

# Synergistic antitumor activity of sorafenib and MG149 in hepatocellular carcinoma cells

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**Advanced hepatocellular carcinoma (HCC) is among the most challenging cancers to overcome, and there is a need for better therapeutic strategies. Among the different cancer drugs that have been used in clinics, sorafenib is considered the standard first-line drug for advanced HCC. Here, to identify a chemical compound displaying a synergistic effect with sorafenib in HCC, we screened a focused chemical library and found that MG149, a histone acetyltransferase inhibitor targeting the MYST family, exhibited the most synergistic anticancer effect with sorafenib on HCC cells. The combination of sorafenib and MG149 exerted a synergistic anti-proliferation effect on HCC cells by inducing apoptotic cell death. We revealed that cotreatment with sorafenib and MG149 aggravated endoplasmic reticulum (ER) stress to promote the death of HCC cells rather than adaptive cell survival. In addition, combined treatment with sorafenib and MG149 significantly increased the intracellular levels of unfolded proteins and reactive oxygen species, which upregulated ER stress. Collectively, these results suggest that MG149 has the potential to improve the efficacy of sorafenib in advanced HCC via the upregulation of cytotoxic ER stress. [BMB Reports 2022; 55(10): 506-511]**

## INTRODUCTION

Sorafenib is a clinically approved anticancer drug that has been

considered the gold standard therapy in advanced hepatocellular carcinoma (HCC) (1). However, the efficacy of sorafenib in HCC is moderate, increasing the overall survival of sorafenib-treated patients by only ~3 months compared with that of the placebo groups (1, 2). For decades, extensive efforts have been made to treat advanced HCC more efficiently by generating novel anti-HCC drugs. However, the efficacies of new drugs barely reach those of sorafenib in advanced HCC (3). Thus, sorafenib remains a major therapeutic option as the first-line therapy for many advanced HCC patients and is even used as a subsequent treatment for patients pretreated with new drugs such as lenvatinib (3). These findings highlight that the value in exploring a therapeutic approach to improve the efficacy of sorafenib in HCC.

Extensive studies have shown that multiple mechanisms, such as the phosphatidylinositol 3-kinase (PI3K)/AKT, Janus kinase/signal transducers and activators of transcription (JAK/STAT), transforming growth factor beta (TGF- $\beta$ ), and Wnt/Notch signaling pathways, are involved in sorafenib resistance in HCC cells (4). These studies showed that upregulation of these pathways is intimately linked to compensating for the anticancer effect of sorafenib. However, simultaneous targeting of these signaling pathways along with sorafenib treatment has not improved the prognosis of HCC patients in phase III clinical trials (5).

The sorafenib-induced unfolded protein response (UPR) coupled with ER stress (6) is another mechanistic axis involved in sorafenib resistance in HCC cells (7-9). ER stress accompanied by autophagy protects cells from stress-induced death, while prolonged ER stress induces cell death (10). In line with this, inhibition of autophagy was shown to increase the sensitivity of HCC cells to sorafenib (11). In addition, suppression of UPR activation via stearyl-CoA desaturase-1 (SCD1) reduced sorafenib resistance in HCC cells (12). These studies suggest that targeting ER stress coupled with UPR is another promising approach to increase sorafenib efficacy in HCC.

To find a new combination partner that showed a synergistic effect with sorafenib in HCC, we explored a focused chemical library and found MG149 to be a promising candidate for modulating the ER stress pathways in sorafenib-treated cells and

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synergistically increase the death of HCC cells.

