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Original Article

Phenformin synergistically sensitizes liver cancer cells to sorafenib by downregulating CRAF/ERK and PI3K/AKT/mTOR pathways

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Abstract: Sorafenib is a first-line drug to treat advanced hepatocellular carcinoma (HCC), which can prolong the median overall survival of patients by approximately 3 months. Phenformin is a biguanide derivative that has been shown to exhibit antitumor activity superior to that of metformin. We herein explored the ability of phenformin to enhance the anti-cancer activity of sorafenib against HCC and the mechanisms underlying such synergy. The Hep-G2 and SMMC-7721 HCC cell lines were treated with sorafenib and/or phenformin, after which the proliferation of these cells was evaluated via MTT and colony formation assays, while invasion and apoptotic cell death were evaluated via Transwell and flow cytometry assays, respectively. In addition, protein levels were assessed by Western blotting, drug synergy was assessed with the CompuSyn software, and xenograft models were established by implanting Hep-G2 cells into nude mice and then assessing drug antitumor efficacy. Sorafenib and phenformin exhibited a synergistic ability to suppress HCC cell proliferation, migration, and survival. Phenformin further bolstered the ability of sorafenib to inhibit the CRAF/ERK and PI3K/AKT/mTOR pathways. Strikingly, the combination of these two drugs achieved better *in vivo* efficacy in a murine model system, without causing significant weight loss or hepatorenal toxicity. Sorafenib and phenformin can synergistically suppress CRAF/ERK and PI3K/AKT/mTOR pathway activation in HCC cells, and may thus represent a promising approach to treating this deadly cancer.

Keywords: Phenformin, sorafenib, hepatocellular carcinoma, co-therapy

Introduction

Hepatocellular carcinoma (HCC) accounts for between 75% and 95% of all primary liver cancer causes, and is among the deadliest forms of cancer globally [1, 2]. While efforts to treat HCC have advanced significantly in recent years, prognosis of HCC patients remains relatively poor, particularly in those with advanced disease not eligible for curative surgical treatment. Sorafenib is a protein kinase inhibitor that can suppress VEGFR, PDGFR, RET, and c-Kit activation, thereby suppressing the activity of the downstream Raf serine/threonine kinase and thus hampering tumor growth [3]. The phase III randomized controlled SHARP (Sorafenib HCC Assessment Randomized Protocol) clinical trial found that sorafenib was able to significantly improve median overall survival (OS) of HCC patients [4-6], and as such, it has been approved by the United States Food and Drug Administration (FDA) for the treatment of advanced HCC [7]. However, many HCC patients exhibit negative reactions following chemotherapy owing to the high drug doses and severe side effects associated with these treatment regimens [8-11], necessitating the discovery of novel therapeutic approaches to increase sorafenib efficacy.

Biguanides, which include metformin and phenformin, are antidiabetic drugs that also exhibit well-established antitumor activities [12], with phenformin being more potent in anti-tumor contexts [13]. We have previously demonstrated that phenformin can inhibit bladder cancer

cell proliferation and reduce AKT and ERK activation in a dose-dependent manner [14].

Herein we sought to explore the anti-tumor efficacy of combined treatment of sorafenib and phenformin, and to explore the molecular basis for any observed combination activity in the context of HCC treatment.

Materials and methods

Reagents

Sorafenib (HY-10201A) was obtained from MedChemExpress (Shanghai, China), Phenformin was from Aladdin Chemistry (Shanghai, China), and the FITC Annexin V Apoptosis Detection kit was from BD Pharmingen (NJ, USA). Anti-β-actin, anti-MAPK (Erk1/2) (Thr-202/Tyr204), anti-c-Raf (Ser338), anti-PI3K (Tyr458)/p55 (Tyr199), anti-Akt (Ser473), anti-4E-BP1 (Thr37/46), anti-mTOR (Ser2448) were all from Cell Signaling Technology, MA, USA.

Cell culture

Human HCC Hep-G2 cells were donated by the Basic Medical College of Xiangya Medical College (Changsha, Hunan, China), while SMMC-7721 cells were provided by the Medical College of Hunan Normal University (Changsha, Hunan, China). All cells were grown in DMEM (Gibco, USA) containing 10% fetal bovine serum (FBS, USA) and 1% penicillin/streptomycin (both from Hyclone) at 37°C in a humidified 5% CO_2 incubator.

MTT assay

MTT assay was used to assess cellular viability. Briefly, cells were added to 96-well plates (8×10^3 cells/well) for 24 h, after which a range of sorafenib and/or phenformin concentrations were added for 72 h. The MTT tetrazolium salt was then added to each well (50 μL; Sigma) for 5 h, after which 150 μL of DMSO (Sigma) was added per well and absorbance at 490 nm was assessed via microplate reader (Biotek, SYNERGY HTX, VT, USA). IC_{50} values were determined based upon dose-response curves with SPSS 16.0 (IBM, IL, USA).

Colony formation assay

How sorafenib and phenformin inhibited HCC cell proliferation was assessed via colony formation assay. Cells were added to 24-well plates (8×10^3 cells/well) for 24 h, after which they were then incubated for 5-7 additional days with a range of sorafenib and/or phenformin concentrations. Next, 10% formaldehyde was used to fix cells, which were then stained for 1 h at room temperature with 0.1% crystal violet. Absorbance at 550 nm was then evaluated via a microplate reader.

Wound healing assay

A wound healing assay was used to detect cell migration. Cells were added to 12-well plates (4×10^5 cells/well) until 90% adherent, after which a range of sorafenib and/or phenformin concentrations were added. Monolayer cells were then scratched in a cross pattern using a 10 μl pipette tip. Images at 0 and 48 h post-wounding were acquired via standard light microscopy (DFC450C; Leica, Wetzlar, Germany).

Migration and invasion assay

Polycarbonate transwell filters were used to evaluate cellular migration and invasion. Briefly, 4×10^4 cells in 200 μL of serum-free DMEM were added to the upper chamber, while DMEM containing 10% FBS was added to the lower chamber. Appropriate phenformin and/or sorafenib were then added for 24 h, after which cells in the upper chamber were removed and the remaining cells were fixed for 30 min with 10% formaldehyde and stained for 2 h with 0.1% crystal violet before being imaged via microscopy.

Invasion assay was conducted using the same approach of migration assay, except that the transwell insert was first coated with Matrigel (BD Biosciences, USA).

Apoptosis analysis

Annexin V-FITC/PI dual-staining assays were used to evaluate cellular apoptosis. Briefly, cells were plated in 6-well plates (6×10^5/well) and incubated for 24 h with a range of doses of phenformin (100 μmol/L for Hep-G2 and 200 μmol/L for SMMC-7721) and/or Sorafenib (2 μmol/L for Hep-G2 and 4 μmol/L for SMMC-7721). Cells were collected and stained with Annexin V-FITC and PI (5 μl each) in a 300 μL volume for 20 minutes in the dark. Cells

were then assessed with a BD FACSCanto™II flow cytometer (Becton-Dickinson).

Western blotting

Protein samples were separated via SDS-PAGE, transferred to membranes, and incubated with appropriate primary antibodies detailed in the Reagents section in a buffer containing bovine serum albumin (BSA) at 4°C overnight. Blots were then washed with PBS containing 0.1% Tween-20 (PBST), stained for 1 h at room temperature with secondary antibodies, and washed thrice in PBST. Protein bands were then detected with Pierce Super Signal chemiluminescent substrate (Rockford, IL) and imaged with a Chemi Doc system (Bio-Rad). ImageJ (NIH, Bethesda, MD) was used for densitometric analyses, with β-actin being used for normalization and with protein expression being assessed relative to the control untreated group.

