



Research report

Sorafenib induces apoptotic cell death in human non-small cell lung cancer cells by down-regulating mammalian target of rapamycin (mTOR)-dependent survivin expression

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ABSTRACT

Sorafenib, a multikinase inhibitor, is emerging as a promising targeted agent that may possess antitumor activity against a broad range of cancers. The mechanism by which sorafenib induces lung cancer cell death and apoptosis, however, is not understood. In the present study, we provide evidence that sorafenib acts through inhibition of mammalian target of rapamycin (mTOR) to down-regulate survivin and promote apoptotic cell death in human non-small cell lung cancer (NSCLC) cells. Sorafenib induced ATF4-mediated Redd1 expression, leading to mTOR inhibition—the upstream signal for down-regulation of survivin. Overexpression of survivin reduced sorafenib-induced apoptosis, whereas silencing survivin using small interfering RNA (siRNA) enhanced it, supporting the interpretation that down-regulation of survivin is involved in sorafenib-induced cell death in human NSCLC cells. Furthermore, sorafenib abolished the induction of survivin that normally accompanies IGF-1-stimulated mTOR activation. We further found that Redd1-induced mTOR down-regulation and ATF4/CHOP-induced expression of the TRAIL receptor DR5 associated with sorafenib treatment helped sensitize cells to TRAIL-induced apoptosis. Our study suggests that sorafenib mediates apoptotic cell death in human NSCLC cells through Redd1-induced inhibition of mTOR and subsequent down-regulation of survivin, events that are associated with sensitization to TRAIL-induced apoptotic cell death.

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1. Introduction

Lung cancer is one of the leading causes of cancer death. More than 80% of lung cancer cases are non-small cell lung cancer (NSCLC), a majority of which present with advanced stage cancer. The prognosis for these patients is poor and few of the normal treatment options are effective, including chemo- and radiotherapy [1]. This has created a need for new therapeutic strategies for treatment of advanced NSCLC.

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that is a downstream target of AKT [2]. It has been shown to integrate diverse signals triggered by growth factors and nutrients, and regulate cell survival, mitogen-stimulated protein synthesis, and cell-cycle progression [3–5]. Cell culture studies

have demonstrated that one of the mechanisms by which mTOR controls protein synthesis is through direct phosphorylation of key translation regulators, including p70S6 kinase (S6K) and eukaryotic initiation factor 4E binding protein 1 (4E-BP1) [6]. Therefore, by inhibiting mTOR, rapamycin causes a decrease in phosphorylation of these effectors and a decrease in protein synthesis, effectively blocking the pro-growth, pro-proliferative, and pro-survival actions of mTOR.

Redd1 (regulated in development and DNA damage responses) was recently shown to negatively regulate mTOR via activation of TSC2 (tuberous sclerosis 2) [7]. Originally identified on the basis of its induction by hypoxia via hypoxia-inducible factor 1 (HIF1) [8], Redd1 is also induced by various cellular stresses, including reactive oxygen species (ROS), endoplasmic reticulum (ER) stress, and 2-deoxyglucose [9]. Redd1 is required to inhibit mTOR activity in response to these stresses. Disruption of Redd1 abrogates the hypoxia-induced inhibition of mTOR, and its overexpression is sufficient to down-regulate phosphorylation of the mTOR target protein S6K in a TSC1/TSC2-dependent manner. It remains to be established whether compounds that induce mTOR activity act through up-regulation of Redd1.

Abbreviations: mTOR, mammalian target of rapamycin; NSCLC, non-small cell lung cancer; 4E-BP1, eukaryotic initiation factor 4E binding protein 1; ATF4, activating transcription factor 4; TRAIL, TNF-related apoptosis-inducing ligand.

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Sorafenib, a multikinase inhibitor, is emerging as a promising targeted agent that may possess significant *in vitro* and *in vivo* antitumor activity against a broad range of cancers, including renal cell carcinoma, hepatoma, and leukemia [10–12]. The antitumor effects of sorafenib in renal cell carcinoma and hepatoma have been ascribed to its anti-angiogenic actions, which are mediated by inhibition of the kinase activity of growth factor receptors [13]. However, recent reports have shown that sorafenib induces cell death in human leukemia cells through a mechanism involving down-regulation of MCL-1 (myeloid cell leukemia sequence 1) [14]. Sorafenib-mediated MCL-1 down-regulation occurs through a post-transcriptional mechanism that may be mediated by signaling related to ER stress [15]. These results suggest that the previously observed antitumor effects of sorafenib involve a combination of inhibition of RAF family kinases, inhibition of receptor tyrosine kinases that signal angiogenesis, and the induction of ER stress signaling [11]. However, very little is known about the mechanism(s) by which sorafenib induces cell death in human NSCLC cells. In the present study, we provide evidence that sorafenib mediates apoptotic cell death in human NSCLCs through REDD1-induced inhibition of mTOR and subsequent down-regulation of survivin. In addition, these events are associated with a sensitizing effect on TRAIL-induced apoptotic cell death.

2. Materials and methods

2.1. Cell culture and reagents

H1299, A549, and H460 human NSCLC cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in recommended growth media (Invitrogen, Carlsbad, CA, USA). Sorafenib (BAY 43-9006) was purchased from Bayer Pharmaceuticals (New Haven, CT, USA) and recombinant human TRAIL/Apo2 ligand was from Alexis Biochemicals (Corgier, Paris, France). Rapamycin and RAD001 were obtained from Selleck Chemicals (Houston, TX, USA) and IGF-1 was from Peprotech Inc. (Rocky Hill, NJ, USA). Anti-XIAP antibody was purchased from BD Biosciences Pharmingen (San Diego, CA, USA); anti-DR5 was from KOMA Biotech (Seoul, Korea); anti-PARP, -Redd1, -S6 and -S6K antibodies were from Cell Signaling Technology (Beverly, MA, USA); and antibodies against survivin, DR4, cIAP-1, cIAP2, ATF4, 4E-BP1 and β -actin were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); CHOP antibody was from Abcam (Cambridge, UK). All si RNA was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Measurement of cell viability

Cell viability was determined by measuring the mitochondrial conversion of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) to a colored product. Cells were treated with drugs for 48 h, after which MTT reagent was added. After incubating for 1 h at 37 °C, the cells were solubilized in DMSO. The amount of converted MTT was determined by measuring the absorbance at 540 nm.

