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# Cryptotanshinone increases the sensitivity of liver cancer to sorafenib by inhibiting the STAT3/Snail/epithelial mesenchymal transition pathway

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## Abstract

**Objective:** Sorafenib resistance has been a major factor limiting its clinical use as a targeted drug in liver cancer. The present study aimed to investigate whether cryptotanshinone can enhance the sensitivity of liver cancer and reduce the resistance to sorafenib.
**Methods:** Sorafenib-resistant cells were established based on HepG2 and Huh7 cell lines. And the anti-tumor effect of sorafenib combined with cryptotanshinone on the sorafenib-resistant cells was verified by MTT, colony formation, transwell assays and tumor growth xenograft model. Moreover, the effects of the combined treatment on the expression of phosphorylated (p)-STAT3, as well as epithelial mesenchymal transition (EMT) and apoptosis related proteins of cells were evaluated by western blot analysis.
**Results:** It was identified that cryptotanshinone inhibited the viability of both HepG2 and Huh7 cells in a dose- and time-dependent manner, and decreased p-STAT3 expression rather than total STAT3 expression at a concentration of 40 μmol/L. In the sorafenib-resistant cells, sorafenib in combination with cryptotanshinone markedly inhibited cell viability, invasion and migration compared with sorafenib alone. In contrast, increased p-STAT3 level by colivelin led to the inhibition of the synergistic effect of cryptotanshinone and sorafenib not only on cell viability, but also on EMT and apoptosis, suggesting that cryptotanshinone and sorafenib may act by downregulating STAT3 signaling. Further, the inhibition of carcinogenicity effect was also verified in xenografted tumor models.
**Conclusion:** The present results indicated that cryptotanshinone could synergize with sorafenib to inhibit the proliferative, invasive, and migratory abilities of sorafenib-resistant cells by downregulating STAT3 signaling.

**Keywords**

cryptotanshinone; sorafenib; sorafenib-resistance; STAT3 signaling; liver cancer

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## 1 Introduction

Liver cancer was the most commonly diagnosed cancer type and the fourth leading cause of cancer-related mortality worldwide in 2018, with 841 000 new cases and 782 000 deaths annually[1]. Especially in cold zones, the cold environment tends to inhibit the immune response of the body[2], which may contribute to the more frequent occurrence of liver cancer. Sorafenib, acting as an inhibitor of serine-threonine kinase Raf-1, is the first line Food and Drug Administration-approved targeted therapeutics for advanced liver cancer, which improves the overall survival by 2.8 months on average and postpones disease progression. However, a low response rate and transient and limited efficacy limit its clinical application, suggesting that innate and acquired sorafenib-resistance exists in liver cancer cells[3-4]. Accumulating evidence supports that STAT3 plays an important role in several malignancies, including liver cancer[5-6]. And sorafenib was reported to activate STAT3 and contribute to the sorafenib-resistance in liver cancer[7]. Thus, inhibition of STAT3 may enhance the carcinogenicity-inhibiting effect and reverse acquired resistance to sorafenib[8].

In searching for candidates that can combat acquired resistance of liver cancer to sorafenib, cryptotanshinone, a currently used therapeutic agent for a variety of conditions with significant STAT3-inhibitory efficacy, has drawn drew our attention.

Cryptotanshinone, isolated from the plant Salvia miltiorrhiza Bunge, is known for its anti-inflammatory, antioxidative, anti-angiogenic and antitumor activities on various malignancies including liver cancer in clinics of China[9-10]. It has been previously shown to inhibit the activity of STAT3 signaling and/or other pathways in certain cancer types[11-14], of note, cryptotanshinone could inhibit liver cancer by blocking the STAT3 signaling pathway[15]. However, to the best of our knowledge, the effect of cryptotanshinone on sorafenib-resistance liver cancer has not been reported. We therefore hypothesized that cryptotanshinone suppresses the STAT3 signaling pathway to mitigate the drug resistance of liver cancer to sorafenib.

Epithelial-mesenchymal transition (EMT) is a crucial biological process in cancer progression, invasion and metastasis[16]. Previous studies have reported that EMT confers drug resistance in cancer cells[17]. Our previous research revealed that EMT occurred in sorafenib-resistant liver cancer cells, which enhanced migratory and invasive abilities compared with the corresponding parental cells[18]. Furthermore, emerging evidence suggests that EMT in liver cancer is associated with the activation of STAT3 and can further promote EMT process[19-20]. Therefore, the present study selected STAT3 as the target to evaluate the antitumor efficacy and molecular mechanism of cryptotanshinone on sorafenib-resistance liver cancer.

## 2 Materials and methods

### 2.1 Cell lines and antibodies

Human hepatoblastoma cell line HepG2 was purchased from the American Type Culture Collection, and human hepatocellular carcinoma cell line Huh7 was obtained from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences. And both cell lines had been authenticated via STR profiling. Cells were routinely cultured at 37˚C in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), l00 μg/mL penicillin and streptomycin under 5% $CO_2$ in an incubator. Antibodies (Abs) against STAT3, phosphorylated (p)-STAT3, E-cadherin, Vimentin and Snail were purchased from Cell Signaling Technology Inc, and Ab against GAPDH was purchased from Santa Cruz Biotechnology Inc. Colivelin, a specific STAT3 activator, was purchased from MedChemExpress and used to test the synergetic effect of STAT3 and cryptotanshinone on sorafenib resistance in liver cancer cells.

Sorafenib and cryptotanshinone were dissolved in DMSO to make a stock solution of 100 mmol/L, which were used in the in vitro assays. The present study was approved by the Ethics Committee of the Second Affiliated Hospital of Harbin Medical University.

