

Arsenic trioxide potentiates the anti-cancer activities of sorafenib against hepatocellular carcinoma by inhibiting Akt activation

Bo Zhai · Xian Jiang · Changjun He · Dali Zhao ·
Lixin Ma · Lishan Xu · Hongchi Jiang · Xueying Sun

Received: 23 September 2014 / Accepted: 11 November 2014 / Published online: 22 November 2014
© International Society of Oncology and BioMarkers (ISOBM) 2014

Abstract Sorafenib is the standard first-line systemic drug for advanced hepatocellular carcinoma (HCC), but it also induces the activation of Akt, which contributes to the mechanisms for the resistance to sorafenib. Arsenic trioxide (ATO) is a currently clinically used anticancer drug and displays its anticancer activities by inhibiting Akt activation. Therefore, we hypothesized that ATO may potentiate the anti-cancer activities of sorafenib against HCC. The results have demonstrated that ATO synergized with sorafenib to inhibit the proliferation and promote the apoptosis of HCC cells by diminishing the increased activation of Akt by sorafenib. ATO was shown to inhibit the expression or activation of Akt downstream factors, including glycogen synthase kinase (GSK)-3 β , mammalian target of rapamycin (mTOR), ribosomal protein S6 kinase (S6K), and eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1), which regulate cell apoptosis and were upregulated or activated by sorafenib. Both sorafenib and ATO downregulated the expression of cyclin D1, resulting in HCC cells arrested at G0/G1

phase. ATO downregulated the expression of Bcl-2 and Bcl-xL and upregulated the expression of Bax, indicating that ATO could induce the apoptosis of HCC cells through the intrinsic pathways; but sorafenib showed little effects on these proteins of Bcl-2 family. ATO synergized with sorafenib to suppress the growth of HCC tumors established in mice by inhibiting the proliferation and inducing the apoptosis of HCC cells *in situ*. These results indicate that ATO may be a potential agent that given in combination with sorafenib acts synergistically for treating HCC.

Keywords Arsenic trioxide · Sorafenib · Hepatocellular carcinoma · Akt · Apoptosis · Proliferation

Introduction

Hepatocellular carcinoma (HCC) is the second most frequent cause of cancer death in men worldwide [1] and is extremely resistant to conventional chemotherapy [2]. Sorafenib has been approved as the standard first-line systemic drug for advanced HCC, but has only demonstrated limited survival benefits with very low response rates [3, 4]. Although the exact mechanisms for the resistance to sorafenib have not yet been fully elucidated, some approaches have been launched by combining it with other anticancer drugs to treat HCC [5].

Sorafenib executes its anticancer activities against HCC largely through its inhibitory actions on the Raf/mitogen-activated protein kinase (MAPK)/extracellular signaling-regulated kinase (ERK) pathway and multiple tyrosine kinase receptors [3]. In the recent explorations on the resistance to sorafenib, the phosphatidylinositol 3-kinase (PI3K)/Akt pathway has become a promising hot spot for developing novel therapeutic agents, though

B. Zhai · L. Xu
Department of General Surgery, The Fourth Affiliated Hospital of
Harbin Medical University, Harbin 150001, China

X. Jiang · D. Zhao · L. Ma · H. Jiang · X. Sun (✉)
The Hepatosplenic Surgery Center, Department of General Surgery,
The First Affiliated Hospital of Harbin Medical University,
Harbin 150001, China
e-mail: kevsun88@hotmail.com

X. Sun
e-mail: k.sun@auckland.ac.nz

C. He
Department of Thoracic Surgery, The Third Affiliated Hospital,
Harbin Medical University, Harbin 150040, China

it is not a direct target of sorafenib. The PI3K/Akt pathway crosstalks with the MAPK/ERK pathway [6] and regulates a large number of molecules involved in all aspects of cancer progression [7]. It represents a key signaling pathway involved in the development and progression of HCC, and is activated in a high proportion of HCC tissues [8]. It has been shown that sorafenib activates Akt and its downstream factors such as ribosomal protein S6 kinase (S6K) and eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) in HCC cells [9, 10], and blockage of the PI3K/Akt pathway augments the effects of sorafenib in treating HCC [9, 11]. Sorafenib-resistant HCC cells had increased expression of phosphorylated Akt (p-Akt) [12, 13], and specific inhibition of Akt synergized with sorafenib to inhibit the proliferation and promote the apoptosis of sorafenib-resistant cells in culture and mice [13, 14]. The results from the above studies indicate that Akt-inhibiting agents may potentiate the anti-cancer activities of sorafenib against HCC.

In searching for candidates that could enhance the efficacy of sorafenib to combat HCC, arsenic trioxide (ATO), a currently clinically used agent, has drawn our attention. ATO has been widely employed to treat acute promyelocytic leukemia (APL) since its original application at our institute in the 1970s [15]. Its therapeutic potential and anti-cancer activity have been tested in a variety of solid tumors including HCC by inducing cell apoptosis and cell cycle arrest [16–18]. ATO has also been investigated in clinical trials though it was not active as a single agent against advanced HCC [19]. More importantly, ATO exhibits its anticancer activities by inhibiting the activation of Akt or downregulating Akt expression [20–22]. Given that ATO possesses abilities to inhibit Akt as well as anticancer activities against HCC, we hypothesized that ATO could synergize with sorafenib in treating HCC.

Materials and methods

Cell culture, antibodies, and reagents

Human HCC HepG2 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), and Huh7 cells from Chinese Academy of Sciences Cell Bank (Shanghai, China). Cells were cultured at 37 °C in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Grand Island, NY, USA) supplemented with 10 % fetal bovine serum. The antibodies

(Abs) against Akt, p-Akt (Ser473), glycogen synthase kinase (GSK)-3 β , phosphorylated GSK3 β (p-GSK3 β) (Ser9), mammalian target of rapamycin (mTOR), S6K, phosphorylated S6K (p-S6K) (Thr389), 4EBP1, and phosphorylated 4EBP1 (p-4EBP1) (Ser65) were purchased from Cell Signaling Technology (Danvers, USA). The Abs against caspase-3, Bcl-2, Bcl-xL, Bax, cyclin D1, and β -actin were purchased from Santa Cruz Biotechnology (CA, USA). The anti-Ki67 Ab was purchased from Abcam (Cambridge, MA, USA). Sorafenib and perifosine were purchased from Jinan Trio Pharmatech Co., Ltd. (Jinan, China). Sorafenib was dissolved in dimethyl sulfoxide (DMSO) to make a stock solution of 100 mM for in vitro assays, and perifosine dissolved in phosphate buffered saline (PBS) to make a stock solution of 30 μ M. ATO was purchased from Yida Pharmaceutical, Harbin Medical University, China. The PI (propidium iodide)/Annexin V-FITC apoptosis detection kit was purchased from BD Biosciences (San Jose, CA, USA). The Cell Counting Kit-8 (CCK-8) kit was purchased from Dojindo Laboratories (Kumamoto, Japan). Terminal deoxynucleotidyl transferase dUTP nick end labeling agent (TUNEL) was purchased from Roche (Shanghai, China). A cell cycle detection kit was purchased from BD Biosciences, Beijing, China.

