British Journal of Pharmacology (2003) 138, 193–201

www.nature.com/bjp

Effector mechanism of magnolol-induced apoptosis in human lung squamous carcinoma CH27 cells

[1]Shu-Er Yang, [1]Ming-Tsuen Hsieh, [2]Tung-Hu Tsai & *,[3]Shih-Lan Hsu

[1]Institute of Chinese Pharmaceutical Sciences, China Medical College Taichung, Taiwan; [2]National Research Institute of Chinese Medicine, Taipei 11221, Taiwan and [3]Department of Education and Research, Taichung Veterans General Hospital, Taichung, Taiwan

1 Magnolol, an active component isolated from the root and stem bark of *Magnolia officinalis*, has been reported to exhibit antitumour effects, but little is known about its molecular mechanisms of action.

2 Magnolol inhibited proliferation of human lung squamous carcinoma CH27 cells at low concentrations (10–40 μM), and induced apoptosis at high concentrations (80–100 μM).

3 Treatment with 80 μM magnolol significantly increased the expression of Bad and Bcl-X_S proteins, whereas it decreased the expression of Bcl-X_L. Overexpression of Bcl-2 protected CH27 cells against magnolol-triggered apoptosis.

4 Magnolol treatment resulted in accumulation of cytosolic cytochrome *c* and activation of caspase-9 and downstream caspases (caspase-3 and -6). Pretreatment with z-VAD-fmk markedly inhibited magnolol-induced cell death, but did not prevent cytosolic cytochrome *c* accumulation.

5 Magnolol induced a modest and persistent JNK activation and ERK inactivation in CH27 cells without evident changes in the protein levels. The responsiveness of JNK and ERK to magnolol suggests the involvement of these kinases in the initiation of the apoptosis process.

6 These results indicate that regulation of the Bcl-2 family, accumulation of cytosolic cytochrome *c*, and activation of caspase-9 and caspase-3 may be the effector mechanisms of magnolol-induced apoptosis.

British Journal of Pharmacology (2003) **138,** 193–201. doi:10.1038/sj.bjp.0705024

Keywords: Magnolol; Bad; Bcl-X_L; Bcl-X_S; cytochrome *c*; caspase; ERK; JNK; apoptosis

Abbreviations: Adv, adenovirus; DAPI, 4′, 6-diamidino-2-phenylindole; DEVD-AFC, Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin; ERK, extracellular signal-regulated kinase; GST, glutathione-S-transferase; IETD-AFC, Ile-Glu-Thr-Asp-7-amino-4-trifluoromethyl coumarin; JNK, c-Jun NH_2-terminal kinase; LEHD-AFC, Leu-Glu-His-Asp-7-amino-4-trifluoromethyl coumarin; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; PARP, poly (ADP-ribose) polymerase; SAPK, stress-activated protein kinase; TUNEL, terminal transferase-mediated dUTP-fluorescensin nick end-labelling; VDVAD-AFC, Val-Asp-Val-Ala-Asp-7-amino-4-trifluoromethyl coumarin; VEID-AFC, Val-Glu-Ile-Asp-7-amino-4-trifluoromethyl coumarin; YVAD-AFC, Tyr-Val-Ala-Asp-7-amino-4-trifluoromethyl coumarin; z-VAD-fmk, z-Val-Ala-Asp-fluoromethyl ketone

Introduction

Lung cancer represents the major cause of cancer-related death in Taiwan, and its incidence continues to rise worldwide. The conventional treatment of lung cancer includes surgery, radiation and chemotherapy. Each of these therapies has serious side effects and other limitations. The long-term results show a high rate of cancer recurrence after these treatments. However, cancer chemotherapy has progressed since its introduction into clinical practice and represents the most promising treatment modality. Various chemotherapy drugs, including doxorubicin, 5-fluorouracil, cisplatin, etoposide and gemcitabine, have been used to treat lung cancer (Scarpati *et al*., 2001; Worden & Kalemkerian, 2000; Yang *et al*., 2002). But all of these anticancer drugs affect not only pathological tumour cells, but also normal cells, especially bone marrow cells or intestinal epithelia with high turnover rate, causing serious complications and toxicity. This has prompted us to search for novel compounds with potent anticancer effect for lung cancer treatment. Recently, many of cancer chemotherapies use alternative medicine, including herbal therapies. It has been observed that traditional Chinese medicine has been time-tested for safety and effectiveness, dating back several thousand years. Several Chinese medicines have been used to treat cancers, but the effective component(s) and their mode of action at cellular and molecular levels are largely unknown.

Magnolol, a phenolic component extracted from the root and stem bark of *Magnolia officinalis* (Chinese name: Houpo), has multiple pharmacological effects, such as antioxidant (Fujita & Taira, 1994; Kong *et al*., 2000; Lee *et al*., 2001), antithrombotic (Teng *et al*., 1991), antimicrobial (Ho *et al*., 2001), antiallergic (Hamasaki *et al*., 1999), antifungal (Bang *et al*., 2000), anti-inflammatory (Wang *et*

*Author for correspondence at: Department of Education & Research, Taichung Veterans General Hospital, No. 160, Section 3, Chung-Gang Road, Taichung 40705, Taiwan;
E-mail: h2326@vghtc.vghtc.gov.tw

al., 1992), xanthine oxidase inhibition (Chang *et al*., 1994), muscle relaxant activity (Watanabe *et al*., 1975), anxiolytic and central depressant effects (Maruyama *et al*., 1998; Watanabe *et al*., 1983). Previous reports have demonstrated that magnolol exhibited remarkable inhibitory effects on mouse skin tumour promotion in an *in vivo* two-stage carcinogenesis (Konoshima *et al*., 1991), inhibition of the growth of human leukaemic HL-60 cells (Hirano *et al*., 1994), and inhibition of human fibrosarcoma HT-1080 invasion (Nagase *et al*., 2001). Recently, magnolol has been reported to inhibit the proliferation of tumour cells *in vitro* and *in vivo* (Lin *et al*., 2002). Magnolol also induced translocation of cytochrome *c* from mitochondria to cytosol, activated caspase-3 and triggered apoptotic cell death in HepG2 cells (Lin *et al*., 2001). Thus far, the signal transduction pathways leading to apoptosis in cells exposed to magnolol have not been clarified. Many lines of evidence have implicated the mitogen-activated protein kinase (MAPK) family molecules in the regulation of apoptosis. In mammalian cells, the distinct MAPK members have been identified, these include extracellular signal-regulated kinases (ERKs), stress-activated protein kinases (SAPKs)/c-Jun NH2-terminal kinases (JNKs), and the p38 kinases, are important signalling in the control of cell proliferation and differentiation. Recent studies suggest that MAPK also regulate the cell cycle and apoptosis (Klekotka *et al*., 2001; Schaeffer & Weber, 1999; Xia *et al*., 1995).

The aims of this study were to investigate the effector mechanism(s) of magnolol-induced apoptosis and the effects of magnolol on the activities of MAPK in human lung cancer CH27 cells. We demonstrated that magnolol activated a complex signalling pathway required for cell death induction, the mechanism of which is in part due to regulation of Bcl-2 family proteins, release of cytochrome *c* from mitochondria into the cytosol and activation of the caspase cascade. Moreover, modulation of MAPK family members had been first evaluated in magnolol-induced apoptotic cells, indicating that magnolol-induced apoptosis is well correlated with the activation of JNK and inactivation of ERK signalling pathway.

