Pien Tze Huang suppresses IL-6-inducible STAT3 activation in human colon carcinoma cells through induction of SOCS3

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Abstract. IL-6/STAT3 is one of the most critical cellular signal transduction pathways known to malfunction in colorectal cancer (CRC). As a target gene of signal transducer and activator of transcription 3 (STAT3) signaling, suppressor of cytokine signaling 3 (SOCS3) can be quickly induced by interleukin-6 (IL-6) stimulation but it then strongly inhibits IL-6-mediated STAT3 activation, functioning as a negative feedback regulator of the IL-6/STAT3 pathway. Aberrant activation of STAT3 and/or reduced expression of SOCS are strongly correlated with carcinogenesis, which therefore becomes a promising target for the development of novel anticancer chemotherapies. Pien Tze Huang (PZH) is a well-known traditional Chinese formula that was first prescribed by a royal physician 450 years ago in the Ming Dynasty. It has been used in China and Southeast Asia for centuries as a folk remedy for various types of cancer including CRC. However, the precise mechanism of its antitumor activity remains largely unclear. In the present study, we found that PZH could significantly and dose-dependently inhibit IL-6-mediated increase of STAT3 phosphorylation levels and transcriptional activity in the human colon carcinoma HT-29 cells, resulting in the suppression of cell proliferation and the induction of apoptosis. In addition, PZH treatment profoundly inhibited IL-6-induced upregulation of cyclin D1 and Bcl-2, two key target genes of the STAT3 pathway. Moreover, PZH treatment increased the expression of SOCS3. These results suggest that PZH could effectively inhibit proliferation and promote apoptosis of human colon carcinoma cells via modulation of the IL-6/STAT3 signaling pathway and its target genes.

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Abbreviations: CRC, colorectal cancer; PZH, Pien Tze Huang; IL-6, interleukin-6; STAT3, signal transducer and activator of transcription 3; SOCS3, suppressor of cytokine signaling 3; TCM, traditional Chinese medicine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Key words: Pien Tze Huang, traditional Chinese medicine, colorectal cancer, IL-6/STAT3 pathway, SOCS3

Introduction

Colorectal carcinoma (CRC) is a serious global health problem, with over one million new cases and half a million mortalities worldwide each year (1). The pathogenesis of CRC is complex, with the involvement of multiple cellular transduction pathways including IL-6/STAT3 signaling. Interleukin-6 (IL-6) is an important pro-inflammatory cytokine that has been shown to play a potential role in CRC. Elevated IL-6 levels have been detected in the serum (2) and cancer tissue (3) in CRC patients. In addition, IL-6 levels are correlated with tumor size and are commonly associated with the disease severity (2,4). IL-6 has also been shown to directly stimulate the proliferation of some colon cancer cell lines *in vitro* (5). Recent studies indicated that IL-6 signal transduction in CRC is remarkably not mediated by the membrane-bound receptor for IL-6 (IL-6R), but the soluble form of the IL-6R (sIL-6R), a process called IL-6 trans signaling (6-8). The IL-6/sIL-6R complex in turn binds to a common signal transducing receptor gp130, promoting dimerization of gp130 and then resulting in activation of the associated Janus kinases (JAKs). Activated JAKs phosphorylate gp130, leading to the recruitment and activation of signal transducer and activator of transcription 3 (STAT3) (9). STAT3 is an important transcription factor that plays an essential role in cell survival and proliferation (10,11). Following activation via phosphorylation at tyrosine 705 by JAKs, STAT3 proteins in the cytoplasm dimerize and translocate to the nucleus where they regulate the expression of various critical genes involved in cell proliferation and survival (12-14). Constitutive activation of STAT3 has been found in many types of human cancer and generally suggests poor prognosis (15-18).

