Combination of the ERK inhibitor AZD6244 and low-dose sorafenib in a xenograft model of human renal cell carcinoma

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Abstract. Sorafenib, a multikinase inhibitor, is currently used as monotherapy for advanced renal cell carcinoma (RCC). However, adverse effects associated with its use have been experienced by some patients. In this study, we examined the antitumor and antiangiogenic activities of low-dose sorafenib in combination with the MEK inhibitor AZD6244 (sorafenib/AZD6244) in a preclinical model of RCC. Primary RCC 08-0910 and RCC 786-0 cells as well as patient-derived RCC models were used to study the antitumor and antiangiogenic activities of sorafenib/AZD6244. Changes of biomarkers relevant to angiogenesis and cell cycle were determined by western immunoblotting. Microvessel density, apoptosis and cell proliferation were analyzed by immunohistochemistry. Treatment of RCC 786-0 cells with sorafenib/AZD6244 resulted in G_1 cell cycle arrest and blockade of serum-induced cell migration. Sorafenib/AZD6244 induced apoptosis in primary RCC 08-0910 cells at low concentrations. *In vivo* addition of AZD6244 to sorafenib significantly augmented the antitumor activity of sorafenib and allowed dose reduction of sorafenib without compromising its antitumor activity. Sorafenib/AZD6244 potently inhibited angiogenesis and phosphorylation of VEGFR-2, PDGFR-β and ERK, p90RSK, p70S6K, cdk-2 and retinoblastoma. Sorafenib/AZD6244 also caused upregulation of p27, Bad and Bim but downregulation of survivin and cyclin B1. These resulted in a reduction in cellular proliferation and the induction of tumor cell apoptosis. Our findings showed that AZD6244 and sorafenib complement each other to inhibit tumor growth. This study provides sound evidence for the clinical investigation of low-dose sorafenib in combination with AZD6244 in patients with advanced RCC.

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Introduction

Renal cell carcinoma (RCC) is the urologic cancer with the highest mortality rate. Approximately one third of patients with RCC exhibit visceral metastasis at the time of diagnosis and approximately 30% of the patients who undergo potentially curative radical nephrectomy develop metastatic disease (1). Cytokine-based immunotherapy with interferon (IFN)-α or interleukin (IL)-2 produces a response rate of only 10-15%. The advent of molecular targeted therapy in the last few years has revolutionized the treatment of metastatic RCC with benefits in terms of disease stabilization, improvement in the quality of life and overall survival (2-5).

The discovery of the molecular links underlying the relationship between Von Hippel-Lindau, hypoxia signaling and VEGF in the biology of clear cell-RCC has led to the development of inhibitors against the VEGF signaling pathway. One of these inhibitors is sorafenib, which inhibits several RTKs including VEGFR-2 and -3, PDGFR-β, the FLT3 and c-KIT receptors (6,7). A randomized phase III trial comparing sorafenib vs. placebo involving approximately 900 patients with clear cell metastatic RCC showed the median progression free survival (PFS) was 5.5 months in the sorafenib arm compared to 2.8 months in the placebo group (3). This study also suggested overall survival benefits in the sorafenib arm compared to placebo (3). However, up to 34% of patients in the sorafenib arm suffered serious adverse events (3), which led to the dose reduction or discontinuation of sorafenib treatment (8). Although the availability of sorafenib is likely to have a considerable clinical impact in RCC, there remains a need for additional treatment options for patients who cannot tolerate full dose sorafenib. This need may be covered by one or more of the novel inhibitors or novel combinations currently being developed.

In a previous study, we reported that sorafenib inhibited RCC xenograft lines regardless of histological subtypes, in a dose-dependent manner. Sorafenib-induced growth suppression was associated with the inhibition of angiogenic targets p-PDGFR-β and p-VEGFR-2 and partial reduction in p-Akt and p-ERK1/2 (9). In the present study, we investigated the effect of a combination of sorafenib and a MEK inhibitor

with a view to achieve sustained antitumor activity with low dose sorafenib, thus minimizing adverse effects. We used a patient-derived RCC xenograft model (9) to test the combination of sorafenib with a MEK inhibitor, AZD6244 (ARRY-142886) (10).

Materials and methods

Drugs and reagents. Research grade Capsitol was purchased from CyDex, Inc., Lenexa, KS, USA. Sorafenib tosylate (BAY 43-9006, Nexavar™, Bayer and Onyx Pharmaceuticals) was purchased from Bayer HealthCare, Leverkusen, Germany. Sorafenib was dissolved in vehicle (30% Capsitol in water) prior to use. AZD6244 was obtained from AstraZeneca (Alderley Park, Macclesfield, UK). Antibodies against p27, S6R, p70S6K, Akt, Rb, VEGFR-2, Raf-1, PDGFR-β, cleaved caspase 3, cleaved PARP, and phosphorylation-specific antibodies against ERK1/2, Rb Ser780 and Ser807/811, Akt Ser473, p70S6KThr421/Ser424, S6R Ser235/236, 4EBP1 Thr70, Histone 3 Ser10, and cdk-2 Thr14/Tyr15 were obtained from Cell Signaling Technology, Beverly, MA, USA. The antibodies against p-PDGFR-β Tyr1021, p-VEGFR-2 Tyr951, ERK1, cyclin B1, cdk-2 and α-tubulin were obtained from Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA. Anti-mouse CD31 antibody was purchased from BioLegend, San Diego, CA, USA.

Cell culture. RCC 08-0910 cells were isolated from RCC 08-0910 tumors as previously described (10). Human RCC 786-0 cells were a gift from Dr Val Macaulay, University of Oxford. They were maintained as monolayer cultures in Hi-Gluc-DMEM (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (growth medium) at 37˚C, 5% CO_2.

To determine the effects of sorafenib/AZD6244 on cell number, RCC 786-0 cells were plated at a density of $2x10^4$ cells per dish and treated with either vehicle, 1 μM sorafenib, 0.5 μM AZD6244, or 1 μM sorafenib plus 0.5 μM AZD6244 in DMEM containing 1% FBS for 72 h. Cell number was determined manually with a hemocytometer. The data are expressed as the mean ± SE.

To study the effect of sorafenib/AZD6244 on the basal expression of the mTOR and ERK signaling pathways, RCC 08-0910 cells were plated at a density of $5x10^6$ cells per 100 mm dish for 24 h. Cells were then treated with either vehicle, 1 μM sorafenib, 0.5 μM AZD6244, or 1 μM sorafenib plus 0.5 μM AZD6244 in DMEM containing 1% FBS. Twenty-four hours post-treatment, cells were harvested for protein extraction and western blot analysis.

