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RESEARCH PAPER

Sorafenib and its derivative SC-49 sensitize hepatocellular carcinoma cells to CS-1008, a humanized anti-TNFRSF10B (DR5) antibody

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BACKGROUND AND PURPOSE

Previously, we have shown that sorafenib sensitizes hepatocellular carcinoma (HCC) to apoptosis induced by TNF-related apoptosis-inducing ligand (TNFSF10; TRAIL). Here, we report that sorafenib and SC-49 sensitize HCC cells to CS-1008, a novel anti-human death receptor 5 (TNFRSF10B) antibody.

EXPERIMENTAL APPROACH

HCC cell lines (PLC5, Huh-7, and Hep3B) were treated with CS-1008 and/or sorafenib and analysed in terms of apoptosis and signal transductions.

KEY RESULTS

SC-49 is a sorafenib derivative, which is devoid of kinase inhibitory activity. Both sorafenib and SC-49 down-regulated the phosphorylation of STAT3 at Tyr^{705} and subsequently reduced the levels of STAT3-regulated proteins, Mcl-1, survivin and cylcin D1, in CS-1008-treated HCC cells. Knockdown of STAT3 by RNA interference overcame apoptotic resistance to CS-1008 in HCC cells, and ectopic expression of STAT3 in HCC cells abolished the sensitizing effects of sorafenib and SC-49 on CS-1008-induced apoptosis, indicating that inhibition of STAT3 mediates the enhancing effects of these compounds when combined with CS-1008. Importantly, inhibition of SHP-1 by adding a specific SHP-1 inhibitor reduced the effects of SC-49 and CS-1008 on p-STAT3 and apoptosis, whereas co-treatment of CS-1008 with SC-49 increased the activity of SHP-1. These data indicate that the combined effects of CS-1008 and SC-49 on HCC are mediated by SHP-1. Moreover, the combination of CS-1008 and SC-49 inhibited HCC xenograft tumour growth *in vivo*.

CONCLUSIONS AND IMPLICATIONS

Sorafenib and its derivative SC-49 sensitize HCC cells to the antitumour effects of CS-1008 through SHP-1-dependent inactivation of STAT3.

Abbreviations

c-FLIP, cellular FLICE-inhibitory protein; FADD, Fas-associated protein with death domain; HCC, hepatocellular carcinoma; TNFSF10 (TRAIL), TNF-related apoptosis-inducing ligand

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world. It is a highly lethal malignancy that has a high recurrence rate despite surgical resection (Tanaka and Arii, 2009; Verslype *et al.*, 2009). Advanced or recurrent HCC is frequently resistant to current chemotherapeutic agents and radiation; therefore, the development of targeted agents with tolerable toxicity is needed to advance anti-HCC therapy (Tanaka and Arii, 2009;). Sorafenib, a multi-kinase inhibitor, has been approved for clinical use in advance HCC after it was found to improve overall survival significantly in two prospective randomized phase III trials for patients with advanced HCC (Llovet *et al.*, 2008; Cheng *et al.*, 2009). Sorafenib inhibits multiple kinases, including the Ras/Raf/MAPK/ERK signalling pathway, the angiogenic pathways VEGFR2, VEGFR3, as well as PDGFRβ and other kinases such as FLT3 and FGFR1 (Adnane *et al.*, 2006; Liu *et al.*, 2006; Wilhelm *et al.*, 2006; Auclair *et al.*, 2007).

Among various targeted strategies for HCC treatment, TNF-related apoptosis inducing ligand (TNFSF10; TRAIL), which targets receptor-mediated apoptosis, represents an attractive option (Johnstone *et al.*, 2008; Wang, 2008; Falschlehner *et al.*, 2009). As a member of the TNF superfamily, TNFSF10 initiates apoptosis by binding to two important death domain-containing death receptors (DRs), TNFRSF10A (DR4) and TNFRSF10B (DR5) (Wang and El-Deiry, 2003; Rowinsky, 2005; Wiezorek *et al.*, 2010). TNFSF10 or TNFSF10 agonists bind to TNFRSF10A or TNFRSF10B and form death-inducing signalling complex (DISC), which is a multi-protein complex consisting of an adaptor molecule, FADD and the initiator of extrinsic pathway caspase-8. (Wang and El-Deiry, 2003; Johnstone *et al.*, 2008) Activated caspase-8 is capable of both initiating an extrinsic apoptotic pathway in type I cells (through activation of caspase-3, -6, and -7) and triggering the intrinsic pathway in type II cells (through activation of Bid) (Li *et al.*, 1998; Johnstone *et al.*, 2008).

CS-1008 is a novel TNFRSF10B agonist that exerts TNFSF10-like activity. It is a humanized anti-human TNFRSF10B antibody manufactured from a murine anti-human TNFRSF10B monoclonal antibody, TRA-8 (Yada *et al.*, 2008). So far, CS-1008 has shown selective cytotoxicity towards tumour cells expressing TNFRSF10B (Yada *et al.*, 2008) and an excellent safety profile in humans (Saleh *et al.*, 2008). CS-1008 monotherapy induces apoptosis in various cancer cells and CS-1008 in combination with some chemotherapeutic agents (such as gemcitabine or docetaxel) has also been found to have enhanced antitumour activity (Yada *et al.*, 2008). In a phase I trial, no dose-limiting toxicity was reported for CS-1008 at doses up to 8 $mg \cdot kg^{-1}$ weekly (Saleh *et al.*, 2008).