Animal experiments

Six to 8-week-old male nude BALB/c mice (H-2b) were obtained from the Animal Research Center, The First Affiliated Hospital of Harbin Medical University, China. This study had been approved (permit SYXK20020009) by the Animal Ethics Committee of Harbin Medical University, in compliance with the Experimental Animal Regulations by the National Science and Technology Commission, China. The experimental protocol has been described previously [13, 23, 24]. Briefly, 5×10^6 of cells were subcutaneously injected into backs of mice. When tumors reached ~ 100 mm³, the mice were assigned to four treatment groups (each group had eight mice), namely control, ATO, sorafenib, and ATO + sorafenib. ATO solution was diluted into 200 μ l of PBS and intraperitoneally injected at a dose of 2.5 mg/kg daily. Sorafenib was suspended in an oral vehicle containing Cremophor (Sigma-Aldrich), 95 % ethanol and water in a ratio of 1:1:6 [13, 23, 24], and orally administrated at a dose of 30 mg/kg by gavage daily. Mice in the control group received oral and intraperitoneal vehicles; mice in sorafenib group, oral sorafenib, and intraperitoneal vehicle; mice in the ATO

group, oral vehicle, and intraperitoneal ATO; and mice in sorafenib + ATO group, oral sorafenib, and intraperitoneal ATO. The treatments lasted for 21 days and the tumor volumes were measured every 3 days. The tumors were harvested at the end of experiments.

Cell viability analysis, in vitro apoptosis assay, visualization of apoptotic cells by laser scanning confocal microscopy, cell cycle distribution assay, assessment of Ki-67 proliferation index, in situ detection of apoptotic cell and immunoblotting

All these methods have been described in details previously [13, 23–25].

Caspase activity assay

The activities of caspase-3 and -9 were measured with a caspase-3 and -9 Activity Kit (Beyotime Institute of Biotechnology, Haimen, Jiangsu, China) according to the manufacturer's instruction [25]. Briefly, cells were harvested and lysed, total protein was extracted, and concentration determined. The mixture of 10 μ l of protein extracts, 80 μ l of reaction buffer, and 10 μ l of caspase-3 substrate (Ac-DEVD-pNA) or caspase-9 substrate (Ac-LEHD-pNA) was incubated on 96-well plates at 37 °C for 4 h. The optical density was measured at 405 nm using a microplate reader. The relative caspase activity was expressed as percentage of enzyme activity compared with the control. The experiments were repeated thrice.

Transfection of Akt siRNA

A double-strand siRNA targeting human Akt (5' - GUGG UCAUGUACGAGAUGATT-3' and 5' -UCAUCUCGUA CAUGACCACTT-3') encoding nucleotides 1006–1025 of Akt1 (GenBank: NM_001014431.1), nucleotides 1210–1229 of Akt2 (GenBank: XM_005336494.1), and nucleotides 955–974 of Akt3 (GenBank: XM_004691046.1) with two introduced thymidine residues at the 3' end was produced by GenePharma Co., Ltd., Shanghai, China. The nonspecific scrambled siRNA (5' -UUCUCCGAACGU GUCACGU-3' and 5' -ACGUGACACGUUCGGAGAA-3') served as a control. Cells were grown to 60–70 % confluence, and incubated with siRNAs at a final concentration of 0.1 μ M by using LipofectamineTM 2000 (Invitrogen, Beijing, China) in a serum-free medium for 24 h and then subjected to assays.

Statistical analysis

The data were expressed as mean values \pm standard deviation. Comparisons were made using one-way analysis of variance followed by Dunnet's *t* test. $P < 0.05$ was considered significant.

Results

Sorafenib inhibits the viability and induces the apoptosis of HCC cells, and increases Akt activation

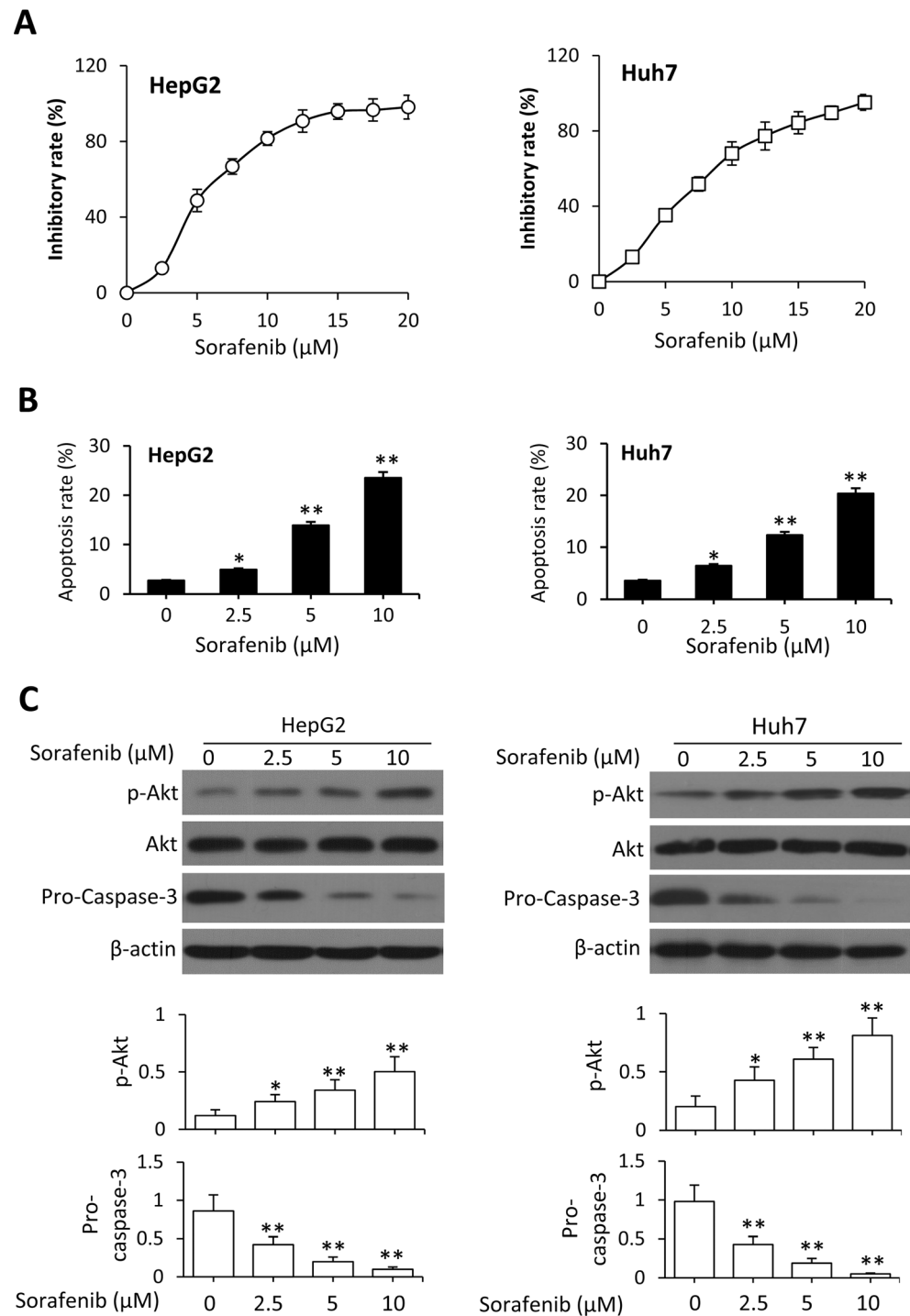
HCC cells were incubated with serial concentrations of sorafenib for 48 h, and then cell viability, and apoptosis were assessed. Sorafenib inhibited the viability of HepG2 and Huh7 cells (Fig. 1a), and increased their apoptosis rates (Fig. 1b), in a concentration-dependent manner, in accordance with previous reports [13, 23, 24]. Immunoblotting analysis showed that sorafenib upregulated the expression of p-Akt, and downregulated the expression of pro-caspase-3, in a concentration-dependent manner, but had no effect on Akt expression (Fig. 1c).

Inhibition of Akt enhances the effects of sorafenib against HCC cells

The above results drove us to investigate the role of Akt in the anticancer activities of sorafenib against HCC cells. First, we incubated control siRNA- and Akt siRNA-transfected Huh7 cells with sorafenib for 48 h, and the expression of p-Akt, Akt, and pro-caspase-3 was examined. Akt siRNA transfection significantly downregulated, but control siRNA had no effect on, the expression of Akt and p-Akt (Fig. 2a). Sorafenib upregulated the expression of p-Akt in control siRNA-transfected cells, but this effect was abolished in Akt-depleted cells (Fig. 2a). Both sorafenib and Akt siRNA transfection downregulated the expression of pro-caspase-3, and sorafenib incubation resulted in an even lower level of pro-caspase-3 in Akt-depleted cells (Fig. 2a).

