

**Brief Report****Apoptosis-inducing effect of cinobufacini, *Bufo bufo gargarizans* Cantor skin extract, on human hepatoma cell line BEL-7402**Fanghua Qi<sup>1</sup>, Anyuan Li<sup>1,\*</sup>, Hong Lv<sup>1</sup>, Lin Zhao<sup>1</sup>, Jijun Li<sup>1</sup>, Bo Gao<sup>2</sup>, Wei Tang<sup>3,\*</sup><sup>1</sup>Department of Traditional Chinese Medicine, Shandong Provincial Hospital, Shandong University, Ji'nan, Shandong, China;<sup>2</sup>Anhui Jinchan Biochemical Co., Ltd., Huaibei, Anhui, China;<sup>3</sup>Hepato-Biliary-Pancreatic Surgery Division, Department of Surgery, Graduate School of Medicine, the University of Tokyo, Tokyo, Japan.

**ABSTRACT:** Cinobufacini, a water-soluble preparation of Chinese medicine, is extracted from the skin of *Bufo bufo gargarizans* Cantor. The present study sought to investigate the effects of Cinobufacini on apoptosis of the hepatocellular carcinoma cell line BEL-7402. Cell viability was measured by methyl thiazolyl tetrazolium assay. Cell morphology was observed by Hoechst 33258 staining. Western blotting analysis was used to detect Bax and Bcl-2 expression. Results indicated that Cinobufacini inhibited the proliferation of BEL-7402 cells in a dose and time-dependent manner. Marked morphological changes indicative of apoptosis were observed after treatment with different concentrations of Cinobufacini. Western blot analysis showed that Bcl-2 expression was down-regulated while Bax expression was up-regulated. Thus, Cinobufacini may have a significant apoptosis-inducing effect on BEL-7402 cells, and this could prove useful for further anti-cancer research.

**Keywords:** Cinobufacini, *Bufo bufo gargarizans* Cantor, Proliferation, Apoptosis, Human hepatoma cell line BEL-7402

**1. Introduction**

Hepatocellular carcinoma (HCC) is one of the most common malignant neoplasms worldwide and its

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incidence has been increasing over the past few decades in areas such as Europe, the US, and eastern Asia (1). Although there are many advanced diagnosis and treatment methods such as surgery, radiation, and chemotherapy, HCC remains a formidable challenge for clinical therapy (2-5). Recently, traditional Chinese medicines have attracted attention as candidates for new cancer therapeutics with a low level of toxicity.

The skin of the Chinese toad, *Bufo bufo gargarizans* Cantor, has been used as an effective traditional Chinese medicine for thousands years in China (6). Cinobufacini, as it is typically designated in Chinese references, is a water-soluble preparation made from toad skin (7). Cinobufacini has been reported to possess a variety of biological effects, such as anti-tumor and anti-virus effects, and enhance physical immunity according to clinical data (8,9). Although Cinobufacini has proven to be effective against a variety of malignancies, and especially gastrointestinal tumors, its anti-tumor mechanisms have yet to be identified for the most part. No detailed data on the role and mechanisms of Cinobufacini in HCC cells have been available thus far. Thus, the current study investigated the effects of Cinobufacini on apoptosis in the human hepatoma cell line BEL-7402 and its mechanisms of action.

**2. Materials and Methods****2.1. Reagents**

Cinobufacini, which was prepared by an extraction of 20 g of toad's skin with boiling water followed by a concentration to 1 mL, was obtained from Anhui Jinchan Biochemical Co., Ltd., Anhui, China. High-glucose Dulbecco's modified Eagle's medium (DMEM) was purchased from Invitrogen, Carlsbad, CA, USA. Fetal calf serum (FCS) was from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd., Hangzhou, China. Hoechst 33258 was purchased from Sigma-Aldrich, St Louis, MO, USA. The anti-Bax, anti-BCL-2 and  $\beta$ -actin antibodies were purchased from

Santa Cruz Biotechnology, Santa Cruz, CA, USA and the second antibodies were purchased from Beijing Zhongshan Jinqiao Biological Technology Co., Ltd., Beijing, China. BCA-100 Protein Quantitative Analysis Kits were obtained from Shenergy BioScience & Technology Company.

