

RESEARCH ARTICLE

Anti-proliferation Effects of Isorhamnetin on Lung Cancer Cells *in Vitro* and *in Vivo*

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Abstract

Background: Isorhamnetin (Iso), a novel and essential monomer derived from total flavones of *Hippophae rhamnoides* that has long been used as a traditional Chinese medicine for angina pectoris and acute myocardial infarction, has also shown a spectrum of antitumor activity. However, little is known about the mechanisms of action Iso on cancer cells. **Objectives:** To investigate the effects of Iso on A549 lung cancer cells and underlying mechanisms. **Materials and Methods:** A549 cells were treated with 10~320 µg/ml Iso. Their morphological and cellular characteristics were assessed by light and electronic microscopy. Growth inhibition was analyzed by MTT, clonogenic and growth curve assays. Apoptotic characteristics of cells were determined by flow cytometry (FCM), DNA fragmentation, single cell gel electrophoresis (comet) assay, immunocytochemistry and terminal deoxynucleotidyl transferase nick end labeling (TUNEL). Tumor models were setup by transplanting Lewis lung carcinoma cells into C57BL/6 mice, and the weights and sizes of tumors were measured. **Results:** Iso markedly inhibited the growth of A549 cells with induction of apoptotic changes. Iso at 20 µg/ml, could induce A549 cell apoptosis, up-regulate the expression of apoptosis genes Bax, Caspase-3 and P53, and down-regulate the expression of Bcl-2, cyclinD1 and PCNA protein. The tumors in tumor-bearing mice treated with Iso were significantly smaller than in the control group. The results of apoptosis-related genes, PCNA, cyclinD1 and other protein expression levels of transplanted Lewis cells were the same as those of A549 cells *in vitro*. **Conclusions:** Iso, a natural single compound isolated from total flavones, has antiproliferative activity against lung cancer *in vitro* and *in vivo*. Its mechanisms of action may involve apoptosis of cells induced by down-regulation of oncogenes and up-regulation of apoptotic genes.

Keywords: Isorhamnetin - apoptosis - A549 - Lewis lung carcinoma - flavones

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Introduction

Cancer, which causes significant morbidity and mortality, is a major public health problem around the world. Worldwide lung cancer is one of the most common and malignant diseases. Although there has been rapid development of antitumor research, there are few effective remedies for lung cancer. Cytotoxic chemotherapy is being used to control and treat this disease, but it remains relatively nonselective and highly toxic to normal tissues. Natural products have been utilized as novel compounds for the treatment of many diseases, including cancer, because of their ability to inhibit cancer cell growth and induce apoptosis (Kuo et al., 2010). Plant-derived compounds are known to have curative potential, so that finding some efficient compounds from herbs and plants for curing such disease is absolutely interesting for traditional medicine (Luo et al., 2014).

The total flavones of *Hippophae rhamnoides* have

long been used as a traditional Chinese medicine for angina pectoris, acute myocardial infarction and exhibit a spectrum of antitumor activity (Wang et al., 2000). Among the total pharmacological activities of *Hippophae rhamnoides*, it has been a hot research on its pharmacological action and mechanism on antitumor activity and caught more and more attention (Yang et al., 2004; Zhu et al., 2004; Li et al., 2008). Several active ingredients have been identified that were effective in protecting against oxidative damage; inhibiting platelet aggregation; and reducing atherosclerosis (Wang et al., 2000; Liu et al., 2007; Teng et al., 2008). Some flavones, in recent years, were found these natural products had good anticancer effect and could induce necrosis in human carcinoma, so it has been researched on its clinical pharmacology and been used to therapy cancer currently (Zhong et al., 2013; Gong et al., 2014). The structure of isorhamnetin (Iso), a methyl group substitute hydroxyl, is similar to those flavones. Could Iso exert cytotoxic effects

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against human cancer cells?

We found that Iso, a novel and essential monomer derived from total flavones of *Hippophae rhamnoides*, exhibited antitumor activities against certain cell lines derived from human carcinomas *in vitro* (Yang et al., 2004; Zhu et al., 2004; Zhu et al., 2006). Aside from its cardiovascular-protective effect, our group found that Iso could induce tumor cell death in cell lines such as lung carcinoma cells, Hela cells, MCF-7 and Eca-109 cell. However, the effect of on human cancer cells, especially on lung cancer, *in vitro* and *in vivo*, the action target, and the antitumor mechanism remain to be elucidated. Much attention has focused on apoptosis as an important cell-death pathway, especially for its prominent role in cancer inhibition, and apoptosis is now recognized as an important mode of cell death in response to chemotherapeutic agents. The apoptotic program is characterized by particular morphological features (Jacobson et al., 1997; Gamet-Payrastre et al., 2000). It is known that growth-deregulating oncoproteins promote apoptosis through the activation of several downstream pro-apoptotic effector pathways. So our study focused on the effects of Iso on the growth of human A549 cell line and mouse Lewis lung cell line, as well as the growth-inhibitory mechanism.

