SC-60, a Dimer-Based Sorafenib Derivative, Shows a Better Anti–Hepatocellular Carcinoma Effect than Sorafenib in a Preclinical Hepatocellular Carcinoma Model

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Abstract

Sorafenib (Nexavar) acts as a tyrosine kinase inhibitor (TKI) against the VEGF receptor (VEGFR) family and Raf-1 (1). According to the survival benefit in several large phase III studies, sorafenib has been proved in renal cell carcinoma and hepatocellular carcinoma since 2006 (2–5). Moreover, sorafenib also displayed a significant clinical benefit for patients with sporadic medullary thyroid cancer (6). However, the low rate of tumor remission and the high cost of treatment prompt us to discover more effective and cheaper therapeutic agents than sorafenib for the treatment of hepatocellular carcinoma. Previously, we have designed a series of sorafenib derivatives and validated the potency of these derivatives as STAT3 inhibitors independent of kinase inhibition (7, 8). We found that SC-60, a dimer-based structure modified from sorafenib, shows a significant anti–hepatocellular carcinoma effect in vitro and in vivo. SC-60 substantially increased SH2 domain-containing phosphatase 1 (SHP-1) phosphatase activity in hepatocellular carcinoma cells and purified SHP-1 proteins, suggesting that SC-60 affects SHP-1 directly. Molecular docking and truncated mutants of SHP-1 further confirmed that SC-60 interferes with the inhibitory N-SH2 domain to relieve the closed catalytic protein tyrosine phosphatase domain of SHP-1. Deletion of N-SH2 domain (dN1) or point mutation (D61A) of SHP-1 abolished the effect of SC-60 on SHP-1, p-STAT3, and apoptosis. Importantly, SC-60 exhibited significant survival benefits compared with sorafenib in a hepatocellular carcinoma orthotopic model via targeting the SHP-1/STAT3–related signaling pathway. In summary, dimer derivative of sorafenib, SC-60, is a SHP-1 agonist and may be a potent reagent for hepatocellular carcinoma–targeted therapy. Mol Cancer Ther; 13(1); 27–36. ©2013 AACR.
access to the catalytic PTP domain (21–25). This closed formation interferes with the exposure of the WPD loop, which contains the active-site residue Asp421 in the catalytic domain (21, 22, 26, 27).

Here, we searched for potent sorafenib derivatives to improve the survival benefit of patients with hepatocellular carcinoma. SC-60, a dimer-based small molecule, provides a new mechanistic option for targeted therapy for hepatocellular carcinoma, and reinforces the role of SHP-1 in tumor proliferation.

Materials and Methods

Cell culture and antibodies

The Huh-7 hepatocellular carcinoma cell line was obtained from the Health Science Research Resources Bank (HSRRB; Osaka, Japan; JCRB0403). The PLC/PRF/5 (PLC5), Sk-Hep-1, HepG2, Huh7, and Hep3B were obtained from American Type Culture Collection (ATCC). All hepatocellular carcinoma cell lines were obtained in 2008. Cells were initially grown and multiple aliquots were cryopreserved and used within 6 months after resuscitation. Sorafenib-resistant cells (SR1) were selected from parental Huh-7 cells after a long-term culture of sorafenib at 10 μmol/L. All cells obtained from HSRRB or ATCC were immediately expanded and frozen such that all the cell lines could be restarted every 3 months from a frozen vial of the same batch of cells. No further authentication was done in our laboratory. Antibodies for immunoblotting such as cyclin D1 and PARP were purchased from Santa Cruz Biotechnology. Other antibodies such as p-STAT3, STAT3, survivin, SOCS-1, SOCS-3, and Myc-tag were from Cell Signaling Technology. SHP-1 antibody was purchased from Abcam.

Reagents

Sorafenib (Nexavar) was kindly provided by Bayer Pharmaceuticals. For cell-based studies, sorafenib at various concentrations was dissolved in dimethyl sulfoxide (DMSO) and then added to the cells in FBS-free Dulbecco’s Modified Eagle Medium. SHP-1 inhibitor (PTP III) was purchased from Calbiochem.