2.3. Measurement of caspase activation

Active caspases were detected using the CaspaTag™ Caspase 3/7 In Situ Assay kit (Chemicon, Temecula, CA, USA) according to the manufacturer's instructions. This kit employs carboxyfluorescein-labeled fluoromethyl ketone peptide inhibitors of caspases 3/7 (FAM-DEVD-FMK). These peptides are cell-permeable and non-cytotoxic fluorochrome inhibitors that covalently bind to a reactive cysteine residue on the large subunit of the active caspase heterodimer, inhibiting enzymatic activity and producing green fluorescence. Thus, the green fluorescent signal directly

corresponds to the amount of active caspases present in the cell at the time the reagent is added. The stained cells were analyzed with a FACS can flow cytometer (BD Biosciences).

2.4. Analysis of apoptosis

Apoptosis was determined using an annexin V fluorescein isothiocyanate/propidium iodide (PI) kit (BD Pharmingen). In brief, cells were washed with ice-cold phosphate buffered saline and resuspended in binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, and 2.5 mM CaCl₂) at a concentration of 1×10^6 cells/ml. Cells were incubated with 5 μ l each of annexin V fluorescein isothiocyanate and PI and analyzed with a FACScan flowcytometer (BD Biosciences).

2.5. Isolation of RNA and RT-PCR analysis

Total RNA was isolated from cells using the easy-BLUE Total RNA extraction kit according to the manufacturer's directions (iNtRON Biotechnology, Seoul, Republic of Korea). cDNA primed with oligo (dT) was prepared from 2 μ g of total RNA using M-MLV reverse transcriptase (Invitrogen). The specific primers for PCR were as follows: survivin, 5'-GGA CCA CCG CAT CTC TAC-3' (forward) and 5'-CAG CCT TCC AGC TCC TTG-3' (reverse); DR5, 5'-CGC TGC ACC AGG TGT GAT TC-3' (forward) and 5'-GTC TCC TCC ACA GCT GGG AC-3' (reverse); CHOP, 5'-CAG ACT GAT CCA ACT GCA G-3' (forward) and 5'-GAC TGG AAT CTG GAG AGT G-3' (reverse); DR4, 5'-CTG AGC AAC GCA GAC TCG CTG TCC AC-3' (forward) and 5'-TCC AAG GAC ACG GCA GAG CCT GTG CCA T-3' (reverse); and β -actin, 5'-GGA TTC CTA TGT GGG CGA CAG-3' (forward) and 5'-CGC TCG GTG AGG ATC TTC ATG-3' (reverse).

2.6. Western blot analysis

Cells were collected, washed with ice-cold phosphate-buffered saline (PBS) and lysed in lysis buffer (40 mM Tris-HCl pH 7.4, 120 mM NaCl, 1% NP-40, 10 mM EDTA) supplemented with protease inhibitor cocktail (Roche, Mannheim, Germany). Insoluble components were removed from the lysate by microcentrifugation at 13,500 rpm for 15 min, and protein concentrations were measured by the Bradford method. Samples (20–40 mg of protein) were separated by SDS-PAGE on 8–14% acrylamide gels and transferred to nitrocellulose membranes. The membranes were incubated with primary antibodies, followed by incubation with horseradish peroxidase-conjugated secondary antibodies. Immunoreactive bands were visualized using enhanced chemiluminescence reagents (Amersham Pharmacia, Uppsala, Sweden).

3. Results

3.1. Sorafenib induces the apoptotic cell death of human NSCLC cell lines

Treatment of human lung cancer cell lines (H1299, A549, H460) with sorafenib for 48 h resulted in a concentration-dependent reduction in cell viability. Fig. 1A and B depicts the decrease in cell viability in the presence of increasing concentrations of sorafenib, as assessed by the tetrazolium-based MTT assay. At concentrations above 5 μ M, the decrease in cell viability was prominent in all tested cell lines. Concomitant with a decrease in viability, Western blotting revealed cleavage of poly(ADP-ribose) polymerase (PARP), a specific substrate of caspase-3, in cells treated with sorafenib. Flow cytometric analysis using a fluorescent inhibitor of active caspase-3/7 showed that caspase-3/7 was markedly activated by treatment with 10 μ M sorafenib and also sorafenib induced cell death, as evaluated with annexin V/PI staining (Fig. 1C and D).

