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Effects of Cinobufacini Injection on Hepatocarcinoma Cell Proliferation, Invasion and Metastasis

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Background & Aims: To explore the effects of Cinobufacini Injection (CI) on proliferation and invasion of hepatocarcinoma cells cultured with lymphatic endothelial cells (LECs), and lymphatic metastasis inhibition in immunocompetent mice. Methods: Trypan blue exclusion assay was used to examine the proliferation of human hepatocacinoma HepG-2 cells; MTT assay was employed to evaluate the heterogeneous adhesive ability of HepG-2 cells; Transwell was used to determine the invasion ability of HepG-2 cells. Protein expression levels of VEGF-C, MMP-2 and MMP-9 in HepG-2 cells were examined by Western-blotting. Tumor growth of mouse hepatocarcinoma HCa-F cell burden mice was evaluated by the size, and lymphatic metastasis were determined by the weight of lymph nodes. Results: The proliferation, invasion and heterogeneous adhesion abilities of HepG-2 cells were significantly greater when they were cultured with LECs than when cultured alone.CI reduced the proliferation, invasion and heterogeneous adhesion of HepG-2 cells with or without cultured with LECs. CI also reduced the expression of VEGF-C, MMP-2 and MMP-9 in HepG-2 cells; Tumor growth and lymphatic metastasis of HCa-F burden mice was significantly inhibited by the treatment of CI. Conclusions: LECs promoted the proliferation, invasion and adhesion of HepG-2 cells; CI inhibited the proliferation, invasion and lymphatic metastasis of hepatocarcinoma cells *in vitro* and *in vivo*. The reduced expression of VEGF-C, MMP-2 and MMP-9 may be one of the mechanisms by which CI inhibited the tumor growth and lymphatic metastasis.

Introduction

Liver cancer is a very common tumor in the world and a major cause of tumor-associated death. However, so far no effective medication is available. Less than 20% of patients respond to chemical medication. Targeted drugs such as sorafenib and sunitinib could not significantly improve the survival time of the patients.[1,2]

Cinobufacini Injection (CI), a sterilization solution of corium bufonis through extraction and processing,[3] is a traditional Chinese medicine approved by the Chinese State Food and Drug Administration (SFDA) (ISO9002). The major pharmacologic constituents of cinobufacini injection are bufodienolides, alkaloids, biogenic amines, peptides and proteins.[4,5] Bufadienolides, a class of cardioactive C-24 steroids with a characteristic α-pyrone ring at C-17, such as bufalin, cinobufagin, resibufogenin, and telocinobufagin (Fig.1), are the major active antitumor compounds of cinobufacini. SU Yong-hua et al[6] determined the concent of CI using high performance liquid chromatography. They have reported that the concent ration of bufalin, cinobufagin and resibufogenin in each 1ml CI were 0.333μg/ml, 0.159μg/ml, and 0.110μg/ml, respectively. CI has been used as an anti-tumor medication in clinical practice in China for decades. Clinical data shows CI alone or in combination with other chemotherapeutic agents significantly inhibits the proliferation and metastasis of various tumors, such as hepatocellular carcinoma, nonsmall-cell lung cancer, pancreatic cancer, and gallbladder carcinoma,[7-9] and meanwhile enhances the immunity as well.[4,10] Research *in vitro* and *in vivo* shows CI and its active compounds exhibit significant antitumor activity such as induction of cell differentiation and tumor apoptosis, disruption of the cell cycle, inhibition of cancer angiogenesis, reversal of multi-drug resistance, and regulation of the immune response.[11,12]

Fig. 1. The active compounds of cinobufacini.
(A) Bufalin. (B) Cinobufagini. (C) Resibufogenin. (D) Telocinobufagin.

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Metastasis represents a critical feature of malignant tumor and is responsible for 90% of cancer-related deaths. Lymphatic metastasis is regarded as the early stage of tumor metastasis, which involves multi-factors and multi-pathways.[13] Early inhibition of lymphatic metastasis is one of the important strategies to inhibit the capacity of cancer cells to dissemination. It has been demonstrated that lymphatic endothelial cells (LECs) in the intratumor or peritumor are involved in the formation of lymphatic tube and the tumor lymphatic metastasis.[14] However, we know little about whether LECs would influence tumor cell malignant behaviors such as proliferation, invasion and the response to medication. In the present study, to better understand the effects of CI on liver cancer lymphatic metastasis, we cultured the human hepatocarcinoma HepG-2 cells with LECs and examined the effects of CI on the proliferation, adhesion and invasion ability of HepG-2 with or without LECs. Then we evaluated the effects of CI on tumor growth and lymphatic metastasis in mouse hepatocacinoma HCa-F burden mice. Finally we investigated the mechanisms by evaluation of the expression of vascular endothelial growth factor C (VEGF-C), MMP-2 and MMP-9 in HepG-2 cells.

Materials and Methods

Ethics Statement

All studies involving mice were approved by the Ethics Committee of Dalian Medical University Laboratory Animal Center (No.2014-0078). All procedures performed in studies involving animals were in accordance with the ethical standards of the institution. All surgeries were performed under general anesthesia and efforts were made to minimize suffering to animals.