## 2.2. Cells and culture conditions

The Bel-7402 cell line, which was established from a specimen obtained from a 53-year-old male with HCC (10), was obtained from the Shanghai Institute of Cell Biology of the Chinese Academy of Science, Shanghai, China. The cells were incubated in DMEM medium supplemented with 10% FCS, 100 U/mL of penicillin and 100 mg/mL of streptomycin in a humidified atmosphere with 5% CO<sub>2</sub> in air at 37°C. Cells in the logarithmic growth phase were collected for the following experiments.

## 2.3. MTT assay

Cells were plated at a density of  $6 \times 10^4$  cells/mL in 96-well plates. Twenty-four hours later, the cultures were incubated with different concentrations of Cinobufacini (0, 0.005, 0.01, 0.05, 0.1, and 0.5 mg/mL) prepared by diluting the Cinobufacini stock solution with serum-free medium. At times of 24 h, 48 h and 72 h after addition of Cinobufacini solutions, 20  $\mu$ L of methyl thiazolyl tetrazolium (MTT: 5 mg/mL) were added to each well and plates were then incubated for 4 h. Water-insoluble formazan was dissolved by adding 100  $\mu$ L dimethyl sulfoxide (DMSO) to each well. Finally, optical densities were monitored at 490 nm with 570 nm as a reference wavelength using an ELISA plate reader. The inhibitory rate (IR) and IC<sub>50</sub> (concentration of drug that inhibits cell growth by 50%) were then calculated.

## 2.4. Hoechst 33258 staining

Hoechst 33258 staining was used to observe the apoptotic morphology of cells. Briefly,  $3 \times 10^5$  cells/mL cells were seeded in six-well plates and incubated for 24 h. Then the cells were treated with Cinobufacini at 0, 0.01, 0.05, and 0.1 mg/mL, respectively, for 48 h. Finally, cells were fixed with 4% formaldehyde in phosphate buffered saline (PBS) for 15 min and stained with Hoechst 33258 at room temperature for 10 min. After cells were washed with PBS, morphological changes including a reduction in volume and nuclear chromatin condensation were observed under a fluorescence microscope and photographed at a magnification of 200 $\times$ .

## 2.5. Western blotting analysis

Cells were seeded in culture dishes (35 mm) at a density of  $6 \times 10^4$  cells/mL and incubated for 24 h.

Then, select cells were treated with Cinobufacini at a concentration of 0.1 mg/mL for 24 h and 48 h. At times of 24 h and 48 h, cells were washed with ice-cold PBS twice and lysed with lysis buffer for 30 min at 4°C, and then debris was removed by centrifugation for 10 min at 20,000  $\times$  g at 4°C. The protein concentrations of supernatant were determined with a BCA-100 Protein Quantitative Analysis Kit. Equal amounts of protein (30  $\mu$ g) were subjected to electrophoresis on 10% SDS-polyacrylamide gels and then transferred to polyvinylidene difluoride membranes by electroblotting. The membranes were first incubated in blocking solution (5% skim milk) for 1 h at room temperature and then incubated overnight at 4°C with the first antibodies: anti-Bax (1:750-dilution) or anti-BCL-2 (1:750-dilution). After they were washed with TBST (10 mM Tris-HCl, 0.15 M NaCl, 8 mM sodium azide, 0.05% tween-20, pH 8.0) three times, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:10,000-dilution) for 1 h and then again washed with TBST three times. Finally, protein bands were visualized with an enhanced chemiluminescence (ECL) detection system. As an internal control,  $\beta$ -actin was detected with anti- $\beta$ -actin antibodies. The ratio of Bax/BCL-2 was analyzed using an Alphamager (IS-2200). (NatureGene Corp., USA).

