Single agent and synergistic activity of the “first in class” dual PI3K/BRD4 inhibitor SF1126 with Sorafenib in hepatocellular carcinoma

Alok R. Singh1,#, Shweta Joshi1, #, Adam M. Burgoyne2, #, Jason K. Sicklick3, Sadakatsu Ikeda2, Yuko Kono4, Joseph R. Garlich5, Guillermo A. Morales5 and Donald L. Durden1, 5, 6

Author’s affiliations: 1Department of Pediatrics, Moores Cancer Center, University of California San Diego, La Jolla, California, USA, 2Division of Hematology-Oncology, Moores Cancer Center, University of California San Diego, La Jolla, California, USA, 3Division of Surgical Oncology, Moores Cancer Center, University of California San Diego, La Jolla, California, USA, 4Division of Hepatology, Department of Medicine, University of California San Diego, La Jolla, California, USA, 5SignalRx Pharmaceuticals, San Diego, California, USA, 6Division of Pediatric Hematology-Oncology, UCSD Rady Children’s Hospital, UC San Diego Health System, La Jolla, CA.

Corresponding Author: Donald L. Durden, UCSD Department of Pediatrics, Moores Cancer Center, UC San Diego Health System, La Jolla, CA- 92093-0819, Phone: (858)534-3355, Fax: (858)822-0022, E-mail: ddurden@ucsd.edu. # ARS, SJ and AMB contributed equally to this work.

Running Title: Synergistic activity of SF1126 and Sorafenib in HCC

Disclosure of potential conflict of interest: The authors DLD, GM and JRG disclose potential conflict of interest in SignalRx Pharmaceuticals and in the SF1126 drug. The relationship between Dr. Durden and SignalRx has been internally reviewed and approved by the University of California, San Diego in accordance with its conflict of interest policies. GM is the CEO of Innoventyx, LLC. JKS reports receiving commercial research grant from Novartis Foundation medicine and Blueprint Medicines and is a consultant/advisory board member for Sirtex Medical Inc. YK has received honoraria from Bayer Pharmaceuticals and Wako Chemicals and is a consultant for Bayer Pharmaceuticals.
Financial Information: This work was supported by NIH grant RO1 CA94233-09, FDA RO1 grant FD-004385, NIH R41 CA192656 to D. L. Durden and grants from ALSF (Springboard Grant) and Hyundai Hope on Wheels Foundation, Hope Grant and the Olivia Hudson Foundation NIH K08 CA168999 to J. K. Sicklick and P30CA023100 to S. Lippmann.

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 PI-3K/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 Hep 3B, Hep G2, 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 co-activator protein from the transcriptional start site of MYC in Huh7 and SK-Hep-1 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 anti-tumor activity in vivo. Our results establish that SF1126, is a dual PI3K/BRD4 inhibitor. This agent has completed a Phase 1 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” PI-3K/BRD4 inhibitor in advanced HCC and a potential combination with Sorafenib.
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 multi-kinase inhibitor of more than dozen kinases at nanomolar potency, including serine/threonine kinases c-Raf and B-Raf, the receptor tyrosine kinases VEGFR2, VEGFR3, platelet-derived growth factor receptor (PDGFR), FLT3, Ret and c-kit (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. PI3K pathway is known to be activated in 30-50% 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 PI3K/AKT/mTOR and Ras/Raf/MAPK pathway might provide benefit in the treatment of HCC. In this regard, other labs have used PI-3K/mTOR inhibitor 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 PI-3K drug which is in clinical trials and can be used to treat HCC patients.
SF1126, a pan-PI-3K inhibitor, has shown anti-tumor and anti-angiogenic activity in a number of xenograft models (20-25). Furthermore, this drug has recently been shown to be safe (no dose limiting toxicity or hepatotoxicity) and have considerable efficacy in B cell malignancies and a variety of solid tumors in a Phase I clinical trial (26). SF1126 is an RGDS-conjugated LY294002 prodrug, which is designed to exhibit increased solubility and bind to specific integrins within the tumor compartment, resulting in enhanced delivery of the active compound to the tumor vasculature and tumor (27). Recent study has demonstrated that LY294002 the active moiety of SF1126, has been cocrystallized in the active site of BRD4 and inhibits BET bromodomain binding to acetylated lysine binding sites within chromatin (28). The bromodomain and extraterminal domain (BET) proteins recently emerged as important therapeutic targets in several types of hematopoietic cancers (29-32). Bromodomains are protein motifs that primarily bind to acetylated lysine residues, including those on histone tails (33). The BET family proteins (BRD2, BRD3, BRD4, BRDT) contain 2 amino-terminal bromodomains and have recently been recognized in the literature as a therapeutic strategy to target c-Myc (32). c-Myc transcription factor is frequently up-regulated in a variety of human cancers (34), including liver cancer and targeting c-Myc is considered as a novel approach to treat HCC (35). Recent studies have shown that BRD4 is overexpressed in HCC tissues and it promotes tumor growth and epithelial mesenchymal transition in HCC (36, 37). Recent studies have shown that JQ1 reduced HCC cell growth by supressing BRD4 using MYC dependent or independent pathway (37, 38). Focusing on the novel dual inhibitory activity of SF1126 towards PI-3K and BRD4, the aim of this study was to evaluate the anti-proliferative effect of SF1126 and Sorafenib as single agents and in combination and evaluate their effects on the PI3K/AKT/mTOR, Ras/Raf/MAPK and Myc signaling pathways in preparation for a Phase II trial in HCC.