Drugs and Reagents

Cinobufacini Injection (Lot: 130402-2, 5ml/pipe, content is measured by one of the ingredients of it, indole alkaloids, as the scalar) were kindly provided by Anhui JingChan Pharmaceutical Co., Ltd (Anhui, China); Dulbecco's Modified Eagle's Medium (DMDM), 1640 medium, fetal bovine serum (FBS, Qualified), and 0.25% trypsin were purchased from Gibco (Life Technologies, USA); Dimethyl sulfoxide (DMSO) was bought in Amersco company (USA); Methyl thiazolyl tetrazolium (MTT) was purchased from Biosharp (Hefei, China); β-actin monoclonal antibody, fluorescein isothiocyanate (FITC) -labeled Goat anti-rabbit IgG (second antibody), horseradish peroxidase (HRP) -labeled Goat anti-mouse sheep IgG, goat anti-rabbit IgG-HRP were purchased from Beijing Zhongshan Jinqiao biological Co., Ltd (Lot: 111009, Beijing, China); VEGF-C mouse anti-human monoclonal antibody, MMP-2 mouse anti-human monoclonal antibody, and MMP-9 mouse anti-human monoclonal antibody were bought from the Santa cruz (USA); Matrigel was from Sigama Company (USA); Transwell was bought by the Corning Company (USA).

Cell lines

The human hepatocarcinoma cell line HepG-2 was bought from Shanghai Institutes for Biological Sciences of China Academic of Science; LECs was bought in American type culture collection (ATCC); The mouse Hca-F hepatocarcinoma cell line (established and stored by Department of Pathology, Dalian Medical University, Dalian, China) has high invasive and lymphatic metastasis potential in intro and *in vivo*.[15]

Trypan blue exclusion assay

HepG-2 cells were treated with different concentrations of CI (20, 10, and 5μg/ml) at the density of 1 x 10^5 /ml and cultured in 96-well plate (100μl), at the same time the negative group was set as control group (without drug). Cells were cultured for 24h, 48h and 72h and collected for staining with 0.4% trypan blue for 3min. Cell growth inhibition rate (CGIR) = (1- the number of viable cells in experiment group/the number of viable cells in control group) ×100%.

Co-culture system of HepG-2 cells and LECs

900ul of LECs (1 x 10^6/ml) were placed into the lower chamber of the transwell plate and cultured for 8-12h for cell adherence. 300μl of HepG-2 cells (1 x 10^5 /ml) treated with CI (20, 10, 5 and 0μg/ml) were added to the upper chamber in triplicate. After cultured for 24h, HepG-2 cells were examined for proliferation by the trypan blue exclusion assay.

Cell adhesion experiment

Preparation of LECs conditioned medium for HepG-2 cells: LECs cells were cultured in a 25 cm^2 flask and observed for their fusion. When they were 80% confluent, culture solution was discarded and cells were washed well with PBS. 3ml of serum-free medium was added, and cells were cultured at 37 ℃ for 24h, afterward, culture medium was collected and used as LECs conditioned medium (LEC-CM).

The artificial matrigel was dissolved at 4℃ overnight and diluted with serum-free medium at a ratio of 1:8 with a final concentration of 50μg/ml. 100μl per well of diluents was added to a 96-well plate and incubated for 4h at 37℃ until the diluents were condensed into gel, then kept at 4℃ overnight. The next day, the residual liquid in the culture plate was suctioned out. 50μl per well of serum-free medium was added and hydrated at 37℃ for 30min.

HepG-2 cells fusion ability. HepG-2 cells cultured in the flask were divided into two groups: one was added with LECs conditioned medium, and the other was added with serum-free DMEM. Both groups were incubated at 37℃ for 24h, and

then added with different concentrations of CI (20, 10, 5 and 0μg/ml) for pre-treatment at 37℃ for 24h. After that, 100μl per well of HepG-2 cells (1x 10^5/ml) was inoculated into 96-well plate covered with artificial matrigel (mentioned above). Five parallel wells were seted for each group, and the experiment was repeated for 3 times.

MTT assay was used to detect optical density (OD) value of cells: For OD value of adhesion cells, after 1h of planking, non-adherent cells were suctioned out carefully, 100μl of medium and 20μl of MTT (5mg/ml) were added to each well. For OD value of total cells, 100μl of medium and 20μl of MTT were added to each well. After incubation at 37℃ for 4h, supernatant was abandoned. 150μl DMSO was added for each well and the plate was shaken for 10min. Then OD value was detected at a wavelength of 570nm. The experiment is repeated for 3 times. Cell adhesion rate (%) = mean OD value of adhesion cells / mean OD value of total cells ×100%.

Cell invasion assay

50μl final artificial matrigel (mentioned above) was added to the polycarbonate microporous membrane of the transwell chamber (24-well plate with the diameter of 8μm). The experiment was performed on ice. The transwell chamber was incubated for 4h at 37℃ until the diluents were condensed into gel, then transferred into a 24-well plate and under UV radiation overnight. The residual liquid in the chamber was suctioned out and 25μl serum-free medium was added into each well and hydrated in incubator at 37℃ for 30min.

HepG-2 cells which cultured in serum-free medium for 24h were treated with different concentrations of CI (20, 10, 5 and 0μg/ml). 200μl of cells (1 x 10^6/ml) was added to the upper chamber of transwell, while 600μl of 10% FBS medium containing 1 x 10^6/ml LECs was added to the lower chamber. For another group, the condition for the upper chamber was same, but for the lower chamber, 600μl of 10% FBS medium was added. All the cells were cultured in the incubator at 37℃ for 24h. 300ul and 900ul of 4% paraformaldehyde was added into the upper and lower chambers respectively following three times of washing with PBS. After 15min in room temperature, 4% paraformaldehyde was discarded and the transwell chamber was inverted to make the lower surface of filter membrane upward and dry naturally. Then 300ul and 900ul of 0.05% crystal violet solution was added to the upper and lower chambers respectively. After 15min in room temperature, the cells were examined under inverted microscope following three times of washing with PBS, and five sections were randomly selected to count cells with dye. Unstained cells in the upper chamber were wiped out gently by cotton swab. Relative numbers of trans-membrane cells represented the cell invasive ability. The experiment was repeated for three times.

