Original Article

The MEK inhibitors enhance the efficacy of sorafenib against hepatocellular carcinoma cells through reducing p-ERK rebound

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Background: Hepatocellular carcinoma (HCC) is one of the most common malignant tumours worldwide and has a poor prognosis. Sorafenib, the only targeted therapeutic agent for HCC, is a multiple kinase inhibitor with targets including RAF and VEGFR-2/3 that display a very limited ability to extend the survival of patients with advanced metastatic HCC for approximately three months. MEK inhibitors including trametinib and selumetinib have shown promising efficacy in combination with sorafenib in clinical trials. However, the mechanisms about the combined effect of these drugs remain unclear.

Methods: Two HCC cell lines (Bel7402 and SMMC7721) were used in the experiments. The protein expression of HCC cell lines was quantified via western blotting. Cell viability was examined by cell counting kit-8 and colony formation assays. Drug interactions between sorafenib and trametinib/selumetinib were determined by the combination index (CI) value.

Results: In this study, we found that short-term sorafenib treatment could inhibit the downstream RAF effectors phosphorylated (p)-MEK and p-ERK in Bel7402 and SMMC7721 cells, while long-term sorafenib treatment could induce a rebound of p-MEK and p-ERK expression in these two human HCC cell lines. We then tested the effect of sorafenib combined with two different FDA-approved MEK inhibitors, trametinib and selumetinib, in the two cell lines. Western blot analysis showed that trametinib/selumetinib could abolish the ERK activation caused by long-term sorafenib treatment. Cell counting kit-8 and colony formation assays indicated that the use of sorafenib or trametinib/selumetinib alone produced a minor effect on the proliferation of these HCC cell lines, while the combination therapies induced strong growth inhibition. CI assays using CompuSyn software indicated that the combined therapies could produce a synergistic effect in these two cell lines. In addition, mechanistic studies revealed that the combination therapies could synergistically reduce the expression of proliferation-related proteins, including cyclin D1 and c-Myc.

Conclusions: Taken together, our study showed that the rebound of p-ERK induced by long-term sorafenib treatment might limit the benefit of sorafenib monotherapy, and the MEK inhibitors trametinib and selumetinib could enhance the efficacy of sorafenib in HCC cells.

Keywords: Hepatocellular carcinoma (HCC); sorafenib; trametinib; selumetinib; combination therapy

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Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and is the third leading cause of death among all cancers worldwide (1). Although surgical therapies, including resection and liver transplantation, can cure some patients in the early stages, patients are often diagnosed at an advanced stage, and these patients have a poor prognosis. Other locoregional therapies for HCC include transarterial embolization (TAE), conventional transarterial chemoembolization (cTACE), drug eluting bead (DEB), transarterial chemoembolization (DEB-TACE), and transarterial radioembolization (TARE). However, these methods have strict indications and are often accompanied by complications (2). Sorafenib was the first molecular-targeted agent that could prolong the median overall survival time for nearly 3 months compared to placebo treatment in HCC (3). However, only approximately 30% of patients can benefit from sorafenib treatment (3,4). Drug resistance limits its effectiveness, and the mechanism of sorafenib resistance has not been fully elucidated. Recent studies showed that activation of the mitogen-activated protein kinase (MAPK) signalling pathway was associated with sorafenib resistance in HCC and that the level of phosphorylated (p)-ERK was also associated with the sensitivity of HCC to sorafenib (5-8). Thus, combination therapy with sorafenib and agents that target the MAPK pathway might be an important therapeutic strategy for HCC treatment.

The *RAS/RAF/MEK/ERK* cascade is the major cascade reaction in the MAPK pathway. Although mutation of *RAS* or *RAF* is rare (9,10), *MEK* and *ERK* are usually activated in HCC (11-13). MEK inhibitors, including trametinib, selumetinib (AZD6244) and refametinib, have been used clinically to treat melanoma as well as several other cancers (14-16). However, the efficacy of MEK inhibitor monotherapy in the treatment of HCC is uncertain, the phase II study of monotherapy with the MEK inhibitor selumetinib in patients with advanced HCC was terminated early because of a lack of adequate antitumour activity (17). In addition, although combination therapy with sorafenib and MEK inhibitors has been widely investigated in HCC patients (18-21), experimental evidence is lacking. Thus, additional studies are needed to confirm the effectiveness of this therapeutic combination.

