

# Combination treatment with sorafenib and wh-4 additively suppresses the proliferation of liver cancer cells

SU-HONG CHEN<sup>1,2</sup>, DAN-DAN XU<sup>2</sup>, PENG-JUN ZHOU<sup>1</sup>, YAO WANG<sup>1</sup>, QIU-YING LIU<sup>1</sup>, ZHE REN<sup>1</sup>, ZHONG LIU<sup>1</sup>, XIA WANG<sup>2</sup>, HUI-QING HUANG<sup>2</sup>, XUE XUE<sup>2</sup>, YING WANG<sup>3</sup> and YI-FEI WANG<sup>1</sup>

<sup>1</sup>College of Life Science and Technology, Jinan University, Guangzhou, Guangdong 510632; <sup>2</sup>College of Biotechnology, Guangdong Food and Drug Vocational College, Guangzhou, Guangdong 510520; <sup>3</sup>College of Food Science and Technology, Zhongkai University of Agriculture and Engineering, Guangzhou, Guangdong 510225, P.R. China

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**Abstract.** Sorafenib is currently used to treat hepatocellular carcinoma (HCC). However, the development of chemoresistance to sorafenib is a major limitation for sorafenib-based therapy in patients with HCC. In the present study, the effect of the combination therapy of sorafenib and wh-4 on the proliferation of liver cancer cells was investigated. The results showed that sorafenib with wh-4 additively suppressed the proliferation of liver cancer cells. The colony formation of liver cancer cells decreased significantly in response to the combination treatment of sorafenib with wh-4, and it also induced the apoptosis of liver cancer cells. Western blot analysis demonstrated decreased expression of Bcl2, and increased expression of Bax in liver cancer cells treated with a combination of sorafenib and wh-4. Moreover, the migration of liver cancer cells was inhibited. The combination treatment of sorafenib with wh-4 reduced the expression levels of *ABCB1* and *ABCG2* which are responsible for resistance. Finally, STAT3 overexpression abolished the proliferation inhibition effect of sorafenib with wh-4 on liver cancer cells, and sorafenib and wh-4 suppressed the proliferation of liver cancer cells by STAT3 pathway. Together, these results suggest that sorafenib-wh4 combination treatment is a potential novel therapeutic approach to suppress the proliferation of liver cancer cells.

## Introduction

Hepatocellular carcinoma (HCC) is the leading cause of cancer-associated deaths and is extremely resistant to chemotherapy (1). Due to poor prognosis and lack of effective drugs, the overall survival rate of patients with HCC is below 30% (2,3). Most patients go undiagnosed until the disease has progressed to an advanced stage (4). Molecular therapy targeted against specific molecules in tumor cells or their niche is currently the standard treatment for patients with advanced liver cancer (5). Sorafenib, a multi-kinase inhibitor, is a Food and Drug Administration (FDA)-approved chemical drug for treating patients with HCC (6-8). However, when treated with sorafenib, the average overall survival of patients is only extended by 2.8 months compared with that of untreated patients. Patients treated with sorafenib either suffer from severe side-effects or show disease progression after the initial response (4,9). Previous studies have shown that sorafenib is no longer effective in patients after months of treatment, which suggests that the shortcomings of sorafenib are associated with the development of drug resistance (10-12). ATP-binding cassette (ABC) transporters are involved in tumor cell multidrug resistance (MDR), such as *ABCB1* and *ABCG2*. ABC proteins can transport a wide variety of anticancer drugs, including inhibitors of tyrosine kinases (13). In HCC cells, ABC proteins are upregulated, which is associated with the activation of survival pathways (14,15). *ABCB1* has been associated with decreased median survival time in patients with HCC and *ABCG2* contributes towards the MDR phenotype in HCC (16,17).

Wh-4, a heat shock protein 90 (Hsp90) inhibitor, was synthesized in the laboratory and was derived from the existing inhibitor, SNX-2112 (18-23). Hsp90 is a member of a highly conserved family of molecular chaperones present in all eukaryotes (24). Although Hsp90 accounts for only 1-2% of total cellular protein content, it is responsible for regulating several activities, including client proteins activity, stability, conformation and function (25,26). Hsp90 facilitates metastasis, rapid cell division, resistance and evasion of apoptosis in cancer cells (27). Many kinases, including PI3K, ERK, vascular endothelial growth factor receptor (VEGFR) and insulin-like growth factor receptor, are Hsp90 client proteins (26). These

*Correspondence to:* Dr Yi-Fei Wang, College of Life Science and Technology, Jinan University, 601 West Huangpu Road, Guangzhou, Guangdong 510632, P.R. China  
E-mail: twang-yf@163.com

Dr Ying Wang, College of Food Science and Technology, Zhongkai University of Agriculture and Engineering, 501 Zhongkai Road, Guangzhou, Guangdong 510225, P.R. China  
E-mail: 39087419@qq.com

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functionally important kinases depend on Hsp90 to achieve an active conformation or to gain increased stability (25). Cancer cells are dependent on Hsp90, and thus Hsp90 has been successfully used as a target in tumor therapeutics in many clinical trials for Hsp90 inhibitors in multiple tumor types (28). For example, the benzoquinone ansamycin Hsp90 inhibitors, including geldanamycin and its derivative 17-AAG (26,29,30), induced cancer cell apoptosis and disrupted the transcriptional function of HIF1 $\alpha$ . Moreover, 17-AAG decreased the colony-formation capacity of lymphoma stem cells (31). In addition, SNX-2112, a novel Hsp90 inhibitor, decreased the cell viability and tumorigenicity of multiple myeloma cells (32).

Although sorafenib, a tyrosine kinase inhibitor, remarkably suppresses the Raf/Ras/MEK/ERK signaling pathway and inhibits receptor tyrosine kinases, including VEGFR, platelet-derived growth factor receptor (PDGFR), and fibroblast growth factor receptor, HCC cells are resistant to sorafenib and its side effects are also severe (33-35). Hsp90 is a molecular chaperone that stabilizes the folding and conformation of proteins in cancer cells. Hsp90 client proteins play an important part in cancer cell proliferation, resistance, and other important cellular processes. According to previous studies, sorafenib interferes with the unfolded protein response (36,37). For example, sorafenib interacts with Hsp90/Hsp70 inhibitors to disrupt the folding of nascent proteins (38,39), which suggests that the combination of sorafenib with wh-4 can effectively inhibit cancer cell proliferation.

