

Isolinderalactone inhibits proliferation of A549 human non-small cell lung cancer cells by arresting the cell cycle at the G₀/G₁ phase and inducing a Fas receptor and soluble Fas ligand-mediated apoptotic pathway

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Abstract. Lung cancer is currently the leading cause of cancer-related mortality worldwide. In Taiwan, lung cancer is also the type of malignancy that is the major cause of cancer-mortality. Investigating the mechanism of apoptosis of lung cancer cells is important in the treatment of lung cancer. In the present study, isolinderalactone was demonstrated to exhibit anticancer effects in A549 human non-small cell lung cancer cells. The effect of isolinderalactone on apoptosis, cell cycle distribution p21 levels and the Fas receptor and soluble Fas ligand (sFasL) were assayed in order to determine the mechanism underlying the anticancer effect of isolinderalactone. It was demonstrated that isolinderalactone may induce p21 expression and then cause the cell cycle arrest of A549 cells. The data of the present study also revealed that the Fas/sFasL apoptotic system is significant in the mechanism of isolinderalactone-induced apoptosis of A549 cells. These novel findings demonstrated that isolinderalactone may cause the cell cycle arrest of A549 cells by induction of p21, and induce apoptosis of A549 human non-small-cell lung carcinoma cells through the Fas/sFasL apoptotic system.

Introduction

Lung cancer is currently the leading cause of cancer-related mortality worldwide. In lung cancer patients, ~85% are diagnosed with non-small cell lung cancer (1,2). The prognosis for patients with non-small cell lung cancer is poor. Surgical intervention, chemotherapy, target therapy and radiotherapy are the treatments of choice for lung cancer (3). However, progression and recurrence of disease often occur after treatment. In addition, lung cancer is often resistant to the current treatment options available. Therefore, there is an requirement for the identification of a novel treatment for non-small cell lung cancer.

Apoptosis is key in numerous diseases, including lung cancer (4). There are intrinsic and extrinsic pathways that are involved in cell apoptosis. In cancer cells, alternations of the upstream regulators of intrinsic and extrinsic pathways are common, which may result in an imbalance between cell proliferation and apoptotic cell death. A number of chemotherapy-related drugs may induce apoptosis of cancer cells (5). Fas is a receptor on the cell surface, which may be activated by its ligand (Fas L) or by a cross-linking antibody. Following activation, oligomerization of the intracellular death domain of Fas is likely to occur (6). Fas results in cleavage of pro-caspase-8 and -3, then pro-caspase-3 turns into activated caspase-3 and induces apoptosis (7).

Lindera aggregata (SIMS) KOSTERM. The Lauraceae family is comprised of ~55 genera and 2,500 species. The majority of them are in the tropics and subtropics (8). In China, it is known as Wu Yao and in Japan, Uyaku. Lauraceae is used for treating renal, cystic and rheumatic diseases (9). *Lindera aggregata*, isolinderalactone, linderalactone and linderane are known sesquiterpenes derived from root tubers. In the present study, the antiproliferative activity of these compounds was determined (Fig. 1) (10) and the effect of isolinderalactone on the cell cycle distribution and apoptosis was examined in A549 human non-small cell lung

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cancer cells. Many anticancer drugs can induce apoptosis of cancer cells. The Fas/FasL system is a key regulator of apoptosis.

Materials and methods

Materials. Isolinderalactone, linderalactone and linderane were purchased from ChemFaces (Wuhan, China). Fetal bovine serum (FBS), penicillin G, streptomycin, amphotericin B and RPMI-1640 were obtained from Gibco-BRL (Gaithersburg, MD, USA). Dimethylsulfoxide (DMSO), ribonuclease and propidium iodide were purchased from Sigma Chemical (St. Louis, MO, USA). p21 Waf1/Cip 1 and APO-1/Fas/CD95 ELISA kits as well as sFas Ligand Immunoassay kits were purchased from Invitrogen Life Technologies (Camarillo, CA, USA). Anti-Fas antibody (ZB4) was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY, USA). Caspase-8 assay kit, and caspase-8 inhibitor, benzyloxy-carbonyl-Val-Ala-Asp-fluoromethylketone (Z-IETD-FMK) were purchased from Calbiochem (Cambridge, MA, USA).

