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Germacrone inhibits the proliferation of breast cancer cell lines by inducing cell cycle arrest and promoting apoptosis

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ABSTRACT

Traditional medicinal herbs are an untapped source of potential pharmaceutical compounds. This study aims to determine whether the proliferation of breast cancer cell lines could be inhibited by germacrone, a natural product isolated from *Rhizoma curcuma*. Germacrone treatment significantly inhibited cell proliferation, increased lactate dehydrogenase (LDH) release, and induced mitochondrial membrane potential ($\Delta\Psi m$) depolarization in both MCF-7 and MDA-MB-231 cells in a dose-dependent manner. Germacrone induced MDA-MB-231 and MCF-7 cell cycle arrest at the G0/G1 and G2/M phases respectively and induced MDA-MB-231 cell apoptosis. Furthermore, germacrone treatment significantly increased Bok expression and cytochrome *c* release from mitochondria without affecting Bcl-2, Bcl-xL, Bax, and Bim protein expressions. In addition, germacrone treatment induced caspase-3, 7, 9, PARP cleavage. We concluded that germacrone inhibited the proliferation of breast cancer cell lines by inducing cell cycle arrest and apoptosis through mitochondria-mediated caspase pathway. These results might provide some molecular basis for the anti-tumor activity of *Rhizoma curcuma*.

1. Introduction

Rhizoma curcuma (*Ezhu* in Chinese) is widely prescribed in traditional Chinese medicine for anti-tumor therapy. Recent studies have determined that the main bioactive constituents of *Ezhu* are its essential oils, which possess anti-tumor (Li et al., 2009), anti-inflammatory (Makabe et al., 2006), and neuroprotective properties (Dohare et al., 2008). At present, many volatile oil components from *Ezhu*, such as β-elemene, curcumol, curdione, neocurdione, etc., have been isolated and identified. However, aside from studies on β-elemene, few reports have focused on the bioactivities of other *Ezhu* oil components. In our previous study, a high content of the sesquiterpene compound germacrone, was found in *Ezhu* essential oils (Yang et al., 2006; Yang et al., 2005). However, studies on its biological activity are limited. A study by Claeson et al. (1993) demonstrated that germacrone exerted a significant anti-inflammatory activity against carrageenan-induced hind paw edema in rats. Matsuda et al. (1998) and Morikawa et al. (2002) reported that germacrone had a potent protective effect on D-galactosamine (D-GalN)/lipopolysaccharide (LPS)-induced and tumor necrosis factor-alpha (TNF-α)-induced acute liver injury in mice). Since *Ezhu* essential oils have been clinically tested for anti-tumor treatment in China (Song and Yin, 2008; Fang et al., 2009) and have been found to inhibit MCF-7 cell proliferation (Pu and Zhao, 2009), we hypothesize that germacrone might be one of the specific bioactive compounds responsible for the anti-proliferative effect. Therefore, in this study, the anti-proliferative effects of germacrone on MCF-7 and MDA-MB-231 breast cancer cell lines are assessed, and the underlying mechanisms are explored.

2. Materials and methods

2.1. Reagents

Germacrone (>96%) was isolated and identified by Prof. Shaoping Li as previously described (University of Macau) (Yang et al., 2005). RPMI-1640 culture medium was obtained from Gibco. Fetal bovine serum (FBS), phosphate-buffered saline (PBS), penicillin–streptomycin (PS), 0.25% (w/v) trypsin/1 mM EDTA, and the APO-BrdU™ TUNEL assay kit were purchased from Invitrogen. Propidium iodide (PI), 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl tetrazolium bromide (MTT), and 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazolylcarbocyanine iodide (JC-1) were purchased from Molecular Probes. RIPA lysis buffer was obtained from Santa Cruz. Lactate dehydrogenase (LDH) release detection kit was purchased from Roche Diagnostics. Primary antibodies against Bcl-2, Bcl-xL, Bax, Bok, Bim, caspase-3,7,9, cleaved caspase 3,7, PARP, cleaved PARP, cytochrome *c*, cdc2, phosphor-Rb (Ser795), phospho-ATR (Ser428), phospho-cdc2 (Tyr15), β-actin, and secondary

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antibodies were obtained from Cell Signaling Technology. Mitochondria/cytosol fractionation kit was purchased from BioVision.

2.2. Cell culture

The human breast cancer line cells, MDA-MB-231 and MCF-7, were obtained from the American Type Culture Collection (ATCC). Cells were cultured in medium containing RPMI-1640, antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin), and 10% (v/v) heat-inactivated FBS at 37 °C under 5% CO_2.