Materials and Methods

Tissue culture, cell lines and reagents
The human HCC cell lines, Hep 3B, Hep G2, SK-Hep1 were obtained from ATCC. 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 suppliers. These cells were cultured in DMEM medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 2 mM 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, pP70S6K, P70S6K and tri-methyl histone H3 (Lys4) were obtained from Cell Signaling Technology (Beverly, MA). Normal rabbit IgG, protein A/G agarose beads and c-Myc antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-BRD4 antibody was obtained from Bethyl Laboratories (Montgomery, TX).

Cell viability and synergy analysis

Hep 3B, Hep G2, SK-Hep1 and Huh7 cells (4 x 10⁴ cells/well) were plated in 96-well plates in DMEM medium containing 10% FBS and 2 mM 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 hr. 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 vs. 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 hrs, followed by caspase-3 activity assay using kit (Roche) or staining with annexin VFITC antibody and propidium iodide (PI) according to manufacturer's instructions (BD, Pharmingen, San Diego,
CA) as reported earlier (25). For cell cycle analysis, DNA content was analyzed with FACS Calibur flow cytometer (BD Biosciences).

Western blot analysis

For all Western blots, 2 x 10^6 cells were plated in 10 cm tissue culture dishes and were allowed to adhere for 36 hrs and were treated with different conc. of SF1126 (1, 5, 10, 20 μM), Sorafenib (1, 10 μM) either alone or in combination (0.5 μM each or 0.25 μM 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 modelling of SF1126/LY294002 in BRD4 BD1 site and BRD4 binding assays.

The crystallographic atomic coordinates of BRD4-BD1 co-crystallized 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, An der Ziegelei 79, 53757 Sankt Augustin, Germany], all water molecules were kept, residues around JQ1 within a grid of 7 Å^3 were selected and used for in silico 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) K5/8/12/16Ac-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, Hilden, Germany) and reverse transcribed using iscript cDNA synthesis kit (Bio-Rad, Hercules, CA). Amplification of cDNA was performed with 1× SYBR green supermix (Bio-Rad, Hercules,
CA) on an CFX96 Real time system (Bio-Rad, Hercules, CA). cDNAs were amplified using specific c-Myc primers and primer sequences will be available upon request.

Chromatin immunoprecipitation (ChIP) studies

Huh7 and SK-Hep1 cells were treated with SF1126 (10 μM), JQ1 (1 μM), or vehicle control for 24 hours. Cells were then harvested for 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 $2^{\Delta \text{Ct MYC} – \text{Ct control antibody}}$ or $2^{\Delta \text{Ct MYC} – \text{Ct negative region}}$, respectively.