IL-6 signal transduction is regulated in a variety of ways. Suppressor of cytokine signaling 3 (SOCS3) is regarded as a key negative regulator of the IL-6/STAT3 pathway. SOCS3 can be rapidly induced by IL-6 stimulation but it then limits IL-6-mediated STAT3 phosphorylation/activation through competitively binding to gp130 and JAKs (9), creating a negative feedback loop of IL-6/JAK/STAT3 signal transduction cascade (19-22). Reduced or silenced SOCS3 has been found in many human types of cancer including CRC (23-25), and restoring SOCS3 expression in cancer cells inhibited IL-6-mediated STAT3 activation, induced tumor cell apoptosis and decreased cell proliferation (23,26). Therefore, suppression of the IL-6/STAT3 pathway via modulation of SOCS3 has been a promising strategy for anticancer therapies.

Despite recent advances in CRC chemotherapy, 5-fluorouracil (5-FU)-based regimens continue to be the international standard chemotherapy for patients with advanced CRC (27). However, due to drug resistance and the unacceptable level of toxicity to normal cells, systemic chemotherapy using 5-FU-based regimens produces objective response rates of less than 40% (28-30). These problems highlight the urgent need for the development of novel cancer chemotherapies. Natural products, such as traditional Chinese herbal medicines (TCMs), have received attention as they have relatively few side-effects and have long been used clinically as significant alternative remedies for a variety of diseases (31-33). Pien Tze Huang (PZH) is a well-known TCM formula that was first prescribed 450 years ago 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 (34). PZH has been used in China and Southeast Asia for centuries as a folk remedy for various types of cancer (35,36), since in the TCM system accumulation of toxic dampness and heat is one of the major causative factors in the pathogenesis of cancer and, therefore, clearing heat and detoxification is a principle of anticancer treatment. We previously reported that PZH can inhibit colon cancer cell growth *in vitro* via promotion of apoptosis (37). In addition, using a CRC mouse xenograft model we found that PZH can suppress tumor growth *in vivo* without apparent adverse effects; PZH treatment also reduces the phosphorylation level of STAT3 in tumor tissues (38). To further elucidate the mechanism of the tumoricidal activity of PZH, herein we investigated its effects on the IL-6-mediated activities in human carcinoma HT-29 cells, such as cell proliferation and apoptosis, phosphorylation level and transcriptional activity of STAT3, as well as the expression of several IL-6/STAT3 signaling target genes including SOCS3.

Materials and methods

Materials and reagents. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, trypsin-EDTA, Lipofectamine™ LTX with PLUS™ reagent, TRIzol reagent were purchased from Invitrogen (Carlsbad, CA, USA). Bcl-2, cyclin D1, SOCS3 and phospho-STAT3 (Tyr^{705}) antibodies, horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Cell Signaling (Beverly, MA, USA). SuperScript II reverse transcriptase and Dual-Luciferase Reporter Assay System were obtained from Promega (Madison, WI, USA). The Hoechst staining kit was obtained from the Beyotime Institute of Biotechnology (Jiangsu, China). Cignal STAT3 Reporter (luc) kit was obtained from SABiosciences (Qiagen, Hilden, Germany). All the other chemicals, unless otherwise stated, were obtained from Sigma Chemicals (St. Louis, MO, USA).

Preparation of PZH. PZH was obtained from and authenticated by the sole manufacturer Zhangzhou Pien Tze Huang Pharmaceutical Co. Ltd., China (Chinese FDA approval no. Z35020242). Stock solution of PZH was prepared immediately prior to use by dissolving the PZH power in phosphate-buffered saline (PBS) to a concentration of 20 mg/ml. The working concentrations of PZH were made by diluting the stock solution in the culture medium.

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 U/ml penicillin and 100 μg/ml streptomycin in a 37˚C humidified incubator with 5% CO_2.

Treatment of PZH and IL-6. HT-29 cells were first grown in complete DMEM (10% FBS) until ~50% confluency and then continuously cultured in FBS-free medium overnight. The medium was replaced with DMEM complete with 10% FBS and cells were pre-treated with various concentrations of PZH for 1 h followed by stimulation with 10 ng/ml of IL-6 for the indicated periods of time in different experiments as described below.

