

Inhibition of cell proliferation by mild hyperthermia at 43°C with Paris Saponin I in the lung adenocarcinoma cell line PC-9

PENGJUN ZHAO^{1*}, HAO JIANG^{2*}, DAN SU³, JIANGUO FENG³, SHENGLIN MA⁴ and XINHAI ZHU⁵

¹Department of Radiation Oncology, Hangzhou Cancer Hospital, Hangzhou, Zhejiang 310002; ²Department of Oncology, Zhejiang Hospital, Hangzhou, Zhejiang 310013; ³Department of Oncology Institute, Zhejiang Cancer Hospital, Hangzhou, Zhejiang 310022; ⁴Department of Oncology, Hangzhou First People's Hospital, Hangzhou, Zhejiang 310006, ⁵Department of Thoracic Surgery, Zhejiang Hospital, Hangzhou, Zhejiang 310013, P.R. China

Received January 30, 2014; Accepted September 12, 2014

DOI: 10.3892/mmr.2014.2655

Abstract. Rhizoma paridis is widely used for cancer therapy due to its potential involvement in the suppression of tumor growth. However, at present there is no clear explanation for the mechanism underlying the inhibitory effects of Rhizoma paridis combined with hyperthermia on tumor growth. The aim of the present study was to evaluate the effects of Paris saponin I (PSI) combined with hyperthermia on a variety of non-small cell lung cancer (NSCLC) cell lines. An MTT assay was used to determine the levels of growth inhibition. The cell cycle was analyzed using flow cytometry and cell apoptosis was analyzed with Annexin V/propidium iodide staining and the Hoechst assay. The morphology of cells during apoptosis was determined using a transmission electron microscope. The expression levels of B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X protein (Bax) and caspase-3 proteins were detected using western blotting. The inhibition rates significantly increased with PSI in combination with hyperthermia at 43°C. PSI with hyperthermia at 43°C caused G2/M phase arrest and significantly induced apoptosis. The expression level of Bcl-2 decreased, while Bax expression increased following treatment with PSI with hyperthermia at 43°C. In addition, the protein expression of caspase-3 was significantly enhanced. PSI combined with hyperthermia is a potent antitumor treatment through the inhibition of proliferation of NSCLC cells and may be developed as a new antitumor therapy. PSI combined with hyperthermia significantly induced apoptosis through a multi regulatory process involving G2/M arrest and

regulation of Bax, Bcl-2 and caspase-3 expression, resulting in cell death and tumor inhibition.

Introduction

Lung cancer is one of the leading causes of cancer-associated mortality worldwide. Non-small cell lung cancer (NSCLC) accounts for ~85% of all cases of lung cancer (1). Patients diagnosed with NSCLC have low survival rates and conventional therapies currently available are rarely beneficial. The optimal chemotherapeutic treatments for NSCLC are often limited by dose-related toxicity (2). Thus, developing new therapeutic agents is urgently required for NSCLC patients. Natural products represent a source of novel antiproliferative agents.

Rhizoma paridis is the root and rhizome of Paris polyphylla var. yunnanensis, which belongs to the Liliaceae family. It is widely used in traditional Chinese medicine for its anti-tumor, antifertility, spermicidal, immunological enhancement and sedative properties (3-5). Several studies have demonstrated that extracts from Rhizoma Paridis possess anti-tumor properties against a variety of malignant cell lines (6,7). The steroidal Paris saponins are the active components of Rhizoma paridis. Five Paris saponins have been identified: Paris saponin I (PSI), also known as polyphyllin D (8-12), Paris saponin V (PSV), Paris saponin VI (PSVI), Paris saponin VII (PSVII) and Paris saponin H (PSH). As the active ingredient, Paris saponin I (PSI) is important in the treatment of cancer due to its anti-tumor activity, cytotoxic effects and anti-angiogenic activity (13-16).