In the present study, we investigated whether the MEK inhibitors trametinib and selumetinib could enhance sorafenib activity in HCC cells and the mechanism underlying this effect.

Methods

Cell culture and reagents

The Bel7402 and SMMC7721 human HCC cell lines were cultured in Dulbecco's modified Eagle's medium containing 10% foetal calf serum (Gibco, USA) at 37 ℃ with 5% CO_2. Sorafenib (BAY439006), trametinib (GSK1120212), and selumetinib (AZD6244) (all from Selleckchem, USA) stock solutions were prepared in DMSO (100 mM) and stored at −20 ℃.

Western blot analysis

The cells were plated and allowed to adhere in complete medium overnight, followed by treatment with the indicated reagent. The cells were then lysed in RIPA buffer containing protease and phosphatase inhibitors (Calbiochem, Darmstadt, Germany). Protein lysates were harvested and centrifuged, and the supernatants were collected and quantified with a BCA protein assay (Pierce Chemical Co., USA). Equal amounts of protein sample (20 μg) were subjected to SDS-PAGE analysis and electrophoretically transferred to nitrocellulose membranes (Millipore, Bedford, USA) using a Bio-Rad semidry transfer system. Protein expression was analysed using an ODYSSEY Infrared Imaging System (LI-COR Biosciences, Lincoln, USA). The primary antibodies included phospho-MEK (Ser217/221) PARP (Cell Signaling Technology, USA), MEK1/2, ERK1/2, phospho-p44/42 MAPK (ERK) (Thr202/Tyr204), c-Myc, bax, cyclin D1 (Abcam, UK), and GAPDH (Santa Cruz, USA).

Cell proliferation and colony formation assays

The cells were plated in a 96-well plate in six replicates at a density of 3,000–5,000 cells per well. The following day, trametinib/selumetinib, sorafenib, or combinations as indicated in the figure legends were added. After 72 hours, cell proliferation was determined by a cell counting kit-8 (Dojindo Molecular Technologies, Japan) assay. The cell viability rate was calculated with the following formula: viability (%) = (average OD value of drug-treated sample/average OD value of control sample) × 100%.

For the colony formation assay, the cells were seeded onto a 35 mm dish. After overnight incubation, the cells

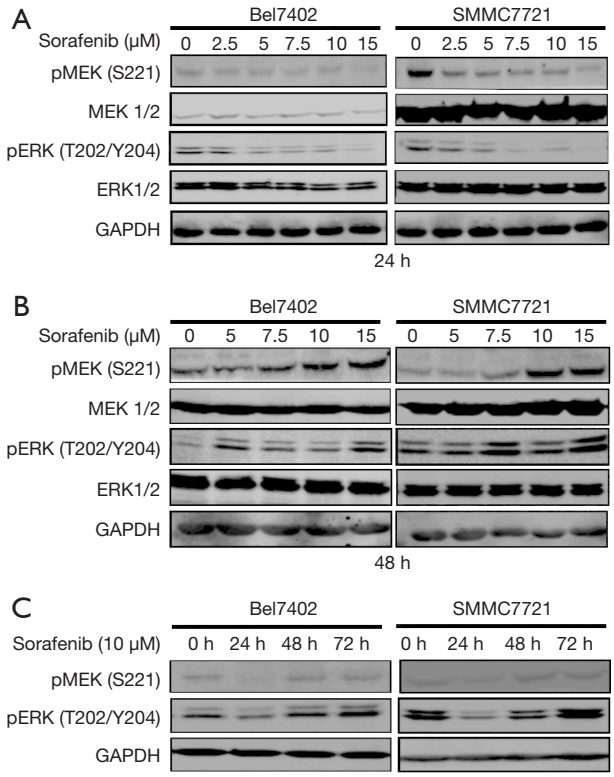

Figure 1 The effect of sorafenib on the ERK signalling pathway in HCC cell lines. (A) HCC cell lines (Bel7402 and SMMC7721) were treated with increasing doses (0, 2.5, 5, 7.5, 10, and 15 μM) of sorafenib for 24 hours. The levels of phosphorylated and total MEK and ERK were evaluated by western blot analysis. (B) HCC cell lines (Bel7402 and SMMC7721) were incubated with increasing doses of sorafenib. After 48 hours, whole-cell extracts were prepared and subjected to immunoblot analysis, and the effect on ERK/MEK signalling was detected with the indicated antibodies. (C) Immunoblot of two HCC cell lines treated with 10 μM sorafenib for various durations (0, 24, 48, and 72 hours). Lysates were analysed for p-ERK and p-MEK expression with the indicated antibodies. HCC, hepatocellular carcinoma.

were cultured in the absence or presence of drugs as indicated. Growth media with or without drug was replaced every 2 days. On day 7, the cells were washed three times with PBS, fixed in 4% paraformaldehyde for 15 min, stained with 0.5% crystal violet for 15 min and then imaged.

