Sorafenib Inhibits Hypoxia-Inducible Factor-1α Synthesis: Implications for Antiangiogenic Activity in Hepatocellular Carcinoma

Li-ping Liu, Rocky L. K. Ho, George G. Chen, and Paul B. S. Lai

Abstract

**Purpose:** The overexpression of hypoxia-inducible factor 1α (HIF-1α) is a common finding in hepatocellular carcinoma (HCC), and it leads to angiogenesis and poor prognosis. Sorafenib, a multikinase inhibitor, has shown significant improvement in survival in patients with advanced HCC in clinical trials. However, the mechanisms that account for the antiangiogenic efficiency of sorafenib have not been fully elucidated. The present study aims to explore the effect of sorafenib on HIF-1α expression and activation in HCC cells and xenografts.

**Experimental Design:** HCC cells and xenografts were treated with sorafenib or vehicles. Western blotting and quantitative PCR array were used to determine protein and mRNA expression, respectively. HIF-1α activity, *de novo* protein synthesis, and VEGF secretions were determined using assay kits.

**Results:** Sorafenib dose dependently decreased the hypoxia-induced accumulation and activation of HIF-1α protein. Further analysis revealed that such reduction of HIF-1α was associated with the inhibition of HIF-1α protein synthesis rather than the promotion of HIF-1α protein degradation or the reduction of HIF-1α mRNA. Moreover, the phosphorylation levels of mTOR, extracellular signal-regulated kinase (ERK), p70S6K, RP-S6, 4E-BP1, and eIF4E were significantly suppressed by sorafenib. In *in vivo* studies further confirmed the inhibitory effect of sorafenib on the expression of HIF-1α and VEGF proteins, leading to a decrease in tumor vascularization and growth of the xenografts.

**Conclusions:** Sorafenib-mediated inhibition of HIF-1α synthesis is associated with previously undefined pathways in which mTOR/p70S6K/4E-BP1 and ERK phosphorylation are downregulated. Our preclinical data expand our understanding of sorafenib’s antiangiogenic mechanism of action by inhibiting HIF-1α and VEGF protein expression. *Clin Cancer Res; 18(20); 5662–71. ©2012 AACR.*
HIF-1α translocates to nuclei and dimerizes with HIF-1β to form a functional transcription factor capable of DNA binding at hypoxia response elements (HRE) and the transcriptional activation of target genes (4, 6).

Although the oxygen-dependent regulation of degradation is the primary mechanism of HIF-1α accumulation, HIF-1α can also be regulated at the translational level (4). An increasing body of evidence indicates that some growth factors, cytokines, and the induction of certain oncogenes can stimulate HIF-1α protein synthesis via the activation of the phosphatidylinositol 3-kinase (PI3K)/Akt/mTOR or the Raf/MAP–extracellular signal-regulated kinase (ERK) kinase (MEK)/ERK pathways (7, 9). Activated mTOR and ERK phosphorylate p70 S6 kinase (p70S6K), which, in turn, phosphorylates the ribosomal protein S6 (RP-S6) and the eukaryotic translation initiation factor 4E (eIF4E) binding protein (4E-BP1; refs. 10, 11). The phosphorylation of 4E-BP1 leads to the activation of eIF4E, initiating cap-dependent mRNA translation (4, 10).

Sorafenib, a multikinase inhibitor, has shown promising results for the treatment of advanced HCC in clinical trials (12, 13). The mechanisms of sorafenib’s antitumor activities have been well explored. Evidence has shown that sorafenib inhibits the Raf/MEK/ERK signal pathways and receptor tyrosine kinases, including vascular endothelial growth factor receptor 2 (VEGFR-2), VEGFR-3, Flt-3, c-KIT, and platelet-derived growth factor receptor (PDGFR; refs. 14, 15). While the blocking of VEGFR and PDGFR may account for the antiangiogenesis effect of sorafenib in HCC, sorafenib, and sunitinib can also inhibit the expression of HIF-1α in neuroblastoma and colon cancer cells (16, 17). These findings may suggest the potential role of HIF-1α in mediating antiangiogenesis effect of sorafenib in HCC. Thus, the present study aims to explore the effect of sorafenib on HIF-1α and VEGF in cultured HCC cells and tumor xenografts in mice.

