




Xiao-Ai-Ping Injection Enhances Effect of Paclitaxel to Suppress Breast Cancer Proliferation and Metastasis via Activating Transcription Factor 3

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Abstract

Chemotherapy is an effective treatment for invasive breast cancer. Paradoxically, many recently published findings showed that the first-line chemotherapeutic agent paclitaxel (PTX) showed pro-metastatic effects in the progress of treating breast cancer. Xiao-Ai-Ping (XAP) injection, composed of a traditional herbal medicine, *Marsdenia tenacissima* extract, is known to exert antitumor effects on various cancers. However, there are few experimental studies on breast cancer. The underlying mechanism of the antitumor effect of XAP combined with chemotherapy agents has not been fully understood. In the present study, we sought to find the antitumor effects of XAP combined with PTX in vitro and in vivo. The data demonstrated that the combination of XAP with PTX resulted in remarkable enhancement of the pro-apoptotic, migration-inhibiting, and anti-invasive effects of PTX in vitro. Significantly, further study showed the overexpression of ATF3 in PTX-treated cell, while XAP counteracted the change of ATF3 induced by PTX. Moreover, it showed that combination treatment could promote the inhibition of tumor growth in MDA-MB-231 cell xenograft mouse model. Compared with PTX treatment, the downregulation of ATF3 indicated that ATF3 played a pivotal role in the combination of XAP with PTX to exert a synergistic effect. Overall, it is expected that PTX combined with XAP may serve as an effective agent for antitumor treatment, and dampening ATF3 maybe a potential strategy to improve the efficacy of PTX.

Keywords

Xiao-Ai-Ping injection, paclitaxel, activating transcription factor 3, breast cancer, herbal medicine

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Introduction

Breast cancer is the most common malignant tumor among women worldwide.¹ Tumor metastasis is a major mortal threat to patients with cancer.² Chemotherapy has remained an effective treatment strategy for invasive breast cancer. Although cytotoxic chemotherapy dramatically decreases the proliferation of cancer cells and reduces the tumor volume, a paradox is emerging suggesting chemotherapeutic promotion of chemoresistance and cancer metastasis.³ Paclitaxel (PTX), a first-line chemotherapeutic agent for the treatment of breast cancer, showed exacerbated metastasis in mouse models of breast cancer.^{4,5} The mechanism might be attributed to PTX-inducing migration and invasion of cancer cells, and regulating the expression of antiapoptotic

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or other related genes. Therefore, identifying the target of PTX, which induced tumor metastasis, and conducting intervention and regulation on it may solve the dilemma.

Activating transcription factor 3 (ATF3) is a member of the ATF/CREB family of transcription factors.⁶ It is a stress-induced gene that participates in cellular processes to adapt to extracellular and/or intracellular changes.⁷ Emerging evidence suggests that ATF3 is critical in regulating cell proliferation and apoptosis that contribute to cancer progression. Interestingly, ATF3 has been demonstrated to play controversial roles in cancer development according to the cell type.^{8,9} Studies have shown that ATF3 was overexpressed in monocytes of metastatic breast cancer patients, which indicated poor prognosis of breast cancer, but this research did not involve any chemotherapy drugs and was not further reported.¹⁰ Recently published data showed that overexpression of ATF3 in breast cancer was associated with PTX-induced tumor metastasis. In addition, increasing evidence indicated that ATF3 was found to be associated with chemotherapy response.¹¹ However, research showed paradoxical results on dampening and strengthening the effect of ATF3 in improving the efficacy of chemotherapy. The role of ATF3 in breast cancer is far from being understood.

Traditional Chinese medicine has played an important role in cancer treatment including during radiotherapy or chemotherapy and in the terminal stage of cancer.¹² In China, it has been increasingly used in the combined therapy of tumors, and it is capable of enhancing the efficacy of chemotherapy by regulating the immune function and improving the sensitivity of tumor to chemotherapeutic agents. Xiao-Ai-Ping (XAP) injection, composed of a traditional herbal medicine, *Marsdenia tenacissima* extract, has exhibited antitumor effects on various cancers such as esophageal cancer, gastric cancer, lung cancer, and liver cancer, as well as the adjuvant treatment of malignant tumors. Studies had shown that *Marsdenia tenacissima* extract enhanced gefitinib efficacy in non-small cell lung cancer cells.¹³⁻¹⁵ Our previous study had shown that XAP increased the exposure to PTX in rats.¹⁶ However, there are few experimental studies on breast cancer, and the underlying mechanism of the antitumor efficacy of XAP combined with chemotherapy agents has not been fully understood.

The present study was designed to reveal how XAP and PTX play synergistic roles in breast cancer and to identify the regulation of ATF3 by XAP. To complete our study, PTX and XAP were administered as monotherapy or combined therapy. We found that XAP potently inhibited proliferation and migration, and it promoted apoptosis in MDA-MB-231 cells, as well showing a synergistic effect on tumor growth *in vivo*, while PTX in the exposed concentration in this study showed a pro-metastatic effect *in vitro*. Significantly, further study showed that the upregulated expression of ATF3 was counteracted by XAP, which indicated that ATF3 plays a pivotal role in the combination of XAP with PTX to exert a synergistic effect.

Materials and Methods

Reagents

Xiao-Ai-Ping injection (1 g crude per mL) was purchased from Nanjing Sanhome Pharmaceutical Co, Ltd (Nanjing, China). PTX injection (6 mg PTX per mL) was purchased from Haikou Pharmaceutical Co, Ltd (Hainan, China). L15 medium was purchased from Hyclone (Logan, UT). Fetal bovine serum was obtained from Gibco (Grand Island, NY). Small interfering RNA interference nucleotide sequence of ATF3 (ATF3-SiRNA) and negative control (NC-SiRNA) were designed by Santa Cruz Biotech Co, (Santa Cruz, CA). Lipofectamine TM 3000 was obtained from Invitrogen (Carlsbad, CA). Chemiluminescence kit was obtained from Millipore (Billerica, MA). ATF3 antibody was obtained from Abcam (Cambridge, MA). Horseradish peroxidase-conjugated secondary antibodies were obtained from Abcam. TRIzol reagent was obtained from Thermo Fisher Scientific (Waltham, MA).