Flow cytometry analysis. RCC 768-0 cells were plated at the density of $5x10^5$ and then treated with either vehicle, 1 μM sorafenib, 0.5 μM AZD6244, or 1 μM sorafenib plus 0.5 μM AZD6244 in DMEM containing 1% FBS for 24 h. Cells were fixed in 70% ethanol at 0˚C for 24 h and stained with propidium iodide. Fluorescence intensity of the stained cells was measured using FACSCalibur flow cytometer (BD, San Jose, CA, USA). Data were analyzed using BD CellQuest Pro software. For every measurement, 10,000 events were collected, and gating was set to exclude cell doublets. DNA contents of certain phases were shown as percentages compared to the total DNA content within the gate.

Wound-healing scratch assay. RCC 786-0 cell monolayers grown to confluence on 100 mm culture dishes were wounded by scratching with a pipette tip and treated with either vehicle, 1 μM sorafenib, 0.5 μM AZD6244, or 1 μM sorafenib plus 0.5 μM AZD6244 in DMEM containing 10% FBS for 24 h. The wounds were photographed (10x objective) at 24 h. Each experiment was performed in triplicate.

Xenograft models. This study received ethical approval from our institutional review board (SingHealth CRIB 2008/094/B). All mice were maintained according to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health, USA.

Primary RCCs have previously been used to create xenograft lines (9), of which the following four lines (RCC19-0809, RCC02-0908, RCC07-0408 and RCC05-1109) were used to establish tumors in male SCID mice (Animal Resources Centre, Canning Vale, Western Australia) aged 9-10 weeks. The histological phenotype of the xenografts is conventional clear-cell (RCC07-0408, RCC05-1109, RCC19-0809), and poorly differentiated clear-cell with sarcomatoid (RCC02-0908) RCC. In comparison with the histological features of the clinical specimens, the four established xenografts retain identical histological characteristics compared to the original tumor (9).

Testing the efficacy of sorafenib and AZD6244 on RCC xenografts. To assess the efficacy of sorafenib and AZD6244 in RCC xenografts, mice bearing indicated xenografts (10 per group) were orally administered 200 μl of vehicle (30% Capsitol), 20 mg/kg/day sorafenib, 16 mg/kg/day of AZD6244, or a combination treatment with 20 mg/kg sorafenib and 16 mg/kg of AZD6244. For dose reduction study, 10 mg sorafenib plus 8 mg/kg AZD6244 was used. Growth of established xenografts was monitored at least twice weekly by Vernier caliper measurement of the length (a) and width (b) of the tumor. Tumor volume was calculated as $(a\text{-}b^2)/2$. Animals were sacrificed at a pre-determined duration after the last treatment dose, and body and tumor weights were recorded, with tumors harvested for further molecular analyses to correlate drug responses with tumor biology. Part of the tumor harvest was fixed in neutral buffer containing 10% formalin for immunohistochemistry.

Western blot analysis. To determine the changes in indicated proteins, 3-4 independent tumors from vehicle and sorafenib-treated mice were homogenized separately in lysis buffer as previously described (11). Proteins (80 μg per sample) were analyzed by western blot analysis as previously described (11). Blots were incubated with the indicated primary antibodies and 1:7500 horseradish peroxidase-conjugated secondary antibodies. All primary antibodies were then visualized with the chemiluminescent detection system (Amersham, Pharmacia Biotech).

Immunohistochemistry. For cleaved PARP and p-Histone 3 Ser10 stainings, tumor samples were processed for paraffin embedding and for CD31 staining, they were embedded in

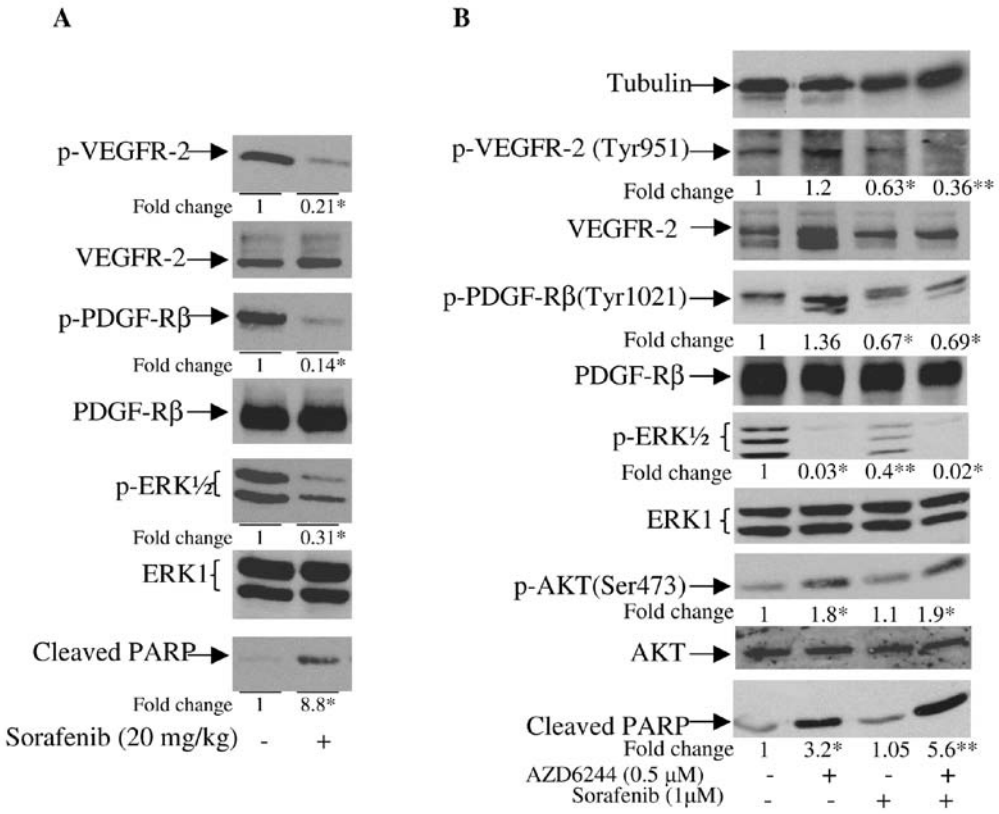


Figure 1. Effects of sorafenib, AZD6244, and sorafenib/AZD6244 on the phosphorylation of VEGFR-2, PDGFR-β, AKT, ERK1/2 and apoptosis. Mice bearing RCC 08-0910 tumors were treated with vehicle or 20 mg/kg sorafenib for 5 days. RCC 08-0910 cells were treated with either vehicle, 1 μM sorafenib, 0.5 μM AZD6244, or 1 μM sorafenib plus 0.5 μM AZD6244 for 24 h, and lysates of tumors or cells were subjected to western blot analysis as described in Materials and methods. Representative blots are shown. The expression levels of protein of interest are expressed as fold of controls (vehicle-treated samples). Asterisks (*) indicate a significant difference among the treatments (P<0.05). Experiments were repeated twice with similar results.

