Involvement of Caspase-3 Activity and Survivin Downregulation in Cinobufocini-Induced Apoptosis in A 549 Cells

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Cinobufocini injection is a preparation containing water-soluble components of the toad skin. The aim of the present study was to investigate the apoptosis of human lung adenocarcinoma cell line A 549 induced by cinobufocini. A 549 or HLF-1(human lung fibroblast) cells were treated with cinobufocini at different concentrations for 24 and 48 h, respectively. The proliferation of cells was detected with the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay. Morphology of cells was carried out with scanning electronic microscopy (SEM) and Hoechst 33258 staining. The apoptosis rate was examined by flow cytometry. The expression of survivin was examined with RT-PCR and Western blot assay. The caspase-3 and caspase-7 activities were detected with caspase colorimetric protease assay. We found that cinobufocini significantly inhibited tumor growth of A 549 cells in a dose- and time-dependent manner without damaging non-cancerous cells (HLF-1) and induced granular apoptotic bodies of A 549 cells. Next, cinobufocini increased the percentage of cells in G1 phase and decreased the percentage of cells in S phase in A 549 cells. Furthermore, cinobufocini downregulated the expression of survivin mRNA and protein. Finally, cinobufocini upregulated caspase-3 activity. We concluded that cinobufocini induces apoptosis of A 549 cells, which is associated with the decreasing expression of survivin mRNA and protein, increasing caspase-3 activity of A 549 cells. Exp Biol Med 234:566–572, 2009

Key words: apoptosis; survivin; caspase-3; A 549 cell; cinobufocini injection

Introduction

Lung cancer is one of the most common cancer-related causes of death worldwide. In light of the very poor 5-year survival, new therapeutic approaches are mandatory (1). It is characterized by its poor prognosis and resistance to the apoptosis activity of antineoplastic drugs both *in vivo and in vitro* (2, 3). Natural products including plants and micro-organisms provide rich resources for anticancer drug discovery. There are many anticancer plants or herbal formulations exist, providing evidence for the identification of new anticancer compounds, and they are receiving increasing scientific attention. Cinobufocini injection is a preparation containing the water-soluble components of Chan Su, a traditional Chinese medicine obtained from the skin and parotid venom glands of toads (4). Chan Su has long been widely applied in Asian countries and is currently used as an alternative medicine (5). The formula of cinobufocini is presented in Figure 1. Cinobufocini and other bufadienolides are cardioactive C-24 steroids that exhibit a variety of biological activities, such as cardiotonic, anaesthetic, and antineoplastic activities (4). Both clinical and laboratorial studies have shown that cinobufocini has anticancer effects (6, 7); however, there is limited information on the global molecular mechanisms of cinobufocini action in lung cancer cells A 549 cells.

Survivin is initially identified as a member of the inhibitor of apoptosis protein (IAP) family and plays a key role in the mechanism of the anti-apoptosis of tumors (8). If survivin activity is suppressed, tumor cells can undergo apoptosis and stop growing (9). In addition, survivin inhibits apoptosis through the caspase enzyme system. Among them, caspase 3 is probably the one that so far best correlates with apoptosis (10). Furthermore, caspase-3 activity can be detected in several human malignancies such as non-small cell lung carcinoma and gastric cancer (11, 12). Cinobufocini is a novel anticancer drug in China. The mechanisms of the anti-cancer effect of cinobufocini have not been well investigated in lung cancer cells. Therefore, the purpose of the present study was to examine

