

Capsaicin and sorafenib combination treatment exerts synergistic anti-hepatocellular carcinoma activity by suppressing EGFR and PI3K/Akt/mTOR signaling

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Abstract. Capsaicin (8-methyl N-vanillyl-6 nonenamide) is a natural plant extract that has antitumor properties and induces apoptosis and autophagy in various types of malignancies, including hepatocellular carcinoma (HCC). Sorafenib is a multi-kinase inhibitor that improves the survival of patients with advanced HCC. In the present study, capsaicin and sorafenib were found to inhibit the growth of LM3, Hep3B and HuH7 cells. In addition, the combination of capsaicin and sorafenib exerted a synergistic inhibitory effect on HCC cell growth. In LM3 cells, capsaicin and sorafenib combination treatment achieved a markedly stronger induction of apoptosis by increasing caspase-3, Bax and poly(ADP-ribose) polymerase activity and inhibiting Bcl-2, and induction of autophagy by upregulating the levels of beclin-1 and LC3A/B II, enhancing P62 degradation. The combination of capsaicin and sorafenib also inhibited cell invasion and metastasis via upregulation of E-cadherin and downregulation of N-cadherin, vimentin, matrix metalloproteinase (MMP)2 and MMP9. Additional studies suggested an association between the abovementioned anticancer activities and inhibition of the epidermal growth factor receptor/phosphoinositide 3 kinase/Akt/mammalian target of rapamycin pathway. Taken together, these data confirm that capsaicin and sorafenib combination treatment inhibits the growth, invasion and metastasis of HCC cells and induces autophagy in a synergistic manner, supporting its potential as a therapeutic option for HCC.

Introduction

Hepatocellular carcinoma (HCC) is the sixth most prevalent type of cancer and ranks third among the most frequent causes of solid tumor-related deaths worldwide (1). HCC is the most common primary malignancy of the liver, and its incidence has increased in recent decades (2). Patients with advanced unresectable or metastatic HCC often have a poor prognosis, and only a few chemotherapeutics have been proven to be effective (3). Only early-stage HCC patients can receive potentially curative therapies, such as surgical resection and liver transplantation. Therefore, there is an urgent need to identify and develop more effective treatments for HCC (4). Sorafenib, the only systemic therapy that improves the survival of patients with advanced HCC, is a multi-kinase inhibitor (5). The phosphoinositide 3 kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) signaling axis, which plays a pivotal role in cell proliferation, colonization and survival (6), is an emerging target in HCC that contributes to disease progression and the development of resistance to sorafenib. However, sensitivity to sorafenib after the development of resistance may be partially restored by PI3K/Akt inhibitors *in vitro* (7). Therefore, targeting PI3K/Akt signaling may considerably improve the management of HCC patients treated with sorafenib (8).

Capsaicin (8-methyl N-vanillyl-6 nonenamide) is a natural plant extract and the major pungent component of hot peppers of the genus *Capsicum* (9). Capsaicin has potential antitumor properties (10) and produces apoptosis in various types of malignancies, including breast cancer (11,12), colon adenocarcinoma (13,14), nasopharyngeal carcinoma (15), esophageal epidermoid carcinoma (16), HCC (17,18) and prostate cancer (19). Capsaicin has been reported to induce apoptosis and autophagy in several types of human carcinoma cells via inhibition of the PI3K/Akt/mTOR signaling pathway (15,18). The activation of PI3K/Akt/mTOR signaling is associated with cancer cell proliferation, colonization and survival. PI3K/Akt/mTOR signaling may inhibit cell apoptosis (20) and autophagy (21), whereas upregulation of this signaling pathway may promote angiogenesis (22), invasion and metastasis (23-25). Therefore, this pathway holds promise as an effective target for the treatment of HCC through the combined use of capsaicin and sorafenib.

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Epidermal growth factor receptor (EGFR) is a growth factor receptor tyrosine kinase, and its isogenous ligands have been found to be commonly affected in multiple cancer types and appear to facilitate solid tumor growth (26). EGFR is located upstream of PI3K/Akt/mTOR and is overexpressed in HCC cells (27). Therefore, the aim of the present study was to investigate the antitumor activity of capsaicin and sorafenib in *in vitro* and *in vivo* studies, alone as well as in combination, in order to determine whether their combination can induce HCC cell apoptosis and autophagy and inhibit HCC cell proliferation, migration and invasion in a synergistic manner.

Materials and methods

Chemicals and antibodies. Capsaicin and sorafenib were purchased from Sigma-Aldrich; Merck KGaA (St. Louis, MO, USA) and Selleckchem (Houston, TX, USA), respectively. Antibodies against GAPDH, Bax, cleaved caspase-3 (Asp175), poly(ADP-ribose) polymerase (PARP), beclin-1, LC3A/B, E-cadherin, vimentin, P-Akt (Ser473), Akt, P-mTOR (Ser2448), mTOR, P-p70S6 kinase (P-p70S6K, Thr389), p70S6K and Ki-67 were obtained from Cell Signaling Technology (Danvers, MA, USA). The P62 antibody was obtained from Proteintech (Rosemont, IL, USA). The antibodies against Bcl-2, N-cadherin, matrix metalloproteinase (MMP)2, MMP9, P-EGFR, EGFR and PI3K p85 α were obtained from Abcam (Cambridge, MA, USA). The details on the antibodies used in the present study are listed in Table I.

Cell lines and culture conditions. The LM3, Hep3B and HuH7 human HCC cell lines were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific Inc.) at 37°C in a humidified atmosphere (5% CO₂, 95% air). The HCC LM3 cell line used in this study has been authenticated by STR profiling.

Cell viability and colony formation assays. To allow cells to attach completely, LM3, Hep3B and HuH7 cells (5,000 cells/well) were seeded in a 96-well plate for 24 h; then, capsaicin and sorafenib were added to the culture media at the indicated concentrations for another 48 h. Next, 10% Cell Counting Kit (CCK)-8 solution was added to the culture media, and the plates were incubated for 4 h. OD450 values were determined by a spectrophotometer, and the results were analyzed to measure cell growth.

For colony formation assays, adherent cells were trypsinized, and 1,000 viable cells were re-seeded in 6-well plates (in triplicate). After cell adherence, cells were allowed to form colonies for 14 days with each of the treatments. To visualize the colonies, the media were discarded, and the cells were submerged in 4% paraformaldehyde for 15 min and dyed with 0.1% crystal violet staining solution.

Cell migration and invasion assays. LM3 cells were seeded and cultured in a 6-well plate for 24 h to adherence and

confluence. The cell layers were scratched with a 200- μ l pipette tip to create a wound and then washed three times with phosphate-buffered saline (PBS) to remove floating cells. The medium was then replaced with serum-free medium. The wound was photographed at 0, 48 and 72 h.

After trypsinization, 2x10⁵ cells were plated on Boyden chambers coated with 10 μ g Matrigel (BD Biosciences, Sparks, MD, USA) per well (for invasion assays), and 5x10⁴ cells were plated on uncoated Boyden chambers (for migration assays) in medium containing 1% FBS. Medium containing 10% FBS was added to the lower chamber as a chemoattractant. Capsaicin, sorafenib or their combination was added to the upper and lower chambers at the indicated concentrations. After 48 h, the cells that had moved to the lower surface of the membrane were fixed with methanol and stained with 0.1% crystal violet solution. Photographs of three random fields of fixed cells were captured, and the cells were counted. Each reported value was estimated from three plates.

Immunofluorescence. Cells (2x10⁵) were cultured on sterile sheet glass and treated with capsaicin, sorafenib or their combination for 48 h. The cells were submerged in 4% paraformaldehyde solution at room temperature for 10 min after treatment, then washed three times in PBS, permeabilized with 0.1% Triton X-100/PBS for 5 min, and then blocked with 10% bovine serum albumin for 1 h. The cells were incubated with primary antibodies overnight at 4°C, washed three times in PBS for 15 min, and incubated with secondary antibodies for 1 h at room temperature. Unbound Ab was removed by washing with 1X TBST four times for 20 min each time; thereafter, the cell nuclei were stained with DAPI (1:20). ProLong® Gold Antifade Mounting Agent (Thermo Fisher Scientific, Inc.) was used for treating the cells, and fluorescence images were captured by a fluorescence microscope.

Western blotting. Following treatment with capsaicin, sorafenib or their combination, cells were lysed in radio-immunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Shanghai, China) containing 1% phenylmethylsulfonyl fluoride (Beyotime Institute of Biotechnology) and 10% phosphatase inhibitor (Roche Diagnostics GmbH, Mannheim, Germany) for 30 min. Subsequently, the lysates were centrifuged at 10,000 x g for 30 min at 4°C and the supernatant was collected in a new tube. The protein concentrations were determined using the BCA Protein Assay Kit (Beyotime Institute of Biotechnology). After denaturation, protein from each group (50 μ g) was fractionated with 8-12% Tris-glycine gel electrophoresis. Subsequently, different proteins were transferred to PVDF membranes and probed with the corresponding primary antibodies for 12 h at 4°C, then incubated with same-species secondary antibodies at 1:5,000 dilution for 1 h. Enhanced chemiluminescence reagent (Thermo Fisher Scientific, Inc.) was used to detect the proteins, and antibody binding was visualized by Image Lab software (<http://www.bio-rad.com/en-cn/product/image-lab-software?ID=KRE6P5E8Z>).