2.3. MTT assay and LDH assay

Exponentially growing MDA-MB-231 (1.5×10^4) and MCF-7 (1.0×10^4) cells in 100 μl medium were seeded in 96-well plates. When cells reach approximately 70–80% confluence, the medium was replaced with a serum-free medium, and the cells were incubated for another 24 h. Different concentrations of germacrone in 0.5% FBS were added to the cells. The cell viability was determined after 24, 48, and 72 h of incubation by adding 100 μl MTT (1 mg/ml) to each well. Then, the MTT-containing medium was carefully aspirated after 4 h, and 100 μl DMSO was added to dissolve the formazan crystals. After shaking for 10 min in the dark, the absorbance at 570 nm was determined with a microplate reader. The LDH release from the cells was evaluated using a commercial kit according to the manufacturers' protocol.

2.4. JC-1 assay

Cells were seeded in 96-well plates, as described above (MDA-MB-231 cells 1.5×10^4/well; MCF-7 cells, 1.5×10^4/well), and treated with different concentration of germacrone for 24 h. The culture medium was removed, and cells were washed twice with 100 μl PBS per well. Then, cells were incubated with 100 μl PBS (containing 2.5 μg/ml JC-1 and 10 mM glucose) at 37 °C for 30 min. The red fluorescence (*Ex*/*Em*, 550/600 nm) and green fluorescence (*Ex*/*Em*, 485/535 nm) were monitored with a microplate reader. The ratio of red fluorescence to green fluorescence was calculated to estimate the mitochondrial membrane potential ($\Delta\Psi m$). Individual JC-1 loaded cells were also observed with a fluorescence microscope and fluorescent images were captured by a digital camera.

2.5. Cell cycle assay

Cells were seeded at the density of 1.0×10^6 cells in 25 ml flasks and exposed to various concentrations of germacrone (0 to 200 μM) for 24 h. After washing twice with PBS, cells were harvested and collected by centrifugation at 350 *g* for 5 min, followed by fixation in ice-cold 70% ethanol at −20 °C overnight. Then, cells were collected by centrifugation and stained with 100 μl PI staining solution (20 μg/ml PI, 8 μg/ml RNase) for 30 min in the dark, followed by analysis with a flow cytometer (BD FACS CantoTM, BD Biosciences, San Jose, USA). The percentages of cell distribution in G0/G1, G2/M, and S phases were measured and the results were analyzed by the ModFit LT software (version 3.0).

2.6. TUNEL assay

A TUNEL assay was performed with the APO-BrdU™ TUNEL assay kit following the manufacturer's instructions. Briefly, MDA-MB-231 cells were seeded in a 25 ml flask as described above. The confluent cells were treated with 0, 50, 100, and 200 μM germacrone for 24 h, then harvested and washed with PBS. Subsequently, cells were fixed with 1% paraformaldehyde for 15 min on ice and washed twice with PBS. Finally, cells were re-suspended in 70% (v/v) ice-cold ethanol and stored at −20 °C overnight. To perform the TUNEL assay, fixed cells were then collected by centrifugation at 300 *g* for 5 min. Cells were washed twice with wash buffer and incubated with DNA labeling solution for 60 min at 37 °C. At the end of the incubation period, cells were washed twice with rinse buffer and collected by centrifugation for 5 min at 300 *g*. The cell pellets were re-suspended in an antibody solution for 30 min at room temperature. After incubation with a staining buffer containing PI plus RNase for an additional 30 min at room temperature, cells were analyzed by flow cytometry.

2.7. Western blotting

After cells were treated with germacrone as described above, total protein was extracted with RIPA lysis buffer containing 1% phenylmethanesulfonylfluoride (PMSF), 1% protease inhibitor cocktail. Sample protein concentrations were determined with a BCA protein assay kit. Equivalent amounts of proteins from each group were separated by SDS-PAGE, followed by transferring onto PVDF membranes. After blocking for 1 h in 5% non-fat dried milk, the membrane was incubated with a specific primary antibody (1:1000), followed by incubation with the corresponding secondary antibody (1:1000). Specific protein bands were visualized using an ECL advanced Western blotting detection kit. Densitometric measurement of band intensity was performed with the Quantity One Software (Bio-Rad).

2.8. Measurement of cytochrome c release

The cytochrome *c* release assay was performed with a mitochondria/cytosol fractionation kit according to the manufacturer's instructions. In brief, germacrone treated cells were harvested and washed twice with PBS. The cells were incubated with cytosol extraction buffer in ice for 10 min, then discarded the pellet through centrifugation at 700 *g* for 10 min at 4 °C. The supernatant was centrifuged at 100,000 *g* for 10 min at 4 °C to obtain the cytosolic fraction. The pellet was resuspended in the mitochondrial extraction buffer to represent the mitochondrial protein. Aliquots of cytosolic or mitochondrial fractions were analyzed of cytochrome *c* using for Western blotting as described above.

