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ORIGINAL ARTICLE

Preclinical efficacy of a novel dual PI3K/mTOR inhibitor, CMG002, alone and in combination with sorafenib in hepatocellular carcinoma

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Abstract

Purpose Sorafenib has been the only first systemic drug that improves survival of patients with advanced hepatocellular carcinoma (HCC). However, because the response rate of sorafenib is relatively low, novel therapeutic strategies are needed to improve survival in patients with HCC. This study investigated the effect of CMG002 alone and in combination with sorafenib on HCC in vitro and vivo.

Methods The effect of a newly developed dual PI3K/mTOR inhibitor, CMG002, on the proliferation of Huh-7 and HepG2 HCC cells was investigated using the MTT assay. Western blotting was performed to assess phosphorylation of the key enzymes in the Ras/Raf/MAPK and PI3K/AKT/mTOR pathways. HepG2 cells were inoculated into mice, which were treated with vehicle, sorafenib, CMG002, and their combinations. Tumor cell proliferation and tumor angiogenesis were evaluated by immunohistochemical analysis of Ki-67 and CD31, respectively. Tumor cell apoptosis was detected by the terminal deoxynucleotidyl transferase dUTP nick end labeling assay. Levels of key enzymes in the Ras/Raf/MAPK and PI3K/AKT/mTOR pathways were evaluated by western blot analysis.

Results The combination of sorafenib and CMG002 additively inhibited Huh-7 and HepG2 cell proliferation compared to single-agent treatment. Sorafenib and CMG002 as single agents differentially inhibited or activated key enzymes in the Ras/Raf/MAPK and PI3K/AKT/mTOR pathways. The combination of sorafenib and CMG002 inhibited all key enzymes in the two pathways. Treatment with CMG002 for 4 weeks alone and in combination with sorafenib strongly inhibited tumor growth. CMG002 inhibited HCC cell proliferation, induced apoptosis, and decreased tumor angiogenesis. Furthermore, these effects were enhanced when CMG002 was combined with sorafenib.

Conclusions The combination of CMG002 and sorafenib significantly inhibited HCC cell proliferation and tumorigenesis by inhibiting the Ras/Raf/MAPK and PI3K/AKT/mTOR pathways. These findings suggest that CMG002 to be a potential novel candidate treatment for HCC.

Keywords Hepatocellular carcinoma · PI3K/AKT/mTOR pathway · Sorafenib · Dual PI3K/mTOR inhibitor

Abbreviations

HCC	Hepatocellular carcinoma
PI	Proliferation index
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and second leading cause of cancer-related mortality worldwide [1, 2]. The prognosis of HCC is poor and curative treatments such as surgical resection, liver transplantation, and loco-ablative therapy can only be applied to a limited subset of patients, as the diagnosis is often made

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at an advanced stage of the disease [3, 4]. Accordingly, new therapeutic strategies for advanced HCC are urgently needed. Sorafenib is the first FDA-approved systemic drug for treating patients with advanced HCC [5, 6]. However, the survival benefit of taking sorafenib for HCC is limited to about 3 months [7, 8]. Thus, new treatment options are needed to improve survival of patients with advanced HCC.

The PI3K/AKT/mTOR pathway is one of the principal pathways regulating cell survival, growth, proliferation, angiogenesis, metabolism, and mortality [9–11]. Deregulation of the PI3K/AKT/mTOR pathway is a common event in human cancer and associated with poor outcomes [12]. [10, 13] As the PI3K/AKT/mTOR pathway is highly activated in most human cancers, this pathway has been investigated as a favorable target for developing anti-cancer drugs [13–15]. Activation of the PI3K/AKT/mTOR pathway is known to occur in 30–50% of HCC tumors [16]. The causes include somatic mutation of PIK3CA, enhancement of Akt and phosphorylated ribosomal protein S6, and decreased PTEN expression [17–20]. Sorafenib blocks tumor cell proliferation through the Ras/Raf/MAPK pathway, but does not directly inhibit the PI3K/AKT/mTOR pathway, which also plays an important role in HCC proliferation [8, 11]. To overcome this problem, combined targeting of the PI3K/AKT/mTOR and Ras/Raf/MAPK pathways might provide a benefit in the treatment of HCC.

To date, several PI3K/AKT/mTOR pathway inhibitors have been developed and demonstrated clinical efficacy in cancer patients [21]. Among these, a dual PI3K/mTOR inhibitor has an advantage for the vertical blockade of the two different crucial nodes of the pathway [21]. Inhibiting all PI3K isoforms can result in subsequent activation of the mTOR pathway [22]. Thus, potent dual inhibition within this pathway is a promising strategy for anti-cancer therapy. Recently, a dual PI3K/mTOR inhibitor, CMG002, was newly developed. Previous study showed the effect of CMG002 on ovarian cancer cells both in vitro and in vivo [23]. This study demonstrated that CMG002 inhibits growth of chemoresistant ovarian cancer cells and re-sensitized these cells to chemotherapeutic agents, suggesting that this might be a promising therapeutic strategy for chemoresistant ovarian cancer [23].

The aim of this study was to evaluate the anti-tumor efficacy of sorafenib and a newly developed dual PI3K/mTOR inhibitor, CMG002, as single agents and in combination, on HCC in vitro and in vivo.

Materials and methods

Cell culture

The human HCC cell line, Huh-7 and HepG2, were purchased from the Korean Cell Line Bank (Seoul, Korea). All cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and 1% penicillin–streptomycin at 37 °C in 5% CO_2 in air.

Drugs

CMG002 is a new product of a dual PI3K/mTOR inhibitor (Fig. 1a), which was developed by CMG Pharmaceutical Co., Ltd. (Seongnam, Republic of Korea). The detailed synthesis process of CMG002 is shown in Supplementary Fig. 1. CMG002 was kindly provided by CMG Pharmaceutical Co., Ltd. Sorafenib was purchased from LC Laboratories (Woburn, MA, USA).