Apoptosis analysis with flow cytometry and TdT-mediated dUTP nick end labeling (TUNEL). For the apoptosis analysis, LM3 cells were treated with capsaicin, sorafenib

Table I. Details of the antibodies used in the present study.

Antibody	Dilution	Catalogue no.	Company details
GAPDH	WB 1:1,000	5174	Cell Signaling Technology ^a
Bax	WB 1:1,000, IHC 1:200	5023	Cell Signaling Technology ^a
Cleaved caspase-3 (Asp175)	WB 1:1,000	9664	Cell Signaling Technology ^a
PARP antibody	WB 1:1,000	9542	Cell Signaling Technology ^a
Beclin-1	WB 1:1,000	3495	Cell Signaling Technology ^a
LC3A/B antibody	WB 1:1,000	4108	Cell Signaling Technology ^a
E-cadherin	WB 1:1,000	3195	Cell Signaling Technology ^a
Vimentin	WB 1:1,000	5741	Cell Signaling Technology ^a
Phospho-Akt (Ser473)	WB 1:1,000, IHC 1:200	4060	Cell Signaling Technology ^a
Akt (pan)	WB 1:1,000	4691	Cell Signaling Technology ^a
Phospho-mTOR (Ser2448)	WB 1:1,000	5536	Cell Signaling Technology ^a
mTOR	WB 1:1,000	2983	Cell Signaling Technology ^a
Phospho-p70 S6 kinase (Thr389)	WB 1:1,000	9234	Cell Signaling Technology ^a
p70 S6 kinase	WB 1:1,000	2708	Cell Signaling Technology ^a
Ki-67	IHC 1:200	12202	Cell Signaling Technology ^a
P62/SQSTM1 antibody	WB 1:1,000, IHC 1:200	18420-1-AP	Proteintech ^b
Anti-N cadherin antibody	WB 1:1,000	ab18203	Abcam ^c
Anti-MMP2 antibody	WB 1:1,000, IHC 1:200	ab37150	Abcam ^c
Anti-PI 3 kinase p85 alpha antibody	WB 1:1,000	ab86714	Abcam ^c
Anti-EGFR antibody	WB 1:1,000, IHC 1:200	ab52894	Abcam ^c
Anti-MMP9 antibody	WB 1:1,000, IHC 1:200	ab38898	Abcam ^c
Anti-Bcl-2 antibody	WB 1:1,000	ab32124	Abcam ^c
Anti-EGFR (phospho Y1068) antibody	WB 1:1,000, IHC 1:200	ab40815	Abcam ^c

Species: Human. ^aDanvers, MA, USA. ^bRosemont, IL 60018, USA. ^cCambridge, UK. WB, western blotting; IHC, immunohistochemistry.

or their combination for the indicated times, trypsinized and collected in tubes, and incubated in binding buffer with propidium iodide and FITC-conjugated Annexin V for 10 min in the dark at room temperature. Flow cytometry analysis was used to estimate the cell apoptosis rate. The TUNEL method was applied to visualize the 3'-OH ends of DNA fragments in apoptotic cells according to the manufacturer's protocol (Roche Diagnostics GmbH). LM3 cells were subjected to different treatments for 48 h and fixed in 4% paraformaldehyde. The cells were then submerged in methanol containing 0.3% H₂O₂ to inhibit endogenous peroxidase activity. Following washing with PBS, cells were covered with proteinase K solution for 10 min. Subsequently, the cells were covered with the TUNEL reaction mixture and incubated for 1 h at 37°C in the dark. After washing in PBS, the cells were placed in Converter-POD (Roche Diagnostics GmbH) and then incubated at 37°C for 30 min. After rinsing in PBS three times for 5 min, the cells were dipped in DAB (Roche Diagnostics GmbH) at room temperature for 10 min and observed under a microscope.

In vivo studies. Five-week-old BALB/C nude mice (SPF grade) were supplied by SLAC Co., Ltd. (Shanghai, China). All the mice were raised in the cabinet with laminar air flow under pathogen-free conditions in a humidity- and temperature-controlled environment with a 12 h light/dark

schedule. The mice had *ad libitum* access to food and water. Prior to the study initiation, the mice were allowed to acclimatize for 1 week. Then, the mice received a subcutaneous injection of 1x10⁷ LM3 cells suspended in 100 µl sterile PBS into the right flank. Two weeks after the inoculation, based on the initial tumor volume, the mice were divided into four groups (n=6 per group) and received daily treatments via i.p. injections: The control group received sterile PBS with 1% dimethyl sulfoxide (DMSO), the capsaicin group was treated with 5 mg/kg capsaicin containing 1% DMSO, the sorafenib group received 50 mg/kg sorafenib containing 1% DMSO, and the combination group was treated with 5 mg/kg capsaicin + 50 mg/kg sorafenib containing 1% DMSO. Tumor volume and tumor weight were measured every other day, and tumor volume was calculated according to the formula V (mm³) = 1/2 (length x width²). The mice were treated with different compounds for 28 days. All the mice were sacrificed by cervical dislocation under pentobarbital sodium anesthesia administered through i.p. injection, the livers and kidneys were harvested for immunohistochemical examination and blood was collected for biochemistry tests. All efforts were made to minimize animal suffering. All animal procedures were conducted in accordance with the guidelines of the National Institutes of Health and were approved by the Ethical Committee of Wenzhou Medical University and the Laboratory Animal

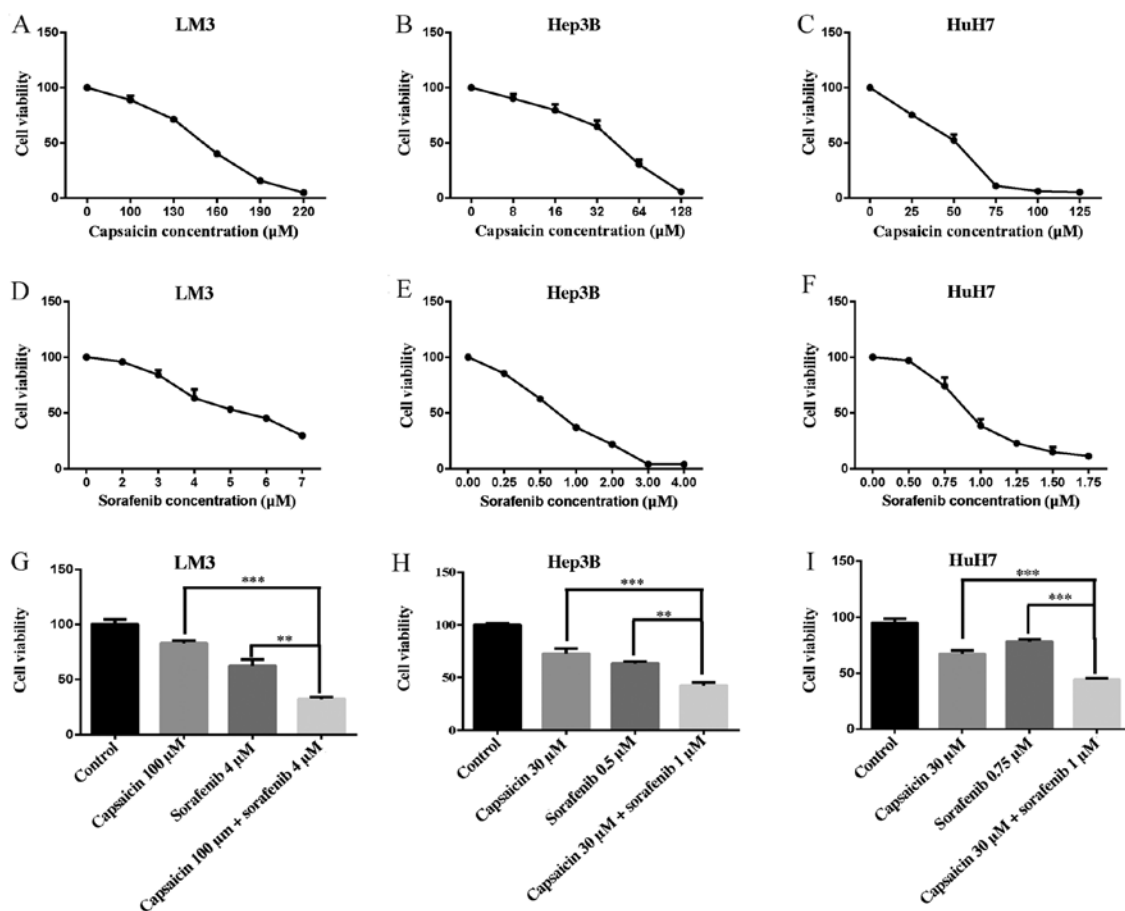


Figure 1. Inhibitory effect of capsaicin, sorafenib and their combined treatment on tumor cell viability. (A-C) Capsaicin dose-dependently reduced the viability of the hepatocellular carcinoma (HCC) cells Hep3b, Huh7 and LM3. (D-F) Sorafenib dose-dependently reduced the viability of the HCC cells Hep3b, Huh7 and LM3. Cells were incubated with different concentrations of capsaicin and sorafenib for 48 h before being subjected to a Cell Counting Kit (CCK)-8 assay. (G-I) Effect of capsaicin and sorafenib combination treatment compared with the single-drug treatment on cell viability in Hep3b, Huh7 and LM3 cell lines. Cells were incubated with capsaicin, sorafenib and their combination for 48 h before being subjected to an CCK-8 assay. The data are presented as the mean \pm standard error of the mean. ** $P < 0.01$, *** $P < 0.005$.

