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Magnolol induces apoptosis in MCF-7 human breast cancer cells through G2/M phase arrest and caspase-independent pathway

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Magnolol, a small-molecule hydroxylated biphenol, isolated from the root and stem bark of *Magnolia officinalis,* has been shown to possess antiproliferative effect on various cancer cell lines. In the current study, we found that magnolol potently inhibited proliferation and induced apoptosis in MCF-7 human breast cancer cells. Further mechanistic studies revealed that induction of apoptosis is associated with cell cycle arrest at G2/M phase, increased generation of reactive oxygen species (ROS), reduced mitochondrial membrane potential (MMP), release of cytochrome c (Cyto c) and apoptosis inducing factor (AIF) from mitochondria to cytosol, upregulation of Bax, p21 and p53, and down-regulation of Bcl-2, cyclin B1 and cyclin-dependent kinase 1 (CDK1). Our findings indicated that magnolol induced apoptosis in MCF-7 cells via the intrinsic pathway with release of AIF from mitochondrial and G2/M phase arrest pathway. Therefore, magnolol might be a potential lead compound in the therapy of breast cancer.

1. Introduction

Currently, the mainstay of breast cancer treatment is surgery, chemotherapy, and radiation therapy. Although, due to improved diagnosis and treatment programs, death rates for breast cancer have been declining in the last few years, there are still approximately 39,520 women were expected to die from breast cancer in 2011 (News-medical). Breast cancer is still the most feared disease among women. Furthermore, breast cancer therapy is associated with lasting side effects including psychological, social, physical, and spiritual distress. Therefore, there is an urgent need to identify highly potent therapeutic agents against this life-threatening disease (Burckhardt and Jones 2005; Sammarco 2003; Leak et al. 2008; Knobf 2007).

Magnolia officinalis is widely used in traditional Chinese medicine for the treatment of diseases such as gastrointestinal disorders, cough, anxiety, and allergic diseases (Maruyama et al. 1998). Magnolol, the main active constituent, has been found to possess various pharmacological activities, including anti-oxidative (Shen et al. 2010), anti-inflammatory, and anti-microbial properties (Jacobo-Salcedo et al. 2011). Previous studies also showed that magnolol had inhibitory effect on various cancer cell lines including human leukemia U937 cells (Ikai et al. 2006), human gastric adenocarcinoma SGC-7901 cells (Rasul et al. 2012), HL-60 cells (Fong et al. 2005), prostate cancer PC-3 cells (Lee et al. 2009), urinary bladder cancer 5637 cells (Lee et al. 2008), hepatoma HepG2 cells, colon cancer COLO 205 cells (Park et al. 2012), and melanoma A375-S2 cells (You et al. 2009). However, the effects of magnolol and its mechanism on human breast cancer cells remain unexplored.

In the current study, we determined the inhibitory effect of magnolol on human breast cancer cells using the MCF-7 cell line. In addition, we have investigated the mechanisms underlying the anti-proliferative effect of magnolol in MCF-7 cells. These findings suggest that magnolol-induced apoptosis resulted from G2/M arrest and mediated via the intrinsic pathway with release of AIF from mitochondria in MCF-7 Cells.

2. Investigations and results

2.1. Magnolol inhibits the growth of MCF-7 cells

The antiproliferative effect of magnolol (chemical structure shown in Fig. 1A) on MCF-7 human breast cancer cells was initially evaluated by MTT methods. MCF-7 cells were treated with various concentrations of magnolol for indicated time points. Our results showed that magnolol inhibited the growth of MCF-7 cells in a time- and dose-dependent manner. The half maximal inhibitory concentration (IC_{50}) values were around 58.27, 53.39, and 49.56 M after 24, 48, and 72 h treatment, respectively (Fig. 1B). The cells morphological changes were observed under a phase-contrast microscope. We found that magnolol treated cells displayed dramatic morphological changes including a reduction in total cell number, an increase in floating cells, and a significant decrease in adherent cells (Fig. 1C). These results indicated that magnolol can act as a potent antiproliferative agent of human breast cancer MCF-7 cells.

Apart from MCF-7 cells, the activity of magnolol was also tested against mouse splenocytes. Interestingly, magnolol was found

