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Down-modulation of Bcl-X_L, release of cytochrome *c* and sequential activation of caspases during honokiol-induced apoptosis in human squamous lung cancer CH27 cells

Shu-Er Yang[a], Ming-Tsuen Hsieh[a], Tung-Hu Tsai[b], Shih-Lan Hsu[c,*]

[a]*Institute of Chinese Pharmaceutical Sciences, China Medical College Taichung, Taiwan, ROC*
[b]*National Research Institute of Chinese Medicine, Taipei 11221, Taiwan, ROC*
[c]*Department of Education and Research, Taichung Veterans General Hospital, No. 160, Section 3, Chung-Gang Road, Taichung 40705, Taiwan, ROC*

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Abstract

Honokiol is a phenolic compound purified from *Magnolia officinalis*, which induced the apoptotic cell death in several types of human cancer cells. In the present study, the molecular mechanism of honokiol-mediated apoptotic process was examined in human squamous lung cancer CH27 cells. Here, we found that honokiol-induced apoptotic cell death was accompanied by upregulation of Bad and downregulation of Bcl-X_L, while honokiol had no effect on the levels of Bcl-2, Bcl-X_S, Bag-1, Bax and Bak proteins. Moreover, honokiol treatment caused the release of mitochondrial cytochrome *c* to cytosol and sequential activation of caspases. Proteolytic activation of caspase-3 and cleavage of PARP, an *in vivo* substrate for caspase-3, were observed in honokiol-treated CH27 cells. Furthermore, treatment with caspase inhibitors z-DEVD-fmk and z-VAD-fmk markedly blocked honokiol-induced apoptosis. These results demonstrated that modulation of Bcl-X_L and Bad proteins, release of mitochondrial cytochrome *c* and activation of caspase-3, participated in honokiol-triggered apoptotic process in human squamous lung cancer CH27 cells.

Keywords: Apoptosis; Bad; Bcl-X_L; Caspase; Cytochrome *c*; Honokiol

1. Introduction

Traditional Chinese medicinal herbs are widely known to be effective in the treatment of many diseases. The root and stem bark of *Magnolia officinalis* (Chinese name: Houpo) has been used as a folk medicine by the Chinese people for the treatment of thrombotic stroke, gastrointestinal complaints, anxiety and nervous disturbance [1]. Honokiol, one of the major phenolic constituents of magnolia bark [2,3], has several pharmacological effects such as anti-oxidant [4], antithrombosis [5], antibacterial [6], xanthine oxidase inhibition [7], and anxiolytic effect [8]. Previous reports have demonstrated that honokiol exhibited remarkable inhibitory effects on mouse skin tumor promotion in an *in vivo* two-stage carcinogenesis [9], and inhibited the growth of human leukemic HL-60 cells [10]. Moreover, honokiol has induced apoptosis as characterized morphologically by DNA fragmentation and apoptotic bodies in human lymphoid leukemia Molt 4B cells [11].

Apoptosis is an essential physiological process required for normal development and maintenance of tissue homeostasis [12]. Insufficient or excessive cell death can contribute to human diseases, including cancer, acquired immunodeficiency syndrome and some neurodegenerative disorders [13]. Apoptosis is genetically regulated, as best demonstrated by studies in the nematode *Caenorhabditis elegans* [14,15]. Three genes necessary for life or death of cells in *C. elegans* are *Ced-3*, *Ced-4* and *Ced-9*. *Ced-9* prevents cell death, while *Ced-3* and *Ced-4* are required for

* Corresponding author. Tel.: +886-4235-925-25x4037; fax: +886-4235-927-05.
E-mail address: h2326@vghtc.vghtc.gov.tw (S.-L. Hsu).

Abbreviations: Adv, adenovirus; DAPI, 4′,6-diamidino-2-phenylindole; PARP, poly(ADP-ribose) polymerase; TUNEL, terminal transferase-mediated dUTP-fluorescensin nick end-labeling; YVAD-AFC, Tyr-Val-Ala-Asp-7-amino-4-trifluoromethyl coumarin; VDVAD-AFC, Val-Asp-Val-Ala-Asp-7-amino-4-trifluoromethyl coumarin; DEVD-AFC, Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin; VEID-AFC, Val-Glu-Ile-Asp-7-amino-4-trifluoromethyl coumarin; IETD-AFC, Ile-Glu-Thr-Asp-7-amino-4-trifluoromethyl coumarin; LEHD-AFC, Leu-Glu-His-Asp-7-amino-4-trifluoromethyl coumarin; z-YVAD-fmk, z-Tyr-Val-Ala-Asp-fluoromethyl ketone; z-DEVD-fmk, z-Asp-Glu-Val-Asp-fluoromethyl ketone; z-VAD-fmk, z-Val-Ala-Asp-fluoromethyl ketone.

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death to occur in cells programmed to die [16]. The *Ced-9* gene product exhibits functional and structural homology to the mammalian Bcl-2 proto-oncogene product. The Bcl-2 and related proteins are key regulators of apoptosis. Some Bcl-2 family members such as Bax, Bad, Bid, Bak, Bcl-X_S, Bik, Bim, Blk, Hrk promote cell death while others inhibit cell death including Bcl-2, Bcl-X_L, Bcl-w, Mcl-1, A-1 [17]. Bcl-2 family members form homo- or hetero-dimers and balance between anti- and pro-apoptotic Bcl-2 family proteins may act as a rheostat for the apoptotic program [18,19].

Ced-3 has homology to a family of at least 14 mammalian enzymes, called caspase [20], which plays a critical role in the execution phase of apoptosis and are responsible for many of the biochemical and morphological changes associated with apoptosis [21,22]. Caspases are normally present in the cell in an inactive proform, and during apoptosis they are preteolytically processed to the active form, usually by other caspases. Caspases may be divided into ''initiator'' caspases with long prodomains, (e.g. caspase-8, -9, and -10), which activate ''effector'' caspases with short prodomains (e.g. caspase-3, -6 and -7), which cleave intracellular substrates, such as PARP, Bcl-2, focal adhesion kinase (FAK), inhibitor of caspase-activated deoxyribonuclease (ICAD), gelsolin, and lamin, cleavage of these proteins results in deregulation of their activity during the execution phase of apoptosis [21,23–25].