### 2.2 Establishment of sorafenib-resistant cells

The sorafenib-resistant liver cancer cells were cultured in the preliminary experiments, as described previously[21]. Parental cells (HepG2 and Huh7) were cultured in 6-well plates at 10 000 cells/well and incubated with sorafenib at a concentration of 4 μmol/L which was just below their respective IC50 (Huh-7 and HepG2, IC50 6-7 μmol/L)[22]. Then, the concentration of sorafenib was slowly increased by 0.25 μmol/L per week. After 6-7 months, two sorafenib-resistant cell lines were established, and termed "HepG2-SR" and "Huh7-SR", which were continuously cultured in the presence of sorafenib to maintain properties of the acquired resistance.

### 2.3 Cell viability assay

Cell viability was analyzed using an MTT (Beyotime Institute of Biotechnology) assay to test whether cryptotanshinone can inhibit liver cancer cells viability. Briefly, the cells were plated in 96-well culture plates at a density of 3 000 cells/well. Cells were pretreated with increasing concentrations of cryptotanshinone for 24, 48 and 72 h, and then cell viability was assessed according to the manufacturer's guidelines. The experiments were repeated in triplicate.

### 2.4 Colony formation assay

Cells were cultured in a 6-well plate at a density of 2 000 cells/well for 48 h, and then continuously cultured after addition of complete media with 5% $CO_2$ at 37 ˚C. The medium was replaced with a fresh medium every 2 days for 14 days. The cell colonies were then analyzed using crystal violet staining (Beyotime Institute of Biotechnology). After 20 min incubation at room temperature, cells were washed, and the number of colonies was counted.

### 2.5 Transwell assays

A 24-well transwell plate (Costar; Corning, Inc.) was used to examine cell migratory or invasive capacities using a Boyden chamber method and polycarbonate membranes with an 8-μm pore size, according to the manufacturer's instructions. Briefly, 50 000 cells were seeded onto the upper chamber with 200 μL serum-free medium, and the bottom chamber contained 600 μL DMEM with 10% FBS. After 48 h, the upper surface cells on the membrane were gently removed and washed three times in PBS. The cells that had migrated to the outer side of the membranes were fixed with 4% paraformaldehyde for 30 min and stained with 0.1% crystal violet for 10 min. Cells were washed with

running water and the number of migrated cells was counted in four fields under ×10 magnification per chamber. A total of three chambers were used in one experiment. The cell invasion assay was performed similarly, except 50 μL Matrigel (BD, Biosciences; diluted 1:6 with serum-free medium) was added to each well and dried at room temperature for 2 h, before 200 000 cells were seeded onto the membrane.

### 2.6 Western-blot analysis

Cells were harvested and subjected to western blot analysis as described previously[21]. Briefly, the total protein among different groups was harvested with the RIPA lysis buffer (Beyotime; China) containing 1 mM PMSF (Beyotime; China). 1 μL RIPA buffer was used to lyse about 10 000 cells and the protein concentration was examined via the BCA kit (Beyotime; China) according to the manufacturer's manual. Next, 50 μg proteins were loaded on the sodium dodecyl sulfonate-polyacrylamide gel for electrophoresis, followed by blotting on the polyvinylidene difluoride (PVDF) membranes (Beyotime; China). Finally, the membranes were blocked with 5% skim milk in PBS-Tween 20 at room temperature for 1 h and then probed with primary antibodies against total STAT3 (Cat number: 12640; CST), *p*-STAT3 (Cat number: 9145; CST), Snail (Cat number: 3879; CST), E-cadherin (Cat number: 3195; CST), vimentin (Cat number: 5741; CST), and GAPDH (Cat number: 5174; CST) at room temperature for 2 h, followed by incubation overnight. Then, membranes were incubated with alkaline phosphatase-conjugated secondary antibody (Cat number: 7054; CST), followed by detection with ECL reagent (Pierce; Thermo Fisher Scientific, Inc.).

### 2.7 Huh7-SR Tumor growth xenograft model

The protocol for the experiments involving tumor growth xenograft tumor and nude mice was approved by the Institutional Animal Care and Use Committee of The Second Affiliated Hospital of Harbin Medical University (Harbin, China). A total of 100 μL of Huh7-SR cells (about 5 000 000 cells) was inoculated subcutaneously into the right armpit of five-week-old female athymic nude mice. The tumor size of 100 $mm^3$ was set as the humane endpoint. When the implanted tumor of nude mice grew to about 5 $mm^3$, 12 mice were randomly divided into 4 groups according to the random number table, with 3 mice in each group. The control group was treated with 100 μL castor oil by gavage and 100 μL castor oil by intraperitoneal injection as well. The sorafenib group was given 100 μL sorafenib (10 mg/kg) by gavage plus 100 μL castor oil by intraperitoneal injection. The cryptotanshinone group received 100 μL castor oil by gavage together with 100 μL cryptotanshinone (100 mg/kg) by intraperitoneal injection. The sorafenib cryptotanshinone group: 100 μL sorafenib (10 mg/kg) by gavage and 100 μL cryptotanshinone (100 mg/kg) by intraperitoneal injection. For all groups, drugs and/or other reagents were administered once a day for 5 consecutive days, and then the animals were let rest for 2 d before further experimental interventions. After 30 d, the largest tumor volume reached 90 $mm^3$ (<100 $mm^3$), and the mice were sacrificed. The mice were euthanized with isoflurane (exposed to >5% isoflurane for >1 min for euthanasia). About 5 min after exposure to isoflurane, death was confirmed by observing the heartbeat, breathing rate, pupils and nerve reflex of the mouse. Finally, tumor grafts were excised, weighed and statistics. And the tumor volume was calculated by the formula of 1/2 (length × $width^2$).

