Cinobufocini inhibits NF-κB and COX-2 activation induced by TNF-α in lung adenocarcinoma cells

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Abstract. The aim of the present study was to investigate the effects of cinobufocini on nuclear factor-κB (NF-κB), cyclooxygenase-2 (COX-2) and the production of cytokines induced by tumor necrosis factor-α (TNF-α) in the A549 cell line. A549 cells were incubated with cinobufocini at different concentrations for 24, 48 or 72 h. Cell proliferation was examined by the WST-8 assay. The expression of NF-κB, COX-2 and inhibitor κBα (IκBα) was studied by western blotting. The NF-κB-dependent luciferase rporter (3xκB-luc) was transfected for 24 h, the cells were treated with the reagents for 24 h, and the transcriptional activity of the NF-κB promoter was detected by a luciferase assay. The levels of IL-6 and IL-8 mRNA were detected by reverse transcription-polymerase chain reaction. We found that cinobufocini inhibited NF-κB p65 expression and the transcriptional activity of the NF-κB promoter induced by TNF-α compared with the control in the nuclei of A549 cells. Moreover, induced COX-2 expression was blocked by cinobufocini and was correlated with a reduction in the activated p65 subunit of NF-κB. Additionally, the levels of IL-6 and IL-8 mRNA induced by TNF-α were significantly suppressed by the addition of cinobufocini. In conclusion, these results suggest that the anti-inflammatory effects of cinobufocini are dependent on the NF-κB/COX-2 pathway in A549 cells, thereby providing a possible anticancer mechanism for the compound.

Introduction

Lung cancer is one of the most common cancer-related causes of death worldwide (1). Natural products, including those from plants and microorganisms, provide rich potential for anticancer drug discovery. The cinobufocini injection is a preparation containing water-soluble components of Chan Su, a traditional Chinese medicine obtained from the skin and parotid venom glands of the toad (2). The chemical formula of cinobufocini is shown in Fig. 1. Cinobufocini and other bufadienolides are cardioactive C-24 steroids that exhibit a variety of biological activities, such as cardiotonic, anesthetic and antineoplastic activities (2). Both clinical and laboratory studies showed that cinobufocini has anticancer effects (3,4); however, there is limited information on the global molecular mechanisms of cinobufocini action in lung cancer cells.

Nuclear factor-κB (NF-κB) is a ubiquitous transcription factor that is activated by a variety of cytokines and mitogens and is a key regulator of cell survival (5). Cyclooxygenases (COX) catalyze the synthesis of prostaglandins from arachidonic acid. The two isoforms of COX are designated COX-1 and COX-2. Whereas COX-1 is expressed constitutively in most tissues and appears to be responsible for housekeeping functions, COX-2 is transcriptionally induced by pro-inflammatory stimuli, including cytokines and bacterial lipopolysaccharide (6). COX-2 has been found in a majority of invasive lung cancers as well as in ductal carcinoma (7). COX-2 overexpression has been implicated in the progression of cancer by increasing proliferation, invasiveness and angiogenesis. The promoter region of the COX-2 gene contains a binding site for the p65 subunit of NF-κB-3, and therefore signals to activate NF-κB have been shown to induce COX-2 expression (8). These correlations imply that the anticancer effect of cinobufocini may be concomitant with the downregulation of COX-2 expression. The purpose of the present study was to examine the effect of cinobufocini on NF-κB, COX-2 activation and the production of IL-6 and IL-8 induced by tumor necrosis factor-α (TNF-α) in the A549 lung adenocarcinoma cell line.

Materials and methods

Reagents. Cinobufocini (injected at 500 mg/ml) was kindly provided by Anhui Jingchan Pharmaceutical Co., Ltd. Fetal bovine serum (FBS) was purchased from the Shanghai Huamei Biotechnology Company. The RPMI-1640 medium and 0.25% trypsin were purchased from Gibco (USA). The recombinant TNF-α and NS-398 were purchased from Calbiochem (Germany). Dimethyl sulfoxide (DMSO) and the COX-2 and NF-κB p65 antibodies were purchased from Santa Cruz Biotechnology, Inc., USA. The transcription factor IIB (TFIIB) antibody was purchased from BD Transduction Laboratories. The inhibitor κBα (IκBα) and β-actin antibodies were purchased from Cell

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Table I. Primers used for RT-PCR analysis.

