

Mechanism of Cryptotanshinone Targeting HK2 to Induce Apoptosis in Mouse Hepatoma H22 Cells

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Abstract

Objective: To observe the anti-liver cancer effect of cryptotanshinone (CTS) in vitro, and to explore the mechanism of targeting hexokinase 2 (HK2) to regulate glycolytic pathway and induce apoptosis in hepatoma carcinoma cells. **Methods:** In in vitro cell experiments, CCK-8 method was used to detect the effects of different concentrations (1, 2, 5, 10, 20, 40, 80, 100, 200 $\mu\text{mol/L}$) of CTS on the viability of H22 cells at different time periods (24 h/48 h). The effects of different concentrations (5, 10, 20 $\mu\text{mol/L}$) of CTS on glucose uptake, lactate production, apoptosis, and the expressions of HK2, Bcl-2 and Cyt-c proteins in H22 cells were detected using GOD-POD method, lactate oxidase method, Annexin V-FITC/PI double staining method and Western blot, respectively, after 48 h of intervention. **Results:** CTS significantly inhibited the proliferation of H22 cells in a concentration- and time-dependent manner, with a 48-hour half-inhibitory concentration (IC_{50}) of 24.43 $\mu\text{mol/L}$. CTS suppressed glucose uptake and lactate production in H22 cells, suggesting that it attenuated the energy metabolism of tumor cells by inhibiting the glycolytic pathway. CTS down-regulated the expressions of HK2 and Bcl-2 proteins, and up-regulated the expression of Cyt-c protein, implying that CTS induced apoptosis in hepatoma cells by repressing the HK2-mediated glycolysis pathway and regulating the mitochondrial apoptosis pathway. **Conclusion:** CTS evidently induces apoptosis in H22 cells by inhibiting the HK2-mediated glycolysis pathway and regulating the mitochondrial apoptosis pathway, which has potential anti-liver cancer effects.



1 Introduction

Liver cancer, as one of the life-threatening diseases, has high morbidity and mortality. In China, the incidence and mortality rates of primary hepatocellular carcinoma (HCC) remain persistently high, and those of liver cancer rank the 5th and 2nd in the world respectively, in 2020, severely impacting health of Chinese people and bringing a heavy economic burden on family and society [1]. In recent years, the incidence of liver cancer in China is increasing annually, higher in males than females; the patient population is also becoming younger, turning into a public health problem that cannot be underestimated [2].

Against the backdrop of the current lack of effective systemic treatment methods, surgical resection and liver transplant are the primary clinical treatment methods, with more than 50% of HCC patients experiencing post-surgical recurrence and metastasis and a 5-year survival rate below 20% [3]; and, these methods are not applicable to patients with advanced HCC. For the majority of advanced HCC patients who are ineligible for surgical treatment, comprehensive therapy and molecular targeted therapy are advocated in clinical practice. Sorafenib, lenvatinib, and regorafenib serve as the principal targeted agents in hepatocellular carcinoma management [4]. Their combination with conventional drugs such as 5-fluorouracil, cisplatin, and gemcitabine yields some therapeutic benefits, but the prognosis often remains poor and the underlying mechanisms are still unclear. Consequently, identifying safer and more effective anti-liver cancer agents has become increasingly urgent in clinical practice.

Previous studies revealed that the additional application of traditional Chinese medicine (TCM) has achieved better outcomes in treating liver cancer [5], and TCM can inhibit the growth and proliferation of liver cancer cells via multiple pathways [6]. As confirmed, TCM can suppress the further progression

of liver cancer by repressing cell proliferation, inducing cell apoptosis, regulating immunity, and improving the tumor inflammatory microenvironment, thereby relieving patient symptoms, prolonging survival, and reducing mortality. This is an indispensable method in the comprehensive treatment of liver cancer, indicating that TCM has unique advantages in treating liver cancer. Screening natural active ingredients with anti-cancer effects is of great significance for the development of new anti-liver cancer drugs [7]. In recent years, the slow progress in the research and development of TCM and its active ingredients for the treatment of liver cancer is mainly due to the lack of basic research on the mechanism of TCM's anti-liver cancer effect, and the unclear targets of the active ingredients.

Disorder of cellular energy metabolism is the main feature of cancer cells [8,9]. Aerobic glycolysis (the Warburg effect) supplies the material basis required for the rapid, sustained proliferation and metastasis of tumor cells. Hexokinase-2 (HK2), the key rate-limiting enzyme of the glycolytic pathway, catalyzes the initial step of glycolysis to convert glucose to glucose-6-phosphate (G-6-P), thereby promoting aerobic glycolysis [10]. HK2 is usually not expressed or lowly expressed in normal tissues, and is specifically overexpressed in tumor cells. It can enhance the glycolysis pathway, bind and protect mitochondria, and boost autophagy in glucose-deficient states to ensure the preferential survival of tumor cells [11]. The inducible expression characteristic confers a safety advantage to anti-tumor drugs targeting HK2. HK2 has been identified as a core participant in the Warburg effect and has been proposed as a metabolic target for cancer therapy development [12].

