Cabozantinib suppresses tumor growth and metastasis in hepatocellular carcinoma by a dual blockade of VEGFR2 and MET

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Translational Relevance

Hepatocellular carcinoma (HCC) patients with an active HGF/MET signaling pathway have a significantly worse prognosis. Moreover, MET activation triggered by anti-angiogenic therapies, such as sunitinib and sorafenib, can contribute to metastasis. In the current study, we first verified an association of phosphorylated MET (p-MET) with resistance to sorafenib as postoperative adjuvant therapy in a set of human HCC samples. In the next set of experiments, we examined anti-tumor effects of cabozantinib, a dual inhibitor of MET and VEGFR2, using cultured HCC cells, mouse xenograft and metastatic models. Both the in vitro and in vivo results showed that cabozantinib could inhibit HCC cells growth and metastasis. Based on an understanding of the drug’s mechanism and its effectiveness in multiple HCC cells and in vivo models, we believe that cabozantinib should be a promising strategy for the treatment of HCC in the future clinical practice.
Abstract

Purpose: MET signaling has been suggested a potential role in hepatocellular carcinoma (HCC) and associated with pro-metastasis during anti-angiogenesis therapy. We investigated the potential association between MET expression and therapeutic response to sorafenib in HCC patients. Anti-tumor effects of cabozantinib, a dual inhibitor of MET and VEGFR2, were examined in cultured HCC cells as well as in vivo models.

Experimental Design: Total MET and phosphorylated MET (p-MET) were measured in 29 resected HCC specimens, and correlated with response to sorafenib as postoperative adjuvant therapy. In the second set of experiments using cultured HCC cells, and mouse xenograft and metastatic models, effects of cabozantinib were examined.

Results: High level of p-MET in resected HCC specimens was associated with resistance to adjuvant sorafenib therapy. In cultured HCC cells that expressed p-MET, cabozantinib inhibited the activity of MET and its downstream effectors, leading to G1 phase arrest. Cabozantinib inhibited tumor growth in p-MET-positive and p-MET-negative HCC by decreasing angiogenesis, inhibiting proliferation, and promoting apoptosis, but it exhibited more profound efficacy in p-MET-positive HCC xenografts. Cabozantinib blocked HGF-stimulated MET pathway and inhibited the migration and invasion of the HCC cells. Notably, cabozantinib reduced the number of metastatic lesions in the lung and liver in the experimental metastatic mouse model.

Conclusions: HCC patients with high level of p-MET are associated with resistance to adjuvant sorafenib treatment. The dual blockade of VEGFR2 and MET by cabozantinib has significant anti-tumor activities in HCC, and the activation of MET in HCC may be a promising efficacy-predicting biomarker.
Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third most common cause of cancer-related death worldwide (1). Despite improvements in diagnostic and therapeutic strategies, the prognosis of HCC still remains poor (2-4). Inhibiting angiogenesis has been used as a strategy in the treatment of HCC (5, 6). For example, sorafenib, a vascular endothelial growth factor receptor (VEGFR) inhibitor with activity against platelet derived growth factor receptor (PDGFR), c-Kit receptor, RAF and p38 signal transduction pathways, has become a standard treatment in patients with advanced HCC (7). Even though sorafenib improves the median survival in advanced HCC, the median overall survival remains less than one year partly due to that many patients eventually become resistant to this drug (8, 9). In addition, sorafenib, like other VEGFR inhibitors such as sunitinib and cediranib, possesses the possibility to increase the invasiveness and/or metastatic potential of tumors (10-12). Thus, developing inhibitors that simultaneously inhibit VEGF and other pathways involved in tumor invasion and metastasis may confer broad and potent anti-tumor efficacy.

MET, a transmembrane tyrosine kinase receptor for hepatocyte growth factor (HGF), has been observed to play an important role in the development of human cancers and drug resistance in cancer cells (13-15). Moreover, overexpression of MET has been reported in various types of human cancers including HCC (13, 14). Blocking MET expression by gene therapy reduces cell proliferation, colony formation, and migration in vitro and suppresses tumor growth, angiogenesis and metastasis in vivo in multiple HCC cell lines (16-18). Interestingly, emerging evidence demonstrated that tumor vascular pruning caused by inhibition of VEGF pathway led to the induction of hypoxia and subsequently triggered MET expression, which enhances tumor invasion.
and metastasis (10, 11, 19). Therefore, blockade of both MET and VEGFR pathways may achieve better treatment outcome in HCC.

