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Sorafenib and Carfilzomib Synergistically Inhibit the Proliferation, Survival and Metastasis of Hepatocellular Carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is one of the most common and deadly human cancers. The five-year survival rate is very low. Unfortunately, there are few efficacious therapeutic options. Until recently, Sorafenib has been the only available systemic drug for advanced HCC. However, it has very limited survival benefits and new therapies are urgently needed. In this study, we investigated the anti-HCC activity of carfilzomib, a second-generation, irreversible proteasome inhibitor, as a single agent and in combination with sorafenib. In vitro, we found that carfilzomib has moderate anticancer activity toward liver cancer cells, but strongly enhances the ability of sorafenib to suppress HCC cell growth, proliferation, migration, invasion and survival. Remarkably, the drug combination exhibits even more potent antitumor activity when tested in animal tumor models. Mechanistically, the combined treatment activates caspase-dependent and ER stress/CHOP-mediated apoptotic pathways, and suppresses epithelial-mesenchymal transition (EMT). In conclusion, our results demonstrate that the combination of carfilzomib and sorafenib has synergistic antitumor activities against HCC, providing a potential therapeutic strategy to improve the mortality and morbidity of HCC patients.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most prevalent malignancies and the third leading cause of cancer death in the world (1), particularly in China and other far eastern countries (2). Despite the overall decreased rate in cancer incident and mortality in the USA,

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Conflicts of Interest

The authors declare no known conflicts of interest.

that of HCC continues to rise due to the epidemic of obesity and non-acoholic fatty liver disease (NAFLD) (3). Surgery is currently considered as the best treatment strategy for early stage HCC, but over 70% of HCC patients are already in the advanced stage when initially diagnosed and they are no longer suitable for surgical resection (3). Unlike other malignancies such as lung or breast cancer, advanced HCC has few therapeutic options because it is highly resistant to cytotoxic chemotherapy or radiotherapy (3). Moreover, recent clinical trials indicate that advanced HCC only has a moderate response rate to immune checkpoint inhibitors (4). Therefore, there is an urgent need to develop new treatments to improve the clinical outcome of this fatal disease.

Over the past two decades, targeted therapies have significantly enhanced our ability to treat cancer. Monoclonal antibodies and small molecules targeting cancer drivers have become standard treatment regimes for solid tumors and hematologic malignancies. Sorafenib is a pan-kinase inhibitor that blocks both cancer cell proliferation and angiogenesis. Sorafenib broadly inhibits multiple oncogenic kinases, including RAF, PDGFR and VEGFR (5). Until last year's US FDA approval of Regorafenib, a Sorafenib-like multikinase inhibitor, it has been the only clinical medicine for treating advanced HCC (6,7). However, its survival benefit is only 3–6 months in overall survival (OS) (6–8). Many patients require a dosage reduction or treatment cessation due to intolerable adverse effects (9). Furthermore, the disease relapses quickly even for the initial responders (10). For these reasons, it is necessary to improve the efficacy and durable response of sorafenib in advanced HCC patients.

Proteasome inhibitors, such as bortezomib, are molecular targeted agents that have significantly improved the overall survival (OS) of patients with multiple myeloma (MM) (11). Bortezomib binds to the catalytic site of the 26S proteasome, preventing degradation of pro-apoptotic factors and promoting programmed cell death of cancer cells (12). In recent years, many clinical trials have been conducted with bortezomib for solid tumors but had little success as a single agent (13,14). Bortezomib has also been tested in combination with chemotherapeutic agents such as docetaxel but failed to achieve desired clinical endpoints (13,14). These unsuccessful clinical trials highlight the need for high quality mechanistic studies that can help design rational drug combinations and improve the outcome of human trials.

Bortezomib was previously shown to induce HCC cell death when used in combination with a tumor-targeting TRAIL (15) or with sorafenib (16). In the latter study, when PLC/PRF/5 cells were pretreated with sorafenib, bortezomib was proposed to induce apoptosis through PP2A-dependent Akt inactivation. These observations suggest that proteasome inhibition has the potential to improve the therapeutic effect of sorafenib in HCC. Carfilzomib is a second-generation proteasome inhibitor. Unlike bortezomib that reversibly inhibits the proteasome, carfilzomib irreversibly binds to the catalytic site of the proteome, resulting in sustained proteosomal inhibition (17). In vitro studies have shown the superiority of carfilzomib over bortezomib for treatment of relapsed multiple myeloma (18). In a chemically induced rat liver cancer model, carfilzomib has preventive benefit against hepatocarcinogenesis (19). However, whether carfilzomib is active against HCC and whether it enhances the anticancer activity of sorafenib remains unresolved. In the present study, we performed a series of

in vitro and *in vivo* experiments to evaluate the preclinical efficacy of carfilzomib alone or in combination with sorafenib. Our results demonstrate that carfilzomib has moderate anticancer activity. However, the combination of carfilzomib and sorafenib displays strong antitumor activity against HCC. Our study indicates that the combinational therapy of carfilzomib and sorafenib is a potentially useful strategy to improve the treatment outcome for HCC patients.

Materials and Methods

Cell culture, drugs and antibodies

HCC cell line Hep3B was obtained from American Type Culture Collection (ATCC, Manassas, VA), and Bel-7402 was kindly provided by Dr. Yue Li, Southern Medical University, Guangzhou, China. The cell lines have been authenticated by the Short Tandem Repeat (STR) analysis. Hep3B and Bel-7402 cells were cultured in Dulbecco's Modified Eagle Medium (GIBCO BRL) supplemented with 10% fetal bovine serum (GIBCO BRL) at 37°C in an atmosphere of 5% CO_2, and *Mycoplasma* contamination was routinely examined by PCR. Cells with low passage numbers (less than 20) were used in this study. Carfilzomib and sorafenib were purchased from Selleck Chemicals. Cleaved caspase-3 (#9664), cleaved caspase-7 (#8438), cleaved caspase-9 (#7237), cleaved PARP (#5625), ATF-4 (#11815), eIF2α (#5324), p-eIF2α (#3398) and GAPDH (#5174) rabbit monoclonal antibodies, anti-CHOP (#2895) mouse antibodies, HRP-conjugated anti-rabbit (#7074) and anti-mouse (#7076) secondary antibodies were purchased from Cell Signaling Technology (USA). PERK (#ab65142), p-PERK (#ab192591), CHOP (#ab11419) and anti-Ki67 antibodies (#ab15580) were purchased from Abcam. Mouse Anti-E-cadherin (#610181), N-cadherin (#610921) and β-catenin (#610154) were purchased from BD Biosciences.

Cell proliferation assay

Cell proliferation was assessed by the MTS method using the CellTiter 96® AQueous One Solution Cell Proliferation Assay kit (G3580, Promega) according to the manufacturer's instructions. Briefly, 2,000 cells were plated into each well of 96-well plates in 200 μL culture medium and incubated overnight for attachment. On the following day, cells were treated with drug or drug vehicle for 5 days. For the MTS assay, 20 μL MTS reagent was added to each well and incubated for another 2 hours. The absorbance was measured at 490 nm with a multifunctional microplate reader.