Recently evidence is emerging that mitochondria participate in the central control or executioner phase of the cell death cascade [26–28]. Cytochrome *c*, which is usually present in the mitochondrial intermembrane space, is released into the cytosol following the induction of apoptosis by many different stimuli including Fas ligand, tumor necrosis factor (TNF), and chemotherapeutic and DNA damaging agents [26,29]. In the cytosol, cytochrome *c* forms a complex with Apaf-1 and procaspase-9 which is then capable of activating procaspase-3 [27]. Cytochrome *c* release from the mitochondria has been shown to be an important mechanism of caspase activation in a number of systems [26,30,31]. Although the mechanism of cytochrome *c* release is not yet understood, it may be mediated by pro-apoptotic members of the Bcl-2/*Ced-9* family.

Proteins of the Bcl-2 family together with caspase, mitochondria, and cytochrome *c* have, among others been identified as essential components of the intracellular apoptotic signaling pathways. Little is known about the cellular and molecular mechanisms of honokiol-induced apoptosis process in human cancer cells. In the current study, the early biochemical events triggered by honokiol and their role in apoptosis have been characterized in human squamous lung cancer CH27 cells. We present evidence that honokiol activates a complex signaling pathway required for cell death induction. In particular, an early downregulation of Bcl-X_L, release of cytochrome *c* from mitochondria into cytosol and the sequential activation of caspases were found to occur in honokiol-induced apoptosis. Activation of caspase-1, -2, -3, -8 and -9 seems to be of pivotal importance in honokiol-induced apoptosis, a process that can be partially blocked by specific and cell-permeable caspase inhibitors.

2. Materials and methods

2.1. Reagents

Honokiol was extracted from magnolia bark [2]. The drug was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 40 mM. Antibodies to various proteins were obtained from the following sources: Bcl-2, Bcl-X_{L-S}, Bad, Bag-1 and Bax were purchased from Santa Cruz Biotechnology; Bak, PARP and cytochrome *c* were purchased from PharMingen. The recombinant Bcl-2-adenoviral and control adenoviral vectors were kindly provided by Song-Kun Shyue at Institute of Biomedical Sciences, Academia Sinica. Caspase-3 was purchased from Transduction Laboratory. DAPI was obtained from Sigma Chemical Company; anti-mouse and anti-rabbit IgG peroxidase-conjugated secondary antibody were purchased from Amersham. Caspase activity assay kits, including the 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. Caspase inhibitors, including caspase-1 inhibitor (z-YVAD-fmk), caspase-3 inhibitor (z-DEVD-fmk) and the broad-spectrum caspase inhibitor (z-VAD-fmk), were obtained from KAMIYA Biomedical Company.

2.2. Cell culture and cell viability assay

Human lung squamous cell line CH27 was kindly provided by Taipei Veterans General Hospital. Human lung non-small cell carcinoma cell lines of H460 and H1299 were purchased from American Type Culture Collection (ATCC). Human fibroblast-like lung cell line WI-38 was purchased from Food Industry Research and Development Institute (FIRDI). Human hepatoma Hep3B, Hep3B/Bcl-2 cells, human cervical carcinoma HeLa-S3 and HeLa-S3/Bcl-2 cells were kindly provided by S.L. Hsieh of Yang Ming Medical College. All the cell lines were maintained in Dulbecco's modified Eagle's medium (Flow Laboratories) except WI-38 (maintained in RPMI medium), supplemented with 5% fetal bovine serum, 2 mM glutamine, and antibiotics (100 unit/mL penicillin and 100 μg/mL streptomycin), at 37° in a humidified atmosphere of 5% CO_2. The medium was changed every 2 days. The cells were seeded on culture plates or coverslips. After 24 hr, cells were treated with 40 μM honokiol for the indicated time periods. Some cells were infected with recombinant Bcl-2-adenoviral and control adenoviral vectors for 24 hr prior to exposure to honokiol. Or cells were treated for 2 hr with caspase inhibitors prior to

exposure to honokiol. For viability assay, cells were seeded at a density of 1×10^5 cells per well onto a 12-well plate 24 hr before drug treatment. Honokiol was added to medium, at various times and concentrations. The control cultures were treated with vehicle (0.1% DMSO). After incubation, cells were washed with PBS then detached by trypsin. The number of viable cells was determined by staining cell populations with Trypan blue. One part of 0.2% Trypan blue dissolved in PBS was added to one part of the cell suspension, and the number of unstained (viable) cells was counted. Dose (or time course) response curve was plotted for honokiol, and a concentration (or time) which gave 50% cell death (LC_{50} or LT_{50}) was calculated.

2.3. Cytochemical staining of apoptotic cells

Cells were seeded on coverslips at a density of 1×10^5 cells per well onto a 12-well plate 24 hr before drug treatment. CH27 cells were treated with 40 μM honokiol for 24 hr. Control cultures were treated with vehicle (0.1% DMSO). For morphological detection of apoptotic nuclei, cells were washed with cold PBS following fixation in 2% paraformaldehyde at room temperature for 30 min. Then the cells were washed twice with PBS, and maintained in PBS solution containing 0.1% Triton X-100, at room temperature for another 30 min. Samples were subsequently incubated in DAPI (1 μg/mL) solution at room temperature for 30 min, washed with PBS and examined under a fluorescence microscope within 24 hr. *In situ* DNA fragmentation was performed by the terminal transferase-mediated dUTP-fluorescensin nick end-labeling (TUNEL) technique according to the manufacturer's instructions (Boehringer Mannheim). Cells were fixed and permeabilized as described above, then exposed to terminal transferase reaction mixture (34 mU/mL terminal transferase, 280 pmol of dATP, 90 pmol of fluorescein-11-dUTP, 30 mM Tris–HCl, 140 mM sodium cacodylate, 1 mM $CaCl_2$, pH 7.2) for 1 hr at 37° in the dark. Cells were subsequently washed with PBS and examined under a fluorescence microscope.