Although TNFSF10 may be applied as anti-HCC strategy, more and more studies have reported that the efficacy of TNFSF10-induced apoptosis in HCC cells is insufficient, often due to resistance to TNFSF10 or its agonists (Shin *et al.*, 2002; Pathil *et al.*, 2006; Chen *et al.*, 2009). Resistance to TNFSF10 may be induced at any step in the apoptosis signalling cascade, from the receptor level (mutations or overexpression of TNFRSF10A or TNFRSF10B) (Zhang and Fang, 2005), or defects in DISC assembly, (Eggert *et al.*, 2001; Okano *et al.*, 2003) through to dysfunctions of the anti-apoptotic Bcl-2 family proteins (Bcl-2, Bcl-xl, Mcl-1, etc) (Fulda *et al.*, 2002; Kim *et al.*, 2008) and pro-apoptotic proteins (Bax or Bak) or defects in mitochondria-derived activator of caspases (Smac/Diablo) (Zhang *et al.*, 2001; Zhang and Fang, 2005). Of particular note, Mcl-1, an anti-apoptotic Bcl-2 family protein, plays a critical role in conferring TNFSF10 resistance (Kim *et al.*, 2008). Data have shown that overexpression of Mcl-1 can neutralize TNFSF10-induced signalling (Meng *et al.*, 2007; Ricci *et al.*, 2007). Moreover, directly or indirectly destabilizing or disabling Mcl-1 can restore TNFSF10 sensitivity (Taniai *et al.*, 2004; Hall and Cleveland, 2007). Interestingly, Mcl-1 is a highly regulated cell death and survival controller that responds to various cytokines and growth factors (Yang-Yen, 2006), and can be regulated by a number of transcription factors, including NF-κB targeting the cAMP response element (CRE-2) motif, and STAT3 targeting the sis-inducible element (SIE) motif of *mcl-1* promoter region (Wang *et al.*, 2003; Yang-Yen, 2006; Kim *et al.*, 2008).

STAT3 is considered a potential anti-cancer therapeutic target because of its crucial role in transcriptional regulation of genes involved in cell proliferation and survival and it is constitutively activated in common human cancers, including HCC (Li *et al.*, 2006; Germain and Frank, 2007; Kusaba *et al.*, 2007). In response to the stimulation of cytokines, growth factors and hormones, STAT3 is phosphorylated (activated) and homodimerizes or heterodimerizes with STAT1 in the cytoplasm; it then translocates to the nucleus to regulate a number of genes, including genes that encode apoptosis-related proteins and cell cycle regulators (i.e. Bcl-2, Bcl-xl, Mcl-1, survivin and cyclin D1). In cancer cells, constitutively activated STAT3 directly contributes to tumourigenesis, invasion and metastasis (Germain and Frank, 2007). Targeting STAT3 using antisense oligonucleotide reduces the growth and metastasis of HCC cells *in vitro* and *in vivo* (Li *et al.*, 2006). Importantly, reducing constitutive STAT3 activity has been shown to sensitize human hepatoma cells to TNFSF10-mediated apoptosis (Kusaba *et al.*, 2007). Moreover, a number of protein tyrosine phosphatases have been shown to negatively regulate STAT3 signalling through direct dephosphorylation of p-STAT3 (Tyr^{705}); these include members of the SH2-domain containing tyrosine phosphatase family (SHP-1 and SHP-2) and protein tyrosine phosphatase 1B (PTP-1B) (Ke *et al.*, 2007; Chen *et al.*, 2008; Kunnumakkara *et al.*, 2009; Pandey *et al.*, 2009). Therefore, activity of protein tyrosine phosphatases may be critical for the regulation of STAT3 phosphorylation in cancer cells.

Recently, we have reported that sorafenib sensitizes HCC cells to TNFSF10 (Chen *et al.*, 2010), and STAT3 is a major kinase-independent target of sorafenib in HCC (Tai *et al.*, 2011). We have discovered that several sorafenib derivatives are novel STAT3 inhibitors (Chen *et al.*, 2011). In this study, we demonstrated that SC-49, a novel sorafenib analogue, is able to sensitize HCC cells to the antitumour effects of CS-1008.

Methods

The receptor nomenclature used in this paper conforms to *Br J Pharmacol's Guide to Receptors and Channels* (Alexander *et al.*, 2011).

Reagents and antibodies

CS-1008 and sorafenib (Nexavar®) were kindly provided by Daiichi Sankyo Co., Ltd. (Tokyo, Japan) and Bayer Pharmaceuticals (West Haven, CT, USA) respectively. For *in vitro* studies, sorafenib at various concentrations was dissolved in DMSO and then added to the cells in 5% FBS-containing DMEM. Antibodies for immunoblotting such as Akt1, Mcl-1 and PARP were purchased from Santa Cruz Biotechnology (San Diego, CA, USA). Other antibodies such as anti-pERK (1/2), ERK2, survivin, cylcin D1, Bcl-xL, Bid, caspase-3, caspase-8, caspase-9, phospho-STAT3 (Tyr^{705}), STAT3 and phosphor-Akt (Ser^{473}) were from Cell Signaling (Danvers, MA, USA).

Cell culture and Western blot analysis

The Huh-7 HCC cell line was obtained from the Health Science Research Resources Bank (HSRRB; Osaka, Japan; JCRB0403). The PLC/PRF/5 (PLC5), Sk-Hep-1, Hep3B and U937 cell lines were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). All cells obtained from HSRRB or ATCC were immediately expanded and frozen such that all cell lines could be restarted every 3 months from a frozen vial of the same batch of cells. No further authentication was done in our lab. Cells were maintained in DMEM supplemented with 10% FBS, 100 U·mL^{-1} penicillin G, 100 μg·mL^{-1} streptomycin sulfate and 25 μg·mL^{-1} amphotericin B in a humidified incubator at 37°C in an atmosphere of 5% CO_2 in air. Lysates of HCC cells treated with drugs at the indicated concentrations for various periods of time were prepared for immunoblotting of caspase-3, PARP, p-STAT3, STAT3, etc. Western blot analysis was performed as previously reported (Chen *et al.*, 2008).

Apoptosis analysis

The following three methods were used to assess drug-induced apoptotic cell death: detection of DNA fragmentation with the Cell Death Detection ELISA kit (Roche Diagnostics, Indianapolis, IN, USA), Western blot analysis of caspase activation and PARP cleavage, and measurement of apoptotic cells by flow cytometry (sub-G1). The ELISA was conducted according to the manufacturer's instructions.

Gene knockdown using siRNA

Smart pool siRNA reagents, including a control (D-001810-10), and mcl-1, STAT3, SHP-1, SHP-2, and PTP-1B were all purchased from Dharmacon Inc. (Chicago, IL, USA). The procedure has been described previously (Chen *et al.*, 2008).