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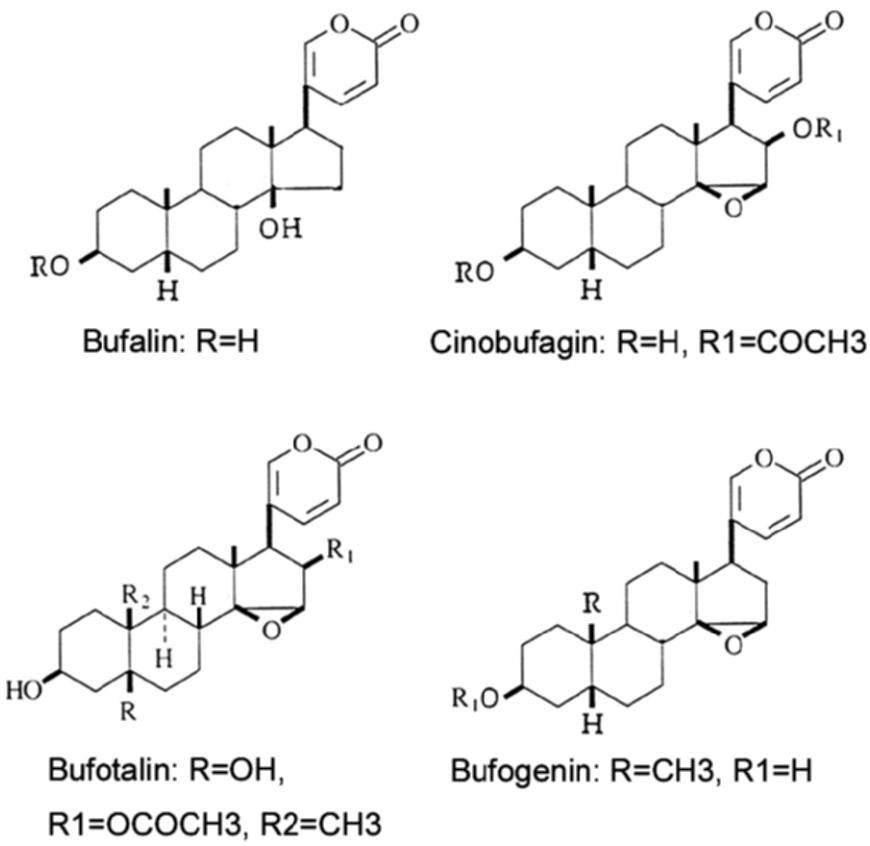


Figure 1. The structural formula of cinobufocini.

the apoptosis of cinobufocini treatment on human lung adenocarcinoma cells (A 549). Here we show that cinobufocini induces apoptosis in A 549 cells and that this is associated with the decreasing expression of survivin and increasing caspase-3 activity of A 549 cells.

Materials and Methods

Reagents. Cinobufocini injection (500 mg/ml) was kindly provided by Anhui Jingchan Pharmaceutical Co., Ltd. (Anhui, China). Dimethyl sulfoxide (DMSO) was purchased from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). Hoechst 33258 was purchased from Sigma (St. Louis, MO, USA).

Cell Culture. The human lung adenocarcinoma cell line A 549 and human fetal lung fibroblast cell line HLF-1 were originally obtained from the American Type Culture Collection (ATCC) and maintained in DMEM medium (Sigma) containing 10% fetal bovine serum (FBS). The cells were maintained at 37°C and 5% CO_2. For experiments, the cells were seeded in 6-well culture plates and grown in complete medium to 90% confluence. Then, the cells were washed with phosphate-buffered saline (PBS) and incubated for 48 h at 37°C in 2 ml of serum free medium containing cinobufocini.

MTT Assay. The cell proliferation was evaluated using a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) colorimetric assay. A 549 cells (1×10^4 cells/well) were seeded into 96-well plates in 100 µl of culture medium overnight, and then treated with various concentrations of cinobufocini at the indicated times. Next, 50 µl of MTT reagent solution (Cell Counting Kit, Keygentec Laboratories, China) was added and incubated for 4 h. After the medium and MTT were removed, 150 µl of DMSO were added, then placed on a plate shaker for 5 min at room temperature. Cell viability was determined according to the manufacturer's instructions. Cell survival rate was calculated as the percentage of MTT inhibition as follows: percentage of survival = (mean experimental absorbance/mean control absorbance) × 100%.

Morphology of Cells by Scanning Electron Microscopy. After treatment with reagents, cells were collected and fixed with 0.25% glutaraldehyde for 45 min, they were subjected to CPD (critical point drying) after extensive dehydration process, then gold coated and observed under scanning electron microscopy (Multimode Nanoscope IIIa, Digital Instrument Company, USA).