Cell Culture

Human tumor-derived MDA-MB-231 cell line was purchased from the cell bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in L-15 medium (Hyclone) and supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin. Cells were subcultured using 0.25% trypsin and incubated in a humidified atmosphere (37°C, 100% air). Drugs were incubated with MDA-MB-231 cells for 24 or 48 hours and treated as follows: XAP at concentrations of 40 mg/mL and 80 mg/mL and PTX at a final concentration of 10 nM, respectively, or combined.

siRNA Transfection

Small interfering RNA interference nucleotide sequence of ATF3 (ATF3-siRNA) was designed (Santa Cruz Biotech Co), and an independent nucleic sequence with the same base number was synthesized as negative control (NC-siRNA). Liposome transfection was in accordance with Lipofectamine TM 3000 (Invitrogen) with 100 nM of NC-SiRNA or ATF3-siRNA according to the manufacturer's instructions. Transfection efficiency was evaluated by ATF3 mRNA and protein level at 24 hours after liposome transfection. Subsequently, the transfected cells were evaluated by wound healing, Transwell migration, and Transwell invasion assays.

Mice

Six-week-old female BALB/c nude mice were purchased from Shanghai Sippr-bk Lab Animal Co Ltd (Shanghai, China; License Number: SCXK (沪) 2013-0016). Animal care and treatment were performed strictly in accordance

with the Provision and General Recommendation of Chinese Experimental Animals Administration Legislation with a 12/12-hour light/dark cycle in a colony room maintained at constant temperature ($22 \pm 1^\circ\text{C}$) with air conditioning. All animals had free access to sterilized food and water. Experimental procedures were in compliance with the *Guide for Care and Use of Laboratory Animals* (National Institutes of Health publication, revised 1996). The protocol was approved by the Animal Care and Use Committee of Shanghai Jiao Tong University Affiliated to Sixth People's Hospital (License Number: SYXK (沪) 2016-0020).

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assay

MDA-MB-231 cell proliferation was determined by the MTT assay. MDA-MB-231 cells were seeded in 96-well plates and treated with increasing concentrations of PTX and XAP monotherapy or combined therapy. Then, the cells were incubated with MTT (0.5 mg/mL) for 4 hours at 37°C . The precipitate was dissolved in dimethyl sulfoxide (150 μL /well). Cell viability was measured with microplate reader at 490 nm.

Cell Apoptosis and Cell Cycle Distribution Assay

A total of 5×10^4 MDA-MB-231 cells plated in 6-well plates were incubated at 37°C overnight and then treated with PTX, XAP, or combined therapy for 24 hours. After treatments, cell apoptosis was analyzed by Annexin V-FITC/PI apoptosis detection kit according to the manufacturer's instructions. Briefly, cells were washed once in phosphate-buffered saline (PBS), then once in binding buffer, and resuspended in the medium at $1 \times 10^6/\text{mL}$. Five microliters fluorochrome-conjugated Annexin V and 5 μL propidium iodide was added to the cell suspension and incubated for 15 minutes at room temperature for cell apoptosis assay. Cells were stained by propidium iodide for cell cycle distribution. After that, 400 μL binding buffer was added to each tube. Subsequently, cell apoptosis was analyzed by flow cytometry (BD FACS Calibur, San Diego, CA) within 1 hour.

Wound Healing Migration Assay

To determine the capacity for migration, a wound healing migration assay was performed. Briefly, MDA-MB-231 cells were seeded in 6-well plates. When cells grew to 90% confluence, wounds were scratched with sterile pipette tips. After washing twice with PBS, cells were treated with PTX or XAP for 48 hours. The cells in the denuded zone of each well were counted and photographed at $20\times$ magnification using an inverted fluorescence microscope (DMi8, Leica, Germany) in a random fashion.

Transwell Migration and Invasion Assay

The chemotactic motility of MDA-MB-231 cells was determined using Transwell migration assay with Transwell plates of 8.0- μm pore (Corning, NY). In brief, 600- μL complete medium was placed in the lower chamber, and 5×10^3 MDA-MB-231 cells were seeded in the upper chamber. Various concentrations of drugs were added in upper chambers to 200 μL total volume. For invasion assay, 1×10^4 cells per well were seeded in the upper chamber coated with Matrigel. After incubation for 24 hours, nonmigrated cells on the top surface of the membrane were gently scraped away with a cotton swab. The migrated cells were fixed with 4% paraformaldehyde for 20 minutes and stained with 0.1% crystal violet. Images were recorded using an inverted fluorescence microscope (DMi8, Leica), and migrated cells were quantified by manual counting.

Subcutaneous Xenograft Mouse Model

A total of 2×10^6 MDA-MB-231 human breast cancer cells prepared in 100 μL PBS were injected subcutaneously on the right axilla of female nude mice. After 7 days, mice injected with MDA-MB-231 cells were randomly divided into 4 groups ($n = 10$) and injected intraperitoneally with normal saline (control group), 20 or 40 mL/kg XAP (experimental group) and intravenously through the tail vein with 10 mg/kg (per 3 day) PTX for 28 days. Tumor volume was assessed by caliper measurement using the formula: volume = length \times width² \times 0.5. Mouse body weights and tumor dimensions were measured every 3 days. Mice were continually observed until sacrificed. Primary tumors and organs were isolated and stored at -80°C for further experiments.

Western Blot Analysis

Cells were plated in 6-well plates and incubated overnight followed by drug treatment with the indicated drug for 24 hours. Protein samples were collected in ice-cold RIPA buffer (50 mM Tris-CL pH 7.5, 150 mM sodium chloride, 1 mM EDTA, 1% Triton-X-100, 0.25% sodium deoxycholate, 0.1% SDS; Beyotime Institute of Biotechnology, Shanghai, China) containing the protease inhibitors 0.1% phenylmethylsulfonyl fluoride (Beyotime Institute of Biotechnology). The supernatant was collected after centrifugation at 12000 rpm for 30 minutes and stored at -80°C . The protein concentration was determined by the BCA protein assay kit (Beyotime Institute of Biotechnology). Protein extracts were loaded and separated by an SDS-polyacrylamide gel electrophoresis, and transferred to an equilibrated polyvinylidene difluoride membrane (Millipore, Bedford, MA) by electroblotting. The membranes were blocked with 5% fat-free milk in Tris-buffered saline for 2 hours at room temperature and incubated at 4°C overnight with primary antibodies, rabbit