Materials and Methods

HCC cell lines and cell cultures

Human HCC cell lines PLC/PRF/5, HepG2, and Hep3B were cultured with Dulbecco’s Modified Eagle Media (DMEM) supplemented with 10% FBS in a humidified atmosphere of 95% air and 5% CO2 at 37°C (normoxic conditions). For hypoxic stimulation, cells were placed in a hypoxia incubator filled with a mixture of 1% O2, 5% CO2, and 94% N2 at 37°C.

Reagents and antibodies

Sorafenib (free base) and sorafenib tosylate were purchased from LC Laboratories. Dimethyl sulfoxide (DMSO), MG132, cycloheximide (CHX), cobalt chloride (CoCl2), and deferoxamine (DFX) were obtained from Sigma-Aldrich. The inhibitors LY294002, rapamycin, and PD98059, and the primary antibodies for phospho-Akt (Ser473), phospho-p70S6K (Thr389), phospho-ERK1/2 (Thr202/Tyr204) were purchased from Cell Signaling Technology. The primary antibodies for HIF-1α, VEGF, CD31, HA, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin, and β-tubulin were purchased from Santa Cruz Biotechnology.

Western blotting

Cells (~80% confluence) in DMEM supplemented with 10% FBS were treated with sorafenib and/or other agents for the indicated periods of time under normoxic (20% O2) or hypoxic (1% O2) conditions. DMSO was applied as vehicle control for sorafenib treatments. The cells were harvested for protein isolation. Western blot analysis was conducted as previously described (18).

Quantitative real-time RT-PCR

Total RNA was isolated and real-time PCR was conducted as previously described (18) with the following primer sets: HIF-1α, 5′-ACTTCTGGATGCTGTATTTG-3′ and 5′-GCTTCGCGTGTGTGTTTTC-3′; VEGF, 5′-TGCTCACTCCACCATGCCA-3′, and 5′-GAAGATGTCACAGGCTCTCG-3′; β-actin, 5′-GTCACCGCAATGCTCTCA-3′, and 5′-TGCTGTCACCTTCACCCGTC-3′.

Transient transfection and luciferase assays

PLC/PRF/5 cells were transfected with empty vector, HA-tagged HIF-1α, P402A/P564A-HIF-1α, or pBl-GV-V6l constructs using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instruction. The expression of HA-tagged HIF-1α and P402A/P564A-HIF-1α was analyzed by Western blotting using an anti-HA antibody. The luciferase assays were conducted with the Luciferase Assay System (Promega).
Enzyme-linked immunosorbent assay

PLC/PRF/5 cells seeded in 6-well plates were treated with various concentrations of sorafenib under normoxic or hypoxic conditions 16 hours later, the supernatants were collected and the cell numbers for each well were counted. VEGF levels in the supernatants were determined with the Quantikine human VEGF ELISA Kit (R&D Systems) and normalized to the cell numbers.

HIF-1α pulse-labeling and immunoprecipitation

PLC/PRF/5 cells were pretreated with a vehicle, CHX, or sorafenib for 4 hours and labeled with Click-IT AHA (Invitrogen) for 2 hours. Whole cell lysates were then prepared using radioimmunoprecipitation assay buffer (RIPA). HIF-1α was immunoprecipitated with anti–HIF-1α monoclonal antibody and analyzed using a Click-IT Biotin Protein Analysis Detection Kit (Invitrogen) and Western blotting, according to the manufacturer’s instructions.

Tumor xenograft assays

All animal procedures were approved by the Animal Experimentation Ethics Committee of the Chinese University of Hong Kong and are in accordance with the Department of Health (Hong Kong) guidelines in Care and Use of Animals. Male nude mice (6–8 weeks old) were purchased from the Laboratory Animal Services Centre of our institute. To generate HCC xenografts, 200 μL of PLC/PRF/5 cell suspension (2.5 × 10⁶/mL) was injected subcutaneously (s.c.) in the left flank of each mouse. Sorafenib tosylate, dissolved in Cremophor EL/95% ethanol (50:50; Sigma; ref. 15), was administered orally daily for 16 days at a dose of 100 mg/kg body weight starting from the 14th day post cells implantation with 10 mice per group. Tumor weight was calculated every 2 days using the equation: [length × (width)²]/2]. Tumor masses were harvested 4 hours after the last treatment, followed by either homogenizing in RIPA for Western blot analysis or fixing in 10% formalin for immunohistochemical staining.