2.4. DNA fragmentation assay

CH27 cells were seeded at a density of 3×10^6 cells onto 10-cm dishes 24 hr before drug treatment, then cells were treated with 40 μM honokiol for 24 hr. Control cultures were treated with vehicle (0.1% DMSO). DNA fragmentation was assayed by the modified procedure of Sandstrom and Buttke [32]. Briefly, all cells (both attached and nonattached) were collected and resuspended in 400 μL of 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, and then centrifuged. Proteinase K (200 μg/mL) was added to the supernatant, which was then incubated at 50° for 2 hr, followed by the addition of 100 μg/mL RNaseA and further incubation at 37° for 2 hr. Fragmented DNA was extracted with phenol/chloroform, and precipitated at −20° with ethanol/sodium acetate. The DNA fragments were electrophoresed on a 2% agrose gel containing 0.1 μg/mL ethidium bromide.

2.5. Protein preparation and Western blot analysis

CH27 cells were seeded at a density of 3×10^6 cells onto 10-cm dish 24 hr before drug treatment, cells were treated with 40 μM honokiol at various time periods. Control cultures were treated with vehicle (0.1% DMSO). After treatment, adherent and floating cells were collected at the indicated times and washed twice in ice-cold PBS. Cell pellets were resuspended in modified protein lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5 mM dithiothreitol, 1% NP-40, 0.3% deoxycholate, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 10 μg/mL soybean trypsin inhibitor, 0.5 mM phenylmethylsulfonylfluoride) for 30 min at 4°. Lysates were clarified by centrifugation at 100,000 *g* for 30 min at 4° and the resulting supernatant was collected, aliquoted (50 μg per tube), and stored at −80° until assay. The protein concentrations were estimated with the Bradford method [33]. For Western blot analysis, samples were separated by various appropriate concentrations of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (8%, PARP; 12%, Bcl-2, Bcl-X_{L-S}, Bag-1, Bax, Bad, Bak, caspase-3; 15%, cytochrome *c*). Then SDS-separated proteins were equilibrated in transfer buffer (50 mM Tris, pH 9.0–9.4, 40 mM glycine, 0.375% SDS, 20% methanol) and electrotransferred to PVDF membrane (Millipore). The membrane was blocked with a solution containing 5% nonfat dry milk in TBST buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl and 0.1% Tween 20) for 1 hr and washed with TBST buffer. For the detection of proteins on PVDF membrane, antibodies to Bcl-2, Bcl-X_{L-S}, Bag-1, Bax, Bad, Bak (0.2 μg/mL), PARP, caspase-3 and cytochrome *c* were diluted 500-, 500-, 500-, 500-, 250-, 5000-, 1000-, 1000- and 500-fold, respectively. After exposure to horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (for Bcl-X_{L-S}, Bak, Bax and Bag-1) or HRP-conjugated goat anti-mouse IgG (for Bcl-2, Bad, caspase-3, PARP and cytochrome *c*) secondary antibody (diluted 20,000-fold) for 1 hr, blots were washed and developed by enhanced chemiluminescence (ECL Kits; Amersham Life Science).

2.6. Caspase activity assay

CH27 cells were seeded at a density of 3×10^6 cells onto 10-cm dish 24 hr before drug treatment. Honokiol was added to medium with 40 μM honokiol at various time periods. Control cultures were treated with vehicle (0.1% DMSO). 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 aprotinin,

1 μg/mL leupeptin) for 20 min at 4° followed by centrifugation (100,000 *g*) for 30 min. Caspase activities were assayed in 50 μL reaction mixtures with fluorogenic report 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 substrate peptide (200 μM) was incubated at 37° with cytosolic extract (5–20 μg of total protein) in reaction buffer (100 mM HEPES, 10% sucrose, 10 mM dithiothreitol, 0.1% 3-[(3-cholamidopropyl) dimethylammoniol] 1-propanesulfonate (CHAPS)). Fluorescence was measured after 2 hr (excitation wavelength, 400 nm; emission wavelength, 505 nm) with a fluorescence plate reader (Fluoroskan Ascent; Labsystems).

2.7. Preparation of cytosolic protein extracts for assessment of cytochrome c

To evaluate mitochondrial cytochrome *c* release, cytosolic protein extracts (S-100) were obtained. Briefly, CH27 cells were seeded at a density of 3×10^6 cells onto 10-cm dish 24 hr before drug treatment. Honokiol was added to medium with 40 μM honokiol, and control cultures were treated with vehicle (0.1% DMSO). At various time points following exposure to honokiol, cells were washed twice with PBS. The cell pellets were resuspended in 100 μL ice-cold buffer containing 20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol and 1 mM PMSF. The cells were allowed to swell on ice and centrifuged at 100,000 *g* for 30 min, then the supernatant was stored at −80° until further analysis.

2.8. Analysis of data

All data are presented in this report as mean ± SD of 12 replicates from four separate experiments. Statistical differences were evaluated using the Student's *t*-test and significance at the $P < 0.001$, $P < 0.01$, $P < 0.05$ levels were considered. All the figures shown in this article were obtained from at least four independent experiments with similar pattern.

3. Results

3.1. Cytocidal effect of honokiol

Treatment of CH27, H460, H1299 human lung cancer cells and WI-38 human fibroblast-like lung cells with honokiol resulted in a dose- and time-dependent cytotoxicity. As shown in Fig. 1A, honokiol-mediated cytotoxicity occurs at a concentration greater than 40 μM for 24 hr. A significant decrease in cell number was seen in the cells treated with honokiol at 40 μM (less than 40% cells survived). The concentration of honokiol leading to a 50% decrease in cell number (LC_{50}) was about 40.9, 41.4, 34.7 and 60.5 μM for CH27, H460, H1299 and WI-38 cells, respectively. Moreover, treatment of lung cancer cell lines (CH27, H460, H1299) and human fibroblast-like lung cell line (WI-38) with 10, 20 and 30 μM resulted in growth inhibition but not cell death, approximately 10–30% growth inhibition was detected after 96 hr incubation (data not shown). Thus, we chose 40 μM concentration of honokiol to detect changes in molecular events during the following experiments using CH27 cells. To examine the kinetics of cell death induced by honokiol treatment, four tested cells were treated with 40 μM honokiol for varying lengths of time (4, 8, 16, 24, 36 and 48 hr). As indicated in Fig. 1B, the cytocidal effect was rapidly observed at 4 hr honokiol treatment. The LT_{50} (50% cell lethal time) was 21.3, 24.2, 20.4 and 32.2 hr for CH27, H460, H1299 and WI-38 cells, respectively. The results showed that human fibroblast-like lung WI-38 cells were more resistant to the honokiol-mediated cytotoxicity than three tested lung cancer cells. Observations of honokiol-treated cells under a inverted phase microscope revealed that cells exhibited morphological features of apoptosis (Fig. 1C), rounded and granulated morphology, and eventually detached from culture plates.

