Biochemical and Biophysical Research Communications 423 (2012) 490–495

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

GSK-3 inhibition *in vitro* and *in vivo* enhances antitumor effect of sorafenib in renal cell carcinoma (RCC)

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ARTICLE INFO

Article history:
Received 25 May 2012
Available online 5 June 2012

Keywords:
Renal cell carcinoma
Glycogen synthase kinase-3
Sorafenib
Tyrosine kinase inhibitor

ABSTRACT

Sorafenib is a multikinase inhibitor approved for the systemic treatment of renal cell carcinoma (RCC). However, sorafenib treatment has a limited effect due to acquired chemoresistance of RCC. Previously, we identified glycogen synthase kinase-3 (GSK-3) as a new therapeutic target in RCC. Here, we observed that sorafenib inhibits proliferation and survival of RCC cells. Significantly, we revealed that sorafenib enhances GSK-3 activity in RCC cells, which could be a potential mechanism of acquired chemoresistance. We found that pharmacological inhibition of GSK-3 potentiates sorafenib antitumor effect *in vitro* and *in vivo*. Our results suggest that combining GSK-3 inhibitor and sorafenib might be a potential new therapeutic approach for RCC treatment.

1. Introduction

Kidney cancer is the sixth and eighth most common cause of cancer for men and women, respectively [1]. Although 5-year survival rate for kidney cancer is steadily increasing from 51% in 1975–77 to 69% in 1999–2005, the increase is not prominent and the survival rate is still far from ideal.

Approximately one-third of patients with renal cell carcinoma (RCC) have metastatic disease at first presentation and up to one half of radically treated patients develop metastatic disease after the operation [2]. Immunotherapy was the only available systemic therapy for metastatic RCC in the cytokine era. However its efficacy was less than 20% [3]. Recently, molecular targeted drugs became available for the treatment of advanced/metastatic RCC. Multiple tyrosine kinase inhibitors (TKI) sunitinib, pazopanib and sorafenib, mTOR inhibitors, and anti-VEGF humanized antibody bevacizumab have been shown to improve progression-free survival and overall survival in randomized trials and are now recommended as the first-line and second-line treatment for systemic therapy of RCC [4–7]. However, the treatment response is not long-standing. Moreover, TKI pose risk of serious adverse events (AE) [8].

Glycogen synthase kinase (GSK)-3 is a serine/threonine protein kinase that has two isoforms GSK-3α and GSK-3β [9,10]. We demonstrated that GSK-3β positively regulates cancer cell proliferation and survival in chronic lymphocytic leukemia [11], pancreatic cancer [12,13] and urological cancers – bladder [14] and kidney [15]. We have reported that nuclear accumulation of GSK-3β could be a novel marker of human RCC and showed that GSK-3β positively regulates RCC cell survival and proliferation via NF-κB–Bcl-2, XIAP pathway [15].

Sorafenib is an orally active TKI. Primarily developed as antineoangiogenic agent sorafenib was demonstrated to directly induce apoptosis in melanoma cell lines [16]. Recently, it was demonstrated that GSK-3β inhibition enhanced Sorafenib-induced apoptosis in melanoma cells [17]. The objective of this study was to examine effect of sorafenib or GSK-3 inhibition on RCC cells *in vitro* and *in vivo* administered as single agents or in combination with each other. We observed that sorafenib treatment induced cell growth retardation and apoptosis, but, on the other hand, it enhanced up GSK-3 activity and resulted in upregulation of antiapoptotic XIAP and Bcl-2, which can be a potential mechanism of acquired resistance to sorafenib. We confirmed that GSK-3 pharmacological inhibition potentiated sorafenib antitumor effect both *in vitro* and *in vivo*. Combination of sorafenib and GSK-3 inhibitor might be a new therapeutic approach for the treatment of RCC.