Animal studies

All procedures involving animals were approved by the University of California San Diego Animal Care Committee. Eight million SK-Hep1 cells or ten 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 callipers. Tumor volume was measured using the following formula: Volume = 0.5 x length x (width)$^2$. For SF1126 and Sorafenib combination studies, treatment was initiated when tumors reached ~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 mg/kg and 25 mg/kg respectively). Mice in each group were treated for six days a week till the termination of experiment.

Statistical analysis
The Students $t$ 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 4 different established HCC cell lines, Hep 3B, Hep G2, SK-Hep1 and Huh7. SF1126 as a single agent inhibited proliferation of all the cell lines tested (Fig. 1). The IC50 of SF1126 for Hep 3B, Hep G2, SK-Hep1 and Huh7 cells was found to be 5.05 µM, 6.89 µM, 3.14 µM and 2.14 µM respectively (Fig. 1A-D). Importantly, these IC50 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. 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 each single agent and combination treatment. For Hep3B cells, IC50 for Sorafenib and combination of SF1126 and Sorafenib was found to be 6.52 µM and 2.74 µM respectively (Fig. 1A). For Hep G2 cells, IC50 for Sorafenib and combination of drugs was found to be 8.6 µM and 1.79 µM respectively (Fig. 1B). For SK-Hep1 cells, IC50 for Sorafenib and combination of SF1126 and Sorafenib was found to be 7.6 µM and 0.52 µM respectively (Fig. 1C). For Huh 7 cells, IC50s are 8.08 µM, and 0.65 µM 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, SKHep-1 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 G0–G1 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 G0-G1 phase with very few cells in S phase (Fig. 2A-D).

In order to determine if the cell cycle arrest phenotype was associated with the induction of apoptosis, Annexin-V FITC staining was performed. Hep 3B, Hep G2, SK-Hep1 and Huh7 cells were incubated with various concentrations of SF1126 and Sorafenib for 48 h and stained by Annexin V and PI. Table 1 and Fig. S1A shows that SF1126 and Sorafenib at 1 and 10µM conc. dose dependently increase apoptotic cells in Sk-Hep-1, 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 Fig. S1A and Table 1). Supplementary Fig. S1B shows the Annexin-V FITC analysis of SF1126 and Sorafenib treated SK-Hep1 cells.

In order to further validate these results, cell death assay and caspase-3 assays were performed on SK-Hep 1 and Huh 7 cells. Supplementary Fig. S2A & B revealed that Huh 7 and SK-Hep-1 cells treated with SF1126 either alone or in combination with Sorafenib had increased cell death. The results shown in Fig. S2C & D illustrate Caspase 3 activity assay performed on Huh 7 and SK-Hep-1 cells suggesting that 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 G1 arrest and apoptosis in a dose-dependent manner in all the cell lines tested but differences in sensitivity exist.
Combination of SF1126 and Sorafenib inhibits multiple key enzymes in PI-3K/AKT/mTOR and Ras/Raf/MAPK signaling pathways