Tivantinib was initially reported as a MET selective inhibitor and demonstrated a nearly doubling of progression-free survival (PFS) and overall survival (OS) in HCC patients with high expression of MET (20). However, subsequent studies confirmed that tivantinib is not a MET inhibitor but an antimitotic agent that kills tumor cells independently of MET (21, 22). Cabozantinib is an oral small-molecule tyrosine kinase inhibitor that blocks phosphorylation of MET and VEGFR2 and also has activity against AXL, RET, and KIT (23). Results from phase II and III clinical trials demonstrated that cabozantinib exhibited encouraging clinical activity in multiple human cancers including castration-resistant prostate cancer, medullary thyroid cancer, and HCC with manageable side effects (24-26). Although cabozantinib has clinical benefit, the anti-tumor mechanism of cabozantinib in HCC has not been fully elucidated. Furthermore, anti-tumor agents that administered to patients before knowing their mechanism of action may be misleading in the development of predictive biomarkers. In the present study, we investigated whether cabozantinib could inhibit tumor growth and metastasis, and explored the molecular mechanism of anti-tumor activity of cabozantinib in HCC.

Materials and Methods

Patients and specimens

Archival HCC specimens were obtained from 29 patients who accepted potentially curative treatment of hepatic resection at Sun Yat-sen Memorial Hospital of Sun Yat-sen University between
January 2008 and December 2012. These patients were pathologically diagnosed as HCC with microvascular invasion. Since microvascular invasion is one of the most powerful factors associated with the recurrence of HCC after resection (27), patients consented to take sorafenib as the adjuvant therapy. The criteria for following-up, definition of sorafenib resistance and high expression of MET were described in the Supplementary Materials and Methods. The characteristics of all patients were summarized in Supplementary Table S1.

**Cell lines and culture conditions**

SK-HEP1 and HepG2 cells were obtained from the American Type Culture Collection (ATCC). MHCC97L and MHCC97H cells were supplied by the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). These cell lines were maintained in DMEM (GIBCO BRL, Grand Island, NY, USA) supplemented with 10% FBS (fetal bovine serum; GIBCO BRL, Grand Island, NY, USA), 100 U/ml penicillin, and 100 U/ml streptomycin. HUVECs (human umbilical vein endothelial cells) were purchased from ScienCell Research Laboratories (San Diego, CA) and were maintained in EBM-2 medium (Cambrex Bioscience, MD, USA) according to the manufacturer’s instructions. The four HCC cell lines were authenticated using short tandem repeat (STR) DNA testing by Beijing Microread Gene Tech. Co., Ltd. (Beijing, China) in 2013. HUVECs were not authenticated by the authors. All cell cultures were maintained at 37°C in a CO_2 incubator with a controlled humidified atmosphere composed of 95% air and 5% CO_2.

**Reagents and antibodies**

Reagents and antibodies used in this study were described in the Supplementary Materials and
Methods.

**Cell viability, colony-formation, cell cycle, and apoptosis analyses**

Cell viability, colony-formation, cell cycle, and apoptosis analyses were performed as described in the Supplementary Materials and Methods.

**Western blot analysis**

Cells or isolated independent tissues (lungs and tumors) from vehicle control- and cabozantinib-treated mice were lysed with RIPA Lysis Buffer (Santa Cruz Biotechnology, Santa Cruz, CA) containing protease inhibitors (Complete; Roche, Mannheim, Germany) and phosphatase inhibitors (PhosStop; Roche). The protein concentration was determined using a BCA assay (Beyotime Biotechnology, Haimen, China) and equalized before loading. A total of 25-50 μg of protein were separated by SDS-PAGE and transferred onto PVDF membranes. Membranes were blocked and blotted with the relevant antibodies. HRP-conjugated secondary antibodies were detected with an enhanced chemiluminescence reagent (Millipore Corp., Bedford, MA). GAPDH was used as a loading control. All antibody dilutions were 1:1,000 except for the GAPDH antibody, which was used at a dilution of 1:5,000.