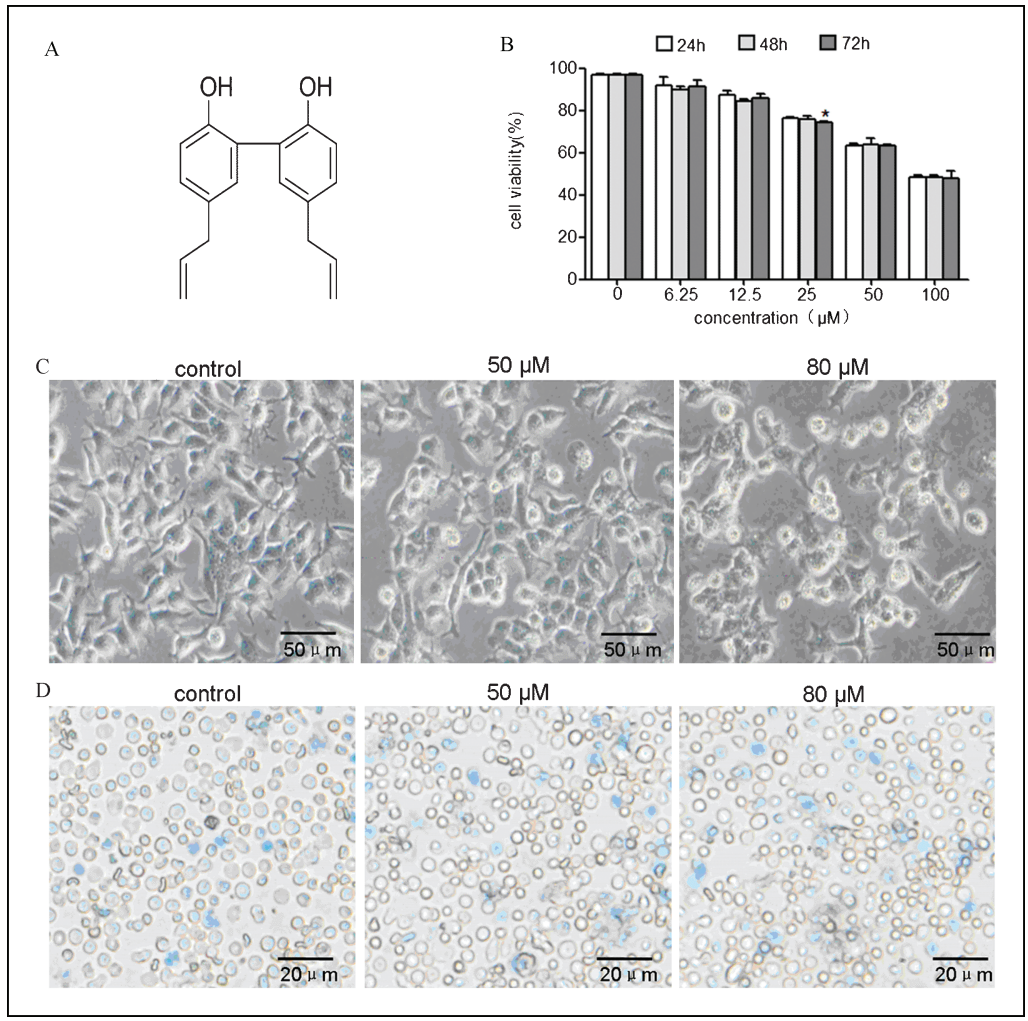

Fig. 1: Effects of magnolol on viability and morphology of human breast cancer MCF-7 cells. (A) Chemical structure of magnolol. (B) MCF-7 cells treated with different concentrations of magnolol for 24, 48, and 72 h, respectively. Data are expressed as Mean ± SEM of three independent experiments. Morphological changes of MCF-7 cells (C) were observed under the phase-contrast microscopy after treating with (0, 50 and 80 μM magnolol for 24 h. (D) Mouse splenocytes were treated with indicated concentrations of magnolol for 24 h and stained with with 0.4% trypan blue and observed dead and live cells microscopically. Dead cells took up trypan blue and stained blue while live cells exclude it.

to be less toxic to mouse splenocytes as compared to MCF-7 cells (Figure 1D).

2.2. Magnolol induced G2/M phase arrest and regulated cell cycle-related proteins in MCF-7 cells

Cell cycle arrest is a possible reason for cell proliferation inhibition. To determine whether the cell growth inhibition is involved in arresting cell cycle at a specific phase, we investigated the effect of magnolol on MCF-7 cell cycle progression. The cells were incubated with different concentrations (50, 80, and 100 M) of magnolol for 24 h, and then stained with PI as described in the Experimental section. Flow cytometry results showed that magnolol dose-dependently arrested MCF-7 cycle at G2/M phase. The percentage of G2/M phase cells was significantly increased to 27.1%, 33%, and 37.5% (vs 18.1% in control group); with a corresponding decrease in G0/G1 phase cells from 59.1% to 51.8%, 44.2%, and 39.2%. There is no significant change in S phase cells (Fig. 2).

It has been fully established that transition from G2 to M phase is controlled by cyclin B1/Cdk1 complex (Choi et al. 2011). We investigated the expression of cyclin B1 and Cdk1 in MCF-7 cells treated with different concentrations of magnolol for 24 h by western blotting method. The data showed that magnolol dose-dependently decreased cyclin B1 and CDK1 levels in the cells (Fig. 3). Because cells with a suppressed cyclin B1/Cdk1 activity are arrested at G2 phase, our data suggested that magnolol induced cell cycle arrest in MCF-7 cells at G2 phase but not at M phase. p53 is a crucial tumor suppressor gene in human cancers, its downstream target, p21, is one of the major cyclin-dependent kinase inhibitors (CDKIs). Activation of p53 may lead to cell cycle arrest at the G2/M phase (Agarwal et al. 1998; Lakin and Jackson 1999; Flatt et al. 2000). We analyzed the expressions of p21 and p53 in MCF-7 cells treated with or without magnolol. We found that magnolol significantly increased the expression of p21 and p53 in a dose-dependent manner (Fig. 3), which further confirmed G2 arrest by magnolol in MCF-7 cells.

2.3. Magnolol induced apoptosis in MCF-7 cells

Apoptotic body is one of the most typical characteristics of apoptosis. So, we analyzed nuclear morphological changes under a fluorescent microscope. As shown in Fig. 4, treatment with 50, 80, and 100 M magnolol for 24 h induced dose-dependent nuclear condensation or nuclear fragmentation in MCF-7 cells. To further determine the percentage of apoptosis in MCF-7 cells, annexin V-FITC and PI double staining methods were used. MCF-7 cells were incubated with different concentrations of magnolol for 24 h; cells were collected and stained with both

