Jolkinolide B induces apoptosis in MDA-MB-231 cells through inhibition of the PI3K/Akt signaling pathway

YU LIN[1,2*], HONGXIA CUI[1*], HUIYU XU[3], LILING YUE[3], HAO XU[3], LIYAN JIANG[3] and JICHENG LIU[3]

[1]Department of Pharmacology, Qiqihar Medical University, Qiqihar 161006; [2]Heilongjiang University of Chinese Medicine, Harbin 150040; [3]The Institute of Medicine, Qiqihar Medical Univeristy, Qiqihar 161006, P.R. China

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Abstract. The phosphoinositol-3-kinase (PI3K)/Akt signal transduction pathway is critically important for tumor cell growth, proliferation and apoptosis. Apoptosis activation has been reported to be a good target in cancer therapies. In this study, we have found that jolkinolide B (JB), a diterpenoid from the traditional Chinese medicinal herb *Euphorbia fischeriana* Steud, strongly inhibited the expression of the PI3K p85 subunit and the phosphorylation of Akt. Furthermore, we evaluated the effects of JB on the proliferation and apoptosis of MDA-MB-231 human breast cancer cells. Our results show significant induction of apoptosis in MDA-MB-231 cells incubated with JB. This effect was enhanced by combination with LY294002. In addition, treatment with JB could induce downregulation of the Bcl-2/Bax ratio, and subsequent promotion of mitochondrial release of cytochrome c and activation of caspase-3. Taken together, JB-induced apoptosis of MDA-MB-231 cells occurs through the mitochondrial pathway. Further, the PI3K/Akt signaling cascade plays a role in the induction of apoptosis in JB-treated cells. These observations suggest that JB may have therapeutic applications in the treatment of cancer.

Introduction

Apoptosis (programmed cell death) has become one of the popular research areas in cancer therapy. It has been shown that inducing tumor cell apoptosis is one of the important mechanism of many chemotherapeutic agents (1,2). The execution of apoptosis is associated with biochemical changes mediated by various genes and cell signaling pathways, such as the phosphoinositide 3-kinase (PI3K)/Akt survival pathway. The PI3K/Akt signaling is a significant transduction pathway which plays a key regulatory function in cell proliferation, apoptosis and the cell cycle (3,4). Class IA PI3K, the type most widely implicated in cancer, is a heterodimeric lipid kinase consisting of a p85 regulatory subunit and a p110 catalytic subunit (5). This pathway is activated by survival signals such as cytokines, growth factors and hormones (6).Activation of PI3K results in the phosphorylation of Akt. Mutation of this pathway has been found in about 70% of breast cancer patients (7). There is an excessive expression and activation of Akt in various malignant tumors, such as gastric and prostate cancer (8,9).

Jolkinolide B (JB), a diterpenoid component, isolated from the dried roots of *Euphorbia fischeriana* Steud, has shown significant antitumor activities against Sarcoma 180 and Ehrlich ascites carcinoma in mice (10). It has been reported that JB regulates proliferation and induces apoptosis in human prostate LNCaP cancer and K562 cells *in vitro* (11,12). In the present study, we aimed to investigate the effects of JB on cell proliferation and the induction of apoptosis in MDA-MB-231 breast cancer cells and to examine whether its effects are associated with the PI3K/Akt signaling pathway.

Materials and methods

Materials. JB (molecular weight of 330.4, purity >99%) (structure, Fig. 1) was kindly provided by Professor Shujun Zhang (Chemical Engineering Institute, Qiqihar University). JB was dissolved in dimethylsulfoxide (DMSO) to make a stock solution at a concentration of 100 mM, which was further diluted to the appropriate concentration with culture medium before each experiment. Control experiments contained DMSO.

Cell line and cell culture. The human breast cancer cell line MDA-MB-231 was purchased from the Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were maintained in L15 medium (Gibco, Invitrogen Corp., USA) supplemented with 10% fetal bovine serum at 37˚C with 5% CO_2 and digested with 0.25% trypsin.

