Apoptosis-inducing activity of compounds screened and characterized from cinobufacini by bioassay-guided isolation

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Abstract. Cinobufacini (huachansu), an aqueous extract from the skin of the toad *Bufo bufo gargarizans* Cantor, is a traditional Chinese medicinal preparation widely used in clinical cancer therapy in China. Here, we screened and identified active compounds of cinobufacini and investigated their apoptosis-inducing effect on $HepG_2$ cells. Screening was performed using bioassay-guided isolation. The effects of different fractions on the proliferation of $HepG_2$ cells were detected by the MTT assay. The extraction and isolation of active fractions were performed by chloroform extraction, silica column chromatography, preparative thin-layer chromatography and high-performance liquid chromatography. Nuclear magnetic resonance (NMR) imaging and electron ionization-mass spectrometry (EI-MS) were used to identify the structure of the active compounds. The extent of cell apoptosis was detected by Hoechst 33258 staining and flow cytometric analysis. Western blot analysis was used to detect the expression of the apoptosis-related proteins Bax and Bcl-2. Through bioassay-guided isolation, two compounds were isolated from cinobufacini. NMR and EI-MS data revealed these compounds to be resibufogenin and cinobufagin. Cinobufagin was determined to be the more efficient of the two in inhibiting the proliferation of $HepG_2$ cells. Hoechst 33258 staining and flow cytometric analysis indicated that cinobufagin induced marked changes in apoptotic morphology and significantly increased the proportion of apoptotic cells in $HepG_2$ cells. Western blot analysis showed that cinobufagin up-regulated Bax expression and down-regulated Bcl-2 expression. In conclusion, we screened and identified two anti-proliferation compounds of cinibufacini, resibufogenin and cinobufagin. The most effective compound, cinobufagin, inhibited cell proliferation by inducing the apoptosis of $HepG_2$ cells. This was potentially triggered by regulation of the Bax/ Bcl-2 ratio.

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Introduction

In recent years, numerous traditional Chinese medicines have been found to possess potent anti-cancer activities, and have attracted considerable interest as potential candidates for the development of novel cancer therapies (1). The skin of the toad *Bufo bufo gargarizans* Cantor, used in traditional Chinese medicine, exhibits antipyretic, detoxicant, diuresis, stasis-eliminative and pus-discharging properties (2-4). Cinobufacini (huachansu), an aqueous extract of *B. bufo gargarizans* Cantor skin, is a traditional Chinese medicinal preparation widely used in clinical cancer therapy in China. It was demonstrated to have significant anti-cancer effects in a variety of cancers, including hepatic, gastric, pancreatic and esophageal carcinoma (5-9). Our previous studies and other related investigations have shown that cinobufacini inhibits cell growth by inducing apoptosis in human hepatoma and gastric neoplasm cells (10-12).

Studying active compounds is important for the development of traditional Chinese medicine (13). The chemical components of cinobufacini have been investigated since the 1980s (14). It has been demonstrated that bufadienolides and indole alkaloids such as bufalin, cinobufagin, resibufogenin, bufothionine, bufotenidine and serotonin are the primary chemical compounds of cinibufacini (14,15). However, to the best of our knowledge, the bioactive compounds of cinobufacini capable of inhibiting cancer cell proliferation and inducing cell apoptosis have yet to be determined.

In our previous study, cinobufacini was shown to inhibit cell growth by inducing apoptosis in $HepG_2$ cells (12). In the present study, we screened and identified the active compounds of cinobufacini with an anti-cancer effect, and investigated their apoptosis-inducing effect in $HepG_2$ cells.

Materials and methods

Preparation of cinobufacini. Cinobufacini, an aqueous extract from the skin of the toad *B. bufo gargarizans* Cantor, was obtained from Anhui Jinchan Biochemical Co., Ltd., Anhui,

China. The preparation process was as follows: *B. bufo gargarizans* Cantor skins (20 g) were boiled twice with distilled water. The resulting decoction was mixed and filtered using filter paper, then the filtered solution was collected, concentrated, lyophilized and extracted two more times with 60 and 85% ethanol. Finally, each decoction from 20 g *B. bufo gargarizans* Cantor skin was concentrated to 1 ml.