PLC5 with ectopic expression of STAT3

STAT3 cDNA (KIAA1524) was purchased from Addgene plasmid repository (http://www.addgene.org/). PLC5 cells with stable expression of STAT3 were then treated with drugs, harvested, and processed for Western blot analysis as described previously (Chen *et al.*, 2008).

Activity of Raf-1 and SHP-1

A tyrosine phosphatase assay kit (R-22067) was used for assessing SHP-1 activity (Molecular Probes, Invitrogen, Grand Island, NY, USA). The Raf-1 kinase cascade assay kit (Upstate-Millipore, Billerica, MA) was used to examine Raf-1 kinase activity. The VEGFR1 kinase activity kit was purchased from Reaction Biology Corp. (Malvern, PA, USA).

Xenograft tumour growth

Male NCr athymic nude mice (5–7 weeks of age) were obtained from the National Laboratory Animal Center (Taipei, Taiwan). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). We used a total of 30 mice for the therapeutic evaluations and each separate experimental group consisted of 6-8 mice. In addition, all nude mice were housed in a light-controlled room with a 12 h day/night cycle and were given free access to water and food. The temperature of the animal room was kept at 25 °C.

All experimental procedures using these mice were performed in accordance with protocols approved by the Institutional Laboratory Animal Care and Use Committee of National Taiwan University. Each mouse was inoculated s.c. in the dorsal flank with 1×10^6 PLC5 cells suspended in 0.1 mL of serum-free medium containing 50% Matrigel (BD Biosciences, Bedford, MA, USA). When tumours reached 200–300 mm^3, mice received an i.v. injection of CS-1008 (200 μg) three times a week, SC-49 (5 mg·kg^{-1}) p.o. once daily or in combination. Controls received vehicle. Tumours were measured weekly using calipers, and their volumes calculated using the following standard formula: width^2 × length × 0.52.

Immunohistochemistry

Immunohistochemical studies were performed on formalin-fixed, paraffin-embedded tissue. The primary monoclonal antibodies used were p-STAT3 (1:50 dilution; Cell Signaling), STAT3 (1:200; Cell Signaling), CD31 (1:40; BD Pharmingen, San Jose CA, USA). Antigen retrieval was performed at pH 9.0 using Epitope Retrieval 2 solution (Leica Microsystems, Wetzlar, Germany) for 30 min at 100°C. Slides were then stained using the Leica Microsystems BONDMAX autostainer according to the manufacturer's protocol.

Statistical analysis

Comparisons of mean values were performed using the independent samples *t*-test in SPSS for Windows 11.5 software (SPSS, Inc., Chicago, IL, USA).

Results

Sorafenib sensitizes resistant HCC cells to CS-1008-induced apoptosis

To investigate the effects of sorafenib and CS-1008 on HCC cells, we first examined the apoptotic effects of both drugs on a panel of three human HCC cell lines Hep3B, PLC5 and Huh-7 at clinically relevant concentrations (≤1000 ng·mL^{-1}); however, the combination of sorafenib and CS-1008 overcame the resistance and induced apoptosis in all cell lines tested, in a dose-dependent manner (Figure 1A). Next, we examined the effect of sorafenib on CS-1008-induced apoptosis, as assessed by DNA fragmentation in all HCC cell lines. DNA fragmentation was determined by cell death ELISA after 48 h of treatment. As shown in Figure 1B, combining sorafenib at 7.5 μM with CS-1008 reversed the resistance in all three cell lines and induced significant apoptosis. Moreover, we further examined the apoptotic pathway by Western blot analysis. Our data indicated that co-treatment with CS-1008 and sorafenib activated caspase-8 then induced cleavage of Bid and subsequently activated caspase-9 and caspase-3 and