Gene	Primer sequence (5'-3')	Size of PCR product (bp)
IL-6	F: 5'-GATGCTACCAAACTGGATATAATC-3'	
	R: 5'-GGTCCTTAGCCACTCCTTCTGTG-3'	269
IL-8	F: 5'-CTGTGTGAAGCTGCAGTTCT-3'	
	R: 5'-TAGGCAGACCTCGTTTCCAT-3'	180
β-actin	F: 5'-AAGTACTCCGTGTGGATCGG-3'	
	R: 5'-TCAAGTTGGGGGACAAAAAG-3'	547

Signaling Technology. The Lipofectamine™ Plus Reagent kit was purchased from Invitrogen. The Dual-Luciferase Reporter Gene Assay System was purchased from Promega. The Lumat 9507 Fluorescence Detector was purchased from the Beijing Xinfeng Electromechanical Technology Company.

Cell culture. The human lung cancer cell line A549 was originally obtained from the American Type Culture Collection (ATCC) and maintained in 5% CO_2 at 37˚C in RPMI-1640 medium containing 10% FBS.

WST-8 assay. Cell proliferation was evaluated using a WST-8 assay. A549 cells ($1x10^4$ 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 (24, 48 or 72 h), 10 μl of WST-8 reagent solution (Cell Counting kit, Dojindo Laboratories, Japan) was added and incubated for 2 h. The optical density (OD) value was detected at a test wavelength of 450 nm according to the manufacturer's instructions. The percentage of the inhibition of proliferation was calculated as follows: percentage of inhibition = (mean control OD - mean experimental OD)/mean control ODx100%.

Extraction of nuclear and whole cell lysates. After a 48-h treatment as described above, the nuclear and cytoplasmic proteins of A549 cells were extracted according to the methods described by Sugimori *et al* (9). Briefly, the cells were harvested in 1 ml of ice-cold PBS and centrifuged for 1 min at 5,000 r/min at 4˚C. The cell pellet was lysed with 0.4 ml of buffer A [10 mmol/l 4-(2-hydroxyethyl-1-piperazine-ethanesulfonic acid) (HEPES), pH 7.9, 10 mmol/l potassium chloride (KCl), 0.1 mmol/l ethylenediamine tetraacetic acid (EDTA), 0.1 mmol/l ethyleneglycol bis (2-aminoethyl ether) tetraacetic acid (EGTA), 1 mmol/l dithiothreitol (DTT) and 1 mmol/l phenylmethylsulfonyl fluoride (PMSF)] for 15 min on ice. Then, 25 μl of 10% Nnonidet P-40 solution was added, and the samples were vortexed for 15 sec before centrifuging at 15,000 r/min for 5 min at 4˚C. The supernatant (cytoplasmic protein) was stored at -80˚C until use. The pellet was washed once with 0.5 ml of buffer A and resuspended in 50 μl of buffer B (20 mmol/l HEPES, pH 7.9, 0.4 mol/l sodium chloride (NaCl), 1 mmol/l EDTA, 1 mmol/l EGTA, 1 mmol/l DTT, and 1 mmol/l PMSF). The lysed nuclei were left on ice for 30 min and then centrifuged at 15,000 r/min for 5 min at 4˚C.

The cell lysates were prepared by adding 500 μl of ice-cold lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nnonidet P-40) containing 1% aprotinin (Bayer, Leverkusen, Germany) and 1 mM PMSF. The lysates were cleared by centrifugation, and the protein concentration was determined using the DC Protein Assay (Bio-Rad, Hercules, CA, USA). The extracts were stored at -80˚C until use.