Immunohistochemical analysis

Tumor sections were cut and deparaffinized. Antigens were retrieved by microwave. The antibodies and dilutions used for immunohistochemical labeling were as follows: anti-CD31 (1:200), anti-HIF-1α (1:100), and anti-VEGF (1:200). Immunohistochemical staining was conducted using the horseradish peroxidase ABC kit and DNA substrates (Vector Laboratories), with diaminobenzidine as the chromogen. For α-smooth muscle actin (SMA) staining, the α-SMA immunohistochemistry kit (Sigma) was used according to the manufacturer’s instructions. The area of CD31 or α-SMA staining was quantified (×200 magnifications, 9 fields from each slide) by Image J software from NIH.

Statistical analysis

Continuous variables were expressed as a mean with SE and analyzed using the Student t test (2-tailed). A P value less than 0.05 was considered to be statistically significant.

Results

Sorafenib inhibits hypoxia-induced HIF-1α protein accumulation in hepatocellular carcinoma cells

To investigate whether sorafenib affects the cellular expression of HIF-1α protein, we conducted both time-course experiments and dose-response experiments to determine HIF-1α protein alteration in the presence of sorafenib. As expected, HIF-1α protein was undetectable under normoxia in PLC/PRF/5 cells, whereas incubating in 1% oxygen induced an increasing accumulation of HIF-1α, which was detectable after 1 hour using Western blotting. The addition of sorafenib to the culture medium markedly inhibited HIF-1α accumulation, and this inhibition persisted as long as the drug was present, at least up to 24 hours (data not shown; Fig. 1A). Dose-response experiments indicated that sorafenib inhibited hypoxia-induced accumulation of HIF-1α in PLC/PRF/5 cells dose dependently, with complete abrogation at 20 μmol/L (Fig. 1B). In addition, some well-characterized hypoxia mimetic reagents, including CoCl2 and DFX, induced accumulation of HIF-1α could also be abrogated by sorafenib (Fig. 1C). To further confirm the effects of sorafenib, 2 other HCC cell lines, HepG2 and Hep3B, were studied and the accumulation of HIF-1α was also inhibited at 10 μmol/L (Fig. 1D).

Sorafenib decreases hypoxia-induced HIF-1α transcriptional activation and VEGF expression

Naturally, accumulated HIF-1α dimerizes with HIF-1β, binds to HREs, and promotes the transcription of target genes (3). We thus further determined whether sorafenib-mediated inhibition of HIF-1α protein expression would lead to the functional suppression of HIF-1α. The transcriptional activity of HIF-1α was examined in PLC/PRF/5 cells transiently transfected with the luciferase reporter gene construct pBI-GL-V6L, containing 6 tandem repeats of the HRE from the human VEGF gene (19). The non-HRE containing luciferase reporter, empty vector pBI-GL, was applied as a control for nonspecific inhibition of luciferase activity. A substantial increase of luciferase activity was observed in cells cultured in hypoxic conditions, whereas sorafenib dose dependently inhibited hypoxia-induced luciferase activity (Fig. 1E). In accordance with the transcriptional activity of HIF-1α, treatment of PLC/PRF/5 cells with sorafenib led to a dose-dependent inhibition of VEGF mRNA levels, measured by real-time reverse transcriptase (RT-PCR) analysis (Fig. 1F). Consistently, the hypoxic induction of VEGF protein expression was dose dependently inhibited by sorafenib (Fig. 1G). The secretory VEGF protein levels in culture supernatants were also examined using ELISA, and the results were consistent with VEGF data obtained by real-time RT-PCR and Western blot (Fig. 1H).

Sorafenib inhibits the protein synthesis of HIF-1α but not its degradation

Generally, the accumulation of HIF-1α is dependent on the balance between its protein synthesis and degradation. HIF-1α is degraded mainly through the ubiquitin-
proteasome pathway after the hydroxylation of prolines 402 and 564 by prolyl PHD2 (4, 6). To determine whether the reduction of HIF-1α accumulation is mediated by a promotion of protein degradation, we examined the effect of sorafenib on the stability of a HIF-1α mutant with double-proline (HA-tagged P402A/P564A-HIF-1α), which cannot be hydroxylated by PHD2 and accumulates in the cells in an O2-independent manner (8). As shown in Fig. 2A, the protein levels of HIF-1α mutant as determined by Western blotting with an anti-HA antibody, were obviously higher than the wild-type HIF-1α in normoxia. However, sorafenib was equally potent at preventing the accumulation of the HIF-1α mutant and the wild-type HIF-1α. Given that P402A/P564A-HIF-1α cannot be degraded via ubiquitin-proteasome pathway, the involvement of sorafenib in the degradation of HIF-1α can be ruled out. To further confirm this finding, the half-life of HIF-1α was measured in normoxia with or without the presence of sorafenib. At the end of the hypoxic treatment, CHX was added to inhibit the protein synthesis and then the cells were exposed to normoxia for increasing periods up to 30 min, with HIF-1α protein monitored by Western blotting. Under these conditions, HIF-1α protein levels prominently reflected the rate of HIF-1α degradation. As shown in Fig. 2B, the degradation of HIF-1α was similar in the sorafenib-treated and the control cells. Therefore, it is confirmed in our experiments that sorafenib does not facilitate or speed up the degradation of HIF-1α.