We next examined whether inhibition of Akt by using perifosine, a specific Akt inhibitor, could potentiate the anticancer activities of sorafenib against HCC. Incubation of both sorafenib and perifosine for 48 h highly significantly inhibited the viability of Huh7 cells; and their combination further reduced cell viability, which was even significantly lower than those treated with sorafenib or perifosine alone (Fig. 2b). The value for the coefficient of drug interaction (CDI) [13, 23, 24] was calculated to be 0.65, indicating a synergistic effect of sorafenib and perifosine in reducing cell viability.

Fig. 1 Sorafenib inhibits the proliferation, induces the apoptosis, and increases Akt activation of HCC cells. **a** HepG2 and Huh7 cells were incubated with vehicle or sorafenib at serial concentrations (2.5, 5, 7.5, 10, 2.5, 15, 17.5, or 20 μM) for 48 h. Cell viability was assessed and the inhibitory rate (in percent) calculated. **b** The above cells incubated with vehicle or sorafenib at concentrations of 2.5, 5, or 10 μM were subjected to flow cytometry for analyzing apoptosis rates. **c** The lysates of cells from (b) were subjected to immunoblotting. The band density of p-Akt was normalized to Akt, and that of pro-caspase-3 normalized to β -actin. Data represent three independent experiments. * $P < 0.05$ and ** $P < 0.001$ indicate a significant difference from vehicle-treated cells

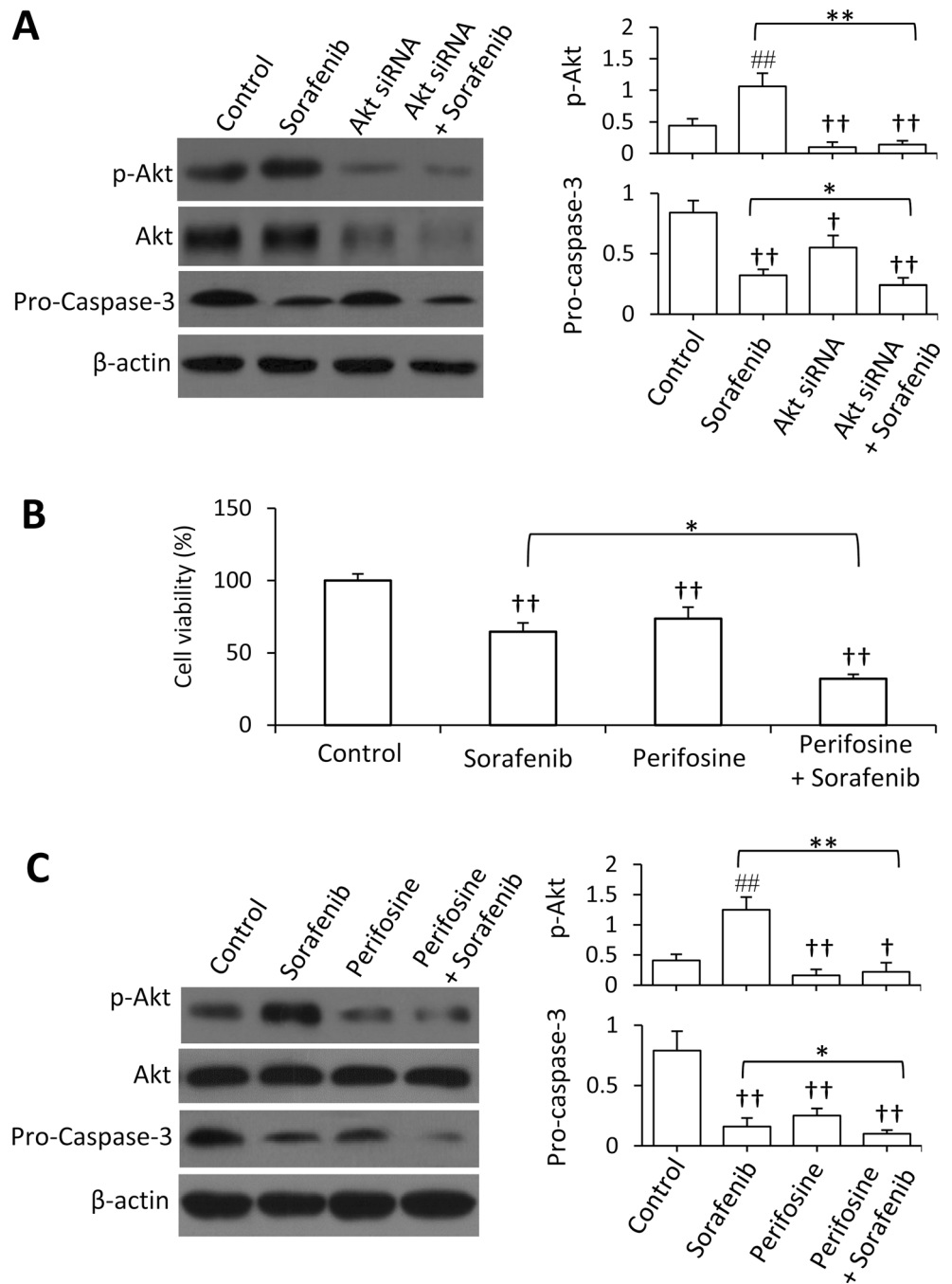


Immunoblotting assay of the above cells showed that sorafenib increased, while perifosine reduced, the expression of p-Akt, though they both downregulated the expression of pro-caspase-3 and had no effect on Akt expression (Fig. 2c). In addition, perifosine diminished the increased expression of Akt induced by sorafenib, and their combination further increased the expression of pro-caspase-3 (Fig. 2c).

ATO reduces the viability, induces the apoptosis of HCC cells and inhibits Akt activation

HCC cells were incubated with serial concentrations of ATO for 48 h, and then cell viability and apoptosis were assessed. ATO significantly reduced the viability of both HepG2 and Huh7 cells (Fig. 3a), and increased their apoptosis rates (Fig. 3b), in a concentration-

Fig. 2 Inhibition of Akt enhances the sensitivity of HCC cells to sorafenib. **a** Huh7 cells transfected with control siRNA (control) or Akt-siRNA (both at a final concentration of 0.1 μ M) for 24 h were incubated in the presence or absence of sorafenib (5 μ M) for 48 h, and then subjected to immunoblotting. The band density was normalized to β -actin. **b** Huh7 cells were incubated for 48 h with sorafenib (5 μ M), perifosine (10 μ M), or the combination. Cell viability (in percent) was assessed and normalized to control cells. **c** The cells in **b** were subjected to immunoblotting. The band density of p-Akt was normalized to Akt, and that of pro-caspase-3 normalized to β -actin. Data represent three independent experiments. * P <0.05 and ** P <0.001 indicate a significant difference. † P <0.05 and †† P <0.001 indicate a significant reduction and ††† P <0.001 a significant increase from controls



