**Single Agent and Synergistic Activity of the "First-in-Class" Dual PI3K/BRD4 Inhibitor SF1126 with Sorafenib in Hepatocellular Carcinoma**

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**Abstract**

Deregulated PI3K/AKT/mTOR, Ras/Raf/MAPK, and c-Myc signaling pathways are of prognostic significance in hepatocellular carcinoma (HCC). Sorafenib, the only drug clinically approved for patients with advanced HCC, blocks the Ras/Raf/MAPK pathway but it does not inhibit the PI3K/AKT/mTOR pathway or c-Myc activation. Hence, there is an unmet medical need to identify potent PI3K/BRD4 inhibitors, which can be used either alone or in combination with sorafenib to treat patients with advanced HCC. Herein, we show that SF1126 (pan PI3K/BRD4 inhibitor) as single agent or in combination with sorafenib inhibited proliferation, cell cycle, apoptosis, and multiple key enzymes in PI3K/AKT/mTOR and Ras/Raf/MAPK pathway in Hep3B, HepG2, SK-Hep1, and Huh7 HCC cell lines. We demonstrate that the active moiety of the SF1126 prodrug LY294002 binds to and blocks BRD4 interaction with the acetylated histone-H4 chromatin mark protein and displaced BRD4 coactivator protein from the transcriptional start site of MYC in Huh7 and SK-Hep1 HCC cell lines. Moreover, SF1126 blocked expression levels of c-Myc in HCC cells. Treatment of SF1126 either alone or in combination with sorafenib showed significant antitumor activity in vivo. Our results establish that SF1126 is a dual PI3K/BRD4 inhibitor. This agent has completed a phase I clinical trial in humans with good safety profile. Our data support the potential future consideration of a phase II clinical trial of SF1126, a clinically relevant dual "first-in-class" PI3K/BRD4 inhibitor in advanced HCC, and a potential combination with sorafenib. Mol Cancer Ther; 15(11): 1–10. © 2016 AACR.

**Introduction**

Human hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver, which represents the fifth most common cancer and second leading cause of cancer-related mortality worldwide (1, 2). Prognosis of HCC is poor, and curative treatments (resection, liver transplantation, local ablation) can only be applied to a limited number of patients as the diagnosis is often made at an advanced stage of the disease (3). Sorafenib (Nexavar) is the first and only FDA-approved drug that is clinically approved for patients with advanced HCC (4–6). Sorafenib is a tyrosine kinase inhibitor that is known to potently inhibit VEGF receptor tyrosine kinases, viz. KDR and FLT4; serine/threonine kinases of RAF family, viz. c-Raf and B-Raf; and various growth factor receptors, specifically platelet-derived growth factor receptor (PDGFR; ref. 7). However, the increased survival benefit related to sorafenib treatment is limited to about 3 months, emphasizing the need for the development of new treatment strategies (8).

Recent publications indicate that HCC cell activation by different factors is known to increase both Ras/Raf/MAPK and PI3K/AKT/mTOR signaling (9). Sorafenib, the only drug for treatment of patients with advanced HCC inhibits Ras/Raf/MAPK pathway (8) but does not directly inhibit the PI3K/AKT/mTOR pathway, which also plays an important role in HCC proliferation. The PI3K pathway is known to be activated in 30% to 50% of HCC cases (10). In HCC, somatic mutation of PIK3CA, enhancement of Akt and phosphorylated ribosomal protein S6, and decrease of PTEN expression have been reported (11–14). These observations suggest that combined targeting of the PI3K/AKT/mTOR and Ras/Raf/MAPK pathway might provide benefit in the treatment of HCC. In this regard, other laboratories have used PI3K/mTOR inhibitors PI-103 and PKI-587 in combination with sorafenib and found that these drugs can synergistically block Ras/Raf/MAPK and PI3K/mTOR pathways (15, 16). However, these drugs are not slated to enter clinical trials for treatment of cancer patients (17–19), hence there is an urgent need to find a PI3K drug that is in clinical trials and can be used to treat HCC patients.