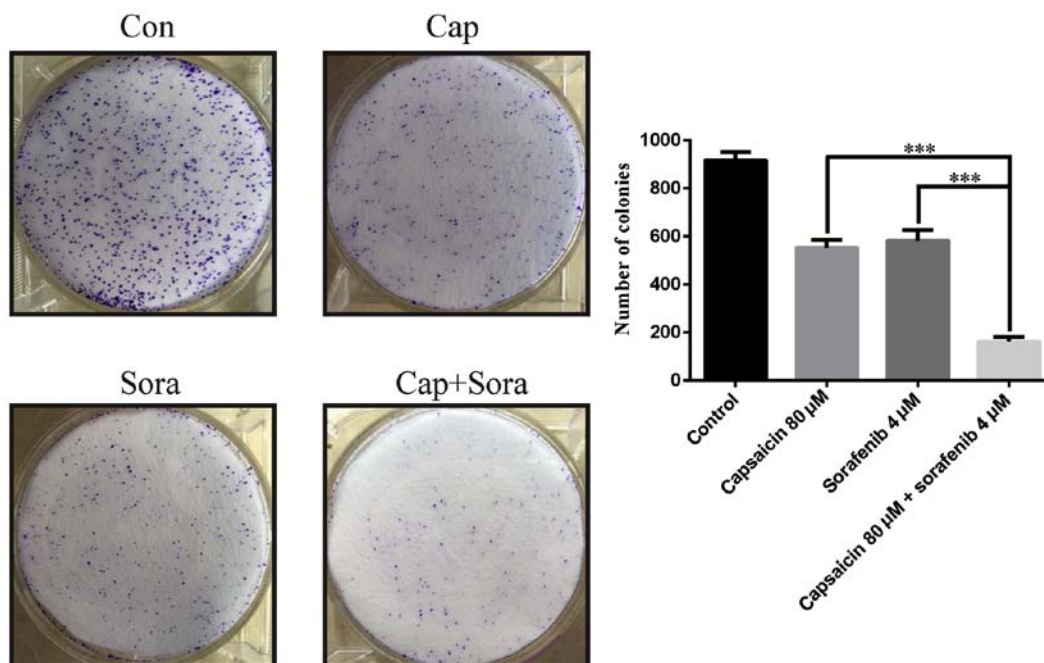


Figure 2. Capsaicin, sorafenib and the IR combination inhibit colony formation in LM3 cells. In the colony formation assay, the formed colonies were counted manually for each group of cells and are presented as the means \pm standard error of the mean from three separate experiments. *** $P < 0.005$. Con, control; Cap, capsaicin 80 μM; Sora, sorafenib 4 μM.

Table II. Cells were incubated with three different concentrations of capsaicin, sorafenib and 9 types of combination for 48 h prior to being subjected to Cell Counting Kit-8 assay.

Capsaicin (μM)	Sorafenib (μM)	OD	Inhibition ratio (%)	CI	IC ₅₀
0	0	1.259±0.217			
80	0	1.169±0.408	8.49921		
100	0	1.038±0.235	20.8752		
120	0	0.8099±0.046	42.5308		
0	2	1.116±0.119	13.4929		
0	3	0.884±0.357	35.4692		3.987
0	4	0.755±0.146	47.7788		
80	2	0.887±0.180	35.1351	0.388557	
80	3	0.754±0.073	47.8033	0.367342	2.989
80	4	0.546±0.106	67.5735	0.283707	
100	2	0.850±0.215	38.7417	0.394444	
100	3	0.633±0.071	59.3175	0.329109	2.590
100	4	0.502±0.064	71.7346	0.279882	
120	2	0.647±0.035	57.9597	0.335968	
120	3	0.402±0.015	81.2204	0.237104	1.854
120	4	0.215±0.006	98.9005	0.137558	

The effect of the drug combination was analyzed using the combination index (CI) method, CI values >1 indicated antagonism, CI values = 1 indicated additivity, CI values <1 indicated synergy, and CI values <0.7 indicated significant synergy. Each CI ratio presented herein was the mean value derived from at least three independent experiments. The data are presented as the mean ± standard error of the mean.

Management Committee of Zhejiang Province (Approval ID: wydw2017-0052).

Immunohistochemistry. The tumors were fixed with 4% paraformaldehyde solution and embedded in paraffin, then cut into 4- μm sections. The slides were incubated with antibodies against P-EGFR, P-Akt, Ki67, Bax, P62, MMP2 and MMP9; then, the slides were washed, stained with secondary antibody, and directly visualized by the ChemMate EnVision Kit (ZSGB-BIO Beijing China). Images of the stained sections were captured under a microscope at a magnification of x400. Histological analysis of liver and kidney sections was conducted with hematoxylin and eosin (H&E) staining. Details on the antibodies used may be found in Table I.

Statistical analysis. The data are presented as the mean ± standard error of the mean for given samples, and were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple range tests using SPSS v.22 statistical software (IBM Corp., Armonk, NY, USA). The significance level was set at $P < 0.05$.

Results

Capsaicin reduces cell viability and potentiates the inhibitory effects of sorafenib in HCC lines. CCK-8 assays were performed to evaluate the effects of capsaicin and sorafenib alone and in combination on three human HCC cell lines, LM3, Hep3B and HuH7 (Fig. 1A-I). The survival and proliferation of these four cell lines decreased with increasing concentrations of capsaicin and sorafenib. The inhibition of cell survival

and proliferation was greatly enhanced by the combination of a low concentration of capsaicin and a moderate concentration of sorafenib.

Different combinations of capsaicin and sorafenib inhibit LM3 cell proliferation to various extents. In colony formation assays, LM3 cells treated with the combination of capsaicin and sorafenib exhibited decreased colony formation ability compared with those treated with monotherapy (Fig. 2). The effects of capsaicin in combination with sorafenib on LM3 cells are further detailed in Table II. In LM3 cells, the IC₅₀ of sorafenib in combination with capsaicin (80, 100 and 120 μM) was significantly decreased from 3.987 μM to 2.989, 2.590 and 1.854 μM , respectively, and significant synergy between sorafenib and capsaicin was observed at 80, 100 and 120 μM capsaicin.

Capsaicin acts synergistically with sorafenib to induce apoptosis in the LM3 cell line. Based on the results in Table II, the combination of 80 μM capsaicin and 4 μM sorafenib was selected for the next series of experiments. To ascertain the extent of apoptosis, cells were examined by TUNEL staining. Following treatment with capsaicin and sorafenib, microscopic examination revealed stained cells, and the combination of capsaicin and sorafenib was associated with a higher number of TUNEL-positive cells (Fig. 3A). To further validate apoptotic cell death induced by capsaicin and sorafenib alone and in combination, apoptosis was evaluated by flow cytometry analysis. The combination treatment triggered apoptosis to a greater extent than either monotherapy. As shown in Fig. 3B, the percentage of apoptotic cells after 48 h of treatment with