Murine xenograft models

To evaluate the antiproliferative effects of phenformin and sorafenib on HCC tumors, a xenograft nude mouse model was developed using female BALB/c-nu mice (4-6 weeks old) from Hunan SJA Laboratory Animal Co., Ltd (Changsha, Hunan, China). Mice were housed under sterile conditions with access to food and water. A total of thirty mice were randomly selected and subcutaneously implanted in the right flank with 5×10^6 Hep-G2 cells. When tumors grew to 50-80 mm^3 in size, mice were randomized into control [100 μl 1% dimethyl sulfoxide (DMSO) plus 5% Tween and 5% Neutral resin], sorafenib (40 mg/kg/day), phenformin (100 mg/kg/day), and combination (phenformin, 100 mg/kg/day plus sorafenib 40 mg/kg/day) groups (n = 5 animals/group). All treatments were administered intragastrically and were administered continuously for 2 weeks. Tumor volumes and body weight were measured every two days, and tumor volume was calculated as follows: volume = 1/2 (length × $width^2$). After this two-week period, mice were euthanized, and tumor tissues were collected. These experiments were consistent with the guidelines of the Institutional Animal Care and Use Committee at Hunan Normal University (Protocol 2020007-B).

Histologic analysis

After the study was completed, animals were euthanized, and organ tissues including the kidney and liver were collected and fixed in 4% neutral-buffered formalin to prepare histologic slides. Samples were then stained with hematoxylin and eosin (H&E), and 7-μm tissue sections were analyzed via standard light microscopy (DFC450C; Leica, Wetzlar, Germany).

Statistical analyses

Data were expressed as means ± standard deviation (SD) and were compared via two-tailed t-tests and two-way ANOVAs as appropriate. Data were given with 95% confidence intervals and were reported with corresponding *P*-values (*P < 0.05, **P < 0.01, ***P < 0.001). GraphPad Prism 6 and SPSS 13.0 were used for all statistical analyses.

Results

Sorafenib inhibited HCC proliferation and suppressed RAF/ERK and PI3K/AKT/mTOR pathway activation

To evaluate the impact of sorafenib on HCC cells, we treated Hep-G2 and SMMC-7721 cells with sorafenib at various concentrations, and witnessed the dose-dependent inhibition of the proliferation of both cell types after 72 h with estimated IC_{50} values of 8.6 μM (Hep-G2) and 17 μM (SMMC-7721). These values were similar to those published by Qiu et al. [15]. Sorafenib similarly suppressed the colony forming activity of these HCC cells in a dose-dependent manner, with this effect being more robust in Hep-G2 cells (**Figure 1A**). We next evaluated protein levels of P-CRAF, P-ERK, P-PI3K, P-AKT, P-mTOR, and P-4EBP1 following sorafenib treatment in Hep-G2 cells (2 and 4 μM) and SMMC-7721 cells (4 and 8 μM) (**Figure 1B**). As previously shown by Li et al. [16], sorafenib treatment reduced P-CRAF, P-ERK, P-PI3K, P-AKT, P-mTOR, and P-4EBP1 levels, with this effect being most pronounced for P-ERK and P-AKT.

Phenformin inhibited HCC proliferation and suppressed RAF/ERK and PI3K/AKT/mTOR pathway activation

Next, we assessed the effects of phenformin on Hep-G2 and SMMC-7721 cells, and the results revealed a dose-dependent inhibition of cell growth after 72 h, with IC_{50} values of 4.3×10^2 μM and 8.7×10^2 μM, respectively, in line with what has previously been demonstrat-

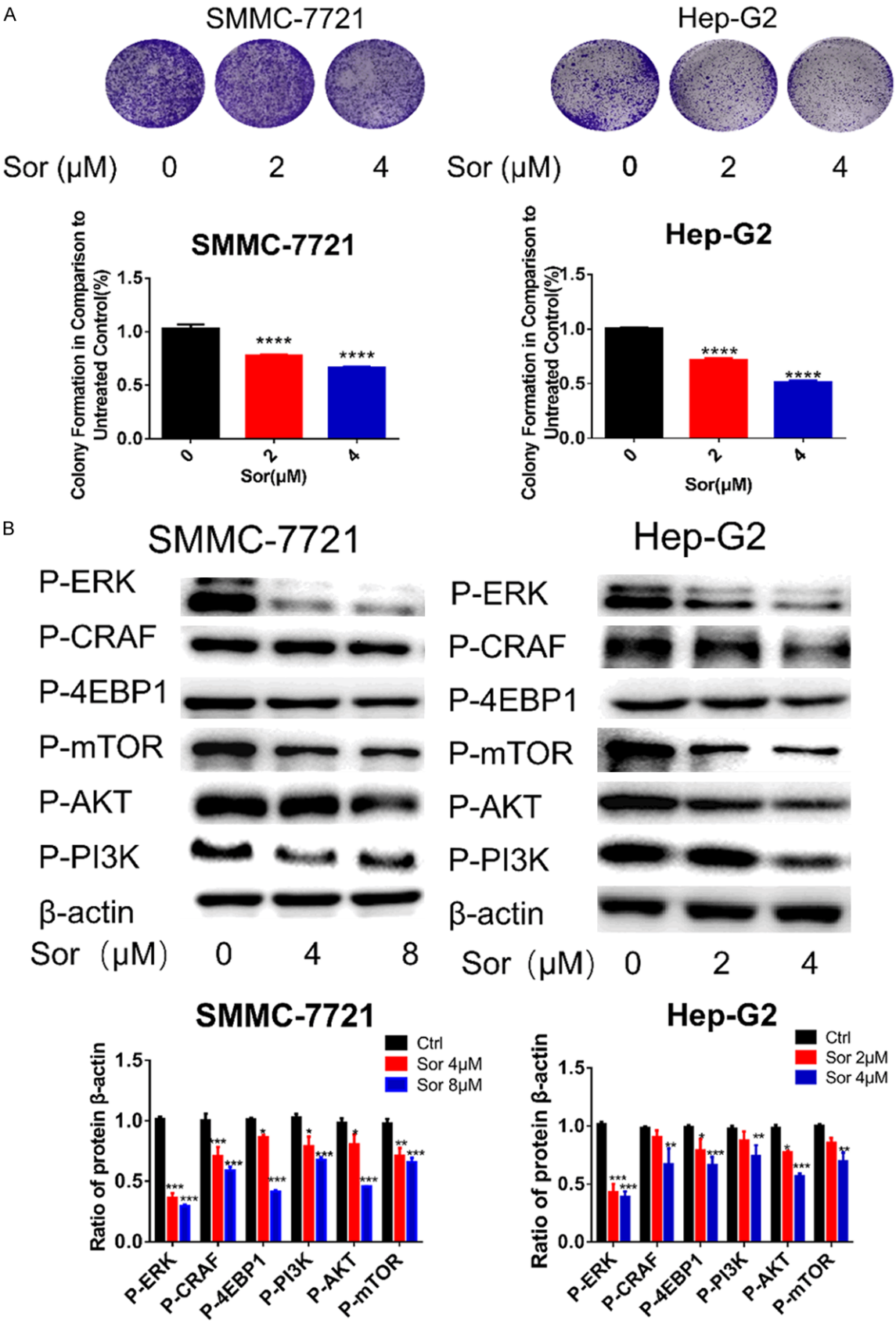

A
SMMC-7721
Hep-G2
Sor (μM) 0 2 4
Sor (μM) 0 2 4
SMMC-7721
Hep-G2
Colony Formation in Comparison to Untreated Control(%)
Sor(μM)
B
SMMC-7721
Hep-G2
P-ERK
P-CRAF
P-4EBP1
P-mTOR
P-AKT
P-PI3K
β-actin
Sor (μM) 0 4 8
Sor (μM) 0 2 4
SMMC-7721
Hep-G2
Ctrl
Sor 4μM
Sor 8μM
Sor 2μM
Ratio of protein β-actin
P-ERK
P-CRAF
P-4EBP1
P-PI3K
P-AKT
P-mTOR

Figure 1. Effects of sorafenib on the colony-forming activity, and protein expression of SMMC-7721 and Hep-G2 cells. A. A colony formation assay was conducted, with wells imaged at 550 nm following a 5-7 day treatment with sorafenib. Data are means ± SD from triplicate experiments. *P < 0.05, **P < 0.01 vs. control (two-tailed t-test). B. How sorafenib impacts RAF/ERK and PI3K/AKT/mTOR signaling was assessed by analyzing levels of phosphorylated (P) proteins in SMMC-7721 and Hep-G2 cells. Control cells were untreated. P-ERK, P-CRAF, P-PI3K, P-AKT, P-mTOR, and P-4EBP1 levels were measured, with β-actin as a loading control (*P < 0.05, **P < 0.01, ***P < 0.001, n = 3).

ed by Hsu et al. [17]. Phenformin also inhibited HCC cell colony formation in a dose-dependent fashion, with this effect being most significant in Hep-G2 cells (**Figure 2A**). Wang et al. previously demonstrated the ability of phenformin to inhibit small cell lung cancer cell proliferation via suppressing PI3K/ERK/mTOR and MEK/ERK pathway activation [18]. We next treated Hep-G2 and SMMC-7721 cells with 100 and 200 μM phenformin, respectively, and evaluated P-CRAF, P-ERK, P-PI3K, P-AKT, P-mTOR, and P-4EBP1 protein levels via Western blotting (**Figure 2B**), and the results showed that phenformin treatment downregulated the phosphorylation of all of these proteins.