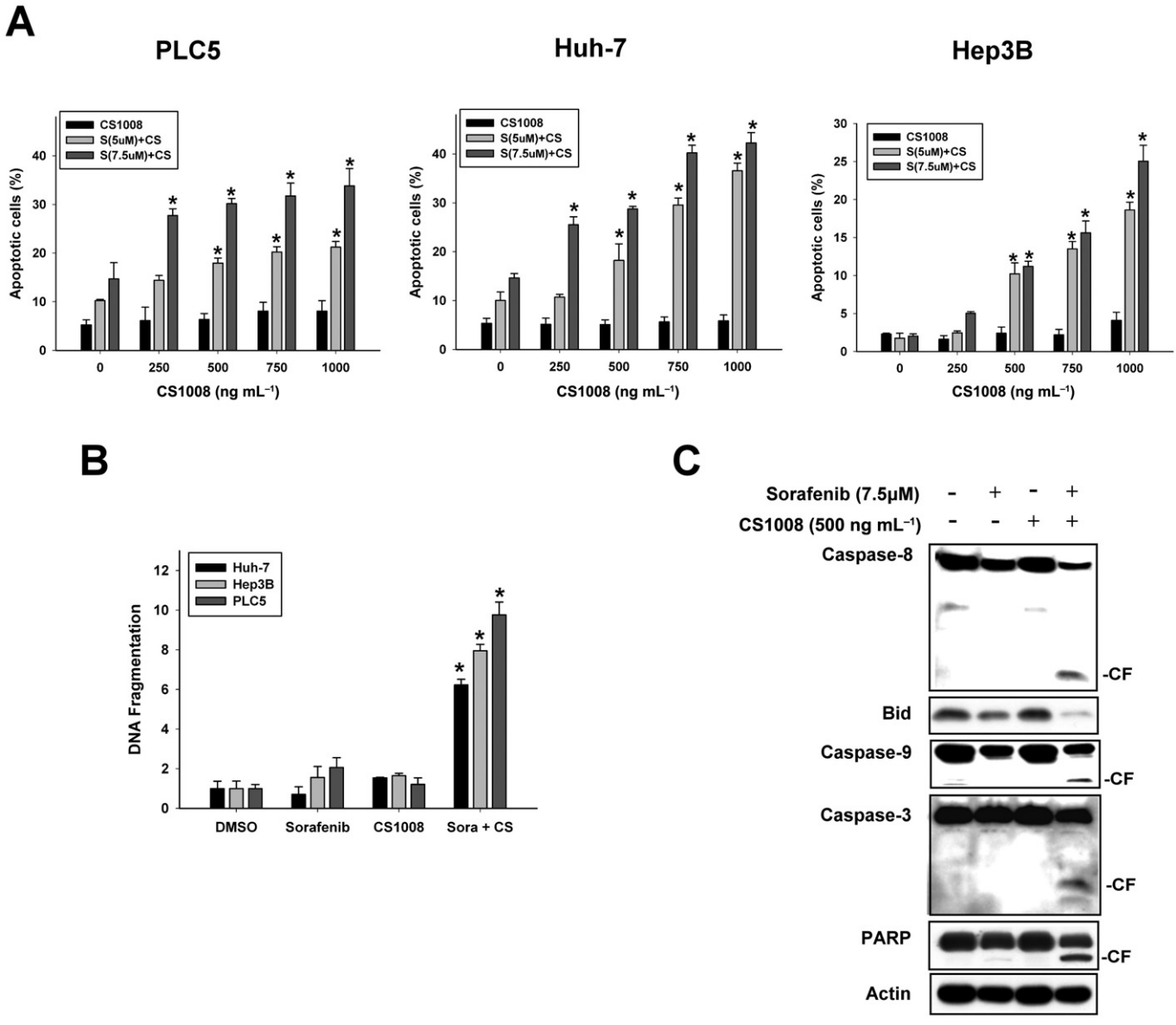

Figure 1

Sorafenib enhances CS-1008-induced apoptosis in resistant HCC cells. (A) Dose escalation effects of a combination of CS-1008 and sorafenib (5 or 7.5 μM) on apoptosis in 3 TNFSF10-resistant HCC cells. Cells were exposed to CS-1008 and/or sorafenib at the indicated doses for 48 h. Apoptotic cells were analysed by flow cytometry (sub-G1). (B) Effects of CS-1008 and sorafenib on DNA fragmentation in three HCC cell lines. Cells were treated with CS-1008 (1000 ng·mL^{-1}) and/or sorafenib (7.5 μM) for 48 h and DNA fragmentation was analysed by using a cell death ELISA kit. (C) Effects of sorafenib on CS-1008-induced apoptosis in PLC5 cells. CF, cleaved form (activated form).

PARP cleavage (Figure 1C). These data suggest that this intrinsic pathway played a role in mediating the combined effect of sorafenib and CS-1008 on apoptosis in HCC cells.

Sorafenib and CS-1008 co-treatment down-regulates p-STAT3 in HCC cells

Previous studies have suggested that Mcl-1, an anti-apoptotic Bcl-2 family protein, may play a role in mediating the sensitizing effect of sorafenib to TNFSF10 in cancer cells (Meng *et al.*, 2007; Ricci *et al.*, 2007; Kim *et al.*, 2008). As STAT3 regulates the expression of Mcl-1, we next examined its related proteins including phospho-STAT3 (p-STAT3), STAT3 and STAT3-regulated proteins, which include Mcl-1, survivin and cyclin D1. As shown in Figure 2A, co-treatment with sorafenib and CS-1008 down-regulated p-STAT3 (Tyr^{705}) and related proteins, Mcl-1, survivin and cyclin D1, in all the cells tested without altering of total protein levels of STAT3. In addition, down-regulation of p-STAT3 was associated with the cleavage of PARP as shown by the analysis of apoptosis induced in cells exposed to sorafenib and CS-1008 for 48 h (Figure 2A). Furthermore, we found that the combination of sorafenib and CS-1008 down-regulated p-STAT3 in PLC5 cells in both a dose- and time-dependent manner (Figure 2B). Interestingly, co-treatment with sorafenib and CS-1008 did not affect phospho-Erk, suggesting that the sensitizing effect of sorafenib on CS-1008 is not associated with its Raf-1 activity (Figure 2C). Notably, the combination of sorafenib and CS-1008 did not alter the expression of Bax and Bcl-xl (Figure 2C).

Validation of STAT3

Several approaches were used to validate the finding that inhibition of STAT3 signals is responsible for the sensitizing effect of sorafenib on CS-1008-induced apoptosis in HCC cells. Firstly, we knocked down the protein expression of Mcl-1 and STAT3 by use of small interference RNA (siRNA). PLC5 cells were transfected with either control, survivin siRNA or STAT3 siRNA for 48 h then exposed to DMSO or CS-1008 at the indicated doses for another 48 h. Silencing Mcl-1 and STAT3 significantly sensitized PLC5 cells to CS-1008-induced apoptosis ($P < 0.05$) (Figure 2D, left and middle), suggesting that inhibition of the STAT3 signalling pathway is important for the sensitivity of HCC cells towards CS-1008. Next, we examined the effects of sorafenib in combination with CS-1008 in both wild-type PLC5 cells and PLC5 cells with ectopic expression (overexpression) of STAT3. Overexpression of STAT3 significantly reduced the combined effects of sorafenib plus CS-1008 on p-STAT3 and apoptosis ($P < 0.05$) (Figure 2D, right). Together, these results confirm the importance of STAT3 inhibition in mediating the combined effect of CS-1008 and sorafenib.