Cryptotanshinone (CTS) is a lipid-soluble quinone compound extracted and isolated from the dried roots and rhizomes of *Salvia miltiorrhiza* Bge belonging to Lamiaceae. It can exert anti-tumor effects by

inhibiting angiogenesis, suppressing cell proliferation, and inducing cell apoptosis [13,14]. Research has shown that CTS can stimulate apoptosis in Hepal-6 liver cancer cells and dampen the growth of subcutaneous transplanted tumor cells in mice [15], demonstrating significant anti-liver cancer effects of CTS *in vitro* and *in vivo*. CTS participates in inhibiting glycolysis and promoting apoptosis in tumor cells. Existing studies have found that CTS can induce apoptosis of tumor cells such as MHCC97-H and Huh7 by inhibiting the PI3K/AKT pathway [16]. The PI3K/AKT signaling pathway is a classic pathway that can regulate various biological behaviors including glucose metabolism, cell apoptosis, and autophagy, and it has been extensively proved to be closely related to the occurrence and development of HCC [17,18]. The activity of HK2 is regulated by the PI3K/AKT pathway, which participates in the anti-apoptotic process of tumor cells. Previous studies have found that CTS can inhibit HK2 expression in liver cancer cells, suggesting that the role of CTS in inducing apoptosis in liver cancer cells may be pertinent to the regulation of HK2 activity and its mediation of glycolysis processes, but the specific regulatory mechanism still needs further clarification. Hence, this study conducted *in vitro* cell experiments to detect the effect of CTS treatment on the viability, glucose uptake, and lactate production of mouse liver cancer cells H22, as well as expressions of proteins related to cell apoptosis rate and mitochondrial apoptosis, with the aim of providing a basis for the discovery of new targeted drugs of CTS active ingredients against liver cancer in the future.

2 Materials

2.1 Drugs

CTS standards (MUST- 14030711, Chengdu Institute of Biology, Chinese Academy of Sciences; B21586- 20 mg, Shanghai Yuanye Biotechnology Co., Ltd., China).

2.2 Reagents and Materials

Mycoplasma-free fetal bovine serum (11012-8611, Hangzhou Tianhang Biotechnology Co., Ltd.); RPMI1640 medium (C11875500BT, Thermo Fisher, USA); PageRuler™ Prestained Protein (26616, Thermo Fisher, USA); RIPA lysis buffer (P0013B), WB Rapid Membrane Transfer Buffer (P0575-10L), 10% SDS-PAGE Gel Fast Preparation Kit (P0012AC), SDS-PAGE Fast Electrophoresis Buffer (P0562-10L), PMSF (ST506), Protease & Phosphatase Inhibitor Cocktail (P1050), CCK-8 Kit (C0037), and Annexin V-FITC/PI Apoptosis Detection Kit (C1062S) (Beyotime Biotechnology Co., Ltd., Shanghai); 1 × PBS Buffer (BL302A), Penicillin-Streptomycin Solution (100X, Dual Antibiotic, BL505A), Ultra-Sensitive ECL Chemiluminescent Substrate Developer (BL520A), Dimethyl Sulfoxide (DMSO, BL165B), and Glucose Assay Kit (GOD-POD Oxidase Method, BL863B) (Beijing Lanjiek Technology Co., Ltd.); Skimmed Milk Powder (S30865, Shanghai Yuanye Biotechnology Co., Ltd.); 6-well Cell Culture Plate (11110), 12-well Cell Culture Plate (11210) and 96-well Cell Culture Plate (11150) (LABSELECT Company); Omni-Easy™ Instant Protein Loading Buffer (Denaturing, Reducing, 5 ×, LT101, Shanghai Yamei Biomedical Technology Co., Ltd.); CheKine™ Lactate Assay Kit (KTB1100, Wuhan Yakeyin Biotechnology Co., Ltd.); Tween 20 (EZ7890B2253, Guangzhou Saiguo Biotechnology Co.); HK2 Antibody (22029-1-AP, Wuhan Sanying Technology Co., Ltd.); Bcl-2 Antibody (ab196495, Abcam, UK); Cytochrome c (Cyt-c) Antibody (ET1610-60) and GAPDH Internal Reference (R1210-1) (Hangzhou Hua'an Biotechnology Co., Ltd.).

2.3 Cell line

The mouse liver cancer cell line H22 was provided by the Laboratory Animal Center of Zhejiang Chinese Medical University.

2.4 Instruments

CO₂ incubator (3111, Thermo Fisher, USA); Biosafety cabinet (Mars-1200, LaboGene, Denmark);

Inverted microscope (Ts-2, Nikon, Japan); Refrigerated centrifuge (ST16R, Thermo Fisher, USA); Multifunctional microplate reader (ELX800, Bio-Tek, USA); Constant temperature metal bath (CHB-100, Shanghai Kehuai Instrument Co., Ltd.); Chemiluminescence imaging system (ChemiDOC XRS+, Bio-Rad, USA); Protein electrophoresis and transfer system (1659033, Bio-Rad, USA); Benchtop high-speed refrigerated centrifuge (Stratos, Thermo, USA); Digital display constant temperature water bath (HH-2, Changzhou Zhiborui Instrument Manufacturing Co., Ltd.); Flow cytometer (C6, BD, USA); Electronic balance (FD-C6002, Shenzhen Feiya Weighing Apparatus Co., Ltd.); Ultrasonic cleaner (KQ-500B, Kunshan Ultrasonic Instrument Co., Ltd.).

3 Methods

3.1 Preparation and dissolution of CTS solution

An appropriate amount of CTS was slowly added to DMSO, followed by thorough vortexing. After sealing with sealing film, the mixture was sonicated in warm water for 30 min to prepare a high-concentration stock solution. The stock solution was aliquoted and stored at -30 °C. Later, it was diluted with 1640 complete medium according to the required specifications.