Here, we investigated the effect of sorafenib in combination with wh-4 on liver cancer cells. In addition, we examined the anti-tumor efficacy of a novel Hsp90 inhibitor, wh-4, in liver cancer cells.

## Materials and methods

**Cell lines.** Liver cancer cell lines SK-HEP-1, which are liver sinusoidal endothelial cells of tumorigenic origin (cat. no. FH0072), Huh7 (cat. no. FH0873) and HepG2 (cat. no. FH0076) were purchased from the FUHENG Biotechnology in Shanghai. The SK-HEP-1 and HepG2 cell lines were authenticated using short-tandem repeat profiling (FUHENG Biotech). The cells were seeded in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 1% penicillin-streptomycin and incubated in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C. Sorafenib (cat. no. Y0002098), Tris, glycine, SDS and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (Merck KGaA). Wh-4 (purity >98.0%) was a kind donation from Professor Huang (Serenex, Inc.; Durham, USA). Wh-4 is a benzamides derivative and was designed to inhibit proteins with purine binding sites, which yielded a novel benzamide hit for Hsp90. Synthetic and modeling analyses of this chemical scaffold prompted effort to combine the benzamide with a 1,2,3,9-tetrahydro-4H-carbazol-4-one moiety. The 1,2,3,9-tetrahydro-4H-carbazol-4-one ring system was established by means of combining 1,3-cyclohexanedione and phenyl hydrazine via the Fischer indole synthesis in a Personal Chemistry microwave apparatus. Use of dimesone or the mono-methyl reagent instead of 1,3-cyclohexanedione yielded the related analogs. The purified tetrahydro-4H-carbazol-4-one was then reacted with the desired 4-fluorobenzonitrile in the

presence of sodium hydride (40). Wh-4 was dissolved in DMSO, and a 10-mM stock solution in DMSO was prepared. For further use, the stock was diluted in cell culture medium.

**MTT assay.** The effect of sorafenib and wh-4 on cell proliferation was determined by the MTT (cat. no. KGT525500; Nanjing KeyGen Biotech Co., Ltd.) uptake method. Approximately 3x10<sup>3</sup> cells were seeded in each well of a 96-well plate and incubated for 12 h. On the next day, the cells were exposed to the following treatments: Various concentrations of wh-4 only, 10  $\mu$ M sorafenib only, or a combination of both drugs. The treatment was carried out at 37°C for 48 h. Finally, MTT (5 mg/ml; cat. no. 96992; Sigma-Aldrich; Merck KGaA) was added to each well and incubated at 37°C for 4 h. The absorbance was measured using a Shimadzu reader (Thermo Fisher Scientific, Inc.) at 570 nm.

**Western blotting.** Cells were lysed in ice-cold 1% SDS buffer and centrifuged at 8,000 x g at 4°C for 10 min. The protein concentration was determined using the BCA method (Beyotime Institute of Biotechnology, Inc.). Then, 20  $\mu$ g protein was separated by 12% SDS-PAGE and transferred to 0.20- $\mu$ m polyvinylidene fluoride membranes (cat. no. ISEQ00010; EMD Millipore). The polyvinylidene fluoride membranes were blocked with 5.0% milk in 0.1% TBST (0.1% Tween-20 in Tris-base buffer, pH 7.0) at room temperature for 1.5 h. Then, the membrane was incubated with primary antibodies at 4°C for 16 h. The primary antibodies used in this study (all purchased from Cell Signaling Technology, Inc.) included anti-Bcl2 (1:1,000; cat. no. 15071), anti-Bax (1:1,000; cat. no. 5023), STAT3 (1:1,000; cat. no. 12640), phosphorylated (p)STAT3<sup>Y705</sup> (1:1,000; cat. no. 9145), caspase-3 (1:1,000; cat. no. 9668), caspase-9 (1:1,000; cat. no. 9508), ABCB1 (1:1,000; cat. no. 13342), ABCG2 (1:1,000; cat. no. 42078) and GAPDH (1:1,000; cat. no. 5174). The membrane was washed with 0.1% TBST buffer three times and subsequently incubated with horseradish peroxidase-conjugated secondary antibody (1:8,000; cat. no. 7074; Cell Signaling Technology, Inc.) for 1 h at 37°C before being treated with the chemiluminescence reagent (EMD Millipore) and exposed to Kodak film.

**Reverse transcription-quantitative (RT-q)PCR analysis.** Total RNA (tRNA) was extracted using the TRIzol Reagent kit (cat. no. DP424; Tiangen Biotech Co., Ltd.) and treated with RNase-free DNase and diethyl pyrocarbonate (Nanjing KeyGen Biotech Co., Ltd.). The extracted tRNA was reverse-transcribed into cDNA using PrimerScript Master mix (Bio-Rad Biotechnology, Inc.) according to the manufacturer's instructions. qPCR was used to evaluate the expression level of genes in the RT-PCR system (CFX96 Real-Time System; Bio-Rad Laboratories, Inc.) using primers (Shanghai GeneChem Co., Ltd.). Primer sequences were as follows: ABCB1, forward, 5'-AGGTGGCGTGGAAGGTCC GGTCC-3', and reverse, 5'-GGTGAGGCCGTGGTAATC GTGA-3'; ABCG2, forward, 5'-GGTCGGACCTGGTAG GTAATG-3', and reverse, 5'-AATGTTGACCGGTGGCAA GTTA-3'; GAPDH, forward, 5'-AGCCACATCGCTCAGAC AC-3, and reverse, 5'-GCCCCAATACGACCAATCC-3. The following PCR conditions were used on the Light Cycler: 95°C for 5 sec, 60°C for 5 sec, followed by 40 cycles of 94°C for

20 sec and 60°C for 1 min in a 25- $\mu$ l reaction volume. Relative expression levels were analyzed by the  $2^{-\Delta\Delta C_q}$  method with GAPDH as the reference gene (41). All experiments were performed three times.