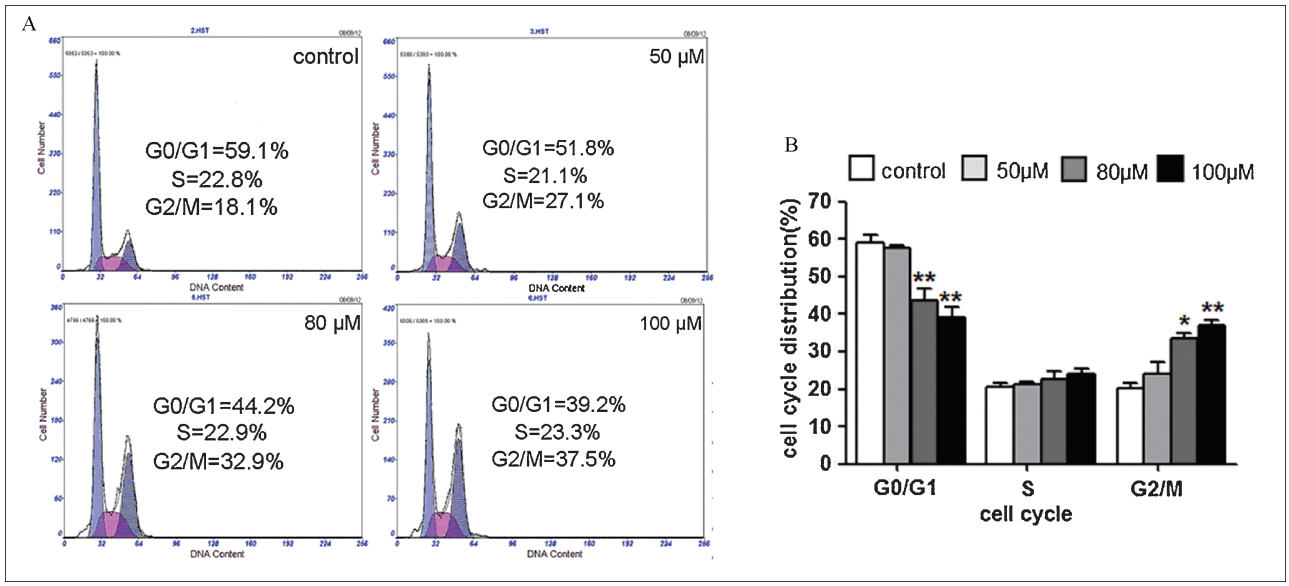

Fig. 2: Effect of magnolol on MCF-7 cell cycle progression. (A) Flow cytometry analysis of cell cycle phase distribution in MCF-7 cells treated with indicated concentrations of magnolol for 24 h. (B) Data are expressed as mean ± SEM. of three independent experiments with the similar results. *$p < 0.05$, **$p < 0.01$ compared with the control.

reagents. Flow cytometry results indicated that there is significant increase in both early and late apoptosis in magnolol treated MCF-7 cells. Early apoptotic rates were increased to 2.48%, 22.75%, and 35.68% after treating the MCF-7 cells with 50, 80, and 100 μM of magnolol for 24 h, respectively, as compared to 0.92 % in the control group. Meanwhile, late apoptosis in the corresponding tests were increased from 1.01% to 1.34%, 3.76%, and 8.84%, respectively (Fig. 5).

2.4. Magnolol disrupts mitochondrial membrame potential in MCF-7 cells

Depolarization in mitochondrial membrane potential is a characteristic feature of apoptosis. Therefore, we determined MMP in MCF-7 cells by using Rho-123 staining and flow cytometry. MCF-7 cells were incubated with different concentrations (50, 80, and 100 μM) of magnolol for 24 h before Rho-123 staining. The data indicated that magnolol significantly decreased MMP from 95.64% (control group) to 94.40%, 89.29% and 77.60% after treatment (Fig. 6).

2.5. Magnolol induces generation of ROS in MCF-7 cells

ROS play important roles in mitochondria-mediated apoptosis pathway. Effect of magnolol on ROS level in MCF-7 cells was measured by DCFH-DA staining. Our results showed that magnolol significantly increased ROS levels from 3.90% (DMSO control) to 19.42%, 45.68%, and 74.89% after 24 h treatment, respectively ($p < 0.01$) (Fig. 7).

2.6. Magnolol modulates the expression of Bcl-2 family proteins

Bcl-2 family proteins play key roles in mitochondrial membrane permeability and membrane potential disruption. Bcl-2 family includes both antiapoptotic (e.g., Bcl-2) and pro-apoptotic (e.g., Bax) members. Ratios of these two groups determine the fate of cells. Thus, we examined the effect of magnolol on Bcl-2 and Bax expression in MCF-7 cells. Western blotting results indicated that magnolol decreased the expression of Bcl-2 and increased the expression of Bax, leading to up-regulation of the ratio of Bax/Bcl-2 (Fig. 8). These results suggest that magnolol

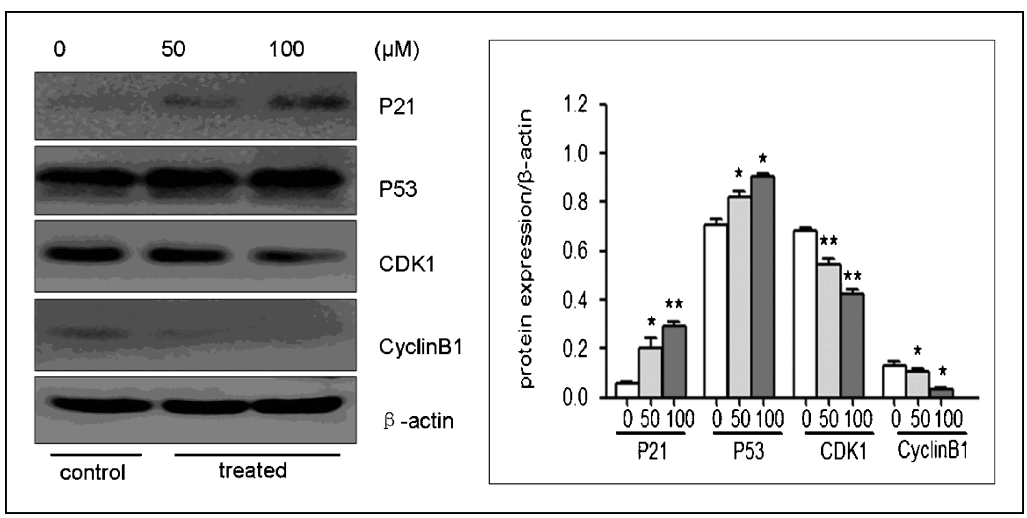

Fig. 3: Effect of magnolol on G2/M phase cell cycle regulators in MCF-7 cells. MCF-7 cells were treated with indicated concentrations of magnolol for 24 h. Western blotting analysis were done as described in the Experimental Section. Data are expressed as mean ± SEM. of three independent experiments with the similar results. *$p < 0.05$, **$p < 0.01$ compared with the control.