3.2. Honokiol induces apoptosis

To obtain further support for the induction of apoptosis by honokiol in cells, *in situ* DAPI staining, TUNEL assay and DNA fragmentation analysis were performed in CH27 cells. As depicted in Fig. 2A, honokiol induced the formation of condensed and segmented nuclei in DAPI-stained CH27 cells. TUNEL assay on the adherent cells revealed that 40 μM honokiol treatment gave green fluorescence under many positive cells after 24 hr, suggesting that the DNA fragmentation was occurring in these cells (Fig. 2A). In addition, treatment with honokiol resulted in degradation of chromosomal DNA into small internucleosomal fragments, evidenced by the formation of a 180–200 bp DNA ladder on agarose gels (Fig. 2B), a hallmark of cell undergoing apoptosis. No DNA ladders were detected in the samples isolated from control cultures. These results indicated that honokiol induced an apoptotic cell death in these cells.

3.3. Effects of honokiol on the expression of Bcl-2 family proteins

The Bcl-2 family proteins is an important regulator of apoptotic pathways [17]. An increasing number of proteins are being reported that either inhibit or promote apoptosis. Bcl-2 and Bcl-X_L have been shown to inhibit apoptosis, whereas Bad, Bcl-X_S and Bax have been reported to enhance apoptosis [17]. To elucidate whether Bcl-2 family molecules were modulated in honokiol-induced apoptosis, we examine the expression of several members of this family by Western blot analysis. As shown in Fig. 3,

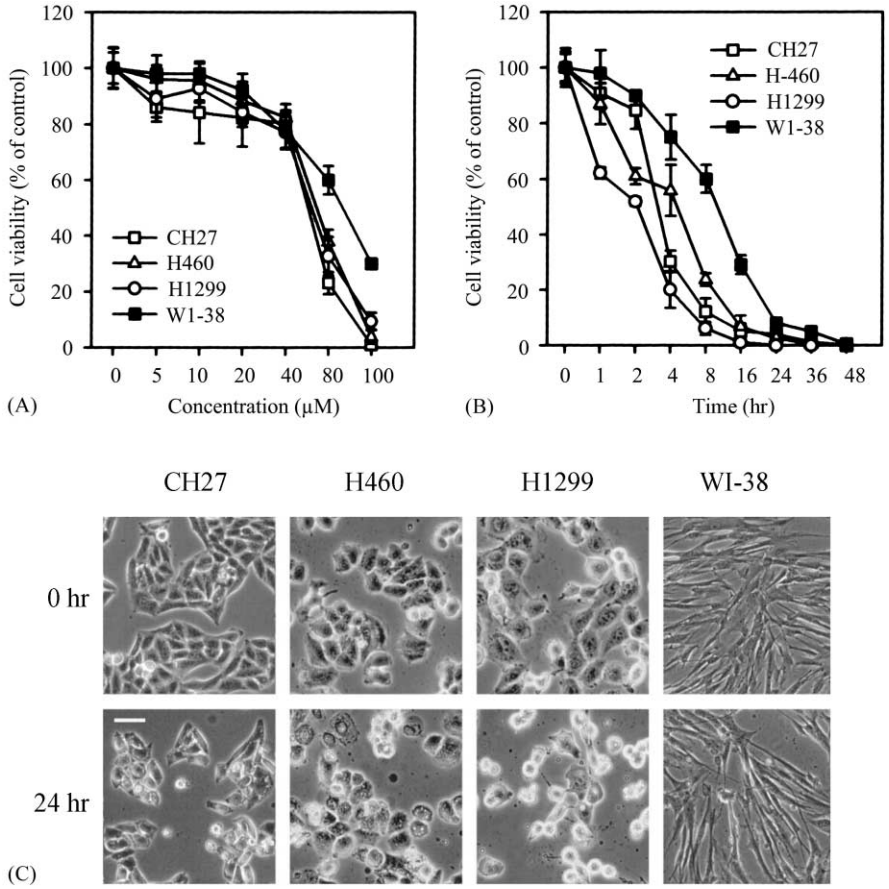

Fig. 1. Cytocidal effect of honokiol. (A) Dose-dependent response: CH27, H460, H1299 and WI-38 cells were treated with a series concentration of honokiol for 24 hr. Cell viability was estimated using direct counting cell number by Trypan blue dye exclusion method. (B) Time-dependent response: CH27, H460, H1299 and WI-38 cells were treated with or without 40 μM honokiol for 4, 8, 16, 24, 36 and 48 hr. After the indicated time periods, cell viability was determined. (C) Morphological changes: CH27, H460, H1299 and WI-38 cells were treated with or without 40 μM honokiol. Phase contrast micrographs were shown for 24 hr treatment (original magnification 200×; scale bar, 20 μm).