2. Materials and methods

2.1. Cell culture and reagents. Measurement of cell viability

The established renal cell cancer cell lines ACHN, KRC/Y, Caki1, Caki2, A704, A498, and KH39 were purchased from ATCC. KU19-20 was kindly provided by Dr. Mototsugu Oya (Department of Urology, School of Medicine, Keio University, Tokyo, Japan). The cells

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http://dx.doi.org/10.1016/j.bbrc.2012.05.147

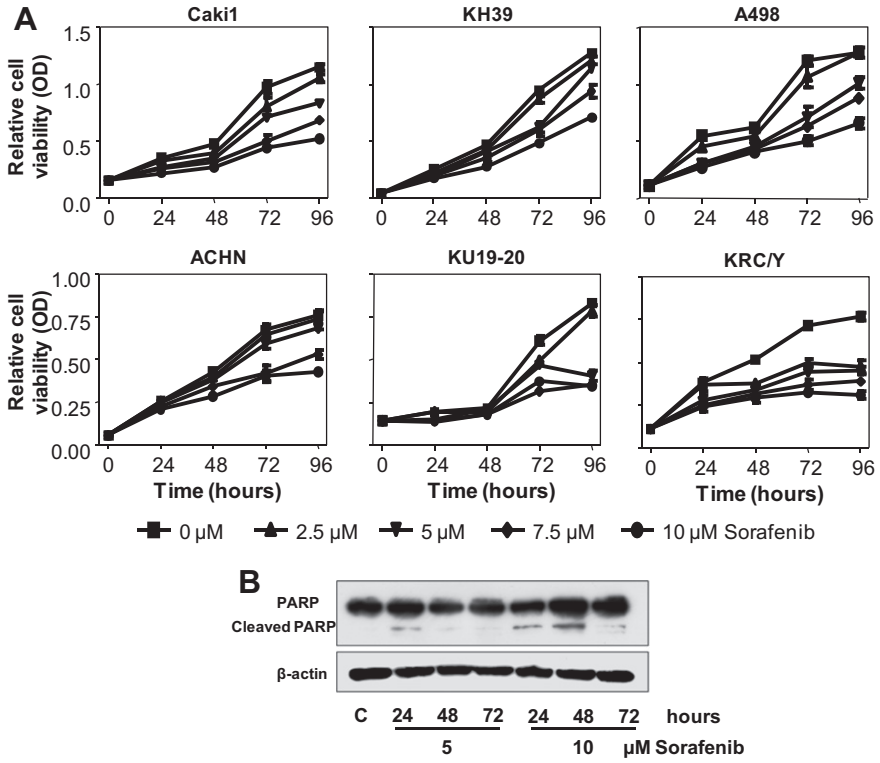

Fig. 1. Sorafenib treatment suppressed proliferation and survival of RCC cells *in vitro*. (A) Relative cell viability was measured by MTS assay in RCC cell lines treated with the indicated concentrations of sorafenib for 24, 48, 72 and 96 h. OD, optical density at 490 nm. (B) Western blotting analysis shows the expression of PARP (cleaved PARP is a marker for apoptosis) in ACHN human RCC cells treated with 5 or 10 μM of sorafenib for 24, 48 or 72 h.

were cultured in RPMI1640 medium as we reported previously [15]. Two ATP-competitive GSK-3 inhibitors were used. AR-A014418 was purchased from Sigma–Aldrich Japan (Tokyo, Japan) and SB-216763 was purchased from Cayman Chemical Company (Ann Arbor, MI). AR-A014418 [18] and SB-216763 [19] are highly specific GSK-3 inhibitors which does not significantly inhibit other kinases [18], [19]. Sorafenib was a kind gift of Bayer HealthCare, Osaka, Japan. Cell viability was examined using a colorimetric MTS assay, the CellTiter 96® assay (Promega, Madison, WI), according to the manufacturer's protocol.

2.2. Xenograft tumors

All experimental procedures using nude mice were performed according to the animal welfare regulations of Yamagata University School of Medicine, and the study protocol was approved by the Animal Subjects Committee of Yamagata University School of Medicine. The investigation conformed to the *Guide for the Care and Use of Laboratory Animals*, published by the National Institutes of Health. Eight weeks old athymic immunocompromised female nude mice were inoculated s.c. with 3 million ACHN or Caki1 renal cancer cells mixed with Matrigel (BD Biosciences). When the tumor volume reached approximately 100 mm^3 the mice were randomized into groups according to experimental protocol. AR-A014418 (30 mg/kg of body weight) [12], SB-216763 (10 mg/kg of body weight) or vehicle (DMSO) was administered by I.P. injections for 14 sequential days. For protein extraction, mice with established xenograft tumors described above were treated with AR-A014418 (I.P. 120 mg/kg; every 12 h for 2 days) followed by protein extraction and Western blotting. Sorafenib (stock solution of 40 mg/ml diluted in Cremophor EL/95% Ethyl alcohol) was administered at 60, 90 mg/kg of body weight or vehicle (solvent only control) by oral gavage once per day for 21 days. The mice body weight and tumor volume (detected with calipers and calculated by standard formula) were measured weekly as we described previously [12]. For combination treatment Sorafenib 30 mg/kg was administered by p.o. gavage five times weekly and AR-A014418 20 mg/kg was administered by I.P. injections three times weekly for 4 weeks. No significant toxicity was observed with these regimens.

