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Reactive oxygen species production and Bax/Bcl-2 regulation in honokiol-induced apoptosis in human hepatocellular carcinoma SMMC-7721 cells

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ABSTRACT

We investigated possible mechanism(s) where honokiol induces apoptosis in human hepatocellular carcinoma SMMC-7721 cells. MTT assay showed that honokiol has strong inhibition on SMMC-7721 cells in a dose-dependent manner. SMMC-7721 cells after honokiol treatment display morphological characteristics such as cell shrinkage, detachment from the culture plate, formation of apoptotic bodies, change to a round shape, and marked nuclear condensation and fragmentation after 32258 staining. Cell apoptosis was measured by Annexin-V/PI staining and alternatively, by the subG0/G1 percentage of the cell cycle analysis followed by FACS. An obvious loss of $\Delta\Psi_m$ and a quick burst of ROS was detected when honokiol reached 4 μg/ml, which was coincident with the high apoptosis percentage in our previous research. Up-regulation of Bax and down-regulation of Bcl-2 were observed, suggesting that honokiol-induced apoptosis was associated with reactive oxygen species (ROS) production and an increase of Bax/Bcl-2 ratios.

1. Introduction

Hepatocellular carcinoma is one of the most dangerous malignancies in China. Detection of hepatocellular carcinomas can be difficult and they are often at an advanced stage when detected. It is unfortunate that hepatocellular carcinomas are highly refractory to conventional chemotherapy, radiation therapy, and even immunotherapy (Ying et al., 2008). According to the World Health Organization, 6,350,000 cancer cases are reported each year, and 4% are hepatocellular carcinoma, 42% of which occur in China (Cai et al., 2003). Thus, novel therapeutic targets need to be sought for the successful treatment of hepatocellular carcinoma.

Traditional Chinese medicine is widely known for its time-tested safety and effectiveness, and has also begun to show promise as a therapy for malignancies. Honokiol, a small phenolic component isolated from the root and stem bark of the traditional Chinese medicinal herb Magnolia officinalis (Chinese name: houpo), has been shown to have anti-angiogenic, anti-invasive, and anti-proliferative activities in several types of human cancer cells (Bai et al., 2003; Nagase et al., 2001). Honokiol was found to exhibit anti-proliferative and apoptotic activities against many cancer cells, including leukemia (Battle et al., 2005; Hibasami et al., 1998; Hirano et al., 1994), human colorectal cancer cell lines (Berry et al., 2008; Chen et al., 2004; Wang et al., 2004a), human prostate cancer cells (Hahm and Singh, 2007; Shigemura et al., 2007), human multiple myeloma (Ishitsuka et al., 2005) and human squamous lung cancer (Yang et al., 2002). Research has demonstrated that honokiol suppresses NF-κB activation and NF-κB-regulated gene expression, which provides a possible mechanism for its anti-tumor actions (Ahn et al., 2006; Tse et al., 2005). But much research is still necessary to support clinical application of honokiol in patients with human hepatocellular carcinoma cell lines.

It has been reported that reactive oxygen species and mitochondria play an important role in apoptosis induction under both physiologic and pathologic conditions (Simon et al., 2000). Mitochondria are crucial regulators of the apoptosis process. Development of cytotoxic drugs that target mitochondria may provide a new strategy to induce apoptosis in tumor cells. Depolarization of the inner mitochondrial membrane potential ($\Delta\Psi_m$) facilitated as one of the opening of large mitochondrial permeability transition pores is regarded as one of the signs of cell death (Hirsch et al., 1997). Reactive oxygen species (ROS), which are the byproducts of normal cellular oxidative processes, have been suggested as regulating the process involved in the initiation of apoptotic signaling (Vaux and Korsmeyer, 1999). An increase of reactive oxygen species (ROS) and a consequent loss of mitochondrial membrane potential

Abbreviations: MTT, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; PI, propidium iodide; $\Delta\Psi_m$, mitochondrial transmembrane potential; FACS, fluorescence activated cell sorter; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2 antagonist X; FITC, annexinV-fluorescein isothiocyanate.

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were reported as typical phenomena in the process of apoptosis related to mitochondria (Ying et al., 2008). Thus, in our work, we have looked at the possibility that ROS and mitochondria may play a role in honokiol-induced apoptosis in SMMC-7721 cells.

In addition, the initial investigations of the Bcl-2 family of proteins have shown that these proteins play an important role in the regulation of mitochondrial-mediated apoptosis (Desagher and Martinou, 2000). So far, over two dozens homologs of Bcl-2 have been identified. These proteins are classified into two subfamilies having opposing roles in apoptosis regulation. Members from one subfamily such as Bcl-2, Bcl-XL, and Bcl-w promote cell survival while members from the other subfamily such as Bax, Bid, and Bak promote cell death. Among the various Bcl-2 homologs identified to date, Bcl-2 and Bax represent the most well characterized members (Hou et al., 2003). It has also been reported that Bcl-2 suppresses ROS-induced apoptosis (Hochenbery et al., 1993) and the over-expression of pro-apoptotic Bax enhances ROS generation (Jurgensmeier et al., 1998).