Cell culture. The human hepatoblastoma cell line $HepG_2$ cells were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA), supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), 100 U/ml of penicillin and 100 μg/ml of streptomycin (Sigma, St. Louis, MO, USA) in a humidified atmosphere with 5% CO_2 in air at 37˚C. Cells in the logarithmic phase of growth were collected for the experiments.

Assessment of the anti-proliferative effects on $HepG_2$ cells. $HepG_2$ cells in the logarithmic phase of growth were collected and plated at a density of $8x10^4$ cells/ml in 96-well plates. After incubation for 24 h, the cells were incubated with different fractions at various concentrations in serum-free high-glucose DMEM as indicated. As the negative control, cells were treated with 0.1% DMSO. After incubation for the indicated times, cell viability was detected using the 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide (MTT) assay (Roche, Mannheim, Germany). IC_{50} values (concentration of drug required to inhibit cell growth by 50%) were then calculated.

Screening and identification of active compounds from cinobufacini. *In vitro* bioactivity-guided isolation was performed to screen for the active anti-cancer compounds of cinobufacini. The purification grade of the cinobufacini at all fractions was monitored by thin-layer chromatography (TLC) (chlorofome/ethyl acetate, 1:1) using 10% sulfuric acid reagent, followed by incubation at 100˚C for 5 min. In this experiment, cinobufacini (50 ml) was first fractionated into the aqueous and chloroform (200 ml) phases. Both the chloroform phase and the remaining aqueous phase were evaporated under a vacuum at 40˚C. Extracted residues were then weighed and their anti-proliferative effects on $HepG_2$ cells were evaluated using the MTT assay.

Subsequently, the more active chloroform fraction (2.2 g) was adsorbed on a silica column of 200 g Wakogel C-200 (Wako Co., Osaka, Japan) and successively eluted by *n*-butyl alcohol/chloroform/acetone (4:3:3) and methanol. The fractions (fractions I-IX) were evaporated in reduced pressure. Extracted residues were weighed and the cytotoxicities of all fractions were evaluated.

Fraction III (154.8 mg) with improved anti-proliferative activity was fractionated again by 40 g silica-gel chromatography (Wakogel C-200), then successively eluted with chloroform/ethyl acetate (1:1) and methanol. Each fraction (fractions 1-13) was dried and weighed, followed by the evaluation of cytotoxicities.

Fraction 8 (38 mg) was found to be the most effective and was further purified by preparative TLC (Wako Co.) and semi-preparative high-performance liquid chromatography (HPLC) with the C-18 μBondasphere column (Waters Inc., Tokyo, Japan). Two purified fractions were obtained. Their purification grades were further detected using the COSMOSIL Cholester reversed-phase C-18 column (Waters, Japan) eluted with 45% acetonitrile.

The structures of the isolated compounds were elucidated by extensive analyses of ^1H nuclear magnetic resonance (NMR), ^{13}C NMR, distortionless enhancement by polarization transfer (DEPT) and electron ionization-mass spectrometry (EI-MS; JMS-SX102A, Jeol, Tokyo, Japan), and identified by spectral data and specific rotations in the literature.

Analysis of cell apoptosis. Hoechst 33258 staining (Dojindo Laboratories, Kumamoto, Japan) was used to observe the morphology of apoptotic cells. Cells ($2x10^5$ cells/ml) were seeded in 12-well plates and incubated for 24 h. After incubation with 0.04 μg/ml (1 μM) cinobufagin for another 24 h, the cells were fixed with 4% formaldehyde in phosphate buffered saline (PBS) for 15 min and stained with Hoechst 33258 solution at room temperature for 10 min. Then, after washing in PBS, the morphological changes of cells including a reduction in volume and nuclear chromatin condensation were observed under a fluorescence microscope and photographed at a magnification of x400.

The apoptosis-inducing effect of cinobufagin was also determined by flow cytometry. Briefly, after incubation with 0.04 μg/ml cinobufagin for 24 h, cells were trypsinized, washed with PBS, fixed in 70% ice-cold ethanol and stored at -20˚C. Prior to analysis, cells were washed again with PBS and treated with DNA-binding propidium iodide (PI; BD Pharmingen™, CA, USA) solution (10 mg/ml in PBS, containing 0.05 mg/ml RNase A) according to the manufacturer's instructions. Finally, the proportions of apoptotic or sub-G1 phase cells were measured by flow cytometry and analyzed using ModFit software (ModFit LT for Mac V2.0).

