Effects of cinobufacini injection on cell proliferation and the expression of topoisomerases in human HepG-2 hepatocellular carcinoma cells

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gargarizans Cantor from the family Bufonidae (1). It has been approved by the Chinese State Food and Drug Administration (ISO9002) as a medicine for tumor therapy (2). Since the 1970s, it has been widely used in tumor therapy and clinical data has demonstrated that cinobufacini injection exhibits efficacy in various types of cancer, including liver, stomach, pancreatic, esophageal (3) and lung cancer. Additionally, it can reverse multidrug resistance (4), particularly in non-responsive cancer, including liver cancer. Cinobufacini injection demonstrates efficacy in a synergistic action when combined with conventional chemotherapy and radiotherapy (5). The major pharmacological constituents of cinobufacini are bufodienolides, which include bufalin, cinobufagin, resibufogenin, bufotalin and lumichrome, alkaloids, biogenic amines, peptides and proteins (6,7).

Hepatic carcinoma is one of the most common types of malignant tumor worldwide. The response of hepatic carcinoma to chemotherapy is known to be <20% (8). Targeted drugs, including sorafenib, demonstrate only a mild increase in the survival rates of patients with hepatic carcinoma. Therefore, an efficient drug for the treatment of hepatic carcinoma is required (9,10).

Clinical practice has indicated that cinobufacini injection has beneficial effects in patients with liver cancer in the control of tumour progression, improvement of signs and symptoms, and enhanced quality of life. However, the molecular mechanisms underlying cinobufacini injection remain to be fully elucidated.

Topoisomerase (TOPO), including TOPO I and TOPO II, are key enzymes in the regulation of nucleic acid topology configurations, and topoisomerases have become important targets for antitumor drugs (11-13). However, there have been no previous reports demonstrating the association between cinobufacini and topoisomerases. Therefore, the present study aimed to investigate the effects of cinobufacini injection on the proliferation, apoptosis, cell cycle arrest, and TOPO I and TOPO II expression levels in human HepG-2 hepatocarcinoma cells.

Materials and methods

Drugs and reagents. Injectable cinobufacini (cat. no. 100207-1), the content of which was measured by using one of its components, indole alkaloids, as a scalar,
was provided by Anhui Jingchan Pharmaceutical Co., Ltd (Anhui, China). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from BioSharp (Hefei, China) and an annexin V (AV) kit was purchased from Molecular Probes (Life Technologies, Carlsbad, CA, USA). A reverse transcription quantitative-polymerase chain reaction (RT-qPCR) kit and PCR amplification primers were purchased from Takara Bio, Inc. (Dalian, China). Mouse anti-human TOPO I monoclonal antibody (cat. no. sc-32736) and mouse anti-human TOPO II monoclonal antibody (cat. no. sc-166934) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), and horseradish peroxidase-labeled rabbit anti-mouse immunoglobulin (Ig) G was purchased from Beijing Zhongshan Jinqiao Biological Biotechnology Co., Ltd. (Beijing, China).

**Cell lines and cultures.** The human HepG-2 hepatocarcinoma cell line was provided by the Central Laboratory of The First Affiliated Hospital of Dalian Medical University (Dalian, China). The cells were incubated in low-carbohydrate Iscove's modified Dulbecco's medium (Gibco Life Technologies, Grand Island, NY, USA), supplemented with 10% fetal bovine serum (Gibco Life Technologies) and cultivated in a humidified atmosphere with 5% CO$_2$ at 37°C. The culture media was replaced every second day, and the cells were digested and passed using 0.25% trypsin (Gibco Life Technologies). When the growth of the HepG-2 cells was at ~90% confluence and a monolayer had formed, the cells in the logarithmic growth phase were collected for use in the subsequent experiments.