Evaluation of cell viability by MTT assay. Viability of HT-29 cells was examined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. HT-29 cells were seeded into 96-well plates at a density of $1x10^4$ cells/well in 0.1 ml medium. Cells were treated with PZH and/or IL-6 as described above for 24 h. MTT (100 μl) (0.5 mg/ml in PBS) was added to each well, and the samples were incubated for an additional 4 h at 37˚C. The purple-blue MTT formazan precipitate was dissolved in 100 μl DMSO. The absorbance was measured at 570 nm using an ELISA reader (Model ELX800; BioTek, Winooski, VT, USA).

Colony formation. HT-29 cells were seeded into 6-well plates at a density of $2x10^5$ cells/well in 2 ml medium. After treatment with PZH and/or IL-6 as described above for 24 h, cells were harvested and diluted in 2 ml fresh medium without PZH and IL-6, and then reseeded into 6-well plates at a density of $1x10^3$ cells/well. Following incubation for 8 days in a 37˚C humidified incubator with 5% CO_2, formed colonies were fixed with 10% formaldehyde, stained with 0.01% crystal violet and counted. Cell survival was calculated by normalizing the survival of the control cells as 100%.

Detection of apoptosis with Hoechst staining. HT-29 cells were seeded into 12-well plates at a density of $1x10^5$ cells/well in 1 ml medium. After treatment with PZH and/or IL-6 as described above for 24 h, cell apoptosis was evaluated by Hoechst staining kit according to the manufacturer's instruc-

tions. Briefly, at the end of treatment, cells were fixed with 4% polyoxymethylene and then incubated in Hoechst solution for 5 min in the dark. The staining images were recorded using a phase-contrast fluorescent microscope (Olympus, Japan). The images were captured at a magnification of x400.

RT-PCR analysis. HT-29 cells were seeded into 6-well plates at a density of $2x10^5$ cells/well in 2 ml medium. After cells were treated with PZH and/or IL-6 as described above for 24 h, total-RNA was isolated with TRIzol reagent. Oligo(dT)-primed RNA (1 μg) was reverse-transcribed with SuperScript II reverse transcriptase according to the manufacturer's instructions. The obtained cDNA was used to determine the mRNA amount of Bcl-2 and cyclin D1 by PCR. GAPDH was used as an internal control.

Western blot analysis. HT-29 cells were seeded into 6-well plates at a density of $2x10^5$ cells/well in 2 ml medium. Cells were treated with PZH and/or IL-6 as described above. IL-6 stimulation was performed for 15 min for pSTAT3 detection, or 24 h for examination of SOCS3, Bcl-2 and cyclin D1 protein expression. Treated cells were lysed with mammalian cell lysis buffer containing protease and phosphatase inhibitor cocktails. The lysates were resolved in 12% SDS-PAGE gels and electroblotted. The PVDF membranes were blocked with 5% skimmed milk and probed with primary antibodies against phosphor-specific STAT3 (Tyr^{705}), SOCS3, Bcl-2, cyclin D1 and β-actin (1:1,000) overnight at 4˚C and then with the appropriate HRP-conjugated secondary antibody followed by enhanced chemiluminescence detection.

Luciferase gene reporter assay. HT-29 cells were seeded into 96-well plates at a density of $1x10^4$ cells/well in 0.1 ml complete DMEM until ~50% confluency and then continuously cultured in FBS- and antibiotics-free medium overnight. Cells were transfected with a mixture of inducible STAT3-responsive firefly luciferase construct and constitutively expressing Renilla luciferase construct using Lipofectamine LTX with PLUS™ reagent. Six hours after transfection the medium was changed back into DMEM complete with FBS, penicillin and streptomycin. After 24 h of transfection, cells were treated with various concentrations of PZH for 1 h followed by IL-6 for another 24 h. Cell extracts were prepared and analyzed using Promega Dual Luciferase Reporter Assay System according to the manufacturer's instructions. The measured firefly luciferase activity was normalized to the activity of Renilla luciferase in the same well.

