博士論文

Traditional Chinese medicine and its active compounds perform inhibitory effect and synergic effect with chemotherapeutics in hepatocellular carcinoma

（漢方薬とその有効成分の肝細胞がんに対する抑制効果及び化学療法併用によるシナジー効果）

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Abstract

Background and Aim: Cinobufacini, a traditional Chinese medicine, has been used widely for cancer treatment, such as hepatocellular carcinoma (HCC), sarcoma, and leukemia. In recent clinical practice, traditional Chinese medicine always was used as a complementary medicine to some chemotherapeutics. As a classical chemotherapeutic, doxorubicin was used in a number of combinations with various kinds of materials, such as traditional Chinese medicine, active compounds, nanoparticles, and other chemotherapeutics. Thus, it was wondered whether the combination of cinobufacini and doxorubicin could achieve better antitumor effect. In addition, there have been reports about antitumor effect of cinobufacini in clinical practice, but the cell functional effect, contained active compounds, and molecular mechanisms of it were not unveiled. Here, we planned to use a series of experiments to isolate active compounds, and illuminate molecular mechanisms and combination therapeutic effects *in vitro* and *in vivo*.

Methods: Firstly, we employed thin-layer chromatography, silica-gel chromatography, and high performance liquid chromatography (HPLC) to isolate active compounds from cinobufacini. Then, we used MTT assay, Hoechst 33258 staining, MitoCapture assay, real-time PCR, and western blot to identify the cell functional activity,

molecular mechanisms, and combination therapeutic effect of cinobufacini and its active compounds. At last, animal experiment was employed to testify the combination therapeutic effect *in vivo*.

Results: Cinobufacini contains two major active compounds, bufalin and cinobufagin. Cinobufacini and its active compounds could induce apoptosis of HCC cells through activating mitochondrial and Fas signaling pathways. The combination of cinobufacini and doxorubicin induced more significant apoptosis by affecting both proteins and RNA of apoptosis-related elements, such as Bcl-2, Bax, Bid, and cytochrome c. Furthermore, cinobufacini, as a mixture of a number of components, had stronger apoptosis-inducing activity than single component or simple mixture of components. And the combination therapeutic effect was proved to be effective both *in vitro* and *in vivo*.

Conclusion: Overall, these results suggested that cinobufacini and its active compounds, bufalin and cinobufagin, could induce apoptosis of HCC cells by activating mitochondrial- and Fas-related apoptotic signaling pathways and combination of cinobufacini and doxorubicin may provide a new strategy to inhibit the proliferation of HCC cells.

1. Introduction

Hepatocellular carcinoma (HCC) is one of the major cancers worldwide, especially in East Asia. Although chemotherapy is one of the major conventional HCC therapies, strong side effects and emergence of drug resistance are gradually becoming serious problems (1).

For thousands of years, traditional Chinese medicine (TCM) and other herbal medicines have been used to fight against various diseases in China, Japan, Korea and other Asian countries (2-6). They are also widely adopted for therapy of cancers because of their advantages of high efficiency, weak side effects, easy availability, and improvement of quality of life (7-10). Recently, in America and Europe, herbal medicines are widely accepted as a form of complementary and alternative medicine (CAM) (11,12).

Cinobufacini (Hua-chan-su) is a TCM composed of the water soluble components from the post-auricular and skin glands of *Bufo bufo gargarizans* Cantor (13-15). Cinobufacini composes a variety of bufadienolide cardiotonic steroids, such as bufalin, cinobufagin, resibufogenin, and telocinobufagin, which are the major active components (16-19). Quantitative analysis showed that the concentrations of bufalin and cinobufagin in cinobufacini are 12.84 μg/mL and 6.13 μg/mL, respectively (20). A number of clinical and experimental studies on cinobufacini have been carried out worldwide to research its anticancer properties and potential mechanisms. In 2005,

cinobufacini was approved by the Chinese State Food and Drug Administration (SFDA) and then wildly accepted to treat patients with cancers at oncologic clinics in China (21-24). Clinical studies have suggested that cinobufacini used alone or in combination with other chemotherapeutic agents had significant activity against cancers, such as HCC, pancreatic cancer, non small cell lung cancer, and gallbladder carcinoma (21,23). Experimental studies have illuminated that cinobufacini has a significant apoptosis-inducing effect on a number of cancer cells. (14). Previous studies by our laboratory suggested that cinobufacini lead to apoptosis of human HCC cells in a dose-dependent manner through the mitochondria-mediated and Fas-mediated pathway with up-regulation of Bax and Cytochrome C and down-regulation of Bcl-2 and Bid. Studies by the same author indicated that bufalin and cinobufagin, which are suggested as active components of cinobufacini, also own the ability to induce apoptosis *via* the mitochondria-mediated and Fas-mediated pathway (25-29). For the complicated mechanisms of traditional medicine, they were usually used as a constituent part of combination therapy but not monotherapy (30). However, there were no studies on antitumor effects of combination therapy of cinobufacini and other chemotherapy drugs by our or other laboratories.

Doxorubicin, a kind of anthracycline antibiotic, is a drug used in cancer chemotherapy. It works by intercalating DNA and induces apoptosis. Doxorubicin has a few adverse effects. The most dangerous side effect of doxorubicin is cardiomyopathy, leading to congestive heart failure (31). Another common and

potentially fatal complication of doxorubicin is typhlitis, an acute life-threatening infection of the bowel (32). Additionally, some patients may develop palmar-plantar erytherodysesthesia, characterized by skin eruptions on the palms of the hand or soles of the feet, swelling, pain and erythema (33). To decrease its side effects, it was reported to be used in combination chemotherapy with various TCMs, such as with *Boswellia serrata* extract in hepatocellular carcinoma, with diterpenes from *Euphorbia piscatoria* in gastric cancer, and with *Eupatorium cannabinum L.* ethanolic extract in colon cancer (34-37). Doxorubicin has determined functional mechanism and side effects, is appropriate for doxorubicin-resistant cell line such as HepG2, and has been widely used as a partner in the study on combination therapy. Thus, we wonder whether a combination of doxorubicin and cinobufacini can obtain a better treatment result than monotherapy.

2. Materials and methods

2.1. Reagents

High glucose Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco/BRL (Gaithersburg, MD, USA). Cell Proliferation Kit I was purchased from Roche Applied Science (Mannheim, Germany). Primary antibody of β-actin was purchased from Sigma (St. Louis, MO, USA). RIPA Lysis Buffer, primary antibodies of Bax, Bcl-2, cytochrome c, Bid, and horseradish peroxidase (HRP)-labeled secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Amersham ECL Western blotting detection reagents and analysis system was purchased from GE Healthcare (Buckinghampshire, UK). Cinobufacini, an aqueous extract from the skins B. gargarizans Cantor, was obtained from Anhui Jinchan Biochemical Co., Ltd. (Anhui, China). Bufalin and cinobufagin both were purchased from Sigma (St. Louis, MO, USA).

2.2. 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.

2.3. 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 HepG2 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 ^{1}H 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.

2.4. Cell lines and maintenance

HepG2 and HLE were maintained in DMEM medium supplemented with 10% fetal bovine serum, 100 unit/mL penicillin, 100 mg/ml streptomycin at 37 ℃ in a humidified incubator with 5% CO2.

2.5. Cell viability

Cells were seeded in 96-well plates at a density of 2×10^4 per well and treated with reagents for 24 hours, 48 hours, and 72 hours. The cytotoxicity of different drugs was analyzed with an MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide] assay using Cell Proliferation Kit I following the manufacturer's instructions. Each experiment was performed in triplicate. Cell viability was expressed as a percentage of the control.

2.6. Hoechst 33258 staining

Hoechst 33258 staining (Dojindo Laboratories, Kumamoto, Japan) was used to observe the morphology of apoptotic cells. Cells (2×10^5 cells/mL) were seeded in 12-well plates and incubated for 24 h. After incubation with 0.1 mg/mL cinobufagin for another 24 h, the cells were fixed with 4% formaldehyde in phosphate buffered saline (PBS) for 20 min and stained with Hoechst 33258 solution at room temperature for 15 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.

2.7. Combination index

The Chou-Talalay method for drug combination is based on the median-effect equation, derived from the mass-action law principle, which is the unified theory that provides the common link between single entity and multiple entities, and first order and higher order dynamics. This general equation encompasses the Michaelis-Menten, Hill, Henderson-Hasselbalch, and Scatchard equations in biochemistry and biophysics. The resulting combination index (CI) theorem of Chou-Talalay offers quantitative definition for additive effect (CI = 1), synergism (CI < 1), and antagonism (CI > 1) in drug combinations. The results were obtained by CompuSyn software which could be downloaded from CompuSyn homepage freely.

2.8. Measurement of mitochondrial membrane potential

Mitochondrial membrane potential was measured by MitoCapture™ Mitochondrial Apoptosis Detection Kit (Enzolifescience, New York, USA). In normal cells, MitoCapture accumulates and aggregates in the mitochondria, giving off a bright red fluorescence, whereas in apoptotic cells MitoCapture cannot aggregate in the mitochondria due to the altered mitochondrial transmembrane potential, and thus it remains in the cytoplasm in its monomer form, fluorescing green. The relative ratio of green/red fluorescence intensity is usually used for quantitative analysis of the

disruption of mitochondrial membrane potential. According to the manufacturer's protocols, cells were seeded in 12-well plates at a density of 2×10^5 cells/mL. After the cells were treated with 0.1 mg/mL cinobufacini for 24h, 200 μL pre-warmed incubation buffer containing 0.2 μL MitoCapture was added to each well and cells were incubated at 37℃ in a 5% CO_2 incubator for 15 min. Afterwards, cells were washed with PBS and observed directly under fluorescence microscopy.

2.9. Quantification of RNA level by real-time PCR

Total RNA was extracted from cells by Trizol reagent (Invitrogen) in accordance with the manufacturer's instructions. The mRNA was then reverse-transcribed to make cDNA by a Reverse Transcription System (Promega, Madison, USA) and oligo dT primer following the manufacturer's instructions. The cDNA was quantified using the StepOneTM Real-Time PCR System (Applied Biosystems, USA). The polymerase chain reaction (PCR) was performed using primers (synthesized by Invitrogen) shown in Table 1. GAPDH was employed as a positive control. FastStart Universal SYBR Green Master (Rox) (Roche) was used to amplify and detect DNA during the reaction. Thermal cycling parameters for the target genes and GAPDH consisted of a hot start for 2 min at 94 ℃ followed by 40 cycles of 94 ℃ for 15 s, 60 ℃ for 30 s, and then 72 ℃ for 30 s. Specificity of the PCR products was verified by melting curve analysis.