**In vitro migration and invasion assays**

Wound healing and Transwell assays were used to examine migration of HCC cells. The invasiveness of cells was determined as described in the Supplementary Materials and Methods.
Animal experiments

Animal care

Female BALB/c athymic nude mice, 5 to 6 weeks old (Experimental Animal Center of Sun Yat-sen University, China), were used for in vivo studies. All animals were fed a standard diet ad libitum and housed in a temperature-controlled animal facility with a 12/12 h light/dark cycle. All procedures were performed according to the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the Bioethics Committee of Sun Yat-sen University.

Tumor implantation and growth

MHCC97H and HepG2 xenograft models were established by s.c. injection of tumor cells (5 × 10^7/ml) in PBS with Matrigel at a 1:1 ratio. The cell suspension was injected in a total volume of 0.2 ml into the right flank of the mice and was allowed to grow for two weeks to reach a tumor size of approximately 80 to 200 mm^3. The mice were then randomized into three groups (n = 8/group): vehicle control (ddH2O, p.o.), cabozantinib (10 mg/kg/d, p.o.), or cabozantinib (30 mg/kg/d, p.o.) for 14 consecutive days. Tumor dimensions and body weights were measured every two days starting with the first day of treatment. Tumor volume (mm^3) was calculated by the following formula: (l × w^2) / 2, where l and w refer to the larger and smaller dimensions collected at each measurement. The mice were sacrificed after 14 days of treatment. Solid tumors were excised, weighed, and either processed for paraffin embedding or snap frozen and stored at -80°C.

Inhibition of Expression of VEGFR2, MET, and its downstream pathway in vivo

For this experiment, treatment was not initiated until tumors reached 300 to 400 mm^3 in size, and cabozantinib (30 mg/kg) or vehicle was administered once daily for only three days. The mice (n = 3/group) were sacrificed 3 h after the last treatment, and lungs and solid tumors were homogenized.
in lysis buffer for Western blot analysis.

**Experimental metastasis**

SK-HEP1 cells (1 × 10^6 cells) in 300 μl PBS were injected directly into the tail veins of 5 to 6-week-old female nude mice (28). This injection was immediately followed by randomization (n = 6/group) and oral treatment with cabozantinib (30 mg/kg), sorafenib (30 mg/kg), or ddH2O. Mice were sacrificed after daily treatment for 28 days, and their livers and lungs were weighed and sampled for tissue sectioning. To examine the metastases, 100 sequential sections (5 μm) were cut from the lungs and livers of each mouse, and every 10th section was stained with hematoxylin and eosin (H&E). Expression of p-MET in each group was determined by immunohistochemistry.

**Immunohistochemical analysis**

Frozen, 5 μm thick sections of tumor samples were prepared to determine vessel density with an anti-CD31 (1:100) antibody. To evaluate proliferation and apoptosis, 5 μm paraffin-embedded sections were stained with anti-Ki67 (1:50) and anti-cleaved PARP (1:50) antibodies, respectively. After blocking endogenous peroxidase activity, the sections were incubated overnight with the primary antibodies at 4°C. Detection was completed with the Polink-2 Plus IHC Detection System (Beijing Zhongshan Biotechnology Co., Beijing, China) according to the manufacturer’s instructions. Sections were visualized by adding diaminobenzidine (DAB kit; Beijing ZhongShan Biotechnology Co.). Negative controls were obtained by omitting the primary antibody. Staining was evaluated by two independent observers. To quantify the mean vessel density (MVD) in sections stained for CD31, 10 random fields per tumor sample at 200× magnification were captured and quantified as CD31-positive area/total area by Image-Pro Plus software (Media Cybernetics, Inc., Bethesda, MD).
For Ki-67 and cleaved PARP, only nuclear immuno-reactivity was considered positive. The proliferation index and apoptosis index corresponded to the number of labeled Ki-67 or cleaved PARP cells among at least 500 cells per region and are expressed as percentages.

**Statistical analysis**

Statistical analyses were performed with mean ± standard deviation (SD) values using Student’s *t*-test and two-way analysis of variance (ANOVA) with the Bonferroni’s correction. Statistical significance was concluded at *P* < 0.05.