Combination index (CI) evaluation

The drug interactions between sorafenib and trametinib/selumetinib were determined by the CI value. The CI was evaluated with CompuSyn software (ComboSyn, Inc., Paramus, NJ, USA) using the method proposed by Chou *et al.* (22). CI values <1, =1, and >1 indicated synergistic, additive and antagonistic effects, respectively.

Statistical analysis

Statistical significance was calculated using GraphPad Prism version 5.01 and was set at *P< 0.05, **P<0.01, and ***P<0.001. Comparisons were analysed using one-way ANOVA. All experiments were performed at least three times.

Results

Sorafenib treatment caused ERK re-activation in HCC cells

Sorafenib, which was first designed as a CRAF inhibitor, is a multikinase inhibitor, and its targets include the RAF kinases (CRAF and BRAF), VEGFR-2/3, PDGFR-β, Flt3 and c-kit (23). Some studies have indicated that the effects of sorafenib on MAPK pathways are both cell type- and context-specific (7,24). Thus, we first explored the effects of sorafenib on the MEK/ERK pathway in the cell lines used in this study (Bel7402 and SMMC7721).

The two HCC cell lines were exposed to increasing concentrations of sorafenib for 24 or 48 hours, and cell lysates were then collected for the detection of MEK/ERK activity (*Figure 1A*,*B*). The cells were also treated with 10 μM sorafenib for 24, 48, and 72 hours, and western blotting was then performed to detect the levels of phosphorylated (p)-ERK) and p-MEK (*Figure 1C*). Our data showed that sorafenib treatment only transiently suppressed p-MEK and p-ERK in the two HCC cell lines, and a rebound in p-ERK was observed by 48 hours, which indicated the re-activation of the MAPK pathway.

MEK inhibitors abolished the ERK activation caused by long-term sorafenib treatment in HCC cells

ERK is the only substrate of MEK. Therefore, we investigated whether MEK inhibitors could abrogate the re-activation of ERK caused by sorafenib. The HCC cell lines were exposed to sorafenib for 48 hours, and two selective and potent MEK inhibitors (trametinib and selumetinib) were then co-administered; after 24 hours, the whole-cell lysates were evaluated by western blotting. As shown in *Figure 2*, combination treatment with either trametinib or selumetinib abolished the rebound in p-ERK, while

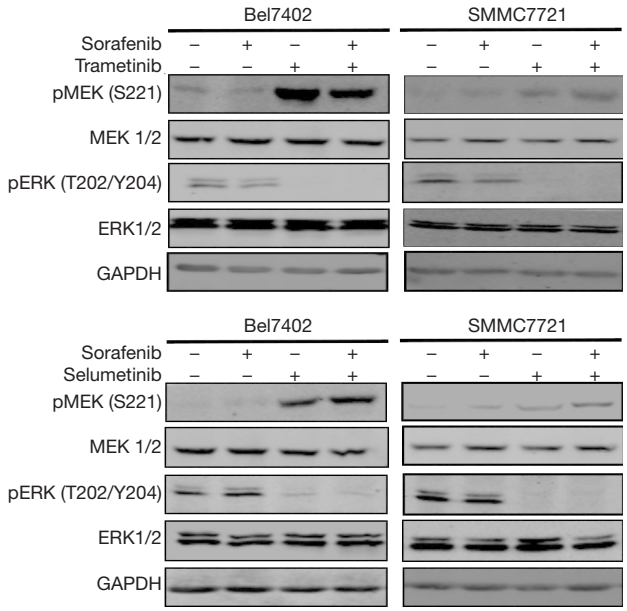

Figure 2 MEK inhibitors (trametinib and selumetinib) abolished the rebound in p-ERK caused by sorafenib in the HCC cell lines. Bel7402 and SMMC7721 cells were treated with 10 μM sorafenib for 48 hours and then co-treated with 10 μM trametinib/10 μM selumetinib for 24 hours. Then, western blotting was performed on the whole-cell extracts. The levels of phosphorylated and total MEK and ERK were detected. HCC, hepatocellular carcinoma.

the p-MEK level was consistent with the feedback loop of MAPK pathway inhibition.

