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Magnolol Induces Apoptosis via Activation of Both Mitochondrial and Death Receptor Pathways in A375-S2 Cells

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Magnolol inhibited proliferation of human malignant melanoma A375-S2 cells. The drug induced oligonucleosomal fragmentation of DNA in A375-S2 cells and increased caspase-3, 8, 9 activities followed by the degradation of caspase-3 substrates, inhibitor of caspase dependent DNase (ICAD) and poly-(ADP-ribose) polymerase (PARP). Pan-caspase inhibitor (z-VAD-fmk), caspase-3 inhibitor (z-DEVD-fmk), capase-8 inhibitor (z-IETD-fmk), caspase-9 inhibitor (z-LEHD-fmk) and caspase-10 inhibitor (z-AEVD-fmk) inhibited magnolol-induced A375-S2 cell apoptosis. The level of anti-apoptotic mitochondrial protein Bcl-2 was up-regulated while the level of pro-apoptotic protein Bax was down-regulated. Taken together, our results indicate that magnolol induces apoptosis by activation of both mitochondrial and death receptor pathways in A375-S2 cells.

Key words: Magnolol, Bax, Bcl-2, Caspase, Fas

INTRODUCTION

Magnolol, a phenolic component extracted from the root and stem bark of *Magnolia officinalis* which has been often used in Traditional Chinese Medicine. It has been shown that magnolol induced cell death in many tumor cell lines including human leukemia U937 cell and HL-60 cell, prostate cancer cell PC3, human nonsmall lung cancer H460 cell, etc. (Ikai et al., 2006; Lee et al., 2009; Zhong et al., 2003; Li et al., 2007). However, its effect on human melanoma remains unexplored.

Many anti-cancer drugs including magnolol exert their therapeutic effect by inducing apoptosis in malignant cells (Ikai et al., 2006; Lee et al., 2009; Zhong et al., 2003; Li et al., 2007; Ferreira et al., 2002). The death receptor pathway and the mitochondrial pathway are the two major signaling pathways that induce apoptosis. The former is initiated by death receptors such as Fas, TNFα receptor and TRAIL and then activate "initiator" caspase, such as caspase-8 and -10. The latter is activated by the opening of permeability transition pore (PTP), molecules, such as cyrochrome *c*, released from mitochondria, and then another "initiator" caspase-9 is activated. Both pathways eventually converge, leading to activation of "executioner" caspases, including caspase-3 and others (Budihardjo et al., 1999; Thornberry and Lazebnik, 1998), which results in cleavage of a number of caspase substrates responsible for apoptotic demise of the cell, including protein kinases, cytoskeleton associated proteins, transcription factors and proteins involved in DNA repair and chromatin structure such as PARP. The Bcl-2 protein family is a large family of apoptosis-regulating proteins that modulate the mitochondrial pathway includes both anti-apoptotic members, such as Bcl-2 and Bcl-X_L, and proapoptotic members such as Bax and Bak (Adams and Cory, 1998). These proteins govern mitochondrial membrane permeability, either promoting or suppressing release of apoptogenic proteins from these organelles (Hunt and Evan, 2001).

We demonstrate that magnolol induced human melanoma A375-S2 cell apoptosis through activation of both death receptor-mediated extrinsic pathway

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and mitochondria-mediated intrinsic pathway.

MATERIALS AND METHODS

Chemical reagents

Magnolol was purchased from Beijing Institute of Biological Product. The purity of magnolol was measured by HPLC and determined to be about 98%. Magnolol was dissolved in dimethyl sulfoxide (Me_2SO) and Me_2SO concentration in all cell cultures was kept below 0.1% which had no detectable effect on cell growth or apoptosis.

Pan-caspase inhibitor (z-VAD-fmk), caspase-3 (z-DEVD-fmk), capase-8 (z-IETD-fmk), caspase-9 (z-LEHD-fmk), and caspase-10 (z-AEVD-fmk) inhibitors were purchased from Enzyme Systems. Anti-Fas antibody CH-11 was obtained from Medical & Biological Laboratories.

Cell culture

The human malignant melanoma A375-S2 cells were obtained from American Type Culture Collection (ATCC). The cells were cultured in RPMI-1640 medium (GIBCO) containing 10% fetal bovine serum (FBS) (Yuanhengshengma Biological Reagent Institute) and 0.03% *L*-glutamine (GIBCO) in 5% CO_2 at 37°C. Cells in the exponential phase of growth were used in the experiments.