SHP-1 plays a role in mediating the effects of apoptosis induced by sorafenib and CS-1008

To elucidate the mechanism by which sorafenib plus CS-1008 down-regulated p-STAT3 in HCC cells, we investigated the roles of several protein phosphatases on the effect of sorafenib plus CS-1008 on p-STAT3 and apoptosis. Firstly, we altered the expression of SHP-1, by using siRNA, in PLC5 cells and showed that silencing SHP-1 significantly reduced the effects of sorafenib plus CS-1008 on p-STAT3 and apoptosis (Figure 3A, left). This suggests that SHP-1 mediates the effects of these drugs on p-STAT3 and apoptosis. Notably, co-treatment with sorafenib and CS-1008 did not affect the expression level of SHP-1 in HCC cells. Therefore, we measured SHP-1 phosphatase activity in PLC5 cells that were treated with sorafenib plus CS-1008. As shown in Figure 3A (right), sorafenib plus CS-1008 significantly increased the activity of SHP-1 ($P < 0.05$). Moreover, as sorafenib is a kinase inhibitor, we examined whether sorafenib plus CS-1008 enhanced SHP-1 activity by affecting the phosphorylation of SHP-1. According to previous reports, phosphorylation of SHP-1 at Tyr^{536} may enhance its activity and phosphorylation at Ser^{591} may down-regulate its activity. However, our data showed that neither sorafenib alone nor co-treatment with CS-1008 altered phospho-SHP-1 at either site (Figure 3B, left). In addition, we examined whether the combination of drugs affected the protein-protein interactions between SHP-1 and STAT3. Our data show that the amount of STAT-SHP1 complex did not alter significantly after co-treatment with the two drugs, suggesting that this combination treatment did not affect the interaction between SHP-1 and STAT3 protein (Figure 3B right). Finally we also examined other protein tyrosine phosphatases such as SHP-2 and PTP-1B that could also regulate the STAT3 signalling pathway. However, neither knockdown of SHP-2 nor silencing of PTP-1B affected the effect of sorafenib plus CS-1008 on p-STAT3 signalling and apoptosis (Figure 3C and D). These data indicate that SHP-2 and PTP-1B are not involved in mediating the effects of sorafenib on p-STAT3 signalling and apoptosis induced by CS-1008.

SC-49, a sorafenib derivative, sensitizes HCC cells to CS-1008

Previously, we showed that STAT3 mediates the anti-tumour effect of sorafenib on HCC, and this effect was not related to inhibition of kinase activity (Tai *et al.*, 2011). We then modified sorafenib and synthesized several new sorafenib analogues, which, by enhancing the activity of SHP-1, are potent inhibitors of STAT3. Here, we investigated the ability of SC-49 to sensitize HCC cells to CS-1008. As shown in Figure 4A, SC-49 is similar to sorafenib structurally but without Raf-1 inhibitory activity. Like sorafenib, the combination of SC-49 and CS-1008 also induced significant apoptosis in TNFSF10-resistant HCC cells. Combining sorafenib at 5 μM with CS-1008 reversed the resistance in all three cell lines and induced significant apoptosis within 24 h (Figure 4B and C). As shown in Figure 4D, co-treatment with SC-49 and CS-1008 activated caspase-8 then induced cleavage of Bid and subsequently activated caspase-9 and caspase-3 and PARP cleavage. It is noteworthy that in comparison with sorafenib, SC-49 sensitized HCC cells to CS-1008 at lower concentrations (5 μM vs. 7.5 μM) and in a shorter period of time (24 h vs. 48 h). These data suggest that SC-49 is more potent than sorafenib in sensitizing HCC cells to CS-1008.

SC-49 showed better apoptotic effects than sorafenib in HCC

To further investigate the effect of SC-49 on angiogenesis, we tested the effect of SC-49 on the activity of VEGFR1 in