Table 1. Primers for RNA quantification

Targets	Sequences (5′-3′)	Number of Bases
Bcl-2 forward	ATGTGTGTGGAGAGCGTCAA	20
Bcl-2 reverse	TTCAGAGACAGCCAGGAGAAA	21
Bax forward	TTTGCTTCAGGGTTTCATCC	20
Bax reverse	CAGTTGAAGTTGCCGTCAGA	20
Bid forward	TCCCGCTTGGGAAGAATAG	19
Bid reverse	CCAGCATGGTCTTCTCCTTC	20
Cytochrome C forward	GTCAGGCCCCTGGATACTCT	20
Cytochrome C reverse	TCTGCCCTTTCTTCCTTCTTC	21
GAPDH forward	AGGTGAAGGTCGGAGTCAAC	20
GAPDH reverse	AGTTGAGGTCAATGAAGGGG	20

2.10. Western blot analysis

After treatment with reagents for 24 h, the harvested cells were lysed on ice for 30 min in RIPA Lysis Buffer. The lysates were centrifuged at 10,000g for 10 min at 4 ℃ and the supernatant was colleted. Protein concentrations of 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–PAGE gels and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were first incubated in blocking solution (5% skim milk) for 1 h and then incubated with desired primary antibodies for 2 h at room temperature or overnight at 4 ℃. After they were washed with TBST (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 1 h and then washed with TBST three more times. Finally, protein bands were visualized by ECL (enhanced chemiluminescence) detection system.

2.11. Detection of caspase activity

Following the protocols of the Caspase Colorimetric Assay Kit, the activity of caspase-3, caspase-8, caspase-9, and caspase-10 were detected by cleavage of chromogenic caspase substrates. Briefly, cells were treated with reagents of each group for 24 h and then harvested and lysed in lysis buffer for 10 min on ice. The lysed cells were centrifuged at 10,000×g for 10 min, and 100μg proteins were incubated with DEVD-pNA (4mM), IETD-pNA (4 mM), LEHD-pNA (4mM) or AEVD-pNA (4 mM) at 37°C for 2 h. Then, the pNA light emission was quantified using a micro plate reader at 405 nm.

2.12. Animals and treatment

Male nude BALB/c nude mice (4-6 weeks old, 18-22 g) (n = 20) were obtained from the Chinese Academy of Sciences. Sub-confluent cultures of HepG2 cells are centrifuged and washed with PBS to prepare to a concentration of 2.5×10^7 cells/mL. And then the cells are stored on ice for injection. Each nude mouse is inoculated with

0.2 mL of (5×10^6 cells) by subcutaneous injection (with a 24-gauge needle) into the left flank. Mice will be weighted every 2 days and tumor volume will be measured once every other day using a caliper when the first tumors form. Tumor volume is estimated according to the following formula: tumor volume (mm^3) = length × $width^2/2$. When tumors reach approximately 100 mm^3 at about 3 weeks, the mice will be assigned randomly to four treatment groups (each group including 5 mice): control (group I), doxorubicin (group II), cinobufacini injection (group III), and cinobufacini injection + doxorubicin (group IV). The control group will receive 200 μL PBS daliy via intraperitoneal injection (i.p.) for 2 weeks. The ADM group is treated with ADM at 5.5 mg/kg once a week intravenously for 2 weeks. In the group III and IV, the mice will be treated with 5 mL/kg for 2 weeks by intraperitoneal injection alone or in combination with doxorubicin at 5.5 mg/kg once a week intravenously for 2 weeks. The treatments lasted 14 days and the size of tumors was recorded every 2 days. Tumor inhibitory efficiency is estimated according to the following formula: tumor inhibitory efficiency (%) = (1- mean tumor volume of the cinobufacini injection or/and DOX administration group)/mean tumor volume of the control group) × 100%. The mice were sacrificed 3 days after the last injection, blood samples were collected via cardiac puncture and chilled in test tubes containing heparin. Tumor tissue was quickly removed and divided into two halves, one half was fixed with 10% buffered formalin, and the other stored at -80 ℃.

2.13. Statistical analysis

All experiments were performed in triplicate and the results were analyzed by ANOVA (one-way analysis of variance) using GraphPad Prism 4, followed by Student's t test using Microsoft Office Excel software. $p < 0.05$ was indicative of significant difference.

3. Results

3.1. Anti-proliferative effects of isolated fractions from cinobufacini on HepG2 cells

Under the bioactivity-guided fractionation, effective anti-cancer components from cinobufacini were purified. As shown in Table 2, the anti-cancer components were efficiently extracted with chloroform, which exhibited a lower IC_{50} (0.21 μg/ml) than water residue (IC_{50} 598.0 μg/ml) and cinobufacini (IC_{50} 116 μg/ml) in HepG2 cells.

Table 2. Cytotoxic activity of cinobufacini, chloroform extract and water residue against HepG2 cells

Extract	Cinobufacini	Chloroform extract	Water residue
Weight (mg)	13,089.3	2,001.1	11,088.2
IC_{50} (μg/ml)	116	0.21	598.0

Further purification of chloroform extract was performed by silica-gel chromatography. The cytotoxicities of fractions I-IX are listed in Table 3. Fractions I, II, V, VII, VIII and IX displayed a weak anti-proliferative effect on HepG2 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 HepG2 cells with the lowest IC_{50} value (5.8 μg/ml). These results suggested that fraction III was a promising candidate for further investigation.

Table 3. Cytotoxic activity of fractions I-IX from chloroform extract against HepG2 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

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 4, 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.

Table 4. Cytotoxic activity of fractions 1-13 from fraction III against HepG2 cells.

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

3.2. 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 reported that two active compounds (Compounds 1 and 2) were obtained (Fig. 1).

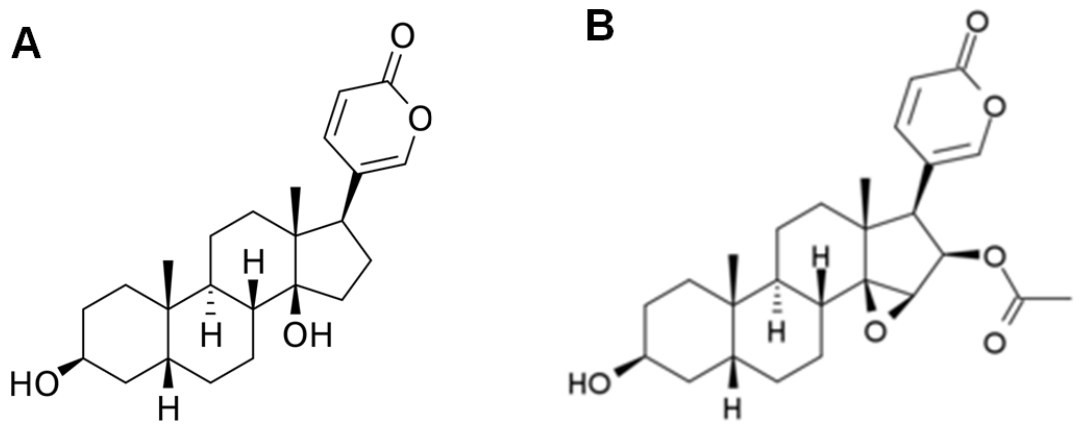

Figure 1. HPLC chromatogram of bufalin (A) and cinobufagin (B) from cinobufacini. HPLC conditions: COSMOSIL Cholester reversed-phase C-18 column; mobile phase, 45% acetonitrile; flow rate, 1 ml/min; detection wavelength, 296 nm. Samples were dissolved in methanol.

3.3. Identification of the active compounds

Compound 1 was obtained as white powder whose EIEI-MS spectrum showed the $(M+H)^+$ ion peak at 386. Combined with the results of ^1H NMR (data not shown) and ^{13}C NMR, DEPT data showed the molecular formula of this compound was $C_{24}H_{34}O_4$ (Mr=386). Compound 2 was obtained as white powder whose EIEI-MS spectrum showed the $(M+H)^+$ ion peak at 443. Combined with the ^1H NMR data (data not shown) and ^{13}C NMR, DEPT data showed the molecular formula of this compound

was $C_{26}H_{34}O_6$ (Mr=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 bufalin (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 (38,39).

3.4. Anti-proliferation effect of cinobufacini and doxorubicin on HCC cell lines

The anti-proliferation effect of cinobufacini and doxorubicin on hepatocellular carcinoma cells was estimated by MTT assay. As shown in Fig. 2, cinobufacini had a significant inhibitive effect on both cell lines. The viability of HLE and HepG2 cells were treated by various concentration of cinobufacini and detected at 24 h, 48 h, and 72 h, respectively (Figs. 2A and B). After treatment with 0.1 mg/mL cinobufacini for 24 h, the growth inhibition rate of HLE cells was 52.05% (Fig. 2C) while that of HepG2 cells was 47.15% (Fig. 2D). As a positive control, inhibition effect of doxorubicin at 24 h was also detected. At a concentration of 800 nM, doxorubicin had a similar inhibition effect as cinobufacini at 0.1 mg/mL (Figs. 2E and 2F). These results suggested that cinobufacini had a significant growth inhibition effect on HLE and HepG2 cells and 0.1 mg/mL cinobufacini was selected to be used in the following experiments.