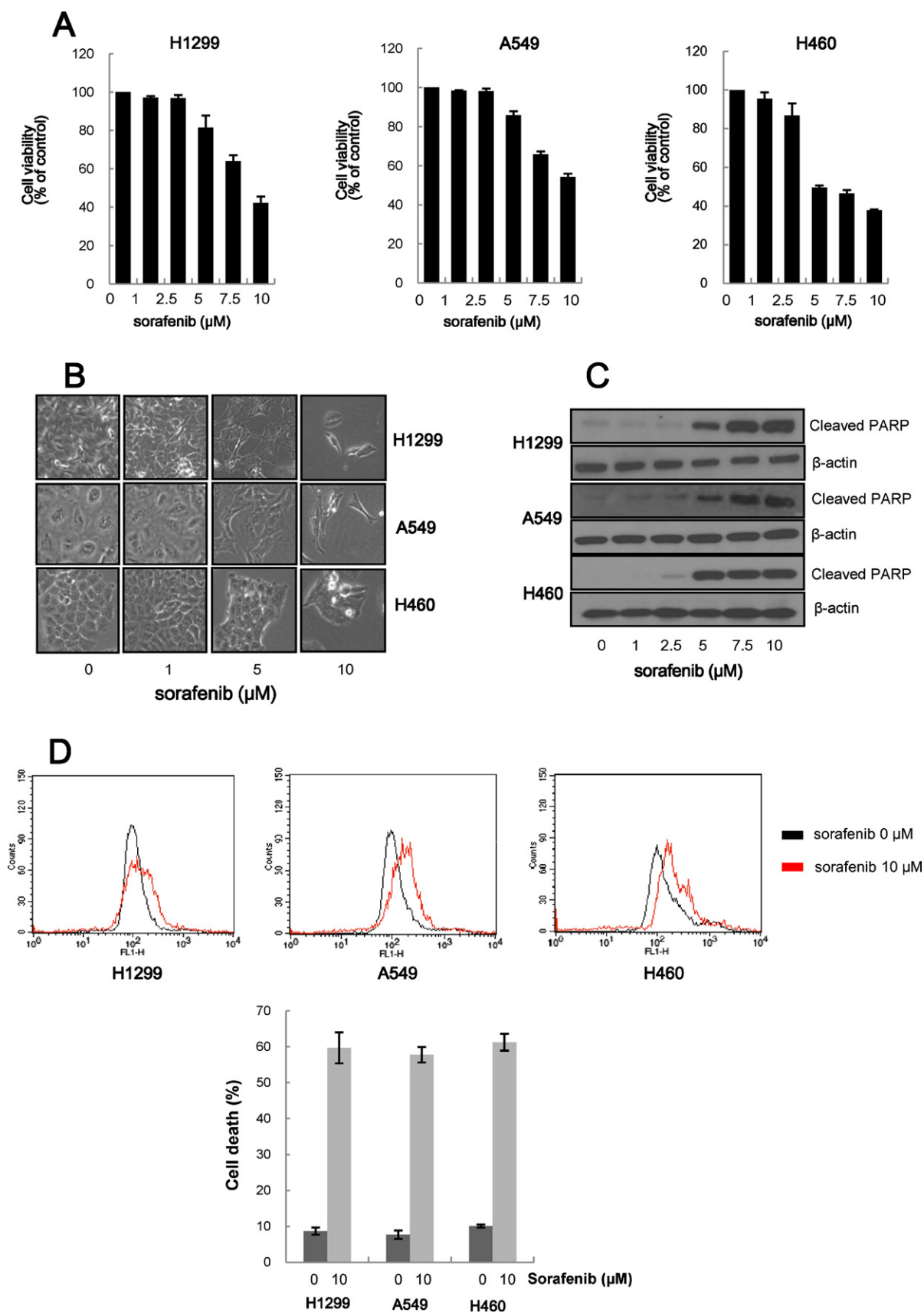
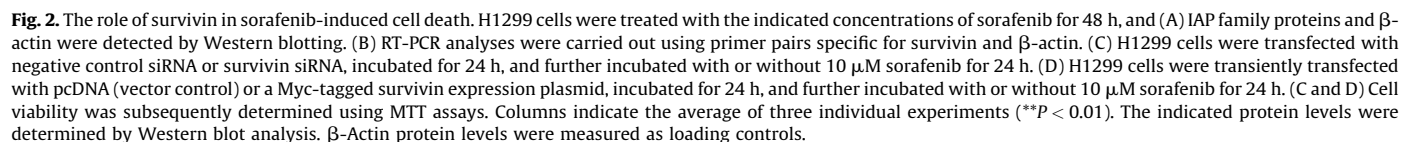


Fig. 1. Cytotoxicity of sorafenib toward NSCLC cells. NSCLC cells were treated with the indicated concentrations of sorafenib for 48 h. (A) Cell viability was measured using MTT assays. (B) Cell morphology was observed after 48 h incubation with the indicated concentrations of sorafenib and photographed by optical microscopy. (C) The levels of PARP protein were determined by Western blot analysis. β -Actin protein levels were measured as loading controls. (D) Apoptosis was measured as the percentage of cells with PI (+) or annexin V (+) using flow cytometry. Caspase activity was detected by staining with fluorescein isothiocyanate-labeled caspase inhibitors followed by flow cytometry.



We next determined the level of survivin protein and mRNA following treatment of H1299 cells with different concentrations

of sorafenib for 48 h using Western blot analysis and real-time reverse transcription-polymerase chain reaction (RT-PCR). Survivin protein levels were reduced by sorafenib treatment in a concentration-dependent manner. The effect of sorafenib was specific for survivin as sorafenib did not change the expression of related IAP (inhibitor of apoptosis) members, cIAP1/2 and XIAP (X-linked IAP) (Fig. 2A). Sorafenib did not modulate survivin mRNA levels (Fig. 2B), suggesting that changes in survivin expression after

exposure to sorafenib did not involve transcriptional regulation of survivin.

We then investigated the role of survivin in sorafenib-induced cell death of lung cancer cells using small interfering RNA (siRNA). Cells transfected with siRNA against survivin displayed significantly elevated cell death compared with control siRNA-treated cells (Fig. 2C). We furthermore examined the effects of sorafenib by transiently transfecting cells with a survivin expression plasmid. Expression of survivin in transiently transfected cells

was confirmed by Western blot analysis. As shown in Fig. 2D, treatment of survivin-overexpressing H1299 cells with sorafenib resulted in a significant statistically decrease in apoptosis compared with control cells, indicating that ectopic expression of survivin in H1299 cells attenuates sorafenib-induced cell death. Collectively, these results indicate that survivin counteracts sorafenib-induced apoptosis in H1299 cells, and suggest that the cell death induced by this drug is due, at least in part, to survivin down-regulation.