Methods

Materials

Magnolol was isolated from bark of *Magnolia officinalis*. The methanol extract of magnolia bark was partitioned between water and chloroform. The chloroform layer was separated repeatedly by column chromatography on silica gel, and magnolol was then extracted. Identification and purity were compared with authentic compound by [^{13}C]-NMR (Bruker, Germany) and HPLC, coupled with photodiode-array detector determination (Tsai *et al*., 1995). The highly purified magnolol was supplied by Dr Tung-Hu Tsai. The stock solution of magnolol was prepared at 80 mM in dimethyl sulphoxide (DMSO) and stored at 4°C.

The antibodies: anti-Bcl-2, anti-Bcl-$X_{L\text{-}S}$, anti-Bax, anti-Bag-1 and anti-JNK1 (C-17) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Anti-Bak, anti-PARP, anti-Caspase-3 and anti-cytochrome *c* were purchased from PharMingen (San Diego, CA, U.S.A.). Anti-Bad was obtained from Transduction Laboratory (Lexington, KY, U.S.A.). Anti-JNK, anti-phospho-JNK, anti-ERK1/2, anti-phospho-ERK1/2, anti-p38, anti-phospho-p38, anti-MEK1/2, anti-phospho-MEK1/2 and glutathione-S-transferase-c-Jun (GST-c-Jun) peptides were purchased from Calbiochem-Novabiochem Corporation (England, U.K.). Anti-c-Jun was obtained from Oncogene Research Products (Boston, MA, U.S.A.). Anti-phospho-c-Jun was obtained from Cell Signaling Technology, Inc (Beverly, MA, U.S.A.). The recombinant Bcl-2-adenoviral vector and control adenoviral vector were kindly supplied by Dr Song-Kun Shyue at the Institute of Biomedical Sciences, Academia Sinica (Taipei, Taiwan). 4′, 6-diamidino-2-phenylindole (DAPI) was obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). Anti-mouse and anti-rabbit IgG peroxidase-conjugated secondary antibody were purchased from Amersham (Buckinghamshire, U.K.). Caspase activity assay kits, including the fluorogenic substrates of caspase-1 (YVAD-AFC), caspase-2 (VDVAD-AFC), caspase-3 (DEVD-AFC), caspase-6 (VEID-AFC), caspase-8 (IETD-AFC) and caspase-9 (LEHD-AFC), were purchased from R&D systems (Minneapolis, MN, U.S.A.). Cell permeable broad-spectrum caspase inhibitor (z-VAD-fmk) was obtained from KAMIYA Biomedical Company (Seattle, WA, U.S.A.).

Cell culture and cytotoxicity assay

Human lung squamous carcinoma cell line (CH27), human lung non-small-cell carcinoma cell lines (H460 and H1299) and human fibroblast-like lung cell line (WI-38) were used in this study. All the cell lines were cultured in Dulbecco's modified Eagle's medium (Flow Laboratories), except WI-38 (cultured in RPMI medium), supplemented with 5% foetal bovine serum, 2 mM glutamine, and antibiotics (100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin) in a 5% CO_2 humidified incubator at 37°C. The medium was changed every 2 days. For cell viability assay, cells were seeded in 12-well plate at a density of 1×10^5 cells well^{-1}. After 24 h incubation, cells were treated with magnolol at various times and concentrations. Some cells were infected with recombinant Bcl-2-adenoviral vector and control adenoviral vector for 24 h, then exposed to 80 μM magnolol. Other cells were pretreated with caspase inhibitor z-VAD-fmk for 2 h prior to 80 μM magnolol treatment. The control cultures were treated with the vehicle (0.1% DMSO). After 24 h incubation, cell viability was assessed by Trypan Blue dye exclusion, and cell number was counted using a haemocytometer.

Morphological and apoptotic cell detection

CH27 cells were treated without or with 80 μM magnolol for 24 h. Control cultures were treated with the vehicle (0.1% DMSO). After treatment, cells were washed with cold PBS and fixed in 2% paraformaldehyde at room temperature for 30 min. After washing twice with PBS, cells were permeabilized with 0.1% Triton X-100/PBS solution at room temperature for another 30 min. Then cells were washed and stained with 1 μg ml^{-1} DAPI solution at room temperature for 30 min. Morphological changes were observed under a fluorescence microscope. For apoptotic cell determination, cells were washed, fixed and terminal transferase-mediated dUTP-fluorescensin nick end-labelling

(TUNEL) assay was performed according to the manufacturer's instructions (Boehringer Mannheim). Then cells were washed with PBS and examined using a fluorescence microscope. TUNEL positive cells were counted as apoptotic cells.

DNA fragmentation assay

CH27 cells were treated without or with 80 μM magnolol for 24 h. DNA fragmentation was assayed as previously described (Sandstrom & Buttke, 1993). Briefly, attached and nonattached cells were harvested and lysed in ice-cold lysis buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 0.3% Triton X-100, incubated on ice for 30 min, then centrifuged. Proteinase K (200 μg ml^{-1}) was added to the supernatant, then incubated at 50°C for 2 h, followed by the addition of 100 μg ml^{-1} RNase A. After incubation at 37°C for 2 h, lysates were extracted twice with phenol/chloroform, and precipitated overnight at −20°C with ethanol/sodium acetate. DNA concentration was determined, and equal amounts of DNA (5 μg) were electrophoresed on a 2% agarose gel containing 0.1 μg ml^{-1} ethidium bromide. DNA banding was examined using a UV Transilluminator Image System (Evergreen, U.K.).

Protein preparation and Western blot analysis

CH27 cells were treated without or with 80 μM magnolol at indicated time points. After treatment, adherent and floating cells were harvested, washed twice with ice-cold PBS and lysed in ice-cold lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5 mM dithiothreitol, 1% Nonidet P-40, 0.3% deoxycholate, 10 μg ml^{-1} leupeptin, 10 μg ml^{-1} aprotinin, 10 μg ml^{-1} soybean trypsin inhibitor, 0.5 mM phenylmethylsulphonylfluoride, 1 mM sodium orthovanadate. After 30 min of incubation on ice, cells were centrifuged at 100,000 × g for 30 min at 4°C, supernatants were collected, and stored at −80°C until assay. Protein concentrations were determined using the Bradford method (Bradford, 1976). For Western blot analysis, equal protein amounts were loaded and separated on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE). Then gels were equilibrated in transfer buffer (50 mM Tris, pH 9.0–9.4, 40 mM glycine, 0.375% SDS, 20% methanol) and electrophoretically transferred to PVDF membrane (Millipore). The membrane was blocked with 5% nonfat dry milk in TBST buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl and 0.1% Tween 20) and incubated overnight at 4°C with specific primary antibodies. After washing with TBST, membrane was incubated with horseradish-peroxidase-conjugated secondary antibodies for 1 h, and proteins were visualized using an enhanced chemiluminescence detection kit (ECL kits; Amersham life science).

Caspase activity assay

CH27 cells were treated without or with 80 μM magnolol at various time periods. Cells were lysed in lysis buffer (1% Triton X-100, 0.32 M sucrose, 5 mM EDTA, 10 mM Tris-HCl, pH 8, 2 mM dithiothreitol, 1 mM PMSF, 1 μg ml^{-1} aprotinin, 1 μg ml^{-1} leupeptin) for 20 min at 4°C and centrifuged at 100,000 × g for 30 min. Caspase activity was estimated according to the manufacture's protocol (R&D systems). Briefly, cell lysates (200 μg total protein) were incubated with reaction mixtures (final volume 50 μl) containing fluorogenic substrate peptides specific for caspase-1 (YVAD-AFC), caspase-2 (VDVAD-AFC), caspase-3 (DEVD-AFC), caspase-6 (VEID-AFC), caspase-8 (IETD-AFC) and caspase-9 (LEHD-AFC). The reaction was performed at 37°C for 2 h. Fluorescence was measured with a fluorescence microplate reader (excitation wavelength, 400 nm; emission wavelength, 505 nm) (Labsystems, Finland).