## RESULTS

### The combination of sorafenib and MG149 mediates synergistic defects in HCC cell viability

Focused chemical libraries have been used for screening potent small molecules to target cancer cells (13, 14). To identify chemical compounds exhibiting a combinatorial effect with sorafenib on suppressing HCC, we screened a Selleckem library composed of inhibitors of different epigenetic regulators (15) for viability of Huh7 HCC cells by measuring cellular ATP with CellTiter-Glo (16). Given that the IC<sub>50</sub> of sorafenib for Huh7 viability was ~3 μM (Fig. 1A), we treated Huh7 cells with 10 μM of each library compound along with 3 μM of sorafenib for 72 hrs and compared the viability of the samples with those of samples treated with a single agent only. Among the 93 different small molecules in the library, anacardic acid and MG149, both of which are histone acetyltransferase (HAT) inhibitors (17, 18), were identified as those of the 4 prominent positive hits (anacardic acid, GSK126, SMI-4A, MG149), the combination of which with sorafenib increased the inhibition effect by 30% compared with treatment with a single reagent alone (Fig. 1B). Anacardic acid, a natural product from cashew nut shell extract, is a non-selective inhibitor of HAT (19). Notably, GSK126, a histone methyltransferase inhibitor, was recently identified as a combination partner with sorafenib in HCC cells (20). Also, the involvement of Pim kinase, the genetic target of

SMI-4A, in sorafenib resistance was previously disclosed (21). Thus, of the other hit compounds, we decided to further investigate the effect of MG149 as a potential combination drug with sorafenib.

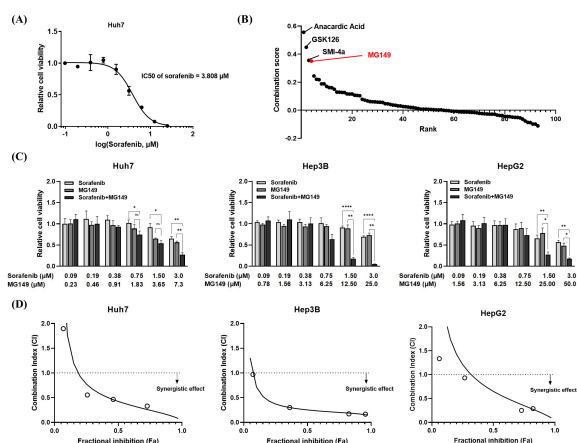
To examine the combined effect of MG149 and sorafenib in different cellular contexts, we determined the IC<sub>50</sub> of sorafenib on the viability of different HCC cell lines (Huh7, Hep3B, and HepG2) with or without MG149 by measuring cellular metabolic activities with EzCytos (22). In all the HCC cells tested, the combination with MG149 decreased the IC<sub>50</sub> of sorafenib (Supplementary Fig. 1). Then, to evaluate whether the combinatorial effect was synergistic, we treated HCC cells with various concentrations of sorafenib, MG149, or combinations of both reagents in a constant ratio. As shown in Fig. 1C, relative cell viability was reduced in all three HCC cell lines treated with the combination of sorafenib and MG149 in a dose-dependent manner. Given these experimental data, we calculated the combination index (CI) with CompuSyn software (23) and found that most of the CI values were less than 1 (Fig. 1D, Supplementary Table 1). Together, these results suggest that sorafenib in combination with MG149 had a synergistic inhibitory effect on HCC cell viability.

### Treatment with MG149 combined with sorafenib significantly induces the apoptotic death of HCC cells

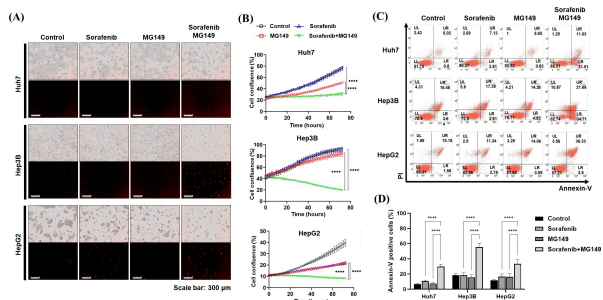
To determine whether the impaired viability of HCC cells cotreated with sorafenib and MG149 was linked to the induction of cell death, we monitored cell growth in real time for 72 hrs in the presence of propidium iodide (PI), which stained dead cells. Compared with treatment with either sorafenib or MG149 alone, the combination of sorafenib and MG149 increased PI uptake in Huh7, Hep3B, and HepG2 cells (Fig. 2A). In contrast, the confluency of HCC cells cotreated with sorafenib and MG149 was significantly lower than that of cells treated with a single reagent (Fig. 2B). These results indicate that the combination of sorafenib and MG149 mediated cell death rather than cell cycle arrest or quiescence.