Western blot analysis. After treatment with 0.04 μg/ml cinobufagin for 24 h, cells were harvested and lysed with RIPA Lysis Buffer (Sigma) for 30 min on ice, then the lysates were centrifuged at 10,000 x g for 10 min and the supernatant was colleted. The protein concentrations of the supernatant were determined with the DC Protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein (30 μg) were separated by electrophoresis on SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. The membranes were first incubated in blocking solution (5% skim milk) for 1 h and then incubated overnight at 4˚C with the primary antibodies anti-Bax or anti-Bcl-2 (1:1,000 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing with TBS-T (Tris buffered saline with Tween-20: 20 mM Tris-HCL, 0.14 M NaCL, 0.1% Tween-20, pH 7.6) three times, the membranes were incubated with HRP-labeled secondary IgG antibodies for another hour and then washed with TBS-T three more times. Finally, protein bands were visualized using an enhanced chemiluminescence detection system. As an internal control, β-actin was detected with anti-β-actin antibodies (Sigma).

Statistical analysis. Experiments were performed in triplicate and the results were expressed as the mean ± SD. Statistical analysis was performed by one-way analysis of variance (ANOVA) using SPSS.15.0 software. $P<0.05$ was indicative of significant difference.

Results

Anti-proliferative effects of isolated fractions from cinobufacini on HepG$_2$ cells. Under the bioactivity-guided fractionation, effective anti-cancer components from cinobufacini were purified. As shown in Table I, the anti-cancer components were efficiently extracted with chloroform, which exhibited a lower IC_{50} (0.18 μg/ml) than water residue (IC_{50} 576.0 μg/ml) and cinobufacini (IC_{50} 1.6 μg/ml) in HepG$_2$ cells. Further purification of chloroform extract was performed by silica-gel chromatography. The cytotoxicities of fractions I-IX are listed in Table II. Fractions I, II, V, VII, VIII and IX displayed a weak anti-proliferative effect on HepG$_2$ cells with an IC_{50} ≥31.1 μg/ml, while fractions IV and VI showed a moderate anti-proliferation effect with an IC_{50} ≥11.2 μg/ml. By contrast, fraction III showed significant cytotoxic activity in the HepG$_2$ cells with the lowest IC_{50} value (5.8 μg/ml). These results suggested that fraction III was a promising candidate for further investigation. Therefore, another silica-gel chromatography was performed to further purify fraction III. Thirteen fractions (fractions 1-13) were separated and their anti-cancer activities were determined. As shown in Table III, fraction 8 displayed a much lower IC_{50} (5.0 μg/ml) than other fractions. Therefore, fraction 8 was selected for the purification of anti-proliferative compounds.

Purification of active compounds in fraction 8. To screen bioactive compounds from fraction 8, preparative TLC and reversed-phase HPLC with the μBondasphere column were employed. Two purified fractions were isolated by preparative TLC and HPLC from fraction 8. The final detection of either of these fractions using HPLC produced a single peak (Fig. 1), indicating that two highly homogeneous compounds (Compounds 1 and 2) were obtained.

Identification of the active compounds. Compound 1 was obtained as white powder whose EI-MS spectrum showed the $(M+H)^+$ ion peak at 385. Combined with the results of ^{1}H NMR (data not shown) and ^{13}C NMR, DEPT data shown in Table IV, the molecular formula of this compound was $C_{24}H_{32}O_4$ (*M*r=384). Compound 2 was obtained as white powder whose EI-MS spectrum showed the $(M+H)^+$ ion peak at 443. Combined with the ^{1}H NMR data (data not shown) and ^{13}C NMR, DEPT data shown in Table IV, the molecular formula of this compound was $C_{26}H_{34}O_6$ (*M*r=442). After analyzing and comparing these data online (SciFinder Scholar) with reported compounds and references, the structures of Compounds 1 and 2 were identified as resibufogenin (Compound 1) and cinobufagin (Compound 2) (Fig. 1), which were previously detected in the parotid secretions of the toads *B. marinus*, *B. gargarizans* and *B. melanostictus* (15,16).