Western blotting. The aliquots of the protein extracts (40 μg total protein each) were separated by 8.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, electrotransferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad), then blocked with 5% nonfat milk in TBS-Tween buffer (20 mmol/l Tris-HCl, pH 7.4, 135 mmol/l NaCl, 0.1% Tween) for 1.5 h at room temperature. The blots were incubated with the appropriate antibody (1:500) overnight at 4˚C and then with horseradish peroxidase-conjugated secondary antibody (1:5,000) for 30 min at room temperature. After extensive washing, the immunoreactive proteins were detected with an Enhanced Chemiluminescence Detection System (ECL; Amersham Biosciences, Piscataway, NJ, USA). TFIIB or β-actin were used as controls.

Recombinant human-specific NF-κB-luciferase reporter plasmid activity detection. The human 3xκB promoter construct (NF-κB-dependent luciferase reporter 3xκB-luc) was kindly provided by Dr Shigeki Miyamoto (10). A549 cells were seeded onto 6-well plates and co-transfected with 1.0 μg of firefly-luciferase reporter plasmid and 0.1 μg of the renilla-luciferase transfection control (phR-CMV; Promega, Southampton, UK) using Lipofectamine™ Plus Reagents. After a 24-h transfection, the cells were treated with reagents for 24 h. The cells were harvested, added to the cell lysate and centrifuged at 15,000 r/min for 5 min at 4˚C. The luciferase activity was measured with the Dual-Luciferase Assay System by Lumat 9507 according to the manufacturer's instructions.

RT-PCR for the detection of IL-6 and IL-8 mRNA expression. Total-RNA was extracted from A549 cells using a TRIzol kit according to the manufacturer's instructions. The concentration and purity of total-RNA obtained were detected by spectrophotometric measurements at 260 and 280 nm. The IL-6 and IL-8 mRNA expression levels in A549 cells were determined by RT-PCR. The primers are presented in Table I. The reaction parameters were as follows: 94˚C for 5 min, (94˚C for 1 min, 55˚C for 1 min, 72˚C for 2 min) for 28-35 cycles, and a 72˚C extension for 10 min. The PCR products were separated on a 2% agarose gel, visualized by staining with ethidium bromide and analyzed using Image 1.62.

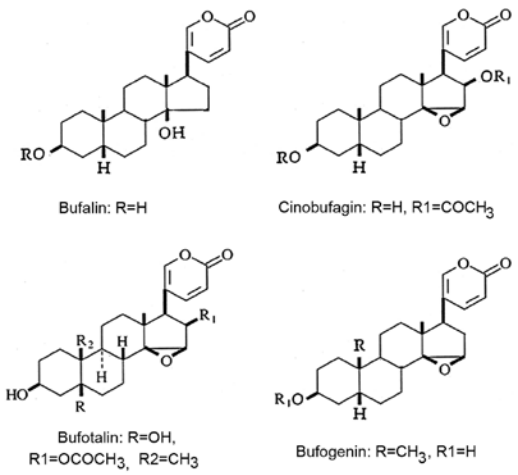

Figure 1. The structural formula of cinobufocini.

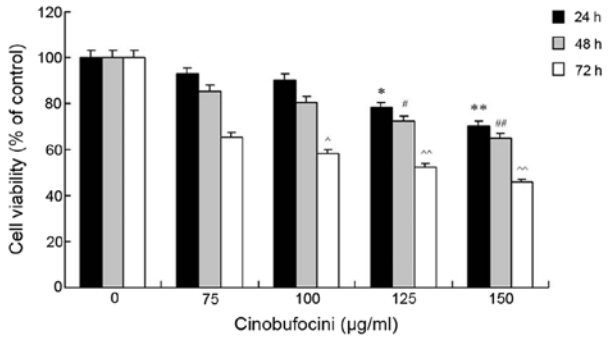

Figure 2. Cinobufocini-mediated growth inhibition of A549 cells. Cells were treated with various concentrations of cinobufocini for the indicated times, and the cell growth rate was determined using the WST-8 assay. The results are expressed as percentages of cell growth relative to untreated control cells. The data represent the means ± SD (n=3), $^{*,\#}$P<0.05; $^{**,\#\#}$P<0.01 vs. control.

Statistical analysis. The data are presented as mean ± SD. Statistical analyses were performed using the SPSS 10.0 statistical software. The comparisons among groups were performed with the one way analysis of variance or the factorial design analysis of variance, and comparisons between groups were performed using the t-test.