## 2.6. Statistical analysis

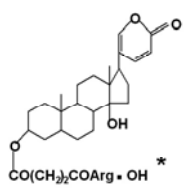
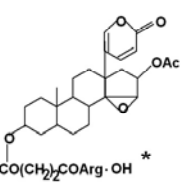
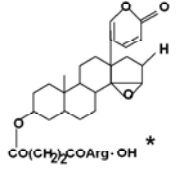
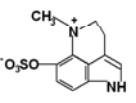
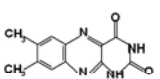
All experiments were performed in triplicate and the results were expressed as mean  $\pm$  S.D. Statistical analysis was performed by ANOVA using SPSS.11.5 software.

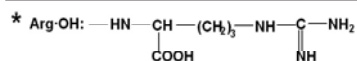
## 3. Results and Discussion

Despite recent advances in exploring the molecular biology of HCC cells and searching for new chemotherapeutic agents for the treatment of this malignant disease, there are still few efficient therapeutic measures for patients in the advanced stages (11-13). Therefore, a significant step would be to find new drugs and effective therapies for the clinical treatment of HCC. Recent reports indicate that traditional Chinese medicines may have a curative potential.

Cinobufacini is a water-soluble preparation of Chinese medicine. Reportedly, there are five mainly compounds that have been isolated from Cinobufacini. Their chemical components and compositions are shown in Table 1 (14). Recent studies have shown that Cinobufacini's active ingredients, bufalin and cinobufagin, have a significant effect on inducing apoptosis in a number of cancer cells such as human leukaemia cell lines HL-60 and U937, human gastric cancer cell line BGC-823, human prostate cancer

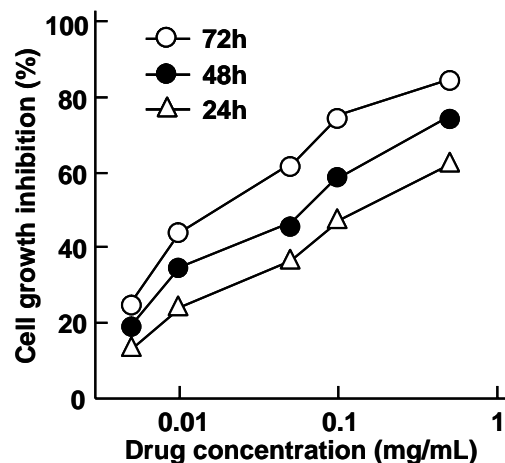
**Table 1.** Major chemical constituents of Cinobufacini

Constituents	Structure
Bufalin 3-succinoyl-arginine ester	
Cinobufagin 3-succinoyl-arginine ester	
Resibufogenin 3-succinoyl-arginine ester	
Bufothionine	
Lumichrome	



cell lines, and human endometrial and ovarian cancer cell lines (15-17). Therefore, further study of the active ingredients in Cinobufacini and their effect on HCC is also of great significance. Clinical data on Cinobufacini indicates that it acts to protect liver function and improves the quality of life for patients with little toxicity and few side effects except when treating tumors (18-20). However, Cinobufacini has also been reported to inhibit growth in a time-dependent manner in the human normal liver L-02 cell line (21). Therefore, another significant step would be to investigate the effects and mechanisms of action of Cinobufacini in normal human liver cells with anti-inflammatory action, anti-viral action, and so on.