Preparation of cytosolic extracts

For examination of mitochondrial cytochrome c release, cytosolic extracts (S-100) were prepared. CH27 cells were treated with or without 80 μM magnolol at various times. After treatment, cells were washed twice with PBS, and lysed in ice-cold lysis buffer containing in mM: HEPES 20, KCl 10, $MgCl_2$ 1.5, EDTA 1, EGTA 1, dithiothreitol 1, PMSF 1 and pH 7.5. After 30 min of incubation on ice, cells were swelled, then centrifuged at 100,000 × g for 30 min, the supernatant was stored at −80°C until analysis.

JNK activity assay

CH27 cells were treated without or with 80 μM magnolol for the indicated times. Immunoprecipitation was performed by mixing of cell lysates (300 μg total protein) with anti-JNK1 polyclonal antibody in the presence of 20 μl of protein A-Sepharose beads and rotated overnight at 4°C. After washing twice with kinase buffer, the JNK activity was determined by incubation of the immunocomplex in 30 μl of kinase reaction buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 2 μg glutathione-S-transferase-c-Jun (GST-c-Jun) peptides, 5 μCi of [γ-^{32}P]-ATP (6000 Ci mmol^{-1}; DuPont NEN), and 5 μM ATP for 30 min at 30°C. The reaction was terminated by the addition of 10 μl of 4× Laemmli sample buffer, followed by boiling for 10 min. Samples were electrophoresed on 12% SDS-polyacrylamide gel, then transferred to PVDF membrane. Phosphorylated GST-c-Jun (1–79) was visualized by autoradiography.

Statistical analysis

All data are presented as mean ± s.d. of twelve replicates from four separate experiments. Statistical differences were evaluated using the Student's t-test and considered significance at $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. All the figures shown in this article were obtained from at least four independent experiments with similar pattern.

Results

Cytotoxic effect of magnolol

Treatment of CH27, H460, H1299 human lung cancer cells and WI-38 human fibroblast-like lung cells with 10, 20, 30 and 40 μM magnolol caused an antiproliferative effect but not cell death, approximately 10–30% growth inhibition was

detected after 96 h incubation (data not shown). However, a significant decrease in cell viability was seen in the tested cells when treated with magnolol at 80 μM for 24 h (less than 50% cell survival) (Figure 1A). Thus, 80 μM concentration of magnolol was chosen to detect changes in molecular events during the following experiments using CH27 cells. To examine the kinetics of cell death induced by magnolol treatment, four tested cells were treated with 80 μM magnolol for varying lengths of time (2, 4, 8, 16, 24, 36 and 48 h). As indicated in Figure 1B, the cytocidal effect was rapidly observed at 8 h magnolol treatment. Moreover, treatment with 80 μM magnolol for 72 h, no viable cell was observed in the three tested cancer cell lines (CH27, H460 and H1299) (Figure 1B). However, approximately 30% of viable cell was detected in magnolol-treated WI-38 cells. These results indicated that human fibroblast-like lung WI-38 cells were more resistant to the magnolol-mediated cytotoxicity than the three tested lung cancer cells.

Magnolol induces apoptosis

The phenotypic characteristics of magnolol-treated cells were evaluated by inverted phase microscope. Treatment with 80 μM magnolol for 24 h resulted in morphological features of apoptosis, rounded morphology, and eventually detachment from the substratum (Figure 2A). To clarify the cell death induced by magnolol in CH27 cells, *in situ* DAPI staining, TUNEL assay and DNA fragmentation analysis were performed. As shown in Figure 2B, treatment with 80 μM magnolol for 24 h caused nuclear condensation in DAPI-stained CH27 cells. Many TUNEL positive cells were observed after 24 h magnolol treatment, suggesting DNA fragmentation was occurring in magnolol-treated cells (Figure 2B). Moreover, treatment with magnolol resulted in internucleosomal DNA fragmentation, evidenced by the formation of a DNA ladder on agarose gels (Figure 2C), a hallmark of cell undergoing apoptosis. These results suggest

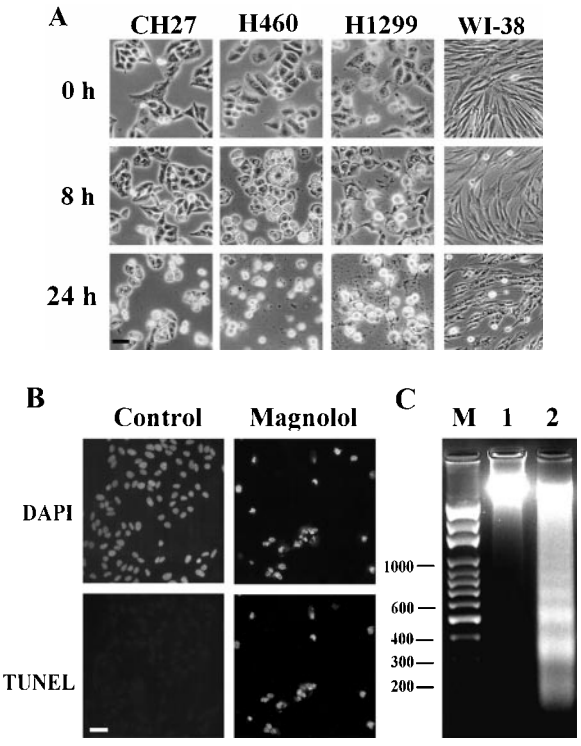

Figure 2 Induction of apoptosis by magnolol. (A) Morphological changes. CH27, H460, H1299 and WI-38 cells were treated with control vehicle (0.1% DMSO) or 80 μM magnolol for 24 h. Phase contrast micrographs were shown (magnification $\times$200; scale bar, 20 μm). (B) Condensed nuclei. CH27 cells were treated with 80 μM magnolol for 24 h, fixed and stained with DAPI, or using TUNEL assay, then examined under a fluorescence microscope. All photographs were taken at magnification $\times$200; scale bar, 20 μm. (C) DNA fragmentation analysis. Adherent and floating CH27 cells were collected 24 h after 80 μM magnolol treatment. Total DNA was isolated and separated by 2% agarose gel electrophoresis. M, size marker (100 base-pair DNA ladder); lane 1, control CH27 cells; lane 2, 80 μM magnolol-treated CH27 cells.