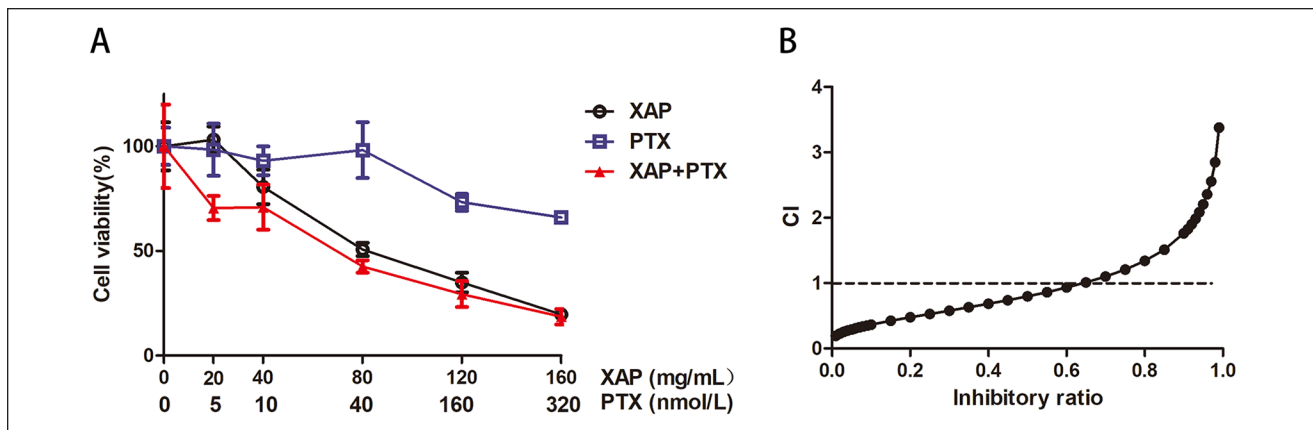


Figure 1. Effect of Xiao-Ai-Ping (XAP) combined with paclitaxel (PTX) on MDA-MB-231 cell proliferation. (A) MDA-MB-231 cells were seeded into 96-well plates and treated with increasing concentrations of XAP (20, 40, 60, 80, and 160 mg/mL) and PTX (5, 10, 20, 40, 160, and 320 nM) for monotherapy or combined therapy. Cell viability rates were examined and compared. The data were expressed in terms of the means \pm SD. The experiments were repeated at least 3 times. *** $P < 0.001$ versus PTX group. (B) Combination index (CI) values were graphed on fraction affected–CI plots. A CI < 1 is a synergistic interaction, CI = 1 is additive, and CI > 1 is antagonistic.

anti-ATF3 (1:1000; Abcam). After washing 3 times with Tris-buffered saline Tween 20, the membranes were incubated with secondary antibody (1:12000; Abcam) for 2 hours at room temperature. Visualization of protein bands was performed using enhanced chemiluminescence kit (Millipore, Billerica, MA) and a gel imaging system (Syngene, Frederick, MD). Beta-actin protein levels were used as an endogenous control to allow the normalization of target proteins. Densitometric analysis of the bands was analyzed by Image J software.

Real-Time Polymerase Chain Reaction (RT-PCR)

Cells were plated in 6-well plates overnight at 37°C and then treated with drugs for 24 hours. Total RNA was extracted from MDA-MB-231 cells using Trizol (Thermo Fisher Scientific). RNA concentrations were quantified using a NanoDrop D-1000 spectrophotometer (Wilmington, DE). The extracted total RNA was reverse-transcribed to cDNA for RT-quantitative PCR. The Applied Biosystems AB 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA) was used to detect amplification. Real-time PCR was performed using Taq Man Gene Expression Assay Primer/Probes for ATF3 (F: TTGCCATCCAGAACAAGCACCTC, R: GCACTCCGTCTTCTCCTTCTTCTTG), the house-keeping gene human β -actin (No. B662102 Sangon Biotech Co, Ltd, Shanghai, China) as per the manufacturer's instructions (Sangon Biotech Co, Ltd). Three independent experiments were performed to determine the average gene expression and standard deviation.

Statistical Analysis

Data were expressed as mean \pm SD. Significance was determined by one-way analysis of variance test. GraphPad

Prism 6 software (GraphPad Software, Inc, San Diego, CA) was used to count the half maximum inhibitory concentration (IC₅₀) values. The criterion for significance was $P < 0.05$. The effect of PTX combined with XAP was calculated using CalcuSyn (Biosoft, Cambridge, UK). Combination index (CI) values were graphed on fraction affected–CI plots. A CI < 1 is a synergistic interaction, CI = 1 is additive, and CI > 1 is antagonistic. All experiments were performed at least 3 times except the animal experiment.

Results

XAP Combined With PTX Suppressed MDA-MB-231 Cell Proliferation

To evaluate the cytotoxicity of XAP, PTX, and the combination of the 2 agents, MDA-MB-231 cells were treated with increasing concentrations of XAP (20, 40, 60, 80, and 160 mg/mL) and PTX (5, 10, 40, 160, and 320 nM) for monotherapy or parallel combined therapy. Data in Figure 1A of MTT assay showed that PTX exhibited an inhibitory effect at concentrations higher than 40 nM, and a weaker inhibitory effect at concentrations lower than 10 nM. XAP treatment resulted in an inhibitory effect at concentrations higher than 20 mg/mL. The inhibitory effect of XAP or PTX on proliferation of MDA-MB-231 cells was dose-dependent with IC₅₀ values of 75.80 mg/mL or 606.05 nM, respectively. When XAP was combined with PTX, the inhibitory effect was strengthened compared with PTX alone. The IC₅₀ of PTX decreased to 29.51 nM. To further evaluate the interaction of PTX and XAP, CI values were graphed on fraction affected–CI plots. When the concentration of XAP and PTX was below 80 mg/mL and 40 nM, respectively, with combined inhibitory ratio $< 60\%$, the CI