Phenformin treatment enhanced HCC cell sensitivity to sorafenib

To evaluate the potential synergy between sorafenib and phenformin, cells were treated with a range of sorafenib and/or phenformin doses, and the results revealed that the combination of these two drugs better inhibited HCC cell growth than either of the single agent alone (**Figure 3A**). The drug combination index for these two agents was computed with the CompuSyn software (**Figure 3B**), and the results showed that there was a degree of synergy between these two drugs (CI < 1). Phenformin and sorafenib treatment may thus be a viable approach to suppressing HCC tumor growth.

Phenformin and sorafenib synergistically suppressed HCC cell colony formation activity

Next, we assessed the combined effects of phenformin and sorafenib on the colony formation activity of HCC cells, with appropriate doses being selected through dose-response curve analyses. This assay revealed that combined treatment of phenformin and sorafenib was able to efficiently inhibit HCC cell colony formation (**Figure 3C**, **3D**).

Phenformin and sorafenib synergistically inhibited HCC cell migration and invasion

We next used wound healing assay and Transwell assay to assess the ability of phenformin and sorafenib to inhibit HepG2 and SMMC-7721 cell migration and invasion, and the results showed that these two drugs in combination were able to better inhibit such migratory (**Figure 4**) and invasive (**Figure 5A**, **5B**) activities than either drug in isolation.

Phenformin and sorafenib induced the apoptotic death of HCC cells

We next tested the impact of the combination of phenformin and sorafenib on HCC cell apoptosis via flow cytometry (**Figure 5C-E**). Either phenformin or sorafenib treatment induced Hep-G2 and SMMC-7721 cell apoptosis relative to untreated cells, whereas the combination of these two drugs induced more robust apoptotic death than that induced by either of the single agent.

Phenformin enhanced the ability of sorafenib to inhibit the CRAF/ERK and PI3K/AKT/mTOR pathways

While both sorafenib and phenformin can inhibit the CRAF/ERK and PI3K/AKT/mTOR pathways, further research is necessary to understand whether they do so via non-overlapping mechanisms. To that end, we assessed CRAF, ERK, PI3K, AKT, mTOR, and 4EBP1 phosphorylation following sorafenib and/or phenformin treatment of SMMC-7721 cells (treated with 4 μmol/L sorafenib and/or 200 μmol/L phenformin) and Hep-G2 cells (treated with 2 μmol/L sorafenib and/or 100 μmol/L phenformin) for 24 hours. As expected, we found that combined treatment of sorafenib and phenformin was sufficient to suppress CRAF/ERK and PI3K/AKT/mTOR pathways activation, suppressing AKT, ERK, and 4EBP1 phosphorylation by > 50% (**Figure 6A**, **6B**). Treatment of Hep-G2 cells with 100 μmol/L

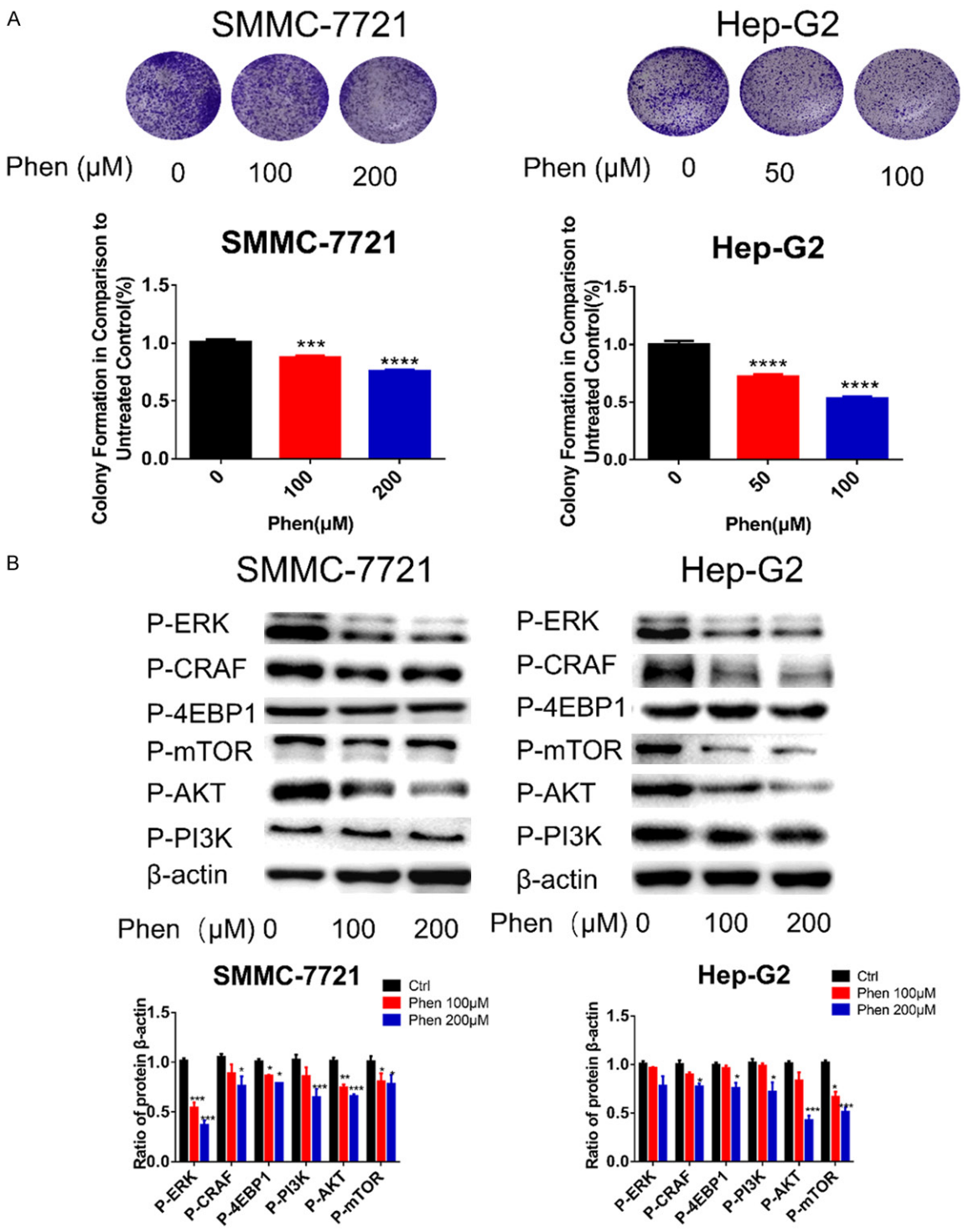


Figure 2. Effects of phenformin on the colony-forming activity, and protein expression of SMMC-7721 and Hep-G2 cells. A. A colony formation assay was conducted, with wells imaged at 550 nm following a 5-7 day treatment with phenformin. Data are means ± SD from triplicate experiments. *P < 0.05, **P < 0.01 vs. control (two-tailed t-test). B. How phenformin impacts RAF/ERK and PI3K/AKT/mTOR signaling was assessed by analyzing levels of phosphorylated (P) proteins in SMMC-7721 and Hep-G2 cells. Control cells were untreated. P-ERK, P-CRAF, P-PI3K, P-AKT, P-mTOR, and P-4EBP1 levels were measured, with β-actin as a loading control (*P < 0.05, **P < 0.01, ***P < 0.001, n = 3).