Our studies suggest that honokiol is a potent agent against human hepatocellular carcinoma cells and honokiol-induced apoptosis is associated with reactive oxygen species (ROS). There may be a major link between the acute production of ROS induced by honokiol and apoptosis in hepatocellular carcinoma cells.

2. Materials and methods

2.1. Materials

Honokiol, purchased from the National Center for Safety Evaluation of Drugs (NCSED, in China), was dissolved in DMSO as a 10 mg/ml stock solution and stored at −20 °C. The structure of the compound is shown in Fig. 1. MTT, Hoechst 32258 and propidium iodide (PI) and Rhodamine-123 were from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) was supplied by the Hangzhou Sijiqing Organism Engineering Material Co. (Hangzhou, China). Mouse anti-Bcl-2, mouse anti-Bax, and rabbit anti-β-actin were purchased from Beijing Zhongshan Golden Bridge Biotech (Beijing, China). Roswell Park Memorial Institute (RPMI) 1640 medium, trypsin, and some other culture reagents were purchased from Hyclone (Logan, UT, USA).

2.2. Cell lines and cell cultures

Human hepatocellular carcinoma SMMC-7721 cells were obtained from the medical school of Peking University. These cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 units/ml). Cells were incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. After confluence, the cells were dissociated with 0.25% trypsin–0.02% ethylenediaminetetraacetic acid (EDTA) at the needed density for treatment.

2.3. Cytotoxicity assay

The effects of cell proliferation by honokiol were investigated by the MTT method. SMMC-7721 cells at a final density of 6×10^4 cells/ml were placed in 96-well cell plates. After 60% confluency, the culture medium was removed and replaced with fresh medium without fetal bovine serum containing honokiol of the desired concentration. After 24 h, 20 μl MTT (5 mg/ml) was added to each well. The supernatants were removed and the formazan crystals were dissolved by the addition of 200 μl DMSO after incubation at 37 °C for 4 h. The optical density (O.D.) in the control and drug-treated wells was measured in an Automated Microplate Reader (Bio-RAD, model 550) at a test wavelength of 570 nm. Experiments were conducted in triplicate.

$$\text{Inhibition rate}\,(\%) = \frac{[\text{A570}\,(\text{control}) - \text{A570}\,(\text{honokiol})]}{\text{A570}\,(\text{control})} \times 100.$$

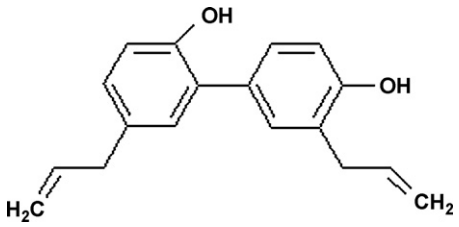

Fig. 1. Chemical structure of honokiol.

2.4. Cell morphological assay

The SMMC-7721 cells in exponential growth were plated at a density of 5×10^4/well in a 6-well cell plate and allowed to attach and proliferate for 4 h. Then they were directly observed under an inverted microscope (Leica, DM-IRB, Germany). After confluency, the cells were harvested and fixed in methanol for 10 min at room temperature. After washing by PBS, they were incubated with Hoechst 32258 (0.1 mg/ml) for 30 min at 37 °C, and subjected to fluorescence microscopy (Leica, DM-IRB, Germany) through a UV filter.

2.5. Apoptosis assays and cell cycle analysis

Apoptosis was determined with staining of FITC-Annexin-V and propidium iodide (PI) according to an Annexin V-FITC Apoptosis Detection Kit (Biosea Biotechnology, Beijing, China). SMMC-7721 cells were cultured in the presence of honokiol at the desired concentration for 12 h. Approximate 1×10^6 cells per experimental condition were harvested, washed with cold PBS twice, and resuspended with 200 μl binding buffer. Cells were then incubated for 15 min at 37 °C or for 30 min at 4 °C in the dark, in the presence of FITC labeled Annexin-V and PI. Another 300 μl binding buffer was added to the cell suspension. The cells were then analyzed by a flow cytometer (Partec, CCA-II).

The cell cycle analysis was performed using a hypotonic solution of propidium iodide (PI). SMMC-7721 cells were cultured in the presence of the desired concentration of honokiol for 12 h. Approximate 1×10^6 cells per experimental condition were harvested, washed with cold PBS twice and fixed with 70% ethanol. The fixed cells were spun down and resuspended in PBS and incubated with RNase A at a final concentration of 100 μg/ml at 37 °C for 30 min. The cell suspension was stained by propidium iodide (PI), before being measured by a flow cytometer (Partec, CCA-II).

