The Role of PI3K/mTOR Inhibition in Combination with Sorafenib in Hepatocellular Carcinoma Treatment

ROBERTO GEDALY[1], PAUL ANGULO[2], CHANGGUO CHEN[1], KATE TOWNSEND CREASY[3,4], BRETT T. SPEAR[3,4], JONATHAN HUNDLEY[1], MICHAEL F. DAILY[1], MALAY SHAH[1] and B. MARK EVERS[1,4]

Departments of [1]Surgery, [2]Internal Medicine, [3]Immunology and [4]Markey Cancer Center, University of Kentucky, College of Medicine, Lexington, KY, U.S.A.

Abstract. *Background: Deregulated RAS/RAF/MAPK and PI3K/AKT/mTOR signaling pathways are found in hepatocellular carcinoma (HCC). This study aimed to test the inhibitory effects of PI-103 (a small molecule inhibitor of PI3K and mTOR) and sorafenib as single agents and in combination on HCC tumorigenesis in an in vivo xenograft model. Materials and Methods: In vitro study: Huh7 proliferation was assayed by 3H-thymidine incorporation and by thiazolyl blue tetrazolium bromide (MTT) assay. Western blots were used to detect phosphorylation of the key enzymes in the two pathways. In vivo study: Human HCC cell line Huh7 was inoculated into nude mice s.c. and the mice were treated with sorafenib (20 mg/kg/day) and PI-103 (5 mg/kg, every 4 days). Tumor size was measured every other day. Tumors were isolated for western blot and Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay detection of apoptosis and signaling pathway enzymes. Results: Our in vitro study found that combination of sorafenib and PI-103 additively inhibited Huh7 proliferation as compared to single-agent treatment. Sorafenib and PI-103 as single agents differentially inhibited or activated key enzymes (MEK, ERK, AKT, mTOR, and S6K) in PI3K/AKT/mTOR and RAS/RAF/MAPK signaling pathways. Combination of sorafenib and PI-103 inhibited all the key enzymes in the two pathways. Our in vivo study demonstrated significant differences between control group, mono-drug groups and drug-combination group ($p<0.05$). Combination of Sorafenib and PI-103 more efficiently inhibited tumorigenesis as compared to mono-drug treatments ($p<0.032$). Conclusion: The combination of PI-103 and sorafenib has the advantage over mono-drug therapy on inhibition of HCC cell proliferation and tumorigenesis by inhibiting both PI3K/AKT/mTOR and RAS/RAF/MAPK signaling pathways.*

Correspondence to: Dr. Roberto Gedaly, 800 Rose Street, Room C453, Lexington, KY 40536-0293, U.S.A. Tel: +1 8593234661, Fax: +1 8592573644, e-mail: rgeda2@uky.edu

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Hepatocellular carcinoma (HCC) is the most common primary neoplasm of the liver and the fifth most common cancer in the world (1). Only a small number of patients with HCC are suitable for curative therapy. The overall median survival time of patients diagnosed with HCC is a few months (2). Recently, transplantation, surgery and ablation, or a combination of transarterial chemoembolization and ablation have been associated with 5-year survival rates in the range from 40 to 70%. There is an important need for the development of adjuvant treatment to continue to improve patient survival. Llovet *et al.* published the impact of sorafenib, a tyrosine kinase inhibitor, on patients with advanced HCC, showing reduced tumor progression and improved survival (3).

Recent studies indicated the genetic and epigenetic impacts that are involved in HCC tumorigenesity from aberrantly activated signal pathways. Among them the RAS/RAF/MAPK and PI3K/AKT/mTOR pathways play an important role in HCC development. The RAS/RAF/MAPK pathway was found in aberrant activity in the majority of HCC at advanced stage as a result of increased signaling from upstream growth factors, and from inactivation of tumor suppressor genes (4). The PI3K/AKT/mTOR pathway is aberrantly activated in 30-50% of HCC cases. The ribosomal protein S6 (RPS6), which is downstream of mTOR/p70S6K signaling, is activated in 50% of patients with HCC (5). Application of novel drugs to target the two pathways may generate promising results in HCC treatment, especially for advanced HCC.

