ACTA ABP BIOCHIMICA POLONICA

Vol. 69, No 4/2022
831–837
https://doi.org/10.18388/abp.2020_6245

Regular paper

Cinobufagin inhibits proliferation and induces apoptosis of hepatocarcinoma cells by activating apoptosis, AKT, and ERK pathways

Wendong Feng[1*], Xiaoyan Zhao[2*], Qiang Yao[1] and Duqiang Li[1]✉

[1]Department of Interventional Therapy for Tumors and Vascular Diseases, Shanxi Bethune Hospital, Taiyuan, China; [2]Breast Surgery, Shanxi Maternity and Child Health Hospital, Taiyuan, China

Cinobufagin is one of the pharmaceutically active ingredients in the parotoid glands of the Chinese toad Bufo *bufo* gargarizans Cantor. This study was conducted to investigate the effect of cinobufagin on viability, migration, and apoptosis of hepatocellular carcinoma (HCC) cells and its mechanisms. Human HCC cells (HepG2) were treated with cinobufagin and assessed for viability, apoptosis, and migration using CCK-8 assay, flow cytometry, and wound healing assay. The expression of genes related to the p53, AKT, and ERK pathways was detected using RT-PCR and Western blot analysis. Cell viability assays showed that cinobufagin reduced the viability of HepG2 cells in a concentration- and time-dependent manner. Significantly increased apoptosis was detected in cinobufagin-treated cells as compared with non-treated cells. The migration ability of HepG2 cells was significantly reduced after they were exposed to cinobufagin as compared with control. RT-PCR and Western blot analyses showed that the expression levels of p53, caspase-3, and Bax were significantly upregulated, and the expression levels of AKT and ERK were significantly downregulated after cinobufagin treatment. Our data demonstrated that cinobufagin reduces the viability and induces apoptosis of HepG2 cells. The cytotoxicity is likely achieved by upregulating the p53 pathway and downregulating the Akt and ERK pathways.

Keywords: cinobufagin, viability, migration, apoptosis, hepatocellular carcinoma, p53 pathway

Received: 03 March, 2022; revised: 05 May, 2022; accepted: 25 May, 2022; available on-line: 10 October, 2022

✉e-mail: app3313@163.com
*Contributed equally to this work.

Abbreviations: AKT, protein kinase B; ERK, extracellular signal-regulated kinase; HCC,hepatocellular carcinoma; RT-PCR, Real-time quantitative reverse transcription polymerase chain reaction; OS, overall survival; JAK, Janus kinase; STAT, signal transducer and activator of transcription; MCF-7, Michigan cancer foundation-7; FGFR4, fibroblast growth factor receptor 4; FRS2α, FGF receptor substrate 2; NF-κB, nuclear factor kappa B; ATCC, American Type Collection Center; EMEM Eagle's minimum essential medium, EMEM; FBS, fetal bovine serum; EDTA, ethylenediaminetetraacetic acid; SDS-PAGE, sodium dodecyl-sulfate polyacrylamide gel electrophoresis; HRP, horseradish peroxidase; ECL, enhanced chemiluminescence; CCK8, cell counting kit 8; OD, optical density; PI, propidium iodide; TBS, tris-buffered saline; ANOVA, analysis of variance; LSD, fisher's least significant difference; LD50, lethal dose, 50%; VDAC, voltage-dependent anion channel; PUMA, p53 upregulated modulator of apoptosis; NOXA, phorbol-12-myristate-13-acetate-induced protein 1; OSCC, oral squamous cell carcinoma cells

INTRODUCTION

Hepatocellular carcinoma (HCC) is the seventh most common primary malignant tumor of the liver and the third major leading cause of cancer-related death (Sung *et al*., 2021). Due to the endemics of hepatitis virus, China has nearly 700000 new HCC cases every year, accounting for nearly 50% of newly diagnosed HCC worldwide (Ding & Wang, 2014; Maluccio & Covey, 2012). Although considerable advances have been made in the diagnosis and treatment of HCC, it is still a chronic disease with high morbidity and mortality (Massarweh & El-Serag, 2017; Villanueva, 2019). At present, surgery, intervention, radiotherapy, chemotherapy, and molecularly targeted therapy are being used to treat HCC patients. For patients with early-stage HCC, surgery is the main option. However, as an occult and rapidly progressing disease, most patients are already in the middle and advanced stages when they are diagnosed and are often too late to be eligible for radical surgery.

Chemotherapy and molecularly targeted therapy are important therapeutic means for these patients. While chemotherapeutic drugs are effective in killing tumor cells, they inevitably damage normal cells and lead to drug resistance and reduced efficacy (Hartke *et al*., 2017; Massarweh & El-Serag, 2017). For example, as the first-line drug, sorafenib prolongs the overall survival (OS) in HCC patients by 7.9 to 10.7 months (Cheng *et al*., 2009; Zhang *et al*., 2012). However, sustained use of sorafenib may trigger drug resistance in HCC cells due to several mechanisms such as abnormal activation of SHP-1 and inhibition of the JAK/STAT signaling pathway (Lachenmayer *et al*., 2012; Zhu *et al*., 2017). Therefore, it is very desirable to explore new chemotherapeutic drugs with low toxicity to normal cells and high efficacy or adjuvant drugs to reduce the cost of chemotherapeutic drugs and to improve overall therapeutic outcomes. For example, sinapine, an alkaloid derived from seeds of the cruciferous species, was found to be able to reverse multi-drug resistance in MCF-7/dox cancer cells by downregulating FGFR4/FRS2α-ERK1/2 pathway-mediated NF-κB activation (Guo *et al*., 2016) and inhibit the proliferation of HCC cells through activating p53 signaling pathway (Gao *et al*., 2021). Pelargonidin, a well-known natural anthocyanidin very commonly presented in berries, strawberries, blueberries, red radishes, and other natural foods has been found to have a variety of beneficial effects including anti-cancer activity due likely to its activation of the Nrf2-ARE signaling pathway and cytoprotective effect (Li *et al*., 2019).