SHP-1 phosphatase activity

After treatment with sorafenib or SC derivatives, PLC5 protein extracts were incubated with anti-SHP-1 antibody in immunoprecipitation buffer overnight. Protein G Sepharose 4 Fast Flow (GE Healthcare BioScience) was added to each sample, followed by incubation for 3 hours at 4°C with rotation. A RediPlate 96 EnzChek Tyrosine Phosphatase Assay Kit (R-22067) was used for SHP-1 activity assay (Molecular Probes, Invitrogen).

Xenograft tumor growth

For the subcutaneous model (n = 10), each mouse was inoculated subcutaneously in the dorsal flank with 1 × 10⁶ PLC5 cells suspended in 0.1 mL of serum-free medium containing 50% Matrigel (BD Biosciences). When tumors reached 100 to 200 mm³, mice received sorafenib or SC-60 (10 mg/kg) orally once daily. Tumors were measured twice weekly using calipers and their volumes were calculated using the following standard formula: width × length × height × 0.523. For the orthotopic model (n = 6), mouse livers were directly inoculated with luc2-expressed PLC5 cells. The treatment initiated when the luciferase activity of mice could be monitored. Mice were randomized into vehicle, sorafenib (10 mg/kg/d), and SC-60 (10 mg/kg/d). The survival curve was determined by the endpoint of treatment.

Other extensive methods are detailed in the Supplementary Methods section.

Results

A dimer-based sorafenib derivative, SC-60, shows antitumor effects in hepatocellular carcinoma cells

To improve the efficiency of sorafenib, we designed a series of dimer-based derivatives and conducted structure–activity relationship studies to define our lead compound, SC-60 (Fig. 1A). First, we assessed growth inhibition in response to SC-60 treatment in a panel of five human hepatocellular carcinoma cell lines: Hep3B, HepG2, PLC5, Huh-7, and sorafenib-resistant hepatocellular carcinoma cells (Huh7-SR; Fig. 1B). SC-60 not only induced significant inhibition of cell viability compared with sorafenib but also demonstrated potent cell death in Huh7-SR cells, which are relatively resistant to sorafenib in long-term exposure. In addition, as shown in Fig. 1C, SC-60 displayed an apoptotic effect at clinically relevant concentrations in a dose-dependent manner in these five cell lines. Although SC-60 did not retain the kinase inhibitory activity of sorafenib, it still demonstrated potent anti–hepatocellular carcinoma activity. We confirmed the kinase-independent function of SC-60 using Raf-1 and VEGFR2 activity (Fig. 1D, left and middle). In contrast with sorafenib, SC-60 did not show potent kinase inhibition. Protein levels of the apoptosis-related molecules, p-Akt, Akt, Bcl-2, Bad, Bax, and Bcl-xL were not changed in cells exposed to SC-60 (Fig. 1D, right). Notably, SC-60 did not significantly downregulate p-ERK, suggesting that the...
effect of SC-60 on Raf-1 may not be important to its anti-hepatocellular carcinoma potency.

Downregulation of p-STAT3 contributes to the apoptotic effect of SC-60 in hepatocellular carcinoma

To elucidate the mechanism by which SC-60 induces apoptosis in hepatocellular carcinoma, we examined the alterations in signal transduction induced by SC-60 in hepatocellular carcinoma. As shown in Fig. 2A, SC-60 downregulated phospho-STAT3 (p-STAT3) at tyrosine 705 in a dose-dependent manner. Therefore, the resistance of Huh7-SR cells to sorafenib may be explained by the activation of p-STAT3 (Fig. 2B). Compared with sorafenib, SC-60 showed significant inhibition of STAT3-related signaling in the submolar range (Fig. 2C). Moreover, SC-60 downregulated p-STAT3 and its related proteins such as cyclin D1 and survivin in a time-dependent manner (Fig. 2D, left). Also, as shown in Fig. 2D (middle), SC-60 showed significant inhibition of STAT3 activity, demonstrated by STAT3 ELISA and luc reporter assay. Importantly, PLC5 cells expressing ectopic STAT3 were insensitive to SC-60–induced STAT3 inhibition and apoptosis (Fig. 2D, right). These results suggest that STAT3 mediates SC-60–induced apoptosis in hepatocellular carcinoma cells.