Optimal Cutting Temperature (Sakura Finetek Inc., Torrance, CA, USA). Sections (5 μm) were stained with CD31, p-Histone 3 Ser10 and cleaved PARP antibodies to assess microvessel density, cell proliferation, and apoptosis, respectively, as previously described (10). The number of p-Histone 3 Ser10 positive cells among at least 500 cells per region was counted and expressed as percentage values. For the quantification of mean micro-vessel density in sections stained for CD31, 10 random fields at a magnification of x100 were captured for each tumor.

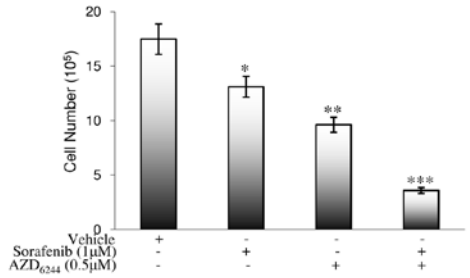
Statistical analysis. For quantification analysis, the sum of the density of bands corresponding to protein blotting with the antibody under study was calculated and normalized with α-tubulin and expressed as fold of controls (the expression level in the vehicle-treated samples). A value greater (lesser) than one indicates that the expression levels of protein of interest was greater (lesser) that in controls. Comparisons of tumor growth over time were performed using ANOVA followed by Student's t-test. Body weight and tumor burden of mice at the point of sacrifice, differences in the levels of protein under study, tumor weight at sacrifice, p-Histone 3 Ser10 index, mean microvessel density, and cleaved PARP-positive cells were compared using Student's t-test. P<0.05 was taken to indicate a statistically significant difference. All the data analysis was performed using statistical software, the Graph-Pad Prism version 4 (GraphPad, CA, USA).

Results

Although sorafenib has been shown to improve overall survival of patients with advanced or metastatic RCC, adverse events are frequent. For some patients, these led to a dose reduction or discontinuation of the treatment (3,8). To search for the signaling pathway that can complement sorafenib, we first performed western blot analysis of primary RCC cells treated with sorafenib. As shown in Fig. 1A, sorafenib was effective in inhibiting phosphorylation of its targeted receptors: VEGFR-2 Tyr951, and PDGFR-β Tyr1021. However, sorafenib only partially inhibited the phosphorylation of ERK1/2, which is one of the key intracellular kinases that regulates cell cycle progression, apoptosis resistance, extracellular matrix remodeling, cellular motility, angiogenesis, and drug resistance (12). These findings suggest that the antitumor effect of sorafenib in RCC could be further improved by combining sorafenib with a MEK inhibitor that abolishes the remaining activity of MEK/ERK.

To test this hypothesis, we first evaluated the *in vitro* activity of sorafenib plus AZD6244 (a MEK inhibitor) using RCC 08-0910 cells. Treatment of RCC 08-0910 cells with sorafenib resulted in the inhibition of the phosphorylation of VEGFR-2 and PDGFR-β (Fig. 1B). Partial inhibition of p-ERK1/2 and elevation of apoptosis, as determined by the levels of cleaved PARP, were also observed. AZD6244 potently inhibited p-ERK1/2 and induced apoptosis. Unlike sorafenib, which had

A



B

Phases of Cell Cycle

Treatment	G_0	G_1	S	G_2/M
Vehicle	0.3 ± 0.1	64.7 ± 5*	14.9 ± 3*	20.1 ± 3.1*
Sorafenib (1μM)	1.0 ± 0.2	56.3 ± 4*	17.0 ± 3.6*	25.9 ± 3.5*
AZD6244 (0.5μM)	0.8 ± 0.2	71.3 ± 4 **	14.8 ± 2.4**	13.2 ± 2.1**
Sorafenib + AZD6244	1.8 ± 0.3*	83.1± 3 ***	5.1 ± 1.6***	10.1 ± 1.7**

C

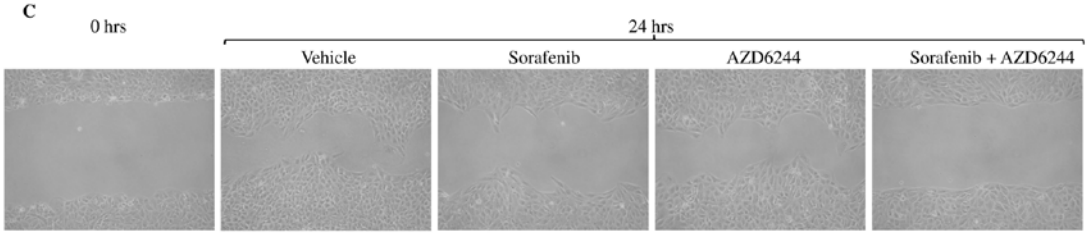


Figure 2. Effects of sorafenib, AZD6244 and sorafenib/AZD6244 on the proliferation and cell cycle progression. RCC768-0 cells were treated with vehicle, 1 μM sorafenib, 0.5 μM AZD6244, or 1 μM sorafenib plus 0.5 μM AZD6244 in DMEM containing 1% FBS. (A) Cell number was determined manually with a hemocytometer. Data are expressed as the mean ± SE. Asterisks (*) indicate significant difference among the treatments (P<0.05). (B) Cell cycle analysis, and (C) cell motility assay are also shown.

insignificant effect on p-AKT, AZD6244 caused elevation of p-AKT. Addition of AZD6244 into sorafenib completely abolished the activity of p-ERK1/2, and significantly augmented the apoptotic activity of sorafenib as determined by the levels of cleaved PARP.

To study the direct effects of sorafenib/AZD6244 on tumor cell growth and cell cycle, the RCC 786-0 cell line was used. As shown in Fig. 2A, treatment of RCC 786-0 cells with vehicle, 1 μM sorafenib, 0.5 μM AZD6244, or 1 μM sorafenib plus 0.5 μM AZD6244 resulted in a 25, 45 and 80% growth inhibition, respectively, when analyzed on Day 3. Furthermore, sorafenib treatment also induced more cells to accumulate in G_2/M and S phases with the reduction in G1 phase (Fig. 2B). By contrast, sorafenib/AZD6244 and AZD6244 caused G_1 phase arrest with reduction in the G_2/M and S phases. A modest increase in G_0 phase was also observed in sorafenib/AZD6244 (Fig. 2B). While sorafenib partially inhibited serum-induced RCC 786-0 cell motility, sorafenib/AZD6244 abolished it as determined by the wound-healing scratch assay (Fig. 2C).