Results

Growth inhibition of A549 cells by cinobufocini. The A549 cells were treated with varying concentrations (from 75 to 150 μg/ml) of cinobufocini for different periods of time (24, 48 or 72 h). The results show that cinobufocini significantly inhibited the proliferation of A549 cells in a dose- and time-dependent manner. These effects were observed as early as 24 h following the treatment of A549 cells with 125 μg/ml of cinobufocini (Fig. 2) when compared to the control. In addition, we observed morphological changes using inverted microscopy. In the control group, monolayer cells were adherently growing with a large transparent cytoplasm and oval-shaped nuclei. In contrast, the treated cells were smaller and detached, with fragmented, dark opaque nuclei after treatment with cinobufocini (data not shown).

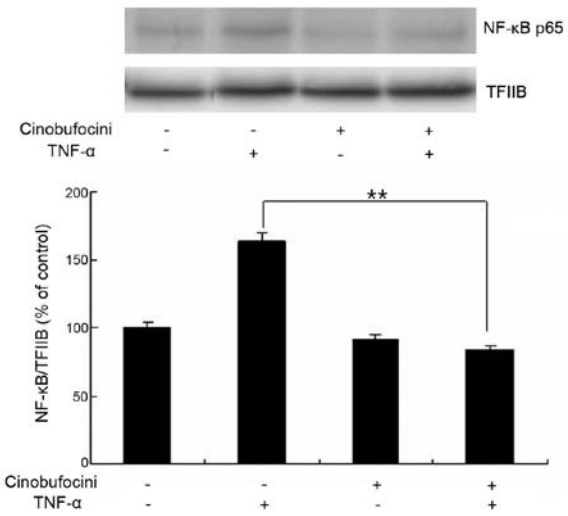

Figure 3. Cinobufocini inhibits NF-κB expression induced by TNF-α in A549 cells. Confluent A549 cells were incubated for 48 h with or without cinobufocini (125 μg/ml) or TNF-α (1 μg/l). The top two panels show the protein expression levels of NF-κB and TFIIB; the bar graphs at the bottom show the expression levels of NF-κB relative to that of TFIIB. The data are represented as mean ±SD (n=3). **P<0.01 compared with TNF-α.

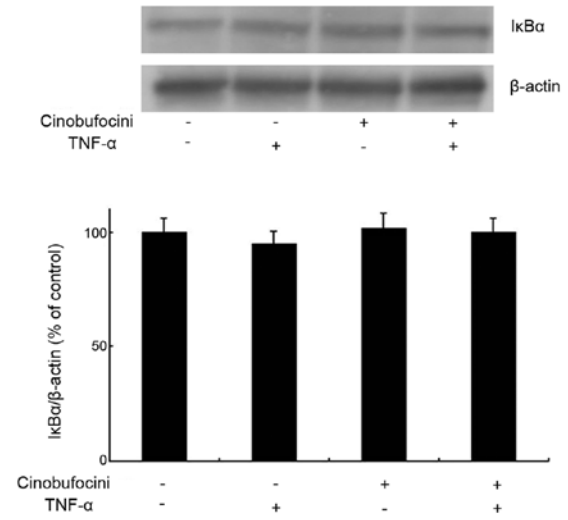

Figure 4. Cinobufocini decreases IκBα expression in A549 cells. Confluent cells were incubated for 48 h with or without cinobufocini (125 μg/ml) or TNF-α (1 μg/l). The two panels show the protein expression levels of IκBα and β-actin; bar graphs at the bottom show the expression levels of IκBα relative to that of β-actin. The data are represented as mean ± SD (n=3).