Huachansu, an injectable preparation from the parotoid glands of the Chinese toad Bufo *bufo* gargarizans Cantor has been approved to treat liver, lung, colon, and pancreatic cancers in China by the Chinese Food and Drug Administration (Meng *et al*., 2009; Qi *et al*., 2010). Cinobufagin is a steroid and one of the active compounds in the preparation derived from the toad. Cinobufagin is shown to be able to suppress the growth of various cancers such as breast cancer (Zhu *et al*., 2018), osteosarcoma (Cao *et al*., 2017; Dai *et al*., 2018a), nasopharyngeal carcinoma (Pan *et al*., 2020), malignant melanoma (Pan *et al*., 2019) and colorectal cancer (Lu *et al*., 2017). Cell-based screening study using a natural product library containing 730 natural products also revealed that cinobufagin downregulates anoctamin1 (ANO1), a calcium-activated chloride channel that is frequently overexpressed in several cancers (Jo *et al*., 2021). However, little is known about its activity against HCC.

This study aimed to investigate the effect of cinobufagin on HCC cells and explore the possible molecular mechanisms underlying the activity. The antiproliferation activity of cinobufagin against HCC cells observed in this study would help develop new treatments for HCC and other cancers.

MATERIALS AND METHODS

Cell line

Human HCC cell line HepG2 (cat no. HB-8065) was purchased from American Type Collection Center (ATCC), Manassas, VA, USA and was cultured in Eagle's minimum essential medium (EMEM) (ATCC cat no. 20-2003) with 10% fetal bovine serum (FBS, (ATCC cat no. 30-2020) as recommended by the manufacturer. Cells were maintained at 37°C and in 5% CO_2 in a humidified incubator.

Reagents and equipment

Cinobufagin (5β,20(22)-bufadienolide-3β,16β-diol-14,15β-epoxy 16-acetate, 14,15β-Epoxy-3β,16β-dihydroxy-5β,20(22)-bufadienolide 16-acetate, cat no. SML3135) was purchased from Sigma-Aldrich, St. Luis, USA. Trypsin-EDTA (0.25%) (cat no. 25200072), Pierce BCA protein assay kit (cat no. 23225), Pierce SDS-PAGE sample prep kit (cat no. 89888), dead cell apoptosis kit with annexin v for flow cytometry (cat no. V13242), TRIzol reagents (cat no. 15596026), Verso cDNA synthesis kit (cat no. AB1453A), microplate reader Multiskan SkyHigh, NanoDrop 2000 spectrophotometer and RIPA buffer (cat no. J63306.AP) were purchased from Thermo Fisher Scientific, Wilmington, Delaware, USA; universal SYBR qPCR master mix (cat no. Q712-02) was obtained from Applied Biosystems Life Technologies, Carlsbad, CA, USA; CFX96 Touch Real-Time PCR detection system was purchased from Bio-Rad, USA. Antibodies against Bax (cat no. ab32503, 1:1000 dilution), p53 (cat no. ab26, 1:1200 dilution), caspase3 (cat no. ab32351, 1:1000 dilution), ERK (cat no. ab184699, 1:1500 dilution) and AKT (cat no. ab8805, 1:2000 dilution), horseradish peroxidase (HRP)-conjugated secondary antibody (cat no. ab6728, 1:1500), ECL kit (cat no. ab133406), and cell counting kit 8 (CCK8, cat no. ab228554) were purchased from Abcam, Waltham, MA, USA. FACSCalibur flow cytometer was a product of Becton Dickinson, USA.

Cell culture

HepG2 cells were cultured in EMEM containing 10% FBS at 37°C and 5% CO_2 in a humidified incubator. For subculture, 80% confluent cells were digested with 0.25% trypsin for 1 min, diluted 1:5 with fresh medium, and subcultured every 3 days.

Cell proliferation assay

Cell proliferation assays were performed using the CCK8 kit according to the manufacturer's instructions. Briefly, HepG2 cells in the logarithmic growth phase were digested with 0.25% trypsin and resuspended in EMEM containing 10% FBS. 100μl of the cell suspension (containing 4×10^4 cells/ml) was inoculated into the wells of 96 well plates, containing 0 (control), 10, 20, 40, 80, 160, and 320 ng/l cinobufagin. The plates were cultured for 12, 24 and 48 h and then added with 10 μl CCK8 reagent. After incubation for 2 h, optical density (OD) at 450 nm was determined using a microplate reader. The assays were independently repeated three times and all assays were performed in triplicate. Each data point was obtained with six measurements.

Wound healing assay

HepG2 cells (3×10^5) were seeded onto the slides and cultured in the wells of 24-well plates for 24 h at 37°C and 5% CO_2 in a humidified incubator to form a monolayer. The confluent monolayers were scratched with a p200 pipette tip and the cellular debris has been removed by washing gently. Following wounding, the culture medium was replaced with a fresh medium containing 100 ng/l cinobufagin and cells were cultured for another 12 h. Images were then acquired with a phase-contrast microscope Axio Obser (Carl Zeiss, Inc., Oberkochen, Germany) and gaps between scratched cells were measured before and after culture using Axio Vision (Carl Zeiss) to determine the migration capability. The assays were independently repeated three times and all assays were performed in triplicate. Each data point was obtained with six measurements.

Detection of apoptosis by flow cytometry

HepG2 cells were pretreated with 100 ng/l cinobufagin for 12 and 24 h, harvested and suspended in PBS.