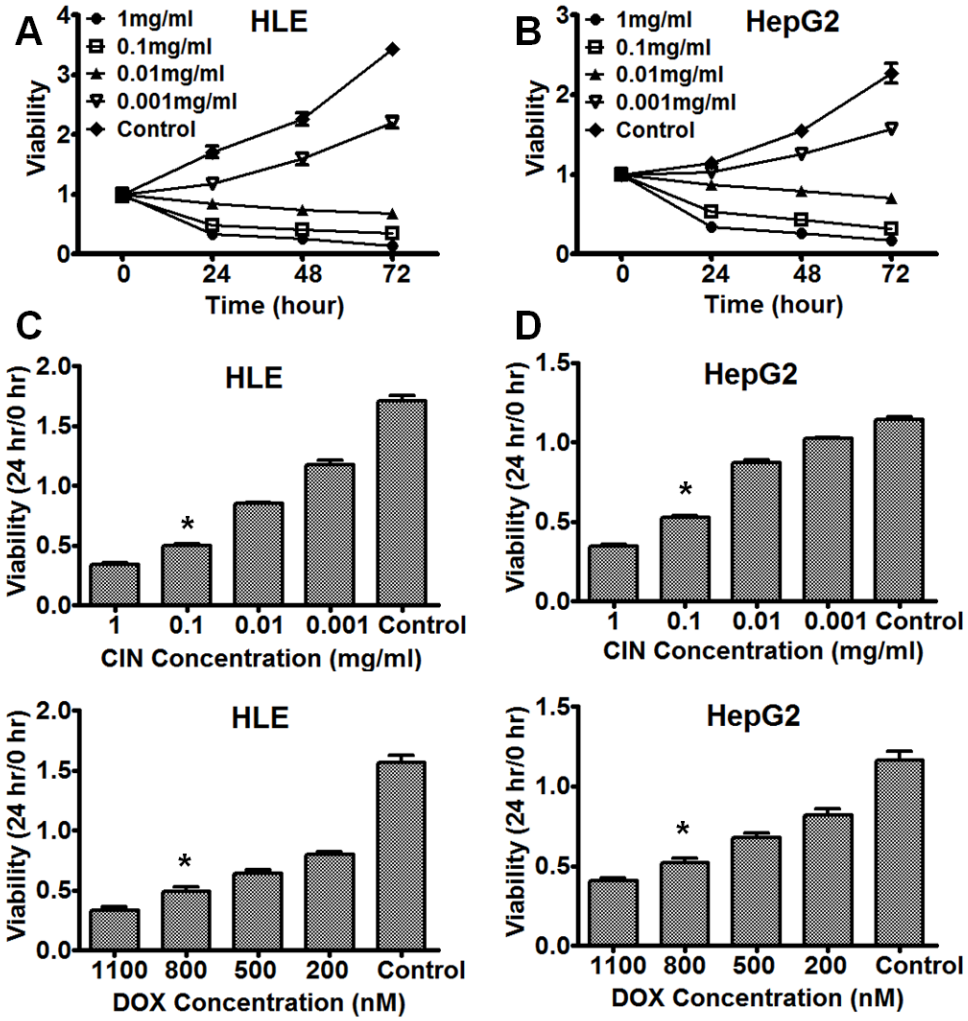

Fig 2. Growth inhibition effect of cinobufacini on HLE (A, C) and HepG2 (B, D) cells. Cells were treated with various concentrations of cinobufacini (1, 0.1, 0.01, 0.001, and 0 mg/mL) for 24 h, 48 h and 72 h, and the cell viability was determined by MTT assay. Growth inhibition effects of doxorubicin are shown in (E) and (F). Cells were treated with different concentrations of doxorubicin (0, 200, 500, 800, 1100 nM) for 24 h, 48 h and 72 h, and the cell viability was detected by MTT assay. The y axis of Figs 1A and 1B means fold change. The results are expressed as percentage of cell growth relative to untreated control cells. The data represent mean ± S.D. (n = 5). * $p < 0.05$ vs. control.

3.5. Anti-Proliferation Effect of Combination of Cinobufacini and Doxorubicin on HCC Cells

The antiproliferation effect of the combination of cinobufacini and doxorubicin on hepatocellular carcinoma cells was assessed by MTT assay. As shown in Fig. 3, there were eight groups: (a) doxorubicin (800 nM), (b) cinobufacini (0.1 mg/mL), (c) doxorubicin and cinobufacini, (d) bufalin (1.284 μg/mL), (e) doxorubicin and bufalin, (f) cinobufagin (0.613 μg/mL), (g) doxorubicin and cinobufagin, (h) untreated group as a control. We choose bufalin and cinobufagin as positive controls because their molecular mechanism of apoptosis has been studied thoroughly. Because quantitative analysis showed that the concentrations of bufalin and cinobufagin in cinobufacini are 12.84 μg/mL and 6.13 μg/mL (Su et al., 2003), the concentration of bufalin and cinobufagin used in this experiment were 1.284 μg/mL and 0.613 μg/mL. After treatment for 24 h, compared to the untreated group, cell viability of the other seven groups of HLE were 27.7%, 29.0%, 23.3%, 46.9%, 27.5%, 47.3% and 27.7%, respectively (Fig. 3B). Those of HepG2 were 49.3%, 56.9%, 48.8%, 81.6%, 55.8%, 82.2% and 57.3%, respectively (Fig. 3C). Morphological changes could be observed with white light microscopy (Fig. 3A). Growth inhibition of group (c) of HLE was stronger than group (a) and group (b) (Fig. 3B), both had a significant difference ($p < 0.05$). That of HepG2 was also stronger than group (a) and group (b) (Fig. 3C), but only the difference between (b) and (c) was significant ($p < 0:05$). Moreover, antiproliferation activity of group (e) was obviously stronger than group (d) and that of group (g) was significantly higher than group (f) in both cell lines ($p < 0.05$). In addition, cinobufacini showed more obvious inhibitive ability than bufalin or

cinobufagin ($p < 0:05$), meanwhile the inhibitive level of cinobufacini combined with doxorubicin was higher than bufalin or cinobufagin combined with doxorubicin in HLE and HepG2 respectively ($p < 0:05$). Through free Chou–Talalay method software, the combination index in HLE and HepG2 cells was analyzed as 0.2098 and 0.2389 respectively (Fig. 3D). As the resulting combination index (CI) theorem of Chou–Talalay offers quantitative definition for additive effect (CI ¼ 1), synergism (CI < 1), and antagonism (CI > 1) in drug combinations, these results means that combination of cinobufacini and doxorubicin produced synergy effects.

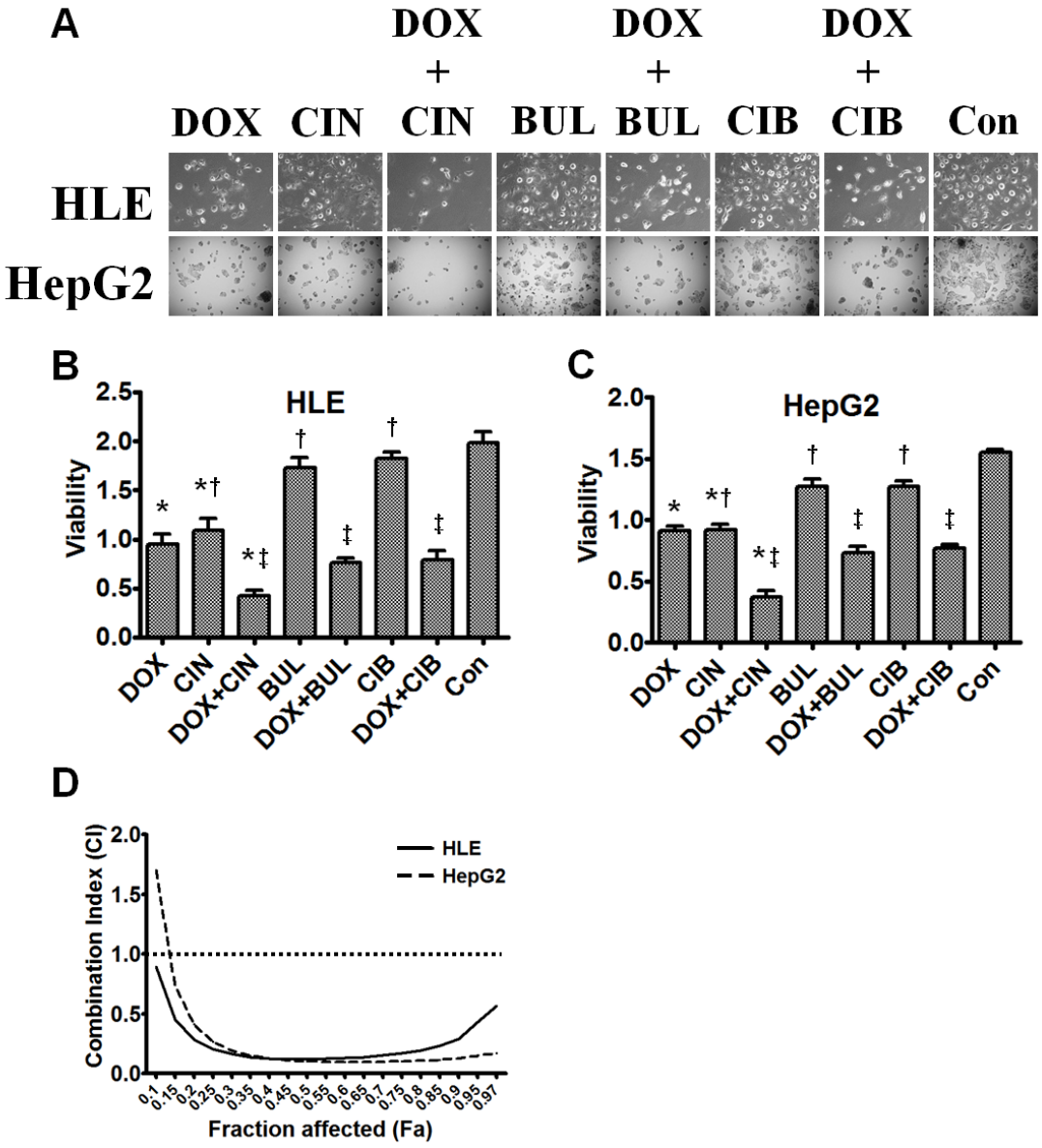

Figure 3. Detection of growth inhibition effect of combination reagents on HCC cells with microscopic examination (A) and MTT assay (B, C). HLE and HepG2 cells were treated with doxorubicin (800nM), cinobufacini (0.1 mg/mL), doxorubicin and cinobufacini, bufalin (1.284 μg/mL), doxorubicin and bufalin, cinobufagin (0.613 μg/mL), doxorubicin and cinobufagin, respectively for 24 h. The results are expressed as percentage of cell growth relative to untreated control cells. The data represent mean ± S.D. (n = 5). * $p < 0.05$ vs. combination of cinobufacini and doxorubicin. † $p < 0.05$ vs. cinobufacini. ‡ $p < 0.05$ vs. combination of cinobufacini and doxorubicin. DOX: doxorubicin; CIN: cinobufacini; BUL: bufalin; CIB: cinobufagin; Con: control.

3.6. Induction of apoptosis in HCC cells

To investigate the effect of each experimental group on the morphology of apoptotic cells, Hoechst 33258 staining was used. After treatment with different medicines for 24h, marked morphological changes in chromatin morphology such as crenation, condensation, and fragmentation were observed in HLE and HepG2cells (Fig. 4A). To further the quantitative analysis of apoptotic cells, we analyzed the fluorescence intensity of the pictures. As shown in Fig. 4B and 4C, the combination group showed higher green/red fluorescence ratio than cinobufacini group and doxorubicin group ($p<0.05$), meanwhile cinobufacini group showed higher ration than bufalin group and cinobufagin group as well ($p<0.05$).