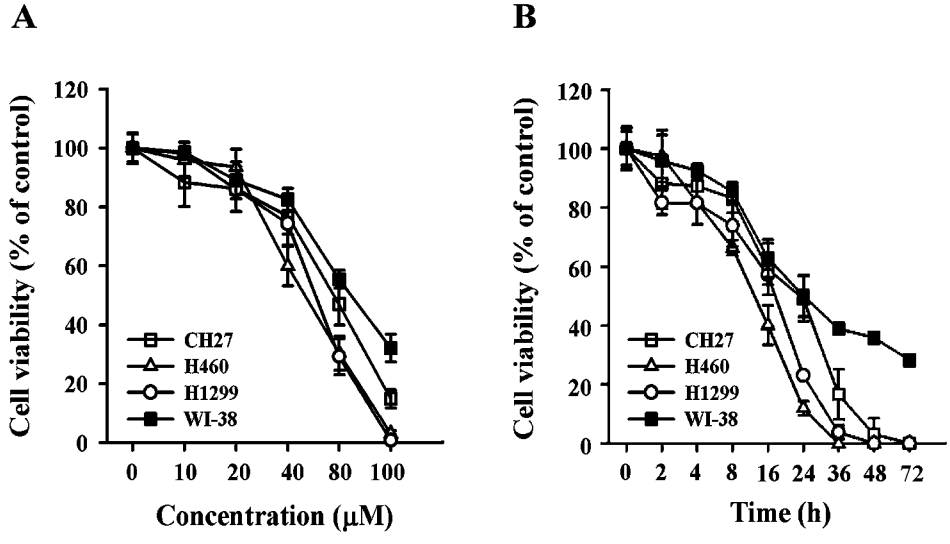

Figure 1 Magnolol-mediated cytotoxicity in CH27, H460, H1299 and WI-38 cells. (A) Dose-dependent response. Cells were treated with various concentrations of magnolol for 24 h. After incubation, the cells were harvested and cell viability was counted by Trypan blue dye exclusion method as described in Methods. (B) Time-dependent response. Cells were treated with control vehicle or 80 μM magnolol for the indicated times. After treatment, cell viability was determined as described above. Data are presented as mean$\pm$s.d. of 12 replicates from four separate experiments.

that magnolol indeed induced apoptotic cell death in these cells.

Involvement of Bcl-2 family proteins in magnolol-induced apoptosis

The products of Bcl-2-related proteins are known to play a role in either inhibition or promotion of apoptotic cell death (Adams & Cory, 1998). To determine whether Bcl-2 family proteins are involved in magnolol-induced apoptosis, we examine the effects of magnolol on the expression of the Bcl-2 family members by Western blot. The results showed that treatment of CH27 cells with 80 μM magnolol caused a down-regulation of Bcl-X_L protein. In contrast, Bad and Bcl-X_S were upregulated by magnolol. The expression levels of Bcl-2, Bag-1, Bax and Bak protein were not affected by magnolol treatment (Figure 3).

It has been reported that expression level of Bcl-2 determines anti- or pro-apoptotic function (Shinoura *et al*., 1999). To examine whether overexpression of Bcl-2 protein affects magnolol-induced apoptosis in human lung cancer cells, we infected CH27 cells with 50 moi (multiplicity of infection) Bcl-2-adenoviral vector and control adenoviral vector. The expression of Bcl-2 protein was analysed by Western blot and the resulting cytotoxicity was measured. As shown in Figure 4, the expression level of Bcl-2 protein was markedly increased by Adv-Bcl-2 infection but not by control adenovector infection (Figure 4). Moreover, Adv-Bcl-2-infected CH27 cells were more resistant to magnolol-induced apoptosis than control Adv-vector infected cells. These results indicated that overexpression of Bcl-2 protein significantly inhibited the magnolol-induced apoptosis.

Caspase activation and cytosolic cytochrome c *accumulation during magnolol-induced apoptosis*

Caspases are a family of cysteine proteases that are activated during the apoptosis (Vaux & Korsmeyer, 1999). Once activated, caspases cleave and activate downstream caspases consequently triggering the apoptotic process. To determine whether the caspase cascade is activated during magnolol-mediated apoptotic process, the caspase specific activity was measured using fluorogenic peptide substrates. As illustrated in Figure 5A, the enhancement of caspase-9 and -3 activities were first observed 4 h after magnolol treatment. Magnolol caused a significant increase in caspase-2, -3, -6 and -9 specific activities, which peaked at 24 h after addition of magnolol. However, the activities of caspase-1 and caspase-8 did not alter during magnolol-induced apoptosis (Figure 5A).

Caspase-3 is involved in the execution of the apoptotic signal, is proteolytically activated by upstream caspases. To confirm the activation of caspase-3 in magnolol-treated CH27 cells, we next examined the proteolysis of caspase-3 and cleavage of downstream molecular PARP by Western blot analysis. As depicted in Figure 5B, active caspase-3 fragments were first observed 4 h after magnolol treatment in parallel with cleavage of PARP (one of the known substrates for caspase-3, molecular mass 116 kDa) to an 85 kDa fragment (Figure 5B). The proteolytic processing of PARP was consistent with a similar time-dependent activation of caspase-3 enzyme activity in magnolol-treated cells (Figure 5A,B). To examine whether the activation of caspase cascade

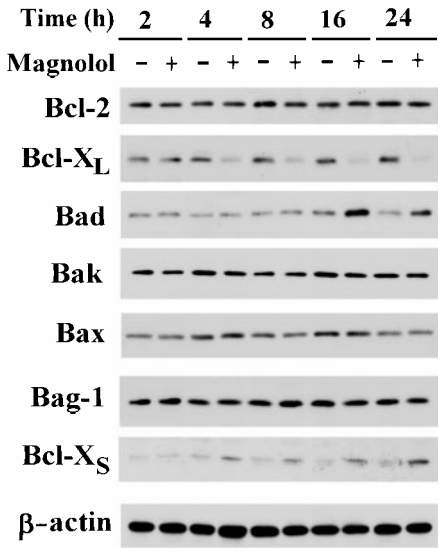

Figure 3 Expression of Bcl-2 family proteins in magnolol-treated CH27 cells. After treatment without or with 80 μM magnolol for indicated time periods, total cellular proteins were extracted and assayed by Western blot, using the antibodies against specific Bcl-2 family proteins. β-Actin was used as an internal loading control.

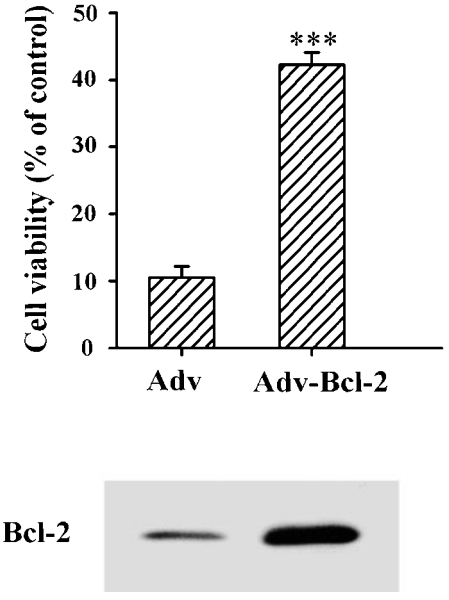

Figure 4 Overexpression of Bcl-2 protein prevented magnolol-induced apoptosis. CH27 cells were infected with Adv-Bcl-2 or Adv-vector for 24 h, then treated with 80 μM magnolol for another 24 h. Total cellular extracts were examined using Bcl-2 specific antibody by Western blot, cell viability was measured by Trypan blue exclusion method.

is responsible for the magnolol-triggered apoptotic process, CH27 cells were pretreated with the broad-spectrum caspase inhibitor z-VAD-fmk 2 h prior to treatment with magnolol. As shown in Figure 5C, combined treatment with z-VAD-fmk significantly inhibited magnolol-induced apoptotic cell death, indicating that the activation of caspases is required for magnolol-induced apoptosis.