SHP-1 plays a vital role in SC-60–induced STAT3 inhibition and apoptosis

To investigate how SC-60 downregulates pSTAT3 in hepatocellular carcinoma cells and overcomes the resistance of sorafenib, we next looked at the role of PTP in the effect of SC-60 on pSTAT3. Our data showed that PTPIII, a specific SHP-1 phosphatase inhibitor, reversed the downregulation of pSTAT3 and the apoptotic effect in PLC5 cells after treatment with SC-60 (Fig. 3A, left), suggesting that SC-60 may downregulate p-STAT3 via modulating SHP-1 activity. We then used the siRNA specific to SHP-1 to examine the role of SHP-1 in the molecular events of SC-60. Knockdown of SHP-1 by siRNA in PLC5 cells abolished the effects of SC-60 on p-STAT3 and apoptosis (Fig. 3A, middle). Conversely, ectopic expression of SHP-1 in SC-60–treated cells induced more apoptotic cells and STAT3 inhibition (Fig. 3A, right). To further clarify the role of SHP-1 in SC-60 induction of apoptosis and STAT3 inhibition, we examined the SHP-1 activity in PLC5 cells, IP-SHP-1–containing extract, and purified SHP-1 recombinant protein. SC-60 significantly enhanced the phosphatase activity of SHP-1 in a dose-dependent manner in PLC5 cell lines (Fig. 3B, left). Also, SC-60 activated SHP-1 in SHP-1–containing immunoprecipitation (IP) extract and purified SHP-1 protein at very low concentrations (nmol/L; Fig. 3B, middle and right), suggesting that SC-60 activates SHP-1 through direct interaction with SHP-1 proteins. Notably, SC-60 did not alter the interactions between SHP-1 and STAT3 (Fig. 3C). Also, SC-60 did not affect the phosphorylation of SHP-1 (Y536 and S591), which has been reported to be involved in the regulation of SHP-1 catalytic activity (Fig. 3D; ref. 28).

SC-60 relieves autoinhibited SHP-1 by interfering with the inhibitory N-SH2 domain

As shown in Fig. 4A, the phosphatase activity of SHP-1 was strongly regulated by the N-SH2 domain and the PTP catalytic domain, such as Asp661 (in N-SH2) and Lys362 (in catalytic PTP; refs. 21, 22, 25, 28). As SC-60 did not alter the phospho-status of SHP-1 to activate phosphatase activity, we further validated whether SC-60 increases SHP-1 activity by interfering with intramolecular inhibition. We found significant induction of SHP-1 activity in SC-60–treated wild-type SHP-1 proteins, suggesting that SC-60 increases SHP-1 activity directly; but, SC-60 did not further increase SHP-1 activity in the dN1 or D61A mutants of SHP-1 (Fig. 4B). Of note, the specific residue D61A may form a “closed” inhibitory form of SHP-1, resulting in blocking the entrance for SC-60. PLC5 cells expressing dN1 and D61A mutants were insensitive to SC-60–mediated STAT3 inhibition and apoptotic effect (Fig. 4C). The effect of SC-60 on the N-terminal SH2 domain was confirmed by the dose-escalation assay of dN1 (Fig. 4D). Consequently, our findings indicate that this dimer-based derivative relieves the inhibitory N-SH2 domain to activate the catalytic activity of SHP-1 and induce a better anti–hepatocellular carcinoma effect than sorafenib.