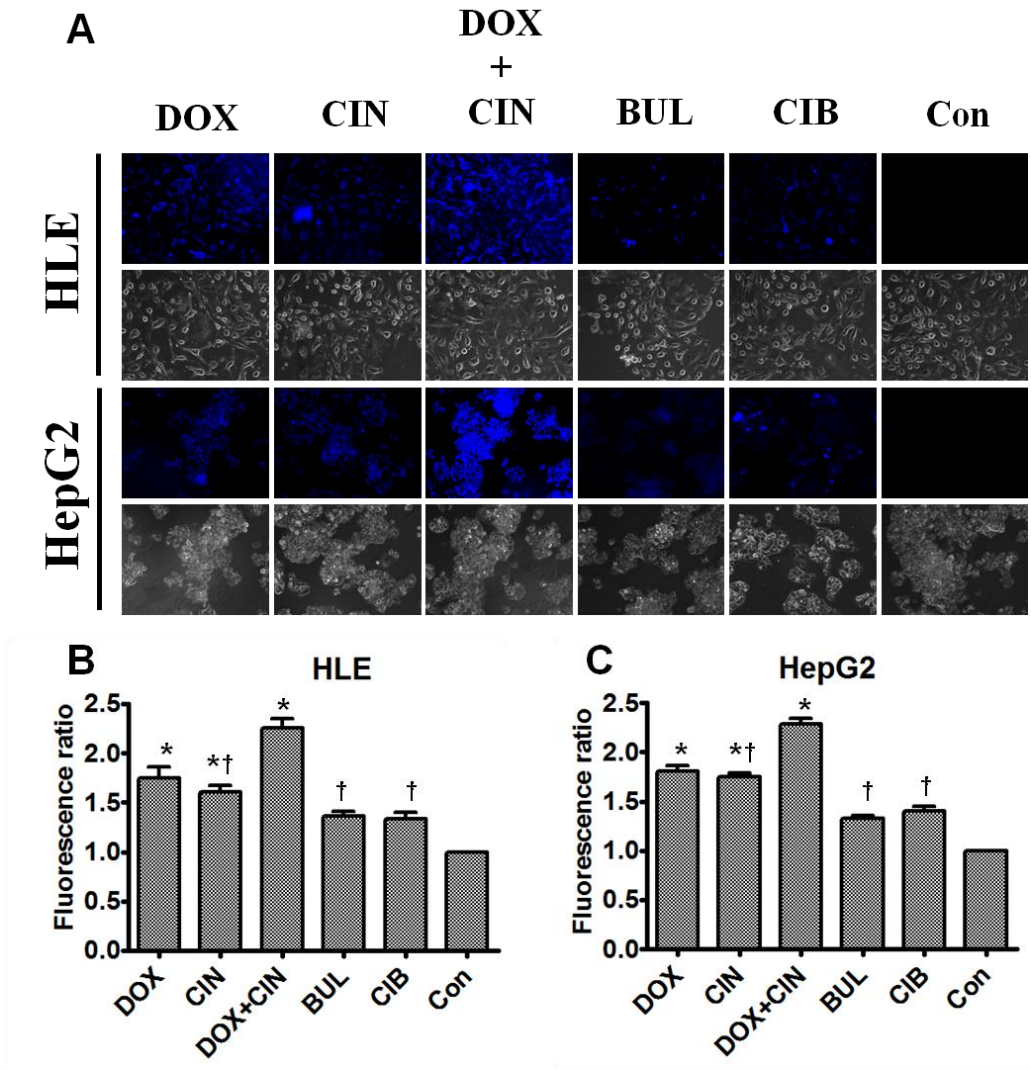

Figure 4. Detection of apoptotic effect of combination reagents on HCC cells with fluorescence microscopic examination. After cells were treated with medicines for 24h, the apoptotic morphology and proportion of apoptotic cells were detected by Hoechst 33258 staining (A). The fluorescence intensity was detected and showed as histograms (B and C). The original magnification is 200×. The data represent mean ± S.D. (n = 3). * $p < 0.05$ vs. combination of cinobufacini and doxorubicin. † $p < 0.05$ vs. cinobufacini.

3.7. Effect of combination reagents on the mitochondrial membrane potential in HCC cells

Disruption of mitochondrial membrane potential is one of the earliest intracellular events that occur following the induction of apoptosis. As shown in Fig. 5A, untreated cells gave off a bright red fluorescence, whereas after the cells were treated with various medicines for 24h, mitochondrial membrane potential loss was observed with fluorescence shifted from red to green. This showed that combination group could induce strongest disruption of mitochondrial function significantly according to the data of relative green-to-red fluorescence intensity ratio ($p<0.05$) (Fig. 5B and 5C).

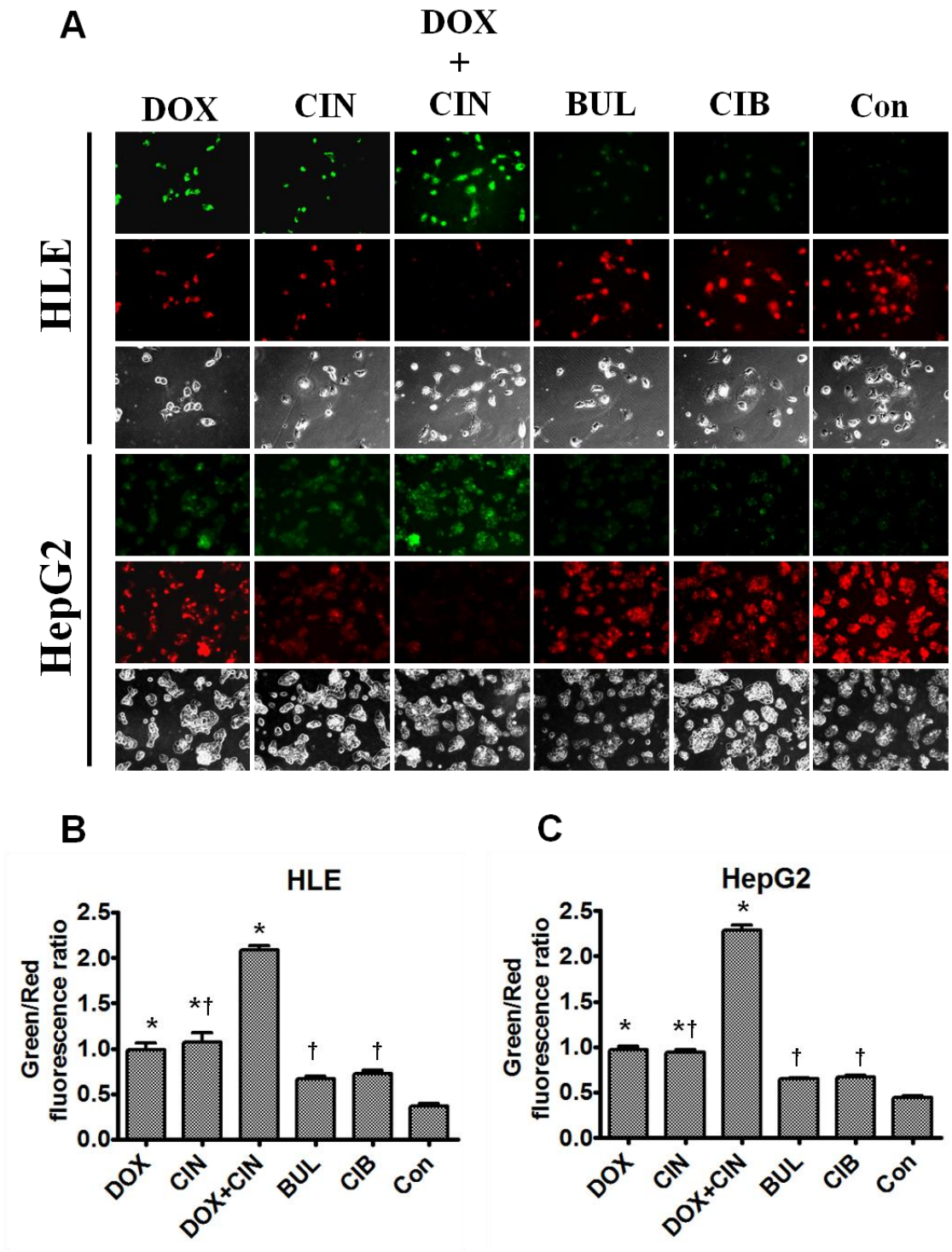

Figure 5. Effects of cinobufacini on mitochondrial membrane potential disruptions in HLE and HepG2 cells. After cells were treated with medicines for 24h, the disruption of mitochondrial membrane potential was observed under a fluorescence microscope. Red fluorescence represents mitochondria with an intact

membrane potential; green fluorescence represents disrupted membrane potential. (A) The changes of mitochondrial membrane potential in HLE and HepG2 cells. Original magnification, 200×. (B and C) The relative ratio of green/red fluorescence intensity in HLE and HepG2 cells. Data are shown by means ± S.D. (n = 3). * $p < 0.05$ vs. combination of cinobufacini and doxorubicin. † $p < 0.05$ vs. cinobufacini.

3.8. Effect of combination reagents on the expression of apoptosis-related mRNAs in HCC cells

The expression changes of apoptosis-related genes after treatment with the combination of cinobufacini and doxorubicin in hepatocellular carcinoma cells were assessed by a real-time PCR system. As shown in Fig. 6, after treatment for 24 h, compared to the untreated group, RNA levels of other groups of Bcl-2 in HLE and HepG2 cells both decreased (Figs. 6A and 6B). For Bax, Bid, and cytochrome c, RNA levels of other groups in HLE and HepG2 cells both increased (Figs. 6C, D, E, F, G, and H).

The expression of Bcl-2, group (c) decreased compared with group (a) and group (b) in HLE and HepG2 cells respectively ($p<0.05$). Group (c) decreased obviously compared to group (d) and group (f) in HLE and HepG2 cells ($p<0.05$). The RNA expression level of group (c) was downregulated compared with group (e) and group (g) ($p<0.05$). The expression of Bax, Bid, and cytochrome c, group (c) increased compared with group (a) and group (b) in HLE and HepG2 cells respectively ($p<0.05$). Group (c) increased strongly in comparison with group (d) and group (f) in both cell

lines ($p<0.05$). The RNA expression level of group (c) was up-regulated obviously compared to group (e) and group (g) ($p<0.05$).