We then studied the antitumor activity of sorafenib/AZD6244 in four RCC xenografts. As shown in Table I, AZD6244 and sorafenib, alone and in combination were well tolerated in the dosage used with no significant weight loss observed in the treated mice. The results of single agent and combination treatment are presented in Fig. 3 and summarized in Table I. As expected, sorafenib at 20 mg/kg resulted in the significant inhibition of tumor growth in all the four xenografts compared with control or AZD6244 alone (P<0.05). By contrast, treatment with AZD6244 alone had either modest (lines RCC19-0809 and RCC05-1109) or no significant (lines RCC02-0908 and RCC07-0408) antitumor activity compared to vehicle-treated mice, which is in agreement with the growth rate results (Fig. 3). Combinatory treatment of AZD6244 and sorafenib resulted in a marked inhibition of tumor growth in all four RCC xenografts tested, compared with the vehicle- and sorafenib-treated groups. Tumor regression was observed in sorafebib/AZD6244-treated groups (Fig. 3). Efficacy of each therapy was further evaluated by comparing the final mean weight of tumors in the drug-treated arm (T) to that of the control arm (C) (T/C ratio) with a value of <0.42 considered an active response (NCI criteria). As shown in Table I, while sorafenib produced T/C ratios ranging from 0.21 to 0.34, sorafenib/AZD6244 resulted in T/C ratios <0.154 in all four RCC lines, namely RCC07-0408 (T/C=0.126), RCC19-0809 (T/C=0.074), RCC05-1109 (T/C=0.099) and RCC02-0908 (T/C=0.154). The results suggest that the addition of AZD6244 into sorafenib significantly improved the antitumor activity of sorafenib.

Subsequently, we probed the mechanistic basis for the synergistic effect of AZD6244 on sorafenib therapy in RCC by evaluating representative tumor sections from four treatment arms using immunohistochemistry and western blotting. Representative staining results for CD31 (a marker of angiogenesis), p-Histone 3 Ser10 (a marker for proliferation) and cleaved PARP (a marker for apoptosis) for RCC07-0409 tumors are shown in Fig. 4 and Table I. Sorafenib alone and AZD6244/sorafenib significantly inhibited cell proliferation and angiogenesis in all four xenograft lines studied compared

Table I. Effects of sorafenib/AZD6244 on tumor burden, angiogenesis, cell proliferation, and apoptosis of RCC xenografts.

Lines of xenografts	Treatments	Body at sacrifice (g)	Tumor weight (mg)	T/C (%)	Microvessel[*1] density	p-Histone Ser10 (%)	Cleaved PARP (%)
RCC07-0408	Vehicle	25.2±1.2	769±65*	100	12.4±5*	6±1*	0.5±0.04*
	Sorafenib	25.6±1.1	182±26**	31.9	1.5±0.8**	0.8±0.5**	5.3±0.8**
	AZD6244	23.9±0.8	798±80*	103.7	13.4±4*	4.5±0.7*	1.3±0.6*
	Sorafenib + AZD6244	24.1±1.1	97±14***	12.6	0.3±0.2***	0.2±0.1***	14.2±3***
RCC19-0809	Vehicle	21.4±0.9	1050±123*	100	9.2±4*	9.2±3*	0.2±0.1*
	Sorafenib	22.3±1.0	317±34**	30.2	2.3±2**	3.6±2**	0.4±0.2*
	AZD6244	23.1±0.8	787±63***	74.9	10.3±3*	7.3±1.1*	0.6±0.3*
	Sorafenib + AZD6244	22.4±0.9	78±16****	7.4	0.8±0.4***	0.7±0.3***	5.3±1.2**
RCC05-1109	Vehicle	21.7±1.4	1823±149*	100	16.4±4*	11.6±3*	0.8±0.4*
	Sorafenib	20.4±1.1	385±58**	21.1	6.8±3**	2.6±0.9**	3.3±0.5**
	AZD6244	19.9±0.8	1125±174***	61.7	14.1±3*	8.5±0.7*	1.7±0.6*
	Sorafenib + AZD6244	19.5±1.1	180±40****	9.9	2.1±0.8***	2.1±0.5***	8.8±1.5***
RCC02-0908	Vehicle	23.2±1.0	492±52*	100	8.8±1.5*	6.4±0.9*	0.2±0.1*
	Sorafenib	22.3±0.8	167±32**	33.9	2.2±0.5**	1.9±0.8**	1.1±0.3**
	AZD6244	21.8±0.9	455±68*	92.4	8.6±0.8*	5.9±0.7*	0.5±0.3*
	Sorafenib + AZD6244	20.1±1.1	76±16***	15.4	1.4±0.2**	0.4±0.3***	6.3±1.5***

[*1], Mean microvessel density of 10 random 0.159mm^2 fields at magnification, x100. Data are expressed as the mean ± SE. Asterisks (*) indicate a significant difference among the treatments (P<0.05, Student's t-test).

