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# Apoptotic effects of a high performance liquid chromatography (HPLC) fraction of *Antrodia camphorata* mycelia are mediated by down-regulation of the expressions of four tumor-related genes in human non-small cell lung carcinoma A549 cell

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ABSTRACT

*Aim of the study: Antrodia camphorata* (niu-chang-chih) is a fungus native to Taiwan which is believed to be effective in preventing diseases. Recent reports demonstrate that *Antrodia camphorata* products induce the apoptosis of various kinds of tumor cells. In this study we determined the inhibitory effects of alcohol extract and individual fractions of alcohol extract on the proliferation of human non-small cell lung carcinoma A549 cell and clarified the mechanism underlying the anti-cancer activities.

*Materials and methods:* Alcohol extracts of *Antrodia camphorata* mycelia were prepared by the serial extraction with the solvents with increasing polarity and fractionated using HPLC. Cell viability was determined by MTT assay. Apoptosis detection was carried out by subG$_1$ analysis and annexin V/propidium iodide staining using flow cytometry. The impacts of HPLC fractions on the expression levels of apoptosis- and cancer-related proteins were evaluated by western blotting.

*Results:* Three HPLC fractions, fractions 5–7, had robust inhibition of human A549 cells and among them fraction 6 (Fr-6) possessed the most potent effectiveness. Apoptotic assay showed that Fr-6-induced human A549 cell apoptosis by triggering the mitochondrial pathway and endothelium reticulum (ER) stress. Immunoblotting results demonstrated that Fr-6 possibly activated ER stress by lowering the expression level of calpain 1/2 small subunit and Fr-6-mediated decrease in cell proliferation might attribute to the suppressive effect on the Erk 1/2 pathway, which arose from Fr-6-derived low galectin-1 expression. Furthermore Fr-6 could diminish Rho GDP dissociation inhibitor α (RhoGDI-α) expression and subsequently activated c-Jun $NH_2$-terminal kinase (JNK) pathway, which is linked to cell apoptosis. Fr-6 also could decrease the production level of eukaryotic translation initiation factor 5A, which is a potential cancer intervention target.

*Conclusion:* These results suggested that the anti-cancer activity of *Antrodia camphorata* might be due to multiple active metabolites, which work together to induce cell apoptosis via various pathways.



## 1. Introduction

Basidiomycete *Antrodia camphorata* in *Polyporaceae*, a native fungus in Taiwan, is commonly used in Taiwanese folk medicine. The fruit body of *Antrodia camphorata* is rare and expensive because it grows only in the inner heart wood wall of the endangered species *Cinnamonum kanehirai* and cultivation is extremely difficult. The bio-active ingredients of its pharmacological functions, including triterpenoids, sesquiterpene lactone, steroid and polysaccharide have been analyzed (Chen et al., 1995; Cherng et al., 1996; Cherng and Chiang, 1995; Chiang et al., 1995; Yang et al., 1996; Wu et al., 1997).

The influences of *Antrodia camphorata* on a variety of biological functions have been studied. Peng et al. (2006, 2007) demonstrated that *Antrodia camphorata* crude extract (ACCE) can inhibit the proliferation of superficial and invasive bladder transitional cell carcinoma (BTCC) cell lines and retard the migration of invasive T24 cell. The methanol extract of mycelia (MEM) from submerged culture of *Antrodia camphorata* can induce HepG2 cell apoptosis possibly via Fas pathway (Song et al., 2005a,b). The experimental results with the fruit bodies of *Antrodia camphorata* showed that the ethylacetate extract (EAC) exhibits the apoptotic effects on HepG2 cells via increasing Fas/APO-1 as well as membrane-bound Fas and initiating the mitochondrial apoptotic pathway, and also on PLC/PRF/5 cells possibly through the inhibition of nuclear factor NF-κB (Hsu et al., 2005, 2007). Furthermore EAC can suppress Hep3B cells by the apoptotic signaling amplified by the cross-talk between the calpain/Bid/Bax and $Ca^{2+}$/mitochondrial pathway (Kuo et al., 2006). The findings observed with the fermented broth of *Antro-*

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*dia camphorata* indicated that the broth can evoke breast cancer cell apoptosis (Hseu et al., 2004, 2007, 2008; Yang et al., 2006). The apoptotic studies conducted with the wild fruit bodies of *Antrodia camphorata* demonstrated that the ethanol extract of fruit body can induce HL-60 cell apoptosis partially through the hypoacetylation (Lu et al., 2009).

In addition to the anticancer activities, *Antrodia camphorata* has other pharmacological activities. Song and Yen (2002) evaluated the antioxidant and free radical scavenging activities of various preparations of mycelia and found that the dry matter of fermented filtrates (DMF) owns the strongest inhibition of lipid peroxidation and DMF as well as water extract (WEM) has the noticeable free radical scavenging activities. Song and Yen (2003) also found that DMF can markedly reduce the $H_2O_2$-induced lipid peroxidation and protect the rat liver from the $CCl_4$-induced damage by up-regulating hepatic GSH-dependent enzymes. Anti-oxidation studies showed that the aqueous extracts of mycelia can avoid the erythrocyte from the damage induced by aqueous preoxyl radical and it also can alleviate the depletion of cytosolic glutathione (Hseu et al., 2002). The results of lipopolysaccharide-induced Raw264.7 cells treated with culture broth of *Antrodia camphorata* showed that the culture broth can lower prostaglandin E2 and nitric oxide production and the expression of iNOS as well as cyclooxygenase-2, suggesting the culture broth possesses the anti-inflammatory activities (Hseu et al., 2005).

Most of the studies investigating the pharmacological activities of *Antrodia camphorata* hitherto utilize the crude extracts. However, only few studies have harnessed the partially or pure compounds isolated from the crude extracts to elucidate the therapeutic effects of *Antrodia camphorata*. Shen et al. (2004) purified zhankuic acid from the fruit bodies to study its anti-inflammatory activities and observed that zhankuic acid can decrease the fMLP or PMA-induced ROS production in the peripheral human neutrophil and inhibit the firm adhesion of neutrophil. Five new maleic and succinic derivatives isolated from the mycelia by Nakamura et al. (2004) are found to show the cytotoxicity against the LLC tumor cell lines. Yeh et al. (2009) purified eight new triterpenes from the fruit bodies and showed that three of these compounds can induce HT-29 human colon cancer cell apoptosis.