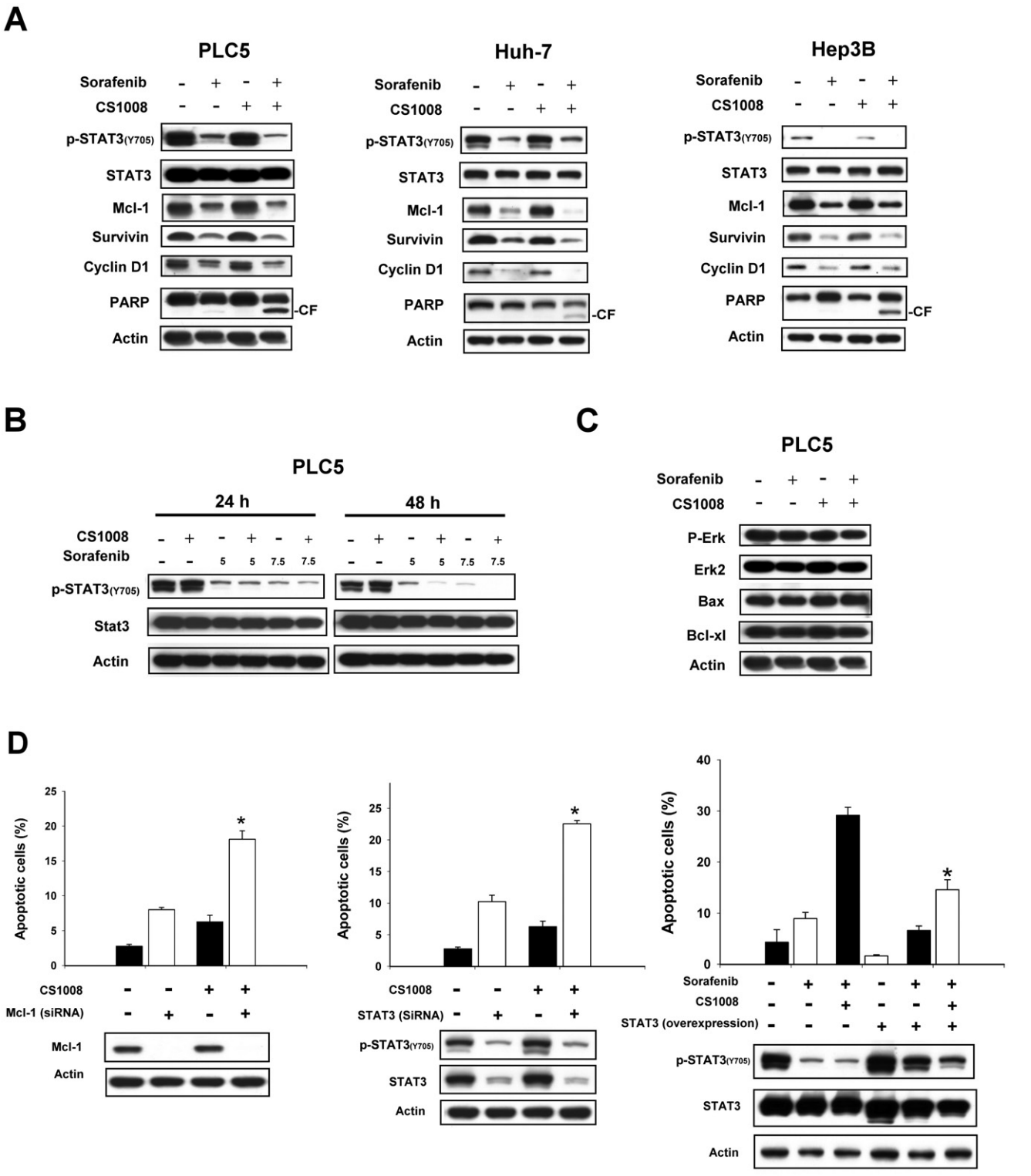

Figure 2

STAT3 mediates the sensitizing effect of sorafenib on CS-1008. (A) Effects of sorafenib (7.5 μM) and/or CS-1008 (1000 ng·mL^{-1}) on STAT3-related proteins. Cells were exposed to the drugs for 48 h. (B) Effects of sorafenib on phospho-STAT3 in PLC5 cells. (C) Effects of sorafenib and/or CS-1008 on p-Erk, Bax and Bcl-xl. Cells were treated with 1000 ng·mL^{-1} CS-1008 and/or sorafenib at 7.5 μM for 48 h. (D) *Left,* down-regulation of mcl-1 by siRNA overcame the resistance to CS-1008 in PLC5 cells. *Middle*, down-regulation of STAT3 by siRNA overcomes resistance to CS-1008 in PLC5 cells. *Right*, ectopic expression of STAT3 reduced apoptosis induced by the combination of CS-1008 and sorafenib in PLC5 cells. Cells (wild-type PLC5 or PLC5 with STAT3 overexpression) were exposed to drugs at the indicated doses for 48 h. Apoptotic cells were analysed by flow cytometry (sub-G1). Columns show means with bars representing SD ($n = 3$). *$P < 0.05$.

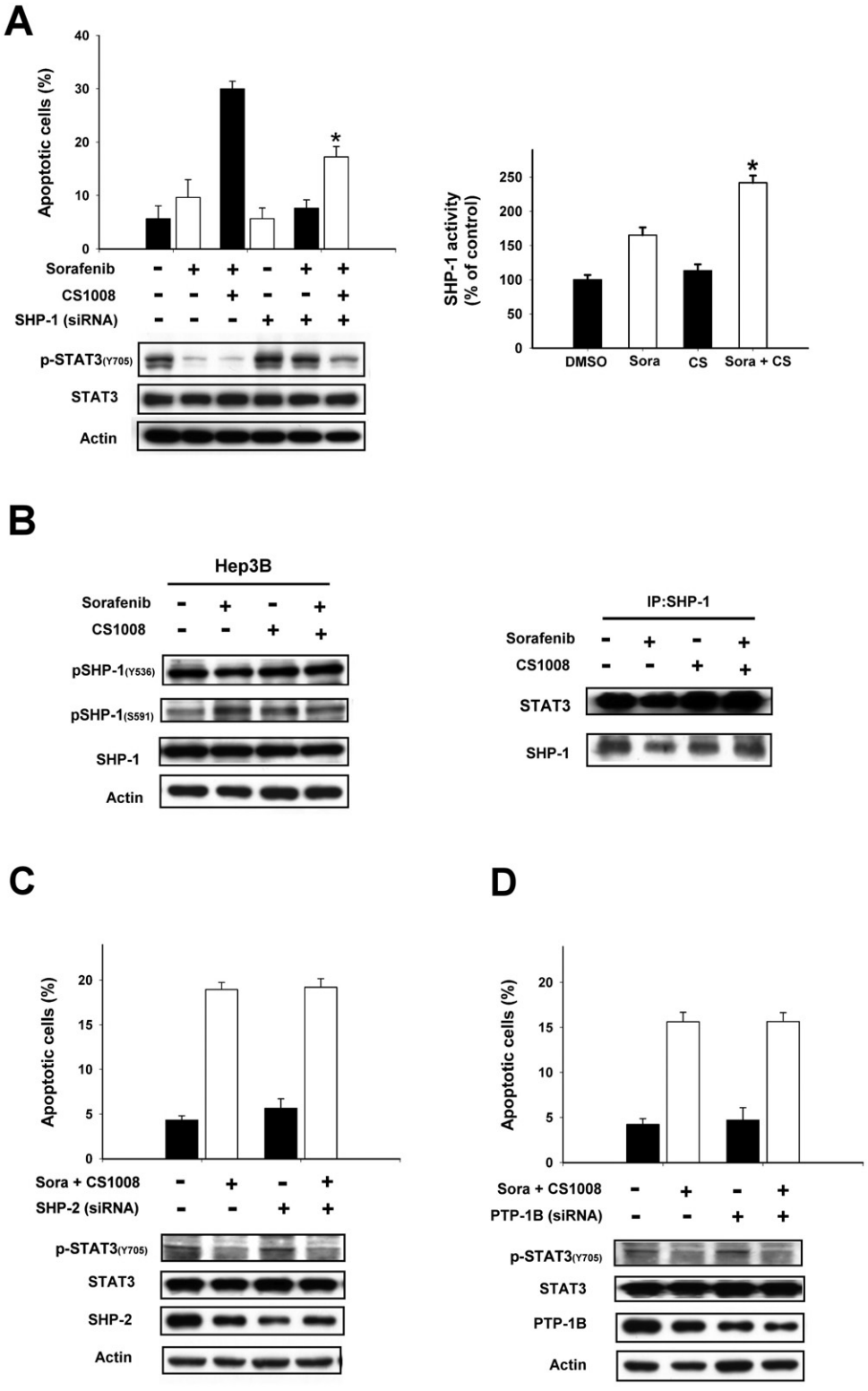

Figure 3

SHP-1 plays a role in mediating the effects of the combination of CS-1008 plus sorafenib on p-STAT3 and apoptosis. (A) *Left,* silencing SHP-1 by siRNA reduced the effects of sorafenib on p-STAT3 in HCC cells. Columns are means and bars represent SD ($n = 3$). *$P < 0.05$. *Right,* co-treatment of CS-1008 and sorafenib enhanced the activity of SHP-1 in PLC5 cells. (B) *Left,* effects of CS-1008 and/or sorafenib on phospho-SHP-1 in Hep3B cells. Columns show means with bars representing SD ($n = 3$). *$P < 0.05$. *Right,* effects of CS-1008 and/or sorafenib on SHP-1 and STAT3 protein interactions. (C) Knock down of SHP-2 did not affect effects of co-treatment of sorafenib and CS-1008 on p-STAT3 and apoptosis. (D) Knock down of PTP-1B did not affect effects of sorafenib on p-STAT3 and apoptosis. Columns show means with bars representing SD ($n = 6$). *$P < 0.05$.