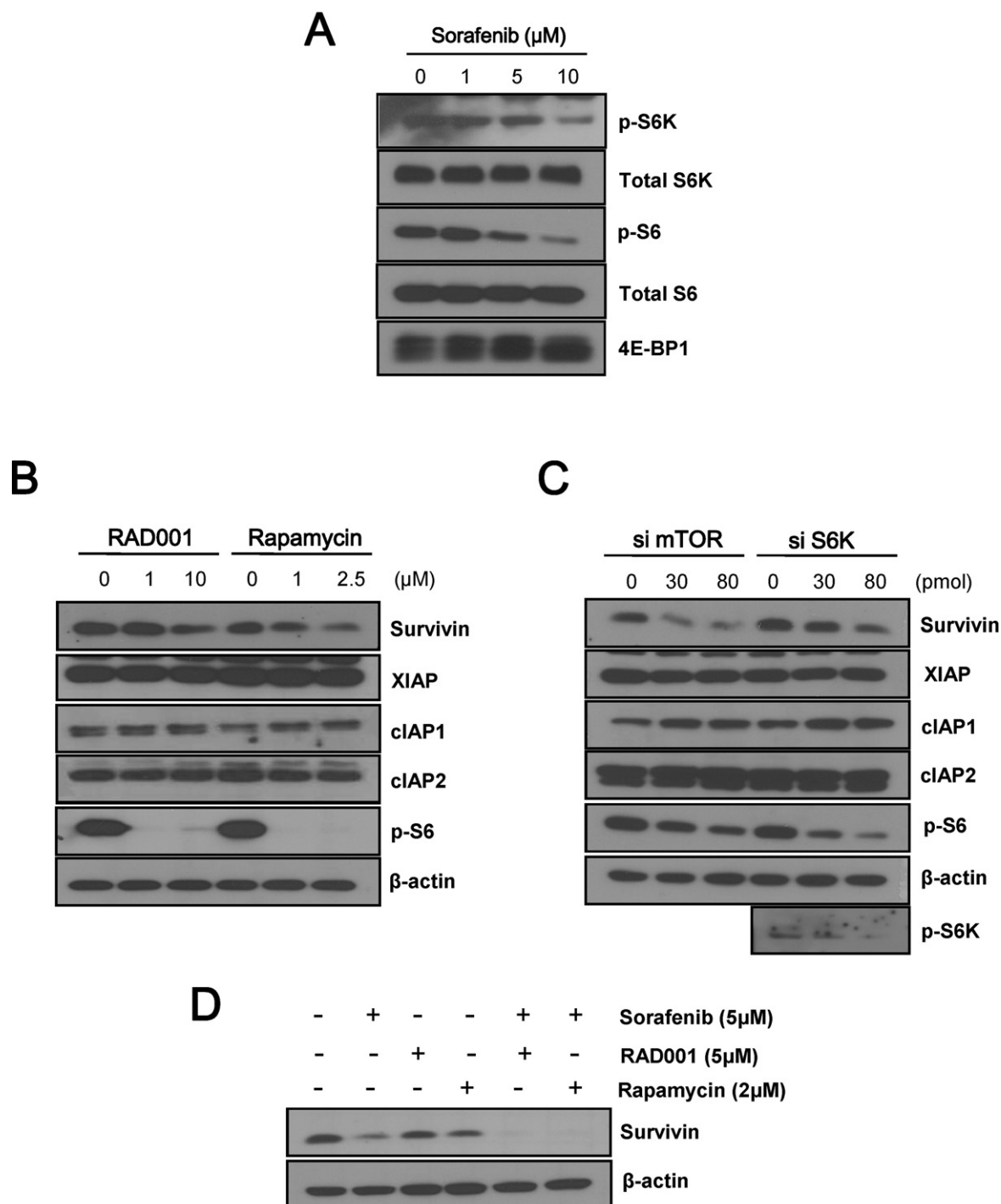


Fig. 3. Inhibition of mTOR activity may be responsible for sorafenib-induced down-regulation of survivin. (A) H1299 cells were treated with the indicated concentration of sorafenib for 48 h. The indicated protein levels were determined by Western blot analysis. (B) H1299 cells were treated with the indicated concentration of RAD001 or Rapamycin for 48 h. (C) H1299 cells were transfected with the indicated concentration of siRNA targeting mTOR or S6K, and incubated for 48 h. (D) H1299 cells were incubated with or without 5 μ M sorafenib, with or without 5 μ M RAD001, and with or without 2 μ M rapamycin for 48 h. (B–D) The indicated protein levels were determined by Western blot analysis. β -Actin protein levels were measured as loading controls.

3.3. Sorafenib suppresses survivin expression by inhibiting mTOR

As a first step to investigate the mechanism(s) underlying sorafenib-induced survivin down-regulation, we used Western blot analysis to examine phosphorylation of the downstream effectors of mTOR, S6K, S6 (p70S6), and 4E-BP1 in H1299 cells after treatment with sorafenib. We found that sorafenib inhibited mTOR activity in a concentration-dependent manner, as evidenced by the disappearance of phosphorylated-S6K/S6, and the appearance of a third faster-migrating 4E-BP1 band that corresponds to the α 4E-BP1 phosphorylated form (Fig. 3A). Next, to determine whether mTOR activation is involved in the regulation of survivin expression, we examined survivin levels after treatment with the mTOR inhibitors, RAD001 and rapamycin. As shown in Fig. 3B, these mTOR inhibitors decreased the levels of survivin protein, as assessed by Western blot analysis, without affecting the levels of other IAP members. We further examined the role of mTOR signaling in survivin expression using siRNA targeting mTOR and S6K. The results showed that survivin expression in H1299 cells was markedly reduced by siRNA-mediated knockdown of mTOR or S6K (Fig. 3C). In addition, combined treatment with sorafenib and mTOR inhibitor decreased survivin expression to a greater extent than treatment with either alone (Fig. 3D). These results demonstrate that sorafenib-induced down-regulation of survivin is mediated by inhibition of mTOR activity in lung cancer cells.

3.4. Sorafenib-induced Redd1 expression inhibits mTOR activity and results in down-regulation of survivin

Redd1, which is induced by activating transcription factor 4 (ATF4) in response to various cellular stresses, including ROS, ER stress and 2-deoxyglucose, is known to negatively regulate mTOR by mediating dissociation of 14-3-3 protein from TSC2 [7,9]. Therefore, we investigated the involvement of Redd1 in sorafenib-induced mTOR inhibition. We first examined the induction of ATF4 and Redd1 expression by sorafenib using Western blot analysis. As shown in Fig. 4A, sorafenib triggered a concentration-dependent increase in the expression of ATF4 and concurrently induced the Redd1 expression. We further investigated whether ATF4 activity was causally linked to sorafenib-induced up-regulation of Redd1 expression by knocking down ATF4 with siRNA. Western blot analyses revealed that ATF4 siRNA blocked sorafenib-induced up-regulation of REDD1 (Fig. 4B), indicating that ATF4 was indeed responsible for REDD1 induction in sorafenib-treated cells. Next, to determine the effects of ATF4-mediated Redd1 induction on sorafenib-induced down-regulation of survivin, we measured the levels of survivin in REDD1 siRNA-expressing cells by Western blot analysis. A significant recovery of survivin expression was evident in Redd1 siRNA-containing cells treated with sorafenib (Fig. 4C). Moreover, S6K phosphorylation levels were increased in Redd1 siRNA-expressing cells exposed to sorafenib compared with