2.3. Western blotting analysis

Western blotting analysis was performed as described previously [15]. Subcellular fractionation was performed using DIGNAM method as described previously [12]. The following antibodies were used: Bcl-2 (DAKO, Japan); GSK-3β, PARP and XIAP (BD Biosciences, Franklin Lades, NJ), Cu/Zn SOD (Stressgen Bioreagents, Ann Arbor, MI), Histone H3 (SIGMA, Saint Louis, MO), and β-actin (Abcam). The proteins were detected using the SuperSignal West Pico Substrate (Pierce, Rockford, IL) with a Light-Capture II Cooled CCD camera system (ATTO, Japan) and analyzed using CS Analyzer version 3.0 for Windows (ATTO, Japan).

2.4. Statistical analysis

Continuous variables are presented as the mean ± SD for *in vitro* and mean ± SE for *in vivo* experiments. Kolmogorov–Smirnov normality test was performed for all data and they met the criteria for a normal distribution. *t*-test or one-way ANOVA were applied to analyze data using GraphPad Prism software package for Windows (GraphPad Software Inc., San Diego, CA) or StatView 5.0 for Windows (Abacus). Two-sided tests were used and all *p*-values less than <0.05 were considered statistically significant.

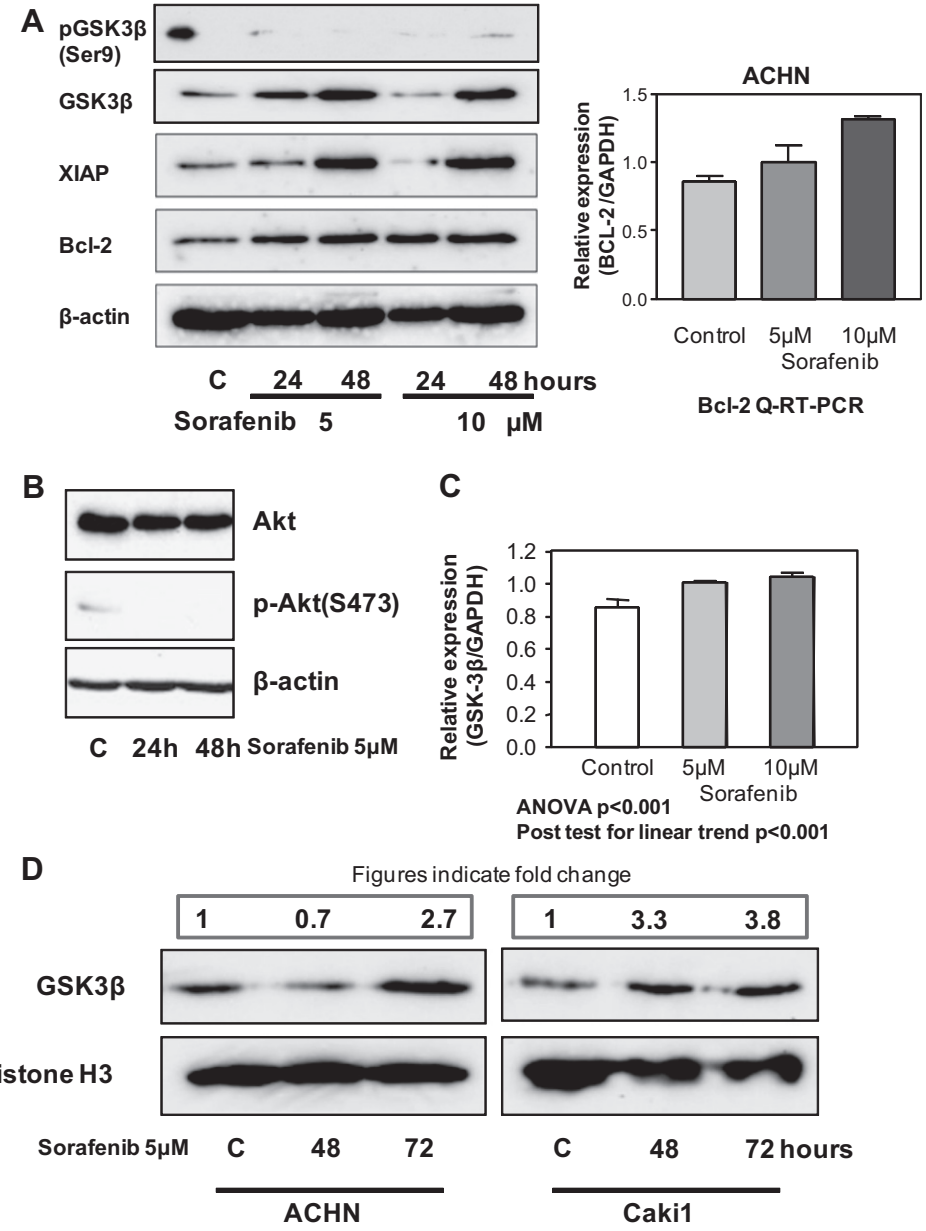

Fig. 2. Sorafenib treatment upregulated Bcl-2, XIAP, total GSK-3β and active GSK-3β nuclear pool presumably by inhibiting Akt and increasing transcription of GSK-3β. (A) Western blotting analysis shows the expression of GSK-3β, pGSK-3β-ser-9 (GSK-3β phosphorylated at Serine 9, an inactive form of GSK-3β), XIAP, and Bcl-2 in ACHN human RCC cells treated with 5 or 10 μM of sorafenib for 24 or 48 h. (B) Western blotting analysis showing decreased phosphorylation of Akt at S473 (p-Akt-S473 is an active form of Akt) to an undetectable levels in ACHN human RCC cells after exposure to 5 μM of sorafenib for 24 or 48 h. (C) mRNA levels of GSK-3β after