SF1126, a pan-PI3K inhibitor, has shown antitumor and antiangiogenic activity in a number of xenograft models (20–25). Furthermore, this drug has recently been shown to be safe (no...
Materials and Methods

Tissue culture, cell lines, and reagents

The human HCC cell lines, Hep3B, HepG2, SK-Hep1 were obtained from ATCC. The HuH7 cell line was obtained from Japanese Collection of Research Bio resources. All cell lines were authenticated by short tandem repeat DNA profiling at the respective cell banks and were maintained as recommended by the manufacturers. These cells were cultured in DMEM (Invitrogen) supplemented with 10% FBS, 2 mmol/L glutamine and 1% penicillin–streptomycin at 37 °C in a 5% CO₂ atmosphere. SF1126 was provided by SignalRx Pharmaceuticals. JQ1 was a gift from James Bradner (Dana-Farber Cancer Institute, Boston, MA). Antibodies specific for AKT, pAKT, ERK, pERK, pT70S6K, P70S6K, and tri-methyl histone H3 (Lys4) were obtained from Cell Signaling Technology. Normal rabbit IgG, protein A/G agarose beads, and c-Myc antibodies were from Santa Cruz Biotechnology, and anti-BRD4 antibody was obtained from Bethyl Laboratories.

Cell viability and synergy analysis

Hep3B, HepG2, SK-Hep1, and HuH7 cells (4 × 10⁴ cells/well) were plated in 96-well plates in DMEM containing 10% FBS and 2 mmol/L L-glutamine. Cells were incubated overnight and treated with DMSO (0.1% final) or different concentrations of SF1126 or sorafenib alone and in combination (1:1) of sorafenib + SF1126 for 48 hours. AlamarBlue (Roche) was added and plates were incubated at 37°C in 5% CO₂ for 6 hours. Fluorescence signals were read as emission at 590 nm after excitation at 560 nm. IC₅₀ values were calculated by plotting fluorescence intensity versus drug concentration. Synergy studies were performed on these cell lines using Chou-Talalay method reported earlier (39) as described in Supplementary Methods.

Cell cycle and apoptosis studies

For apoptosis studies, cells were treated with inhibitor for 24 hours, followed by caspase-3 activity assay using kit (Roche) or staining with Annexin V FITC antibody and propidium iodide (PI) according to the manufacturer’s instructions (BD Pharmingen) as reported earlier (25). For cell-cycle analysis, DNA content was analyzed with FACSCalibur flow cytometer (BD Biosciences).

Western blot analysis

For all Western blots, 2 × 10⁶ cells were plated in 10-cm tissue culture dishes, were allowed to adhere for 36 hours, and were treated with different concentrations of SF1126 (1, 5, 10, 20 μmol/L), sorafenib (1, 10 μmol/L) either alone or in combination (0.5 μmol/L each or 0.25 μmol/L each) for 30 minutes. Whole cell lysates were prepared using RIPA buffer containing protease inhibitor cocktail (Roche Molecular Biochemicals). Clarified lysates were resolved in 10% SDS-PAGE, transferred to PVDF membrane, and probed for different antibodies.

Molecular modeling of SF1126/LY294002 in BRD4 BD1 site and BRD4 binding assays

The crystallographic atomic coordinates of BRD4-BD1 cocrystallized with JQ1 (PDB code 3MXF) were obtained from the Protein Data Bank (40). To model the binding of LY294002 and JQ1 at the key acetyl-lysine recognition pocket, the PDB file was imported into LeadIT (BioSolveIT GmbH), all water molecules were kept, residues around JQ1 within a grid of 7 Å³ were selected and imported into LeadIT’s standard parameters. Compounds LY294002 and JQ1 were docked using a novel docking calculations. The 3D structures of LY294002 and JQ1 (all hydrogens included) were docked using LeadIT’s standard parameters. Compounds LY294002 and JQ1 were tested for BRD4-1 and BRD4-2 activity by using Histone H4 peptide (1-21) KS/8/12/16Ac-Biotin as a ligand in alpha screen binding assay. The test was performed in collaboration with Reaction Biology.

RNA extraction and reverse transcription PCR

Total RNA was extracted using the Qiagen RNAeasy Kit (Qiagen) and reverse transcribed using iscript cDNA Synthesis Kit (Bio-Rad). Amplification of cDNA was performed with 1 × SYBR Green supermix (Bio-Rad) on a CFX96 Real time system (Bio-Rad). cDNAs were amplified using specific c-Myc primers and primer sequences will be available upon request.