### 2.8 Statistical analysis

The data are presented as the mean ± SD. The statistical difference between the two experimental groups was determined using the independent-samples *t*-test, and one-way ANOVA followed by Tukey's test was used for multiple comparisons. $P < 0.05$ was considered to indicate a statistically significant difference.

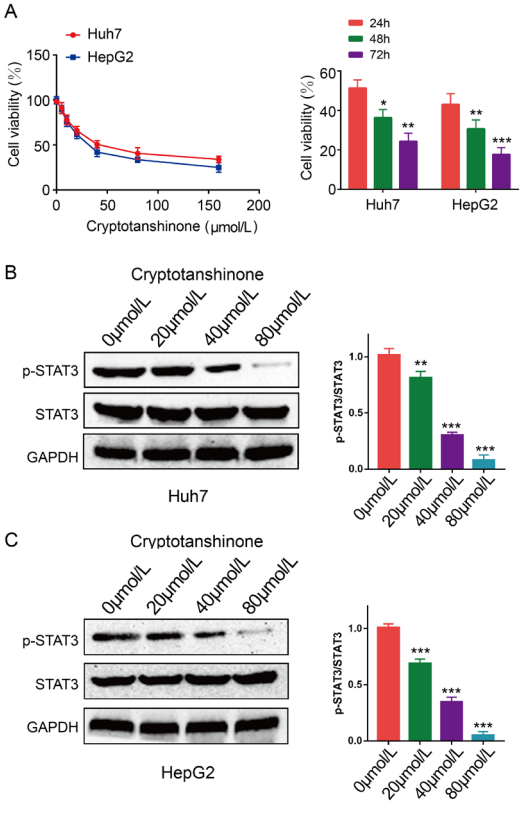


***Fig. 1*** Cryptotanshinone inhibits the proliferation and STAT3 activation of liver cancer cells.

(A) HepG2 and Huh7 cells were exposed to different concentrations (0, 5, 10, 20, 40, 80, 160 and 320 μmol/L) of cryptotanshinone for 24, 48 and 72 h. (B, C) The expression levels of p-STAT3 and STAT3 were measured via western blotting. The band density of p-STAT3 was normalized to STAT3. The data are representative of three independent experiments. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

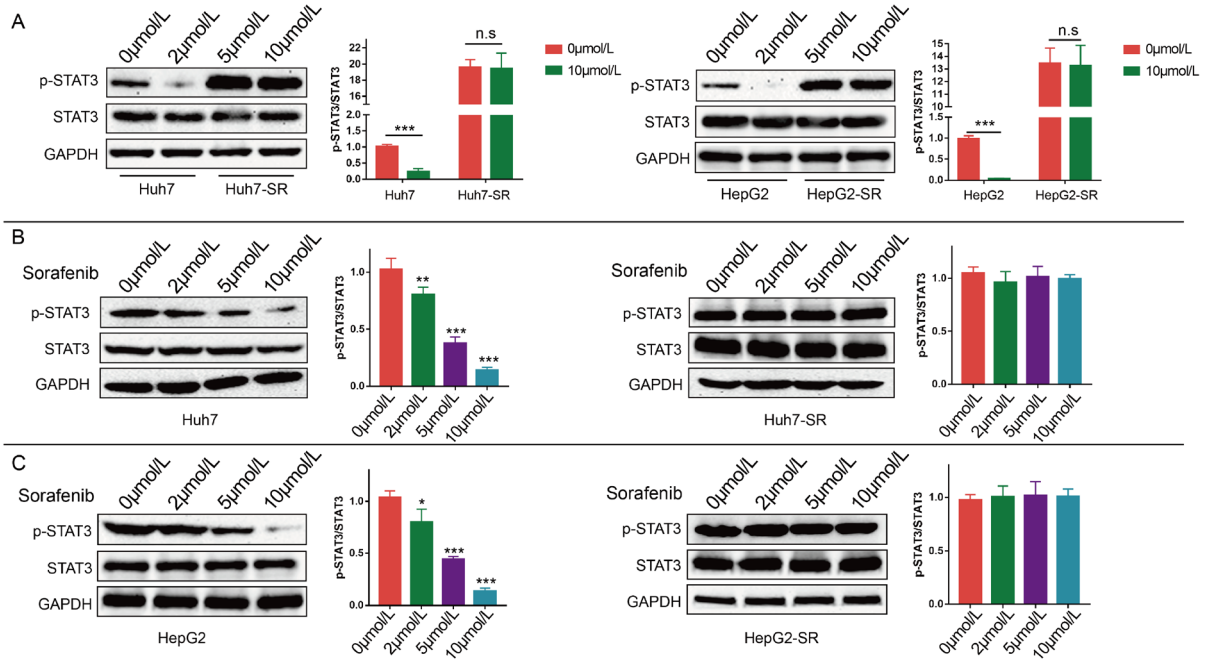


***Fig. 2*** Sorafenib inhibits STAT3 activation in sorafenib-sensitive cells but not in sorafenib-resistant liver cancer cells.

The expression levels of p-STAT3 and STAT3 were measured by western blot analysis. (A) Sorafenib-resistant cells and parental cells were incubated with or without 10 μmol/L sorafenib for 24 h. (B, C) Sorafenib-resistant cells and parental cells were incubated with sorafenib at serial concentrations of 0, 2, 5 or 10 μmol/L for 24 h. The protein levels were detected via western blot analysis. GAPDH served as the loading control. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

## 3 Results

### 3.1 Cryptotanshinone inhibits liver cancer cells proliferation by suppressing the STAT3 pathway

HepG2 and Huh7 cells were pretreated with increasing concentrations of cryptotanshinone for 24h, 48h and 72 h, and then cell viability was assessed. As presented in Fig. 1A, cryptotanshinone inhibited cell viability in dose- and time-dependent manners. The IC50 was 35 μmol/L in HepG2 cells and 51 μmol/L in Huh7 cells.