Since mitochondrial cytochrome *c* release is controlled by Bcl-2 family molecules, we next characterized the effects of

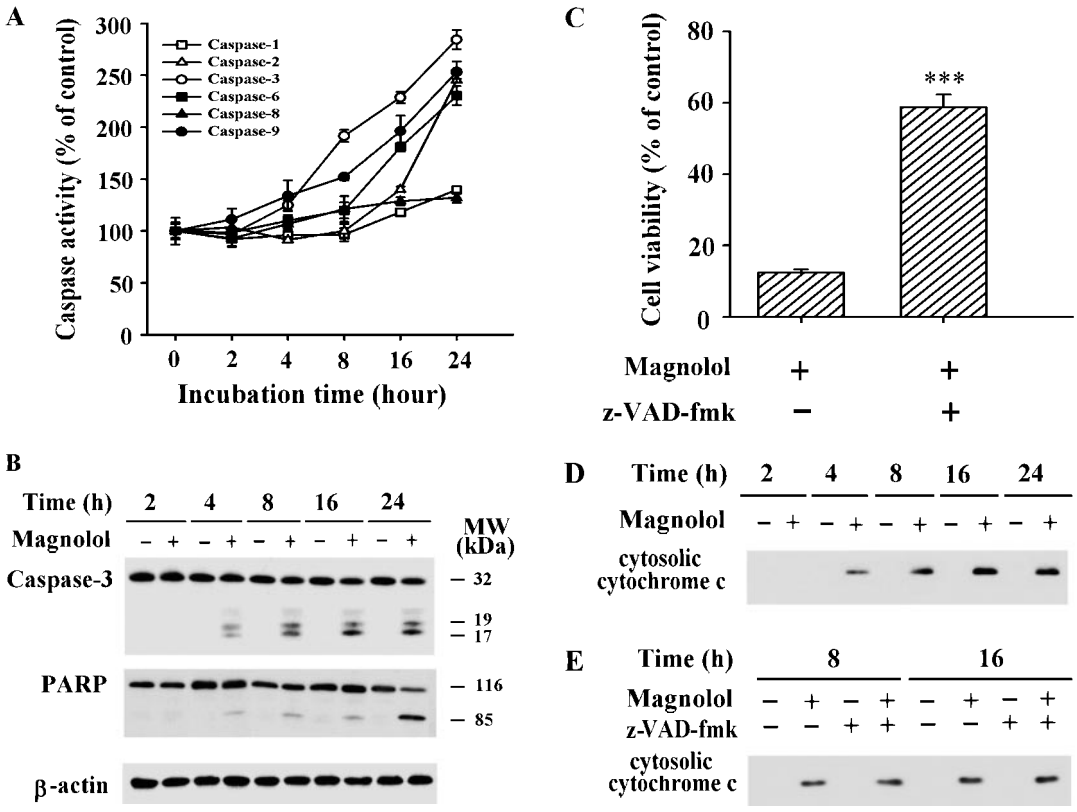

Figure 5 Effect of magnolol on caspase activity and cytochrome *c*. (A) Caspase activity analysis. Extracts from untreated or magnolol-treated CH27 cells were assayed for caspase activity using fluorogenic peptide substrates. (B) Proteolytic cleavage of caspase-3 and PARP were analysed by Western blot analysis. β-Actin was used as an internal loading control. (C) Inhibition of magnolol-induced apoptotic cell death by z-VAD-fmk. CH27 cells were treated with cell permeable broad-spectrum caspase inhibitor (z-VAD-fmk) 2 h prior to 80 μM magnolol treatment. Cell viability was estimated. $^{***}P<0.001$, compared with the 80 μM magnolol-treated group. (D) Effect of magnolol on cytosolic cytochrome *c*. Cytosolic fractions were isolated and analysed by Western blot for cytochrome *c* specific antibody. (E) z-VAD-fmk did not affect magnolol-induced cytosolic cytochrome *c* accumulation. CH27 cells were pretreated with 100 μM z-VAD-fmk for 2 h, then treated with vehicle or with 80 μM magnolol for 8 or 16 h. After treatment, cytosolic extracts were analysed by Western blot for cytochrome *c*.

magnolol and z-VAD-fmk (broad-spectrum caspase inhibitor) on the release of mitochondrial cytochrome *c*. Results from Western blot analysis demonstrated that a gradual increase in cytosolic cytochrome *c* was observed within 4 to 24 h of magnolol treatment (Figure 5D). These data indicate that the accumulation of cytosolic cytochrome *c* preceded the activation of caspase-2, -3, -6 and -9. Moreover, treatment with z-VAD-fmk markedly inhibited the magnolol-induced cell death (Figure 5C), but did not prevent the accumulation of cytosolic cytochrome *c* (Figure 5E). These results suggest that the target caspase(s) of z-VAD-fmk in magnolol-induced apoptosis is downstream of mitochondria.

Modulation of MAPK molecules by magnolol

MAPK signalling pathways have been shown to play an important role in the regulation of apoptosis (Schaeffer & Weber, 1999; Xia *et al*., 1995). To determine whether the regulation of MAPK cascade is involved in the magnolol-induced apoptosis, we examined the effect of magnolol on the activities of MAPK family proteins. As shown in Figure 6, the expression levels of JNK, ERK, p38 and MEK1/2 proteins were not affected by magnolol. However, administration of magnolol significantly and persistently increased

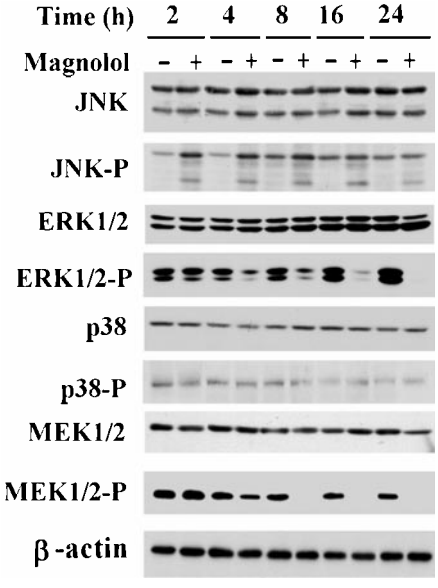

Figure 6 Regulation of MAPK molecules by magnolol. CH27 cells were treated with 80 μM magnolol for the indicated times. Cell extracts were analysed by Western blot, using the indicated antibodies. β-Actin was used as an internal loading control.

the phosphorylated JNK, whereas it markedly decreased the phosphorylation of ERK1/2 and MEK1/2 proteins. The phosphorylation status of p38 was not affected by magnolol. To further confirm whether JNK was activated by magnolol, the downstream molecule of JNK, transcription factor c-Jun, was detected by Western blot analysis. As depicted in Figure 7A, magnolol increased the phosphorylated form of c-Jun, but did not alter the expression levels of c-Jun protein. We also used GST-c-Jun fusion protein as the substrate to determine JNK activity. As shown in Figure 7B, the JNK activity (phosphorylation of GST-c-Jun) was increased approximately 2–3 fold by treatment of cells with magnolol. These results demonstrated that JNK was modestly and persistently activated and MEK/ERK was inactivated in magnolol-induced apoptotic CH27 cells.

Discussion

Magnolol has been found to prevent mouse skin tumour promotion in an *in vivo* two stage carcinogenesis (Konoshima *et al*., 1991), inhibition of the growth of human leukaemic HL-60 cells (Hirano *et al*., 1994), and inhibition of human fibrosarcoma HT-1080 invasion (Nagase *et al*., 2001). Recent study indicated that the concentrations of magnolol less than 50 μM strongly inhibited growth and proliferation, and 100 μM magnolol indeed induced apoptosis in colon and liver cancer cells (Lin *et al*., 2001). However, the cellular and molecular mechanism of apoptotic cell death induced by magnolol is poorly understood. The present study demonstrated the molecular mechanisms of cell death induced by magnolol. Consistent with a previous study, we also found that magnolol inhibited cell growth at low concentrations (10–40 μM) and induced cell death at high concentrations (>80 μM). In addition, human lung fibroblast WI-38 cells were more resistant to magnolol-induced cell death than three lung cancer cell lines (H460, H1299 and CH27). Data from TUNEL assay and DNA fragmentation analysis confirmed that magnolol induced apoptotic cell death in the tested human lung cancer cells.