treating ACHN human RCC cells with 5 or 10 μM of sorafenib for 72 h were measured by real time PCR using TaqMan probe technique. (D) Western blotting of nuclear fraction from ACHN and Caki1 human RCC cells. The cells were treated with 5 μM of sorafenib for 48 or 72 h. Histone-H3 is a nuclear marker.

3. Results

3.1. Sorafenib treatment suppresses RCC cell proliferation and survival and upregulates GSK-3β

Using MTS assay, here we demonstrate that sorafenib reduced cell viability in a panel of human RCC cell lines in a dose- and time-dependent manner (Fig. 1A). It also induced apoptosis, as confirmed by PARP cleavage (Fig. 1B).

On the other hand, it has been shown *in vitro* using melanoma cell lines that sorafenib activates GSK-3β [17]which in turn alleviates sorafenib antitumor action and could be a cause of acquired resistance to sorafenib. Here, we observed an increase of total GSK-3β protein expression levels (Fig. 2A). Moreover, we found that phospho-GSK-3β (Ser9), an inactive form of GSK-3β, decreased after sorafenib treatment (Fig. 2A). Sorafenib is known to target multiple kinases, blocking receptor tyrosine kinase autophosphorylation and preventing activation of downstream MAP, Raf, PI3K kinases. Phosphorylation of Akt at serine 473 was completely abolished by sorafenib treatment (Fig. 2B). It was shown that Akt inactivates GSK-3β by phosphorylation at Serine 9 [9]. Here we also observed moderate but statistically significant increase in mRNA levels of GSK-3β after exposing RCC cells to sorafenib (Fig. 1C). Increase in nuclear GSK-3β fraction was also noted (Fig. 2D). We have previously demonstrated that GSK-3β nuclear pool represents active kinase form as inactive GSK-3β rapidly undergoes intranuclear proteosomal degradation [12]. We have demonstrated previously that GSK-3β facilitates transcription of XIAP and Bcl-2, NF-κB