2.9. Data analysis

All data represent the mean of three separately performed experiments and were presented as mean ± S.D. The significance of intergroup differences was evaluated by one-way analyses of variance (one-way-ANOVA) using SPSS11.5 software. Statistical differences were considered significant at $P<0.05$.

3. Results

3.1. Germacrone inhibited breast cancer cell proliferation

The cytotoxicity of germacrone was first assessed by MTT assay. As shown in Fig. 1, germacrone treatment significantly inhibited the proliferation of both MDA-MB-231 cells (Fig. 1A) and MCF-7 cells (Fig. 1B) in a dose-dependent manner (0–400 μM) after 24, 48, and 72 h of incubation. Furthermore, the inhibitory effect was also observed to occur in a time-dependent manner in germacrone treated MDA-MB-231 (Fig. 1A) cells but not in germacrone treated MCF-7 cells (Fig. 1B).

3.2. Germacrone increased LDH release from breast cancer cells

After treatment with germacrone for 24 h, the LDH release from both cell lines was significantly increased from 28.34% to 66.85% in MDA-MB-231 cells and from 21.64% to 72.76% in MCF-7 cells (Fig. 2A). A dose-dependent behavior was also observed in both cell lines.

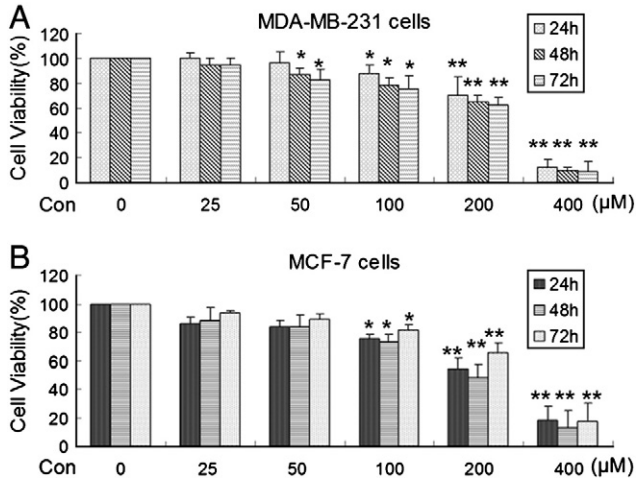

Fig. 1. Effect of germacrone on breast cancer cell proliferation. The human breast cancer cell lines MDA-MB-231 and MCF-7 cells were treated with different concentration of germacrone (0–400 μM) for 24, 48, and 72 h. Cell viability was determined by MTT assay. Germacrone significantly inhibited the proliferation of breast cancer cells in a dose-dependent manner. Con, concentration. Data were expressed as mean ± S.D. $^{*}P<0.05$ *vs.* control and $^{**}P<0.01$ *vs.* control.

3.3. Germacrone decreased ΔΨm in breast cancer cells

Compared with the control group, the JC-1 fluorescence ratio (red/green) was dramatically decreased after 24 h of germacrone incubation in both cell lines (Fig. 2B), suggesting a decrease of $\Delta\Psi m$. The decrease of $\Delta\Psi m$ occurred in a concentration-dependent manner, and a significant loss of $\Delta\Psi m$ was observed at 50 μM. After treating the cells with 400 μM germacrone, the $\Delta\Psi m$ of both cell lines is decreased by more than 60%.

Similarly, fluorescence microscopy images demonstrated intense red fluorescence in intact MDA-MB-231 and MCF-7 cells (Fig. 2C and F) indicating normal $\Delta\Psi m$. Following a 24 h treatment with 200 μM germacrone, however, the red fluorescence was significantly deceased, while the green fluorescence increased (Fig. 2D and G). In groups treated with 400 μM germacrone (Fig. 2E and H), only sporadic red fluorescence was observed against the intense green fluorescence, suggesting a significant decrease in $\Delta\Psi m$. These results clearly demonstrated that germacrone could decrease $\Delta\Psi m$ in a dose-dependent manner.

3.4. Germacrone induced G0/G1 and G2/M cell arrest in breast cancer cells

The effect of germacrone on the cell cycle was illustrated in Fig. 3. The flow cytometric assay showed that germacrone induced both MDA-MB-231 (Fig. 3A and B) and MCF-7 (Fig. 3C and D) cell cycle arrest at the G0/G1 and G2/M phases in a dose-dependent manner. Following a 24 h exposure to 200 μM germacrone, the fraction of MDA-MB-231 cells at the G0/G1 phase increased from 59.37% to 71.24%, while the fraction of cells in S phase decreased from 34.27% to 18.93%, and the fraction in G2/M phase increased from 6.36% to 15.89% (Fig. 3A and B). Under the same condition, the number of MCF-7 cells at the G0/G1 phase increased from 55.35% to 77.08%, those at the S phase decreased from 39.47% to 10.58%, and those at the G2/M phase increased from 5.18% to 12.36% (Fig. 3C and D).