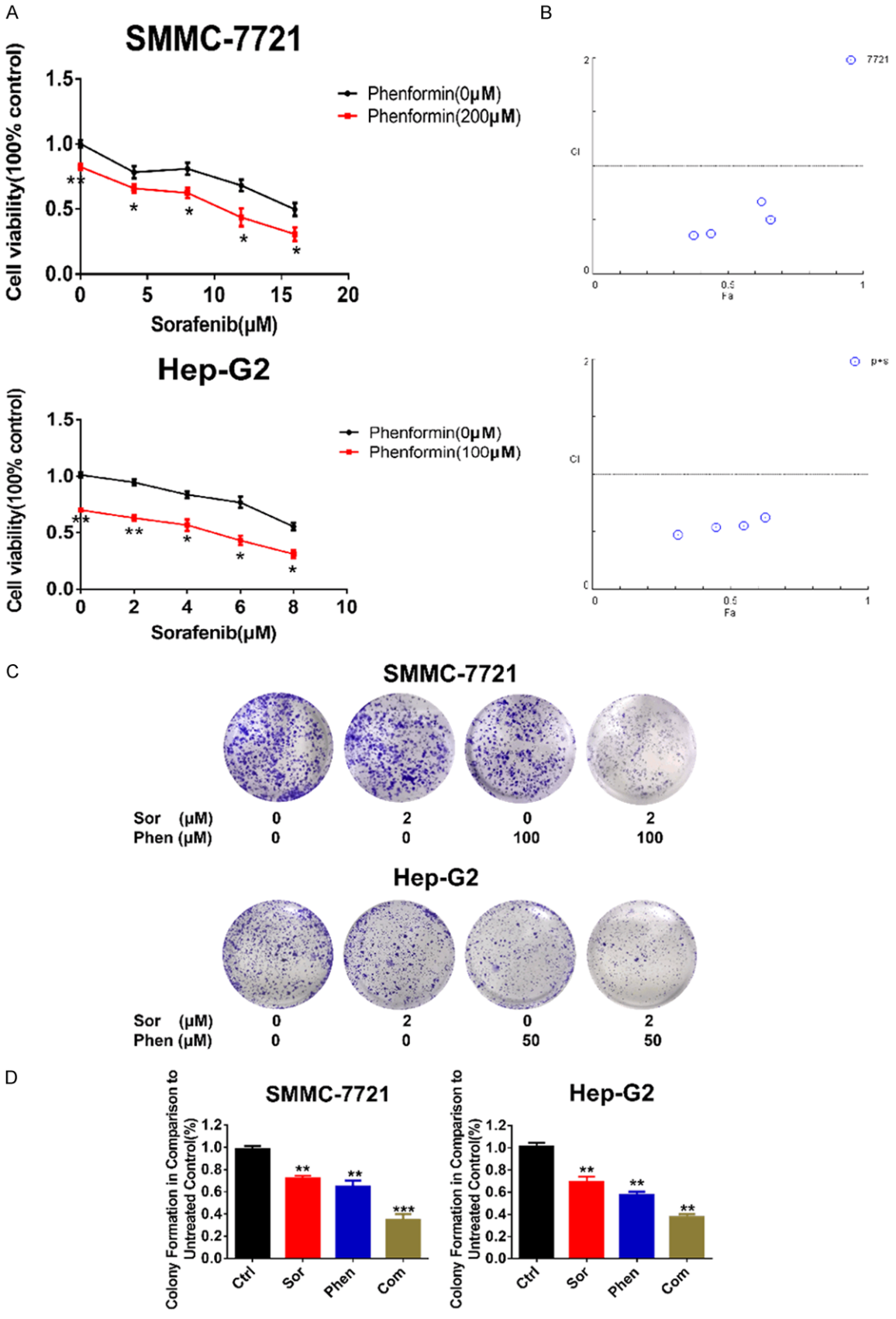

A
SMMC-7721
Cell viability(100% control)
Phenformin(0μM)
Phenformin(200μM)
Sorafenib(μM)
Hep-G2
Phenformin(0μM)
Phenformin(100μM)
Sorafenib(μM)
B
7721
CI
Fa
p+s
C
SMMC-7721
Sor (μM) 0 2 0 2
Phen (μM) 0 0 100 100
Hep-G2
Sor (μM) 0 2 0 2
Phen (μM) 0 0 50 50
D
SMMC-7721
Hep-G2
Colony Formation in Comparison to Untreated Control(%)
Ctrl
Sor
Phen
Com

Figure 3. Effects of sorafenib in combination with phenformin on SMMC-7721 and Hep-G2 cell proliferation and colony formation activity. (A) Sorafenib and phenformin synergistically suppress the proliferation of SMMC-7721 and Hep-G2 cells, as measured at 72 h post-treatment with phenformin and/or sorafenib. (B) Synergy between these drugs was assessed based upon combination index (CI) values, with additive, antagonistic, and synergistic interactions respectively indicated by values of CI = 1, CI > 1, and CI < 1. In almost all cases, CI values were below 1, suggesting a moderate level of synergy. 1 is indicative of synergism. CI values for nearly all combinations were less than 1, consistent with moderately strong synergism. (C) The ability of phenformin and sorafenib to suppress colony formation was assessed following a 5-7 day treatment with one or both of these agents, with results quantified in (D) following analysis at 550 nm. (*P < 0.05, **P < 0.01, ***P < 0.001, n = 3).

