Antitumor effects of Xi Huang pills on MDA-MB-231 cells *in vitro* and *in vivo*

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Abstract. The management of patients with triple-negative breast cancer is challenging due to the lack of effective therapeutic options, aggressive behavior and relatively poor prognosis. Xi Huang pills (XHP) are a well-known traditional Chinese medicine that demonstrate anticancer activities. The aim of the present study was to investigate the antitumor effects of XHP on MDA-MB-231 cells *in vitro* and *in vivo*, and its potential underlying molecular mechanisms. In the present study, an MTT assay was used to evaluate the antiproliferative activity of XHP on MDA-MB-231 cells. In order to investigate the effects further, cell cycle distribution, apoptosis and mitochondrial membrane potential assays were performed, as well as western blot analyses. In addition, a tumor xenograft model was employed to investigate the effects of XHP *in vivo*. The results of the MTT assay demonstrated that the viability of MDA-MB-231 cells was markedly inhibited by XHP in a dose- and time-dependent manner. The inhibitory effect of XHP on the viability of MDA-MB-231 cells was greater when compared with MCF-10A cells. An increase in apoptosis and loss of mitochondrial membrane potential was observed following 4, 8 and 12 mg/ml XHP treatment of MDA-MB-231 cells. The protein expression levels of cleaved caspase-3 were increased by 1.62-, 2.13- and 2.19-fold, respectively, when compared with the untreated controls, whereas no effects on the expression of B-cell lymphoma 2 (Bcl-2) or Bcl-2-associated X protein (Bax) were observed. The results of the cell cycle distribution assay analysis demonstrated that XHP treatment arrested cells at the G_2/M phase. In addition, XHP treatment decreased the expression of cyclin A and increased the expression of $p21^{Cip1}$. *In vivo* experiments revealed that XHP inhibited the growth of MDA-MB-231 xenograft tumors without body weight loss, and demonstrated similar effects on the protein expression levels of cleaved caspase 3, cyclin A and $p21^{Cip1}$ as observed *in vitro*. In conclusion, the viability of MDA-MB-231 cells was inhibited by XHP in a dose-dependent, time-dependent and cell-selective manner *in vitro*, and the potential underlying mechanisms may involve apoptosis and cell cycle arrest at the G_2/M phase. XHP may induce apoptosis in MDA-MB-231 cells via the intrinsic pathway, which does not involve the Bcl-2/Bax ratio. G_2/M phase arrest may have been due to the integrated action of decreased cyclin A expression and increased $p21^{Cip1}$ expression. In addition, XHP inhibited the growth of xenograft tumors in the absence of body weight loss *in vivo*.

Introduction

Breast cancer is the second most common cancer worldwide and the most common cancer among women (1). Triple-negative breast cancer (TNBC), which comprises ~10-15% of all breast cancer cases (2), is characterized by the absence of the estrogen receptor, progesterone receptor, and the human epidermal growth factor receptor-2 (3). Management of TNBC is challenging due to a current lack of targeted therapies, aggressive behavior and relatively poor prognosis (4). There are currently few therapeutic options, and conventional chemotherapy is one of the treatments, which may be effective for patients following surgery (5); however, it is associated with severe side effects. Therefore, it is important to identify novel and effective therapeutic agents with low levels of toxicity for the treatment of patients with TNBC. Traditional Chinese medicine has been used for the treatment of patients with cancer, either alone or in combination with western medicines (6). It is a promising field for research and development.

Xi Huang pills (XHP) are a Chinese formula first mentioned in the Life-saving Manual of Diagnosis and Treatment of External Diseases, written by Hongxu Wang in the year 1740 (7). XHP contains Niu Huang (*Calculus bovis*), She Xiang (*Moschus berezovskii*), Ru Xiang (*Resina olbani*) and Moyao (*Commiphora myrha*). The primary function of XHP

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is detoxification, as well as relieving swelling and pain. It is used primarily for the treatment of furunculosis, scrofula and neoplasms. XHP is a well-known traditional Chinese medicine that demonstrates anticancer properties. It has been reported that XHP may be efficacious against breast cancer, liver cancer, leukemia and additional malignancies (8). However, evidence for the antitumor effects of XHP against TNBC is scare. Pan *et al* (9) used the MDA-MB-231 TNBC cell line in 2013, and demonstrated the cytotoxic effects of aqueous XHP extracts on these cells. The potential underlying mechanisms were thought to have been associated with apoptosis; however, Pan *et al* (9) did not investigate the molecular mechanisms further or study the effects *in vivo*. Therefore, the aim of the present study was to investigate the antitumor effects of XHP on MDA-MB-231 cells *in vitro* and *in vivo*, and elucidate the potential underlying molecular mechanisms.

Materials and methods

Chemicals and antibodies. XHP was purchased from Tong Ren Tang Technologies Co., Ltd. (Beijing, China). Dimethyl sulphoxide and MTT reagent were purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). Human insulin, epidermal growth factor, cholera toxin and hydrocortisone were purchased from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). The human caspase-3 monoclonal antibody (cat. no. ab32351; 1:3,000 dilution) was purchased from Abcam (Cambridge, UK). The human cyclin A (cat. no. 4656; 1:5,000 dilution), p21^{Cip1} (cat. no. 2947; 1:5,000 dilution), caspase-8 (cat. no. 9746; 1:4,000 dilution), Bcl-2-associated X protein (Bax; cat. no. 5023; 1:5,000 dilution) and B-cell lymphoma 2 (Bcl-2; cat. no. 2870; 1:5,000 dilution) monoclonal antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The β-actin rabbit monoclonal antibody (cat. no. TDY051; 1:5,000 dilution) was purchased from Beijing TDY Biotech Co., Ltd. (Beijing, China). The horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibodies (cat. no. ZB-2306; 1:5,000 dilution) and goat anti-mouse IgG secondary antibodies (cat. no. ZF-0312; 1:5,000 dilution) were purchased from OriGene Technologies, Inc. (Beijing, China).

Drug preparation. A total of 3 g XHP was dissolved in 15 ml cold distilled water, and mixed by Test Tube Rotary Mixer (Thermo Fisher Scientific, Inc.) for 2 h at 4˚C. XHP was then fragmented using an ultrasound oscillator (40 kHz) for 2 h at 37˚C, and the sample was centrifuged at 1,500 x g for 5 min at 4˚C. The supernatant was centrifuged again at 4,800 x g for 15 min at 4˚C, and the final supernatant was filtered through a sterile microporous membrane (0.45-μm in diameter) before storing at -20˚C. XHP was diluted in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) to the desired concentrations prior to treatment of the cells.

For the *in vivo* experiments, a total of 3 g XHP was dissolved in 22.5 or 45 ml cold distilled water, rotated for 2 h at 4˚C. XHP was then fragmented with an ultrasound oscillator (40 kHz) for 2 h at 37˚C, and stored at -20˚C until required. XHP was warmed to room temperature and manually agitated prior to intragastric administration of nude mice with the XHP solution.