Recently, we studied the effect of sorafenib and PI-103, a dual PI3K/mTOR inhibitor, on inhibition of the proliferation of HCC cell line Huh7. We found that sorafenib and PI-103 as single agents significantly inhibited Huh7 growth by blocking the RAS/RAF/MAPK and PI3K/AKT/mTOR pathways respectively. Combination of the two drugs caused

an additive inhibition of tumor cell growth by blocking both RAS/RAF/MAPK and PI3K/AKT/mTOR pathways (6). The aim of this study was to evaluate the antitumor efficacy of sorafenib and PI-103, both as single agents and in combination, and the mechanisms involved in a Huh7 tumor xenograft model.

Materials and Methods

Cell culture. The human HCC cell line, Huh7 (a gift from Dr. Guangxiang Luo in our Institution) was cultured in Dulbeco's Modified Eagle Media (DMEM) (Invitrogen, Carlsbad, CA, USA) plus 10% heat inactivated fetal bovine serum (FBS) in an incubator at 37˚C with 5% CO_2 in air.

Chemicals and antibodies. Sorafenib p-toluenesulfonate salt (sorafenib tosylate, purity >99%) was purchased from LC Laboratories, Woburn, MA, USA). PI-103 (purity>98%) was purchased from Cayman Chemicals (Ann Arbor, MI, USA). Methyl-^{3}H-thymidine (2 Ci/mmol) was purchased from MP Biomedicals (Costa mesa, CA, USA). Human EGF was purchased from Insight Genomics (Falls Church, VA, USA). Primary antibodies against AKT, phospho-AKT (Ser473), mTOR, phosphor-mTOR (Ser2448), ERK1/2, phosphor-ERK1/2 (Thr 202/204), MEK1/2, phosphor-MEK1/2 (Ser 217/221), p70-S6K, phospho-p70-S6K (Thr389) and secondary goat anti-rabbit-HRP conjugated antibody were purchased from Cell Signaling Technology (Danvers, MA, USA). Positive and negative controls for phosphor-MEK1/2 and phosphor-ERK1/2, protein markers and LumiGlo reagent A/B were also purchased from Cell Signaling Technology (Danvers, MA, USA). Protease and phosphatase inhibitor cocktail and Restore-Plus Stripping Buffer for western blot were purchased from Pierce (Rockford, IL, USA). MTT, GW5074, LY294002, U0126, mouse anti-β-actin antibody, rabbit anti-mouse antibody conjugated with HRP and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

^{3}H-Thymidine incorporation, MTT assay, EGF treatment and western blot. The methods of ^{3}H-Thymidine incorporation, MTT assay, EGF treatment and western blot were performed as previously described (6).

Tumor xenograft model. Female NU/NU nude mice were purchased from Harlan Animal Research Laboratory (Indianapolis, IN, USA), and housed and maintained in the animal facility of the Division of Laboratory Animal Resources in our Institution. Mice received sterile water and irradiated food *ad libitum*. Tumors were generated by harvesting Huh7 cells from mid-log phase cultures using 0.25% trypsin-EDTA (Invitrogen). Cells were then washed and resuspended in a 50% mixture of Matrigel (BD Biosceince, San Diego, CA, USA) in culture medium to final cell number of 3.25×10^7/ml. A volume of 0.2 ml (6.5×10^6) of the cell suspension was injected subcutaneously in the right flank of each mouse (7). The mice were checked for tumor growth every other day and mouse weight was measured every week. When tumors reached 300-500 mm^3 in volume (9 days after inoculation), the mice were divided randomly into four groups (n=5) and drug treatments started. The treatments in the four groups were: control (drug vehicle); sorafenib (20 mg/kg/day); PI-103 (5 mg/kg/every four days); and combination of sorafenib (20 mg/kg/day) and PI-103 (5 mg/kg/every four days). Sorafenib was dissolved in mixture of Cremophor EL and 95% ethanol (50:50) and formulated according to a published method (8). Sorafenib was administered by oral gavage daily. PI-103 was dissolved in 100% dimethyl sulfoxide (DMSO) and administered by intraperitoneal injection every four days. All groups of the mice had the same number of vehicle controls. Tumors were measured using an optical caliper and tumor size was calculated using the following formula: length × $(\text{width})^2$ × 0.4 according to a published method (9). At the end of the experiment (when statistical difference of the drug treatments were observed, or tumor size was too big), sorafenib and PI-103 were administered three hours before euthanizing the mice (7). The tumors were isolated from the mice immediately after euthanizing. Each tumor was cut into two halves, one half was fixed in neutral buffered 10% formalin solution; the other half was used for isolation of tumor lysate.