3.2 Cell thawing and culture

The water bath was preheated to 37 °C, and the 1640 complete medium containing 10% fetal bovine serum and 1% penicillin-streptomycin solution was also placed in the water bath for preheating. Using forceps, the hepatocarcinoma H22 cells were retrieved from the cryostorage box and rapidly thawed in the preheated water bath. During thawing, the cryovial was gently shaken. The cell suspension was completely thawed and transferred to a centrifuge tube, and 5 ml of complete medium was added for centrifugation (800 rpm, 5 min) to thoroughly remove residual DMSO from the cell suspension. After centrifugation, an appropriate amount of complete

medium was added, and gently pipetted to mix evenly. The cell suspension was then transferred to a culture flask and incubated in a constant temperature incubator at 37 °C with 5% CO₂.

After 2 to 3 days of culture, the cell growth morphology and medium turbidity were observed under an inverted microscope. When cell density reached 80%, cell passage was performed. In brief, the cell suspension in the flask was transferred to a centrifuge tube and centrifuged (800 rpm, 5 min). Then, the supernatant was discarded. An appropriate amount of complete medium was added and gently mix evenly with a pipette. The cell suspension was evenly distributed into 2 to 3 new culture flasks, with additional complete medium added as needed. After cell density and growth morphology were observed under an inverted microscope, the flasks were placed in the incubator for further culture. Subsequently, cells in the logarithmic growth phase were selected for experiments.

3.3 CCK-8 method

Hepatocarcinoma H22 cells in the logarithmic growth phase were seeded into a 96-well plate at a density of 8×10^3 cells/well, with 100 μL of cell suspension per well. After cells adapted to the well environment, the CTS stock solution was diluted and added to the corresponding wells according to a concentration gradient, resulting in final concentrations of 1, 2, 5, 10, 20, 40, 80, and 100 μmol/L, totaling 8 treatment groups. Additionally, a blank group and a control group were set up, with 5 replicate wells per group. The 96-well plate was cultured in the incubator for 24/48 h, after which 10 μL of CCK-8 working solution was added to each well under light-protected conditions. The plate was then re-placed to the incubator for an additional 4 h, with medium color observed every 30 min. The absorbance value (OD) of each well was measured at a wavelength of 450 nm using a microplate reader, and the cell apoptosis rate and

half-maximal inhibitory concentration (IC₅₀) value were calculated. Based on the IC₅₀ value, the CTS concentration was selected for subsequent experiments. The cell inhibition rate (%) = [(OD value in Control group – OD value in Treatment group) / (OD value in Control group – OD value in Blank group)] × 100%.

3.4 GOD-POD Method

Hepatocarcinoma H22 cells in the logarithmic growth phase were seeded into a 6-well plate at a density of 2×10^4 cells/well, with 2 mL of cell suspension per well. A blank control group and CTS treatment groups using CTS of 5, 10, and 20 μmol/L were established, with 3 replicate wells per group. Following incubation for 48 h, cells from each group were collected, washed with pre-cooled PBS, ultrasonically lysed, and centrifuged (12,000 rpm, 10 min, 4 °C). The supernatant was collected and processed according to the instructions. The final solution (200 μL) was added to a 96-well plate, and incubated for 30 min in the dark. The OD value was measured at a wavelength of 520 nm. Glucose content was calculated according to the instructions (Glucose Assay Kit, GOD-POD Oxidase Method, BL863B).

3.5 Lactate oxidase method

H22 mouse liver cancer cells in the logarithmic growth phase were plated into a 6-well plate at a density of 2×10^4 cells per well, with 2 mL of cell suspension per well. A blank group and CTS treatment groups using CTS of 5, 10, and 20 μmol/L were established, each with 3 replicate wells. After incubation for 48 h, cells from each group were collected, washed with pre-cooled PBS, ultrasonically lysed, and centrifuged (12,000 rpm, 10 min, 4 °C). The supernatant was collected and processed as per the instructions, and the final solution (100 μL) was added to a 96-well plate. Following incubation in the dark for 30 min, the OD value was measured at a wavelength of 450 nm. Lactate production was calculated based on the

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manual (CheKine™ Lactate Assay Kit, KTB1100).

3.6 Annexin V-FITC/PI double staining

H22 mouse liver cancer cells (1.0×10^5 cells/well) were seeded into a 6-well plate, with 2 mL of cell suspension per well. A blank group and CTS treatment groups using CTS of 5 (low), 10 (middle), and 20 (high) μmol/L were established, each with 3 replicate wells. Post culture for 48 h in the incubator, cells from each group were collected, washed twice with pre-cooled PBS, and centrifuged (800 rpm, 5 min, 4 °C). The supernatant was discarded, and the cells were resuspended in 195 μL of 1 × Binding buffer to prepare a single-cell suspension. The suspension was aliquoted into flow cytometry tubes, and according to the groups, 5 μL of Annexin V-FITC and 10 μL of PI dye were added. The mixture was gently pipetted to ensure even distribution and was incubated at room temperature in the dark for 15 min. After the cells were resuspended, the samples were placed on ice, filtered, and analyzed using a flow cytometer to detect apoptosis. The apoptosis rate was calculated accordingly.