Colony formation assay

Cells were seeded onto 6-well plates (1,000 cells/well) and cultured at 37°C with 5% CO_2 overnight. The cells were then treated with drug or drug vehicle for about 2 weeks until colonies were visible. Cell culture media with different concentration of sorafenib and/or carfilzomib were changed every three days during this period. It has been suggested that free plasma sorafenib concentrations are likely to be in the sub-μM range due to extensive plasma binding (20). So we performed this assay using sorafenib concentrations of 0.1 μM or 0.5 μM. The colonies were washed with PBS twice, fixed with 4% paraformaldehyde for 15 min and stained with 0.1% crystal violet solution for 30 min. After washing with PBS, the number of colonies (> 50 cells/colony) was counted under the microscope.

Analysis of apoptosis by flow cytometry

Apoptosis was analyzed using the Annexin V-FITC/PI apoptosis kit (KGA108, KeyGEN Biotech, China) according to the manufacturer's instruction. Briefly, cells were harvested and washed in cold phosphate-buffered saline (PBS). 500 μL binding buffer was added to each sample. Cells were mixed with annexin-V-FITC and PI solution, and incubated at room temperature for 15 min. The stained cells were analyzed by flow cytometry (Novocyte, ACEA Biosciences, China) at the fluorescence emission of 530 nm.

Transwell migration and invasion assays

Cell migration and invasion assays were performed on transwell chambers with 8-μm pore-size filters without (for migration) or with (for invasion) coated matrigel (Falcon). Cells were trypsinized and resuspended in serum-free medium. 250 μL of cell suspension (1 × 10 5 cells) was added to the upper chambers in a transwell insert, and the upper chambers were then placed into the wells of a 24-well plate. 750 μL culture medium containing 20% FBS was added to the lower chamber. After transwell inserts were cultured at 5% CO_2 at 37°C for 24 h, cells on the top of the membrane were removed with a cotton swabs. Cells attached on the underside of the membrane were fixed and stained with 0.1% crystal violet. After washing with PBS, the number of cells was counted in three random microscopic fields under the microscope.

Caspase activity assay

The activity of caspase 3/7 was measured using a Caspase-Glo® 3/7 Assay kit (G8091, Promega) with a modified protocol. Briefly, the proluminescent substrate containing the DEVD sequence is cleaved by caspase 3/7, respectively. After caspase cleavage, the substrate for luciferase (aminoluciferin) was released, inducing the luciferase reaction, which was analyzed in a total volume of 200 μl in 96-well plates. 100 μl reagent was added into each well of a white-walled 96-well plate containing 100 μl of blank, control or treated cells in culture medium. The mixture was incubated at room temperature for 2 hours and measured for luminescence by a plate-reading luminometer.

Immunoblot

Equal amount of protein extracts from HCC cells or tissues were separated by 10% SDS-PAGE, and transferred onto Immobilon-P membrane (Millipore). After blocking with 5% non-fat milk in TBST (with 0.1% Tween-20), the membrane was incubated with the indicated primary antibodies overnight at 4°C, washed with TBST, incubated with secondary antibodies at room temperature for 60 min, and detected by the Western Lightning Plus-ECL Kit (Thermo, USA) according to manufacturer's instruction.

In vivo studies and immunohistochemistry

The animal protocol was approved by the Animal Ethics Committee of Sun Yat-Sen University Cancer Center. Subcutaneous xenograft models were established and drug treatments were carried out as previously described (21–25). Four-week-old female BALB/c nude mice were used in this study. Log phase Hep3B cells were harvested and resuspended in PBS at a density of 3 × 107 cells/ml. 0.1 ml cell suspension containing 3 × 106 cells

were injected subcutaneously into the right flank of each mouse. Mice were then randomly divided into four groups and were treated with drug vehicle, 4 mg/kg carfilzomib, 15 mg/kg sorafenib, and carfilzomib plus sorafenib, respectively. Carfilzomib was diluted with physiological saline and a volume of 200μL was intraperitoneally injected twice weekly. Sorafenib was resuspended in an oral vehicle containing Cremophor EL (Sigma-Aldrich), 95% ethanol and water in a ratio of 1:1:6, and a volume of 200μL was orally administrated by gavage daily. Mice in the combination group received both drugs treatment given the same as in the single treatment group concurrently. Mice in the control group received drug vehicles only.

Tumor volumes and mice weight were measured every three days. Tumor volumes were calculated using the following formula: volume (mm3) = length× width2 × 0.5. After 3 weeks of treatment, the mice were euthanized by cervical dislocation. The subcutaneous tumors were harvested. Part of the tumors was frozen in liquid nitrogen for immunoblot analysis. The remainder was fixed in 4% formalin for HE or immunohistochemistry (IHC) staining. For IHC staining, antibody against Ki-67 was used to evaluate cell proliferation rate. The Ki-67 staining score was determined using the following formula: overall scores = intensity score × percentage of positive cells. The intensity scores were graded as 0–3 (0 = negative staining, 1 = weakly positive staining, 2 = moderately positive staining and 3 = strongly positive staining). The percentage of positive cells was graded from 0 to 100%. The staining results were observed in three random microscopic fields under the microscope.

Xenograft mouse model was used to evaluate sorafenib/carfilzomib effect with or without CHOP expression. Hep3B cells expressing CHOP shRNA or control shRNA were injected subcutaneously into the athymic nude mice. After tumors were developed, mice were treated using combined sorafenib/carfilzomib as mentioned above. For IHC staining, anti-CHOP antibody was used to detect the CHOP expression in the tumor tissues. Cell proliferation was detected by Ki67 staining. Apoptotic cells in tumor tissues were detected by Terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick end labeling (TUNEL) assay with In Situ Cell Death Detection Kit (Roche). The cell nuclei with brown color were considered as cells undergoing programmed death.

Statistical analysis

One-way ANOVA was used to measure difference among treated groups versus control group. Unpaired t-test was used to compare the differences between each treated group to the combination group in colony formation, apoptosis assay and migration assays. The difference in tumor growth rate between the 4 different groups of nude mice was determined by repeated measures ANOVA. Results are shown as mean ± SD. A p value of less than 0.05 is considered statistically significant. All statistical analyses were conducted using the SPSS17.0 statistical software (Version 17.0 SPSS Inc.) and GraphPad Prism 6.0 (GraphPad Software).

Results

Carfilzomib has anti-HCC activity and displays synergy with sorafenib

Carfilzomib is a second generation of proteasome inhibitor that can irreversibly target the proteasome. To evaluate its therapeutic potential toward liver cancer, we studied carfilzomib in Hep3B and Bel-7402, two commonly used liver cancer cell lines. Indeed, carfilzomib displays excellent anticancer activity as measured by the MTS assay, with an IC_{50} value at 40.80 nM and 61.95 nM, respectively, which compare favorably with sorafenib that shows an IC50 of 5.35 μM and 8.62 μM for Hep3B and Bel-7402, respectively (Fig. 1A). We further evaluated the combinational activity of carfilzomib with sorafenib. The results show that carfilzomib significantly enhances sorafenib anti-HCC activity in a dosage-dependent manner, though there is a slight variation between the two HCC cell lines (Fig. 1B). We therefore evaluated their synergism using the Chou-Talalay method with the Calcusyn software (Biosoft, Cambridge, UK). A combination index (CI) less than 1 indicates the drug combination has synergism. The results show that the combination index (CI) value can reach as low as 0.506, demonstrating a strong synergistic activity for this drug combination (Fig. 1C, Table 1).