Effects of resibufogenin and cinobufagin on cell proliferation. Cell growth inhibition by resibufogenin and cinobufagin treatment was determined by the MTT assay. As shown in Fig. 2, both resibufogenin and cinobufagin treatment induced growth inhibition in HepG$_2$ cells in a dose- and time-dependent manner. However, cinobufagin showed more significant cytotoxicity on HepG$_2$ cells than resibufogenin. At 24 h of drug treatment, the IC_{50} value of cinobufagin was 0.55 μg/ml, while that of resibufogenin was 6.9 μg/ml. Therefore, cinobufagin was selected for further investigation because of its significant cytotoxicity.

Table I. Cytotoxic activity of cinobufacini, chloroform extract and water residue against HepG$_2$ cells.

Extract	Cinobufacini	Chloroform extract	Water residue
Weight (mg)	13,089.3	**2,021.2**	11,068.1
IC_{50} (μg/ml)	1.6	**1.8x10^{-1}**	576.0

Table II. Cytotoxic activity of fractions I-IX from chloroform extract against HepG$_2$ cells.

Fraction	I	II	III	IV	V	VI	VII	VIII	IX
Weight (mg)	103.4	148.9	**171.3**	65.1	205.7	65.7	49.1	154.9	980.0
IC_{50} (μg/ml)	433.5	112.6	**5.8**	11.2	43.4	14.4	31.1	51.0	105.3

Table III. Cytotoxic activity of fractions 1-13 from fraction III against HepG$_2$ cells.

Fraction	Weight (mg)	IC_{50} (μg/ml)	Fraction	Weight (mg)	IC_{50} (μg/ml)
1	5.6	101.7	**8**	**42.0**	**5.0**
2	8.7	276.5	9	13.0	23.6
3	25.8	574.1	10	19.4	13.1
4	9.8	295.8	11	7.3	508.7
5	1.0	664.4	12	2.0	865.3
6	2.3	347.7	13	11.0	126.6
7	7.1	124.5	-		

Table IV. ^{13}C NMR and DEPT data for Compounds 1 and 2[a].

Carbon	Compound 1 (δ_C-DEPT)	Compound 2 (δ_C-DEPT)
1	29.4-CH_2	29.4-CH_2
2	27.9-CH_2	27.9-CH_2
3	66.8-CH	66.7-CH
4	33.3-CH_2	33.2-CH_2
5	36.0-CH	35.8-CH_0
6	25.8-CH_2	25.6-CH_2
7	20.7-CH_2	20.9-CH_2
8	33.5-CH	33.0-CH
9	39.3-CH	39.2-CH
10	35.5-CH_0	35.5-CH_0
11	21.1-CH_2	20.6-CH_2
12	39.4-CH_2	40.0-CH_2
13	45.3-CH_0	45.2-CH_0
14	74.7-CH_0	72.5-CH_0
15	59.8-CH	59.4-CH
16	32.4-CH_2	74.7-CH
17	47.8-CH	50.3-CH
18	16.9-CH_3	17.2-CH_3
19	23.8-CH_3	23.7-CH_3
20	122.2-CH_0	116.2-CH_0
21	149.5-CH	151.2-CH
22	147.0-CH	148.3-CH
23	115.3-CH	113.9-CH
24	162.0-CH_0	161.7-CH_0
$CH_3\underline{C}O$	-	170.2-CH_0
$\underline{C}H_3CO$	-	20.5-CH_3

[a]Recorded in chloroform-*d*.