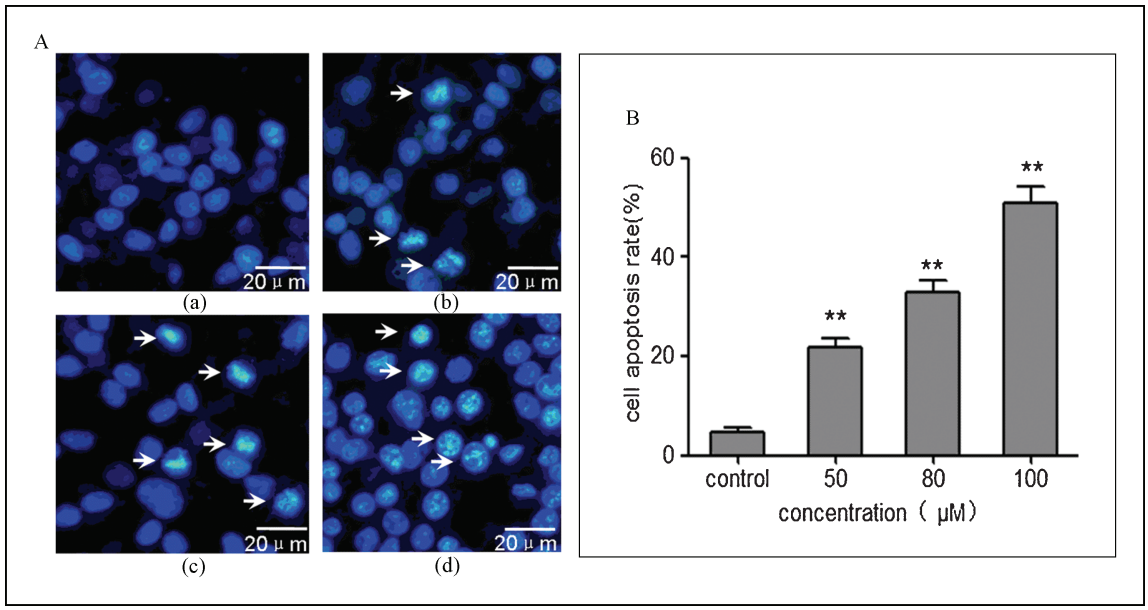

Fig. 4: Induction of apoptosis by magnolol in MCF-7 cells. (A) Cells were treated with 0 (a), 50 (b), 80 (c), and 100 μM (d) magnolol for 24 h respectively. Nuclear morphological changes were observed using Hoechst 33258 staining and fluorescence microscope. Arrows indicate the condensed and fragmented nuclei. Scale bar = 20 μm. (B) Histograms show number of cleaved nuclei (apoptotic cells) counted microscopically from 100 nuclei. Data are expressed as mean ± SEM of three independent experiments with the similar results. **$p < 0.01$ compared with the control.

disrupts mitochondrial membrane potential via modulation of the Bcl-2 family proteins.

2.7. *Magnolol stimulates AIF and cytochrome c release*

Disruption of MMP can lead to the release of cytochrome c from intermembrane space to cytosol. To further define the apoptosis pathway, we measured the level of cytochrome c in cytosol in MCF-7 cells. We found that magnolol increased the cytochrome c level in cytosol, and induced cleavage of PARP (Fig. 8).

A previous study (Ikai et al. 2006). indicated that magnolol induced apoptosis in U937 cells via the intrinsic pathway with release of AIF from mitochondria. Therefore, we investigated the effect of magnolol on translocation of AIF from mitochondria to cytosol. Western blotting analysis revealed that of released active AIF at 24 h after the magnolol treatment was significantly increased in dose-dependent manner (Fig. 8).

3. Discussion

Previous studies indicated that magnolol has inhibitory activities against various cancer cells (Ikai et al. 2006; Rasul et al. 2012; Fong et al. 2005; Lee et al. 2009; Lee et al. 2008; Park et al. 2012; You et al. 2009). However, its effect on human breast cancer cells remained undefined. In the present study, we addressed the