Cell viability assay. To measure cell viability, cells were seeded into 96-well plates ($5x10^3$ cells/well) and incubated overnight. Subsequently, they were incubated with different

Correspondence to: Dr Jicheng Liu, The Institute of Medicine, Qiqihar Medical Univeristy, 333 BuKui Street, JianHua District, Qiqihar 161006, P.R. China
E-mail: qyybliu@sohu.com

*Contributed equally

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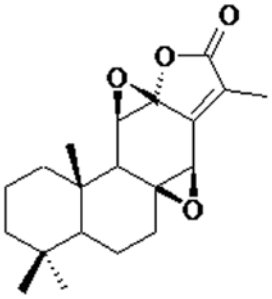

Figure 1. Chemical structure of jolkinolide B.

concentrations of JB for 12, 24 or 48 h. After treatment, 20 μl of MTT [3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] solution (final concentration, 500 μg/ml) was added in each well and incubated continuously at 37˚C for 4 h. Next, the medium was removed from all the wells and mixed with 150 μl of DMSO. The absorbance for each well was measured at the wavelength of 570 nm by a microplate reader (Safire2, Tecan Group Ltd., Maennedorf, Switzerland). Cell viability was determined as a percentage of viable cells in JB-treated vs. untreated controls. The MTT assay was performed at each time point for three independent experiments.

Annexin V-FITC/PI flow cytometry analysis. Cell apoptosis analysis was performed using the Annexin V-FITC/PI Apoptosis Detection kit (Becton-Dickinson, San Jose, CA). Cells were seeded into 6-well plates at a density of $1x10^6$ cells/well. After overnight incubation, cells were treated with different concentrations of JB (0, 10, 20 or 40 μM) for 24 h. LY294002 (50 μM) (Sigma, St. Louis, MO, USA), a selective PI3K inhibitor, was added to the culture 1 h prior to JB (40 μM).Then, cold PBS was used to wash the cells twice and the cells were resuspended in 500 μl of binding buffer. In the next step, 5 μl of Annexin V-FITC and 5 μl of PI were added, and the mixture was incubated for 15 min at room temperature in the dark. After staining, the cell apoptosis was measured immediately by flow cytometry (BD Biosciences, San Jose, CA, USA). Flow cytometry analysis was performed using the FlowJo software (Tree Star, Ashland, OR, USA).

Measurement of mitochondrial membrane potential ($\Delta\Psi m$). The loss of $\Delta\Psi m$ was monitored with the dye Rh123 (Sigma). About $1x10^6$ cells were treated with JB (0, 20 or 40 μM) for 24 h or pretreated with LY294002 (50 μM) for 1 h prior to JB (40 μM), then harvested and washed in ice-cold PBS followed by incubation with 50 μM Rh123 at 37˚C for 30 min in the dark. Cells were washed with PBS again and analyzed by flow cytometry at 488 nm of excitation wave.

Western blot analysis. The cells were treated as described above for the cell apoptosis analysis. The total protein of the cells was extracted with lysis buffer [20 mM Tris-HCl at pH 7.4, 0.2 mM EDTA, 1 mM EGTA, 50 μg/ml leupeptin, 250 mM sucrose, 20 mM phenylmethylsulfonyl fluoride (PMSF)]. Cytoplasmic protein was isolated with lysis buffer (10 mM HEPES pH 7.9, 250 mM sucrose, 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 10% NP-40). Cell lysates were centrifuged at 12,000 x g for 20 min at 4˚C and the protein

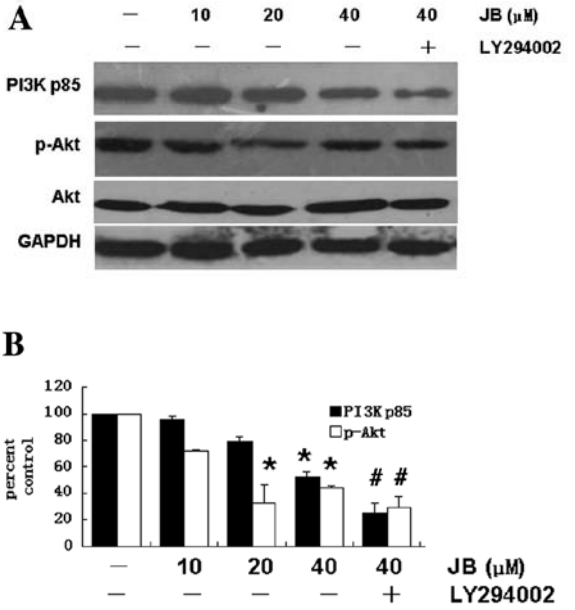

Figure 2. Effect of JB on the expression of PI3K p85 and p-Akt in MDA-MB-231 cells. MDA-MB-231 cells were treated with various (0, 10, 20 or 40 μM) JB alone and/or pretreated with 50 μM LY294002 for 1 h followed by co-incubation with JB (40 μM) for 24 h. (A) Proteins were extracted, then PI3K p85, Akt and p-Akt expressions were determined by western blot analysis. (B) Densitometric values of protein bands were normalized to those of GAPDH. The intensity of each band was quantified by densitometry, where the control group was assigned the value of 100%. The data represent the mean ± SD of three independent determinations. $^*p<0.05$ compared with the untreated cells. $^\#p<0.05$ compared to JB alone.