3.7 Western blot analysis

Cells in the logarithmic growth phase were inoculated into a 6-well plate at a density of 8×10^4 cells/well, with 2 mL of cell suspension per well. A blank group and CTS treatment groups using CTS of 5, 10, and 20 μmol/L were established, each with three replicate wells. After 48-h incubation in the incubator, the cells from each group were collected and centrifuged (800 rpm, 5 min), and the supernatant was discarded. The cells were washed twice with an appropriate amount of PBS, and lysed in a lysis buffer containing protease and phosphatase inhibitors on ice for 45 min. Following centrifugation (12,000 rpm, 20 min, 4 °C), the supernatant was collected. Protein concentration was measured using an ultra-micro UV spectrophotometer. Based on the concentration and loading quantity of sample, the volume balance was

calculated, and protein loading buffer was added. The samples were denatured by being heated in a metal bath at 95 °C for 10 min, aliquoted, sealed with sealing film, and stored at -20 °C. The glass plates were washed, dried, inserted into a gel-producing apparatus, and aligned at the bottom. A 10% separation gel was prepared according to the instructions and added between the glass plates using a pipette, followed by water sealing. The separation gel was solidified for 30-45 min at room temperature, and the water was discarded. A stacking gel was prepared as per the instructions and added between the glass plates, and a comb was inserted quickly. The stacking gel was solidified for 30 min at room temperature, and the comb was removed. The glass plates were placed in the electrophoresis tank, with electrophoresis buffer added. Protein samples were loaded into the lanes, and electrophoresed at 80 V for 130 min until all proteins reached the bottom of the gel. Filter paper and the gel were cut and immersed in transfer buffer. A PVDF membrane was cut, activated with methanol, and soaked in transfer buffer with sponges, the gel, and filter paper, which were stacked in a "sandwich" style in sequence, avoiding air bubbles, and underwent membrane transfer in the wet transfer membrane tank under constant voltage (100 V) and constant current (200 mA) for 35 min (adjusted based on the molecular weight of the target proteins) on ice. The target strips were cut, placed in 5% skimmed milk, and blocked on a shaker for 2 h, followed by three washes with PBST (8 min each). Primary antibodies were diluted in primary antibody dilution buffer (dilutions: HK2 at 1:1000; Bcl-2 at 1:1000; Cyt-c at 1:1000; GAPDH at 1:5000). The strips were incubated with the primary antibodies at 4 °C overnight, washed thrice with PBST (15 min each), cultivated with a secondary antibody (dilution 1:5000) on a shaker at room temperature for 2 h, and washed again thrice with PBST (15 min each). The developing solution was prepared in the same volume

as specified in the manual and evenly applied to cover the target strips, which were placed in a dark box for reaction. Exposed images were captured using a gel imaging system and archived. The gray values of the target strips were semi-quantitatively analyzed using Image J software.

3.8 Molecular docking

Molecular docking and the binding energy between receptor proteins (HK2, Bcl-2, Cyt-c) and ligand compounds (cryptotanshinone) were performed using CB-Dock2

(<http://cadd.labshare.cn/cb-dock2/index.php>) and AutoDock Vine. The main steps of molecular docking are as follows. (1) Ligand preparation: Download the cryptotanshinone SDF file from PubChem, convert it to PDB format and add hydrogens using OpenBabel; add charges, identify rotatable bonds using AutoDock Tools, and generate the PDBQT format. (2) Protein processing: Obtain the receptor structure from the PDB database, remove water molecules, ions, and inactive chains using PyMOL; add polar hydrogens in AutoDock and generate the PDBQT format. (3) Docking and visualization: Set docking parameters using AutoDock, run the docking with AutoDock Vina to generate PDBQT results; Then, load the receptor and ligand in PyMOL to visualize the docking outcomes.

3.9 Statistical analysis

All experimental data were analyzed with GraphPad Prism 8.0. Measurement data were presented as mean $\pm$ standard deviation ($\bar{X} \pm s$). Comparisons between two groups were performed using independent samples *t*-tests, while those among multiple groups were conducted using one-way analysis of variance (ANOVA). A *P* value < 0.05 was considered statistically significant.

4 Results

4.1 The effect of CTS on the viability of H22 liver

cancer cells in mice

The CCK-8 test results were shown in Figure 1. Hepatocarcinoma H22 cells were exposed to different concentrations of CTS (1, 2, 5, 10, 20, 40, 80, 100, 200 $\mu\text{mol/L}$) for 24 and 48 h, respectively. The cell inhibition rate was elevated with the increase of CTS

concentration, and gradually increased with the prolongation of administration time. When the administration concentration reached 20 $\mu\text{mol/L}$, the cell inhibition rate was only slightly augmented. The IC_{50} was 24.43 $\mu\text{mol/L}$. Based on the experimental results, concentrations of 5, 10, and 20 $\mu\text{mol/L}$ were selected for subsequent experiments.