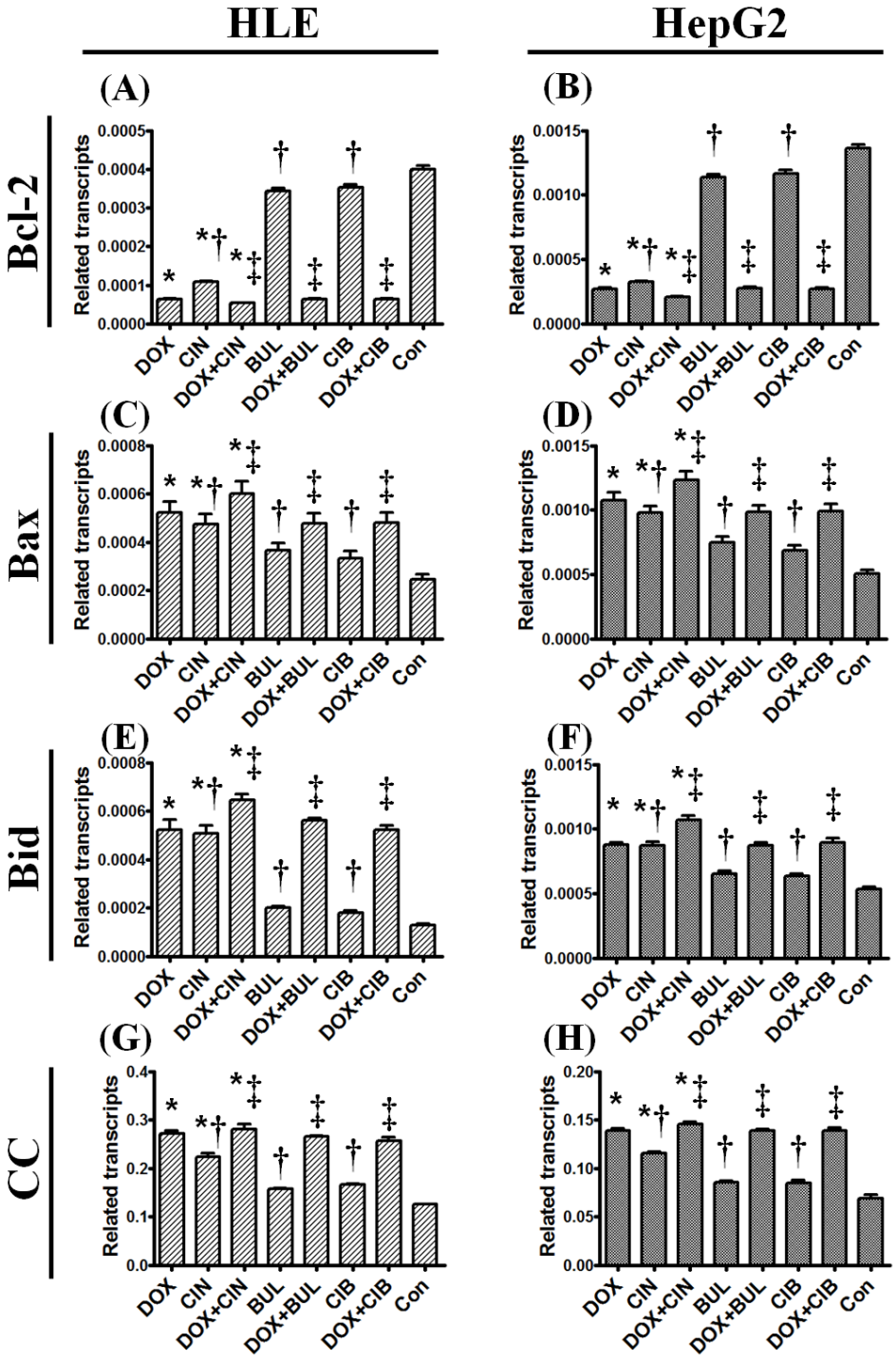

Figure 6. Effect of a combination of reagents on transcription of apoptosis-related genes. After cells were treated for 24 h, the RNA expression levels of Bcl-2, Bax, Bid, and Cytochrome c (CC) were detected by real-time PCR. The results are expressed as percentage of cell growth relative to untreated control cells. The data represent mean ± S.D. (n = 3). * $p < 0.05$ vs. combination of cinobufacini and doxorubicin. † $p < 0.05$ vs. cinobufacini. ‡ $p < 0.05$ vs. combination of cinobufacini and doxorubicin. DOX: doxorubicin; CIN: cinobufacini; BUL: bufalin; CIB: cinobufagin; Con: control.

3.9. Effect of combination reagents on the expression of apoptosis-related proteins in HCC cells

To clarify the changing situation of apoptosis-related proteins affected by combination reagents in HLE and HepG2 cell lines, expression levels of apoptosis-related proteins Bcl-2, Bax, Bid, and cytochrome c were measured by Western blot analysis. As shown in Fig. 7, after treatment for 24 h, compared to the untreated group, protein levels of group (c) of Bcl-2 and Bid decreased compared to group (a) and group (b) in HLE and HepG2 cells respectively (Figs. 7A2, B2, A4, and B4). Protein levels of group (c) of Bax and cytochrome c increased in comparison with group (a) and group (b) in HLE and HepG2 cells respectively (Figs. 7A3, A5, B3, and B5).

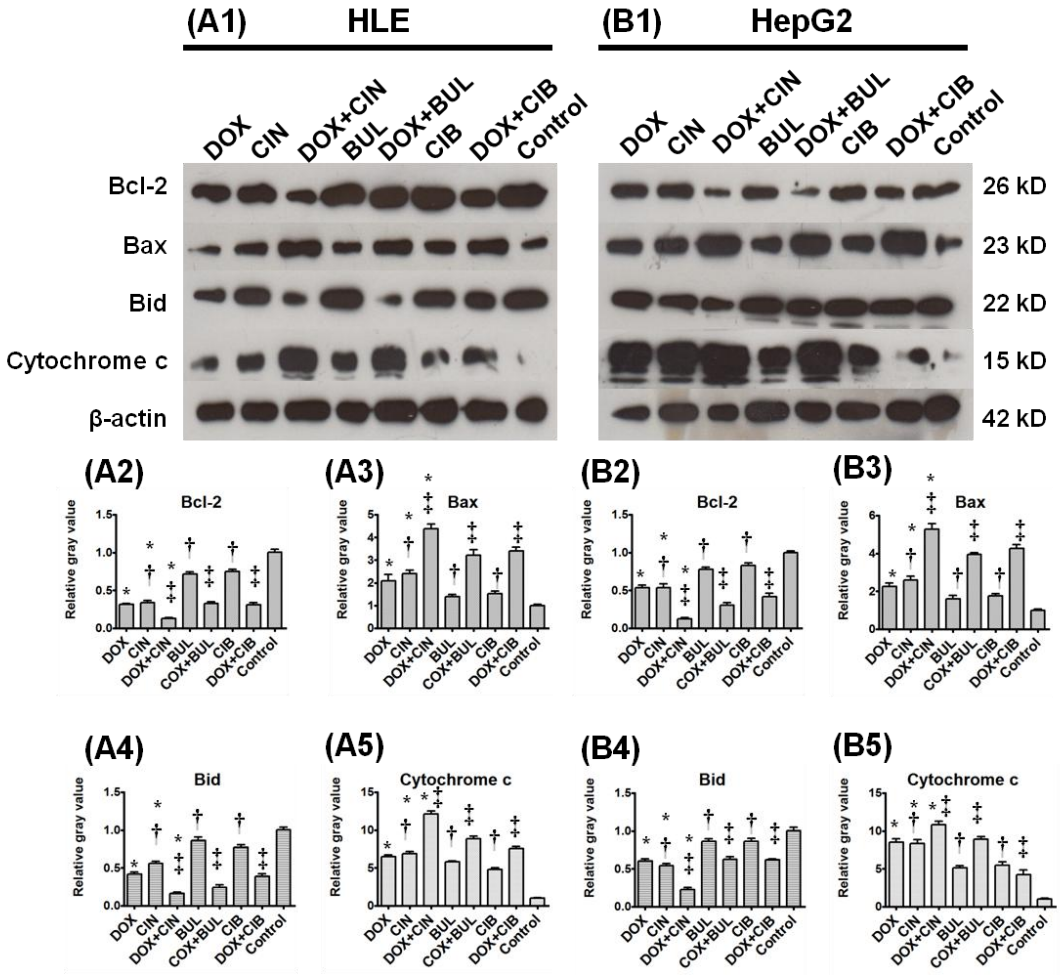

Figure 7. Changes of apoptotic proteins after treatment with a combination of reagents in HCC cells. After cells were treated for 24 h, the protein expression levels of Bcl-2, Bax, Bid, and Cytochrome c (CC) were detected by Western blot. The data represent mean ± S.D. (n = 3). * $p < 0.05$ vs. combination of cinobufacini and doxorubicin. † $p < 0.05$ vs. cinobufacini. ‡ $p < 0.05$ vs. combination of cinobufacini and doxorubicin. DOX: doxorubicin; CIN: cinobufacini; BUL: bufalin; CIB: cinobufagin.

3.10. Effect of combination reagents on the expression of apoptosis-related caspases in HCC cells

To investigate the changing situation of apoptosis-related caspases affected by combination reagents in HLE and HepG2 cell lines, expression levels of apoptosis-related proteins caspase-3, caspase-8, caspase-9, and caspase-10 were measured by colorimetric assay. As shown in Fig. 8, after treatment for 24 h, the expression of four caspases, group (c) increased compared with group (a) and group (b) in HLE and HepG2 cells respectively ($p<0.05$). Group (c) increased obviously compared to group (d) and group (f) in HLE and HepG2 cells ($p<0.05$). The expression level of group (c) was upregulated compared with group (e) and group (g) ($p<0.05$).

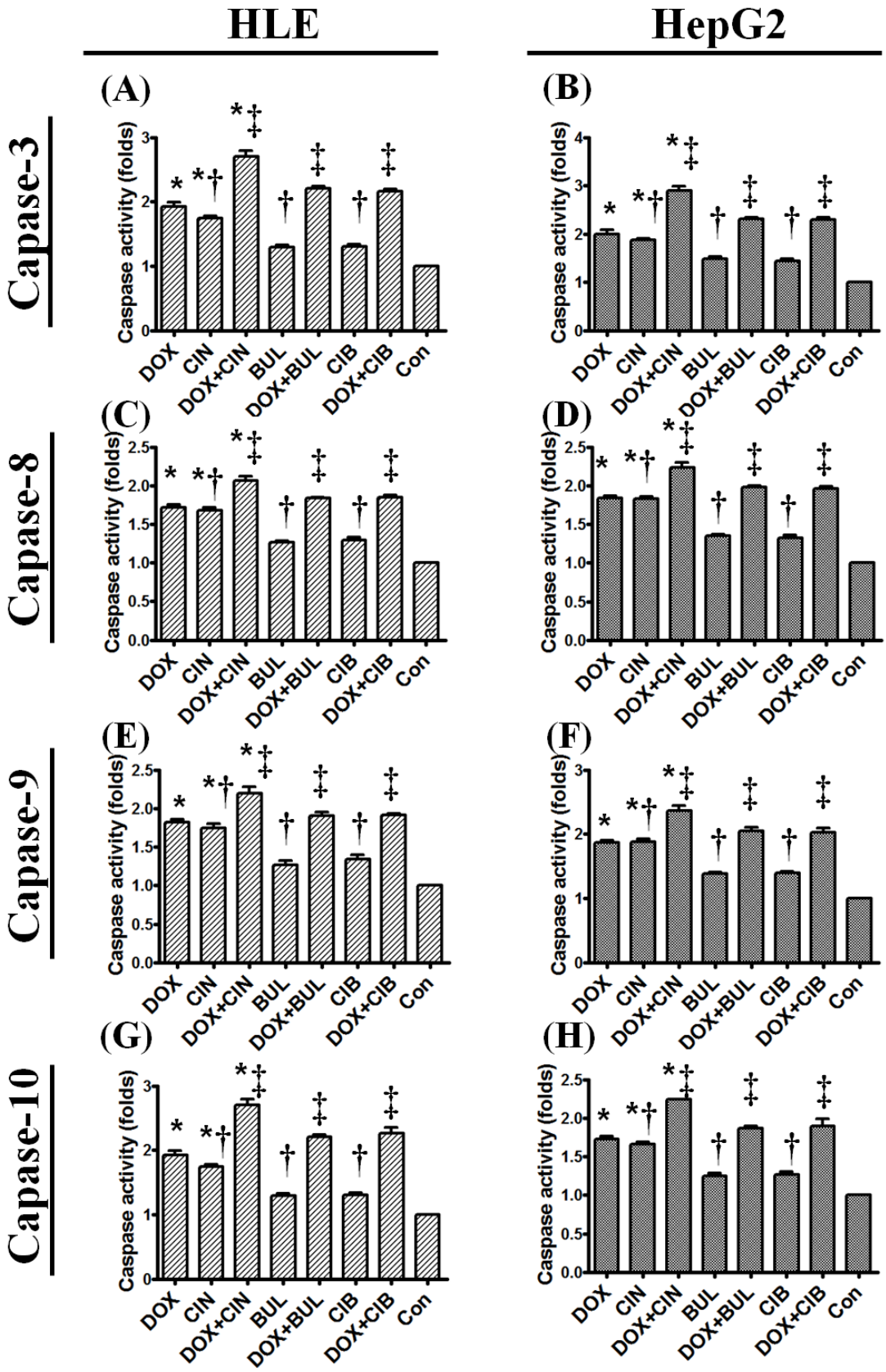
HLE
HepG2
Capase-3
Capase-8
Capase-9
Capase-10
(A)
(B)
(C)
(D)
(E)
(F)
(G)
(H)
Caspase activity (folds)
DOX
CIN
DOX+CIN
BUL
DOX+BUL
CIB
DOX+CIB
Con