Molecular modeling of the SC-60–SHP-1 complex

In contrast with SC-60, another dimer-based sorafenib derivative, SC-68, did not display a significant antitumor effect in hepatocellular carcinoma cells (Fig. 5A and B), and in agreement, the effect of STAT3 and SHP-1 activity on SC-60 was not found in SC-68–treated hepatocellular carcinoma cells (Fig. 5C). Furthermore, we applied molecular docking to elucidate the discrepancy effect on SC-60 and SC-68 in SHP-1 activation (Fig. 5D). SC-60 was docked into the interface of N-SH2 and PTP domain, which is a key point of SHP-1 activation. The urea structure of SC-60 interacted with the N280 of PTP domain via a hydrogen bond, leading to unveil the catalytic site and elevate SHP-1 catalytic activity (Fig. 5D; ref. 28). Consequently, our findings indicate that this dimer-based derivative relieves the inhibitory N-SH2 domain to activate the catalytic activity of SHP-1 and induce a better anti–hepatocellular carcinoma effect than sorafenib.

SC-60 shows more significant survival benefit and tumor inhibition compared with sorafenib in a preclinical hepatocellular carcinoma model

To evaluate the antitumor effect of SC-60 on hepatocellular carcinoma, we established a preclinical hepatocellular carcinoma orthotopic model using luc2–expressed PLC5 cells inoculated into the liver of nude mice.
Figure 2. Downregulation of p-STAT3 (Y705) is associated with sensitizing effects of SC-60 in hepatocellular carcinoma cells. A, dose-dependent effects of SC-60 on STAT3-related proteins. B, activated-STAT3 was found in sorafenib-resistant Huh7 cells. C, dose-dependent effects of SC-60 and sorafenib on STAT3-related proteins. PLC5 cells were exposed to the indicated doses for 24 hours. D, left, time-dependent assay of STAT3-related signaling pathway in SC-60-treated PLC5 cells. Middle, SC-60 decreases STAT3 activity in the submicro molar range. Top, dose-escalation effects of SC-60 on pSTAT3 in PLC5 cells; bottom, STAT3 reporter analysis of SC-60 in PLC5 cells. PLC5 cells were exposed to the indicated drugs for 24 hours (top) or 12 hours (bottom) for reporter assay. Right, ectopic STAT3 reverses the apoptotic effect of SC-60. PLC5 cells stably expressed STAT3 with Myc-tag, were treated with SC-60 5 μmol/L for 24 hours, and the percentage of apoptosis was measured by sub-G1 analysis. *, P < 0.05; **, P < 0.01.
Figure 3. Inhibition of SHP-1 reverses the effects of SC-60 on p-STAT3 and apoptosis. A, PLC5 cells were pretreated with a specific SHP-1 inhibitor (PTPIII) for 30 minutes before SC-60 treatment. Middle, silencing SHP-1 by siRNA reduces the effects of SC-60 on p-STAT3 in hepatocellular carcinoma cells. PLC5 cells were transfected with control siRNA or SHP-1 siRNA for 24 hours and then treated with sorafenib or SC-1 for another 24 hours. Right, ectopic SHP-1 induces more apoptosis with SC-60. Apoptotic assay was performed by sub-G1 analysis. Columns, mean; bars, SE (n ≥ 3); *, P < 0.05. B, SC-60 induces SHP-1 activity directly. Left, SC-60 increases the phosphatase activity of SHP-1 in hepatocellular carcinoma cell lines. Middle, SC-60 increases the SHP-1 activity in IP-SHP-1–containing cell extract. Right, SC-60 increases the SHP-1 activity in purified SHP-1 recombinant protein. Columns, mean; bars, SE (n ≥ 3); *, P < 0.05; **, P < 0.01. C, SC-60 did not affect the interaction status between SHP-1 and STAT3. D, SC-60 did not affect the phospho-status of SHP-1 (Y536 and S591).
Importantly, SC-60–treated mice displayed significant survival benefits compared with mice treated with vehicle or sorafenib (Fig. 6A). Also, SC-60 had an evident anti–hepatocellular carcinoma effect in PLC-bearing subcutaneous mice with 10 mg/kg/d treatment (Fig. 6B). Compared with sorafenib, SC-60 treatment had an inhibitory effect on tumor growth and the average tumor sizes of animals were less than half of those of control mice at the end of treatment. SC-60–induced molecular events of SHP-1/STAT–related signaling were also found in tumor samples (Fig. 6B, middle and left). Notably, mice inoculated with PLC5 cells expressing STAT3 were insensitive to SC-60 (Fig. 6C). These findings suggest that SC-60 acts as a potent STAT3 inhibitor and SHP-1 enhancer, and thus induces its anti–hepatocellular carcinoma effect via a STAT3–related signaling pathway. To further validate the possibility that SHP-1/STAT3–related signaling is a biomarker in patients with hepatocellular carcinoma, we examined the expression status of SHP-1 and p-STAT3 in clinical patients with hepatocellular carcinoma. STAT3 showed strong cytoplasmic expression in liver cancer cells but mild cytoplasmic expression and negative nuclear expression in adjacent noncancer liver cells. On the contrary, SHP-1 showed moderate cytoplasmic expression with negative nuclear expression in liver cancer cells but mild cytoplasmic expression with negative nuclear expression in adjacent noncancer liver cells (Fig. 6D). Collectively, these results confirm that SC-60, a dimer-based sorafenib derivative, had increased SHP-1 activity that repressed the p-STAT3–related signaling and is involved in tumor inhibition in PLC5 xenograft.