2.6. Measurement of mitochondrial membrane potentials ($\Delta\Psi_m$).

SMMC-7721 cells were cultured in the presence of the desired concentration of honokiol for 12 h and harvested with trypsin/EDTA. After washing by cold PBS, they were incubated with Rhodamine-123 at a final concentration of 10 μg/ml at 37 °C or for 30 min in the dark, rinsed by cold PBS twice, and analyzed by a flow cytometer (Partec, CCA-II). The probes were excited with a laser at 488 nm and the fluorescence emission was measured through a 520 nm bandpass filter.

2.7. Detection of ROS

Intracellular ROS production was measured by use of a reactive oxygen species assay kit (Applygen Technologies Inc., Beijing, China). Briefly, SMMC-7721 cells were seeded at a density of 5×10^5/well in a 6-well culture plate and allowed to attach and proliferate for 24 h. These cells were exposed to the desired concentration of honokiol for the indicated times. After exposure, cells were harvested by trypsinization and washed with PBS solution, and finally resuspended in 0.5 ml 2′,7′-dichlorofluorescin diacetate (DCFH-DA) solution (a final concentration of 10 μM). The cell suspensions were incubated at 37 °C or for 30 min, and analyzed for fluorescence intensity by flow cytometry (Partec, CCA-II).

2.8. Western blot analysis

SMMC-7721 cells (1×10^6/dish) were seeded in 10-cm dishes. After 24 h for incubation, cells were treated with different concentrations of honokiol for 12 h. Cells were scraped from the culture, washed twice with PBS, and suspended in 200 μl cell lysis solution (CellChip Biotechnology, Beijing, China). After centrifugation at 14,000 × *g* for 5 min, the supernatants were collected, and the protein amount in each sample was assayed by BCA™ protein Assay kit (Thermo Scientific, USA). Equal amounts of samples were subjected to electrophoresis on 12.5% SDS-PAGE. Following electrophoresis, protein blots were transferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk in TBST solution, and incubated overnight with the corresponding primary antibodies in the blocking solution at 4 °C. After washing three times with TBST solution, the membrane was incubated at room temperature for 1 h with horseradish peroxidase-conjugated secondary antibody diluted with TBST solution (1:1000). The protein signals were visualized using an HRP-DAB development kit (Tiangen Biotech Co. Ltd., Beijing, China). The color was observed within 10 min and the reaction was stopped by the addition of distilled water.

2.9. Statistical analysis

Data are presented as means ± SD. The comparisons were made with a test and the difference was considered to be statistically significant if the p value was <0.05. All data points represented the means of triplicates.

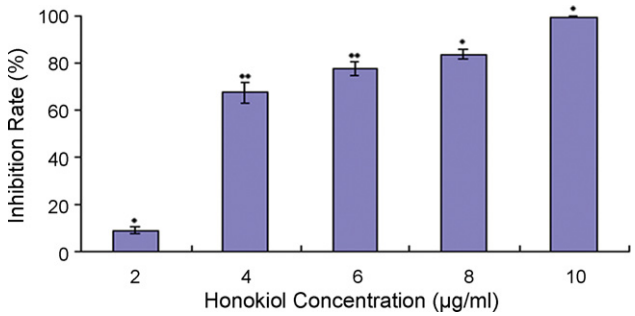

Fig. 2. Cytotoxicity effect of honokiol on SMMC-7721 cells in intro. Cells in logarithm growing period were treated with 2–10 μg/ml honokiol for 24 h. Cell viability was then determined by MTT assay as described in Section 2. The data was expressed as means ± SD of three separate experiments. Significant differences from untreated control were indicated by $^{*}p < 0.05$; $^{**}p < 0.01$.

3. Results

3.1. Cytotoxic effect of honokiol on SMMC-7721 cells.

MTT assay was used as an indirect measure to determine the cell viability of SMMC-7721 cells after treatment with different honokiol concentrations. As shown in Fig. 2, MTT assay showed that honokiol had significant cytotoxicity toward SMMC-7721 cells in a dose-dependent manner. It was noted that the inhibition was very distinct when the honokiol concentration reached 4 μg/ml.

One of the characteristics of honokiol-treated cells was cell morphology change. For further research, both the control cells and cells treated with honokiol for 4 h were observed under an inverted microscope (Fig. 3A) and a fluorescence microscope (Fig. 3B). Shown in Fig. 3A (1–4), cells treated with 2 μg/ml honokiol did not have any obvious morphological changes but cells treated with 4 μg/ml honokiol displayed drastic morphological changes; for example the cells shrank, detached from the culture plate, and became round, and apoptotic bodies were formed. Cells treated with 6 μg/ml honokiol became totally detached from the culture dish. As shown in Fig. 3B (1–2), the SMMC-7721 cells exposed to honokiol (4 μg/ml) revealed marked nuclear condensation and fragmentation after