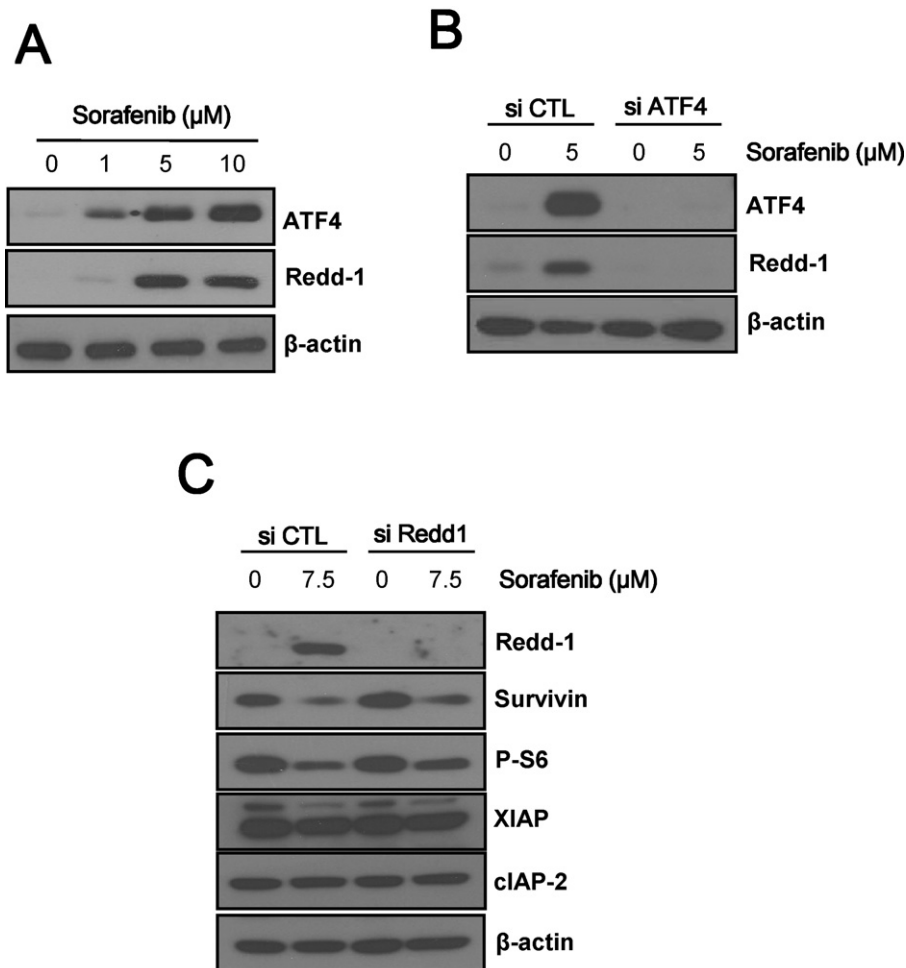


Fig. 4. Sorafenib-induced Redd1 expression inhibits mTOR activity. (A) H1299 cells were stimulated with the indicated concentrations of sorafenib for 48 h. The effects of sorafenib treatment were analyzed by Western blotting. β -Actin protein levels were measured as loading controls. (B and C) H1299 cells were transfected with negative control siRNA or siRNA against ATF4 or Redd1 for 24 h. Cells were then treated with the indicated concentrations of sorafenib for 24 h. The indicated protein levels were determined by Western blot analysis. β -Actin protein levels were measured as loading controls.

control cells. These results strongly suggest that ATF4-induced Redd1 is required for sorafenib-induced inhibition of mTOR activity and down-regulation of survivin expression.

3.5. Sorafenib suppresses IGF-1-induced survivin expression

It was recently reported that stimulation with insulin-like growth factor-1 (IGF-1) results in increased survivin expression in prostate cancer cells via activation of mTOR signaling [16,17]. Therefore, we first examined the expression of survivin and mTOR activation in H1299 lung cancer cells after treatment with different concentrations of IGF-1. Stimulation of serum-starved cells with IGF-1 or fetal bovine serum (FBS) markedly increased survivin expression (Fig. 5A), in agreement with a previous report. In contrast, IGF-1 did not modulate XIAP or cIAP2 levels compared with untreated or FBS-treated cultures, suggesting that the effect of IGF-1 was specific for survivin in lung cancer cells. Phosphorylation levels of S6 were also increased by IGF-1 in a pattern that paralleled survivin expression.

We next asked whether treatment with sorafenib affected IGF-1-induced survivin expression. Treatment of H1299 cells with sorafenib nearly completely abolished IGF-1-induced survivin expression. As expected, the mTOR inhibitor RAD001 markedly suppressed the IGF-1-induced expression of survivin (Fig. 5B). These results indicate that the increase in survivin expression induced by IGF-1 activation of mTOR is abolished by sorafenib in human lung cancer cells.

3.6. mTOR down-regulation by sorafenib sensitizes lung cancer cells to TRAIL-induced apoptotic cell death

To investigate whether down-regulation of survivin by sorafenib sensitizes lung cancer cells to TRAIL (TNF-related apoptosis-inducing ligand), we first examined the sensitizing effect of sorafenib on TRAIL-induced apoptosis. H1299 lung cancer cells which are resistant to TRAIL, were used as a model. Treatment of H1299 cells with increasing concentrations of sorafenib for 24 h sensitized cells to concurrent exposure to TRAIL, as evidenced by the concentration-dependent decrease in viability observed in MTT assays; very little cell death occurred in cells exposed to TRAIL alone (Fig. 6A). Similar inferences were drawn from an examination of optical microscopic images of cells (Fig. 6B). These results suggest that sorafenib synergizes with TRAIL to enhance TRAIL-induced cell death in H1299 cells. Next, to test the involvement of Redd1-induced down-regulation of survivin in this synergistic effect, we knocked down Redd1 or survivin using siRNA. As shown in Fig. 6C, cell death induced by treatment with the sorafenib/TRAIL combination was decreased in Redd1 siRNA-expressing H1299 cells compared with control cells. In contrast, the combination of sorafenib/TRAIL promoted cell death to a greater extent in cells expressing survivin siRNA than in control cells. Based on these results, we propose that Redd1-induced mTOR down-regulation may contribute to the sensitizing effect of sorafenib on TRAIL-induced apoptosis.