Hoechst 33258 Staining. To observe nuclear changes occurring during apoptosis, the chromation-specific dye Hoechst 33258 was used (13). Cultures were fixed for 5 min with 4% formaldehyde in PBS at 37°C, and then permeabilized by treatment with a mixture of ethanol/acetic acid (3:1) for 10 min at 25°C. After being washed with PBS,

the cells were stained with 1 μg/ml Hoechst 33258 in PBS for 10 min at room temperature and then washed again. Apoptosis was determined morphologically after staining the cells with Hoechst 33258 using fluorescence microscopy.

Flow Cytometric Analysis. To evaluate the effect of cinobufocini on the A 549 cell apoptosis and cell cycle, A 549 cells were plated onto 6-well plates at a density of 1×10^6/well and allowed attachment for 12 h. Then the medium was replaced with an equal volume of fresh medium containing cinobufocini 125 μg/ml for desired periods of time (24 h and 48 h). After the treatment, the attached cells were harvested by trypsinization, washed twice with ice-cold PBS, resuspended in ice-cold PBS, and fixed with 70% ethanol. When ready to stain with propidium iodide (PI), the cells were centrifuged. After the ethanol was removed, the cells were washed once in PBS. The cell pellets were then resuspended in 1 ml of PI/Triton X-100 staining solution (0.1% Triton X-100 in PBS, 0.2 mg/ml RNase A, and 10 (g/ml propidium iodide) and incubated in the dark for at least 30 min at room temperature. The stained cells were analyzed using a FACScan flow cytometer in combination with BD Lysis II Software (Becton Dickinson).

RT-PCR for the Detection of Survivin mRNA Expression. Survivin mRNA expression in A 549 2 cells was determined by RT-PCR analysis. To prepare the samples for the analysis, we isolated total RNA from A 549 cells using TRIZOL (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. Survivin sense primer: 5′-GGA CCA CCG CAT CTC TAC AT-3′; antisense primer: 5′-GCA CTT TCT TCG CAG TTT CC-3′ (14). GAPDH sense primer: 5′-ACC ACA GTC CAT GCC ATC AC-3′; antisense primer: 5′-TCC ACC ACC CTG TTG CTG-3′. The reaction parameters of survivin were as follows: 95°C 5 min, 94°C 1 min, 54°C 1 min, 72°C 1 min for 30 cycles, 72°C extension 10 min. The PCR products were separated in 2% agarose gels, visualized by staining with ethidium bromide, and analyzed with Image 1.62.

Western Blotting for Survivin Expression. Cell lysates were prepared by adding 500 μl of ice-cold lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40) containing 1% aprotinin (Bayer, Leverkusen, Germany) and 1 mM phenylmethylsulfonyl fluoride. The lysates were cleared by centrifugation, and protein concentration in the supernatant was determined by the DC Protein assay (Bio-Rad, Hercules, CA, USA). Aliquots of cell lysates (20 μg of protein each) were separated by 8.5% SDS-PAGE, electrotransferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad), blocked with 5% nonfat milk in TBS-Tween buffer (20 mM Tris-HCl, pH 7.4, 135 mM NaCl, 0.1% Tween) for 1.5 h at room temperature, and incubated with the appropriate antibody overnight at 4°C, and then with horseradish peroxidase conjugated for a secondary antibody for 30 min at room temperature. After extensive washing, immunoreactive proteins were detected with an Enhanced Chemiluminescence Detection System (ECL; Amersham Biosciences Corp., Piscataway, NJ, USA). The following primary antibodies were used: antibodies for survivin and β-actin from Santa Cruz Biotechnology (USA).

Caspase-3, -7 Activities Assay. To analyze caspase-3, -7 activities, A 549 cells (1×10^6 cells) were treated with regents for 48 h. The caspase-3 and caspase-7 activities were measured with a Caspase Colorimetric Protease assay kit (Medical & Biological Laboratories Co., Ltd., Japan) according to the manufacturer's instructions.