MTT assay

The cell lines were plated in 96-well plates at 5000 cells/well ($n = 3$) in 100 µl of DMEM + 10% FBS and cultured for 24 h. Sorafenib or CMG002 was added to the cells and cultured for another 48 h. Carrier DMSO was used as a vehicle

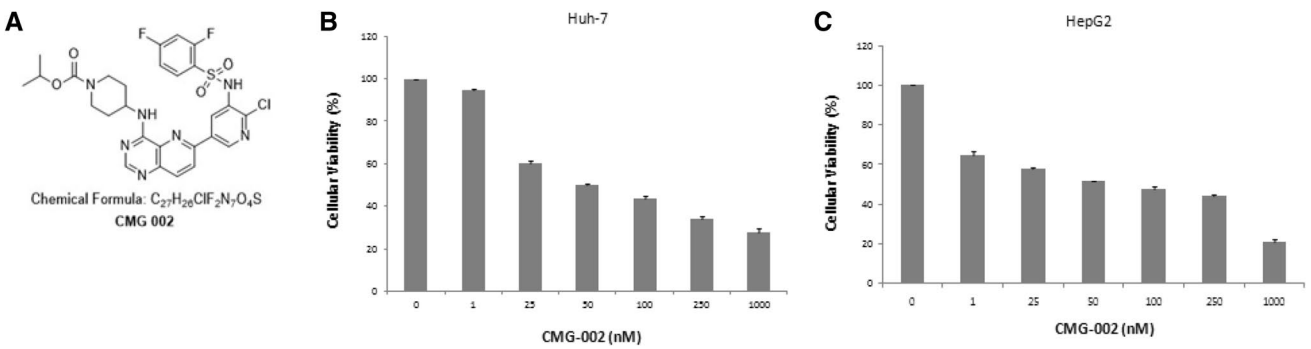

Fig. 1 The chemical structure of CMG002 and effect of CMG002 on HCC proliferation in vitro. The chemical structure of CMG002 was shown in **a**. After the 48 h treatment, CMG002 decreased viability of the two cell lines in a dose-dependent manner (**b** and **c**)

control (< 0.1% final concentration). Ten µl of MTT (5 mg/ml) were added to the wells and incubated for 4 h. After aspiration, 100 µl of 20% SDS/50% *N*, *N*-dimethylformamide (DMF) was added to each well and incubated for 2 h at 37 °C to solubilize the bio-reduced colored MTT-formazan and to lyse the cells. The optical density value was read at 570 nm in a microplate reader, according to the manufacturer's instructions (Insight Genomics, Falls Church, VA, USA) with a minor modification of the cell-lysis time from overnight to 4 h at 37 °C.

Western blot analysis

Cells ($2–4 \times 10^5$) were seeded in 6-well plates. The next day, the medium was discarded and replaced with 2 ml fresh medium, and the cells were treated with the compounds at the indicated concentrations for 0–48 h. Floating and adhered cells were washed twice with ice-cold PBS and then lysed in the appropriate volume of lysis buffer (25 mM Tris–HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS; Thermo Scientific, Waltham, MA, USA). Protein concentrations were determined using a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). The cell lysates were separated by 7.5–12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to PVDF membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat dry milk in TBS-T [10 mM Tris–HCl (pH 7.4), 150 mM NaCl, and 0.05% Tween-20] for 1 h and incubated with the following primary antibodies: anti-Akt, anti-p-Akt, anti-S6, anti-p-S6, anti-ERK, anti-p-ERK, cleaved poly (ADP-ribose) polymerase (PARP), cleaved caspase 8, and GAPDH followed by the secondary antibody reaction (all from Cell Signaling Technology, Danvers, MA, USA). The bands were visualized with enhanced chemiluminescent western blotting detection reagents.

Tumor xenografts

Six-to eight-week-old male BALB/c nude mice were purchased from OrientBio (Seongnam, Republic of Korea). The mice were maintained in the animal facility of the Division of Laboratory Animal Resources at CHA University (Seongnam, Republic of Korea). All animal experiments were approved by the Institutional Animal Care and Use Committee of CHA University and were carried out in accordance with the approved protocols. HepG2 cells (1×10^6) were injected subcutaneously into the right flank of each mouse. The body weight and tumor volume were measured every 3 days from the day of cell injection. Tumor volume was estimated using the standard formula: $(\text{length} \times \text{width}^2)/2$. After tumor volume reached 50 mm^3, the mice were divided randomly into four groups ($n = 4–6$ per group) and drug treatment started. The mean time for tumor volume to reach 50 mm^3 was 5.6 [standard deviation (SD) 0.3] days after cells injection. The treatments in the four groups were: control (drug vehicle); sorafenib (30 mg/kg/day); CMG002 (20 mg/kg/day); and combination of sorafenib (30 mg/kg/day) and CMG002 (20 mg/kg/day). All drugs were administered by oral gavage. The tumors were isolated from the mice immediately after euthanasia. The tumor was cut into two halves, one half was fixed in 10% neutral buffered formalin solution and the other half was used to isolate the tumor lysate. The lysates were used for the western blot analysis.

Immunohistochemical staining

Paraffin-embedded tissue sections were deparaffinized in xylene and rehydrated through a gradual decrease in ethanol concentration. Antigens were unmasked using sodium citrate buffer. The sections were incubated overnight at 4 °C with primary antibodies against Ki67 (Abcam, Cambridge, MA, USA), CD31 (Abbiotec; San Diego, CA, USA). The sections were incubated with biotinylated secondary antibody. The color reaction was visualized with diaminobenzidine, and the tissues were counterstained with hematoxylin. The proliferation index (PI) was determined by counting the number of Ki67- positive cells among at least 1000 cells in five randomly selected fields (magnification 200×) and expressed as a percentage. The number of CD31-positive cells was determined by counting five randomly selected fields (magnification 200×) from each section.

Apoptosis detection

Apoptosis was detected using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. Tissues were embedded in paraffin blocks, and 4-µm sections were cut and stained with the ApopTag Peroxidase In Situ Apoptosis Detection Kit (Cell Signaling Technology). The stained tumor sections from each group were reviewed, and the apoptosis index, determined by TUNEL staining, was calculated by counting at least 1000 cells in five randomly selected fields (magnification, 200×) and expressed as percentage values.