Cell culture. A549 human non-small cell lung cancer cells were incubated at 37°C in a 5% CO₂-containing incubator. Minimum essential medium with 10% FBS, 100 U/ml penicillin G, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B, non-essential amino acids and 0.1 mM sodium pyruvate was used. The medium was changed every 2-3 days and we ensured the cells reached a distinct cell density.

Cell proliferation. Briefly, the cells were plated in 96-well culture plates (1x10⁴ cells/well), and after 24 h incubation, treated with vehicle alone (0.1% DMSO) and various concentrations of isolinderalactone, linderalactone and linderane for 48 h. A549 proliferation was determined by Premixed WST-1 Cell Proliferation Reagent (Clontech Laboratories Inc., Mountain View, CA, USA) according to the manufacturer's instructions. In brief, the cells were incubated with premixed WST-1 cell proliferation reagent for 0.5-4 h. Tetrazolium salt WST-1 was cleaved to a formazan-class dye by mitochondrial succinate-tetrazolium reductase in viable and metabolically active cells, thereby quantitating the formazan dye by measuring the absorbance at 450 nm in a multiwell plate reader (Multiskan EX, Labsystems, Helsinki, Finland) providing measurement of cell proliferation. The percentage of inhibition was calculated using the following formula: % inhibition = [100 - (ODt/ODs) x 100] ODt and ODs indicate the optical density of the test substances and the solvent control, respectively.

Cell cycle analysis. In order to determine cell cycle distribution, 5x10⁵ cells were plated in 60-mm dishes and treated with vehicle alone (0.1% DMSO) and isolinderalactone (40 µM) for 24 h. Following treatment, the cells were collected by trypsinization, fixed in 70% ethanol, washed in phosphate-buffered saline (PBS), re-suspended in 1 ml PBS containing 1 mg/ml ribonuclease and 50 µg/ml propidium iodide, incubated in the dark for 30 min at room temperature and analyzed by an EPICS flow cytometer (Beckman Coulter, High Wycombe, UK). The data were analyzed using

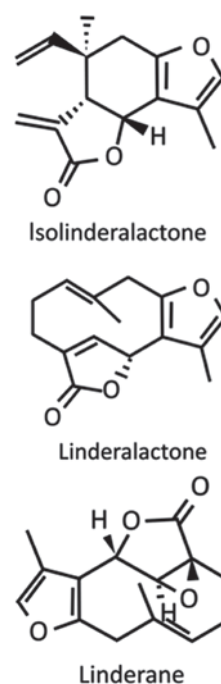


Figure 1. Chemical structure of isolinderalactone, linderalactone and linderane.

the Multicycle software (Phoenix Flow Systems, San Diego, CA, USA).

Analysis of apoptosis. A quantitative analysis of apoptotic cells was undertaken by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) method, which examines the DNA-strand breaks during apoptosis using the BD ApoAlert™ DNA fragmentation assay kit (BD Biosciences Clontech, Palo Alto, CA, USA). Briefly, the cells were incubated with vehicle alone (0.1% DMSO) and isolinderalactone (40 µM) for the indicated times, then trypsinized, fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. Following washing, the cells were incubated with the reaction mixture for 60 min at 37°C. The stained cells were then analyzed with an EPICS flow cytometer and a fluorescence microscope (Nikon Eclipse TE 300, Nikon, Tokyo, Japan) at magnification, x20.

