Original research paper

Paeoniflorin inhibits the growth of bladder carcinoma *via* deactivation of STAT3

JIANHUI-YANG
YU REN
ZHONG-GUAN LOU
XUE WAN
GUO-BIN WENG*
DONG CEN*

Laboratory of Kidney Carcinoma
Ningbo Urology and Nephrology
Hospital, Urology and Nephrology
Institute of Ningbo University, Ningbo
Zhejiang 315000, P.R. China

Bladder cancer (BCa) is one of the most common urinary cancers. The present study aims to investigate whether Paeoniflorin (Pae) can exert inhibitory effects on BCa. The results showed that Pae inhibited proliferation of human BCa cell lines in a concentration- and time-dependent manner. Pae and cisplatin (Cis) synergistically inhibited the growth of tumours in RT4-bearing mice. Pae treatment neutralized the body loss induced by Cis. Moreover, Pae induced apoptosis in RT4 cells and increased the activities of caspase3, caspase8 and caspase9. Western blotting and immunohistochemical analysis revealed that the phosphorylated signal transducer and activator of transcription-3 (p-STAT3) level were decreased in Pae-treated RT4 cells and Pae-treated tumour-bearing mice. Furthermore, STAT3 transcriptional target B-cell lymphoma-2 was decreased in Pae-treated RT4 cells. Interestingly, Pae prevented translocation of STAT3 to the nucleus in RT4 cells. Collectively, Pae inhibits the growth of BCa, at least in part, *via* a STAT3 pathway.

Bladder cancer (BCa) is one of the most common malignancies in the western world. In the United States, it was estimated that there were 76,960 new cases and 16,390 deaths in 2016 (1). It was previously reported that approximately 30 % of BCa patients had muscle and invasive cancer cells and 10 % of patients suffered from metastatic disease, which resulted in a poor prognosis (2). Current application of chemotherapy, radiotherapy and radiochemotherapy has made great progress in preventing BCa, and the overall survival has been improved. However, recurrence and metastasis of BCa occurs frequently, leading to unsatisfactory survival rates. Moreover, poor prognosis has persisted due to fatigue, pain and side effects induced by chemotherapy (3). Thus, there is a demand to develop a safe and effective drug for improving treatment and prognosis of BCa patients.

Signal transducer and activator of transcription-3 (STAT3), one of the STAT family members, is a downstream effector of various cytokines, growth factors and hormones (4). Fol-

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* Correspondence; e-mail: cengdong2002@163.com, wgbningbo@126.com

lowing activation, phosphorylated STAT3 (p-STAT3) dimerizes and translocates to the nucleus, where it finally regulates the expression of numerous critical genes that control cell proliferation and survival (5–6). A number of studies have been performed on BCa, which have highlighted STAT3 as a potential therapeutic target. In clinical specimens, it has been confirmed that urothelial BCa displays elevated expression as well as activation of STAT3 (7). Silencing of STAT3 suppresses the proliferation of T24 cells *in vitro* and *in vivo* compared to the controls, and overexpression of STAT3 prevents cells from undergoing morphological changes and apoptosis triggered by the *Ha-ras* oncogene (8–9). Moreover, inhibition of STAT3 by small interfering RNA or a specific inhibitor restores chemosensitivity in T24 cells (10), suggesting that STAT3 could be a major target for increasing chemosensitivity in patients.

Interest in the identification of novel anticancer agents derived from natural sources has been growing exponentially. Paeoniflorin (Pae) is the main active ingredient of Radix *Paeoniae alba* (RPA), which is used in traditional Chinese medicine to treat a range of inflammatory diseases (11). Today, there is interest in studying the therapeutic effects of Pae on different cancer types. In previous pharmacological investigations, Pae was confirmed to inhibit the growth of human pancreatic cancer, gastric carcinoma cells, colorectal carcinoma cells, and glioma cells (12–15). A new study (16) recently reported the anti-proliferative properties of RPA extraction in treating BCa, both *in vitro* and *in vivo*. However, it is unknown whether Pae could inhibit BCa. Therefore, the main purpose of the present study was to estimate the anti-tumour activity of Pae in BCa.

EXPERIMENTAL

Cell lines and culture

Human BCa cell lines (RT4, 253J and J82) were originally obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were maintained in Dulbecco's Modified Eagle Medium (Gibco-BRL, USA), supplemented with 10 % foetal bovine serum (Sigma-Aldrich, USA), 4 mmol L^{-1} glutamine and 100 U mL^{-1} penicillin, at 37 °C in 5 % CO_2.