Cinobufocini inhibits NF-κB p65 expression by TNF-α. There is evidence that cell survival and the progression to malignancy are regulated by NF-κB (11). We examined whether cinobufocini inhibits NF-κB. Compared with the control, there was no significant difference in NF-κB expression with cinobufocini treatment, whereas TNF-α strongly induced NF-κB expression. However, the TNF-α-induced NF-κB expression was significantly suppressed by the addition of cinobufocini (125 μg/ml) (Fig. 3). To assess whether the downregulation of NF-κB expression was mediated by the degradation of IκBα expression by cinobufocini, IκBα expression was quantified in the cytosolic protein fraction of A549 cells. We observed

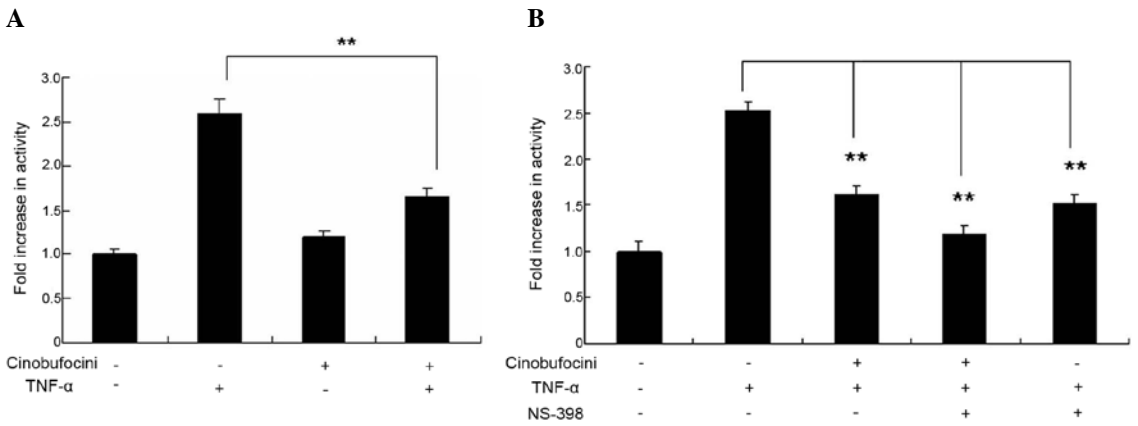

Figure 5. Cinobufocini reduces NF-κB promoter activity induced by TNF-α in A549 cells. Cells plated in 6-well plates were transfected with 3xκB-luc plasmid. One day after transfection, the cells were treated with cinobufocini (125 μg/ml), (A) TNF-α (1 μg/l) , or (B) NS-398 (25 μM) for 24 h; then, luciferase activity in the cell lysates was measured. The experiments were performed in triplicate. The data are represented as mean ± SD (n=3), **P<0.01.

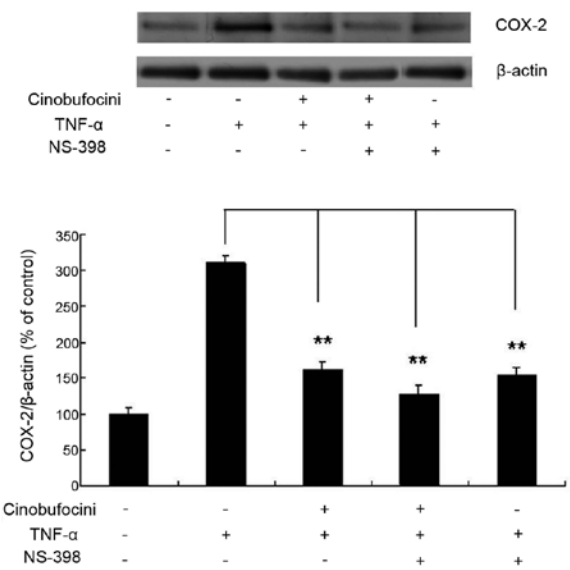

Figure 6. Cinobufocini reduces COX-2 expression by TNF-α in A549 cells. Confluent cells were incubated for 48 h with cinobufocini (125 μg/ml), TNF-α (1 μg/l), or NS-398 (25 μM). COX-2 expression was measured by western blotting. The two panels show the protein expression levels of COX-2 and β-actin; bar graphs at the bottom show the expression levels of COX-2 relative to that of β-actin. The data are represented as mean ± SD (n=3).

