# Pien Tze Huang suppresses the stem-like side population in colorectal cancer cells

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**Abstract.** Accumulating evidence suggests that a small population of cells termed cancer stem cells (CSCs) are crucial in tumor development and drug resistance, leading to cancer relapse and metastasis and eventually the failure of clinical cancer treatment. Therefore, targeting CSCs is a promising approach for anticancer therapies. Due to the drug resistance and adverse effects of currently used chemotherapies, traditional Chinese medicines (TCM) have recently received attention due to the relatively few side-effects. Thus, they have been used as important alternative remedies for various diseases, including cancer. Pien Tze Huang (PZH), a well-known TCM formula that was first prescribed more than 450 years ago in the Ming Dynasty, has been used in China and Southeast Asia for centuries as a folk remedy for various types of cancer. Previously, it was reported that PZH inhibits colon cancer growth via the promotion of cancer cell apoptosis and inhibition of cell proliferation and tumor angiogenesis, which is probably mediated by its regulatory effect on multiple intracellular signaling pathways. To elucidate the mechanism of the tumoricidal activity of PZH, the aim of the present study was to investigate the effect of PZH on CSCs that were isolated as the side population (SP) from the HT-29 colorectal cancer cell line. The results demonstrated that PZH significantly and dose-dependently reduced the percentage of the colorectal cancer stem-like SP cells, decreased the viability and sphere-forming capacity of HT-29 SP cells, indicating that PZH is potent in suppressing the growth of colorectal cancer stem cells. Moreover, PZH treatment in HT-29 SP cells markedly inhibited the mRNA levels of ABCB1 and ABCG2, which are members of the ABC transporter superfamily, thereby contributing to the SP phenotype and multi-drug resistance. Findings of the present study suggest that inhibiting the growth of CSCs is a potential mechanism by which PZH can be used in cancer treatment.

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*Abbreviations:* CRC, colorectal cancer; PZH, Pien Tze Huang; CSCs, cancer stem cells; SP, side population

*Key words:* Pien Tze Huang, traditional Chinese medicine, colorectal cancer, cancer stem cells, side population

## Introduction

Colorectal cancer (CRC) is one of the most common malignancies, with over one million new cases and more than half a million deaths each year worldwide (1,2). Although complete resection of the tumor is the best prognosis for long-term survival, CRC patients frequently present with metastatic disease at the time of their diagnosis, and surgery cannot always extirpate the recurrence of advanced CRC (3,4). Therefore, chemotherapy remains one of the major therapeutic approaches for patients with invasive and metastatic CRC, with 5-fluorouracil (5-FU)-based regimens being considered as standard chemotherapy treatment. However, due to drug resistance and unacceptable levels of toxicity against normal cells, systemic chemotherapy using 5-FU-based regimens produces objective response rates of only 10-20% (5-8). Thus, novel therapeutic strategies should be developed and antitumor agents should be identified.

Malignant tumors arise from a small fraction of cancer cells that possess stem cell features and are therefore termed cancer stem cells (CSCs) (9). The existence of CSCs has been demonstrated in the majority of leukemias and many solid tumors including CRC (10-14). Similar to normal stem cells, CSCs possess properties of continuous self-renewal and multi-directional differentiation, which confers CSCs the ability of unlimited proliferation facilitating the long-term maintenance of the cancer, and differentiation into different cell types to develop new tumors (15). More importantly, it has been

shown that CSCs are naturally resistant to chemotherapeutic agents through various mechanisms (16). CSCs specifically overexpress the ATP-binding cassette (ABC) family of transporter proteins such as ABCB1 and ABCG2, which pump various xenobiotics out of the cell, reducing the intracellular accumulation of chemotherapeutic drugs (17-19). In addition, CSCs usually contain high levels of DNA repair mechanism and anti-apoptotic proteins such as Bcl-2 and survivin, further conferring CSCs a survival advantage (20,21). CSCs also exhibit relative cell cycle quiescence resulting in a slow rate of cell turnover, thereby assisting CSCs to evade the cytotoxic effects of conventional chemotherapies that are designed to target rapidly replicating cells (22,23). Therefore, CSCs are, not only responsible for tumor initiation and progression, but also play important roles in drug resistance leading to cancer relapse and metastasis and eventually the failure of clinical treatment. Thus, targeting CSCs is a promising approach for anticancer treatments.