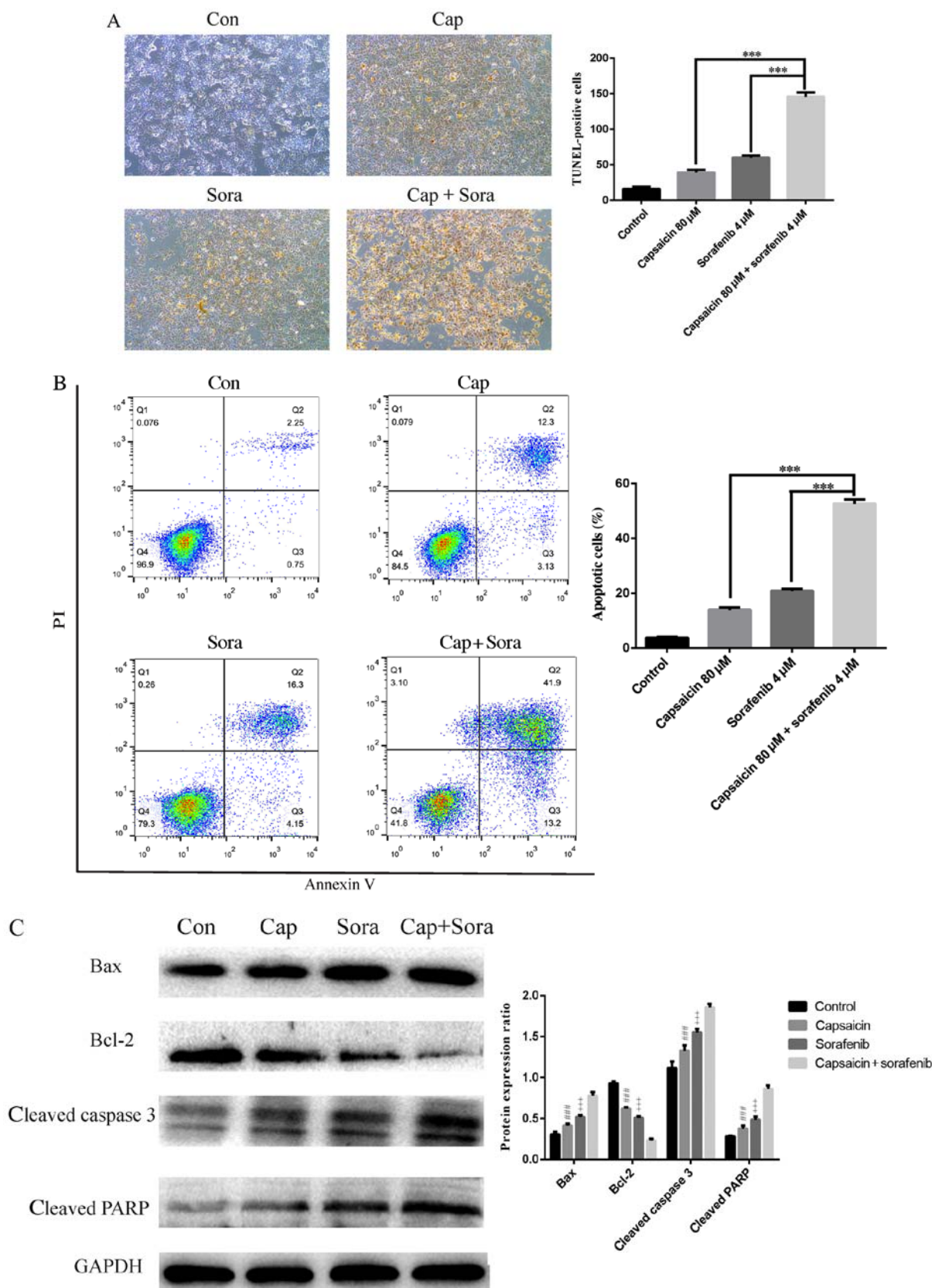


Figure 3. Capsaicin and sorafenib induce apoptosis in LM3 cells, and their combination exerts a synergistic effect on cell apoptosis. (A) TUNEL staining was used to visualize the 3'-OH ends of DNA fragments in apoptotic cells. Representative photomicrographs of cells under control or drug treatments are shown (magnification, $\times 100$). (B) LM3 cells were treated with capsaicin, sorafenib and their combination for 48 h and were then stained with Annexin V and propidium iodide (PI). The early apoptotic cells (Annexin V-positive, PI-negative) and the late apoptotic cells (Annexin V-positive, PI-positive) are indicated as the percentage of gated cells. The histogram represents the apoptotic cells for each dose. Data are presented as the mean \pm standard error of the mean (SEM) of three experiments. (C) Western blot analysis of Bax, Bcl-2, cleaved caspase-3 and cleaved PARP after capsaicin, sorafenib and combination treatment for 12 h. The results are representative of three separate experiments. GAPDH was set as control. The data are presented as the mean \pm SEM. *** $P < 0.005$; #, Capsaicin vs. combination; +, sorafenib vs. combination. ###/####/+++/++++: $P < 0.005$. Con, control; Cap, capsaicin 80 μ M; Sora, sorafenib 4 μ M; PI, propidium iodide; PARP, poly(ADP-ribose) polymerase.

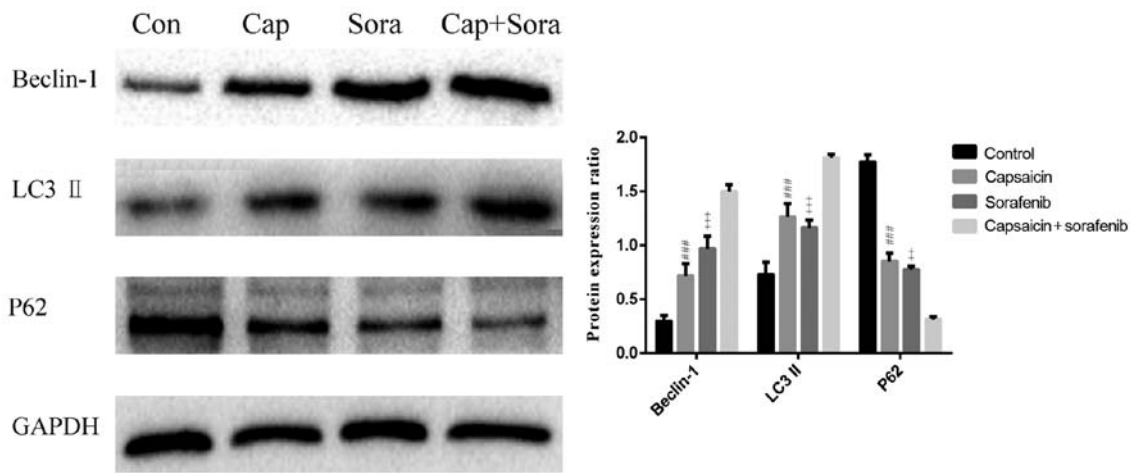


Figure 4. Capsaicin and sorafenib induce autophagy in LM3 cells and have a synergistic effect on autophagy. LM3 cells were treated with capsaicin, sorafenib and their combination for 12 h, and then autophagy-associated proteins beclin-1, LC3 II and P62 were detected by western blot analysis. GAPDH served as a loading control. The results are representative of three separate experiments. #, Capsaicin vs. combination; +, sorafenib vs. combination. The data are presented as the mean \pm standard error of the mean. ++ and ###/+++: $P < 0.01$ and 0.005 , respectively. Con, control; Cap, capsaicin $80 \mu\text{M}$; Sora, sorafenib $4 \mu\text{M}$.

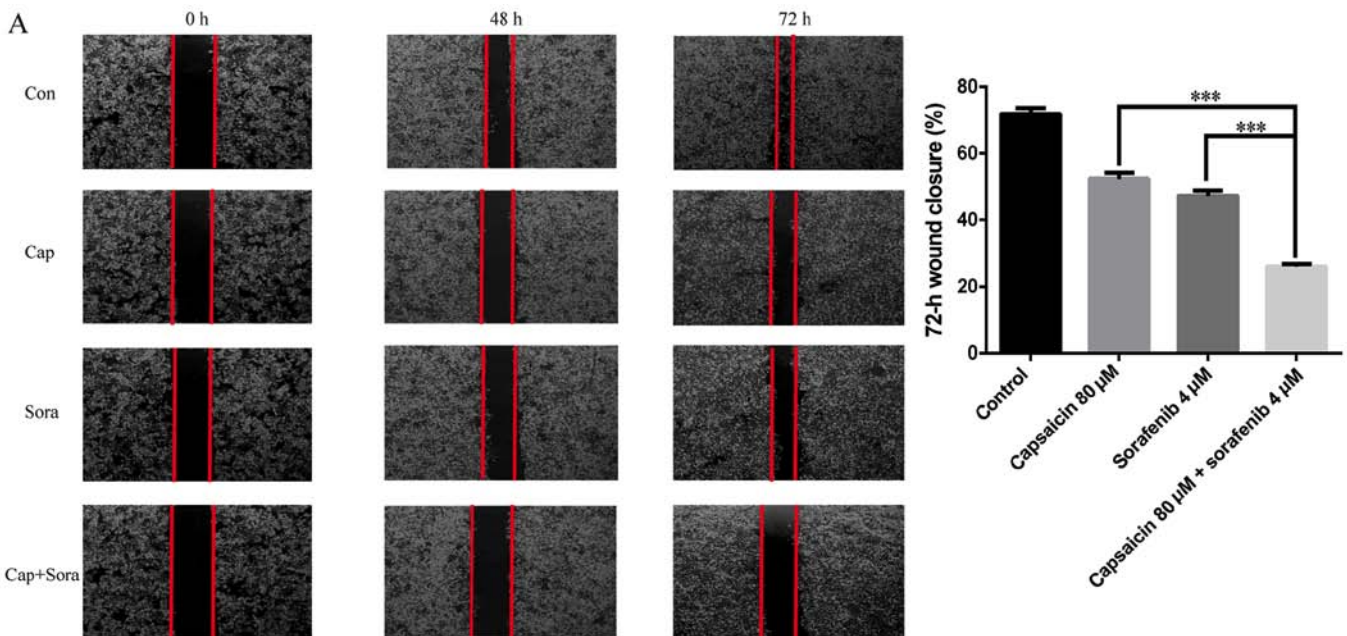


Figure 5. Capsaicin and sorafenib inhibit the invasion and migration of LM3 cells, and the combination treatment can inhibit invasion and migration synergistically. (A) Capsaicin, sorafenib and their combination inhibited migration of LM3 cells. The cell migration was assessed by wound healing assay (magnification, $\times 40$). Con, control; Cap, capsaicin $80 \mu\text{M}$; Sora, sorafenib $4 \mu\text{M}$.