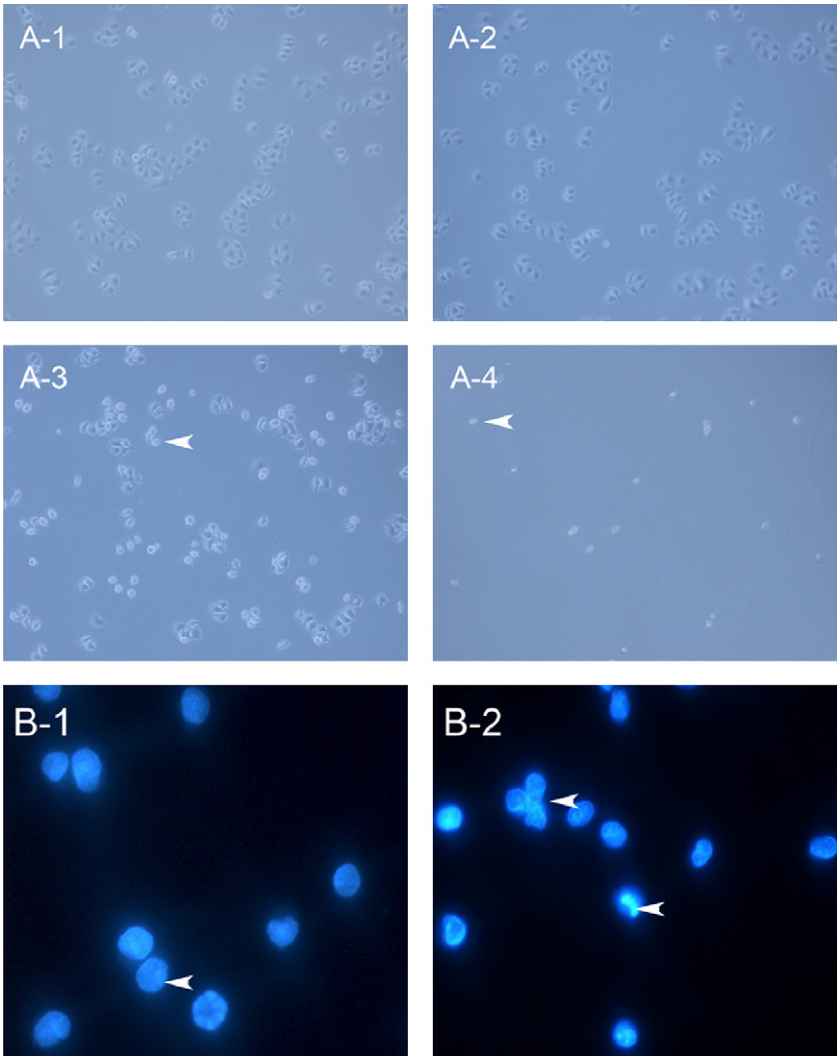

Fig. 3. Morphology changes of cells treated by honokiol. Cells were treated without honokiol (A1) or with honokiol at 2 μg/ml (A2), 4 μg/ml (A3), 6 μg/ml (A4) for 4 h, then directly observed under an inverted microscope (A1–A4, magnification 100×). Arrows in A3 shows morphological-changed SMMC-7721 cells. After 24 h, both the control cells and 4 μg/ml honokiol-treated cells were loaded with Hoechst 32258, and then observed under a fluorescence microscope (B1–B2, magnification 100×). Arrows in B1 (control) show a common cell, and Arrows in B2 (treated) show condemned chromatin. Each experiment was performed in triplicate (magnification 100×).