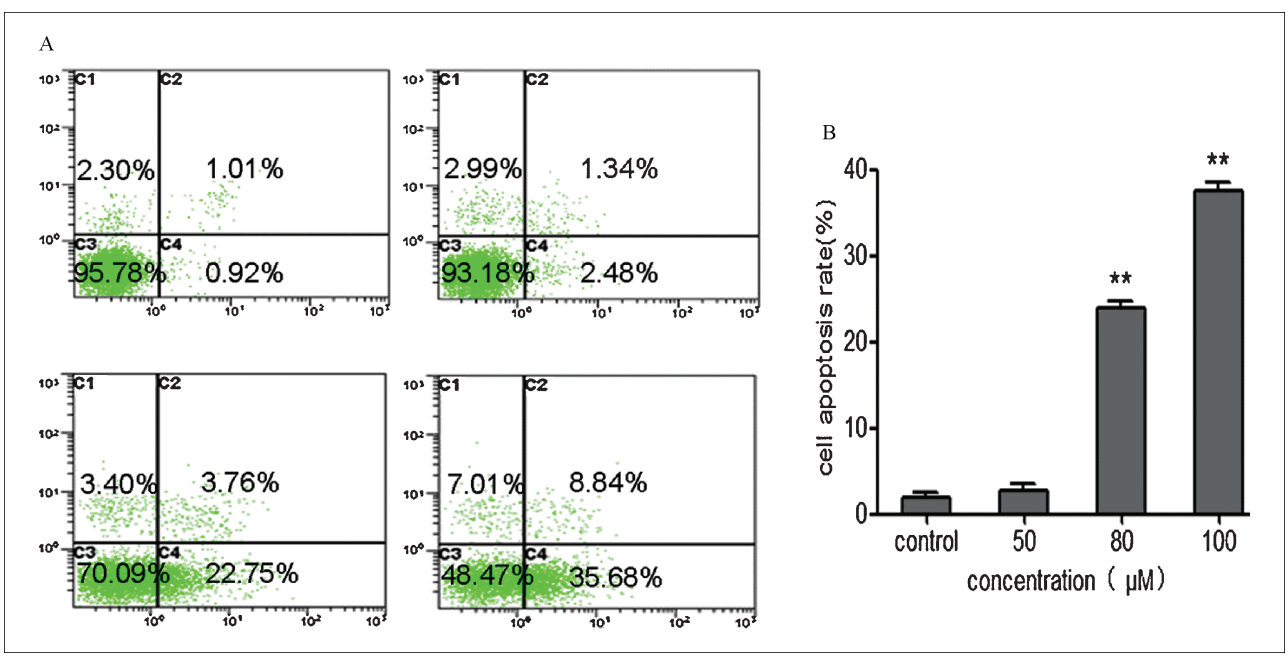

Fig. 5: Flow cytometry analysis of apoptosis in MCF-7 cells treated with magnolol. MCF-7 Cells were treated with 0 (a), 50 (b), 80 (c), and 100 μM (d) magnolol for 24 h. The cells were then stained with FITC-conjugated annexin V and PI for flow cytometric analysis. X-axis and y-axis represents annexin V-FITC staining and PI, respectively. Cell populations shown in the lower right (annexin V+/PI-) represents apoptotic cells, upper right (annexin V+/PI+) represents necrotic cells.

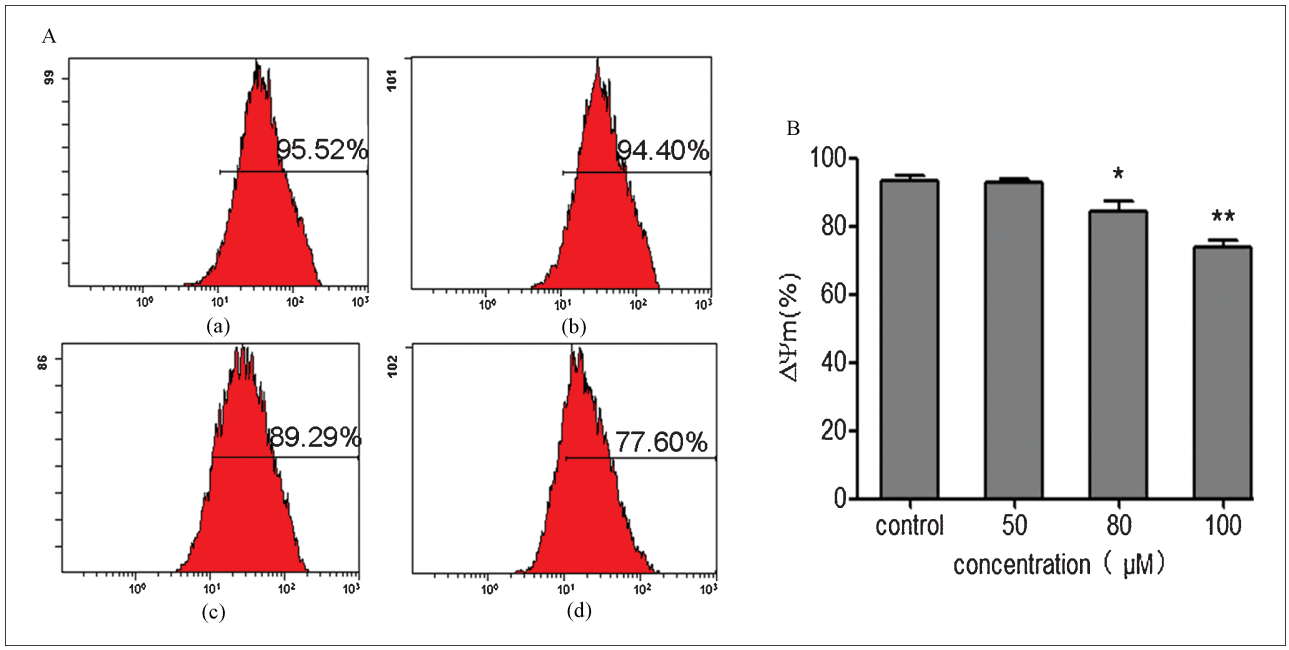

Fig. 6: Effect of magnolol on MMP in MCF-7 cells. (A) Flow cytometry results of MMP in MCF-7 cells treated with 0 (a), 50 (b), 80 (c), and 100 μM magnolol for in 24 h. (B) Data are expressed as mean ± SEM of three independent experiments with the similar results. *p < 0.05, **p < 0.01 compared with the control.

question of whether or not magnolol could induce apoptosis in MCF-7 human breast cancer cells. We investigated the antiproliferative effect and related mechanism of magnolol on MCF-7 cell line using MTT assay, flow cytometry and Western blotting methods. Our results showed that magnolol significantly inhibited proliferation and induced apoptosis and arrested the cell cycle at G2 phase in MCF-7 cells. Further mechanistic studies indicated that induction of apoptosis by magnolol is mediated by a caspase-independent pathway.