3.7. Sorafenib induces DR5 expression at the transcriptional level

To further explore the mechanism that may be responsible for the enhancement of TRAIL-induced apoptosis by sorafenib, we examined expression of TRAIL receptors in H1299 cells. As shown in Fig. 7A, sorafenib increased expression of DR5 (TRAIL-R2/TNFRSF10B) mRNA in a dose-dependent manner but had no effect on DR4 (TRAIL-R1/TNFRSF10A) expression. DR5 protein levels were also increased in parallel with the increase in mRNA levels. These findings suggest that DR5 up-regulation could be a mechanism by which sorafenib enhances TRAIL-mediated cell

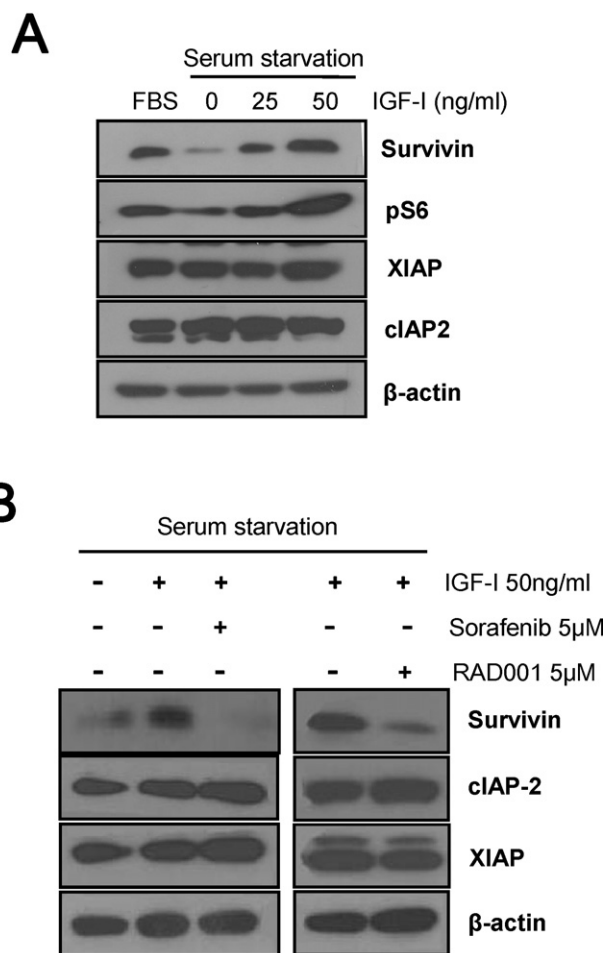


Fig. 5. Regulation of survivin expression by IGF-1. (A) Serum-deprived H1299 cells were stimulated with the indicated concentrations of IGF-1 or 10% FBS, and analyzed after 48 h by Western blotting. (B) Serum-starved H1299 cells were pretreated with the indicated concentrations of IGF-1 for 2 h, and then incubated for 48 h with or without sorafenib and with or without RAD001. Changes in survivin expression were analyzed by Western blotting.

death in H1299 cells. Several studies have shown that up-regulation of DR5 is mediated by CHOP ([DDIT3 (DNA-damage-inducible transcript 3)]). Consistent with this relationship, we found that sorafenib induced CHOP expression at both mRNA and protein levels (Fig. 7A).

To elucidate the role of CHOP in sorafenib-induced up-regulation of DR5, we silenced CHOP using siRNA and examined sorafenib effects on DR5 expression. Transfection with CHOP siRNA almost completely abrogated sorafenib-induced DR5 expression (Fig. 7B), indicating that CHOP plays a major role in DR5 up-regulation.

As shown above (see Fig. 4A), sorafenib induced the expression of ATF4, which is known to up-regulate CHOP, suggesting that ATF4 might play a role in CHOP-mediated DR5 expression. To determine whether knockdown of ATF4 blocks CHOP and DR5 induction, we transfected H1299 cells with a specific siRNA against ATF4. The results indicated that siRNA-mediated knockdown of ATF4 significantly suppressed sorafenib-induced up-regulation of CHOP, but had no effect on DR4 expression (Fig. 7C).

Finally, we further investigated the relationship between sorafenib-induced DR5 expression and the enhancement of TRAIL-mediated cell death. We found that siRNA-mediated knockdown of DR5 restored cell viability in sorafenib-treated cells (Fig. 7D), indicating that sorafenib-induced up-regulation of DR5 expression enhances TRAIL-induced apoptosis.