Statistical analyses

Data are expressed as mean ± SD. Differences between groups were analyzed using Student's *t* test. The Kaplan–Meier method was used to determine the probability of survival as a function of time, and the differences between groups were compared using the two-sided log-rank test. *P* values less than 0.05 were regarded to indicate statistical significance. Statistical analyses were performed using SPSS software (ver. 18.0; SPSS Inc., Chicago, IL, USA).

Results

Effect of CMG002 on HCC proliferation in vitro

To determine whether CMG002 could affect the viability of HCC, Huh-7, and HepG2 cells were treated with CMG002 at various concentrations (0–1000 nM) for 48 h. Cell viability rates were analyzed by the MTT assays. After the 48 h treatment, CMG002 decreased viability of the two cell lines in a dose-dependent manner (Fig. 1b, c). The IC_{50} value of CMG002 was 53.8 ± 1.9 nM for Huh-7, and 86.1 ± 7.0 nM for HepG2 cells, respectively.

Effect of CMG002 alone and in combination with sorafenib on the PI3K/AKT/mTOR and Ras/Raf/MAPK pathways in the HCC cell lines

Western blot experiments demonstrated that CMG002 (54 nM for Huh-7, and 85 nM for HepG2) inhibited phosphorylation of the key enzymes, Akt and S6, in the PI3/AKT/mTOR pathway in both cell lines (Fig. 2). In contrast, sorafenib (5 μM for Huh-7 and 4 μM for HepG2) did not inhibit phosphorylation of Akt and S6 in either cell lines. Sorafenib inhibited phosphorylation of ERK in both cell lines, whereas CMG002 showed no effect on phosphorylation of ERK in either cell lines (Fig. 2). Combination therapy (54 nM CMG002 plus 5 μM sorafenib for Huh-7, and 85 nM CMG002 plus 4 μM for HepG2) resulted in decreased phosphorylation of Akt, S6, and ERK in both cell lines (Fig. 2).

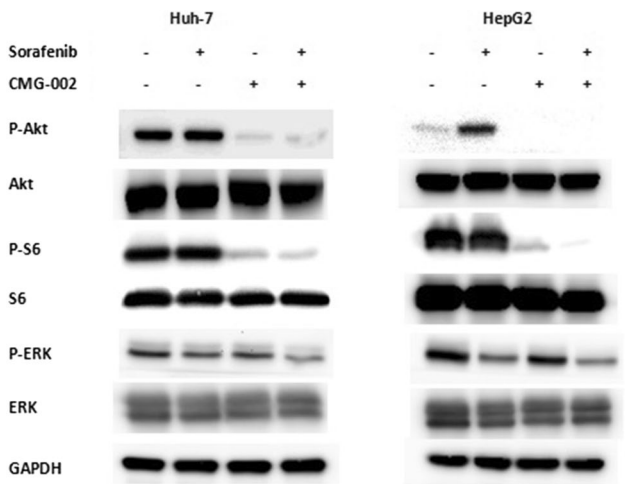

Fig. 2 Effect of CMG002 alone and in combination with sorafenib on the PI3K/AKT/mTOR and Ras/Raf/MAPK pathways in the HCC cell lines. CMG002 inhibited phosphorylation of the key enzymes, Akt and S6, in the PI3/Akt/mTOR pathway in both cell lines. Sorafenib inhibited phosphorylation of ERK in both cell lines. Combination therapy resulted in decreased phosphorylation of Akt, S6, and ERK in both cell lines

Inhibition of tumor growth by CMG002 and sorafenib in a mouse model of HCC

CMG002 and sorafenib alone and in combination inhibited tumor growth during the 4 weeks of drug administration. Average tumor volume in the CMG002 and combination groups decreased significantly compared with that in the control group ($P = 0.001$). Tumor volume tended to decrease in the sorafenib group compared with the control group, but the difference was not statistically significant ($P = 0.095$). The combined treatment of CMG002 and sorafenib significantly reduced tumor volume compared to the sorafenib alone group ($P = 0.029$), but not to the CMG002 group ($P = 0.211$) (Fig. 3a).

Effect of CMG002 alone and in combination with sorafenib on the PI3K/AKT/mTOR and Ras/Raf/MAPK pathways in vivo

Changes in the phosphorylation levels of key proteins in PI3K/AKT/mTOR and Ras/Raf/MAPK pathways of the excised tumors were determined by western blot analysis. CMG002 alone and combined with sorafenib significantly decreased levels of phosphorylated Akt, compared to the control group. Levels of phosphorylated S6, a downstream target of Akt, also decreased in the CMG002 and combination groups. Even though sorafenib suppressed Akt phosphorylation, the level of phosphorylated S6 did not decrease by sorafenib alone. The level of phosphorylated ERK decreased in the sorafenib group compared to the control group. However, the level of phosphorylated ERK did not decrease in the CMG002 group (Fig. 3b). These results suggest that the combined CMG002 and sorafenib treatment suppressed HCC tumor growth by downregulating the PI3K/AKT/mTOR and Ras/Raf/MAPK pathways in vivo.

Effect of CMG002 alone and in combination with sorafenib on tumor cell proliferation

To determine whether the observed suppression of tumor growth was caused by inhibited cell proliferation, the effect of CMG002 alone and in combination with sorafenib on tumor cell proliferation was investigated by Ki67 staining. Mice in all drug-treated groups showed a decrease in the number of Ki67-positive cells compared to the control group (Fig. 4a). As shown in Fig. 5b, the mean PI based on Ki67 staining was 14.2 ± 1.8 for the control group, which decreased significantly in the sorafenib group (4.4 ± 0.2; $P < 0.001$, compared to the control group) or CMG002 (3.5 ± 0.2; $P < 0.001$, compared to the control group). In addition, the effect of CMG002 on tumor cell proliferation was significantly greater than that of sorafenib ($P < 0.001$). The effect of the combination treatment on tumor cell