Cell cycle arrest and apoptosis are the two main causes of proliferation inhibition. Cell cycle control is the major regulatory process of cell growth. Cell cycle is controlled by a complex regulatory network to ensure the cell cycle happen in the right order: entry into mitosis, chromosomal segregation, and cytokinesis (Schafer 1998; Spugnini et al. 2007). Many chemotherapeutic agents induce apoptotic cell death through cell cycle interfering (Maddika et al. 2007; Batsi et al. 2009; Komata et al. 2003; Muschel and McKenna 1996). In the present paper, we found that magnolol potently inhibited proliferation of MCF-7 human breast cancer cells. Further flow cytometry studies manifested that the inhibition was accomplished by arresting the MCF-7 cells at the G2/M phase. It has been fully revealed that the cell cycle is governed by many extra- and intra-cellular stimuli, in which the most important ones are the cyclin-dependent kinases (CDKs) (Giono and Manfredi 2006). CDKs play major roles in the cell cycle checkpoint's control in which each of the CDKs controls a specific checkpoint. Transition from G2 to M phase is controlled by formation of cyclin B1/Cdk1 complex. Cells with a suppressed cyclin B1/Cdk1 activity are arrested at the G2 phase (Gavet and Pines 2010; Enomoto et al. 2009). Our data

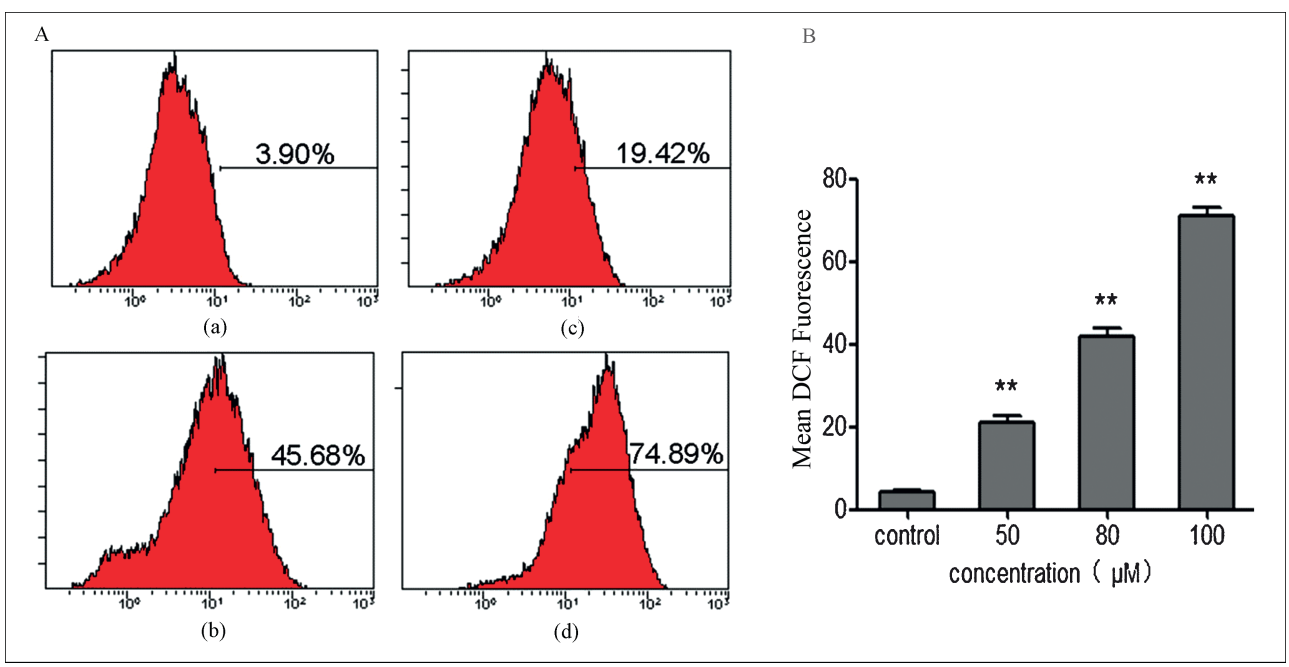

Fig. 7: Effect of magnolol on ROS grenération in MCF-7 cells. (A) Flow cytometry results of ROS generation in MCF-7 cells treated with 0 (a), 50(b), 80 (c), and 100 μM magnolol for in 24 h. (B) Data are expressed as meanm ± mSEM of three independent experiments with the similar results. **p < 0.01 compared with the control.

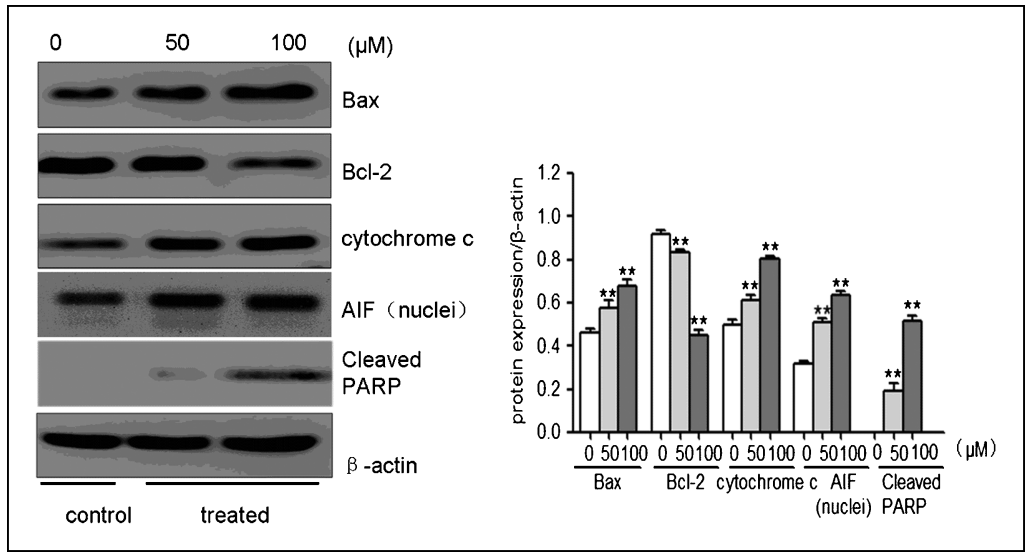

Fig. 8: Effect of magnolol on apoptosis regulators in MCF-7 cells. MCF-7 cells were treated with indicated concentrations of magnolol for 24 h. Protein extraction and Western blotting analysis were done as described in the Experimental section. Data are expressed as mean ± SEM of three independent experiments with the similar results. **$p < 0.01$ compared with the control.