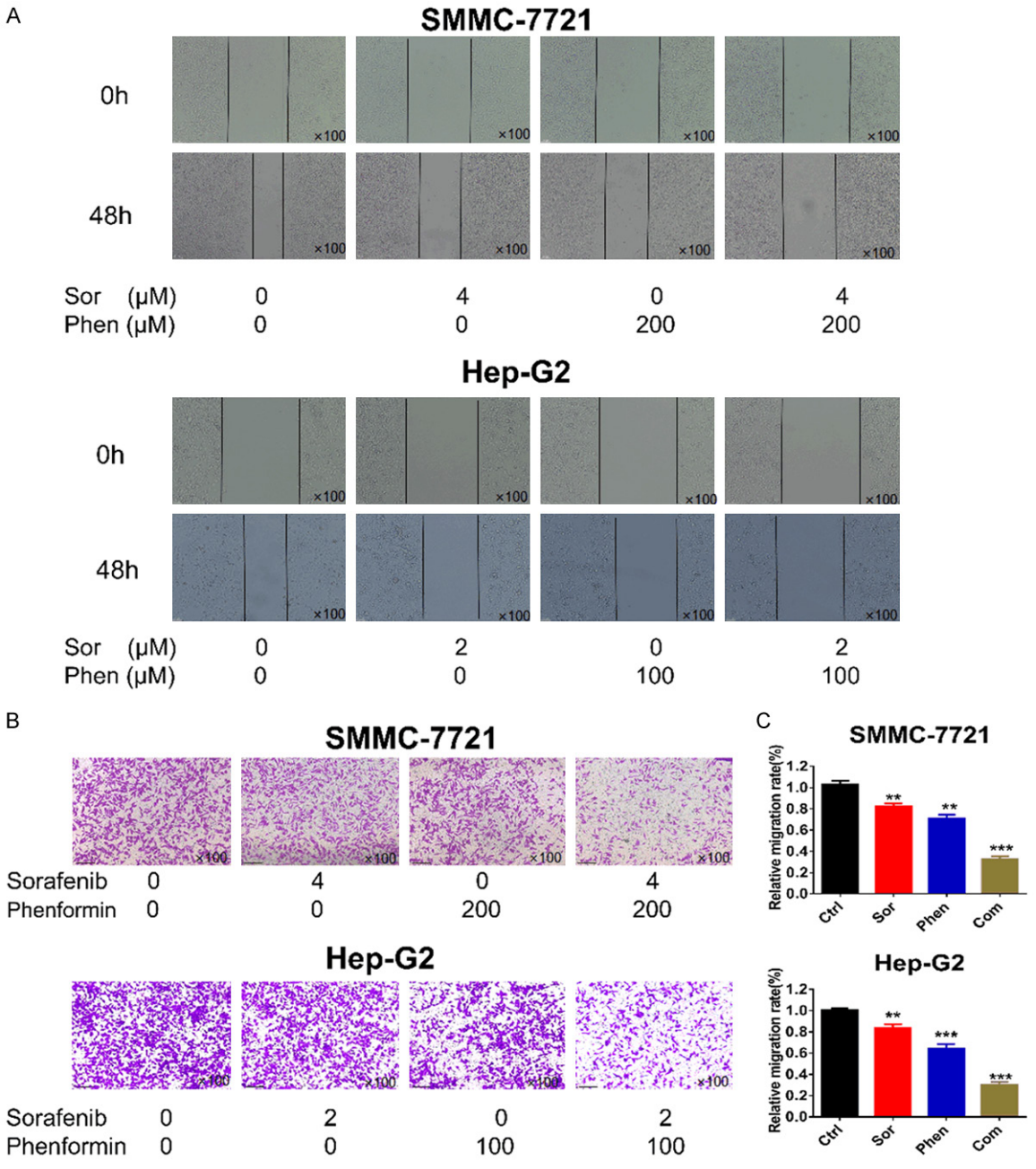
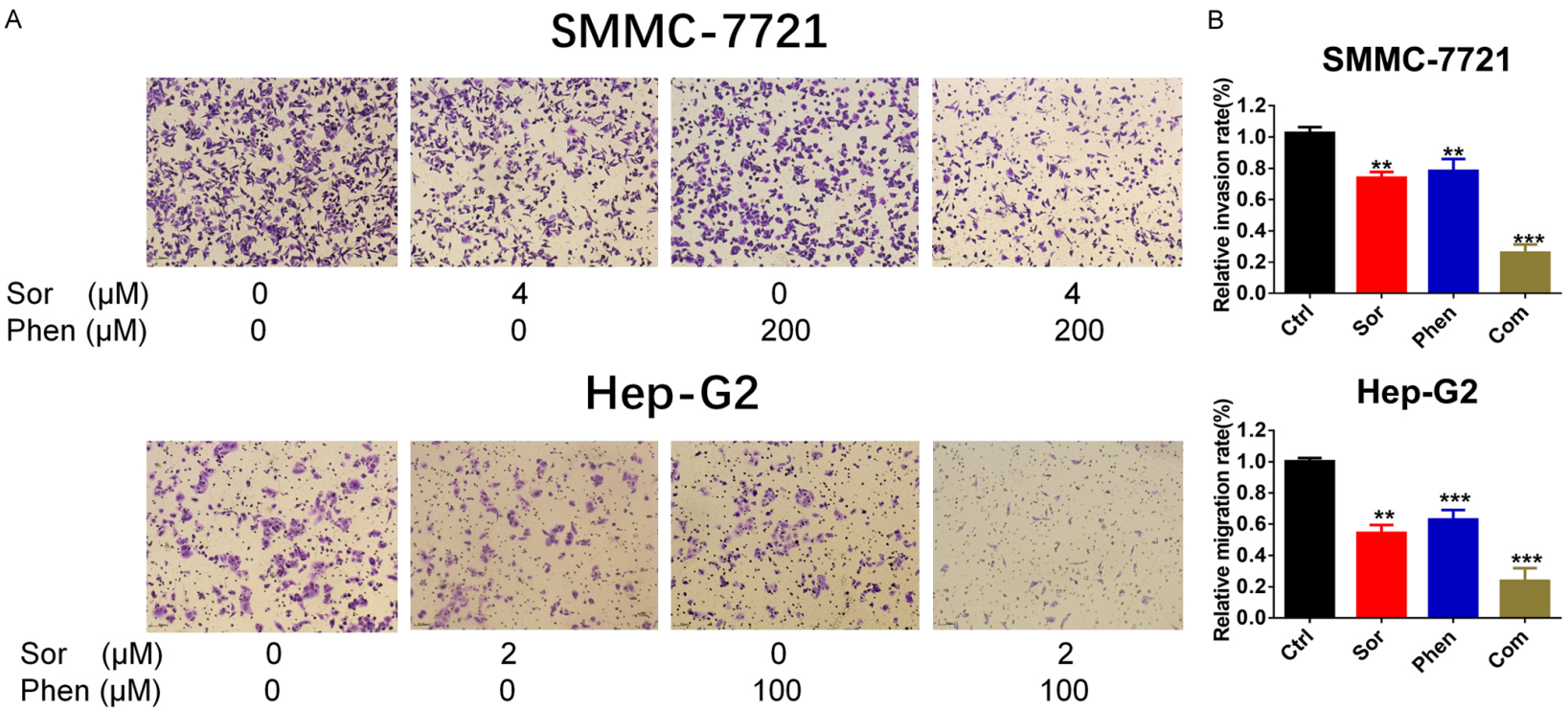
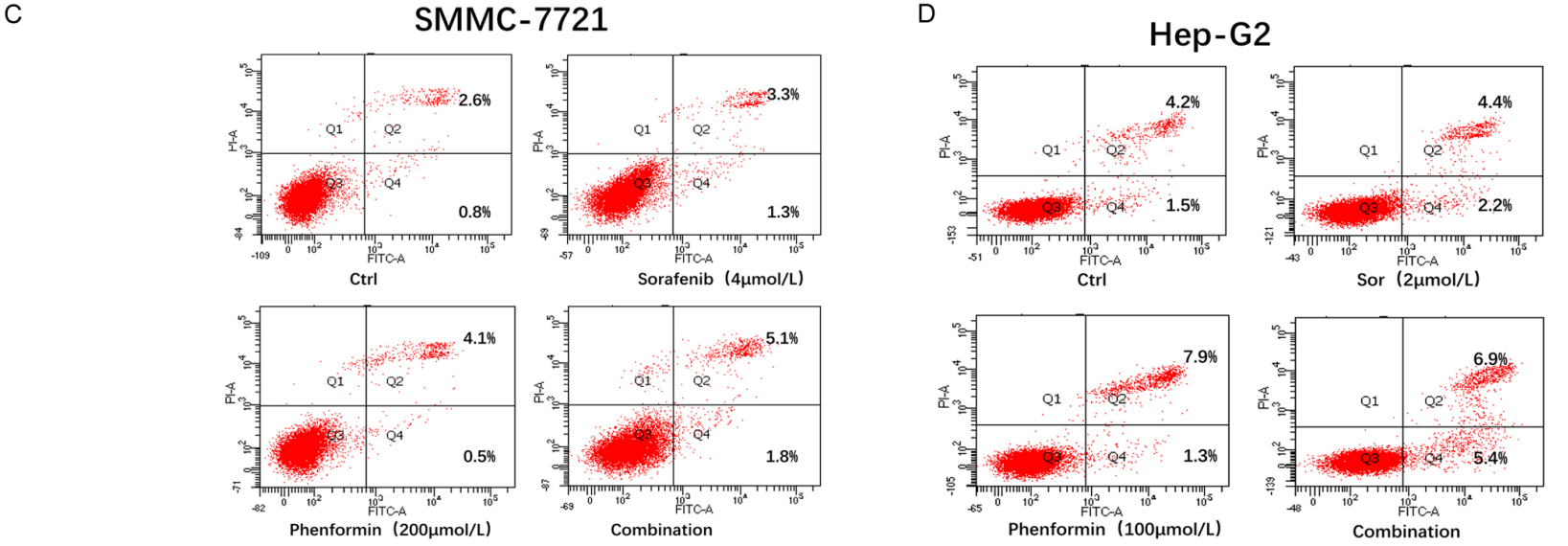


Figure 4. Effects of sorafenib in combination with phenformin on the migration of SMMC-7721 and Hep-G2 cells. (A) Combination of sorafenib and phenformin treatment suppressed the migratory activity of SMMC-7721 and Hep-G2 cells in a wound healing assay. (B) The ability of phenformin and sorafenib to suppress the migration of these two cell types in a transwell assay, with data quantified in (C). (*P < 0.05, **P < 0.01, ***P < 0.001, n = 3).