**MTT assay for cell proliferation.** Cell proliferation was evaluated using an MTT colorimetric assay. The HepG-2 cells in the logarithmic growth phase were seeded at a density of 5,000 cells/well in 96-well plates and incubated in a CO$_2$ incubator at 37°C to allow the cells to adhere. Following incubation for 24 h in culture medium, different concentrations of cinobufacini (0.105, 0.21 and 0.42 mg/l) were added to the treated group via injection, and the cells were incubated for an additional 24, 48 and 72 h at 37°C. For each drug concentration, eight repeat wells were used. Cells without cinobufacini were used as the control group for each plate. Following incubation, 20 µl MTT reagent (5 g/l) was added to each well and the cells were incubated for 4 h at 37°C. The medium and MTT test was subsequently removed and 150 µl dimethylsulfoxide (Amresco, Waltham, MA, USA) was added to each well, and the plates were incubated at room temperature to dissolve the water-insoluble formazan. Finally, the absorbance (A) was measured at 570 nm using an optical spectrophotometer (BioSharp). The inhibitory rate (IR) was calculated using the following formula: IR% = \((1 - \frac{A_{\text{treated}}}{A_{\text{control}}}) \times 100\)%

**RT-qPCR assays to assess the mRNA expression levels of TOPO I and TOPO II.** The cells in the logarithmic growth phase (5x10$^5$ cells/well) were seeded into 24-well plates and cultured for 48 h. Cinobufacini injections at different concentrations (0, 0.105, 0.21 and 0.42 mg/l) were added to each group. Following incubation for 48 h, the cells were collected. The total cellular RNA was extracted using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions, and the concentration of total RNA was measured using a spectrophotometer (ND2000; Thermo Fisher Scientific, Waltham, MA, USA). Deionized water was added to the RNA samples to result in a final concentration of 500 ng/µl. The total RNA from each sample was reverse-transcribed using a Takara RNA PCR kit (AMV) Ver3.0 (Takara Biotechnology Co., Ltd.). The resulting cDNA was then amplified by qPCR. The following primers were used: TOPO I, forward 5'-CGCTATCTGGAAGGTGGAC-3' and reverse 5'-GAATCCATTGTAGATGG-3'; TOPO II, forward 5'-CTGGAGGAGGAGAAGGAACC-3', and reverse 5'-CTGGAGGAGGAGAAGGAACC-3'. The qPCR reaction conditions for TOPO I and TOPO II were as follows: Predenaturation at 94°C for 5 min; 10 cycles of denaturation at 94°C for 30 sec
Western blot analysis to determine the protein expression levels of TOPO I and II. The cells in the logarithmic growth phase were seeded into four 25 cm² culture flasks at a density of 5x10⁴ cells/ml, and incubated for 24 h. Following incubation, the cells were treated with cinobufacini injection at different concentrations (0, 0.105, 0.21 and 0.42 mg/l) for 24 h, washed three times with ice-cold PBS and lysed with prepared lysis buffer (Keygen Biotech. Co., Ltd.) for 30 min on ice. The debris was subsequently removed by centrifugation at 12,000 x g for 12 min at room temperature. The protein concentrations of the supernatants were determined using the Bradford method (14,15). Equal quantities of protein (50 µg/well) were subjected to electrophoresis using sodium dodecyl sulfate-polyacrylamide gel (Keygen Biotech. Co., Ltd.) electrophoresis (5% stacking gel + 8% separating gel), which were then transferred onto polyvinylidene difluoride membranes (Sigma-Aldrich, St. Louis, MO, USA). The membranes were incubated in blocking solution (5% skim milk; BD Biosciences, Franklin Lakes, NJ, USA) for 1 h and were subsequently incubated overnight with mouse monoclonal anti-human primary antibody against TOPO I or TOPO II at 4°C. The membrane was washed three times with Tris-buffered saline (10 mM Tris-HCl, 0.15 M NaCl and 8 mM sodium azide), containing 0.05% Tween-20 (TBST; Solarbio Science & Technology Co., Ltd., Beijing, China), and incubated with horseradish peroxidase-labeled rabbit anti-mouse IgG secondary antibody for 1 h at room temperature. Following incubation, the membrane was washed with TBST three more times. The protein bands were visualized on Kodak X-ray film using an enhanced chemiluminescence system (Amersham; GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). The intensity ratios of the bands, compared with those of the control were analyzed using ImageJ v2.1.4.7 software (National Institutes of Health, Bethesda, MA, USA). The experiments were repeated three times.