Cell culture. The MDA-MB-231 human breast cancer cell line was purchased from the Cell Resource Center of the Peking Union Medical College (Beijing, China). The cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Solarbio Science & Technology Co., Ltd., Beijing, China). Cells were incubated in a humidified chamber at 37˚C and 5% CO_2.

MCF-10A human breast epithelial cells were a generous gift from Professor Liu Zhihua (Cancer Hospital Chinese Academy of Medical Sciences, Beijing, China). The cells were cultivated, maintained and treated in Dulbecco's modified Eagle's medium/F-12 (1:1; Gibco; Thermo Fisher Scientific, Inc.), supplemented with human insulin (10 μg/ml), epidermal growth factor (20 ng/ml), cholera toxin (100 ng/ml), hydrocortisone (0.5 μg/ml), 5% horse serum (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA), 100 U/ml penicillin and 100 μg/ml streptomycin.

In vivo tumor xenograft model. Female BALB/c nude mice (n=30, weight 18-20 g, mean 19 g; 5-8 weeks old) were obtained from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The animals were housed in laminar airflow cabinets under pathogen-free conditions with a 12-h light/dark cycle, and were fed autoclaved standard food and water *ad libitum*. The animal experiment protocol was approved by Peking University Animals Research Committee (Beijing, China) and was conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals (10). Human MDA-MB-231 cells (4x10^6) were diluted in 0.1 ml RPMI-1640 medium, mixed at a 1:1 ratio with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) to make the percentage of Matrigel 50%. The cells were inoculated at 2x10^6 cells/mouse subcutaneously into the right flank of the mice. Tumor growth was measured, and tumor volume was calculated using the following formula: Tumor volume (mm^3)=0.5 x length x width^2. When the tumor volume had reached 50-100 mm^3, the mice were randomly divided into three different treatment groups (n=10/each group): Distilled water/control group, 20 mg/day group and 40 mg/day group. The mice and administrated with distilled water, 20 mg/day XHP and 40 mg/day XHP intragastrically, respectively. The total dose of the drug was administrated twice per 12 h for 15 days. The body weight and tumor volume of the mice were measured every 5 days following XHP administration. The mice were sacrificed 15 days after XHP administration under 1.5% pentobarbital sodium (1 ml/kg; Lianshuoinc, Shanghai, China) and tumor tissues were stored at -80˚C for protein expression analysis.

MTT assay. MDA-MB-231 cells (1x10^4) were seeded in 96-well plates (Corning Incorporated, Corning, NY, USA) and treated on the following day with different concentrations of XHP (0, 4, 8, 12, 16 mg/ml) for 6, 12, 24 and 48 h, respectively. MCF-10A cells (1x10^4) were seeded in 96-well plates and treated on the following day with XHP (12 mg/ml) for 12 h. The MTT assay was performed using the methods described previously (11), and the optical density (OD) was read at 570 nm using a 96-well microplate reader (Bio-Rad

Laboratories, Hercules, CA, USA). As reduction of MTT only occurs in metabolically active cells, the OD values were used to provide a measure of cell viability. The percentage cell viability was calculated according to the following formula: $(OD_{treatment}/OD_{control}) \times 100$.

Apoptosis assay. MDA-MB-231 cells (3.2×10^5) were seeded on 30-mm culture dishes (Corning Incorporated). The following day, cells were treated with 0, 4, 8 or 12 mg/ml XHP for 24 h. Cells were subsequently trypsinized (Gibco; Thermo Fisher Scientific, Inc.), washed with cold phosphate-buffered saline (PBS), and stained using an Annexin V/fluorescein isothiocyanate/propidium iodide (PI) staining kit (BestBio Company, Shanghai, China). The cells were detected by flow cytometry analysis using a BD FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and the results were analyzed by ModFit software version 6.0 (BD Biosciences).

Mitochondrial membrane potential assay. The loss of mitochondrial membrane potential was detected using a mitochondrial membrane potential assay kit (Beyotime Institute of Biotechnology, Haimen, China). Briefly, MDA-MB-231 cells (3.2×10^5) were seeded on 30-mm culture dishes. The following day, the cells were treated with 0, 4, 8 and 12 mg/ml XHP for 24 h. The cells were then trypsinized and stained with JC-1 at 37°C for 15 min. The cells were subsequently washed with JC-1 staining buffer twice and analyzed immediately by flow cytometry using a BD FACSCalibur flow cytometer (BD Biosciences), results of which were analyzed by ModFit software version 6.0 (BD Biosciences).

Cell cycle distribution assay. MDA-MB-231 cells (8×10^5) were seeded on 50-mm culture dishes. The following day, cells were treated with 0, 4, 8 and 12 mg/ml XHP for 48 h. The cells were then trypsinized, washed with PBS and fixed in 1 ml ice-cold 70% ethanol overnight at 4°C. The cells were centrifuged (1,000 x g, 5 min, 4°C), washed with cold PBS and treated with PI/RNase Staining buffer (BD Pharmingen, San Diego, CA, USA) according to the manufacturer's protocols for 15 min at room temperature in the dark. The cell cycle distribution was determined by flow cytometry using a BD FACSCalibur flow cytometer. The percentage of cells in G_1, S or G_2/M phases was calculated using ModFit software version 6.0 (BD Biosciences).

Western blot analysis. MDA-MB-231 cells (3.06×10^6) were seeded on 100-mm culture dishes. The following day, the cells were treated with 0, 4, 8 and 12 mg/ml XHP for 24 or 48 h. Cells were then harvested, washed with cold PBS and homogenized with radioimmunoprecipitation assay lysis buffer (TDY Biotech Co., Ltd, Beijing, China). In the mice tumor xenograft model, following the sacrifice of the mice, tumor tissues were stored at -80°C for protein expression analysis. Upon protein extraction, tumor tissues were homogenized with radioimmunoprecipitation assay lysis buffer (TDY Biotech Co., Ltd, Beijing, China). The concentration of total protein extracts was determined using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc.). The protein samples were subsequently mixed with 5X loading buffer (CWBIO,

Beijing, China) and were boiled for 5 min. Protein samples (35 μg) were separated by 10% SDS-PAGE, and transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). Membranes were blocked for 2 h at room temperature with 5% fat-free milk or bovine serum albumin in Tris-buffered saline-Tween-20 (TBST) containing 10 mM Tris-HCl, 0.1 M NaCl and 0.1% Tween-20 (pH 7.4). The membranes were then incubated with specific primary antibodies, as detailed above in 'chemicals and antibodies' at 4°C overnight with gentle agitation. Following washing with cold TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Visualization of the protein bands was accomplished using the Immobilon Western Chemiluminescent HRP substrate (EMD Millipore, Billerica, MA, USA). The protein band intensities were measured using ImageJ software version 2.0 (National Institutes of Health, Bethesda, MD, USA), normalized to that of β-actin and compared to the control.