The current study found that Cinobufacini extracted from the skin of *Bufo bufo gargarizans* Cantor inhibited proliferation and induced apoptosis in the human hepatoma cell line BEL-7402. MTT assays were performed to investigate the effect of Cinobufacini on the proliferation of BEL-7402 cells. As shown in Figure 1, after treatment with different concentrations (0.01, 0.05, and 0.1 mg/mL) of

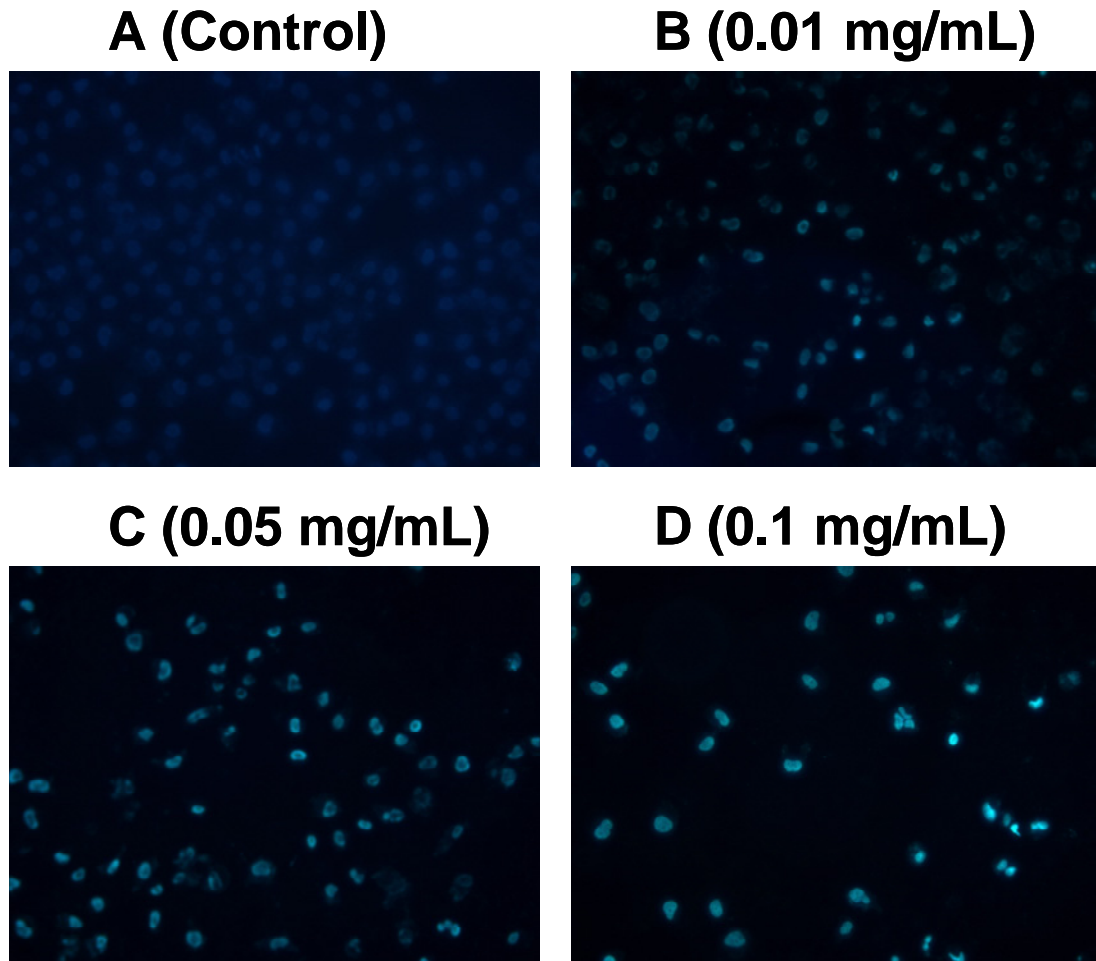


**Figure 1.** Growth-inhibiting effects of Cinobufacini on Bel-7402 cells. Cells were treated with different concentrations of Cinobufacini as indicated.