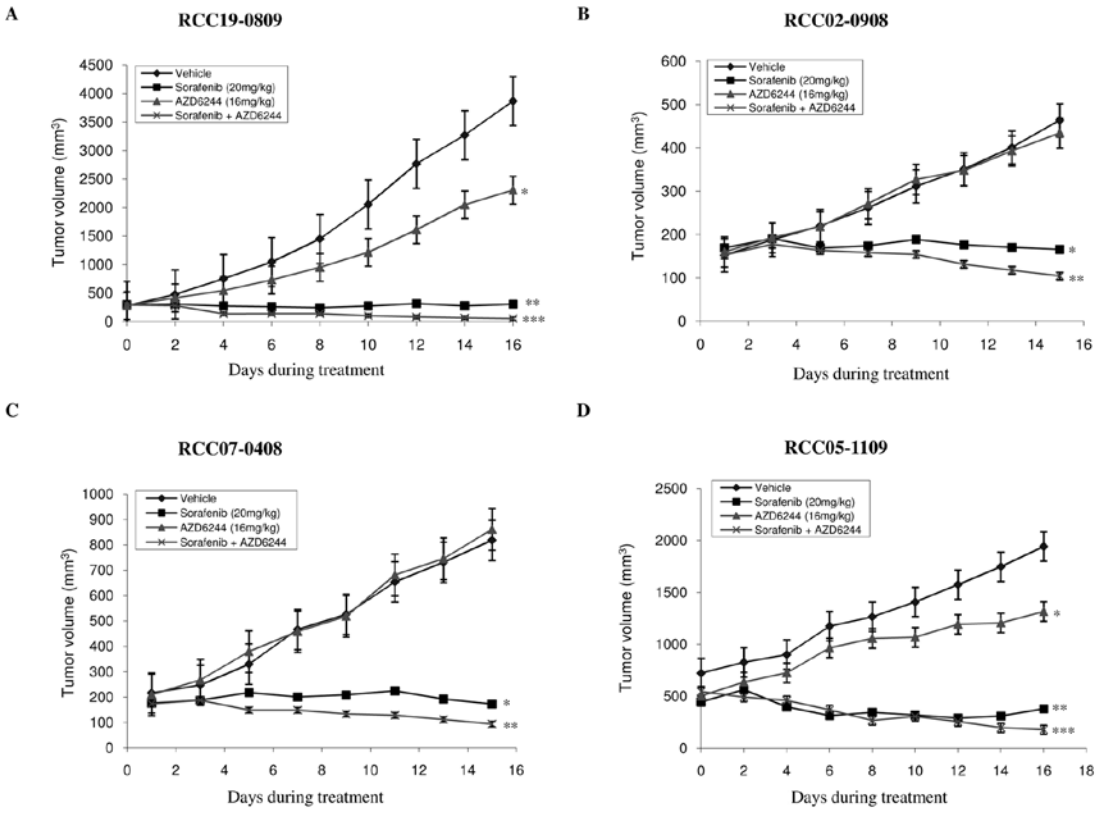


Figure 3. Effects of sorafenib/AZD6244 on the growth rate of patient-derived RCC xenografts. Mice bearing tumors (10 mice/group) were treated with vehicle, AZD6244 (16 mg/kg/day), sorafenib (20 mg/kg/day), or sorafenib/AZD6244 for the indicated number of days, as described in Materials and methods. Mean tumor volume at given time points for (A) RCC19-0809, (B) RCC02-0908, (C) RCC07-0408, and (D) RCC05-1109 lines are shown. Data are expressed as the mean ± SE. Asterisks (*) indicate a significant difference among the treatments (P<0.05). Experiments were repeated twice with similar results.

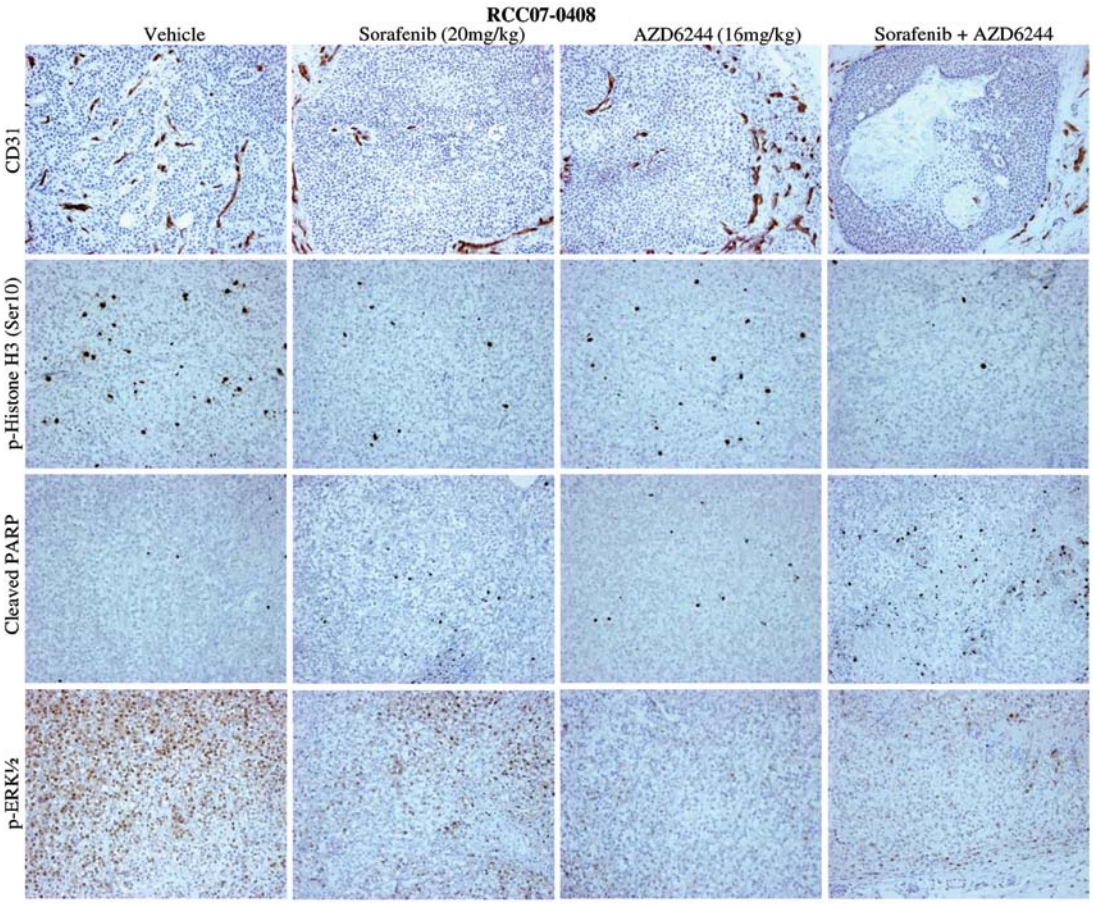


Figure 4. Effects of sorafenib, AZD6244, and sorafenib/AZD6244 on angiogenesis, cell proliferation and apoptosis in the RCC07-0408 xenograft. Mice bearing RCC07-0408 tumors (10 mice/group) were treated with vehicle, AZD6244 (16 mg/kg/day), sorafenib (20 mg/kg/day), or sorafenib/AZD6244 for 16 days, as described in Materials and methods. Representative pictures of vehicle- and drug-treated tumors are shown. Proliferative cells are stained with anti-phospho-Histone Ser10, blood vessels with anti-CD31, apoptotic cells with anti-cleaved-PARP and p-ERK1/2 are stained with p-ERK antibodies. Magnification, x200. Experiments were repeated twice with similar results.

with the control (Table I; P<0.05). An increase in apoptosis was observed in sorafenib-treated tumors. AZD6244, on the other hand, inhibited p-ERK1/2, modestly reduced cell proliferation but had an insignificant effect on microvessel density and apoptosis. As expected, sorafenib/AZD6244 abolished p-ERK1/2, significantly reduced microvessel density and cell proliferation but induced apoptosis at the dose studied (P<0.05). AZD6244/sorafenib also showed a significant reduction in tumor cell proliferation and elevated levels of apoptosis when compared with either sorafenib or AZD6244 alone (Table I; P<0.05), suggesting that sorafenib and AZD6244 complement each other and the addition of AZD6244 to sorafenib promotes the antitumor, antiangiogenic and apoptotic activities of either single agent.