It has been demonstrated that hyperthermia can cause regression of tumors and may be applicable to a wide range of cancer types (17-20). Hyperthermia is an adjuvant therapeutic modality to treat cancer by maintaining the temperature of the tumor region in order to inhibit the regulatory and growth processes of cancer cells. In addition, hyperthermia causes cancer cells to become more sensitive to the effects of radiation and certain anticancer drugs (17-20). Improvement in clinical outcome involving induced hyperthermia is suggested to be associated with its ability to inhibit DNA repair, promote intracellular accumulation of chemical agents, alter cellular Ca²⁺ homeostasis, induce cell cycle arrest and apoptosis,

Correspondence to: Dr Xinhai Zhu, Department of Thoracic Surgery, Zhejiang Hospital, 12 Lingyin Road, Hangzhou, Zhejiang 310013, P.R. China
E-mail: zjyylk@163.com

*Contributed equally

Key words: Paris saponin I, hyperthermia, non-small cell lung cancer, apoptosis

increase membrane permeability and rearrange the cytoskeleton (21-23). Hyperthermia also has direct cytotoxic effects, which provide a number of other clinical advantages, including activation of the immune system against tumors, improvement of oxygenation and positive effects on drug delivery (24-26). However, the exact underlying mechanisms remain to be elucidated.

In our preliminary experiments, it was demonstrated that PSI significantly inhibited the growth of PC-9 cells in a dose- and time-dependent manner by causing G2/M arrest, inducing apoptosis and regulating the B-cell lymphoma 2 (Bcl-2)/Bcl-2-associated X protein (Bax) ratio (27). In the present study, hyperthermia in tandem with PSI was selected as a combination therapy to increase the antitumor activity of PSI. In order to further elucidate the biological effects and mechanisms by which hyperthermia combined with PSI functions and to improve the effects of this novel anticancer agent, the role of PSI at different temperatures in inhibiting NSCLC cell growth was investigated and cell cycle arrest, apoptosis and the expression of key proteins were evaluated. This may provide a theoretical and practical basis for their prospective use in cancer therapy.

Materials and methods

Drugs and reagents. Paris Saponin I ($C_{44}H_{70}O_{16}$; Fig. 1) was obtained from the ZheJiang Institute for Food and Drug Control (Hangzhou, China; cat. no. 111590). It was dissolved in dimethyl sulfoxide (DMSO) as a 100 $\mu\text{g}/\mu\text{l}$ stock solution and stored at -20°C . This was diluted in Dulbecco's modified Eagle's medium (DMEM) to achieve the final concentration indicated for each experiment. DMEM and fetal calf serum were obtained from HyClone Laboratories, Inc. (Logan, UT, USA). A polyclonal rabbit anti-rat Bax antibody used at 1:2,000 dilution and a monoclonal mouse anti-rat Bcl-2 used at 1:2,000 dilution were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) (17). A polyclonal rabbit anti-rat caspase 3 antibody, used at 1:25 dilution was purchased from Abcam (Cambridge, MA, USA). Cycle Test™ Plus DNA Reagent kit and Annexin V-fluorescein isothiocyanate and propidium iodide (PI) Apoptosis Detection kits were purchased from BD Biosciences (Franklin Lakes, NJ, USA) and Hoechst 33258 was obtained from Beyotime Institute of Biotechnology. (Jiangsu, China). All other chemical reagents were purchased from Sigma (St. Louis, MO, USA). The present study was approved by the ethics committee of Zhejiang Hospital (Hangzhou, Zhejiang, China).

Cell lines and culture. The NSCLC cell line PC-9, was cultured in Dulbecco's modified Eagle's minimum essential medium (Hyclone Laboratories, Inc.) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Inc.), in a humidified incubator (Thermo Fischer Scientific, Inc., Houston, TX, USA) containing 5% CO_2 at 37°C .

MTT assay. The MTT assay was performed to detect cell proliferation following exposure to PSI with hyperthermia. Following harvesting by trypsinization, the PC-9 cells (100 $\mu\text{l}/\text{well}$) were seeded in 96-well plates at a density of 1×10^4 cells/ml. Each group had three wells with a non-treated group as the control.

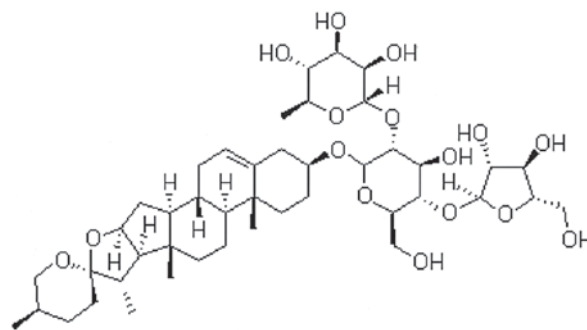


Figure 1. Chemical structure of Paris saponin I.