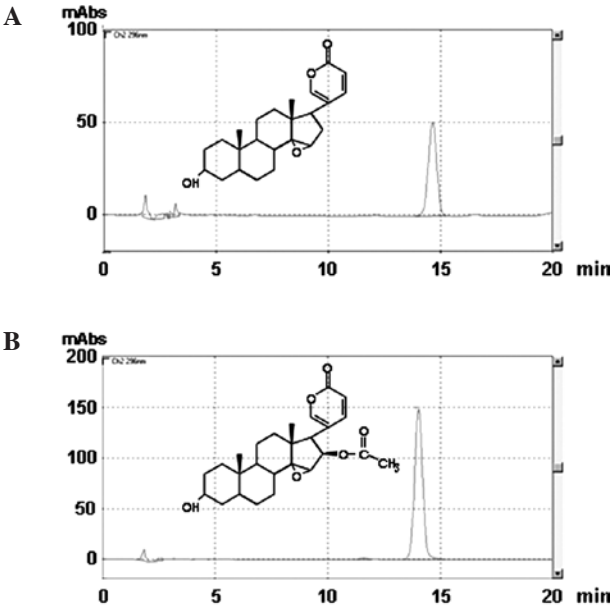

Figure 1. HPLC chromatogram of resibufogenin (A) and cinobufagin (B) from cinobufacini. HPLC conditions: COSMOSIL Cholester reversed-phase C-18 column; mobile phase, 45% acetonitrile; flow rate, 1 ml/min; detection wavelength, 296 nm. Samples were dissolved in methanol.

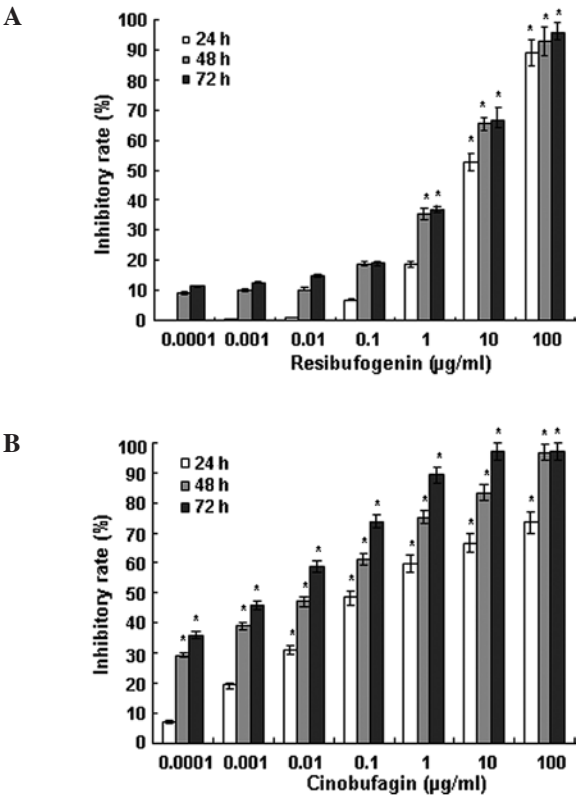

Figure 2. Time- and dose-dependent effects of resibufogenin (A) and cinobufagin (B) on the proliferation of $HepG_2$ cells. Cells were treated with various concentrations of regibufogenin or cinobutagin (0.0001-100 μg/ml) for 24, 48 and 72 h. The inhibition of cell proliferation was measured by the MTT assay. Each value is the mean ± SD of three determinations. *Significant inhibition ($P<0.05$) compared to the control.

Effects of cinobufagin on cell apoptosis. To investigate the effect of cinobufagin on the morphology of apoptotic cells, Hoechst 33258 staining was used. After treatment with 0.04 μg/ml cinobufagin for 24 h, marked morphological changes in chromatin morphology, such as crenation, condensation and fragmentation, were observed in $HepG_2$ cells (Fig. 3A). For further quantitative analysis of apoptotic cells, the population in the sub-G1 phase was analyzed, and the apoptotic cell population was estimated. As shown in Fig. 3B, cinobufagin treatment resulted in an increased accumulation of sub-G1 cells. After treatment with 0.04 μg/ml cinobufagin for 24 h, the apoptotic cell population of $HepG_2$ cells was 22.24%. These results suggested that cinobufagin markedly induces apoptosis in $HepG_2$ cells.

Effects of cinobufagin on the expression of apoptosis-related proteins Bax and Bcl-2. To clarify the mechanisms of apoptosis caused by cinobufagin, the protein expression of Bax and Bcl-2 was examined in $HepG_2$ cells after treatment with 0.04 μg/ml cinobufagin for 24 h. As shown in Fig. 4A, the expression of the pro-apoptotic protein Bax was up-regulated, while the expression of the anti-apoptotic protein Bcl-2 was down-regulated. The ratio of Bax/Bcl-2 was significantly increased in cinobufagin-treated cells compared to the control (Fig. 4B).