concentration was determined using the Bicinchoninic Acid (BCA) Protein Assay kit (Beyotime Biotechnology, Haimen, China) according to the protocol provided by the manufacturer. Equal amounts of protein (40 μg) were separated by SDS-PAGE and electrotransferred onto nitrocellulose filter membranes. The membranes were blocked with a solution of 5% non-fat dry milk for 2 h at room temperature, followed by incubation overnight at 4˚C with primary antibodies against PI3K p85, Akt and p-Akt (Cell Signaling, Danvers, MA, USA), caspase-3, Bcl-2, Bax, cytochrome c (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membranes were further incubated for 1 h at room temperature with horseradish peroxidase-conjugated respective secondary antibodies (Cell Signaling). Following washing, the specific proteins were detected using an ECL western blotting kit (Amersham Biosciences, Piscataway, NJ, USA) as recommended by the manufacturer. Each experiment was repeated three times. An internal control was used to confirm that an equal amount of protein was loaded in each lane. Band intensities were quantified by an AlphaImager™ 2200 using the Spot Denso function of the AlphaEaseFC™ Software version 3.1.2 (Witec, Littau, Switzerland).

Statistical analysis. The data are expressed as mean ± SD from at least three experiments and were analyzed using the SPSS13.0 statistical software. Differences between two treatment groups were evaluated by the t-test and intergroup differences were evaluated by one-way ANOVA. Statistically significant differences were considered those with $p<0.05$.