Due to the drug resistance and cytotoxicity of currently used chemotherapies, traditional Chinese medicines (TCM) have received attention due to few side-effects as compared to modern chemotherapeutics and have been used for thousands of years as important alternative remedies for various diseases (24,25). 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 that exert their therapeutic activities in a more holistic way. Pien Tze Huang (PZH) is a well-known TCM formula that was first prescribed by a royal physician >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, reduction of blood stasis, dissipation of hard mass, detumescence and analgesia (26). In the TCM system, accumulation of toxic dampness and heat is one of the major causative factors in the pathogenesis of cancers, and therefore clearing heat and detoxification is a principle of anticancer treatment. Thus PZH, which has also been used in China and Southeast Asia for centuries as a folk remedy for various types of cancer, is believed to be effective for anticancer treatment. Modern pharmacological studies suggested that PZH exhibits therapeutic effects in clinical trials of tumors such as hepatocellular carcinoma and colon cancer (27,28). In addition, in experimental animals PZH inhibits the growth of Ehrlich-Ascites tumor, gastric carcinoma, and hepatoma (29). Moreover, it was reported that PZH inhibits colon cancer growth *in vivo* and *in vitro* via the promotion of cancer cell apoptosis and inhibition of cell proliferation and tumor angiogenesis, which is probably mediated by its regulatory effect on multiple intracellular pathways (30-35). To elucidate the mechanism of the tumoricidal activity of PZH, the effect of PZH on colorectal cancer stem cells was investigated.

## Materials and methods

*Materials and reagents*. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), B27 supplement (50X), penicillin-streptomycin, trypsin-EDTA and TRIzol reagent were purchased from Invitrogen (Carlsbad, CA, USA). SuperScript II reverse transcriptase was obtained from Promega (Madison, WI, USA). Any other chemicals used, unless otherwise stated, were obtained from Sigma Chemicals (St. Louis, MO, USA).

*Preparation of PZH*. PZH was obtained from and authenticated by 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 powder in phosphate-buffered saline (PBS) to a concentration of 40 mg/ml. The working concentrations of PZH were obtained by diluting the stock solution in the culture medium.

*Cell culture*. Human colon carcinoma HT-29 cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were grown in DMEM containing 10% (v/v) FBS, and 100 U/ml penicillin and 100 $\mu$g/ml streptomycin in a 37˚C humidified incubator with 5% $CO_2$.

*Side population analysis*. Based on the protocol by Goodell *et al* (36), HT-29 cells were digested with 0.25% trypsin-EDTA, and resuspended in DMEM culture (supplemented with 2% FBS) at a concentration of $2.5x10^6$ cells/ml. Fresh Hoechst 33342 dye (10 $\mu$g/ml; final concentration) was added for 30 min at 37˚C in a rotary shaker. As a control, some cells were incubated with Hoechst 33342 dye in the presence of 50 $\mu$M verapamil. At the end of incubation, the cells were washed and resuspended in cold PBS, 1 mg/ml propidium iodide was added, and the cells were kept at 4˚C in the dark. Cell analysis and sorting were performed on Moflo XDP™ cell sorter flow cytometry (Beckman Coulter, Fullerton, CA, USA). Excitation of Hoechst dye was performed using a UV laser at 355 nm, and the fluorescence was measured with a 450±25 nm filter (Hoechst blue) and a 620±15 nm filter (Hoechst red). Doublets and dead cells were gated out. Inhibition of the SP phenotype by verapamil was used as guidance for drawing of sorting gates.

*Stem cell culture*. Stem cells were cultured in serum-free stem cell culture medium, which contains DMEM/F12 culture medium, B27 (1X), 20 ng/ml EGF and 20 ng/ml bFGF, to prevent differentiation of the stem cells.