In our previous studies (Wu et al., 2006), we found that the ethanol extracts of *Antrodia camphorata* (SACE) can evoke human lung cancer A549 cell apoptosis through the endothelium reticulum stress (ER stress) possibly mediated by the down-regulation of human galectin-1, human eukaryotic translation initiation factor 5A (eIF5A), human Rho GDP dissociation inhibitor α (RhoGDI-α), human calcium-dependent protease (calpain) small (regulatory) subunit and human annexin V genes in human A549 cells. In this study the ethanol extracts of *Antrodia camphorata* mycelia were fractionated by high performed liquid chromatography into 8 fractions and fraction 6 (Fr-6) had the highest inhibitory effectiveness against human A549 cells. The detail apoptotic studies found that Fr-6 might provoke human A549 cell apoptosis through the under-expression of galectin-1 and subsequently inhibition of cell proliferation-related Erk-1/2 pathway. Besides Fr-6 could decrease the expression of RhoGDI-α, which led to the activation of apoptosis-evoking JNK pathway.

## 2. Materials and methods

### 2.1. Cell lines

Human non-small cell lung carcinoma A549 cell and primary human fetal lung fibroblast MRC-5 were purchased from ATCC. Both A549 and MRC-5 cells were cultured at 37 °C in DMEM (Invitrogen Life Technologies Inc., Grand Island, NY, USA), supplemented with 10% fetal bovine serum (Biological Industries Ltd., Kibbutz Beit Haemek, Israel).

### 2.2. Preparation of the ethanol extract of Antrodia camphorata cultivated by solid-state fermentation

Air-dried *Antrodia camphorata* mycelia grown by solid culture were provided by Biotechnology Research Center Southern Taiwan University, Tainan, Taiwan. 554.6 g of mycelia was first mixed with n-hexane at a ratio of 1:3 (w/v) and soaked for 24 h at room temperature. Then the suspension was filtered through the filter paper (Qualitative Filter Paper, NO. 1) and the filtrate was concentrated under reduced pressure to give n-hexane extraction (48 g). After filtration, the residue was extracted sequentially with ethylacetate (EtOAc), ethanol (EtOH) and water as described in n-hexane extraction. After extraction, 20.1 g, 16 g and 127.6 g of the products were obtained respectively in the EtOAc, EtOH and water extraction experiments.

### 2.3. Separation of the ethanol extract of Antrodia camphorata by high performance liquid chromatography (HPLC)

The HPLC system consisted of a Hitachi liquid chromatograph equipped with one pump (Hitachi L-2130), an autosampler injector (Hitachi L-2200) and a UV photodiode-array detection (Hitachi L-2450). The chromatographic separation was performed with Hypersil Gold column (250 mm × 4.6 mm, 5 μm, Thermo Scientific) to dissect the ethanol extract. The extract was eluted at the flow rate of 0.9 ml/min for 147 min using the water/acetonitrile gradient system which was changed every 10 min. Elute was collected every 15 min for each fraction. Totally 8 fractions were obtained and the resulting fractions were examined for the inhibitory activities. 21.3 mg, 52 mg and 38 mg of fraction-5, -6 and -7 (Fr-5, -6 and -7) were produced respectively by separation of 380 mg of dried ethanol extract. Each fraction was dissolved in dimethyl sulfoxide (DMSO) and stored at −20 °C for future use.

### 2.4. MTT assay

Appropriate concentrations of the HPLC fractions were added to a 96-well plate already seeded with 3000 human A549 cells or 1000 MRC-5 cells per well. After exposure for the indicated time duration, 20 μl of MTT solution (Merck, Damstadt, German) (5 mg/ml PBS) was added to each well and the plate was incubated at 37 °C for 4 h. After medium removal, 200 μl of DMSO was added to each well and the plate was gently shaken for 5 min. The absorbance was determined at 540 nm. Quadruplicate wells were applied to each concentration for a specific time period. 0.1% (v/v) DMSO-treated human A549 cells were employed as the control. As to cell survival experiments incorporated with JNK inhibitor SP600125 (Sigma, Saint Louis, MO, USA), human A549 cells were incubated with 50 μg/ml of Fr-6 in the presence or absence of 15 μM of SP600125 at the indicated time periods. Cell viability was determined by MTT assay.

### 2.5. Analysis of Fr-6 by gas chromatography/mass spectrometry (GC/MS)

The chemical composition of Fr-6 was analyzed by GC/MS using a SHIMADZU QP2010 GC/MS with a DB-5 column (30 m, film 0.25 μm, ID 0.25 mm). The temperature of the column was programmed from 45 °C to 270 °C at 5 °C/min and the injector or detector temperature for the analysis was about 250 °C. Helium was used as the carrier gas at a flow rate of 0.82 ml/min. The mass spectrometer was operated in electron-impact ionization (EI) mode

with 70 eV energy. The identification of the chemical constituents was based on matching their recorded mass spectra with those obtained from the FFNSC1.2, NIST05 and NIST05s library spectra provided by the software of GC/MS system.

### 2.6. SubG$_1$ cell analysis of Fr-6-treated human A549 cells

Human A549 cells were plated onto a 6-well plate at $8 \times 10^5$/well and cultivated overnight. Then 50 μg/ml of Fr-6 was added onto each well and the cells were harvested with trypsinization at the indicated time period. The harvested cells were incubated with 500 μl of ice-cold 70% ethanol/well at 4 °C overnight. After ethanol treatment, the cells were washed with 1 ml ice-cold PBS/well, re-suspended in 100 μl PBS/well and incubated with 300 μl propidium iodide (PI) (Sigma, Saint Louis, MO, USA) solution (3 μl RNase and 20 μg PI per ml)/well in the dark at 37 °C for 30 min. The stained cells are analyzed by FACSCalibur flow cytometer (Becton Dickinson). 0.1% (v/v) DMSO-treated human A549 cells were used as the control and analyzed as described above.

### 2.7. Annexin V/PI staining of Fr-6-exposed human A549 cells

Human A549 cells were plated onto a 6-well plate at $4 \times 10^5$/well and cultivated overnight. Then 50 μg/ml of Fr-6 was added onto each well and the cells were harvested with trypsinization at the indicated time period. The harvested cells were washed with 1 ml ice-cold PBS/well and re-suspended in 100 μl binding buffer/well. Then 2 μl of annexin V conjugated with FITC (Strong Biotech Corporation, Tainan, Taiwan) plus 2 μl of PI solution (Sigma, Saint Louis, MO, USA) were mixed with the re-suspended cells and incubated in the dark on ice for 15 min. After staining, the cells were analyzed using FACSCalibur, Becton Dickinson. 0.1% (v/v) DMSO-treated human A549 cells were utilized as the control and analyzed as described above.