Table 1. PCR primers

Gene		Primer sequence (5′ -3′)
Bax F	Forward	5′-GATGGCCTCCTTTCCTACTTC-3′
Bax R	Reverse	5′-CTTCTTCCAGATGGTGAGTGAG-3′
P53 F	Forward	5′-GAGGTTGGCTCTGACTGTACC-3′
P53 R	Reverse	5′-TCCGTCCCAGTAGATTACCAC-3′
β-actin F	Forward	5′-ACAGGATGCAGAAGGAGATTC-3
β-actin R	Reverse	5′-ACAGTGAGGCCAGGATAGA-3′
Caspase 3 F	Forward	5′-ACAGTGGAACTGACGATGATATG-3′
Caspase 3 R	Reverse	5′-TCCCTTGAATTTCTCCAGGAATAG-3′
AKT F	Forward	5′- TGGACTACCTGCACTCGGAGAA -3′
AKT R	Reverse	5′- GTGCCGCAAAAGGTCTTCATGG-3′
ERK F	Forward	5′- ACACCAACCTCTCGTACATCGG -3′
ERK R	Reverse	5′- TGGCAGTAGGTCTGGTGCTCAA -3′

Apoptosis was detected using a dead cell apoptosis kit (annexin V and propidium iodide (PI) staining) following the manufacturer's instructions on a FACSCalibur flow cytometer. The quantitation of apoptotic cells was calculated by CellQuest software.

Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

HepG2 cells were cultured in EMEM with 10% FBS and 100 ng/l cinobufagin for 24 h, harvested by centrifugation at 500×*g* for 10 min at room temperature. Total RNA was extracted from the cells using the TRIzol reagents according to the manufacturer's instructions. Extracted RNA was quantified using a Nanodrop spectrophotometer and reversely transcripted into cDNA using Verso cDNA synthesis kit according to the manufacturer's recommendations. qRT-PCR was run with universal SYBR qPCR Master Mix on CFX96 Touch Real-Time PCR Detection System using primers listed in Table 1. The PCR was carried out in a total volume of 15 µl containing 1 µl of diluted and pre-amplified cDNA and 10 µl of TaqMan gene expression master mix. The cycling conditions were 96ºC for 10 min followed by 40 cycles, each consisting of 15 s at 95ºC and 1 min at 57ºC. The relative mRNA levels were determined using the $2^{-\Delta\Delta Ct}$ method after normalization with β-actin as an internal reference (Livak & Schmittgen, 2001). All assays were performed in triplicate.

Western blotting

HepG2 cells were cultured in EMEM with 10% FBS and 100 ng/l cinobufagin for 24 h, harvested by centrifugation at 500×*g* for 10 min at room temperature. Cells (10^9) were lysed with RIPA buffer and proteins in the lysates were quantitated using a BCA protein assay kit according to the manufacturer's instructions. After denatured by boiling at 100ºC for 5 min, 60 µg protein per lane was subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were transferred to PVDF membranes, blocked with 5% non-fat dry milk in 1× TBS – 0.1% Tween 20 buffer for 6 h at room temperature. The blots were then incubated with primary antibodies against Bax, p53, caspase 3, AKT, and ERK at above-specified dilutions at 4°C overnight, rinsed three times with 1X TBS-0.1% Tween 20 buffer, and then with the (HRP)-conjugated secondary at 25°C for 2 h. Immunoreactive bands were visualized using the ECL kit as instructed by the supplier. Quantity One software (version v4.6.6; Bio-Rad Laboratories, Inc.) was used to measure the relative gray density of the bands using β-actin as the internal control. All assays were performed in triplicate. Each data point was obtained with three measurements.

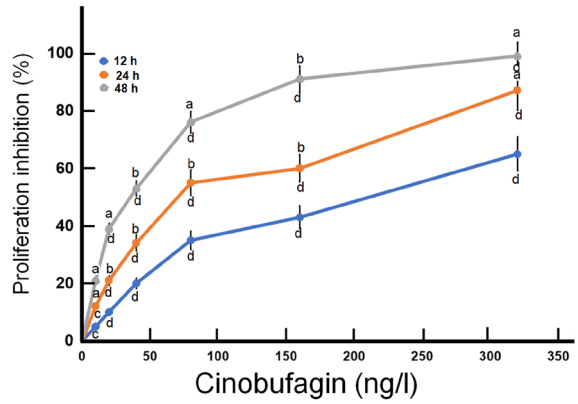

Figure 1. Inhibition of HepG2 proliferation after exposure to cinobufagin at various times.
HepG2 cells were cultured in EMEM containing 10% FBS and 0 to 320 ng/l cinobufagin for 12, 24 and 48 hours, and the cells were assessed for viability using commercial CCK8 kit from cell counting kit 8 from Abcam The assays were independently repeated three times and all assays were performed in triplicate. (a) and (b) denote $P<0.01$ and $P<0.001$ compared to 12-hour cinobufagin exposure. (c) and (d) denote $P<0.01$ and $P<0.001$ compared to 0 ng/l cinobufagin (control)

Statistical analysis

Statistical analysis was performed using SPSS 21.0 software. Measurement data are expressed as the mean ± standard error of the mean obtained from at least three independent experiments. One-way ANOVA with Tukey's post hoc tests was used for the comparison among groups, and the LSD t-test was used for the comparison between the two groups. The difference was considered statistically significant if $P<0.05$.