We next addressed the possibility that sorafenib may affect HIF-1α production. Because sorafenib did not reduce HIF-1α mRNA levels detected by using real-time RT-PCR in PLC/PRF/5 cells (Fig. 2C), this finding implied that sorafenib may inhibit the translation of HIF-1α mRNA into protein. To test this hypothesis, we labeled the de novo synthesis of HIF-1α with CHX, a global protein synthesis inhibitor, as a positive control. Collectively, the inhibitory effect of sorafenib on HIF-1α accumulation is mediated...
by a decrease in protein synthesis rather than an increase in protein degradation.

**Downregulation of mTOR/p70S6K/4E-BP1 and MAPK signaling by sorafenib correlates with inhibition of HIF-1α synthesis**

A great number of factors contribute to the important biological event of translation initiation. To reveal the underlying mechanisms responsible for the sorafenib-mediated inhibition of the HIF-1α synthesis, we first detected the phosphorylation of these translation initiation factors. As shown in Fig. 3A, in parallel with the alteration of HIF-1α protein, sorafenib dose dependently inhibited the expression of phospho-p70S6K, phospho-RP-S6, phospho-eIF4E, and phospho-4E-BP1 in both normoxia and hypoxia. The protein levels of phospho-mTOR, as the upstream kinases of p70S6K (10, 11), and phospho-ERK, were also inhibited by sorafenib, whereas the phosphorylation level of Akt was significantly increased (Fig. 3B). Similar to sorafenib, rapamycin, an inhibitor of mTOR, suppressed the expression of phospho-mTOR, phospho-p70S6K, and phospho-RP-S6, and induced the phosphorylation level of Akt as well (Fig. 3C). Interestingly, rapamycin also boosted the expression of phospho-4E-BP1 and phospho-eIF4E.

**Sorafenib inhibits the growth and vascularization of HCC xenograft**

To further reveal the effect of sorafenib on the expression of HIF-1α and VEGF in vivo, we treated the HCC xenograft bearing nude mice with 100 mg/kg body weight of sorafenib tosylate for 16 days. As expected, sorafenib tosylate produced significant growth inhibition of s.c. implanted PLC/PRF/5-cell tumor xenografts in nude mice, as shown in Fig. 4A. Tumors were harvested 4 hours after the last treatment and the representative tumor masses are shown in Fig. 4B. Compared with the control group treated with a vehicle, the tumors treated with sorafenib tosylate showed significantly smaller sizes, fewer blood vessels on the tumor surfaces on naked-eye observation and more extensive areas of necrosis with hematoxylin and eosin (H&E) staining. Meanwhile, sorafenib tosylate evidently decreased the microvessel density (MVD) of tumors analyzed by the immunostaining of CD31, a specific endothelial cell marker (22), and
Because of the key roles of HIF-1α and VEGF in tumor angiogenesis, we studied their expression in the tumor tissue sections by immunohistochemistry. Consistent with the findings in cultured cells, sorafenib tosylate significantly decreased the protein levels of HIF-1α and VEGF in tumor tissues (Fig. 5A). In addition, a remarkable reduction in the phosphorylation levels of p70S6K, RP-S6, elf4E, 4E-BP1, mTOR, and ERK was also observed in the tumor lysates from the tumor bearing mice treated with sorafenib tosylate (Fig. 5B). Taken together, our study showed that sorafenib inhibits the synthesis of HIF-1α, likely through the repression of the translation initial factors, and the downregulation of HIF-1α by sorafenib contributes to the inhibition of tumor growth and angiogenesis in HCC. Besides of these findings, several limitations in the in vivo study on sorafenib should not be ignored. Firstly, end of treatment analysis of tumors (day 16 of dosing) was applied instead of using satellite tumor bearing animals that are dosed for 2 to 3 days to determine persistent drug effects on HIF-1 and VEGF expression. Secondly, relatively high dose of sorafenib (100 mg/kg) was tested in our in vivo study as sorafenib has been shown antitumor efficacy between 10 to 30 mg/kg in a HCC preclinical model study (24). Finally, use of biotin based immunohistochemistry detection system potentially increased the background of staining.