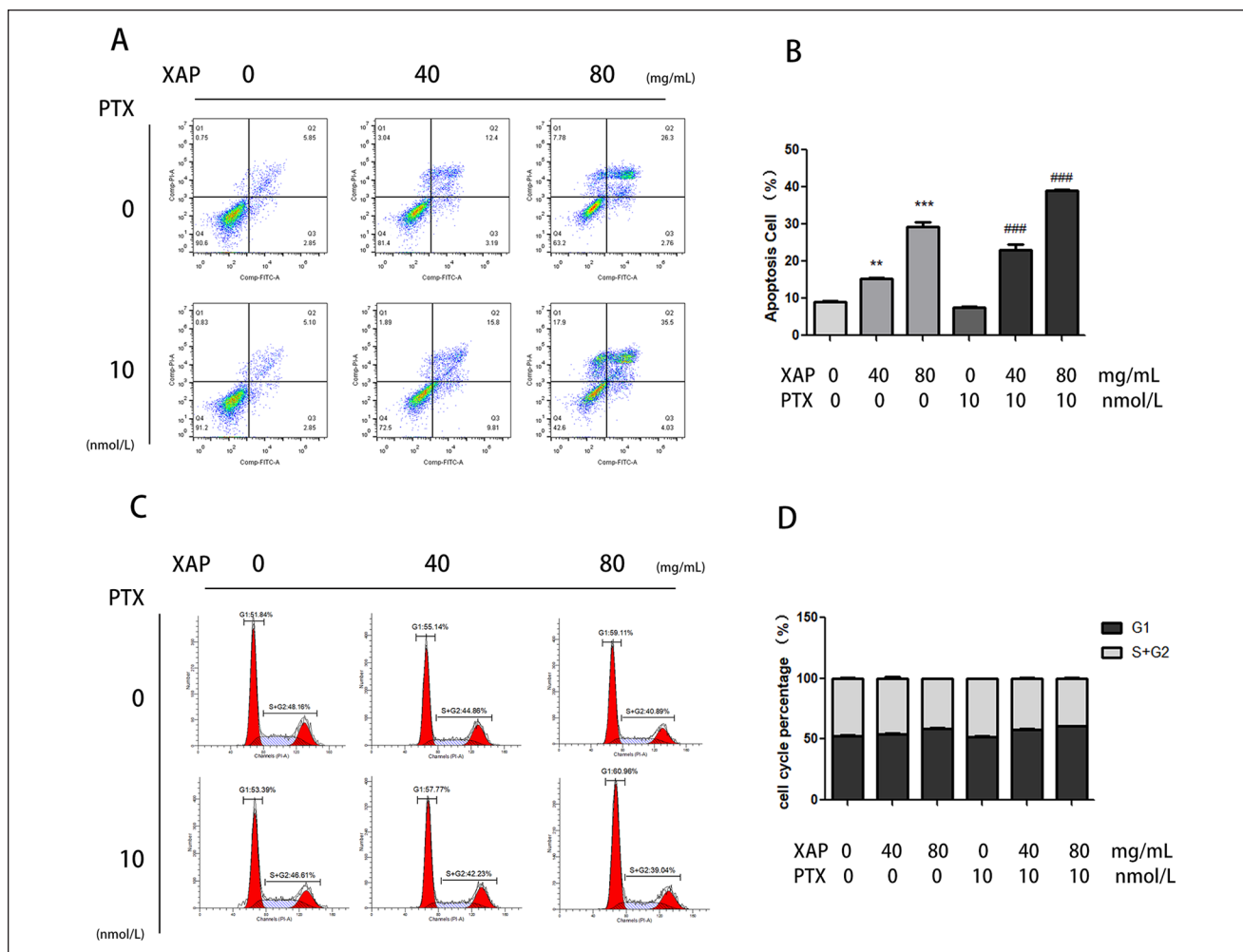


Figure 2. Effect of Xiao-Ai-Ping (XAP) combined with paclitaxel (PTX) on MDA-MB-231 cell apoptosis. MDA-MB-231 cells were plated into 6-well plates and treated with PTX (10 nM) or XAP (40 and 80 mg/mL) or 2 drugs combined for 24 hours. (A) Cell apoptosis stained by Annexin V/PI and detected by flow cytometry. (B) Histogram of MDA-MB-231 cell apoptotic rate. (C) Cell cycle distribution stained by propidium iodide and detected by flow cytometry. (D) Histogram of MDA-MB-231 cell cycle distribution. Results were presented as mean \pm SD, and the error bars represent the SD of 3 independent experiments. ** $P < 0.01$, *** $P < 0.001$ versus control group. ### $P < 0.001$ versus PTX group.

was <1 , which indicated the synergistic effect of XAP and PTX on MDA-MB-231 cell proliferation (Figure 1B).

XAP Combined With PTX Induced MDA-MB-231 Cell Apoptosis

To illustrate how XAP suppressed the growth of MDA-MB-231 cells, we examined cell viability through inducing cell apoptosis or cell cycle arrest. As shown in Figure 2A, flow cytometry analysis showed that cell apoptosis rate did not change dramatically after exposure to 10 nM PTX. XAP effectively induced 15.36% and 29.41% cell apoptosis ratio, when MDA-MB-231 cells were exposed to 40 mg/mL and 80 mg/mL XAP for 24 hours, respectively. When XAP was combined with PTX in the aforementioned

concentrations, cell apoptosis ratio increased to 20.03% and 39.15%, respectively. A histogram of MDA-MB-231 cell apoptotic rate is shown in Figure 2B. The results indicated that XAP combined with PTX caused a dose-dependent induction of apoptosis in MDA-MB-231 cells. However, it did not affect the cell cycle distribution as shown in Figure 2C and D.

XAP Combined With PTX Suppresses Breast Cancer Cell Migration and Invasion

Tumor cell migration and invasion leading to tumor metastasis is the main cause for the death of breast cancer patients. To further evaluate the anti-migration and anti-invasion effects of XAP on MDA-MB-231 cells, wound healing,

Transwell migration, and Matrigel-coated Transwell invasion assays were conducted. As shown in Figure 3A, the number of migrating cells were decreased greatly in the presence of XAP alone and XAP combined with PTX. However, the migrating cell number did not significantly decrease after exposure to 10 nM of PTX. In the Transwell migration and invasion assay, the cells penetrating through to the lower surface were stained. Cell invasion was suppressed by XAP and PTX in a dose-dependent manner, especially when exposed to the combined treatment group.

