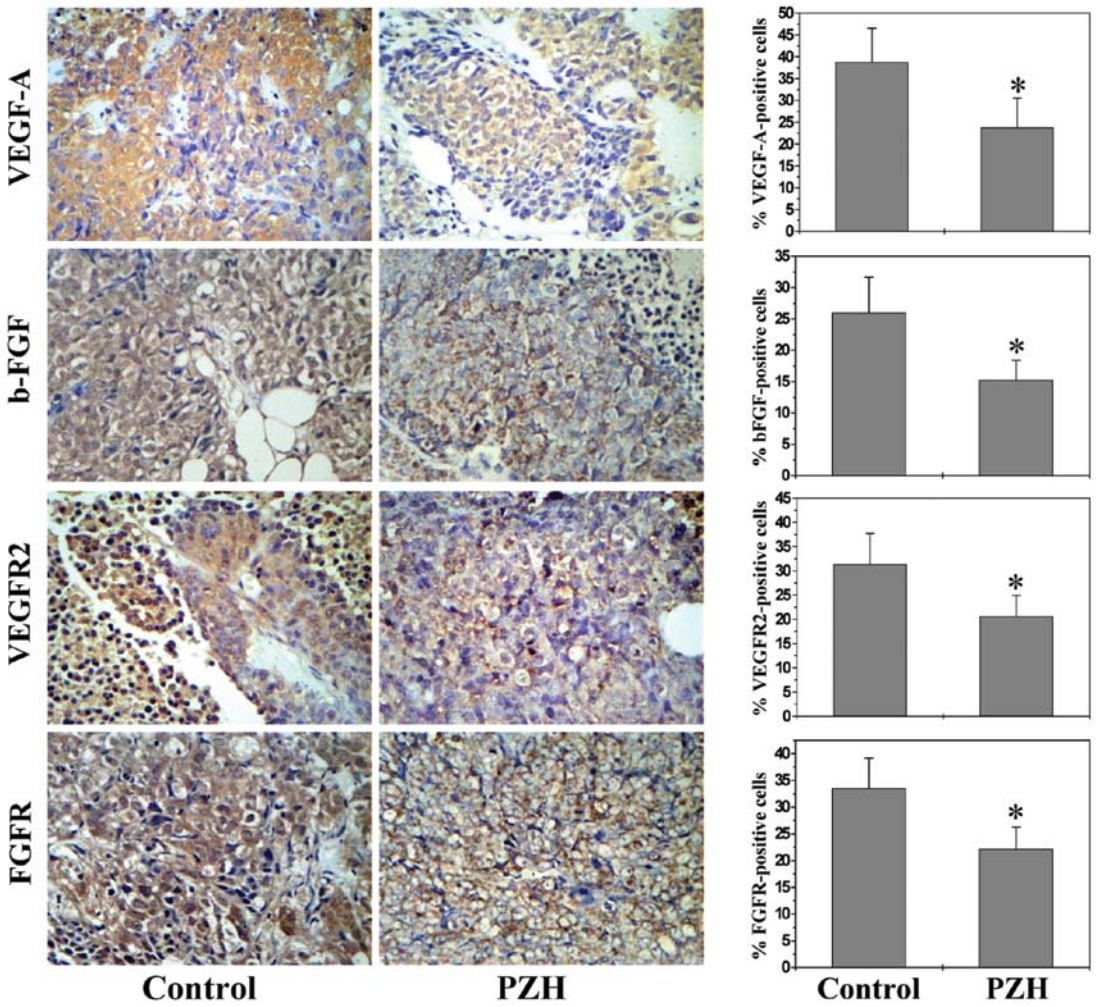

Figure 3. Effect of PZH on the expression of VEGF-A, VEGFR2, bFGF and bFGFR in CRC xenograft mice. Tumor tissues were processed for IHS for VEGF-A, VEGFR2, bFGF and bFGFR. The photographs are representative images taken at a magnification of x400. Quantification of IHS assay is presented as percentage of positively-stained cells. Data shown are the average with SD (error bars) from 10 mice in each group. *P<0.05, vs. controls.