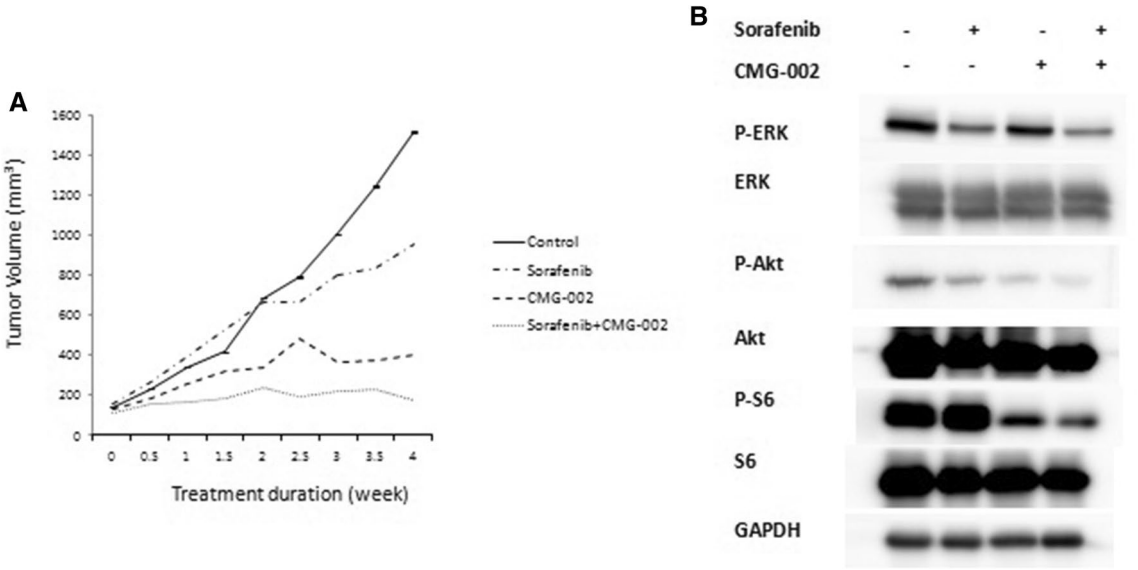

Fig. 3 **a** Effect of CMG002 alone and in combination with sorafenib in vivo. Average tumor volume in the CMG002 and combination groups decreased significantly compared with that in the control group ($P=0.001$). **b** Effect of CMG002 alone and in combination with sorafenib on the PI3K/AKT/mTOR and Ras/Raf/MAPK pathways. CMG002 alone and combined with sorafenib significantly decreased levels of phosphorylated Akt, compared to the control group. The level of phosphorylated ERK decreased in the sorafenib group compared to the control group

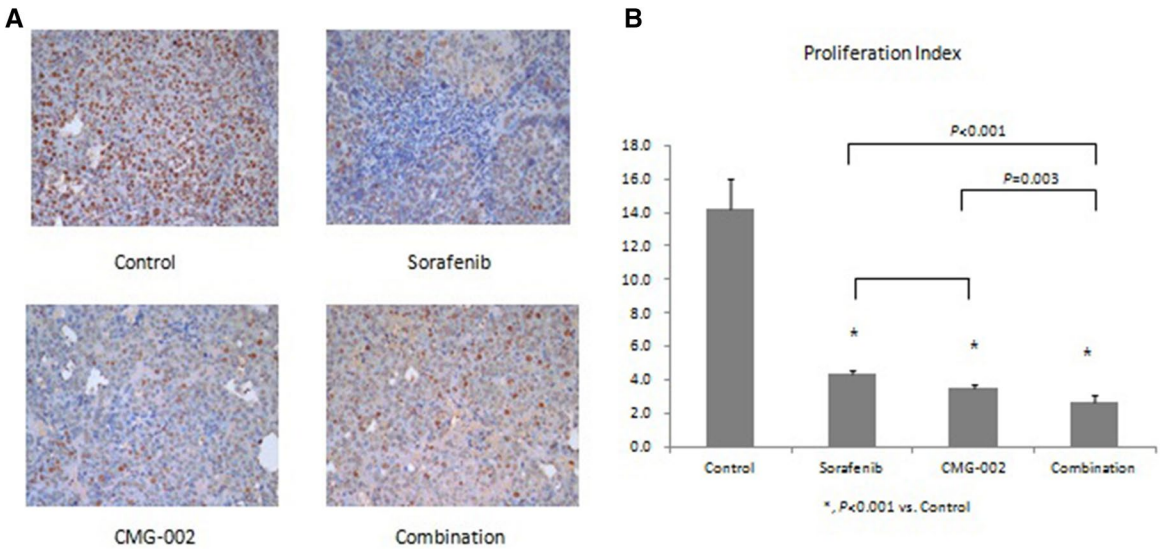

Fig. 4 Effect of CMG002 alone and in combination with sorafenib on tumor cell proliferation. **a** Mice in all drug-treated groups showed a decrease in the number of Ki67-positive cells compared to the control group. **b** The effect of the combination treatment on tumor cell proliferation (PI, 2.7 ± 0.4) was significantly greater than that of CMG002 alone or sorafenib alone (all $P<0.005$)

proliferation (PI, 2.7 ± 0.4) was significantly greater than that of CMG002 alone or sorafenib alone (all $P<0.005$) (Fig. 4b).