SF1126 as a single agent is known to inhibit PI3K/AKT/mTOR pathway in various cell lines (23, 27, 42). SF1126 at 1 μM and 10 μM conc. significantly suppressed phosphorylation of AKT, p70S6K, and 4EBP1 in Hep 3B, Hep G2, SK-Hep1 and Huh7 cells in a dose dependent manner as determined by Western blot analysis (Fig. 3). SF1126 supressed phosphorylation of ERK in Hep3B and Huh 7 cells while there is 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. 3 B-D). Interestingly, Sorafenib increased PI-3K signaling in Hep 3B cells (Fig. 3A). Similar to this result, 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 if Sorafenib alone or in combination with SF1126 can block the key enzymes of PI3K/AKT/mTOR and Ras/Raf/MAPK pathway. As shown in Fig. 3, treatment of cells with combination of SF1126 and Sorafenib at 0.5 μM and 2.5 μM 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 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 in 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) (28). We utilized in silico modeling of BRD4-BD1 with LY294002 compared to JQ1 (PDB code: 3MXF) to map the comparative binding orientation and free energy of binding ($\Delta G°$, kcal/mole) of these two
small molecules in the BRD4-BD1 active site (Fig 4A, Left panel). We discovered that docking LY294002 (BRD4-BD1 IC$_{50}$ = 5.3 μM) and JQ1 (BRD4-BD1 IC$_{50}$ = 33 nM) into the binding sites of 3MXF predicted the expected binding conformations of these 2 compounds as found in their corresponding BRD4-BD1 crystal structures. The predicted binding affinity trend for LY294002 and JQ1 were 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 and JQ1 using Histone H4 peptide (1-21) K5/8/12/16Ac-Biotin as a ligand (Fig 4A, lower panel) of 5 μM and 33 nM, respectively for BD1. Since the literature supports c-MYC as a clinically relevant target for the treatment of HCC (35) we first determined if SF1126 can block c-Myc expression in HCC cells. Fig. 4B-E showed that SF1126 significantly decreased c-Myc expression in Hep 3B, Hep G2, SK-Hep1 and Huh7 cell lines. In consistency with this results, SF1126 blocked c-MYC protein levels in all the cell lines as shown by Western blot analysis (Fig. 3). It was previously shown that BET bromodomain inhibitors displace BRD4 from the MYC transcriptional start site, so we next investigated if SF1126 is able to displace BRD4 from the MYC promoter. Using chromatin immunoprecipitation (ChIP) with a BRD4 antibody followed by quantitative real-time PCR with primers designed against the MYC transcriptional start site, we observed marked BRD4 localization to the MYC transcriptional start site (>10-fold enrichment in comparison to a non-specific locus upstream of MYC and >100-fold enrichment in comparison to a non-specific control antibody). This BRD4 binding to the MYC transcriptional start site was significantly abrogated by treatment with SF1126 by 10-fold in comparison to a non-specific locus upstream of MYC and by 45-fold in comparison to a non-specific control antibody (Fig. 4 F & G). The BRD4 inhibitor JQ1 was used as a positive control with similar efficacy. These results demonstrate that SF1126 is a dual PI-3K/BRD4 inhibitor that targets multiple signaling nodes dysregulated in HCC.

Antitumor activity of SF1126 and Sorafenib in human xenografts
The anti-tumor activity of SF1126 in vivo was investigated in a xenograft model using SK-Hep1 and Huh7 cells. Fig. 5A & C illustrates the antitumor efficacy of SF1126 either alone or in combination with Sorafenib in SK-Hep-1 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, 6 times a week) (administered subcutaneously) and Sorafenib (administered intraperitoneally) (25 mg/kg, 6 times a week) significantly blocked SK-Hep 1 and Huh 7 HCC tumor growth in vivo (Fig. 5A & 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 h post-drug administration (Fig. 5 B, D). These results suggest that combined SF1126 and Sorafenib treatment suppresses HCC tumor growth through the down-regulation of c-MYC, PI3K/AKT/mTOR and Ras/Raf/MAPK pathways in vivo.

Discussion

The present 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 PI-3K/mTOR/Ras/Raf pathways and anti-tumor activity in vivo. The conceptual framework for the investigation SF1126 alone or in combination with Sorafenib in HCC emerged from the literature in that: 1) PI-3K pathway is known to be activated in 30-50% of HCC cases (14) 2) Sorafenib, the only drug FDA approved for use in advanced HCC treatment, does not directly inhibit the PI3K/AKT/mTOR pathway 3) the MYC oncogene has been implicated in the pathogenesis and prognosis of HCC. This lead to the hypothesis that a novel dual PI-3K/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 which has shown anti-tumor and anti-angiogenic 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 co-crystalized 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-Hep-1 HCC cells, that SF1126 displaced BRD4 co-activator protein from the transcriptional start site of MYC. Our molecular modelling 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 the 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 Huh7 cells. The synergistic inhibition by the drug combination treatment 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 is 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 stimulates the PI3K/AKT/mTOR pathway. Indeed, Sorafenib stimulated HGF secretion in HCC cells promoted c-Met, S6K, and 4EBP1 phosphorylation (43). Increased HCC activation and mobility by HGF were reported to be mediated through PI-3K signaling (44).