When the cells had anchored to the plates, various concentrations of PSI (0.5, 1, 2, 3 and 4 $\mu\text{g}/\text{ml}$) were added and the plates were incubated at different temperatures (37, 39, 41 and 43°C) for 1 h in a humidified atmosphere containing 5% CO_2 . After 24 h, 20 μl of 0.5% MTT was then added to each well and cultured for another 4 h. Following this, the supernatant was discarded, MTT formazan precipitates were dissolved in 150 μl DMSO, agitated mechanically for 10 min and then the absorbance (A) value was measured at 492 nm using a multiscanner autoreader (Thermo Fisher Scientific, Inc.) The following formula was used: Inhibition rate (%) = $(1 - \text{average A value of the experimental samples}) / \text{average A value of the control}) \times 100\%$.

Cell cycle analysis by flow cytometry. The experimental groups included the control group, PSI group, hyperthermia group and PSI + hyperthermia group. Cells were treated with 1.21 $\mu\text{g}/\text{ml}$ PSI and incubated at 43°C , then harvested at 24, 48 and 72 h, fixed with 70% ethanol and stored overnight at -20°C . The following day, cells were incubated in 10 $\mu\text{g}/\text{ml}$ RNase for 30 min at 37°C and then stained in 50 $\mu\text{g}/\text{ml}$ propidium iodide (PI) for 1 h at 4°C in the dark. Cell cycle analysis was performed on a fluorescence-activated cell sorting (FACS) Calibur flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA) and the data were analyzed using BD Cell Quest Pro software, version 5.1 (Becton-Dickinson, Franklin Lakes, NJ, USA). The experiments were repeated three times.

Apoptosis analysis by flow cytometry. Cell apoptosis was examined by PI/Annexin V double staining and Hoechst staining. The experimental groups included the control group, PSI group, hyperthermia group and PSI + hyperthermia group. Cells were treated with 1.21 $\mu\text{g}/\text{ml}$ PSI and incubated at 43°C , harvested at 24 and 48 h and then stained with PI and Annexin V. The apoptotic fraction was detected by flow cytometry (Beckman Coulter, Inc., Miami, FL, USA). Cells were washed in phosphate-buffered saline (PBS), stained with Hoechst 33528 (5 $\mu\text{g}/\text{ml}$ in PBS) for 15 min at room temperature and then observed under an Olympus BX60 fluorescence microscope (Olympus, Tokyo, Japan) equipped with 356 nm excitation and 492 nm emission bandpass filters.

Western blotting. The experimental groups included the control group, PSI group, hyperthermia group and PSI + hyperthermia group. Cells were treated with 1.21 $\mu\text{g}/\text{ml}$ PSI and incubated at 43°C , then harvested at 48 h. Samples containing equal

Table I. Effect of PSI with hyperthermia on the G2/M phase of PC-9 cells (% , $\bar{x}\pm s$).

Group	24 h	48 h	72 h
Control	8.17±1.88	11.34±2.46	10.22±1.63
PSI ^a	21.47±2.75	26.71±2.58	29.11±2.92
Hyperthermia	11.33±2.17	10.67±1.65	14.52±2.18
PSI + Hyperthermia ^b	33.59±2.24	38.18±3.73	42.58±3.14

^aP<0.01 vs. control group; ^bP<0.01 vs. PSI group. PSI, Paris saponin I.

quantities of proteins were electrophoresed on 10% SDS-PAGE gel and transferred onto polyvinylidene difluoride membranes and then incubated with specific primary antibodies. The blots reacted with horseradish peroxidase conjugated secondary antibodies and were detected using the ECL system (Santa Cruz Biotechnology, Inc.). The density of the band was quantified by densitometry exposed to X-ray film (Eastman-Kodak, Rochester, NY, USA) using GAPDH as a control.

Statistical analysis. The experiments were repeated three times. The data are presented as the mean ± standard deviation. Groups were compared using one-way analysis of variance. P<0.05 or P<0.01 was considered to indicate a statistically significant difference.

Results

PSI with hyperthermia inhibits proliferation of PC-9 cells. PSI with hyperthermia inhibited the growth of PC-9 cells in a dose-dependent manner with increasing concentrations between 0.5 and 4 µg/ml at different temperatures for 24 h. The half maximal inhibitory concentration (IC50) was 2.69 µg/ml (37°C, control), 3.27 µg/ml (39°C), 3.28 µg/ml (41°C) and 1.21 µg/ml (43°C), respectively. The effect of PSI with hyperthermia on the growth of PC-9 cells using an MTT assay is shown in Fig. 2.