Effect of CMG002 alone and in combination with sorafenib on tumor cell apoptosis

To investigate further the mechanism for the observed suppression of tumor growth, the effect of CMG002 alone and in combination with sorafenib on tumor cell apoptosis was

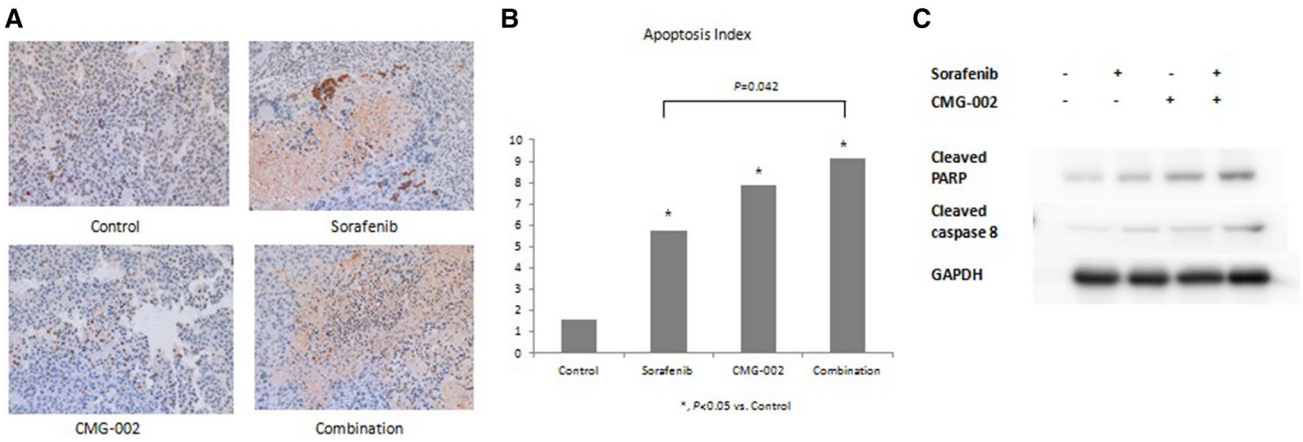

Fig. 5 Effect of CMG002 alone and in combination with sorafenib on tumor cell apoptosis. **a** All drug-treated groups had an increase in the number of TUNEL-stained cells compared to the control group. **b** The effect of the combination treatment on apoptosis (apoptosis index, 9.1 ± 1.6) was significantly greater than that of sorafenib alone (all $P = 0.042$). **c** The combination treatment significantly increased cleavage of cleaved caspase 8 and cleaved PARP

examined by the TUNEL assay. All drug-treated groups had an increase in the number of TUNEL-stained cells compared to the control group (Fig. 5a). The mean apoptosis index on the percentage of TUNEL-stained cells was 1.6 ± 0.8 for the control group, and increased significantly in the sorafenib group (5.7 ± 2.7; $P < 0.05$, compared to the control group) and the CMG002 group (7.9 ± 3.0; $P < 0.05$, compared to the control group). The apoptosis index was not different between the CMG002 and sorafenib groups ($P = 0.269$). The effect of the combination treatment on apoptosis (apoptosis index, 9.1 ± 1.6) was significantly greater than that of sorafenib alone (all $P = 0.042$) (Fig. 5b).

Tumor cell apoptosis was also evaluated by western blot analysis of cleaved PARP, and cleaved caspase 8, which are critical components of caspase-mediated apoptosis [24]. Although the levels of cleaved caspase-8 and cleaved PARP increased by sorafenib or CMG002 alone, the single-drug treatments had little effect on the cleavage of other apoptotic markers. However, the combination treatment significantly increased cleavage of cleaved caspase 8 and cleaved PARP (Fig. 5c).

Effect of CMG002 alone and in combination with sorafenib on tumor angiogenesis

Tumor sections were stained with antiCD31 antibody to determine the effect of CMG002 alone and in combination with sorafenib on tumor angiogenesis. Mice in all drug-treated groups had fewer CD31-positive cells than those in the control group (Fig. 6a). The mean number of CD31-positive cells in the control group was 44.2 ± 6.5, which decreased significantly by treatment with sorafenib (14.4 ± 2.9) or CMG002 (7.6 ± 1.1) (all $P < 0.001$). Both the CMG002 and combination groups (7.2 ± 0.8) showed significantly fewer CD31-positive cells than the group treated with sorafenib alone (all $P < 0.005$). The CMG002 and combination groups did not show significant difference in CD31-positive cells ($P = 0.545$) (Fig. 6b).

Discussion

This study focused on the effect of CMG002 alone or in combination with sorafenib in preclinical HCC models. First, we determined that CMG002 inhibited HCC cell proliferation, and the PI3K/AKT/mTOR pathway in vitro. Then, anti-tumor activity, phosphorylation of key enzymes in the PI3K/AKT/mTOR and Ras/Raf/MAPK pathways, HCC proliferation, apoptosis, and angiogenesis were investigated in vivo. This is the first study showing the efficacy of CMG002 as a novel dual PI3K/mTOR inhibitor in HCC. Second, the synergistic efficacy of CMG002 in combination with sorafenib was investigated in vitro and in vivo.

Our cell viability assay indicated that CMG002 inhibited cell proliferation in both Huh-7 and HepG2 cell lines. CMG002 inhibited phosphorylation of Akt and S6 in both cell lines. Sorafenib inhibited phosphorylation of ERK in both cell lines. However, CMG002 did not affect phosphorylation of ERK, and sorafenib did not inhibit phosphorylation of Akt or S6. These in vitro results strongly suggest that the drug combination of CMG002 and sorafenib had synergistic effects on HCC tumor growth. Our xenograft HCC model indicated that CMG002 and sorafenib alone significantly inhibited tumor growth. We also demonstrated the synergistic anti-tumor efficacy of CMG002 and sorafenib.

Three hypotheses were considered to explain these effects. First, CMG002 may directly inhibit tumor cell proliferation by inhibiting phosphorylation of the PI3K/