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay

A total of 2×10^3 cells were seeded in a 96-well plate and then incubated with different concentrations of Pae (purity > 98 %; Chengdu Biopurify Phytochemicals Ltd., China) for 24–72 h. The cells were treated with 20 µL of MTT (5 g L^{-1}, Sigma-Aldrich) solution and incubated at 37 °C for 4 h. After discarding the medium, absorbance was recorded by a microplate reader (Biotek, Vermont, USA). Each cell viability assay was performed in triplicate. The relative cell viability (%) was determined by comparing the absorbance with the control, which was treated with a phosphate buffered solution (PBS). The IC_{50} value was determined according to the growth curve of the cells using the GraphPad Prism 5.0 software.

Apoptosis assay

Apoptosis in RT4 cells was detected using a cell apoptosis detection kit (BD Pharmingen, San Diego, CA, USA). Briefly, the cells were seeded 2.5×10^5 per well in 6-well plates for 24 h and were then treated with Pae (25 or 50 µM) for 48 h. The cells were labelled by

Annexin V-fluorescein isothiocyanate (FITC) and stained using a binding buffer for 30 min in the dark according to the instructions. Subsequently, 10 μL of propidium iodide (PI) was added to the cells and incubated for 15 min at room temperature in the dark. The apoptotic rate was measured by flow cytometry (FACS Calibur, BD Biosciences).

Caspase activity assay

Activities of caspase-3, caspase-8 and caspase-9 in Pae-treated RT4 cells (48 h) were detected by caspase activity assay kits from Beyotime Biotechnology (China).

Western blotting

RT4 cells were pre-treated with Pae (25 or 50 μM) in a serum-free medium for 48 h. Nuclear protein was extracted using a nuclear and cytoplasmic protein extraction kit provided by Beyotime Biotechnology (Nantong, China). The amount of protein was quantified by the bicinchoninic acid assay (Thermo Fisher Scientific, USA). Equal amounts of extract were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Gibco-Life Technologies, USA). The membranes were incubated with a primary antibody and then probed with a secondary antibody and visualized by enhanced chemiluminescence. All western blot experiments were performed at least three times. The primary antibodies used were as follows: anti-B-cell lymphoma-2 (Bcl-2), anti-Bcl-2 associated X protein (Bax) and anti-p-STAT3, all purchased from Santa Cruz Biotechnology (USA). β-actin and histone were used as internal controls for relative quantification.

In vivo *tumour xenograft study*

Male BALB/c nude mice (20–22 g) were from the Shanghai Laboratory Animal Center Co., Ltd. (China). The animals were housed in temperature-controlled rooms (22 ± 2 °C), with relative humidity of 55 ± 5 % and a 12 h light/dark cycle. All protocols involved in animal experiments were approved by the Animal Care and Use Committee of Ningbo Urology and Nephrology Hospital (Qianhe road, No. 1, Yinzhou, Ningbo, China). Moreover, the animal experiments were also conducted according to the U.K. Animals (Scientific Procedures) Act, of 1986 and associated guidelines. RT4 cells (5×10^6) in 100 μL of PBS were subcutaneously injected into the right flank area of the mice. After the tumours grew to an area of about 100 mm³, the tumour-bearing mice were randomly divided into the control group, Pae group (purity > 98 %, 100 mg kg⁻¹, Aladdin Ltd.), cisplatin (Cis, purity > 99 %, 5 mg kg⁻¹, Sigma-Aldrich,) group, and Pae (100 mg kg⁻¹) + Cis (5 mg kg⁻¹) group, with five mice in each group. They were given an intraperitoneal injection daily for 20 days. Body weight and tumour growth were measured every four days. The size of tumours was measured according to the following formula: tumour volume = $\pi/6 \times L \times W^2$, in which L and W represent the major and minor diameter, respectively. At the end of the study, the mice were sacrificed and tumour tissue was weighed prior to immunohistochemistry.