Statistical Analysis. Data are presented as means ± SD. Statistical analysis was performed by one-way analysis of variance (ANOVA) using SPSS 11.0 software for Windows and $P < 0.05$ was considered statistically significant.

Results

Growth Inhibition of A 549 Cells by Cinobufocini. Since there is not standard concentration for the use of cinobufocini in A 549 cell culture for experiments, we used several different concentrations to test the effect of cinobufocini on the growth of A 549 and HLF-1 cells at various time points. The cells were treated with different concentrations (from 75 μg/ml to 150 μg/ml) of cinobufocini for different periods of time (24 and 48 h). The results illustrated that cinobufocini inhibited significantly the proliferation of A 549 cells in a dose- and time-dependent manner, which was observed as early as 24 h in A 549 cells after treatment with 125 μg/ml cinobufocini (Fig. 2A) without damaging non-cancerous cells (HLF-1, Fig. 2B) compared with control.

Apoptosis in A 549 Cells Induced by Cinobufocini. To determine whether the decrease in cell proliferation was associated with cell cycle arrest or apoptosis, cells were treated with 125 μg/ml cinobufocini for 24 or 48 h, then cell cycle and apoptosis were analyzed by flow cytometry. Cinobufocini led to an increase in the percentage of cells in the G1 phase and a decrease in the S phase at 24 h in A 549 cells, but there was no significant change in the proportion of cells in the G2/M phase. Moreover, the apoptosis rate gradually increased time-dependently in A 549 cells (Fig. 5). These results were consistent with the growth inhibition of A 549 cells, suggesting that the antiproliferation effect of cinobufocini should be associated with cell cycle G1 arrest and apoptosis in lung cancer cells. Furthermore, we also observed the morphologic changes by scanning electron microscope. In the control group, A 549 cells were round and homogeneously stained (Fig. 3A, 3B). However, A 549 cells grew slowly, the smooth surface of A 549 cell became uneven punch and irregular shapes treated with cinobufocini (Fig. 3C, 3D). In addition, we also observed changes to the nucleus by Hoechst 33258 staining. Cinobufocini treated A 549 cells showed more granular apoptotic bodies as compared with the control group (Fig. 4). These results demonstrate that cinobufocini induces apoptosis of A 549 cells.

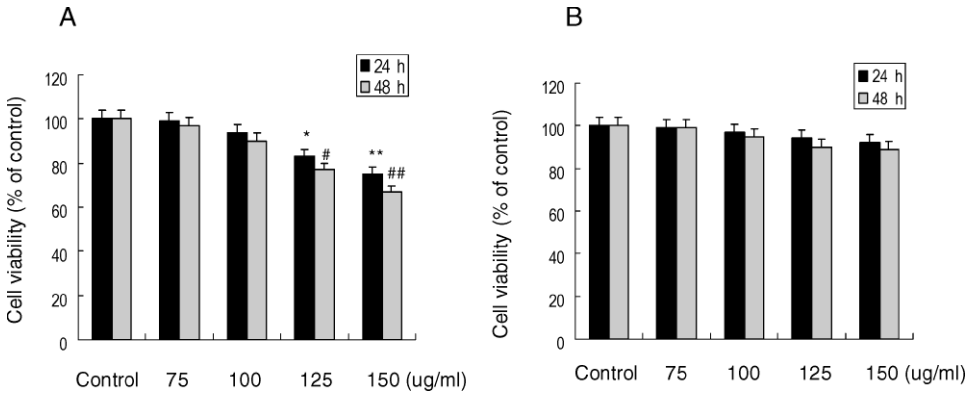


Figure 2. Growth inhibition of cinobufocini in A 549 (A) and HLF-1 (B) cells. Cells were treated with different concentrations of cinobufocini for indicated times, and the cell growth was determined using a MTT assay. The results are expressed as percentages of cell growth relative to untreated control cells. The data represent the means ± SD ($n = 3$), *, $^{\#}P < 0.05$; **,$^{\#\#}P < 0.01$ vs. control.