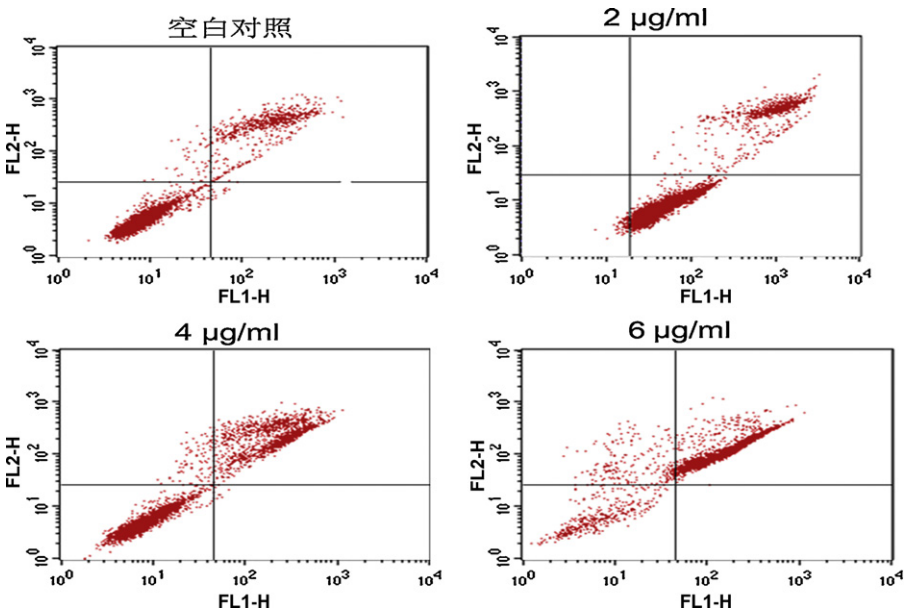

Fig. 4. Effects of honokiol on apoptosis of SMMC-7721 cells. SMMC-7721 cells were incubated with 0 μg/ml (A), 2 μg/ml (B), 4 μg/ml (C), 6 μg/ml (D) honokiol for 24 h. Apoptosis was measured by annexin-V and PI double staining. The FL1-H axis denotes annexin-V, while the FL2-H axis denotes PI. The numbers indicated represent the percentage (mean ± SD) of gated viable cells, and the percentage of annexin-V^+/PI^- and annexin-V^+/PI^+ cells was indicated. Each experiment was performed in triplicate.

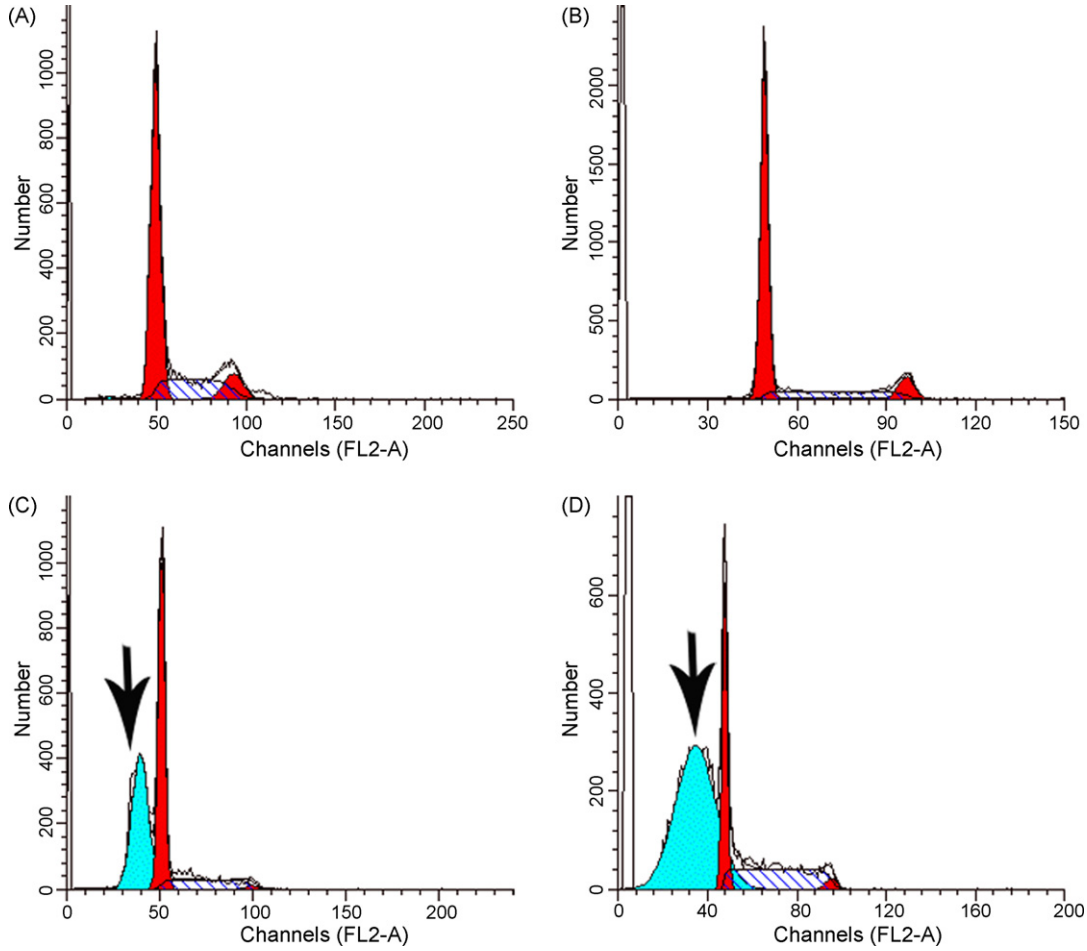

Fig. 5. Effects of honokiol on cell cycle distribution in SMMC-7721 cells. SMMC-7721 cells were incubated with 0 μg/ml (A), 2 μg/ml (B), 4 μg/ml (C), 6 μg/ml and (D) honokiol for 24 h and then stained with PI (1 mg/ml) and analyzed by flow cytometry as described in Section 2. The cell population in subG0/G1 phase (apoptotic peak) was indicated by arrow. Each experiment was performed in triplicate.