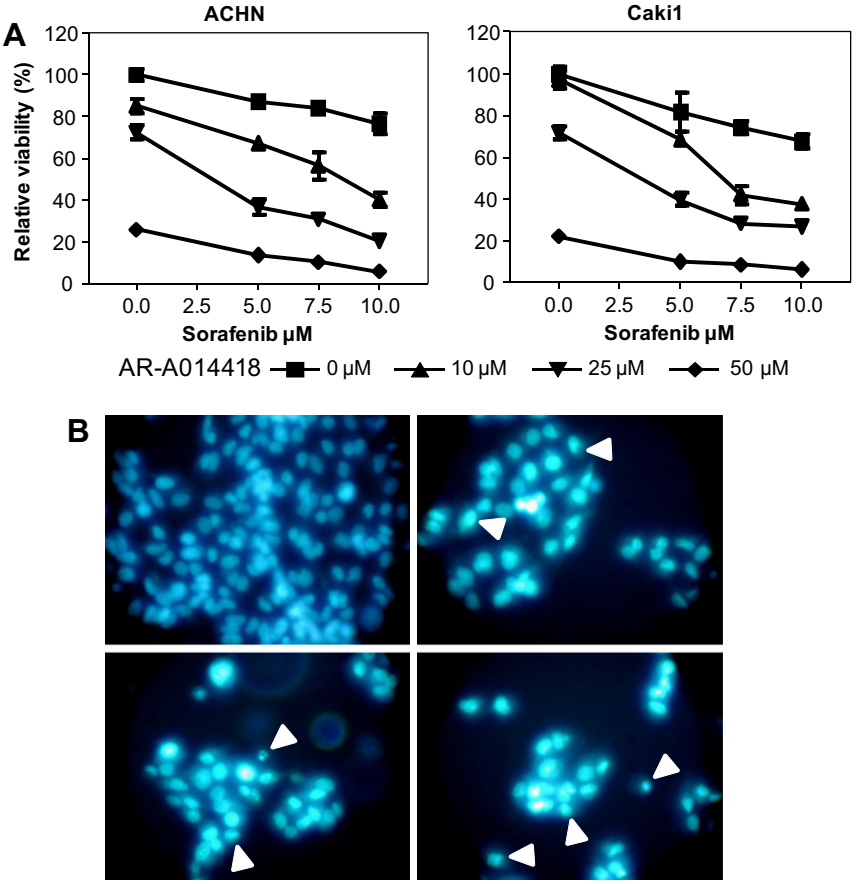

Fig. 3. Synergistic effect of sorafenib and GSK-3β pharmacological inhibition by AR-A014418 to suppress proliferation and survival of RCC cells. (A) Relative cell viability was measured by MTS assay in ACHN (left panel) and Caki1 (right panel) RCC cell lines treated with the indicated concentrations of sorafenib and AR-A014418 for 72 h. OD, optical density at 490 nm. (B) ACHN renal cancer cells were cultured in the presence of DMSO (upper left panel), 25 μM AR-A014418 (upper right panel), 5 μM sorafenib (lower left panel) or combination of the both (lower right panel) for 72 h, followed by Hoechst 33342 staining. Arrowheads point at apoptotic cells.

downstream genes, through chromatin remodeling in RCC [15]. Consistent with this, XIAP and Bcl-2 protein levels also increased (Fig. 2A).

3.2. Synergistic effect of sorafenib and GSK-3β inhibition in suppression of proliferation and survival of RCC cells

Because of our findings that sorafenib treatment of RCC cells *in vitro* resulted in a significant increase in total and active nuclear fraction of GSK-3β, we treated cultured RCC cells with either sorafenib, AR-A014418 or a combination of the both. As shown in the Fig. 3A, double treatment resulted in synergistic effect to suppressed RCC cells proliferation and survival *in vitro*. This was associated with increase in apoptotic cells as confirmed by Hoechst 33342 staining (Fig. 3B).

3.3. Pharmacologic inhibition of GSK-3 suppressed xenograft RCC tumor growth in vivo

We have previously demonstrated antitumor effect of GSK-3 pharmacological inhibition of RCC cells *in vitro* [15]. Here we studied effect of two specific GSK-3 inhibitors on RCC mice xenograft model. We found that daily injections of AR-A014418 [18] or SB-216763 [19] suppressed tumor growth (Fig. 4A–C). Moreover, *in vivo* treatment with AR-A014418 decreased levels of antiapoptotic proteins c-IAP-1, XIAP, Bcl-xL, and Bcl-2 in ACHN xenograft tumors (Fig. 4D).