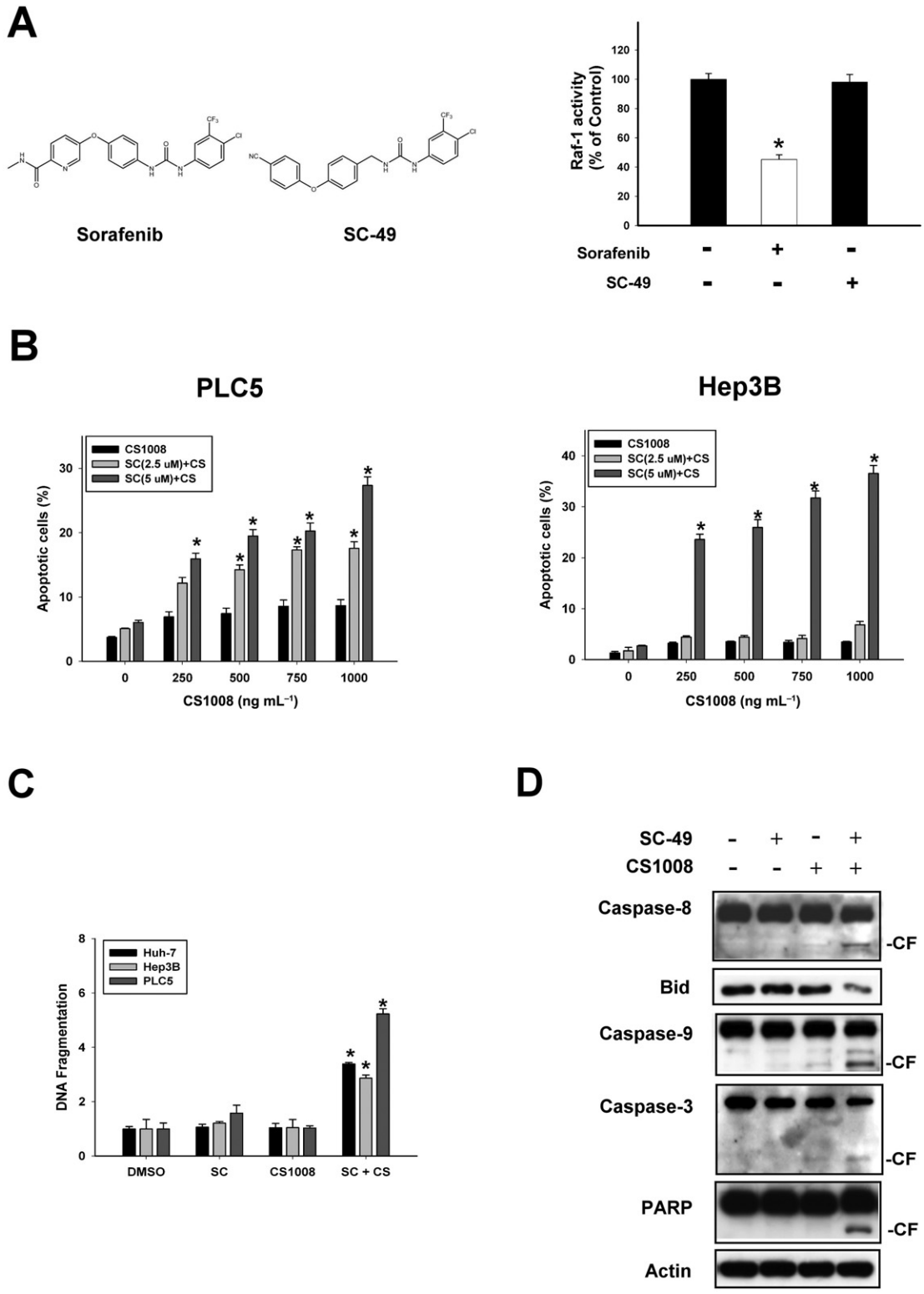

Figure 4

SC-49 sensitized HCC cells to CS-1008. (A) *Left*, chemical structures of sorafenib and SC-49. *Right,* effect of sorafenib and SC-49 on Raf-1 activity. PLC5 cells were exposed to sorafenib or SC-49 at 10 μM for 24 h. Points show means with bars representing SD ($n = 6$). (B) Dose escalation effects of a combination of CS-1008 and SC-49 (2.5 μM or 5 μM) on apoptosis in HCC cells. Cells were exposed to CS-1008 and/or SC-49 at the indicated doses for 24 h. (C) Effects of CS-1008 and SC-49 on DNA fragmentation in three HCC cell lines. Cells were treated with CS-1008 (1000 ng·mL^{-1}) and/or sorafenib (5 μM) for 24 h and DNA fragmentation was analysed by using a cell death ELISA kit. (D) Effects of SC-49 on CS-1008-induced apoptosis in PLC5 cells. CF, cleaved form (activated form).