control, $80 \mu\text{M}$ capsaicin, $4 \mu\text{M}$ sorafenib and the combination of $80 \mu\text{M}$ capsaicin and $4 \mu\text{M}$ sorafenib was 3.43 ± 0.536 , 16.58 ± 0.629 , 21.18 ± 0.809 and $52.13 \pm 1.602\%$, respectively. To further elucidate the mechanism underlying the increased apoptosis of LM3 cells, the protein levels of two key apoptosis-related protein, Bcl2 and Bax, were investigated. The pro-apoptotic protein Bax expression was upregulated by the capsaicin and sorafenib combination, and the expression of the anti-apoptotic protein Bcl2 was reduced (Fig. 3C). Caspase-3 was also examined by western blot assays. As shown, exposure to $80 \mu\text{M}$ capsaicin and $4 \mu\text{M}$ sorafenib for 24 h increased the cleavage of caspase-3. The results mentioned above indicate that capsaicin combined with sorafenib can modulate the activation of apoptotic signaling pathways in LM3 cells.

Capsaicin combined with sorafenib enhances autophagy in the LM3 cell line. To determine the effect of the capsaicin and sorafenib combination treatment on cell autophagy, the protein expression of the autophagy-related genes beclin-1, P62 and LC3A/B-II was examined by western blotting (Fig. 4). Beclin-1 and LC3A/B-II levels were increased by the combination treatment compared with monotherapy. Compared with the monotherapy groups, the autophagy-specific substrate P62 was obviously reduced in the combination group.

The capsaicin and sorafenib combination markedly inhibits LM3 cell invasion and migration. Cell scratch assays were used to determine whether capsaicin and sorafenib inhibit HCC cell migration. As shown in Fig. 5A, the LM3 cell-free area

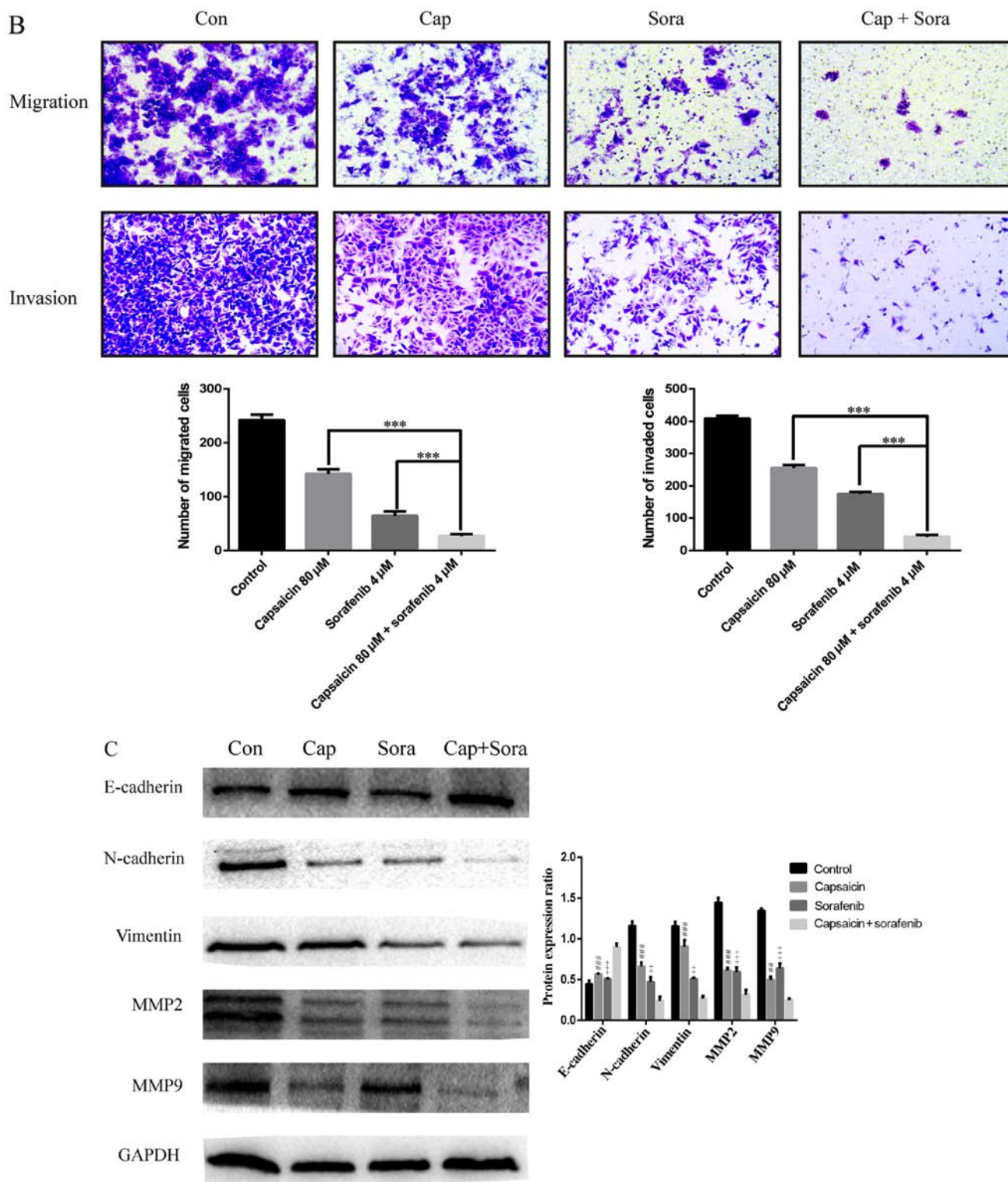


Figure 5. Continued. Capsaicin and sorafenib inhibit the invasion and migration of LM3 cells, and the combination treatment can inhibit invasion and migration synergistically. (B) Effects of capsaicin on invasion and migration in LM3 cells. Cells were pretreated with capsaicin, sorafenib and their combination for 48 h. After 48 h, cells on the lower side of the filter were counted (magnification, x200). (C) LM3 cells were treated with capsaicin, sorafenib and their combination for 12 h, and the expression of E-cadherin, N-cadherin, vimentin, matrix metalloproteinase (MMP)2 and MMP9 was examined by western blot analysis. GAPDH served as a loading control. The results are representative of three separate experiments. The data are presented as the mean \pm standard error of the mean. *** P <0.005. #, Capsaicin vs. combination; +, sorafenib vs. combination. ##/++ and ###/+++; P <0.01 and 0.005, respectively. Con, control; Cap, capsaicin 80 μ M; Sora, sorafenib 4 μ M.

after the combination treatment was wider compared with that after monotherapy, and the wound was wider in the treatment groups compared with that in the control group at 48 and 72 h. This result demonstrated that capsaicin and sorafenib inhibit

LM3 cell migration in a synergistic manner. Transwell migration assays and Matrigel invasion assays (Fig. 5B) revealed that capsaicin and sorafenib can inhibit both the migration and invasion of LM3 cells, and the combined treatment was