### 2.8. Western blotting

After treatment as indicated in Section 3, human A549 cells were harvested and lysed in the sample buffer [0.1 M Tris (pH 6.8), 2% (w/v) SDS, 0.2% (v/v) β-mercaptoethanol, 10% (v/v) glycerol and 0.0016% (w/v) bromophenol blue]. Total cell lysates (50 μg of protein) were separated using 10% (w/v) acrylamide gel electrophoresis and transferred onto the PVDF membrane (Stratagene, La Jolla, CA, USA). The membrane was blotted with primary antibody overnight followed by incubation with HRP-conjugated secondary antibodies (1:15,000), and visualized using chemiluminescence (Amersham-Pharmacia Biotech Inc., Piscataway, NJ). For JNK inhibitor SP600125 (Sigma, Saint Louis, MO, USA) experiments, human A549 cells were first treated with 15 μM SP600125 (Sigma, Saint Louis, MO, USA) for 24 h and then 50 μg/ml Fr-6 was administrated into the medium. At the indicated time period, the cells were harvested for western blotting with JNK antibody. The antibodies were purchased from the following sources: human eukaryotic translation initiation factor 5A from BD Biosciences, San Diego, CA, USA; human Rho GDP dissociation inhibitor from Upstate Biotechnology, Lake Placid, NY, USA; human calcium-dependent protease (calpain) 1/2 small subunit from EMD Bioscienecs Inc., San Diego, CA, USA; human galectin-1 from CytoLab Ltd., Rehovot, Israel; human actin from Chemicon International, Temecula, CA, USA; human caspase-3 from PharMingen, San Diego, CA, USA; human caspase-12 from Rockland, Philadelphia, PA, USA; human Bax from Santa Cruz, Santa Cruz, CA, USA; human Bcl-2 from Santa Cruz, Santa Cruz, CA, USA; human Erk-1/2 from AbFrontier, Seoul, Korea; human phospho-Erk-1/2 from AbFrontier, Seoul, Korea; human JNK from BD Biosciences, San Jose, CA, USA; human phospho-JNK from Cell signaling, Danvers, MA, USA.

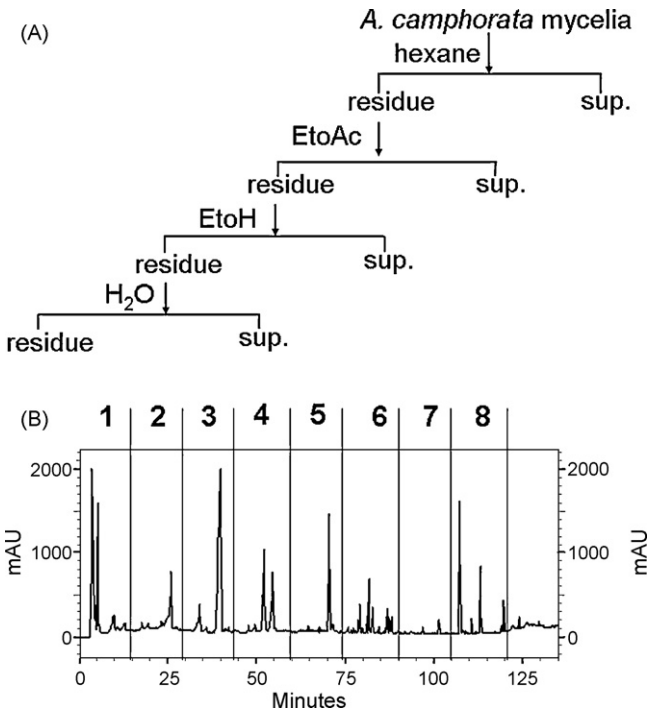


**Fig. 1.** Isolation of Fr-6 by HPLC. The mycelia of *Antrodia camphorata* was extracted sequentially by the solvent with increasing polarity (supernatant, abbreviated sup.) (A), and the ethanol fraction was further separated into 8 fractions by HPLC as described in Section 2 (B).

## 3. Results

### 3.1. Separation of the ethanol fraction of Antrodia camphorata by HPLC

In this work, the mycelia of *Antrodia camphorata* were sequentially extracted using the solvent with increasing polarity as shown in Fig. 1A. Our previous results implied that the ethanol fraction may have robust suppressive activity against human A549 cells (Wu et al., 2006). Therefore, in order to explore the effective compounds present in the ethanol fraction this layer was further separated into 8 fractions (fractions 1–8) using HPLC according to retention time as described in Section 2 (Fig. 1B). Each HPLC fraction was examined for the inhibitory effectiveness against human A549 cells.

### 3.2. The inhibitory activities of HPLC fractions against human A549 cell

The results of cell survival assay demonstrated that Fr-5 to -7 exhibited obvious suppressive impacts on human A549 cells (Fig. 2A). The does-dependent invetigations showed that Fr-6 was the most potent fraction (Fig. 2B) because at 25 μg/ml it already could heavily retard human A549 cell proliferation. In addition, this inhibitory activity was time-dependent as indicated in Fig. 2C. However, much higher concentration of Fr-6 was required to efficiently suppress MRC-5 cells (Fig. 2D), suggesting that Fr-6 exhibited the selectivity toward human A549 and MRC-5 cells. In order to know the active compounds present in Fr-6, Fr-6 was subjected to GC/MS analyses and the data were shown in Table 1. The GC/MS results showed that alkanes, fatty alcohols, fatty esters and fatty acid amide were present in the fraction.