**Results**

The expression of p-MET in resected HCC specimens is associated with resistance to adjuvant sorafenib therapy

The expression of MET in HCC specimens were detected by immunostaining. We observed that MET is overexpressed in most of tumor tissues (data not shown), whereas p-MET is highly expressed in approximately 27.6% HCC patients. Notably, we found that positive p-MET staining was associated with the therapeutic response to sorafenib. Specifically, seven of 12 (58.3%) cases with sorafenib resistance have high p-MET expression, whereas only one of 17 (5.88%) sorafenib sensitive cases were observed to have positive staining for p-MET (Supplementary Figure S1).

Anti-proliferative effect of cabozantinib on HCC cells *in vitro*

The effect of cabozantinib on proliferation in each HCC cell line is shown in Figure 1. Cabozantinib inhibited cell growth in a concentration-dependent manner in MHCC97L and MHCC97H cells, with
IC$_{50}$ values of 13.47 and 9.466 nM, respectively. SK-HEP1 and HepG2 cells were much less sensitive to cabozantinib (IC$_{50}$ = 4,306 nM and 5,040 nM, respectively). Similar results were obtained from the colony-formation assay (Supplementary Figure S2). To analyze the mechanisms by which cabozantinib inhibited cell proliferation, flow cytometric analysis was conducted to analyze the cell cycle and apoptosis of cells after treatment with various concentrations of cabozantinib. As shown in Figure 2A, in both MHCC97L and MHCC97H cells, cabozantinib markedly increased the percentage of cells in the G1 phase while decreasing the percentage of cells in the S phase. Cyclin D1 is a critical regulator of the G1-S transition (29). Up-regulation of cyclin D1 results in rapid growth of a subset of HCC (30). Western blot analysis indicated that cyclin D1 expression in MHCC97L and MHCC97H cells was reduced after treatment with cabozantinib for 24 h (Supplementary Figure S3A). Notably, after treatment with cabozantinib, SK-HEP1 and HepG2 cells showed a decrease in the G1 phase and an increase in the G2 phase (Figure 2B). Cabozantinib induced apoptosis in SK-HEP1 and HepG2 cells, but not in MHCC97L and MHCC97H cells (Figure 2C, Figure 2D and Supplementary Figure S3B). The data presented above collectively suggest that different mechanisms appear to be involved in the anti-proliferative effect of cabozantinib on MHCC97L, MHCC97H, SK-HEP1, and HepG2 cells.

**Cabozantinib inhibits MET and VEGFR2 phosphorylation and their downstream effectors in vitro**

Western blot analysis demonstrated that cabozantinib-sensitive MHCC97L and MHCC97H cells displayed a dramatic elevation in MET phosphorylation, compared with SK-HEP1 and HepG2 cells. No VEGFR2 expression was detected in cultured HCC cells (Figure 3A), which indicates that the
inhibitory activity of cabozantinib on VEGFR2 is not involved in its anti-proliferative effects on these HCC cells in vitro.

Next, we investigated the effect of cabozantinib treatment on MET-dependent signaling pathways. Marked suppression of p-MET was observed in MHCC97L and MHCC97H cells tested after 4 h incubation with cabozantinib at concentrations as low as 10 to 100 nM. Moreover, treatment with these doses also effectively abrogated the phosphorylation of downstream effectors, such as STAT3, AKT, and ERK1/2 (Figure 3B). Thus, constitutive activation of these proliferative and survival effectors in MHCC97L and MHCC97H cells appears to depend specifically on MET signaling. In contrast, in SK-HEP1 and HepG2 cells, where MET is not constitutively phosphorylated, cabozantinib at a dose of 100 nM had no demonstrable effect on the phosphorylation of STAT3, AKT, or ERK1/2, indicating that these proliferative and survival effectors are likely activated through alternative growth factor receptors. An increase in the concentration of cabozantinib, up to 5,000 and 10,000 nM, had a significant impact on the phosphorylation of STAT3, AKT, and ERK1/2 in both SK-HEP1 and HepG2 cells (Figure 3B). This effect may be because cabozantinib has a non-specific inhibitory effect on these effectors or exerted through the inhibition of other cancer-specific cabozantinib targets, such as AXL, RET, and KIT.