To further explore the anti-proliferation activity of sorafenib in combination with carfilzomib, we analyzed their time-dependent effect on Hep3B and Bel-7402 cells. Sorafenib and carfilzomib together exhibits much more potent and durable inhibition of HCC cell proliferation than either drug alone ($p < 0.05$) (Fig. 2A). When evaluated by the colony formation assay, 0.01μM carfilzomib and 0.5 μM sorafenib individually only moderately inhibit the colony formation, and 0.1 μM sorafenib alone showed no significant effect (Fig. 2B). In contrast, their combination results in far more significant reduction in the number of colonies (all p values < 0.05) (Fig. 2B). These findings demonstrate that carfilzomib has anticancer activity toward HCC and has the ability to significantly enhance the therapeutic effect of sorafenib.

Carfilzomib promotes the apoptotic activity of sorafenib in HCC

Inducing cancer cell death is an important anticancer mechanism. We asked if carfilzomib and sorafenib have this therapeutic property. To this end, we used flow cytometry to analyze Hep3B and Bel-7402 cells treated with carfilzomib and sorafenib individually or in combination. After treatment for 24 hours, both carfilzomib and sorafenib result in significant apoptotic cell population, which is further increased when two drugs were used together ($p < 0.05$) (Fig. 3A). We next investigated whether the induced cell death is caspase-dependent. By immunoblot analysis, we found that in HCC cells treatment with sorafenib and carfilzomib, or in combination, leads to an elevated cleavage of caspase-3, caspase-7, caspase-9 and PARP (Fig. 3B), indicating that the two drugs cause apoptosis through a caspase-dependent manner.

Carfilzomib and sorafenib inhibit HCC cell migration and invasion

Increased cancer cell motility and invasion are associated with liver cancer progression, which accounts for the morbidity and mortality of HCC patients. We next performed experiments to investigate the effect of carfilzomib and sorafenib on HCC cell migration

and invasion. Transwell migration assay shows that each drug has moderate inhibition of HCC cell migration, but their combination has much more potently inhibitory effect ($p < 0.05$) (Fig. 4A). Carfilzomib and sorafenib individually only have a weak effect on HCC cell invasion ($p < 0.05$), but their combinational treatment, however, produces much stronger blockage of HCC cell invasion, especially for BEL-7402 cells ($p < 0.05$) (Fig. 4B). Taken together, these results show that the combination of carfilzomib and sorafenib markedly improves the anti-metastatic activity against HCC cells.

Epithelial to mesenchymal transition (EMT) is a biological process that converts epithelial cells to mesenchymal cells. During this process, epithelial cells lose cell-cell adhesion and gain an increased migratory and invasive ability (26). Because EMT is a major contributor to cancer cell invasiveness and metastasis, we studied how carfilzomib and sorafenib affect the EMT process in HCC cells. The results show that each drug alone moderately increases the epithelial marker E-Cadherin while decreases the expression of the mesenchymal markers N-Cadherin and β-Catenin (Fig. 4C). When the two drugs are used together, they produce much stronger inhibitory effect on EMT (Fig. 4C). These results indicate that carfilzomib and sorafenib have excellent combinational activity to block EMT in HCC cells.

Carfilzomib and sorafenib potently inhibit the growth of HCC xenograft tumors

To evaluate the antitumor activity of carfilzomib and sorafenib in vivo, we established Hep3B xenograft tumors in nude mice. In this experiment, tumor bearing nude mice were randomized into four groups that were treated with vehicle control, sorafenib, carfilzomib or drug combination (n = 6 per group) for 3 weeks. Tumor volume was monitored throughout the study to evaluate the antitumor activity. As expected, sorafenib shows moderate antitumor activity (Fig. 5A). Carfilzomib has a slightly better antitumor effect than sorafenib (Fig. 5A). In contrast, the combination of sorafenib plus carfilzomib generates markedly higher antitumor activity than either drug alone, resulting in minimal tumor growth (Fig. 5A). Consistently, the drug combination group has the lowest tumor burden (Fig. 5B and 5C). Notably, all treated mice maintain similar body weight to the untreated animals and display no obvious signs of toxicity throughout the study, indicating that carfilzomib and sorafenib combination is well tolerated (Fig. 5D). Ki-67 staining of tumor sections from treated animals shows much more reduced cell proliferation in the drug combination group and individual drug groups (Fig. 5E and 5F), which is supported by the reduced mitotic cells as shown by HE staining (Fig. 5E). Moreover, caspase cleavage is higher in the drug combination group as determined by immunoblot (Fig. 5G). These data indicate that carfilzomib significantly enhances the antitumor activity of sorafenib in vivo.

Carfilzomib and sorafenib trigger ER stress-mediated apoptosis through the PERK/eIF2α/ATF4/CHOP Pathway

Endoplasmic reticulum (ER) stress is an important mechanism for cancer cell apoptosis (27). During ER stress, the ER stress kinase PERK is activated, which transduces the ER stress signal through phosphorylation of its downstream effectors eIF2α, and increased expression of ATF4 and CHOP. Caspase-7 is the main caspase that responds to ER stress (28). We found that caspase-7 is induced by the combinational treatment of carfilzomib and sorafenib (Fig. 3B and 5G), suggesting that ER stress is involved. These observations are consistent

with the idea that proteasome inhibitor targets the ER-stress pathway (29–32). Hence, we investigated if ER stress is involved in the antitumor response by carfilzomib and sorafenib. The results show that both ATF4 and CHOP are up-regulated by sorafenib in a concentration dependent manner (Fig. 6A). When HCC cells are treated with sorafenib and carfilzomib together, the level of p-PERK and p-eIF2α is significantly increased, while total PERK and elF2α remain unchanged (Fig. 6B). Concomitantly, the expression of ATF4 and CHOP is increased (Fig. 6B). These data demonstrate that carfilzomib and sorafenib act together to induce ER-stress and ER-stress mediated apoptosis.

CHOP up-regulation is critical for ER-stress induced apoptosis (33). To ask if CHOP has a role in carfilzomib/sorafenib-induced apoptosis, we knocked down CHOP in Hep3B and Bel-7402 cells by a CHOP-specific siRNA (Fig. 6C). CHOP down-regulation abrogates caspase-3/7 cleavage (Fig. 6D). To further verify the role ER stress in carfilzomib/sorafenib-induced apoptosis, we treated HCC cells with salubrinal, a selective inhibitor of eIF2α dephosphorylation and ER-stress induced apoptosis (34). Salubrinal significantly attenuates caspase 3/7 cleavage induced by the carfilzomib and sorafenib combination (Fig. 6E). A previous study suggested that the combinational treatment with sorafenib and bortezomib enhances apoptosis in HCC cells through inhibition of AKT (16). To ask if AKT is involved in the anti-HCC effects of sorafenib and carfilzomib combination, we investigated the effect of these drugs on AKT phosphorylation, an indicator of AKT activation status. On the contrary, sorafenib and carfilzomib alone or in combination not only do not inhibit AKT phosphorylation, they actually induce robust increase in the p-AKT level (Fig. 6F). These results demonstrate that ER-stress, rather than AKT inhibition, is a major mechanism for carfilzomib/sorafenib-induced apoptotic cell death.