Assaying the levels of p21, Fas and sFasL. p21 Waf1/Cip 1 and APO-1/Fas/CD95 ELISA kits and an sFas ligand immunoassay kit were used to detect p21, Fas receptor and sFasL, respectively. Briefly, the A549 cells were treated with vehicle alone (0.1% DMSO) and isolinderalactone (40 µM) for 6, 12, 24, and 48 h. The samples of the cell lysate were placed in 96-well microtiter plates that were coated with monoclonal detector antibodies and incubated for 1 (Fas) or 2 h (p21 and sFasL) at room temperature. Each sample is assessed in triplicate. Following removal of the unbound material by washing with washing buffer (50 mM Tris, 200 mM NaCl and 0.2% Tween-20), the horseradish peroxidase-conjugated streptavidin, which can bind to detector antibody, was added and incubated at room temperature for 30 min. Horseradish peroxidase catalyzed the conversion of a chromogenic substrate (tetramethylbenzidine) to a colored solution with

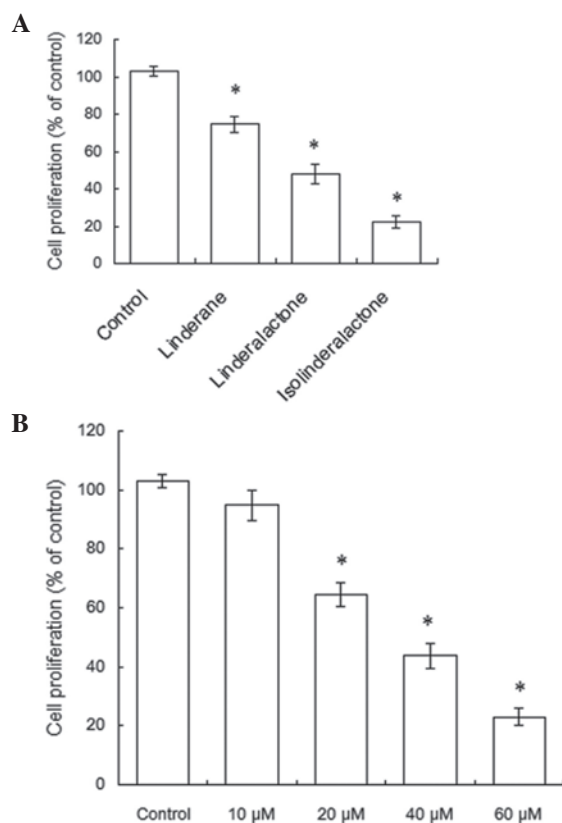


Figure 2. Antiproliferative effect of three known sesquiterpenes from the root tubers of *Lindera aggregata* (isolinderalactone, linderalactone and linderane) in the A549 non-small-cell lung cancer cell line. (A) Adherent cells plated in 96-well plates (1×10^4 cells/well) were incubated with 60 μ M isolinderalactone, linderalactone or linderane for 48 h. (B) Adherent cells plated in 96-well plates (1×10^4 cells/well) were incubated with different concentrations of isolinderalactone for 48 h. Cell proliferation was determined by the WST-1 assay. The results are expressed as the percentage of cell proliferation relative to the proliferation of the control. Each value is presented as the mean \pm standard deviation of three determinations. * $P < 0.05$, compared with the control group, the proliferation of isolinderalactone-treated cells was significantly suppressed, as analyzed by one-way analysis of variance with post hoc Student's t-test.

color intensity proportional to the quantity of protein present in the sample. The absorbance of each well was measured at 450 nm, and concentrations of p21, Fas and sFasL were determined by interpolating from standard curves obtained with known concentrations of standard proteins.

Assay for caspase-8 activity. The assay is based on the ability of the active enzyme to cleave the chromophore from the enzyme substrate, Ac-IETD-pNA. The cell lysates were incubated with peptide substrate in assay buffer [100 mM NaCl, 50 mM HEPES, 10 mM dithiothreitol, 1 mM EDTA, 10% glycerol, 0.1% CHAPS, (pH 7.4)] for 3 h at 37°C. Each sample was assayed in triplicate. The release of *p*-nitroaniline was monitored at 405 nm using a multiwell plate reader (Multiskan EX). The results are presented as the percentage change of the activity compared with the untreated control.

Statistical analysis. All results are expressed as the mean \pm standard deviation and analyzed by one-way analysis of variance. The differences between the experimental and control groups were analyzed using Student's t-test.