In a cytokine-stimulated tyrosine kinase activity assay, we found that cabozantinib treatment resulted in the marked inhibition of cytokine-stimulated phosphorylation of MET and VEGFR2 and their resultant downstream effectors in HUVECs (Figure 3C). In a concentration-dependent manner, cabozantinib eliminated HGF-induced MET phosphorylation and its downstream effectors STAT3, Akt, and Erk-1/2 in both SK-HEP1 and HepG2 cells (Figure 3D).
Cabozantinib inhibits HGF-induced cell motility and invasion

Because MET is not necessary for proliferation of SK-HEP1 and HepG2 cells, we utilized these two cell lines to test whether cabozantinib has an effect on motility and invasion. We observed that HGF enhanced the migration and invasion of SK-HEP1 and HepG2 cells as evaluated by a wound healing and Transwell assays. Moreover, at a concentration that has minimal impact on growth, cabozantinib inhibited HGF-induced migration and invasion in both SK-HEP1 and HepG2 cells (Figure 4). These findings reflect the potential anti-metastatic effect of cabozantinib in HCC cells.

In vivo efficacy and mechanism of cabozantinib against MHCC97H and HepG2 xenografts

To examined that cabozantinib inhibits VEGFR2 and MET signaling activity in vivo, established MHCC97H xenografts (n = 3/group) were treated daily with an oral dose of vehicle or cabozantinib at 30 mg/kg for three days. As shown in Supplementary Figure S4, administration of cabozantinib resulted in significant inhibition of VEGFR2 and MET phosphorylation in mice lungs and tumors, respectively, compared with the vehicle-treated control group. Moreover, the inhibition of downstream MET effectors, such as STAT3, AKT, and ERK1/2, was also detected in MHCC97H tumors.

The results in Supplementary Table S2 and Figure 5A demonstrate that cabozantinib at concentrations of both 10 mg/kg and 30 mg/kg displayed a good anticancer effect on MHCC97H xenografts; their tumor growth inhibition (TGI) rates were 53.4% and 84.6%, respectively. Treatment of HepG2 xenografts with an identical treatment scheme led to 27.5% and 59.1% TGI, compared with the vehicle-treated control group (Supplementary Table S2 and Figure 5B). These differences in efficacy imply that the overexpression of p-MET may identify a sensitivity index for cabozantinib.
treatment in HCC.

We next evaluated the anti-angiogenic, anti-proliferative, and pro-apoptotic effects of cabozantinib in treated tumor xenografts. Immunohistochemical analyses revealed that cabozantinib decreased the mean vessel density (MVD) in MHCC97H xenografts by 49.9% and 90% at doses of 10 mg/kg and 30 mg/kg, respectively, compared with the vehicle-treated control (Figure 6). MVD in HepG2 xenografts was reduced by 58.3% and 87.1% at doses of 10 mg/kg and 30 mg/kg, respectively, compared with the vehicle-treated control. In addition, MHCC97H and HepG2 xenografts treated with cabozantinib revealed a significant reduction in proliferation (percentage of Ki-67-positive cells) and an increase in apoptosis (percentage of cleaved PARP-positive cells).

**Cabozantinib prevents metastasis of SK-HEP1 cells to the lung and liver**

To determine whether cabozantinib treatment could reduce metastasis, SK-HEP1 cells were directly injected into the tail vein of female nude mice. After injection, mice received sorafenib or cabozantinib treatment for four weeks. We found that the formation of metastases in the lung and liver was reduced by 53.7% and 52.9% in cabozantinib-treated group, respectively, compared with the vehicle-treated group (Figure 7). Interestingly, lungs and livers from mice in the sorafenib-treated group displayed an apparent increase in metastatic foci. Additional evidence of the inhibition of metastasis in cabozantinib-treated group was confirmed by the significant difference in whole lung wet weights among control, sorafenib and cabozantinib treatment groups (Figure 7A). Comparison of p-MET, as assessed by immunohistochemistry, in mice treated with ddH$_2$O or sorafenib corroborated the known positive relationship between VEGF signaling inhibition and MET activation (31-33). After sorafenib treatment for 28 days, staining for phosphorylated MET was strong and widespread.
in lung tissue and metastatic foci in the liver. Staining for p-MET was weak or absent in the cabozantinib-treated group (Supplementary Figure S5). Cabozantinib treatment was well tolerated, as determined by stable body weights throughout the 28 days treatment period.