Expression of VEGF-C, MMP-2 and MMP-9 by Western blotting

After 24h treatment with different concentrations of CI (20, 10, 5 and 0μg/ml), total proteins of HepG-2 cells were extracted and the concentration was determined by Biorad. 40μg sample was loaded onto 10% SDS-PAGE electrophoresis to examine the proteins. After electrophoresis, Size-separated proteins were transferred to the PVDF membrane. PVDF membrane was probed with primary antibodies of VEGF-C (1:150), MMP-2 (1:1000), and MMP-9 (1:500) respectively at 4ºC overnight and washed by PBST. Then the membrane was incubated in PBST with goat anti-mouse IgG-HRP (1:10000) at 37ºC for 1h, and washed by PBST for 3 times. Finally substrates for HRP were added to membranes and luminous films were exposed on the X-ray for about 15min. Then it was developed. The experiment was repeated for 3 times.

***In Vivo* Tumor Metastasis Assay**

HCa-F cell line was used to examine its growth and metastasis ability *in vivo*. Eight-week-old male 615 mice (specific pathogen-free) were obtained from the Dalian Medical University Experimental Animal Center. *In vivo* tumor metastasis assay was performed as previously described.[16] Sixteen 615 mice were equally assigned into two groups. HCa-F cells (2x106, 0.05ml) were inoculated to the left footpads of the mice. After 48h, normal saline (NS, control group, 0.2ml) or CI (treated groups, 5 mg/kg, 0.2ml) was respectively injected into the abdominal cavity of each group once a day. All mice were terminated with either anesthesia and tumor was examined by size and sacrificed in the end to isolate the lymph nodes. The weight of the lymph nodes was measured by scale. The longest and shortest diameter (longest diameter:LD, shortest diameter:SD) of each tumor was measured by vernier caliper, and the tumor volume was calculated with the following formula. Tumor volume = LD × SD^2/2.

Statistical analysis

The SPSS 17.0 statistical software was used for data analysis, and normally distributed data are shown as the mean number ± standard deviation (x ± s). The data between multiple groups were compared using one-way analysis of variance, and the data between two groups were compared using two-tailed Student t test. P<0.05 was considered statistically significant.

Results

CI inhibited the LECs-induced proliferation in HepG-2 cells

First, as shown in Fig.2A, we examined the effect of LECs on the proliferation of HepG-2 cells. We found that the proliferation of HepG-2 cells was significantly greater when they were cultured with LECs than when cultured alone (153±6.5 vs 132±2.4, P<0.05) (Fig.2B). Then we examined

whether CI could inhibit the proliferation of HepG-2 cells cultured with LECs. HepG-2 cells cultured alone or with LECs were treated with different concentrations of CI (20, 10, 5 and 0μg/ml), trypan blue exclusion assay was used for evaluation of the proliferation. The results revealed that, both for HepG-2 cells cultured alone and cultured with LECs, there was significantly less HepG-2 cells per visual field in CI treated groups than the control, and showed in a dose-dependent manner (Fig.2C).

Fig.2. CI inhibited the LECs-induced proliferation in human hepatocarcinoma HepG-2 cells.

(A) HepG-2 cells decreased with the increasing concentrations of CI (5, 10, and 20μg/ml) with or without LECs after trypan blue staining.

(B) The proliferation of HepG-2 cells was significantly greater when they were cultured with LECs than when cultured alone (P<0.05).

(C) Both for HepG-2 cells cultured alone and cultured with LECs, there was significantly less HepG-2 cells per visual field in CI treated groups than the control, and showed in a dose-dependent manner (P<0.05).

CI inhibited the LECs-induced heterogeneous adhesion in HepG-2 cells

First, we examined the effect of LECs on the heterogeneous adherence ability of HepG-2 cells. We found that the adhesion rate of HepG-2 cells was significantly greater when cultured with LECs than that cultured alone (98.3±2.1 vs 88.7±2.4, P<0.05) (Fig.3A). Then we examined whether CI could inhibit the heterogeneous adherence ability of HepG-2 cells when cultured with LECs. After 24h pretreatment with CI (20, 10 and 5μg/ml), the adhesion rate of HepG-2 cells cultured alone or with LECs was observed. Results showed that both in HepG-2 cells cultured alone and cultured with LECs, there were significantly less HepG-2 cells in CI treated groups than those in the control (Fig.3B). Moreover, the adhesion rate of HepG-2 cells cultured alone and with LECs were almostly similar following treatment of CI at 5μg/ml (65.6±3.5 vs 69.6±0.6, P>0.05), 10μg/ml (61.7±1.5 vs 64.3±3.1, P>0.05) and 20μg/ml (30.1±3.1 vs 39.0±1.0, P>0.05), indicating that CI could totally block the LECs-enhanced heterogeneous adherence ability of HepG-2 cells.

Fig.3. CI inhibited the LECs-induced heterogeneous adhesion in human hepatocarcinoma HepG-2 cells.

(A) The adhesion rate of HepG-2 cells was significantly greater when cultured with LECs than that cultured alone (P<0.05).