3.5. Germacrone induced MDA-MB-231 cell apoptosis

Germacrone-induced apoptosis in MDA-MB-231 cells was determined by a TUNEL assay. As expected, germacrone increased the TUNEL-positive cell population of MDA-MB-231 cells undergoing apoptosis (Fig. 4). The percentage of TUNEL-positive cells in the positive control group was 51.40% (Fig. 4A). Compared with negative control (0.77%) (Fig. 4B), a low concentration of germacrone (50 μM) showed no significant effect on apoptosis (0.23%) (Fig. 4C), while 100 μM germacrone induced a low but statistically significant increase in apoptotic rate (2.10%) (Fig. 4D and F). However, the percentage of TUNEL-positive cells was significantly increased by 200 μM germacrone treatment (45.13%) for 24 h (Fig. 4E and F).

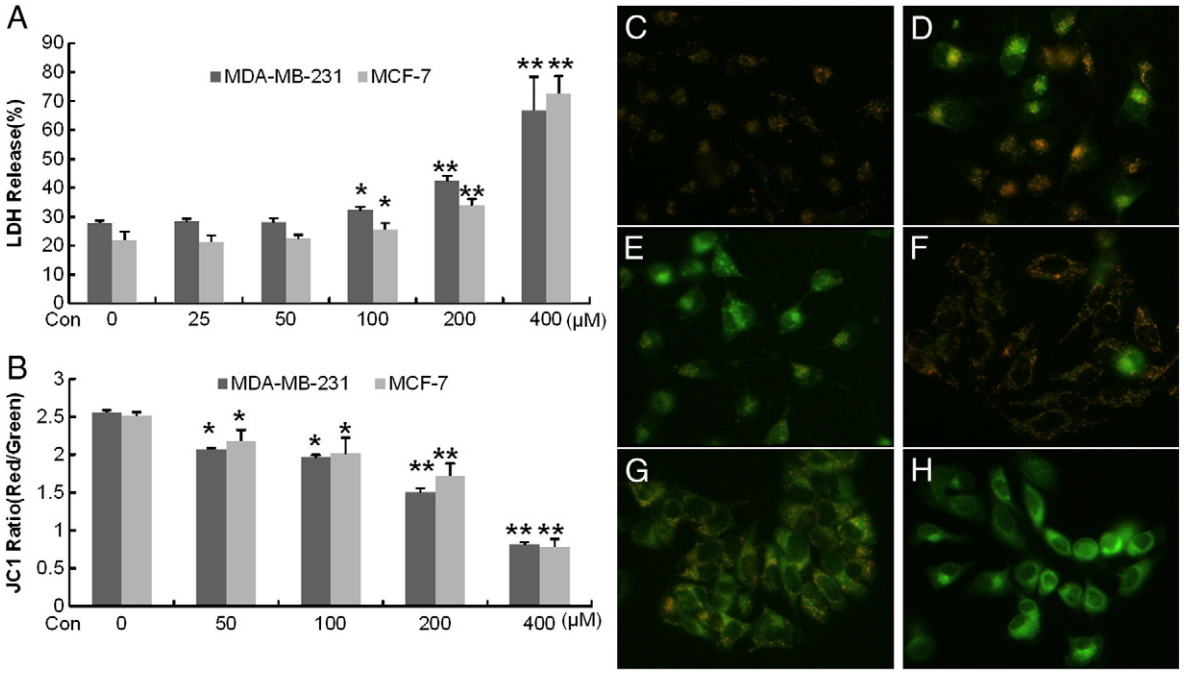

Fig. 2. Effect of germacrone on LDH release (A) and $\Delta\Psi m$ in breast cancer line cells (B–H). MDA-MB-231 and MCF-7 cells were treated with different concentration of germacrone (0–400 μM) for 24 h, and the LDH release to culture medium was determined by a LDH assay kit. The $\Delta\Psi m$ was monitored by JC-1 (C, D, E, F, G, and H) staining, and the red/green fluorescent ratio was calculated (B). C, D, and E stand for germacrone at 0, 200, and 400 μM, respectively, for MDA-MB-231cells; F, G, and H stand for germacrone at 0, 200, and 400 μM, respectively, for MCF-7 cells. Con, concentration. Data were expressed as mean ± S.D. $^{*}P<0.05$ *vs.* control and $^{**}P<0.01$ *vs.* control.