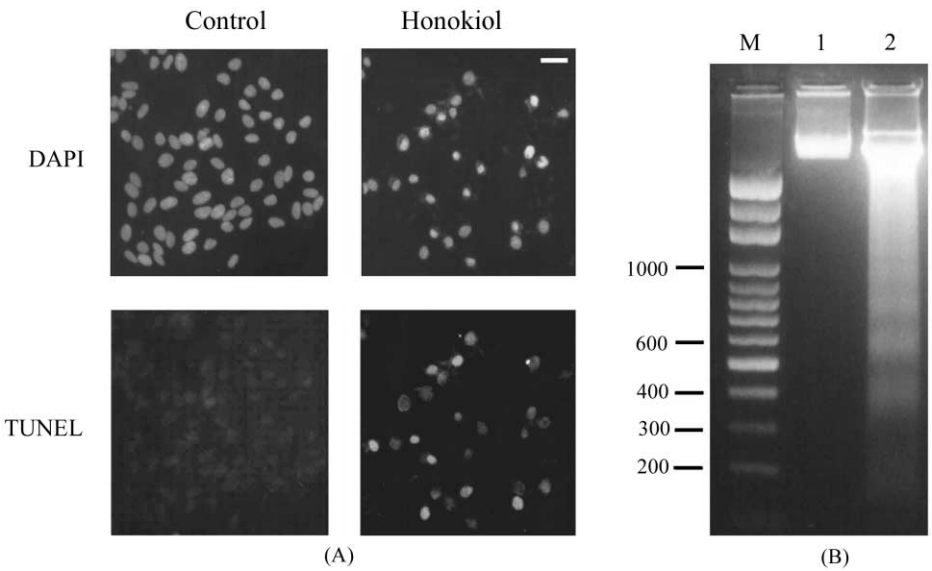

Fig. 2. Induction of apoptosis by honokiol. (A) Apoptotic nuclei: CH27 cells were treated with 40 μM honokiol for 24 hr, then fixed and stained with DAPI, or using TUNEL assay, and investigated under a fluorescence microscope (original magnification 200×; scale bar, 20 μm). (B) DNA fragmentation: adherent and floating cells were collected 24 hr after 40 μM honokiol treatment. DNA was isolated and separated on a 2% agarose gel. M, size marker (100 base-pair DNA ladder); lane 1, control culture; lane 2, 40 μM honokiol treatment.

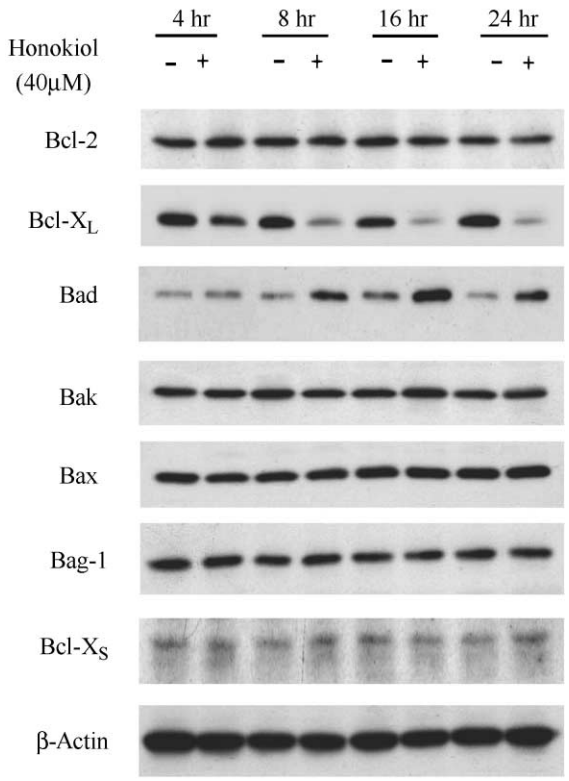

Fig. 3. Effect of honokiol on the expression of Bcl-2 family proteins. CH27 cells were treated with 40 μM honokiol for 4, 8, 16 and 24 hr. After treatment, cell lysates were extracted, and the level of Bcl-2 family proteins were analyzed by Western blot analysis. β-Actin was used as an internal loading control.

exposure of CH27 cells to 40 μM honokiol resulted in downregulation of Bcl-X_L expression. In contrast, honokiol significantly upregulated the expression of Bad, while did not alter the expression level of Bcl-2, Bcl-X_S, Bag-1, Bax and Bak proteins (Fig. 3).

3.4. Effect of Bcl-2 overexpression on honokiol-induced apoptosis

Recent reports have been suggested that expression level of Bcl-2 determines anti- or pro-apoptotic function [34]. To examine whether overexpression of Bcl-2 shows an anti- or pro-apoptotic effect on honokiol-induced apoptosis, Bcl-2 overexpressed cell lines Hep3B/Bcl-2 (human hepatoma cells) and HeLa-S3/Bcl-2 (human cervical carcinoma cells) were used. As depicted in Fig. 4, overexpression of Bcl-2 in these cell lines were determined by Western blot analysis. In both cell lines expression of Bcl-2 at a high level accelerated honokiol-induced apoptosis. To evaluate the effect of Bcl-2 protein on honokiol-induced apoptosis in human lung cancer cells, we infected CH27 cells with 50 moi (multiplicity of infection) Bcl-2-adenoviral and control adenoviral vectors. The expression of Bcl-2 protein was analyzed by Western blot analysis and the resulting cytotoxicity was assayed by cell number counting. Results showed that the expression level of Bcl-2 protein was increased by Adv-Bcl-2 infection but not by control adenovector infection (Fig. 4). Moreover, overexpression of Bcl-2 protein by Adv-Bcl-2 vector infection significantly protected CH27 cells against honokiol-induced apoptosis.

3.5. Involvement of caspase activation in honokiol-induced apoptosis

Activation of caspases is a central mechanism of apoptosis, and caspases are considered to be the ‘‘executioners’’