A
SMMC-7721
Sor (μM) 0 4 0 4
Phen (μM) 0 0 200 200
Hep-G2
Sor (μM) 0 2 0 2
Phen (μM) 0 0 100 100
B
SMMC-7721
Relative invasion rate(%)
Ctrl
Sor
Phen
Com
Hep-G2
Relative migration rate(%)
Ctrl
Sor
Phen
Com


C
SMMC-7721
2.6%
0.8%
Ctrl
3.3%
1.3%
Sorafenib (4μmol/L)
4.1%
0.5%
Phenformin (200μmol/L)
5.1%
1.8%
Combination
D
Hep-G2
4.2%
1.5%
Ctrl
4.4%
2.2%
Sor (2μmol/L)
7.9%
1.3%
Phenformin (100μmol/L)
6.9%
5.4%
Combination

E

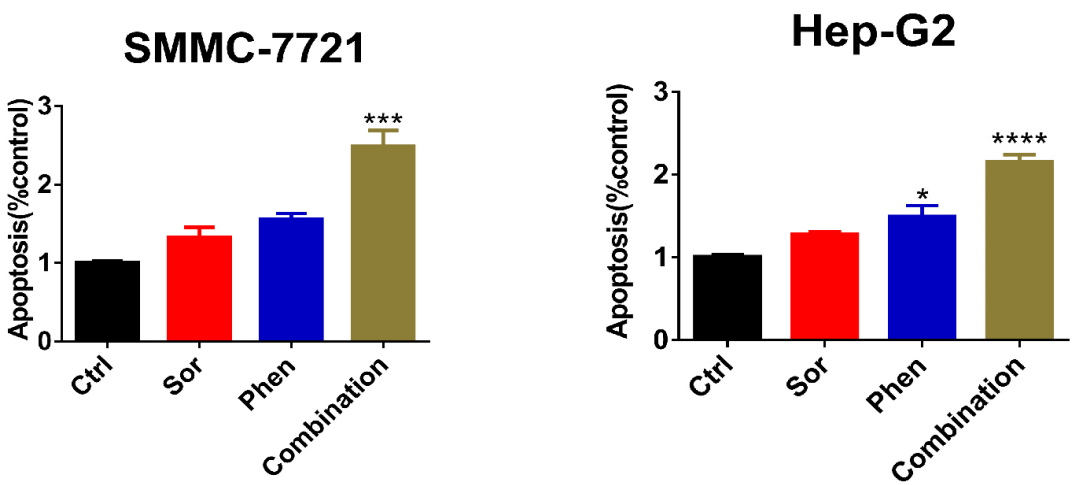


Figure 5. Effects of sorafenib in combination with phenformin on the invasion of SMMC-7721 and Hep-G2 cells. (A) Combination of sorafenib and phenformin treatment suppressed the invasive activity of SMMC-7721 and Hep-G2 cells in a transwell assay, with data quantified in (B). (C, D) Representative flow cytometry plots corresponding to cells stained with propidium iodide (y-axis) and Annexin V-FITC (x-axis), with data quantified in (E). (*P < 0.05, **P < 0.01, ***P < 0.001, n = 3).

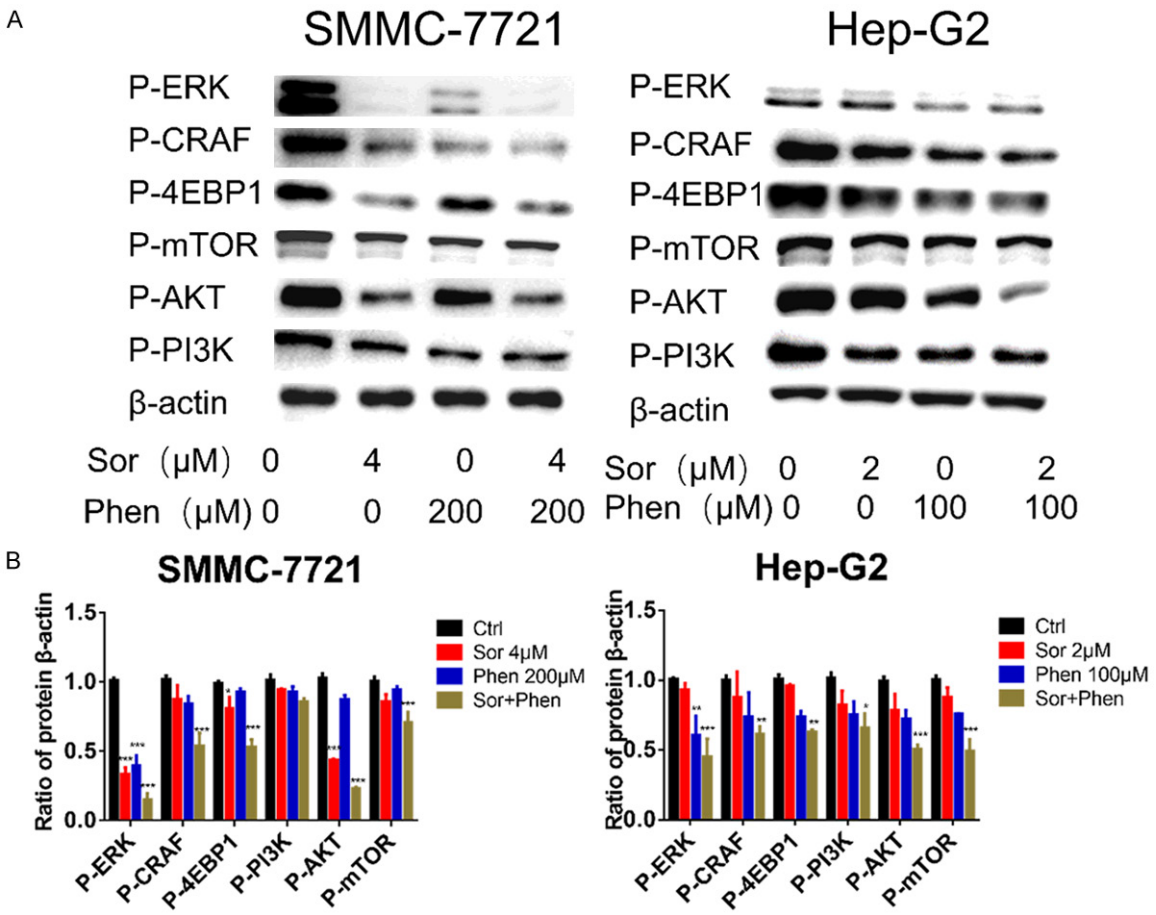


Figure 6. The effects of single-agent and combined treatment of sorafenib and phenformin on PI3K/AKT/mTOR and CRAF/ERK signaling. Phosphorylated (P) levels of proteins associated with these signaling pathways were assessed via Western blotting following treatment with sorafenib and/or phenformin in two HCC cell lines. A. P-ERK, P-CRAF, P-PI3K, P-AKT, P-mTOR, and P-4EBP1 levels were measured, with β-actin as a loading control. B. Relative protein levels (*P < 0.05, **P < 0.01, ***P < 0.001, n = 3).

phenformin more efficiently suppressed AKT, ERK, and 4EBP1 phosphorylation. The suppression of CRAF phosphorylation was also more pronounced in SMMC-7721 cells relative to Hep-G2 cells, whereas AKT and 4EBP1 phosphorylations were more significantly suppressed in Hep-G2 cells. No significant reductions in mTOR or PI3K phosphorylation were observed following combined treatment relative to single-agent treatment, potentially as a consequence of the selected drug dosage.

ERK inhibitor (AZD6244) and AKT inhibitor (MK2206) treatment enhanced the responses of HCC cells to sorafenib and phenformin

To evaluate whether the regulation of the CRAF/ERK and PI3K/AKT/mTOR pathways can affect the response of HCC cells to sorafenib and phenformin, we treated Hep-G2 and SMMC-7721 cells with AZD6244 or MK2206 for 72 h, and the effect on the proliferation of both cell types was determined (Figure S1A). We next evaluated p-ERK protein level following AZD6244 (0.5 μM) treatment for 24 h and p-AKT protein level following MK2206 (0.5 μM) treatment for 24 h in both cell lines. AZD6244 treatment reduced p-ERK levels, whereas MK2206 treatment reduced p-AKT levels (Figure S1B). Both HCC cell lines were then treated with a range of sorafenib or phenformin doses in combination with the above inhibitors, revealing that the inhibition of either ERK or AKT can enhance the anticancer activities of sorafenib and phenformin for these HCC cells, with this effect being most pronounced after MK2206 treatment (Figure S1C, S1D). Zhai et al. previously demonstrated that the regula-

tion of the RAF/ERK and AKT pathways can affect the responses of HCC cells to sorafenib [19]. We have also previously shown that the regulation of the CRAF/ERK and PI3K/AKT/mTOR pathways can influence the responses of bladder cancer cells to phenformin [14]. Furthermore, we revealed that ERK inhibitor (AZD6244) and AKT inhibitor (MK2206) treatments were sufficient to enhance the response of HCC cells to sorafenib and phenformin (Figure S1).