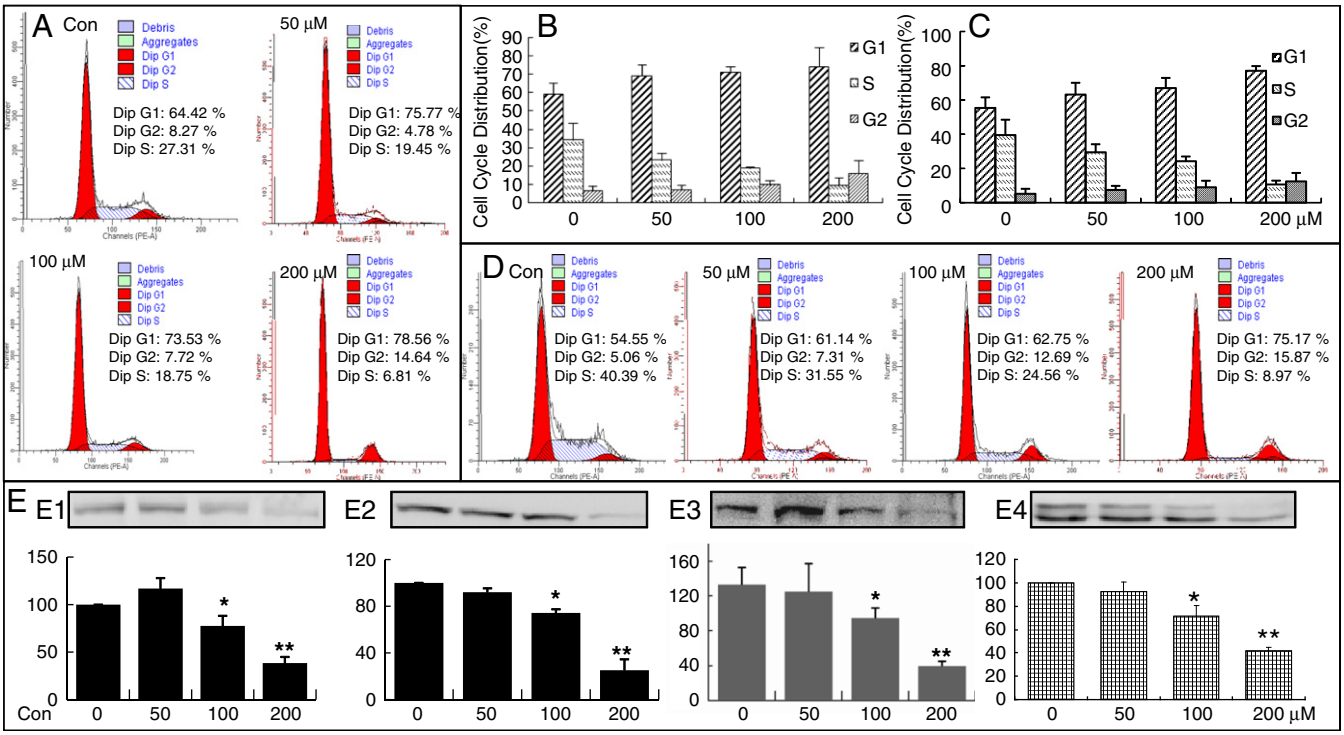

Fig. 3. Effect of germacrone on the cell cycle progression of breast cancer cell lines. MDA-MB-231 and MCF-7 cells were treated with germacrone (0–200 μM) for 24 h. The various phases of the cell cycle were evaluated by flow cytometry. Related protein expressions were determined by Western blotting. Germacrone dose-dependently induced G0/G1 and G2/M phase arrests in MDA-MB-231 (A and B) and MCF-7 (C and D) cells and significantly decreased phosphor-ATR (Ser428) (E1), phospho-cdc2 (Tyr15) (E2), phospho-Rb (Ser795) (E3), and cdc2 (E4) expression. Con, concentration. Data were expressed as mean ± S.D. $^{*}P<0.05$ *vs.* control and $^{**}P<0.01$ *vs.* control.

3.6. Germacrone regulated expression of cell cycle and apoptotic related proteins

To explore the potential mechanisms of germacrone induced cell cycle arrest and toxicity, the expression of several proteins related to cell cycle regulation and apoptosis was determined by Western blotting. Germacrone induced a significant decrease of phospho-ATR (Ser428) (Fig. 3E1), phospho-cdc2 (Tyr15) (Fig. 3E2), phospho-Rb (Ser795) (Fig. 3E3), and cdc2 (Fig. 3E4) protein expressions in a dose-dependent manner. Compared with untreated cells, germacrone treatment did not result in a significant alteration of Bcl-2, Bcl-xL, Bax, and Bim protein expressions. However, the expression of Bok was dramatically up-regulated in a dose-dependent manner (Fig. 4G).

3.7. Germacrone increased mitochondria cytochrome c release and activated caspases pathway

As shown in Fig. 4G, germacrone treatment dramatically promoted the translocation of cytochome *c* from the mitochondria (Fig. 4G Cyto a) to cytosol (Fig. 4G Cyto b). Germacrone significantly activated caspases 3, 7, and 9, as demonstrated by the appearance of the cleaved fragments at 200 μM. Furthermore, germacrone also triggered PARP cleavage as indicated by the appearance of PARP fragments (Fig. 4G).The full length of PARP was dose-dependently increased after germacrone treatment.