(B) Both in HepG-2 cells cultured alone and cultured with LECs, there were significantly less HepG-2 cells in CI treated groups than those in the control (P<0.05); The adhesion rate of HepG-2 cells cultured alone and with LECs were almostly similar following treatment of CI at 5μg/ml, 10μg/ml and 20μg/ml (P>0.05).

CI inhibited the LECs-induced invasion in HepG-2 cells

Transwell chamber was used to observe *in vitro* invasion ability of HepG-2 cells cultured alone or with LECs (Fig.4A). Cells that penetrated polycarbonate microporous membrane covered by the artificial Matrigel and adhered to the lower surface of the membrane reflected the invasion ability of HepG-2 cells. We found that the numbers of HepG-2 cells per visual field that adhered to the lower surface of the membrane were significantly more when cultured with LECs than those cultured alone (108.0+2.0 vs 89.7±0.6, P<0.05) (Fig.4B). Then we examined whether CI could inhibit LECs-induced invasive ability of HepG-2 cells. After 24h pretreatment with CI (20, 10 and 5μg/ml), the numbers of HepG-2 cells per visual field adhered to the lower surface of the membrane were observed. Results showed that both for HepG-2 cells cultured alone and cultured with LECs, there was significantly less HepG-2 cells per visual field in groups treated with CI than those in control (Fig.4C). Moreover, the number of HepG-2 cells cultured alone and cultured with LECs were similar following treatment of CI at 5μg/ml (75.3±2.5 vs 71.0±1.0, P>0.05), 10μg/ml (50.7±1.1 vs 51.7±1.5, P>0.05) and 20μg/ml (32.0±2.0 vs 37.7±1.5, P>0.05), suggesting that CI could totally block the LECs-enhanced invasive ability of HepG-2 cells.

Fig. 4. CI inhibited the LECs-induced invasion in human hepatocarcinoma HepG-2 cells.

(A) HepG-2 cells decreased with the increasing concentrations of CI (5, 10, and 20μg/ml) with or without LECs in the invasion transwell assay.

(B) The numbers of HepG-2 cells were significantly more when cultured with LECs than those cultured alone (P<0.05).

(C) Both for HepG-2 cells cultured alone and cultured with LECs, there was significantly less HepG-2 cells per visual field in groups treated with CI than those in control (P<0.05); The number of HepG-2 cells cultured alone and cultured with LECs were almostly similar following treatment of CI at 5μg/ml, 10μg/ml and 20μg/ml (P>0.05).

CI reduced expression of VEGF-C, MMP-2 and MMP-9 in HepG-2 cells

HepG-2 cells were lysed following treatment with different concentrations of CI and proteins were loaded on the gel and examined by VEGF-C, MMP-2, and MMP-9 antibodies respectively, membrane was stripped and reprobed with β-actin for equal loading. Following treatment with CI (5, 10 and 20 μg/ml), the ratios of VEGF-C/β-actin (0.589±0.019, 0.263±0.022 and 0.144±0.022, respectively) were significantly lower compared with that observed in the control group (1.005±0.030; P<0.05); the ratios of MMP-2/β-actin (0.532±0.021, 0.443±0.011 and 0.146±0.015, respectively) were significantly lower compared with that observed in the control group (1.001±0.016; P<0.05); the ratios of MMP-9/β-actin (0.836±0.031, 0.310±0.054 and 0.056±0.041, respectively) were significantly lower compared with that observed in the

control group (1.033±0.089; P<0.05; Fig.5). These results suggested that CI inhibited the protein expression levels of VEGF-C, MMP-2, and MMP-9 in the HepG-2 cells, and demonstrated a dose-dependent manner.

Fig.5. CI reduced the protein expression of VEGF-C, MMP-2 and MMP-9 in human hepatocarcinoma HepG-2 cells.

(A) Protein expression levels of VEGF-C, MMP-2 and MMP-9 following treatment with different doses of CI (5, 10 and 20 μg/ml) in HepG-2 cells.

(B) Ratios of the protein expression levels of VEGF-C/β-actin. The ratios of VEGF-C/β-actin following treatment with 5, 10 and 20μg/ml CI were significantly lower compared with that of the control group (P<0.05).

(C) Ratios of the protein expression levels of MMP-2/β-actin. The ratios of MMP-2/β-actin following treatment with 5, 10 and 20μg/ml CI were significantly lower compared with that of the control group (P<0.05).

(D) Ratios of the protein expression levels of MMP-9/β-actin. The ratios of MMP-9/β-actin following treatment with 5, 10 and 20μg/ml CI were significantly lower compared with that of the control group (P<0.05).

CI Inhibited Cell Growth and Metastasis on HCa-F *In Vivo*

Hca-F is a mouse hepatocarcinoma cell line and has high lymphatic metastasis potential when inoculated subcutaneously into footpads of 615 mice [15]. We evaluated the effect of CI on tumor cell growth and metastasis using HCa-F burden 615 mice. The results showed that tumor volumes have significantly reduced in Hca-F burden 615 mice treated with CI than that without CI treatment (Fig.6A). The mean weight of metastasis lymph nodes in HCa-F burden 615 mice treated with CI was significantly lower than that without CI treatment (control group) (0.31±0.04 vs 0.48±0.12, P<0.05, Fig.6B).

Fig.6. CI Inhibited Cell Growth and Metastasis on HCa-F In Vivo.