Chromatin immunoprecipitation studies

Huh7 and SK-Hep1 cells were treated with SF1126 (10 μmol/L), JQ1 (1 μmol/L), or vehicle control for 24 hours. Cells were then harvested for chromatin immunoprecipitation (ChIP) assay as previously reported (32) and described in Supplementary Methods. ChIP and input DNA were analyzed by real-time PCR.
analysis as described before (24) using previous published primers against the MYC transcriptional start site (41) and a negative region upstream of MYC (32). Fold enrichment over control antibody and over the negative region upstream of MYC was determined from duplicate PCR reactions according to the formula 
$$2^{\left(\frac{C_{t \text{MYC}} - C_{t \text{control antibody}}}{C_{t \text{negative region}} - C_{t \text{negative region}}}\right)}$$  

**Animal studies**

All procedures involving animals were approved by the University of California San Diego Animal Care Committee. Eight million SK-Hep1 cells or 10 million Huh-7 cells in 100-μL PBS were injected subcutaneously into the right flank of NSG mouse. Tumor dimensions were recorded regularly using Vernier calipers. Tumor volume was measured using the following formula: volume = 0.5 × length × (width)^2. For SF1126 and sorafenib combination studies, treatment was initiated when tumors reached approximately 50 mm^3. After 15 days of implantation of SK-Hep1 and 25 days of Huh-7 tumor implantation, mice were divided into four groups (n = 7–8 mice per group). Mice in group 1 were treated with acidified water (vehicle control), Group 2 treated with SF1126 (50 mg/kg/day), Group 3 treated with sorafenib (25 mg/kg), and Group 4, SF1126 + sorafenib (50 and 25 mg/kg, respectively). Mice in each group were treated for 6 days a week till the termination of the experiment.

**Statistical analysis**

The Student t test was used to evaluate differences observed between experimental groups and to compare tumor volume differences between SF1126 treated, combination treatment of SF1126 and sorafenib, and vehicle-treated controls.

**Results**

SF1126 and sorafenib as single agents or in combination inhibited HCC proliferation in a dose-dependent fashion

To test the effect of SF1126 on HCC cell proliferation, we chose four different established HCC cell lines, Hep3B, HepG2, SK-Hep1, and Huh7. SF1126 as a single agent inhibited proliferation of all the cell lines tested (Fig. 1). The IC_{50} of SF1126 for Hep3B, HepG2, SK-Hep1, and Huh7 cells was found to be 5.05, 6.89, 3.14, and 2.14 μmol/L, respectively (Fig. 1A–D). Importantly, these IC_{50} concentrations are well within the pharmacokinetic levels of SF1126 achieved in the human phase I trial (26). Next, we evaluated the potency of the combination of SF1126 and sorafenib in HCC proliferation.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** SF1126 and sorafenib as single agents or in combination inhibited HCC proliferation. A–D, 4 × 10^4 cells (Hep3B, HepG2, SK-Hep1, and Huh7) were grown in 96-well plates overnight, then treated with SF1126 or sorafenib (0.0978–100 μmol/L) alone and in combination of SF1126 + sorafenib (1:1) for 48 hours followed by addition of Alamar Blue as described in Materials and Methods.
Figure 2.
SF1126 and sorafenib inhibit cell-cycle progression by inducing apoptosis. Hep3B, HepG2, SK-Hep1, and Huh7 cells were treated with SF1126 or sorafenib (1, 5, and 10 μmol/L concentrations) and in combination of SF1126 + sorafenib (1:1) at 0.5 and 2.5 μmol/L concentrations and were incubated for 24 hours. Cell-cycle distribution was determined by DNA staining with PI and flow cytometry. A-D, left shows the fraction of cells in each phase (M, S, and G1 phase) of the cell cycle in Hep 3B, Hep G2, SK-Hep1, and Huh7, respectively. Right, the fraction of cells in G1 phase in all the cell lines. Data are shown as mean ± SEM. *P < 0.05, **P < 0.01; and ***P < 0.001 vs. vehicle, t-test.
All four cell lines were treated with different concentrations of SF1126 and sorafenib. The combination of SF1126 and sorafenib resulted in an increased inhibition of HCC proliferation (Fig. 1). As expected, differences were noted in the sensitivity for each cell line for single agent and combination treatment. For Hep3B cells, IC<sub>50</sub> for sorafenib and combination of SF1126 and sorafenib was found to be 6.52 and 2.74 μmol/L, respectively (Fig. 1A). For HepG2 cells, IC<sub>50</sub> for sorafenib and combination of drugs was found to be 8.6 and 1.79 μmol/L, respectively (Fig. 1B). For SK-Hep1 cells, IC<sub>50</sub> for sorafenib and combination of SF1126 and sorafenib was found to be 7.6 and 0.52 μmol/L, respectively (Fig. 1C). For Huh7 cells, IC<sub>50</sub> are 8.08 and 0.65 μmol/L for sorafenib alone or in combination with SF1126, respectively (Fig. 1D). Analysis of the enhanced cytotoxic effect in combination versus SF1126 or sorafenib alone was performed using the Chou–Talalay method for calculating the combination index (CI), with values <1 suggesting synergism. Combination of SF1126 and sorafenib in Hep G2, SK-Hep1, and Huh7 cells resulted in CI values of 0.6, 0.7, and 0.5, which is consistent with synergistic inhibition of proliferation. For Hep 3B, the CI value is 1.049 suggests an additive effect of the drug combination (Supplementary Table S1).