Discussion

The use of TKIs as hepatocellular carcinoma–targeted therapies is an actively researched area. But, in light of several failed clinical trials of VEGFR inhibitors such as sunitinib and brivanib, there are still open questions about what exactly drives drug efficiency. Consequently, further study of determinant factors in addition to their kinase activity is necessary. Here, we put forward a detailed molecular mechanism to explain the resistance of sorafenib, and present a novel dimer-based sorafenib derivative, SC-60, which overcomes this resistance.

Having clarified the molecular mechanism by which sorafenib inhibits the STAT3–related signaling pathway in liver cancer cells, the next step is to translate this knowledge into the clinic. Future studies should focus on evaluating the therapeutic potential of SC-60 in clinical settings. Additionally, understanding the mechanism of resistance to sorafenib and how SC-60 overcomes this resistance could provide valuable insights into developing more effective treatment strategies for hepatocellular carcinoma.

Figure 4. SC-60 potentially relieves autoinhibited SHP-1 to induce apoptosis in hepatocellular carcinoma cells. A, schematic representation of deletion and single mutants of SHP-1. B, SC-60 significantly increases SHP-1 activity in wild-type SHP-1 recombinant proteins in comparison with deletion N-terminal inhibitory domain (dN1) and single mutants of SHP-1 (D61A). SC-60 was incubated with purified SHP-1 protein for 30 minutes at 4°C. C, dN1 and D61A impair SC-60–induced STAT3 signaling and apoptotic effect. Apoptotic assay was performed by sub-G1 analysis. Columns, mean; bars, SE (n ≥ 3); *P < 0.05. D, dN1 affects the SC-60–induced inhibition of STAT3 in a dose–dependent manner.
hepatocellular carcinoma, we then designed novel dimer-based sorafenib derivatives that act as potent STAT3 inhibitors. Screening revealed that SC-60 has better anti-hepatocellular carcinoma effects than sorafenib 

in vitro and in vivo. Notably, SC-60 could further sensitize the sorafenib-resistant hepatocellular carcinoma cells and exhibited better survival benefit than sorafenib in a hepatocellular carcinoma orthotopic model. Moreover, we found that SC-60 is a potent SHP-1 enhancer and that elevated SHP-1 activity significantly correlates to the anti-hepatocellular carcinoma effect. SC-60 strongly activates the purified SHP-1 protein, suggesting that SC-60 affects SHP-1 phosphatase activity directly. Molecular docking and truncated SHP-1 mutants further confirmed our hypothesis about the mechanism by which SC-60 increases SHP-1 activity. In this study, we observed that SC-60 had a docking potential between the inhibitory N-SH2 domain and catalytic PTP of SHP-1. This dimer structure strongly interferes with the "closed" form of SHP-1 and activates its phosphatase activity.