To elucidate the cell death mechanisms induced by the combination of sorafenib and MG149, we then analyzed dead cells by staining with PI and annexin-V, which indicates early apoptotic status. Combined treatment with sorafenib and MG149 resulted in 11.03%, 21.68%, and 36.33% of Huh7, Hep3B, and HepG2 cells costained with PI and annexin-V, respectively (Fig. 2C). The double-stained fractions were higher than those treated with either sorafenib or MG149 alone. The population of annexin-V-stained cells with or without PI staining was also significantly higher in cells cotreated with sorafenib and MG149 than in those treated with each single reagent (Fig. 2D).

Dead cells under sorafenib and MG149 treatment were further examined by a TUNEL assay visualizing fragmented DNA in apoptotic cells. We observed that the levels of TUNEL staining significantly increased when HCC cells were treated with a combination of sorafenib and MG149 compared with those treated with a single agent alone (Supplementary Fig. 2).



**Fig. 1.** Synergistic effect of sorafenib and MG149 on liver cancer cell proliferation. (A) Huh7 cells were treated with various concentrations of sorafenib for 72 hrs to determine the IC<sub>50</sub> value for sorafenib. (B) A total of 93 small molecules in the focused chemical library were ordered by 'Combination score'. (C) Cotreatment of sorafenib and MG149 in HCC cells decreased cell viability in a dose-dependent manner. (D) Synergism between sorafenib and MG149 was confirmed in all HCC cell lines. The CI value was calculated using CompuSyn software (n = 3) (28). \*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.0001, ns, not statistically significant.



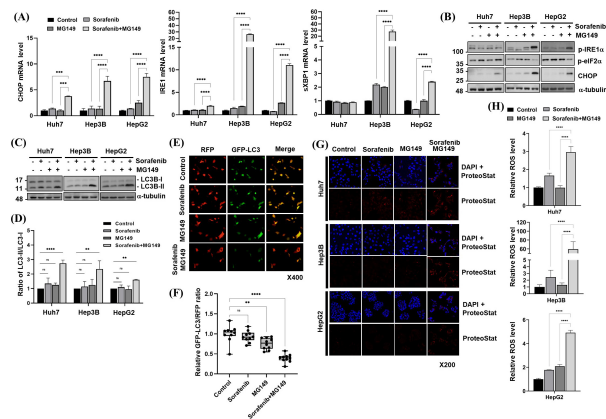
**Fig. 2.** Combination of sorafenib with MG149 induces synergistic liver cancer cell death. (A) Cotreatment with sorafenib and MG149 facilitates cell death in all HCC cell lines. Representative images were acquired after 72 hrs of incubation with DMSO (0.1%) or drugs. Scale bars denote 300  $\mu$ m. (B) Cell confluence under the indicated treatment for 72 hrs was determined by live-cell imaging analysis ( $n = 3$ ). (C) Apoptotic cell death was analyzed by flow cytometry. Representative data are displayed. (D) The percentage of cells positively labeled with annexin-V was quantified from three independent FACS analyses ( $n = 3$ ). \*\*\*\* $P < 0.0001$ .

Together, these results demonstrate that the combinatorial effects of sorafenib and MG149 on the apoptotic death of HCC cells were larger than those mediated by sorafenib or MG149 alone.