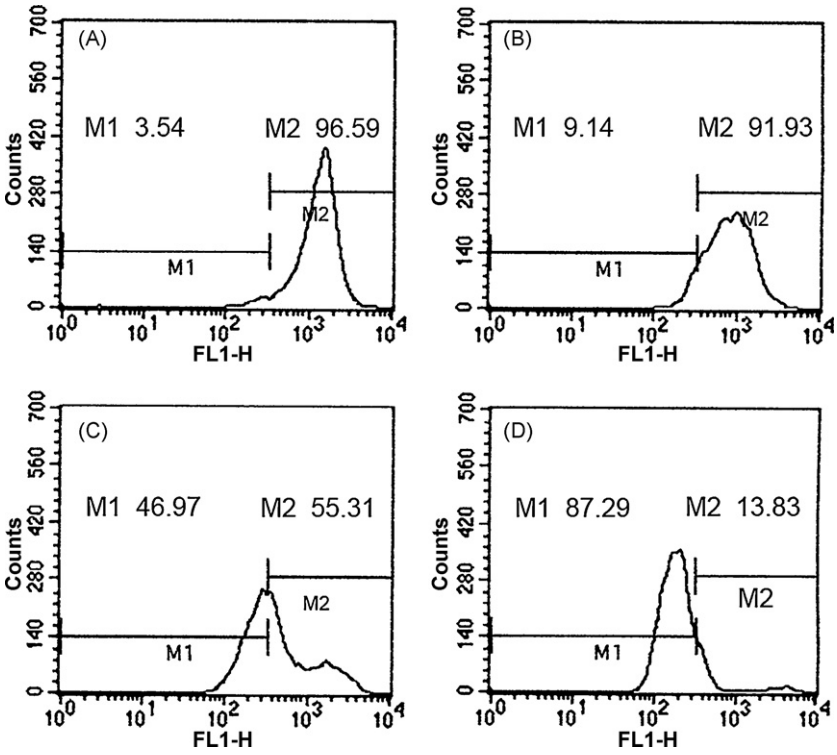

Fig. 6. Mitochondrial membrane potential changes of cells treated by honokiol. SMMC-7721 cells were incubated with 0 μg/ml (A), 2 μg/ml (B), 4 μg/ml (C) and 6 μg/ml (D) for 24 h and quantitation of $\Delta\Psi_m$ was analyzed by FACS. The FL1-H axis (or the *x*-axis) denotes mitochondrial membrane potential and cell count on the *y*-axis. For the analysis the M1 gate was set on the cells showing low mitochondrial membrane potential and the M2 gate was set on the cells showing high mitochondrial membrane potential ($\Delta\Psi_m$). Numbers in charts represent the percentages of cells in each gate. Data in this figure are representative of three independent experiments.

32258 staining, as indicated by arrows. All of these changes are typical characteristics of apoptotic cell death. In contrast, the control cells did not exhibit such alterations. All these results suggested that honokiol had significant cytotoxicity toward SMMC-7721 cells.

3.2. Honokiol inhibits the proliferation of SMMC-7721 cells via apoptosis

Phosphatidylserine (PS) externalization is an extremely early event of cell apoptosis. In the present study, SMMC-7721 cells were incubated with 0, 2, 4 and 6 μg/ml honokiol for 24 h, and then stained with annexin-V and PI to examine whether honokiol caused PS externalization. The results of dual-color flow cytometry analysis showed that honokiol can induce dose-dependent apoptosis in SMMC-7721 cells (Fig. 4). The numbers indicated represent the percentage (mean ± SD) of gated viable cells, and the percentage of annexin-V^+/PI^- and annexin-V^+/PI^+ cells is indicated in Fig. 4. The annexin-V positive and PI negative cells were early-stage apoptotic cells, whereas annexin-V positive and PI positive cells were late stage apoptotic cells. Notably, the percentage of apoptotic cells changed significantly when the concentration of honokiol reached 4 μg/ml.

In addition, cell cycle analysis by FACS was carried out for both the control cells and cells treated with honokiol for 24 h. The cells in the subG0/G1 phase constituted the apoptotic peak and the percentage of the subG0/G1 cells represented the apoptotic percentage. The results presented in Fig. 5 showed that honokiol dose-dependently increased the subG0/G1 cell population in SMMC-7721 cells. An obvious apoptotic peak was observed when the concentration of honokiol was 4 μg/ml.

Considering these results together, honokiol induced apoptosis in SMMC-7721 cells in a dose-dependent manner and treated cells had a significantly higher apoptosis percentage than the control group when the concentration of honokiol reached 4 μg/ml.

3.3. Honokiol induces loss of $\Delta\Psi_m$ and increase of ROS

Recent investigations have shown that an alteration in mitochondrial $\Delta\Psi_m$ was implicated in the induction of apoptosis. In our work, we investigated the change of $\Delta\Psi_m$ of SMMC-7721 treated by honokiol using rhodamine-123 retention. Rhodamine-123 is a cationic fluorescent dye, which is localized in the mitochondria of viable cells because of the relatively high negative electric potential across the mitochondria inner membrane (Johnson et al., 1980). Depolarization of $\Delta\Psi_m$ drives the down-regulation of the accumulation in mitochondria of cationic dyes such as rhodamine-123. Thus, the amount of rhodamine-123 uptake of mitochondria in cells is proportional to mitochondrial membrane potential.