demonstrated that magnolol decreased the expression of both cyclin B1 and Cdk1, which indicated that magnolol induces G2 arrest in MCF-7 cells.

The tumor suppressor protein p53, plays a key role in cell cycle regulation. Activation of p53 may lead to cell cycle arrest at G0/G1, S or G2/M phase through its target genes (Reinhardt and Schumacher 2012). The p53-target gene, p21 (waf-1/cip-1), is one of the major cyclin-dependent kinase inhibitors (CDKIs) which has a broad-spectrum of specificity in the cell cycle proteins. Up-regulation of the expression of p21 may block the cyclin/CDK complexes, thus leading to cell cycle arrest and inhibiting differentiation (Gartel et al. 1996). Our data indicated that magnolol significantly elevated the level of p21 in MCF-7 cells well consistent with the suppressed expression of the cell-cycle-related protein cyclin B1 in Figure 3 and previous reports (Chen et al. 2009; Hsu et al. 2009).

Apoptosis occurs through two main pathways: the death receptor pathway and the intrinsic or mitochondrial pathway (Schultz and Harrington 2003). The intrinsic apoptotic pathway is mediated by Bcl-2 family proteins (Zinkel et al. 2006). which include both anti-apoptotic and pro-apoptotic members. Ratio of anti-apoptotic (e.g., Bcl-2) and pro-apoptotic proteins (e.g., Bax) regulate the passage of small molecules such as cytochrome c, which activate the caspase cascade through the mitochondrial transition pore (Gottlieb 2001; Antico Arciuch et al. 2012). Previous reports indicated that magnolol induced intrinsic apoptosis in U 937 and SGC-7901 human gastric adenocarcinoma cells (Ikai et al. 2006; Rasul et al. 2012); we here examined whether magnolol had an effect on this pathway in MCF-7 cells. Western blotting analysis indicated that magnolol significantly decreased the expression of Bcl-2 and increased the expression of Bax, leading to up-regulation of the Bax/Bcl-2 ratio. These results suggest that magnolol induced apoptosis in MCF-7 cells via modulation of the Bcl-2 family proteins. Involvement of the mitochondrial pathway in Bax/Bcl-2-mediated apoptosis was further confirmed by observing disruption of MMP. A significant reduction in MMP suggests an increased opening state of mitochondrial permeability transition pores which can lead the release of cytochrome c and AIF from intermembrane space to cytosol. Cytosolic cytochrome c binds and activates initiator caspases (Schultz and Harrington 2003; Zinkel et al. 2006; Gottlieb 2001). The activated caspases cleaved effector proteins including PARP, and induced DNA fragmentation in the nucleus which eventually leads to cell death (Soldani and Scovassi 2002). Our western blotting results indicated that magnolol induced the release of cytochrome c into the cytosol and increased the cleavage of PARP in MCF-7 cells. AIF is a known potent factor in the caspase-independent apoptosis. Induction of translocation of AIF from mitochondria to cytosol suggests that magnolol induces apoptosis in MCF-7 cells via the intrinsic pathway, which is in agreement with previous studies (Ikai et al. 2006). Cytochrome c release from mitochondria is largely mediated by direct or indirect ROS action. Magnolol has been shown to induce ROS generation in endothelial cells. We here examined whether magnolol increases the intracellular level of ROS in MCF-7 cells. The data showed that magnolol increased the level of ROS in MCF-7 cells in a dose-dependent manner.

In conclusion, our data demonstrated that magnolol inhibited the proliferation of MCF-7 human breast cancer cells by arresting the cell cycle at the G2/M phase and by induction of apoptosis. G2/M phase arrest was found to be associated with up-regulation of p53 and p21 and down-regulation of cyclin B1/CDK1 complex. Induction of apoptosis was associated with increased ROS generation, disruption of MMP, release of cytochrome c and AIF, modulation of Bcl-2 family proteins and cleavage of PARP. Thus, magnolol might be a leading candidate in the development of chemotherapeutic or chemopreventive drug for breast cancer.

4. Experimental

4.1. Chemicals and reagents

Manolol was obtained from the National Institute for the Control of Pharmaceutical and Biological Products in China (purity >99% as determined by analytical HPLC). Dimethyl sulfoxide (DMSO) and 3'-(4,5-dimethylthiazol-2-yl)-2,5-dipheny tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Rhodamine 123 was purchased from Eugene Co. (OR, USA). Compounds were dissolved as 20 mM mother solution in DMSO and stored at -80 °C. Final concentration of DMSO was <1% in all experiments. Fetal bovine serum (Characterized) was purchased from HyClone. Hoechst 33258, Dulbecco's Modified Eagle's Medium (DMEM), penicillin and streptomycin were purchased from Sigma. Annexin V-FITC Apoptosis Detection Kit and Reactive Oxygen Species (ROS) Assay Kit were purchased from Beyotime Institute of Biotechnology (Shanghai, China). Mouse polyclonal anti-human Bcl-2, Rabbit polyclonal anti-human Bax, cytochrome c, p53, cyclin B1 and caspase-3 antibodies were purchased from Cell Signaling Technology. Mouse anti-β-actin and anti-rabbit antibodies were purchased from Santa Cruz Biotechnology. Rabbit polyclonal anti-human p21 and mouse polyclonal anti-human CDK1

antibodies were purchased from Boster Biological Technology Co., Ltd. (Wuhan, China). Other reagents were of analytical grade or higher.