### The combination of sorafenib and MG149 hyperactivates endoplasmic reticulum (ER) stress signaling in HCC cells

Sorafenib is known to induce ER stress (6), which can mitigate the efficacy of sorafenib and mediate apoptosis resistance (8, 9). Considering these previous findings, we hypothesized that MG149 might suppress sorafenib-induced ER stress involved in cell survival and thus accelerate the cytotoxic effect of sorafenib. To test this hypothesis, we determined the activity of the ER stress response in HCC cells by assessing the transcript levels of the well-known ER stress markers CHOP, IRE1 $\alpha$ , and sXBP1 (24, 25). Unexpectedly, while sorafenib or MG149 alone slightly increased the transcript levels, the combination of sorafenib and MG149 significantly elevated the expression of these ER stress markers (Fig. 3A). Consistently, phosphorylation of IRE1 $\alpha$  (p-IRE1 $\alpha$ ) and abundance of CHOP protein, which reflect the activity of the ER stress pathway (26), were also prominently enhanced in HCC cells cotreated with sorafenib and MG149 (Fig. 3B). These results demonstrate that the combination of sorafenib and MG149 hyperactivated the ER stress response in HCC cells.

We note that cotreatment with sorafenib and MG149 largely decreased the phosphorylation of eIF2 $\alpha$  (p-eIF2 $\alpha$ ) in Hep3B and HepG2 cells, while treatment with either sorafenib or MG149 alone slightly induced p-eIF2 $\alpha$  levels. Unlike the findings in Hep3B and HepG2 cells, the combination of sorafenib and MG149 did not reduce p-eIF2 $\alpha$  in Huh7 cells. Prolonged ER stress has been known to trigger negative feedback for eIF2 $\alpha$  activity through the expression of GADD34, a regulatory subunit of the eIF2 $\alpha$  phosphatase PP1 (27). Given this finding, we



**Fig. 3.** Combination of sorafenib and MG149 enhances ER stress response. (A) The relative mRNA levels of CHOP, IRE1, and sXBP1 (splicing form of XBP1) were determined by qPCR analysis ( $n = 3$ ). (B) The protein levels of IRE1 $\alpha$ , phospho-eIF2 $\alpha$ , and CHOP in cells were determined by western blots. (C) The expression level of LC3B was examined using western blot analysis. (D) The ratio of LC3B-II and LC3B-I was calculated from the densitometric analysis of Fig. 3C ( $n = 3$ ). (E) Representative fluorescent images revealed increased autophagic flux in the combination treatment (reduced intensity of GFP). (F) The relative GFP-LC3/RFP ratio was quantified by analysis of the intensity of GFP and RFP signals from treated cells in Fig. 3E ( $n = 10$ ). (G) In all HCC cells, augmentation of aggregates in combination with sorafenib and MG149 was detected by a PROTEOSTAT<sup>®</sup> aggregate detection kit. (H) Increased intracellular ROS levels were found after combination treatment with sorafenib and MG149 compared with single drug treatment. \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ , ns, not statistically significant.

postulated that the combination of sorafenib and MG149 might highly upregulate GADD34 expression and thus dephosphorylate eIF2 $\alpha$  via negative feedback. Consistent with this hypothesis, we observed that cotreatment with sorafenib and MG149 highly elevated GADD34 expression in HepG2 and Hep3B cells but not in Huh7 cells (Supplementary Fig. 3). These findings imply that combination treatment of sorafenib and MG149 hyperactivates ER stress signaling in HCC cells and that the magnitude of ER stress induction by the combination is dependent on cell context.

### Autophagy activity is elevated by combination treatment with sorafenib and MG149 in HCC cells

Autophagy is an essential protective mechanism to alleviate ER stress-induced cell death (28, 29). To examine whether down-regulation of autophagy might underlie the synergistic cytotoxic activity of sorafenib and MG149, we determined the effect of both drugs on the level of LC3B protein, which reflects autophagy activity (Fig. 3C). In contrast to treatment with sorafenib alone, the combination of sorafenib and MG149 significantly increased the ratio of LC3B-II to LC3B-I (Fig. 3D). This finding demonstrates that cotreatment with sorafenib and MG149 amplified the autophagy activity in HCC cells (30).