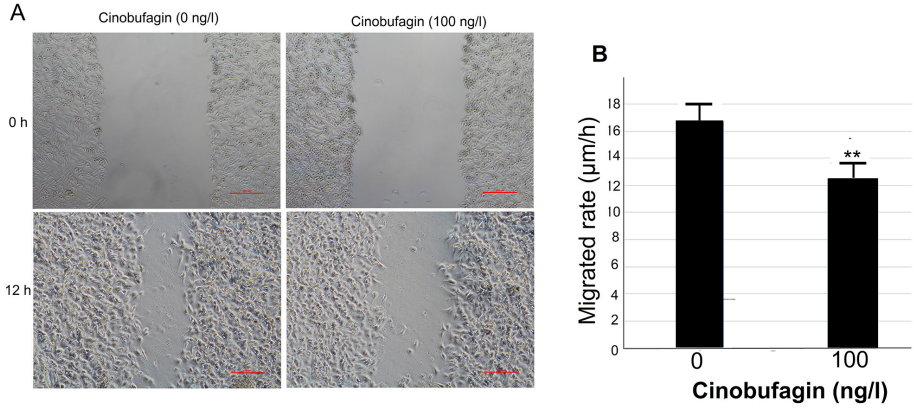

Figure 2. Wound healing assays of HepG2 proliferation after exposure to 100 ng/l cinobufagin for 12 hours.
HepG2 cells (3×10^5) were cultured for 24 h at 37°C and 5% CO_2 in a humidified incubator to form a monolayer. The confluent monolayers were scratched and cultured in fresh medium containing 100 ng/l cinobufagin for another 12 h. (**A**) microphotos of wound healing assays, (**B**) migration rate. The assays were independently repeated three times and all assays were performed in triplicate. $^{**}P<0.01$ compared to control (0 ng/l cinobufagin).

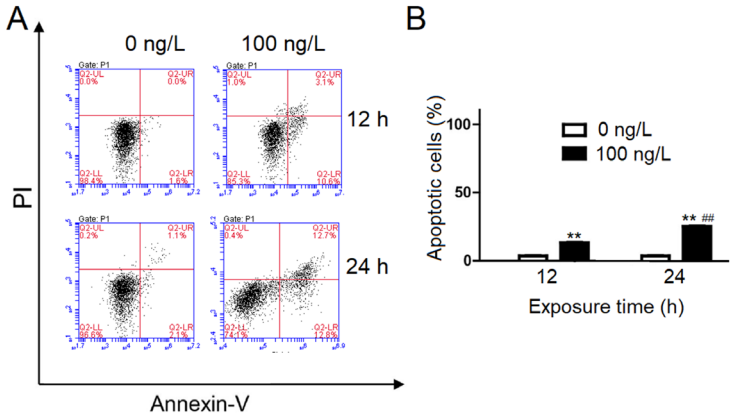

Figure 3. Apoptosis of HepG2 cells after exposure to cinobufagin for 12 and 24 hours.
HepG2 cells were pretreated with 100 ng/l cinobufagin, harvested, and stained with annexin V and propidium iodide (PI) using a commercial dead cell apoptosis kit from Thermo Fisher Scientific and analyzed on a FACSCalibur flow cytometer (Becton Dickinson, USA). (**A**) flow cytometry results, (**B**) apoptotic percentage. The assays were independently repeated three times and all assays were performed in triplicate. ** and ##P<0.01 *vs.* control and 12-hour exposure, respectively.

RESULTS

Cinobufagin inhibits the proliferation of HepG2 cells

To assess the effect of cinobufagin on the growth of HCC, HepG2 cells were cultured in EMEM medium containing various concentrations (0 to 320 ng/l) of cinobufagin for 12, 24 and 48 hours and assessed for cell viability using a CCK-8 kit. The results showed that when cinobufagin concentration increased from 0 to 320 ng/l, the inhibition of HepG2 cells increased from 0% to 65% at 12 hours, to 87% at 24 hours and to 99% at 48 hours (Fig. 1). The LD_{50} of cinobufagin was estimated to be 170, 78 and 40 ng/l, respectively, after the cells were exposed to cinobufagin for 12, 24 and 48 hours. These data indicated that cinobufagin inhibits the proliferation of HepG2 cells in a concentration- and time-dependent manner and has cytotoxicity to HepG2 cells even at very low concentrations (10 ng/l, Fig. 1)

Cinobufagin reduces the migration ability of HepG2 cells

Wound healing assays were then used to assess the migration capacity of HepG2 cells after exposure to cinobufagin. After being exposed to 100 ng/l cinobufagin for 12 hours, the migration rate of HepG2 cells was significantly reduced as compared to control (12.5 *vs* 16.7 μm/h, P<0.01, Fig. 2), suggesting that cinobufagin reduces the migration ability of HepG2 cells.

Cinobufagin induces apoptosis in HepG2 cells

Since cinobufagin reduced the viability of HepG2 cells, we investigated the apoptosis of HepG2 cells after exposure to cinobufagin for 12- and 24-hours using flow cytometry. The results showed after exposure to 100 ng/l cinobufagin for 12 hours, there was a significant increase in the percentage of apoptotic cells as compared to control (0 ng/l cinobufagin, 1.6% *vs* 13.6%) and the increase was more remarkable and highly statistically significant after 24 h exposure (3.2% *vs* 25.5%, P<0.01, Fig. 3).