Combination treatment with sorafenib and MEK1/2 inhibitors produced synergistic suppression of HCC cell proliferation

Because the MEK inhibitors selumetinib and trametinib abrogated the induction of ERK activation by sorafenib in the HCC cell lines, we accordingly hypothesized that MEK inhibitors could act synergistically with sorafenib to reduce the viability of HCC cells. The two HCC cell lines were treated with a range of concentrations of sorafenib and trametinib/selumetinib alone or combined in a fixed-ratio concentration (1:1). After 72 hours, cell viability was determined by a cell counting kit-8 assay. We first calculated the CI values using CompuSyn software (ComboSyn, Inc., Paramus, NJ, USA). According to the method proposed by Chou *et al.* (22), CI values <1, =1, and >1 indicated synergistic, additive and antagonistic effects, respectively. Our data showed that combination treatment with sorafenib and trametinib/selumetinib resulted in a synergistic effect in the two HCC cell lines (*Figure 3*). In addition, as shown in *Figures 4,5*, combination treatment was more effective in limiting colony formation and cell growth than sorafenib

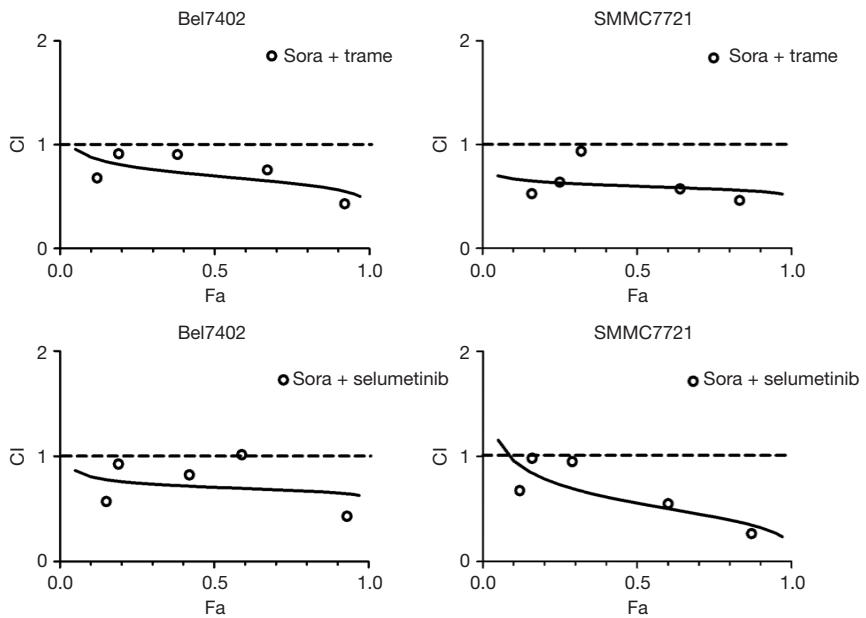

Figure 3 Median effect analysis was used to investigate the CIs for sorafenib and trametinib/selumetinib co-treatment in HCC cells. A representative figure for each cell line is shown. Fa denotes the fraction of cell death. CIs, combination indexes; HCC, hepatocellular carcinoma.