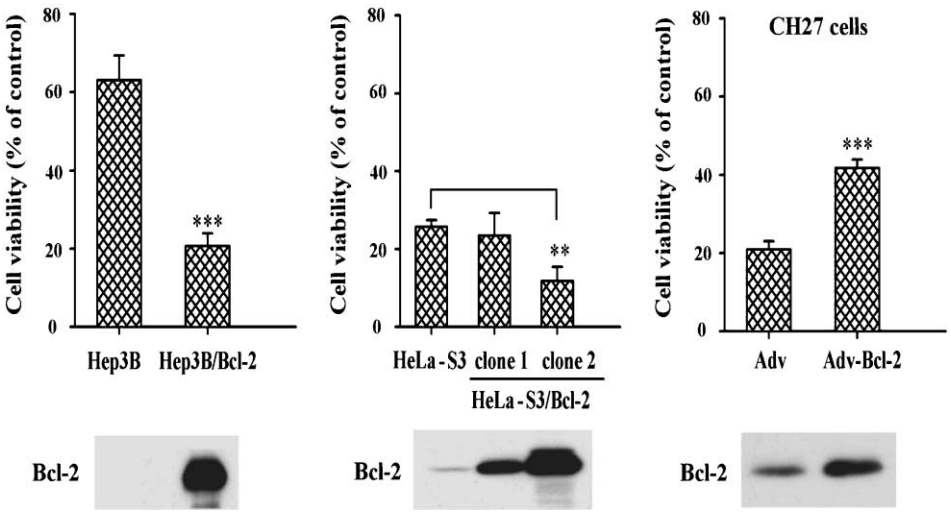

Fig. 4. Effect of Bcl-2 overexpression on honokiol-induced apoptosis, Bcl-2 expression and cell viability. Total proteins were extracted from Hep3B, Hep3B/Bcl-2, HeLa-S3 and HeLa-S3/Bcl-2. The expression levels of Bcl-2 were determined by Western blot analysis. Cells were treated with 40 μM honokiol for 24 hr and cell viability was determined by direct cell number counting. $^{***}P < 0.001$, compared with the 40 μM honokiol-treated Hep3B cell group. $^{**}P < 0.01$, compared with the 40 μM honokiol-treated HeLa-S3 cell group. CH27 cells were infected with Adv-Bcl-2 and Adv-vector for 24 hr, and then total proteins were extracted. The expression levels of Bcl-2 were determined by Western blot analysis. CH27 cells were infected for 24 hr, and then treated with 40 μM honokiol. After 48 hr treatment, cell viability was determined by direct cell number counting. $^{***}P < 0.001$, compared with the 40 μM honokiol-treated CH27 cell group.

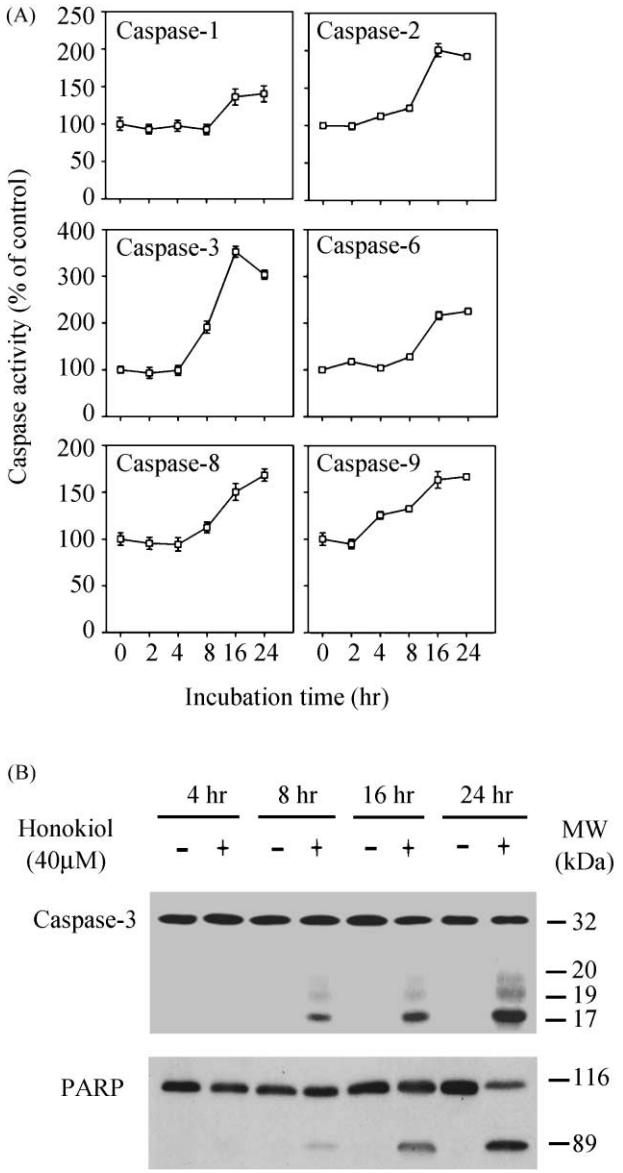

Fig. 5. Effect of honokiol on caspase activation. CH27 cells were treated with 40 μM honokiol for the indicated time periods. Extracts from untreated or honokiol-treated cells were assayed for (A) caspase proteolytic activities using fluorogenic peptide substrates, or (B) analyzed for proteolytic cleavage of caspase-3 and PARP by Western blot analysis.

of cell death [35]. To determine whether caspase family proteases are activated in the apoptotic process induced by honokiol, the proteolytic activity of specific caspase was measured using fluorogenic peptide substrates. As shown in Fig. 5A, in CH27 cells, honokiol treatment caused a time-dependent activation of caspase-1, -2, -3, -6, -8 and -9 proteolytic activities. Activation of caspase-9 was first observed 4 hr after honokiol treatment. Moreover, after 8 hr there was a marked increase in caspase-3 activity, reaching a maximum after 16 hr of honokiol treatment. Whereas, caspase-1, -2, -6 and -8 were significantly activated at 16 hr honokiol treatment. These results suggest that caspase cascade may be responsible for apoptotic characteristics induced by honokiol.

Since caspase-3 is a central effector of apoptosis, and was drastically activated in honokiol-treated CH27 cells, we next examined the proteolytic processing of caspase-3 and its downstream target PARP protein by Western blot analysis. As shown in Fig. 5B, in control vehicle-treated CH27 cells, only the intact ~32 kDa caspase-3 proform was observed. Whereas, induction of honokiol-mediated apoptosis resulted in loss of the proform of caspase-3 and appearance of two processing fragments, ~17 and 19 kDa, following initial cleavage at Asp-175 and then at Asp-9 and Asp-28 [36], processing was first detected after 8 hr treatment with honokiol, and markedly increased at 24 hr. PARP was identified *in vivo* substrate for caspase-3, and cleavage of PARP is a commonly used measure of caspase-3 enzyme activity. Fig. 5B showed that PARP proform (molecular mass 116 kDa) was cleaved to give a ~89 kDa fragments in honokiol-treated cells, but not in control cultures. Taken together, the proteolytic processing of caspase-3 and PARP was consistent with a similar time-dependent activation of caspase-3 enzyme activity (Fig. 5A and B). To further evaluate the role of caspase in honokiol-mediated apoptotic process, we used highly specific cell-permeable inhibitor of caspases to provide further evidence of involvement of specific caspase in the execution phase of honokiol-mediated apoptotic process. CH27 cells were pretreated with caspase inhibitors for 2 hr, and then induced to undergo apoptosis by treatment with honokiol. Results indicated that administration of CH27 cells with caspase inhibitors alone did not affect the cell viability (data not shown). However, z-DEVD-fmk (caspase-3 inhibitor) and z-VAD-fmk (broad-spectrum caspase inhibitor), significantly inhibited honokiol-induced caspase-3 activation (data not shown) and cell death in CH27 cells (Fig. 6), suggesting the involvement of caspase-3-like activity in honokiol-induced apoptotic