Cinobufagin upregulates apoptosis pathways

To investigate the possible molecular mechanisms underlying the cytotoxicity, RT-PCR and Western blotting analysis were used to detect the expression of genes related to apoptosis pathways. The results showed that Bax, p53, and caspase 3 expressions were significantly up-regulated at both mRNA and protein levels after the HepG2 cells were exposed to 100 ng/l

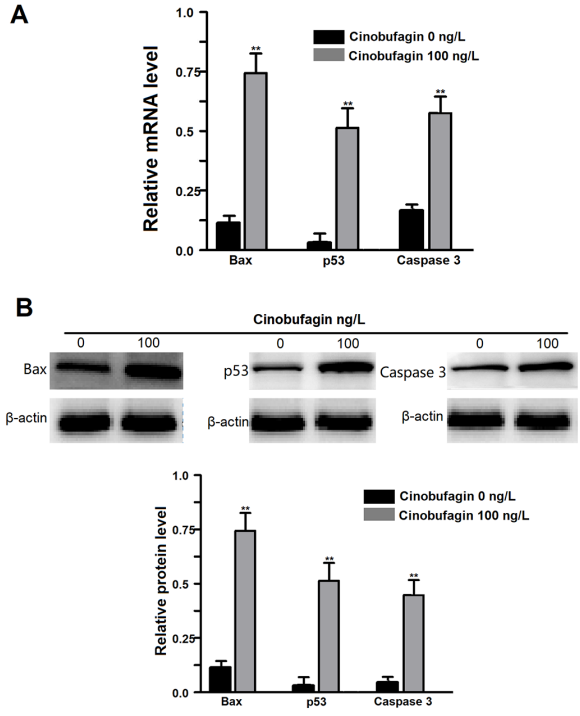

Figure 4. Expression of the Bax, p53 and caspase 3 genes in HepG2 cells after exposure to cinobufagin.
(**A**) HepG2 cells were cultured in EMEM with 10% FBS and 100 ng/l cinobufagin for 24 h, harvested and extracted for total RNA and protein with TRIzol reagents and RIPA buffer. Total RNAs were reversely transcribed to cDNA for quantification of mRNA in PCR. The proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and reacted with relevant antibodies after being transferred to PVDF membranes. The bands on the blots were visualized commercial ECL kit from Abcam and quantified using Quantity One software. A. relative mRNA level, (**B**) upper panel: representative Western blots, lower panel: relative protein level. The assays were repeated three times. **P<0.01 compared to control.

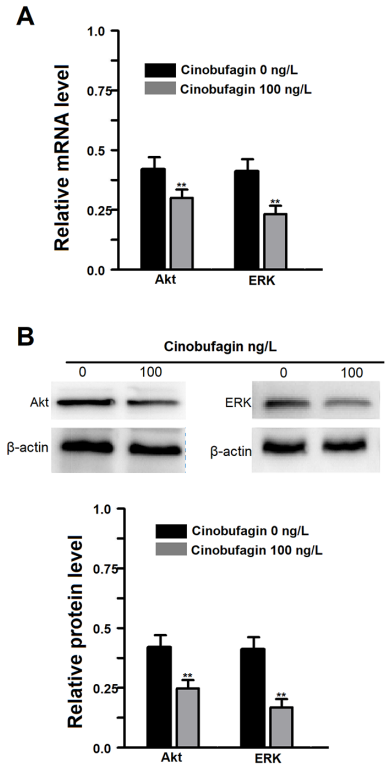

Figure 5. Expression of the Akt and ERK genes in HepG2 cells after exposure to cinobufagin.
HepG2 cells were cultured in EMEM with 10% FBS and 100 ng/l cinobufagin for 24 h, harvested and extracted for total RNA and protein with TRIzol reagents and RIPA buffer. Total RNAs were reversely transcribed to cDNA for quantification of mRNA in PCR. The proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and reacted with relevant antibodies after being transferred to PVDF membranes. The bands on the blots were visualized commercial ECL kit from Abcam and quantified using Quantity One software. (**A**) relative mRNA level, (**B**) upper panel: representative Western blots, lower panel: relative protein level. The assays were repeated three times. $^{**}P<0.01$ compared to control.

cinobufagin as compared to non-treated cells where these genes had a low level of expression (Fig. 4, $P<0.01$).

Cinobufagin downregulates Akt and ERK pathways

Wound healing assays showed that cinobufagin reduced the migration ability of HepG2 cells. We then profiled the expression of genes in the Akt and ERK pathways at both mRNA and protein levels. The result showed that Akt and ERK mRNA and proteins were significantly downregulated in the cells following exposure to 100 ng/l cinobufagin for 24 hours as compared to control (Fig. 5, $P<0.01$).

DISCUSSION

Cinobufagin is one of the major compounds present in an injection preparation Cinobufacini from the toad skin has antitumor activity. Our study showed that cinobufagin has potent cytotoxicity to HepG2 cells. It induces apoptosis in the cells and reduces the migration ability of HepG2 cells, upregulates the expression of apoptosis-related genes and downregulates the expression of migration-related genes in HepG2 cells.

Liver cancer is a common malignant tumor with complicated etiologies, including alcohol drink, water contamination, exposure to nitrosamines, and aflatoxins (Jackson & Groopman, 1999; Milligan *et al.*, 1990). Cancer progresses rapidly, leading to a short course of the disease and a short survival time for middle- and late-stage patients (Craig *et al.*, 2020; Jiang *et al.*, 2019). Liver cancer is less sensitive to chemotherapy, resulting in limited therapeutic effects. Targeted molecular therapy, while effective, is not applicable or available to many subtypes of HCC (Anwanwan *et al.*, 2020). Cinobufacini prepared from the skin of Chinese toad contains mainly cinobufagin, indole alkaloids and bufalin. It has been demonstrated to have antitumor activities against several tumors such as gastric lung cancer(Xiong *et al.*, 2018), colon cancer (Wang *et al.*, 2020) and osteosarcoma (Cao *et al.*, 2017; Dai *et al.*, 2018b). However, the molecular mechanisms underlying the antitumor activity against liver cancer are largely unclear. In this study, we investigated the effect and mechanisms of cinobufagin against liver cancer using HepG2 cells.