(A) growth curve of HCa-F cells in mice treated with CI (5mg/ml) or without CI in control group. Tumor volumes were calculated and plotted at various times after engraftment as indicated on the bottom of the graph (A). Tumor volumes have significantly reduced in Hca-F burden 615 mice treated with CI than that without CI treatment.

(B) Weights of lymph nodes excised from mice treated with CI (5mg/ml) or without CI in control group. The mean weight of metastasis lymph nodes in HCa-F burden 615 mice treated with CI was significantly lower than that without CI treatment (P<0.05).

Discussion

Lymphatic metastasis is a frequent event in tumor development and is regarded as an earlier event in tumor metastasis. Recent studies showed that lymphangiogenesis driven by growth factors, such as VEGF-C and VEGF-D, play important roles in promoting tumor metastasis to lymph nodes. VEGF-C is highly expressed in various malignant tumors such as breast cancer, gastric cancer, lung cancer, cholangiocarcinoma, colorectal cancer, prostate cancer, cervical cancer, thyroid cancer and ovarian cancer,[17-23] and it is an independent factor of poor prognosis.[24] Binding of VEGF-C to VEGFR-3 expressed in LECs of intratumor and peritumor induces the proliferation and migration of LECs through the activation-induced extracellular signal-regulated kinase (ERK),[25] resulting in new lymphatic vessels that increase lymphatic diffusion approaches of tumor cells.

Besides their roles in tumor lymphangiogenesis, however, we know little about whether LECs would influence tumor cell malignant behaviors such as proliferation, invasion, and tumor response to medication. We cultured the human hepatocacinama HepG-2 cells with LECs, for the first time we demonstrated that LECs enhanced proliferation, heterogeneous adherence and invasion ability of HepG-2 cells, and CI inhibited the LECs induced proliferation, heterogeneous adherence and invasion ability of HepG-2 cells. Invasion of malignant tumor is a complex process involving multiple factors, the increase of heterogeneous adhesion ability between tumor cells and matrix is one of the key steps of the invasion, and contributes to the metastases of tumor cells.

Metastasis is the leading cause of tumor related death. A large number of reports confirm that almost 1/3 of the patients with liver cancer develop lymph nodes metastases, which is secondary to the number of pulmonary metastases.[26,27] At present, no effective medicine is available to inhibit liver cancer lymphatic metastasis. In the present study, the fact that CI totally blocked the LECs-enhanced proliferation, heterogeneous adherence and invasion ability of HepG-2 cells helped to explain its inhibition role for hepatocarcinoma lymphatic mestastasis. We adopted an immunocompetent mice model to confirm the effects of CI on proliferation and lymphatic metastasis. HCa-F is a mouse hepatocarcinoma cell line with high potential of lymphatic metastasis.[28] HCa-F cells metastasize only to lymph nodes and do not disseminate to other organs when they're inoculated into footpads of 615 mice. Preclinical testing of various human cancer therapeutic approaches is usually carried out using human cancer xenografts in nude mice. But these models do not mimic the normal immunocompetent host. Specifically immune-mediated response cannot be tested effectively in nude mice models. We found that CI inhibited HCa-F cells proliferation and lymphatic metastasis in the immunocompetent mice.

As the vital subtypes of MMPs family, MMP-2 and MMP-9 are involved in the degradation of extracellular matrix (ECM) and play important roles in tumor invasion and metastasis.[29,30] It has been suggested that down regulation of MMP-2 and MMP-9 can significantly inhibit the malignant degree of tumor cells.[31,32] Shi Dongmei, et al. found that CI of 2.5 mg/ml can significantly reduce the expression of MMP-2 in ovarian cancer

3AO cells, thus inhibiting the invasive growth of 3AO cells.[33,34] Our data demonstrated that CI reduced the expression of MMP-2 and MMP-9 in HepG-2 cells. In the present study, it was also observed that CI could reduce the expression of VEGF-C in HepG-2 cells. The inhibition degree was positively correlated with the drug concentration, indicating that influencing the expression of VEGF-C, MMP-2, and MMP-9 in HepG-2 cells was one of the mechanisms by which CI inhibited the tumor metastasis through the lymphatic system. That may account for the mechanism of CI. In summary, our data strongly supported that CI had anti-proliferation and lymphatic metastasis effects on hepatocarcinoma cells and would provide cancer patients with an alternative treatment.

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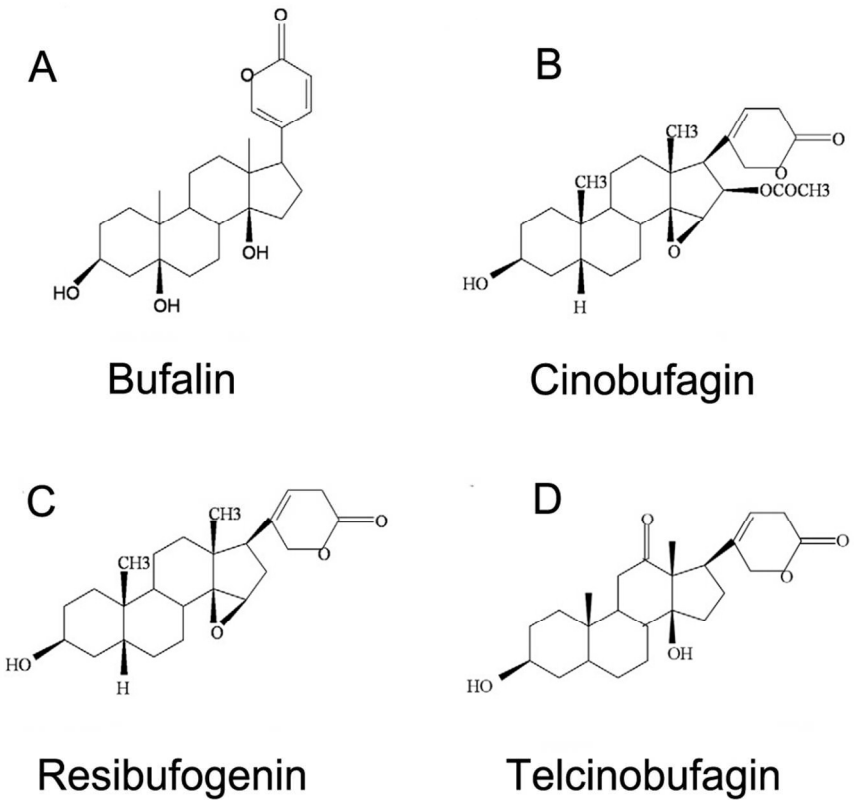