PSI with hyperthermia induces G2/M arrest of PC-9 cells. Flow cytometric analysis revealed the effect of PSI (IC50=1.21 µg/ml) at 43°C on cell cycle distribution. PSI alone could induce G2/M arrest in a time-dependent manner. The percentage of cells at the G2/M phase increased from 21.47 to 29.11% compared with the control group (P<0.01), however, hyperthermia at 43°C significantly altered cell cycle distribution of PSI-treated cells leading to cell cycle arrest at the G2/M phase in a time-dependent manner. The percentage of cells at the G2/M phase increased from 33.59 to 42.58% compared with the PSI group (P<0.01; Table I).

PSI with hyperthermia induces apoptosis in PC-9 cells. The ability of PSI combined with hyperthermia to induce apoptosis in PC-9 cells was assessed using the Annexin-V/PI double staining and Hoechst staining assay. PSI (IC50=1.21 µg/ml) induced significant levels of apoptosis in PC-9 cells and PSI (IC50=1.21 µg/ml) with hyperthermia at 43°C increased the apoptotic ratio at 24 and 48 h (P<0.01; Table II). From the Hoechst staining assay, it was demonstrated that the cells in

Table II. Effect of PSI with hyperthermia on the apoptosis of PC-9 cells (% , $\bar{x}\pm s$).

Group	24 h	48 h
Control	2.43±0.67	5.47±1.91
PSI ^a	18.27±2.45	29.17±2.55
Hyperthermia	8.16±0.97	13.05±0.31
PSI + Hyperthermia ^b	28.82±2.46	39.63±2.18

^aP<0.01 vs. control group; ^bP<0.01 vs. PSI group. PSI, Paris saponin I.

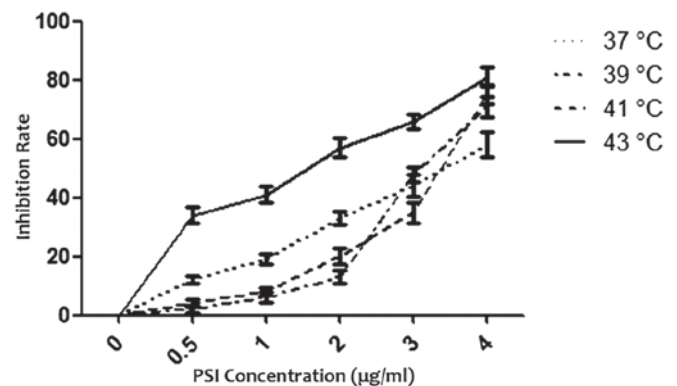


Figure 2. Inhibition rates of PSI with hyperthermia at different temperatures in PC-9 cells. Percentage of cell viability was determined using an MTT assay. The inhibition rate was significantly increased in the 43°C treatment group compared with the 37, 39 and 41°C treatment groups (P<0.01) in a dose-dependent manner for 24 h. PSI, Paris saponin I.

the control and hyperthermia at 43°C groups were morphologically normal and the nuclei were regularly-shaped with even staining. However, typical morphological alterations associated with apoptosis were identified, including nuclear shrinkage, DNA condensation and chromatin fragmentation in the PSI (IC50=1.21 µg/ml) and PSI (IC50=1.21 µg/ml) with hyperthermia at 43°C group (Fig. 3). This indicates that hyperthermia at 43°C can further increase the apoptosis induced by PSI.

Effects of PSI with hyperthermia on the levels of Bcl-2, Bax and caspase-3 in PC-9 cells. In order to examine the potential signaling pathways by which PSI induces apoptosis and cell cycle arrest, western blotting was used to evaluate the expression of the Bcl-2 family and caspase-3 protein. The level of Bcl-2 protein decreased, while the level of Bax and caspase-3 protein