As shown in Fig. 5, p-VEGFR-2, PDGF-Rβ and, to a lesser extent, p-ERK1/2 in sorafenib-treated tumors was significantly reduced (P<0.05). A decrease in p-cdk-2, survivin, p-Rb Ser807/811 and an elevation of p27 and Bim in sorafenib-treated tumors were also observed. AZD6244, on the other hand, inhibited phosphorylation of ERK1/2. A modest elevation of p-VEGFR-2, p-PDGFR-β, Bad and Bim, but a decrease in cyclin B1, p-70S6K, p-4EBP1 and p-p90RSK were seen in AZD6244-treated tumors. In sorafenib/AZD6244, the addition of AZD6244 to the sorafenib treatment not only led to the inhibition of phosphorylation of VEGFR-2 and PDGFR-β but also resulted in further reduction of p-ERK and p-p90RSK compared to sorafenib monotherapy. The levels of Bad, Bim and cleaved PARP were also elevated in tumors treated with sorafenib/AZD6244 compared to treatment with sorafenib or AZD6244 alone, suggesting that sorafenib/AZD6244 treatment caused apoptosis. Elevation of p27, and hypophosphorylated Rb coupled with a significant reduction in cyclin B1, survivin and p-cdk-2 were also observed in sorafenib/AZD6244-treated tumors, supporting the observation that sorafenib/AZD6244 induced cell cycle arrest. In addition, sorafenib/AZD6244 also reduced the levels of p-S6R, p-p70S6K and p-4EBP1, which are downstream targets of the mTOR pathway. These data suggest that the mTOR pathway is impaired by sorafenib/AZD6244 treatment. Similar results were obtained with the other three xenografts (data not shown).

As 20 mg/kg sorafenib plus 16 mg/kg AZD6244 was quite potent in inhibiting tumor growth and angiogenesis, we sought to determine if half a dose of each (10 mg/kg of sorafenib plus 8 mg/kg AZD6244) could produce similar antitumor activity as a 20 mg/kg dose of sorafenib. If that is the case then adverse effects associated with a full dose of sorafenib in some patients (3) could potentially be reduced by administering a lower dose of sorafenib/AZD6244. To test this hypothesis, we treated mice bearing RCC19-0809 tumors with the combination of 10 mg/kg sorafenib and

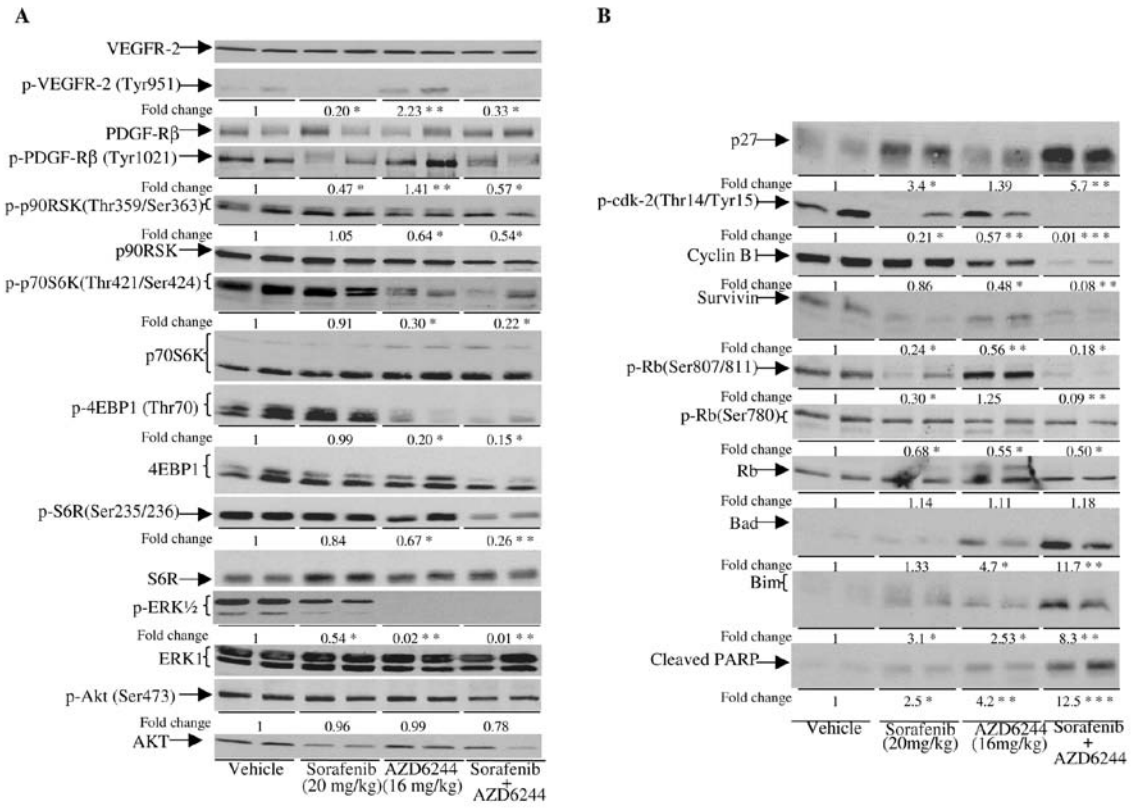


Figure 5. Effects of sorafenib, AZD6244, and sorafenib/AZD6244 on biomarkers relevant to angiogenesis, cell cycle, and apoptosis. Mice bearing RCC07-0408 tumors were randomized (10 mice/group) and treated with vehicle, AZD6244 (16 mg/kg/day), sorafenib (20 mg/kg/day), or sorafenib/AZD6244 for 16 days. Lysates of 4 tumors from one group were pooled. Each lane represented one protein pool and two pools per group were subjected to western blot analysis, as described in Materials and methods. Representative blots and quantification analysis (expressed as fold of controls) are shown. Asterisks (*) indicate a significant difference among the treatments (P<0.05). Experiments were repeated twice with similar results.