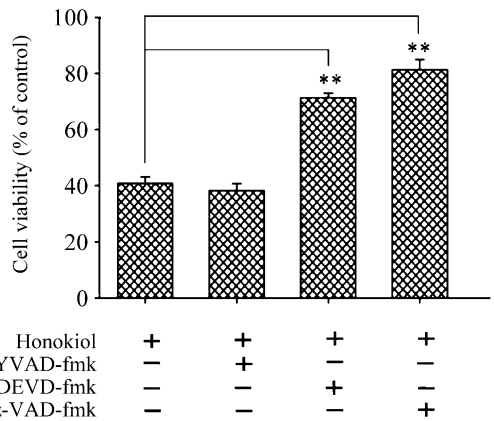

Fig. 6. Inhibition of honokiol-induced apoptotic cell death by the caspase inhibitors. CH27 cells were treated for 24 hr with vehicle (0.1% DMSO, control) or with 40 μM honokiol following pretreatment for 2 hr in the presence or absence of 100 μM caspase inhibitors (z-YVAD-fmk, z-DEVD-fmk and z-VAD-fmk). After treatment, cell viability was determined. $^{**}P < 0.01$, compared with the 40 μM honokiol-treated CH27 cell group.

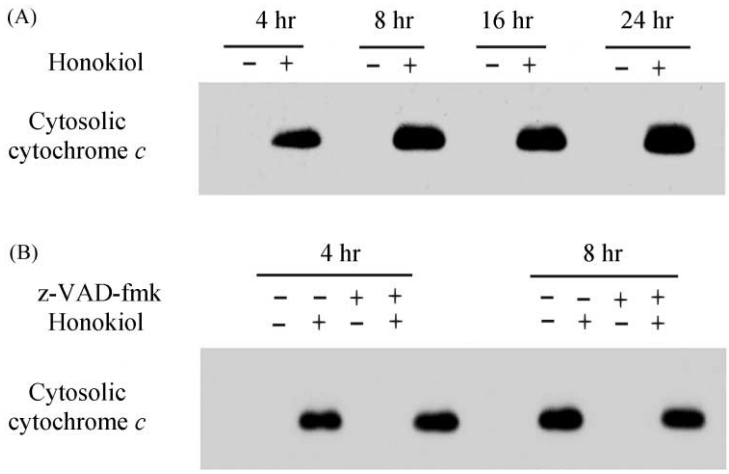

Fig. 7. The effects of honokiol and z-VAD-fmk on mitochondrial cytochrome *c* release. (A) CH27 cells were treated with vehicle or with 40 μM honokiol for the indicated time points. After treatment, cytosolic fractions were isolated and analyzed by Western blot analysis using cytochrome *c* specific antibody. (B) CH27 cells were pretreated for 2 hr of 100 μM z-VAD-fmk, then treated with vehicle or with 40 μM honokiol for 4 and 8 hr, then cytosolic extracts were analyzed by Western blot analysis for cytochrome *c*.

process. In contrast, a caspase-1 inhibitor (z-YVAD-fmk) had no effect at similar concentration (Fig. 6).

3.6. Release of cytochrome c occurs prior to the activation of caspase

Recently studies have implied a key role for the release of mitochondrial cytochrome *c* in activation of caspase in apoptosis [26,29,31]. We therefore investigated the importance of mitochondrial cytochrome *c* release in the induction of apoptosis by honokiol in CH27 cells and the effects of broad-spectrum caspase inhibitor z-VAD-fmk on the release of mitochondrial cytochrome *c*. Western blot analysis revealed a marked increase in cytosolic cytochrome *c*, detected within 4 to 24 hr honokiol treatment, most probably due to release of mitochondrial cytochrome *c* (Fig. 7A). These data indicated that cytochrome *c* release preceded the activation of caspase-1, -2, -3, -6, -8 and -9. Moreover, treatment with z-VAD-fmk markedly blocked the honokiol-induced cell death (Fig. 6), but did not prevent the accumulation of cytosolic cytochrome *c* (Fig. 7B). These results further support the hypothesis that the target caspase(s) of z-VAD-fmk in honokiol-induced apoptosis is downstream of mitochondria.

4. Discussion

Current antineoplastic therapies, chemotherapy and radiation-therapy, are likely to be affected by the apoptotic tendencies of cells; thus, this process has obvious therapeutic implications [37]. During apoptosis, certain characteristic morphologic events, such as nuclear condensation, nuclear fragmentation, and cell shrinkage, and biochemical events, such as DNA fragmentation, occur [32]. In this study, we showed that honokiol-induced CH27 cell apoptosis was confirmed by DNA ladder, DAPI staining and TUNEL assay. These honokiol-mediated apoptotic cell death was not restricted to CH27 cells, similar results was obtained with honokiol-treated H460, H1299 and WI-38 cell lines. It is interesting that human fibroblast-like lung WI-38 cells were more resistant to the honokiol-mediated cytotoxic activity than the three tested lung cancer cell lines.

The commitment of a cell to apoptosis requires the integration of numerous inputs involving multiple signal transduction pathways. Some cellular pathways and molecular regulators/effectors of apoptosis have been identified [38–40]. It is well known that Bcl-2 family members play a pivotal role in the regulation of cell death and survival [17]. In this study, honokiol-induced apoptosis of CH27 cells was accompanied by upregulation of Bad and downregulation of Bcl-X_L, but did not involve the regulation of Bcl-2 or Bax protein expression. Bad, a Bcl-2-related molecule, promotes cell death by hetero-dimerization with Bcl-2 or Bcl-X_L, which eventually results in the release of free Bax and induction of apoptosis [41,42]. Overexpression of Bad in murine FL5.12 cells could abrogate the protective capacity of coexpressed Bcl-X_L [41]. Bcl-X_L, a mitochondrial membrane protein [43], has been shown to block apoptosis induced by a variety of stimuli [44,45] and to be a stronger protector against apoptosis than Bcl-2 under certain circumstances [46,47]. Low levels of Bcl-X_L expression correlated with a greater tendency to undergo apoptosis [48]. Recent studies have demonstrated that Bcl-X_L exerts its anti-apoptotic function by association with caspase-9 [49], caspase-1 and caspase-8 [50] to inactivate these caspases in mammalian cells. Based on these results, we suggest that upregulation of Bad and downregulation of Bcl-X_L are associated with honokiol-induced apoptosis. Moreover, induction of Bcl-2 protein expression in the CH27 cells by Adv-Bcl-2 significantly blocked honokiol-induced apoptosis. In contrast, overexpression of Bcl-2 in