The aim of this study was to gain a better understanding of the antitumor effect and the molecular mechanism underlying the previously unknown apoptotic activity of the Iso. To these aims, cell cycle and PCNA (proliferating-cell nuclear antigen) were analyzed; and the relative genes with apoptosis, such as caspase-3, Bcl-2, Bax, and P53 were also analyzed.

Materials and Methods

Drugs and chemicals

Iso was isolated, purified and given by the Department of Pharmacy at SU, purity 95%. Details of the extraction procedure and the structure of the monomer were reported elsewhere (Wang et al., 2000). Iso liquor was dissolved in 0.1% NaOH to obtain extract at different concentrations. Polyclonal/monoclonal anti-Bax, anti-Bcl-2, anti-caspase-3, anti-p53, anti-PCNA antibodies, anti-rabbit/mouse anti-bodies were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). TUNEL detection kit was purchased from Boehringer Mannheim, Germany. Others standards reagents were from Sigma-Aldrich. The remaining chemicals were purchased from local firms and were of the highest purity grade.

Cell cultures

The human lung carcinoma A549 cell lines were kindly provided by Dr. Boyao Wang (from ATCC, Manassas, VA, USA). Mouse lung carcinoma Lewis cells were kindly provided by the Department of Cell Biology (SU). Cells were routinely grown in RPMI-1640 medium or DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin, in a humidified atmosphere of 5% CO₂-95% air at 37 °C. Cells grown in drug-free medium (0.1% NaOH) served as control.

MTT assay, clonogenic assay and growth curve assay

Cytotoxicity and viability were determined with a standard MTT assay, which is based on the ability of viable cells to reduce a soluble yellow tetrazolium salt (MTT) to blue formazan crystals (Ling et al., 2002). In brief, cells at a density of 1×10⁴/ml were cultured for indicating time points in a 96-well-plate. After treatment with various concentrations of Iso (eight points ranging from 0 to 640 µg/ml) for 48 h and each concentration was performed at least 4 times in duplicate. MTT solution (5 mg/ml in PBS) was added to each well, and cells were further incubated at 37 °C for 4 h. Absorbance of the MTT-formazan product dissolved in DMSO was estimated at 540 nm with an ELISA plate reader. Growth inhibiting curves were then generated, and the concentration of drug that inhibited 50% of cell growth (50% inhibitory concentration, IC₅₀) was determined.

For clonogenic assay (Miyata et al., 2001; Vasilevskaya et al., 2004), cells were plated in wells at a density of 2×10⁵ per well in a 6-well-plate, and after 24 h cells were exposed to various concentrations of the test drugs or drug-free medium for 48 h. After removal of drug-containing media, cells were plated at density of 500 per well in a 60-mm dish, and incubated for 10 days under the standard culture conditions as described above. Formed colonies were fixed in 4% paraformaldehyde, stained with Giemsa blue solution for 5 min. Colony-forming efficiency was calculated as the average of triplicate experiments by counting the number of colonies that consisted of more than 50 cells.

Taking the growth curve methods (Singh et al., 2003; Chang et al., 2004), cells in the logarithmic phase were cultured at an initial density of 2×10⁴ cells/well in 100-mm dishes under the standard culture conditions as described above. Day one after seeded, the cells were exposed to various concentration of test drugs or drug-free medium for different times. Each treatment and time point had three plates. After 1-7 days of this treatment, the attached cells were collected by trypsinization, and a trypan blue dye assay was used to evaluate the drug's effect on cell growth, and counted in duplicate with a hemocytometer. Each treatment and time point had three independent plates. The representative data shown in this study were reproducible in three independent experiments.

Morphology change

Morphological analysis was performed by Giemsa staining and Hoechst 33258 nucleus staining. Cells were plated at low density (1.5×10⁵ cells/well) in a 6-well plate and treated with the drugs for 24 or 48 h. At the end of the experiment, cells were washed twice with PBS at room temperature and then fixed with ice-cold 4% paraformaldehyde for 10 min. Fixed cells were rinsed with PBS and stained with Giemsa (0.75 mg/mL) or Hoechst 33258 (10 µg/ml) for 15 min. Finally, cells were washed with PBS and analyzed under a fluorescence microscope with a UV light filter (Gamet-Payrastre et al., 2000). To deeply observe the morphological changes of A549 cells treated by Iso, the cells were collected by trypsinization, fixed with 3% glutaraldehyde in 0.1 M sodium cacodylate buffer for 2 h. After washed for three times by the same

buffer, cells were postfixed with 1% osmium tetroxide and then dehydrated in graded ethanol. The 100% ethanol solution was then replaced by propylene oxide and embedded in Epon 812. Sections were stained with 5% uranyl acetate and 2.7% lead citrate and then examined with a Jeol 1200EX electron microscope (Adachi et al., 1998; Singh et al., 2005). Random fields were selected for photography. To observe the morphological changes of Lewis lung cells treated with Iso, HE staining was performed.