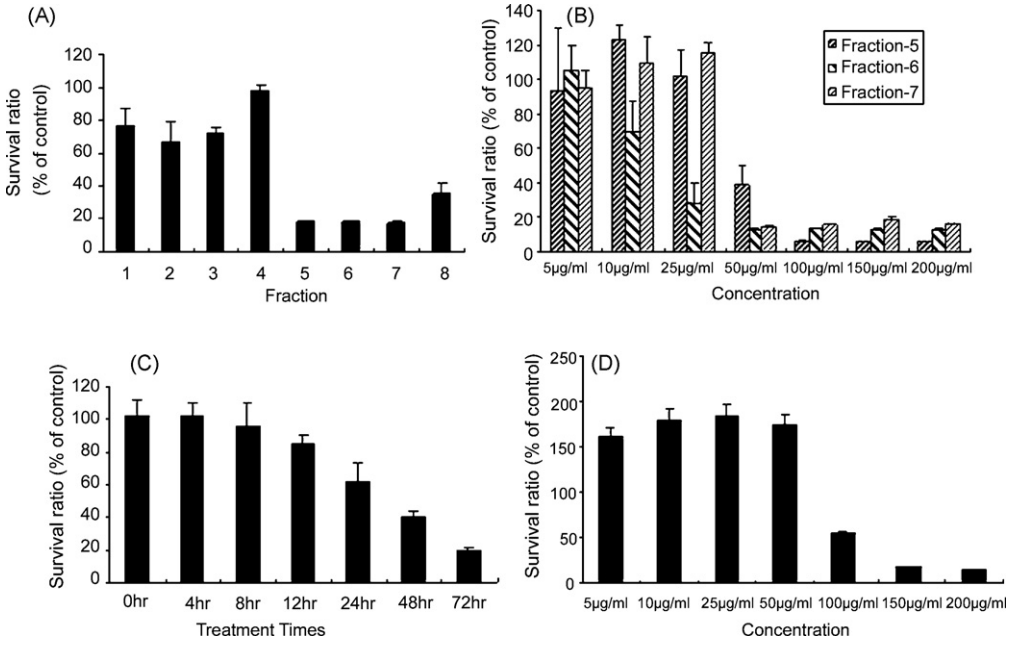


**Fig. 2.** The inhibitory impacts of HPLC fractions on human A549 cells. Human A549 cells were incubated with each HPLC fraction (200 μg/ml) for 72 h and cell viability was measured by MTT assay as described in Section 2 (A). Dose–response experiments of Fr-5 to -7 were carried out by treating human A549 cells with the indicated concentrations for 72 h (B). Time-dependent studies of Fr-6 against lung cancer cells were performed by incubating human A549 cells with 50 μg/ml of Fr-6 for various time periods (C). The selectivity of Fr-6 was determined by dose–response against MRC-5 done by exposing the cells in the presence of the indicated concentrations for 72 h (D). All the data were expressed as mean ± standard error of the mean of four wells performed in triplicate.

### 3.3. Fr-6 could evoke human A549 cell apoptosis

To prove that Fr-6 inhibited human A549 cell proliferation by induction of the apoptosis, Fr-6-treated human A549 cells were examined with the subG$_1$ cell analysis and annexin V/PI staining using flow cytometry. Flow cytometric analyses demonstrated that 50 μg/ml of Fr-6 could induce the formation of hypodiploid subG$_1$ peak in a time-dependent manner (Fig. 3A and B), implicating that Fr-6 could cause the DNA fragmentation in human A549 cells, which is a typical phenomena of late apoptotic cell. Besides, the results of annexin V/PI staining showed that Fr-6 could induce the phosphatidylserine (PS) translocation in the cell membrane, which is an initial sign of apoptosis and causes the subsequent membrane leakage (late apoptotic cell) (Fig. 3C and D).

### 3.4. Fr-6 caused the apoptosis in human A549 cell by triggering the mitochondrial pathway and ER stress

The above results implicated that Fr-6 might induce human A549 cell apoptosis. However, cytometric analysis could not determine whether Fr-6 led to the apoptosis by death receptor signaling, mitochondrial damage or ER stress. To explore the mechanism by which Fr-6 invoked the apoptosis, the processing and activation of caspase-3, which is considered to play a central role in death receptor signaling and mitochondrial damage, was examined by western blotting with anti-caspase-3 antibody. As shown in Fig. 4A, the quantity of procaspase-3 was found to decrease in human A549 cells treated with 50 μg/ml Fr-6, implying that caspase-3 was activated and the apoptosis might be initiated via death receptor signaling or mitochondrial damage. To further clarify the apoptotic mechanism mediated by Fr-6, the expression of Bcl-2 and Bax in Fr-6-exposed human A549 cells was examined by western blotting. The results of western blotting indicated that Bax/Bcl-2 ratio was increased in Fr-6-administrated human A549 cells (Fig. 4B), suggesting that Fr-6 could induce human A549 cell apoptosis through the mitochondrial pathway. Besides the mitochondrial pathway, western blotting with anti-caspase-12 antibody was carried out to explore ER stress evoked by Fr-6 treatment. The experimental results of western blotting showed that the amount of procaspase-12 markedly decreased due to the processing for activation in cells treated with 50 μg/ml Fr-6 (Fig. 4C). Taken together, the above results suggested that Fr-6

**Table 1**
The composition of Fr-6 evaluated by GC/MS.

| Constituents | Peak area % | Constituents | Peak area % |
|---|---|---|---|
| 2-Methyl-2-hexanol | 33.08 | Palmitic acid ethyl ester | 1.44 |
| 3-Ethyl-3-methylheptane | 2.04 | Stearic acid methyl ester | 2.45 |
| 4,6-Dimethyldodecane | 1.35 | Tetradecanoic acid ethyl ester | 0.60 |
| 3-Methyl-5-propylnonane | 0.66 | Tributyl acetylcitrate | 0.45 |
| Isodecyl methacrylate | 0.56 | 8-Heptylpentadecane | 0.44 |
| Farnesan | 1.56 | Eicosanoic acid methyl ester | 0.75 |
| Tridecane | 0.79 | Oleamide | 2.78 |
| Pentadecane | 0.87 | Nonadecanamide | 2.02 |
| 2,6,11-Trimethyldodecane | 0.84 | Octadecane | 0.59 |
| Eicosane | 0.84 | Methyl behenate | 0.66 |
| Heneicosane | 0.97 | Docosane | 1.02 |
| Heptadecane | 0.40 | Palmitic acid methyl ester | 4.84 |
| Total | 62.00 | | |