The Bcl-2 family is composed of several members that play key roles in regulation of apoptosis (Adams & Cory, 1998). In this study, we found that magnolol induced up-regulation of Bad and Bcl-X_S and down-regulation of Bcl-X_L. Bad, a proapoptotic member of the Bcl-2 family, causes cell death through displacing Bax from binding to Bcl-2 and Bcl-X_L (Kelekar *et al*., 1997; Yang *et al*., 1995). Bcl-X has two alternatively spliced forms, the apoptotic Bcl-X_S and the antiapoptotic Bcl-X_L. Bcl-X_S has been shown to induce apoptosis through antagonizing the protective effect of Bcl-2 and Bcl-X_L (Fridman *et al*., 2001; Minn *et al*., 1996). Therefore, we suggest that magnolol-triggered apoptosis is mediated by up-regulation of Bad and Bcl-X_S, and down-regulation of Bcl-X_L. Overexpression of Bcl-2 and Bcl-X_L has been found to abolish apoptosis induced by diverse stimuli (Kim *et al*., 1997; Miyake *et al*., 2001). Intriguingly, overexpression of Bcl-2 by Adv-Bcl-2 infection markedly blocked magnolol-induced cell death. These results suggest that modulation of Bcl-2 related molecules is involved in magnolol-induced apoptosis. Bcl-2 and Bcl-X_L reside on the mitochondrial membrane (Gonzalez-Garcia *et al*., 1994) and mediate mitochondrial membrane permeability transition to release cytochrome *c* (Kharbanda *et al*., 1997; Yang *et al*., 1997). Interestingly, magnolol-mediated cytosolic cytochrome *c* accumulation was accompanied by down-regulation of Bcl-X_L and up-regulation of Bad and Bcl-X_S. This result is consistent with many previous reports in which regulation of Bcl-2 family proteins and release of cytochrome *c* were involved in the apoptosis process (Gonzalez-Garcia *et al*., 1994; Kharbanda *et al*., 1997; Yang *et al*., 1997).

Caspase is a family of cysteine proteases that plays a critical role in apoptosis. In the cytosol, cytochrome *c* interacts with Apaf-1, dATP (or ATP), and procaspase-9, thus activating caspase-9, which in turn cleaves and activates caspase-3 and other downstream caspases (Li *et al*., 1997; Zou *et al*., 1997). Activation and cleavage of caspase-3 served as a convergence point for apoptosis (Porter & Janicke, 1999). In this study, we showed an early release of cytochrome *c* and activation of caspase-9 prior to the activation of caspase-2, -3 and -6 upon magnolol treatment. Magnolol also induced proteolysis of caspase-3 and cleavage of downstream molecular PARP. Moreover, combined treatment of CH27 cells with magnolol and z-VAD-fmk markedly inhibited magnolol-induced apoptosis, but failed to prevent the release of cytochrome *c*, suggesting that magnolol-triggered cytochrome *c* release preceded caspase activation, and caspase activation plays a critical role in the magnolol-mediated apoptotic pathway. Fas/FasL is known to initiate drug-induced apoptosis *via* caspase-8 activation (Friesen *et al*., 1996; Fulda *et al*., 1997). However, magnolol neither induced caspase-8 activation nor Fas/FasL expression (data not shown). These data indicated that magnolol-induced caspase activation and apoptosis is independent of the Fas/FasL pathway. A previous study demonstrated that magnolol induced down-regulation of Bcl-2, accumulation of cytosolic cytochrome *c*, activation of caspases-3, -8, -9 in HepG2 cells (Lin *et al*., 2001). In the present study, we showed that magnolol-induced apoptosis was accompanied by up-regulation of Bcl-X_S and down-regulation of Bcl-X_L, while Bcl-2 and caspase-8 were not affected by magnolol in CH27 cells. These inconsistent observations suggest that the molecular events of magnolol-induced apoptosis may be dependent on the cell type, concentration, incubation time and method of analysis.

The MAPK superfamily consists of three distinct members including ERK, p38 and JNK. It has been proposed that a

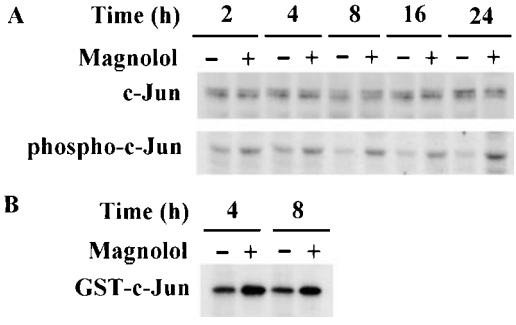

Figure 7 Effect of magnolol on c-Jun and the JNK activity. CH27 cells were treated with 80 μM magnolol for the indicated times. (A) Phosphorylated c-Jun was analysed by Western blot using phospho-c-Jun specific antibody. (B) JNK activity was measured in kinase reaction buffer using GST-c-Jun as substrate.

balance between the ERK and JNK-p38 pathways might play an important role in the regulation of apoptosis (Xia *et al*., 1995), since activation of ERK prevents apoptosis, whereas activation of JNK and p38 promotes it. The data presented here showed that magnolol caused apoptosis in parallel with the inactivation of ERK1/2 and the activation of JNK in human lung squamous carcinoma CH27 cells. Recent reports indicated that inhibition of the ERK signal transduction was required for chemotherapeutic agent-induced apoptosis (Iwama *et al*., 2001; McDaid & Horwitz, 2001; Rice *et al*., 2001). It has also demonstrated that NGF withdrawal led to the induction of apoptosis accompanied by activation of JNK and p38 kinase, and inhibition of ERK (Xia *et al*., 1995). In this report, we found that both ERK1/2 and MEK1/2 (which act upstream of ERK) were inactivated upon magnolol treatment. These results indicated that inhibition of ERK signalling pathway might promote magnolol-induced apoptosis. Although p38 can be activated *via* phosphorylation induced by diverse cellular stress (Minden & Karin, 1997; Xia *et al*., 1995), magnolol did not affect the expression and activation of p38, indicating p38 is not involved in magnolol-induced apoptosis. The JNK pathway can be activated by a variety of cellular stresses including genotoxic stress, free radicals, heat shock, ischaemia and proinflammatory cytokines such as tumour necrosis factor-α and interleukin-1β (Ip & Davis, 1998; Minden & Karin, 1997). When activated, JNK can phosphorylate c-Jun or other transcription factors including ATF-2 and Elk-1 and augment their transcriptional activity (Davis, 2000; Minden & Karin, 1997). Overexpression of a dominant-interfering c-Jun mutant lacking the NH_2 terminus inhibits stress-induced apoptosis, suggesting that phosphorylated c-Jun by JNK may be necessary for the activation of downstream events in the cell death programme (Verheij *et al*., 1996). Growing evidence suggests that JNK activation serves as an important upstream event in the decision to undergo apoptosis (Davis, 2000). Our results showed that magnolol induced a delayed and persistent activation of JNK. This result is consistent with a previous report that γ-radiation induced a delayed and persistent JNK1 activation, the ability of γ-radiation to induce JNK1 activation and cell apoptosis were correlated (Chen *et al*., 1996). Chen *et al*. (1996) suggest that the delay of JNK activation may be due to the time needed for accumulation of cellular damage, the existence of unrepairable damage could be the reason for persistent JNK activation, this aberrant kinase activation may have detrimental effects and lead to cell death. Therefore, the responsiveness of JNK to magnolol suggests the involvement of JNK activation in the initiation of magnolol-induced apoptosis process.