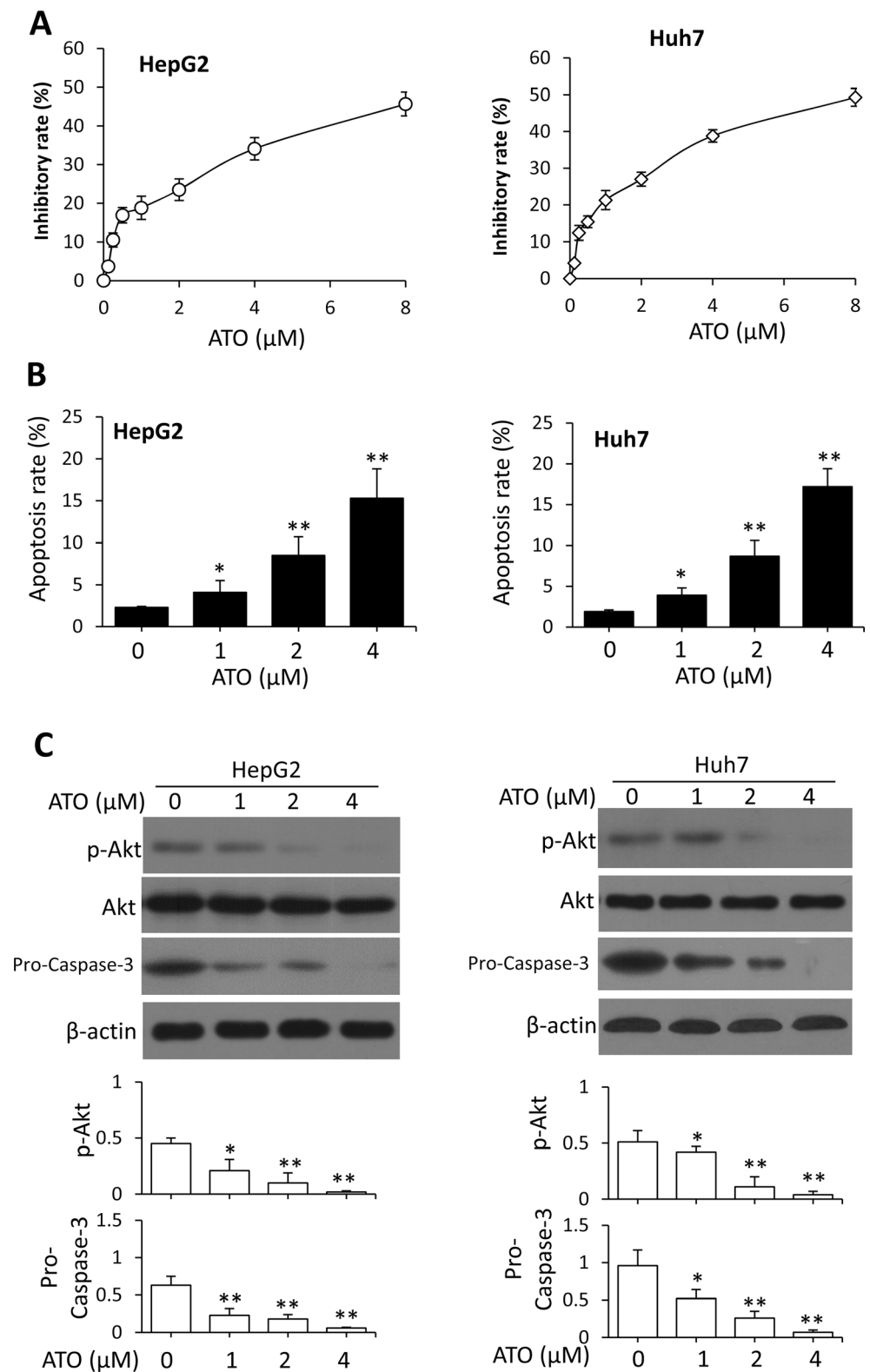
dependent manner. Immunoblotting analysis showed that ATO reduced the expression of p-Akt and pro-caspase-3, in a concentration-dependent manner; but had no effect on Akt expression (Fig. 3c).

ATO synergizes with sorafenib to inhibit the proliferation of HCC cells by regulating the Akt pathway in vitro

Huh7 cells were incubated with sorafenib (5 μ M), ATO (2 μ M) or the combination for 24, 48, or 72 h, and then cell

viability was assessed. As shown in Fig. 4a, ATO synergized with sorafenib in inhibiting cell viability in a time-dependent manner. The value of CDI was calculated to be 0.91, 0.87, and 0.69, when cells were incubated for 24, 48, or 72 h, respectively, indicating their inhibitory effects were synergistic. The above cells incubated for 48 h were subjected to a cell cycle detection assay and immunoblotting. ATO or sorafenib induced cells arrested at G0/G1 phase, and their combination resulted in a significantly bigger percentage of cells arrested at G0/G1 phase, compared with ATO or sorafenib alone

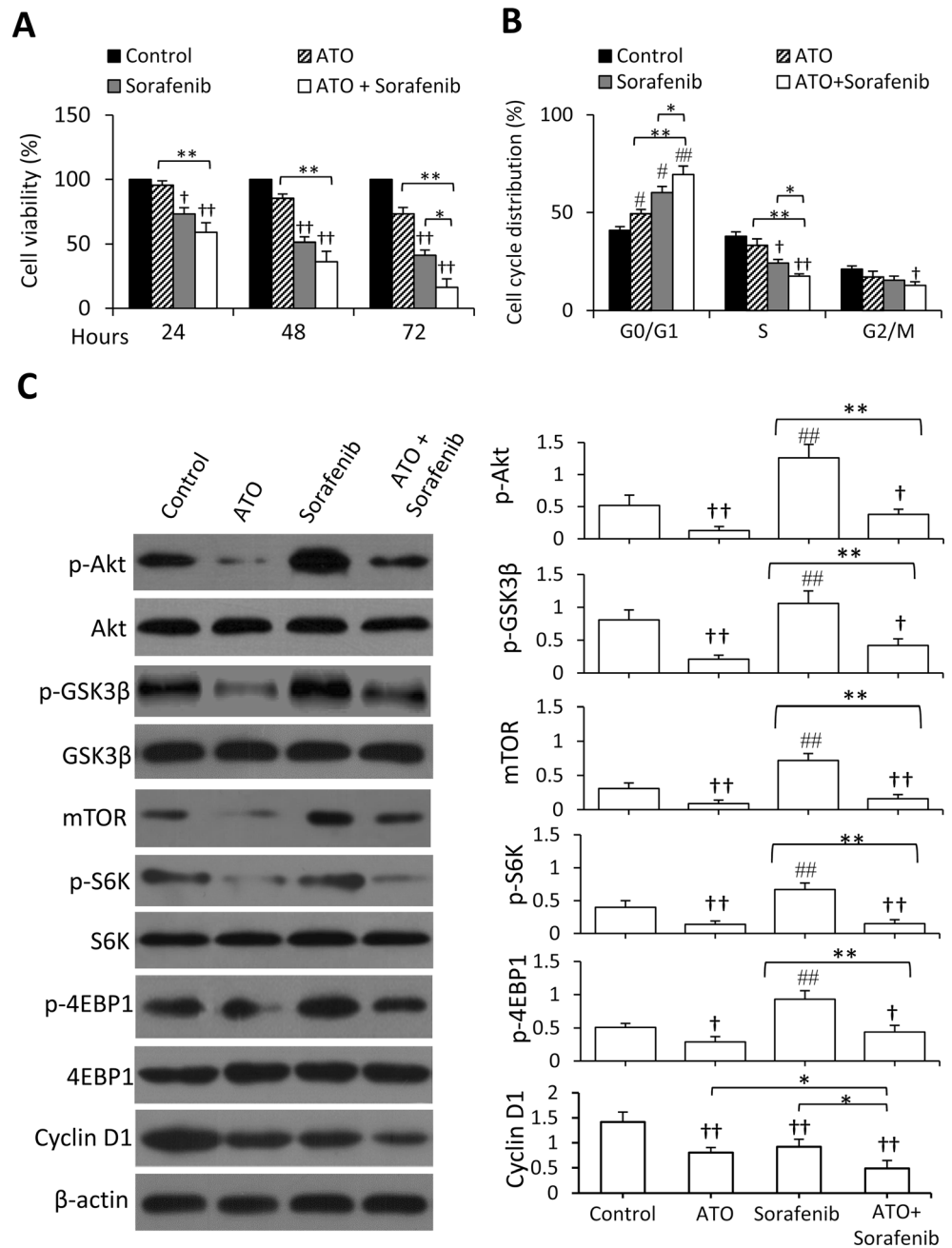
Fig. 3 ATO inhibits the growth, and induces the apoptosis and inhibits Akt activation of HCC cells. **a** HepG2 and Huh7 cells were incubated with vehicle or ATO at serial of concentrations (0.125, 0.25, 0.5, 1, 2, 4, or 8 μM) for 48 h. Cell viability was assessed and the inhibitory rate (in percent) calculated. **b** The above cells incubated with vehicle or ATO at concentrations of 1, 2, or 4 μM were subjected to flow cytometry for analyzing apoptosis rates. **c** The lysates of cells from **b** were subjected to immunoblotting. The band density of p-Akt was normalized to Akt, and that of pro-caspase-3 normalized to β -actin. Data represent three independent experiments. * $P < 0.05$ and ** $P < 0.001$ indicate a significant difference from vehicle-treated cells