Fig. 1. The active compounds of cinobufacini.
(A) Bufalin. (B) Cinobufagini. (C) Resibufogenin. (D) Telocinobufagin.

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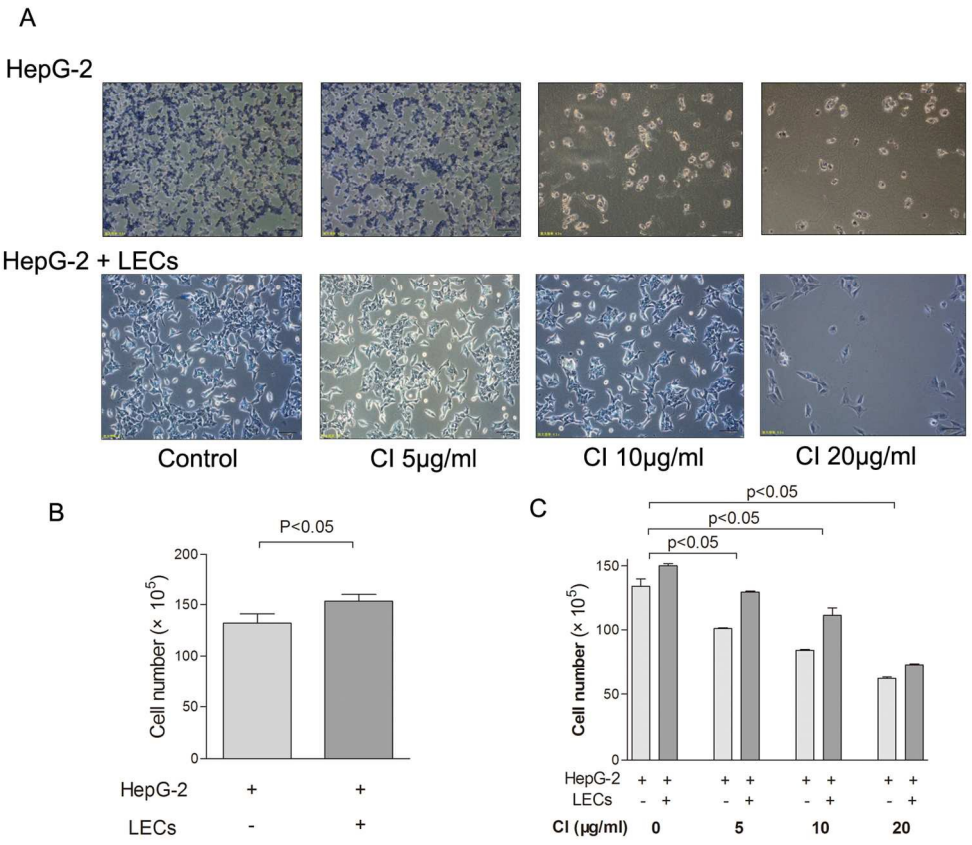

Fig.2. CI inhibited the LECs-induced proliferation in human hepatocarcinoma HepG-2 cells.
(A) HepG-2 cells decreased with the increasing concentrations of CI (5, 10, and 20μg/ml) with or without LECs after trypan blue staining.
(B) The proliferation of HepG-2 cells was significantly greater when they were cultured with LECs than when cultured alone ($P<0.05$).
(C) Both for HepG-2 cells cultured alone and cultured with LECs, there was significantly less HepG-2 cells per visual field in CI treated groups than the control, and showed in a dose-dependent manner ($P<0.05$).

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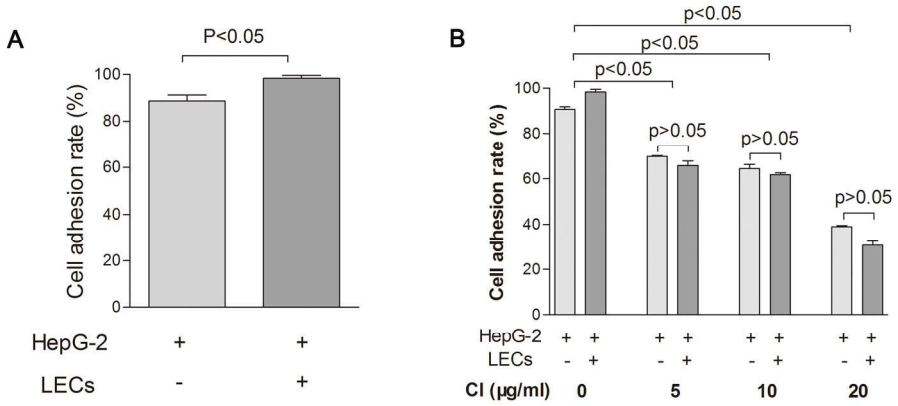