*Sphere formation assay*. Sorted cells were seeded at a density of 1,000 cells/well in 6-well plates (Corning, Lowell, MA, USA) and cultured in the above-mentioned serum-free stem cell culture medium in a humidified incubator (5% $CO_2$) at 37˚C. The medium was added every 3 days. When the spheroids sufficiently large (>50 cells within a sphere was considered to be a full sphere), they were collected and transferred onto a 96-well dish, and images were captured by BD Pathway™ 855 under x10 objective in the form of 10x10 montage (BD Biosciences Bioimaging, Rockville, MD, USA).

*Cell viability evaluation*. Cell viability was assessed by the WST-1 assay. HT-29 stem-like cells (20,000/well in 96-well plates) were incubated with the above-mentioned serum-free stem cell culture medium at 37˚C for 48 h. The cells were then

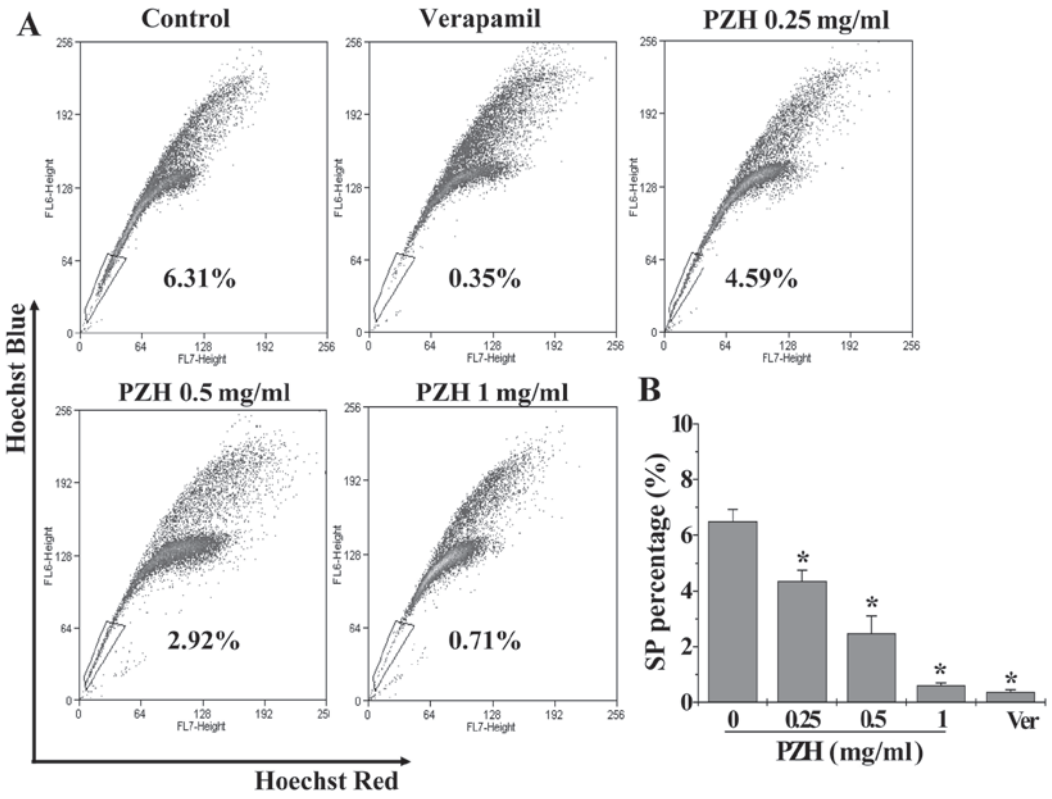


Figure 1. Effect of Pien Tze Huang (PZH) on the percentage of side population (SP) in human colorectal cancer HT-29 cells. (A) Subsequent to treatment with various concentrations of PZH for 24 h, HT-29 cells were stained with Hoechst 33342 and percentages of SP were analyzed by FACS. As an ABC transporter inhibitor, verapamil was used for the confirmation of SP identity. (B) Quantification of FACS analysis. Data are expressed as the average with SD (error bars) from three independent experiments. *P<0.05, vs. untreated control cells.