(Fig. 4b). ATO significantly reduced the phosphorylation of Akt, and inhibited the expression of mTOR and the phosphorylation of GSK3 β , S6K, and 4EBP1; and diminished the

increased expression or phosphorylation of the above molecules induced by sorafenib (Fig. 4c). The results of cell cycle distribution were supported by the reduced expression

Fig. 4 ATO synergizes with sorafenib to inhibit the proliferation of HCC cells. Huh7 cells were incubated for 48 h with vehicle (control), sorafenib (5 μ M), ATO (2 μ M) or the combination. **a** Cell viability (in percent) was assessed and normalized to control cells. **b** Cell cycle distribution was analyzed by flow cytometry. **c** Cells were subjected to immunoblotting. The band density of p-Akt, p-GSK3 β , p-S6k, or p-4EBP1 was normalized to Akt, GSK3 β , S6K, or 4EBP1, respectively; and that of mTOR or cyclin D1 was normalized to β -actin. Data represent three independent experiments. * P <0.05 and ** P <0.001 indicate a significant difference. † P <0.05 and †† P <0.001 indicate a significant reduction and # P <0.05 and ## P <0.001 a significant increase from controls



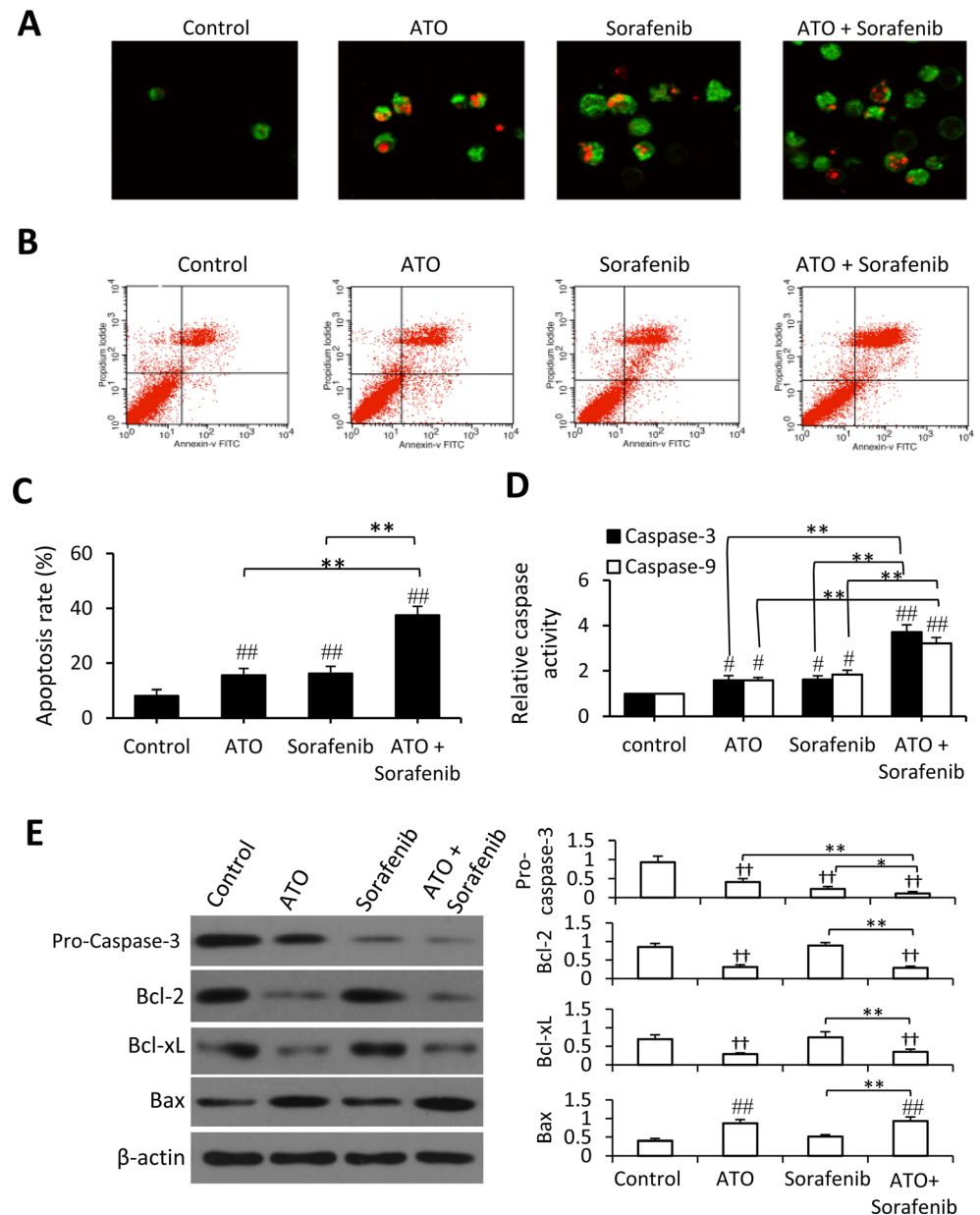
of cyclin D1 by ATO and sorafenib alone or in combination as examined by the immunoblotting assay (Fig. 4c).

ATO synergizes with sorafenib to induce the apoptosis of HCC cells via the intrinsic apoptotic pathway in vitro

Huh7 cells were incubated for 48 h with sorafenib (5 μ M), ATO (2 μ M) or the combination, and then stained with Annexin V/PI and examined under laser confocal microscopy. The early-stage apoptotic cells

(with green fluorescence in membrane) and late-stage apoptotic cells (with green fluorescence in membrane and red in nuclei) were abundant in ATO or sorafenib-treated cells, but few in the untreated control cells; incubation of the combination of ATO and sorafenib resulted in more apoptotic cells, compared with ATO or sorafenib alone (Fig. 5a). The results were supported by the flow cytometry, thus the apoptosis rates were 15.5, 16.2, and 37.5 % in Huh7 cells treated with ATO, sorafenib, or ATO + sorafenib, respectively, which were significantly higher than that of untreated

Fig. 5 ATO synergizes with sorafenib to induce the apoptosis of HCC cells. Huh7 cells were incubated for 48 h with vehicle (control), sorafenib (5 μ M), ATO (2 μ M) or the combination. **a** Representative images were taken from cells stained with Annexin V/PI, and viewed by laser scanning confocal microscopy. **b** Representative histograms were shown. **c** Apoptosis rates (in percent) were plotted. **d** The activities of caspase-3 and -9 were measured. **e** Cell lysates were immunoblotted to detect the expression of pro-caspase-3, Bcl-2, Bcl-xL, and Bax. Band densities were normalized to β -actin. Data represent three independent experiments. * P <0.05 and ** P <0.001 indicate a significant difference. †† P <0.001 indicate a significant reduction and # P <0.05 and ### P <0.001 a significant increase, from controls