Immunohistochemistry and immunocytochemistry

RT4 cells were treated with 50 μM Pae for 48 h and fixed in 4 % paraformaldehyde for 20 min at room temperature. The cells were incubated with anti-STAT3 overnight at 4 °C

(1:100, Santa Cruz), followed by incubation with FITC-conjugated secondary antibodies for 1 h at room temperature. Finally, the cells were labelled with a 4′,6-diamidino-2-phenylindole (DAPI) solution and observed with a confocal laser scanning microscope (TCS SP8, Leica Microsystems, Germany). Moreover, tumour tissues were fixed with 10 % formaldehyde for 24 h. Paraffin-embedded slides were conventionally prepared. The sections were deparaffinised in xylene, rehydrated by descending grades of ethanol, and incubated with an antibody against p-STAT3 (Santa Cruz Biotechnology). After washing with PBS, slides were incubated with the biotinylated secondary antibody (Dako). The immunostained cells were quantified by counting the brown cells and the total number of cells in five randomly selected fields at 400× magnification.

Statistical analysis

The data are expressed as the mean ± SD. The statistical difference was determined by one-way ANOVA tests and Student's *t*-test. A *P* value less than 0.05 was considered statistically significant. All statistical analyses were performed using the SPSS version 13.0 statistical software (SPSS, USA).

RESULTS AND DISCUSSION

The cytotoxic effect of Pae on BCa cells was evaluated by the MTT assay after exposure to Pae. As shown in Fig. 1b,c, Pae treatment decreased RT4, J82 and 253J cell viability
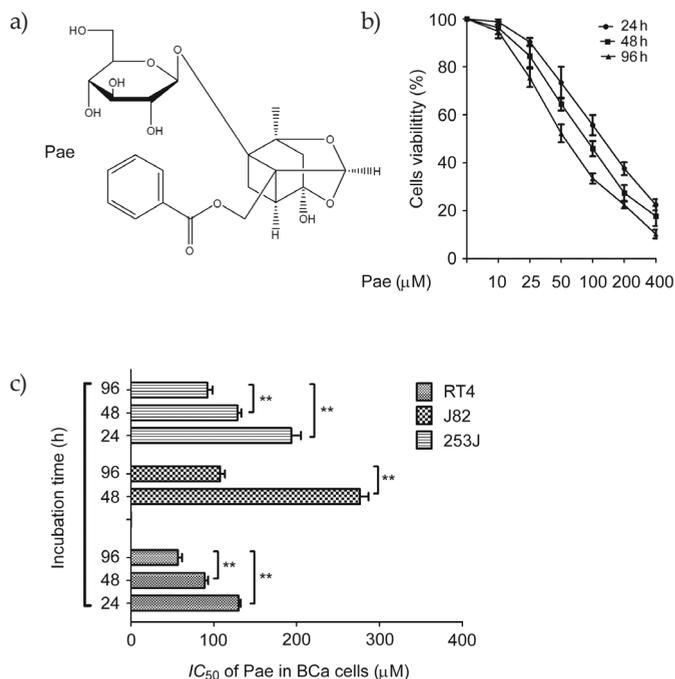

Fig. 1. Cytotoxicity of Pae in BCa cells. a) Pae chemical structure, b) RT4 cells were incubated with different concentrations of Pae for 24, 48 and 96 h, c) IC_{50} of Pae. Data are presented as the mean ± SD, *n* = 3; [b] *p* < 0.01 compared to the 96 h group.
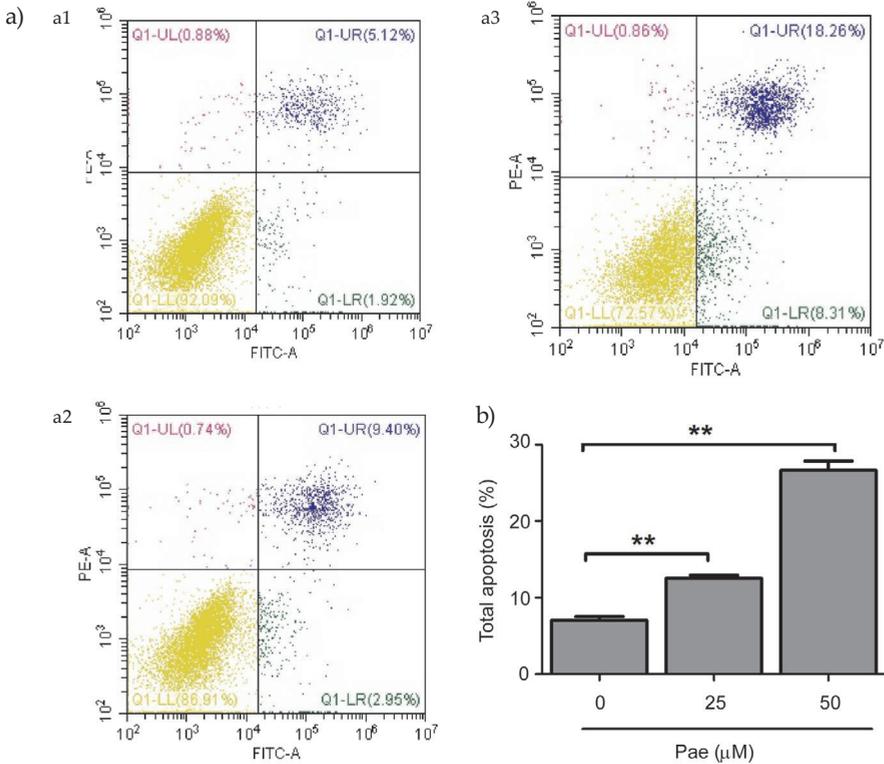
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Fig. 2. Effects of Pae on apoptosis of RT4 cells. a) Representative apoptosis data analysed by flow cyto-metry using an AnnexinV-FITC in RT4 cells are shown for a1 (control group), a2 (Pae 25 µM group) and a3 (Pae 50 µM group), b) quantitative apoptosis data in Pae treated RT4 cells are shown. Data are pre-sented as the mean ± SD, $n = 3$; **$p < 0.01$ compared to the control group (without Pae treatment).