Fig.3. CI inhibited the LECs-induced heterogeneous adhesion in human hepatocarcinoma HepG-2 cells. (A) The adhesion rate of HepG-2 cells was significantly greater when cultured with LECs than that cultured alone ($P<0.05$).
(B) Both in HepG-2 cells cultured alone and cultured with LECs, there were significantly less HepG-2 cells in CI treated groups than those in the control ($P<0.05$); The adhesion rate of HepG-2 cells cultured alone and with LECs were almostly similar following treatment of CI at 5μg/ml, 10μg/ml and 20μg/ml ($P>0.05$).

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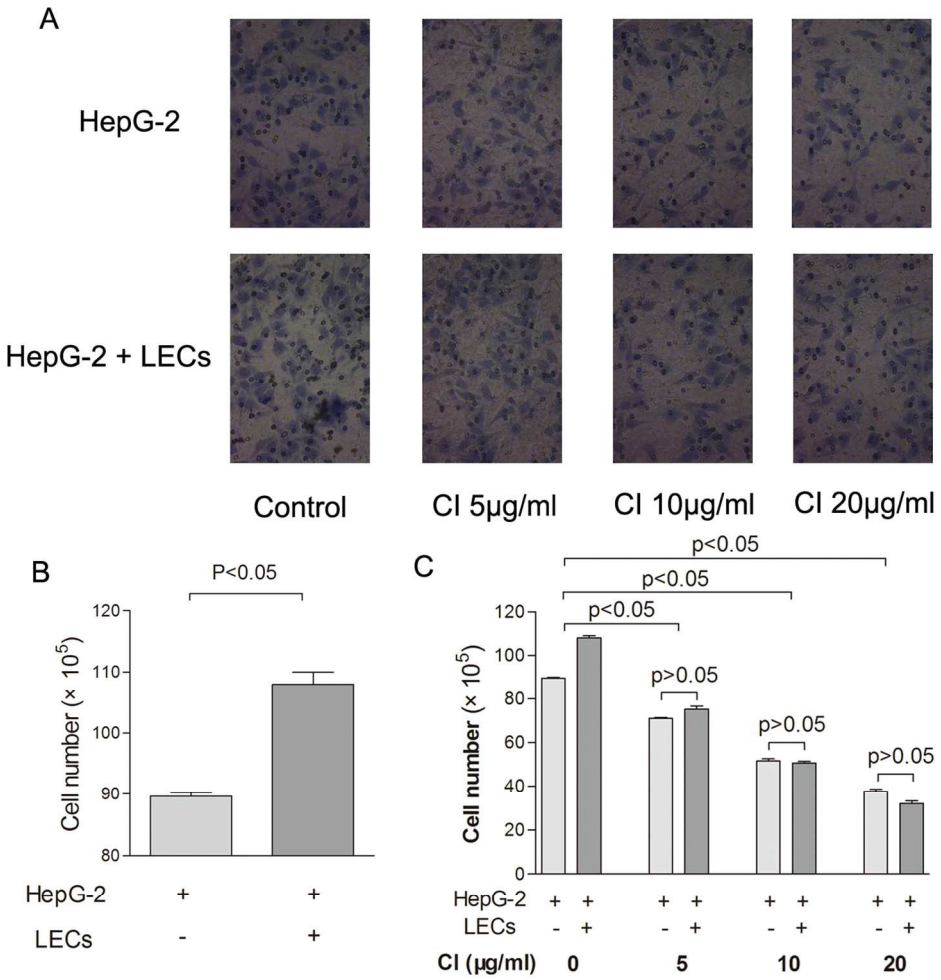

Fig. 4. CI inhibited the LECs-induced invasion in human hepatocarcinoma HepG-2 cells.
(A) HepG-2 cells decreased with the increasing concentrations of CI (5, 10, and 20μg/ml) with or without LECs in the invasion transwell assay.
(B) The numbers of HepG-2 cells were significantly more when cultured with LECs than those cultured alone ($P<0.05$).
(C) Both for HepG-2 cells cultured alone and cultured with LECs, there was significantly less HepG-2 cells per visual field in groups treated with CI than those in control ($P<0.05$); The number of HepG-2 cells cultured alone and cultured with LECs were almostly similar following treatment of CI at 5μg/ml, 10μg/ml and 20μg/ml ($P>0.05$).