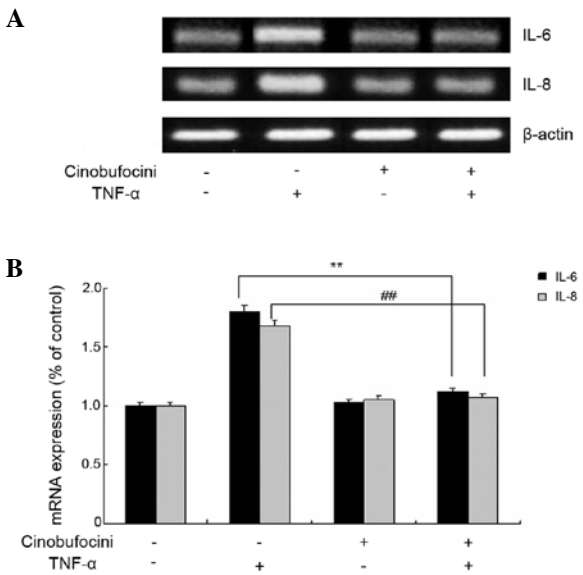

Figure 7. Cinobufocini decreases IL-6 and IL-8 mRNA expression by TNF-α. A549 cells were treated for 48 h with cinobufocini (125 μg/ml) or TNF-α (1 μg/l). RNA was then extracted, and the levels of cytokines and β-actin mRNA were examined by RT-PCR. (A) The three top panels show the expression levels of IL-6, IL-8 and β-actin mRNA; (B) the bar graphs at the bottom show the expression levels of IL-6 or IL-8 relative to those of β-actin. The experiments were performed in triplicate. The data represent means ± SD (n=3), **P<0.01; $^{\#\#}$P<0.01.

no significant change in IκBα expression levels compared with the control (P>0.05, Fig. 4). These results suggest that the inactivation of NF-κB by cinobufocini is not dependent on IκBα degradation.

Cinobufocini decreases the NF-κB promoter transcriptional activity induced by TNF-α. To further investigate the effects of NF-κB promoter activity, we performed dual-luciferase assays with a reporter vector containing the NF-κB promoter in A549 cells. As shown in Fig. 5A, compared with the control, there was no significant difference in luciferase reporter activity upon the addition of cinobufocini. However, upon stimulation with TNF-α, the luciferase reporter activity was increased by approximately 2.5-fold. Cinobufocini significantly inhibited the NF-κB promoter transcriptional activity induced by TNF-α.

Inactivation of NF-κB by cinobufocini is dependent on COX-2 function. COX-2 is an NF-κB-inducible gene that plays important roles in both inflammation and carcinogenesis (8). To assess whether the downregulation of NF-κB expression was mediated by COX-2 expression induced by cinobufocini, the cells were treated with cinobufocini in the presence of the COX-2 inhibitor NS-398. As shown in Fig. 5B, both NS-398 and cinobufocini reduced the effect of NF-κB activity. In addition, NS-398 decreased the NF-κB activity that was induced by TNF-α. We also measured the COX-2 expression in A549 cells by western blotting. Compared with the control, TNF-α strongly induced COX-2 expression by approximately 3-fold. However, the induced expression of COX-2 was significantly suppressed by the addition of cinobufocini or NS-398 (Fig. 6). These results

suggest that the inactivation of NF-κB by cinobufocini is dependent on COX-2 degradation.

Cinobufocini decreases the expression of IL-6 and IL-8 mRNAs. The activation of NF-κB has been shown to play an important role in enhancing the expression of several cytokine genes (12). To investigate the effect of cinobufocini on IL-6 and IL-8 expression in A549 cells, we examined the expression of IL-6 and IL-8 mRNAs by RT-PCR. Compared with the control, TNF-α strongly induced IL-6 and IL-8 mRNA expression; however, the induced IL-6 and IL-8 mRNA expression levels were significantly suppressed by the addition of cinobufocini (Fig. 7).

Discussion

The NF-κB signaling pathway is important for a variety of cell differentiation and proliferation processes. The pivotal role of the NF-κB signaling pathway in tumor promotion and progression, together with the occurrence of constitutively activated NF-κB in various solid and hematopoietic malignancies, strongly suggests that NF-κB inhibitors may be useful in cancer therapy. A close relationship between NF-κB and cancer has been proposed and recently reviewed (13-15). Furthermore, it has been reported that NF-κB p65 expression was significantly higher and was associated with disease progression in 394 cases of lung cancer by immunohistochemisty when compared with controls (16). Importantly, proliferation in the setting of chronic inflammation predisposes humans to carcinoma of the large bowel, lung, liver, breast, bladder, gastric mucosa, prostate, ovary and skin (17).