Downregulation of Survivin Expression by Cinobufocini in A 549 Cells. Survivin, a member of the IAP family, is a bifunctional protein that suppresses apoptosis and regulates cell division (6, 14). To determine the mechanism of apoptosis by cinobufocini, the effect of cinobufocini on survivin expression was studied. Forty-eight hours after the treatment with reagents, the level of survivin mRNA was detected by RT-PCR (Fig. 6A), and the expression of survivin was examined by Western blot (Fig. 6B). The statistical analysis showed that both mRNA and protein of survivin expression in A 549 cells were downregulated significantly by cinobufocini at 125 μg/ml compared with control.

Induction of Caspase-3 Activity by Cinobufocini in A 549 Cells. Caspases, the cytoplasmic aspartate-specific cysteine protease, have been shown to play a central role in the apoptotic signaling pathway. Finally, we examined whether caspase-3, –7 activities were increased

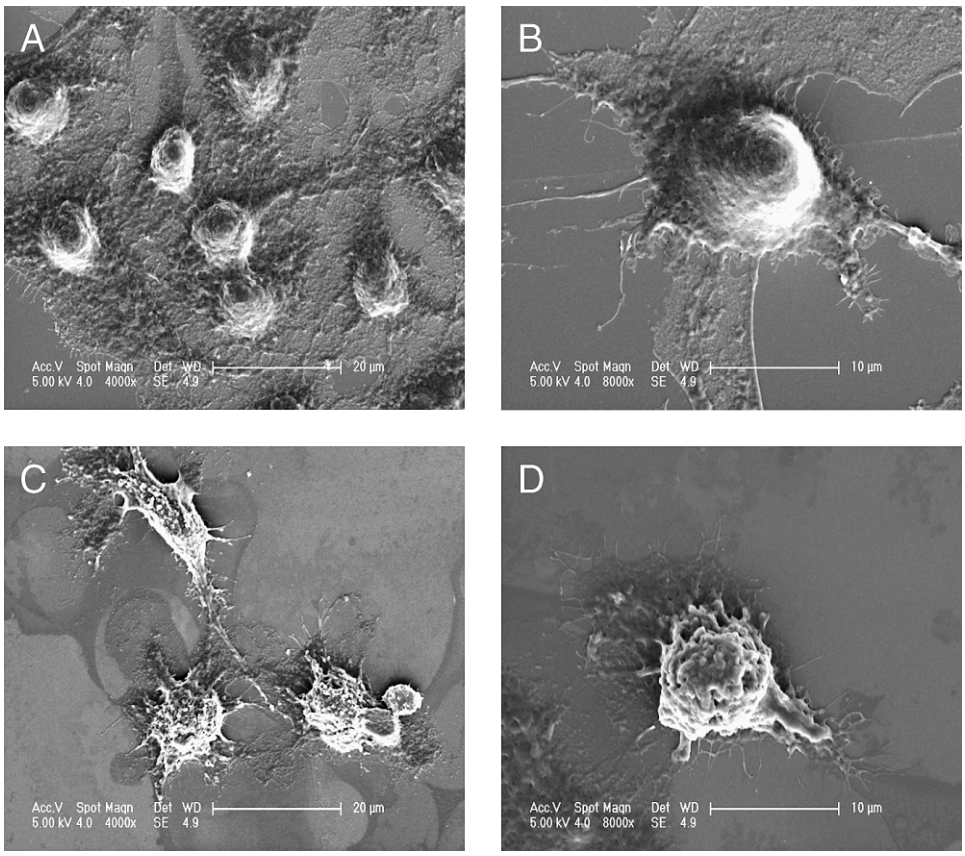


Figure 3. Photomicrograph of A 549 cells by scanning electron microscopy. The assessment of morphology of the cell was performed after A 549 cells were incubated for 48 h with vehicle (A ×4000; B ×8000), with 125 μg/ml cinobufocini (C ×4000; D ×8000).