**Discussion**

Targeting angiogenesis has become an established therapeutic approach to fighting solid tumor growth in cancer patients, and the systemic therapy with sorafenib represents a milestone in advanced HCC. However, the benefit of sorafenib in clinical therapy is marginal and transient (8, 9). The MET pathway has been found involved in gefitinib resistance in lung cancer (15). In line with this, we revealed that high level of activated MET in HCC is associated with resistance to adjuvant sorafenib treatment. Moreover, we demonstrated that cabozantinib, a dual Inhibitor of MET and VEGFR2, could inhibit the growth, migration, invasion and metastasis of HCC both *in vitro* and *in vivo*.

In tumor cell growth inhibition assays, cabozantinib could inhibit the growth of p-MET expressing cells (MHCC97L and MHCC97H) at low concentrations, but required much higher concentration in p-MET negative cells (SK-HEP1 and HepG2). Flow cytometry analysis revealed that cabozantinib suppressed the proliferation of MHCC97L and MHCC97H cells by causing G1-phase cell cycle arrest without inducing apoptosis. The expression of cyclin D1, which is a critical regulator of the G1-S transition, was markedly blocked by cabozantinib. These results are supported by the study of Sheng-Zhou Zhang et al., who showed that MET knockout induces significant G1 arrest and a decrease of cyclin D1 in MHCC97L and MCC97H cells (16). Cabozantinib displayed a significant concentration-related anti-proliferative effect on MHCC97L and MHCC97H cells at ~10 nM, which
was accompanied by a reduction of phosphorylation of MET and its downstream effectors STAT3, Akt, and Erk1/2. These findings highlight that cabozantinib suppressed the proliferation of MHCC97L and MHCC97H cells by impeding the MET pathway.

Consistent with the *in vitro* study, cabozantinib showed an anti-MET and -VEGFR2 pathway activity in MHCC97H xenografts model. The treatment of MHCC97H xenografts with cabozantinib resulted in a more pronounced TGI compared with its efficacy on HepG2 xenografts, suggesting that p-MET amplification may be a molecular marker of susceptibility to cabozantinib treatment in HCC cells. Both MHCC97H and HepG2 xenografts treated with cabozantinib showed reduced microvessel density, suppressed proliferation, and increased apoptosis. Based on our findings, we propose that the anti-tumor effect of cabozantinib on p-MET-positive MHCC97H xenografts appears to be mediated by inhibiting tumor angiogenesis (anti-VEGFR2 effect) and by directly inhibiting tumor cell proliferation (anti-MET effect). For p-MET-negative HepG2 xenografts, impeding stromal angiogenesis through VEGFR2 inhibition may contribute to the dominant abrogation of tumor growth. Because HCC patients may have MET-negative disease (34), we propose that MET activation may be a useful biomarker in cabozantinib clinical trials in identifying HCC patients with potential for the greatest benefit, by predicting durable tumor shrinkage and increased tumor response rate. However, the degree to which inhibition of the MET signaling pathway contributes to anti-angiogenesis and inhibition of other cabozantinib targets contributes to tumor growth inhibition *in vivo* remain to be clarified.

It has been reported that cabozantinib suppressed cell migration and invasion in various types of tumor cells (23, 35). Consistently, our results showed that cabozantinib inhibited HGF-stimulated migration and invasion in HCC cell lines. Notably, treatment of SK-HEP1 and HepG2 cells with low
concentration of cabozantinib had minimal impact on growth but strongly reduced migration and invasion potential, suggesting a role of cabozantinib on cell motility and invasion without affecting proliferation.