SF1126 and sorafenib suppresses proliferation of HCC cells by inducing cell-cycle arrest and induced apoptosis

To study the growth-inhibitory mechanisms of SF1126 and sorafenib on HCC cells, we evaluated the effect of these inhibitors on cell-cycle progression and apoptosis. Cell-cycle analysis revealed that SF1126 or sorafenib as single-agent treatment resulted in cell-cycle arrest with a proportional increase in G<sub>0</sub>–G<sub>1</sub>, and a decrease in the number of cells in the S-phase in Hep 3B, Hep G2, SK-Hep1, and Huh7 cells (Fig. 2A–D). The combined targeting of SF1126 and sorafenib on SK-Hep1 cells resulted in substantial increase in the number of cells in G<sub>0</sub>–G<sub>1</sub> phase with very few cells in S-phase (Fig. 2A–D).

To determine whether the cell-cycle arrest phenotype was associated with the induction of apoptosis, Annexin-V FITC staining was performed. Hep3B, HepG2, SK-Hep1, and Huh7 cells were incubated with various concentrations of SF1126 and sorafenib for 48 hours and stained with Annexin V and PI. Table 1 and Supplementary Fig. S1A show that SF1126 and sorafenib at 1 and 10 μmol/L, respectively, dose dependently increase apoptotic cells in SK-Hep1, Huh-7, and Hep3B cell lines. Interestingly, HepG2 cells are not sensitive to the apoptosis-inducing effects of SF1126 or sorafenib either alone or in combination (Supplementary Table S1; Table 1). Supplementary Fig. S1B shows the Annexin-V FITC analysis of SF1126 and sorafenib-treated SK-Hep1 cells.

To further validate these results, cell death assay and caspase-3 assays were performed on SK-Hep1 and Huh7 cells. Supplementary Fig. S2A and S2B revealed that Huh 7 and SK-Hep1 cells treated with SF1126 either alone or in combination with sorafenib had increased cell death. The results shown in Supplementary Fig. S2C and S2D illustrate caspase-3 activity assay performed on Huh 7 and SK-Hep1 cells suggesting that our combined treatment of SF1126 and sorafenib induce apoptosis in both cells. Taken together, these results suggest that SF1126 and sorafenib inhibited cell proliferation through the induction of G<sub>0</sub> arrest and apoptosis in a dose-dependent manner in all the cell lines tested while differences in sensitivity exist.

Combination of SF1126 and sorafenib inhibits multiple key enzymes in PI3K/AKT/mTOR and Ras/Raf/MAPK signaling pathways

SF1126 as a single agent is known to inhibit the PI3K/AKT/mTOR pathway in various cell lines (23, 27, 42). SF1126 at 1 and 10 μmol/L concentrations significantly suppressed phosphorylation of AKT, p70S6K, and 4EBP1 in Hep3B, HepG2, SK-Hep1, and Huh7 cells in a dose-dependent manner as determined by Western blot analysis (Fig. 3). SF1126 suppressed phosphorylation of ERK in Hep3B and Huh 7 cells, whereas there was no effect or mild effect of this drug on pERK levels in SK-Hep1 and HepG2 cells, respectively (Fig. 3). As expected, sorafenib showed no effect on phosphorylation of AKT, p70S6K, and 4EBP1 in HepG2, SK-Hep1, and Huh7 cells (Fig. 3B–D). Interestingly, sorafenib increased PI3K signaling in Hep3B cells (Fig. 3A). Similar to this result, a previous report has demonstrated that blockage/inhibition of only one of the main pathways PI3K/AKT/mTOR or Ras/Raf/MAPK, separately, can result in activation of the other pathway (16). We next tested whether sorafenib alone or in combination with SF1126 can block the key enzymes of PI3K/AKT/mTOR or Ras/Raf/MAPK, separately. As shown in Fig. 3, treatment of cells with combination of SF1126 and sorafenib at 0.5 and 2.5 μmol/L, suppress phosphorylation of AKT, p70S6K, 4EBP1, and ERK in all the cell lines. These results suggest that combined treatment of SF1126 and sorafenib blocked multiple key enzymes in