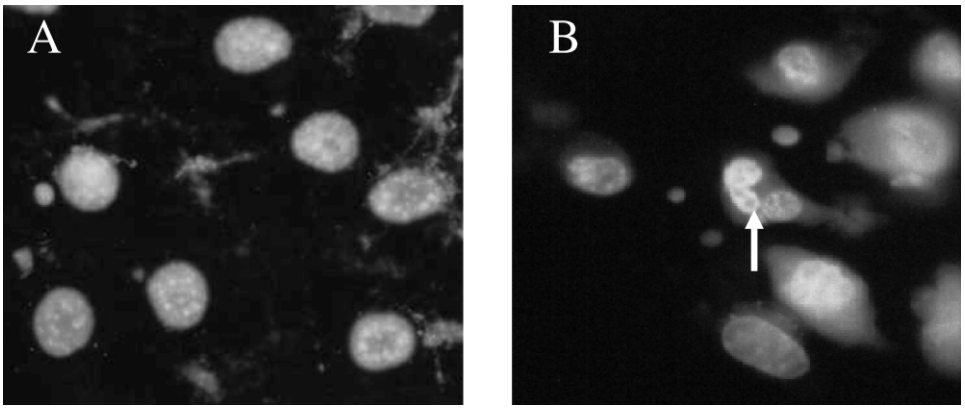
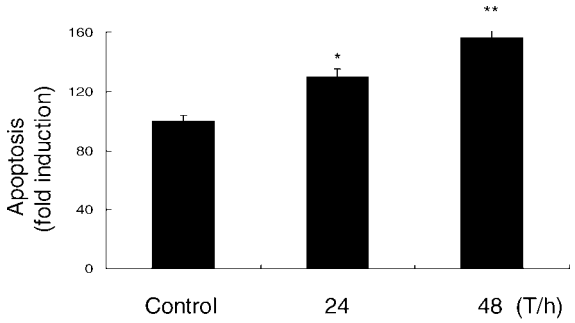


Figure 4. Fluorescence photomicrographs of A 549 cells with Hoeschst 33258 staining. The assessment of nuclear morphology of the cell was performed after A 549 cells were incubated for 48 h with vehicle (A), with 125 μg/ml cinobufocini (B). →: nuclear fragmentation (Hoechst 33258 ×400).

during cinobufocini-induced apoptosis in A 549 cells. Figure 7 shows the appearance of the caspase-3 activity following exposure to 125 μg/ml cinobufocini for 48 h without effect of caspase-7 activity compared with control. Caspase-3, a member of the caspase family, was shown to play an essential role in apoptosis induced by a variety of stimuli (15–17). These results indicated that cinobufocini efficiently activated caspase-3 in A 549 cells.

Discussion

The balance between apoptosis and anti-apoptosis signal pathways plays a role in the pathogenesis of a variety of cancers. It has been demonstrated that the inhibition of apoptosis promotes the mitotic progression in cancer cells (18). Tumor development and progression as well as resistance to most oncologic therapies result mainly from a lack in the response to apoptotic stimuli (19). Due to metabolic and morphological similarities, monocytic A 549 cells have been accepted as a good model of lung carcinoma. Cinobufocini is a preparation containing water soluble components of the toad skin used in Chinese traditional medicine. In the present study, cinobufocini significantly suppressed the proliferation of A 549 cells. Moreover, the nuclear morphology and apoptosis rate analyzed quantitatively by flow cytometry suggested that an apoptotic cell death mechanism was also potentially involved in this direct cytotoxicity.



T (h)	G0/G1 (%)	S (%)	G2/M (%)
0	42.14± 2.04	34.31± 3.04	23.73± 1.56
24	56.85± 2.56*	22.35± 2.13*	20.8± 2.21
48	62.67± 3.02**	17.23± 2.56**	20.1± 2.45

Figure 5. Cell-cycle analysis of A 549 cells treatment with cinobufocini by flow cytometry. Subconfluent monolayers of A 549 cells were treated with cinobufocini (125 μg/ml) for 48 h. The percentage of nonapoptotic and apoptotic cells within each cell cycle was observed by flow cytometry. The data represent means ± SD ($n = 3$), $^*P < 0.05$, $^{**}P < 0.01$ vs. control.