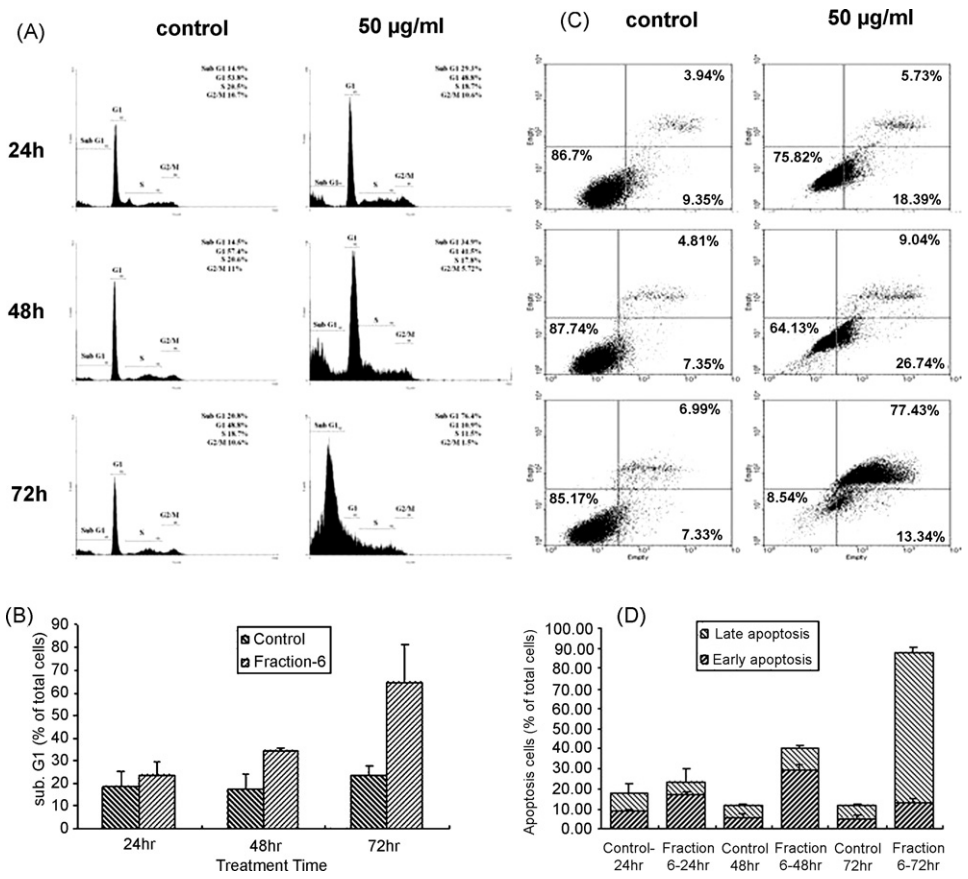


**Fig. 3.** Induction of human A549 cells apoptosis by Fr-6. Human A549 cells were incubated with 50 μg/ml of Fr-6 for the indicated time durations and subG$_1$ analyses were determined by flow cytometry as described in Section 2. The representative diagram was shown in (A), and the mean ± standard error of the mean of three independent experiments was indicated in (B). Apoptotic effect was also observed by treating human A549 cells with 50 μg/ml of Fr-6 for the indicated time periods and the effect was measured by the annexin V/PI staining as described in Section 2. The representative data was posted in (C), and the mean ± standard error of the mean of three independent experiments was demonstrated in (D).

might evoke human A549 cell apoptosis via mitochondrial damage and ER stress.

### 3.5. The impact of Fr-6 on the expressions of four tumor-related genes

Our previous examinations observed that the ethanol extracts of *Antrodia camphorata* mycelia (SACE) can evoke the apoptosis in human A549 cell possibly by the down-regulation of human galectin-1, human eIF5A, human RhoGDI-α, human calpain small (regulatory) subunit and human annexin V gene expression (Wu et al., 2006). Four of these genes (galectin-1, eIF5A, calpain small subunit and RhoGDI-α) are closely associated with the tumorigenesis. To investigate if Fr-6 owns the same effect on the expression of these four tumor-related genes, western blotting with the antibody against each of these four proteins was performed. The experimental results of western blotting demonstrated that the production of galectin-1, RhoGDI-α, calpain small subunit and e-IF5A was diminished by the exposure of human A549 cell to 50 μg/ml in a time-dependent manner (Fig. 5). The aforementioned findings suggested that Fr-6 likely induced the apoptosis of human A549 cell via the suppression of these cancer-related genes.

Recent findings showed that the recruiting of H-*Ras* to the cell membrane is dependent on galectin-1 and galectin-1 binds H-*Ras* to stimulate the transformation (Belanis et al., 2008; Paz et al., 2001). The well-documented evidences indicate that *Ras* takes part in the Erk pathway and Erk is the downstream of *Ras* (Belanis et al., 2008; Paz et al., 2001). In addition, our work found that Fr-6 could knock-down the galectin-1 gene expression. Therefore we anticipated that Fr-6 was able to interfere with the Erk signaling pathway. To examine this anticipation, western blotting with the antibodies against Erk and phospho-Erk (p-Erk) was implemented to examine if the Erk pathway was interrupted by Fr-6. As demonstrated in Fig. 6, Fr-6 inhibited the expressions of Erk-1 and -2 while a more serious influence was observed on Erk-2. In addition to the effect on Erk-1 and -2, the formation of phospho-Erk-1 and -2 (activated Erk-1 and -2) was also decreased by Fr-6 and the inhibition of p-Erk-2 by Fr-6 was more profound than p-Erk-1.

Recently the investigation by Park et al. (2009) observed that the inhibition of RhoGDI-α production invokes the apoptosis of insulin-secreting cells by the activation of c-Jun $NH_2$-terminal kinase (JNK) pathway. Since RhoGDI-α was suppressed in Fr-6-exposed human A549 cells, in this investigation we also explore if the JNK pathway was triggered in Fr-6-incubated human A549 cells. As indicated in Fig. 7A, the level of JNK was decreased by Fr-6 but phospho-JNK was up-regulated in Fr-6-treated human A549 cells, suggesting that the JNK pathway was activated by Fr-6 and Fr-6-induced cell death might occur through an increase in the expression level of activated JNK mediated by the inhibition of RhoGDI-α expression.

To further examine if down-regulation of RhoGDI-α induced apoptosis via activation of JNK, Fr-6-administrated human A549 cell was incubated with JNK inhibitor SP600125. The experimental results of inhibitor administration demonstrated that SP600125 could retard Fr-6-evoked inhibition of JNK levels and phosphor-JNK formation (Fig. 7B) while the down-regulation of RhoGDI-α by Fr-6 was not affected by the inhibitor (Fig. 7C). In addition, cell