Cytotoxicity assay

All the cells were cultured at 1×10^4 cells/well in 96-well plates (NUNC). After preincubation with caspase inhibitors, z-VAD-fmk, z-DEVD-fmk, z-IETD-fmk, z-LEHD-fmk, z-AEVD-fmk and Ac-YVAD-cmk at given concentrations for 1 h, the cells were incubated with or without magnolol for 12 h. In another experiment, the cells were incubated with mangnolol and anti-Fas antibody CH-11 for 6 h. Cell growth was measured at different time points by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as previously described with a plate reader (Tecan) (Takano et al., 2003).

Observation of morphological changes

A375-S2 cells in RPMI-1640 containing 10% FBS were seeded into 25 mL culture bottles and cultured overnight. Magnolol 100 μM was added to the cell culture and the cellular morphology was observed using phase contrast microscopy (LEICA) at 0, 12, 24, 36 and 48 h.

Nuclear damage observed by Hoechst 33258 staining

A375-S2 cells in RPMI-1640 containing 10% FBS were seeded into a 6 well plate and cultured overnight. Magnolol 100 μM was added to the cell culture and cultured for 12, 24 and 36 h. Cells were fixed with 3.7% paraformaldelyde at room temperature for 1 h, then washed twice with PBS and stained with Hoechst 33258 (Sigma) 167 μM at 37°C for 15 min. At the end of incubation, nuclear morphology of the cells was observed of using fluorescence microscope (LEICA) (Zhang et al., 2004).

DNA extraction and detection of DNA fragments

Floating and attached A375-S2 cells (1×10^6 cells) were harvested and centrifuged at $1{,}000\times g$ for 10 min, and washed once in Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (PBS). Cell pellets were lysed in 100 μL cell lysis buffer (10 mM Tris-HCl pH 7.4, 10 mM EDTA pH 8.0, 0.5% Triton X-100) at 4°C for 15min, and centrifuged at $15{,}000\times g$ for 20 min. The supernatants were incubated with 40 μg/mL proteinase K (Merck) and 40 μg/mL RNase A (Sigma) at 37°C for 2 h. The lysate was extracted with 0.5 M NaCl and 50% 2-propanol and incubated at -20°C overnight, and centrifuged at $15{,}000\times g$ for 20 min. The pellets were suspended in TE buffer (10 mM Tris-HCl pH 7.4 and 1 mM EDTA pH 8.0). DNA was separated by 2% agarose gel electroporesis at 100 V for 40 min and stained with 0.1 μg/mL ethidium bromide (Kitamura et al., 2003).

Caspase-3, caspase-8 and caspase-9 activities assay

A375-S2 cells were cultured at subconfluent and treated with or without magnolol 100 μM for different time periods. Both adherent and non-adherent A375-S2 cells were collected. After brief rinsing in PBS, pellets were frozen at -80°C until analysis. The pellets were suspended in ice-cold lysis buffer obtained from Alexis, placed on ice for 10 min then centrifuged at $13{,}000\times g$ at 4°C for 15 min. 100 μg of protein was diluted in 50 μL of lysis buffer and added to 40 μL of reaction buffer. 10 μL of the appropriate caspase substrate was then added to give a final concentration of 100 μM. The plates were incubated in a 5% CO_2 incubator at 37°C for 3 h. The substrates for each caspase were as follows: Ac-DEVD-*p*NA for caspase 3-like activity (Alexis), Ac-LEHD- *p*NA (Calbiochem) for caspase 9 activity, and Z-IETD- *p*NA (Calbiochem) for caspase 8 activity. The substrates were prepared as 1 mM stocks according to the manufacturer's instructions and stored at -20°C. Amounts of released *p*-nitroanilide (*p*NA) were measured by measuring absor-

bance at 405 nm.

Western blotting analysis

Immunoblotting of cell lysates was performed as previously described (Suzuki et al., 2001). Both adherent and non-adherent cells were harvested, washed twice with ice-cold phosphate buffered saline (PBS), then lysed in lysis buffer (50 mM Hepes pH 7.4, 1% Triton X-100, 2 mM sodium orthovanadate, 100 mM sodium fluoride, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 10 μg/mL aprotinin and 10 μg/mL leupeptin) at 4ºC for 60 min. The lysate was centrifuged at 15,000 g for 10 min and the supernatant was used for Western blotting analysis. Equivalent amounts of protein were separated by SDS-PAGE and wet-electrotransferred onto nitrocellulose manbranes, and equivalent loading was confirmed by Bio-Rad protein assay. Proteins were detected with antibodies against Bcl-2, Bax, ICAD (Santa Cruz Biotechnology), and PARP (Upstate biotechnology) followed by horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology) and visualized by using 3, 3-diaminobenzidine tetrahydrochloride as the HRP substrate.