Previously, cinobufagin was shown to inhibit the growth of cancer cells but did not reduce the viability of noncancerous cells such as human L-O2 liver and NCM460 colon epithelial cell lines (Dai *et al.*, 2018b; Niu *et al.*, 2021), demonstrating that cinobufagin is selectively toxic to cancerous cells. Our study showed that the cytotoxicity of cinobufagin to HepG2 cells is concentration- and time-dependent, high concentration and long exposure generated more cytotoxicity, suggesting that cinobufagin might interact with HepG2 directly and indirectly. One of the mechanisms by which chemotherapy agents kill cancer is to trigger apoptosis in cancer cells, leading to reduced proliferation and death of cancer cells. To investigate if the reduced cell viability observed after cinobufagin exposure is due to apoptosis, we assessed apoptosis in cinobufagin-exposed HepG2 cells and found that there was a significant increase in apoptotic cells after cinobufagin exposure. This is consistent with earlier studies, in which cinobufagin was reported to induce apoptosis in osteosarcoma cells via the mitochondria-mediated apoptotic pathway with increased reactive oxygen species (ROS) production (Dai *et al.*, 2018c) and increased apoptosis in SGC-7901 human gastric cancer cells with suppressed autophagy and increased expression of Bax, cytosolic cytochrome c, cleaved PARP, caspase-3 and caspase-9 (Xiong *et al.*, 2019). To further confirm that the gene expressions in apoptosis pathways are altered following exposure to cinobufagin, we assessed the expression of several key genes such as Bax, p53 and caspase-3 involved in apoptosis pathways. These genes were found upregulated after the HepG2 cells were exposed to cinobufagin at both mRNA and protein levels, confirming that cinobufagin has an impact on apoptosis pathways. Since the expression changes occur at both mRNA and protein levels, it is likely that cinobufagin may interact directly or indirectly with DNA to regulate its expression. One possible mechanism is that cinobufagin binds to DNA to inactivate its transcription or to damage DNA to suppress gene expression (Jin *et al.*, 2020). It may also bind to the transcription factor SF-1 to suppress the expression of the StAR protein to inhibit the synthesis of aldosterone and cortisol to disturb cellular metabolism (Kau *et al.*, 2012). The expression of pro-apoptotic genes, caspase-3, Bax (the BCL-2-associated X apoptosis regulator) and p53 is upregulated after HepG2 cells were exposed to cinobufagin. These genes regulate apoptosis *via* various mechanisms. Increased cleaved caspase-3 would result in proteolysis and apoptosis (Choud-

hary *et al*., 2015; Crowley & Waterhouse, 2016), elevated Bax level increases the opening of the mitochondrial voltage-dependent anion channel (VDAC), leading to decreased mitochondrial membrane integrity and secretion of cytochrome *c* and cell death (Kuwana *et al*., 2020; Maes *et al*., 2019) and p53 activation may be leading to transcriptional activation of PUMA plus NOXA, and subsequent induction of apoptosis as observed in our experiments(Aubrey *et al*., 2018).

We also observed that cinobufagin reduces the migration ability of HepG2 cells. Previously, cinobufagin was found to inactivate the expression of metalloproteinases, leading to reduced migration of invasion of human gastric cancer cell line MGC-803 (Ni *et al*., 2018) and reduced cell proliferation and invasion of endometrial cancer cells (Zheng & Wang, 2017). RT-PCR and Western blot analysis showed that compared with the control, the expression of Akt and ERK was significantly downregulated, implying that cinobufagin may inactivate Akt and ERK signaling pathways, leading to reduce cell proliferation and migration. Recurrence and metastasis are the major obstacles to the successful treatment of HCC and many other cancers. Inhibition of cancer cell migration is an indication that cinobufagin would be able to inhibit the metastasis of HCC, likely through downregulating genes involved in the Akt and ERK signaling pathways, as well as relevant pathways.

Although increasing evidence indicates that cinobufagin is a potential natural product effective for liver cancer, its molecular targets remain unclear. Cinobufagin has been shown to target ANO1 to exert anticancer activity against oral squamous cell carcinoma cells (OSCC) (Jo *et al*., 2021). Since ANO1 is frequently overexpressed in various cancers such as breast cancer, head and neck squamous cell carcinoma, gastric cancer, and colorectal cancer (Zhang *et al*., 2012), it is proposed as a potential candidate for targeted anticancer therapy. Although cinobufagin was shown to downregulate the expression of ANO1 and reduce the growth of OSCC cells (Jo *et al*., 2021), it remains to be investigated if cinobufagin exerts anticancer activity in liver cancer *via* ANO1.

Taken together, our study demonstrated that cinobufagin inhibits the growth and reduces the migration of HepG2 cells. It causes apoptosis in the cells with upregulated expression of pro-apoptotic genes and downregulated expression of migration-related genes. Further studies, including in vivo study, are needed to further define the role of cinobufagin as an anticancer agent.

Declarations

Availability of data and material: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests: none.

Funding: none.

Authors' contributions: WF, XZ, and DL: Project conceptualization. WF, XZ and QY: data collection, analysis, and methodology development. All authors wrote and approved the final version of the manuscript.

Acknowledge: Not applicable.