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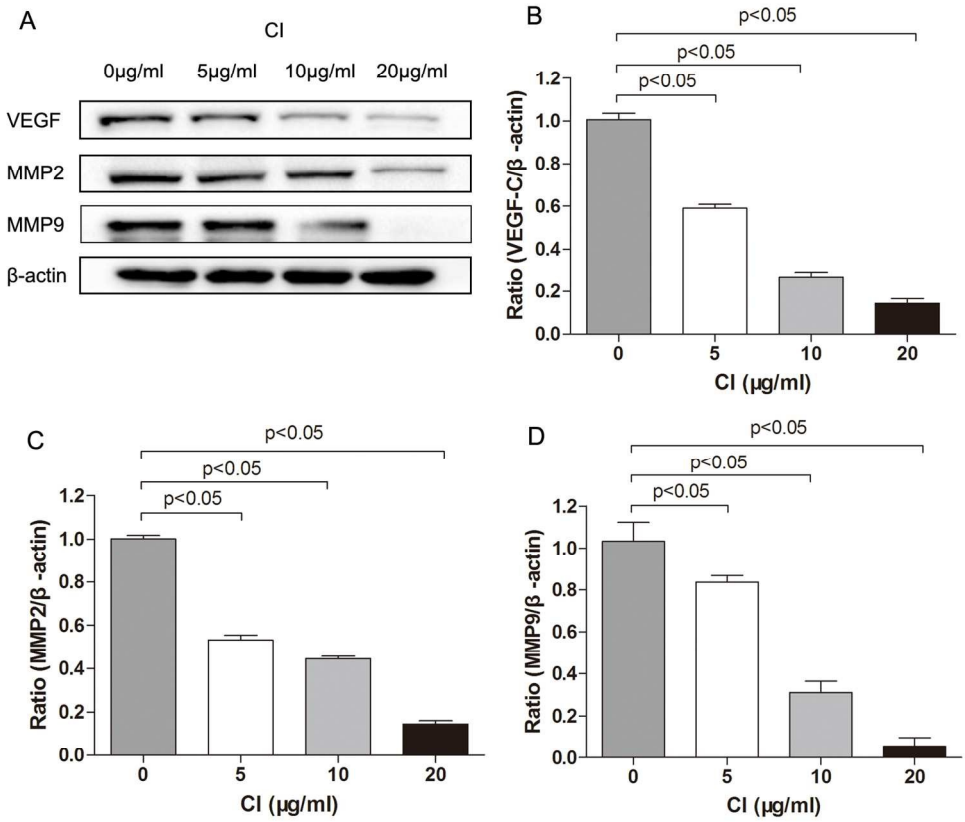

Fig.5. CI reduced the protein expression of VEGF-C, MMP-2 and MMP-9 in human hepatocarcinoma HepG-2 cells.
(A) Protein expression levels of VEGF-C, MMP-2 and MMP-9 following treatment with different doses of CI (5, 10 and 20 μg/ml) in HepG-2 cells.
(B) Ratios of the protein expression levels of VEGF-C/β-actin. The ratios of VEGF-C/β-actin following treatment with 5, 10 and 20μg/ml CI were significantly lower compared with that of the control group ($P<0.05$).
(C) Ratios of the protein expression levels of MMP-2/β-actin. The ratios of MMP-2/β-actin following treatment with 5, 10 and 20μg/ml CI were significantly lower compared with that of the control group ($P<0.05$).
(D) Ratios of the protein expression levels of MMP-9/β-actin. The ratios of MMP-9/β-actin following treatment with 5, 10 and 20μg/ml CI were significantly lower compared with that of the control group ($P<0.05$).

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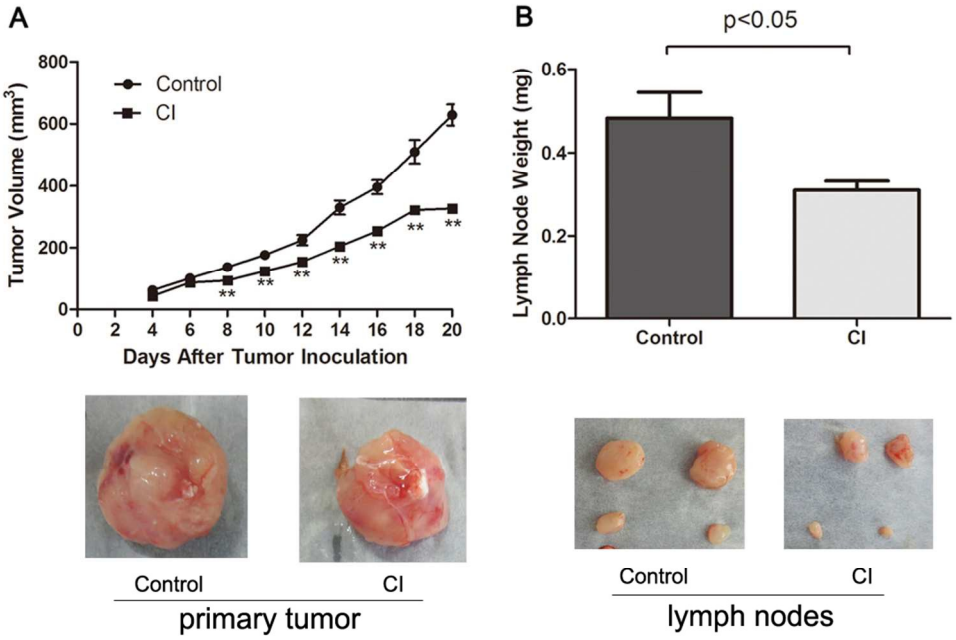

Fig.6. Effects of CI on HCa-F Cell Growth and Metastasis In Vivo.
Mouse hepatocarcinoma Hca-F cells were implanted into footpads of mice and examined for growth (A) and metastasis (B). Figure A depicted the growth curve of HCa-F cells in mice treated with CI (5mg/ml) or without CI in control group. Tumor volumes were calculated and plotted at various times after engraftment as indicated on the bottom of the graph (A). Weights of lymph nodes excised from mice treated with CI (5mg/ml) or without CI in control group as indicated on the bottom were represented by the height of each bar in the graph (B). The error bars provided the S.E.M. of 4 mice. P indicated the statistical significance as assessed by Student's t test.

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