Statistical analysis. The results are presented as the mean ± standard deviation of at least three independent experiments. The data were analyzed by one-way analysis of variance and Tukey as the post hoc test with SPSS version 13.0 (SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

XHP inhibited the viability of MDA-MB-231 cells. To evaluate the cytotoxic effects of XHP on MDA-MB-231 cells, the viability of cells treated with different concentrations (from 0 to 16 mg/ml) of XHP was measured using an MTT assay. The results demonstrated that XHP reduced cell viability in a dose- and time-dependent manner (Fig. 1A and B). The inhibition of MDA-MB-231 cell viability following exposure to XHP was observed as early as 6 h following exposure (Fig. 1A). The percentage cell viability following exposure to XHP for 6, 12, 24 and 48 h at the highest concentration was significantly decreased from 100% to 84.66±5.03%, 74.49±11.53%, 31.01±9.03% and 5.15±3.28%, respectively. By contrast, no statistical difference between the control and the 4 mg/ml XHP-treated group was observed at all time points (Fig. 1B).

The MCF-10A cell line was used to represent normal human breast epithelial cells, while the MDA-MB-231 cell line was used to represent human TNBC cells for the purposes of the present study. These cell lines were treated with 12 mg/ml XHP for 24 h, and the viability of MDA-MB-231 and MCF-10A cells significantly decreased to 52.24±3.63% and 72.41±9.79% respectively, compared with untreated controls, (Fig. 1C). The results indicated that XHP reduced cell viability in a cell-selective manner.

XHP induced apoptosis in MDA-MB-231 cells. In order to determine whether the antiproliferative effects of XHP on MDA-MB-231 cells may involve induction of apoptosis, the Annexin V/propidium iodide double-staining method combined with flow cytometry analysis was employed. Following treatment with 4, 8 and 12 mg/ml XHP for 24 h, the percentage of cells in early apoptosis increased from
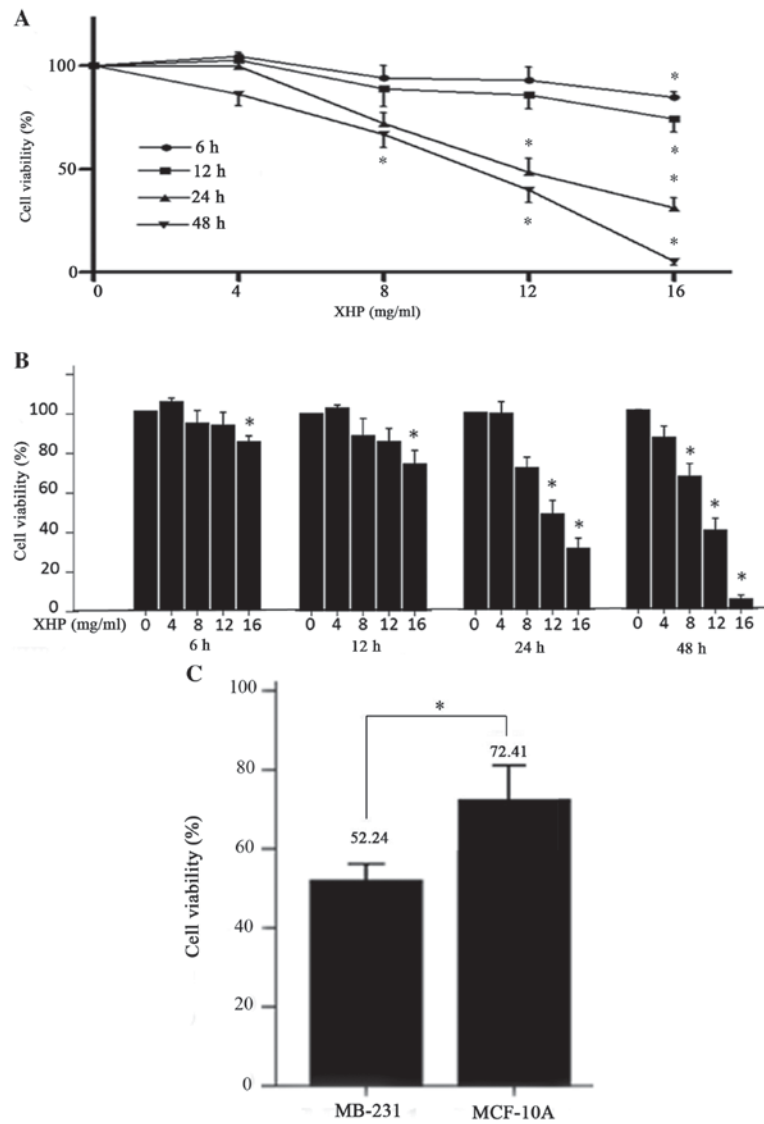
Figure 1. Effect of XHP on the viability of MDA-MB-231 cells. MDA-MB-231 cells were treated with different concentrations of XHP (0, 4, 8, 12 and 16 mg/ml) for 6, 12, 24 and 48 h. Cells treated with medium (0 mg/ml XHP) were used as a control. (A) XHP decreased cell viability in a dose-dependent manner. *P<0.05 vs. control. (B) XHP decreased cell viability in a time-dependent manner. *P<0.05 vs. control. (C) XHP decreased cell viability in a cell-selective manner. MDA-MB-231 and MCF-10A cells were treated with 12 mg/ml XHP for 24 h and the percentage viability was compared. Values represent the mean ± standard deviation (n=3). *P<0.05 MDA-MB-231 vs. MCF-10A. XHP, Xi Huang pills.

2.7% (untreated cells) to 2.8, 4.6 and 8.8%, respectively, whereas the percentage of cells in late apoptosis increased from 7.3% (untreated cells) to 11.9, 15.7 and 20.6%, respectively (Fig. 2A). A statistically significant increase in the rate of apoptosis was observed following treatment of cells with 8 and 12 mg/ml XHP when compared with the controls (Fig. 2B). Therefore, XHP induced apoptosis in MDA-MB-231 cells in a dose-dependent manner.

XHP induced the depletion of mitochondrial membrane potential in MDA-MB-231 cells. In order to determine whether the increased apoptosis rate of MDA-MB-231 cells was associated with a depletion of mitochondrial membrane potential, flow cytometry analysis of MDA-MB-231 cells treated with different concentrations of XHP was performed to measure alterations in the mitochondrial membrane potential. Following treatment with XHP (4, 8 and 12 mg/ml) for 24 h,

the percentage of cells with a low mitochondrial membrane potential increased from 4.2% (untreated cells) to 6.0, 10.5 and 19.2%, respectively (Fig. 3A). XHP induced significant mitochondrial membrane potential depletion in MDA-MB-231 cells following treatment with 12 mg/ml XHP (Fig. 3B). The results indicate that XHP induced a dose-dependent reduction in mitochondrial membrane potential.

XHP induced apoptosis via the intrinsic pathway. Apoptosis is executed via two major pathways, known as the intrinsic and extrinsic pathways (12). These pathways lead to caspase-3 activation. The caspase family is the most prominent protease family involved in apoptosis (13), and is divided into two functional groups; the apoptosis initiators (caspase-8, -9 and -10) and the apoptosis executors (caspase-3, -6 and -7) (14).