Table 1. Annexin-V FITC analysis of SF1126 and sorafenib-treated HCC cells

<table>
<thead>
<tr>
<th>Apoptosis</th>
<th>Cells</th>
<th>Control</th>
<th>SF 1 μmol/L</th>
<th>SF 10 μmol/L</th>
<th>Sora 1 μmol/L</th>
<th>Sora 10 μmol/L</th>
<th>SF + Sora 0.5 μmol/L</th>
<th>SF + Sora 2.5 μmol/L</th>
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<tr>
<td>Early stage</td>
<td>Hep3B</td>
<td>0.2 ± 0.05</td>
<td>11.3 ± 1.3</td>
<td>43.9 ± 3.7</td>
<td>10.4 ± 1.6</td>
<td>18.5 ± 2.5</td>
<td>45 ± 4.3</td>
<td>59.6 ± 2.9</td>
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<tr>
<td>Profile</td>
<td>HepG2</td>
<td>0.2 ± 0.01</td>
<td>3.7 ± 0.5</td>
<td>4.4 ± 0.7</td>
<td>0.6 ± 0.2</td>
<td>0.5 ± 0.07</td>
<td>4.5 ± 1.1</td>
<td>4.8 ± 1.6</td>
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<td>Hep3</td>
<td>0.5 ± 0.3</td>
<td>6.8 ± 0.8</td>
<td>16.8 ± 1.8</td>
<td>16 ± 0.1</td>
<td>11.6 ± 2.1</td>
<td>40.1 ± 2.0</td>
<td>39.8 ± 1.7</td>
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<tr>
<td>HepG2</td>
<td>0.5 ± 0.1</td>
<td>11.8 ± 2.1</td>
<td>15.9 ± 2.3</td>
<td>4.7 ± 0.3</td>
<td>16.4 ± 2.4</td>
<td>19.5 ± 1.8</td>
<td>28.6 ± 1.5</td>
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<tr>
<td>Hep3B</td>
<td>0.5 ± 0.2</td>
<td>39.0 ± 3.6</td>
<td>31.6 ± 3.3</td>
<td>12 ± 2.7</td>
<td>34.1 ± 3.1</td>
<td>29 ± 2.5</td>
<td>26 ± 1.1</td>
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<td>HepG2</td>
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<td>15.1 ± 2.1</td>
<td>9.8 ± 1.1</td>
<td>7.0 ± 0.6</td>
<td>8.9 ± 0.8</td>
<td>10.9 ± 1.6</td>
<td>10.4 ± 0.9</td>
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<tr>
<td>Hep3B</td>
<td>0.5 ± 0.0</td>
<td>8.6 ± 0.4</td>
<td>11.7 ± 0.9</td>
<td>2.0 ± 0.03</td>
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<td>44.3 ± 3.2</td>
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<td>HepG2</td>
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<td>35.1 ± 2.4</td>
<td>20.9 ± 1.6</td>
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<td>Hep3B</td>
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<tr>
<td>Hep3B</td>
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<td>HepG2</td>
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<td>43.5 ± 3.8</td>
<td>25.6 ± 11</td>
<td>10.3 ± 2.9</td>
<td>53.4 ± 5.0</td>
<td>53.2 ± 2.2</td>
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<tr>
<td>Hep3B</td>
<td>0.5 ± 0.0</td>
<td>25.6 ± 11</td>
<td>10.3 ± 2.9</td>
<td>53.4 ± 5.0</td>
<td>53.2 ± 2.2</td>
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</table>

NOTE: Induction of apoptosis in Hep3B, HepG2, SK-Hep1, and Huh7 cells treated with different concentrations of SF1126 and sorafenib, either alone or in combination, was determined by flow cytometric analysis using Annexin V/PI staining. Table shows the percentage of cells in early and late stage of apoptosis. Data are presented as mean ± SEM of three independent experiments.