We further investigated the role of sorafenib/carfilzomib in ER-stress induced apoptosis using xenograft model. Subcutaneous xenograft tumors were established from Hep3B cells with stable CHOP knockdown by shRNA or a control shRNA. The growth rate of Hep3B tumors with CHOP knockdown is considerably higher than the control tumors, (Fig. 6G). While the combination of sorafenib and carfilzomib shows significant antitumor activity in the control group, it has no discernible effect toward the CHOP knockdown tumors (Fig. 6G). IHC staining shows that combinational treatment of sorafenib/carfilzomib increases CHOP expression, reduced proliferation (Ki67 staining) and elevated apoptosis (TUNEL staining) in the control tumors (Fig. 6H). In contrast, the combinational treatment has little effect on cancer cell proliferation and apoptosis in the CHOP knockdown tumors (Fig. 6H). These results indicate that carfilzomib and sorafenib together produce antitumor activity through ER stress/CHOP-mediated growth inhibition and apoptosis in vivo.

Discussion

Sorafenib has been the only available systematic drug for unresectable HCC until the recent US FDA approval of Regorafenib. Unfortunately, both Sorafenib and Regorafenib have limited clinical benefits. Developing new liver cancer drugs has proven to be especially challenging. Thus, considerable efforts have been directed at improving the treatment outcome for sorafenib. Combinational drug therapy is an useful approach to enhance their anticancer activity while minimizing the adverse effects due to reduced drug dosage of each

agent. They can also reduce the potential drug resistance because each drug attacks cancer cells through distinct mechanisms. The proteasome inhibitors bortezomib and carfilzomib are FDA-approved oncology drugs for multiple myeloma. Proteasome targeting has also been recognized as a promising approach for solid tumors (35). For example, bortezomib showed modest single-agent activity in patients with relapsed or refractory advanced non-small-cell lung cancer (36). Objective clinical response for carfilzomib combined with irinotecan had 19% partial response and 6% stable disease in small cell lung cancer in a phase I clinical study (37). However, there is a strong need to improve the antitumor activity of proteasome inhibitors in order to advance them in the clinic for solid tumors. Our study demonstrates that carfilzomib and sorafenib display excellent synergistic anticancer action against HCC in vitro and in vivo. Further development of this therapeutic strategy may lead to a new treatment for HCC, and possibly other solid tumors.

Endoplasmic-reticulum stress (ER Stress), also known as unfolded protein response (UPR), happens when unfolded or misfolded proteins accumulate in the ER lumen (38). In response to ER stress, cells up-regulate molecular chaperones to restore normal protein folding and ameliorate the source of ER stress. In the event that unfolded proteins cannot be corrected, cells commit to apoptosis. The proteasome plays a critical role in clearing unfolded/misfolded proteins. Emerging evidence suggests that targeting proteasome is a promising strategy for anticancer therapy due to its ability to selectively kill malignant cells (39). Bortezomib was previously reported to induce ER-stress mediated apoptosis in multiple myeloma (MM) by increasing the expression of UPR genes BiP, CHOP and XBP-1 (29,40). In this study, we found that the combination of carfilzomib and sorafenib leads to activation of PERK, phosphorylation of eIF2α, and increased expression of ATF4 and CHOP, as well as enhanced caspase-dependent apoptosis. Thus, pharmacological inhibition of proteasome by carfilzomib enhances the ability of sorafenib to promote ER stress-dependent cell death pathway.

The combination of carfilzomib and sorafenib displays remarkably stronger inhibition of HCC cell migration and invasion than each drug individually, suggesting that they are especially useful for treatment of late stage liver tumors that are invasive and metastatic. Mechanistically, the drug combination inhibits EMT, an oncogenic process required for the metastatic colonization of HCC (41). Although sorafenib has been reported to inhibit the EMT and metastasis of HCC cells (42–44), its effect is relatively weak (Fig. 4). Here we show for the first time that carfilzomib also moderately inhibits EMT, migration and invasion of HCC cells. The combination of carfilzomib and sorafenib produces a much more robust blockage of EMT, which is consistent with the inhibitory effect on HCC cell migration and invasion.

There are several new observations in our study with carfilzomib/sorafenib compared with the previous study with bortezomib/sorafenib. First, the previous study used sorafenib pretreatment to prevent AKT activation by bortezomib. In our study, however, carfilzomib, sorafenib and their combination all activate AKT. This observation is consistent with the notion that blockage of ERK pathway leads to feedback activation of the PI3K-AKT pathway (45). Second, carfilzomib/sorafenib strongly inhibits the epithelial-to-mesenchymal transition (EMT), and migration/invasion of HCC cells, which was not observed in the

previous study. Third, carfilzomib/sorafenib triggers ER stress and ER stress-induced activation of apoptosis, which was not observed in the previous study. Finally, Bortezomib is a first-generation proteasome inhibitor that reversibly inhibits both the chymotrypsin-like (CT-L) and caspase-like (C-L) activities of the proteasome, and also inhibits the serine proteases chymase, dipeptidyl peptidase II, HtrA2/Omi, and cathepsins A and G (46–48). It is limited clinically due to acquired resistance and severe toxicities, including peripheral neuropathy (46,49–54). Moreover, recent studies indicate that these adverse events are due to off-target, nonproteasomal effects of bortezomib (46,55). In contrast, carfilzomib is highly selective for the CT-L activity. Compared with bortezomib, carfilzomib has higher affinity to proteasome and lower off-target toxicity. It does not exhibit inhibitory activity against the multiple serine proteases targeted by bortezomib, and has a markedly reduced rate of peripheral neuropathy in patients (46). For these reasons, it is important to explore the combinational therapy of carfilzomib and sorafenib in liver cancer.

In conclusion, the present study shows that the combinational treatment with carfilzomib and sorafenib synergistically inhibit HCC cells proliferation, migration and invasion. Moreover, they induce HCC cells to undergo apoptosis through ER stress and activation of the PERK/eIF2α/ATF4/CHOP pathway (Fig. 6I). Because carfilzomib and sorafenib are both FDA-approved oncology drugs, this combinational therapy is readily translatable into the clinic, improving the mortality and morbidity of liver cancer patients.