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. Villanueva et al. used a rapamycin analogue, everolimus, and an EGF/VEGF inhibitor, AEE788, to block these two pathways in the Huh7 HCC cell line, which resulted in the inhibition of mTOR signaling in vitro and decreased tumor progression and increased survival in a xenograft model. The combination of everolimus and AEE788 enhanced tumor suppression compared with everolimus monotherapy (46). Newell et al. used Sorafenib and rapamycin to target mTOR and Ras/Raf/MAPK signaling and decreased proliferation and induced apoptosis in HCC cell lines. In a xenograft mouse model, the combination of rapamycin and Sorafenib enhanced tumor necrosis and ulceration compared with Sorafenib alone (47). The failure of a novel MEK inhibitor (AZD6624) to show any clinical benefits in HCC treatment indicates that inhibition of Ras/Raf/MAPK signaling pathway alone is not sufficient in HCC therapy (10).

In the present study, we found that SF1126 blocks both Ras/Raf/MAPK and PI3K/AKT/mTOR pathway in the HCC cell lines. Moreover, SF1126 blocks c-Myc expression and displaces BRD4 from MYC transcriptional start site in HCC cell lines. Moreover, the active moiety of SF1126, LY294002 blocks BRD4 interaction with acetylated lysine residues Histone H4 peptide (1-21) K5/8/12/16Ac-Biotin and has been co-crystallized in the active site of BRD4 and we have executed extensive molecular modeling of this chemotype as compared to JQ1 to develop additional more potent dual inhibitors of PI3K and BRD4 (48). Our data show that SF1126 either alone or in combination with Sorafenib significantly suppressed tumor growth in vivo in SK-Hep1 cells and Huh 7 cells. SF1126 exhibits potent dual inhibition of PI3K and mTOR (mTORC1 and mTORC2). It has high bioavailability and
biosafety. SF1126 has been used in preclinical studies to inhibit 50 human cancer types. Finally, several components are in place to rapidly translate our findings into a Phase II trial in HCC: 1) SF1126 has completed a Phase 1 clinical trial in humans with good safety profile i.e. no MTD was reached and no hepatotoxicity observed (26) 2) a well-established GMP manufacturing and formulation plan has been established 3) a GMP drug supply and an active IND are in place to rapidly move SF1126 into a Phase II trial in advanced HCC and 4) based on the data generated in this report, 3 months ago we submitted a Phase II clinical trial protocol to IND#744451 at the FDA to begin a Phase II clinical trial of SF1126 in advanced HCC. In conclusion, we demonstrate that SF1126 has potent anti-HCC activity in vitro and in vivo. Moreover, the application of SF1126 in combination with the FDA approved agent, Sorafenib, shows synergistic antitumor activity in vitro and in vivo a result which correlates with the inhibition of both canonical Ras/Raf/MAPK and PI3K/AKT/mTOR pathways 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.

Acknowledgements: The authors thank Muamera Zulcic for ordering and maintaining the mice needed for the experiment.
References:

Legend to Table:

Table 1: Annexin-V FITC analysis of SF1126 and Sorafenib treated HCC cells: Induction of apoptosis in Hep 3B, Hep G2, SK-Hep1 and Huh7 cells treated with different conc. 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.