Phenformin bolstered the ability of sorafenib to suppress xenograft HCC tumor growth in mice

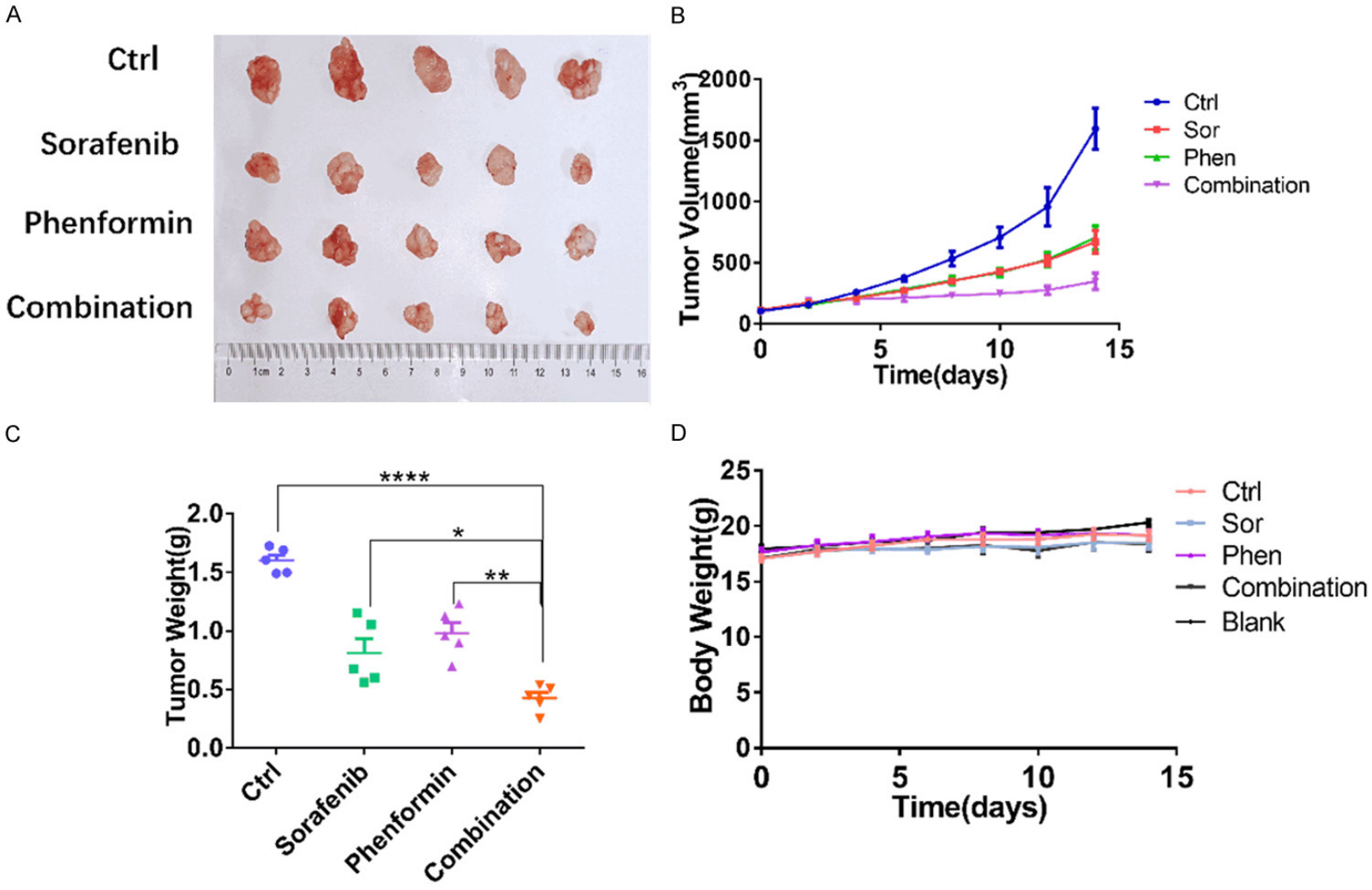
To explore the *in vivo* efficacy of combined treatment of sorafenib and phenformin on HCC, nude mice were subcutaneously implanted with Hep-G2 cells, and mice were subsequently treated with one or both of these drugs. While single-agent treatment exhibited moderate efficacy (**Figure 7A**, **7B**), combined treatment more significantly inhibited tumor growth. Consistent with these results, we observed significant differences in tumor weight among the control, sorafenib, phenformin, and combined treatment groups (1602, 809, 983, and 428 mg, respectively) ($P < 0.01$) (**Figure 7C**). No significant weight loss was detected in any treatment group (**Figure 7D**). Combined treatment also failed to cause any renal or hepatic damage in treated mice (**Figure 7E**). Overall, these data indicate that combination of phenformin and sorafenib can synergistically suppress HCC tumor growth *in vivo* more effectively than either agent in isolation.

Discussion

HCC is the fourth deadliest cancer globally, and over 80% of HCC-related deaths occur in developing nations with limited medical and social resources [20, 21]. The prognosis of advanced HCC is poor, and until sorafenib was approved, there were no available pharmacological agents for the systemic treatment [22-25]. However, sorafenib resistance has emerged as a primary barrier to treatment of advanced HCC [26, 27], which is associated with serious side effects that can result in dose interruption [10, 11]. Exploring novel approaches to treat HCC is thus essential.

Phenformin was developed as an antidiabetic drug but was withdrawn from the market in the 1970s owing to its high risk of inducing fatal lactic acidosis. Nonetheless, phenformin has exhibited robust antitumor activity, enabling it to suppress the growth and proliferation of cancers including melanoma, lung cancer, prostate cancer, breast cancer, and glioblastoma [28]. Combining phenformin with other anti-tumor drugs can also facilitate synergistic treatment efficacy [29]. Guo et al. determined that phenformin was able to suppress ErbB2, AKT, ERK, and mTOR activity more effectively than rapamycin [30]. However, only one single phase I clinical trial assessing the safety of combining phenformin with standard chemotherapy for the treatment has been conducted. This study, which was scheduled to run for two years, was initiated by Paul Chapman of MD Memorial Sloan Kettering Cancer Center (NCT03026517) and was first published on January 20, 2017. The results of this study will guide future efforts to treat cancer via a combination approach that leverages phenformin. The specific mechanisms whereby phenformin inhibits HCC cell invasion remain to be clarified. Herein, we found that phenformin was able to increase HCC cell sensitivity to sorafenib via the suppression of the PI3K/AKT/mTOR pathway activation such that lower sorafenib doses were required to achieve a comparable level of tumor inhibition following phenformin treatment. We further confirmed that sorafenib and phenformin synergistically suppressed HCC cell colony formation activity. RAS/Raf/ERK pathway activation in HCC cells has been documented previously [31, 32], and we further found that phenformin was able to suppress CRAF/ERK expression. Such suppression may be linked to the observed synergistic activity. Further work, however, will be necessary to fully understand these mechanisms underlying the sensitization of sorafenib by phenformin. In addition, we conducted *in vivo* experiments which confirmed that sorafenib and phenformin treatment was able to suppress Hep-G2 tumor growth in a synergistic fashion without reducing murine body weight, suggesting that this combination treatment approach was both safe and effective.