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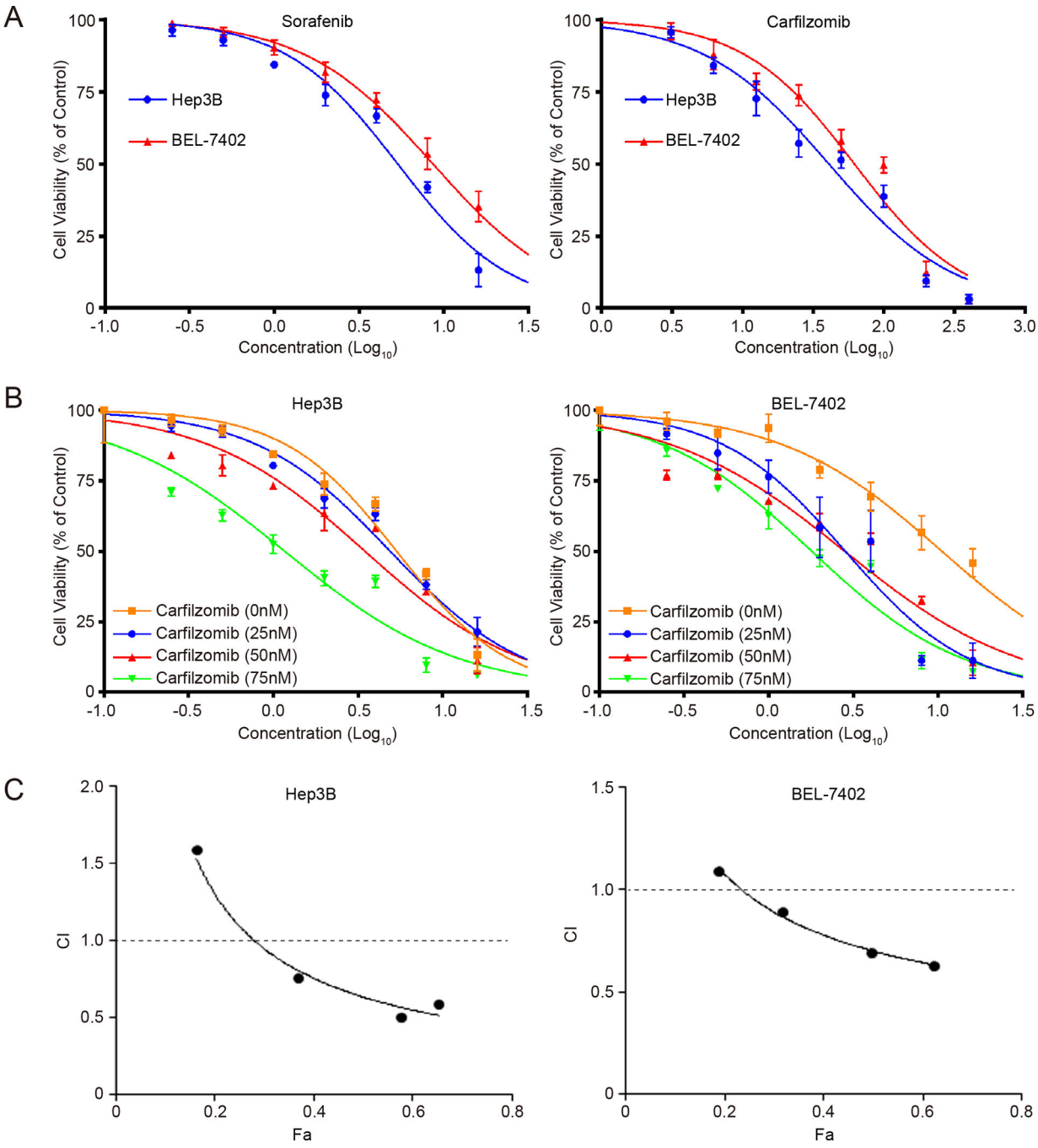

Figure 1. Sorafenib and carfilzomib synergistically inhibit the proliferation of HCC cells.

(A) Hep3B and Bel-7402 cells were treated with various concentrations of sorafenib or carfilzomib for 48h. Cell proliferation was determined by the MTS assay in triplicate. Data represent mean ± SD. IC_{50} value of sorafenib and carfilzomib in HCC cell lines was then determined.

(B) Sorafenib inhibition of HCC cell proliferation is enhanced by carfilzomib. Hep3B and Bel-7402 cells were treated with sorafenib and various concentrations of carfilzomib for

48h. Cell proliferation was determined by the MTS assay. The assays were performed in triplicate. Data represent mean ± SD.

(C) CalcuSyn software was used to determine whether there was synergism between sorafenib and carfilzomib. Combination index (CI) of the combination of sorafenib and carfilzomib is shown. The assays were repeated in triplicate.

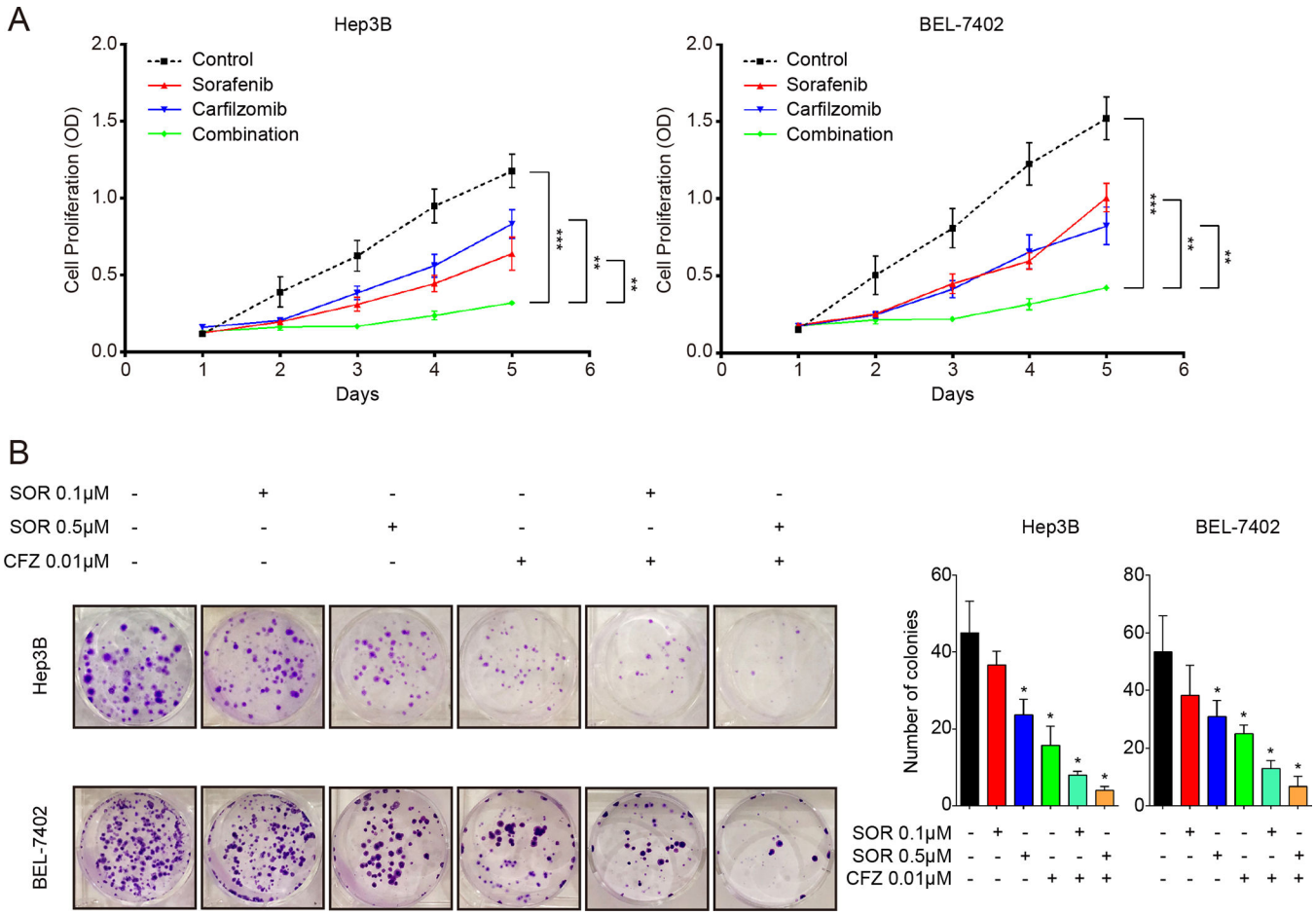

Figure 2. Sorafenib and carfilzomib synergistically inhibit oncogenic growth of HCC cells.