Statistical analysis

Statistical analysis was conducted using Student's *t*-test for analysis of significance between the different values. Values were expressed as the mean ± S.D. and they were considered significant at a P of less than 0.05. All Ps are two tailed.

RESULTS

Cytotoxic effects of magnolol on tumour cells

As shown in Fig. 1, magnolol inhibited A375-S2 cell growth in a dose- and time-dependent manner, and after 24 h treatment. By 36 h after magnolol 100 μM treatment, A375-S2 cell growth inhibition rate reached nearly 100% (Fig. 1A). When incubated for 12 h, however, magnolol induced more death in cells with high density (Fig. 1B).

Magnolol induces A375-S2 cell death through apoptotic pathways

The morphological changes were observed in A375-S2 cells treated with magnolol 100 μM for 24 h, compared with the untreated control cells marked changes were observed. The majority of magnolol-treated cells became round, some of these cells showed membrane blebbing which indicate the formation of apoptosis body (Fig. 2A). The morphological changes of nuclei were observed by Hoechst 33258 staining. In the

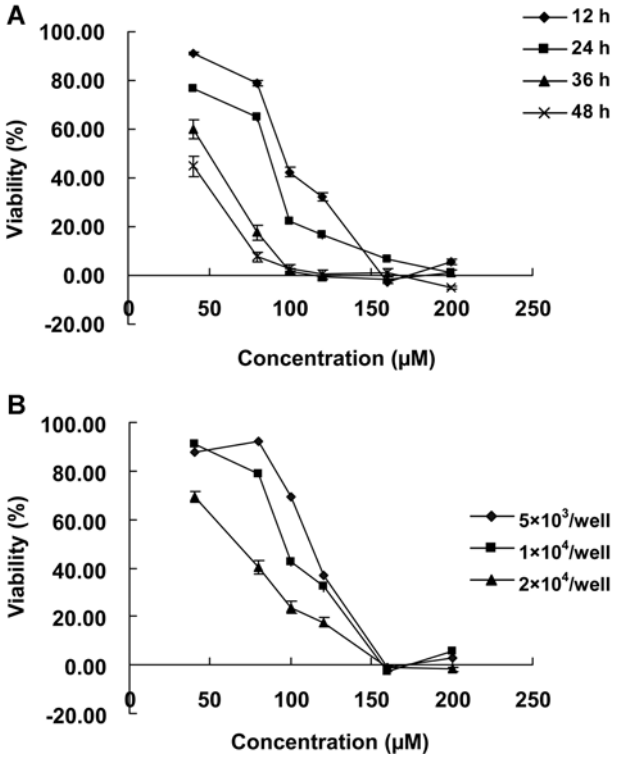

Fig. 1. (**A**) Dose-dependent inhibitory effect of magnolol on A375-S2 cells. The cells were cultured for 12 (closed diamond), 24 (closed square), 36 (closed triangle), and 48 (open square) h with different concentrations of magnolol. (**B**) Cells with different densities were cultured with different concentrations of magnolol for 12 h. $n = 3$. Mean ± S.D.

control group, A375-S2 cell nuclei were round in shape and stained homogeneously with Hoechst 33258. After 24 h treatment with 100 μM magnolol, apoptotic characteristics in A375-S2 cells were observed with condensed and marked blebbing nuclei (Fig. 2B). After 12, 24, or 36 h treatment with 100 μM magnolol, A375-S2 cells began to exhibit a typical DNA fragmentation which is another hallmark of apoptosis (Fig. 2C). The results above suggest that magnolol induced A375-S2 cell death through apoptotic pathways.