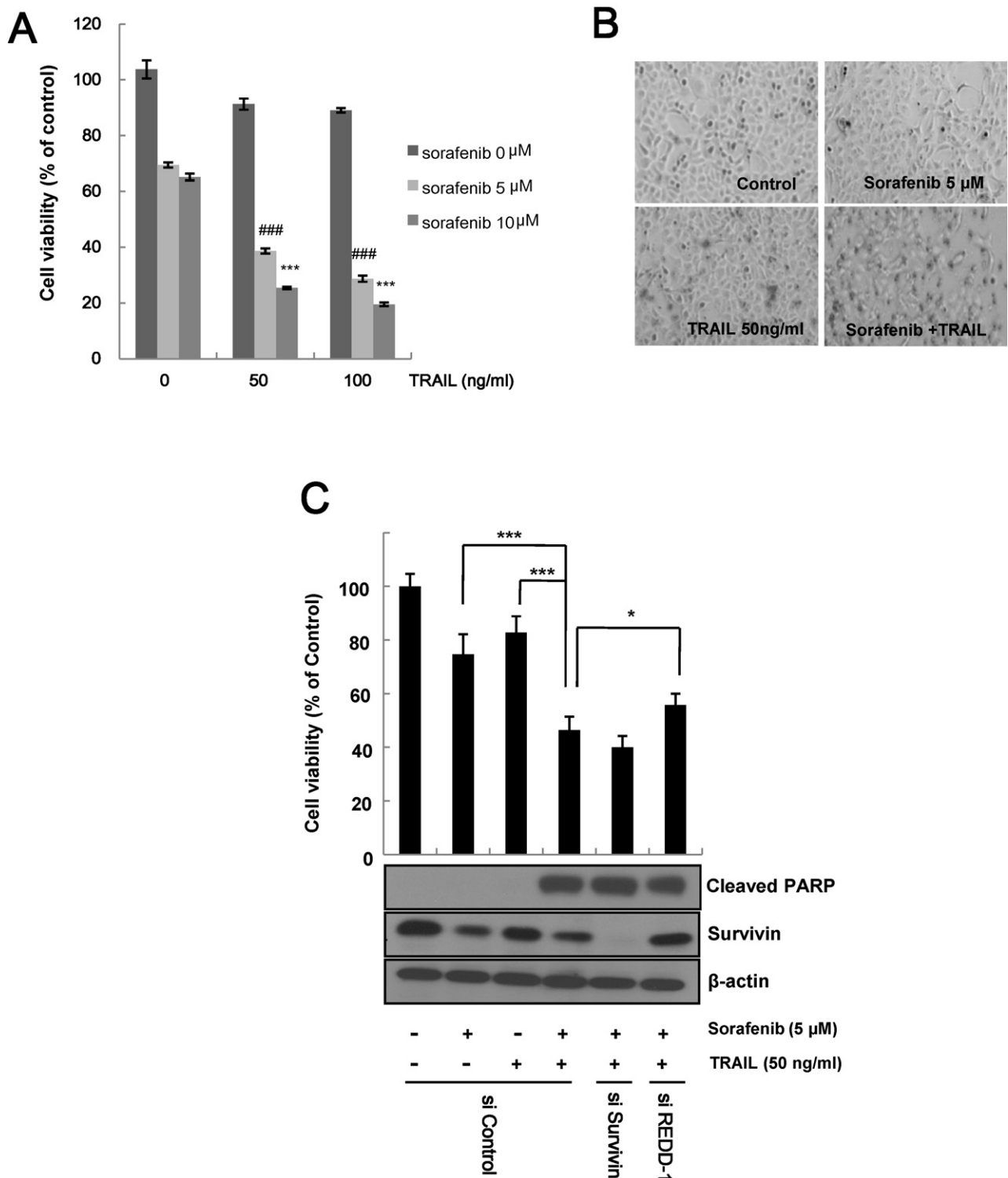


Fig. 6. Sorafenib sensitizes H1299 cells to TRAIL-induced apoptosis through survivin down-regulation. (A) H1299 cells were co-treated with TRAIL and the indicated concentrations of sorafenib for 24 h and then cell viability was assessed using MTT assays. Columns indicate the average of three individual experiments (### $P < 0.001$ versus TRAIL untreated and 5 μ M sorafenib-treated groups; *** $P < 0.001$ versus TRAIL untreated and 10 μ M sorafenib-treated groups). (B) H1299 cells were co-treated indicated concentrations of sorafenib and TRAIL for 24 h. And then, cell morphology was photographed by optical microscopy. (C) H1299 cells were transfected with negative control siRNA or siRNA against survivin or Redd1 for 24 h, and then incubated with the indicated concentrations of sorafenib and/or TRAIL for 24 h. Cell viability was determined using MTT assays. Columns indicate the average of three individual experiments (*** $P < 0.001$, * $P < 0.05$). The indicated protein levels were determined by Western blot analysis.

4. Discussion

The mTOR pathway constitutes a sensor network for nutrients and stress conditions that affects cell growth and cell survival [18].

Therefore, inhibiting mTOR is an attractive therapeutic target for cancer therapy, as exemplified by current clinical trials of rapamycin analogues. In this study, we report that sorafenib exerts significant anti-tumor activity through inhibition of mTOR