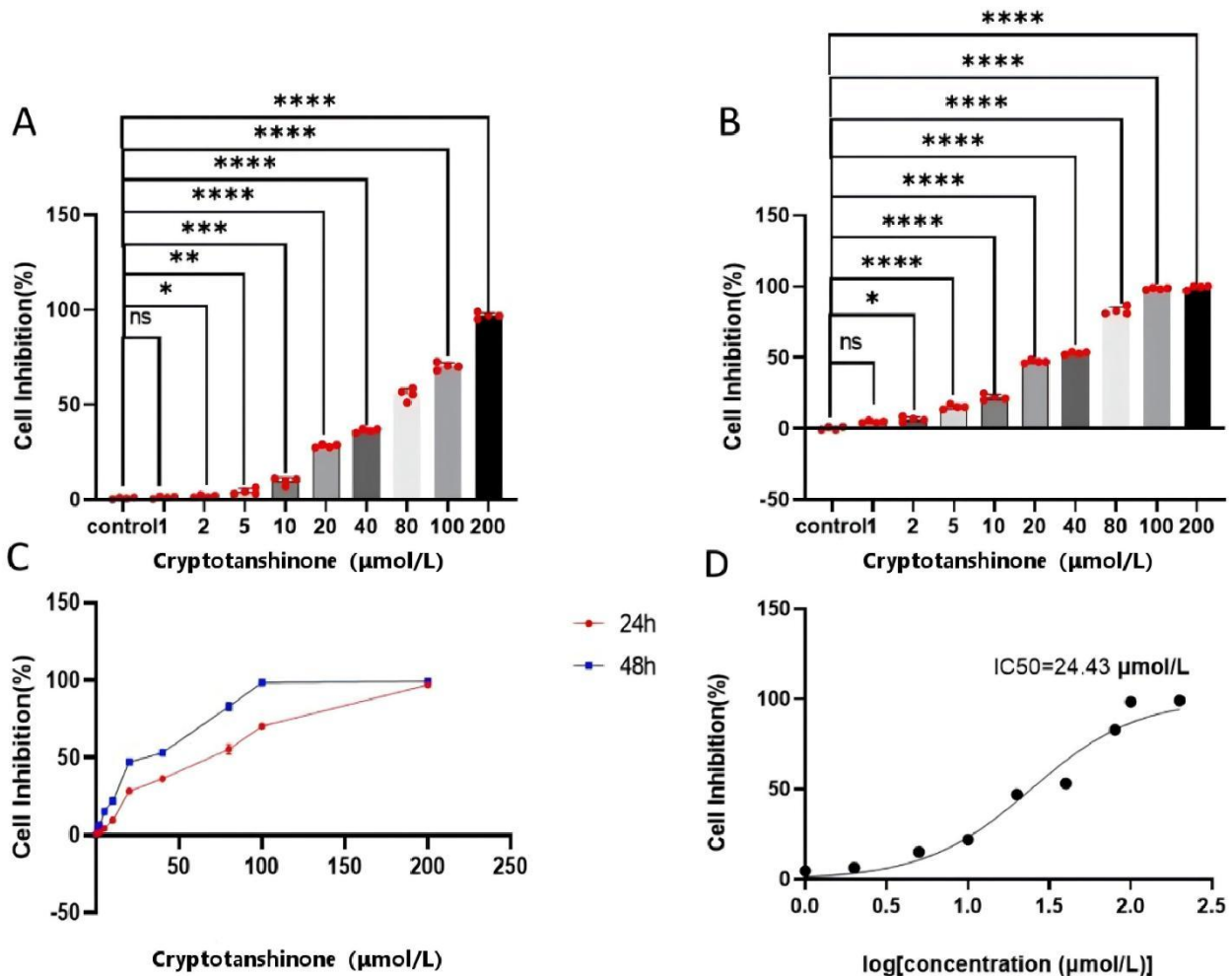


Figure 1 Effect of cryptotanshinone on the proliferation of H22 mouse liver cancer cells ($\pm S$, $n = 3$).

A: Inhibitory effect of different concentrations of cryptotanshinone on H22 mouse liver cancer cell proliferation after 24 h; **B:** Inhibitory effect of different concentrations of cryptotanshinone on H22 mouse liver cancer cell proliferation after 48 h; **C:** Comparison of the inhibitory effects of different concentrations of cryptotanshinone on H22 mouse liver cancer cell proliferation at 24 h and 48 h; **D:** Inhibitory curve of different concentrations of cryptotanshinone on H22 mouse liver cancer cell proliferation after 48 h. (Compared with the control group, *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$)

4.2 The effect of CTS on glucose uptake and lactate production abilities of H22 liver cancer cells in mice

The results of glucose content detection and analysis

were displayed in Figure 2A. After 48 h of treatment, the glucose uptake by the hepatocarcinoma H22 cells was gradually decreased with the increase of CTS

concentrations, indicating that CTS can inhibit the glucose uptake.

Meanwhile, the concentration of lactate generated by the hepatocarcinoma H22 cells was gradually decreased with the increase of CTS concentration,

proving that CTS can suppress the production of lactate in H22 cells. Combined with the results in above section, the treatment of CTS was further verified to curb the aerobic glycolysis cycle of H22 cells in mice.