**Colony-formation assay.** Approximately  $5 \times 10^3$  cells were seeded in each well of a 6-well dish. On the next day, the cells were treated with different concentrations of wh-4 only, sorafenib only, or a combination of both drugs (5  $\mu$ M sorafenib, 5  $\mu$ M wh-4) and incubated at 4°C for another 48 h. The plates were subsequently incubated at 37°C in a humidified incubator for 21 days. Culture media was replenished every 3 days. The colonies of more than 40 cells was visualized as positive and stained with 0.5% crystal violet for 30 min at 37°C (cat. no. KGA229; Nanjing KeyGen Biotech Co., Ltd.). The numbers of positive colonies were counted under a light microscope (Nikon Corporation) and calculated. The number of more than 40 cells was divided by 5,000, in order to obtain the colon-forming efficiencies. The experiments were repeated three times.

**Scratch wound healing assay.** The combined effects of sorafenib and wh-4 treatment on cell migration were examined using a scratch wound healing assay. Cells were counted, and  $2 \times 10^5$  cells were seeded into 60-mm cell culture plates (Corning, Inc.) in DMEM medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd.). Upon reaching 80% confluence, the bottom of the plates was scratched gently and slowly with a sterile pipette tip, and the gap created in the attached monolayer of cells was photographed (Nikon Corporation). Then, the cells were cultured in DMEM medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd.). After 48 h, the migration distance of the cells was captured under a light microscope (Nikon Corporation) and calculated by subtracting the gap distance recorded at 0 h from the current gap distance. Data were collected from three independent experiments.

**Cell apoptosis analysis.** The cells were exposed to the following treatments: Various concentrations of wh-4 only, sorafenib only, or a combination of both drugs. The cells were incubated with the drugs at 37°C for 48 h. Subsequently, the cells were harvested and washed three times with phosphate buffer, followed by the addition of 0.5 ml binding reagent and 5  $\mu$ l Annexin V-FITC (cat. no. KGAV116; Nanjing KeyGen Biotech Co., Ltd.). After 30 min, the cells were stained with 5  $\mu$ l 7-AAD (cat. no. KGAV116; Nanjing KeyGen Biotech Co., Ltd.) for 15 min at room temperature according to the manufacturer's instructions. Apoptosis in the cells was examined using flow cytometry (BD FACSCalibur). All data were analyzed using the FlowJo 10 software (FlowJo, Becton, Dickinson & Company).

**Cell Ki-67 analysis.** A total of 50,000 cells were seeded in 6-cm plates (Corning, Inc.). They were exposed to the following treatments at 37°C: wh-4 only, sorafenib only, or a combination treatment of both drugs. After 48 h, the cells

were collected, washed, and incubated with Alexa Fluor 488-conjugated Ki-67 antibody (1:100; cat. no. ab197234; Abcam) at room temperature for 1.5 h. Images were captured (Nikon Corporation). Image-Pro Plus was used to calculate the fluorescence intensity of Ki-67 cells (Media Cybernetics).

**Plasmid construct, siRNA sequence and transient transfection.** STAT3 mRNA was extracted from HepG2 cells and cloned into the pcDNA3.1 vector (Shanghai GenePharma Co., Ltd.). STAT3 DNA sequencing was performed by Sangon Biotech Co., Ltd. The vectors were purified using a plasmid filter maxiprep kit (cat. no. K210027; Thermo Fisher Scientific Inc.). The STAT3 recombinant plasmid (pcDNA3.1-STAT3) was transfected using the 5  $\mu$ l Lipofectamine<sup>®</sup> 3000 reagent (cat. no. L300-015; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. pcDNA3.1-STAT3 recombinant was transfected using a serum-free medium, and after 4 h, the medium was replaced with normal medium. Synthetic small interfering RNA (si)-GFP, si-ABCB1 and ABCG2 had the following sequences: GFP sense, 5'-GCAUCAAGG UGAACUCAA-3'; GFP antisense, 5'-UUGAAGUUCACC UUGAUGC-3'; ABCB1 sense, 5'-GCGGUUAACCAUCGA GUUA-3'; ABCB1 antisense, 5'-UAAUCUGAUGGUUAA CCGC-3'; ABCG2 sense, 5'-GCAAUCAGACCUGGAACA AUU-3'; ABCG2 antisense, 5'-AAUUGUCCAGGUCU GAUUGC-3'. Then, 100 pmol siRNA were transfected using the 5  $\mu$ l Lipofectamine<sup>®</sup> 3000 reagent (cat. no. L300-015; Thermo Fisher Scientific, Inc.) in 5% CO<sub>2</sub> at 37°C according to the manufacturer's instructions. Also, 8  $\mu$ g/ml polybrene (cat. no. G04001; Shanghai GenePharma Co., Ltd.) was used to improve the transfection efficiency. After 4 h the medium were replaced with normal medium. 72 h later, the cells were harvested.

**Statistical analysis.** Statistical analysis was performed using SPSS 19.0 software (SPSS, Inc.). All data are presented as the mean  $\pm$  SD for at least three independent experiments. Student's t-test was used for two-group comparisons, whilst comparisons among multiple groups were performed using a one-way ANOVA followed by Tukey's test. Results with  $P < 0.05$  were considered to indicate a statistically significant difference.