Figure 8. Effect of combination reagents on the expression of apoptosis-related caspases in HCC cells. After cells were treated for 24 h, the RNA expression levels of caspase-3, -8, -9, and -10 were detected by colorimetric assay. The results are expressed as percentage of cell growth relative to untreated control cells. The data represent mean ± S.D. (n = 3). * $p < 0.05$ vs. combination of cinobufacini and doxorubicin. † $p < 0.05$ vs. cinobufacini. ‡ $p < 0.05$ vs. combination of cinobufacini and doxorubicin. DOX: doxorubicin; CIN: cinobufacini; BUL: bufalin; CIB: cinobufagin; Con: control.

3.11. Effect of cinobufacini and mixture of components on the expression of apoptosis-related mRNAs in HCC cells

The expression changes of apoptosis-related genes after treatment with cinobufacini and mixture of bufalin and cinobufagin in hepatocellular carcinoma cells were tested by real-time PCR assay. As shown in Fig. 9, there were six groups: (a) doxorubicin (800 nM), (b) cinobufacini (0.1 mg/mL), (d) bufalin (1.284 μg/mL), (f) cinobufagin (0.613 μg/mL), (i) bufalin (1.284 μg/mL) and cinobufagin (0.613 μg/mL), (h) untreated group as a control.

After treatment for 24 h, on expression of Bcl-2, group (b) decreased compared with group (d), group (f) and group (i) in HLE and HepG2 respectively ($p<0.05$) (Figs. 9A and B). On expression of Bax, Bid, and cytochrome c, group (b) increased compared with group (d), group (f) and group (i) in HLE and HepG2 cells respectively ($p<0.05$) (Figs. 9C, D, E, F, G, and H).

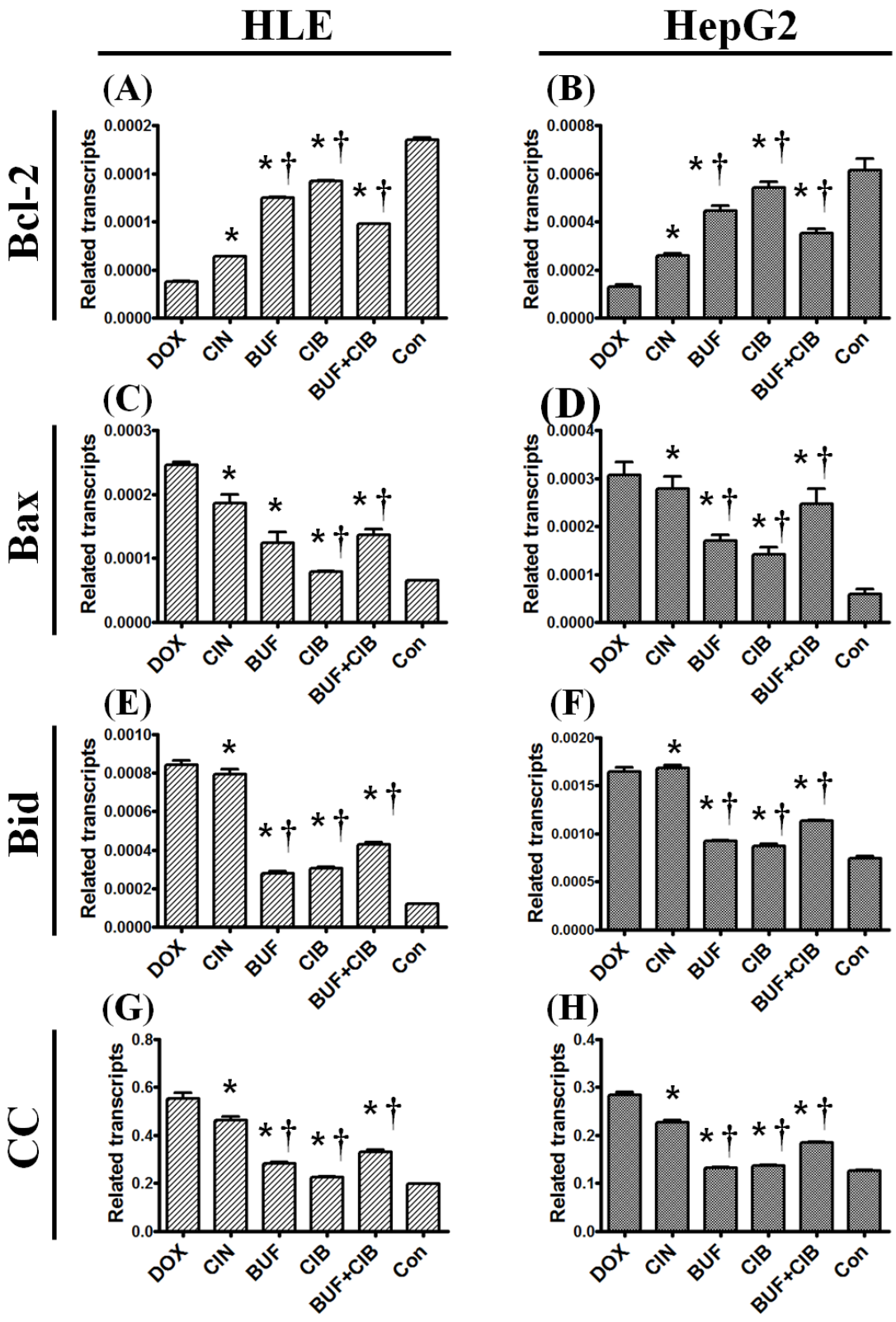

Figure 9. Effect of reagents on transcription of apoptosis-related genes. HLE and HepG2 cells were treated with doxorubicin (800nM), cinobufacini (0.1 mg/mL),

bufalin (1.284 μg/mL), cinobufagin (0.613 μg/mL), bufalin and cinobufagin, respectively for 24 h. After 24 h, the RNA expression levels of Bcl-2, Bax, Bid, and Cytochrome c were detected by real-time PCR. The results are expressed as percentage of cell growth relative to untreated control cells. The data represent mean ± S.D. (n = 3). * $p < 0.05$ vs. cinobufacini. † $p < 0.05$ vs. bufalin and cinobufagin. DOX: doxorubicin; CIN: cinobufacini; BUL: bufalin; CIB: cinobufagin; Con: control.

3.12. Effect of cinobufacini and mixture of components on the expression of apoptosis-related proteins in HCC cells

To investigate the expression of apoptosis-related proteins affected by cinobufacini and mixture of components in HLE and HepG2 cell lines, expression level of apoptosis-related proteins Bcl-2, Bax, Bid, and cytochrome c was measured by Western blot method. As shown in Fig. 10, after treatment for 24 h, compared to untreated group, protein levels of group (b) of Bcl-2 and Bid decreased compared to group (d), group (f), and group (i) in HLE and HepG2 cells respectively ($p<0.05$) (Figs. 10A2, B2, A4, and B4). Protein levels of group (b) of Bax and cytochrome c increased compared to group (d), group (f), and group (i) in HLE and HepG2 cells respectively ($p<0.05$) (Figs. 10A3, B3, A5, and B5).