We further examined the effect of sorafenib and MG149 on

autophagic flux in HepG2 cells by assessing GFP-LC3-RFP protein, a fluorescent reporter used to measure autophagic degradation (31). At 48 hrs after drug treatment, we observed that the combination of sorafenib with MG149 prominently reduced cellular GFP signals (Fig. 3E). The intensity of GFP over the internal control RFP was significantly reduced by simultaneous treatment with sorafenib and MG149 (Fig. 3F). Altogether, these findings indicate that the combination of sorafenib and MG149 largely elevated rather than suppressed autophagic activity in HCC cells.

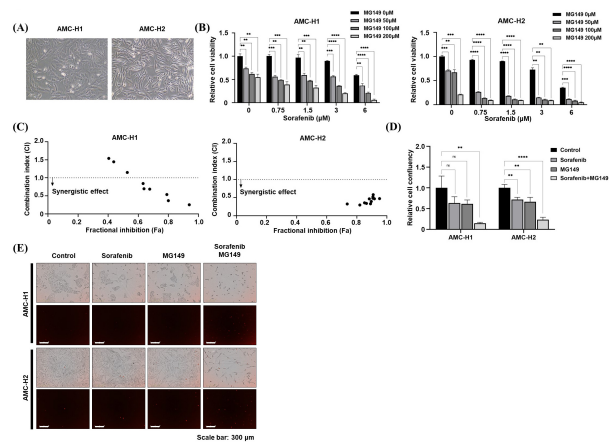
### Cotreatment with sorafenib and MG149 induces oxidative stress and misfolded protein aggregates

The accumulation of unfolded proteins in the ER is a major signal that activates the ER stress response pathway to cope with proteotoxic stress (29). Given that the combination of sorafenib and MG149 hyperactivated ER stress accompanied by autophagy activity, we examined whether the combination generated a greater unfolded protein burden in HCC cells. To identify whether misfolded proteins were increased by the combination treatment, we stained Huh7, HepG2, and Hep3B cells with a molecular rotor dye, ProteoStat, which becomes fluorescent when intercalated into structures of misfolded protein aggregates (32). In all three HCC cell lines, the combination of sorafenib and MG149 mediated the accumulation of misfolded protein aggregates in the cytoplasm (Fig. 3G). Aggregates detected by fluorescence imaging analysis were quantified and normalized to the number of cells measured by DAPI signals. All the cells treated with the combination displayed levels of aggresomes higher than five times that of those treated with sorafenib or MG149 alone (Supplementary Fig. 4). This finding implies that the combination of sorafenib and MG149 robustly induces protein unfolding, which can activate ER stress engaged with autophagy activity.

Since protein folding is highly redox-dependent, excessive reactive oxygen species (ROS) can mediate protein misfolding to generate aggresomes (33). Thus, we measured the effect of the sorafenib/MG149 combination on the level of intracellular ROS in HCC cells. As previously reported, ROS generation was elevated in all HCC cells treated with sorafenib alone (34), but treatment with MG149 alone increased the ROS level in HepG2 cells only (Fig. 3H). In contrast, when sorafenib was coupled with MG149, the levels of ROS in HCC cells were considerably higher than the treatment of each agent alone. Collectively, these findings demonstrate that cotreatment with sorafenib and MG149 exerted an impact on the accumulation of misfolded proteins and ROS production in HCC cells.