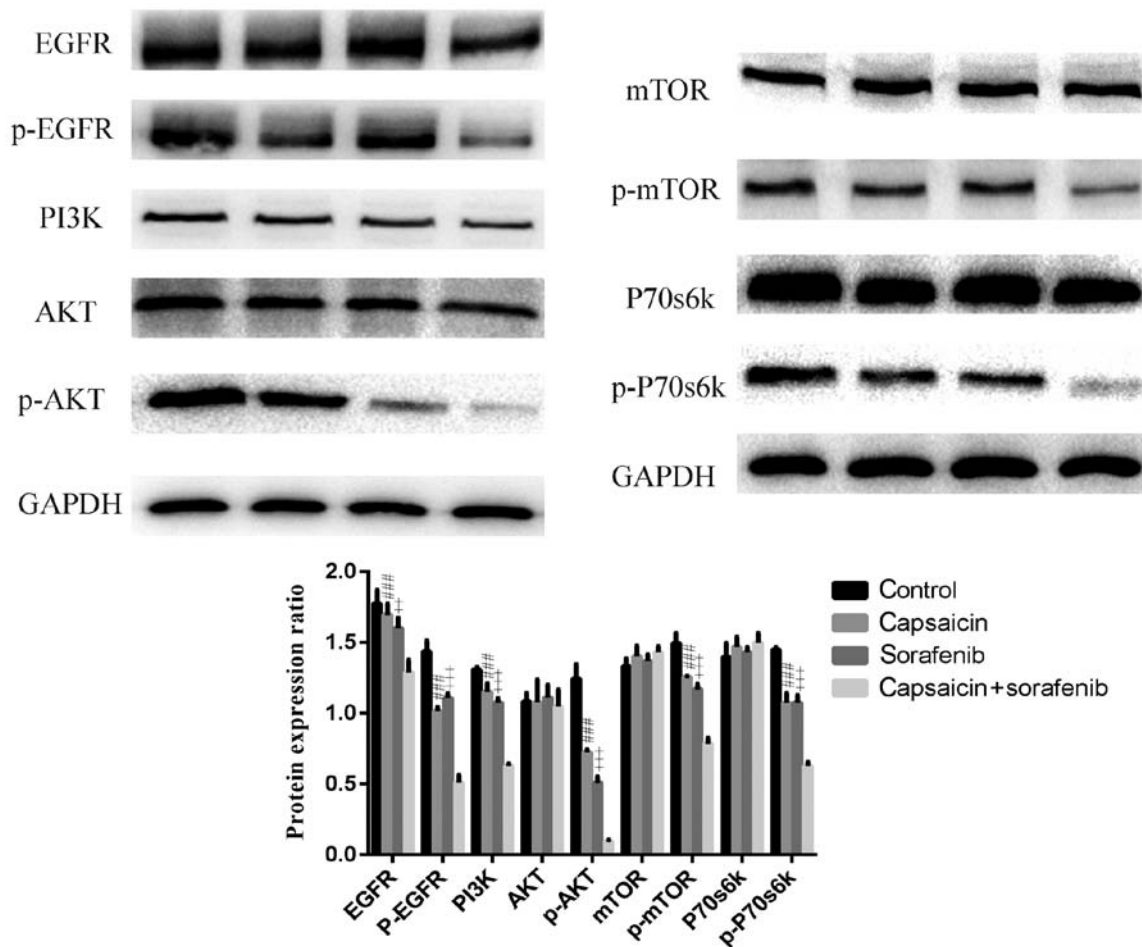


Figure 6. Capsaicin and sorafenib reduce the expression of EGFR, PI3K/Akt/mTOR pathway components. Capsaicin and sorafenib downregulate EGFR, PI3K/Akt/mTOR signaling and the client protein P-p70s6k in LM3 cells. The combination of capsaicin and sorafenib exerted a synergistic effect on this pathway. Cells were serum-starved for 24 h and then treated with capsaicin, sorafenib and their combination for 12 h. Protein lysates were analyzed by western blotting with the indicated antibodies. GAPDH served as a loading control. Values represented the mean \pm standard error of the mean from three independent experiments. #, Capsaicin vs. combination; +, sorafenib vs combination. ++ and ###/+++, $P < 0.01$ and 0.005 , respectively. EGFR, epidermal growth factor receptor; PI3K, phosphoinositide-3 kinase; mTOR, mammalian target of rapamycin.

more effective than either monotherapy. Therefore, we next examined the expression levels of E-cadherin, N-cadherin, vimentin, MMP2 and MMP9 in the monotherapy and combination groups through western blotting. The N-cadherin, vimentin, MMP2 and MMP9 levels were markedly decreased in LM3 cells after treatment with the combination of capsaicin and sorafenib, while the E-cadherin levels were increased (Fig. 5C).

Capsaicin combined with sorafenib obviously inhibits the expression of EGFR and downstream effectors of the EGFR/PI3K/Akt signaling pathway in LM3 cells. To elucidate the mechanism underlying the synergistic effects of capsaicin and sorafenib, we investigated PI3K/Akt/mTOR signal transduction, which is critically implicated in the effects of capsaicin and sorafenib. Capsaicin and sorafenib treatment decreased the levels of PI3K, P-Akt, P-mTOR and P-p70S6K, and these levels were lower in LM3 cells after combination treatment compared with after either monotherapy (Fig. 6). PI3K/Akt/mTOR signaling may be activated by multiple stimuli. Growth factor receptor family proteins are major upstream molecules of PI3K/Akt/mTOR signaling (28). A

number of solid tumors display high or abnormal EGFR expression. EGFR is associated with tumor cell proliferation, angiogenesis, invasion, metastasis and inhibition of apoptosis (29). Therefore, P-EGFR and EGFR levels were examined in LM3 cells following treatment with capsaicin, sorafenib, or their combination. The P-EGFR and EGFR levels decreased in LM3 cells treated with capsaicin and sorafenib, and the combination treatment synergistically downregulated P-EGFR and EGFR levels, which were lower compared with those in the monotherapy groups. Therefore, it was concluded that EGFR and PI3K/Akt/mTOR signaling was inhibited synergistically by capsaicin and sorafenib.

Capsaicin and sorafenib restrain the growth of hepatocellular tumors synergistically in vivo. To further confirm capsaicin and sorafenib as synergistic inhibitors of tumor growth *in vivo*, 1×10^7 LM3 cells were subcutaneously inoculated in nude mice. Capsaicin and sorafenib treatment was administered i.p. for 28 days starting at the 7th day post-inoculation. It was observed that the capsaicin and sorafenib combination treatment exerted an obvious inhibitory effect on tumor volume (Fig. 7A and B). However, there were no significant differences

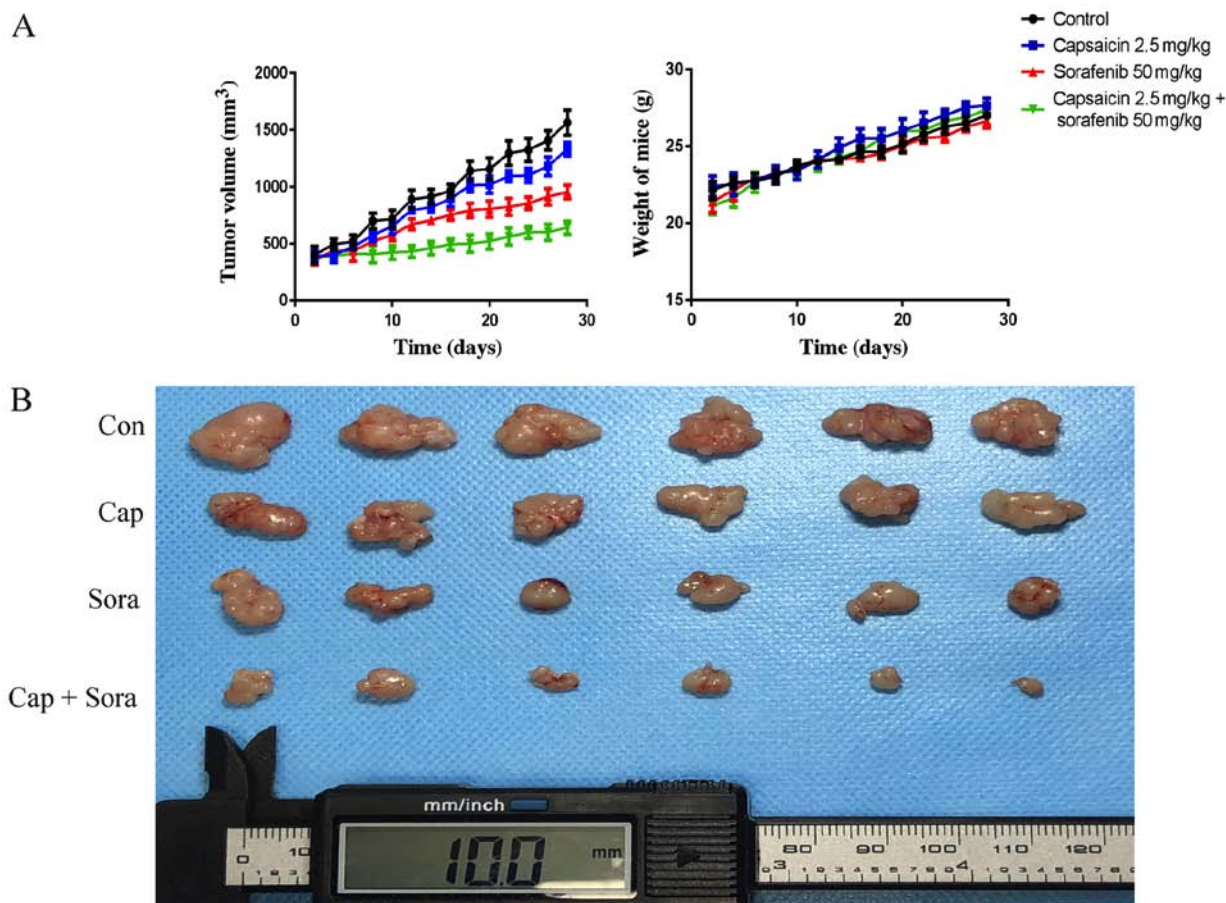


Figure 7. Capsaicin and sorafenib inhibit HCC cancer cell growth, invasion and metastasis synergistically *in vivo*. (A) 1×10^7 LM3 cells were inoculated into BALB/c-nude mice. The mice were randomized into 4 groups ($n=6$) and treated with PBS (with DMSO), capsaicin (Cap), sorafenib (Sora) and their combination daily for 28 days. Tumor volumes and body weight were measured every 2 days. (B) The tumors were excised after the last treatment. EGFR, epidermal growth factor receptor; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide.