From our research, we could see that honokiol did cause a decline in rhodamine-123 uptake of mitochondria in a dose-dependent manner, as compared with the control (Fig. 6). The percentages of cells in the M2 gate (high mitochondrial membrane potential) decreased obviously and showed an obvious loss of $\Delta\Psi_m$ in SMMC-7721 cells. $\Delta\Psi_m$ loss can induce the opening of permeability transition pores in mitochondria, and several apoptogenic factors are released from mitochondria to cytosol by apoptosis-inducing stimuli (van Loo et al., 2002).

$\Delta\Psi_m$ loss is an early event in apoptosis induction and this reduction in $\Delta\Psi_m$ is often accompanied by the production of reactive oxygen species (ROS) (Kroemer et al., 1997). To further address the possibility that honokiol-induced apoptosis could be related to contributions from the mitochondrial pathway, we detected the production of ROS induced by honokiol. Induction of ROS was detected by flow cytometry using 2′,7′-dichlorofluorescein

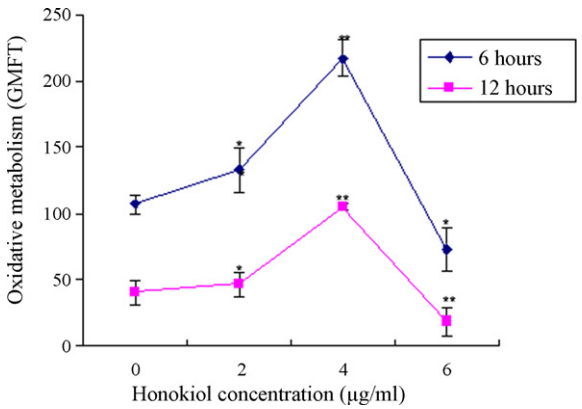

Fig. 7. Reactive oxygen species (ROS) generation in SMMC-7721 cells. ROS production was observed after 6 h, 12 h exposure to honokiol of different concentration compared with untreated cells. ROS activity was calculated and expressed as the geometric mean fluorescence intensity (GMFI) in SMMC-7721 cells. $^{**}p < 0.01$, $^{*}p < 0.05$ compared to untreated cells.

diacetate (DCFH-DA). This dye is a stable nonpolar compound that readily diffuses into the cells. In the cytoplasm it is hydrolyzed by intracellular esterases, producing DCFH, which is a compound that is trapped within the cells. Hydrogen peroxide or low molecular weight peroxides oxidize DCFH, resulting in the production of a highly fluorescent compound, 2′,7′-dichlorofluorescein. Thus, the intensity of fluorescence in cells treated with DCFH-DA is proportional to the amount of ROS (Thannickal and Fanburg, 2000). The production of ROS increased at low concentrations of honokiol and decreased at high concentrations of honokiol in our work (Fig. 7). A drastic increase of reactive oxygen species in SMMC-7721 cells between 2 and 4 μg/ml and a decrease between 4 and 6 μg/ml suggest that honokiol-induced apoptosis is associated with reactive oxygen species (ROS) production.

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

The properties of Bcl-2 also extend to its ability to function as an antioxidant, exerting a particular buffering effect on mitochondrial ROS production (Agostinis, 2003). In order to study the positive involvement of Bcl-2 and Bax (the anti- and pro-apoptotic proteins of the Bcl-2 family) in honokiol-induced apoptosis, SMMC-7721 cells were exposed to honokiol (0, 2, 4, and 6 μg/ml) for 12 h and the expression of Bcl-2 and Bax was investigated by western blot analysis. The results showed that honokiol could alter the balance between pro-apoptotic Bax and anti-apoptotic Bcl-2 proteins at the mitochondrial membrane. The Bcl-2 protein level was decreased and the Bax protein level was increased after cells were treated with 4 μg/ml honokiol (Fig. 8).

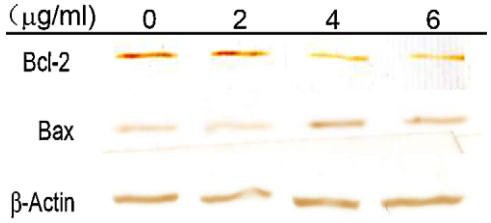

Fig. 8. Effect of honokiol on the expression of Bcl-2 family proteins. SMMC-7721 cells were treated with different concentrations of honokiol (0, 2, 4, and 6 μg/ml) for 12 h. After treatment, cell lysates were extracted. Bcl-2 family proteins, anti-apoptotic Bcl-2 and pro-apoptotic Bax were examined by western blot analysis. β-Actin was used as an internal loading control.

4. Discussion

The bark of *M. officinalis* is a rich source of honokiol (Wang et al., 2004b) and has been widely used as a folk remedy in China, Korea, and Japan for gastrointestinal disorders, cough, anxiety, allergy and other diseases (Tse et al., 2005). Recently, more and more researchers have begun to focus on the effects of honokiol in cancer treatment. Honokiol is considered a new potent antitumor compound (Amblard et al., 2006). However, there is still much to learn regarding the mechanism of apoptosis induced by honokiol.