## Results

**Combination of sorafenib with wh-4 demonstrates an inhibitory effect on the proliferation of liver cancer cells.** The chemical structure of wh-4 is shown in Fig. 1A. The MTT assay was used to evaluate the inhibitory effect of the drugs on the proliferation of liver cancer cells. Liver cancer cells were treated with various concentrations of sorafenib or wh-4 for 24 h. It was found that the IC<sub>50</sub> values for wh-4 and sorafenib at 24 h were 4.90 and 4.62  $\mu$ M, respectively, in SK-HEP-1 (Fig. 1B). In addition, the IC<sub>50</sub> values for wh-4 and sorafenib at 24 h in Huh7 cells were 4.32 and 5.35  $\mu$ M, respectively (Fig. 1C). Furthermore, a colony-formation assay was performed to evaluate the anti-tumor effect of the drugs, and a similar outcome was observed (Fig. 2A and B). The colony-formation experiments showed that the combination of 5  $\mu$ M sorafenib and 5  $\mu$ M wh-4 significantly inhibited colony

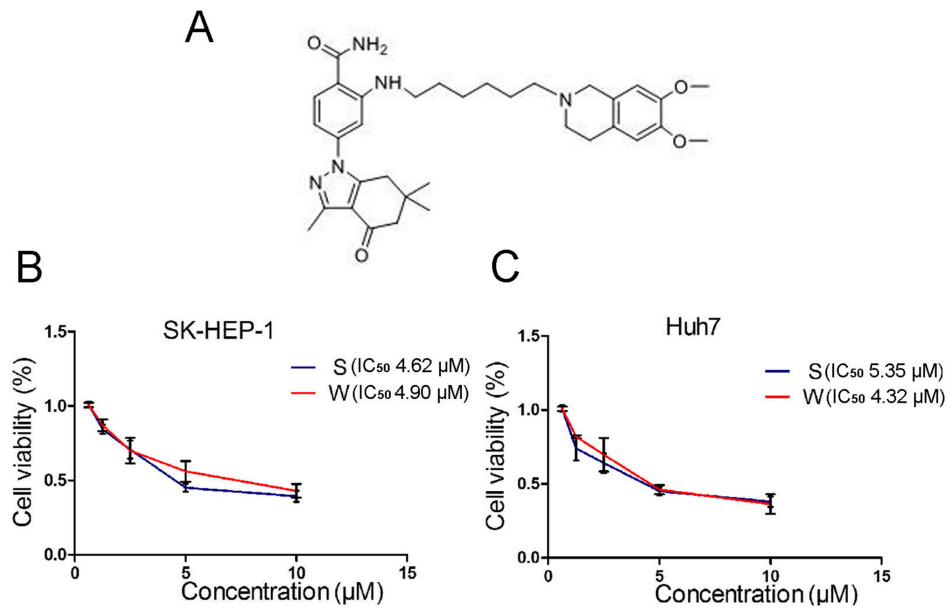


Figure 1. Cell viability of liver cancer cells analyzed by MTT assay. (A) Formal chemical structure of wh-4. (B and C) Three thousand cells were seeded in a 96-well dish and incubated for ~12 h. Cells were then treated with the indicated doses of sorafenib and wh-4 for 48 h. The effect of sorafenib, wh-4 and combination treatment on the cell viability of Huh7, SK-HEP-1 cells was evaluated by the MTT assay.  $\text{IC}_{50}$ , half-maximal inhibitory concentration; S, sorafenib; W, wh-4.

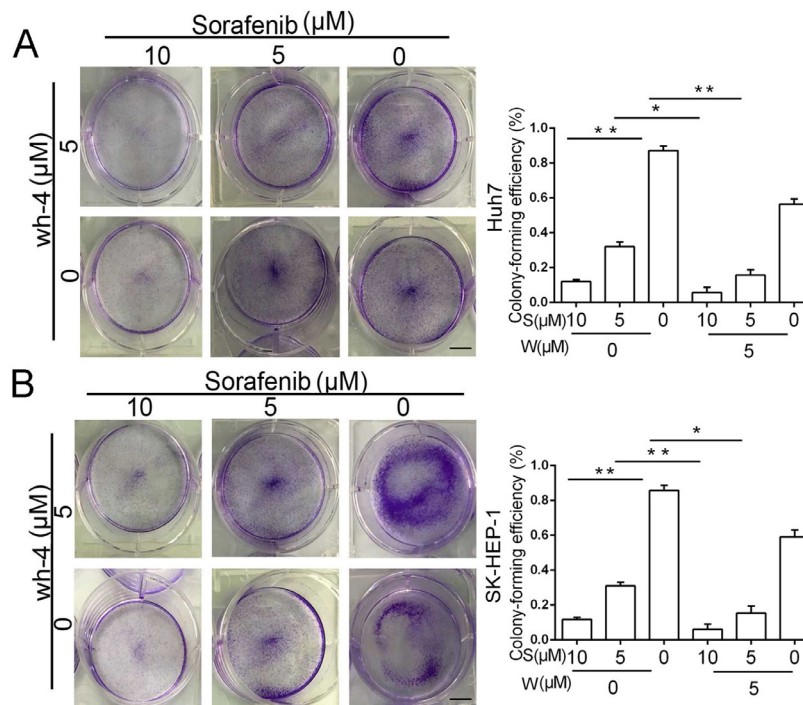


Figure 2. Sorafenib combined with wh-4 additively decreases the viability of liver cancer cells. (A and B) Fifty thousand cells were seeded in a 6-well dish, and the cells were treated with sorafenib, wh-4 or their combination for 48 h. The plates were incubated at 37°C in a humidified incubator for 21 days. Culture media was replenished every 3 days. The colonies of more than 40 cells were visualized as positive and stained with 0.5% crystal violet for 30 min. Scale bar, 10 mm. \* $P < 0.05$  and \*\* $P < 0.01$ . S, sorafenib; W, wh-4.

formation of liver cancer cells. The combination treatment decreased the efficiency of the colony formation more significantly than with sorafenib or wh-4 alone.