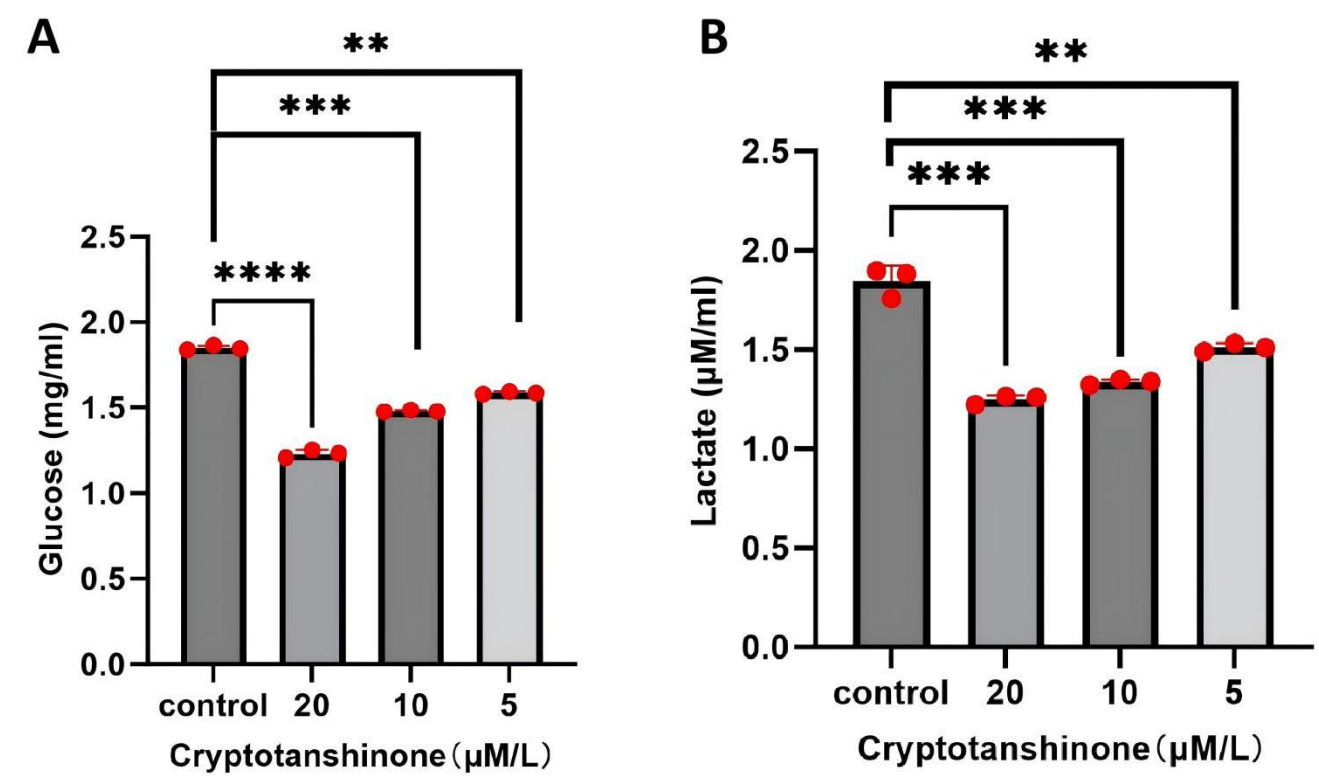


Figure 2 Effect of Cryptotanshinone on glucose uptake and lactate production in H22 mouse liver cancer cells ($\pm S$, $n = 3$).

A: Glucose uptake measured by GOD-POD method after 48 h of treatment with indicated concentrations of Cryptotanshinone; **B:** Lactate production measured by lactate oxidase method after 48 h of treatment with indicated concentrations of Cryptotanshinone. (Compared with the control group, **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$).

4.3 Effect of CTS on apoptosis in H22 mouse liver cancer cells

The results of Annexin V-FITC/PI double staining method were presented in [Figures 3A-E](#). Following 48 h of treatment with CTS at concentrations of 5 (low), 10 (middle), and 20 (high) $\mu\text{mol/L}$ in H22 mouse liver cancer cells, the apoptosis rates were significantly increased in 10 (middle) and 20 (high) $\mu\text{mol/L}$ groups in a concentration- dependent manner compared with the control group. Although an upward trend in

apoptosis was observed at 5 (low) $\mu\text{mol/L}$, no statistically significant difference was detected.

To further observe the effect of CTS on the endogenous mitochondrial apoptosis pathway, Western blot was conducted to quantify the expressions of mitochondrial Bcl-2 and cytoplasmic Cyt-c protein. As shown in [Figure 3F, G](#), compared with the control group, cells treated with different concentrations of CTS for 48 h displayed downregulated Bcl-2 protein and upregulated Cyt-c

protein in H22 cells. The results of the binding energy analysis also indicated that there was a strong interaction between CTS and BCL2, as well as Cyt-c. This hinted that CTS can promote apoptosis of H22 cells by inhibiting the expression of Bcl-2 protein and promoting the expression of Cyt-c protein.

4.4 Effect of CTS on HK2 protein expression in H22 mouse liver cancer cells

Figure 4 shows the results of Western blot analysis and

the binding energy between CTS and HK2 protein. After treatment with different concentrations of CTS for 48 h, the HK2 protein expression levels in H22 cells were diminished to varying degrees. Meanwhile, the results of the binding energy analysis indicated a strong interaction between CTS and HK2 protein, implying that CTS can enhance apoptosis in H22 mouse liver cancer cells via inhibiting the expression of HK2 protein.