REFERENCES

Anwanwan D, Singh SK, Singh S, Saikam V, Singh R (2020) Challenges in liver cancer and possible treatment approaches. *Biochim Biophys Acta Rev Cancer* **1873**: 188314. https://doi.org/10.1016/j.bbcan.2019.188314

Aubrey BJ, Kelly GL, Janic A, Herold MJ, Strasser A (2018) How does p53 induce apoptosis and how does this relate to p53-mediated tumour suppression? *Cell Death Differ* **25**: 104–113. https://doi.org/10.1038/cdd.2017.169

Cao Y, Yu L, Dai G, Zhang S, Zhang Z, Gao T, Guo W (2017) Cinobufagin induces apoptosis of osteosarcoma cells through inactivation of Notch signaling. *Eur J Pharmacol* **794**: 77–84. https://doi.org/10.1016/j.ejphar.2016.11.016

Cheng AL, Kang YK, Chen Z, Tsao CJ, Qin S, Kim JS, Luo R, Feng J, Ye S, Yang TS, Xu J, Sun Y, Liang H, Liu J, Wang J, Tak WY, Pan H, Burock K, Zou J, Voliotis D, Guan Z (2009) Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomised, double-blind, placebo-controlled trial. *Lancet Oncol* **10**: 25–34. https://doi.org/10.1016/S1470-2045(08)70285-7

Choudhary GS, Al-Harbi S, Almasan A (2015) Caspase-3 activation is a critical determinant of genotoxic stress-induced apoptosis. *Methods Mol Biol* **1219**: 1–9. https://doi.org/10.1007/978-1-4939-1661-0_1

Craig AJ, von Felden J, Garcia-Lezana T, Sarcognato S, Villanueva A (2020) Tumour evolution in hepatocellular carcinoma. *Nat Rev Gastroenterol Hepatol* **17**: 139–152. https://doi.org/10.1038/s41575-019-0229-4

Crowley LC, Waterhouse NJ (2016) Detecting cleaved caspase-3 in apoptotic cells by flow cytometry. *Cold Spring Harb Protoc* **2016**: https://doi.org/10.1101/pdb.prot087312

Dai G, Zheng D, Guo W, Yang J, Cheng AY (2018a) Cinobufagin induces apoptosis in osteosarcoma cells *via* the mitochondria-mediated apoptotic pathway. *Cell Physiol Biochem* **46**: 1134–1147. https://doi.org/10.1159/000488842

Dai G, Zheng D, Guo W, Yang J, Cheng AY (2018b) Cinobufagin induces apoptosis in osteosarcoma cells *via* the mitochondria-mediated apoptotic pathway. *Cell Physiol Biochem* **46**: 1134–1147. https://doi.org/10.1159/000488842

Ding J, Wang H (2014) Multiple interactive factors in hepatocarcinogenesis. *Cancer Lett* **346**: 17–23. https://doi.org/10.1016/j.canlet.2013.12.024

Gao Y, Li H, Han D (2021) Study on effect of sinapine on proliferation and apoptosis of hepatocarcinoma cells and its mechanism. *Chinese J Immunol* **23**: 2845–2849. https://doi.org/10. 3969/j.issn.1000-484X

Guo Y, Ding Y, Zhang T, An H (2016) Sinapine reverses multidrug resistance in MCF-7/dox cancer cells by downregulating FGFR4/FRS2alpha-ERK1/2 pathway-mediated NF-kappaB activation. *Phytomedicine* **23**: 267–273. https://doi.org/10.1016/j.phymed.2015.12.017

Hartke J, Johnson M, Ghabril M (2017) The diagnosis and treatment of hepatocellular carcinoma. *Semin Diagn Pathol* **34**: 153–159. https://doi.org/10.1053/j.semdp.2016.12.011

Jackson PE, Groopman JD (1999) Aflatoxin and liver cancer. *Baillieres Best Pract Res Clin Gastroenterol* **13**: 545–555. https://doi.org/10.1053/bega.1999.0047

Jiang Y, Han QJ, Zhang J (2019) Hepatocellular carcinoma: Mechanisms of progression and immunotherapy. *World J Gastroenterol* **25**: 3151–3167. https://doi.org/10.3748/wjg.v25.i25.3151

Jin X, Wang J, Zou S, Xu R, Cao J, Zhang Y, Guo J, Wen X, Deng S, Zheng Y, Zhu Y, Wang F, Xu Z (2020) Cinobufagin triggers defects in spindle formation and cap-dependent translation in liver cancer cells by inhibiting the AURKA-mTOR-eIF4E axis. *Am J Chin Med* **48**: 651–678. https://doi.org/10.1142/S0192415X20500330

Jo S, Yang E, Lee Y, Jeon D, Namkung W (2021) Cinobufagin exerts anticancer activity in oral squamous cell carcinoma cells through downregulation of ANO1. *Int J Mol Sci* **22**: https://doi.org/10.3390/ijms222112037

Kau MM, Wang JR, Tsai SC, Yu CH, Wang PS (2012) Inhibitory effect of bufalin and cinobufagin on steroidogenesis *via* the activation of ERK in human adrenocortical cells. *Br J Pharmacol* **165**: 1868–1876. https://doi.org/10.1111/j.1476-5381.2011.01671.x

Kuwana T, King LE, Cosentino K, Suess J, Garcia-Saez AJ, Gilmore AP, Newmeyer DD (2020) Mitochondrial residence of the apoptosis inducer BAX is more important than BAX oligomerization in promoting membrane permeabilization. *J Biol Chem* **295**: 1623–1636. https://doi.org/10.1074/jbc.RA119.011635

Lachenmayer A, Toffanin S, Cabellos L, Alsinet C, Hoshida Y, Villanueva A, Minguez B, Tsai HW, Ward SC, Thung S, Friedman SL, Llovet JM (2012) Combination therapy for hepatocellular carcinoma: additive preclinical efficacy of the HDAC inhibitor panobinostat with sorafenib. *J Hepatol* **56**: 1343–1350. https://doi.org/10.1016/j.jhep.2012.01.009

Li S, Li W, Wang C, Wu R, Yin R, Kuo HC, Wang L, Kong AN (2019) Pelargonidin reduces the TPA induced transformation of mouse epidermal cells -potential involvement of Nrf2 promoter demethylation. *Chem Biol Interact* **309**: 108701. https://doi.org/10.1016/j.cbi.2019.06.014

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta C(T)) Method. *Methods* **25**: 402–408. https://doi.org/10.1006/meth.2001.1262