*Combination of sorafenib with wh-4 induces apoptosis in liver cancer cells.* Flow cytometry results demonstrated that the percentage of Huh7 cells undergoing apoptosis was

(54.4±5.64)% when treated with 5  $\mu\text{M}$  wh-4 and (58.7±6.51)% when treated with 5  $\mu\text{M}$  sorafenib (Fig. 3A). The fraction of apoptotic cells after the combination treatment of sorafenib and wh-4 was (66.6±6.22)%, which was higher than that after single-drug therapy (Fig. 3A). Furthermore the effect of combination treatment with the two drugs were examined in SK-HEP-1 cells. The percentage of SK-HEP-1 cells



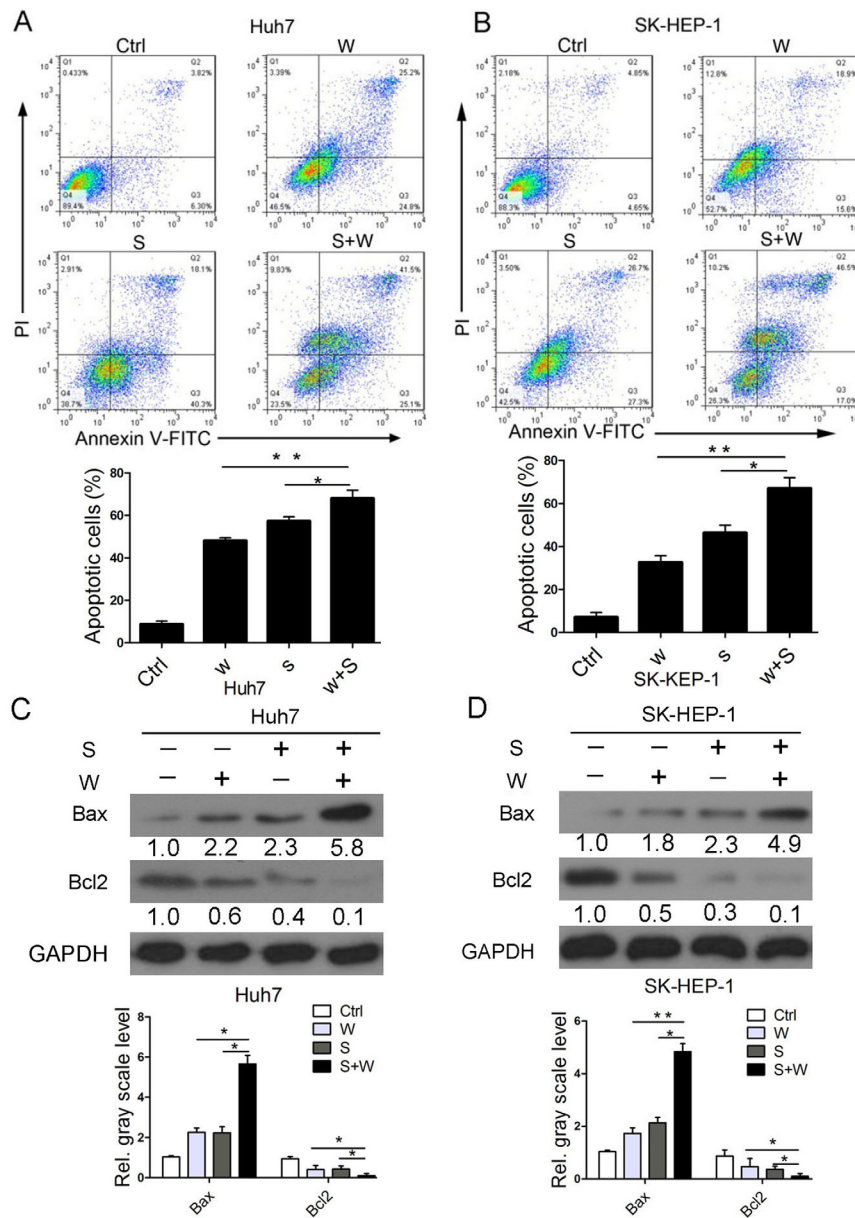


Figure 3. Flow cytometry and western blot assays demonstrates that combination treatment with sorafenib and wh-4 additively induces liver cancer cell apoptosis. (A and B) Analysis of liver cancer cells treated with sorafenib (5  $\mu$ M), wh-4 (5  $\mu$ M) and their combination by flow cytometry. The combination contained 5  $\mu$ M each of sorafenib and wh-4. After 24 h, cells were collected and the effects were analyzed by flow cytometry. (C and D) Analysis of liver cancer cells treated with 5  $\mu$ M sorafenib, 5  $\mu$ M wh-4 and their combination (5  $\mu$ M concentration of each drug) by western blotting. GAPDH was considered as the loading control. \*P<0.05 and \*\*P<0.01. S, sorafenib; W, wh-4; Ctrl, control; Rel., relative.

undergoing apoptosis after the combination treatment was (63.5 $\pm$ 5.85)%, which was higher than that after single-drug therapy with sorafenib or wh-4. The fraction of apoptotic SK-HEP-1 cells after treatment with sorafenib or wh-4 alone was (54.0 $\pm$ 6.34)% and (34.5 $\pm$ 4.89)%, respectively (Fig. 3B). The aforementioned results demonstrated that the Bax levels in Huh7 and SK-HEP-1 cells notably increased when subjected to combination treatment compared to those with either drug alone (Fig. 3C and D). The levels of Bcl2 in Huh7 and SK-HEP-1 cells were significantly decreased after combination treatment with the two drugs (Fig. 3C and D). In addition, the caspase-3 and caspase-9 levels were not significantly different (Fig. S1). Collectively, the aforementioned results suggested that combination treatment with sorafenib and wh-4 increased apoptosis in liver cancer cells.

*Sorafenib with wh-4 suppresses liver cancer cell proliferation and migration.* The scratch wound healing assay demonstrated that both sorafenib and wh-4 inhibited the migration of liver cancer cells. The data suggested that the combination treatment with sorafenib and wh-4 significantly inhibited Huh7 migration (Fig. 4A). A similar additive effect of sorafenib and wh-4 was also observed in SK-HEP-1 cells (Fig. 4B). In addition, the Ki-67 assay demonstrated that sorafenib and wh-4 combination treatment remarkably decreased the proliferation of Huh7 and SK-HEP-1 cells (Fig. 4C and D). The fluorescence intensity level in sorafenib with wh-4 combination treatment was notably decreased (Fig. 4C and D). The aforementioned observations indicated that sorafenib with wh-4 suppressed liver cancer cell proliferation.