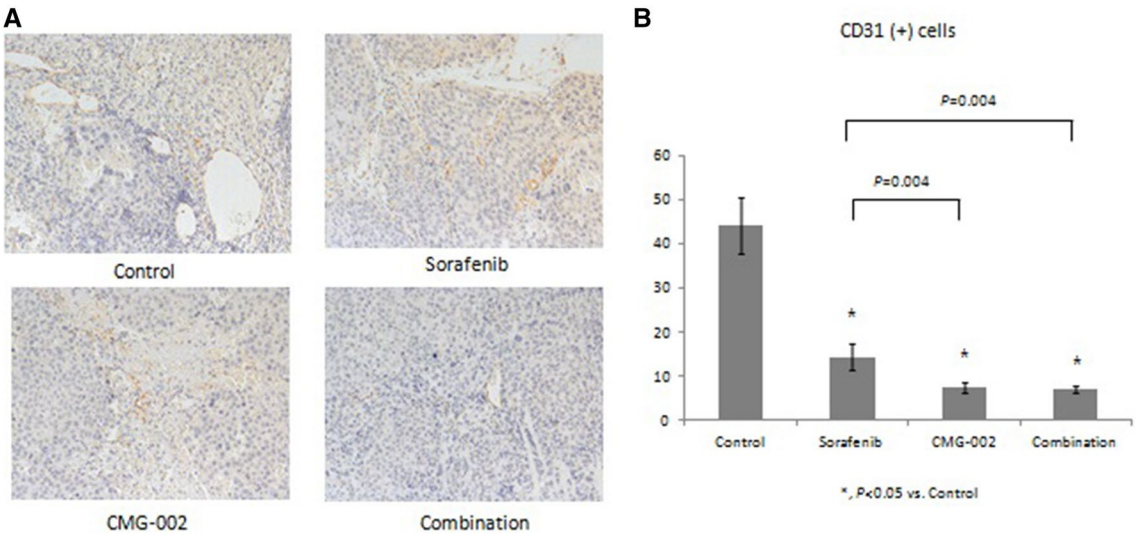

Fig. 6 Effect of CMG002 alone and in combination with sorafenib on tumor angiogenesis. **a** Mice in all drug-treated groups had fewer CD31-positive cells than those in the control group. **b** Both the CMG002 and combination groups (7.2 ± 0.8) showed significantly fewer CD31-positive cells than the group treated with sorafenib alone (all $P < 0.005$)

AKT/mTOR pathway and its downstream effector S6. This hypothesis is supported by the results of both in vitro and in vivo studies. In addition, Ki67 immunohistochemistry showed increased inhibition of cell proliferation in the combination treatment (Fig. 4). This synergistic effect indicates the important roles of the PI3K/AKT/mTOR and Ras/Raf/MAPK signaling pathways in HCC cell proliferation.

Previous studies have shown that the Ras/Raf/MAPK and PI3K/AKT/mTOR pathways are the dominant signaling pathways activated in HCC cells [25], suggesting that concomitant inhibition of these two pathways may be a promising treatment strategy for advanced HCC. In addition, several studies have demonstrated that the activation of PI3K/AKT/mTOR pathway is associated with sorafenib use [26–28]. This can be another rationale to combine sorafenib with dual PI3K/mTOR inhibitor.

Second, apoptosis induced by the drugs alone and in combination may have induced the observed anti-tumor effect of CMG002 alone and in combination with sorafenib, as indicated by the TUNEL assay results (Fig. 5). In addition, the role of apoptosis was further confirmed by western blot, which revealed a significant increase in the cleavage of caspase-8 and PARP in the combination treatment.

Finally, angiogenesis is another essential mechanism for HCC growth, and CMG002 may inhibit angiogenesis. Sorafenib also inhibits the vascular endothelial growth factor receptor, so it has been classified as an antiangiogenic drug. We investigated CD31 immunohistochemistry, and showed fewer CD31-positive cells in HCC tumors of CMG002-treated mice compared to those in the untreated controls. This effect was enhanced by the combination treatment (Fig. 6).

The mechanisms of action of sorafenib on the PI3K/AKT/mTOR pathway are not fully elucidated. Gedaly et al. showed that sorafenib stimulated Akt and mTOR phosphorylation in Huh-7 cells [29]. However, Zhang et al. found that sorafenib inhibited hepatic tumor growth by downregulating PI3K/AKT signaling pathway in Human SMMC-7721 HCC cells [30]. This result was consistent with another study using LM3 human HCC cells [31]. Furthermore, Cervello et al. found no variation of p-Akt in both Huh-7 and HepG2 cell lines treated with sorafenib [32]. In our results, suppression of p-Akt and p-S6 was not shown in Huh-7 and HepG2 cell lines. The action of sorafenib on the PI3K/Akt/mTOR pathway should be further studied with specifically designed in vitro study focusing on this aim.

Also, our study showed the different results in regulating the phosphorylation of Akt between in vitro and in vivo with HepG2 cells. Although the phosphorylation of Akt was not suppressed in sorafenib-treated HepG2 cell line, the level of p-Akt slightly decreased in sorafenib-treated HepG2 xenograft mouse model (Fig. 3b). One possibility of this difference is that co-activator or co-suppressor of Akt exists in the different manner between in vitro and in vivo [33, 34]. Studying the mechanistic difference between in vitro and in vivo can show further information for choosing more proper HCC cell lines to show drug effect.

For in vivo study, Huh-7 and HepG2 cell lines were chosen in this study. Several cell lines derived from human

hepatoma cells, such as Huh-7, HepG2, and Hep3B, retain some morphological features and functions of hepatocytes. Also they are well defined and commercially available, and have been widely used to study a variety of treatment options for HCC when implanted in recipient mice [35, 36]. However, with regard to the high metastatic feature of HCC in human, undifferentiated cell lines, such as HA22T/VGH or JHH6, can show more similar results for high aggressive forms of HCC. Previous studies showed that the proteasome inhibitor bortezomib affected differently on major cell cycle regulatory genes in HepG2 and JHH6 cell lines [37, 38]. Future studies with undifferentiated cell lines will be used to guide the selection of HCC cell lines to fit the study aim and open important insights for strategy against advanced HCC.

In conclusion, CMG002 showed an inhibitory effect in HCC tumor growth. This is the first in vitro and in vivo study demonstrating that CMG002 inhibits HCC tumor growth. Furthermore, the combination with sorafenib enhanced the anti-tumor effect of CMG002. The preclinical effects shown here represent a strong rationale for development and clinical trials using CMG002 alone and in combination with sorafenib to treat patients with HCC.

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Compliance with ethical standards

Conflicts of interest Nothing to declare for all authors.