3.4. Pharmacologic inhibition of GSK-3 enhanced antitumor effect of sorafenib in vivo

Daily treatment with sorafenib 60 or 90 mg/kg for two weeks suppressed ACHN xenograft tumor growth (Fig. 4E). Moreover, alongside with decreased tumor diameter prominent central necrosis and cysts were observed in xenograft tumors consistent with reported findings in xenograft models [20] and our observation of clinical RCCs (personal unpublished data). This was presumably due to impeded vascular support to the tumor with following necrosis [20] and cystic degeneration. Combination of GSK-3 small molecule inhibitor AR-A014418 (20 mg/kg three times weekly) with sorafenib (30 mg/kg five times weekly) for 4 weeks was superior to each single agent treatment in suppressing ACHN xenograft tumor growth in mice (ANOVA $p < 0.001$) (Fig. 4F).

4. Discussion

Recently, we reported on nuclear accumulation of GSK-3β being a new marker of human RCC and identified that GSK-3 positively regulated RCC cell survival/proliferation [15]. Previously, we have demonstrated that GSK-3 pharmacological inhibition and genetic depletion suppressed RCC cells viability and induced apoptosis [15]. We have also showed antitumor effect of GSK-3 inhibition in pancreatic cancer mice xenograft model [12].

Sorafenib has been approved for the first-, second-line treatment of advanced/metastatic RCC. Antiangiogenic action has been considered the main effect of sorafenib in clinical RCCs [20–22]. A