Abbreviations: SF, SF1126; Sora, sorafenib.
PI3K/AKT/mTOR and Ras/Raf/MAPK pathway (Fig. 3). This is correlated with greater antitumor activity in vivo.

The active moiety of SF1126, LY294002 binds to the active site of BRD4 (BD1), inhibits BRD4 binding to acetyl lysine of H4 chromatin mark protein, and displaces BRD4 from the MYC transcriptional start site in HCC

Herein, we investigated the molecular interaction between LY294002, the active moiety of SF1126, and the BRD4 bromodomain binding domain 1 (BD1; ref. 28). We utilized in silico modeling of BRD4-BD1 with LY294002 compared with JQ1 (PDB code: 3MXF) to map the comparative binding orientation and free energy of binding (ΔG, kcal/mol) of these two small molecules in the BRD4-BD1 active site (Fig. 4A, left). We discovered that docking LY294002 (BRD4-BD1 IC50 = 5.3 μmol/L) and JQ1 (BRD4-BD1 IC50 = 33 nmol/L) into the binding sites of 3MXF predicted the expected binding conformations of these two compounds as found in their corresponding BRD4-BD1 crystal structures. The predicted binding affinity trend for LY294002 and JQ1 was in accordance when docked in 3MXF (binding scores = −14.808 and −24.956 kcal/mol, respectively). Alpha screen binding assay performed in collaboration with Reaction Biology showed BRD4 activity of LY294002 (0.5 and 2.5 μmol/L concentrations each) for 30 minutes followed by Western blot analysis. The membrane was probed with antibodies for pAKT(S473), AKT, p70S6K(Thr 389), P70S6K, p4EBP1(Thr 37/46), 4EBP1, pERK(Thr 202/Tyr204), ERK, c-MYC, and β-actin.

Antitumor activity of SF1126 and sorafenib in human xenografts

The antitumor activity of SF1126 in vivo was investigated in a xenograft model using SK-Hep1 and Huh7 cells. Fig. 5A and C illustrate the antitumor efficacy of SF1126 either alone or in combination with sorafenib in SK-Hep1 and Huh-7 cells, respectively. Mice bearing 50 mm3 tumors were treated subcutaneously with SF1126 at 50 mg/kg, six times a week for 3 weeks. The combined treatment of SF1126 (50 mg/kg, six times a week; administered subcutaneously) and sorafenib (administered intraperitoneally; 25 mg/kg, six times a week) significantly blocked SK-
Hep1 and Huh 7 HCC tumor growth in vivo (Fig. 5A and C). SF1126 either alone or with sorafenib produced tumor growth inhibition with no evidence of toxicity, as measured by weight loss relative to control animals or drug related lethality (data not shown). Interestingly, the levels of c-MYC, pAKT, and pERK were decreased in tumors excised from the SF1126 or sorafenib or drug combination treated mice at 1 hour after drug administration (Fig. 5B and D). These results suggest that combined SF1126 and sorafenib treatment suppresses HCC tumor growth through the downregulation of c-MYC, PI3K/AKT/mTOR, and Ras/Raf/MAPK pathways in vivo.

Discussion

This study was focused on the effect of SF1126 alone or in combination with sorafenib in preclinical HCC models. We evaluated HCC proliferation, cell cycle, apoptosis, phosphorylation of the key enzymes in the PI3K/mTOR/Ras/Raf pathways, and antitumor activity in vivo. The conceptual framework for the investigation SF1126 alone or in combination with sorafenib in HCC emerged from the literature in that: (i) PI3K pathway is known to be activated in 30% to 50% of HCC cases (14); (ii) Sorafenib, the only drug FDA approved for use in advanced HCC treatment, does not directly inhibit the PI3K/AKT/mTOR pathway; (iii) the MYC oncogene has been implicated in the pathogenesis and prognosis of HCC. This led to the hypothesis that a novel dual PI3K/BRD4 inhibitor, SF1126 used alone or in combination with sorafenib would demonstrate potent antitumor activity in HCC. Importantly, SF1126 is an IND-enabled drug currently in clinical trials that has shown antitumor and antiangiogenic activity in a number of xenograft models and has recently showed considerable efficacy in B-cell
malignancies in a phase I clinical trial (26). A recent report that the active moiety of SF1126, LY294002 can be cocrystalized in the acetyl-lysine binding pocket of BRD4 prompted us to evaluate the effect SF1126 on the potential BRD4–MYC interaction. We observe in Huh7 and SK-Hep1 HCC cells that SF1126 displaced BRD4 coactivator protein from the transcriptional start site of MYC. Our molecular modeling and BRD4 binding studies demonstrate LY294002 binding to the BRD4 BD1 bromodomain. These findings suggest a novel and interesting function of this drug as it targets both PI3K and MYC transcription in HCC via inhibition of BRD4 interaction with MYC enhancer–promoter elements.