(A) Hep3B and Bel-7402 cells were treated with sorafenib (2.5 μM) and carfilzomib (0.025 μM) individually or in combination for various times. HCC cell proliferation was determined by the MTS assay.

(B) Carfilzomib enhances the ability of sorafenib to inhibit colony formation of HCC cells. Hep3B and Bel-7402 cells were treated with sorafenib (0.1 μM or 0.5 μM) and carfilzomib (0.01 μM) individually or in combination for about two weeks. The inhibition effects were observed based on the number of colonies formed. The results shown are representative of three independent experiments. All Data were shown as mean ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

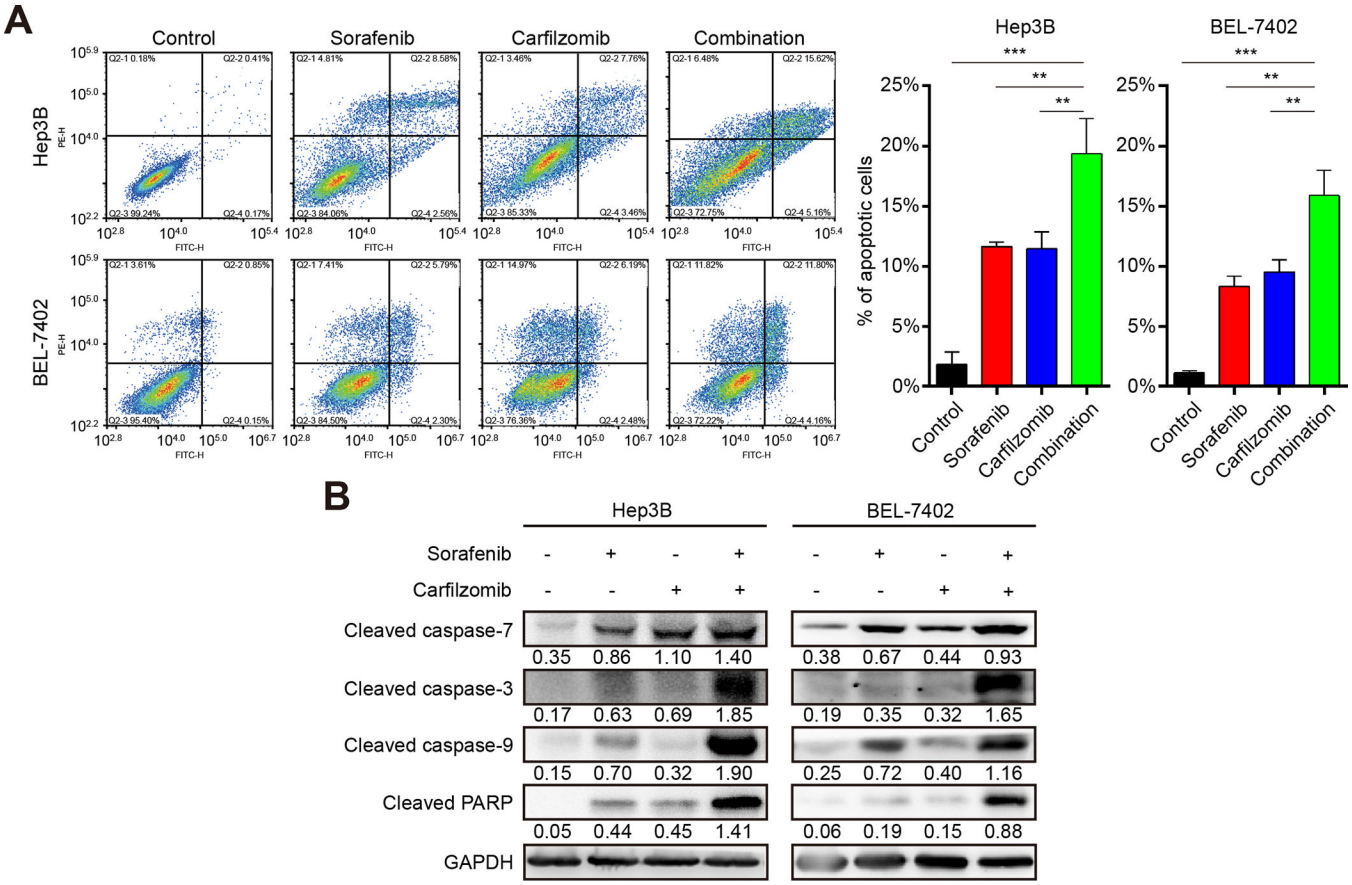

Figure 3. Carfilzomib and sorafenib induce apoptosis of HCC cells in a caspase-dependent manner.

(A) Hep3B and Bel-7402 cells were treated with carfilzomib (0.1 μM) and sorafenib (7.5 μM) individually or in combination for 24 h and analyzed for cell death by flow cytometry. The experiments were performed in triplicate and data represent mean ± SD. *$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$.

(B) Cleaved caspase-3, cleaved caspase-7, cleaved caspase-9 and cleaved PARP were used to analyze apoptotic cell death as detected by immunoblot. GAPDH was used as a loading control. Number indicates relative abundance (arbitrary unit).