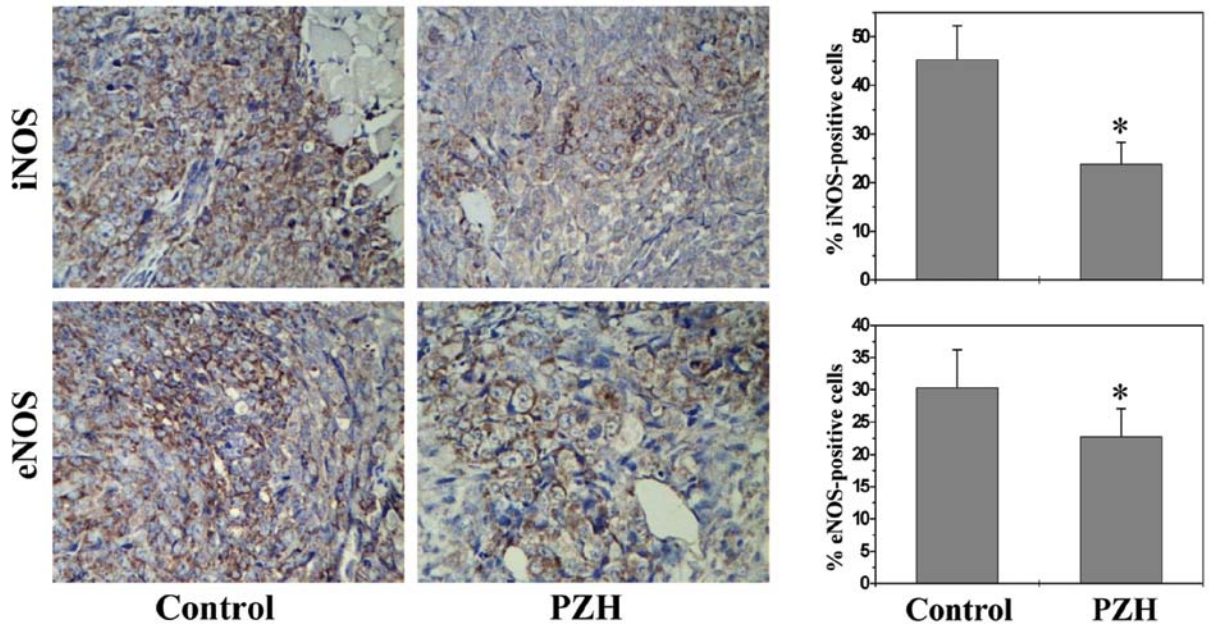

Figure 4. Effect of PZH on the expression of iNOS and eNOS in CRC xenograft mice. Tumor tissues were processed for IHS for iNOS and eNOS. The photographs are representative images taken at a magnification of x400. Quantification of IHS assay is presented as percentage of positively-stained cells. Data shown are the average with SD (error bars) from 10 mice in each group. *P<0.05, vs. controls.