DNA fragmentation and Single cell gel electrophoresis (comet) assay

A549 cells were incubated in culture medium in the presence or absence of Iso for the indicated time. For the DNA fragmentation analysis, 5×10^5 cells were pelleted by centrifugation, and DNA was isolated from the cell pellets as described (Ling et al., 2002; Miyoshi et al., 2004). Cells were harvested from culture and lysed with lysis buffer containing 0.5% Triton X-100, 100 mM Tris-HCl, pH 7.5, and 25 mM EDTA at 4°C for 60 min. After centrifugation at 15,000g for 10 min, the supernatants were collected and treated with 100 µg/ml of RNase I and 100 µg/ml of proteinase K at 50°C for 30 min, and then with phenol and chloroform. The fragmented DNA was precipitated in the presence of sodium acetate and ethanol, dissolved in Tris/EDTA buffer, and then the DNA was subjected to electrophoresis in 1% agarose gel, DNA was stained with ethidium bromide and visualized by UV light illumination.

The comet assay was performed as previously described (Passagne et al., 2003; Zhu et al., 2005). Results were expressed as the percentage of DNA in the comet tail and as the tail length (Wang et al., 1998).

Cell cycle analysis and detection of apoptosis by flow cytometry

A549 cells were incubated with different concentrations of Iso for 24h. After treatment, cells were washed with PBS and fixed in ice-cold 75% ethano. Lewis cells, as harvested from tumor-bearing mice, were fixed with *p*-formaldehyde, permeabilized with Triton X-100. Cellular DNA was stained with 0.05% propidium iodide (PI) for 20 min at 4°C in darkness. The cell cycle distribution and apoptotic cells were detected with FACScan (Becton-Dickinson, San Jose, CA).

Immunocytochemistry and terminal deoxynucleotidyl transferase nick end labeling (TUNEL)

A549 cells plated on cover slips were treated with different concentrations of Iso for 24 h. After treatment, cells were fixed in fixing with 4% paraformaldehyde for 15 min and washed with PBS. Cells were then blocked with 0.5% bovine serum albumin and 0.15% glycine in PBS and further incubated with normal goat serum (1:20 dilution with PBS containing 0.5% bovine serum albumin and 0.15% glycine) containing anti-PCNA, anti-caspase-3, anti-p53 antibody (1:400 dilution) for 2 h at room temperature. After being washed with PBS, cells were reincubated with Alexa Fluor 488-conjugated secondary antibody (1:1000 dilutions) for 1 h. After washing Cells were observed with a microscope (Chang

et al., 2004). TUNEL (TdT-mediated dUTP Nick End Labeling) staining was performed using an in situ cell detection kit POD, according to the manufacturer's instructions (Boehringer Mannheim, Germany). The cells were plated on chamber slides, grown overnight, and then treated either without or with drug. Both untreated and treated cells were washed, fixed in 4% paraformaldehyde solution, and incubated with hydrogen peroxide to block endogenous peroxidase. After rinsing, the cells were incubated in a TUNEL reaction mixture. Cells were washed and incubated with Converter-POD (anti-fluorescein antibody conjugated to horseradish peroxidase) for 30 min at 37 °C, then incubated with DAB-substrate solution and counter-stained with hematoxylin stain (Qin and Ng, 2002). Cells were observed with an Olympus BX50 fluorescence microscope.

Tumor models and treatment

All animal experiments were performed according to Principles of Laboratory Animal Care, as well as specific laws on Protection of Animals under the provision of authorized investigators. C57BL/6 mice, 18~22 g, were subcutaneously inoculated with 0.2 ml/each of Lewis cells (1×10^7 cells/ml) below the right forelimb armpit. After a two-week tumor inoculation, the lung xenograft cancer model was established when the size of tumor grew to about 65 mm³. Then 32 C57BL/6 mice with xenograft tumor were randomly divided into four groups, each groups had 8 female mice. Each group animals were treated as follows: Group one, as a control group, animals were tumor-bearing set, 0.1%NaOH by subcutaneous injection for 7 days; Group two, animals were not only tumor-bearing set, but also treated by Iso for 14 days (Iso was administered orally (50 mg/kg/d) to animals for 7 days, and then was administered by subcutaneous injection (50 mg/kg/d) for 7 days); Group three, animals also were tumor-bearing set and treated by Iso for 7 days (Iso was administered by subcutaneous injection(50 mg/kg/d) for 7 days); Group four, animals were tumor-bearing set and treated by cisplatin which was administered by subcutaneous injection (25 mg/kg/d) for 7 days. After treatment, samples of tumor were collected. The sections of tumor were observed under light microscope and electron microscope. Cell apoptosis in situ was examined by a TUNEL assay, and expression of some apoptosis proteins was detected by immunohistochemistry.

Statistical analysis

Values are shown as the standard error of the mean, except where otherwise indicated. The statistical package for social sciences version 13.0 (SPSS Inc., Chicago, IL) was used for standardl analysis including one-way variance analysis and student's t-test. Results were considered significant when $P < 0.05$.