Tumor lysates and immunoblotting. Dissected tumor tissue was minced and 0.3 g of the minced tissue were immediately homogenized in 1 ml tumor lysis buffer [25 mM Hepes, pH 7.5, 100 mM NaCl, 20 mM β-glycerophosphate, 1.5 mM $MgCl_2$, 0.5 mM EGTA, 0.25 mM EDTA, 1% NP-40, 0.1% 2-mercaptomethanol, 1× proteinase and phosphotase inhibitor cocktail (Pierce, Rockford, IL, USA) plus 1μg/ml of pepstatin A] in a 100×160 mm tube using a probe homogenizer (Ultra-Turrax S10N-5G connected to Disperser T10 Basic from IKA Works, Inc. Wilmington, NC, USA) (10). The crude lysates were transferred to micro-centrifuge tubes and centrifuged at 16,000 g-force (rcf) for 15 min at 4˚C. The supernatants between the lipid layer and the debris were withdrawn with a syringe and kept at –80˚C until use. Equal amounts of proteins (100 μg/lane) were used for western blot analysis for key enzymes in the two pathways and for apoptosis marker of cleaved poly-ADP-ribose-polymerase (PARP) using our published method (6).

TUNEL assay. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining for tumor sections was performed according to the protocol of the TdT-FragEL™ DNA fragmentation detection kit from CalBiochem (San Diego, CA, USA). TUNEL-positive and -negative- areas were determined according to the CalBiochem protocol.

Statistics. All analyses were performed using the software SPSS version 18 (International Business Machines Corporation, Endicott, NY, USA). Data are presented as the mean±SE. One-way ANOVA followed by Tukey correction was used to compare means. The level of statistical significance was set at $p<0.05$.

Results

In vitro study of PI-103 and sorafenib on HCC proliferation and cell signaling. Our *in vitro* study found that PI-103 and sorafenib as single agents inhibited HCC cell line Huh7 proliferation as assayed by ^{3}H-thymidine incorporation and by MTT assay. PI-103 and sorafenib in combination caused additive inhibition of HCC proliferation. Western blot experiments demonstrated that sorafenib as a single agent inhibited key enzymes MEK and ERK1/2 in the RAS/RAF/MAPK signaling pathway; however sorafenib stimulated mTOR and AKT phosphorylation 17% and 41% as compared to controls. PI-103 as a single agent inhibited