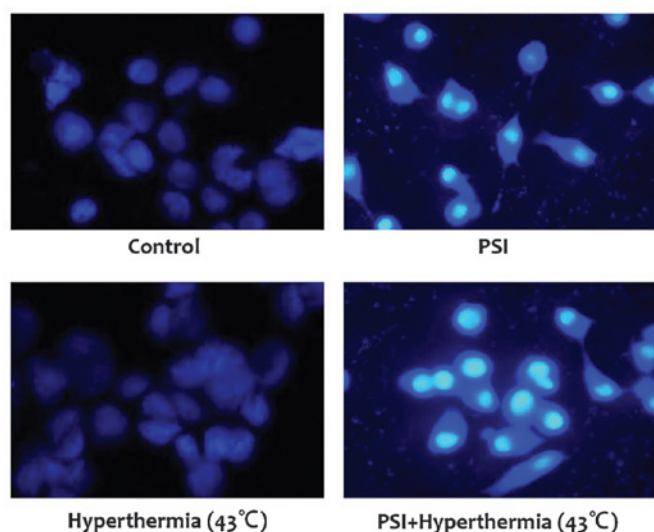


Figure 3. Fluorescent imaging of nuclei in the control group, PSI group, hyperthermia (43°C) group and PSI + hyperthermia (43°C) group by Hoechst 33258 staining. PC-9 cells stained with the fluorescent dye Hoechst 33258 were visualized by fluorescence microscopy. Condensed and fragmented nuclei were observed in the PSI group and PSI + hyperthermia (43°C) group, however, not in the control group and hyperthermia (43°C) group. PSI, paris saponin 1.

increased following treatment with PSI (IC₅₀=1.21 μg/ml) for 48 h, which is a significant increase to PSI (IC₅₀=1.21 μg/ml) with hyperthermia at 43°C (Fig. 4A-C).

Discussion

Rhizoma Paridis and its components have been extensively used in China as antibacterial, antifungal, antimicrobial, anti-inflammatory and hemostatic regulatory medications (28). PSI has been approved for cancer therapy due to its potential involvement in the suppression of tumor growth (13-16). The inhibitory effects of PSI were associated with increased levels of pro-apoptotic Bax, cytochrome c, active caspase-9 and active caspase-3. PSI also decreased anti-apoptotic Bcl-2 expression levels and phosphorylated extracellular signal-regulated kinase 1/2 in treated cells (16,29-31).

Hyperthermia can be a highly effective cancer treatment, particularly when combined with chemotherapy, radiotherapy or immunotherapy (32-34). Hyperthermia can inhibit DNA repair, promote intracellular accumulation of chemical agents, alter cellular Ca²⁺ homeostasis, induce cell cycle arrest and apoptosis, increase membrane permeability and rearrange the cytoskeleton (20-25). Thus, hyperthermia in combination with PSI was selected to increase the antitumor activity of PSI.

In our previous study, PSI exhibited anti-tumor effects in PC-9 cells (27). However, in the present study, four temperatures were selected combined with PSI on PC-9 cells in order to decide which is the optimum temperature for PSI to inhibit the growth of NSCLC cells and elucidate the potential mechanisms.

Current literature reported that hyperthermia affects various cellular targets, including DNA, proteins, membranes and the cytoskeleton of carcinoma cells following exposure to temperatures between 41.5 and 45.5°C (35). There are a number