Statistical analysis. Data were analyzed using the SPSS package for Windows (Version 11.5). Statistical analysis of the data was performed with the Student's t-test and one-way ANOVA. Differences with $P<0.05$ were considered statistically significant.

Results

PZH inhibits IL-6-mediated STAT3 activation in HT-29 cells. Several cultured human cancer cell lines including HT-29 do not express constitutively phosphorylated STAT3 *in vitro*; we therefore stimulated STAT3 activation with IL-6 in HT-29 cells. We first determined STAT3 activation by performing western blotting to examine its phosphorylation level using an antibody that recognizes phosphorylated STAT3 (pSTAT3) at Tyr^{705}. As shown in Fig. 1, stimulation with 10 ng/ml of IL-6 for 15 min significantly increased the level of pSTAT3 in HT-29 cells, which, however, was profoundly inhibited by PZH in a dose-dependent manner. The levels of non-phosphorylated STAT3 remained unchanged after the treatment with IL-6 and/or PZH. To further confirm the inhibitory effect of PZH on the activation of STAT3, we performed Dual Luciferase Reporter Assay to examine STAT3 transcriptional activity. Results in Fig. 2 showed that PZH significantly and dose-dependently inhibited IL-6-stimulated increase of STAT3 transcriptional activity. Taken together, our data suggest that PZH is potent in inhibiting IL-6-mediated STAT3 activation in human colon carcinoma cells.

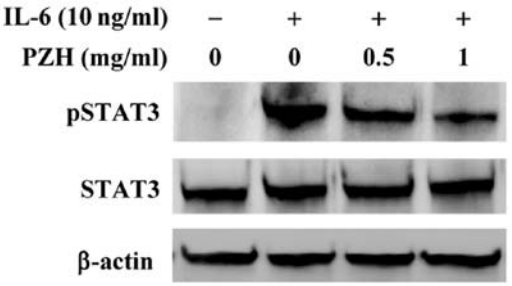

Figure 1. Effect of PZH on IL-6-mediated STAT3 phosphorylation in HT-29 cells. Cells were pre-treated with various concentrations of PZH for 1 h followed by stimulation with 10 ng/ml of IL-6 for 15 min. STAT3 phosphorylation was determined by western blotting using an antibody that recognizes phosphorylated STAT3 at Tyr^{705}. β-actin was used as the internal control. Data are representative of three independent experiments.

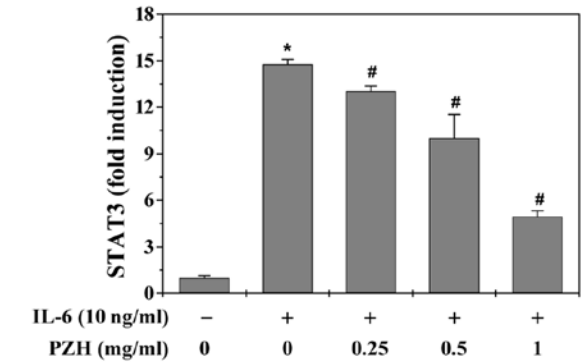

Figure 2. Effect of PZH on IL-6-mediated STAT3 transcriptional activity in HT-29 cells. Cells were transfected with a mixture of inducible STAT3-responsive firefly luciferase construct and constitutively expressing Renilla luciferase construct (40:1). After 24 h of transfection, cells were treated with various concentrations of PZH for 1 h followed by IL-6 for another 24 h. The transcriptional activity of STAT3 was measured by DLR assay. Data are averages with SD (error bars) from at least three independent experiments. *$P<0.05$ vs. control cells; #$P<0.05$ vs. cells treated with IL-6 but without PZH.