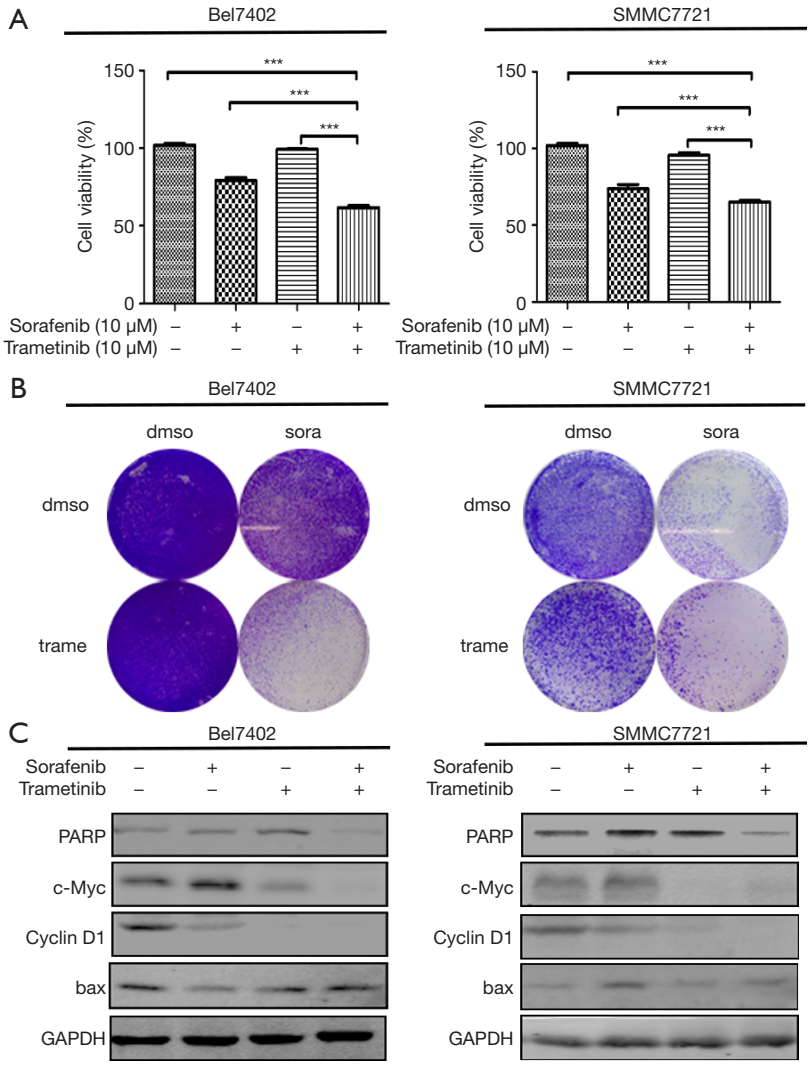

Figure 4 The combination of sorafenib and trametinib synergistically inhibited HCC cell growth. (A) The HCC cell lines were treated with sorafenib (10 μM), trametinib (10 μM), a combination of sorafenib and trametinib or DMSO (control) for 72 hours. Cell viability was measured using a cell counting kit-8 (Dojindo Laboratories, Kumamoto, Japan) assay according to the manufacturer's instructions. The data are representative of similar results obtained in three independent experiments performed for both groups. Statistical significance was calculated using GraphPad Prism version 5.01 and was set at ***P<0.001. (B) Colony formation assay for the two HCC cell lines treated with sorafenib (10 μM), trametinib (10 μM), a combination of sorafenib and trametinib or DMSO (control) for 7 days. (C) Bel7402 and SMMC7721 cells were treated with sorafenib (10 μM), trametinib (10 μM), both sorafenib and trametinib, or vehicle for 72 hours, and we detected the expression of proliferation- and apoptosis-related proteins in the whole-cell lysates. HCC, hepatocellular carcinoma.

monotherapy, while trametinib or selumetinib alone had little effect on HCC cell lines. The expression levels of proteins related to proliferation (c-Myc, cyclin D1) and apoptosis (PARP, bax) were determined by western blot analysis (*Figures 4C,5C*). We found that trametinib/selumetinib treatment produced a significant downregulation of c-Myc and cyclin D1, while no significant alteration was observed in the level of bax following either treatment. In addition, a decrease in PARP expression was found in the combination group, but cleaved PARP was not observed.