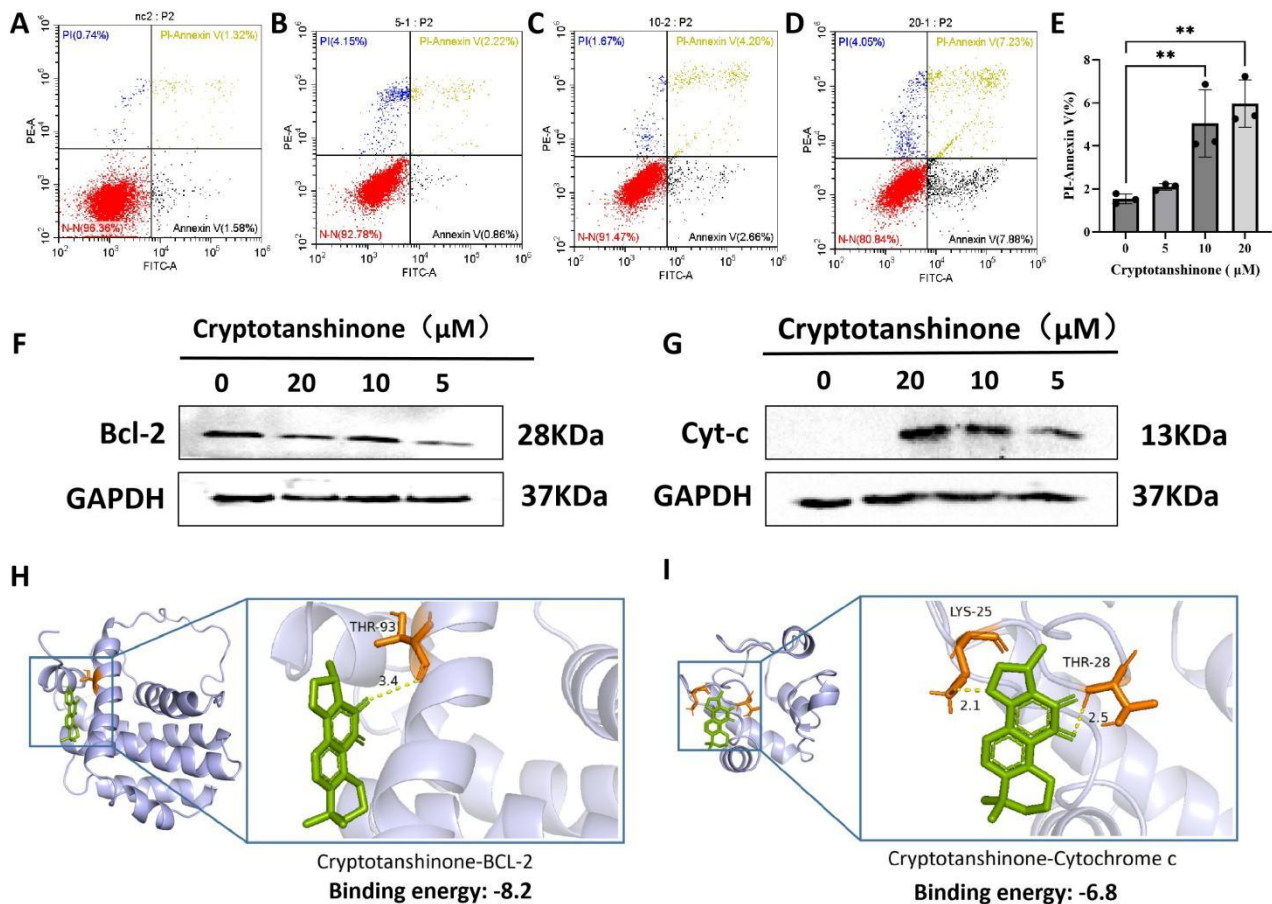


Figure 3 Effect of Cryptotanshinone on apoptosis and Bcl-2/Cyt-c expression in H22 mouse liver cancer cells.

A-E: Representative flow cytometry plots and quantitative bar charts of Annexin V-FITC/PI staining after 48 h of Cryptotanshinone treatment; **F-G:** Western blot analysis of Bcl-2 and Cyt-c protein expression; **H-I:** Molecular docking models and binding energies between cryptotanshinone and Bcl-2/Cyt-c. (Compared with the control group, **: $p < 0.01$)

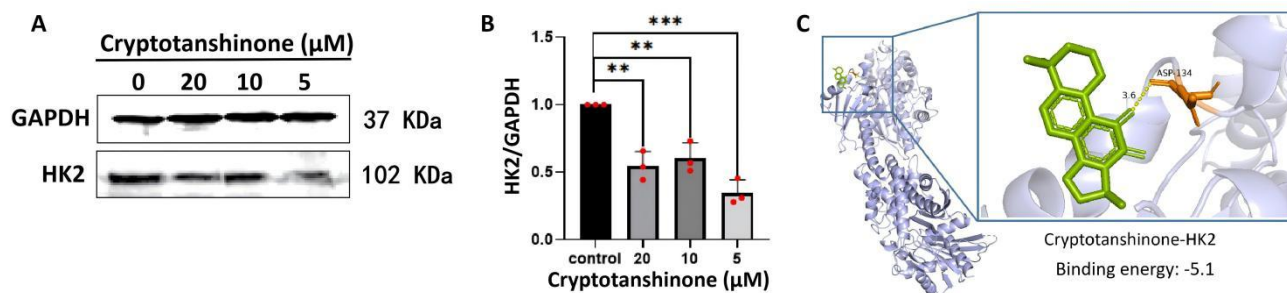


Figure 4 Effect of Cryptotanshinone on HK2 expression and molecular docking with HK2 in H22 cells ($\pm$ S, n = 3).

A: Representative Western blot bands of HK2 and GAPDH after 48 h of treatment with indicated concentrations of CTS; **B:** Quantitative analysis of HK2 protein expression normalized to GAPDH; **C:** Molecular docking model and binding energy between cryptotanshinone and HK2. (Compared with the control group, **: $p < 0.01$; ***: $p < 0.001$).

5 Discussion

According to statistical data from 2022, liver cancer has become the third leading cause of cancer-related deaths worldwide, secondary only to lung cancer and colorectal cancer [19]. The incidence of liver cancer is high in China, with HCC constituting about 90% of the patients. Early symptoms of the disease are insidious, and most patients are diagnosed in the middle and late stages. Surgical resection treatment has relatively limited effectiveness [20]. For advanced patients, systemic therapy is particularly important, currently mainly including chemotherapy, targeted therapy, and immunotherapy [21,22]. Although commonly used chemotherapy drugs in clinical practice play a significant role in improving patient prognosis, they also have the drawback of significant toxic side effects, and patients are prone to developing drug tolerance [23], which is not conducive to long-term treatment. Research has found that due to the imbalance of yin and yang, qi and blood dysregulation, and organ dysfunction in patients with liver cancer, TCM can be used as an adjuvant therapy [24,25]. Compared with the currently popular Western medicines, TCM has prominent advantages of high efficiency, low toxicity, safety, and multi-targets, which can address the treatment difficulties.