<table>
<thead>
<tr>
<th>Apoptosis Cells</th>
<th>Control</th>
<th>SF 1 µM</th>
<th>SF 10 µM</th>
<th>Sora 1 µM</th>
<th>Sora 10 µM</th>
<th>SF + Sora 0.5 µM</th>
<th>SF + Sora 2.5 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<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>
</tr>
<tr>
<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±0.6</td>
</tr>
<tr>
<td>Sk-Hep1</td>
<td>0.2±0.03</td>
<td>6.8±0.8</td>
<td>16.8±1.8</td>
<td>1.6±0.1</td>
<td>11.6±2.1</td>
<td>40.1±2.0</td>
<td>39.8±1.7</td>
</tr>
<tr>
<td>Huh 7</td>
<td>0.2±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>
</tr>
<tr>
<td>Late stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hep3B</td>
<td>0.0</td>
<td>29.1±3.6</td>
<td>31.6±3.3</td>
<td>41.2±2.7</td>
<td>34.1±3.1</td>
<td>29±2.5</td>
<td>26±1.1</td>
</tr>
<tr>
<td>HepG2</td>
<td>0.0</td>
<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>
</tr>
<tr>
<td>Sk-Hep1</td>
<td>0.0</td>
<td>8.6±0.4</td>
<td>11.7±0.9</td>
<td>2.2±0.03</td>
<td>15.1±1.7</td>
<td>8.9±0.3</td>
<td>44.3±3.2</td>
</tr>
<tr>
<td>Huh 7</td>
<td>0.0</td>
<td>31.7±3.3</td>
<td>35.1±2.4</td>
<td>20.9±1.6</td>
<td>17.3±1.9</td>
<td>33.9±2.8</td>
<td>24.8±1.7</td>
</tr>
<tr>
<td>Total apoptotic cells</td>
<td>0.2±0.05</td>
<td>40.4±4.2</td>
<td>75.5±5.6</td>
<td>51.6±3.5</td>
<td>52.6±4.8</td>
<td>74±5.3</td>
<td>85.6±3.7</td>
</tr>
<tr>
<td>Hep3B</td>
<td>0.2±0.01</td>
<td>18.8±2.4</td>
<td>14.2±1.7</td>
<td>7.6±0.9</td>
<td>9.4±1.1</td>
<td>15.4±2.2</td>
<td>15.2±1.3</td>
</tr>
<tr>
<td>HepG2</td>
<td>0.2±0.03</td>
<td>15.4±1.3</td>
<td>28.5±2.0</td>
<td>3.6±0.2</td>
<td>26.7±2.4</td>
<td>48.7±2.8</td>
<td>84.1±2.6</td>
</tr>
<tr>
<td>Sk-Hep1</td>
<td>0.2±0.1</td>
<td>43.6±3.8</td>
<td>51.0±3.9</td>
<td>25.6±1.1</td>
<td>33.7±2.9</td>
<td>53.4±3.0</td>
<td>53.2±2.2</td>
</tr>
<tr>
<td>Huh 7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Legends to Figures:

Figure 1. SF1126 and Sorafenib as single agents or in combination inhibited HCC proliferation. A-D, 4 x 10^4 cells (Hep 3B, Hep G2, SK-Hep1 and Huh7) were grown in 96 well plate for overnight, then treated with SF1126 or Sorafenib (0.0978 µM - 100 µM) alone and in combination of SF1126 + Sorafenib (1:1) for 48 hrs followed by addition of Alamar Blue as described in Methods.

Figure 2. SF1126 and Sorafenib inhibit cell cycle progression by inducing apoptosis. Hep 3B, Hep G2, SK-Hep1 and Huh7 cells were treated with SF1126 or Sorafenib (1, 5 & 10 µM conc.) and in combination of SF1126 + Sorafenib (1:1) at 0.5 & 2.5 µM conc. and were incubated for 24 hours. Cell cycle distribution was determined by DNA staining with Propidium iodide and flow cytometry. A-D, Left panel 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 panel shows the fraction of cells in G1 phase in all the cell lines. Data is shown as mean ± SEM, *P <0.05, **P <0.01 and ***P <0.001 vs. vehicle, t test.

Figure 3. Combination of SF1126 and Sorafenib inhibits multiple key enzymes in PI-3K/AKT/mTOR and Ras/Raf/MAPK signaling pathways: A-D, Effect of SF1126 or Sorafenib alone and in combination on Hep 3B, Hep G2, SK-Hep1 and Huh7 hepatocellular carcinoma cells. Cells were treated with SF1126 and Sorafenib (1 and 10 µM conc.) alone or in combination (0.5 & 2.5 µM concentrations each) for 30 minutes followed by Western blot analysis. The membrane was probed with antibodies for pAKT (S473), AKT, pP70S6K (Thr 389), P70S6K, p4EBP1 (Thr 37/46), 4EBP1, pERK (Thr 202/Tyr204), ERK, c-MYC and β-actin.