Lu XS, Qiao YB, Li Y, Yang B, Chen MB, Xing CG (2017) Preclinical study of cinobufagin as a promising anti-colorectal cancer agent. *Oncotarget* **8**: 988–998. https://doi.org/10.18632/oncotarget.13519

Maes ME, Grosser JA, Fehrman RL, Schlamp CL, Nickells RW (2019) Completion of BAX recruitment correlates with mitochondrial fission during apoptosis. *Sci Rep* **9**: 16565. https://doi.org/10.1038/s41598-019-53049-w

Maluccio M, Covey A (2012) Recent progress in understanding, diagnosing, and treating hepatocellular carcinoma. *CA Cancer J Clin* **62**: 394–399. https://doi.org/10.3322/caac.21161

Massarweh NN, El-Serag HB (2017) Epidemiology of hepatocellular carcinoma and intrahepatic cholangiocarcinoma. *Cancer Control* **24**: 1073274817729245. https://doi.org/10.1177/1073274817729245

Meng Z, Yang P, Shen Y, Bei W, Zhang Y, Ge Y, Newman RA, Cohen L, Liu L, Thornton B, Chang DZ, Liao Z, Kurzrock R (2009) Pilot study of huachansu in patients with hepatocellular carcinoma, nonsmall-cell lung cancer, or pancreatic cancer. *Cancer* **115**: 5309–5318. https://doi.org/10.1002/cncr.24602

Milligan RA, Whittaker M, Safer D (1990) Molecular structure of F-actin and location of surface binding sites. *Nature* **348**: 217–221. https://doi.org/10.1038/348217a0

Ni T, Li D, Wang H (2018) Cinobufagin inhibits invasion and migration of human gastric adenocarcinoma cell line MGC-803 by regulating matrix metalloproteinases. *Chinese J Exp Prescript* **24**: 106–111.

Niu J, Wang J, Zhang Q, Zou Z, Ding Y (2021) Cinobufagin-induced DNA damage response activates G2/M checkpoint and apoptosis to cause selective cytotoxicity in cancer cells. *Cancer Cell Int* **21**: 446. https://doi.org/10.1186/s12935-021-02150-0

Pan Z, Zhang X, Yu P, Chen X, Lu P, Li M, Liu X, Li Z, Wei F, Wang K, Zheng Q, Li D (2019) Cinobufagin induces cell cycle arrest at the G2/M phase and promotes apoptosis in malignant melanoma cells. *Front Oncol* **9**: 853. https://doi.org/10.3389/fonc.2019.00853

Pan Z, Luo Y, Xia Y, Zhang X, Qin Y, Liu W, Li M, Liu X, Zheng Q, Li D (2020) Cinobufagin induces cell cycle arrest at the S phase and promotes apoptosis in nasopharyngeal carcinoma cells. *Biomed Pharmacother* **122**: 109763. https://doi.org/10.1016/j.biopha.2019.109763

Qi F, Li A, Zhao L, Xu H, Inagaki Y, Wang D, Cui X, Gao B, Kokudo N, Nakata M, Tang W (2010) Cinobufacini, an aqueous extract from Bufo bufo gargarizans Cantor, induces apoptosis through a mitochondria-mediated pathway in human hepatocellular carcinoma cells. *J Ethnopharmacol* **128**: 654–661. https://doi.org/10.1016/j.jep.2010.02.022

Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, Bray F (2021) Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J Clin* **71**: 209–249. https://doi.org/10.3322/caac.21660

Villanueva A (2019) Hepatocellular carcinoma. *N Engl J Med* **380**: 1450–1462. https://doi.org/10.1056/NEJMra1713263

Wang X, Zeng H, Hu W (2020) Cinobufagin alleviate bone cancer pain *via* inhibiting CCL2/CCR2 pathway in spinal cord and activating microglia. *Acta Pharm Sinica* **55**: 67–73 (in Chinese). https://doi.org/10.16438/j.0513-4870.2019-0479

Xiong F, Shi Y, Shen J, Cheng H, Hang Y (2018) Inhibitory effect and mechanism of cinobufagin on NCI-A549 transplanted lung cancer in nude mice. *Chinese J Clin Pharmacol Therap* **23**: 1103–1108. (in Chinese) https://doi.org/10.12092/j.issn.1009-2501.2018.10.004

Xiong X, Lu B, Tian Q, Zhang H, Wu M, Guo H, Zhang Q, Li X, Zhou T, Wang Y (2019) Inhibition of autophagy enhances cinobufagininduced apoptosis in gastric cancer. *Oncol Rep* **41**: 492–500. https://doi.org/10.3892/or.2018.6837

Zhang X, Yang XR, Huang XW, Wang WM, Shi RY, Xu Y, Wang Z, Qiu SJ, Fan J, Zhou J (2012) Sorafenib in treatment of patients with advanced hepatocellular carcinoma: a systematic review. *Hepatobiliary Pancreat Dis Int* **11**: 458–466. https://doi.org/10.1016/s1499-3872(12)60209-4

Zheng J, Wang X (2017) Effect of cinobufagin on biological behavior of two endometrial cancer cells. *J Shizhen Med* **28**: 2380–2382.

Zhu L, Chen Y, Wei C, Yang X, Cheng J, Yang Z, Chen C, Ji Z (2018) Anti-proliferative and pro-apoptotic effects of cinobufagin on human breast cancer MCF-7 cells and its molecular mechanism. *Nat Prod Res* **32**: 493–497. https://doi.org/10.1080/14786419.2017.1315575

Zhu YJ, Zheng B, Wang HY, Chen L (2017) New knowledge of the mechanisms of sorafenib resistance in liver cancer. *Acta Pharmacol Sin* **38**: 614–622. https://doi.org/10.1038/aps.2017.5