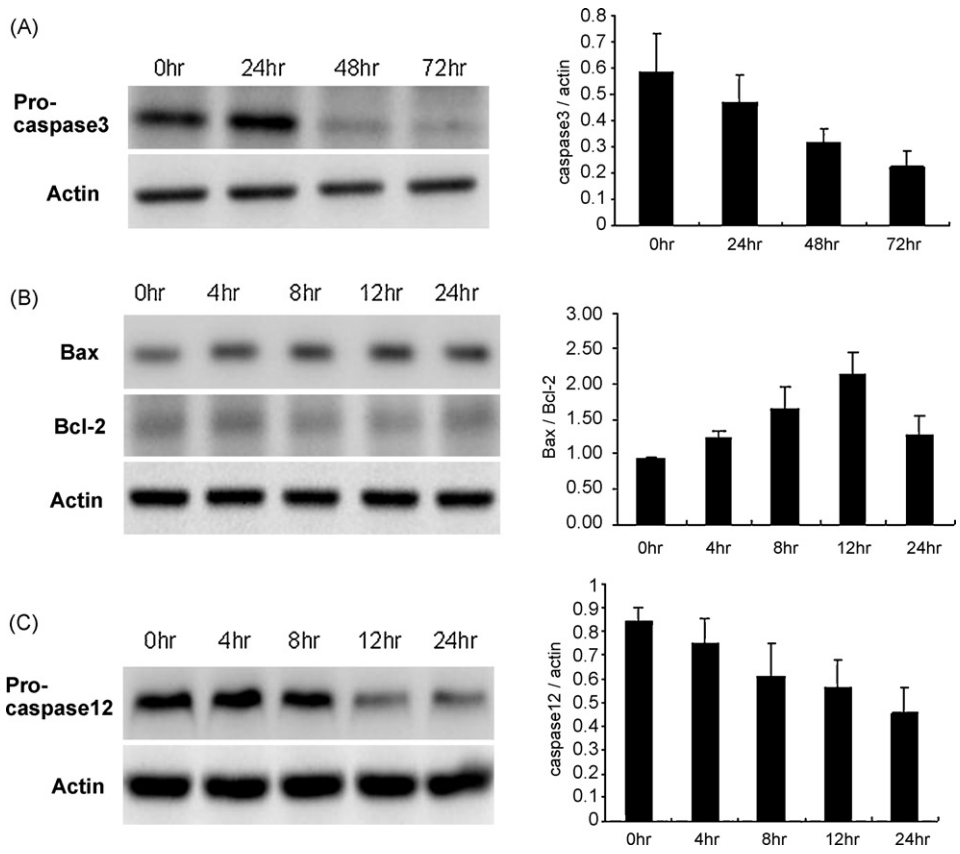


**Fig. 4.** Fr-6-evoked human A549 cell apoptosis via the mitochondrial pathway and ER stress. Human A549 cells were treated with 50 μg/ml of Fr-6 for different time durations. Procaspase-3, bax, bcl-2 and procaspase-12 in cell lysates were detected by western blotting with the antibodies against procaspase-3 (A), bax (B), bcl-2 (B) and procaspase-12 (C) respectively as described in Section 2. The blot in each diagram was the representative result of three independent experiments and the densitometer-intensity data (mean ± standard error) were shown under each blot.

survival experiments showed that SP600125 could antagonize the inhibitory effect of Fr-6 on human A549 cell proliferation (Fig. 7D). The aforementioned data implicated that suppression of RhoGDI-α expression level might provoke human A549 cell apoptosis via increase in JNK activity.

## 4. Discussion

*Antrodia camphorata* is a native fungus to Taiwan and is regarded as a precious herbal cure in Taiwanese folk medicine. *Antrodia camphorata* is thought to be a potent herbal medicine in cancer-related cases. This investigation found that a HPLC fraction, e.g. fraction 6 of ethanol extract of *Antrodia camphorata* could impede the proliferation of human lung cancer A549 cells in 72 h. Additionally, the results of time- and dose-dependent response of human A549 cell and those of human fetal lung fibroblast MRC-5 cells to Fr-6 showed that human A549 cell was more vulnerable than MRC-5 to Fr-6. In summary, Fr-6 was obviously selective to human A549 cells at concentration of 5–50 μg/ml within 72 h (Fig. 1). The results of flow cytometry and western blotting implicated that Fr-6 might invoke apoptosis in human A549 cells by triggering the mitochondrial pathway and ER stress.

In our previous study, we implemented the proteomics scheme to search globally for the differentially expressed proteins in human non-small cell lung carcinoma A549 cells affected by *Antrodia camphorata* and identified five de-regulated proteins in cells treated with *Antrodia camphorata*. Among these, human galectin-1 is a member of the galectin family, proteins with conserved carbohydrate-recognition domains that bind β-galactoside. Functional studies have shown that galectins are implicated in cell–cell (Hughes, 2001) and cell–extra-cellular matrix interactions (Moiseeva et al., 1999). Increasing evidence suggests that galectin-1 plays an important role in cell growth, apoptosis and angiogenesis (Perillo et al., 1998). Galectin-1 is substantially expressed in breast, colon and brain cancer (Lahm et al., 2001). Recent examination on galectin-1 has indicated that melanoma cells can secrete galectin-1 to escape from T-cell dependent immunity by inducing of activated T-cell apoptosis, thus conferring immune privilege to tumor cells (Rubinstein et al., 2004). As well as its role as a negative regulator of immunity, galectin-1 has been demonstrated to bring H-*Ras* to cell membrane for H-*Ras* to function in the cells to stimulate the transformation (Belanis et al., 2008; Paz et al., 2001). Our experimental results revealed that Fr-6 could decrease the expression of galectin-1. Therefore it is likely that the Erk pathway in which H-*Ras* participate was interrupted by Fr-6. Western blotting with anti-Erk protein showed that Fr-6 could interfere with the phosphorylation of Erk-1/2 and with the expression of Erk-1/2. Taken the apoptotic data together, the above findings implied that Fr-6 might evoke apoptosis in human A549 cells by suppressing the production of galectin-1 and subsequently blockading Erk-regulated cellular proliferation and survival.

Human calcium-dependent proteases (calpain) represent a family of nonlysosomal calcium-dependent cysteine protease including two ubiquitously expressed isoforms, μ-calpain (calpain 1) and m-calpain (calpain 2); several tissue-specific isoforms and a small 28-kDa regulatory subunit (calpain 4) (Goll et al., 2003). ER-induced apoptosis has been shown to be associated with the activation of capspase-12 and calpain (Nakagawa et al., 2000; Orrenius et al., 2003) and calpain can activate caspase-12 in ER stress (Nakagawa and Yuan, 2000). Kuo et al. (2006) reported that