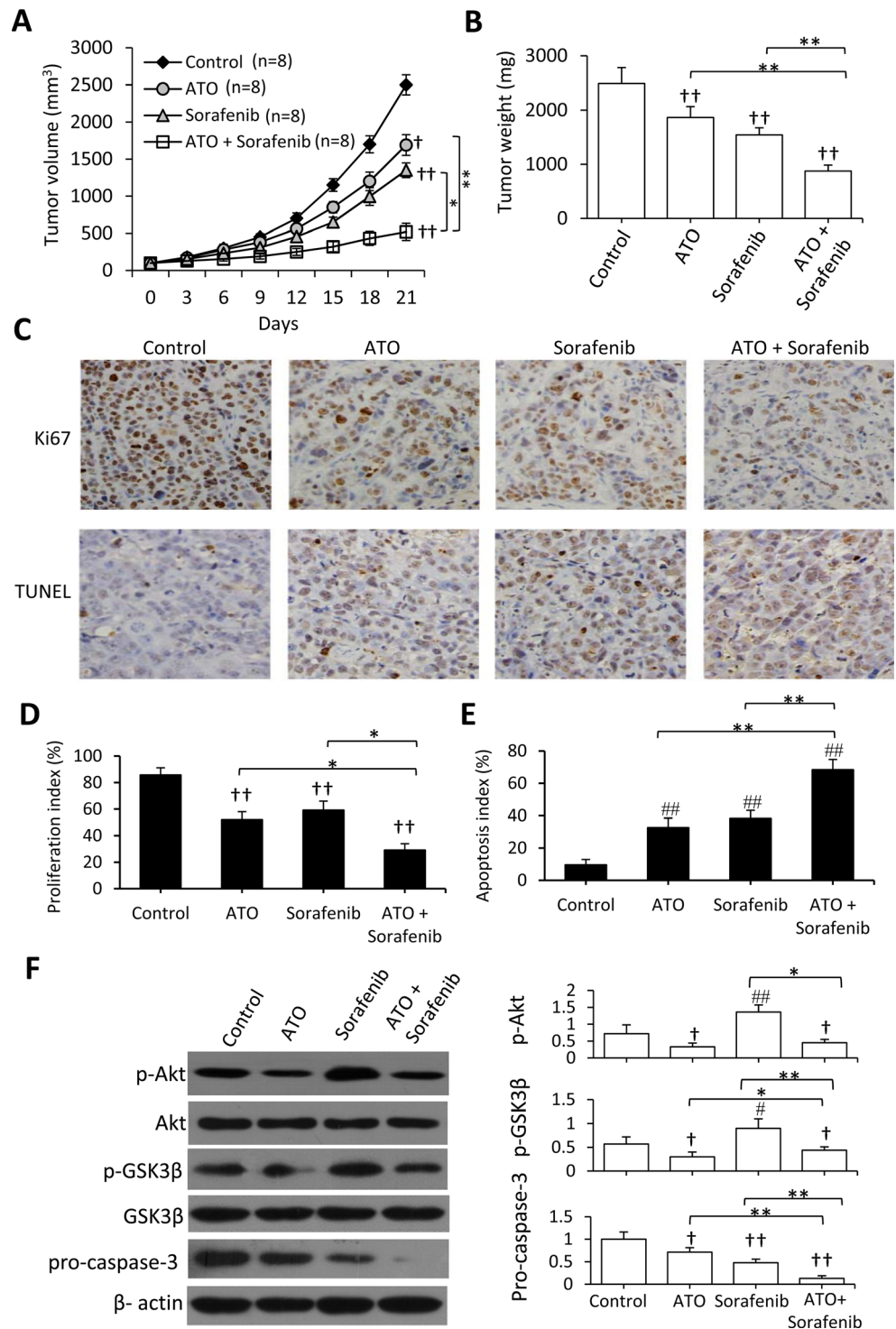
cells (8.1 %) (Fig. 5b, c). The value of CDI was 0.87, indicating that ATO synergized with sorafenib to induce the apoptosis of HCC cells. Incubation of ATO or sorafenib significantly increased cellular activities of caspase-3 and -9, compared with untreated cells; and the combination treatment resulted in even higher activities of caspase-3 and -9 than ATO or sorafenib alone (Fig. 5d). Immunoblotting results showed that ATO synergized with sorafenib to downregulate the expression of pro-caspase-3 (Fig. 5e). We also examined the expression of Bcl-2, Bcl-xL, and Bax, the three key Bcl-2 family members that regulate the intrinsic apoptotic pathway. ATO alone or in combination with sorafenib downregulated the expression of Bcl-2

and Bcl-xL, and upregulated the expression of Bax, but sorafenib alone had little effect on their expression (Fig. 5e).

ATO synergizes with sorafenib to suppress HCC tumors by inhibiting cell proliferation and promoting apoptosis

As shown in Fig. 6a, the tumors from ATO and sorafenib-treated mice were significantly smaller by 32.3 and 46.0 %, respectively, than those from vehicle-treated controls. The tumors from mice treated with the combinational therapy were even smaller by 79.2 % than those from vehicle-treated controls and significantly smaller than those treated with ATO or

Fig. 6 ATO synergizes with sorafenib to suppress HCC tumors in mice. Huh7 tumors were established in mice, which received different treatments as described in “Materials and Methods section”. **a** The size (cubic millimeter) of tumors was recorded. **b** Tumors were weighed at the end of experiment. **c** Representative images from tumor sections stained with an anti-Ki67 antibody (upper panel) or TUNEL (lower panel; magnification $\times 200$). Proliferation index (**d**) and apoptosis index (**e**) were quantified. *n* indicates the number of samples. **f** Tumor tissue lysates were immunoblotted. The band density of p-Akt or p-GSK3 β was normalized to Akt or GSK3 β , respectively, and that of pro-caspase-3 was normalized to β -actin. * $P < 0.05$ and ** $P < 0.001$ indicate a significant difference. † $P < 0.05$ and †† $P < 0.001$ indicate a significant reduction and # $P < 0.05$ and ## $P < 0.001$ a significant increase from controls



sorafenib alone (Fig. 6a). The weights of tumors harvested from mice at the end of experiments showed a similar trend to the volume of tumors (Fig. 6b). The values of CDI were 0.57 (for tumor volume) and 0.64 (for tumor weight), indicating that the synergistic effect of ATO and sorafenib was significant in suppressing the growth of HCC tumors. ATO significantly synergized

with sorafenib to inhibit cell proliferation (Fig. 6c, d) and induce cell apoptosis (Fig. 6c, e) as the values of CDI were 0.66 (for proliferation index) and 0.70 (for apoptosis index). Immunoblotting analysis of tumor lysates showed the similar alteration of expression of p-Akt, p-GSK3 β , and pro-caspase-3 (Fig. 6f), compared with that shown in vitro (Figs. 4a and 5e).

Discussion

Since no alternative effective systemic therapy against HCC is available so far after failure of sorafenib therapy [26–28], sorafenib still retains a unique pharmacotherapeutic option for the treatment of HCC, though the response rate is very low [3]. The present study has demonstrated that ATO, a currently used anticancer drug, synergizes with sorafenib to suppress the growth of HCC cells in vitro and in vivo. The main rationale for the synergistic effects of the two agents is that sorafenib-induced Akt activation, which contributes to the resistance of HCC cells, could be diminished by ATO, and both of them can individually inhibit the proliferation and induce the apoptosis of HCC cells. The encouraging results presented herein warrant future investigation of the use of ATO for combating HCC, especially in combination with sorafenib.