in a dose-dependent and time-dependent manner. The IC_{50} values of Pae in the RT4 group were lower than that in J82 and 253J cells ($p < 0.01$). Consequently, RT4 was used for the following study.

We further examined whether Pae could induce apoptosis in RT4 cells. As seen in Fig. 2, Pae treatment induced total apoptosis in RT4 cells. Moreover, we also noticed that Pae-treated RT4 cells showed higher levels of early and late apoptotic rates compared to those of the control group ($p < 0.01$, Table I). To determine the mechanism underlying the Pae-mediated apoptosis in RT4 cells, we investigated the effects on caspase3, caspase8 and caspase9 activities. The results showed that Pae treatment increased the activities of caspase3, caspase8 and caspase9 in RT4 cells ($p < 0.01$, Table II). Moreover, it should be noted that Bax expression increased and Bcl-2 expression decreased in RT4 cells that were exposed to Pae ($p < 0.01$, Fig. 3a,b).

As STAT3 plays an important role in BCa cell proliferation and survival, we investi-gated whether Pae modulates constitutive STAT3 activation in RT4 cells. Fig. 4A-4B show

Table I. Effects of Pae on early and late apoptosis of RT4 cells

Concentration (μM)	Early apoptosis (%)	Late apoptosis (%)
0	1.88 ± 0.26	5.20 ± 0.62
25	3.07 ± 0.34**	9.50 ± 0.52**
50	8.45 ± 0.41**	18.20 ± 1.30**

Data are presented as the mean ± SD, $n = 3$; [**]$p < 0.01$ compared to the control group (without Pae treatment).

Table II. Effects of Pae on caspase activities of RT4 cells

Concentration (μM)	Caspase-3 activity (IU)	Caspase-8 activity (IU)	Caspase-9 activity (IU)
0	1.88 ± 0.26[a]	1.51 ± 0.16	2.20 ± 0.41
25	4.62 ± 0.50[b]	6.90 ± 0.75[b]	5.08 ± 0.52[b]
50	12.2 ± 0.92[b]	8.56 ± 0.56[b]	11.50 ± 3.73[b]

Data presented as the mean ± SD, $n = 3$; [b]$p < 0.01$ compared to the control group (without Pae treatment).

that Pae incubation inhibited STAT3 phosphorylation in RT4 cells ($p < 0.01$). Moreover, in view of the nuclear translocation of STAT3 being central to the biological function, we determined whether Pae suppressed nuclear translocation of STAT3. As shown in Fig. 4c, Pae treatment inhibited the translocation of STAT3 to the nucleus in RT4 cells.