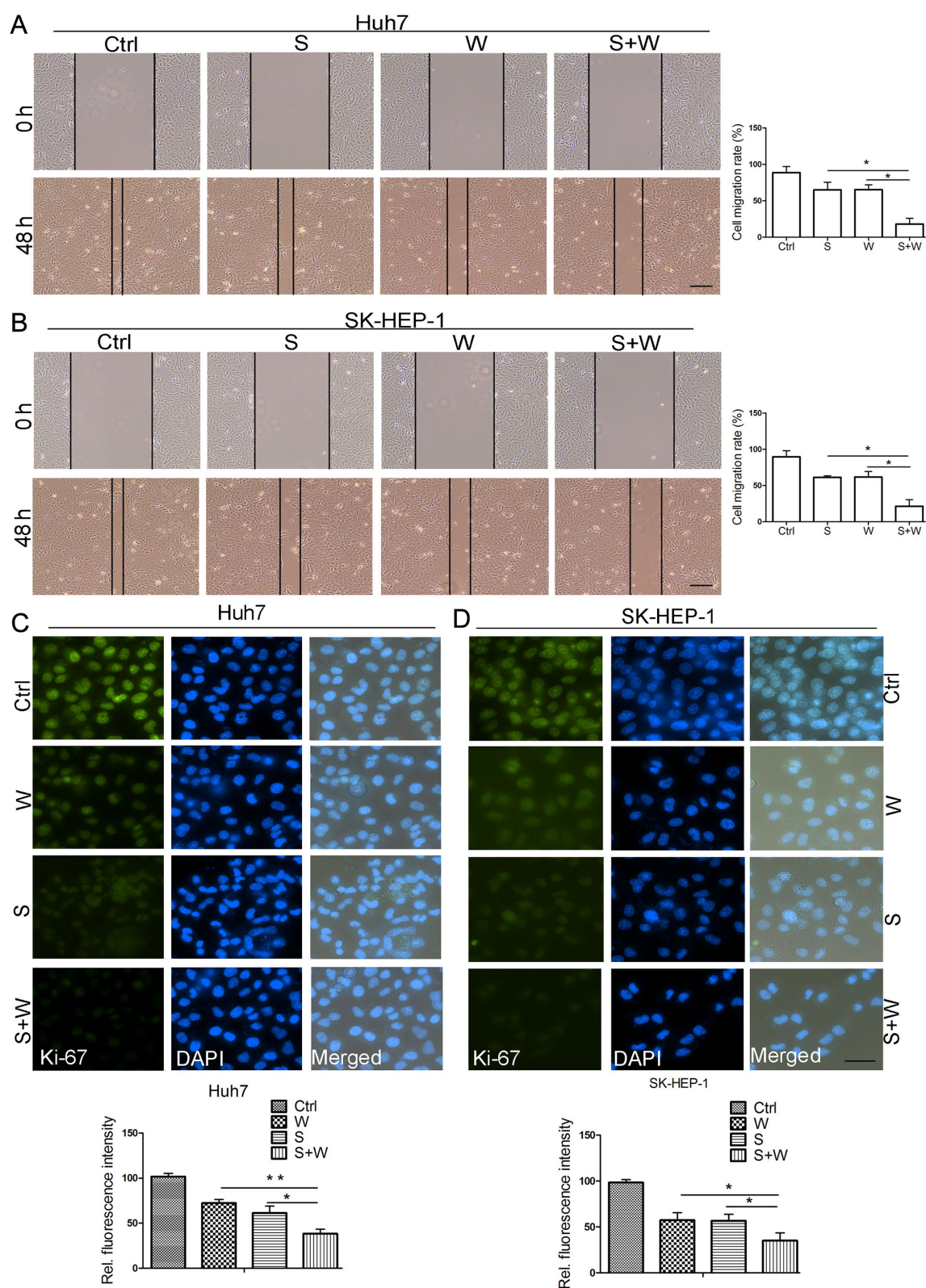


Figure 4. Sorafenib with wh-4 suppresses cell migration. (A and B) Cells were subjected to 6-well plates. The cells were treated with 5  $\mu$ M sorafenib, 5  $\mu$ M wh-4 and their combination (5  $\mu$ M each of sorafenib and wh-4) for 24 h. A 200- $\mu$ l sterile pipette tip was used to scratch the dish bottom. After 48 h, the width was recorded using captured images. The combination contained sorafenib (5  $\mu$ M) and wh-4 (5  $\mu$ M). Scale bar, 50  $\mu$ m. (C and D) Ki-67 analysis of cell proliferation. Cells were treated with sorafenib (5  $\mu$ M), wh-4 (5  $\mu$ M) and their combination (5  $\mu$ M concentration of each drug) for 24 h. Scale bar, 100  $\mu$ m. \* $P$ <0.05 and \*\* $P$ <0.01. S, sorafenib; W, wh-4; Ctrl, control; Rel., relative.

Combination treatment with sorafenib and wh-4 decreases the levels of ABC transporter genes. ABCB1 and ABCG2 play an important part in liver cancer cells proliferation (42,43).

The si-ABCB1 and ABCG2 silencing efficiency was evaluated (Fig. S2). Knockdown of ABCB1 and ABCG2 inhibited the proliferation of liver cancer cells (Fig. 5A and B). However,