Discussion
Numerous stimulating factors contribute to tumor angiogenesis and VEGF is one of the most potent angiogenic factors currently known (25). The overexpression of VEGF is frequently detected in HCC tissues and its expression level is tightly associated with tumor microvessel density, invasion, and metastasis (26, 27). After VEGF binds to its receptors, it functions not only as a proliferating factor, but also as an antiapoptotic for vascular endothelial cells (27). In this study, we have shown that sorafenib suppresses the expression of VEGF in HCC cells and xenografts. Our findings suggest that the inhibition of the angiogenesis via the downregulation of VEGF could be one of the anti-HCC mechanisms offered by sorafenib.

Intratumoral hypoxia is a common finding in solid tumors. Tumor cells adapt to low O2 concentrations by inducing the accumulation of HIF-1α, which dimerizes with HIF-1β and activates the transcription of numerous target genes. The overexpression of HIF-1α in HCC is significantly associated with tumor angiogenesis, invasion and metastasis, treatment resistance and poor prognosis, implying the therapeutic effect via the blocking of HIF-1α (4, 6). This study shows that sorafenib inhibits hypoxia-induced HIF-1α protein accumulation and activation, leading to the decreased expression of VEGF protein and lower MVD. It is worth noting that the inhibition of HIF-1α by sorafenib can contribute not only to the blockade of tumor angiogenesis, but also to the transcriptional inhibition of other genes, involving in tumor growth, invasion and metastasis (4, 6). Thus, HIF-1α appears to play a key role in sorafenib-mediating tumor inhibition. A recent study by Feng and colleagues showed that sorafenib is effective at suppressing postsurgical intrahepatic recurrence and abdominal metastasis of HCC in an orthotopic mouse model (28). Our data are in agreement with this report.
and extends these findings, providing a mechanism by which sorafenib decreases angiogenesis and metastasis of tumor cells.

The level of HIF-1α in cells is dependent on the balance between its protein degradation and protein synthesis. HIF-1α is oxygen-sensitive and is degraded mainly by ubiquitin-proteasome systems (3). Here, we have revealed that sorafenib neither facilitates the degradation of HIF-1α, nor suppresses the expression of HIF-1α mRNA. Interestingly, sorafenib significantly inhibited the synthesis of HIF-1α protein. A variety of oncoproteins, growth factors, and cytokines can regulate HIF-1α protein translation through the activation of the PI3K/Akt/mTOR and Ras/MEK/ERK pathways (29, 30). As a central serine/threonine protein kinase, mTOR controls protein synthesis via the phosphorylation of 2 downstream effectors: the p70S6K that activates RP-S6 and the 4E-BP1, inactivation of which induces the release of eIF4E, promoting cap-dependent mRNA translation (31, 32). In the Ras/MEK/ERK pathway, ERK can activate p70S6K by direct phosphorylation. Moreover, ERK also activates MNK, which phosphorylates eIF4E and stimulates its activity (33, 34). We have shown that sorafenib suppresses the phosphorylation of mTOR and ERK, in addition to their downstream effectors p70S6K, RP-S6, 4E-BP1, and eIF4E, paralleled with the loss of HIF-1α expression. Therefore, given the vital roles of these pathways in regulating HIF-1α translation, our findings strongly indicate that the sorafenib induced suppression of mTOR/p70S6K/4E-BP1 and Ras/MEK/ERK pathways might be involved in the inhibition of HIF-1α synthesis. In addition to effects on HIF-1α synthesis, suppression of the Raf/MEK/ERK signaling pathway may also inhibit the transcriptional activity of HIF-1α because the phosphorylation of the coactivator CBP/p300 by ERK plays a vital role in the transactivation domains interaction (35). Inhibition of eIF4E has been shown to downregulate HIF-1α in various human cancer cell lines (36, 37). In line with these reports, we here uncovered that sorafenib-induced inhibition of HIF-1α was paralleled with the suppression of eIF4E phosphorylation, highlighting the potential role of eIF4E in regulating HIF-1α synthesis. However, if sorafenib suppresses the phosphorylation of eIF4E directly or through blocking of its upstream kinases, 4E-BP1 and ERK, is an interesting issue deserved further investigation.