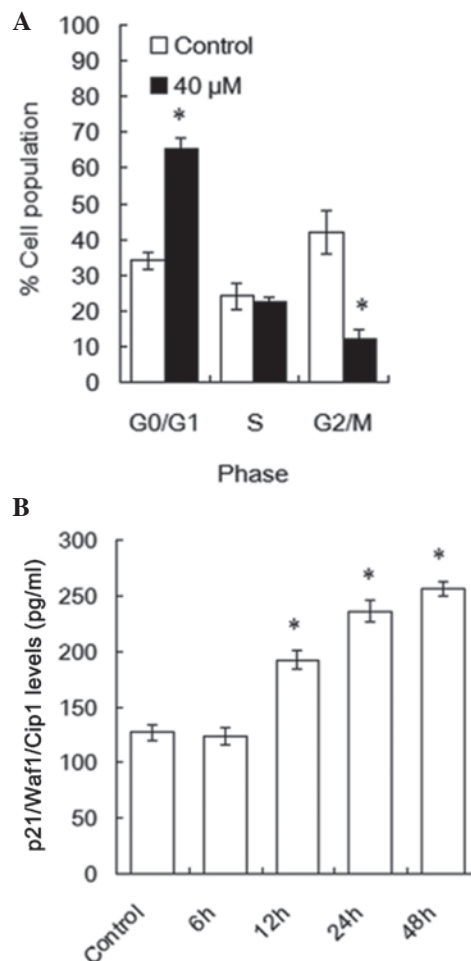


Figure 3. Effects of isolinderalactone on (A) cell cycle distribution as well as (B) p21 in A549 cells. A549 cells following treatment with vehicle alone (0.1% DMSO) and isolinderalactone (40 μ M) for 24 h were fixed and stained with propidium iodide, and the cell cycle distribution was then analyzed by flow cytometry. The A549 cells were treated with vehicle alone (0.1% DMSO) and isolinderalactone (40 μ M) for the indicated time. The p21 levels were determined by the p21 Waf1/Cip 1 ELISA kit. The detailed procedure is described in the Materials and methods. Each value is presented as the mean \pm standard deviation of three determinations. The asterisk indicates a significant difference between the control and isolinderalactone-treated cells, as analyzed by one-way analysis of variance post hoc Student's t-test. DMSO, dimethylsulfoxide.

$P < 0.05$ was considered to indicate a statistically significant difference.

Results

Cell proliferation of A549 human non-small-cell lung cancer cells is inhibited by isolinderalactone. Isolinderalactone, linderalactone and linderane were tested on A549 non-small-cell lung cancer cells. These compounds are sesquiterpenes derived from the root tubers of *Lindera aggregata*. Fig. 2A demonstrated that after 48 h treatment, these three compounds (60 μ M) inhibited the proliferation of human non-small-cell lung cancer cells. Compared with linderalactone and linderane, isolinderalactone exhibited a greater effect on the inhibition of cell proliferation. Due to the aforementioned results, isolinderalactone was selected for the investigation of the mechanism of *Lindera aggregata* in