PZH inhibits HT-29 cell proliferation. The effect of PZH on HT-29 cell viability in the presence of IL-6 was determined by MTT assay. As shown in Fig. 3A, although IL-6 stimulation increased the viability of HT-29 cells to 115% compared to control cells ($P<0.05$), treatment with 0.25-1 mg/ml of PZH for

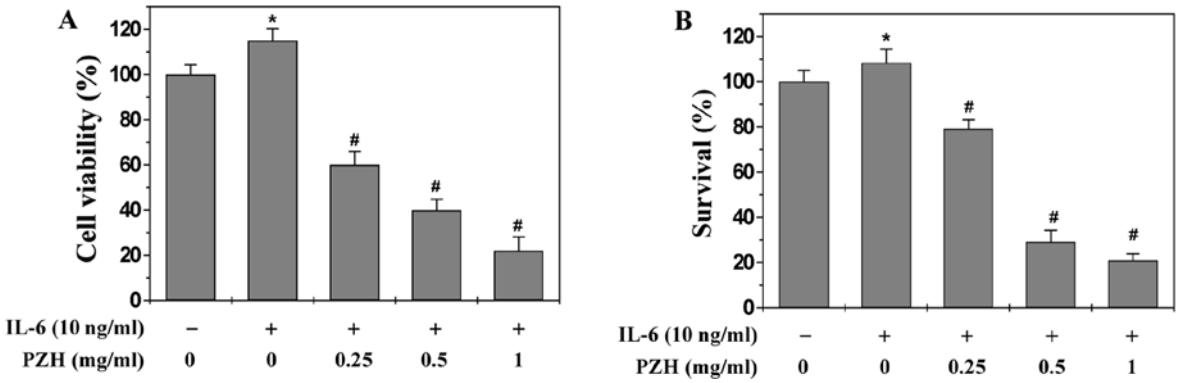

Figure 3. Effect of PZH on HT-29 cell proliferation. Cells were pre-treated with various concentrations of PZH for 1 h followed by stimulation with 10 ng/ml of IL-6 for 24 h. (A) Cell viability was determined by the MTT assay. (B) Cell survival was evaluated by colony formation assay. The data were normalized to the viability or survival of control cells. Data are averages with SD (error bars) from at least three independent experiments. *P<0.05 vs. control cells; #P<0.05 vs. cells treated with IL-6 but without PZH.

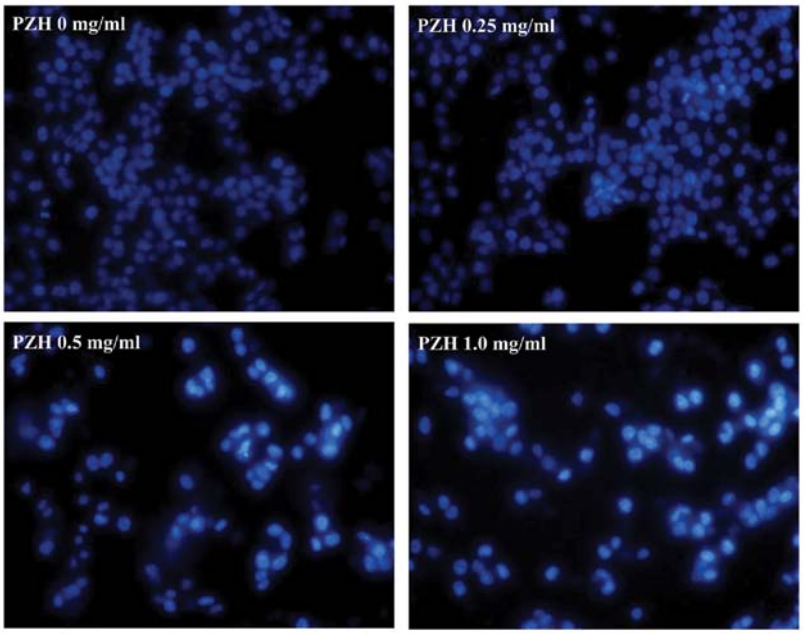

Figure 4. Effect of PZH on HT-29 cell apoptosis. Cells were pre-treated with various concentrations of PZH for 1 h followed by stimulation with 10 ng/ml of IL-6 for 24 h. Cell apoptosis was evaluated by Hoechst staining. The staining images were recorded using a phase-contrast fluorescent microscope and the images were captured at magnification, x400. Images are representative of three independent experiments.