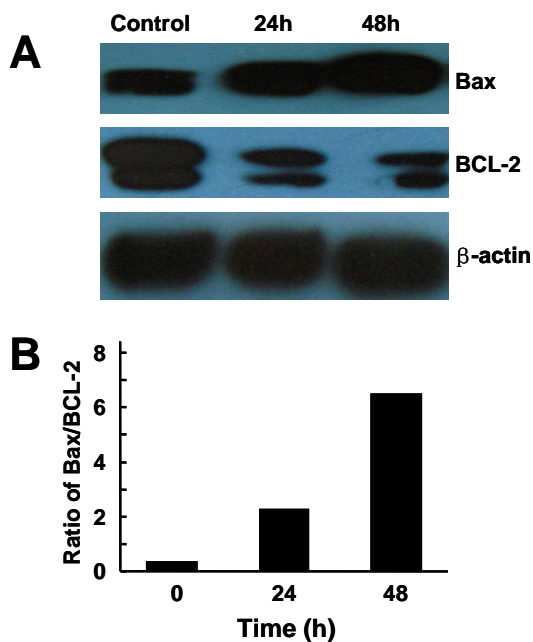
Cinobufacini for 48 h, the growth inhibition rates of cells were 34.8%, 45.5% and 58.5%, respectively, while the growth inhibition rates of cells treated with 0.1 mg/mL of Cinobufacini for 24 h, 48 h, and 72 h were 47.0%, 58.5% and 74.9%, respectively. In addition, the  $IC_{50}$  of BEL-7402 cells at times of 24 h, 48 h, and 72 h were 0.15, 0.06, and 0.02 mg/mL, respectively. These findings indicate that Cinobufacini has a significant growth-inhibiting effect on cells in a dose and time-dependent manner. After treatment with different concentrations (0.01, 0.05, and 0.1 mg/mL) of Cinobufacini for 48 h, marked morphological changes of cell apoptosis including chromatin aggregation, nuclear and cytoplasmic condensation, and partition of cytoplasm and nucleus into membrane-bound vesicles (apoptotic bodies) were observed with Hoechst 33258 staining (Figure 2).

To clarify the mechanisms of apoptosis caused by Cinobufacini, the protein expression of BCL-2 and Bax was examined in BEL-7402 cells after treatment with 0.1 mg/mL Cinobufacini for 24 h and 48 h. As shown in Figure 3A, Western blotting analysis showed that anti-apoptotic protein Bcl-2 expression was down-regulated while pro-apoptotic protein Bax expression was up-regulated in a time-dependent manner. Moreover, the ratio of Bax/BCL-2 significantly increased at all measured times compared to the control (Figure 3B). Apoptosis is now recognized as a key step in the evolution of tumors (22). Inducers of apoptosis have recently been used in cancer therapy, and activation of apoptosis pathways is a significant mechanism by which cytotoxic drugs kill tumor cells (23). The ratio of Bax to BCL-2, rather than BCL-2 alone, is crucial to the survival of drug-induced apoptosis (24). The current results indicated that an increased ratio of Bax to BCL-2 may be a significant mechanism by which Cinobufacini induces apoptosis of BEL-7402 cells.

In conclusion, the present study indicated that



**Figure 2.** Apoptosis observed with Hoechst 33258 staining. Cells were treated with 0 (A), 0.01 (B), 0.05 (C), and 0.1 mg/mL (D), respectively, for 48 h. Original magnification, 200 $\times$ .



**Figure 3.** Western blotting analysis of apoptosis-related factors. (A) Effects of Cinobufacini treatment on protein levels of Bax, BCL-2, and  $\beta$ -actin. Cells were treated with 0.1 mg/mL for 24 h and 48 h. (B) The ratio of Bax/BCL-2.

Cinobufacini significantly inhibited the proliferation and induced the apoptosis of BEL-7402 cells. Moreover, apoptosis induced by Cinobufacini may be regulated by the expression of Bax and BCL-2. However, further study is needed to clarify the mechanisms by which Cinobufacini and its active ingredients induce apoptosis.

#### Acknowledgments

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