XAP Suppresses PTX-Induced ATF3 Expression in MDA-MB-231 Cells

ATF3 has been demonstrated to play controversial roles in different cancer types. To explore the emerging role of ATF3 in breast cancer, ATF3 mRNA and protein expression in PTX and XAP alone and in combination treated MDA-MB-231 cells were determined. PTX treatment resulted in induced expression of ATF3 mRNA, while XAP combined with PTX reduced the expression of ATF3 mRNA (Figure 4A). Similarly, the ATF3 protein was significantly downregulated by the combination of XAP and PTX compared with PTX treatment alone (Figure 4B). The results indicated that the ATF3 might exert a pivotal role in XAP-treated MDA-MB-231 cells.

ATF3 Regulated MDA-MB-231 Cell Migration and Invasion

To further understand the effect of ATF3 on the metastasis of breast cancer after XAP intervention, MDA-MB-231 cells were transfected with siRNA. The results of RT-PCR and western blot showed repressed expression of ATF3 mRNA (Figure 5A and B), which indicated the success of transfection. The cell viability, wound healing, Transwell migration, and invasion were inhibited after siATF3 transfection (Figure 5C and D). These findings indicated that ATF3 down-expression could inhibit MDA-MB-231 wound healing, migration, and invasion. The synergetic effects of XAP combined with PTX might associate with the down-regulation of ATF3.

XAP Combined With PTX Inhibited the Growth of MDA-MB-231 Cell Xenograft Tumors in Nude Mice

MDA-MB-231 cell xenograft model was established in BALB/c nude mice to evaluate the antitumor efficacy of XAP and PTX combined treatment in breast cancer. Twenty-eight days of XAP delivered intraperitoneally combined with PTX delivered intravenously significantly reduced the growth of tumor implanted in a dose-dependent manner.

The tumor volume reached 1600 mm³ in the control group and 355 mm³ in PTX treatment group on average, while the tumor volume was just 198 mm³ and 147 mm³ in the dose of 20 mL/kg and 40 mL/kg XAP combined with PTX, respectively (Figure 6A). Combined administration of PTX and XAP did not significantly reduce the body weight of the mice (Figure 6B). In addition, after 28 days treatment, tumors were harvested and weighed. The results showed that PTX and XAP co-treatment further decreased the tumor weight and tumor size compared with PTX alone (Figure 6C and D). Furthermore, the tumor specimens were examined using western blot. The results showed that the expression of ATF3 protein was upregulated in the PTX treated group, while the level was downregulated in PTX combined with XAP, especially in the concentration of 20 mL/kg XAP combined with PTX (Figure 6E).

Discussion

In recent times, therapies of patients suffering from cancer have evolved from chemotherapy and radiotherapy to targeted therapy and immunotherapy, which have considerably improved the clinical prognosis.^{17,18} However, these burgeoning therapies also experience intractable issues, such as acquired resistance, low tumor response rates, and unaffordable medical expenses.¹⁹ For triple-negative breast cancer, there is no target for therapy in the clinic so far. Chemotherapy with PTX continues to play a pivotal role in oncotherapy. Considering the widely reported limitations of PTX, combining ingredients from traditional Chinese medicine with chemotherapeutic drugs, have received heightened attention.²⁰⁻²² The combination has been reported to exert synergetic effects, reduce side effects, and reverse drug resistance.²³ In the present study, we found that the combination of XAP with PTX resulted in remarkable enhancement of the pro-apoptotic, migration-inhibiting, and anti-invasive effects of PTX, and it revealed a novel target, ATF3, a stress gene, as essential for the synergistic effect of PTX on triple-negative breast cancer therapy.

Xiao-Ai-Ping injection is a common herbal medicine for adjuvant therapy after chemotherapy and radiotherapy in various types of cancer in clinic.²⁴ Our previous pharmacokinetic study has shown that XAP increased the exposure to PTX in rats.¹⁶ Further study should be performed to evaluate the efficacy of the combination therapy in improving results. To address this, the present study aimed to refine XAP improvement of antitumor efficacy of PTX. Considering that both XAP and PTX showed antitumor effect, the IC₅₀ value of PTX was compared with XAP combination. The data showed that XAP reduced the IC₅₀ of PTX from 606 nM to 30 nM, indicating that XAP could enhance the antiproliferative effect of PTX. To explore whether the combination showed synergistic effect, CI values were calculated. It is interesting that when the inhibitory ratio was <60%, the