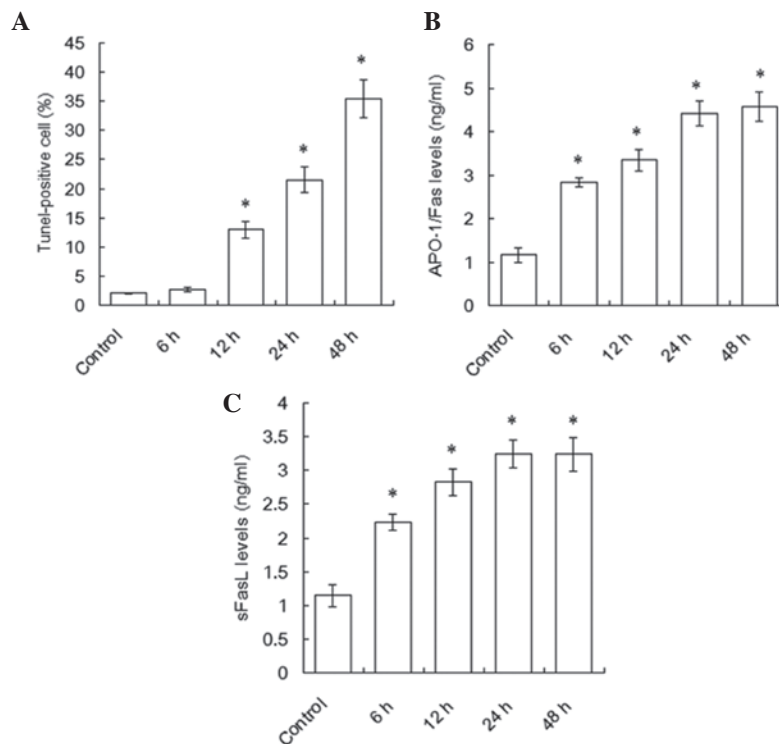


Figure 4. Fas/soluble Fas ligand (sFasL) apoptotic system was involved in isolinderalactone-mediated apoptosis in A549 cells. (A) Isolinderalactone-induced apoptosis in A549 cells. (B) The effects of isolinderalactone on the levels of Fas receptor and (C) the levels of sFasL in A549 cells. The A549 cells were treated with vehicle alone (0.1% dimethylsulfoxide) and various concentrations of isolinderalactone for the indicated times, and the apoptotic cells were then stained using the terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling assay. Fas and sFasL levels were determined by the APO-1/Fas/CD95 ELISA kit and sFas ligand immunoassay kit respectively. The detailed procedure is described in the Materials and methods. Each value is presented as the mean \pm standard deviation of three determinations. The asterisk indicates a significant difference between the control and isolinderalactone-treated cells, as analyzed by one-way analysis of variance with post hoc Student's t-test.

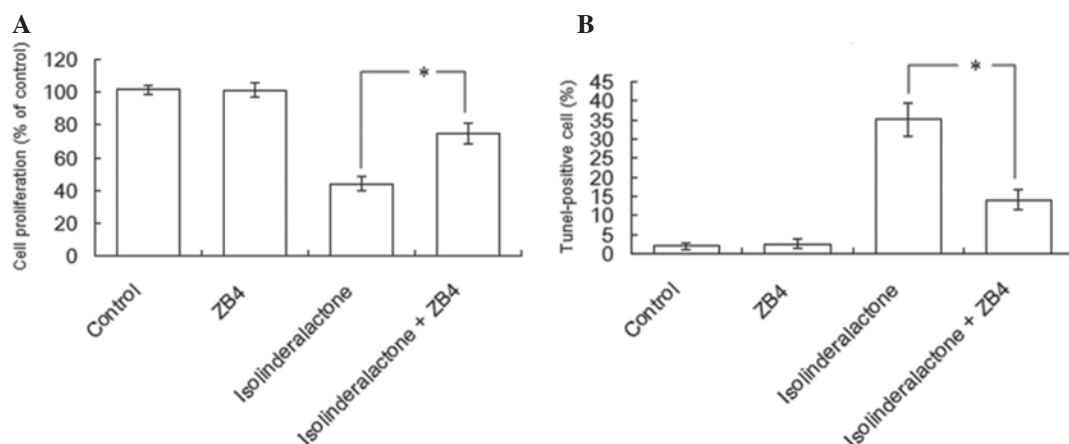


Figure 5. Effect of antagonistic anti-Fas antibody (ZB4) on isolinderalactone in A549 cells. (A) The antiproliferative and (B) apoptotic effect of isolinderalactone was decreased by Fas antagonist ZB4. For the blocking experiments, the cells were pre-incubated with 250 ng/ml ZB4 for 1 h and then treated with 40 μ M isolinderalactone for 48 h. Cell viability and apoptosis induction were examined by a WST-1 and BD ApoAlert™ DNA fragmentation assay kit. Each value is presented as the mean \pm standard deviation of three determinations. The asterisk indicates a significant difference between the two test groups, as analyzed by one-way analysis of variance with post hoc Student's t-test.