Our cell viability assay experiments indicated that SF1126 or sorafenib, as single agents, inhibited HCC cell proliferation. A combination of SF1126 and sorafenib resulted in synergistic inhibition of HCC cell proliferation in HepG2, SK-hep1, and Huh 7 cells. The synergistic inhibition by the drug combination suggests that both the Ras/Raf/MAPK and PI3K/AKT/mTOR pathways play an important role in HCC cell proliferation. In addition, the results of cell-cycle analysis and apoptosis assays showed that G1 arrest and apoptosis were induced by SF1126 treatment alone and these effects were augmented in combination with sorafenib. These results suggest that the antiproliferative effects of these agents are due to G1 arrest and apoptosis in the HCC cell lines and that the combination increased G1 arrest and apoptosis in vitro.

Western blot experiments demonstrated potent inhibition of key enzymes of both Ras/Raf/MAPK and PI3K/AKT/mTOR pathways when cells were treated with SF1126 and sorafenib. SF1126 monotherapy strongly inhibited phosphorylation of S6K, 4EBP1, and AKT (Ser473). Sorafenib, as single agent, inhibited ERK phosphorylation, but interestingly it stimulated AKT (Ser473) phosphorylation. These results confirm the previous findings that inhibition of one of the canonical pathways may cause stimulation of the pathway in HCC cells (15, 16). This supports the hypothesis that monotherapy in HCC treatment may not be efficacious. Presumably, sorafenib may induce other signals, such as HGF/HGFR, that further stimulate the PI3K/AKT/mTOR pathway. Indeed, sorafenib stimulated HGF secretion in HCC cells promoted c-Met, S6K, and 4EBP1 phosphorylation (43).

Recent studies have found that the Ras/Raf/MAPK and PI3K/AKT/mTOR are the dominant signaling pathways activated in HCC cells (45). Several attempts have been made to target both of these pathways as therapy. In one study, everolimus, known rapamycin analogue, and AEE788, EGF/VEGF inhibitor, blocked tumor growth in Huh7 HCC cells in combination compared with everolimus monotherapy (46). In another study, Newell and colleagues used sorafenib and rapamycin to target mTOR and Ras/Raf/MAPK signaling and decreased proliferation and induced apoptosis in HCC cell lines. In a
Our data show that SF1126, either alone or in combination with additional more potent dual inhibitors of PI3K and BRD4 (48), modeling of this chemotype and compared it with JQ1 to develop active site of BRD4 and we have executed extensive molecular (1-21) K5/8/12/16Ac-Biotin, it has been cocrystallized in the interaction with acetylated lysine residues Histone H4 peptide over, the active moiety of SF1126, LY294002, blocks BRD4 from the MYC transcriptional start site in HCC cell lines. Moreover, SF1126 blocks c-Myc expression and displaces BRD4 MAPK and PI3K/AKT/mTOR pathways in the HCC cell lines. In conclusion, we demonstrate that SF1126 has potent anti-HCC synergistic antitumor activity in vitro and in vivo. The results suggest the consideration of a phase II clinical trial of SF1126 in advanced HCC and a potential combination phase I trial with sorafenib.

Disclosure of Potential Conflict of Interest
J.K. Sicklick reports receiving a commercial research grant from Novartis Pharmaceuticals, Inc., Foundation Medicine, Inc., and Blueprint Medicines, is a consultant/advisory board member for Sirtex Medical Inc. (SIR-Spheres microspheres Advisory Board). Y. Kono has received speakers bureau honoraria from Bayer and Wako and is a consultant/advisory board member for Bayer. J.R. Garlich has ownership interest (including patents) in SignalRx Pharmaceuticals. G.A. Morales is CEO at Innoventyx, LLC. D.L. Durden has ownership interest (including patents) in and is a consultant/advisory board member for SignalRx Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

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References
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