Figure 4. SF1126 displaces BRD4 from the MYC transcriptional start site in HCC A. Upper panel shows molecular modelling diagram depicting molecular interaction between LY294002, the active moiety of SF1126, and the BRD4 bromodomain binding domain 1 (BD1). The figure shows LY294002 and JQ1 docked at the key acetyl-lysine recognition pocket of BRD4-BD1. LY294002 is displayed with brown carbons and JQ1 with magenta carbons. Fig. shows the expected hydrogen-bond interaction between the carbonyl of LY294002 and N140. Water molecules are not displayed for clarity but they were included in the in silico docking experiments. Lower panel shows Alpha screen binding assay showing BRD4 activity of LY294002 and JQ1 using Histone H4 peptide (1-21) K5/8/12/16Ac-Biotin as a ligand. B-E Hep 3B, Hep G2, SK-Hep1 and Huh7 cells were treated with 10 µM and 20 µM of SF1126 for 24 hrs followed by RNA isolation and RTPCR analysis of c-Myc. cMyc levels are normalized expressed relative to Gapdh expression. Data is shown as mean ± SEM, *P <0.05, **P <0.01 and ***P <0.001 vs. control, t test. F, BRD4 binding to the MYC transcriptional start site is abrogated by SF1126. Huh-7 and SK-Hep-1 cells were treated with SF1126 (10 µM), JQ1 (1 µM), or vehicle control for 24 hours and then proceeded for ChIP analysis as described in Methods. Purified immunoprecipitated DNA and input DNA was amplified by real-time quantitative PCR and analysed for enriched binding of BRD4 to
the MYC transcriptional start site in comparison with a non-specific locus upstream of MYC (F) and in comparison with non-specific control antibody (G). Data were normalized and plotted as percentage of vehicle control binding. Asterisks denote p ≤ 0.05 in comparison to vehicle control by unpaired student's t-test.

Figure 5. Anti-tumor effect of SF1126 and Sorafenib in human xenografts: A, C 8 X 10^6 SK-Hep-1 cells (A) or 10 X 10^6 Huh7 (C) cells were injected subcutaneously in NSG mice. After 15 days of SK-Hep1 or 25 days of Huh 7 tumor implantation when tumor volume reached up to ~50 mm^3 mice were divided into four groups (n=7-8 mice per group). Mice in group 1, treated with acidified water (vehicle control), Group 2, treated with SF1126 (50 mg/kg/day), injected subcutaneously Group 3, treated with Sorafenib (25 mg/kg) injected intraperitoneally, and Group 4, SF1126 + Sorafenib (50 mg/kg and 25 mg/kg, respectively), six days a week for 3 weeks. Arrow indicates the treatment start date. (p ≤ 0.001). B, D One hour after the last treatment, the SK-Hep 1 (B) or Huh 7 (D) HCC xenografts were removed and the expression level of pAKT, pERK and c-MYC were analysed by Western blot analysis. Data in A and C is shown as mean ± SEM, *P <0.05, **P <0.01 and ***P <0.001 vs. vehicle control, t test.
Fig. 1

(A) Hep 3B

- SF1126 (IC50 = 5.05 µM)
- Sorafenib (IC50 = 6.52 µM)
- SF1126 + Sorafenib (IC50 = 2.74 µM)

(B) Hep G2

- SF1126 (IC50 = 6.89 µM)
- Sorafenib (IC50 = 8.6 µM)
- SF1126 + Sorafenib (IC50 = 1.79 µM)

(C) SK-Hep1

- SF1126 (IC50 = 3.1 µM)
- Sorafenib (IC50 = 7.6 µM)
- SF1126 + Sorafenib (IC50 = 0.521 µM)

(D) Huh-7

- SF1126 (IC50 = 2.14 µM)
- Sorafenib (IC50 = 8.08 µM)
- SF1126 + Sorafenib (IC50 = 0.658 µM)
Molecular Cancer Therapeutics

Single agent and synergistic activity of the "first in class" dual PI3K/BRD4 inhibitor SF1126 with Sorafenib in hepatocellular carcinoma

Alok R. Singh, Shweta Joshi, Adam M. Burgoyne, et al.

Mol Cancer Ther Published OnlineFirst August 5, 2016.

Updated version Access the most recent version of this article at: doi:10.1158/1535-7163.MCT-15-0976

Supplementary Material Access the most recent supplemental material at: http://mct.aacrjournals.org/content/suppl/2016/08/05/1535-7163.MCT-15-0976.DC1

Author Manuscript Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.