24 h decreased the viability of IL-6-stimulated cells from 60 to 22% (P<0.05 vs. PZH-untreated cells). To further verify these results, we examined the effect of PZH on HT-29 cell survival using a colony formation assay. As shown in Fig. 3B, treatment with 0.25, 0.5 and 1 mg/ml of PZH for 24 h dose-dependently reduced the survival rate of IL-6-stimulated cells by 27, 73 and 81% (P<0.05). Collectively, these data demonstrate that PZH inhibits HT-29 cell proliferation in the presence of IL-6 stimulation.

PZH induces HT-29 cell apoptosis. Cell apoptosis was evaluated by observing nuclear morphological changes by staining the cell nuclei with DNA-binding dye Hoechst. As shown in Fig. 4, PZH-treated cells showed condensed chromatin and fragmented nuclear morphology that are typical apoptotic morphological features, whereas the untreated cell nuclei showed homogenous staining and were less intense than PZH-treated cells, suggesting that PZH promotes HT-29 cell apoptosis in the presence of IL-6 stimulation.

PZH downregulates the expression of Bcl-2 and cyclin D1 and upregulates SOCS3 expression in HT-29 cells. To further investigate the underlying mechanism of PZH's activities, we performed RT-PCR and western blot analyses to examine the effect of PZH on the expression of the pro-proliferative cyclin D1 and the anti-apoptotic Bcl-2, two important target genes of the STAT3 signaling pathway. The results in Fig. 5 show that the mRNA and protein expression of cyclin D1 and Bcl-2 was clearly increased by IL-6 stimulation. However, PZH treatment profoundly inhibited IL-6-induced upregulation of cyclin D1 and Bcl-2 expression, at both the transcriptional and translational levels.

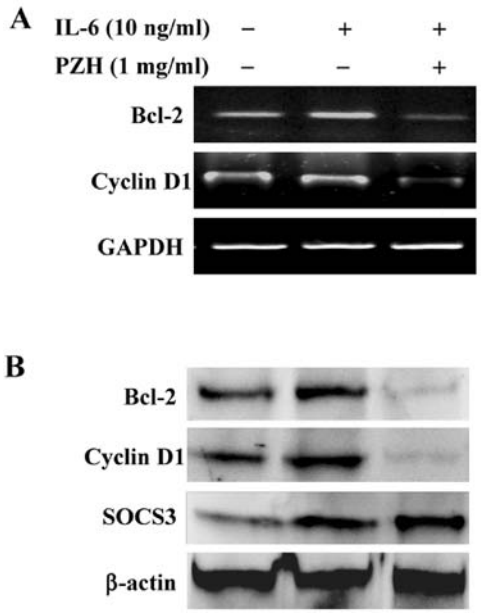

Figure 5. Effect of PZH on the expression of Bcl-2, cyclin D1 and SOCS3 in HT-29 cells. Cells were pre-treated with various concentrations of PZH for 1 h followed by stimulation with 10 ng/ml of IL-6 for 24 h. (A) The mRNA levels of Bcl-2 and cyclin D1 were determined by RT-PCR. (B) The protein expression levels of Bcl-2, cyclin D1 and SOCS3 were determined by western blot analysis. GAPDH and β-actin were used as the internal controls for the RT-PCR or western blot assays, respectively. Data are representative of three independent experiments.