anticancer agent development. The A549 cells were treated with isolinderalactone at varying concentrations. Following treatment with isolinderalactone, the proliferation of A549 cells was observed to decrease. Following treatment of the A549 cells with 60 μ M isolinderalactone for 48 h, the proliferation of A549 cells was inhibited by 78%. The IC_{50} values were 37.3 μ M (Fig. 2B). Thus, a total concentration of 40 μ M

isolinderalactone was selected for detecting the mechanism of the antiproliferative effect in A549 cells.

Isolinderalactone-mediated cell cycle arrest may operate through the induction of p21 protein in A549 human non-small-cell lung cancer cells. The cell cycle distribution was assessed in order to clarify the mechanism of the

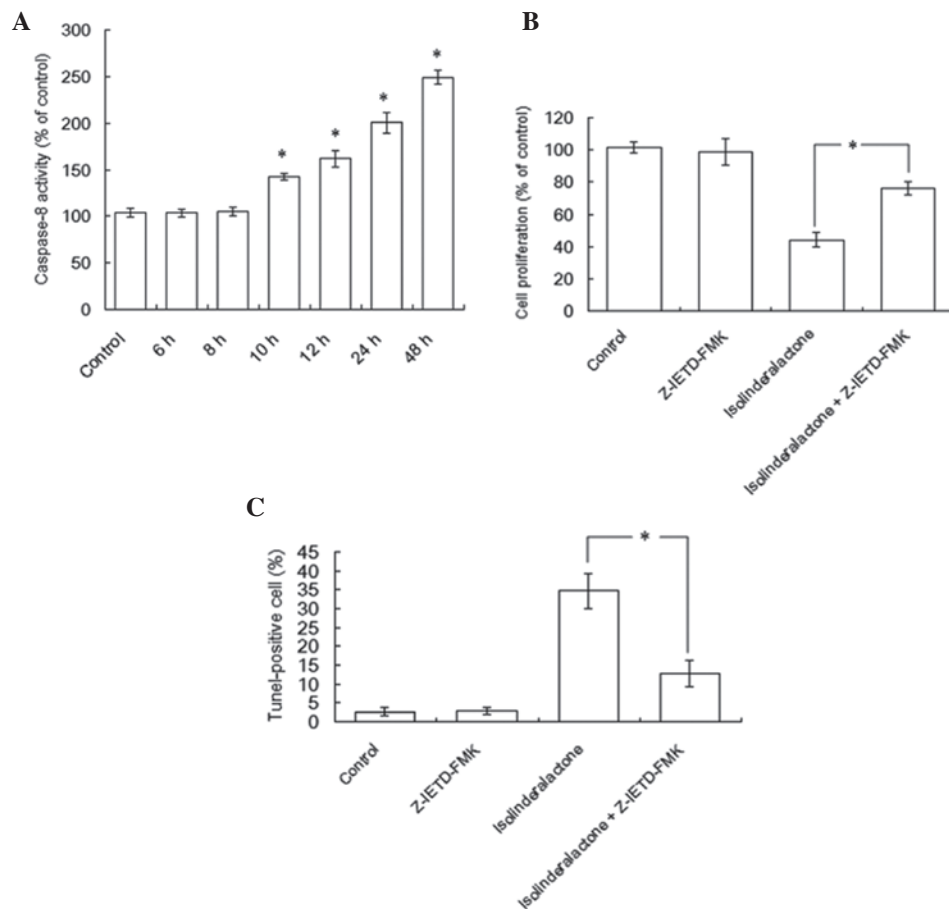


Figure 6. (A) Activation of caspase-8 in A549 cells induced by isolinderalactone. (B) Effect of caspase-8 inhibitor on isolinderalactone-mediated antiproliferation. (C) Effect of caspase-8 inhibitor on isolinderalactone-induced apoptosis. The A549 cells were incubated with various concentrations of isolinderalactone for the indicated times. For the blocking experiments, the cells were pre-incubated with Z-IETD-FMK (10 μ M) for 1 h prior to the addition of 40 μ M isolinderalactone. After 48 h of treatment, the cell viability and apoptosis induction were examined by the WST-1 and BD ApoAlert™ DNA fragmentation assay kit. Each value is presented as the mean \pm standard deviation of three determinations. The asterisk indicates a significant difference between the two test groups, as analyzed by one-way analysis of variance with post hoc Student's t-test.