Results

Morphologic changes following iso treatment

Treatment with Iso for 24 h or 48 h at low dosage (5 µg/ml) showed lightly morphological changes. Small numbers of shrunken cells and apoptotic bodies were

observed. When A549 cells were treated with Iso at a 20 µg/ml dosage, obvious morphologic changes were observed and the number of shrunken cells and apoptotic bodies increased. Morphological changes of A549 cells exposed to Iso (20 µg/ml) were examined at various times by Giemsa stain (Figure 1). In the control cells, the structure of the nucleus, as well as the size and shape of the mitochondria, was normal. In contrast, in cells treated with Iso, characteristics of apoptosis namely, cell detachment, membrane blebbing fragmented nuclei and nuclear chromatin condensation were observed.

Nuclear chromatin condensation is considered to be part of the cellular events involved in apoptosis. Therefore, we analyzed the fluorescence of the nuclei of cells stained with the DNA-specific dye Hoechst 33258. Pictures were taken after 48 h of treatment. In untreated cells, we observed normal nuclei staining (Figure 1). By contrast, Iso-treated cells (20 µg/ml) displayed typical condensed chromatin and fragmented nuclei. Some cytoplasmic vacuoles were found, and some cells began to round up. At higher dosages, there was a significant increase in the percentage of rounded cells with progressive nuclear shrinkage, especially when the dosage exceeded 40 µg/ml. The cells eventually detached from the culture dish, died, and some cells were observed cytoplasmic vacuolation and cellular fragmentation, especially at high dosages (>80 µg/ml).

Effects on cell growth and proliferation of Iso

To assess the growth inhibition effects of Iso on human lung cancer cells, the MTT assay, clonogenic assay and growth curve assay were performed. The A549 cells were incubated with 10~320 µg/ml Iso for 48h. As seen in Figure 2, a 48 h exposure to Iso triggered a dose-dependent loss in cell viability in A549 cells with an IC₅₀ of approximately 44.45±6.21 µg/ml by MTT assay with the Bliss method. Although the MTT assay is more

accurate as an assessment of growth inhibition, MTT methodology does not allow one to distinguish between the living cells and those committed to death (Vasilevskaya IA et al., 2004). We wished to confirm the results of the MTT assay and to evaluate the cytotoxicity of Iso with a clonogenic assay. The results presented were the percentages of surviving colonies compared to untreated controls after treatment with various concentrations of Iso and cisplatin (Figure 2B). The 20 µg/ml Iso obviously inhibited A549 cell proliferation. The 20µg/mL Iso, 40 µg/ml Iso and cisplatin groups inhibited the A549 cell line growth with Growth Curve Assays. The inhibition was dose- and time-dependent over 7 days.

Effects of Iso on cell apoptosis and cell cycle arrest

The antiproliferative effect of Iso was determined in various human cancer cell lines including oral, nasopharyngeal, breast, lung, colon, stomach, and brain tumor cells (data not shown). To determine whether cell death induced by Iso is associated with apoptosis or not, the nuclear morphology of cells were evaluated. 20 µg/ml Iso-treated cells displayed typical condensed chromatin and fragmented nuclei characteristic of apoptotic cell death were seen in A549 cells after 48 h (Figure 1) and were further increased at later time points. Cell shrinkage with a condensed cytoplasm, and vesicle formation (abundant vacuoles with multivesicular bodies) appeared (Figure 1). We also observed swelling of the endoplasmic reticulum cisternae. The compaction and margination of nuclear chromatin into an amorphous mass osmophilic was quite obvious. Moreover, the extent of nuclear chromatin condensation could be correlated with changes in mitochondria structure. At 24 h after the addition of Iso, the mitochondrial changes could be characterized with interruption and/or absence of the cristae and with loss of matrix density (Figure 1).