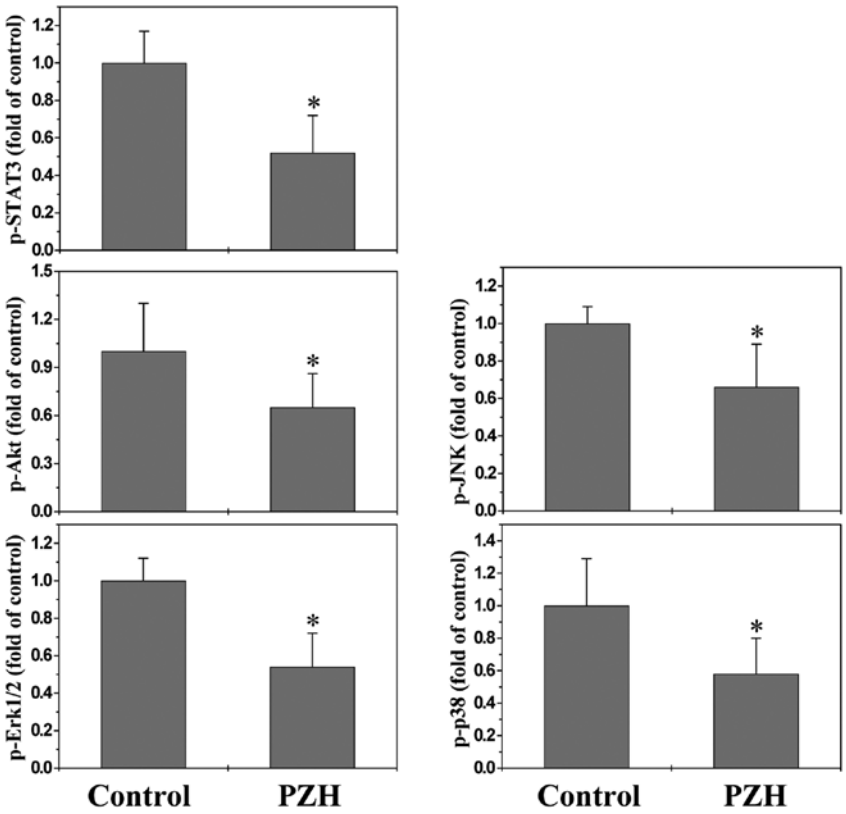

Figure 5. Effect of PZH on phosphorylation of STAT3, Akt, Erk1/2, JNK and p38 in CRC xenograft mice. The phosphorylation levels of STAT3, Akt, Erk1/2, JNK and p38 in tumor tissues were determined by Bio-Plex phosphoprotein assay. The data are normalized to the phosphorylation level within controls and presented as fold of control. Data are the average with SD (error bars) from 10 mice in each group. *P<0.05, vs. controls.

and 33.50±5.67%, whereas that in PZH-treated mice was 26.0±5.67, 20.60±4.32, 15.25±3.17 and 22.20±4.07% (Fig. 3; P<0.05).

PZH inhibits expression of iNOS and eNOS. Nitric oxide synthases (NOSs), particularly the inducible (iNOS) and endothelial (eNOS) isoforms, have been strongly implicated in tumor angiogenesis (21,22,37-41); we therefore determined the effect of PZH on NOS expression. As shown in Fig. 4, the percentage of iNOS- or eNOS-positive cells in control group was, respectively, 45.33±6.93 and 30.33±5.87%, while that in PZH-treated mice was 23.80±4.52 and 22.75±4.34% (P<0.05), suggesting that iNOS and eNOS could be potential molecular targets for the antitumor and anti-angiogenic effects of PZH.

PZH suppresses the activation of multiple signaling pathways. Cancer development is tightly regulated by multiple intracellular signaling pathways, including STAT3, Akt, ERK, JNK and p38. Aberrant activation of these pathways alters the expression of various critical target genes, such as above-mentioned angiogenic factors and NOSs, leading to the promotion of tumor angiogenesis. To further elucidate the underlying mechanisms of antitumor activity of PZH, we determined its effect on the activation of STAT3, Akt, ERK, JNK and p38 pathways. The activation (phosphorylation) of STAT3, Akt, Erk1/2, JNK and p38 in xenograft tumor tissues was determined by Bio-Plex phosphoprotein assay. As shown in Fig. 5, after PZH treatment the phosphorylation levels of STAT3, ERK, Akt, JNK and p38 in tumors were decreased as compared to controls (P<0.05), suggesting that PZH exerts its antitumor activity probably through affecting multiple intracellular targets.

In conclusion, in the present study, we demonstrate for the first time that PZH inhibits colorectal cancer growth via suppression of multiple intracellular signaling pathways leading to the inhibition of tumor angiogenesis, which may in part explain its anticancer activity.

Acknowledgements

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