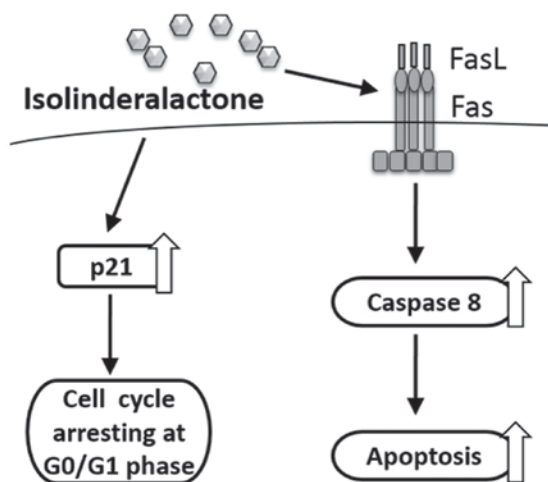


Figure 7. Pathway of isolinderalactone-induced apoptosis of A549 non-small cell lung cancer cells.

antiproliferative effect. Fig. 3 shows the results on the effects of isolinderalactone on the cell cycle progression of the A549 cells. As compared with the control group, the population of the G₀/G₁ phase in A549 cells treated with 40 μ M isolinderalactone

increased from 34.1-65.6% (Fig. 3A). The levels of the p21 protein were assayed by a p21 Waf1/Cip 1 ELISA kit in order to investigate whether p21 is involved in the mechanism of the isolinderalactone-induced arrested cell cycle in the A549 cells. Fig. 3B shows the p21 protein level in A549 cells treated with 40 μ M isolinderalactone increased when the period of 40 μ M isolinderalactone treatment was increased (6, 12, 24 and 48 h). Therefore, it is hypothesized that the induction of p21 is involved in isolinderalactone-mediated cell cycle arrest.

Fas/sFasL apoptotic system may be a possible pathway of isolinderalactone-mediated apoptosis. Due to the consideration that the mechanism of the antiproliferative effect of isolinderalactone is associated with apoptosis, TUNEL was used to detect DNA-strand breaks as a quantitative evaluation. A concentration of 40 μ M isolinderalactone induced apoptosis in 35.5% of A549 cells at 48 h (Fig. 4A). The expression of the Fas receptor and sFasL in A549 cells increased in a dose-dependent manner after 6 h treatment with isolinderalactone. The APO-1/Fas/CD95 ELISA and sFas ligand immunoassay kits were used in this process (Fig. 4B and C). The sFasL level reached the peak value after 24 h of treatment. It was hypothesized that the Fas/sFasL system is involved in the isolinderalactone-induced apoptosis of A549 cells, due

to the time correlation between the expression of Fas/sFasL at 6 h of treatment and the initiation of apoptosis at 12 h of treatment. In order to investigate this further, the A549 cells were pretreated with ZB4, which is an antagonistic anti-Fas antibody for inhibiting the antiproliferative and proapoptotic effects of isolinderalactone. At 48 h in A549 cells pretreated with ZB4 and treated with 40 μ M isolinderalactone, the isolinderalactone-induced inhibition of cell proliferation inhibition decreased from 56.1 to 25.1% in the A549 cells (Fig. 5A). At the same time, the induction of apoptosis by 40 μ M of isolinderalactone decreased from 35.1 to 13.9%.