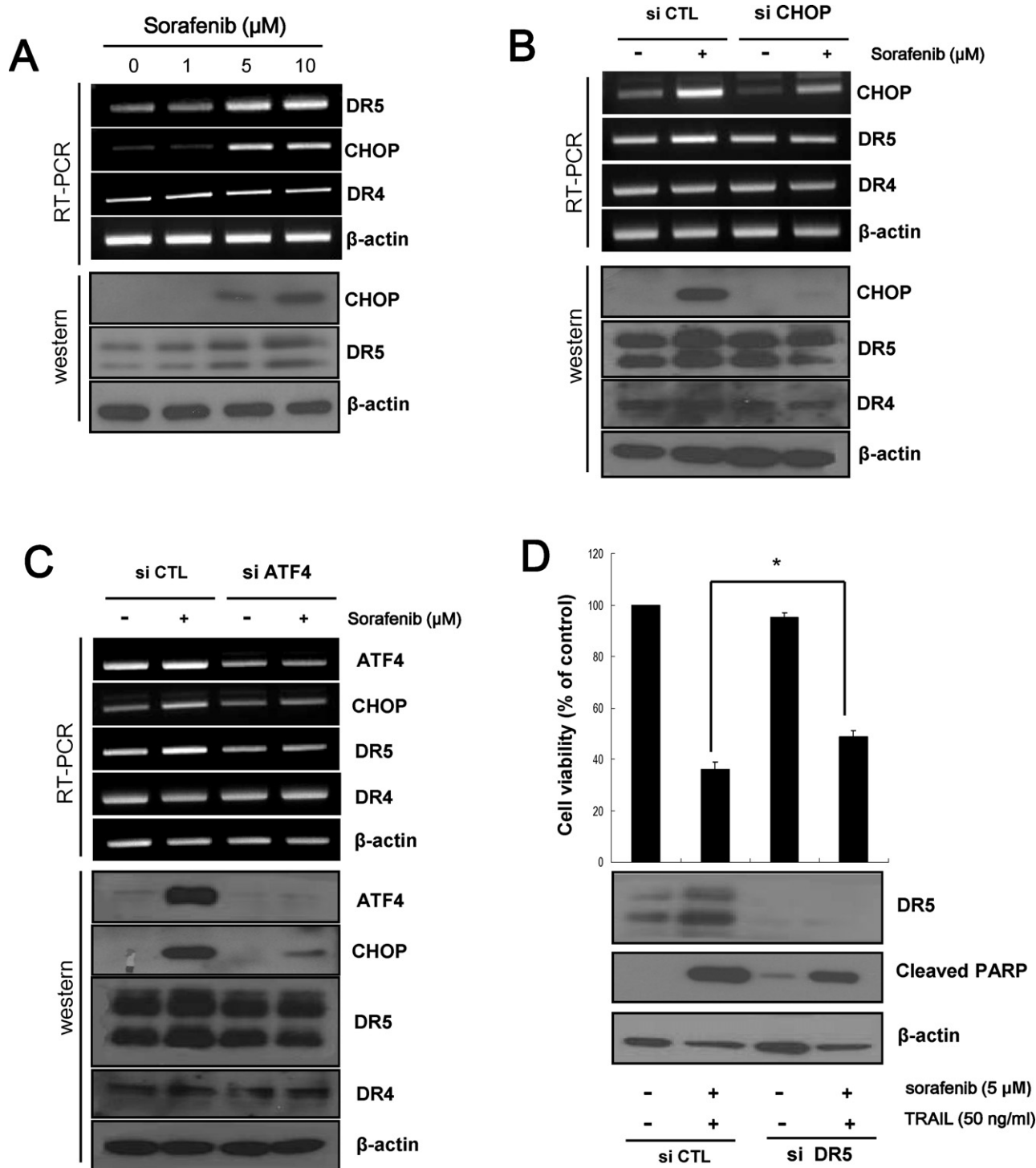


Fig. 7. ATF4/CHOP mediates sorafenib-induced DR5 up-regulation. (A) H1299 cells were stimulated with the indicated concentrations of sorafenib for 48 h. The effects of sorafenib treatment were analyzed by RT-PCR using primer pairs specific for DR5, DR4, and CHOP. DR5 and CHOP protein levels were determined by western blotting. β -Actin was used as a loading control. (B and C) H1299 cells were transfected with negative control siRNA or siRNA against ATF4 or CHOP for 24 h, and then incubated with or without 5 μ M sorafenib for 24 h. The indicated mRNA and protein levels were determined by RT-PCR and Western blot analysis, respectively. (D) H1299 cells were transfected with negative control siRNA or DR5 siRNA. After 24 h, cells were co-treated with the indicated concentrations of sorafenib and TRAIL for 24 h. Cell viability was then assessed using MTT assays, and the indicated protein levels were determined by Western blot analysis. Columns indicate the average of three individual experiments (* $P < 0.05$).

signaling and subsequent down-regulation of survivin expression in human NSCLC cells. We found that the effect of sorafenib on mTOR inhibition was mediated by Redd1, a hypoxia-induced gene that acts as a negative regulator of mTOR. Increased levels of Redd1 were associated with ER stress and activation of ATF4, a transcription factor induced by ER stress through increased phosphorylation of eIF2 α . We show that sorafenib increased

ATF4 expression, and specific knockdown of ATF4 abolished sorafenib-induced Redd1 expression. Therefore, the effect of sorafenib on mTOR inhibition is mediated by ATF4-induced Redd1 expression.

One notable event associated with sorafenib treatment was the marked inhibition of survivin protein expression. Survivin belongs to the IAP protein family, whose members act as inhibitors of cell

apoptosis. There is considerable evidence showing that increased survivin expression is associated with poor prognosis in many malignancies, and that down-regulation of survivin expression by siRNA or antisense oligonucleotides increases basal levels of apoptosis and sensitizes cancer cells to various apoptotic stimuli [19]. Considering its pleiotropic roles in cell proliferation and apoptosis, survivin is a promising therapeutic target. Survivin protein levels can be increased by IGF-1 stimulation in prostate and lung cancer cells through mTOR signaling effects on translation [16,17]. Therefore, inhibitors targeting this pathway may reduce cellular anti-apoptotic activity by down-regulating survivin expression, thereby increasing the efficacy of co-therapies in lung cancers. Sorafenib has such an action, suppressing mTOR signaling and down-regulating survivin expression in lung cancer cells. Indeed, targeted knockdown of survivin increased the apoptotic cell death induced by sorafenib; conversely, survivin overexpression blunted the apoptotic response to sorafenib. Furthermore, our study found that knockdown of Redd1 reduced the ability of sorafenib to downregulate mTOR activity and survivin expression. These results indicate that Redd1 is intimately involved in sorafenib-induced cell death and blockade of survivin expression via negative regulation of mTOR. Collectively, our data suggest that inhibition of the mTOR signaling pathway and suppression of associated protein translation events play important roles in the anticancer mechanism of sorafenib in human NSCLC cells.

We found that sorafenib enhances TRAIL-induced cell death in human lung cancer cells and identified the underlying sensitization mechanisms. One mechanism of sensitization is the regulation of survivin. Sorafenib down-regulated the expression of survivin, which has been linked to tumor cell resistance to TRAIL. Indeed, down-regulation of survivin expression has been shown to sensitize tumor cells to TRAIL [20]. We found that, in addition to down-regulating cell-survival proteins, sorafenib selectively induced DR5 expression. We showed that DR5 up-regulation plays a role in the sensitization of lung cancer cells to TRAIL-induced apoptosis, as evidenced by the fact that siRNA-mediated knockdown of this TRAIL receptor abolished the effect of combined treatment with sorafenib and TRAIL. Numerous mechanisms have been described for the induction of this death receptor [21–23]. We found that sorafenib-induced DR5 up-regulation was mediated through CHOP induction. CHOP is among the genes that are highly induced during ER stress, in which the PERK-eIF2 α -ATF4 branch of the unfolded-protein response plays an essential role. CHOP is a direct target gene of ATF4 [24]. Our data showed that ATF4 and CHOP were up-regulated by sorafenib, and siRNA-mediated silencing of CHOP blocked DR5 expression at both mRNA and protein levels. Our findings are consistent with those of previous studies, which have shown that CHOP binds to the DR5 promoter and up-regulates DR5 expression [23]. Knocking down ATF4 with siRNA abolished the effect of sorafenib on CHOP and DR5 induction. Thus, we suggest that ER stress may be an important upstream signal linking sorafenib to the induction of DR5.

In conclusion, we demonstrated that sorafenib is a potential chemotherapeutic agent that effectively induces apoptosis of human NSCLC cells. Although further research is needed to determine the full range of molecular changes associated with sorafenib exposure, the effectiveness of sorafenib on lung cancer cells *in vitro* was associated with down-regulation of the mTOR pathway via ER stress-induced ATF4 activation. Therefore, we demonstrated that sorafenib exerts effective anti-tumor activity against lung cancer cells by acting as an inhibitor of the mTOR pathway—events that are associated with the sensitizing effect of sorafenib on TRAIL-induced apoptotic cell death. These results establish sorafenib as a promising therapeutic agent in the treatment of patients with lung cancer.

Conflicts of interest

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2011.04.011.

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