Bax is a well-known proapoptotic protein and Bcl-2 is an anti-apoptotic protein, and the Bcl-2/Bax ratio serves a
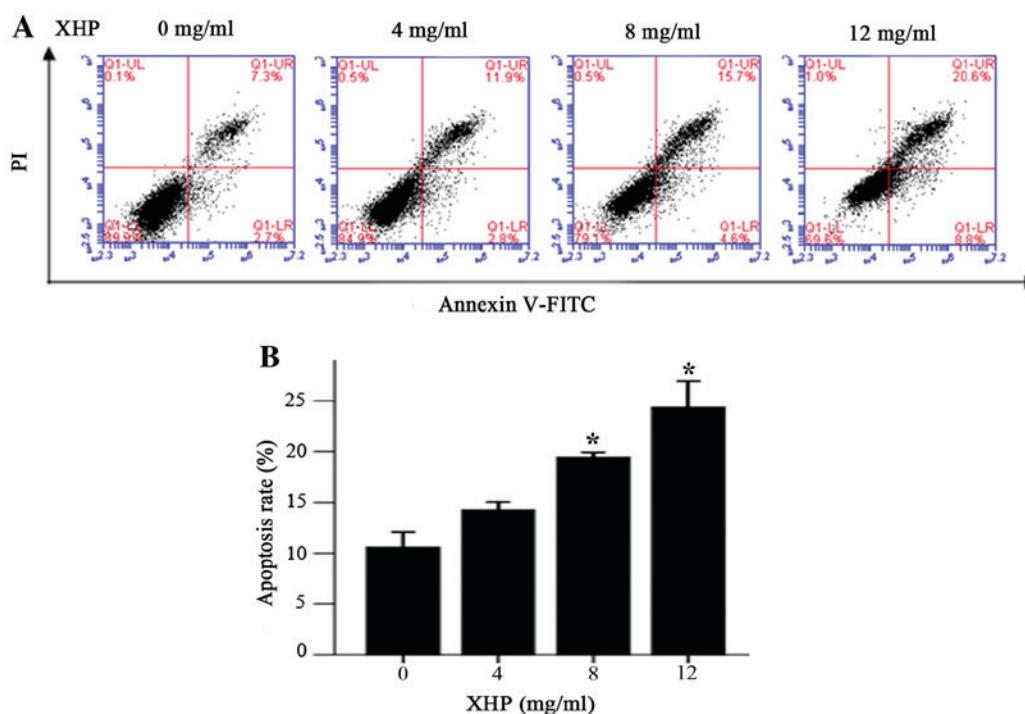
Figure 2. Apoptosis of MDA-MB-231 cells treated with XHP. (A) Cell apoptosis as determined by flow cytometry analysis. Cells were treated with XHP (4, 8 and 12 mg/ml) for 24 h. Cells treated with medium (0 mg/ml XHP) were used as a control. The fluorescence intensity of Annexin V-FITC (x-axis) against the PI (y-axis) staining is shown. (B) XHP induced apoptosis in MDA-MB-231 cells in a dose-dependent manner. The apoptosis rate was defined as the early apoptosis rate plus the late apoptosis rate. The results represent the mean ± standard deviation (n=3). *P<0.05 vs. control. XHP, Xi Huang pills; FITC, fluorescein isothiocyanate; PI, propidium iodide.
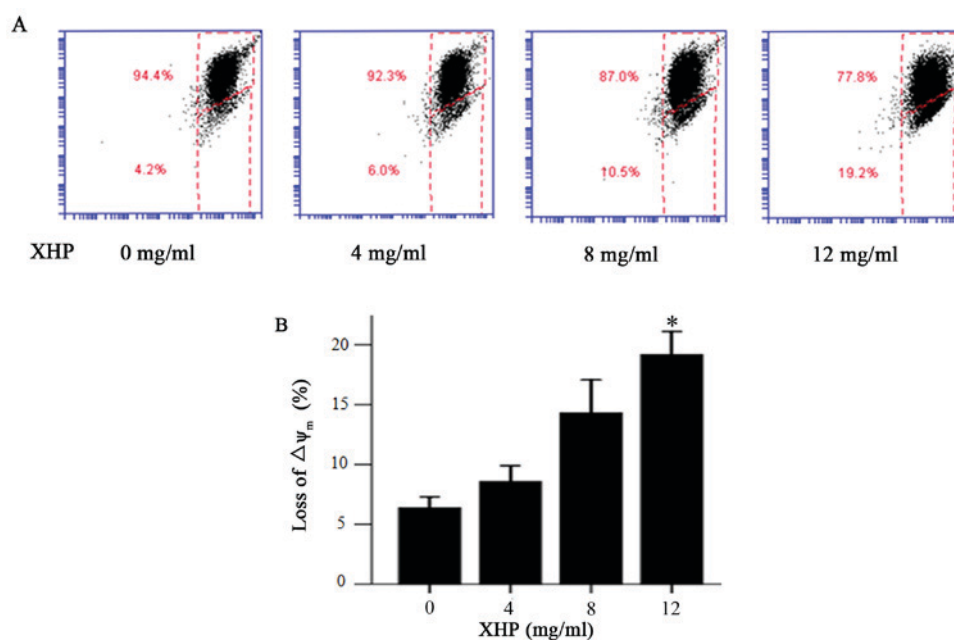

Figure 3. XHP induced Δψm depletion in MDA-MB-231 cells. (A) MDA-MB-231 cells were treated with different concentrations of XHP (4, 8 and 12 mg/ml) for 24 h and Δψm was analyzed by flow cytometry. Cells treated with medium (0 mg/ml XHP) were used as a control. The lower part of the circle shown the percentage of cell with lost Δψm after XHP treatment. (B) XHP induced the depletion of Δψm in a dose-dependent manner. Values represent the mean ± standard deviation (n=3). *P<0.05 vs. control. XHP, Xi Huang pills; Δψm, mitochondrial membrane potential.