Because cinobufocini could inhibit growth and induce G1 arrest and apoptosis in A 549 cells, more attention has been paid to its potential mechanism. Survivin is one of the top four transcripts uniformly upregulated in human cancers but not in normal tissues (20). Based on its specific overexpression in a vast majority of solid cancers and its anti-apoptotic function, survivin represents a suitable target for antitumor approaches. The inhibition of survivin would block the anti-apoptosis and mitotic progression in tumor cells. The overexpression of survivin appears to correlate with aggressive tumor behavior and poor prognosis in non-small cell lung cancers (21). Additionally, survivin overexpression is also correlated with insensitivity to chemotherapy and radiotherapy in cancers (22). Inhibiting the expression of survivin can induce tumor cell apoptosis, sensitize tumor cells to chemotherapy and radiotherapy, and inhibit tumor angiogenesis (23, 24). Survivin has become an ideal target for the diagnosis and treatment of cancer. To determine whether the apoptosis of A 549 cells by cinobufocini was mediated by a decrease in survivin expression, A 549 cells were treated with cinobufocini (125 μg/ml) for 48 h, as shown in Figure 6. RT-PCR and Western blot findings revealed that cinobufocini suppressed survivin mRNA and protein expression levels compared with control. This finding demonstrates that the apoptosis of A 549 cells by cinobufocini is associated with down-regulation of survivin expression.

In addition, the mechanisms by which survivin inhibits cell apoptosis and cell division are still highly controversial

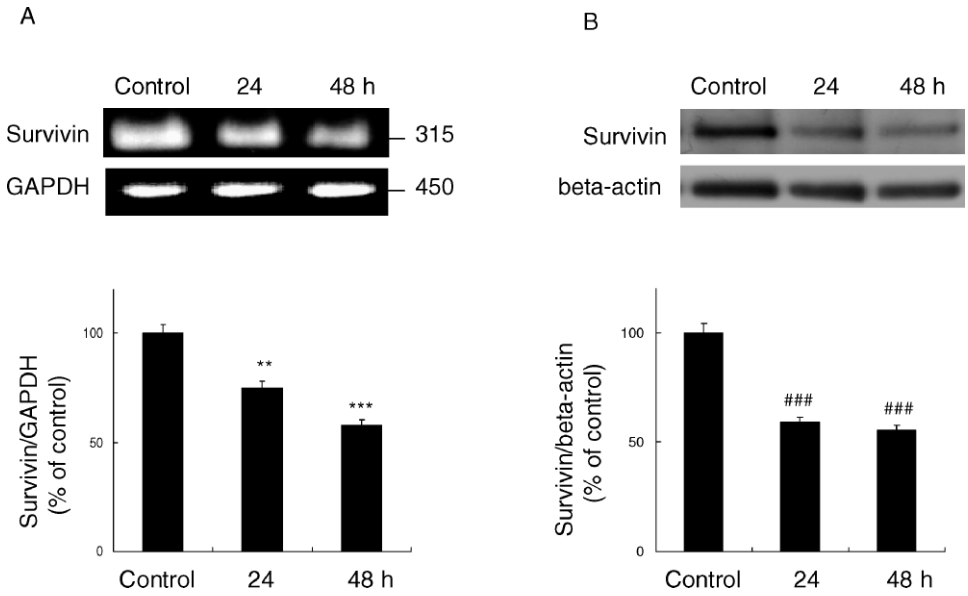


Figure 6. Survivin expression following treatment with cinobufocini. Subconfluent monolayers of A 549 cells were treated with cinobufocini (125 μg/ml) for 48 h. After the treatment, (A) RNA was extracted. Levels of survivin and GAPDH mRNA were examined by RT-PCR. The two panels on top show the expression levels of survivin and GAPDH mRNA; bar graphs at the bottom show the expression levels of survivin relative to those of GAPDH. (B) The cells were lysed, and survivin was examined by Western blot with antibody specific for survivin form. The two panels on top show the expression levels of survivin and β-actin; bar graphs at the bottom show the expression levels of survivin relative to those of β-actin. The data represent means ± SD ($n = 3$), $^{**}P < 0.01$, ***, $^{###}P < 0.001$ vs. control.