In the TUNEL assay, the presence of DNA

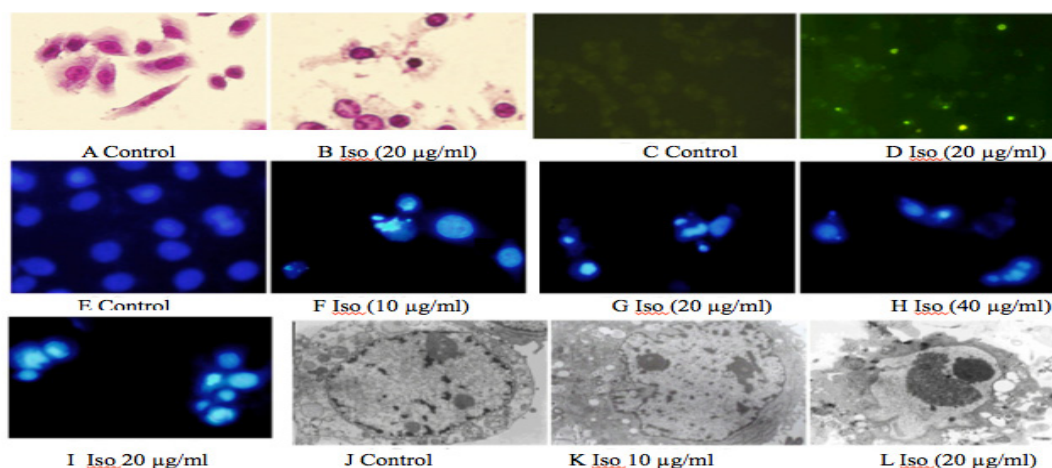


Figure 1. Morphological Changes of A549 Cells by Iso Treatment. Microscope analysis of control cells (A) and Iso-treated cells (B) by Gimesa-staining ; cells became round, and typical apoptotic changes such as membrane blebbing and condensed nuclear chromatin (B) were observed in Iso-treated cells for 48 h. The positive cells (D) was increased and the presence of DNA fragmentation was noted compared the control cell (C) by the TUNEL assay. Condensed nuclear chromatin , vesicle formation , fragmented nuclei and increased apoptotic bodies appeared in Iso-treated cells for 24 h (F,G,H) or in for 48h (I) compared with control cells (E) were demonstrated by Hoechst 33258 staining. Detected by electron microscope, control cells with apparent desmosome and microvilli (J). Membrane blebbing was quite obvious after Iso treatment for 24h (K). Vesicle formation and abundant vacuole formation, multivesicular bodies appeared for 48h. Mitochondrial changes could be characterized by the interruption and/or absence of the cristae and the loss of matrix density (L)

fragmentation was noted in A549 cells and in our mouse model's Lewis cells treated with Iso (Figure 1D). With high dosage treatments, the number of TUNEL-positive cells increased in a dose-dependent manner (20~80 $\mu\text{g/ml}$).

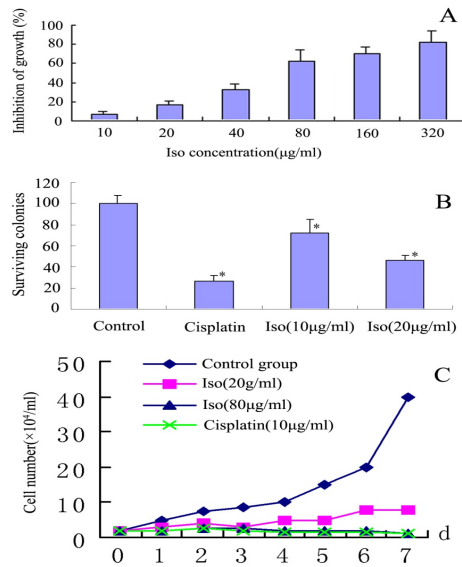


Figure 2. Inhibitory Effects of Iso on A549 Cells. For the studies assessing the effect of Iso on exponentially growing A549 cell growth (A), 1 day after seeding, cells were treated with increasing concentrations of Iso for 48 h. At the end of the incubation, cell viability in control or in Iso-treated cells was measured using the MTT assay. Results were expressed as the percentage of viable cells of the untreated cells and were the mean \pm SD of four separate experiments. By using clonogenic assays (B), colony-forming efficacy was calculated as an average of triplicate experiments. * $p < 0.05$ versus control. Cell viability in control or in different concentration Iso-treated cells was measured every day by trypan blue dye assay (C). Results are expressed as the percentage of viable cells at the beginning of the experiment

The results of comet assay in A549 cells are summarized in Figure 5, and representative examples of comets were showed in Figure 3D. We observed that Iso and cisplatin produced damage in terms of the percentage of DNA in the comet tail. We noticed a high percentage (>25%) of typical apoptotic comets with Iso, whereas cisplatin induced very few apoptotic comets (<5%) and more damage in terms of the percentage of DNA in the comet tail. The percentage of typical apoptotic comets with control, Iso and cisplatin are summarized in Figure 3D.

DNA fragmentation analysis, flow cytometric

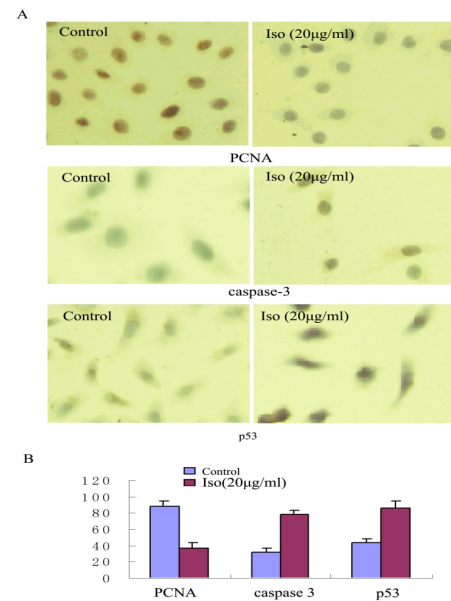


Figure 4. Effect of Iso on Inducing Cellular Apoptosis and Apoptosis-related Genes. Using immunocytochemistry method, A549 cells plated on cover slips were treated with different concentrations of Iso for 24 h. The images were representative of two independent experiments