Our results highlight a potentially viable approach to treat advanced HCC. Lohmeyer et al. previously demonstrated that sorafenib can inhibit HCC proliferation by suppressing CRAF/ERK pathway activity [33]. Targeting this


A
Ctrl
Sorafenib
Phenformin
Combination
B
Tumor Volume(mm3)
2000
1500
1000
500
0
0
5
10
15
Time(days)
Ctrl
Sor
Phen
Combination
C
Tumor Weight(g)
2.0
1.5
1.0
0.5
0.0
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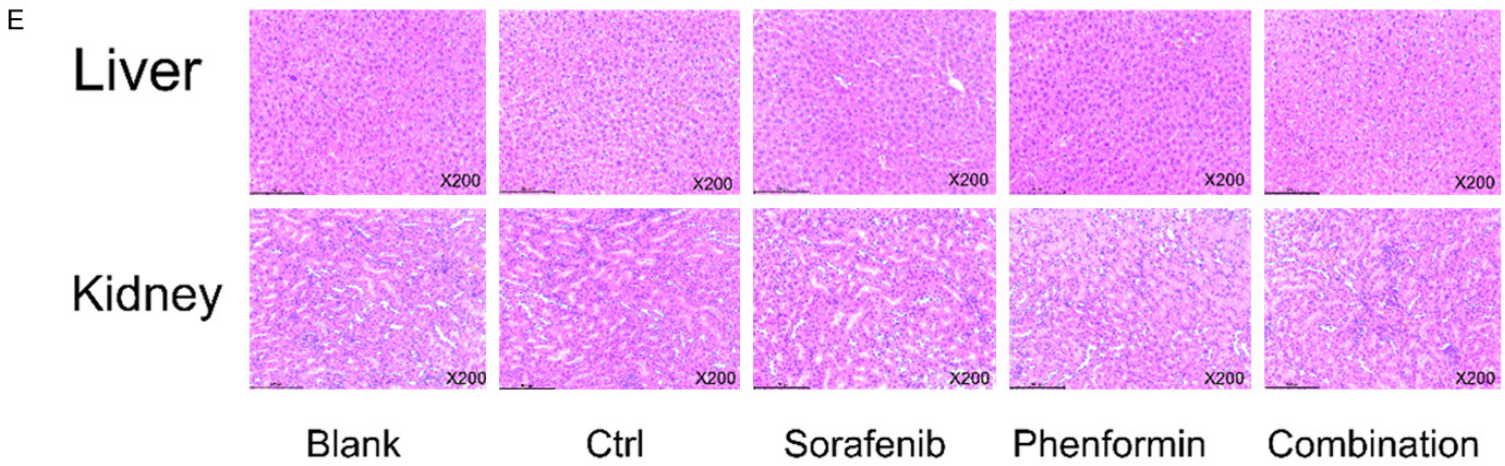

Figure 7. In vivo assessment of the effects of sorafenib and phenformin on Hep-G2 xenograft tumor growth. A. Tumor images. B. Tumor volume changes. C. Differences in tumor weight. D. Murine body weight over time. E. H&E staining of the liver and kidney (*P < 0.05, **P < 0.01, ***P < 0.001, n = 5).

pathway may thus be a viable anti-tumor strategy. No prior studies to our knowledge have evaluated the combined effects of sorafenib and phenformin as an anti-tumor therapeutic approach, underscoring the novelty of our findings.

Together, our data suggest that phenformin can enhance the ability of sorafenib to inhibit HCC cell proliferation, migration, and survival. These two compounds may exhibit synergistic anticancer activity by modulating the PI3K/AKT/mTOR and CRAF/ERK pathways. As such, combined treatment of sorafenib and phenformin may be a safe and effective approach to treat primary HCC.

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Disclosure of conflict of interest

None.

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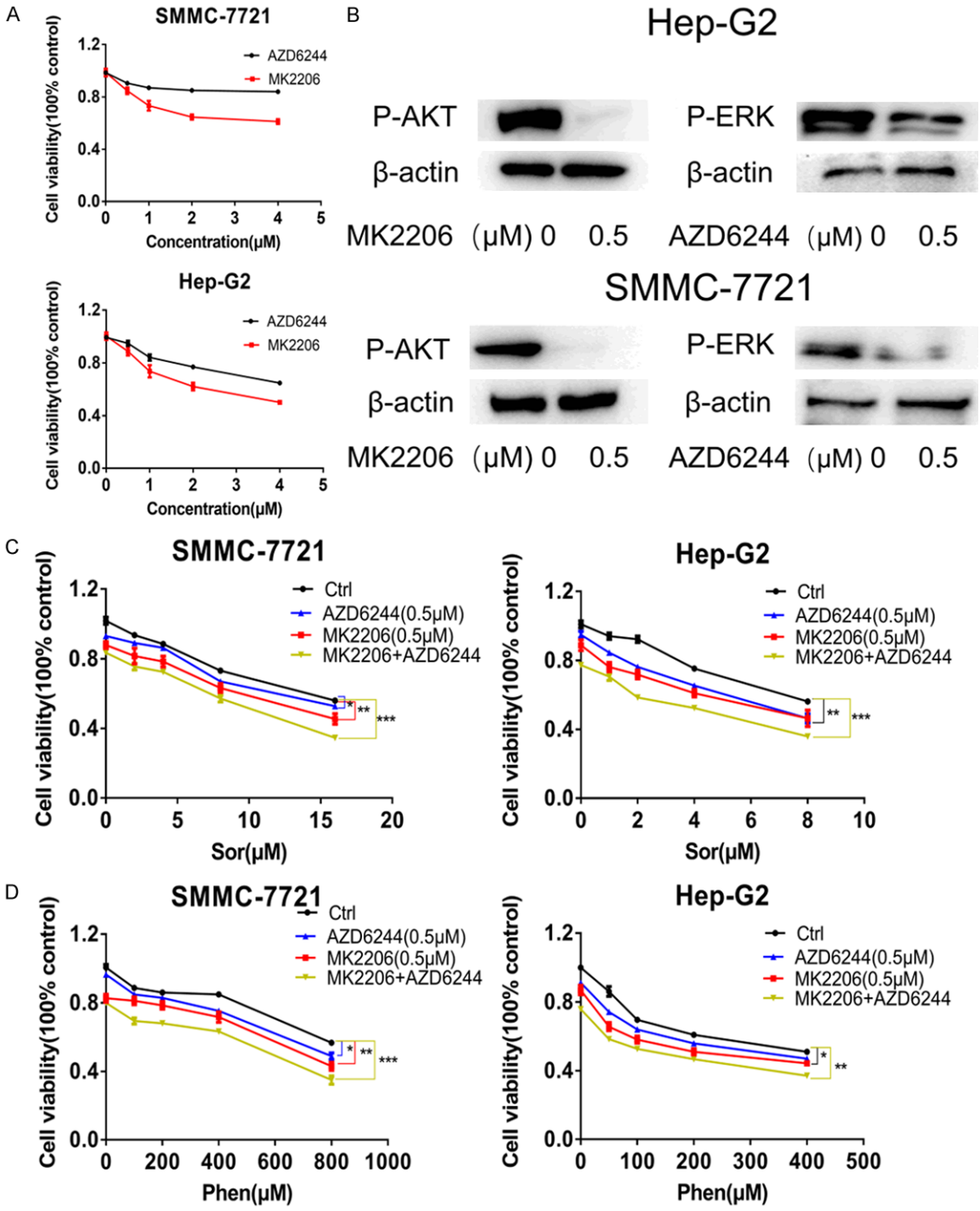

Figure S1. ERK inhibitor (AZD6244) and AKT inhibitor (MK2206) treatments affect the response of HCC cells to sorafenib and phenformin. A. The viability of SMMC-7721 and Hep-G2 cells was assessed at 72 h after AZD6244 and MK2206 treatment. B. The inhibitory effects of AKT inhibitor and ERK inhibitor treatment (0.5 μM) on P-AKT and P-ERK were evaluated. C. Sorafenib and these two inhibitors synergistically suppressed the proliferation of SMMC-7721 and Hep-G2 cells, as measured at 72 h post-treatment. D. Phenformin and these two inhibitors synergistically suppressed the proliferation of SMMC-7721 and Hep-G2 cells, as measured at 72 h post-treatment. (*P < 0.05, **P < 0.01, ***P < 0.001, n = 3).