Although it is not a direct target of sorafenib, the PI3K/Akt pathway plays an important role in the mechanism of sorafenib resistance since it crosstalks with the MAPK/ERK pathway, the major sorafenib-targeted signaling pathway [6]. It has been previously reported that sorafenib activates the PI3K/Akt pathway and blockage of the PI3K/Akt signaling pathway enhances the efficacy of sorafenib [9–11]. Here, we have again shown that sorafenib induces the activation of Akt in HCC cells, in accordance with our previous report [13]. Akt regulates the expression of mTOR and the phosphorylation GSK3 β , which mediates cell apoptosis; and mTOR regulates the apoptotic proteins S6K and 4EBP1 [7, 26, 29]. Therefore, blockage of Akt by a specific inhibitor, siRNA, or ATO could dysregulate the above downstream factors, leading to the apoptosis of HCC cells.

The Bcl-2 family plays a key role in the intrinsic apoptotic pathway, as they regulate the activation of caspase-9 and -3 by controlling the release of cytochrome c from mitochondria to the cytosol and the sequential proteolytic processing [30]. Among the Bcl-2 family members, Bcl-2 and Bcl-xL are the major antiapoptotic proteins as they block the function of proapoptotic proteins, such as Bax [18]. Although it has been reported that sorafenib induces cell apoptosis through the intrinsic apoptosis pathway, its effects on the Bcl-2 family remains unclear. Sorafenib has shown little effect on Bcl-2 and Bcl-xL in chronic lymphocytic leukemia cells [31] and HCC cells [32–34]. Our results have again revealed that sorafenib had little effects on the expression of Bcl-2, Bcl-xL, and Bax in HCC cells. Many lines of studies have shown that ATO induces cell apoptosis by regulating Bcl-2, Bcl-xL, and Bax in multiple solid tumors including HCC [18, 30, 35]. In accord, we have demonstrated here that ATO inhibited the expression of Bcl-2 and Bcl-xL, and increased the expression of Bax in HCC cells. In support of our results that ATO synergizes with sorafenib to promote cell apoptosis by regulating different apoptotic molecules, inhibition of Bcl-2 antiapoptotic

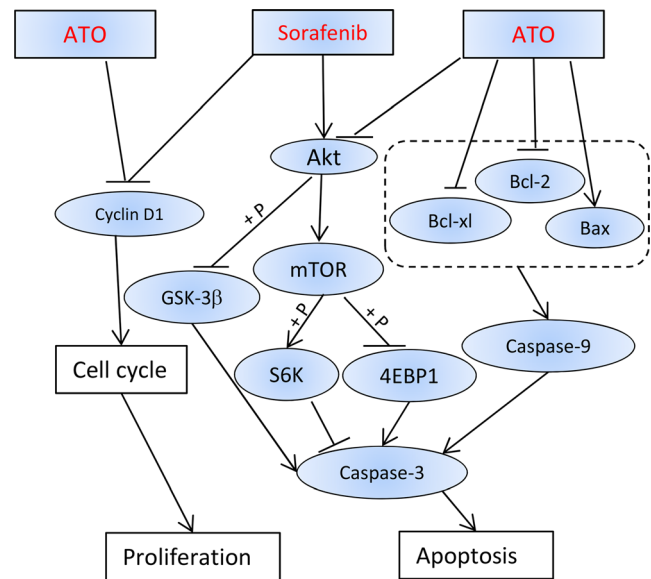


Fig. 7 Proposed mechanisms by which ATO synergizes with sorafenib to execute antitumor activity. “→” indicates positive regulation or activation; “⊥”, negative regulation or blockade; +P, regulation by phosphorylation. Abbreviations: *AKT/PKB*, protein kinase B; *ATO*, arsenic trioxide; *4EBP1*, eukaryotic translation initiation factor 4E-binding protein 1; *GSK-3 β* , glycogen synthase kinase 3 β ; *mTOR*, mammalian target of rapamycin; *S6K*, ribosomal protein S6 kinase

members enhances sorafenib-induced apoptosis in human myeloid leukemia cells [36].

Cyclin D1 is an essential molecule for driving the G1/S transition of cell cycle [37], and is overexpressed in HCC tissues [38]. It has been reported that ATO suppresses mantle cell lymphoma by downregulation of cyclin D1 [39], and sorafenib induces cell cycle arrest at G1 phase by downregulating the expression of cyclin D1 in mantle cell lymphoma [40] and HCC [41]. Accordingly, our results revealed that ATO synergized with sorafenib to downregulate the expression of cyclin D1, resulting in cell cycle arrested at G0/G1 phase in HCC cells.

The proposed mechanism by which ATO synergizes with sorafenib to suppress the growth and induce the apoptosis of HCC cells is summarized in Fig. 7. Sorafenib induces the activation of Akt, which contributes to the resistance of HCC to sorafenib [9–11]. ATO blocks Akt activation [12, 13], thus inhibiting the expression or phosphorylation of the downstream factors of Akt, such as GSK3 β , mTOR, S6K, and 4EBP1 [7, 26, 29]. ATO downregulates the expression of Bcl-2 and Bcl-xL, and upregulates Bax expression, thus leading to the activation of caspase-9 and -3, and sequential apoptotic processing. Both ATO and sorafenib downregulate the expression of cyclin D1, resulting in cell cycle arrest and proliferation inhibition.

Acknowledgments This work was supported by grants from the National Natural Scientific Foundation of China (81172331, 81472321, and

81401975), and Heilongjiang Provincial Scientific Fund for Youths in China (QC2013C103 and QC2013C098).