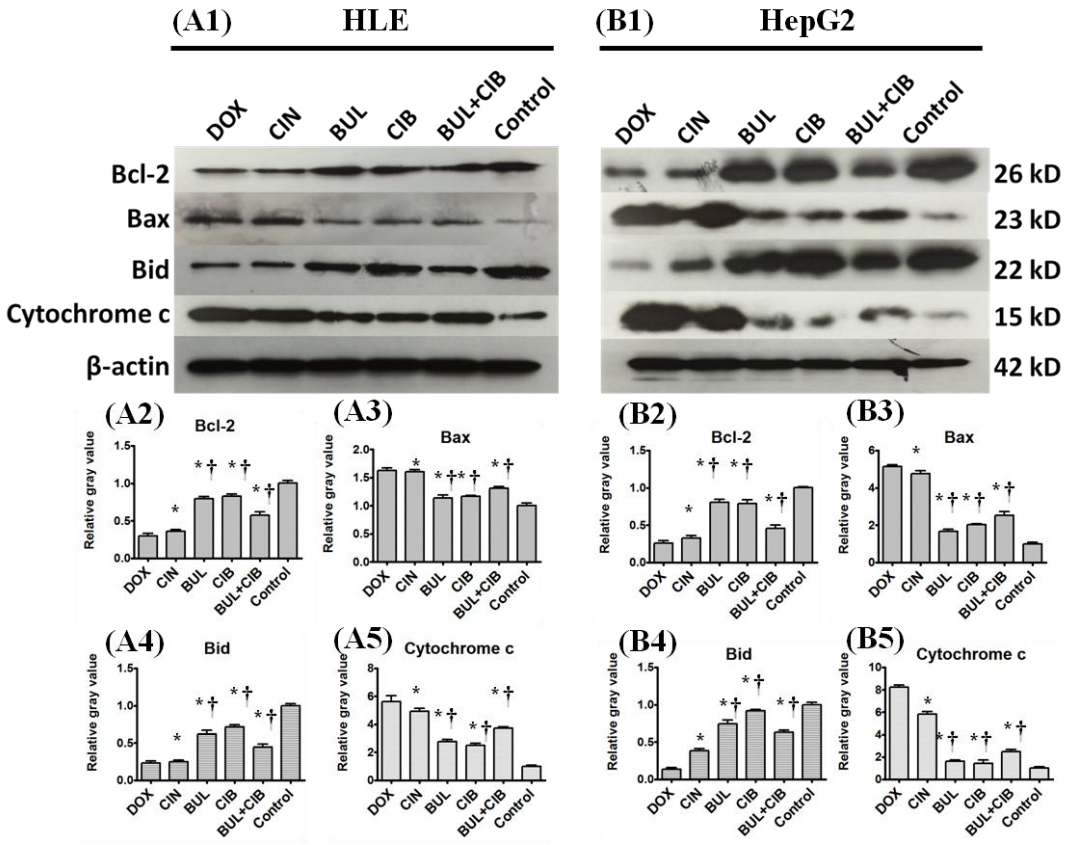

Figure 10. Induction of apoptosis activation by cinobufacini and mixed compounds in HCC cells. After cells were treated for 24 h, the protein expression levels of Bcl-2, Bax, Bid, and Cytochrome c were detected by Western blot. The data represent mean ± S.D. (n = 3). * $p < 0.05$ vs. cinobufacini. † $p < 0.05$ vs. bufalin and cinobufagin. DOX: doxorubicin; CIN: cinobufacini; BUL: bufalin; CIB: cinobufagin.

3.13. Effect of combination reagents on the expression of apoptosis-related proteins in HCC xenograft model

Based on the above results, the key in vitro findings were validated in vivo in a xenograft mouse model of human HepG2 liver cancer. The inhibitive effect of combination of cinobufacini and doxorubicin was evaluated for two weeks. As shown

in Figure 11A, exposure to combination group led to a significant suppression of tumor growth ($p < 0.01$) and decreased the final tumor weight by 68.8% (Figure 11B, $p < 0.05$).

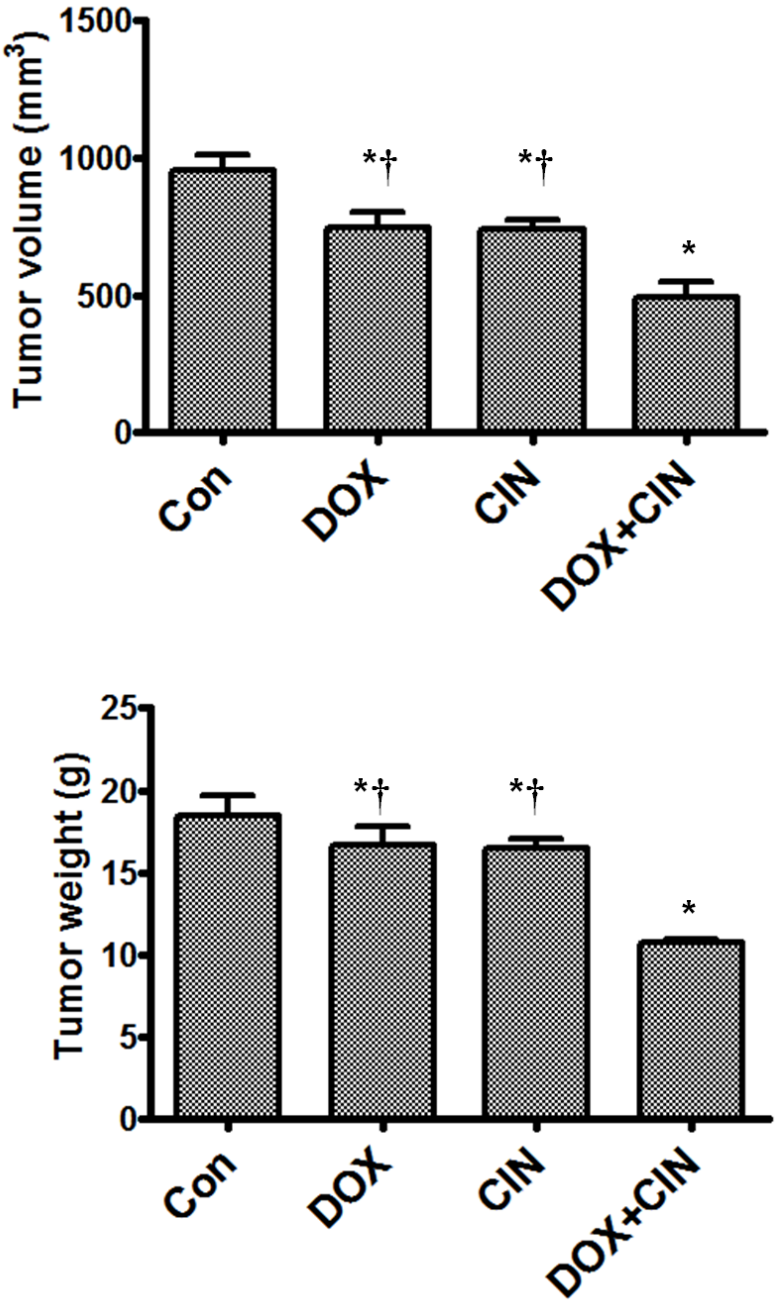

Figure 11. Volume and Weight of tumors. * $p < 0.05$ vs. control. † $p < 0.05$ vs. doxorubicin+cinobufacini. DOX: doxorubicin; CIN: cinobufacini; CON: control.

The expression changes of apoptosis-related genes after treatment with each group in hepatocellular carcinoma xenograft were tested by real-time PCR assay. After treatment, on expression of Bcl-2, group IV decreased significantly compared with group I, group II and group III ($p<0.05$) (Figs. 12A). On expression of Bax, Bid and cytochrome c, group IV increased significantly compared with group I, group II and group III ($p<0.05$) (Figs. 12B, C, and D).

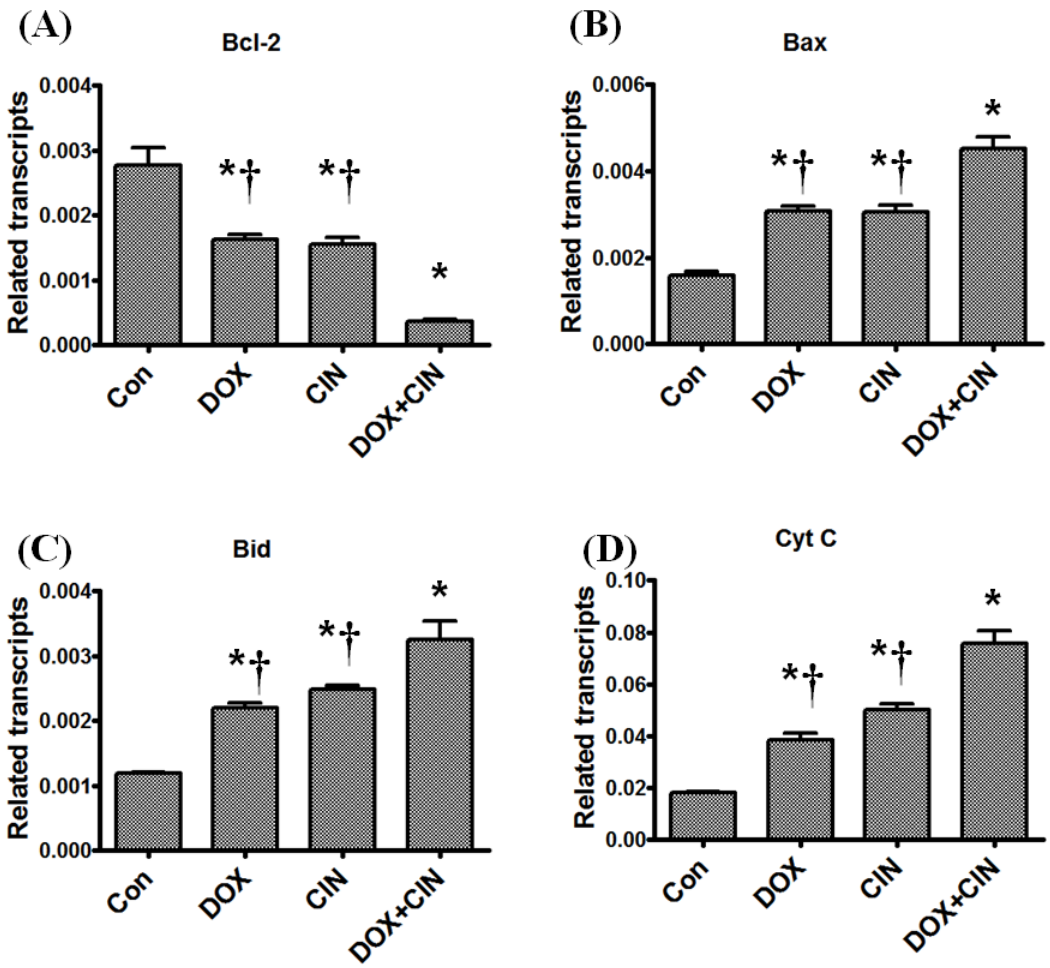

Figure 12. Effect of reagents of each group on the expression of apoptosis-related genes in the xenograft. * $p < 0.05$ vs. control. † $p < 0.05$ vs. doxorubicin+cinobufacini. DOX: doxorubicin; CIN: cinobufacini; CON: control.

To clarify the changing situation of apoptosis-related proteins affected by combination reagents, expression levels of apoptosis-related proteins Bcl-2, Bax, Bid, and cytochrome c were measured by Western blot analysis. As shown in Fig. 13A and C, protein levels of group IV of Bcl-2 and Bid decreased compared to group I, group II and group III. Protein levels of group IV of Bax and cytochrome c increased in comparison with group I, group II and group III (Figs. 13B and D).