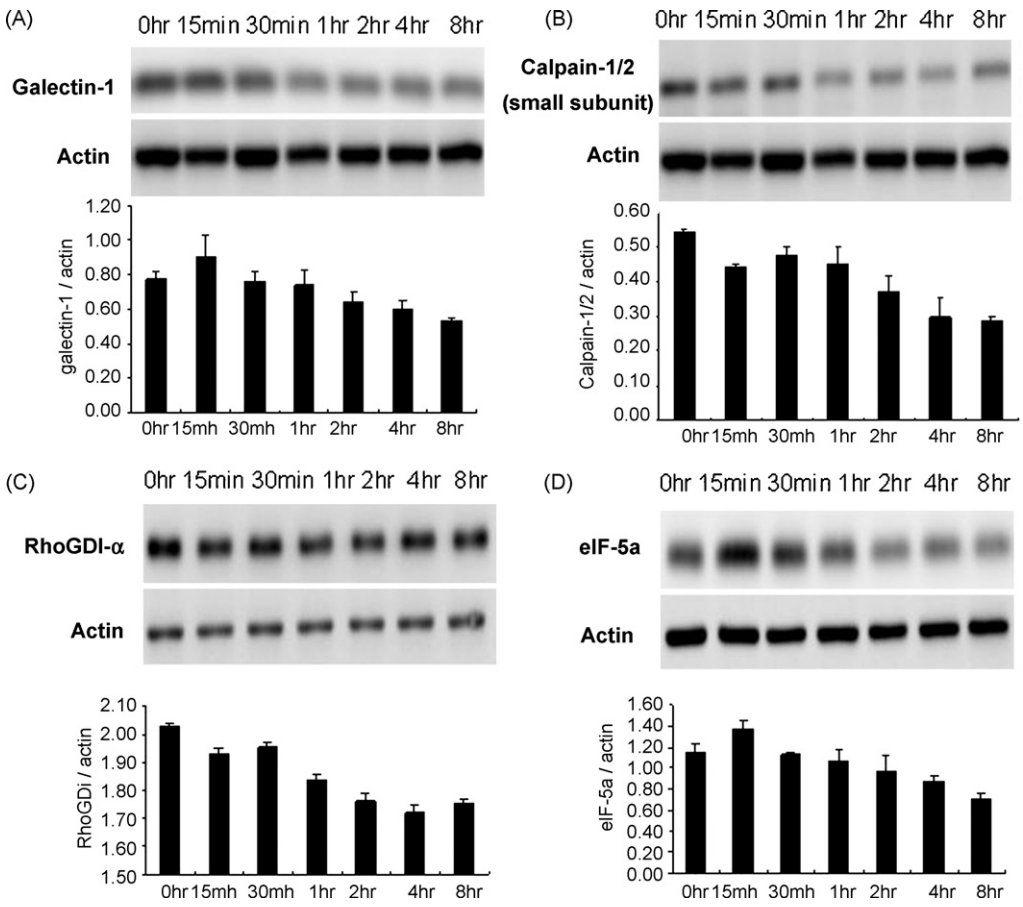


**Fig. 5.** The effects of Fr-6 on the expression levels of galectin-1, calpain-1 small subunit, RhoGDI-α and eIF-5A. Human A549 cells were exposed in the presence of 50 μg/ml of Fr-6 for the indicated time periods. Galectin-1, calpain-1 small subunit, RhoGDI-α and eIF-5A in cell lysates were detected by western blotting with the antibodies against galectin-1 (A), calpain-1 small subunit (B), RhoGDI-α (C) and eIF5A (D) respectively as described in Section 2. The blot in each figure was the typical data of at least three independent studies and the densitometer-intensity data (mean ± standard error) were indicated under each blot.

the ethylacetate extract (EAC) of *Antrodia camphorata* fruit body increases the calcium level in the cytoplasm of EAC-affected Hep 3B cells and triggers the subsequent activation of caspase-12 and calpain. Consistent with the activation of calpain, the small subunit is decreased in EAC-treated Hep 3B cells. Autolysis of the small subunit is relevant to the activation of calpain during apoptosis (Daniel et al., 2003). They also found that the apoptotic signal is amplified by the cross-talk between the calpain and mitochondrial apoptotic pathways. Similar to the above results, in Fr-6-treated human A549 cells the expression of calpain regulatory subunit subsided and

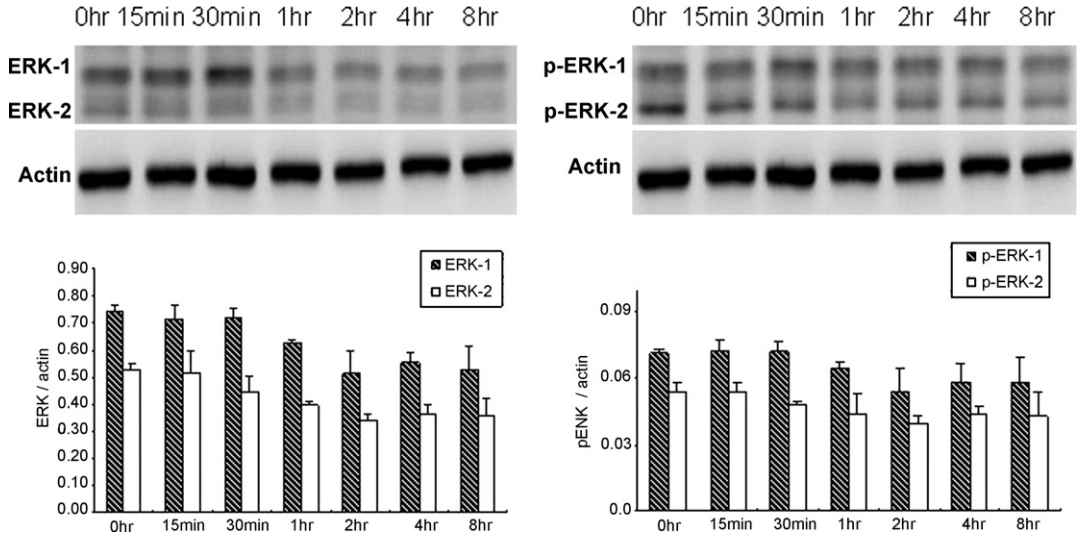


**Fig. 6.** The influence of Fr-6 on the expression levels of Erk-1 and -2. Human A549 cells were incubated with 50 μg/ml of Fr-6 for various time durations. The expression levels of Erk-1 and -2 and phosphor-Erk-1 and -2 were observed by western blotting with the antibodies against Erk-1/2 (A) and phosphor-Erk-1/2 (B) as described in Section 2.