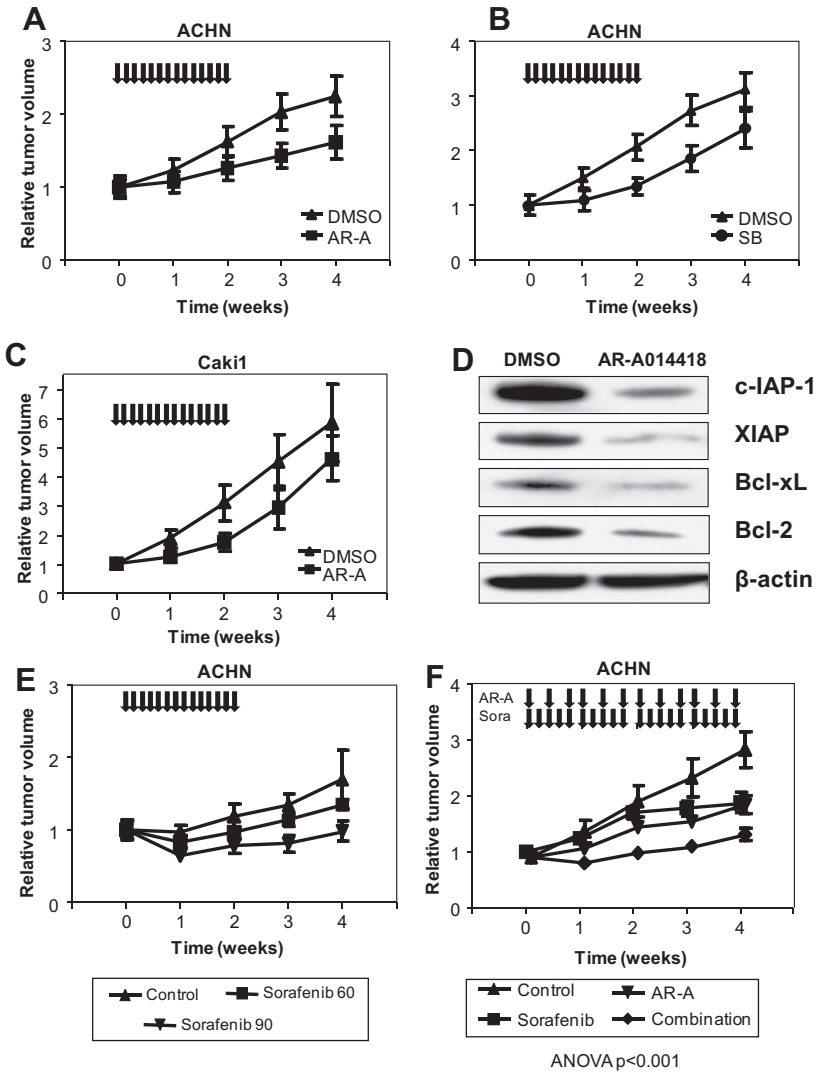

Fig. 4. In vivo effect of pharmacological inhibition of GSK-3, sorafenib or the combination of both to suppress mice xenograft tumor growth. Growth curves of ACHN (A and B) and Caki1(C) RCC xenograft tumors. Mice were treated with GSK-3 inhibitors (A and C, AR-A014418; B, SB216763) or vehicle control. Treatment was initiated on day 0 with i.p. injections of either diluent (DMSO), 30 mg/kg AR-A014418 or 10 mg/kg SB216763 and animals were injected daily for two weeks as indicated by arrows. Filled triangles, diluent (DMSO); filled squares, AR-A014418, filled circles SB216763. Data points represent the mean relative tumor volume ± SE. (D) Western blotting analysis. Mice bearing ACHN xenograft tumors were injected i.p. with DMSO or AR-A014418 (120 mg/kg; every 12 h for 2 days) followed by protein extraction and Western blotting. (E) Growth curves of ACHN human RCC cells xenograft tumors subjected to daily sorafenib oral gavage for 2 weeks as indicated by arrows. Filled triangles, vehicle control; filled squares, sorafenib 60 mg/kg; filled inverted triangles, sorafenib 90 mg/kg. (F) Mice were treated with GSK-3 small molecule inhibitor AR-A014418 20 mg/kg three times weekly (filled inverted triangles) or sorafenib 30 mg/kg five times weekly (filled squares) for 4 weeks as indicated by arrows. Combination of the both (filled rhombus) further suppressed tumor growth in mice (ANOVA $p < 0.001$). Filled triangles, vehicle control. Data points represent the mean relative tumor volume ± SE.

growing body of evidence also suggests a direct antitumor effect of sorafenib [16,23,24]. Although the precise mechanisms are not completely understood and some or all of these mechanisms could be cell-type specific, it has been shown recently that sorafenib induced cell cycle arrest and promoted cell death in RCC cell lines *in vitro* [25]. Combining sorafenib with other molecular targeted agents may improve its activity while maintaining acceptable toxicity, thus providing additional benefits to RCC patients.

Consistent with our previous *in vitro* findings, here we observed tumor growth retardation by GSK-3 pharmacological inhibition using two different specific small molecule inhibitors [18,19] (Fig. 4A–C). This was associated with decreased expression of major antiapoptotic molecules of IAP and Bcl-2 family (Fig. 4D).

Both antiangiogenic [20–22] and non-antiangiogenic [16,23,24] effects were reported for sorafenib. Here we observed antitumor effect of sorafenib both *in vitro* and *in vivo* using xenograft model. However, sorafenib treatment of cultured cell lines results in induction of GSK-3β, increase its active nuclear pool and elevation of antiapototic proteins XIAP and Bcl-2 which might alleviate sorafenib antitumor effect. Sorafenib prevents Akt phosphorylation on S473, resulting in Akt inactivation which in turns decreases levels of inactive GSK-3β phosphorylated at Ser9 (Fig. 2A and B). We also observed mild but statistically significant increase in GSK-3β transcription (Fig. 2C). Thus, sorafenib treatment increased GSK-3β levels and its active fraction by several mechanisms. Pharmacological inhibition of GSK-3 and sorafenib had synergistic effect to induce cell growth retardation both *in vitro* (Fig. 3) and *in vivo* (Fig. 4E and F).

Our data demonstrate that sorafenib activates GSK-3 via several mechanisms in RCC cells. GSK-3 is a negative regulator of sorafenib antitumor effect in RCC. Here we demonstrated synergistic effect of combinatory treatment of RCC cells with sorafenib and GSK-3 inhibitor. This finding can have future clinical implication because coadministration of sorafenib with GSK-3 inhibitor can potentiate

an antitumor effect of sorafenib overcoming acquired resistance. Applying agents with different molecular targets can further alleviate adverse effects due to different spectrum of adverse reactions. This combination treatment could be a new potential therapeutic approach in advanced and metastatic RCC by increasing efficacy and preventing or delaying acquired resistance to sorafenib.

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