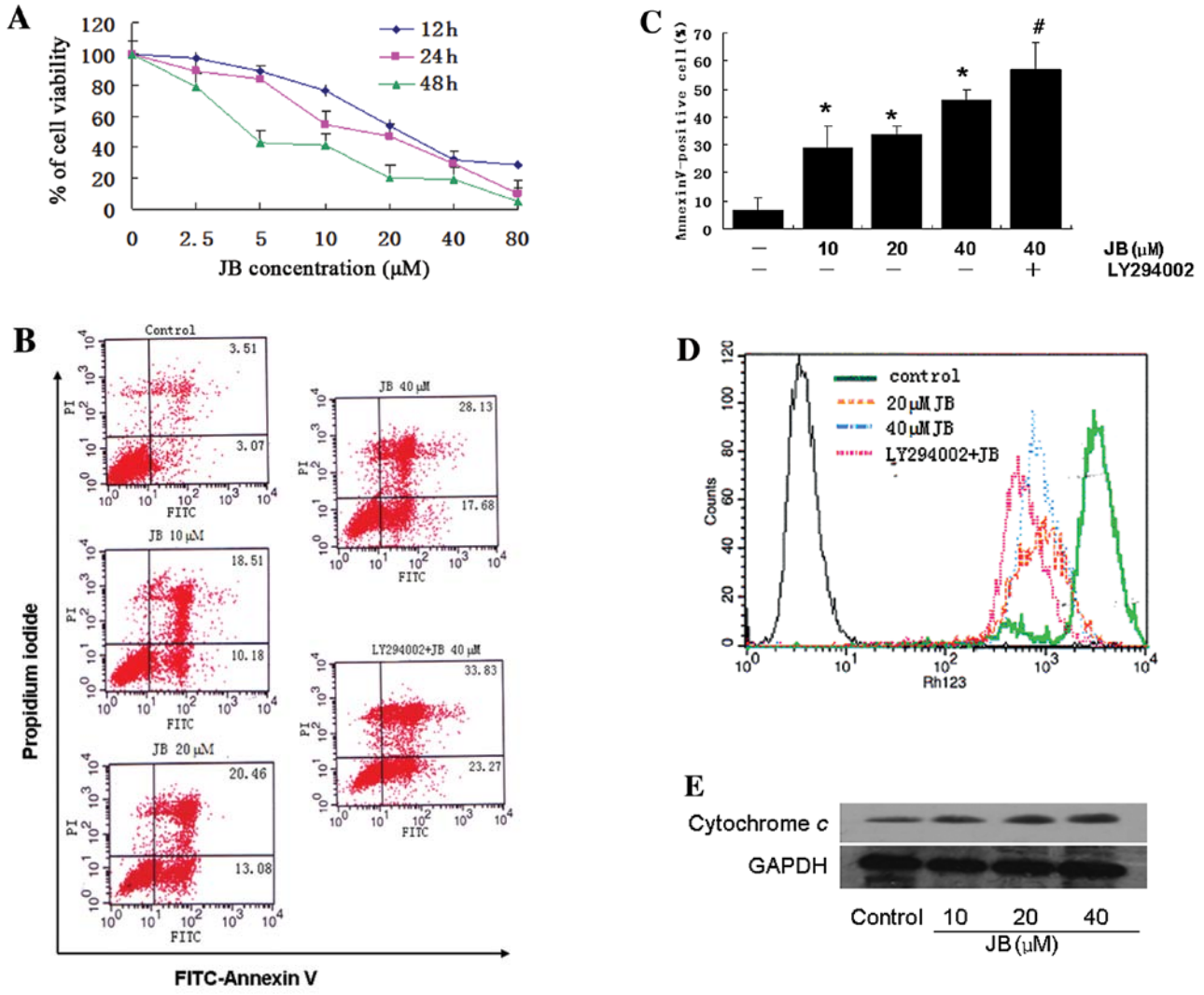

Figure 3. Effect of JB on the proliferation and apoptosis of MDA-MB-231 cells. (A) Proliferation of MDA-MB-231 cells was analyzed by the MTT assay. Cells were treated with various concentrations of JB for 12, 24 or 48 h. Cell viability was detected by the MTT assay. Values are expressed as means ± SD from three independent experiments. (B) Apoptosis induced by JB was assessed with Annexin V-FITC/PI double staining. Cells were treated with JB (0, 10, 20 or 40 μM) for 24 h and/or pretreated with LY294002 (50 μM) for 1 h prior to JB (40 μM). Cells were then harvested and stained for Annexin V-FITC/PI and analyzed by flow cytometry. The numbers indicate the percentage of cells in each quadrant. (C) The bar chart describes the percent distribution of apoptotic cells. Data were obtained from three separate experiments and are expressed as mean ± SD. $^{*}p<0.05$ compared with the untreated cells. $^{\#}p<0.05$ compared with JB alone. (D) JB induces the depletion of the mitochondrial membrane potential ($\Delta\Psi m$). Cells treated with the indicated reagent or combination were harvested and stained with the Rh123 dye and then analyzed by flow cytometry. (E) Western blot analysis for cytochrome c in the cytosolic extract of cells treated with JB (0, 10, 20 or 40 μM).

Results

JB suppresses PI3K/Akt phosphorylation. In order to determine the effect of JB on the PI3K/Akt signaling pathway, MDA-MB-231 cells were treated with various concentrations of JB (0, 10, 20 or 40 μM) for 24 h and analyzed for the expression of the PI3K/Akt by western blotting. As shown in Fig. 2, protein levels of the PI3K p85 subunit and of phosphorylated Akt were significantly and concentration-dependently suppressed by JB treatment compared with untreated cells.

To further confirm the effect of JB on the PI3K/Akt pathway, cells were pretreated with LY294002 (a specific PI3K inhibitor) prior to JB exposure. The combined treatment with LY294002 (50 μM) and JB (40 μM) had a synergistic inhibitory effect on PI3K/Akt compared with treatment with JB alone in MDA-MB-231 cells.

JB inhibits growth and induces apoptosis of MDA-MB-231 cells. The PI3K/Akt pathway plays an important role in apoptosis (13). Therefore, we next investigated the effects of JB on the growth and proliferation of MDA-MB-231 cells, and examined whether JB could induce apoptosis. The MTT assay was performed to examine whether JB inhibits growth of MDA-MB-231 cells. As shown in Fig. 3A, after treatment with various concentrations of JB for 12, 24 or 48 h, cell viability and proliferation was markedly inhibited in a dose- and time-dependent manner compared with the control group. The calculated IC_{50} concentrations at 12, 24 and 48 h were 23.82±4.39, 15.29±2.67 and 4.68±1.85 μM, respectively.