in body weight between the control and treatment groups (Fig. 7A). Furthermore, the expression levels of proteins related to proliferation, apoptosis, autophagy, invasion and metastasis in xenograft tumor tissues were evaluated by immunohistochemistry. Proliferation, invasion and metastasis were inhibited in xenograft tumors by capsaicin, sorafenib and their combination, while apoptosis and autophagy were activated (Fig. 7C). Ki67 and Bax indicated the presence of more apoptotic cells and obviously fewer proliferative cells in tumors treated with the combination of capsaicin and sorafenib. In addition, the highest autophagy level among the four groups, as measured by P62, was observed in tumors of the combination treatment group. Capsaicin and sorafenib decreased MMP2 and MMP9 expression in the tumors, and the combination treatment enhanced this effect. Moreover, P-EGFR and P-Akt levels were evaluated in xenograft tumors and it was observed that the capsaicin and sorafenib combination decreased P-EGFR and P-Akt levels to the greatest extent. The biochemical function of the liver and kidney was monitored, and there were no significant differences among the four groups (Fig. 7D). To further evaluate treatment-related toxicity, liver and kidneys from the control and drug-treated groups were stained with H&E. The histological structure of the liver and kidneys was compared under the microscope, and no considerable histological changes were observed following

treatment with capsaicin, sorafenib, or their combination (Fig. 7E). These results suggest that capsaicin and sorafenib combination treatment can effectively inhibit the growth, invasion and metastasis of xenograft hepatocellular tumors *in vivo* in a synergistic manner, with well-tolerated toxicity.

Discussion

The multi-kinase inhibitor sorafenib is the only systemic therapy that improves the survival of patients with advanced HCC, but its efficacy is not satisfactory. The aim of the present study was to ascertain the synergistic effect of capsaicin and sorafenib against HCC. It was observed that capsaicin and sorafenib exerted synergistic antitumor effects on HCC cells *in vitro* as well as *in vivo*. Capsaicin induces apoptosis and autophagy through the PI3K/Akt/mTOR pathway (15) and suppresses EGF-induced invasion and metastasis of tumor cells (30). Sorafenib inhibits HCC growth by decreasing the expression of PI3K/Akt/mTOR pathway components (31). In addition, inhibition of the PI3K/Akt/mTOR pathway enhances sorafenib-induced autophagy in HCC cells (32,33). EGFR is overexpressed in HCC cells (27). Overexpression or mutation of EGFR leads to activation of the PI3K/Akt/mTOR pathway. In addition, activated Akt affects a variety of biological processes via phosphorylation cascades involving numerous

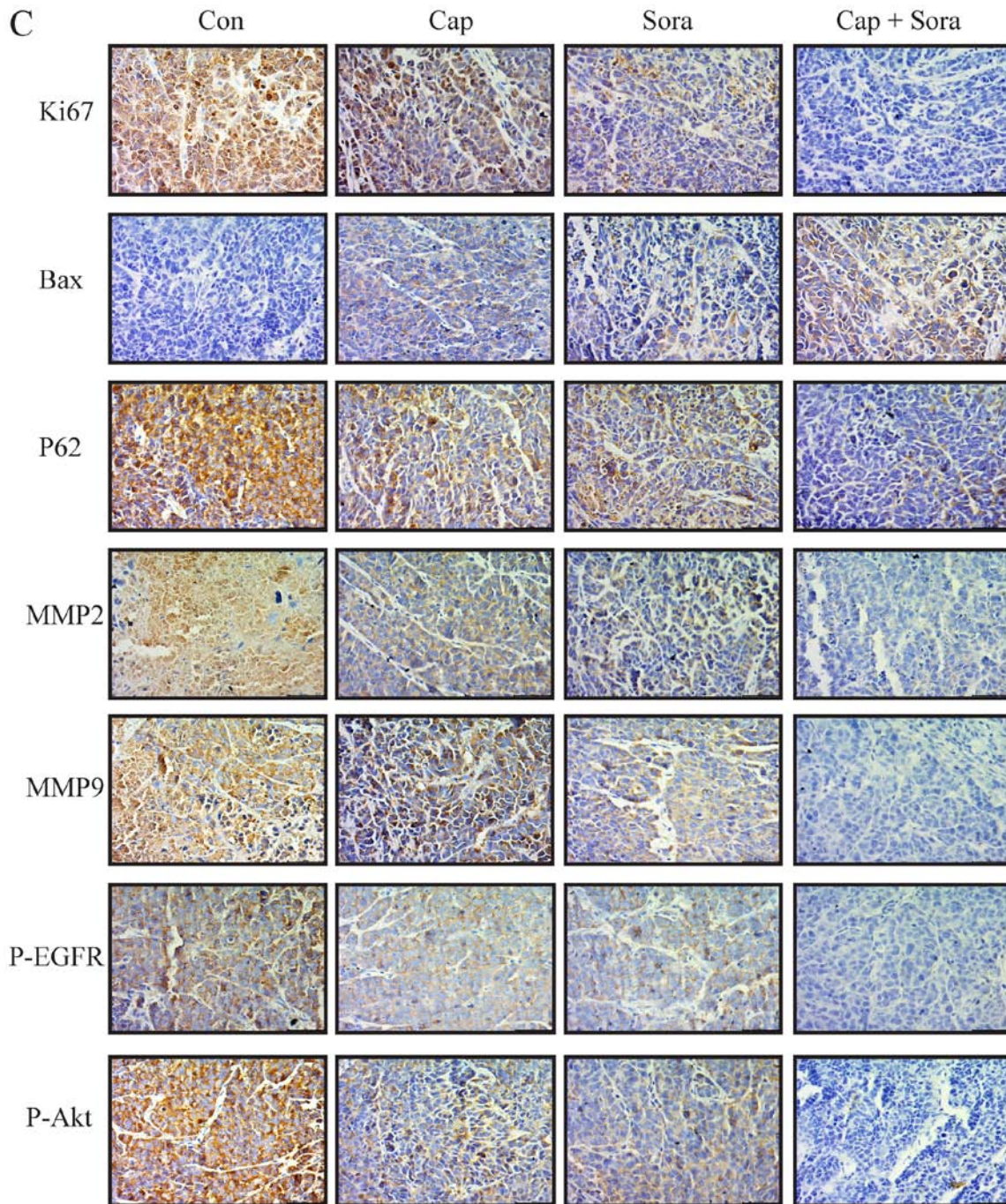


Figure 7. Continued. Capsaicin and sorafenib inhibit HCC cancer cell growth, invasion and metastasis synergistically *in vivo*. (C) The apoptosis, autophagy, invasion, metastasis and phosphorylation of Ki67, Bax, P62, matrix metalloproteinase (MMP)2, MMP9, P-Akt and P-EGFR in xenograft tumor tissues were detected by immunohistochemistry (magnification, x400). Con, control; Cap, capsaicin; Sora, sorafenib.

proteins; Akt activation promotes tumor cell growth, proliferation, invasion and metastasis, regulates tumor angiogenesis and inhibits apoptosis (29).

In the present study, four HCC cell lines were examined to observe the effects of capsaicin, sorafenib and their combination on cell proliferation. Capsaicin inhibited the growth of three HCC cell lines at 48 h, with IC_{50} values between 44.7 μ M (HuH7) and 95.7 μ M (LM3). Sorafenib inhibited HCC cell growth at 48 h, with IC_{50} values between 0.99 μ M (HuH7) and 4.02 μ M (LM3). Then, HCC cells were treated with a low concentration of capsaicin and a moderate concentration of sorafenib, and synergistic inhibitory effects were observed.