Traditional Chinese medicine (TCM) may facilitate the identification of novel lung cancer therapeutic compounds. Cinobufocini is one of the major active compounds that is widely used in TCM. Previously, we reported that cinobufocini induces the apoptosis of A549 cells without damaging non-cancerous cells (18). In the present study, we found that cinobufocini markedly inhibited A549 cell proliferation. In addition, the inactivation of NF-κB by cinobufocini is not dependent on IκBα degradation. IκB activity is regulated by the IκB kinase (IKK) complex. IKK, including IKK-α, IKKβ and IKK-γ, is also known as the NF-κB essential modifier (NEMO). When IKK is activated, the IKK complex causes the rapid phosphorylation, ubiquitination and hydrolysis of IκB, which then releases NF-κB from the cytoplasm to the nucleus (19).

COX-2 is overexpressed in malignant tissues including lung and breast tumors. Visscher *et al* (20) analyzed 235 archived breast biopsies of atypical ductal hyperplasia for COX-2 expression by IHC and found that the risk of the subsequent diagnosis of breast cancer increased with increased COX-2 expression. This is clinically significant because COX-2 plays a major role in pulmonary and intestinal inflammation, conditions that are correlated with tumor progression in these cancer models (21). Because NF-κB induction was abrogated by cinobufocini in A549 cells, we looked further down the signal cascade to the activation of COX-2. Both NS-398 and cinobufocini reduced the expression of COX-2, consistent with the inhibition of induced NF-κB activity. These results suggest that the inactivation of NF-κB by cinobufocini is dependent on COX-2 degradation.

The mixture of cytokines that is produced in the tumor microenvironment has an important role in cancer pathogenesis. Cytokines are released in response to infection, inflammation and immunity, which can inhibit tumor development and progression. Alternatively, cancer cells can respond to host-derived cytokines that promote growth, attenuate apoptosis and facilitate invasion and metastasis. A group of cytokine proteins, including IL-1, IL-6, TNF and RANKL, activate inflammation and are known to augment the ability of tumor cells to metastasize by affecting several steps of cell dissemination and implantation at secondary sites (22). Inflammatory cytokines lie downstream of NF-κB, which is known as the 'master' gene transcription factor for promoting inflammation. NF-κB is itself activated by these same inflammatory cytokines (23,24). The serum levels of cytokines have been reported to be elevated in patients with breast, colorectal and lung cancers (25-28). IL-6, which has diverse biological effects on immune and inflammatory responses, is produced in response to infection or injury in a variety of cells, including monocytes, lymphocytes, fibroblasts, ECs and keratinocytes. The activation of NF-κB has been shown to play an important role in enhancing IL-6 expression (22). To evaluate the effect of cinobufocini on the production of the inflammatory markers, we measured the levels of IL-6 and IL-8 mRNA in A549 cells. TNF-α-induced IL-6 and IL-8 expression levels were suppressed by the addition of cinobufocini.

Cinobufocini has four main active components: bufalin, bufotalin, cinobufagin and bufogenin. High-pressure liquid chromatography (HPLC) analysis determined the relative concentrations of the components; bufalin was present at the highest concentration, followed by moderate levels of cinobufagin and low concentrations of bufogenin (29). Bufalin and cinobufagin, even at low concentrations, remarkably inhibit hepatoma cancer cell (HepG2) and prostate cancer cell (PC3, DU145) growth and induce apoptosis. The anticancer effect of bufalin is more potent than that of cinobufagin, while bufogenin has a weak antitumor effect (30-32). Our findings demonstrate that cinobufocini can inhibit the activation of NF-κB and COX-2; moreover, it can also decrease the expression levels of the pro-inflammatory cytokines IL-6 and IL-8. These results provide a possible anticancer mechanism of cinobufocini through the NF-κB/COX-2 pathway in human lung cancer cells.

Acknowledgements

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