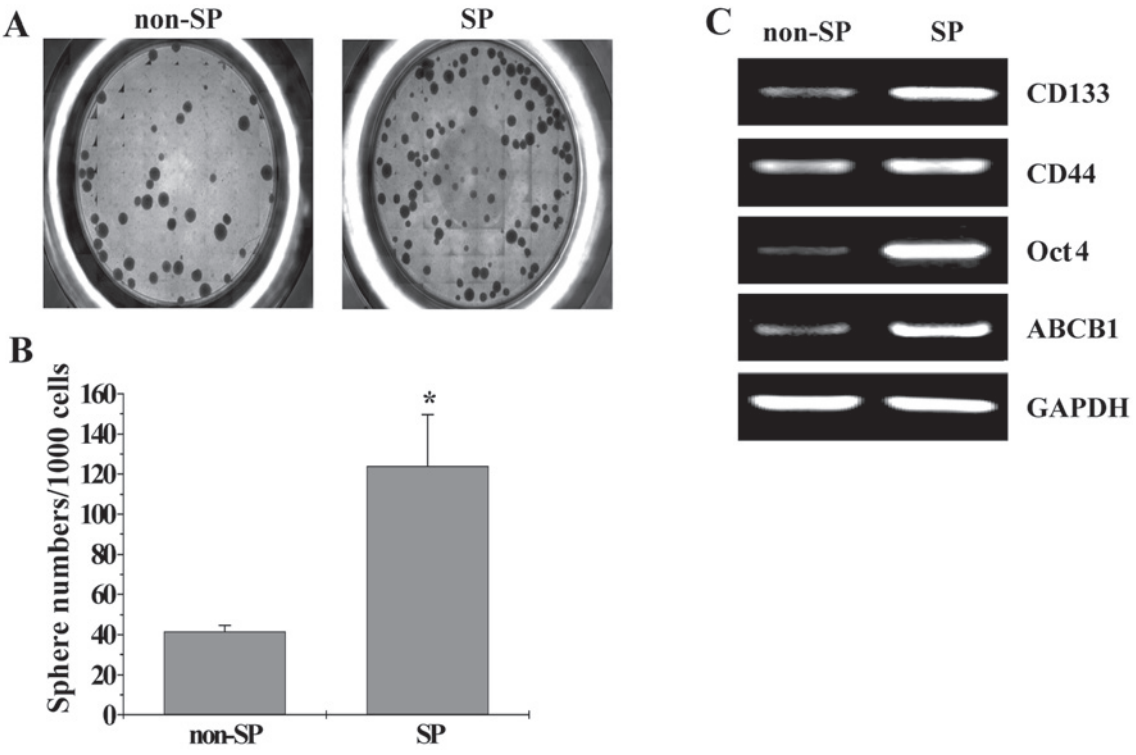


Figure 2. Phenotypic identification. (A) Sphere-formation ability of sorted side population (SP) and non-SP cells was analyzed. Cells were seeded at a density of 1,000 cells/well and grown in serum-free stem cell culture medium. After 15 days, when the spheroids were sufficiently large (>50 cells), they were counted and photographed. (B) Quantification of sphere formation analysis. Data are shown as averages with SD (error bars) from three independent experiments. *P<0.05. (C) The mRNA levels of CD133, CD44, Oct4 and ABCB1 in SP cells and non-SP cells were determined by RT-PCR. GAPDH was used as the internal control. Images are representative of three independent experiments.