References

1. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F (2015) Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 136(5):E359–E386. https://doi.org/10.1002/ijc.29210
2. Sartorius K, Sartorius B, Aldous C, Govender PS, Madiba TE (2015) Global and country underestimation of hepatocellular carcinoma (HCC) in 2012 and its implications. Cancer Epidemiol 39(3):284–290. https://doi.org/10.1016/j.canep.2015.04.006
3. European Association For The Study Of The Liver, European Organisation For Research And Treatment Of Cancer (2012) EASL-EORTC clinical practice guidelines: management of hepatocellular carcinoma. J Hepatol 56(4):908–943. https://doi.org/10.1016/j.jhep.2011.12.001
4. Yang JD (2019) Detect or not to detect very early stage hepatocellular carcinoma? The western perspective. Clin Mol Hepatol. https://doi.org/10.3350/cmh.2019.0010
5. Omata M, Cheng AL, Kokudo N, Kudo M, Lee JM, Jia J, Tateishi R, Han KH, Chawla YK, Shiina S, Jafri W, Payawal DA, Ohki T, Ogasawara S, Chen PJ, Lesmana CRA, Lesmana LA, Gani RA, Obi S, Dokmeci AK, Sarin SK (2017) Asia-Pacific clinical practice guidelines on the management of hepatocellular carcinoma: a 2017 update. Hep Intl 11(4):317–370. https://doi.org/10.1007/s12072-017-9799-9
6. Kim TS, Kim JH (2017) Complete response of advanced hepatocellular carcinoma to sorafenib: another case and a comprehensive review. Clin Mol Hepatol 23(4):340–346. https://doi.org/10.3350/cmh.2016.0070
7. Dika IE, Abou-Alfa GK (2017) Treatment options after sorafenib failure in patients with hepatocellular carcinoma. Clin Mol Hepatol 23(4):273–279. https://doi.org/10.3350/cmh.2017.0108
8. Llovet JM, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, de Oliveira AC, Santoro A, Raoul JL, Forner A, Schwartz M, Porta C, Zeuzem S, Bolondi L, Greten TF, Galle PR, Seitz JF, Borbath I, Haussinger D, Giannaris T, Shan M, Moscovici M, Voliotis D, Bruix J (2008) Sorafenib in advanced hepatocellular carcinoma. N Engl J Med 359(4):378–390. https://doi.org/10.1056/NEJMoa0708857
9. Courtney KD, Corcoran RB, Engelman JA (2010) The PI3K pathway as drug target in human cancer. J Clin Oncol 28(6):1075–1083. https://doi.org/10.1200/jco.2009.25.3641
10. Wymann MP, Zvelebil M, Laffargue M (2003) Phosphoinositide 3-kinase signalling–which way to target? Trends Pharmacol Sci 24(7):366–376. https://doi.org/10.1016/s0165-6147(03)00163-9
11. Kim MN, Ro SW, Kim DY, da Kim Y, Cho KJ, Park JH, Lim HY, Han KH (2015) Efficacy of perifosine alone and in combination with sorafenib in an HrasG12 V plus shp53 transgenic mouse model of hepatocellular carcinoma. Cancer Chemother Pharmacol 76(2):257–267. https://doi.org/10.1007/s00280-015-2787-7
12. Sharp ZD, Bartke A (2005) Evidence for down-regulation of phosphoinositide 3-kinase/Akt/mammalian target of rapamycin (PI3K/Akt/mTOR)-dependent translation regulatory signaling pathways in Ames dwarf mice. J Gerontol Ser A Biol Sci Med Sci 60(3):293–300
13. Morgensztern D, McLeod HL (2005) PI3K/Akt/mTOR pathway as a target for cancer therapy. Anticancer Drugs 16(8):797–803
14. Brown RE (2005) Morphoproteomics: exposing protein circuitries in tumors to identify potential therapeutic targets in cancer patients. Expert Rev Proteom 2(3):337–348. https://doi.org/10.1586/14789450.2.3.337
15. Yip PY (2015) Phosphatidylinositol 3-kinase-AKT-mammalian target of rapamycin (PI3K-Akt-mTOR) signaling pathway in non-small cell lung cancer. Transl Lung Cancer Res 4(2):165–176. https://doi.org/10.3978/j.issn.2218-6751.2015.01.04
16. Faivre S, Bouattour M, Raymond E (2011) Novel molecular therapies in hepatocellular carcinoma. Liver Int 31(Suppl 1):151–160. https://doi.org/10.1111/j.1478-3231.2010.02395.x
17. Lee JW, Soung YH, Kim SY, Lee HW, Park WS, Nam SW, Kim SH, Lee JY, Yoo NJ, Lee SH (2005) PIK3CA gene is frequently mutated in breast carcinomas and hepatocellular carcinomas. Oncogene 24(8):1477–1480. https://doi.org/10.1038/sj.onc.1208304
18. Hu TH, Huang CC, Lin PR, Chang HW, Ger LP, Lin YW, Changchien CS, Lee CM, Tai MH (2003) Expression and prognostic role of tumor suppressor gene PTEN/MMAC1/TEP1 in hepatocellular carcinoma. Cancer 97(8):1929–1940. https://doi.org/10.1002/cncr.11266
19. Zhou L, Huang Y, Li J, Wang Z (2010) The mTOR pathway is associated with the poor prognosis of human hepatocellular carcinoma. Med Oncol (Northwood, London, England) 27(2):255–261. https://doi.org/10.1007/s12032-009-9201-4