SHP-1 was first identified in hematopoietic cells and is involved in various signaling pathways in the immune system. However, the underlying molecular mechanism by which SHP-1 participates in carcinogenesis and proliferation is still poorly understood. Recently, SHP-2, which shares 70% to 80% sequence similarity with SHP-1, was reported to act as a tumor suppressor in hepatocellular carcinoma carcinogenesis (29). In this study, we validated that the elevated SHP-1 activity further induces an apoptotic effect in hepatocellular carcinoma cells through STAT3 inhibition, and a strong correlation between inhibition of SHP-1 and overexpression of p-STAT3 was found.

Figure 5. Another dimer-based sorafenib derivative, SC-68, did not show an anti-hepatocellular carcinoma effect compared with SC-60. A, SC-68 did not induce significant cell toxicity in hepatocellular carcinoma cell lines. B, SC-68 did not display an apoptotic effect compared with SC-60 in hepatocellular carcinoma cell lines. C, SC-68 did not affect STAT3 or SHP-1 activity in a dose-dependent manner. *, P < 0.05; **, P < 0.01. D, molecular docking model of SC-60, sorafenib, and SC-68 docked into N-SH2 and PTP domain of SHP-1. The N-SH2 domain is shown in gold and the PTP domain is in hot pink. The CDOCKER interaction energy (CDOCKER docking score) for SC-60, sorafenib, and SC-68 are 37.1, 37.4, and 31.7, respectively.
in our clinical hepatocellular carcinoma samples. These data suggest that SHP-1 agonists may have potential in hepatocellular carcinoma–targeting therapy. In conclusion, we generated a new dimer-based sorafenib derivative, SC-60, which is a potent SHP-1 enhancer that showed better antitumor effects than sorafenib in a preclinical hepatocellular carcinoma model.

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

Figure 6. In vivo effects of SC-60 on hepatocellular carcinoma (HCC) xenograft and orthotopic animal models. A, SC-60 treatment resulted in significant tumor growth inhibition and survival benefits in a hepatocellular carcinoma orthotopic model. Left, survival curve of hepatocellular carcinoma orthotopic mice receiving different adjuvant therapies at indicated times. PLC5/luc2–bearing orthotopic mice received sorafenib, SC-60, or vehicle orally at 10 mg/kg/day (n = 6). Right, tumor growth was monitored by IVIS imaging system at the indicated times. B, SC-60 treatment had a significant antitumor effect on subcutaneous PLC5 tumor–bearing mice. Left, mice received SC-60 at 10 mg/kg/day and tumor growth was measured twice weekly. Points, mean; bars, SE (n = 10); **, P < 0.01. Middle, analysis of p-STAT3 and STAT3 in PLC5 tumors. Right, SHP-1 phosphatase activity in SC-60–treated tumor sample. C, SC-60 did not induce a significant tumor inhibition effect in a STAT3–overexpression hepatocellular carcinoma model. D, representative immunohistochemical patterns showed a expression of p-STAT3 and suppressed status of SHP-1 in hepatocellular carcinoma samples (high magnification, ×200).
Grant Support

This study was supported by grants, NSC100-2325-B-002-036 (to K.F. Chen) and NSC100-2325-B-010-007 (to C.W. Shiau), from the National Science Council, Taiwan.

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Received July 23, 2013; revised November 12, 2013; accepted November 14, 2013; published OnlineFirst November 25, 2013.
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