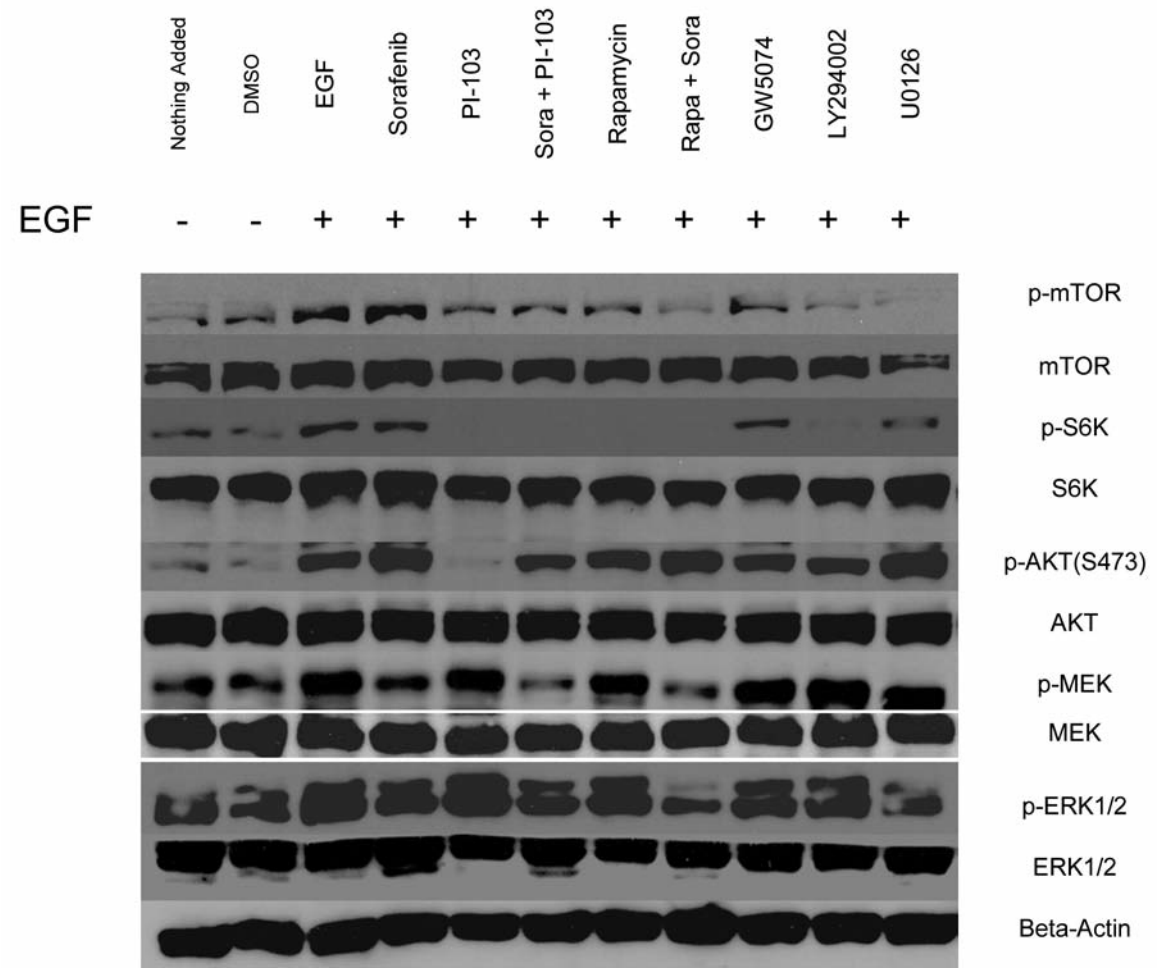

Figure 1. *Sorafenib and PI-103 differentially inhibited or activated phosphorylation of several key enzymes in the RAS/RAF/MAPK and PI3K/AKT/mTOR pathways. Western blot for phosphorylated mTOR (p-mTOR Ser2448), p-S6K (Thr389), p-AKT (Ser473), p-MEK1/2 (Ser217/221) and p-ERK1/2 (Thr202/204) level after different treatments. Also shown are endogenous mammalian target of rapamycin (mTOR), p70S6 kinase (S6K), protein kinase B (AKT), mitogen-signal regulated kinase1/2 (MEK1/2) and extracellular signal regulated kinase 1/2 (ERK1/2). Positive and negative controls for p-MEK and for p-ERK were included in the experiments but are not shown. Phosphorylated kinase band densities were assessed by Scion Image software (Scion Corporation, Frederick, MD, USA) and normalized by β-actin. (Previously published in Anticancer Research in 2010 (6), with permission to present here).*

phosphorylation of key enzymes AKT, mTOR and S6K, however, PI-103 did not inhibit the RAS/RAF/MAPK pathway, instead, PI-103 stimulated phosphorylation of MEK 1/2 (Ser217/221) and ERK1/2 (Thr202/204) 11% and 33%. Combination of PI-103 and sorafenib strongly inhibited both RAS/RAF/MAPK and PI3K/AKT/mTOR pathways. EGF stimulated phosphorylation of MEK1/2 (Ser217/221), ERK1/2(Thr202/204), AKT (Ser473), mTOR (Ser2448) and S6K (Thr389) was inhibited by 70%, 21%, 17%, 54% and 91% respectively (Figure 1) (6). Our *in vitro* experiments indicated that PI-103 as a single agent targets PI3K and mTOR complex 1 and complex 2. Sorafenib as a multi-kinase inhibitor inhibits MEK and ERK phosphorylation. Combination of the two drugs led to a dramatic inhibition over single agent treatment at specific concentrations by preventing increased activity of non-targeted pathways.