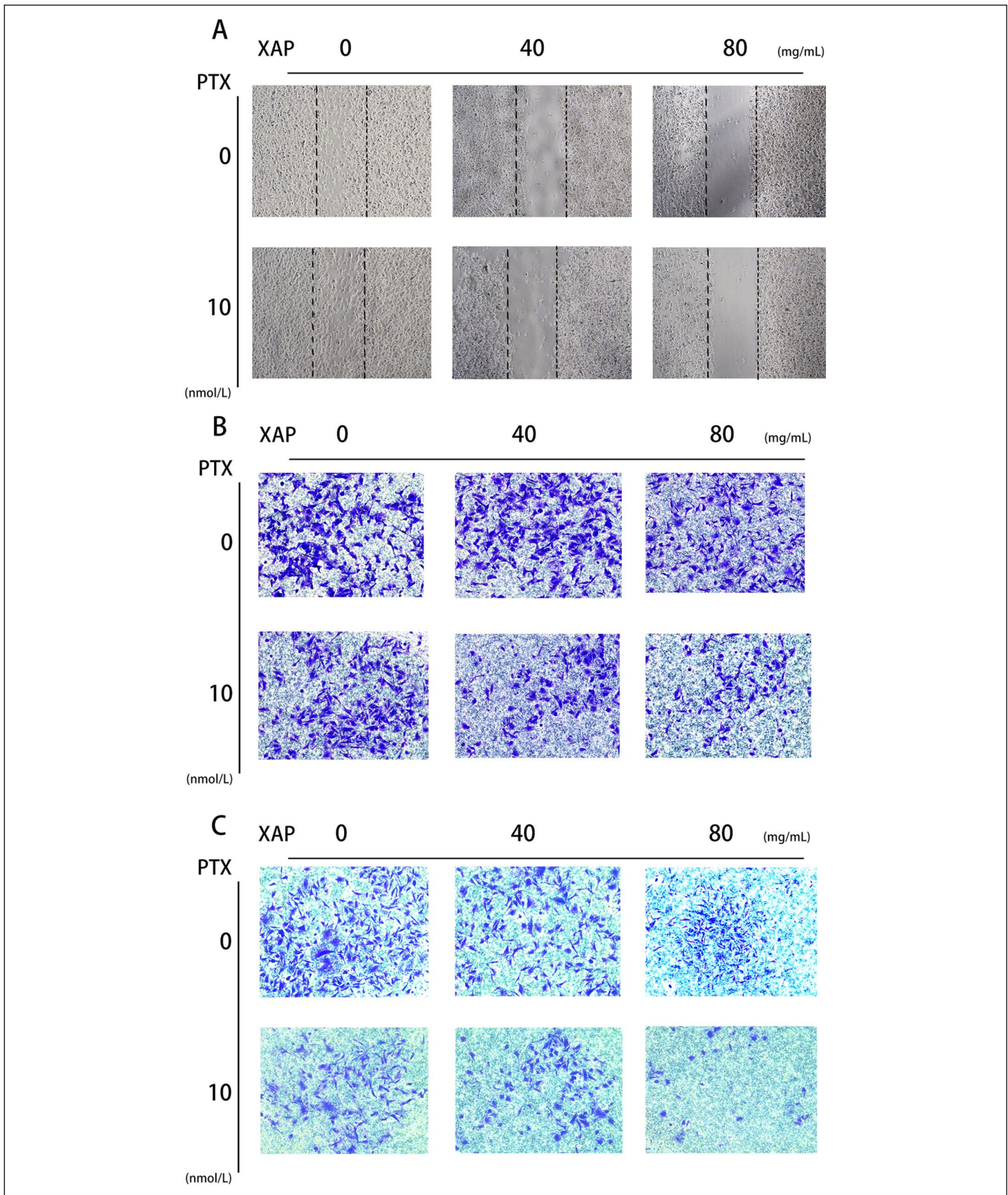


Figure 3. Effect of Xiao-Ai-Ping (XAP) combined with paclitaxel (PTX) on breast cancer cells migration and invasion. (A) MDA-MB-231 cells were seeded in 6-well plates for wound healing migration assay. Cells were treated with PTX (10 nM) or XAP (40 and 80 mg/mL) or 2 drugs combined for 48 hours. The denuded zone of each well was counted and photographed. MDA-MB-231 cells were seeded in the upper chamber of Transwell plates with and without Matrigel coat for (B) Transwell migration assay and (C) invasion assay, respectively. Cells penetrating through to the lower surface were stained with crystal violet and photographed.

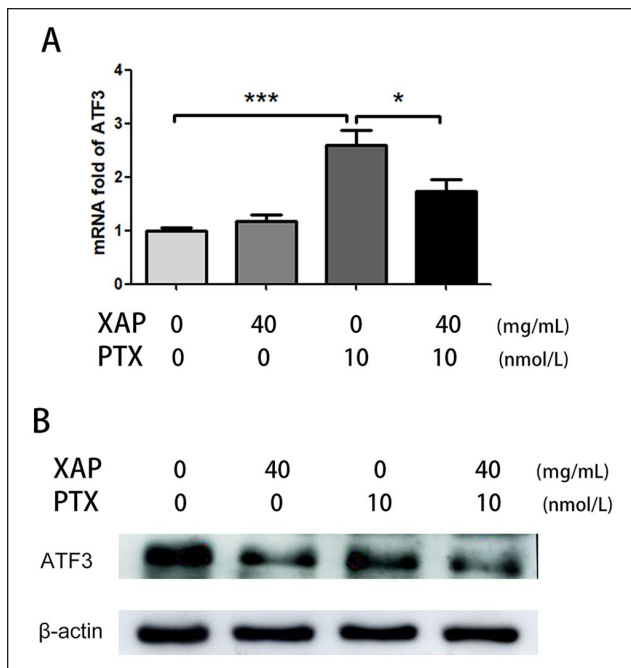


Figure 4. Effect of Xiao-Ai-Ping (XAP) on paclitaxel (PTX)-induced ATF3 expression. MDA-MB-231 cells were pretreated with PTX (10 nM) or XAP (40 and 80 mg/mL) or 2 drugs combined for 24 hours. (A) ATF3 mRNA expression was determined by real-time polymerase chain reaction. (B) ATF3 protein expression was determined by western blot. Results were presented as mean \pm SD, and the error bars represent the SD of 3 independent experiments. *** $P < 0.001$ versus control group. * $P < 0.05$ versus PTX group.

CI was < 1 , indicating XAP and PTX showed a synergistic interaction. Therefore, the corresponding concentrations in this range were chosen for the subsequent experiment. It should be noted that XAP in the experimental concentration inhibited cell growth intensively, while showing a slight effect on cell migration and invasion. However, combination of XAP with PTX suppressed cell migration and invasion significantly. We wondered why combination of XAP with PTX showed synergetic antitumor effects on cell proliferation and migration and invasion.

In vitro, it is puzzling that PTX treatment induced ATF3 expression, but XAP showed slight effect on ATF3. When XAP was combined with PTX, the overexpression of ATF3 induced by PTX was suppressed. ATF3 is expressed at a low basal level, but it is greatly increased by a wide spectrum of stress signals.^{25,26} Although various evidence has indicated that ATF3 plays controversial roles in various cancer cells, few studies investigated the significance of ATF3 in triple-negative breast cancer until recently.²⁷⁻³⁰ Reported research has shown that chemotherapy of PTX, as a stressor, could counteract its own efficacy by inducing a stress response.³¹ The data in this research *in vitro* indicated that ATF3 played a critical role in mediating this process, especially in MDA-MB-231 cell migration and invasion. This finding was consistent with recent research, which showed that ATF3 was upregulated by doxorubicin.³² However, ATF3 was only considered to be associated with doxorubicin cytotoxicity, and the cell migration and invasion was not investigated in the published research. To further investigate