In TCM, liver cancer belongs to the categories of “abdominal masses,” “swelling,” and “pain in the subcostal region.” The key pathogenesis of the onset and development of liver cancer is the disorder of qi activity, Zang-fu organ dysfunction, qi stagnation and blood stasis, prolonged stasis transforming into toxins, and the mutual accumulation of blood stasis and toxins. The basic method of treating liver cancer in TCM is to promote blood circulation, remove blood stasis, and treat blood stasis and toxins simultaneously. *Salvia miltiorrhiza* (Danshen in Chinses) is a TCM herb classified as a blood-activating agent, with effects of promoting blood circulation, removing blood stasis, relieving pain through unblocking meridians, cooling blood, and eliminating carbuncles. It is often used to treat liver diseases and hematological disorders [26,27]. CTS is one of the main active ingredients of Danshen, which can inhibit tumor cell growth, induce cell apoptosis, improve tumor inflammatory microenvironment, block tumor invasion and metastasis via regulating various signaling pathways, and has good anti-tumor activity. Existing research has proved that CTS can hinder the deterioration and development of lung cancer, liver cancer, breast cancer, colon cancer, stomach cancer, bladder cancer, leukemia, neuroma and other diseases [28,29], thus improving the treatment effect.

This study explored the *in vitro* anti-liver cancer effect of CTS with a cell line of H22 mouse liver cancer cells, and primarily illuminate the potential mechanism of CTS on HK2-mediated glycolysis. The results showed that CTS could dose- and time-depend inhibit the proliferation of H22 mouse liver cancer cells.

Lactate is one of the typical substances produced by tumor cells through glucose glycolysis, and active glycolysis can generate substantial lactate to form lactate accumulation. It was found in this study that after intervention for 48 h, the CTS could inhibit glucose uptake and lactate production in H22 cells, indicating that CTS weakened the energy metabolism of tumor cells by inhibiting the glycolysis pathway and thus suppressed cell growth and proliferation.

As the first key rate-limiting enzyme in the glycolysis pathway, HK2 is typically highly expressed in tumor cells and closely related to the anti-apoptotic ability of tumor cells. HK2 binds to the voltage-dependent anion channel (VDAC) on the outer mitochondrial membrane via its N-terminal hydrophobic peptide, forming an HK2-VDAC complex. Mitochondrial-bound HK2 preferentially utilizes adenosine triphosphate (ATP) produced by mitochondria, promotes the coupling between glycolysis and oxidative phosphorylation, and forms an efficient glycolytic energy supply mechanism in tumors. Meanwhile, HK2 preferentially interacts with the mitochondrial permeability transition pore (mPTP) through VDAC, inhibiting its opening. This process not only interferes with the binding of the pro-apoptotic protein Bax to VDAC, blocking its pro-apoptotic function, but also further suppresses the release of Cyt-c, ultimately antagonizing tumor cell apoptosis and promoting malignant progression. In this study, we found that CTS downregulated HK2 protein, implying that CTS might induce cell apoptosis by inhibiting the HK2-mediated glycolytic pathway.

Cyt-c, as a key protein in the mitochondrial electron transport chain, participates in cellular respiration and

ATP generation and maintains cellular energy supply by transferring electrons. When cells are stimulated by apoptotic signals, the permeability of the outer mitochondrial membrane is increased, and Cyt-c is released from the mitochondria into the cytoplasm, forming an apoptotic body with Apaf-1 and caspase-9 to activate the caspase cascade reaction and leading to cell apoptosis. Bcl-2, an anti-apoptotic protein, inhibits outer mitochondrial membrane permeability by interacting with pro-apoptotic proteins such as Bax and Bak, preventing pore formation and thereby blocking the release of Cyt-c to suppress apoptosis. In addition, Bcl-2 can mediate mitochondrial function and metabolism, maintain mitochondrial membrane potential and ATP production, and support cell survival. The results of Western blot confirmed that CTS downregulated Bcl-2 protein expression and upregulated Cyt-c protein expression, suggesting that CTS may induce apoptosis through the mitochondrial pathway.

Several limitations of this study should be acknowledged. First, the findings are derived exclusively from *in vitro* experiments conducted in a H22 cell line; consequently, their generalizability to other liver cancer subtypes or to the complex *in vivo* tumor microenvironment remains uncertain. Second, the absence of animal experiments precludes any assessment of *in vivo* efficacy, pharmacokinetic properties, or potential toxicity of CTS. Third, direct evidence supporting HK2 as a specific molecular target—such as gain- or loss-of-function studies—is lacking; the proposed mechanism therefore relies primarily on correlative protein expression data and *in silico* docking predictions. Given these constraints, the therapeutic potential of CTS should not be overstated at this stage. Future investigations incorporating animal models, multiple cell lines, and genetic manipulation strategies are warranted to validate the anti-hepatocellular carcinoma activity and safety profile of CTS prior to any translational consideration.

In conclusion, this study preliminarily revealed that CTS induces apoptosis of H22 liver cancer cells via repressing HK2-mediated glycolysis pathway and regulating mitochondrial apoptosis pathway. This discovery not only provides basis for developing HK2-targeting anti-liver cancer drugs, but also establishes a theoretical basis for CTS as a potential anti-liver cancer drug candidate, while offering a fresh perspective on tumor-targeting studies of active components from TCM, which is expected to provide new ideas for optimizing clinical therapeutic strategies against liver cancer.

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Not applicable.

Conflicts of Interest

The authors declare that there is no conflict of interests.

Author Contributions

P.X.: Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing - original draft. H.H.: Validation, Data curation, Writing - original draft. H.Z.: Validation, Data curation, Investigation. Z.Lou.: Investigation. L.Z.: Data curation, Visualization. Z.Liu.: Conceptualization, Writing - review & editing. All authors have read and approved the final version of the manuscript.

Ethics Approval and Consent to Participate

Not applicable.

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Availability of Data and Materials

The data presented in this study are available on

request from the corresponding author.

Supplementary Material

Not applicable.

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