4. Discussion

Since ancient times, *Ezhu* has been prescribed by traditional Chinese medical practitioners as a "*Huoxue Huayu*" herb, which means promoting blood circulation and removing blood stasis. Modern research methods have determined that the essential oils isolated from *Ezhu* exhibit multiple bioactive properties, including anti-tumor activities. However, except for β-elemene, which has been relatively well-studied as an anti-tumor substance both *in vitro* and *in vivo*, the bioactivities of other components in *Ezhu* essential oil have not been clearly established. In the present study, the anti-proliferative effects of germacrone on two breast cancer cell lines were tested. To the best of our knowledge, it is the first study on the anti-tumor potential of germacrone *in vitro*.

The MTT assay confirmed that germacrone significantly suppressed MDA-MB-231 cell proliferation in a dose- and time-dependent manner. However, no time-dependent inhibition was observed for MCF-7 cell proliferation. While the reason for this is unclear, MCF-7 and MDA-MB-231 cells have many distinguishing phenotypes. For example, MCF-7 is an estrogen receptor (ER)-positive human breast cancer line cell while MDA-MB-231 is an ER-negative one. The large amount of LDH released from both cell lines after germacrone treatment indicated significant damage to plasma membrane integrity. Taken together, germacrone is clearly cytotoxic in both breast cancer cell lines.

The fluorescent dye JC-1 is a very useful reagent for investigating mitochondrial function, and it is widely used to monitor $\Delta\Psi m$ alteration. The dye undergoes a reversible change in fluorescence emission from green to red as $\Delta\Psi m$ increases. Cells with high membrane potential promote the formation of dye aggregates, which exhibit red fluorescence. Cells with low $\Delta\Psi m$ contain monomeric JC-1 and exhibit green fluorescence. Consequently, mitochondrial depolarization is indicated by a decrease in red/green fluorescence intensity ratio. The reduction or loss of $\Delta\Psi m$ is an early indicator of apoptosis and a key indicator of cellular viability. Our results revealed that germacrone treatment significantly induced MDA-MB-231 and MCF-7 $\Delta\Psi m$ loss, suggesting that germacrone might induce apoptosis in these cells, which has been confirmed by TUNEL assay indicating an increased number of apoptotic cells after germacrone treatment. Apoptosis plays a critical role in eliminating genetically altered cells or cells that have been improperly stimulated for hyperproliferation. The induction of apoptosis protects organisms against neoplastic development. Thus, germacrone-induced apoptosis might partially contribute to its cytotoxic and anti-tumor effects in breast cancer cells.

Normally, the cell cycle is precisely controlled by cell cycle checkpoint, which ensures the fidelity of cell division in eukaryotic cells. However, cell cycle arrest triggered by various exogenous and endogenous