Our study has shown that sorafenib could induce the phosphorylation of Akt, which plays a critical role in controlling cellular survival and apoptosis. This finding is consistent with previous investigations showing the activation of Akt as a result of mTOR suppression in several human cancer cells (38). It is well established that mTOR can form 2, multiprotein complexes (mTORC1 and mTORC2) that regulate different aspects of mTOR signaling. The suppression of mTORC1 may lead to the activation of mTORC2, increasing Akt activity by direct phosphorylation. Meanwhile, the inhibition of p70S6K also activates Akt by relieving the negative feedback produced by p70S6K, which has been shown to inhibit the P13k/Akt pathway by phosphorylation and inactivating insulin receptor substrate-1 (39). We here show that LY294002, an inhibitor

Figure 4. Sorafenib inhibits the growth and vascularization of HCC xenograft. A, PLC/PRF/5 cells were implanted s.c. in the left flanks of nude mice, which were administered orally daily with vehicle (n = 10) or sorafenib tosylate (100 mg/kg, n = 10) starting from 14th day. Tumor weight was calculated every 2 days. Values represent mean ± SE. *P < 0.05 versus vehicle group. B, representative tumor masses of 2 groups, which were harvested 4 hours after the last treatment. C, tumor sections were stained with H&E or analyzed by immunohistochemistry for CD31 and SMA. The stained areas in 9 fields were quantified and shown with mean ± SE. **P < 0.01 versus vehicle group. Arrows indicate the necrotic areas (black arrows) and positive staining (blue arrows).
of P13K, suppresses the phosphorylation of Akt, mTOR, and subsequent translation initiation factors. Therefore, these findings may indicate a potential benefit if the cancer therapy can use sorafenib in combination with PI3K or Akt signaling inhibitors.

Although the effect of sorafenib on HIF-1α in HCC has not been reported previously, there are experiments examining the relationship between HIF-α subunits and sorafenib or sunitinib, a multiple receptor tyrosine kinase inhibitor, in colon cancer and neuroblastoma cells [16, 17]. Both Nilsson and colleagues and our studies found that sorafenib reduced hypoxia-induced HIF-1α protein [17]. We used cycloheximide to show that sorafenib did not affect the degradation of HIF-1α protein. However, Nilsson and colleagues did not study the stability of HIF-1α. Instead, they used MG132, a proteasome inhibitor, to show that the stability of HIF-2α protein was reduced by sunitinib but not by sorafenib [17]. Shin and colleagues used sunitinib in their study whereas we tested sorafenib [16]. Sorafenib and sunitinib have similar drug profiles and overlapping targets but they have some differences [40]. It appears that both sorafenib and sunitinib can inhibit the level of HIF-1α without affecting the stability of HIF-1α protein [16]. However, Shin and colleagues showed that the reduction of HIF-1α production by sunitinib was associated with the inhibition of the phosphorylation of Akt in colon cancer cells [16]. Our data revealed that sorafenib suppressed HIF-1α synthesis through inhibiting the phosphorylation of mTOR, p70S6K, 4E-BP1, eIF4E, and ERK signalings. We also showed that the expression of phospho-Akt was increased rather than decreasing, which is in line with other studies [41, 42]. The increased level of phospho-Akt is known to serve as a negative feedback mechanism responding to the suppression of mTOR [21, 43]. Thus, the underlying mechanisms of blocking the synthesis of HIF-1α between sorafenib and sunitinib are different. When we try to interpret mechanisms deduced from the results of these experiments, it should bear in mind that these studies are conducted in different types of cells.

In conclusion, through the suppression of mTOR/p70S6K/4E-BP1 and Raf/MEK/ERK signaling pathways, sorafenib inhibits the protein synthesis of HIF-1α to downregulate the expression of VEGF in HCC. The result of our study should expand our understanding of the antiangiogenic mechanisms of sorafenib, and it may help the optimization of anti-HCC treatments.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: L.-P. Liu, G.G. Chen, P. Lai
Development of methodology: L.-P. Liu
 Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L.-P. Liu, G.G. Chen
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L.-P. Liu, P. Lai
Writing, review, and/or revision of the manuscript: L.-P. Liu, G.G. Chen, P. Lai
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L.-P. Liu, R.L.K. Ho, P. Lai
Study supervision: G.G. Chen

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