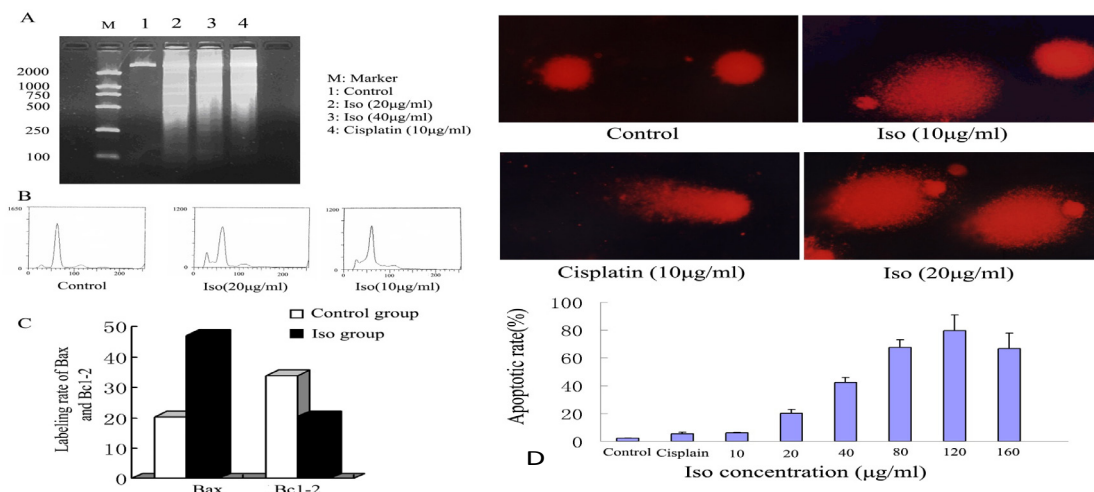


Figure 3. Effects of Iso on the Apoptosis, Cell Cycle Progression and Expression of Apoptosis-Related Genes of A549 Cells. (A) DNA fragmentation in the Iso-treated cells, A549 cells were treated with free or drugs at each concentration as indicated (Lanes 2–5) for 24 h. lane M, DNA marker; lane 1, untreated cells; lane 2, cells treated with 20 $\mu\text{g/ml}$ Iso; lane 3, treated with 40 $\mu\text{g/ml}$ Iso; lane 4, treated with 10 $\mu\text{g/ml}$ cisplatin. (B) Effect of Iso on the cell cycle progression of A549 cells. Cell cycle phase distribution (%) was measured by flow cytometry. Data were representative shown from one of three independent experiments. (C) Expression of bcl-2 and bax of A549 cells treated with 0 or 20 $\mu\text{g/ml}$ Iso for 24 h were analyzed using a flow cytometer. (D) DNA fragmentation of A549 cells treated with Iso or cisplatin for 24h by comet assay. The percentage of typical apoptotic comets was evaluated on 50 cells. Values were mean \pm SD (n=10). Photos were representative of observed comets.

detection, Comet assays, and TUNEL assays were performed to detect apoptosis. The cells were treated with different concentrations (20, 40, 80 $\mu\text{g/ml}$) of Iso, or cisplatin (10 $\mu\text{g/ml}$) for 24 h. DNA fragmentation, shown by the formation of a DNA ladder, was observed in A549 cells treated with Iso (Figure 3A). Apoptosis was further confirmed by flow cytometric detection of DNA fragmentation in Iso-treated A549 cells (Figure 3B). The results of all of our studies indicated apoptosis induced by Iso not only in A549 cells, but also in other human solid tumor cells (data not shown).

The effect of different concentrations of Iso (10–20 $\mu\text{g/ml}$) on the cell-cycle progression of A549 cells was studied after 24 h of drug exposure. Iso treatment resulted in an accumulation of A549 cells in the G₀/G₁ phase with concomitant losses in the S phase (Figure 3B). Similar results were obtained using other cell lines (e.g., Hela and MCF-7 cells; data not shown).

Effect of iso on the expression of cell cycle regulators and apoptosis-related genes

After confirming that Iso induces apoptosis and changes the cell cycle, we attempted to unveil the underlying mechanisms. It was well recognized that various pro- and anti-apoptotic proteins or genes play an important role in cell survival, as well as in programmed cell death. We examined the effect of Iso on the expression of these regulators and apoptosis-related genes. Compared with the negative control group, flow cytometric analysis showed that Bcl-2 decreased and Bax increased significantly, thereby the ratio of Bcl-2/Bax decreased in Iso treating group (Figure 3C), and H-ras, c-myc expression levels slightly decreased but p53, C-fos expression levels slightly increased (data not shown) in flow cytometry measurements. Besides, in Iso groups the Bcl-2 expression and the ratio of Bcl-2/Bax were downregulated in A549 cells, CyclinD1 expression levels slightly decreased that could induced G₁ phase inhibition as measured by immunocytochemistry (data not shown). Using immunocytochemistry method to determine the effect of Iso, it displayed that Iso could significantly induce PCNA protein expressions decrease and caspase-3 and p53 expressions increase, and the changes were dose-dependent (Figure 4), same results were found by western-blot methods. The same results were found in the animal model.