decisive role in apoptosis induction (15,16). To determine whether caspase-3, caspase-8, Bax and Bcl-2 proteins were involved in apoptosis induction, the authors of the present study examined the expression of these proteins in MDA-MB-231 cells treated with XHP by western blot

analysis. The results indicated that the protein expression levels of the cleaved caspase-3 were increased by 1.62-, 2.13- and 2.19-fold, when compared to the control following 4, 8 and 12 mg/ml XHP treatment for 24 h, respectively (Fig. 4A and B). However, the expression levels of cleaved caspase-8,
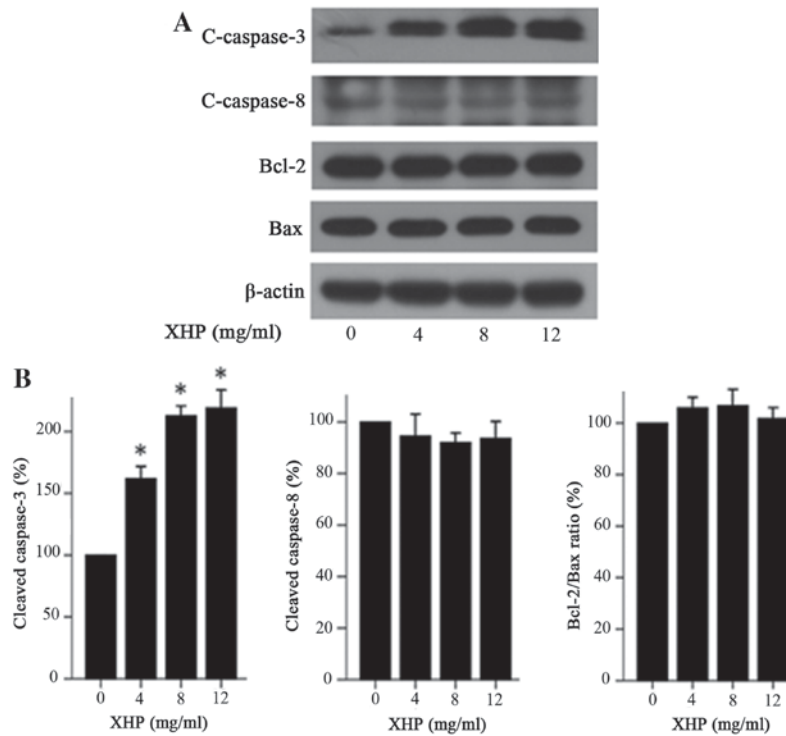
Figure 4. Protein expression levels of apoptosis-associated proteins. (A) MDA-MB-231 cells were treated with different concentrations of XHP (4, 8 and 12 mg/ml) for 24 h, prior to western blot analysis for the expression of c-caspase-3, c-caspase-8, Bcl-2 and Bax. Cells treated with medium (0 mg/ml XHP) were used as a control. (B) A histogram showing the normalized protein band densities of c-caspase-3, c-caspase-8 and the Bcl-2/ Bax ratio. Values represent the mean ± standard deviation (n=3). *P<0.05 vs. control. XHP, Xi Huang pills; c-caspase, cleaved caspase; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein.
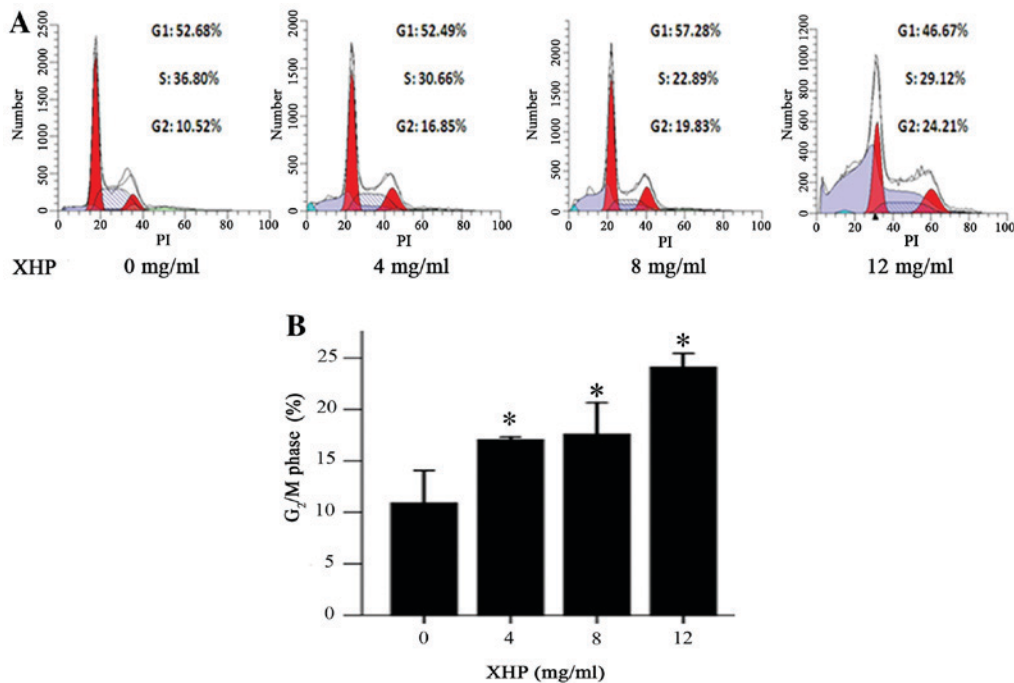

Figure 5. Effect of XHP on the cell cycle distribution of MDA-MB-231 cells. (A) MDA-MB-231 cells were treated with different concentrations of XHP (4, 8 and 12 mg/ml) for 48 h, and then harvested to determine cell cycle distribution by flow cytometry analysis. Cells treated with medium (0 mg/ml, XHP) were used as a control. (B) XHP induced cell cycle arrest at G_2/M phase in a dose-dependent manner. Values represent the mean ± standard deviation (n=3). *P<0.05 vs. control. XHP, Xi Huang pills; FL3AFL3A, propidium iodide signal.