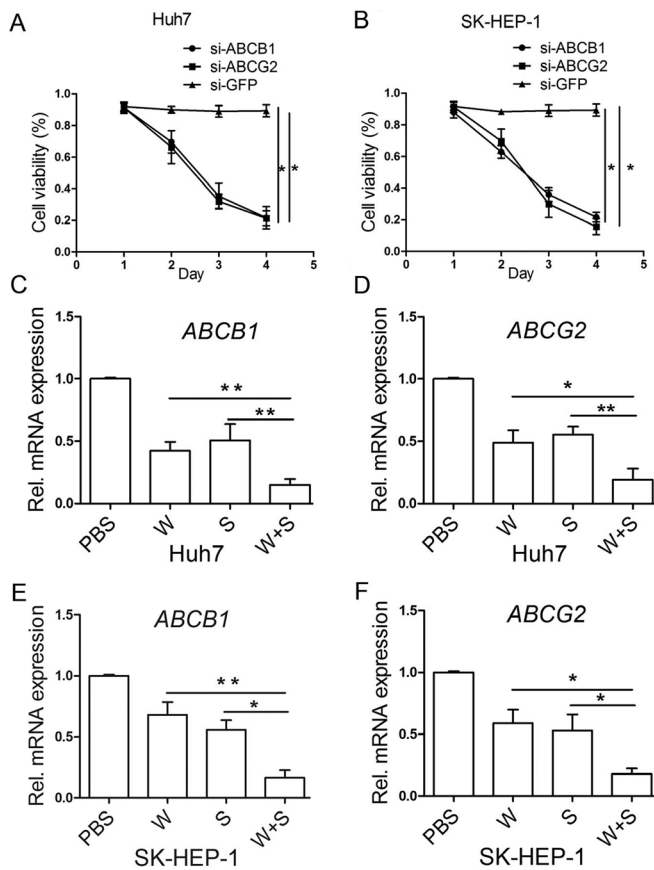


Figure 5. Sorafenib with wh-4 decrease the levels of chemoresistant genes. (A and B) Cell proliferation analysis of liver cancer cells after *ABCB1* and *ABCG2* knockdown. (C and D) Cell viability analysis of liver cancer cells by MTT assay. The liver cancer cells were knockdown with siRNA. (E and F) Reverse transcription-quantitative PCR analysis of *ABCB1* and *ABCG2*. Tumor cells were treated with sorafenib (5  $\mu$ M), wh-4 (5  $\mu$ M) and their combination (5  $\mu$ M concentration of each drug) for 24 h. \* $P$ <0.05 and \*\* $P$ <0.01. S, sorafenib; W, wh-4; siRNA/si-, small interfering RNA; Rel., relative.

the mechanism by which sorafenib leads to the development of resistance remains unclear. After examining the effects of the drugs on cell proliferation, the expression of ABC transporter genes that are responsible for drug resistance were further investigated. The results demonstrated that the combination treatment with sorafenib and wh-4 significantly decreased the expression levels of *ABCB1* and *ABCG2* in Huh7 cells (Fig. 5C and D). Next, the levels of *ABCB1* and *ABCG2* were examined in SK-HEP-1 cells. The levels of *ABCB1* and *ABCG2* were also decreased after combined treatment with sorafenib and wh-4 (Fig. 5E and F). However, the changes in the expression level of P-glycoprotein (P-gp)-encoded and breast cancer resistance protein-encoded *ABCG2* were not observed after the liver cancer cells were treated with the chemicals (Fig. S3). The aforementioned results demonstrated that the combination treatment decreased the resistance level in treated cells.

**Sorafenib and wh-4 additively inhibit liver cancer cell proliferation by suppressing the STAT3 signaling pathway.** The potential molecular mechanism of the additive inhibition of liver cancer cell proliferation by combination treatment with sorafenib and wh-4 was further explored. Individual treatment with sorafenib and wh-4 decreased the phosphorylation

level of p-STAT3<sup>Y705</sup> in both Huh7 and SK-HEP-1 cells (Fig. 6A and B). To investigate whether STAT3 mediates the proliferation of cells treated with a combination of sorafenib and wh-4, a STAT3 overexpression vector was constructed. The pcDNA3.1-STAT3 vector efficiency was analyzed (Fig. S4). As shown in the soft agar assay in Fig. 6C, STAT3 overexpression remarkably reversed the apoptosis induced by combination treatment with sorafenib and wh-4. Similar effects were observed in SK-HEP-1 cells (Fig. 6D), indicating that this reversal of apoptosis was not a cell-line-specific effect. The aforementioned results suggested that sorafenib with wh-4 may suppress the proliferation of liver cancer cells by the STAT3 pathway.

## Discussion

The present study reported that treatment with sorafenib suppressed the proliferation of live cancer cells. This inhibitory effect of sorafenib was significantly enhanced in combination with the Hsp90 inhibitor wh-4, suggesting an additive mechanism of action of these drugs on the inhibition of liver cancer cell proliferation.

Patients with HCC are diagnosed at intermediate or advanced stages when therapies are no longer effective (44). Sorafenib is the first anti-tumor drug approved by the FDA for treating patients with HCC (45). Clinical trials demonstrated that sorafenib prolonged the median overall survival time of patients by about 3-5 months (9,46,47). However, the side effects of this treatment, including anorexia, diarrhea, vomiting and squamous cell carcinoma are apparent (48). In addition, the drug is not effective for all patients with liver cancer, and some patients develop resistance (9). The ABC transporters play a key role in liver cancer cells proliferation (49). In the present study, it was found that the expression of ABC transporter genes was decreased in Huh7 and SK-HEP-1 cells (Fig. 5), suggesting that the chemoresistance was partially limited. In addition, knockdown of *ABCB1* and *ABCG2* inhibited the proliferation of liver cancer cells in the present study. Combination treatment with sorafenib and wh-4 additively decreased the resistance in liver cancer cells. In Fig. 5A and B, si-GFP was used as a control and the Fig. S2 results demonstrated that the si-ABCB1 partly decreased the expression level of *ABCB1*. Also, si-ABCG2 demonstrated the same silencing effect.

The development of drug resistance is a major challenge in the treatment of patients with HCC. Thus, a more rational treatment plan should focus on combining two or more therapeutic methods. Wh-4 is a derivative of SNX-2112, and SNX-2112 inhibits target proteins, such as Akt, p38, MAPK and Erk that play a crucial role in regulating cell survival, proliferation, resistance and homeostasis (25). In addition, the anticancer activity of sorafenib is attributed to its multi-kinase inhibitory function on several signaling pathways, such as Raf-1, B-Raf, and the receptor tyrosine kinase activity of VEGFRs and PDGFR- $\beta$  (50). Induction of apoptosis in HCC cells suggests that sorafenib might promote apoptosis in other cancer cells such as prostate, breast and colorectal cancer cells (51-53). According to previous studies, the anti-tumor activity of sorafenib on cancer cell proliferation and viability may be useful in combination with other therapies or signaling