Caspases were activated during magnolol induced-A375-S2 cell death

As caspases are well reported to participate in both initiation and execution of apoptosis, the effects of caspase inhibitors on magnolol-induced A375-S2 cell death were investigated. Caspases inhibitors, z-VAD-fmk, z-DEVD-fmk, z-IETD-fmk, z-LEHD-fmk and z-AEVD-fmk effectively inhibited 100 μM magnolol-induced A375-S2 cell death (Fig. 3A). Then the activities of caspase 3, 8 and 9 after magnolol-treatment were examined, the activity of caspases-8 and -9 reached peaks at 12 h followed by a further increase of

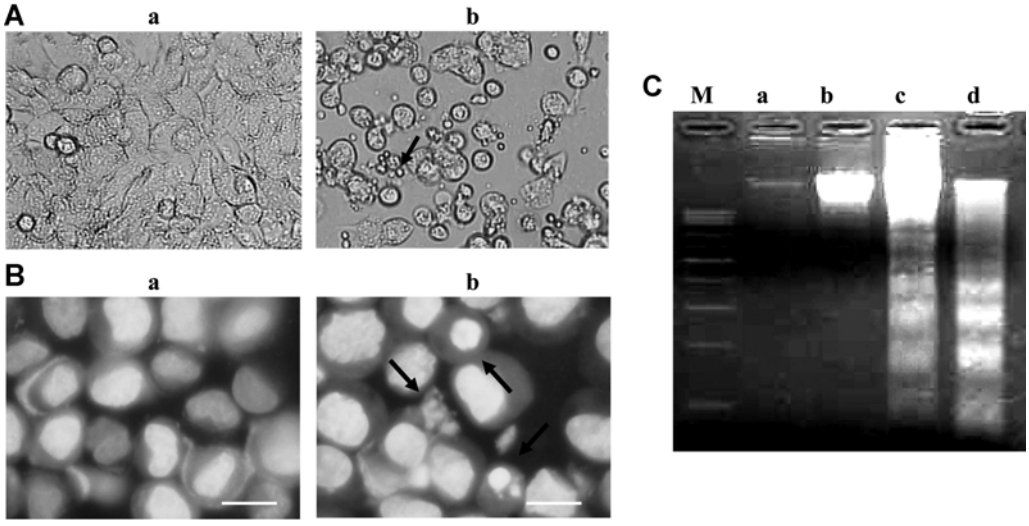

Fig. 2. Magnolol-induced apoptotic morphological changes and DNA fragmentation of A375-S2 cells. (**A**) A375-S2 cells were incubated in the medium alone for 12 h (a) or the medium containing 100 μM magnolol for 12 h (b) (magnification = 200). Arrow indicates multiblebbing cells and apoptotic bodies. Scale bar represents 200 μm. (**B**) A375-S2 cells were incubated in the medium alone for 36 h (a) (magnification = 200) or the medium containing 100 μM magnolol for 12 h (b) (magnification = 200, arrow indicates vesicle like area), Hoechst 33258 staining. Scale bar represents 100 μm. (**C**) A375-S2 cells were cultured with magnolol 100 μM for 0 (a), 12 (b), 24 (c) and 36 (d) h. Hae-III-digested phage øx-174 DNA fragments were used as molecular markers (M).