To evaluate whether JB inhibited the survival of MDA-MB-231 cells through the induction of apoptosis, cells were treated with different doses of JB (0, 10, 20 or 40 μM) for 24 h. Then cells were stained with Annexin V-FITC/PI to assess the apop-

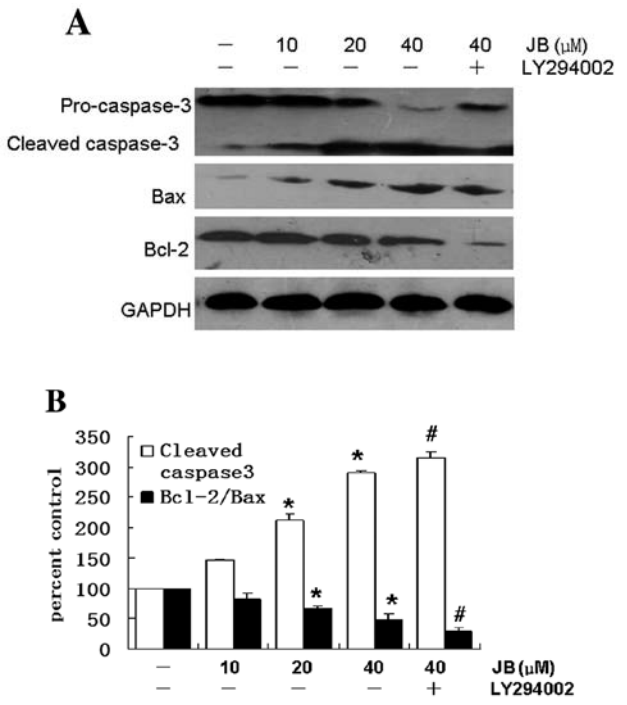

Figure 4. Effect of JB on the expression of caspase-3 and Bcl-2/Bax protein in MDA-MB-231 cells. Cells were treated with increasing doses of JB for 24 h. LY294002 (50 μM) was added to the culture 1 h prior to JB (40 μM). (A) Whole cell extracts were prepared for western blot analysis of the indicated proteins. (B) Quantitative results of caspase-3 and Bcl-2/Bax are presented relative to control. Densitometric analysis of western blot obtained from three independent experiments and are expressed as mean ± SD. $^{*}p<0.05$ compared with untreated cells. $^{\#}p<0.05$ compared to JB alone.

totic and necrotic cell population by flow cytometry analysis. Treatment with JB led to marked cell apoptosis indicated by the percentage of Annexin V-positive cells compared with the untreated control (Fig. 3B and C). Pretreatment of MDA-MB-231 cells with LY294002 at 50 μM, prior to JB (40 μM) exposure, significantly enhanced the JB-induced cell apoptosis.

Potential changes in mitochondrial membrane potential ($\Delta\Psi$m) were analyzed by flow cytometry with the Rh123 dye. MDA-MB-231 cells treated with JB were found to display a concentration-dependent decrease in $\Delta\Psi$m and increase in cytochrome c levels in the cytosol (Fig. 3D and E). LY294002 (50 μM) pretreatment encouraged JB-induced loss of $\Delta\Psi$m.

Effect of JB on protein expression of genes related to apoptosis. Bcl-2 family members are important regulators of the apoptotic pathway (14,15). Therefore, we determined the effect of JB on Bcl-2 and Bax protein levels and the role of the PI3K/Akt signaling pathway. JB treatment significantly decreased Bcl-2 protein levels and increased Bax expression. Meanwhile, the combined treatment with JB and LY294002 (50 μM) showed a synergistic effect on Bcl-2 and Bax protein expression (Fig. 4).

We further determined the activation of caspase-3 by western blot analysis (Fig. 4). Cells treated with JB (0, 10, 20 or 40 μM) significantly increased the cleavage of pro-caspase-3 to the active form in a dose-dependent manner. In addition, pretreatment with LY294002 (50 μM) resulted in an increase in cleaved caspase-3.