In the present study, MTT assay showed that cells treated with 2–10 μg/ml honokiol underwent obvious viability changes (Fig. 2). The inhibition was most distinct when the honokiol concentration reached 4 μg/ml. Morphological changes observed in Fig. 3 were typical characteristics of apoptotic programmed cell death, suggesting that honokiol-induced cytotoxicity may be activated by apoptosis Pathway. Cell apoptosis was measured by Annexin-V/PI staining and alternatively, by the subG0/G1 percentage of the cell cycle analysis followed by FACS. We could notice that the percentage of apoptotic cells changed significantly (Fig. 4) and an obvious apoptotic peak (Fig. 5) was observed when the concentration of honokiol reached 4 μg/ml.

It has been reported that $\Delta\Psi_m$ is a component of the overall moving proton force that drives the ATP generation in mitochondria (Polyak et al., 1997). Recent investigations have shown that an alteration in mitochondrial $\Delta\Psi_m$ is implicated in the induction of apoptosis. $\Delta\Psi_m$ loss can induce the opening of permeability transition pores in mitochondria, and several apoptogenic factors are released from mitochondria to cytosol by apoptosis-inducing stimuli (van Loo et al., 2002). We observed an obvious loss of $\Delta\Psi_m$ in SMMC-7721 cells in our research (Fig. 6).

$\Delta\Psi_m$ loss is an early event in the apoptosis induction and this reduction in $\Delta\Psi_m$ is accompanied by the production of reactive oxygen species (ROS) (Kroemer et al., 1997). ROS production plays an important role in apoptosis, and several groups have shown that molecules that stimulate the formation of ROS can result in apoptosis (Heussler et al., 1999; Kelso et al., 2001). An increase of reactive oxygen species (ROS) and a consequent loss of mitochondrial membrane potential were reported as typical phenomena in the process of apoptosis related to mitochondria (Vaux and Korsmeyer, 1999). The production of ROS increased at low concentrations of honokiol and decreased at high concentrations of honokiol in our work (Fig. 7). A drastic increase of reactive oxygen species in SMMC-7721 cells treated by honokiol between 2 and 4 μg/ml and a decrease between 4 and 6 μg/ml suggest that honokiol-induced apoptosis is associated with reactive oxygen species (ROS) production. It was observed that the ROS production was high when the concentration of honokiol reached 4 μg/ml, which was coincident with the high apoptosis percentage in our research.

Apoptosis is a genetically regulated biological process with two major pathways: the death-receptor-induced extrinsic pathway and the mitochondria-apoptosome-mediated apoptotic intrinsic pathway (Hu and Kavanagh, 2003). Bcl-2 family proteins have a central role in controlling the mitochondrial pathway. The pro-apoptotic proteins and anti-apoptotic proteins of the Bcl-2 family may turn apoptosis on and off, because of the formation of heterodimers among these proteins (Antonsson and Martinou, 2000; Reed, 1997). This heterodimerization results in mutual neutralization of the bound pro- and anti-apoptotic proteins. Therefore, the balance between the expression levels of the protein units (e.g., Bcl-2 and Bax) is critical for cell survival or death. There are reports showing that honokiol-induced apoptotic cell death was accompanied by up-regulation of Bad and down-regulation of Bcl-XL, while honokiol had no effect on the levels of Bcl-2 or Bax proteins in human squamous lung cancer (Yang et al., 2002). But in our research, up-regulation of Bax and down-regulation of

Bcl-2 were observed, suggesting that an increase of Bax/Bcl-2 ratios might be involved in apoptosis induced by honokiol (Fig. 8). It has also been reported that Bcl-2 suppressed ROS-induced apoptosis (Hochenbery et al., 1993) and the over-expression of pro-apoptotic Bax enhanced ROS generation (Jurgensmeier et al., 1998). These events may also support the theory that honokiol-induced death signaling is mediated through a mitochondrial oxygen stress pathway.

Another noteworthy study found that honokiol potentiates apoptosis through modulation of the nuclear factor–kappa B activation pathway, which provides a possible mechanism for its anti-tumor actions (Ahn et al., 2006; Tse et al., 2005). ROS have been regarded as inhibitors for NF-κB activation (Bowie and O'Neill, 2000; Karin et al., 2001; Li and Karin, 1999). Thus, we guess that honokiol potentiates apoptosis through a mitochondrial oxygen stress pathway, which need further investigations to confirm it.

Mitochondrial reactive oxygen species (ROS) have become a target for drug discovery in recent years since their production is characteristic of the early stages of apoptosis [37]. Our results showed that honokiol is a potent agent against human hepatocellular carcinoma, suggesting that reactive oxygen species production and Bax/Bcl-2 regulation are involved in honokiol-induced apoptosis.

Conflict of interest

None.

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