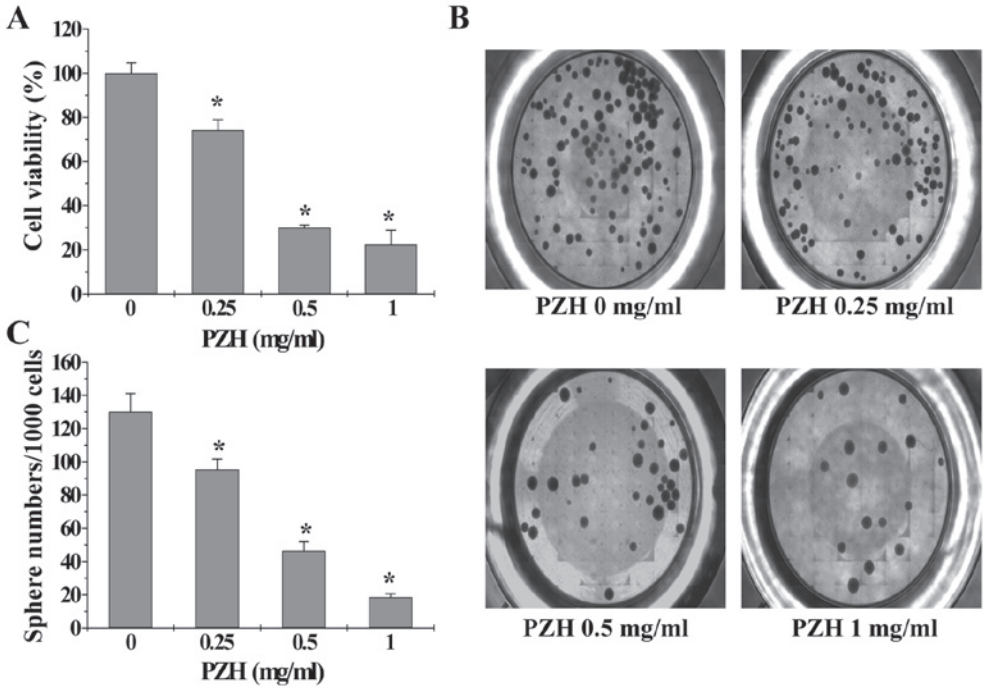


Figure 3. Effect of Pien Tze Huang (PZH) on the growth of isolated HT-29 side population (SP) cells. (A) SP cells were treated with the indicated concentrations of PZH for 24 h. Cell viability was determined by WST-1 assay. Data are averages with SD (error bars) from three independent experiments. *P<0.05, vs. untreated control cells. (B) After treatment with the indicated concentrations of PZH for 24 h, the sphere-formation capacity of SP cells was analyzed. (C) Quantification of sphere formation analysis. Data are shown as averages with SD (error bars) from three independent experiments. *P<0.05, vs. untreated control cells.

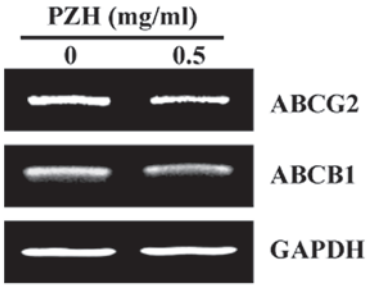


Figure 4. Effect of Pien Tze Huang (PZH) on the mRNA expression of ABCB1 and ABCG2 in isolated HT-29 SP cells. Following treatment with 0.5 mg/ml of PZH for 24 h, the mRNA levels of ABCB1 and ABCG2 in isolated HT-29 SP cells were determined by RT-PCR. GAPDH was used as the internal control. Images are representative of three independent experiments.

treated with various concentrations of PZH for 24 h. At the end of the treatment, 10 $\mu$l WST-1 was added to each well, and the samples were incubated for an additional 2 h at 37˚C. The absorbance was measured at 450 nm using an ELISA reader (Model ELX800, BioTek, USA).

*RT-PCR analysis*. Total RNA was isolated with TRIzol reagent. Oligo(dT)-primed RNA (1 $\mu$g) was reverse-transcribed with SuperScript II reverse transcriptase (Promega) according to the manufacturer's instructions. The obtained cDNA was used to determine the mRNA amount of CD133, CD44, Oct4, ABCB1, ABCG2 by PCR. GAPDH was used as an internal control.

*Statistical analysis*. Data were analyzed using the statistical software SPSS13.0. Statistical analysis of the data was performed using the Student's t-test and one-way analysis of variance (ANOVA). P<0.05 was considered statistically significant.

## Results and Discussion

*Human colorectal cancer cell line HT-29 contains stem-like side population cells*. One of the commonly used techniques for the identification and isolation of CSCs is the flow cytometric side population (SP) analysis (37), which is based on the ability of CSCs to efflux Hoechst dye due to the overexpression of ABC transporter proteins (17-19). SP cells have been identified in various types of cancer including CRC; and cells in SP exibit stem cell-like characteristics, such as the ability for self-renewal and tumorigenicity (38-46). Moreover, it has been shown that the percentage of SP cells in tumors is correlated with tumor grade and patient prognosis (46). Therefore, in the present study, the stem-like cells from the CRC HT-29 cell line were isolated as SP using fluorescence-activated cell sorting (FACS) after staining with Hoechst 33342. As shown in Fig. 1, the percentage of SP in HT-29 cells was 6.50±0.42%. Following treatment with verapamil, a multi-drug transporter inhibitor, the SP was reduced to 0.38±0.08% (P<0.05).