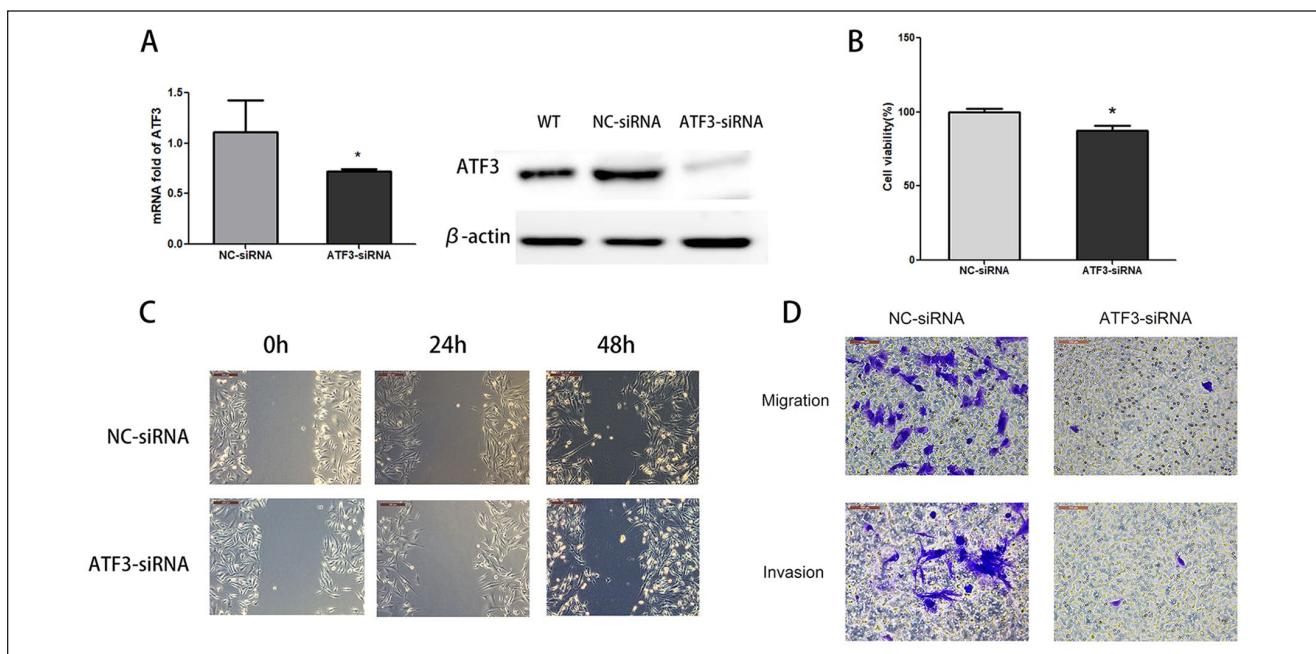


Figure 5. Effect of ATF3 on MDA-MB-231 cell migration and invasion. MDA-MB-231 cells were transfected with ATF3-siRNA. (A) After 24 hours of transfection, the ATF3 mRNA and protein expression were determined by real-time polymerase chain reaction and western blot. (B) Cell viability was determined by MTT assay. (C) Cell wound healing migration was assayed. (D) Cell Transwell migration and invasion were assayed. Data were expressed as means \pm SD. * $P < 0.05$ versus NC-siRNA.