20. Minguez B, Tovar V, Chiang D, Villanueva A, Llovet JM (2009) Pathogenesis of hepatocellular carcinoma and molecular therapies. Curr Opin Gastroenterol 25(3):186–194. https://doi.org/10.1097/MOG.0b013e32832962a1
21. Yap TA, Bjerke L, Clarke PA, Workman P (2015) Drugging PI3K in cancer: refining targets and therapeutic strategies. Curr Opin Pharmacol 23:98–107. https://doi.org/10.1016/j.coph.2015.05.016
22. Elkabets M, Vora S, Juric D, Morse N, Mino-Kenudson M, Muranen T, Tao J, Campos AB, Rodon J, Ibrahim YH, Serra V, Rodrik-Outmezguine V, Hazra S, Singh S, Kim P, Quadt C, Liu M, Huang A, Rosen N, Engelman JA, Scaltriti M, Baselga J (2013) mTORC1 inhibition is required for sensitivity to PI3K p110alpha inhibitors in PIK3CA-mutant breast cancer. Sci Transl Med 5(196):196ra199. https://doi.org/10.1126/scitranslmed.3005747
23. Choi HJ, Heo JH, Park JY, Jeong JY, Cho HJ, Park KS, Kim SH, Moon YW, Kim JS, An HJ (2019) A novel PI3K/mTOR dual inhibitor, CMG002, overcomes the chemoresistance in ovarian cancer. Gynecol Oncol 153(1):135–148. https://doi.org/10.1016/j.ygyno.2019.01.012
24. Fei HR, Chen G, Wang JM, Wang FZ (2010) Perifosine induces cell cycle arrest and apoptosis in human hepatocellular carcinoma cell lines by blockade of Akt phosphorylation. Cytotechnology 62(5):449–460. https://doi.org/10.1007/s10616-010-9299-4
25. Llovet JM, Bruix J (2008) Molecular targeted therapies in hepatocellular carcinoma. Hepatology (Baltimore, MD) 48(4):1312–1327. https://doi.org/10.1002/hep.22506
26. Gedaly R, Angulo P, Hundley J, Daily MF, Chen C, Koch A, Evers BM (2010) PI-103 and sorafenib inhibit hepatocellular carcinoma cell proliferation by blocking Ras/Raf/MAPK and PI3K/AKT/mTOR pathways. Anticancer Res 30(12):4951–4958
27. Huynh H, Ngo VC, Koong HN, Poon D, Choo SP, Thng CH, Chow P, Ong HS, Chung A, Soo KC (2009) Sorafenib and rapamycin induce growth suppression in mouse models of hepatocellular carcinoma. J Cell Mol Med 13(8b):2673–2683. https://doi.org/10.1111/j.1582-4934.2009.00692.x
28. Piguet AC, Saar B, Hlushchuk R, St-Pierre MV, McSheehy PM, Radojevic V, Afthinos M, Terracciano L, Djonov V, Dufour JF (2011) Everolimus augments the effects of sorafenib in a syngeneic orthotopic model of hepatocellular carcinoma. Mol Cancer Ther 10(6):1007–1017. https://doi.org/10.1158/1535-7163.mct-10-0666
29. Gedaly R, Angulo P, Chen C, Creasy KT, Spear BT, Hundley J, Daily MF, Shah M, Evers BM (2012) The role of PI3K/mTOR inhibition in combination with sorafenib in hepatocellular carcinoma treatment. Anticancer Res 32(7):2531–2536
30. Zhang CZ, Wang XD, Wang HW, Cai Y, Chao LQ (2015) Sorafenib inhibits liver cancer growth by decreasing mTOR, AKT, and PI3K expression. J BUON 20(1):218–222
31. Dai N, Ye R, He Q, Guo P, Chen H, Zhang Q (2018) Capsaicin and sorafenib combination treatment exerts synergistic antihepatocellular carcinoma activity by suppressing EGFR and PI3K/Akt/mTOR signaling. Oncol Rep 40(6):3235–3248. https://doi.org/10.3892/or.2018.6754
32. Cervello M, Bachvarov D, Lampiasi N, Cusimano A, Azzolina A, McCubrey JA, Montalto G (2012) Molecular mechanisms of sorafenib action in liver cancer cells. Cell cycle (Georgetown, Tex) 11(15):2843–2855. https://doi.org/10.4161/cc.21193
33. Ho C, Wang C, Mattu S, Destefanis G, Ladu S, Delogu S, Armbruster J, Fan L, Lee SA, Jiang L, Dombrowski F, Evert M, Chen X, Calvisi DF (2012) AKT (v-akt murine thymoma viral oncogene homolog 1) and N-Ras (neuroblastoma ras viral oncogene homolog) coactivation in the mouse liver promotes rapid carcinogenesis by way of mTOR (mammalian target of rapamycin complex 1), FOXM1 (forkhead box M1)/SKP2, and c-Myc pathways. Hepatology (Baltimore, MD) 55(3):833–845. https://doi.org/10.1002/hep.24736
34. Yan G, Ru Y, Yan F, Xiong X, Hu W, Pan T, Sun J, Zhang C, Wang Q, Li X (2019) MIIP inhibits the growth of prostate cancer via interaction with PP1alpha and negative modulation of AKT signaling. Cell Commun Signal 17(1):44. https://doi.org/10.1186/s12964-019-0355-1
35. Shi J, Wang X, Lyu L, Jiang H, Zhu HJ (2018) Comparison of protein expression between human livers and the hepatic cell lines HepG2, Hep3B, and Huh7 using SWATH and MRM-HR proteomics: focusing on drug-metabolizing enzymes. Drug Metab Pharmacokinet 33(2):133–140. https://doi.org/10.1016/j.dmpk.2018.03.003
36. Friedman D, Baird JR, Young KH, Cottam B, Crittenden MR, Friedman S, Gough MJ, Newell P (2017) Programmed cell death-1 blockade enhances response to stereotactic radiation in an orthotopic murine model of hepatocellular carcinoma. Hepatology 47(7):702–714. https://doi.org/10.1111/hepr.12789
37. Baiz D, Pozzato G, Dapas B, Farra R, Scaggiante B, Grassi M, Uxa L, Giansante C, Zennaro C, Guarnieri G, Grassi G (2009) Bortezomib arrests the proliferation of hepatocellular carcinoma cells HepG2 and JHH6 by differentially affecting E2F1, p21 and p27 levels. Biochimie 91(3):373–382. https://doi.org/10.1016/j.biochi.2008.10.015
38. Baiz D, Dapas B, Farra R, Scaggiante B, Pozzato G, Zanconati F, Fiotti N, Consoloni L, Chiaretti S, Grassi G (2014) Bortezomib effect on E2F and cyclin family members in human hepatocellular carcinoma cell lines. World J Gastroenterol 20(3):795–803. https://doi.org/10.3748/wjg.v20.i3.795