Inhibition of tumor growth by PI-103 and sorafenib in xenograft model. For the first 8 and 10 days of drug administration, PI-103 and sorafenib in monotherapy, and in combination therapy inhibited tumor growth. The tumor sizes in drug treated groups were significantly different from the ones of the control group ($p<0.05$). However, in this 8- to 10-day period, we did not observe significant differences between monotherapy and drug combination therapy. After we euthanized the mice in the control group (due to tumor

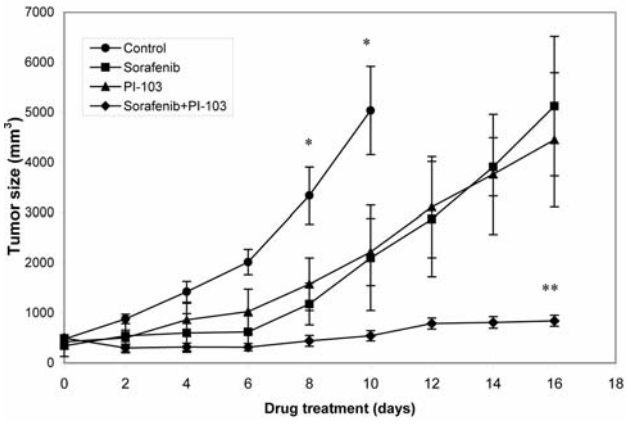

Figure 2. *Growth curves of tumor xenografts. Mice were treated with sorafenib and PI-103 as single agents or in combination. *Control significantly different from mono-drug or drug-combination treatments ($p<0.05$); **drug-combination treatment significantly different from mono-drug treatments ($p<0.032$).*

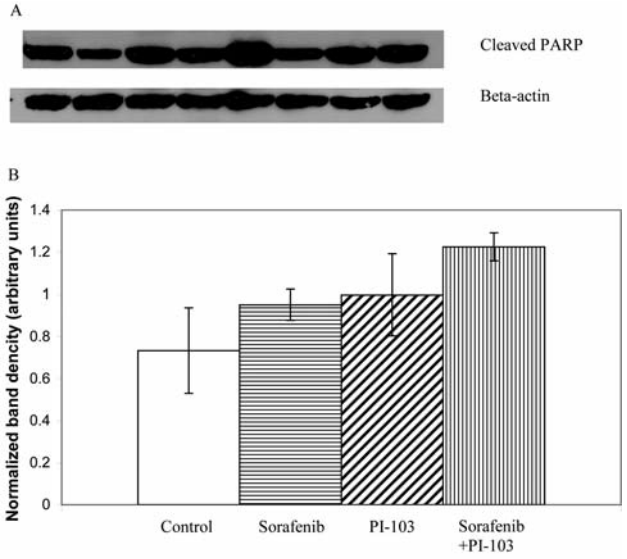

Figure 3. *Sorafenib- and PI-103- induced apoptosis in xenograft tumors assayed by cleaved poly-ADP-ribose-polymerase (PARP). A: Western blot for cleaved PARP (Asp214) in the tumor lysates of the four treated groups. Two individual tumor lysates (100 μg protein/lane) are shown for each treatment. The cleaved PARP band densities were assessed by Scion Image software and normalized by that of β-actin. B: A plot generated based on β-actin-normalized cleaved PARP band densities shown in A.*

development) and extended drug treatment for the monotherapy groups and combination therapy group for another six days, we observed that PI-103 and sorafenib in combination therapy statistically and additively inhibited tumor growth as compared to the monotherapy groups ($p<0.032$) (Figure 2). Mouse weight was not significantly different between the four groups.

Drug induced apoptosis in the tumors. Sorafenib and PI-103 monotherapy and in combination, increased cleaved PARP, marker of apoptosis, in the tumors as compared with the control group. Drug combination increased cleaved PARP by 23% in the tumors compared to that in the monotherapy groups (Figure 3 A, B). This drug increased apoptosis was also observed in TUNEL assay experiments as demonstrated in Figure 4.

Drug inhibition of PI3K/AKT/mTOR pathway in the tumors. We found that AKT was activated in the tumors of control group. Sorafenib had almost no effect on AKT phosphorylation at Ser473 (marker for mTOR complex-2 activity). PI-103 monotherapy and drug combination therapy both noticeably reduced AKT phosphorylation by 65% and 70% respectively (Figure 5 A, B).