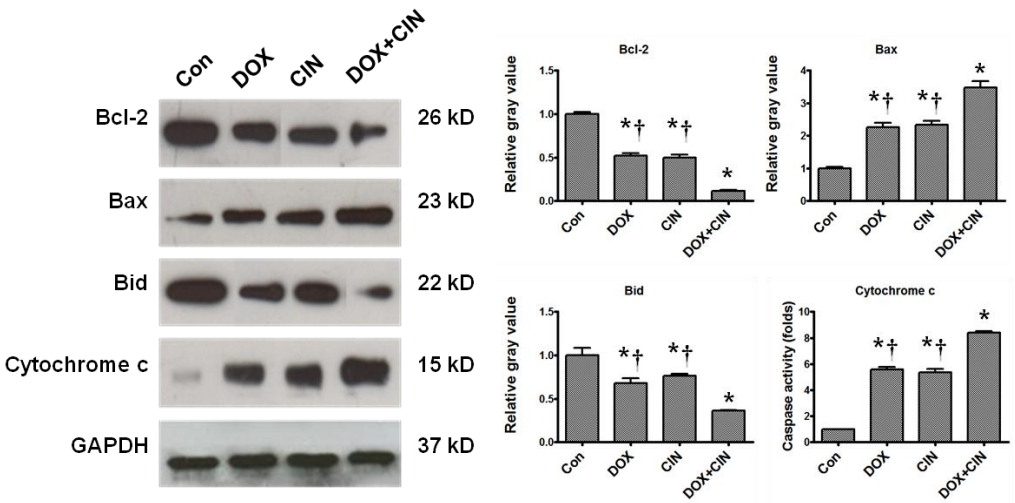

Figure 13. Change of expression level of apoptosis-related proteins in xenograft. * $p < 0.05$ vs. control. † $p < 0.05$ vs. doxorubicin+cinobufacini. DOX: doxorubicin; CIN: cinobufacini; CON: control.

4. Discussion

HCC is one of the most severe cancers worldwide with high morbidity and mortality (40). Surgery and chemotherapy are the preferred therapeutic strategies for advanced HCC. However, for metastasis and the drug resistant nature of terminal HCC, patients with advanced HCC are not suitable for surgery and there have been few effective chemotherapeutic agents for HCC treatment (41,42). Thus, exploration of combination chemotherapeutic strategies is considered to contribute to the development of chemotherapy for HCC. The previous studies of our laboratory illuminated that cinobufacini and its compounds bufalin and cinobufagin could inhibit HCC growth by inducing Fas- and mitochondria-mediated apoptosis pathways (25,27,43). So we wondered whether combination of cinobufacini and doxorubicin could achieve better antitumor effects than monotherapy.

In the beginning, doxorubicin was used in combination with cinobufacini, bufalin, and cinobufagin respectively. The experimental results suggested that combination of cinobufacini and doxorubicin showed a stronger growth inhibition ability than either cinobufacini or doxorubicin alone, and the results had significant differences ($p<0.05$). Meanwhile, bufalin and cinobufagin have been proven to have weaker inhibitory activity than cinobufacini on HCC cells, and the combination of them with doxorubicin had a similar suppressive effect compared to doxorubicin and cinobufacini.

In comparison, there was an interesting result that cinobufacini had a better anti-proliferation activity than peer-to-peer concentrations of bufalin and cinobufagin, and cinobufacini combined with doxorubicin also did a better job than bufalin or cinobufagin combined with doxorubicin. As we know, cinobufacini contains a number of bufadienolide cardiotonic steroids, such as bufalin, cinobufagin, resibufogenin, and telocinobufagin, which play a role as tumor inhibitors together (44). Thus, we supposed that cinobufacini as a mixture could cause a better anti-tumor effect than particular components alone and even a simple mixture of two components such as mixture of bufalin and cinobufagin.

Going a step further, we added a mixture of bufalin and cinobufagin to our project. The experimental results exhibited that the inhibitory effect of cinobufacini was stronger than the mixture of components, and the mixture of components caused a better effect than one component alone. In addition, the suppressive activity of the mixture could not compare to all of the components. This means the effect of bufalin and cinobufagin could not reach two fold of bufalin or cinobufagin separately. Because bufalin and cinobufagin target the same pathway, it may be that a limited number of targets limit the effect of their mixture (45).

Previous research by our laboratory proved that cinobufacini, bufalin and cinobufagin all could induce apoptosis by activating mitochondria-mediated and Fas-mediated apoptosis pathways (28). In our previous research we focused on expression of protein but not on RNA expression, and we were wondering whether

cinobufacini and its components could affect the transcription of apoptosis-related genes. Thus, we picked up four apoptosis-related genes and their corresponding proteins, Bcl-2, Bid, Bax, and cytochrome c which are major elements in apoptotic pathways. The real-time PCR assay and Western blot assays were performed synchronously. In the results of the present study, the RNA expression level of Bcl-2 decreased, but that of Bax, Bid, and cytochrome c increased. The protein expression level of Bcl-2 and Bid decreased, but that of Bax and cytochrome c increased. Bid should transfer to t-Bid molecules to get into mitochondria, and the decreasing amount of Bid suggested that an increasing amount of it penetrated as the t-Bid form. The low level of Bcl-2 attenuated its inhibitory effect on apoptosis, and meanwhile the high level of Bax further antagonized the Bcl-2 and promoted the apoptosis pathway in mitochondria. Cytochrome c was released by mitochondria into cytoplasm. In the cytoplasm, it activated downstream elements of caspase-9, caspase 3, and PARP, and then caused DNA fragmentation. Based on the previous studies of our laboratory, the change of Bid was associated with activation of the Fas pathway, and this pathway also could activate a caspase-8/10/3-related cascade reaction. Through both Fas- and mitochondria-mediated pathways, combination reagents induced apoptosis of HCC cells. As a result, these reagents could not only affect protein expression but also interrupt RNA expression. To do both things simultaneously made the anti-tumor activity more efficient. Although the change of these apoptosis-related genes and proteins were observed, how cinobufacini regulate the expression level of these was

still unclear.

Above all, our research suggests that cinobufacini and its active compounds could inhibit HCC cells by activating mitochondrial and Fas-related signaling pathways, the combination of cinobufacini and doxorubicin could reach a better inhibitory effect than any other reagents respectively and the apoptotic effect of cinobufacini was stronger than particular bufalin, cinobufagin, or a mixture of them alone. Thus, the combination of cinobufacini with the chemotherapeutic drug could be a better way to improve the treatment effect and life quality of HCC patients. However, a few of clinical reports suggested that cinobufacini could lead to stimulation on vessels, anaphylaxis, and drug fever in very few patients. Thus, reasonable application of cinobufacini clinically should attract enough attention (46). In the future, more traditional medicines and related active compounds would involve in the combination therapy for various cancers and other diseases (47).

5. Conclusion

Overall, these results suggested that cinobufacini and its active compounds, bufalin and cinobufagin, could induce apoptosis of HCC cells by activating mitochondrial- and Fas-related apoptotic signaling pathways which included bcl-2, bax, bid, cytochrome c, caspase-3, caspase-8, caspase-9, and caspase-10 (Figure 14). These reagents could not only affect protein expression but also interrupt RNA expression. To do both things simultaneously made the anti-tumor activity more efficient.The combination of cinobufacini and doxorubicin showed more obvious inhibitive activity on HCC cells and xenograft than monotherapies with cinobufacini or doxorubicin. Then, the cinobufacini as a mixture of various active compounds showed higher inhibitive activity on HCC cells than monotherapies with bufalin or cinobufagin. That indicates there would be other active compounds functioning in the inhibition of HCC cells.

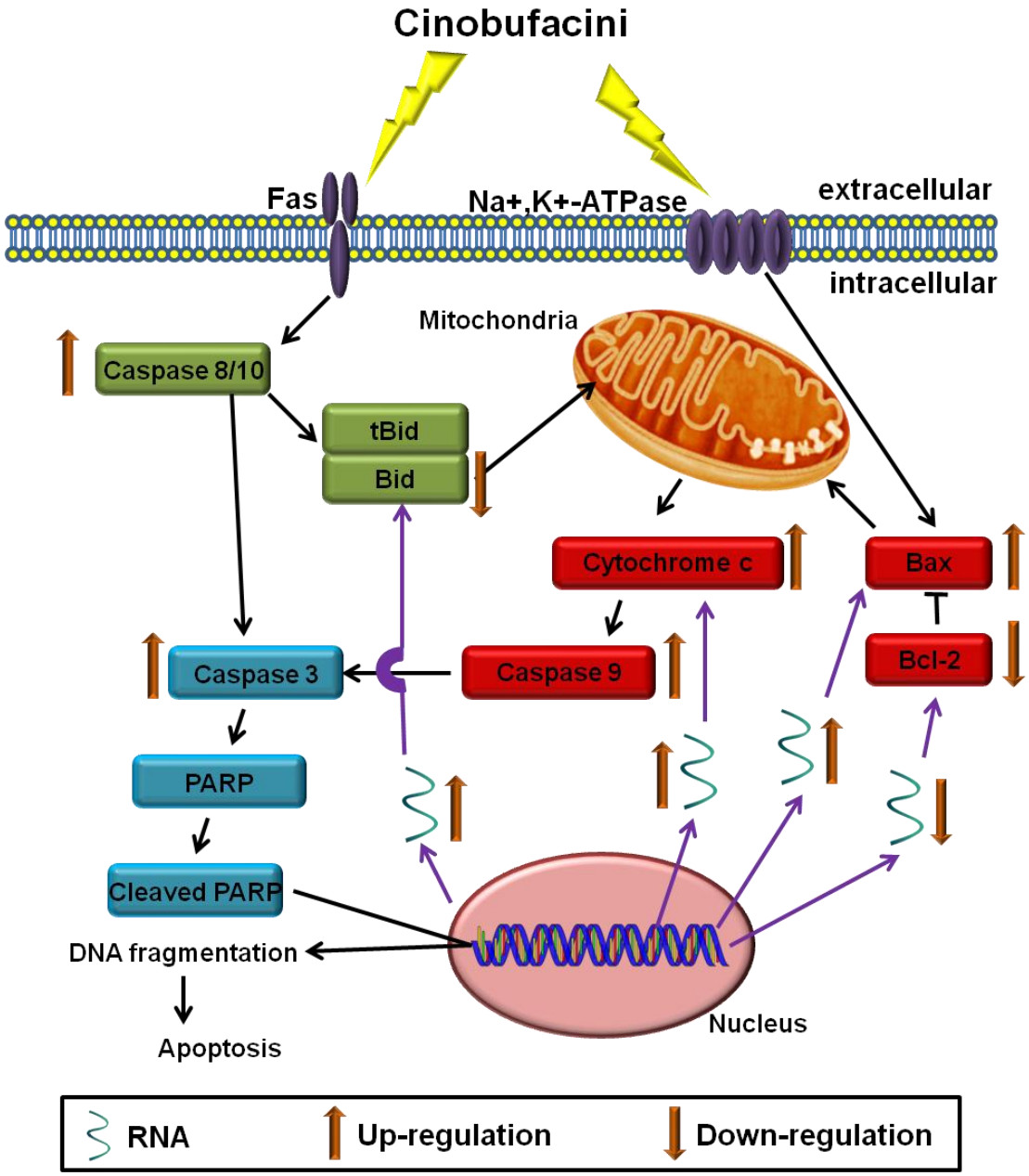

Figure 14. Signaling pathways involved in cinobufacini, bufalin, and cinobufagin induced apoptosis of HCC cells.

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