Conflicts of interest None

References

- Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin.* 2011;61:69–90.
- Zhu AX. Systemic treatment of hepatocellular carcinoma: dawn of a new era? *Ann Surg Oncol.* 2010;17:1247–56.
- Llovet JM, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, et al. Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med.* 2008;359:378–90.
- Bruix J, Sherman M. American Association for the Study of Liver Diseases. Management of hepatocellular carcinoma: an update. *Hepatology.* 2011;53:1020–2.
- Kelley RK, Nimeiri HS, Munster PN, Vergo MT, Huang Y, Li CM, et al. Temsirolimus combined with sorafenib in hepatocellular carcinoma: a phase I dose-finding trial with pharmacokinetic and biomarker correlates. *Ann Oncol.* 2013;24:1900–7.
- Mendoza MC, Er EE, Blenis J. The Ras-ERK and PI3K-mTOR pathways: cross-talk and compensation. *Trends Biochem Sci.* 2011;36:320–8.
- Hennessy BT, Smith DL, Ram PT, Lu Y, Mills GB. Exploiting the PI3K/AKT pathway for cancer drug discovery. *Nat Rev Drug Discov.* 2005;4:988–1004.
- Zhou L, Huang Y, Li J, Wang Z. The mTOR pathway is associated with the poor prognosis of human hepatocellular carcinoma. *Med Oncol.* 2010;27:255–61.
- Gedaly R, Angulo P, Hundley J, Daily MF, Chen C, Koch A, et al. PI-103 and sorafenib inhibit hepatocellular carcinoma cell proliferation by blocking Ras/Raf/MAPK and PI3K/AKT/mTOR pathways. *Anticancer Res.* 2010;30:4951–8.
- Huynh H, Ngo VC, Koong HN, Poon D, Choo SP, Thng CH, et al. Sorafenib and rapamycin induce growth suppression in mouse models of hepatocellular carcinoma. *J Cell Mol Med.* 2009;13:2673–83.
- Piguet AC, Saar B, Hlushchuk R, St-Pierre MV, McSheehy PM, Radojevic V, et al. Everolimus augments the effects of sorafenib in a syngeneic orthotopic model of hepatocellular carcinoma. *Mol Cancer Ther.* 2011;10:1007–17.
- Chen KF, Chen HL, Tai WT, Feng WC, Hsu CH, Chen PJ, et al. Activation of phosphatidylinositol 3-kinase/Akt signaling pathway mediates acquired resistance to sorafenib in hepatocellular carcinoma cells. *J Pharmacol Exp Ther.* 2011;337:155–61.
- Zhai B, Hu F, Jiang X, Xu J, Zhao D, Liu B, et al. Inhibition of Akt reverses the acquired resistance to sorafenib by inducing autophagic cell death in hepatocellular carcinoma. *Cancer Mol Ther.* 2014;13:1589–98.
- Lin J, Sampath D, Nannini MA, Lee BB, Degtyarev M, Oeh J, et al. Targeting activated Akt with GDC-0068, a novel selective Akt inhibitor that is efficacious in multiple tumor models. *Clin Cancer Res.* 2013;19:1760–72.
- Antman KH. Introduction: the history of arsenic trioxide in cancer therapy. *Oncologist.* 2001;6:1–2.
- Luo L, Qiao H, Meng F, Dong X, Zhou B, Jiang H, et al. Arsenic trioxide synergizes with B7H3-mediated immunotherapy to eradicate hepatocellular carcinomas. *Int J Cancer.* 2006;118:1823–30.
- Alarifi S, Ali D, Alkahtani S, Siddiqui MA, Ali BA. Arsenic trioxide-mediated oxidative stress and genotoxicity in human hepatocellular carcinoma cells. *Oncol Targets Ther.* 2013;6:75–84.
- Chen G, Wang K, Yang BY, Tang B, Chen JX, Hua ZC. Synergistic antitumor activity of oridonin and arsenic trioxide on hepatocellular carcinoma cells. *Int J Oncol.* 2012;40:139–47.
- Lin CC, Hsu C, Hsu CH, Hsu WL, Cheng AL, Yang CH. Arsenic trioxide in patients with hepatocellular carcinoma: a phase II trial. *Investig New Drugs.* 2007;25:77–84.
- Guilbert C, Annis MG, Dong Z, Siegel PM, Miller Jr WH, Mann KK. Arsenic trioxide overcomes rapamycin-induced feedback activation of AKT and ERK signaling to enhance the anti-tumor effects in breast cancer. *PLoS One.* 2013;8:e85995. doi:10.1371/journal.pone.0085995.
- Mann KK, Colombo M, Miller Jr WH. Arsenic trioxide decreases AKT protein in a caspase-dependent manner. *Mol Cancer Ther.* 2008;7:1680–7.
- Xue P, Hou Y, Zhang Q, Woods CG, Yarborough K, Liu H, et al. Prolonged inorganic arsenite exposure suppresses insulin-stimulated AKT S473 phosphorylation and glucose uptake in 3T3-L1 adipocytes: involvement of the adaptive antioxidant response. *Biochem Biophys Res Commun.* 2011;407:360–5.
- Zhao D, Zhai B, He C, Tan G, Jiang X, Pan S, et al. Upregulation of HIF-2 α induced by sorafenib contributes to the resistance by activating the TGF- α /EGFR pathway in hepatocellular carcinoma cells. *Cell Signal.* 2014;26:1030–9.
- Ma L, Li G, Zhu H, Dong X, Zhao D, Jiang X, et al. 2-Methoxyestradiol synergizes with sorafenib to suppress hepatocellular carcinoma by simultaneously dysregulating hypoxia-inducible factor-1 and -2. *Cancer Lett.* 2014. doi:10.1016/j.canlet.2014.09.011.
- Wei Z, Jiang X, Liu F, Qiao H, Zhou B, Zhai B, et al. Downregulation of Skp2 inhibits the growth and metastasis of gastric cancer cells in vitro and in vivo. *Tumor Biol.* 2013;34:181–92.
- Zhai B, Sun XY. Mechanisms of resistance to sorafenib and the corresponding strategies in hepatocellular carcinoma. *World J Hepatol.* 2013;5:345–52.
- Villanueva A, Llovet JM. Second-line therapies in hepatocellular carcinoma: emergence of resistance to sorafenib. *Clin Cancer Res.* 2012;18:1824–6.
- Santoro A, Rimassa L, Borbath I, Daniele B, Salvagni S, Van Laethem JL, et al. Tivantinib for second-line treatment of advanced hepatocellular carcinoma: a randomised, placebo-controlled phase 2 study. *Lancet Oncol.* 2013;14:55–63.
- Serova M, de Gramont A, Tijeras-Raballand A, Dos Santos C, Riveiro ME, Slimane K, et al. Benchmarking effects of mTOR, PI3K, and dual PI3K/mTOR inhibitors in hepatocellular and renal cell carcinoma models developing resistance to sunitinib and sorafenib. *Cancer Chemother Pharmacol.* 2013;71:1297–307.
- Jiang H, Ma Y, Chen X, Pan S, Sun B, Krissansen GW, et al. Genistein synergizes with arsenic trioxide to suppress human hepatocellular carcinoma. *Cancer Sci.* 2010;101:975–83.
- Fecteau JF, Bharati IS, O'Hayre M, Handel TM, Kipps TJ, Messmer D. Sorafenib-induced apoptosis of chronic lymphocytic leukemia cells is associated with downregulation of RAF and myeloid cell leukemia sequence 1 (Mcl-1). *Mol Med.* 2012;18:19–28.
- Cervello M, Bachvarov D, Lampiasi N, Cusimano A, Azzolina A, McCubrey JA, et al. Molecular mechanisms of sorafenib action in liver cancer cells. *Cell Cycle.* 2012;11:2843–55. doi:10.4161/cc.21193.
- Tai WT, Shiau CW, Chen HL, Liu CY, Lin CS, Cheng AL, et al. Mcl-1-dependent activation of Beclin 1 mediates autophagic cell death induced by sorafenib and SC-59 in hepatocellular carcinoma cells. *Cell Death Dis.* 2013;4:e485.
- Shimizu S, Takehara T, Hikita H, Kodama T, Miyagi T, Hosui A, et al. The let-7 family of microRNAs inhibits Bcl-xL expression and potentiates sorafenib-induced apoptosis in human hepatocellular carcinoma. *J Hepatol.* 2010;52:698–704.

35. Rangwala F, Williams KP, Smith GR, Thomas Z, Allensworth JL, Lyerly HK, et al. Differential effects of arsenic trioxide on chemosensitization in human hepatic tumor and stellate cell lines. *BMC Cancer*. 2012;12:402.
36. Rahmani M, Aust MM, Attkisson E, Williams Jr DC, Ferreira-Gonzalez A, Grant S. Inhibition of Bcl-2 antiapoptotic members by obatoclax potently enhances sorafenib-induced apoptosis in human myeloid leukemia cells through a Bim-dependent process. *Blood*. 2012;119:6089–98.
37. Malumbres M, Barbacid M. Cell cycle, CDKs and cancer: a changing paradigm. *Nat Rev Cancer*. 2009;9:153–66.
38. Parekh P, Rao KV. Overexpression of cyclin D1 is associated with elevated levels of MAP kinases, Akt and Pak1 during diethylnitrosamine-induced progressive liver carcinogenesis. *Cell Biol Int*. 2007;31:35–43.
39. Lo RK, Kwong YL. Arsenic trioxide suppressed mantle cell lymphoma by downregulation of cyclin D1. *Ann Hematol*. 2014;93:255–65.
40. Xargay-Torrent S, López-Guerra M, Montraveta A, Saborit-Villarroya I, Rosich L, Navarro A, et al. Sorafenib inhibits cell migration and stroma-mediated bortezomib resistance by interfering B-cell receptor signaling and protein translation in mantle cell lymphoma. *Clin Cancer Res*. 2013;19:586–97.
41. Plataras JP, Kim SH, Liu YY, Dicker DT, Dorsey JF, McDonough J, et al. Cell cycle dependent and schedule-dependent antitumor effects of sorafenib combined with radiation. *Cancer Res*. 2007;67:9443–54.