Discussion

The incidence of HCC has increased coincident with the increase in hepatitis C incidence. Currently, excellent outcomes have been reported after liver transplantation for HCC, with 5-year survival rates close to 70% (11, 12). Unfortunately, only few patients qualify for transplantation. Most centers in the US and in Europe use the Milan criteria (one lesion less than 5 cm or fewer than three lesions less than 3 cm each), as selection criteria for patient with HCC (13). At the same time, most patients with HCC have cirrhosis and portal hypertension, making liver resection a risky procedure, associated with high morbidity and mortality. It has been reported that fewer than 30% of patients are eligible for surgical resection. Consequently several other treatments have been developed, such as radiofrequency ablation, alcohol ablation and transarterial chemoembolization. More recently, new efforts have been made to use even novel therapies or combination treatments in order to assess their efficacy. It was only recently that Llovet *et al*. published findings of the effect of sorafenib, a multikinase inhibitor, as a single agent in patients with advanced HCC, demonstrating reduced tumor progression and improved survival (3). This was an important step in the treatment of HCC patients, since it was the first time that any drug showed significant response and survival advantage for this patient population at any stage. Recently, the same group with the intent to develop new treatment options for HCC, published data on a xenograft model utilizing a combination of treatment using the mTOR inhibitor rapamycin with sorafenib (9).

Our previous *in vitro* results confirm the theoretical advantage of using drug combinations in HCC treatment to target the two main pathways, RAS/RAF/MAPK and PI3K/AKT/mTOR pathway. We demonstrate that if one pathway is inhibited, the other is activated, which could lead

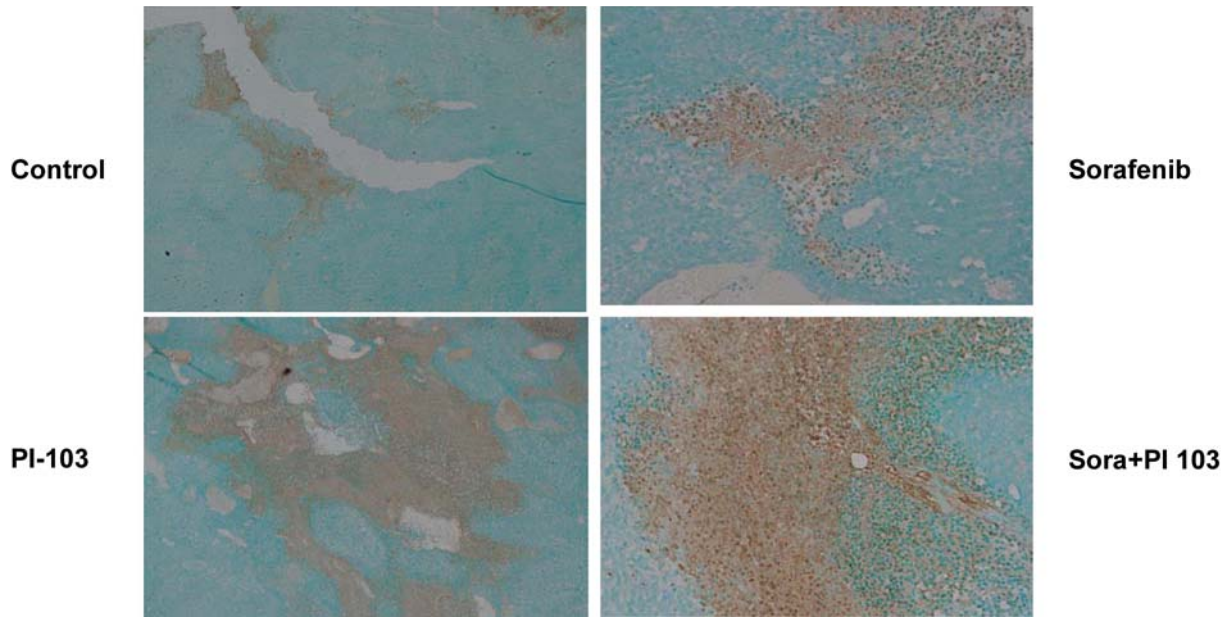

Figure 4. *TUNEL assay indicates drug induced apoptosis in the tumors. Combination of the two drugs induced more apoptosis than mono-drug treatment.*

to cancer cell survival and proliferation. Additionally, we found that if rapamycin is used in combination with sorafenib to treat HCC cells, inhibition of mTOR complex 1 is observed. No inhibition of mTOR complex 2 was observed with this combination, demonstrating undesirable reactivation of AKT which cause cell survival and replication (6). Based on these findings we hypothesized that a drug combination using sorafenib and a dual PI3K/mTOR inhibitor should have a better inhibitory effect on tumor growth, apoptosis and microvascular development. In two different *in vitro* studies, using two different drugs (PI-103 and PKI-587) with a similar mechanism of action (PI3K/mTOR inhibition), we were able to demonstrate an additive inhibitory effect of the two drugs in combination with sorafenib in Huh7 HCC cells (6, 14).