Recent studies have demonstrated that anti-angiogenesis agents could increase local invasion and distant metastasis during or after treatment (11, 12, 36, 37). For example, VEGFR inhibitors, such as sorafenib and sunitinib, can result in upregulation of MET, leading to promotion of metastasis (19, 32, 38). In support of this, we observed that sorafenib treatment promoted metastasis in the lung and liver, and accompany with activation of MET in experimental metastasis models with SK-HEP1 cells. This finding argues that simultaneously targeting MET and VEGFR2 may circumvent the “metastatic escape pathways”. Indeed, mice treated with cabozantinib had fewer metastatic foci in lung and liver tissues compared to control and sorafenib treated groups, suggesting that cabozantinib could reduce tumor metastasis mainly through inactivation of MET. Interestingly, our observations raise the possibility that MET could be activated during tumor progression and sorafenib treatment, leading to enhanced metastasis, thereby identifying a potential target for therapeutic intervention.

In summary, our study revealed that the high level of p-MET in HCC tissue could be a prognosticator of resistance to adjuvant sorafenib therapy. The inhibition of both VEGFR2 and MET signaling pathway by cabozantinib could have considerable therapeutic effects in HCC in vitro and in vivo. Also, the presence of MET activation in HCC may be a promising biomarker for predicting the response to cabozantinib treatment. Altogether, cabozantinib could be a useful agent for inhibiting tumor growth, angiogenesis, and metastasis in HCC with dysregulated MET and VEGFR2 signaling pathway.
Acknowledgment

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References


Figure legends

Figure 1. Cabozantinib inhibits HCC cell proliferation. MHCC97L, MHCC97H, SK-HEP1, and HepG2 cells were treated with different concentration of cabozantinib for 72 h in DMEM containing 10% FBS. Each point represents the mean ± SD for three independent experiments. IC$_{50}$ was calculated by nonlinear regression analysis using GraphPad Prism software.

Figure 2. Effects of cabozantinib on cell cycle progression and apoptosis in HCC cells. A and B, Effect of cabozantinib on the cell cycle. MHCC97L, MHCC97H, SK-HEP1, and HepG2 cells were treated with either 0.1% DMSO or cabozantinib for 24 h. After treatment, cells were harvested, fixed, and stained with PI for flow cytometric analysis. Data were analyzed using ModFit and were reported as the mean ± SD. C and D, Apoptosis of HCC cells detected by Annexin V-FITC/PI binding assay. Cells were treated with either 0.1% DMSO or cabozantinib for 48 h at the indicated concentrations, and then stained with Annexin V-FITC and PI. The rate of apoptosis was determined using a flow cytometer, and data were analyzed using Kaluza software and were reported as the mean ± SD. The results are representative of three independent experiments. *P < 0.05 and **p < 0.01, vs. control.

Figure 3. Expression profile of receptor tyrosine kinases (RTKs) and the effect of cabozantinib on RTKs signaling in HUVECs and HCC cells. A, Western blot analysis was performed to detect the expression profile of VEGFR2 and MET in HUVECs and HCC cells. B, Western blot analysis was conducted to measure effect of cabozantinib on phosphorylation of MET and downstream effectors in MHCC97L, MHCC97H, SK-HEP1, and HepG2 cells. Cells were treated with either 0.1%
DMSO or the indicated concentrations of compounds in DMEM containing 10% FBS for 4 h before protein extraction. C, HUVECs were grown with either 0.1% DMSO or the indicated doses of cabozantinib for 4 h, followed by the addition of 50 ng/ml VEGF or HGF 10 min before cell lysis for Western blot analysis. D, Cells were starved in medium containing 1% FBS for 12 h before adding either 0.1% DMSO or cabozantinib. After incubation for 4 h and stimulation with 50 ng/ml HGF for 10 min, cells were lysed for Western blot analysis. Independent experiments were performed at least three times, and the results from a representative experiment are shown.

Figure 4. Cabozantinib depresses HGF-induced cell migration and invasion of SK-HEP1 and HepG2 cells. A, Cabozantinib inhibits HGF (50ng/ml) stimulated cell migration in the wound healing assay. Shown here are representative images of three independent experiments. B and C, Cabozantinib inhibits HGF (50ng/ml) stimulated SK-HEP1 and HepG2 cells migration and invasion in a Transwell assay. The representative photographs of migration and invasion cells were shown as B and C, respectively. Bar graphs represent the average number of stained cells was calculated from three independent experiments, with ten fields counted per experiment, and are reported as the mean ± SD. *P < 0.05 and **p < 0.01. Scale bars, 50 μm.