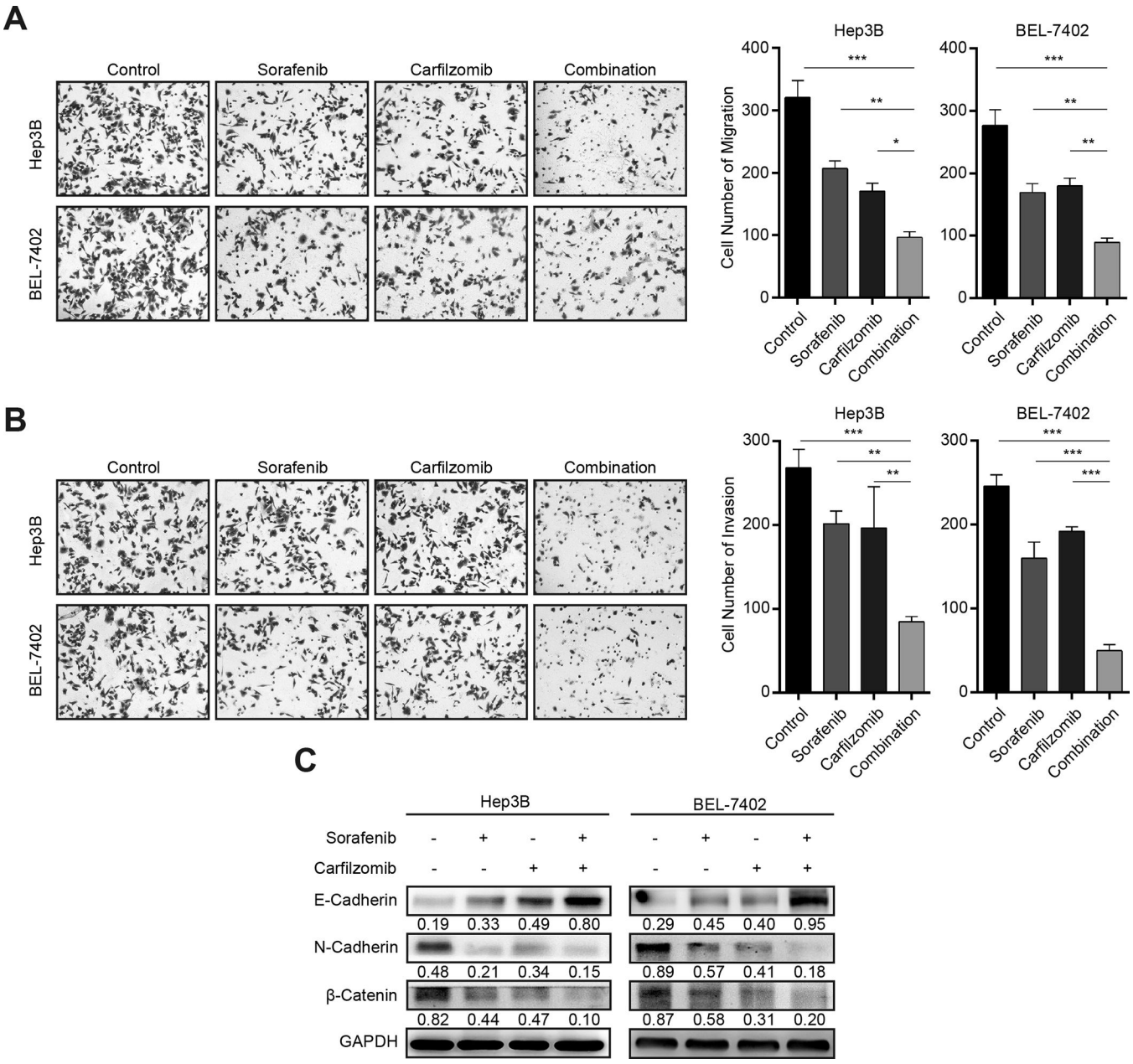

Figure 4. Carfilzomib and sorafenib inhibit HCC cell migration, invasion and EMT.

(A) Hep3B and Bel-7402 cells were treated with carfilzomib (0.05 μM) and sorafenib (7.5 μM) individually or in combination for 24 h. Transwell migration assays were performed to determine the migratory ability of Hep3B and Bel-7402 cells. The experiments were performed in triplicate and data represent mean ± SD. *$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$. (B) Hep3B and Bel-7402 cells were treated with carfilzomib (0.05 μM) and sorafenib (7.5 μM) individually or in combination for 24 h. Transwell invasion assays were performed in metrigel to determine the invasion ability of Hep3B and Bel-7402 cells. The experiments were performed in triplicate and data represent mean ± SD. *$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$

(C) Hep3B and Bel-7402 cells were treated with carfilzomib (0.05 μM) and sorafenib (7.5 μM) individually or in combination for 24 h. The expression of the epithelial marker E-cadherin, and the mesenchymal markers N-Cadherin and β-Catenin was analyzed by immunoblot. GAPDH was used as a loading control. Number indicates relative abundance (arbitrary unit).

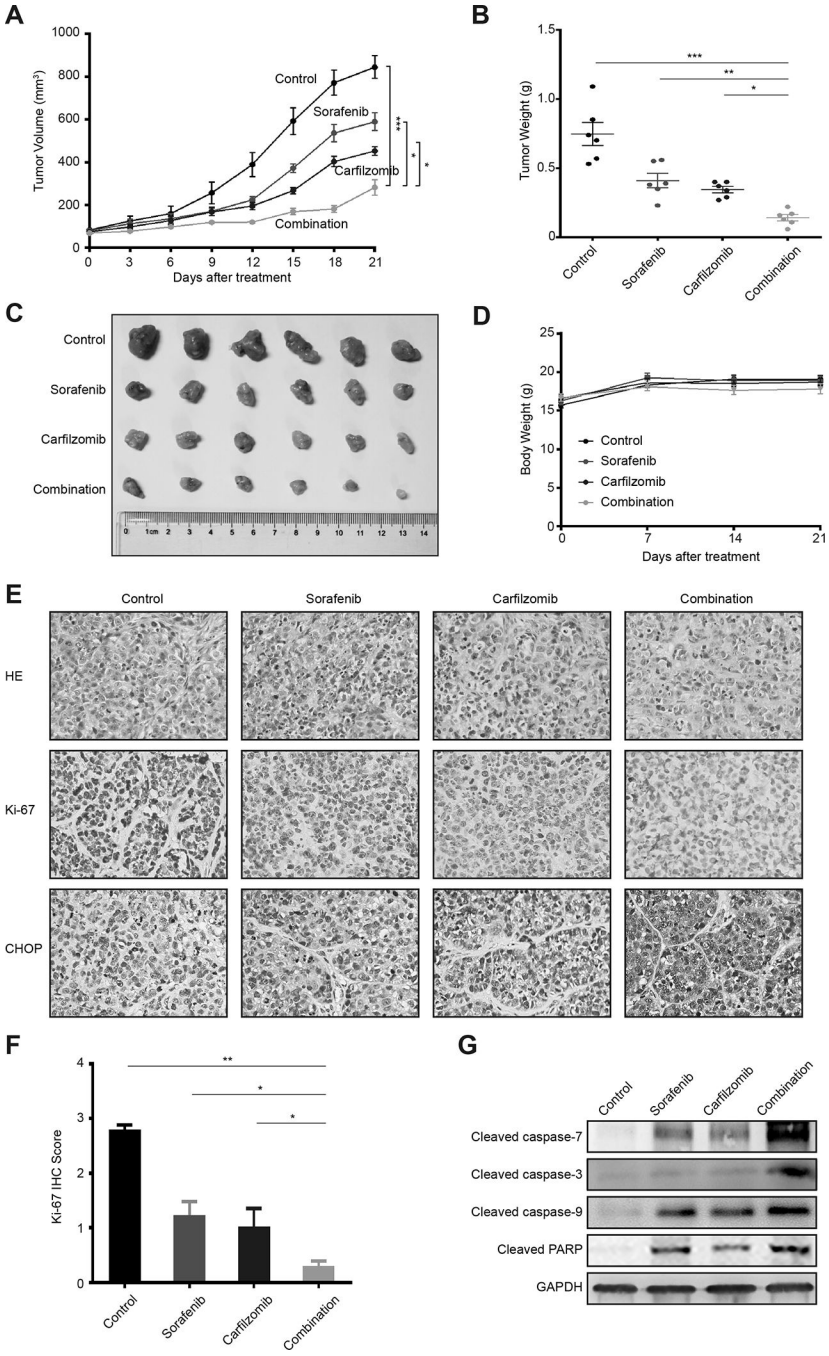

Figure 5. Carfilzomib and sorafenib strongly attenuate HCC tumor growth in vivo.

(A) Xenograft tumors generated from Hep3B cells were treated with carfilzomib and sorafenib individually or in combination, or with a drug vehicle control. Growth of xenograft tumors was measured by tumor volume. Data represent mean ± SD (n = 6). Statistical analyses were performed by two-way ANOVA and sample-paired t-test. *$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$.

(B, C) Shown are the weights and images of xenograt tumors at the end of the experiment. Data represent mean ± SD (n = 6). Statistical analyses were performed by one-way ANOVA and sample-paired t-test. *$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$.

(D) The body weight +/− SD of mice in different treatment groups.

(E) Upper panel: Representative images of HE staining of HCC xenografted tumor sections in different treatment groups (200 × magnification); Lower panel: Representative images of Ki67 staining of HCC xenografted tumor sections in different treatment groups (200 × magnification).