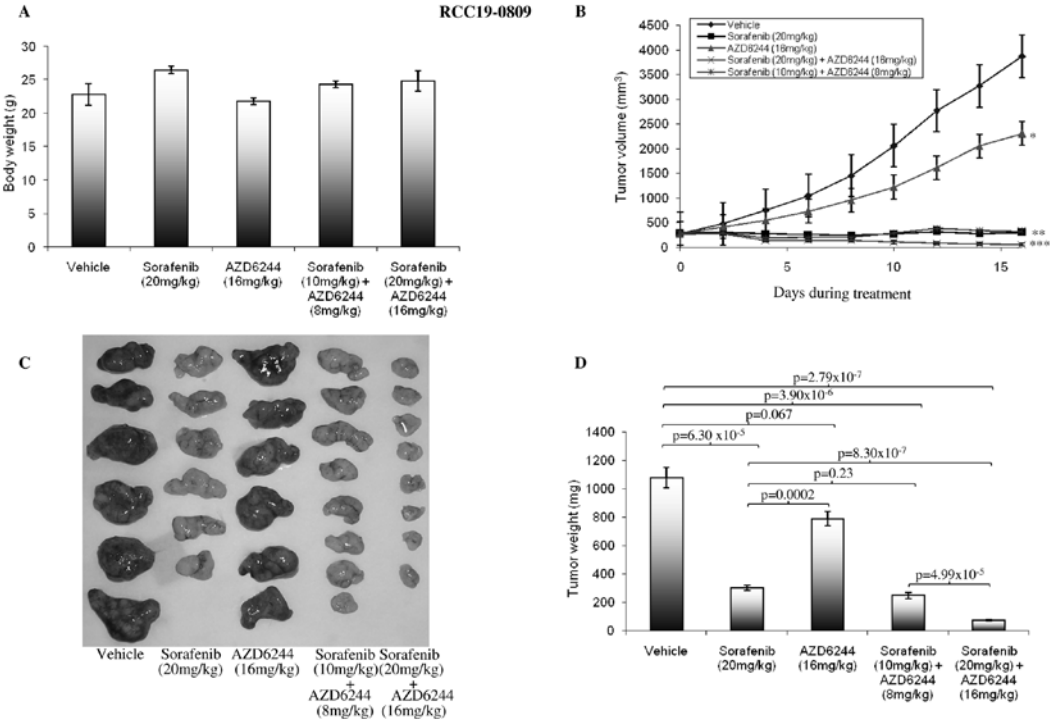


Figure 6. Effects of sorafenib, AZD6244 sorafenib/AZD6244 on the growth rate of patient-derived RCC19-0809 xenografts. Mice bearing tumors (10 mice group) were treated daily with vehicle, 16 mg/kg AZD6244, 20 mg/kg sorafenib (20 mg/kg/day), 16 mg/kg AZD6244 plus 20 mg/kg sorafenib or 10 mg/kg sorafenib plus 8 mg/kg AZD6244 for 16 days, as described in Materials and methods. (A) Mean of body weight at sacrifice, (B) mean of tumor volume ± SE at given time points, (C) representative vehicle- and drug-treated tumors, and (D) the corresponding tumor weight and statistical analysis for the RCC19-0809 xenograft are shown. Data are expressed as the mean ± SE. Asterisks (*) indicate a significant difference among the treatments (P<0.05). Experiments were repeated twice with similar results.

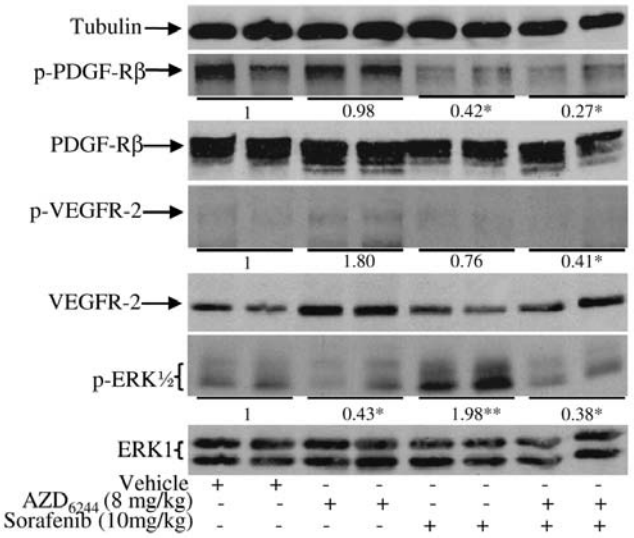


Figure 7. Effects of sorafenib, AZD6244, and sorafenib/AZD6244 on p-VEGFR-2, p-PDGFR-β and p-ERK in RCC19-0809 xenografts. Mice bearing tumors (10 mice per group) were treated daily with vehicle, 8 mg/kg AZD6244, 10 mg/kg sorafenib or 10 mg/kg sorafenib plus 8 mg/kg AZD6244 for 16 days, as described in Materials and methods. Lysates of 4 tumors from one group were pooled. Each lane represented one protein pool and two pools per group were subjected to western blot analysis, as described in Materials and methods. Representative blots and quantification analysis (expressed as fold of controls) are shown. Asterisks (*) indicate a significant difference among the treatments ($P<0.05$). Experiments were repeated twice with similar results.

8 mg/kg AZD6244. As shown in Fig. 6, sorafenib at the dose of 20 mg/kg produced good antitumor activity with more than 60% tumor growth inhibition. AZD6244 at the dose of 16 mg/kg had insignificant antitumor activity. Combination of AZD6244 at 16 mg/kg and sorafenib at 20 mg/kg achieved more than double the antitumor activity of sorafenib alone at 20 mg/kg. For monotherapy, 40±5% and 5±1.3% tumor growth inhibition were observed for 10 mg/kg sorafenib and 8 mg/kg AZD6244, respectively. However, combination sorafenib at 10 mg/kg and AZD6244 at 8 mg/kg produced similar tumor growth inhibition compared to sorafenib as single-agent therapy at 20 mg/kg (Fig. 6). Although sorafenib increased p-ERK1/2 at the dose of 10 mg/kg, sorafenib/AZD6244 still inhibited phosphorylation of VEGFR-2, PDGFR-β and ERK1/2 (Fig. 7). Notably, the 10 mg dose of sorafenib used in this study was lower than the dose given to patients, which is approximately 13 mg/kg. These findings suggest that AZD6244/sorafenib treatment allows for the dose reduction of sorafenib without compromising the antitumor efficacy of the optimal sorafenib dose. Similar results were obtained for the other RCC xenografts (data not shown). Combination therapy with low doses of AZD6244 and sorafenib was tolerated well by the animals with no significant adverse effects observed as manifested by weight loss, unkempt appearance, mortality or distress behavior.