Treatment of the tumor-bearing mice with iso

To investigate the effect of Iso on lung tumor growth, cell proliferation and apoptosis, the tumors in C57BL/6 mice, growing by transplanting of lung cancer of Lewis cell line were checked. After treatment of the tumor-bearing mice with Iso or cisplatin, the tumor weight was significantly lighter and the tumor size was smaller than that of the control group (Figure 5). To determine whether cell death induced by Iso is associated with apoptosis *in vivo*, the nuclear morphology of cells was evaluated. Just as seen in A549 cells, transplanting of Lewis lung cancer cells line treatment by Iso displayed typical condensed chromatin, cell shrinkage with a condensed cytoplasm and fragmented nuclei characteristic of apoptotic cell death

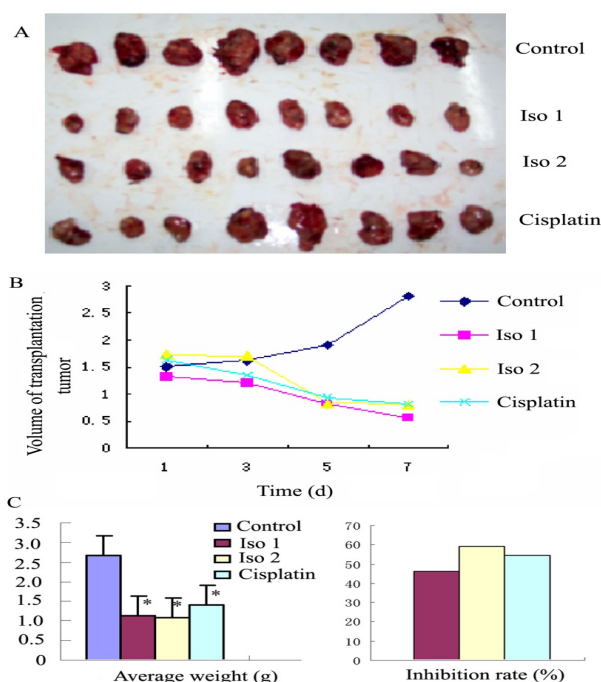


Figure 5. Effect of Iso on the Xenograft Lewis Lung Cancer Model. (A) Inhibitory effects of Iso and cisplatin on mouse tumor. (B) Growth curve of mouse transplantable lung tumors. So compared to the control group, the tumor weight was lower, tumor size was smaller and the inhibition rate was increased (C). Data were the mean \pm SD of two independent experiments

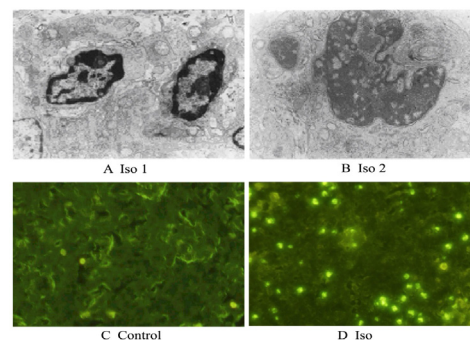


Figure 6. Morphological Apoptosis Changes of Transplanted Lewis Lung Cells Treated with Iso *in vivo*. Obviously typical apoptosis changes, such as condensed nuclear chromatin in Iso-treated cells observed by electron microscope (A,B); typical condensed chromatin and fragmented nuclei characteristic of apoptotic cell death in Iso-treated group (D) compared negative control (C) by TUNEL detection

compared control cells (Figure 6). Apoptosis was further confirmed by TUNEL detection of apoptotic bodies in Iso-treated Lewis cells compared control cells (Figure 6). The results of apoptosis-related genes, PCNA and other protein expression levels of transplanted Lewis cells were the same as those of A549 cells *in vitro* (data not shown).

Discussion

The total flavones and Iso, the novel essential monomer derived from total flavones of Hippophae rhamnoides, have long been used as a traditional Chinese herb medicine in the treatment of cardiovascular diseases and chronic hepatitis, and cancer.