(25). Survivin may regulate apoptosis by directly inhibiting the activity of caspases and mainly suppress the activity of caspase-3 and caspase-7 (26). Disruption of survivin-microtubule interactions could result in loss of survivin's anti-apoptosis function and increase caspase-3 activity, a mechanism involved in cell death during mitosis (27). Caspase-3 is the ultimate executioner caspase that is essential for the nuclear changes associated with apoptosis (28). In the present study, we found that the cinobufocini-induced apoptosis of A 549 was also related to the increase in caspase-3 activity and consistent with the data in Figure 5 which show the appearance of apoptotic cells, but did not detect the activation of caspase 7 (Fig. 7). The results were inconsistent with others (29) and may be caused by differences in drug formulations, experiment design, treatment time, and cell lines.

The vast history of experience of traditional Chinese medicine (TCM) may facilitate the identification of novel anti-lung cancer compounds. Cinobufocini is one of the major active compounds and is a widely used TCM. Cinobufocini has four main active components: bufalin, bufotalin, cinobufagin, and bufogenin. High-pressure liquid chromatography (HPLC) analysis results showed that the concentration of bufalin was the highest, that of cinobufagin was higher, and that of bufogenin was the weakest in cinobufocini (30). Bufalin and cinobufagin, even at low

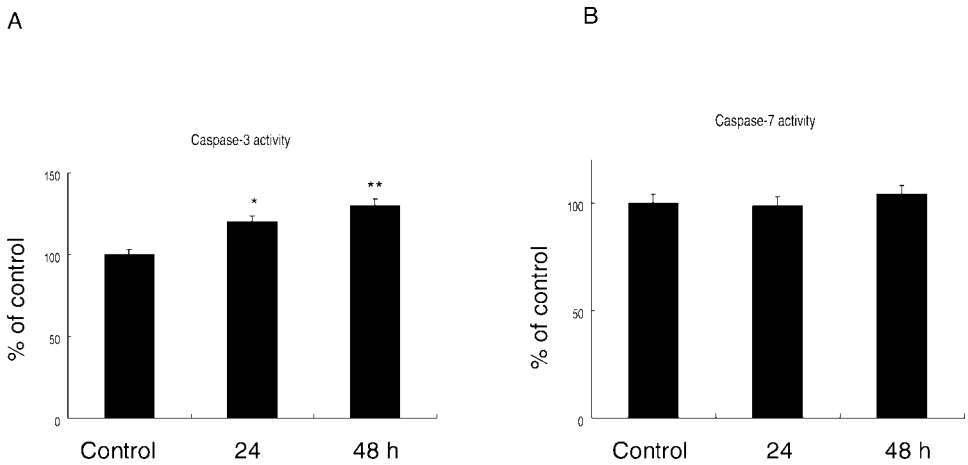


Figure 7. Cinobufocini-induced activity of caspase. Subconfluent monolayers of A 549 cells were exposed to cinobufocini (125 μg/ml) for 48 h. The caspase-3 (A) or caspase-7 (B) activity was measured with a colorimetric protease assay, respectively. The results are expressed as percentages of caspase activity relative to untreated control cells. The data represent means ± SD ($n = 3$), $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control.

concentration, remarkably inhibited hepatoma cancer cell (SMMC-7721, BEL-7402) and prostate cancer cell (PC3, DU145) growth and induced apoptosis of the cells and the effect of bufalin was stronger than that of cinobufagin, while bufogenin has weak antitumor effect (31, 32). In the present study, we found that cinobufocini induced apoptosis of A 549 cells. However, the active component in cinobufocini that affects apoptosis in the A 549 cells needs to be characterized more detail on apoptosis in A 549 cells.

In summary, we have shown that cinobufocini, which is a well-characterized and well-established pharmaceutical, induces apoptosis of A 549 cells through nuclear degradation, suppressing the survivin mRNA and protein and increasing caspase-3 activity, which may provide a molecular explanation for its potent *in vitro* anticancer properties. In addition, the effects of cinobufocini treatment on apoptosis in A 549 need to be characterized in more detail.

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