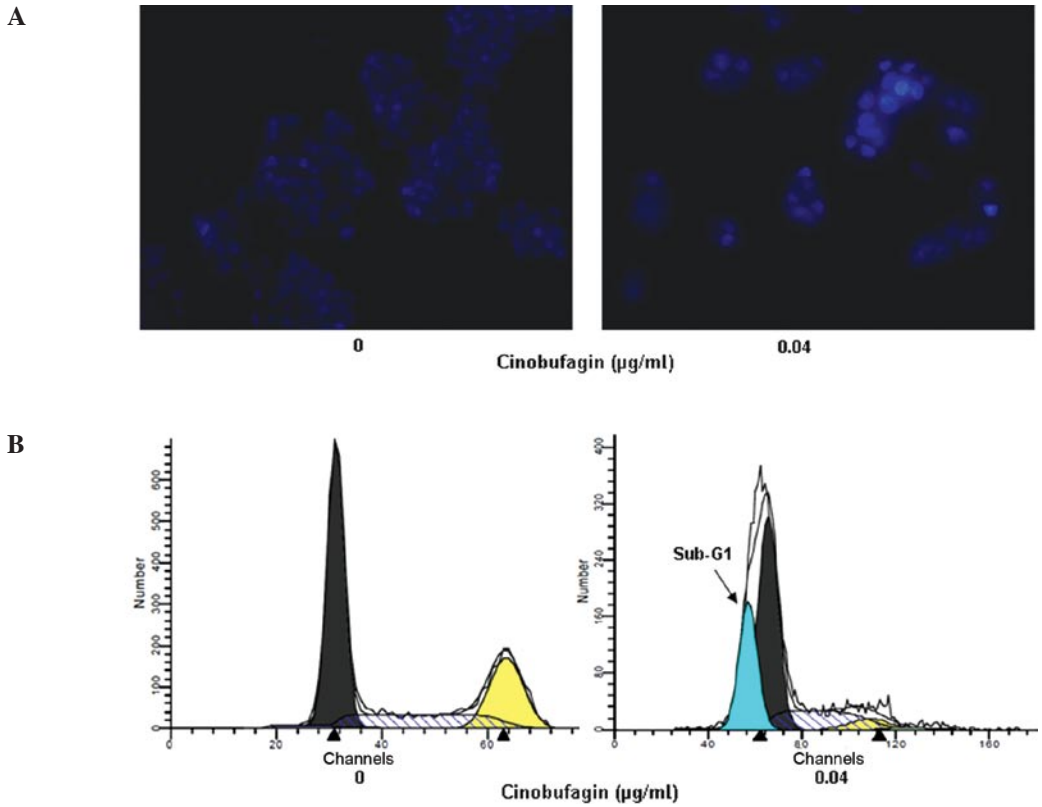

Figure 3. Effect of cinobufagin on apoptosis induction in HepG2 cells. (A) Effect of cinobufagin on the morphology of apoptotic cells. Hoechst 33258 staining was used. Original magnification, x200. (B) Quantitative analysis of apoptotic cells. The population in the sub-G1 phase was calculated by flow cytometry to estimate the apoptotic cell population. Cells were treated with 0 or 0.04 μg/ml cinobufagin for 24 h. The blue area represents the sub-G1 cells.

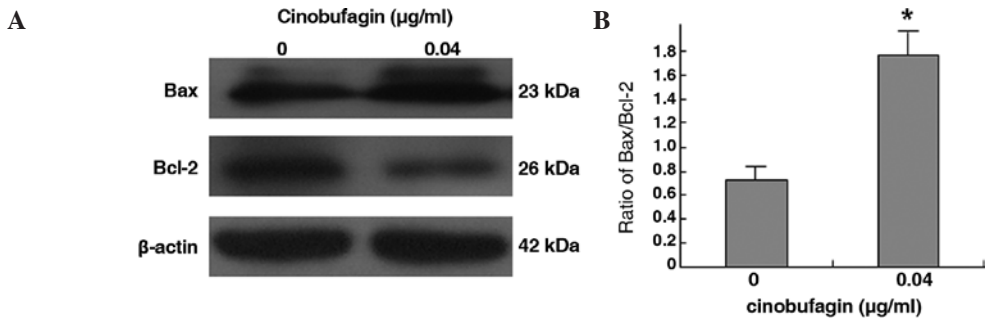

Figure 4. Effect of cinobufagin on the expression of Bax and Bcl-2 in $HepG_2$ cells. (A) Western blot analysis of the protein levels of Bax and Bcl-2. (B) The ratio of Bax/Bcl-2. Cells were treated with 0 or 0.04 μg/ml cinobufagin for 24 h. Each value is the mean ± SD of three determinations. *Significant inhibition ($P<0.05$) compared to the control.