Bcl-2, Bax and the Bcl2/Bax ratio in MDA-MB-231 cells were not significantly different among cells treated with 0, 4, 8 and 12 mg/ml XHP (Fig. 4). These results demonstrated that XHP may induce apoptosis via the intrinsic pathway and
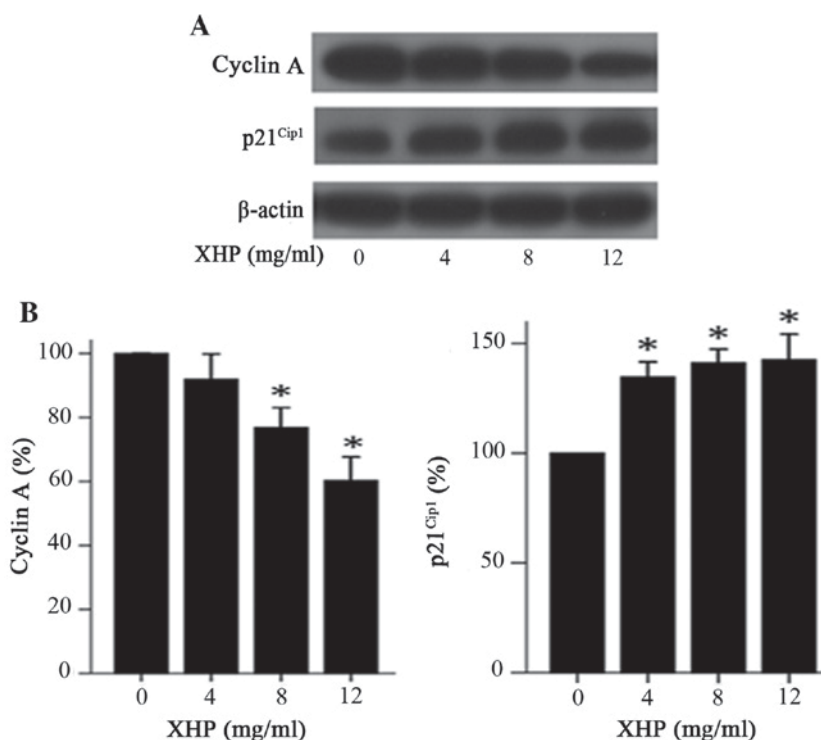
Figure 6. Expression levels of cell cycle regulatory proteins. (A) MDA-MB-231 cells were treated with different concentrations of XHP (4, 8 and 12 mg/ml) for 48 h, and the cells were collected for western blot analysis. (B) A histogram showing the normalized band densities of each cell cycle regulatory protein is shown. Values represent the mean ± standard deviation (n=3). *P<0.05 vs. control. XHP, Xi Huang pills.
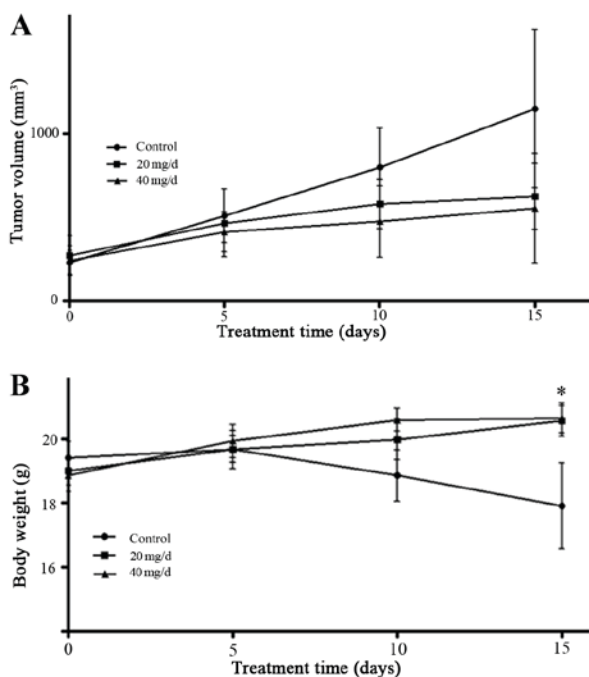

Figure 7. Body weight and tumor volume of female nude mice injected with MDA-MB-231 cells and treated with XHP. MDA-MB-231 cells were subcutaneously inoculated into nude mice, which were injected with XHP (20 and 40 mg/day, the total dose of the drug was administrated twice every 12 h for 15 days. intragastrically when the tumor volume reached ~50-100 mm^3, and the body weight and tumor volume was recorded every five days following XHP administration. The group treated with distilled water was used as control. (A) The tumor volume of nude mice following XHP treatment. A statistically significant difference in tumor volume was not observed among groups at all time points. (B) The body weight of nude mice following XHP treatment. Values represent the mean ± standard deviation (n=10). *P<0.05 vs. control. XHP, Xi Huang pills.

not the extrinsic pathway, which is associated with Bcl-2/Bax ratio.

XHP induced cell cycle arrest at the G_2/M phase. Cell cycle regulation is important for cell proliferation. Numerous antitumor drugs induce cell cycle inhibition by impeding cell proliferation (17,18). In order to examine whether the antiproliferative effects of XHP was associated with cell cycle arrest in the present study, the cell cycle distribution of XHP-treated MDA-MB-231 cells was examined. Cells in the G_2/M phase increased from 10.52% in the untreated group to 16.85, 19.83 and 24.21% following treatment with 4, 8 and 12 mg/ml XHP, respectively (Fig. 5). Consistent with these alterations, the percentages of cells in the G_1 or S phases were concomitantly decreased (Fig. 5A). The results indicated that XHP treatment significantly affected the cell cycle distribution of MDA-MB-231 cells, leading to cell cycle arrest at G_2/M phase in a dose-dependent manner (Fig. 5B).

Effect of XHP on the expression levels of cell cycle regulatory proteins. To explore the mechanism by which XHP induced cell cycle arrest at G_2/M phase in MDA-MB-231 cells, western blot analysis was performed to determine whether XHP modulates the expression of cell cycle regulatory molecules in the present study. The results demonstrated that treatment with different concentrations of XHP (4, 8 and 12 mg/ml) for 48 h resulted in a decrease in the expression of cyclin A and an increase in the expression of p21^{Cip1} when compared with the control group (Fig. 6A and B). These results suggested that the decreased expression of cyclin A and the increased expression of p21^{Cip1} may have contributed to the cell cycle
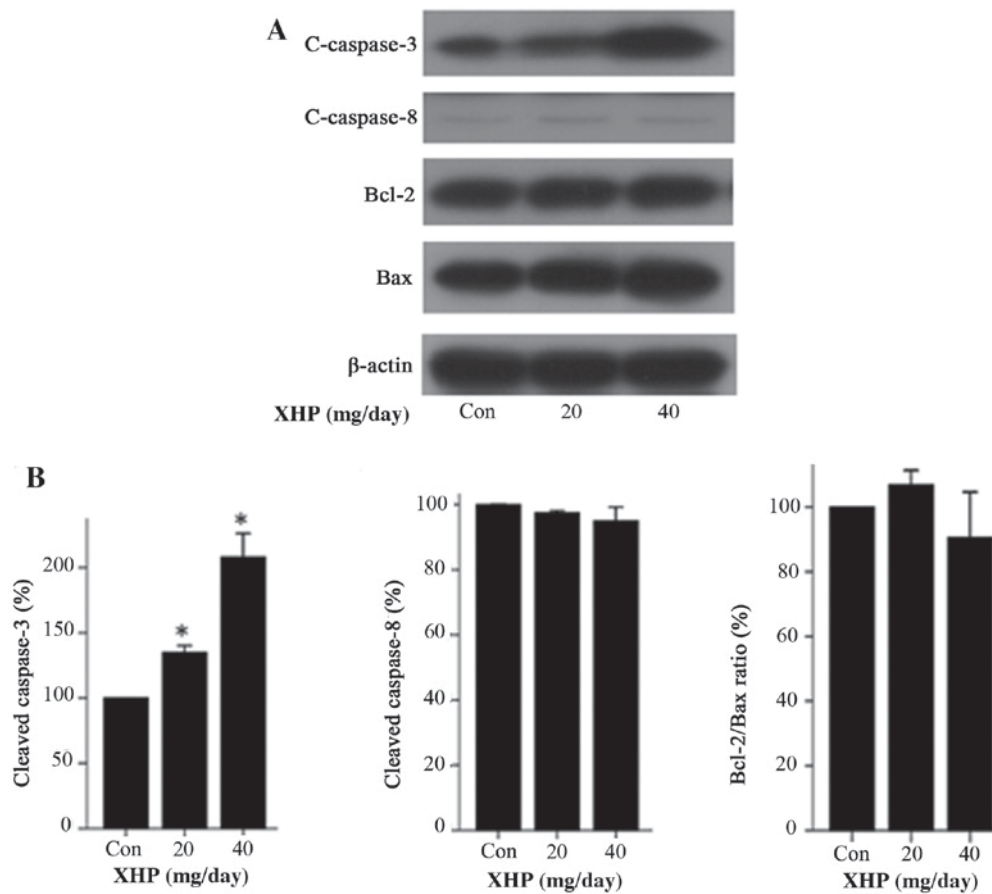
Figure 8. Expression of apoptosis-associated proteins in mouse MDA-MB-231 xenograft tumor tissues. (A) Nude mice with tumor xenografts were treated with different concentrations of XHP (20 and 40 mg/day) for 15 days, and the tumor tissues from mice in each group were collected for western blot analysis of c-caspase-3, c-caspase-8, Bcl-2 and Bax expression. The con group was treated with distilled water only. (B) A histogram showing the normalized band densities of c-caspase-3, c-caspase-8 and the Bcl-2/Bax ratio, relative to the con group. Values represent the mean ± standard deviation (n=3). *P<0.05 vs. control. XHP, Xi Huang pills; con, control; c-caspase, cleaved caspase; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein.