Figure 5. Cabozantinib abrogates tumor growth in xenograft mouse model. A and B, MHCC97H and HepG2 cells were s.c. implanted in nude mice as described in the Materials and methods. Mice bearing tumors xenografts were treated daily with vehicle or either 10 mg/kg or 30 mg/kg of cabozantinib for 14 days. The image shows the tumor size of MHCC97H and HepG2 xenografts at the end of the experiment. Data represent the mean ± SD of the tumor volume and...
tumor weight for each group of eight experimental animals. * $P < 0.05$ and **$p < 0.01$, treated group vs. control group.

**Figure 6. Effects of cabozantinib on angiogenesis, cell proliferation, and apoptosis of MHCC97H and HepG2 xenografts.** Mice bearing tumor xenografts were treated as described in the Materials and methods. A, Representative pictures of blood vessels stained with anti-CD31; B, proliferative cells stained with Ki-67; C, apoptotic cells stained with anti-cleaved-PARP antibodies in tumors. The bar graph represents the mean ± SD of quantification of CD31-positive areas, Ki-67- and PARP-positive cells from immunohistochemical analysis of tumors. * $P < 0.05$ and **$p < 0.01$, treated group vs. control group. Scale bars, 100 μm.

**Figure 7. Effect of sorafenib and cabozantinib on the metastasis of SK-HEP1 in nude mice.** Following the experimental design as described in the Materials and Methods, nude mice were sacrificed at day 28 to evaluate lung and liver metastases. The image is a representative hematoxylin and eosin (H&E)-stained section of (A) lung and (B) liver metastases, and the average number of foci is presented as the mean ± SD. * $P < 0.05$ and **$p < 0.01$, treated group vs. control group. Scale bars in A and B 40×, 500 μm. Scale bars in A and B 200×, 100 μm.
Figure 1

A. MHCC97L

B. MHCC97H

C. SK-Hep1

D. HepG2

Survival rate (%) vs. Cabozantinib (nM)

IC_{50} = 13.47 nM
IC_{50} = 9.466 nM
IC_{50} = 4306 nM
IC_{50} = 5040 nM
Figure 2

A

C

MHCC97L

MHCC97H

G1  S  G2

MHCC97L

MHCC97H

Cell number

DNA Content

Annexin V-FITC

Cell population (%)

Apoptosis rate (%)

Caboza

B

SK-HEP1

HepG2

DNA Content

Annexin V-FITC

Cell population (%)

Apoptosis rate (%)

Caboza

D

SK-HEP1

HepG2

Cell population (%)

Apoptosis rate (%)

Caboza
Figure 4

A. Cellular morphology of SK-HEP1 and HepG2 cells treated with HGF and Cabozantinib. 

B. Migration assay showing the number of migrated cells under different conditions. 

C. Invasion assay showing the number of invasive cells under different conditions.
Figure 6

A. CD31 staining

Area CD31 density (%)

B. Ki-67 staining

C. Cleaved PARP staining

Proliferation index (%)

Apoptosis index (%)

HepG2  MHCC97H

Control  Cabozantinib (10 mg/kg)  Cabozantinib (30 mg/kg)

MHC97H

Control  Cabozantinib (10 mg/kg)  Cabozantinib (30 mg/kg)

HepG2

Control  Cabozantinib (10 mg/kg)  Cabozantinib (30 mg/kg)

MHCC97H

Control  Cabozantinib (10 mg/kg)  Cabozantinib (30 mg/kg)
Figure 7

A. Control, Sorafenib (30 mg/kg), Cabozantinib (30 mg/kg)

40X, 200X magnification images

B. Control, Sorafenib (30 mg/kg), Cabozantinib (30 mg/kg)

Images of tumors and lung sections

Bar graphs showing:
- No. of metastasis foci
- Lung wet weight (mg)

- Control
- Sorafenib (30 mg/kg)
- Cabozantinib (30 mg/kg)

Significance levels indicated by asterisks: *

* indicates p < 0.05
** indicates p < 0.01
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Cabozantinib suppresses tumor growth and metastasis in hepatocellular carcinoma by a dual blockade of VEGFR2 and MET

Qingfeng Xiang, Weiqiang Chen, Men Ren, et al.

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