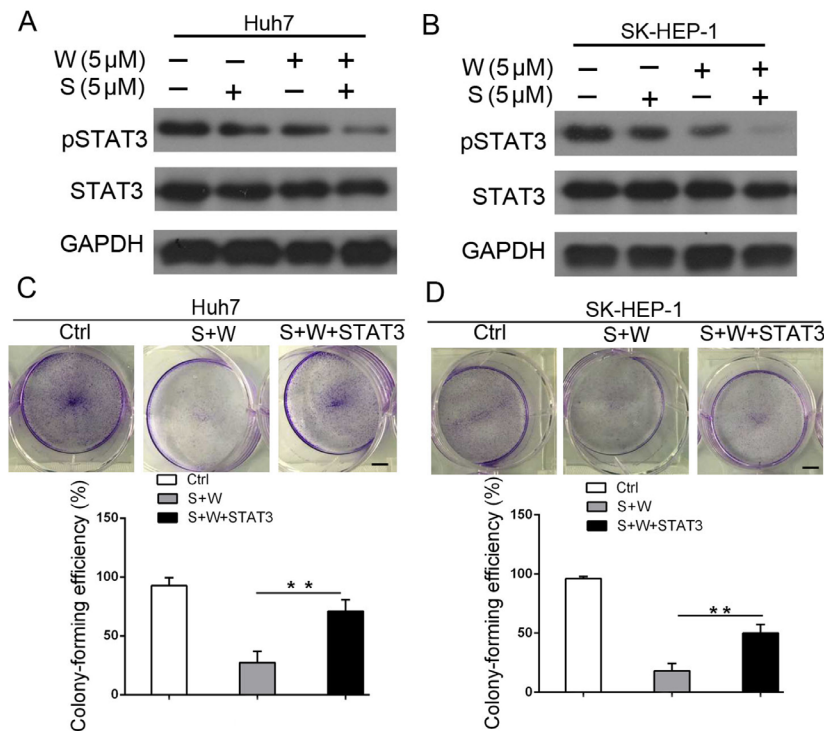


Figure 6. STAT3 signaling pathway mediates liver cancer cell apoptosis induced by sorafenib with wh-4. (A and B) Analysis of STAT3 levels in cells treated with sorafenib (5  $\mu$ M), wh-4 (5  $\mu$ M) and their combination (5  $\mu$ M sorafenib and 5  $\mu$ M wh-4) for 24 h. GAPDH served as the loading control. (C and D) A colony-formation assay was used to analyze whether STAT3 was key to apoptosis induced by sorafenib with wh-4. Colony formation was detected in cells treated with sorafenib (5  $\mu$ M), wh-4 (5  $\mu$ M) and their combination (5  $\mu$ M sorafenib and 5  $\mu$ M wh-4) for 48 h. Colonies were stained with 0.5% crystal violet. Scale bar, 10 mm. \*\* $P$ <0.01. S, sorafenib; W, wh-4; Ctrl, control; pSTAT3, phosphorylated STAT3.

transduction pathway inhibitors (38). Therefore, functional inhibition of Hsp90 target proteins in combination with targets of sorafenib may be an effective cancer treatment strategy. In the present study, it was found that combination treatment with sorafenib and wh-4 additively inhibited the proliferation of liver cancer cells. In addition, it was significant to investigate the additive effect of sorafenib with wh-4 on liver cancer cells. Drug concentration is critical for anti-tumor effects and it was found the effect of one drug was different at different concentration (54,55). It is, therefore, meaningful to adjust the concentration the sorafenib and wh-4.

The changes in the levels of STAT3 were also investigated. Among the STAT family members, STAT3 has received the most attention because it plays a central role in many oncogenic signaling pathways and controls signal transduction pathways in several inflammatory cytokines and growth factors that are implicated in liver damage and repair mechanisms (56). In normal cells, STAT signaling is critical for embryonic development, organogenesis, regulation of cell differentiation, proliferation, growth, and apoptosis, whereas constitutive activation of STAT3 is found in many human types of cancer cell lines and primary tumors including liver, prostate, breast, lung, gastric and head and neck cancer (57-59). STAT3 plays a key role in HCC initiation and progression, and it has been found that its phosphorylation is highly positive in the analysis of HCC biopsies (60-62). Previous more studies demonstrated that STAT3 is an attractive molecular target for the prevention of proliferation and treatment of HCC (56,63). In the present study, the STAT3 pathway was found to mediate apoptosis induced by combination treatment with sorafenib and wh-4.

The results demonstrated that STAT3 is implicated in signal transduction that induces apoptosis in liver cancer cells upon combination treatment with sorafenib and wh-4.

In addition, the limitation of the present study was that *in vivo* experiments in animal were not conducted. Resistance to sorafenib is a major obstacle for clinical treatment. The present *in vitro* study demonstrated that sorafenib with wh-4 combination treatment significantly inhibited liver cancer cells proliferation and reduced ABCB1 and ABCG2 expression levels which were responsible for liver cancer cells resistance partly. However, it was not known whether sorafenib with wh-4 had the antitumor effect *in vivo*. It was desirable to investigate the effect of sorafenib with wh-4 treatment on liver cancer cells *in vivo*.

The present study showed that combination treatment with sorafenib and wh-4 inhibits the proliferation of liver cancer cells and suppresses the development of drug resistance. A novel treatment regimen was also identified to improve the efficacy of sorafenib in patients with liver cancer by targeting the STAT3 pathway. This study demonstrates that combination treatment with sorafenib and wh-4 may present a promising strategy for further clinical therapy of patients with liver cancer.

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#### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

#### Authors' contributions

SHC carried out most of the experiments and wrote the manuscript. DDX and PJZ analyzed the data and results. YaW and QYL read and revised the manuscript and contributed to data collection and statistical analysis. ZR and ZL provided technical assistance with several experiments. XW participated in the study design and drafted the paper. HQH conceived the study. YiW and XX participated in the design and coordination of the study. YFW designed the study and revised the manuscript. All authors have read and approved the final manuscript. YFW and SHC have confirmed the authenticity of all the raw data.

#### Ethics approval and consent to participate

Not applicable.

#### Patient consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interests.

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