arrest of MDA-MB-231 cells in the G_2/M phase following exposure to XHP.

XHP inhibited the tumor growth in vivo without body weight loss. The results presented so far indicate that XHP may reduce cell viability *in vitro*. Therefore, the next aim of the present study was to determine whether XHP inhibits the growth of xenograft tumors *in vivo*. MDA-MB-231 cells were subcutaneously inoculated into nude mice, which were subsequently administered with a total dose of 20 and 40 mg/day XHP administratd intragastrically twice every 12 h for 15 days when the tumor volume had reached ~50-100 mm³. The body weight and tumor volume were recorded every 5 days. The results indicated that, despite the lack of statistical significance, 20 and 40 mg/day XHP inhibited the growth of xenograft tumors in nude mice when compared with the controls (Fig. 7A). A 7.7% decrease in the weight of mice in the control group was observed compared with the weight at the commencement of treatment, whereas an increase in body weight of 8.1 and 9.4% in mice treated with 20 and 40 mg/ml XHP, respectively, was observed compared with the weight at the commencement of treatment (Fig. 7B).

Expression of apoptosis-associated proteins and cell cycle regulatory proteins in vivo. The results of *in vitro* analysis

demonstrated that XHP affected the expression of apoptosis-associated proteins and cell cycle regulatory proteins (Figs. 4 and 6). Therefore, the authors of the present study investigated whether XHP demonstrated the same effect on the expression of these molecules *in vivo*. The results indicated that, following treatment with 20 and 40 mg/day XHP, the expression of cleaved caspase-3 was increased by 1.35- and 2.08-fold, respectively, when compared with the control group (Fig. 8). By contrast, the expression levels of cleaved caspase-8, Bcl-2, Bax and the Bcl-2/Bax ratio were not significantly different among cells treated with different concentrations of XHP (Fig. 8A and B). The expression levels of cyclin A decreased by 0.58- and 0.26-fold following treatment with 20 and 40 mg/day XPA, respectively when compared with the control (Fig. 9). The expression levels of p21[Cip1] increased by 1.18- and 1.41-fold following treatment with 20 and 40 mg/day XHP, respectively, when compared with the control (Fig. 9). These results demonstrated that similar alterations in the expression of apoptosis and cell cycle regulatory proteins were observed *in vivo* and *in vitro*.

Discussion

The aim of the present study was to investigate the antitumor effects of XHP on MDA-MB-231 cells *in vitro* and *in vivo*,
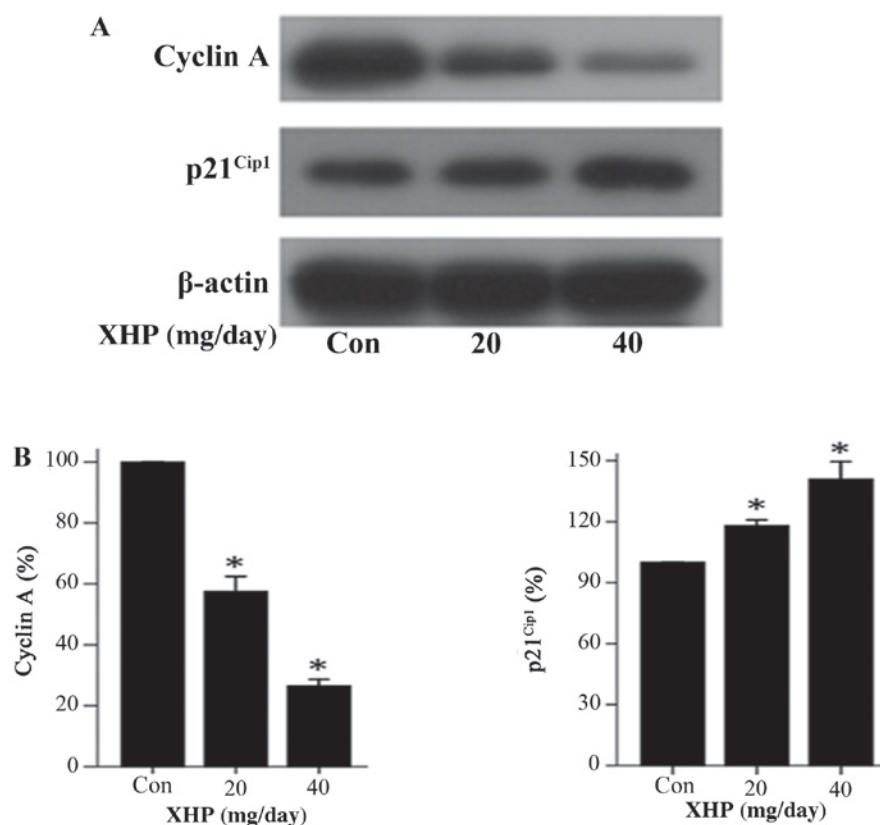
Figure 9. Expression of cell cycle regulatory proteins in xenograft tumor tissues. (A) Nude mice with MDA-MB-231 tumor xenografts were treated with different concentrations of XHP (20 and 40 mg/day) for 15 days, and the tumor tissues from mice in each group were collected for western blot analysis of cyclin A and p21^{Cip1} expression. The con group was treated with distilled water only. (B) A histogram showing the normalized band densities of each cell cycle regulatory protein relative to the con group. Values represent the mean ± standard deviation (n=3). *P<0.05 vs. control. XHP, Xi Huang pills; con, control.

as well as the potential underlying molecular mechanisms involved. Therefore, MTT, apoptosis, cell cycle distribution and western blot assays were conducted, and a xenograft tumor model in nude mice was established.

The results of the MTT assay demonstrated that XHP inhibited the viability of MDA-MB-231 cells in a dose- and time-dependent manner. Based on the difference in MDA-MB-231 and MCF-10A cell viability following XHP treatment, it is possible that XHP inhibited the viability of MDA-MB-231 cells in a cell-selective manner, which is a significant factor in cancer treatment.