Next, Huh7 and HepG2 cells were incubated with increasing concentrations of cryptotanshinone for 24 h and then subjected to western blotting analysis. Cryptotanshinone inhibited the activation of STAT3, as reflected by significant downregulation of the phosphorylated or activated form of STAT3 protein (p-STAT3) in a concentration-dependent manner but did not affect the protein level of total STAT3 after 24 h treatment at a concentration of 40 μmol/L (Fig. 1B, C).

### 3.2 p-STAT3 is highly expressed in sorafenib-resistant cells

To further examine the role of p-STAT3 in the acquired drug resistance to sorafenib, the protein expression levels of total STAT3 and p-STAT3 were detected by western blot analysis. The results demonstrated that p-STAT3 level was higher in sorafenib-resistant cells than in parental cells. However, the level of total STAT3 was not different between the groups (Fig. 2A). As depicted in Fig. 2B and C, sorafenib downregulated the expression of p-STAT3 in a concentration-dependent manner, but this effect was abolished in HepG2-SR and Huh7-SR cells.

### 3.3 Cryptotanshinone increases the sensitivity of sorafenib in resistant cells by decreasing p-STAT3 and EMT

To investigate the effect of co-treatment with sorafenib and cryptotanshinone on cell proliferation, HepG2-SR and Huh7-SR cells were incubated with increasing concentrations of cryptotanshinone and/or sorafenib for 24, 48 or 72 h. Cell viability was determined using MTT assay. As demonstrated in Fig. 3A and B, the viability of sorafenib-resistant cells was decreased with the increasing concentrations of sorafenib and cryptotanshinone. The values of drug interaction coefficients were 0.86, 0.89 and 0.83 for incubation durations of 24, 48 and 72 h, respectively. The data indicated that cryptotanshinone synergized with sorafenib to inhibit cell viability in dose- and time-dependent manners, and cotreatment with cryptotanshinone

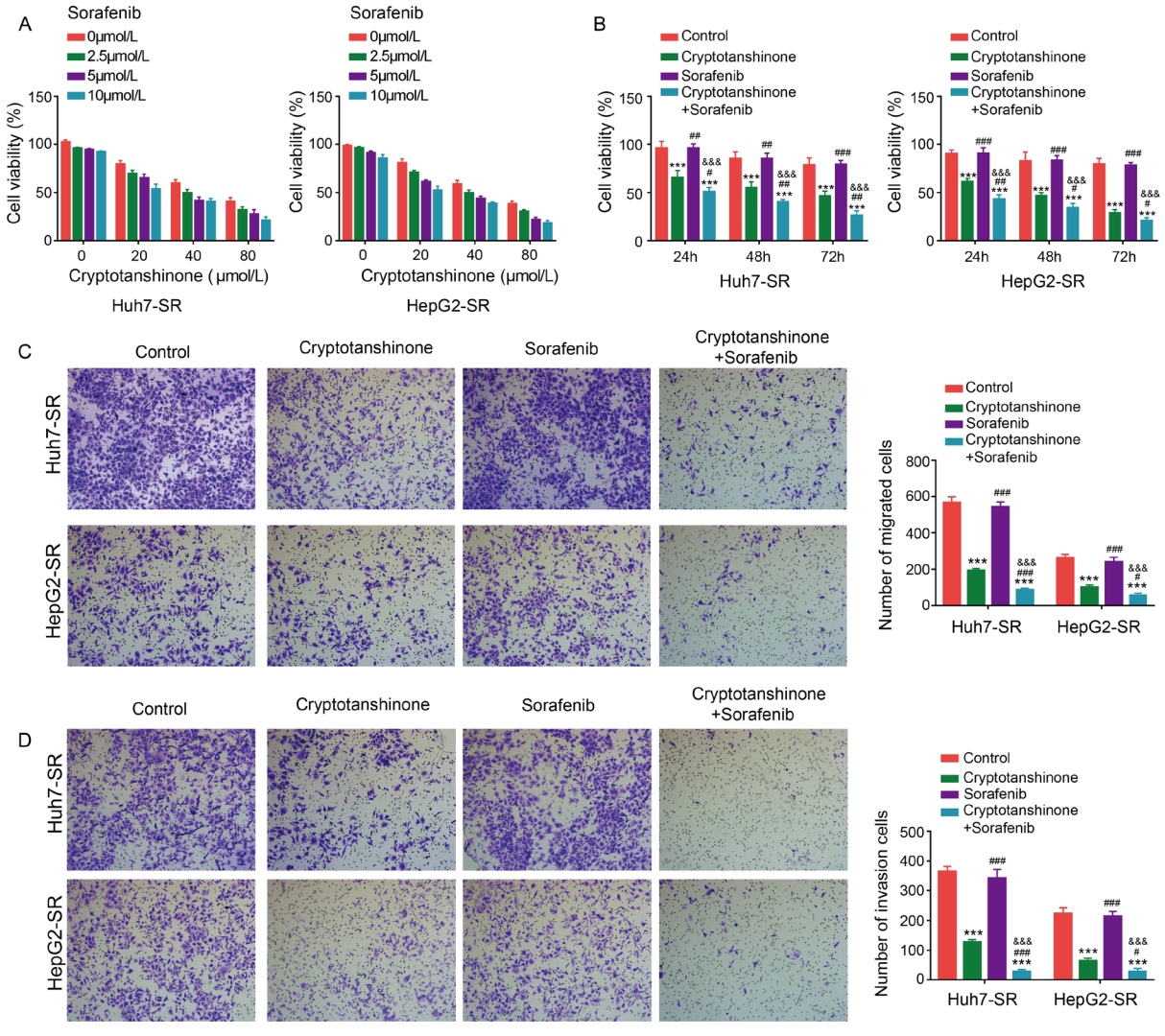


***Fig. 3*** Cryptotanshinone synergizes with sorafenib to inhibit the malignant biological behavior of sorafenib-resistant cells.