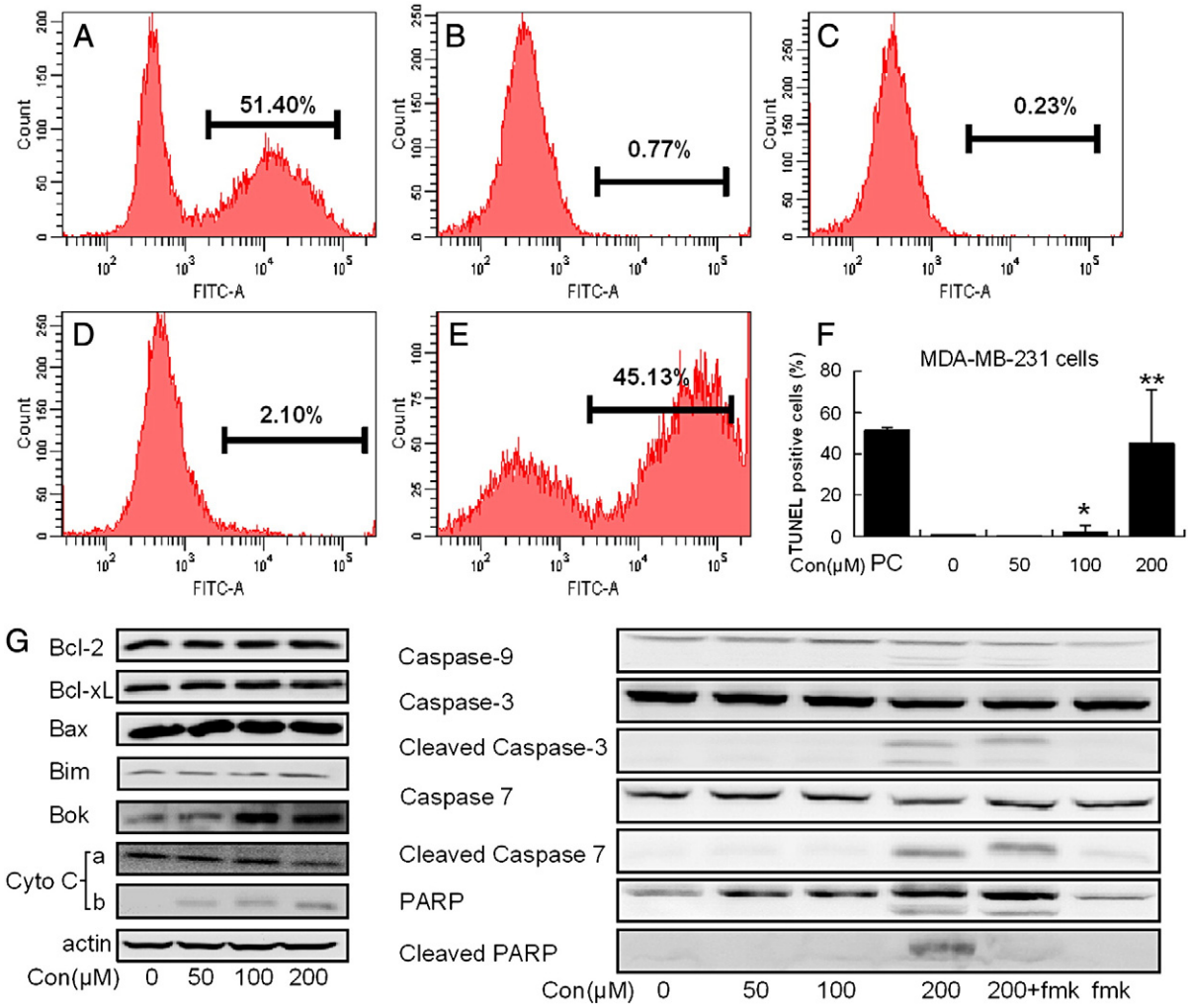

Fig. 4. Germacrone induced MDA-MB-231 cell apoptosis, regulated Bcl-2 family proteins expression, activated caspases, and cleaved PARP. Cells were treated with different concentration of germacrone (0–200 μM) for 24 h, and apoptosis was determined by a TUNEL assay. The protein expression was determined by Western blotting. (A) Positive control; (B) negative control; (C) germacrone 50 μM; (D) germacrone 100 μM; (E) germacrone 200 μM; (F) statistical results of A to E; and (G) expression of proteins belonging to the Bcl-2 family, activation of several caspases and cleavage of PARP. Cytochrome *c* release from mitochondria was evaluated by analyzing both the mitochondrial (a) and cytosolic fractions (b). Data were expressed as mean ± S.D. $^{*}P<0.05$ *vs.* negative control and $^{**}P<0.01$ *vs.* negative control.

stimulating factors could result in the breakdown of cell division, cell death and/or apoptosis. In present study, germacrone treatment induced G0/G1 and G2/M phase arrests in the cell cycle progression, indicating another possible mechanism by which germacrone inhibits the proliferation of breast cancer cells.

To explore the potential mechanisms of germacrone-induced cell cycle arrest and apoptosis, cell cycle and apoptosis-related protein expression were examined. Ataxia telangiectasia and rad3-related kinase (ATR) is a member of a class of protein kinases involved in sensing DNA damage and activating the DNA damage checkpoint, leading to cell cycle arrest (Sancar et al., 2004). Phosphorylation of the retinoblastoma tumor suppressor protein (Rb) plays a pivotal role in cell cycle regulation and cell proliferation through the restriction point within the G1 phase of the cell cycle (Sherr, 1996). Activation of cdc2 kinase induces all eukaryotic cells to enter into mitosis. The activation of cdc2 is controlled through the phosphorylation of Thr161 by cdc2-activating kinase and the dephosphorylation of Thr14 and Tyr15 by cdc25C phosphatase (Pines, 1999). Recent studies have demonstrated that a genistein metabolite induced G2/M cell cycle arrest in T47D tumorigenic breast epithelial cells *via* a mechanism involving the activation of ATR by phosphorylation at Ser428 (Vauzour et al., 2007). Anti-estrogen-induced G1 cell cycle arrest and anti-proliferative effects are the consequence of an early decline in Rb phosphorylation (Watts et al., 1995). Cdc2 kinase plays an important role in G2/M phase arrest in human breast cells (Frey et al., 2001; Balabhadrapathruni et al., 2000). In the present study, we found that germacrone treatment decreased Rb, cdc-2, and total cdc-2 phosphorylation in a dose-dependent manner, which might account for the germacrone-induced G0/G1 and G2/M phase arrests in MDA-MB-231 cells. Unexpectedly, we also found that germacrone decreased ATR phosphorylation, suggesting that germacrone might have a comprehensive effect on cell cycle regulation.