Many classes of cancer chemopreventive agents, including naturally occurring and pharmaceutical compounds, were studied for efficacy *in vivo* and *in vitro*. In this study, Iso inhibited cell growth and induced apoptosis in both the cancer cells coming from human lung carcinoma A549 cell line and the Lewis lung carcinoma transplanting cells. The effect of Iso on A549 cell growth was observed at dosages as low as 5 µg/ml, and the maximal effect was seen at 320 µg/ml, the IC₅₀ was 44.5 µg/ml. We also found Iso could inhibit other carcinoma cell lines such as MCF-7, SMMC7721, PC3, K562, Caco-2, and etc the IC₅₀ was from 57.2 µg/ml to 129.1 µg/ml, but we found more growth inhibition effect on lung cell line A549. Very lucky we found that, the IC₅₀ of mice normal tracheal epithelial cells by primary culture and human keratinocytes cell line (HaCaT cells) were very high, we could not test out the value by MTT assay (Data not shown), that means Iso had no obviously growth inhibition on normal cell such as Primary normal mice airway epithelial cells, HaCaT cell line. Our results clearly also showed that Iso induced cell cycle arrest and subsequent apoptotic death in the human lung cancer cell line A549 in a dose-dependent manner. A549 cells treated with Iso for 24 h resulted in a concentration-dependent accumulation in G0/G1 phase with concomitant losses in the S phase, which indicated that cells continued mitosis but could not pass the G1 phase to enter the S phase by inhibit cyclinD1 and failed to trigger DNA synthesis. In our model *in vitro*, we clearly observed many of the typical structural and ultrastructural modifications that happened during the apoptotic pathway. The classical DNA fragmentation on Iso treatment was also observed. Iso induced apoptosis in a concentration-dependent manner, as seen in flow cytometry data, TUNEL assays, and comet assays, and was further confirmed by an immunocytochemical study. We further demonstrated *in vivo* antitumor activity of Iso in mouse model. By western-blot methods, we found same results, we also found NF-κB expression slightly increased by Iso, but there was no dose-dependent effect on A549 cell line. We need more research to know the deeply mechanism of Iso. Based on those results, it indicated that Iso, a novel plant-derived agent, was a potent trigger of the apoptosis of tumor cells and thus holds promise as a chemotherapeutic compound. Iso was a potent anticancer agent. Its ability to induce apoptosis in different cancer cell lines indicated the possibility of developing Iso as a universal cancer prophylactic agent. One of the major signaling pathways involves in apoptotic cell death including the intracellular caspases, a family of cysteine proteases structurally related (Salvesen and Dixit, 1997). Caspase activity is responsible, either directly or indirectly, for cleavage of cellular proteins, which is characteristically proteolyzed during apoptosis. Activating cytosolic caspases, including caspase-3, results in the rapid dismantling of recognition signals for neighboring phagocytes (Uthaisang et al., 2004). In the mitochondria dependent pathways, cytochrome C release stimulates an apoptotic protease cascade involving caspases, which are cysteine proteases active during the effector phase of apoptosis (Takagi et al., 2004). The Bcl-2 family members reside primarily in the membranes

of the mitochondria, but Bcl-2 is also localized to the membranes of the endoplasmic reticulum and the nucleus. It has been known that the Bcl-2 family plays a central role in the control of apoptosis, which includes a number of proteins that have amino acid sequence homology, including anti-apoptotic members such as Bcl-2 and Bcl-xL, as well as pro-apoptotic members including Bax and Bad (Scatena et al., 1998; Zhong et al., 2013). Over expression of Bcl-2 is shown to prevent chemotherapeutic agent-induced apoptosis associated with altering mitochondria transmembrane potential (Kim et al., 2003; Bhattacharyya et al., 2004; Das et al., 2012). In addition, Bcl-2 is displayed to prevent the release of cytochrome C from the mitochondria while Bax promotes its release (Huigsloot et al., 2003). Furthermore the protein P53 plays an important role in pathogenesis of neoplasia (Qin and Ng, 2002). The mechanism involved entails a rapid increase in p53 protein level and the mediation of several cellular responses including G1 arrest, DNA damage repair and induction of apoptosis (Radhakrishna et al., 2004). PCNA is found in the nucleus and is at the very heart of many essential cellular processes, such as DNA replication, repair of DNA damage, chromatin structure maintenance, chromosome segregation and cell-cycle progression (Stoimenov and Helleday, 2009). The higher the degree of tumor cell proliferation, tumor growth is faster, more prone to transfer. Therefore, PCNA expression assessed as a useful indicator of tumor prognosis (Chen et al., 2010). The results of apoptosis-related genes, PCNA and other protein expression levels of transplanted Lewis cells were the same as those of A549 cells *in vitro*. After treated with Iso, Bcl-2 and PCNA protein expressions decreased significantly, and Bax and caspase-3 increased, thereby resulting in a decrease in Bcl-2/Bax ratio, but p53 expression levels slightly decreased.

In conclusion, we demonstrated that Iso, a natural single compound isolated from total flavones, can inhibit A549 cell proliferative activity, whereas it also has significant antitumor effects *in vivo*. Our results have suggested the Iso effect of anti-cancer by inducing apoptosis of cells, may alter the expression of apoptosis-related genes such as downregulating Bcl-2 expression and the ratio of Bcl-2/Bax via modulating Bcl-2 family members. These results suggested that Iso was an attractive candidate for cancer therapy. Further exploration of Iso as an anticancer compound is worthwhile.

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