We next investigated the effect of PZH on another STAT3 transcriptional target, the SOCS3 protein, a critical negative feedback inhibitor of the IL-6/STAT3 pathway. As shown in Fig. 5B, IL-6 stimulation induced SOCS3 expression, which is consistent with previous studies (39,40). Notably, PZH treatment further increased the protein expression of SOCS3, suggesting that PZH suppresses the IL-6/STAT3 pathway in HT-29 cells partially via promoting SOCS3 expression.

Discussion

Colorectal cancer (CRC) is a complex and heterogeneous tumor involving multiple cellular signaling transduction pathways. It is noteworthy that these signaling pathways usually have functional redundancy. In addition, there is crosstalk between these pathways, forming a complicated and robust cellular signal transduction network that is regulated by compensatory mechanisms. Therefore, specific inhibitors that target only one single pathway might not always be effective on the complex tumor systems; also, the long-term use of many single-target-based agents will often generate unsatisfactory drug resistance and side-effects, which is possibly one of the major reasons why overall CRC patient response to chemotherapy is less than 40% despite advances in this area. These problems highlight the urgent need for the development of novel cancer chemotherapies. Natural products, such as traditional Chinese herbal medicines (TCMs), have relatively fewer side-effects compared to modern chemotherapeutics and have long been used clinically for cancer treatment. Pien Tze Huang (PZH), a well-known and important TCM formula, has been demonstrated to be clinically effective in treating various types of cancer including CRC. However, the mode of action for its antitumor effect is largely unknown.

IL-6/STAT3 is one of the most critical cellular signal transduction pathways known to malfunction in CRC. IL-6 transduces its signal through a common signaling receptor gp130, eventually resulting in the activation of STAT3. The transcription factor STAT3 is an oncogenic protein that is constitutively activated in most tumor cells but not in normal cells (1-14). Activation of STAT3 is mediated by phosphorylation at tyrosine 705, leading to its homodimerization, nuclear translocation and DNA binding, which in turn upregulates the expression of various critical genes involved in cell proliferation and survival, such as the pro-proliferative cyclin D1 and the anti-apoptotic Bcl-2. Markedly, as another target gene of STAT3 signaling, SOCS3 can be quickly induced by IL-6 stimulation but it then strongly inhibits IL-6-mediated STAT3 activation, functioning as a negative feedback regulator of the IL-6/STAT3 pathway. Aberrant activation of STAT3 and/or reduced expression of SOCS3 facilitate unregulated increase in cell proliferation and reduction in cell apoptosis resulting in cancer development. Therefore, modulation of IL-6/STAT3/SOCS3 signaling has been a promising target for the development of anticancer therapies.

In the present study, we stimulated the human colon carcinoma HT-29 cells with IL-6 and found that STAT3 was quickly activated upon IL-6 stimulation, leading to a significant increase in its phosphorylation level and transcriptional activity. However, the IL-6-mediated STAT3 activation could be profoundly inhibited by PZH treatment in a dose-dependent manner. Consequently, PZH treatment significantly inhibited IL-6-induced upregulation of cyclin D1 and Bcl-2, two key target genes of the STAT3 pathway. Moreover, the inhibitory effect of PZH on the IL-6-mediated STAT3 activation and cyclin D1/Bcl-2 expression resulted in the suppression of HT-29 cell proliferation and the induction of cell apoptosis. Furthermore, PZH treatment increased the expression of SOCS3. In conclusion, our data demonstrate that PZH could effectively inhibit proliferation and promote apoptosis of human colon carcinoma cells via modulation of the IL-6/STAT3 signaling pathway and its target genes.

Several TCM formulas including PZH are composed of many natural products, each of which contains numerous chemical compounds. TCM formulas are thus considered to be multi-component and multi-target agents that exert their therapeutic function in a more holistic manner. However, it remains unknown whether PZH can affect other cancer-related cellular signal transduction pathways, such as Hedgehog, Ras/ERK, PI3K/Akt and Wnt signalings, as well as STAT3. This issue could be addressed in future studies to fully elucidate the molecular mechanism by which PZH is involved in cancer treatment and in order to develop better multi-target drugs for cancer therapy.

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