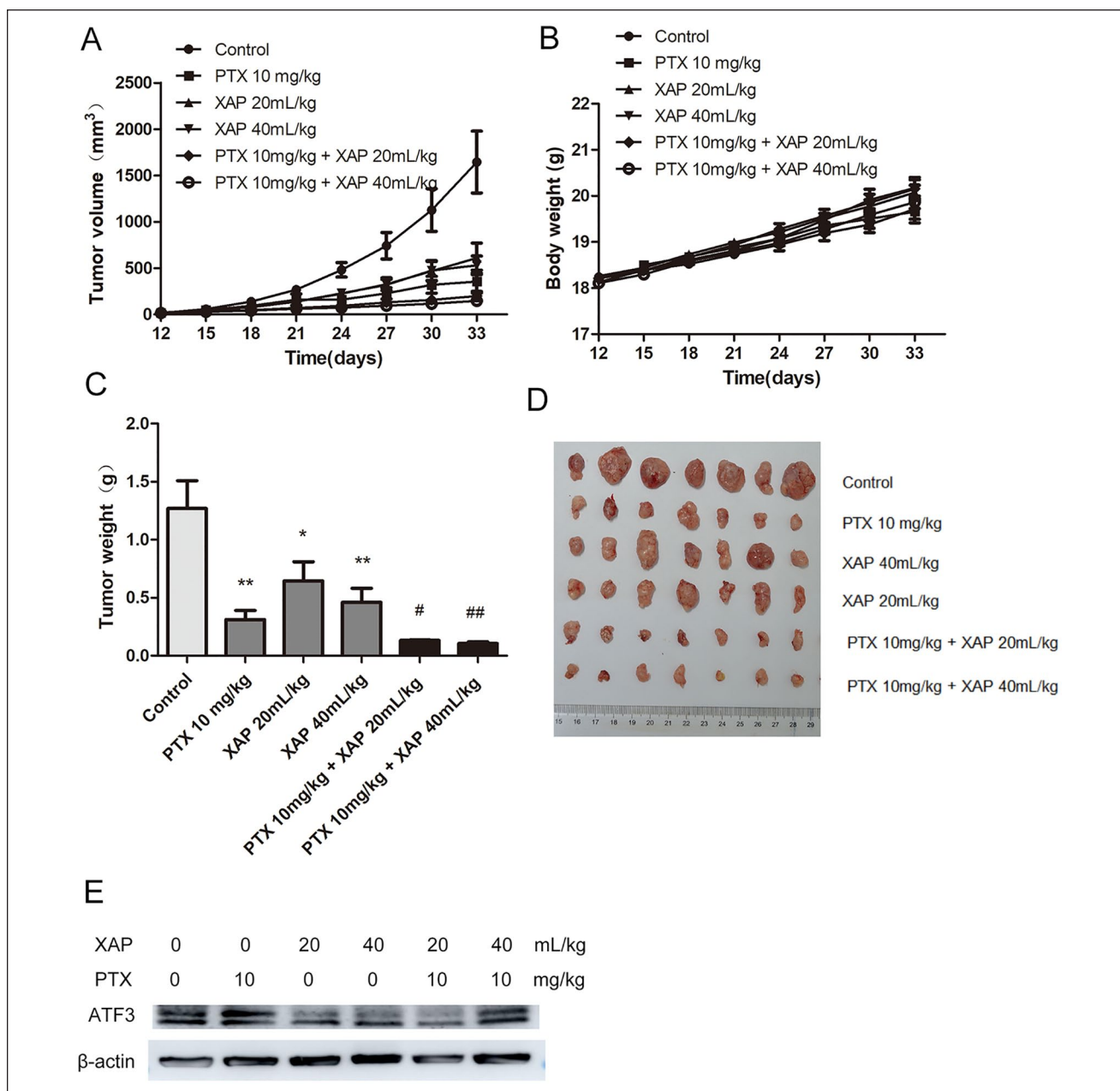


Figure 6. Effect of Xiao-Ai-Ping (XAP) combined with paclitaxel (PTX) on the growth and metastasis of MDA-MB-231 cell xenograft tumors in nude mice. MDA-MB-231 cells were subcutaneously injected into the right axillary fossa of nude mice to establish the xenograft tumor model. The mice were treated with PTX (10 mg/kg) or XAP (20 and 40 mL/kg) or 2 drugs combined at the indicated concentrations for 28 days. After treatments, (A) the tumor volume, (B) body weight, and (C) tumor weight were measured. (D) Tumor size was depicted. (E) The expressions of ATF3 in xenograft tumor was analyzed by western blot. Data were expressed as means \pm SD. * $P < 0.05$ versus control group. ** $P < 0.01$ versus control group. # $P < 0.05$ versus PTX-treated group. ## $P < 0.01$ versus PTX-treated group.

whether the change of ATF3 would affect the cell growth, cell migration, and invasion, ATF3-siRNA was transfected into MDA-MB-231 cells to silence the expression of ATF3. The results indicated that ATF3 down-expression could inhibit MDA-MB-231 wound healing, migration, and invasion. The synergetic effects of XAP combined with PTX

might involve with the downregulation of ATF3. In addition, we further investigated the antitumor effect of XAP combined with PTX *in vivo*. Our research found that XAP enhanced the antitumor effect of PTX, which might be partially mediated by the inhibition of ATF3 mRNA expression. However, it is not clear whether the underlying mechanisms

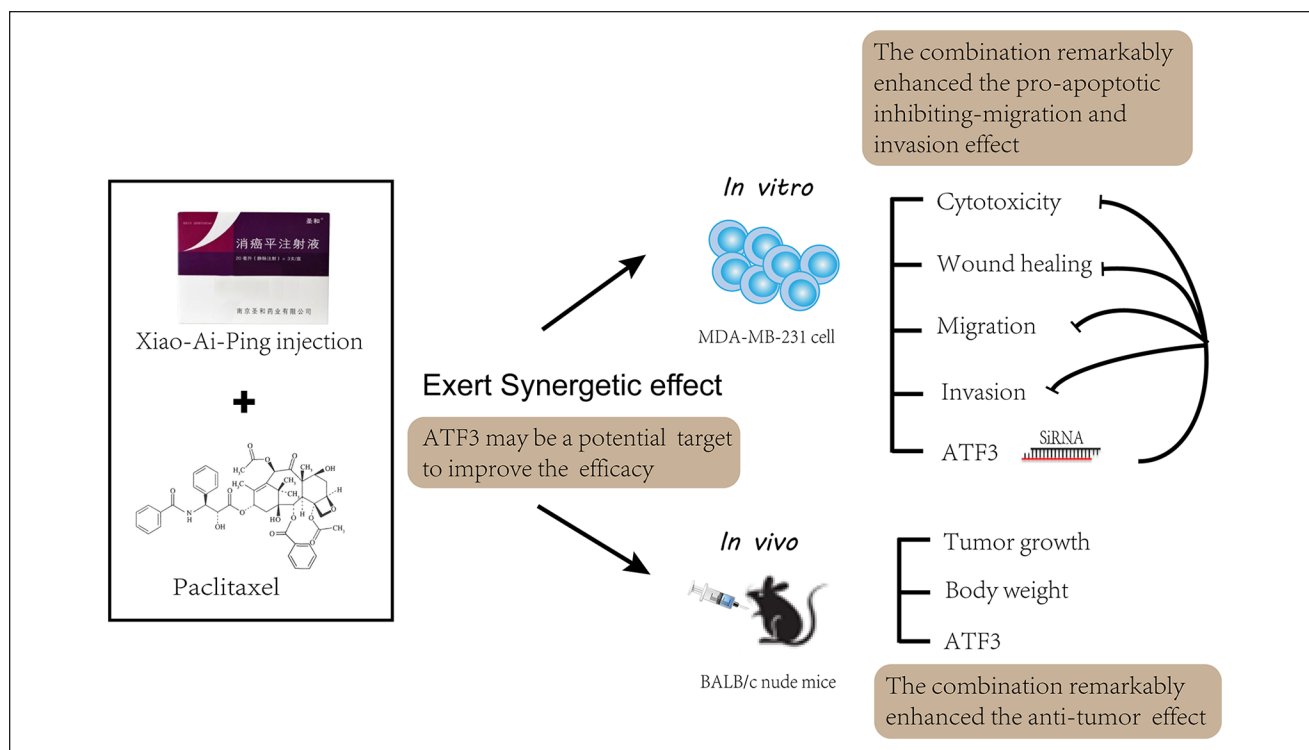


Figure 7. The underlying mechanism of the synergetic antitumor efficacy was mostly ascribed to the significant suppression of ATF3 by Xiao-Ai-Ping (XAP) in breast cancer.

for various cell types are consistent with this finding. Further investigation is required to address this issue.

Conclusion

In conclusion, the present study confirmed that XAP and PTX acted synergistically to suppress cell growth, shown by lower IC_{50} of PTX and $CI < 1$. Meanwhile, the inhibition of cell apoptosis, cell migration, and invasion were greatly promoted by combined therapy. The underlying mechanism of the remarkable antitumor efficacy was mostly ascribed to the significant suppression of ATF3 by XAP in breast cancer cells (Figure 7). Our results suggest that XAP is a promising herbal medicine to improve PTX efficacy in triple-negative breast cancer. However, the effect of XAP in PTX-resistant MDA-MB-231 cells needs further research. Since PTX is an important treatment for triple-negative breast cancer, it is expected that combination with XAP may serve as an effective antitumor agent in treatment, and that dampening ATF3 may be a potential strategy to improve the efficacy of PTX.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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