Hep3B and HeLa-S3 cells drastically accelerated honokiol-induced cell death. These results suggest that the effect of Bcl-2 overexpression on honokiol-induced cell death depends on the types of cell and the amount of Bcl-2. Recently, an organic compound, HA14-1 has been demonstrated to bind to surface functional pocket of Bcl-2 protein and induce apoptosis of tumor cells [51]. It is intriguing that honokiol showed some chemical similarity with HA14-1, and therefore whether honokiol can directly bind to Bcl-2 and induce apoptosis in Bcl-2 overexpressed cells remains to be elucidated.

Previous reports have indicated that mitochondria play an important role in inducing apoptosis [26–28]. Bcl-X_L is a mitochondrial membrane protein, which extending across the mitochondrial membrane can bind with cytochrome *c*, blocks the release of mitochondrial cytochrome *c*, and consequently protects cells from apoptosis [44]. In the present study, we found that the release of cytochrome *c* from mitochondria to cytosol as well as the downregulation of Bcl-X_L, are the early events in the honokiol-induced apoptotic process, preceding morphological signs of apoptosis. After or at the time of the mitochondrial cytochrome *c* efflux, the caspases were activated and PARP is cleaved in honokiol-treated cells. This is in accord with the notion that cytochrome *c* may be required for caspase activation during the induction of apoptosis, at least in some situations [52].

Many lines of evidence demonstrated that activation of caspase is a central mechanism of apoptosis, and caspases are considered to be the ''executioners'' of cell death [35]. Upon treatment with honokiol, the activation of caspases occurred after the release of mitochondrial cytochrome *c*. Activation of caspase-9 occurred very early, first observed 4 hr after honokiol treatment, and appeared to precede the activation of caspase-1, -2, -3, -6 and -8. Recent report indicated that caspase-9 and caspase-2 are found in the intermembrane space of mitochondria, and released in a Bcl-2-inhibitable fashion upon induction of permeability transition in isolated mitochondria and upon apoptosis induction in cells [53,54]. It is reported that during or after the release from mitochondria, the procaspase-9 and procaspase-2 become proteolytically processed and enzymatically active [53,54]. Some Bcl-2 family molecules are mitochondrial membrane proteins which can alter the permeability of mitochondrial membrane and trigger the release of cytochrome *c*, caspase-9 and -2, then activate postmitochondrial caspases, including caspase-3, -6 and -7 [55,56]. Caspase-8 activation was found both upstream and downstream of mitochondria [56,57]. In the present study, upon treatment with honokiol, caspase-9 was activated downstream of mitochondria. Consistence with our observation, several studies have demonstrated that chemical-induced apoptosis goes through caspase-9-dependent pathway [58–61]. In contrast, Ferreira *et al*. [62] reported that the activation of caspase-8 involved in DNA-damaging agents-inducible apoptotic pathway. These results suggested that the molecular events of chemical-induced apoptosis may depend on the kind of tested cell types and actions of different chemicals. Moreover, we made an attempt to dissect cytochrome *c* release from caspase activation to decipher the sequence of apoptotic events triggered by honokiol. Cells were preincubated with broad-spectrum caspase inhibitor (z-VAD-fmk) before the addition of honokiol and analyzed by Western blot analysis for cytochrome *c*. Results showed that pre-incubation of cells with z-VAD-fmk effectively inhibited the caspase activities (data not shown) and prevented the honokiol-induced apoptosis, but failed to block the release of mitochondrial cytochrome *c*, suggesting a possible sequence of events in which honokiol first trigger the release of mitochondrial cytochrome *c*, which activates caspase-9, leading to downstream activation of executioner caspase-3, -6 and -8, and consequent cell death.

A variety of drugs have been observed to induce upregulation of Fas/FasL expression, followed by the subsequent induction of Fas-dependent apoptosis [63,64]. However, there are also reports indicating that anticancer drugs induce apoptosis in the absence of Fas engagement [65–67]. In this study, we also found that honokiol did not alter the expression of the Fas and FasL, and interference with Fas/FasL interaction by blockage of Fas did not inhibit honokiol-induced apoptosis (data not shown). Furthermore, wild-type p53 is considered to participate in apoptosis in response to DNA damage in many tumor cells [68], and it also been reported as a transactivator of the *Bax* gene [69], suggesting that p53 may be responsible for activation of certain apoptosis pathways. However, p53-independent apoptotic cascade after administration of anticancer drugs or γ-irradiation have been described [70,71]. In the present study, honokiol-induced apoptosis in both wild-type p53 expressing cells (CH27 and H460) and no p53 expressed cells (H1299). These results suggest that honokiol-induced caspase activation and apoptosis is entirely controlled by a p53- and Fas/FasL-independent mitochondrial pathway.

In conclusion, the schematic representation of honokiol-induced apoptotic cascade is described as following. Treatment of human lung cancer CH27 cells with honokiol induced an unknown cell death-transducing signals that regulate the mitochondrial membrane permeability by downregulation of Bcl-X_L and upregulation of Bad, triggering the cytochrome *c* release from mitochondria to cytosol. Upon entering the cytosol, cytochrome *c* triggers a p53- and Fas/FasL-independent activation of caspases (caspase-9 pathway), then activates downstream executioner caspase-3 and -6, and consequently cleaves specific substrates leading to process apoptotic changes (nuclear condensation, degradation of caspase substrates, and DNA fragmentation). Taken together, our observations indicated that downregulation of Bcl-X_L and upregulation of Bad triggering the accumulation of cytosolic cytochrome *c* may play a central role in the regulation and activation of the executioner phase of honokiol-induced apoptosis. Further

investigations will focus on the identification of the upstream signals modulation the expression of Bcl-X_L and Bad.

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