Discussion

Although the phase II study of the MEK inhibitor

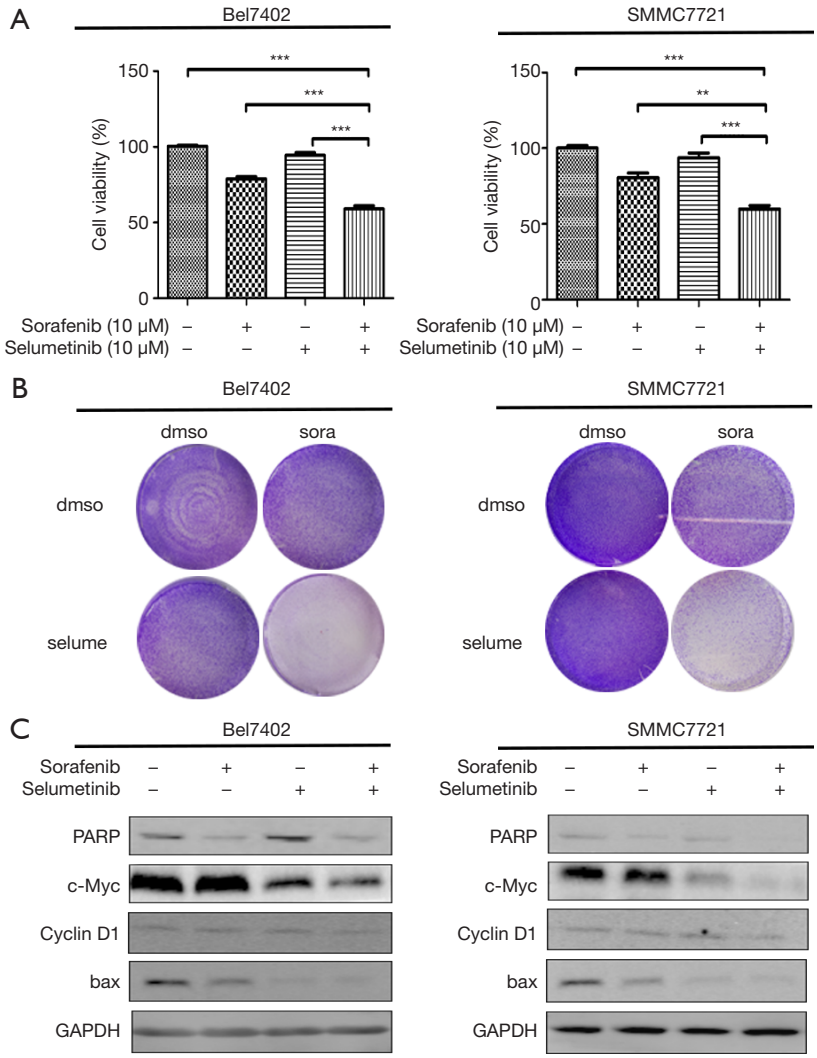

Figure 5 The combination of sorafenib and selumetinib synergistically inhibited HCC cell growth *in vitro*. (A) The two HCC cell lines were treated with sorafenib (10 μM), selumetinib (10 μM), a combination of sorafenib and selumetinib or DMSO (control) for 72 hours. Cell viability was measured using a cell counting kit-8 (Dojindo Laboratories, Kumamoto, Japan) assay according to the manufacturer's instructions. The data are representative of similar results obtained in three independent experiments performed for both groups. Statistical significance was calculated using GraphPad Prism version 5.01 and was set at **P<0.01, and ***P<0.001. (B) Colony formation assay for the two HCC cell lines treated with sorafenib (10 μM), selumetinib (10 μM), a combination of sorafenib and selumetinib or DMSO (control) for 7 days. (C) Bel7402 and SMMC7721 cells were treated with sorafenib (10 μM), selumetinib (10 μM), both sorafenib and selumetinib, or vehicle for 72 hours, and the expression of proliferation- and apoptosis-related proteins in the whole-cell lysates was detected.

selumetinib showed minimal single-agent activity in patients with advanced HCC (17), MEK is still an intriguing target for the treatment of human HCC. Some clinical trials have shown that the combination of sorafenib and MEK inhibitors presents promising activity against HCC (18,20,21). Recently, the phase I clinical study of combined treatment with the MEK inhibitor trametinib and sorafenib showed good safety and efficacy for patients with advanced HCC (19), but pre-clinical evidence is still lacking. Thus, in this study, we evaluated the effect of two MEK inhibitors (trametinib and selumetinib) combined with sorafenib on HCC cells, and furthermore, we clarified the molecular mechanism underlying this effect, ultimately providing a reasonable basis for clinical treatment.

Here, we selected two different MEK inhibitors and investigated their antitumour effects combined with sorafenib in HCC cells. The first drug was trametinib, a selective MEK inhibitor that plays a role in advanced melanoma treatment but has an unclear role in HCC. The second drug was selumetinib, and its efficacy in HCC has been demonstrated in animal models and clinical trials (18,25). By comparing the effect of these two MEK inhibitors in combination with sorafenib, we aimed to obtain new experimental evidence to support the therapeutic strategy of combining MEK inhibition with sorafenib treatment in HCC.