Discussion

The dried roots of *Euphorbia fischeriana* Steud have a long history of being used as folk medicine. Its antitumorigenic constituents, such as the diterpenoid compound 17-acetoxyjokinolide B and Jolkinolide B, have been identified (10). However, its molecular mechanism remains largely unknown. In the present study, JB inhibited the proliferation of MDA-MB-231 cells and markedly induced apoptosis. Meanwhile, JB significantly induced downregulation of the Bcl-2/Bax ratio, thus facilitating cytochrome c release, loss of $\Delta\Psi$m and caspase-3 activation. In addition, we found that the Akt-dependent signaling pathway could be involved in the effect of JB in MDA-MB-231 cells.

The execution of apoptosis is an important mechanism of anticancer drugs. It can be initiated via two crucial pathways: the death receptor-mediated and the mitochondrial-mediated pathways. The mitochondrial death pathway is controlled by Bcl-2 family proteins (16). It consists of pro-apoptotic (Bim, Bax and Bid) and anti-apoptotic members (Bcl-2, Bcl-xL) (17-19). During apoptosis, the Bcl-2 family can regulate the release of apoptotic factors and induce or prevent apoptosis. Alteration in the levels of anti- and pro-apoptotic Bcl-2 family members can influence apoptosis. In this study, JB inhibited the proliferation of MDA-MB-231 cells in a dose- and time-dependent manner. Furthermore, JB could markedly promote apoptosis of MDA-MB-231 cells, and was associated with downregulation of the Bcl-2/Bax ratio.

Caspase family proteins are critical enzymes to execute apoptosis (20). Although caspase-3 has been documented to play a key role as an executor for apoptosis in mammalian cells (21,22), it is not clear whether caspase-3 is involved in JB-induced apoptosis of MDA-MB-231 cells. As shown in Fig. 4, JB dose-dependently resulted in the activation of caspase-3 in JB-treated MDA-MB-231 cells. The results indicate that caspase-3 may modulate JB-induced apoptosis of MDA-MB-231 cells.

Mitochondria play an important role in regulation of apoptosis. The mitochondrial membrane potential is an important measure for mitochondrial functions. The decrease in $\Delta\Psi$m is one of the earliest events in cell apoptosis (23). Disruption of mitochondrial integrity is one of the early events leading to apoptosis (24-26). Therefore, to determine whether JB affects the function of mitochondria, potential changes in the mitochondrial membrane were analyzed by flow cytometry with the dye Rh123. Rh123 is a cell permeable cationic fluorescent probe. It can pass through cell membranes and assemble in living mitochondria. During apoptosis, the $\Delta\Psi$m and the fluorescence intensity decrease. In this study, $\Delta\Psi$m loss and the release of cytochrome c were increased in JB-treated cells (Fig. 3D and E). The measurements for $\Delta\Psi$m further demonstrated that JB may induce apoptosis of MDA-MB-231 cells, which are common mechanisms proposed for the cytotoxic effects of JB.

The PI3K/Akt signal transduction pathway was reported to play an essential role in various cellular processes including apoptosis, survival, proliferation and differentiation (27,28). Activated PI3K initiates the activation of the downstream kinase Akt. Moreover, phosphorylated Akt could facilitate cell survival and proliferation and regulate various signaling

pathways (29-31). It has been shown that the activation of PI3K and Akt could inhibit autophagy (32). Yuan *et al* confirmed that there is a close relationship between the excessive expression and activation of Akt kinase and the malignant biological behavior of ovarian cancer (33). Pretreatment with LY294002 induced apoptosis of ovarian cancer cells (3). The PI3K/Akt signaling pathway has been closely related to the occurrence of human malignant tumors, such as endometrial cancer and non-small cell lung cancer (34,35). In this study, JB treatment of MDA-MB-231 cells decreased the expression of the PI3K p85 subunit, and of the phosphorylated Akt in a concentration-dependent manner (Fig. 2). Additionally, pretreatment with the PI3K pharmacological inhibitor LY294002 (50 μM) before JB (40 μM) enhanced the effects of JB on the expression of PI3K/Akt, inducing apoptosis, downregulation of the ratio of Bcl-2/Bax or activation of caspase-3 and the release of cytochrome c.

In conclusion, our findings demonstrate that JB induces apoptosis of MDA-MB-231 cells, at least in part, by inhibition of the PI3K/Akt signaling pathway. This seems to be the important mechanism of the JB-induced suppression of the growth of the breast cancer cells. Therefore, JB may serve as a novel promising compound in cancer therapy. Future studies are geared toward evaluating the antitumor activity of JB in animal models.

Acknowledgements

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