(F) Quantification of IHC scores for Ki-67 staining in Hep3B xenograft tumors. Data represent mean ± SD (n = 6). Statistical analysis was performed by sample-paired t-test. *$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$.

(G) Xenograft tumor tissues from different drug treatment groups were analyzed for cleaved caspase-3, cleaved caspase-7, cleaved caspase-9 and cleaved PARP by immunoblot. GAPDH was used as a loading control.

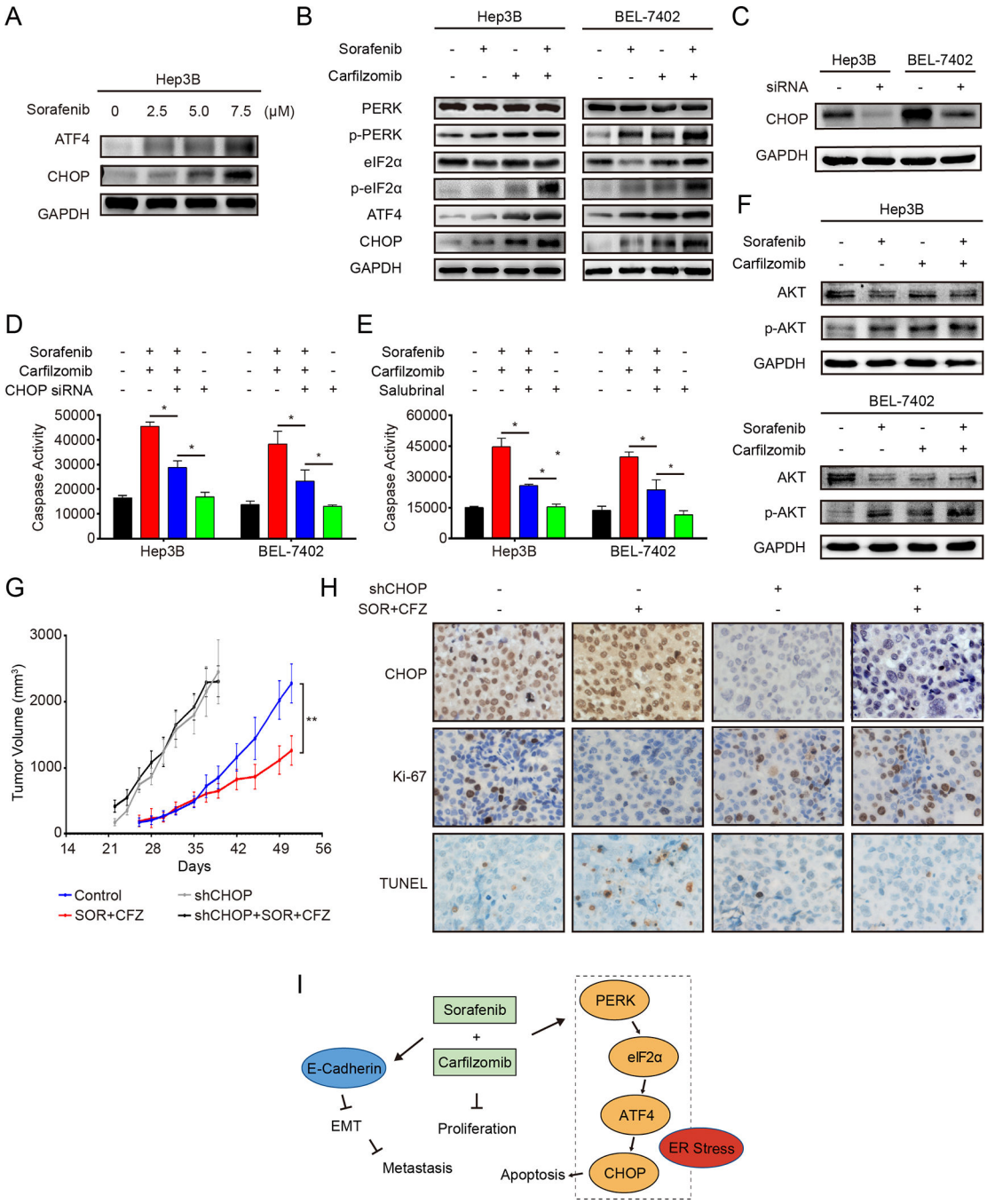

Figure 6. Carfilzomib and sorafenib treatments trigger ER stress-mediated apoptosis through the PERK/eIF2α/ATF4/CHOP pathway.

(A) Hep3B cells were treated with different concentrations of sorafenib (2.5, 5.0, 7.5 μM) for 24 h. The expression of ATF4 and CHOP was analyzed by immunoblot. GAPDH was used as a loading control.

(B) Hep3B and Bel-7402 cells were treated with carfilzomib (0.1 μM) and sorafenib (7.5 μM) individually or in combination for 24 h. Activation of the PERK/eIF2α/ATF4/CHOP pathway was analyzed by the expression of various marker proteins of the pathway by immunoblot.

(C) Shown is the knockdown efficiency of CHOP by siRNA as determined by immunoblot in Hep3B and Bel-7402 cells.
(D) Knockdown of CHOP decreases the activity of caspase-3/7 induced by carfilzomib and sorafenib. Hep3B and Bel-7402 cells were transfected with CHOP siRNA for 48 h before treated with carfilzomib (0.1 μM) and sorafenib (7.5 μM) individually or in combination for another 24 h. The activity of caspase-3/7 was determined by the Caspase-Glo assay. The experiments were performed in triplicate and data represent mean ± SD. *$p < 0.05$.
(E) Salubrinal suppressed caspase-3/7 activities induced by carfilzomib and sorafenib. Hep3B and Bel-7402 cells were pretreated with salubrinal (20 μM) for 12 h before treated with carfilzomib (0.1 μM) and sorafenib (7.5 μM) individually or in combination for another 24 h. The activity of caspase-3/7 was determined by the Caspase-Glo assay. The experiments were performed in triplicate and data represent mean ± SD. * $p < 0.05$.
(F) Hep3B and Bel-7402 cells were treated with carfilzomib (0.1 μM) and sorafenib (7.5 μM) individually or in combination for 24 h. Protein levels of p-AKT and AKT were analyzed by immunoblot.
(G) Xenograft tumors generated from Hep3B cells expressing shCHOP or control shRNA were treated with combined carfilzomib/ sorafenib or a drug vehicle. Growth of xenograft tumors was measured by tumor volume. Data represent mean ± SD (n = 8). Statistical analyses were performed by two-way ANOVA and sample-paired t-test. ** $p < 0.01$.
(H) Representative images of IHC staining for CHOP (upper panels), Ki67 (middle panels) and TUNEL (lower panels) in HCC xenograft tumor sections in different treatment groups (400 × magnification).
(I) A working model illustrating the inhibitory mechanism of carfilzomib and sorafenib against HCC.

Table 1.

The combination index calculated by CalcuSyn software

Sorafenib (μM)	Carfilzomib (nM)	Hep3B		Bel-7402	
		Fa	CI	Fa	CI
1.25	12.5	0.162	1.589	0.187	1.091
2.5	25	0.368	0.757	0.315	0.895
5.0	50	0.575	0.506	0.494	0.692
7.5	100	0.651	0.591	0.620	0.632