The key biochemical event involved in the apoptotic process is the up-regulation of pro-apoptotic proteins and/or the down-regulation of anti-apoptotic protein molecules. Among these proteins, the Bcl-2 family of proteins, including Bcl-2, Bcl-xL, Bim, and Bax proteins, serve as critical regulators of the mitochondrial pathway involved in apoptosis, acting to either inhibit or promote cell death (Reed, 1998). The Bcl-2 and Bcl-xL proteins have been identified as anti-apoptotic proteins, which bind to the outer membrane of the mitochondria and prevent the release of cytochrome *c*. Bax and Bim are thought to be pro-apoptotic effector proteins and are responsible for permeabilizing the membrane due to damaging cellular stress (Green and Chipuk, 2008; Ewings et al., 2007). Dohare et al. (2008) reported that the volatile oil of *Curcuma* suppressed the elevated protein level of Bax and aided mitochondrial

translocation and activation of Bcl-2 by altering $\Delta\Psi m$. Unexpectedly, germacrone showed no significant effects on Bcl-2, Bcl-xL, Bim, and Bax expressions in MDA-MB-231 cells in this study, suggesting that modulation of apoptosis by germacrone was not mediated by altering Bcl-2, Bcl-xL, Bcl-2/Bax ratio, or Bim. Further study revealed that germacrone could dramatically induce the expression of Bok, another member of the pro-apoptotic Bcl-2 family protein that induces apoptosis through strong interaction with Mcl-1 and BHFR1 but not with Bcl-2 or Bcl-xL (Hsu et al., 1997; Inohara et al., 1998). A recent study also showed that the nuclear translocation of Bok induced apoptosis in HEK 293 T cells, HeLa cells, and breast cancer cells (Bartholomeusz et al., 2006). Our results suggested that Bok protein might play a uniquely important role in germacrone-induced apoptosis. An increase in the level of Bax and/or a decrease in Bcl-2 leads to a loss of $\Delta\Psi m$ and an opening of the mitochondrial permeability transition pores (Crompton, 2000). Therefore, the loss of $\Delta\Psi m$ after germacrone treatment might be mediated by its alteration of Bok. Further studies are needed to elucidate this pathway.

Caspases are a family of proteases involved as the central component of a proteolytic system in the apoptotic process. These enzymes take part in a cascade that results in cell disassembly due to a trigger in response to pro-apoptotic signals. This culminates in the cleavage of a set of proteins (Thornberry and Lazebnik, 1998).Caspases are classified into two groups according to their function and structure: the initiator caspases (caspase-2, 8, 9, 10) and the executioner caspases (caspase-3, 6, 7) (Kuribayashi et al., 2006). Caspase-3 is a critical executioner of apoptosis and is either partially or totally responsible for the proteolytic cleavage of many key proteins, such as the nuclear enzyme poly (ADPribose) polymerase (PARP), which is an important protein that appears to be involved in DNA repair, maintenance of chromosomal stability, and programmed cell death. PARP induction may be an early signal of apoptosis in human tumor (Bursztajn et al., 2000). In the present study, we found that germacrone induced a significant cleavage of caspase-3, 7, and 9 suggesting the involvement of caspase-3, 7, and 9 in this process. Furthermore, germacrone triggered relocalization of cytochrome *c* suggesting that the release of cytochrome *c* from mitochondria. In addition, germacrone also induced PARP cleavage, another apoptotic related event, possibly catalyzed by caspase-3. Taken together, these results suggested that germacrone induced apoptosis might be through mitochondria mediated pathway.

In the 2010 version of Chinese pharmacopeia, germacrone content is one of the mandatory indexes for *Ezhu* essential oil quality control. However, the *in vivo* concentration of germacrone in human being remains to be clear. A recent animal study in rat revealed that the blood C_{max} for germacrone was about 0.42 μg/ml (~2 μM) after oral administration of *Ezhu* oil essential (500 mg/kg) (Y. Zhao et al., 2010; X.L. Zhao et al., 2010). The blood germacrone concentration was less than 10 μg/ml (~50 μM) even after intravenous administration (100 mg/kg) of *Ezhu* oil essential in rat (Y. Zhao et al., 2010; X.L. Zhao et al., 2010). In view of the germacrone content in *Ezhu* essential oil (~8%) (Zhang et al., 2009), it is hard to reach the concentrations used in the present study if *Ezhu* essential oil was administrated. However, these concentrations might be achievable when pure germacrone was administrated.

In summary, our results demonstrated that a natural product germacrone significantly inhibited human breast cancer cell proliferation *in vitro* possibly through the induction of cell cycle arrest and apoptosis.

Acknowledgments

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