Discussion

In this study, we aimed to isolate active compounds from cinobufacini, an extract used in traditional Chinese medicine. Cinobufacini compounds were primarily divided into two fractions through chloroform extraction. It was shown that, although the chloroform fraction contributed only 15.4% of the total amounts of cinobufacini, this less hydrophilic fraction exhibited much lower IC_{50} values than the aqueous fraction of cinobufacini in $HepG_2$ cells. These results suggest that the less hydrophilic compounds of cinobufacini may play the most important role in anti-proliferation in $HepG_2$ cells.

Through bioassay-guided isolation, two active compounds were isolated from the less hydrophilic ingredients of cinobufacini. These two active compounds were identified as resibufogenin and cinobufagin, which are members of the bufadienolide family. Bufadienolides, including resibufogenin, cinobufagin, bufalin, bufotalin and arenobufagin, are a type of steroidal cardiac glycosides (17) that exhibit a variety of biological effects, including cardiotonic, anesthetic and anti-neoplastic activities (18). Bufalin, as a major active compound of bufadienolide, has been demonstrated to possess significant anti-proliferative activities and apoptosis-inducing effects on human leukaemia, liver, prostatic, endometrial and ovarian cancer cells (19,20). In the present study, we found that another two bufadienolides, resibufogenin and cinobufagin (derived from cinobufacini), inhibit the proliferation of $HepG_2$ cells in a dose- and time-dependent manner. Cinobufagin induced a

more marked anti-proliferative effect than resibufogenin in the $HepG_2$ cells, indicating that cinobufagin was the more effective anti-cancer agent of the two, and that its mechanisms required further investigation.

Apoptosis, also called programmed cell death, plays an important role in the formation and evolution of tumors (21). The induction of apoptosis represents a major protective mechanism against cancer. The present study found that cinobufagin induces the apoptosis of $HepG_2$ cells. Marked morphological changes indicative of cell apoptosis were clearly observed when the cells were treated with 0.04 μg/ml cinobufagin for 24 h. Furthermore, according to flow cytometric analysis, the apoptotic cell population increased significantly after cinobufagin treatment.

Based on this finding, we further investigated the possible mechanisms of apoptosis induced by cinobufagin. Apoptosis is regulated by numerous apoptosis-related molecules including the Bcl-2 family (e.g., Bcl-2, Bcl-xl, Bax and Bad), p53, Fas and FasL (22). The Bcl-2 family plays a crucial role in the control of apoptosis and is mainly comprised of two functionally distinct groups: anti-apoptotic proteins, such as Bcl-2 and Bcl-xL, which protect cells from apoptosis, and pro-apoptotic proteins, such as Bax and Bad, which trigger apoptosis (23). It was found that decreased expression of Bcl-2 promotes apoptosis induced by diverse apoptotic stimuli, while increased expression of Bax induces apoptosis by suppressing the activity of Bcl-2. Thus, the ratio of Bax to Bcl-2, rather than Bcl-2 alone, is crucial to the survival of drug-induced apoptosis (23). Our study showed that cinobufagin markedly up-regulated the expression of Bax and down-regulated the expression of Bcl-2, eventually leading to an increase in the ratio of Bax/Bcl-2 levels. These results suggest that the up-regulation of Bax expression and the down-regulation of Bcl-2 expression may be one of the important apoptotic inducing mechanisms of cinobufagin.

In summary, we screened and identified two active anti-cancer compounds, resibufogenin and cinobufagin, of cinibufacini. These two compounds exhibited significant anti-proliferative activity against $HepG_2$ cells. Furthermore, cinobufagin was found to inhibit cell proliferation by inducing the apoptosis of $HepG_2$ cells. The up-regulation of Bax and down-regulation of Bcl-2 expression may be among the key mechanisms of apoptosis induction. However, further studies are necessary to clarify the underlying apoptotic signal pathways induced by cinobufagin and other major active compounds of cinobufacini.

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