In order to understand the mechanisms underlying the antiproliferative effects of XHP *in vitro*, the level of apoptosis and the cell cycle distribution of MDA-MB-231 cells treated with different concentrations of XHP were determined. The results demonstrated that XHP induced apoptosis and cell cycle arrest at the G$_2$/M phase in MDA-MB-231 cells, which was consistent with the results of the MTT assay. A previous study involving the Hs578T human TNBC cell line, demonstrated similar effects of XHP *in vitro*, whereby XHP inhibited the viability of Hs578T cells, induced apoptosis and cell cycle arrest in S-phase, not the G$_2$/M phase (19). Previous studies have demonstrated the anti-tumor effect of XHP, in which XHP inhibited the proliferation of human tumor cell lines SMMC7721 (liver cancer cell line), T24 (bladder cancer cell line), A549 (lung cancer cell line), LoVo (colorectal cancer cell line) and LAC (human lung cancer stem cell line) *in vitro* (20-22), and XHP could induce H22

cell (mouse liver cancer cell line) and Bel-7402 cell (human liver cancer cell line) apoptosis by downregulating Bcl-2 expression in tumor-bearing mice (23,24). All these studies, including the present study, indicated that XHP possessed anti-tumor activity in a wide range of cancer types. In order to elucidate the mechanisms underlying the antiproliferative effects of XHP, further studies have been performed, and beside the apoptosis and cell cycle arrest noted in the present study, the anti-tumor mechanisms elucidated included the suppression of the invasion, migration and metastasis of tumor cells (21,25,26), inhibition of angiogenesis (26,27) and modulation of the tumor immune microenvironment (26,28-30). However, there remains further studies to be performed to elucidate the anti-tumor mechanisms of XHP treatment on MDA-MB-231.

In the present study, the protein expression levels of caspase-8 and caspase-3 were detected by western blot analysis, in order to elucidate the mechanism by which XHP induces apoptosis in MDA-MB-231 cells *in vitro*. A significant increase in the expression of cleaved caspase-3 was observed, whereas there were no significant alterations in the expression of cleaved caspase-8. Therefore, XHP may induce apoptosis via the intrinsic apoptotic pathway and not the extrinsic pathway. In addition, it is possible that the observed decrease in mito-chondrial membrane potential following XHP treatment may have induced the intrinsic pathway and led to apoptosis.

In human cells, Bax is a constituent of the ion channel in the mitochondrial membrane which causes the loss of $\Delta\psi_m$,

and Bcl-2, an oncogene with many anti-apoptotic functions, and which combines with Bax to prevent formation of the ion channel (31). Thus, the Bcl-2/Bax ratio serves an important role in the induction of apoptosis. The results of western blot assay demonstrated there were no alterations in the expression of Bax, Bcl-2 or the Bcl-2/Bax ratio in MDA-MB-231 cells *in vitro*. Therefore, the authors of the present study concluded that the depletion of mitochondrial membrane potential induced by XHP was not associated to alterations in the Bcl-2/Bax ratio, and deduced that it may instead be associated with factors that damage the mitochondrial membrane directly, or activate Bax or additional Bcl-2 family members. This requires further investigation in future studies.

Cell cycle progression is orchestrated by a complex network of interactions between proteins, among which are cyclins, cyclin-dependent kinases (CDKs), E3 ubiquitin ligase complexes, CDK activating kinase, cell division cycle 25 phosphatases and CDK inhibitors (32). In order to explore the mechanisms by which XHP induces cell cycle arrest at the G_2/M phase in MDA-MB-231 cells, a western blot assay was used to determine whether XHP modulates the expression of cell cycle regulatory proteins. The results indicated that XHP treatment was associated with decreased expression of cyclin A and increased expression of p21^{Cip1}. The G_2/M phase arrest may be explained by the decreased expression of cyclin A, as cyclin A is a regulatory protein specific to the late stage of the S phase and the early stage of G_2 phase (18). As p21^{Cip1} forms a heterotrimeric complex with cyclin D-, cyclin E- and cyclin A-dependent kinases, this leads to the inhibition of their activities (33), and the increase in the expression of p21^{Cip1} may therefore arrest the cell cycle at all stages.

In order to investigate the antitumor effect of XHP on MDA-MB-231 cells *in vivo* in the present study, a mouse xenograft tumor model was established. The results indicated that, despite the lack of statistical significance, treatment with 20 and 40 mg/day XHP inhibited the growth of xenograft tumors in nude mice when compared with controls, which was in accordance with the *in vitro* MTT assay results. In addition, weight loss was observed in the untreated control group. By comparison, a significant increase in the weight of mice treated with 40 mg/day XHP was observed, which suggested that XHP may be safe and non-toxic. This is consistent with the results of previous studies that have examined the clinical use of XHP in cancer treatment (34,35). The expression levels of apoptosis-associated and cell cycle regulatory proteins in xenograft tumor tissues were analyzed by western blotting in the present study. The results demonstrated that the expression of these molecules was altered in a similar manner *in vitro* and *in vivo*. Therefore, the observed alterations in protein expression *in vivo* provides further evidence of the molecular mechanisms of apoptosis and cell cycle arrest induced by XHP *in vitro*. In addition, the authors detected the expression levels of CDK1 and cyclin B *in vitro* and *in vivo* (data not shown). The CDK1 and cyclin B expression were increased when the XHP concentration increased *in vivo*, while in the *in vitro* experiment the CDK1 expression was decreased and the cyclin B expression was unchanged when the XHP concentration increased. The explanation for this observation may be due to the complex composition of XHP which has numerous of compounds to be determined in the future. All these data, *in vitro* and *in vivo* demonstrated the complex effects of XHP, which merits further study.

In conclusion, MDA-MB-231 cell viability was significantly inhibited by XHP treatment in a dose-dependent, time-dependent and cell-selective manner *in vitro*. The potential underlying mechanisms may involve induction of apoptosis and cell cycle arrest at the G_2/M phase. XHP may induce apoptosis of MDA-MB-231 cells via the intrinsic pathway, which is not associated with alterations in the Bcl-2/Bax ratio. The observed cell cycle arrest in G_2/M phase may have been due to the integrated action of decreased cyclin A expression and increased p21^{Cip1} expression. In addition, XHP inhibited the growth of xenograft tumors in nude mice without decreasing body weight *in vivo*. Therefore, the results of the present study indicated that XHP demonstrates antitumor effects on the MDA-MB-231 TNBC cell line, which provides evidence that XHP may be a useful antitumor agent for the treatment of patients with TNBC.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author's contributions

WZ, MC, SJ, XH and XL performed cell culture, MTT assay, apoptosis detection, cell cycle detection and western-blot respectively. WZ performed the *in vivo* experiment and wrote the manuscript. SH and PL designed the experiment and led the team. HD performed the flow cytometry.

Ethics approval and consent to participate

The animal experiment protocol was approved by Peking University Animals Research Committee (Beijing, China) and was conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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