Subsequent to this, the downstream caspase of the Fas/sFasL system was measured. Caspase-8 activity was observed to increase at 10 h. The maximum induction effect appeared at 48 h in 40 μ M isolinderalactone treated A549 cells (Fig. 6A). The activation of caspase-8 (at 10 h) was compared earlier with the production of DNA fragmentation (at 12 h). This finding suggests that caspase-8 activation is involved in isolinderalactone-induced apoptosis. To prove this hypothesis, the A549 cells were pretreated with a caspase-8 inhibitor (Z-IETD-FMK) and isolinderalactone, compared the A549 cells that were only treated with isolinderalactone, there was increased cell proliferation and fewer tunnel-positive cells. The results indicated that inhibition of caspase-8 may decrease the antiproliferative activity of isolinderalactone (Fig. 6B) and evidently decrease the induction of apoptosis of the A549 cells (Fig. 6C).

Discussion

The protein p21 is classified as a member of the cip/kip family, which function as cyclin-dependent kinase (CDK) inhibitors (11). Upon stimulation of the cells, the expression of p21 is regulated at the transcriptional and post-translational levels (12). The p21 protein has the ability to inhibit the cyclin-CDK2 and CDK1 complexes. Therefore, it may regulate the cell cycle progression at the G₁ phase, and may also inhibit the phosphorylation of the retinoblastoma protein. For this reason, p21 may inhibit the G₁-S phase transition. Following treatment of A549 cells with isolinderalactone, the quantity of p21 was observed to increase. In addition, flow cytometric analysis revealed that the A549 cells were arrested in the G₀/G₁ phase by isolinderalactone. Based on the aforementioned findings, we hypothesize that the increase in the level of p21 protein is involved in the blockade of cell cycle progression. However, p21 may also inhibit apoptosis (13). The data of the present study may reveal that p21 has a greater effect in inhibiting the G₁-S phase transition compared with the inhibition of apoptosis following treatment of the A549 cells with isolinderalactone.

Apoptosis may be induced by intrinsic and extrinsic pathways (14). In the extrinsic pathway, the Fas/FasL system is a significant signal transduction pathway of apoptosis in cancer cells. The Fas receptor is a cell surface receptor and is significant in triggering the apoptotic pathway. Following stimulation with FasL, the Fas-associated death domain (FADD) is recruited to the cytoplasmic domain of Fas (15). FADD is an adaptor protein and tumor necrosis factor receptor family-mediated apoptosis is associated with FADD. FADD is involved in cell proliferation, survival and embryonic

death (16). Certain anticancer drugs activate FasL, when FasL binds Fas in an auto-/paracrine manner, it triggers the extrinsic pathway through activation of caspase-8 (17). Celastrol, a natural compound derived from Chinese traditional herbs, induces apoptosis of the A549 lung cancer cells by cleavage of caspase-9, -8, -3, and the PARP protein, increasing the expression of Fas and FasL, and reducing the mitochondrial membrane potential (17).

The present study demonstrates that sFasL increased in the A549 cells that were treated with isolinderalactone. The levels of Fas and the activity of caspase-8 increased in A549 cells with upregulated sFasL. When the Fas/sFas ligand system was inhibited with ZB4, the cell growth inhibition and the proapoptotic effect of isolinderalactone were observed to decrease. The apoptotic induction and cell growth inhibition of isolinderalactone was observed to decrease in the A549 cells that were treated with caspase-8 inhibitor.

These findings indicate that the Fas/sFasL system has a significant role in isolinderalactone-mediated A549 cellular apoptosis. Finally, the present study demonstrated that isolinderalactone induces cytotoxic activity in the A549 human non-small cell lung cancer cells. To the best of our knowledge these findings are the first to demonstrate that the Fas/sFasL system is significant with regard to isolinderalactone-mediated A549 cellular apoptosis and that isolinderalactone inhibits proliferation of A549 human non-small cell lung cancer cells by arresting the cell cycle at the G₀/G₁ phase (Fig. 7). Thus, isolinderalactone may have the potential to be a novel agent for future treatment of non-small-cell lung cancer.

Acknowledgements

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