Cancer is currently the most common cause of death in Taiwan (Chen *et al*., 2002). Despite surgery, radiotherapy and traditional chemotherapy, the prognosis of advanced cancer has not significantly improved over the past 10 years (Chen *et al*., 2002). Therefore, the development of novel agents with selectivity against critical targets necessary for the control of apoptosis may provide a rational approach to management of cancers. Conventional Chinese medicines have been time-tested for safety and effectiveness to treat cancers, but the effective component(s) and their molecular action remains unclear. The data presented here showed that magnolol (an active component of Houpo) drives cell death by modulating MAPK family signals, may be these signals become integrated at Bcl-2 family protein containing complexes where the decision to undergo apoptosis is made and signalled to apoptosis effector molecules (caspases), which subsequently cleave key cellular proteins to generate the apoptotic morphology.

This work was supported by Taichung Veterans General Hospital Grant TCVGH-907304C and National Science Council Grant NSC90-2311-B-075A-001, Taiwan, Republic of China. We thank Dr Song-Kun Shyue for supplying Adv-Bcl-2 and control adenoviral vectors.

References

ADAMS, J.M. & CORY, S. (1998). The Bcl-2 protein family: arbiters of cell survival. *Science,* **281,** 1322 – 1326.

BANG, K.H., KIM, Y.K., MIN, B.S., NA, M.K., RHEE, Y.H., LEE, J.P. & BAE, K.H. (2000). Antifungal activity of magnolol and honokiol. *Arch. Pharm. Res.,* **23,** 46 – 49.

BRADFORD, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.,* **72,** 248 – 254.

CHANG, W.S., CHANG, Y.H., LU, F.J. & CHIANG, H.C. (1994). Inhibitory effects of phenolics on xanthine oxidase. *Anticancer Res.,* **14,** 501 – 506.

CHEN, C.J., YOU, S.L., LIN, L.H., HSU, W.L. & YANG, Y.W. (2002). Cancer epidemiology and control in Taiwan: a brief review. *Jpn J. Clin. Oncol.,* **32,** S66 – S81.

CHEN, Y.R., MEYER, C.F. & TAN, T.H. (1996). Persistent activation of c-Jun N-terminal kinase 1 (JNK1) in gamma radiation-induced apoptosis. *J. Biol. Chem.,* **271,** 631 – 634.

DAVIS, R.J. (2000). Signal transduction by the JNK group of MAP kinases. *Cell,* **103,** 239 – 252.

FRIDMAN, J.S., PARSELS, J., REHEMTULLA, A. & MAYBAUM, J. (2001). Cytochrome c depletion upon expression of Bcl-XS. *J. Biol. Chem.,* **276,** 4205 – 4210.

FRIESEN, C., HERR, I., KRAMMER, P.H. & DEBATIN, K.M. (1996). Involvement of the CD95 (APO-1/FAS) receptor/ligand system in drug-induced apoptosis in leukemia cells. *Nat. Med.,* **2,** 574 – 577.

FUJITA, S. & TAIRA, J. (1994). Biphenyl compounds are hydroxyl radical scavengers: their effective inhibition for UV-induced mutation in Salmonella typhimurium TA102. *Free Radic. Biol. Med.,* **17,** 273 – 277.

FULDA, S., SIEVERTS, H., FRIESEN, C., HERR, I. & DEBATIN, K.M. (1997). The CD95 (APO-1/Fas) system mediates drug-induced apoptosis in neuroblastoma cells. *Cancer Res.,* **57,** 3823 – 3829.

GONZALEZ-GARCIA, M., PEREZ-BALLESTERO, R., DING, L., DUAN, L., BOISE, L.H., THOMPSON, C.B. & NUNEZ, G. (1994). Bcl-XL is the major bcl-x mRNA form expressed during murine development and its product localizes to mitochondria. *Development,* **120,** 3033 – 3042.

HAMASAKI, Y., KOBAYASHI, I., ZAITU, M., TSUJI, K., KITA, M., HAYASAKI, R., MURO, E., YAMAMOTO, S., MATSUO, M., ICHIMARU, T. & MIYAZAKI, S. (1999). Magnolol inhibits leukotriene synthesis in rat basophilic leukemia-2H3 cells. *Planta Med.,* **65,** 222 – 226.

HIRANO, T., GOTOH, M. & OKA, K. (1994). Natural flavonoids and lignans are potent cytostatic agents against human leukemic HL-60 cells. *Life Sci.,* **55,** 1061 – 1069.

HO, K.Y., TSAI, C.C., CHEN, C.P., HUANG, J.S. & LIN, C.C. (2001). Antimicrobial activity of honokiol and magnolol isolated from Magnolia officinalis. *Phytother. Res.,* **15,** 139 – 141.

IP, Y.T. & DAVIS, R.J. (1998). Signal transduction by the c-Jun N-terminal kinase (JNK) – from inflammation to development. *Curr. Opin. Cell. Biol.,* **10,** 205 – 219.

IWAMA, K., NAKAJO, S., AIUCHI, T. & NAKAYA, K. (2001). Apoptosis induced by arsenic trioxide in leukemia U937 cells is dependent on activation of p38, inactivation of ERK and the Ca2+-dependent production of superoxide. *Int. J. Cancer,* **92,** 518–526.

KELEKAR, A., CHANG, B.S., HARLAN, J.E., FESIK, S.W. & THOMPSON, C.B. (1997). Bad is a BH3 domain-containing protein that forms an inactivating dimer with Bcl-XL. *Mol. Cell. Biol.,* **17,** 7040–7046.

KHARBANDA, S., PANDEY, P., SCHOFIELD, L., ISRAELS, S., RONCINSKE, R., YOSHIDA, K., BHARTI, A., YUAN, Z.M., SAXENA, S., WEICHSELBAUM, R., NALIN, C. & KUFE, D. (1997). Role for Bcl-xL as an inhibitor of cytosolic cytochrome C accumulation in DNA damage-induced apoptosis. *Proc. Natl. Acad. Sci. U.S.A.,* **94,** 6939–6942.

KIM, C.N., WANG, X., HUANG, Y., IBRADO, A.M., LIU, L., FANG, G. & BHALLA, K. (1997). Overexpression of Bcl-X(L) inhibits Ara-C-induced mitochondrial loss of cytochrome c and other perturbations that activate the molecular cascade of apoptosis. *Cancer Res.,* **57,** 3115–3120.

KLEKOTKA, P.A., SANTORO, S.A., WANG, H. & ZUTTER, M.M. (2001). Specific residues within the alpha 2 integrin subunit cytoplasmic domain regulate migration and cell cycle progression via distinct MAPK pathways. *J. Biol. Chem.,* **276,** 32353–32361.

KONG, C.W., TSAI, K., CHIN, J.H., CHAN, W.L. & HONG, C.Y. (2000). Magnolol attenuates peroxidative damage and improves survival of rats with sepsis. *Shock,* **13,** 24–28.

KONOSHIMA, T., KOZUKA, M., TOKUDA, H., NISHINO, H., IWASHIMA, A., HARUNA, M., ITO, K. & TANABE, M. (1991). Studies on inhibitors of skin tumor promotion, IX. Neolignans from Magnolia officinalis. *J. Nat. Prod.,* **54,** 816–822.