The LM3 cell line was selected for subsequent studies, as it has a strong capacity for growth and is sensitive to both capsaicin and sorafenib. The 80 μ M capsaicin and 4 μ M sorafenib combination was selected for further studies after analyzing various combinations of capsaicin and sorafenib in LM3 cells.

We investigated apoptosis, autophagy, invasion and metastasis in LM3 cells treated with capsaicin and/or sorafenib, and it was concluded that capsaicin and sorafenib inhibit proliferation, invasion and metastasis and induce apoptosis and autophagy in LM3 cells in a synergistic manner. Therefore, the expression of EGFR/PI3K/Akt/mTOR signaling components was further examined in LM3 cells. The results demonstrated that capsaicin

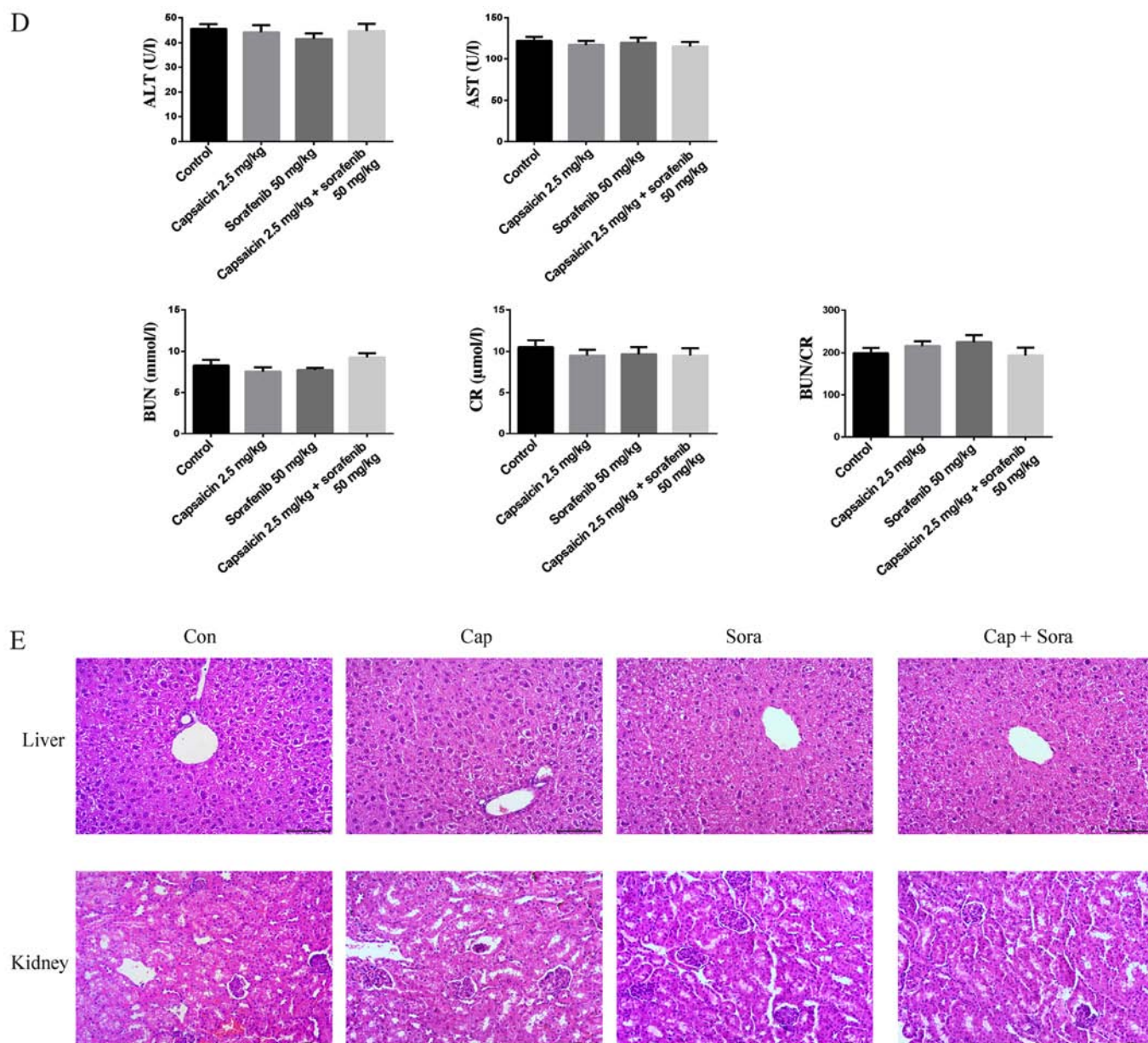


Figure 7. Continued. Capsaicin and sorafenib inhibit HCC cancer cell growth, invasion and metastasis synergistically *in vivo*. (D) The liver and kidney biochemical functions were evaluated. The AST, ALT, BUN, CR and BUN/CR levels of were detected in mouse blood by ELISA. (E) The liver and kidneys from the control and the three treatment groups were stained with hematoxylin and eosin to evaluate the toxicity after treatment. The histological structures of the liver and kidney were observed and compared microscopically (magnification, x200). AST, aspartate aminotransferase; ALT, alanine aminotransferase; BUN, blood urea nitrogen; CR, creatinine; Con, control; Cap, capsaicin; Sora, sorafenib.

and sorafenib inhibited EGFR/PI3K/Akt/mTOR signaling and that their combination exerted a synergistic inhibitory effect on EGFR/PI3K/Akt/mTOR signaling in LM3 cells.

Compared with monotherapy, the capsaicin and sorafenib combination treatment induced apoptosis and autophagy synergistically, as evidenced by western blotting, TUNEL staining and immunofluorescence staining. Apoptosis and autophagy play key roles in cancer progression. Moreover, the same synergistic effect on invasion and metastasis was observed. Cell scratch, Transwell migration, Matrigel invasion and immunofluorescence assays all demonstrated that capsaicin and sorafenib inhibited LM3 cell invasion and metastasis in a synergistic manner. Activation of PI3K/Akt/mTOR signaling increases tumor cell apoptosis, invasion and metastasis;

therefore, inhibiting PI3K/Akt/mTOR signaling can inhibit tumor cell apoptosis, invasion and metastasis (34,35). Additionally, it has been demonstrated by previous studies that mTOR (particularly mTORC1) plays an important role in regulating autophagy, with PI3K/Akt signaling as the key upstream effector (36,37). In addition, it was reported that autophagy in HCC cells can be induced by inhibiting the PI3K/Akt/mTOR pathway (38,39). The present study demonstrated that the capsaicin and sorafenib combination decreased the expression levels of P-Akt, PI3K, P-mTOR and P-p70S6K and exerted a synergistic inhibitory effect on PI3K/Akt/mTOR signaling in LM3 cells. It is likely that capsaicin and sorafenib combination treatment inhibits LM3 cell proliferation, invasion and metastasis and enhances apoptosis and autophagy

synergistically through the PI3K/Akt/mTOR pathway. The *in vivo* experiments yielded the same results: The combination treatment exerted synergistic effects on tumor proliferation, invasion and metastasis. P-EGFR and EGFR levels were next investigated in LM3 cells and found that treatment with capsaicin or sorafenib alone decreases p-EGFR and EGFR levels, whereas the combination treatment exerts a synergistic effect. The combination treatment also decreased P-EGFR and P-Akt levels in a synergistic manner in mouse xenograft tumors.

In conclusion, a strong growth inhibitory effect of capsaicin and sorafenib combination was observed in LM3 cells by decreasing EGFR levels and PI3K/Akt/mTOR downstream signaling. Capsaicin acted synergistically with sorafenib to inhibit LM3 cell growth, invasion and metastasis and to enhance apoptosis and autophagy *in vitro* as well as *in vivo*. The combination treatment is associated with the inhibition of EGFR and PI3K/Akt/mTOR signaling, with concomitant increases in cleaved caspase-3, Bax, cleaved PARP, beclin-1, LC3A/B-II and E-cadherin, and decreases in Bcl-2, P62, N-cadherin, MMP2, MMP9 and vimentin. Therefore, this combination may be a promising approach to the treatment of patients with advanced HCC.

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Availability of data and materials

All data generated/analyzed in the present study are available from the corresponding author on reasonable request.

Authors' contributions

QH contributed to the conception of the study; PG contributed significantly to the analysis and manuscript preparation; ND and RY performed the data analyses and wrote the manuscript; HC helped perform the analysis with constructive discussions; QZ was involved in the conception of the study and approved the final version of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal procedures were conducted in accordance with the guidelines of the National Institutes of Health and were approved by the Ethical Committee of Wenzhou Medical University and the Laboratory Animal Management Committee of Zhejiang Province (Approval ID: wyd2017-0052).

Patient consent for publication

Not applicable.

Competing interests

All the authors declare that they have no competing interests to disclose.

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