Notably, we observed a rebound in p-ERK signalling in the human HCC cell lines Bel7402 and SMMC7721 after long-term treatment with sorafenib. Therefore, we tested whether the MEK inhibitors trametinib and selumetinib could augment the efficacy of sorafenib through inhibiting re-activation of ERK. In addition, this hypothesis was further confirmed by western blotting, which showed that trametinib/selumetinib could downregulate the p-ERK expression induced by sorafenib, while the level of p-MEK was consistent with the feedback loop of MAPK pathway inhibition. A similar phenomenon was also observed in a previous study (26).

Furthermore, co-treatment with sorafenib and MEK inhibitors could synergistically decrease the expression of proliferation-related proteins (c-Myc and cyclin D1). However, no significant change in apoptosis-related proteins such as bax or cleaved PARP was found, but a decrease in total PARP was observed with combination treatment. The synergistic effect was demonstrated by the CI; the CI for the fixed-ratio concentration (1:1) was evaluated using CompuSyn software, and the CI values for the two cell lines were both less than 1, which indicated a synergistic effect according to the method of Chou *et al.* (22). However, some differences were observed between the two cell lines; the data showed that Bel7402 cells were more sensitive to the combination of an MEK inhibitor and sorafenib. The results of the colony formation assay also showed a strong antitumour effect in co-treated cells. As some RAF-inhibiting drugs could also induce paradoxical ERK pathway activation in cells with wild-type *BRAF* by transactivating RAF dimers (27,28), we hypothesized that a similar mechanism was responsible for the sorafenib-induced activation of ERK. Therefore, the re-activation of p-ERK caused by sorafenib in HCC cells could be reversed by treatment with the MEK inhibitors trametinib and selumetinib. In HCC, ERK overexpression and overactivation could lead to increased tumour cell proliferation, survival and invasion (11,13). Thus, dual inhibition of the MAPK pathway has a theoretical advantage in improving the activity of sorafenib in HCC treatment.

However, we also found that HCC cells were insensitive to trametinib/selumetinib as single agents. These results are consistent with data obtained from the clinical trial of selumetinib monotherapy (17). However, they are not consistent with the results of Zhou *et al.*'s study (29). They found that trametinib could inhibit the viability and proliferation of HCC cells, and in vivo, trametinib treatment inhibited HepG2 xenograft tumour growth and attenuated tumour invasion into surrounding tissues. Klein *et al.*'s study also showed that the MEK inhibitor PD184161 inhibited tumour formation in nude mice but not in tumourigenic mice (30). In addition, HCC patients rarely harbour mutations in *KRAS* or *BRAF* (9,10), while the cytotoxicity of MEK inhibitors is highly dependent on these mutations (31,32). Another important limitation may be that the effects of sorafenib and MEK inhibitors on ERK activation are both cell type- and context-specific; sorafenib has been shown to reduce the level of p-ERK in tumour cell lines that contained *RAS* or *RAF* mutations (7,33,34), and studies have also shown that *BRAF*-mutated cells are more sensitive to MEK inhibition (31), but the incidence of *RAS* and *RAF* gene mutations in HCC is low. Some researchers have also shown that tumours expressing higher baseline p-ERK levels were more sensitive to sorafenib (8,35). All of these observations suggest the complexity of the use of sorafenib for HCC treatment.

Conclusions

Taken together, the results of our study demonstrate that MEK inhibitors combined with sorafenib produce synergistic inhibition of the growth and survival of HCC cells. Our results supplement the accumulating evidence for this combination as a novel therapeutic strategy for HCC. Furthermore, research also shown that *MEK1* shRNA transfection notably increases sorafenib-mediated lethality in lymphoma cells (36). The combination of sorafenib and AZD6244 also produced a synergistic effect in preclinical studies in renal cell carcinoma (RCC) (37) and medullary thyroid cancer (MTC) patients (38), therefore, the combination of sorafenib and MEK inhibitors may have potent antitumour activity in several cancers. In addition, further clinical investigations are urgently needed to provide more definitive evidence for the use of this combination for

HCC treatment.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2019.07.11). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The institutional ethical approval and informed consent were waived.

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