LEE, Y.M., HSIAO, G., CHEN, H.R., CHEN, Y.C., SHEU, J.R. & YEN, M.H. (2001). Magnolol reduces myocardial ischemia/reperfusion injury via neutrophil inhibition in rats. *Eur. J. Pharmacol.,* **422,** 159–167.

LI, P., NIJHAWAN, D., BUDIHARDJO, I., SRINIVASULA, S.M., AHMAD, M., ALNEMRI, E.S. & WANG, X. (1997). Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell,* **91,** 479–489.

LIN, S.Y., CHANG, Y.T., LIU, J.D., YU, C.H., HO, Y.S., LEE, Y.H. & LEE, W.S. (2001). Molecular mechanisms of apoptosis induced by magnolol in colon and liver cancer cells. *Mol. Carcinog.,* **32,** 73–83.

LIN, S.Y., LIU, J.D., CHANG, H.C., YEH, S.D., LIN, C.H. & LEE, W.S. (2002). Magnolol suppresses proliferation of cultured human colon and liver cancer cells by inhibiting DNA synthesis and activating apoptosis. *J. Cell. Biochem.,* **84,** 532–544.

MARUYAMA, Y., KURIBARA, H., MORITA, M., YUZURIHARA, M. & WEINTRAUB, S.T. (1998). Identification of magnolol and honokiol as anxiolytic agents in extracts of saiboku-to, an oriental herbal medicine. *J. Nat. Prod.,* **61,** 135–138.

MCDAID, H.M. & HORWITZ, S.B. (2001). Selective potentiation of paclitaxel (taxol)-induced cell death by mitogen-activated protein kinase kinase inhibition in human cancer cell lines. *Mol. Pharmacol.,* **60,** 290–301.

MINDEN, A. & KARIN, M. (1997). Regulation and function of the JNK subgroup of MAP kinases. *Biochim. Biophys. Acta.,* **1333,** F85–F104.

MINN, A.J., BOISE, L.H. & THOMPSON, C.B. (1996). Bcl-x(S) anatagonizes the protective effects of Bcl-x(L). *J. Biol. Chem.,* **271,** 6306–6312.

MIYAKE, H., HARA, S., ARAKAWA, S., KAMIDONO, S. & HARA, I. (2001). Overexpression of Bcl-2 regulates sodium butyrate- and/or docetaxel-induced apoptosis in human bladder cancer cells both in vitro and in vivo. *Int. J. Cancer,* **93,** 26–32.

NAGASE, H., IKEDA, K. & SAKAI, Y. (2001). Inhibitory effect of magnolol and honokiol from Magnolia obovata on human fibrosarcoma HT-1080. Invasiveness in vitro. *Planta Med.,* **67,** 705–708.

PORTER, A.G. & JANICKE, R.U. (1999). Emerging roles of caspase-3 in apoptosis. *Cell Death Differ.,* **6,** 99–104.

RICE, P.L., GOLDBERG, R.J., RAY, E.C., DRIGGERS, L.J. & AHNEN, D.J. (2001). Inhibition of extracellular signal-regulated kinase 1/2 phosphorylation and induction of apoptosis by sulindac metabolites. *Cancer Res.,* **61,** 1541–1547.

SANDSTROM, P.A. & BUTTKE, T.M. (1993). Autocrine production of extracellular catalase prevents apoptosis of the human CEM T-cell line in serum-free medium. *Proc. Natl. Acad. Sci. U.S.A.,* **90,** 4708–4712.

SCARPATI, M.D., VICIDOMINI, G. & SANTINI, M. (2001). Gemcitabine and small cell lung cancer. *Lung Cancer,* **34,** 313–314.

SCHAEFFER, H.J. & WEBER, M.J. (1999). Mitogen-activated protein kinases: specific messages from ubiquitous messengers. *Mol. Cell. Biol.,* **19,** 2435–2444.

SHINOURA, N., YOSHIDA, Y., NISHIMURA, M., MURAMATSU, Y., ASAI, A., KIRINO, T. & HAMADA, H. (1999). Expression level of Bcl-2 determines anti- or proapoptotic function. *Cancer Res.,* **59,** 4119–4128.

TENG, C.M., KO, F.N., WANG, J.P., LIN, C.N., WU, T.S., CHEN, C.C. & HUANG, T.F. (1991). Antihaemostatic and antithrombotic effect of some antiplatelet agents isolated from Chinese herbs. *J. Pharm. Pharmacol.,* **43,** 667–669.

TSAI, T.H., TSAI, W.J., CHOU, C.J. & CHEN, C.F. (1995). Magnolol inhibits collagen-induced platelet serotonin release. *Thromb. Res.,* **78,** 265–270.

VAUX, D.L. & KORSMEYER, S.J. (1999). Cell death in development. *Cell,* **96,** 245–254.

VERHEIJ, M., BOSE, R., LIN, X.H., YAO, B., JARVIS, W.D., GRANT, S., BIRRER, M.J., SZABO, E., ZON, L.I., KYRIAKIS, J.M., HAIMOVITZ-FRIEDMAN, A., FUKS, Z. & KOLESNICK, R.N. (1996). Requirement for ceramide-initiated SAPK/JNK signalling in stress-induced apoptosis. *Nature,* **380,** 75–79.

WANG, J.P., HSU, M.F., RAUNG, S.L., CHEN, C.C., KUO, J.S. & TENG, C.M. (1992). Anti-inflammatory and analgesic effects of magnolol. *Naunyn Schmiedebergs Arch. Pharmacol.,* **346,** 707–712.

WATANABE, K., WATANABE, H., GOTO, Y., YAMAGUCHI, M., YAMAMOTO, N. & HAGINO, K. (1983). Pharmacological properties of magnolol and honokiol extracted from Magnolia officinalis: central depressant effects. *Planta Med.,* **49,** 103–108.

WATANABE, K., WATANABE, H.Y., GOTO, Y., YAMAMOTO, N. & YOSHIZAKI, M. (1975). Studies on the active principles of magnolia bark. Centrally acting muscle relaxant activity of magnolol and honokiol. *Jpn J. Pharmacol.,* **25,** 605–607.

WORDEN, F.P. & KALEMKERIAN, G.P. (2000). Therapeutic advances in small cell lung cancer. *Expert. Opin. Investig. Drugs,* **9,** 565–579.

XIA, Z., DICKENS, M., RAINGEAUD, J., DAVIS, R.J. & GREENBERG, M.E. (1995). Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science,* **270,** 1326–1331.

YANG, C.H., TSAI, C.M., WANG, L.S., LEE, Y.C., CHANG, C.J., LUI, L.T., YEN, S.H., HSU, C., CHENG, A.L., LIU, M.Y., CHIANG, S.C., CHEN, Y.M., LUH, K.T., HUANG, M.H., YANG, P.C. & PERNG, R.P. (2002). Gemcitabine and cisplatin in a multimodality treatment for locally advanced non-small cell lung cancer. *Br. J. Cancer,* **86,** 190–195.

YANG, E., ZHA, J., JOCKEL, J., BOISE, L.H., THOMPSON, C.B. & KORSMEYER, S.J. (1995). Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death. *Cell,* **80,** 285–291.

YANG, J., LIU, X., BHALLA, K., KIM, C.N., IBRADO, A.M., CAI, J., PENG, T.I., JONES, D.P. & WANG, X. (1997). Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. *Science,* **275,** 1129–1132.

ZOU, H., HENZEL, W.J., LIU, X., LUTSCHG, A. & WANG, X. (1997). Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3. *Cell,* **90,** 405–413.

(Received July 16, 2002
Revised September 24, 2002
Accepted September 30, 2002)