To determine if Pae could inhibit tumour growth *in vivo*, we studied the effects of Pae on RT4 cell xenografts in nude mice. As shown in Fig. 5B-5C, administration of Pae significantly reduced the weight and volume of tumour tissue ($p < 0.01$). Interestingly, we noticed that Pae and Cis showed synergistically inhibitory effects on the growth of tumour
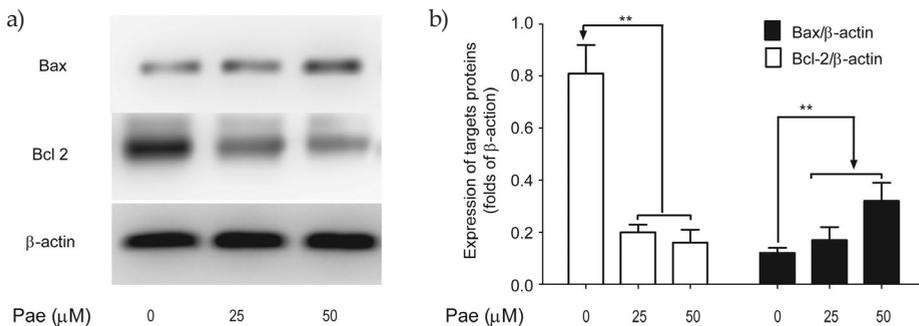

Fig. 3. Effects of Pae on Bax and Bcl-2 levels. a) Representative immunoblotting bands of Bax and Bcl-2. β-actin was used as an internal reference, b) quantitative data of the levels of Bax and Bcl-2. Data are presented as the mean ± SD, $n = 3$; ** $p < 0.01$ compared to the control group (without Pae treatment).
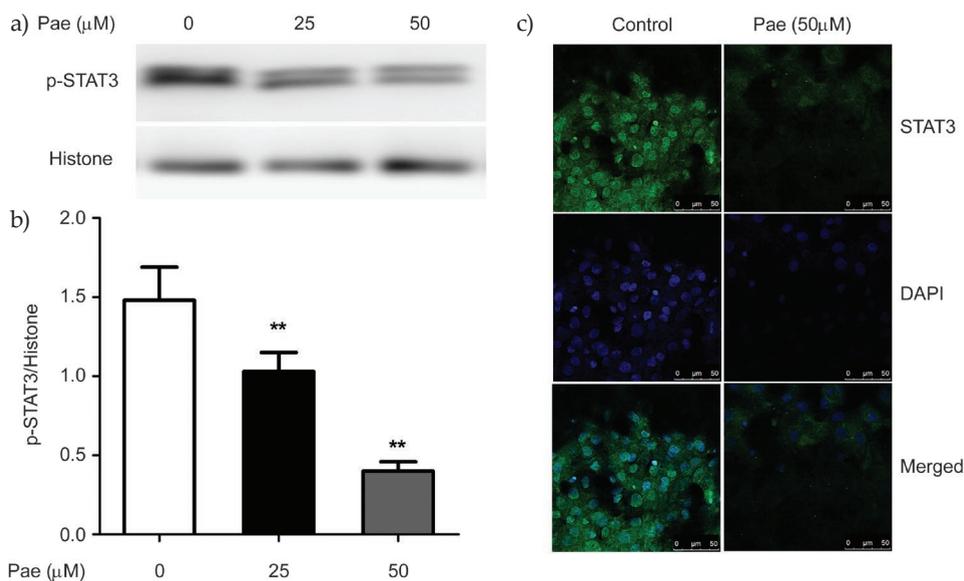
Fig. 4. Effects of Pae on p-STAT3 expression and nuclear translocation *in vitro*. a) Representative immunoblotting bands of p-STAT3. Histone was used as an internal reference, b) quantitative data of p-STAT3 expression, c) RT4 cells were treated with 50 μM Pae for 48 h. Data are presented as the mean ± SD, *n* = 3; ** *p* < 0.01 compared to the control group (without Pae treatment).

tissue ($p < 0.01$). In addition, Pae treatment had no effects on the body mass of tumour-bearing mice, while Cis significantly reduced the weight (Fig. 5a, $p < 0.01$). Furthermore, the body weight of the combined group was not statistically different from that of the control group and the Pae group at the end of the study. Moreover, in Fig. 5d, the rates of p-STAT3-positive cells in control or Pae-treated mice were 46.0 ± 4.5 % and 12.0 ± 1.3 %, respectively ($p < 0.01$), suggesting that Pae significantly suppresses the activation of STAT3 in RT4-bearing mice.