Statistical analysis. SPSS 11.5 statistical software (SPSS, Inc., Chicago, IL, USA) was used for data analysis, and the normally distributed data are expressed as the mean ± standard deviation. The data between multiple groups were compared using one-way analysis of variance. P<0.05 was considered to indicate a statistically significant difference.

Results

Cinobufacini injection decreases the viability and growth of HepG-2 cells. The effect of cinobufacini on the viability of HepG-2 cells was determined using an MTT assay. As shown in Fig. 1, the proliferation rate of the cells was affected by the concentration of cinobufacini and the duration of treatment. The cinobufacini injection significantly decreased the growth of the HepG-2 cells compared with the control group (0.86±0.03, 0.08±0.01 and 0.04±0.06 mg/l for 24, 48 and 72 h, respectively), and the inhibitory effect was dose- and time-dependent. The data are expressed as the mean ± standard deviation.

Cinobufacini injection induces dose- and time-dependent apoptosis in HepG-2 cells. The effect of cinobufacini injection on the apoptosis of HepG-2 cells was assessed using AV/PI staining. Following treatment with different concentrations (0, 0.105, 0.21 and 0.42 mg/l) of cinobufacini for 24 h and 48 h, AV/PI staining was performed to quantify the percentage of apoptotic cells in the total cell population using flow cytometry. The percentages of apoptotic cells following injection with 0.42, 0.21 and 0.105 mg/l of cinobufacini were 35.35±1.87, 27.52±1.22 and 17.51±1.03% following 24 h treatment, and 67.87±1.49, 59.22±2.65 and 37.02±1.58% following 48 h treatment, respectively. These values were significantly higher compared with those of the control group following 24 h (1.25±0.53%; P<0.05) and 48 h (1.25±0.53%; P<0.05) treatment and demonstrated dose- and time-dependency (Fig. 2). These data suggested that cinobufacini injection effectively induced dose- and time-dependent apoptosis in the HepG-2 cells.

Cinobufacini injection induces cell cycle arrest at the S phase in HepG-2 cells. Following treatment with cinobufacini injection at different concentrations (0, 0.105, 0.21 and 0.42 mg/l) for 24 h, the cell cycle distributions of the HepG-2 cells were analyzed using flow cytometry. The percentages of cells in S phase were 24.85±0.85, 31.46±0.98 and 42.66±1.12% following 24 h treatment with 0.105, 0.21 and 0.42 mg/l cinobufacini injection, respectively. All these values were markedly higher compared with the control.
Cinobufacini injection inhibits the mRNA expression levels of TOPO I and TOPO II in HepG-2 cells. Following treatment with cinobufacini injection at different concentrations (0.105, 0.21 and 0.42 mg/l) for 48 h, a dose-dependent decrease in the mRNA expression levels of TOPO I and TOPO II was observed in the HepG-2 cells, as shown in Fig. 4. The ratios of TOPO I/β-actin following treatment with 0.105, 0.21 and 0.42 mg/l cinobufacini (0.73±0.04, 0.55±0.02 and 0.46±0.03, respectively) were significantly lower compared with that observed in the control group (0.91±0.04; P<0.05), and the ratios of TOPO II/β-actin (0.94±0.05, 0.79±0.03 and 0.44±0.02, respectively) were significantly lower compared with the control group following 24 h treatment (19.16±0.76%; P<0.05), which suggested that treatment with cinobufacini led to an accumulation of HepG-2 cells in the S phase and demonstrated dose-dependency (Fig. 3). This result suggested that cinobufacini injection effectively induced cell cycle arrest at the S phase in the HepG-2 cells.
with that observed in the control group (1.041±0.06; P<0.05). These results demonstrated that cinobufacini injection inhibited the transcription of TOPO I and TOPO II in the HepG-2 cells, which was observed to occur in a dose-dependent manner.
**Cinobufacini injection inhibits the protein expression levels of TOPO I and TOPO II in HepG-2 cells.** Following treatment with cinobufacini injection (0.105, 0.21 and 0.42 mg/l), the ratios of TOPO I/β-actin (0.954±0.047, 0.816±0.031 and 0.592±0.036, respectively) were significantly lower compared with that observed in the control group (1.157±0.045; P<0.05), and the ratios of TOPO II/β-actin (0.967±0.017, 0.897±0.027 and 0.667±0.015, respectively) were significantly lower compared with that observed in the control group (1.047±0.043; P<0.05; Fig. 5). These results suggested that cinobufacini injection inhibited the protein expression levels of TOPO I and TOPO II in the HepG-2 cells, and that this occurred in a dose-dependent manner.