4.2. Cell culture

Human breast cancer MCF-7 cells (ATCC) were cultured in DMEM (supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 g/ml streptomycin) at 37 °C with 5% CO_2 in humidified atmosphere.
Mouse splenocytes were isolated from Kunming mice (Central Research Laboratory, Jilin University Bethune Second Hospital, China). The splenocytes were cultured in RPMI 1640 medium supplemented with 10% (v/v) FBS and maintained in a CO_2 incubator (5% CO_2 and 95% air, 95% humidity).

4.3. MTT assay

MCF-7 cells were plated into a 96-well plate (Costar, Corning, NY, USA) at a density of 10^5/well. After 24 h, the cells were incubated with different concentrations of magnolol for specified duration. After treatment, the cells were further incubated with 10 μl MTT solution (5 mg/ml) at 37 °C for 4 h. After incubation, 150 μl DMSO was added in each well and plate was gently shaked for 15 min to dissolve formazen crystals. The absorbance (OD_{570}) of each well was recorded by using a micro-plate reader ((ELX 800, BIO-TEK Instruments). Details are described elsewhere(Jacobo Saludo et al. 2011). Percentage cell viability was calculated as follows:
Cell viability (%) = (A570sample– A570blank) / (A570control–A570blank) × 100
The IC_{50} Values were calculated by using GraphPad Prism 5.

4.4. Cell Morphological changes

Cells were treated with test compounds for 24 h, and then morphological changes of the cells were observed under a phase-contrast microscope and photographed with a CCD camera (Olympus 1X71).

4.5. Flow cytometric analysis of apoptosis

MCF-7 cells were seeded in 6-well plates at a density of 10^5 and incubated in CO_2 incubator (37 °C, 5% CO_2, 95% humidity) for 24 h, then exposed to test compounds for 24 or 48 h. The cells were harvested and washed twice with phosphate buffered saline (PBS). Cell pellets were resuspended in PBS, and then labeled sequentially with Annexin V-FITC and PI by using the Annexin V-FITC Apoptosis Detection Kit as manufacture's instructions. Flow cytometric analysis was done on Beckman FC400 MPL flow cytometry (USA).

4.6. Hoechst 33258 staining

DNA Fragmentation was determined with the Hoechst 33258 staining method. After incubation with 50, 80, and 100 μM magnolol for 24 h, the MCF-7 cells were fixed with 4% paraformaldehyde for 30 min at room temperature, and then stained with Hoechst 33258 (50 μg/mL) at 37 °C for 20 min in the dark. The cells were washed and resuspended in PBS and nuclear morphological changes were observed under fluorescence microscope (Olympus 1x71).

4.7. Flow cytometric analysis of cell cycle

Flow cytometric cell cycle analysis was done as described previously (Rasul et al. 2011). Briefly, MCF-7 cells were seeded in a 6-well tissue culture plate and incubated with test compound for 24 h. The cells were harvested and washed with PBS (3 times). The pellets were fixed with 70% ethanol at -20 °C overnight. The cells were centrifuged to remove alcohol, washed with PBS and stained with 50 g/ml PI (containing 100 μg/ml RNase A) at 4 °C for 30 min in the dark. Data acquisition was done on Flow Cytometry and analyzed by Cell Quest software.

4.8. Determination of mitochondrial membrane potential ($\Delta\Psi m$)

ΔΨm was determined by flow cytometric method as reference (Ji et al. 2011). Briefly, MCF-7 cells were seeded into 6-well tissue culture pates and incubated with test compound for 24 h. The cells were collected and washed twice with PBS to remove medium, then resuspended in 500 μl PBS. The cells were stained with 1 μM Rhodamine 123 (Rho-123) at 37 °C for 20 min, washed twice with PBS, and then resuspended in 0.5 ml of PBS. Rho-123 fluorescence was measured using flow cytometry (λex = 488, λem = 530).

4.9. Determination of reactive oxygen species (ROS) generation

ROS generation was measured by using ROS Assay Kit (Beyotime, Shanghai, China) as manufacture's instruction. Briefly, MCF-7 cells were cultured in 6 well plates overnight. After exposure to test compound for 24 h, the cells were incubated with 10 μmol/L DCFH-DA at 37 °C for 30 min. The DFC-labeled MCF-7 cells were harvested, rinsed, and resuspended in PBS. DFC fluorescence was measured by flow cytometry.

4.10. Western Blot Analysis.

MCF-7 cells were incubated with test compound at 37 °C for 24 h. The cells were collected, rinsed twice with PBS, and then lysed on ice with RIPA Lysis Buffer (Beyotime, Shanghai, China). The lysates were centrifuged at 12000 rpm for 15 min. Protein concentrations of the supernatants were determined by NanoDrop 1000 spectrophotometer (Thermo Scientific, USA). Subcellular fractionations were prepared (Akao et al. 1994). Proteins were size fractionated in a 12.5% SDS-PAGE and transferred to PVDF membranes according to standard protocols. The membranes were sequentially blocked with 5% (w/v) nonfat milk, washed with Tris buffered saline-tween solution (TBST), incubated with specific primary antibodies overnight and anti-rabbit or anti-mouse horseradish peroxidase (HRP) conjugated secondary antibodies for 1 h. Signals were detected using ECL plus chemiluminescence kit on X-ray film (Millipore Corporation, Billerica, USA).

4.11. Statistical Analysis

The data are expressed as Mean ± SEM. Student's *t* test was used to compare test and control values, $P < 0.05$ was considered statistically significant.

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