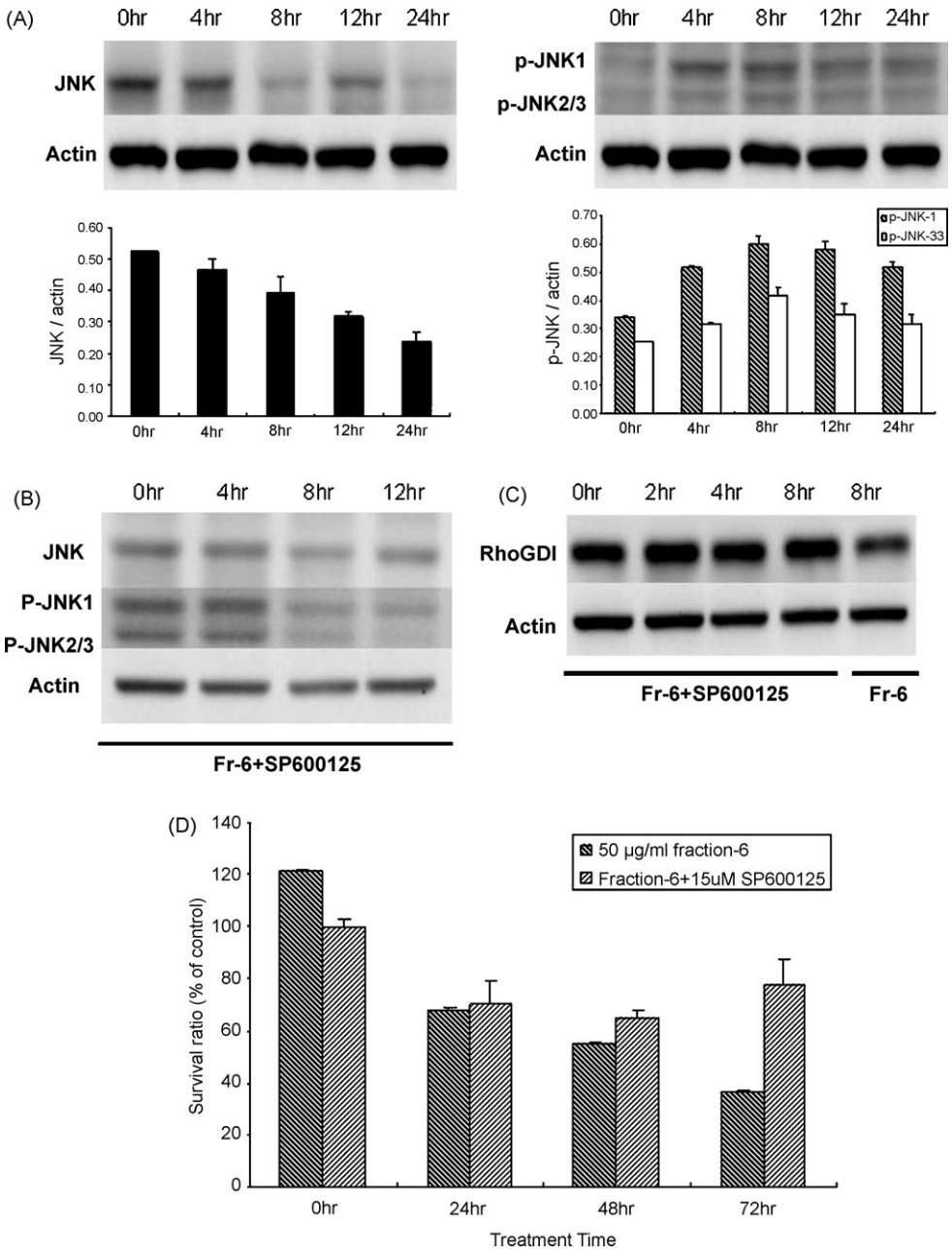


**Fig. 7.** The activation of JNK by Fr-6. Human A549 cells were incubated with 50 μg/ml of Fr-6 for various time periods. The expression levels of JNK and phosphor-JNK were observed by western blotting with the antibodies against JNK and phosphor-JNK (A). For the counteract study of JNK inhibitor SP600125, human A549 cells were incubated with 15 μM of SP600125 for 24 h and then 50 μg/ml of Fr-6 was incorporated with the inhibitor-administrated cells for various time durations. After incubation, the cells were collected for western blotting with RhoGDI-α (B) and JNK antibodies (C). Furthermore, the antagonizing effects of JNK inhibitor on cell survival were carried out by exposing human A549 cells in the presence of 50 μg/ml of Fr-6 as well as 15 μM of SP600125 and cell survival was measured using MTT assay (D).

was observed before caspase-12 was processed (Figs. 4C and 5B), indicating that caspase-12 processing/activation might attribute to calpain activation. The impacts of Fr-6 on calpain small subunit in this examination implied that Fr-6-evoked human A549 cell apoptosis probably occurred through the activation of calpain and subsequently triggering caspase-12-mediated ER stress.

RhoGDI-α regulates the Rho family of proteins. Using proteomic profiling combined with siRNA, MacKeigan et al. (2003) discovered that in Taxol/MEK inhibitor-treated lung cancer H157 cell the expression of RhoGDI-α is diminished and RhoGDI-α expression knockdown by siRNA enhances the apoptosis of control, taxol- and MEK inhibitor-treated H157 cells. Park et al. (2009) also found that the decreased RhoGDI-α expression level can induce the apoptosis of insulin-secreting cells through the activation of JNK pathway. Many investigations have suggested that the expression level of RhoGDI-α closely modulates the JNK activity, which is strongly related to the apoptotic cell death (Muñoz-Alonso et al., 2008; Park et al., 2009). In this study we found that Fr-6 could knockdown the RhoGDI-α gene expression in human A549 cells and JNK inhibitor SP600125 could abrogate Fr-6-mediated JNK activation and cell death. Our experimental results and the above findings implicated that Fr-6 reduced the RhoGDI-α expression level in human A549 cell and invoked subsequent cell apoptosis by JNK activation.

eIF5A is involved in cell proliferation and apoptosis (Caraglia et al., 2000, 2001). A special amino acid hypusine is necessary for functionality (Chen and Liu, 1997; Park et al., 1993). Many works have indicated that inhibition of hypusine synthesis or down-regulation of eIF5A gene expression impedes cancer cell growth (Balabanov

et al., 2007; Caraglia et al., 1999, 2003; Takeuchi et al., 2002; Taylor et al., 2007), strongly indicating that eIF5A may be an appropriate new target for cancer intervention. In this work Fr-6 was observed to be capable of decreasing eIF5A production, indicating that *Antrodia camphorata* might impede human A549 cell growth by down-regulation of eIF5A.

The experimental results of this work shed light on the anticancer mechanism of *Antrodia camphorata* from a molecular perspective and identify possible targets for cancer intervention. We found that a HPLC fraction (Fr-6) isolated from the mycelia of *Antrodia camphorata* might evoke human A549 cell apoptosis by (1) interfering with the Erk pathway via down-regulation of galectin-1; (2) activation of JNK pathway due to the reduced level of RhoGDI-α; (3) invoking ER stress through activation of calpain induced by the decreased production of calpain small subunit; and (4) inhibition of eIF5A synthesis.

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