### Sorafenib and MG149 display synergistic cytotoxic effects in patient-derived HCC cells

We then examined the combinatorial effect of sorafenib and MG149 on patient-derived HCC cells, AMC-H1 and AMC-H2, which displayed distinctive morphology (Fig. 4A) (35). Consistent with the findings of the conventional HCC cell lines de-



**Fig. 4.** The combination of sorafenib with MG149 synergistically suppresses the viability of patient-derived liver cancer cells. (A) Cell morphology of patient-derived liver cancer cell lines (AMC-H1 and AMC-H2). (B) MG149 enhanced the sensitivity to sorafenib at various concentrations in two patient-derived liver cancer cell lines. ( $n = 3$ ). (C) At various concentrations, the synergistic effect between sorafenib and MG149 was corroborated by combination index (CI) values. (D) Relative cell confluency was considerably decreased in sorafenib + MG149 in AMC-H1 and AMC-H2 cells ( $n = 3$ ). (E) PI images were acquired from IncuCyte Zoom software at the end of drug treatment. \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ , ns, not statistically significant.

scribed above, combined sorafenib and MG149 treatments significantly reduced the viability of AMC-H1 and AMC-H2 cells compared with sorafenib treatment alone (Fig. 4B). In addition, CI calculated based on these data showed synergistic interactions between sorafenib and MG149 in patient-derived liver cancer cells (Fig. 4C and Supplementary Table 2). Real-time cell growth monitoring in the presence of PI showed that cotreatment with sorafenib and MG149 impaired cellular proliferation (Fig. 4D) and increased the population of PI-stained dead cells compared with treatment with either agent alone (Fig. 4E). These findings suggest that the combinatorial cytotoxic effect of sorafenib and MG149 would be efficacious in diverse HCC cells with different phenotypic features.

## DISCUSSION

ER stress and autophagy are intimately connected cellular processes that can result in both survival and death induction (36). Mild ER stress promotes cell survival by facilitating self-protection processes such as refolding and/or autophagic removal of misfolded proteins upon UPR and ER stress activation. Unresolvable ER stress leads to the induction of proapoptotic pathways (37). In contrast to previous studies showing that sorafenib induces ER stress to promote the adaptation and survival of HCC cells (7, 8), we showed that sorafenib combined with MG149 hyperactivated ER stress accompanied by autophagy and led to the death of HCC cells. In addition, simultaneous

treatment with MG149 and sorafenib elevated protein aggregate levels, implying that the combination of these compounds failed to refold and/or remove misfolded proteins. These results suggest that MG149 and sorafenib cooperate to upregulate the unresolvable UPR-ER stress axis and thereby result in synergistic apoptosis. Accordingly, MG149 appears to confer sorafenib-induced ER stress in HCC cells being off balance to death rather than survival. These findings suggest that enhancing ER stress can surpass sorafenib resistance in HCC cells.

MG149 is reported to be a potent inhibitor against MYST family histone acetyltransferases (HATs), Tip60 and MOF (18). Thus, our initial postulation was that inactivation of Tip60 or MOF might phenocopy the effect of MG149 on the sensitivity of HCC cells to sorafenib. However, knockdown of neither Tip60 nor MOF affected sorafenib sensitivity in HCC cells (data not shown). Simultaneous loss of Tip60 and MOF also failed to elevate sorafenib sensitivity in HCC cells (data not shown). These observations implied that the combinatorial effect of MG149 and sorafenib is unlikely to be dependent on the activity of Tip60 and MOF. Many cancer drugs have been shown to induce cytotoxic effects independent of their reported targets (38). We speculate that MG149 may interact with unidentified molecular targets and thus be involved in enhancing sorafenib sensitivity in HCC cells.

Together, we demonstrated that MG149 is a potent small molecule that combines with sorafenib to induce the synergistic death of HCC cells via the upregulation of unresolvable ER stress. Future studies to define the precise molecular targets of MG149 involved in the combinatorial effect with sorafenib on HCC cells will help develop improved therapeutic strategies to treat advanced HCC.

## MATERIALS AND METHODS

Details on the used methods are provided in the expanded Materials and Methods section in the online data supplement.

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## CONFLICTS OF INTEREST

The authors have no conflicting interests.

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