Naturally derived compounds are a prime source of highly effective conventional anti-cancer drugs. Pae, extracted from RPA, is a natural anti-cancer medicine. Many pharmacological studies have reported that Pae exerts anti-cancer and anti-proliferative activity in cultured tumour cells, as well as in tumour xenograft models (12–15). In 2016, Lin *et al.* (16) reported the anti-proliferative properties of RPA extract on BCa, both *in vitro* and *in vivo*. In their research, they also found that the extract showed lower cytotoxicity to normal cells compared to that in BCa cells. It is well known that Pae is the main active ingredient in the RPA extract (17). Therefore, the evidence suggests that Pae may inhibit the growth of BCa tumours. Our investigation first explored the anti-tumour effects of Pae in BCa in both *in vitro* and *in vivo* models. Our data demonstrated that Pae effectively inhibits BCa cell (RT4, J82 and 253J) growth *in vitro*. The IC_{50} of Pae for inhibiting RT4 growth is 88.93 μM, which is similar to previous studies conducted in BxPC-3 cells (12). In the *in vivo* study, we observed that oral administration of Pae significantly inhibited tumour growth and that Pae & Cis showed synergistically inhibitory effects on the growth of tumours. Some
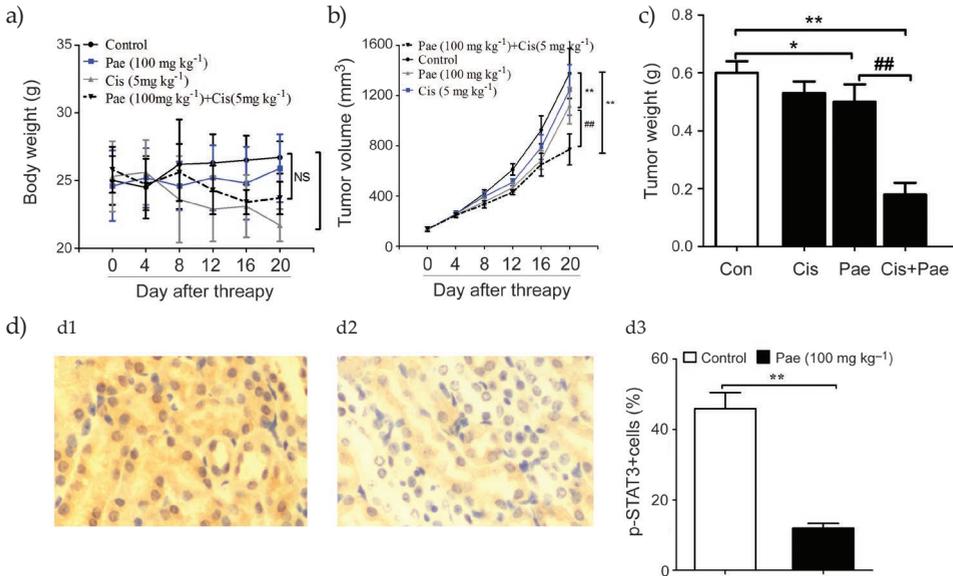
Fig. 5. Effects of Pae on tumour growth in RT4 xenograft mice. a) Body mass, b) tumour volume, c) tumour mass, d) tumour tissues from the control (d1) and Pae group (d2) were stained with pSTAT3 antibody. Representative images were recorded at a magnification of 400×, and quantification of the immunohistochemical assay is presented as the percentage of positively stained cells in d3. Data are presented as the mean ± SD, $n = 5$; *$p < 0.05$, **$p < 0.01$ compared to the control group; ##$p < 0.01$ compared to the Pae group; NS: No significance.

previously published papers have shown similar results. For instance, in 2008, Wu *et al.* (18) reported that Pae enhanced 5-fluorouracil-induced apoptosis in human gastric carcinoma cells, and Fang *et al.* (19) concluded that Pae at non-toxic concentrations effectively modulated multidrug resistance of a human gastric cancer cell line *via* inhibition of nuclear factor-kappa B activation. Similarly, Hao *et al.* (12) previously confirmed that Pae enhanced the effect of erlotinib in pancreatic cancer cells by directly suppressing human epidermal growth factor receptor-3 activation. Based on these findings, we may speculate that Pae has a synergistic effect with chemotherapeutic drugs regarding the molecular mechanisms or intracellular pharmacokinetic processes. An unexpected finding in the present study was that Pae had no effect on body weight but neutralized body mass loss induced by Cis. These interesting results may be explained as follows: Pae shows high selectivity with an anti-tumour dose and inhibits the growth of tumour cells rather than normal cells. It was shown in a previous study that Pae used against human colon carcinoma in a xenograft tumour did not lead to body weight loss or alterations in food consumption, while docetaxel resulted in a 23.6 ± 3.1 % mass loss during the same time (20). Moreover, Pae did not show any effects on the numbers of leukocytes, erythrocytes and haemoglobin, while docetaxel significantly reduced those of leukocytes (20), indicating the safety of Pae. Moreover, Pae shows favourable dual immunomodulatory effects and normalizes the function of the immune system in various disease states. For example, Pae was shown to enhance and