**Discussion**

Clinical trials since the 1970s have demonstrated that cinobufacini injection exhibits high efficacy against hepatocarcinoma (16) and it has been used in Phase II clinical trials at the MD Anderson Cancer Center (Houston, USA) (17). Previous clinical studies have indicated that cinobufacini injection exhibits high efficacy against tumor tissues, either alone or in combination with conventional treatment, including chemotherapy and radiotherapy. Additionally, cinobufacini increases physical immunity and improves the quality of life of patients (18). Preliminary studies have demonstrated that cinobufacini injection can induce tumor cell apoptosis, regulate the expression of tumor-associated genes and inhibit tumor angiogenesis and metastasis (19-22). However, to the best of our knowledge, no previous studies have reported the impact of cinobufacini injection on topoisomerases.

Mammalian topoisomerases are enzymes, which are important in regulating the breakage and religation of DNA, providing swivel points for the transcription and replication of DNA, and facilitating the segregation of chromatids prior to mitosis (23). They are classified as TOPO I and TOPO II. TOPO I introduces single strand breaks in the DNA, whereas TOPO II causes transient breaks in pairs of DNA strands (24). Previous studies have demonstrated that the content and activity of topoisomerases in tumor cells is significantly higher than in normal somatic cells. The content of TOPO I is significantly higher in leukemia, colon cancer, cervical cancer and ovarian cancer (25-27), compared with normal cells. TOPO II is highly expressed in laryngeal squamous cell carcinoma, Hodgkin's lymphoma, diffuse large B-cell lymphoma and breast cancer (28). The independent contribution of each enzyme to the regulation of nucleic acid topology configuration is often difficult to assess (29). The fact that topoisomerases are a key requirement in the mammalian cell division cycle makes them important targets for cancer chemotherapy (23). Inhibitors of DNA topoisomerase, including Hydroxycamptothecine, topotecan, irinotecan, etoposide and teniposide, are widely used as clinical anticancer drugs (30).

In the present study, the effects of cinobufacini injection on the expression of topoisomerases in human hepatocarcinoma HepG-2 cells were investigated. The results indicated that cinobufacini injection significantly inhibited HepG-2 cell proliferation, induced apoptosis and cell cycle arrest at the S phase and downregulated the mRNA and protein expression levels of TOPO I and TOPO II in a dose-dependent manner. The S phase is the phase of the cell cycle in which DNA is replicated, occurring between the G1 and G2 phases. Topoisomerases are significant during the S phase, and the expression of topoisomerases are key in DNA replication. Therefore, topoisomerases interfere with the replication of DNA in the S phase and induce cell cycle arrest at this phase, which may lead to apoptosis. As the S phase is the stage at which DNA replication occurs, and topoisomerase is the key enzyme regulating nucleic acid topology, it is important in DNA replication during the S phase (31). Decreased expression levels of TOPO I and TOPO II may interfere with DNA synthesis, thereby inducing cell arrest in the S phase. If the accumulated cells at the S phase fail to pass the S/G2 checkpoint, then the cells may enter the apoptotic program (32,33). Therefore, the results of the present study demonstrated that cinobufacini injection induced HepG-2 arrest at the S phase and initiated the apoptotic program by interfering with the expression levels of TOPO I and TOPO II. Therefore, cinobufacini offers a promising drug for the treatment of hepatic carcinoma.

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**References**