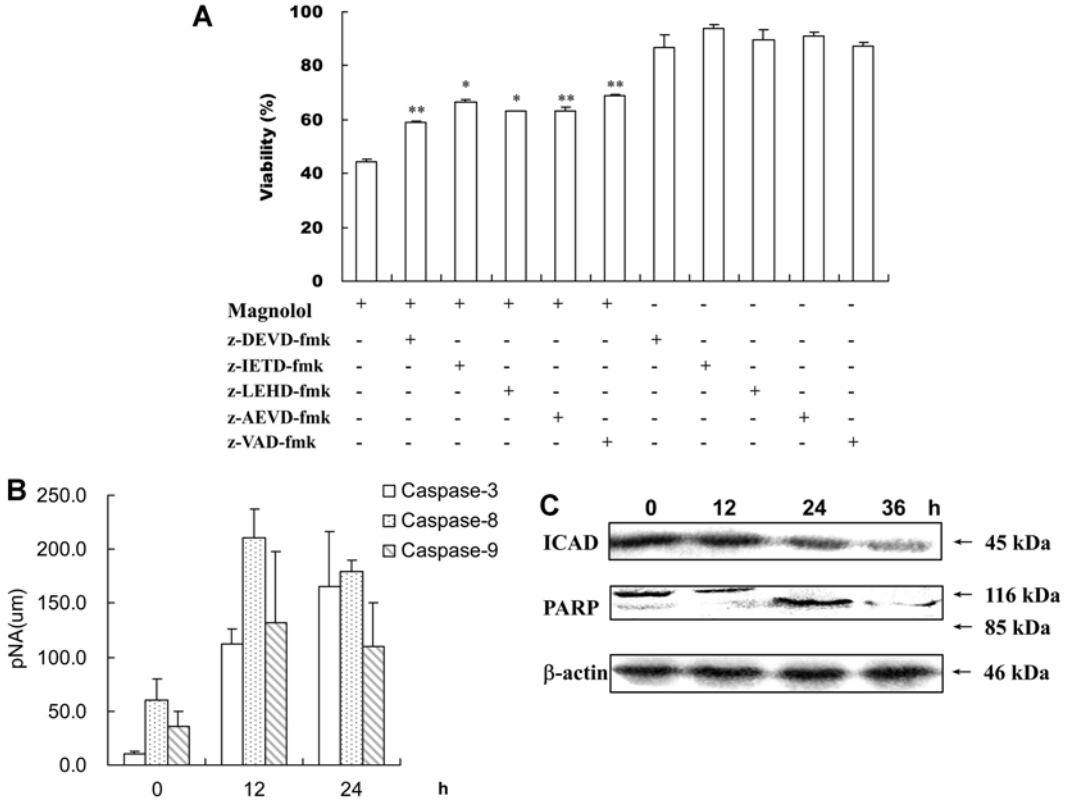

Fig. 3. Involvement of caspases during magnolol-induced apoptosis in A375-S2 cells. (**A**) Effects of caspase inhibitors on 100 μM magnolol induced A375-S2 cell death. The cells were cultured in the absence or presence of caspase inhibitors, 1 h prior to the addition of magnolol, then incubated for 12 h. *$p < 0.05$, **$p < 0.01$ *vs.* magnolol 100 μM group. (**B**) Activities of caspase-3, -8 and -9 in magnolol-treated A375-S2 cells. The cell lysate were assayed after treatment with magnolol 100 μM for 12, 24 and 36 h. (**C**) Degradation of ICAD and PARP in magnolol-treated A375-S2 cells. The cells were treated with magnolol 100 μM for the indicated times. ICAD and PARP were analysed by Western Blotting.

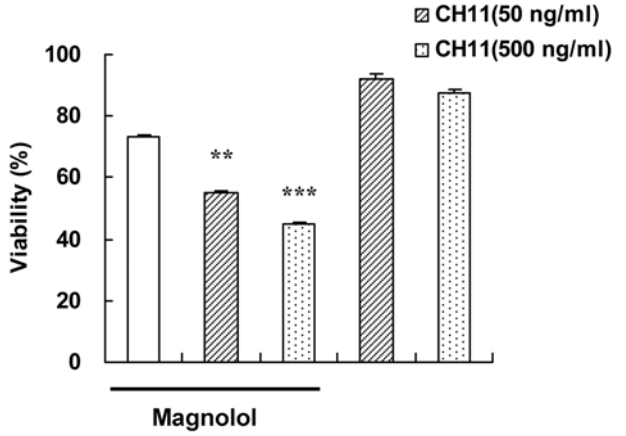

Fig. 4. Synergeic effect of magnolol with agonistic anti-Fas antibody CH-11. A375-S2 cells were treated with magnolol 100 μM simultaneously with CH-11 for 6 h. $n = 3$. Mean ± S.D. $^{**}p < 0.01$ and $^{***}p < 0.001$ *vs.* magnolol 100 μM group.

caspase-3 activity at 24 h (Fig. 3B).

Caspase 3's role in magnolol induced A375-S2 cell was further confirmed by the examination of two of caspase-3 substrate, ICAD and PARP. ICAD is the inhibitor of a caspase dependent DNase referred to as CAD, and is cleaved to be inactivated and allow the DNase to execute the characteristic fragmentation of DNA (Enari et al., 1998). After exposure to 100 μM magnolol for 24 h, the level of ICAD decreased significantly and was barely detected at 36 h. Cleavage of another substrate for caspase-3, PARP, was detected. By 24 h, the 116 kDa protein began to decline (Fig. 3C). Together with the fragmentation of DNA, the caspase cascade was activated in magnolol-induced A375-S2 cell apotosis.

Synergeic effect of magnolol with agonistic anti-Fas antibody CH-11

Since the inhibitor of caspase-8 and -10 effectively protect A375-S2 cells from magnolol-induced apoptosis, an agonistic anti-Fas antibody CH-11 was cocultured with magnolol. As expected, CH-11 significantly increased magnolol induced A375-S2 cell death (Fig. 4).