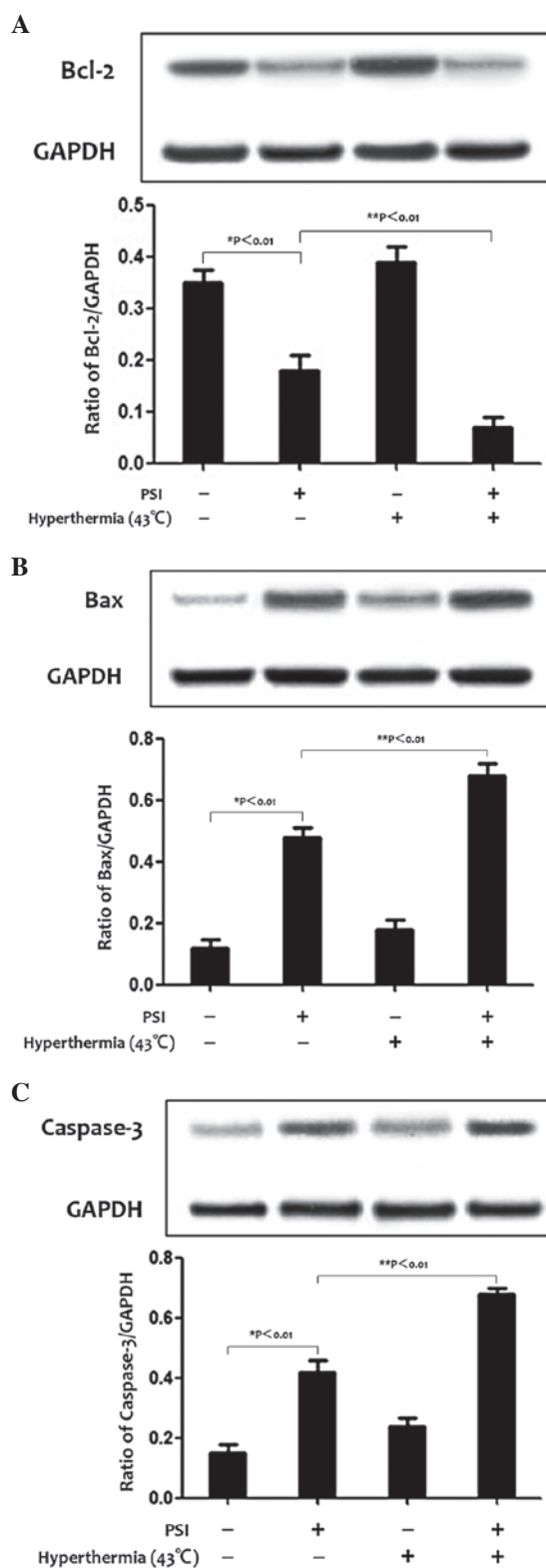


Figure 4. Effect of PSI with hyperthermia on the levels of Bcl-2, Bax and caspase-3 protein expression in the control group and treatment groups. Protein levels were detected by western blotting and the graphs show the percentage of protein levels in the treatment and control groups. Values are presented as the means of triplicate analysis. Error bars show the standard deviations. *P<0.01 and **P<0.01, significant differences between the treatment group compared with the control group and between treatment groups, respectively. (A) PSI decreased the expression of Bcl-2 in PC-9 cells, however, this decrease was more marked in the PSI with hyperthermia at 43°C treatment group. (B and C) PSI increased the expression of Bax and caspase-3, however, this effect was more marked in the PSI with hyperthermia at 43°C treatment group. PSI, paris saponin 1; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein.

of studies demonstrating that hyperthermia at 43°C could improve the inhibitory effect of antitumor agents, including curcumin and triptonide in Hep-2 cells (36,37). In the present study it was demonstrated that PSI with hyperthermia inhibited the growth of PC-9 cells in a dose-dependent manner with increasing concentrations between 0.5 and 4 µg/ml at different temperatures, and the IC₅₀ of PSI was 2.69 µg/ml (37°C, control), 3.27 µg/ml (39°C), 3.28 µg/ml (41°C) and 1.21 µg/ml (43°C), respectively. It was revealed that hyperthermia at 43°C was the most effective temperature to enable PSI to inhibit the PC-9 cells. Therefore, hyperthermia at 43°C with PSI (IC₅₀=1.21 µg/ml) was used to further elucidate the potential underlying mechanisms.

Cell cycle regulation is important for cell proliferation. In the present study, it was found that PSI altered the cell cycle distribution of PC-9 cells, leading to cell cycle arrest at the G₂/M phase, but hyperthermia at 43°C altered the cell cycle distribution of PSI-treated cells further, leading to cell cycle arrest at the G₂/M phase in a time-dependent manner. The percentage of cells at the G₂/M phase increased from 33.59 to 42.58% compared with the PSI group. PSI can also increase apoptosis in PC-9 cells. PSI can induce significant apoptosis in PC-9 cells and in combination with hyperthermia at 43°C can increase the apoptotic ratio further. Caspases are crucial mediators of apoptosis. Among them, caspase-3 is a frequently activated death protease, catalyzing the specific cleavage of numerous key cellular proteins (38). The Bcl-2 family, which comprise of anti-apoptotic (Bcl-2 and Bcl-x1) and pro-apoptotic members (Bax and Bak), was the main controller and mediator of cell apoptosis (39,40). The high Bcl-2/Bax ratio is considered a crucial factor of cell resistance to apoptosis (41,42). In the present study, the results indicated that the level of Bcl-2 protein decreased, while the level of Bax and caspase-3 protein increased following treatment with PSI and increased further in PSI with hyperthermia at 43°C. Thus, hyperthermia at 43°C increases the number of cells arresting at the G₂/M phase and promotes apoptosis induced by PSI through the association between Bcl-2 and Bax and caspase-3, eventually leading to inhibition of cell proliferation.

In conclusion, PSI is a potent antitumor agent and hyperthermia at 43°C can significantly enhance the inhibitory effect of PSI on PC-9 cells. This occurs through inducing G₂/M arrest and apoptosis via a decrease of Bcl-2 expression and increase in the protein expression of Bax and caspase-3. This modality may have significant therapeutic potential in clinical settings.

Acknowledgements

The study was supported by grants from the National Natural Science Foundation of China (nos. 81303274 and 81202947).

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