Our xenograft model indicates that PI-103 and sorafenib as single agents can significantly inhibit tumor growth. The combination of the two drugs caused additive inhibition of tumor progression as compared with monotherapy.

In this study we used a lower concentration of sorafenib (20 mg/kg/day) compared to the 30 mg/kg/day used by Newell *et al*. (9) and 10 to 100 mg/kg/day used by Liu *et al*. (7). The dosage of 5 mg/kg/every four days of PI-103 has been safely used previously in a glioma xenograft model (15) and worked well in our HCC xenograft model. We initiated drug treatments when the tumor size was at 300-500 mm^3. This is different from previously published data in which drug treatment was started at 100-300 mm^3 (7, 9). The initiation of treatment at a larger tumor size of 300-500 mm^3

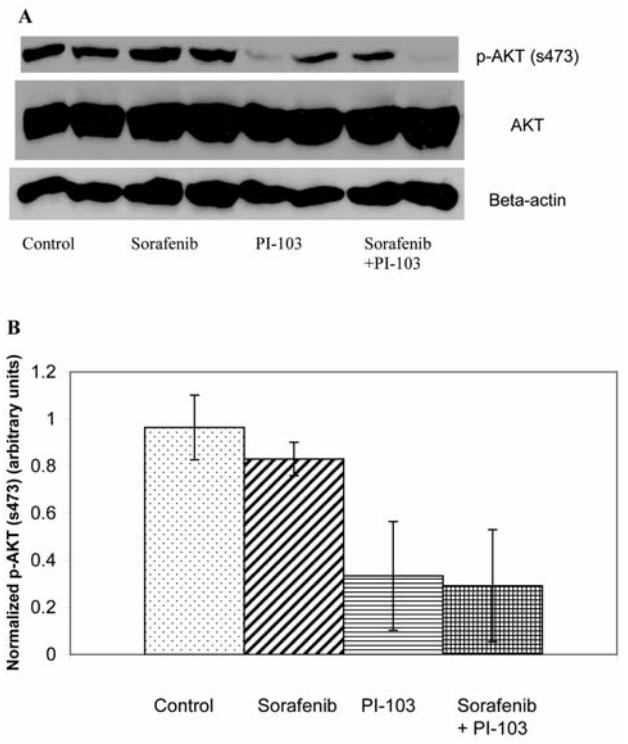

Figure 5. *Sorafenib and PI-103 as single agents and in combination differentially inhibited protein kinase B (AKT) phosphorylation. A: Western blot for p-AKT (Ser473), endogenous AKT and β-actin in the tumor lysates of the four treated groups. The experimental condition and analysis method was as indicated in Figure 3. B: A plot generated based on β-actin-normalized p-AKT band densities shown in A.*

may mimic therapy at advanced HCC stages. We felt it was important to use a larger tumor size in the xenograft model to more accurately reflect the late presentation, common in such tumors treated with chemotherapy without resection or ablation. Significant inhibition of tumor progression was demonstrated by sorafenib and PI-103 as single agents, and in combination ($p<0.05$). However, prolonged treatment was needed to reveal the additive effect of the drug combination treatment.

The apoptosis induced by the drugs in monotherapy and in combination was expected, since PI-103 and sorafenib were previously reported to cause apoptosis in tumor cells (7, 16). The additive effect on apoptosis found by the drug combination treatment is a novel discovery and was demonstrated here twice with western-blot and immunohistochemistry. PI-103 monotherapy and PI-103/sorafenib combination therapy inhibited AKT (s473) phosphorylation in the tumors, which correlates with our *in vitro* findings. Our findings in the xenograft model are relevant to our previous findings *in vitro* (6). However, due to the complexity of tumor development *in vivo*, some of the observations found in vitro, such as the stimulatory effect of sorafenib on the PI3K/AKT/mTOR pathway, and of PI-103 on the RAS/RAF/MAPK pathway were not found in this xenograft model. Our *in vitro* and *in vivo* studies are complementary and illustrate the significance of targeting these two pathways with combination therapy, causing effective suppression of HCC progression and apoptosis. These findings support further investigation in clinical treatment of HCC with a dual PI3K/mTOR inhibitor and sorafenib.

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