(A) HepG2-SR and Huh7-SR cells were exposed to different concentrations (0, 20, 40 and 80 μmol/L) of cryptotanshinone and/or different concentrations (0, 2, 5 and 10 μmol/L) of sorafenib for 24 h. Cell viability was assessed and normalized to the values from control cells. (B) Huh7-SR and HepG2-SR cells were incubated for 48 h with sorafenib (10 μmol/L), cryptotanshinone (40 μmol/L) or a combination. Cell viability was assessed and normalized to control cells. $^{***}P < 0.001$ *vs.* Control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs.* Cryptotanshinone; $^{\&\&\&}P < 0.001$ *vs.* Sorafenib alone. (C, D) Cells described in panel (A) were subjected to Transwell assay for 48 h, and cell migration and invasion were evaluated. $^{***}P < 0.001$ *vs.* Control; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ *vs.* Cryptotanshinone; $^{\&\&\&}P < 0.001$ *vs.* Sorafenib alone.

and sorafenib exhibited a significantly greater antitumor effect compared with treatment with sorafenib or cryptotanshinone alone.

To examine the ability of cryptotanshinone and sorafenib to inhibit the cell invasive and migratory capacities, HepG2-SR and Huh7-SR cells after 48 h incubation were subjected to transwell assay. Cryptotanshinone combined with sorafenib significantly suppressed the invasion and migration of HepG2-SR and Huh7-SR cells relative to those treated with cryptotanshinone or sorafenib alone (Fig. 3C and D). Moreover, the western-blot results indicated that cryptotanshinone combined with sorafenib downregulated the expression of p-STAT3 (Fig. 4A).

As reported, EMT and apoptosis are involved in the activation of STAT3 in liver cancer initiation and progress[23,24]. To elucidate whether cryptotanshinone inhibits the EMT phenotype and promotes the apoptosis of the sorafenib-resistant cells, the protein expression levels of E-cadherin,

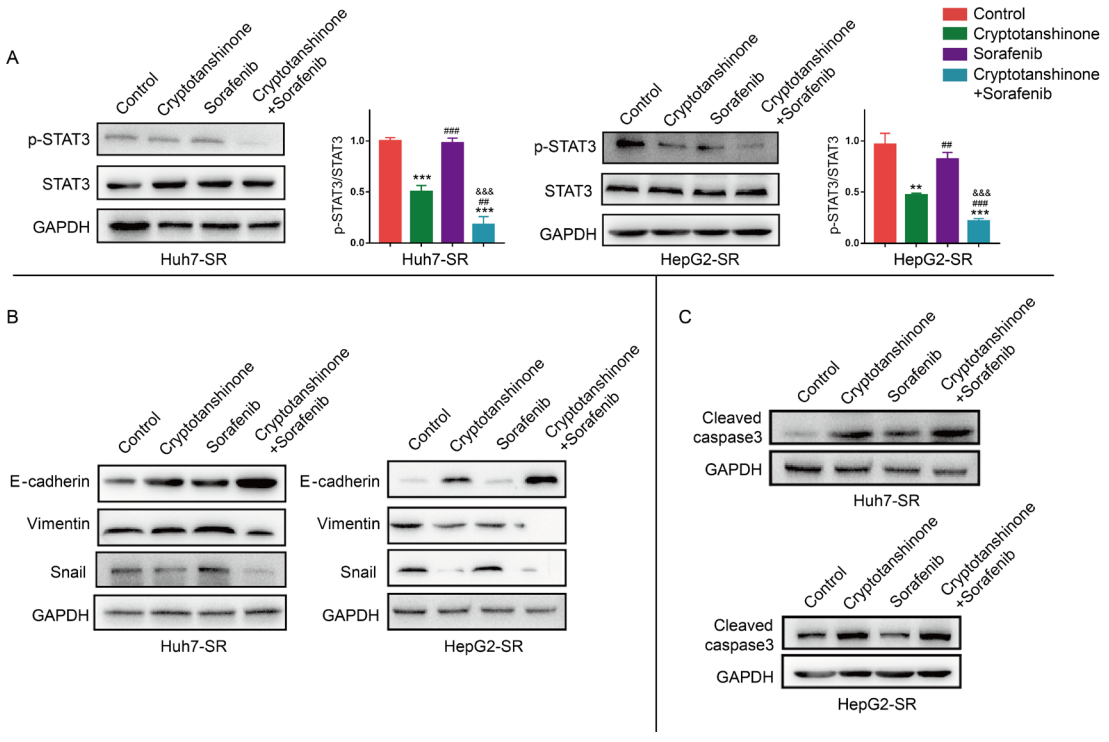


***Fig. 4*** Cryptotanshinone synergizes with sorafenib to inhibit p-STAT3, EMT and promote apoptosis in sorafenib-resistant cells.