To determine whether the SP cells were enriched for cancer stem cells (CSCs), the ability of the SP cells to undergo *in vitro* sphere formation was examined. The results in Fig. 2A show that the SP cells had a higher ability to undergo sphere formation compared with non-SP cells after 15 days in culture (P<0.05). To verify these observations, an RT-PCR assay was performed to analyze the expression of proteins that are either CSC surface markers or specifically and/or highly expressed

in CSCs (CD133, CD44, Oct4 and ABCB1). As shown in Fig. 2B, the mRNA expression level of CD133, CD44, Oct4 and ABCB1 in SP cells was markedly higher than that in non-SP cells. Taken together, these data suggest that human colorectal cancer HT-29 cells contain a stem-like population.

*PZH reduces the percentage of SP in HT-29 cells*. CSCs are naturally resistant to conventional chemotherapy treatments, leading to cancer relapse and metastasis and eventually the failure of clinical anticancer treatment. Therefore, development of novel therapeutic agents targeting CSCs holds hope for improvement of effectiveness of anticancer therapies. Natural products, including traditional Chinese medicine (TCM), have long been used as alternative remedies for cancer. Recently, natural products received attention in stem cell biology as some compounds have been reported to attack cancer stem-like cells as well as to improve the efficacy of conventional chemotherapies (47,48). PZH is a well-known TCM formula that has been used to clinically treat various types of cancer. Previous studies have reported that PZH likely exerts its anticancer activities via the modulation of multiple intracellular signaling pathways (30-35). To elucidate the mechanism of the tumoricidal activity of PZH, its effect was evaluated on colorectal cancer stem-like cells by examining the size of SP in HT-29 cells following treatment with various concentrations of PZH. As shown in Fig. 1, the percentage of SP cells following treatment with 0, 0.25, 0.5 and 1 mg/ml of PZH was 6.50±0.42, 4.35±0.39, 2.47±0.64 and 0.61±0.09%, respectively ($P<0.05$, vs. untreated control cells), suggesting that PZH possesses anti-CSC activity.

*PZH inhibits the viability and sphere formation capacity of SP in HT-29 cells*. To determine the effect of PZH on the growth of colorectal cancer stem-like cells, we treated isolated HT-29 SP cells with PZH and then examined cell viability via WST-1 assay. As shown in Fig. 3A, treatment with 0.25-1 mg/ml of PZH for 24 h dose-dependently reduced the viability of HT-29 SP cells by 26.83-77.67% compared with the untreated control cells ($P<0.05$). To confirm the growth suppressive activity of PZH in cancer stem-like cells, we investigated its effect on the sphere formation ability of SP cells. Data in Fig. 3B and C show that PZH dose-dependently suppressed sphere formation in isolated HT-29 SP cells ($P<0.05$). These results demonstrate that PZH is potent in suppressing the growth of colorectal cancer stem cells.

*PZH inhibits the expression of ABCB1 and ABCG2 of SP in HT-29 cells*. ATP-binding cassette (ABC) transporter proteins belong to the superfamily of membrane pumps that expel various xenobiotics out of cells, such as chemotherapeutic drugs and lipophilic fluorescent dyes, contributing to the SP phenotype and chemotherapy resistance. ABC transporters are commonly overexpressed in multi-drug-resistant tumors and CSCs. To explore the mechanism whereby PZH inhibited the growth of colorectal cancer stem-like cells, we examined the mRNA expression of ABCB1 and ABCG2, which are considered the best-characterized ABC members. Results from RT-PCR showed that PZH treatment markedly reduced the mRNA levels of ABCB1 and ABCG2 in HT-29 SP cells (Fig. 4).

In conclusion, our findings in this study suggest that attacking CSC is a potential mechanism by which PZH exerts its anticancer activities.

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