Discussion

While the clinical efficacy of tyrosine kinase inhibitors (TKIs), such as sorafenib, in patients with mRCC is impressive in some patients, only approximately 60% of patients with mRCC respond to this agent (3). For those treated with sorafenib, many adverse effects associated with its use were observed (3). The common adverse effects of sorafenib include fatigue, weight loss, desquamation, hand-foot skin reaction, diarrhea, nausea and abdominal pain. Rarely, it can also cause hematological abnormalities, including leukopenia, anemia, neutropenia and thrombocytopenia; and severe cardiovascular adverse event such as hypertensive crisis, myocardial ischemia and congestive heart failure. It has been reported that almost a third of the patients on a full dose of sorafenib (400 mg/BD) required a dose reduction and 10-17% of patients ceased treatment with the drug due to serious adverse events (8,13). Based on this, a combination treatment strategy with sorafenib and an agent that has synergistic effects is being evaluated. In particular, the addition of a drug that would allow the reduction of the sorafenib dosage while maintaining its overall antitumor effects would lead to a decrease in the risk of adverse events, thus increasing the tolerability of the treatment.

In the present study, we showed that sorafenib/AZD6244 is considerably more effective than the single agents in suppressing the growth of human RCC xenografts. Sorafenib/AZD6244 effectively suppresses tumor growth, induces apoptosis, and inhibits angiogenesis *in vivo*. We also observed that sorafenib inactivates mTOR targets. This combination is significantly superior to either drug in suppressing tumor growth with minimal toxicity. Our findings provide further insights into the limited benefits observed in clinical trials using sorafenib or MEK inhibitors as single agents and exemplify how the efficacy of sorafenib or MEK inhibitors could be improved in the treatment of RCC. Combined sorafenib and ERK inhibition may be a promising drug combination as one third of RCCs are driven by signals generated from angiogenesis, the Ras/Raf/ERK, PI3K/Akt/mTOR signaling pathways and this combination can reduce side effects due to the high dose of the sorafenib treatment. The addition of AZD6244 complements well with the effect of sorafenib in that it allows reducing the optimal dose of sorafenib in half without reducing the antitumor activity. Markedly, we found that AZD6244 alone, and combination sorafenib/AZD6244 at a full dosage (20 and 16 mg/kg respectively) and half doses (10 and 8 mg/kg) were well tolerated by the animals. This finding has important clinical implications as it suggests that the sorafenib/AZD6244 combination is a potentially more effective clinical treatment strategy for patients with advanced RCC.

The significance of the molecular inhibition of both VEGFR-2/PDGFR-β and MEK/ERK pathways by combination sorafenib/AZD6244 treatment was evidenced by a more profound inhibition of angiogenesis, cell proliferation and induction of apoptosis as shown in the immunohistochemical staining of the treated RCC xenografts. We had previously identified survivin as an oncogene that plays an important role in the pathogenesis of RCC (9). Of note, sorafenib/AZD6244 combination treatment leads to marked depletion of survivin, in addition to more inhibition of cell cycle proteins and the mTOR signaling pathway compared to sorafenib alone. It is hypothesized that combination sorafenib/AZD6244 treatment resulted in more profound inhibition of both VEGFR-2/

PDGFR-β and MEK/ERK pathways compared to sorafenib alone in RCC, leading to significantly more inhibition of mTOR, survivin, cell cycle proteins and apoptosis induction, thus enhancing the antitumor activity of sorafenib in RCC.

Although sorafenib/AZD6244 is effective in preclinical models of human RCC, the molecular mechanisms responsible for this cooperativity remain unknown. Due to the fact that VEGF-induced migration and proliferation of endothelial cells are in part mediated by the activation of the MEK/ERK signaling cascades and recruitment of pericytes into tumors involves VEGFR/PDGFR system, pronounced inhibition of VEGFR-2, PDGFR-β and the MEK/ERK signaling pathway by sorafenib/AZD6244 would effectively inhibit tumor angiogenesis and ultimately tumor growth *in vivo*. Since cyclin B1 and Cdk-2 are required for cell cycle progression, inhibition of cyclin B1 and Cdk-2 coupled with upregulation of p27 by sorafenib/AZD6244 would cause cell cycle arrest. Our present study shows that the pro-apoptotic activity of sorafenib is significantly enhanced when combined with AZD6244. However, the mechanism(s) responsible for this effect has yet to be fully elucidated. Bim has been implicated in the regulation of apoptosis (10). It is possible that upregulation of Bad and Bim by sorafenib/AZD6244 would allow more Bim and Bad to bind to and antagonize anti-apoptotic effect of the Bcl-2 and Bcl-xL, leading to Bax-dependent apoptogen release, caspase activation and cell death.

The clinical effects of sorafenib in advanced RCC is primarily disease stabilization, thereby leading to the prolongation of progression-free survival with only marginal overall survival clinically. In this patient RCC-derived xenograft model, combination therapy with sorafenib/AZD6244 resulted in more tumor regression compared to monotherapy with either AZD6422 or sorafenib monotherapy. The antitumor effects resulting from the addition of AZD6422 to sorafenib may bring about significant survival benefit not seen with the sorafenib monotherapy clinically thus far. Despite favorable *in vivo* data suggesting the beneficial effects of combination sorafenib/AZD6244 treatment in RCC, a study using a murine xenograft model has its inherent limitations. Firstly, clinical conclusions based on data derived from a limited number of RCC xenografts should be interpreted with caution. Secondly, even though we demonstrated that the xenografts we generated retain identical histological features compared to the original specimens, tumor behavior in a non-orthotropic environment in mice may not resemble the micro-environment of RCC in patients. Nevertheless, our molecular data generated using RCC-derived xenografts is consistent and reproducible. A recent pilot study of treatment guided by personalized tumor xenografts in patients with advanced cancer demonstrated a remarkable correlation between drug sensitivity in the model and clinical outcome, both in terms of resistance and sensitivity (14). Although we caution direct extrapolation of these data to the clinical setting, these data suggest that the sorafenib/AZD6244 combination is potentially beneficial; the extent of these benefits require a clinical trial to be verified.

In summary, our study shows that combination sorafenib/AZD6244 treatment enhances the antitumor activity of sorafenib and allows for a dose reduction of sorafenib without compromising its antitumor activity.

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