Western blot analysis of protein levels of p-STAT3 (A), and EMT- (B) and apoptosis-associated markers (C) in Huh7-SR and HepG2-SR. Band densities were normalized to GAPDH. Data represent three independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* Control; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ *vs.* Cryptotanshinone; $^{\&\&\&}P < 0.001$ *vs.* Sorafenib alone.

vimentin, Snail and cleaved-caspase3 were detected in HepG2-SR and Huh7-SR cells following the treatment as described above (Fig. 4B and C). As anticipated, cryptotanshinone alone or in co-treatment with sorafenib upregulated E-cadherin and cleaved-caspase3 expression and downregulated the expression levels of vimentin and Snail, indicating a reversed EMT phenotype and promotion of apoptosis, whereas sorafenib alone had opposite effect. These findings indicated that the cryptotanshinone/sorafenib combination suppresses cell proliferative, invasion and migration.

### 3.4 Pharmacological activation of STAT3 inhibits the antitumor activity of cryptotanshinone in HepG2-SR and Huh7-SR cells

We then went on to examine whether activation of STAT3 by colivelin can potentiate the anticancer activities of cryptotanshinone in combination with sorafenib in liver cancer. Specifically, HepG2-SR and Huh7-SR cells were exposed to cryptotanshinone and/or 10 μmol/L sorafenib for 48 h in the presence or absence of colivelin. As illustrated in Fig. 5A and B, cell viability and colony formation assays demonstrated that colivelin significantly inhibited the antitumor activity of cryptotanshinone by inhibiting cell proliferation either in the presence or in the absence of sorafenib, and coincidently, colivelin increased the resistance to sorafenib. Out transwell assay demonstrated that colivelin abrogated the antitumor activity of cryptotanshinone with or without sorafenib by inhibiting cell invasive and migratory abilities (Fig. 5C and D).

To evaluate the molecular mechanism by which STAT3 activation mitigated the anticancer activities of cryptotanshinone, the protein levels of EMT- and apoptosis-associated markers were detected by western blotting. The data revealed that colivelin reversed the cryptotanshinone-induced p-STAT3 downregulation and significantly increased the protein level of p-STAT3 (Fig. 6A). Furthermore, colivelin significantly reversed cryptotanshinone-induced up-regulation of E-cadherin and cleaved-caspase3 and down-regulation of Snail and vimentin, indicating a reversal of the EMT and apoptosis in colivelin treated sorafenib-resistant cells (Fig. 6B and C). These results demonstrated that upregulation of p-STAT3 (enhanced activation of STAT3) enhances the acquired resistance to sorafenib and reverses the antitumor activity of cryptotanshinone by suppressing EMT and apoptosis.