Involvement of Bcl-2 and Bax in magnolol-induced apoptosis

Bcl-2 family members play a critical role in the regulation of mitochondrial pathway, so the expression of the Bcl-2 family proteins, Bcl-2, and Bax, were detected by Western blot analysis. At 12 h after treatment with magnolol, the expression of Bcl-2 decreased, but that of Bax increased (Fig. 5). Together with the activation of caspase-9, these results suggest the activation of mitochondrial pathway in magnolol-induced A375-S2 cell apotosis.

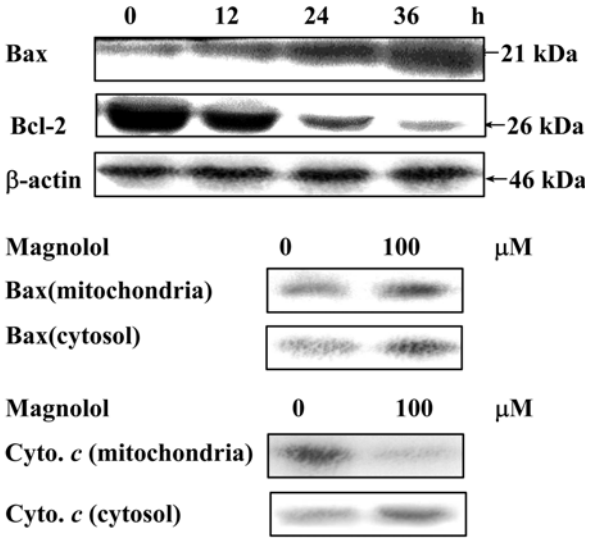

Fig. 5. The expression of Bax and Bcl-2 in magnolol-treated A375-S2 cells. The cells were treated with magnolol 100 μM for the indicated times. Bax, Bcl-2, and Cyto. c were analysed by Western Blotting.

DISCUSSION

Our present findings demonstrated that magnolol has an antiproliferative effect on human melanoma A375-S2 cells. Based on the morphological changes of A375-S2 cells and DNA fragmentation, we concluded that magnolol induced A375-S2 cell death through apoptotic pathways.

The results in this study showed that pretreatment with z-VAD-fmk, effectively inhibited 100 μM magnolol-induced cell apoptosis, indicating the participation of caspase family mamber. Caspase-8 inhibitor z-IETD-fmk and caspase-10 inhibitor z-AEVD-fmk partially dampened A375-S2 cell apoptosis that suggest the activation of death receptor pathway, a synergistic effect of Fas against antibody CH-11 with magnolol may further support this conclusion. As to a higher inhibition rate in cells with higher density, we presume it might be associated with an up-regulation of Fas or FasL, even soluble FasL.

A balance between members of the Bcl-2 family is thought to determine whether mitochodria remain intact or become permeabilized and release proteins that promote cell death (Huang and Strasser, 2000). Activation of Bax results in oligomerization at the outer mitochondrial membrane and causes the release of a variety of pro-apoptotic molecules, including cytochrome *c*, from the intermitochondrial membrane space into the cytosol (Korsmeyer et al., 2000). Released cytochrome *c* then binds to Apaf-1 form the apoptosome, then recruit and activate the inactive pro-

caspase-9, and in turn activates the effector caspases (caspase-3, -6, and -7). Bcl-2 forms heterodimers with a variety of proapoptotic proteins thereby prevent Bax/Bak mediated pore formation in the outer mitochondrial membrane and the subsequent release of pro-apoptotic factors (Cheng et al., 2001). In this study we found that, the level of the anti-apoptotic protein Bcl-2 decreased followed by the up-regulation of pro-apoptotic protein Bax. Caspase-9 inhibitor z-LEHD-fmk inhibited magnolol induced A375-S2 cell apoptosis and the activity of caspases-9 reached peaks at 12 h followed by a further increase of caspase-3 activity at 24 h, and the caspase-3 substrates, ICAD and PARP were cleaved at the same time point. These indicate that magnolol induces A375-S2 cell apoptosis by the down-regulation of Bcl-2 and up-regulation of bax, initiates the mitochondria pathways.

Melanoma accounts for less than 5% of skin cancer cases but causes a large majority of skin cancer deaths. Despite surgery, radiotherapy and immunotherapy, the development of novel agents against melanoma are still being expected. Conventional Chinese medicines have been historically tested for safety and effectiveness to treat cancers, thus we hope the present results may provide a basic view for a further use of magnolol for the treatment of melanoma.

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