inhibit spleen lymphocyte proliferation at different concentrations (21). Unlike Pae, it is well known that Cis can attack and destroy the growth of tumour cells and immune cells simultaneously. In addition, Pae attenuated Cis-induced body loss in tumour-bearing mice, indicating that Pae reduced chemotherapy -induced toxicity to the immune system. In previous investigations, Pae was shown to decrease myelosuppression induced by chemotherapy (22). We suspect that Pae could increase the defensive power of the immune system in response to chemotherapy. These results indicate that Pae is a potential anti-tumour adjuvant in the prevention of BCa development.

Apoptosis is a process of programmed cell death. In general, two major apoptotic pathways have been described for mammalian cells. The first pathway, called the extrinsic pathway, is characterized by activation of caspase-8. The second pathway, called the intrinsic pathway, is characterized by activation of caspase-9. Activation of caspase-8 and caspase-9 could trigger activation of the downstream caspase-3. Moreover, the intrinsic pathway is also modulated by Bcl-2 family members, which play key regulatory roles in apoptosis (23–24). In previous studies, Pae induced apoptosis in several types of tumour cells with activation of caspases. For instance, Wang *et al.* (14) reported that Pae induced apoptosis in HT 29 cells and activated caspase-3 and caspase-8. Wang *et al.* (25) found that Pae effectively increased cell apoptosis and augmented the activation of caspase-3 and caspase-9 in SKO-007 cells. In our study, we found that induction of apoptosis was observed in RT4 cells exposed to Pae, followed by activation of caspase-3, caspase-8 and caspase-9. Furthermore, Pae also increased the level of Bax and simultaneously reduced the level of Bcl-2. These results demonstrated that both the extrinsic and intrinsic pathways seemed to be engaged in the Pae-treated RT4 cell types, which is in agreement with previously published results.

Many studies have been conducted to reveal the mechanisms of the Pae anti-tumour action. Some previous studies reported that Pae exerted its anti-tumour activity *via* suppressing extracellular regulated protein kinases and phosphatidylinositol 3-kinase/protein kinase B signalling pathways (26–27). Moreover, other studies concluded that Pae induced apoptosis in hepatocellular carcinoma cells by down-regulating prostaglandin E receptor 2 (28). It is accepted that the STAT3 signalling pathway is over-activated and contributes to tumour progression in malignancies. Constitutive activation of STAT3 resulted in cell growth and survival in human solid tumour malignancies and the up-regulation of anti-apoptotic proteins (Bcl-2, Survivin) (29–30). Therefore, deactivation of STAT3 is associated with the induction of apoptosis and conveyance of anti-tumour effects. In 2015, Zheng *et al.* (15) demonstrated that Pae significantly inhibited total expression of p-STAT3 in MGC-803 cells and that over-expression of STAT3 reversed the effect of Pae on the proliferation of MGC-803. As the tumour-promoting effects are ultimately regulated by the STAT3-dependent transcriptional regulation of downstream oncogenes, we focused on the nuclear level of p-STAT3. Our results showed that Pae reduced the expression of nuclear p-STAT3 and demonstrated that Pae also decreased p-STAT3-positive cells numbers *in vivo*. As expected, we also found that Pae inhibited translocation of STAT3 to the nucleus in RT4 cells. It is noteworthy that a recent study conducted by Nie *et al.* (13) found rapid down-regulation of STAT3 that was consistent with its accelerated degradation, and the pro-degradation effect of Pae on STAT3 was mainly through the ubiquitin-proteasome pathway. Therefore, these findings clearly indicate that deactivation of STAT3 by Pae contributes to its inhibitory effects on BCa development.

CONCLUSIONS

The present study demonstrated that Pae caused growth inhibition in human BCa cells, both *in vivo* and *in vitro*. To the best of our knowledge, our data support the hypothesis that Pae may be an effective anti-tumour agent for the treatment of human BCa. Its action is regulated, at least in part, by deactivation of STAT3 and inhibition of subsequent nuclear translocation.

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