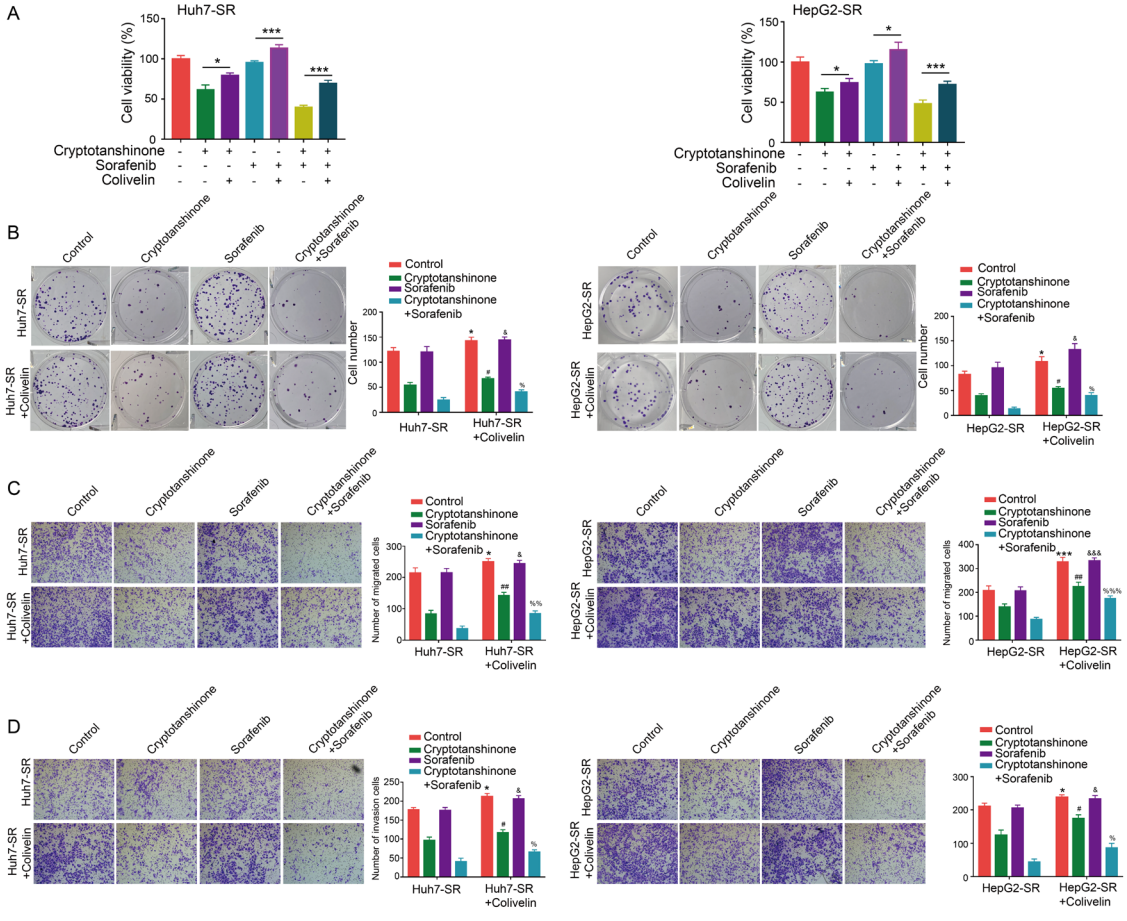


***Fig. 5*** Activation of STAT3 enhances the malignant biological behavior of sorafenib-resistant cells.

(A) HepG2-SR and Huh7-SR cells were incubated with 0 or 10 μmol/L colivelin for 0.5 h, and then incubated with 40 μmol/L cryptotanshinone and/or 10 μmol/L sorafenib for 24 h. Cell viability was assessed and compared with the corresponding untreated cells. $^{*}P < 0.05$, $^{***}P < 0.001$. (B) Cells were subjected to colony formaiton assay. $^{*}P < 0.05$ *vs.* Control; $^{\#}P < 0.05$ *vs.* Cryptotanshinone; $^{\&}P < 0.05$ *vs.* Sorafenib; $^{\%}P < 0.05$ *vs.* combination. (C, D) The cells were subjected to Transwell assay for 48 h, and then cell migration and invasion were measured. $^{*}P < 0.05$, $^{***}P < 0.001$ *vs.* Control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* Cryptotanshinone; $^{\&}P < 0.05$, $^{\&\&\&}P < 0.001$ *vs.* Sorafenib; $^{\%}P < 0.05$, $^{\%\%}P < 0.01$, $^{\%\%\%}P < 0.001$ *vs.* combination.

### 3.5 Cryptotanshinone increases drug sensitivity in sorafenib-resistant cells in vivo

In order to further verify the inhibitory effect of cryptotanshinone on sorafenib-resistant liver cancer cells, Huh7-SR xenograft tumor model was developed. After 30 d of growth and observation, the shortest and longest diameters of the biggest tumor reached to 5 mm and 10 mm, separately (Fig. 7A). Cryptotanshinone combined with sorafenib produced much greater suppression of tumor growth than cryptotanshinone or sorafenib monotherapy, as manifested by the greater magnitudes of decreases in tumor volume (Fig. 7B) and tumor mass (Fig. 7C), which was consistent with the results from the in vitro experiments. Therefore, these in vivo results verified the synergy between cryptotanshinone and sorafenib in inhibiting the growth of sorafenib-resistant cells.

## 4 Discussion

Although sorafenib has become a new standard therapy for patients with advanced liver cancer, long-term sorafenib treatment could likely result in enhanced tumor growth or distant metastasis, which is commonly ascribed to the intrinsic or acquired resistance to the drug[25]. It has been documented that sorafenib can activate STAT3[26-27], and p-STAT3 is a mediator of sorafenib-resistance in liver cancer[25,28], suggesting that STAT3 activity is associated with sorafenib-resistance.

Accumulating evidence has shown that EMT is a prominent feature in liver cancer and is associated with sorafenib-resistance[7,17]. Our preliminary research revealed that HepG2-SR and Huh7-SR cells have enhanced metastatic capacities and increased invasive activities with the epithelial marker E-cadherin being

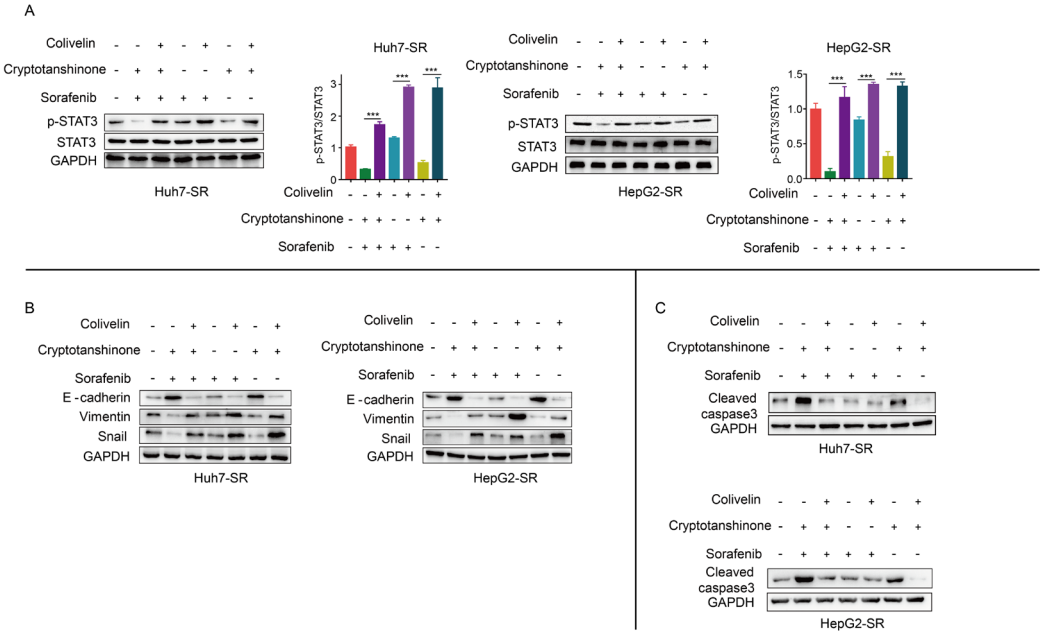


***Fig. 6*** Activation of STAT3 enhances EMT activation and inhibits apoptosis of the sorafenib-resistant liver cancer cells.

Western blot analysis of the protein levels of p-STAT3 (A) and EMT- (B) and apoptosis-associated markers (C) in Huh7-SR and HepG2-SR. Band densities were normalized to GAPDH. Data represent three independent experiments. ***$P < 0.001$.

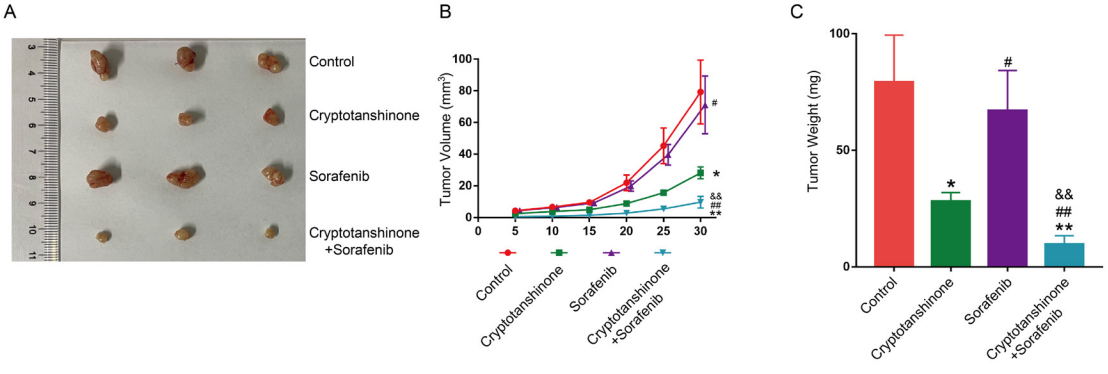


***Fig. 7*** Cryptotanshinone increases the sensitivity to sorafenib in sorafenib-resistant cells *in vivo*.

(A) Xenograft tumor images from different groups ($N = 3$). (B) Measurement of tumor volume ($N = 3$). (C) Measurement of the tumor weight of different groups ($N = 3$). *$P < 0.05$, **$P < 0.01$ *vs.* Control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ *vs.* Cryptotanshinone; $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ *vs.* Sorafenib alone.

downregulated and the mesenchymal markers vimentin and Snail being upregulated relative to their corresponding parental liver cancer cells[18]. There is a crosstalk between the STAT3 and MAPK/ERK signaling pathways indicating that a latent compensatory mechanism of the STAT3 signaling pathway might contribute to EMT in sorafenib-resistant liver cancer cells[29-30].

The present study demonstrated that sorafenib-resistant liver cancer increased the level of p-STAT3 and induced EMT compared with the parental cells, which was consistent with a study reported by Chen *et al.*, where increased p-STAT3 expression and enhanced EMT might be an unwanted side effect for sorafenib therapy to suppress liver cancer progression[31]. It is likely that suppression of p-STAT3 by combination chemotherapy is a mechanism for the synergetic action to overcome the sorafenib-induced p-STAT3 upregulation[17,25,28]. However, the exact mechanism for the synergy remains unknown.

Cryptotanshinone is widely used for the treatment of several conditions, including high blood pressure, fibrosis and malignant

tumors. The antitumor mechanism of cryptotanshinone is complex and multifaceted: inducing cell cycle arrest and/or apoptosis. According to Ke *et al*., cryptotanshinone could induce apoptosis and cell cycle arrest in cholangiocarcinoma cells by suppressing both the JAK2/STAT3 and PI3K/Akt/NF-κB signaling pathways[32]. Moreover, cryptotanshinone was reported to produce dual inhibitory effects on p-STAT3 and p-STAT5 to suppress key oncogenic proliferation and drug-resistance pathways in chronic myeloid leukemia[33]. Although STAT3/5 are also involved in the progression of liver cancer, only STAT3 has been found to be involved in the drug resistance induced by sorafenib in liver cancer[34]. Therefore, whether cryptotanshinone can act on sorafenib-resistant liver cancer through STAT3 mechanism has not been clarified, to the best of our knowledge.

In the present study, liver cancer cell lines (Huh7 and HepG2) were used to establish the sorafenib-resistant cells (Huh7-SR and HepG2-SR). Consistent with previous reports, p-STAT3 was highly expressed in both Huh7-SR and HepG2-SR cells. And cryptotanshinone elicited anti-cancer effects in sorafenib-resistant liver cancer cells in dose- and time-dependent manners. Moreover, cryptotanshinone also suppressed the proliferation, invasion, and migration in sorafenib-resistant liver cancer by inhibiting EMT and promoting apoptosis via targeting STAT3 activation. However, the inhibitory effect of cryptotanshinone on sorafenib-resistant liver cancer could be partially abolished by STAT3 activator colivelin. Most notably, the synergetic anti-tumor property of the cryptotanshinone/sorafenib combination in the context of growth inhibition of tumors induced by sorafenib-resistant liver cancer cells could be consistently reproduced in vivo in xenograft tumor model.

In conclusion, the present study provides supportive evidence that cryptotanshinone can partly reverse the acquired resistance to sorafenib in liver cancer by suppressing sorafenib-induced STAT3 activation, so as to suppress cell proliferation, EMT, and anti-apoptosis in sorafenib-resistant of liver cancer. The findings may facilitate the development of treatment strategies for drug-resistance of liver cancer. However, the potential mechanism for the reversal of sorafenib-resistance by cryptotanshinone is complex, and whether cryptotanshinone could inhibit other STAT family members remains unsolved in the present study. Further mechanistic studies and animal experiments are warranted to let deeper insight into the therapeutic effect of cryptotanshinone combined with sorafenib on liver cancer.

## Conflicts of interest

For the remaining authors none were declared.

## Ethical Approval

The present study was approved by the Ethics Committee of The Second Affiliated Hospital of Harbin Medical University (Harbin, China).

## Author Contribution

ZL and DW substantially contributed to the conception and the design of the study. ZL, KC and CC contributed to data acquisition and statistical analysis. ZL and YW were responsible for interpretation of experiment data, manuscript writing, reviewing and revision of the manuscript. ZL and DW confirmed the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

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