HUVEC cells. As shown in Figure 5A (left), sorafenib as a kinase inhibitor significantly inhibited the activity of VEGFR1 in HUVEC cells. However, unlike sorafenib, SC-49 did not affect the activity of VEGFR1 in HUVEC cells. Next, we examined the effect of SC-49 on p-STAT1 and p-STAT5; SC-49 down-regulated both p-STAT1 and p-STAT5 in a dose-dependent manner (Figure 5A, right). Furthermore, in HCC cell lines, Huh-7 and Hep3B, we found that SC-49 induced more apoptotic cell death than sorafenib (Figure 5B). In addition, SC-49 was more effective at down-regulating p-STAT3 than sorafenib in HCC cells (Figure 5C). These data suggest that SC-49, a sorafenib derivative without kinase inhibitory activity, is a more potent anti-tumour agent than sorafenib and that its effect is induced by targeting the STAT3 signalling pathway. Notably, a recent study has shown that depletion of tumour-associated macrophages may be associated with the effect of sorafenib on a liver metastasis model (Zhang *et al.*, 2010). In this regard, we tested the effect of SC-49 on U937, a human macrophage cell line (Passmore *et al.*, 2001) and again found that SC-49 down-regulated p-STAT3 in a dose-dependent manner in these cells (Figure 5D).

SHP-1-dependent inhibition of STAT3 mediates the effect of SC-49

We next examined the mechanism by which SC-49 sensitized HCC cells to CS-1008. Our data showed that the combination of SC-49 and CS-1008 decreased p-STAT3 in three TNFSF10-resistant cell lines. Co-treatment with SC-49 plus CS-1008 also down-regulated STAT3-driven proteins, Mcl-1, survivin and cylcin D1, in all three HCC cell lines (PLC5, Huh-7 and Hep3B) (Figure 6A). As shown in Figure 6B, overexpression of STAT3 reversed the sensitizing effects of SC-49 on CS-1008, suggesting that STAT3 plays a role in mediating the effect of SC-49. We next employed a specific SHP-1 inhibitor to test whether SHP-1 mediates the effect of SC-49 on p-STAT3, and found that the SHP-1 inhibitor abolished the sensitizing effect of SC-49 on the action of CS-1008 significantly, indicating that SHP-1 is a mediator of this effect of SC-49 (Figure 6C). As shown in Figure 6D (left), SC-49 at 5 μM enhanced the activity of SHP-1. Notably, SC-49 did not affect the phosphorylation of SHP-1 (Figure 6D, right). To further explore the mechanism by which SC-49 affected the activity of SHP-1, we tested the effect of SC-49 on SHP-1-containing cell lysates to determine whether SC-49 enhances the activity of SHP-1 by affecting its interactions with other proteins. Briefly, PLC5 cells were immunoprecipitated with anti-SHP-1 antibody. Protein extract which included SHP-1 complex was further incubated with SC-49 at 7.5 μM and/or CS-1008 at 1000 ng·mL^{-1} for 30 min and then SHP-1 phosphatase activity assay was performed. Our data showed that SC-49 alone or in combination with CS-1008 increased the phosphatase activity of SHP-1-containg lysates (Figure 6E). These data suggest that SC-49 enhances the activity of SHP-1 by directly interacting with it. However, further work is needed to elucidate details of the interactions between SC-49 and SHP-1.

In vivo *effects of SC-49 in Huh-7 xenograft tumours*

To further examine the effect of SC-49, we next tested the effect of SC-49 on Huh-7 xenograft tumours *in vivo*. As shown in Figure 7A (left), treatment of mice with SC-49 at a dose of 10 mg·kg^{-1}·day^{-1} p.o. significantly reduced the growth of the Huh-7 tumour and this anti-tumour effect was better than that of sorafenib *in vivo*. As shown in Figure 7A (right), animals had stable body weights throughout the course of study. In addition, SC-49 down-regulated p-STAT3 in Huh-7 tumours (Figure 7B). SC-49 and sorafenib enhanced the activity of SHP-1 in Huh-7 tumours (Figure 7C). Immunohistochemical staining for STAT3 showed no obvious significantly different cytoplasmic expression in all groups (Figure 7D). The treatment of both sorafenib and SC-49 decreased the nuclear expression of P-STAT3 (Figure 7D). From the immunohistochemical stain for CD-31, all the groups showed a similar vascular density in the tumour areas (Figure 7D).

These data indicate that SC-49 exhibited better *in vivo* effects than sorafenib through an SHP-1-dependent inhibitory effect on STAT3.

The effect of the combination of SC-49 and CS-1008 in vivo

To confirm whether the sensitizing effect of SC-49 in resistant cell lines has potentially relevant clinical implications, we assessed the effect of the combination of CS-1008 plus SC-49 on the growth of PLC5 tumours *in vivo*. Tumour-bearing mice were treated with vehicle or CS-1008 i.v. at a dose of 200 μg three times a week or SC-49 p.o. at a dose of 5 mg·kg^{-1}·day^{-1}, or a combination of the two, for the duration of the study. All animals tolerated the treatments well without an observable signs of toxicity and had stable body weights throughout the course of study. No gross pathological abnormalities were noted at necropsy.

Tumour growth was significantly inhibited by co-treatment with CS-1008 and SC-49 for 2 weeks (vs. control, $P < 0.05$), and tumour size in the co-treatment group was only one third of that of the control group at the end of the study (Figure 8A). Treatment with CS-1008 had no significant effect on PLC5 tumour growth. SC-49 alone showed modest effects on tumour growth. In addition, co-treatment of SC-49 and CS1008 significantly down-regulated p-STAT3 in PLC5 tumours (Figure 8B). Moreover, as shown in Figure 8C, co-treatment with SC-49 and CS-1008 enhanced SHP-1 activity significantly, indicating that SHP-1 plays a role in mediating the effects of the combination of drugs on PLC5 tumours. Together, these data indicate that a combination of CS-1008 and SC-49 exhibits good anti-tumour activity *in vivo*. Further clinical investigations are warranted.

Discussion

Previous literature has consistently shown that sorafenib is capable of sensitizing various cancer cells, including HCC, to TNFSF10-induced apoptosis (Hall and Cleveland, 2007; Ricci *et al.*, 2007; Koehler *et al.*, 2009; Huang and Sinicrope, 2010; Llobet *et al.*, 2010). For that reason, CS-1008 is currently undergoing phase II trials for the treatment of advanced HCC (NCI clinical trial: NCT01033240). The results should provide informative *in vivo* evidence of the activity of this combination strategy.

Although the mechanisms employed by various tumours to evade TNFSF10-induced apoptosis are heterogeneous

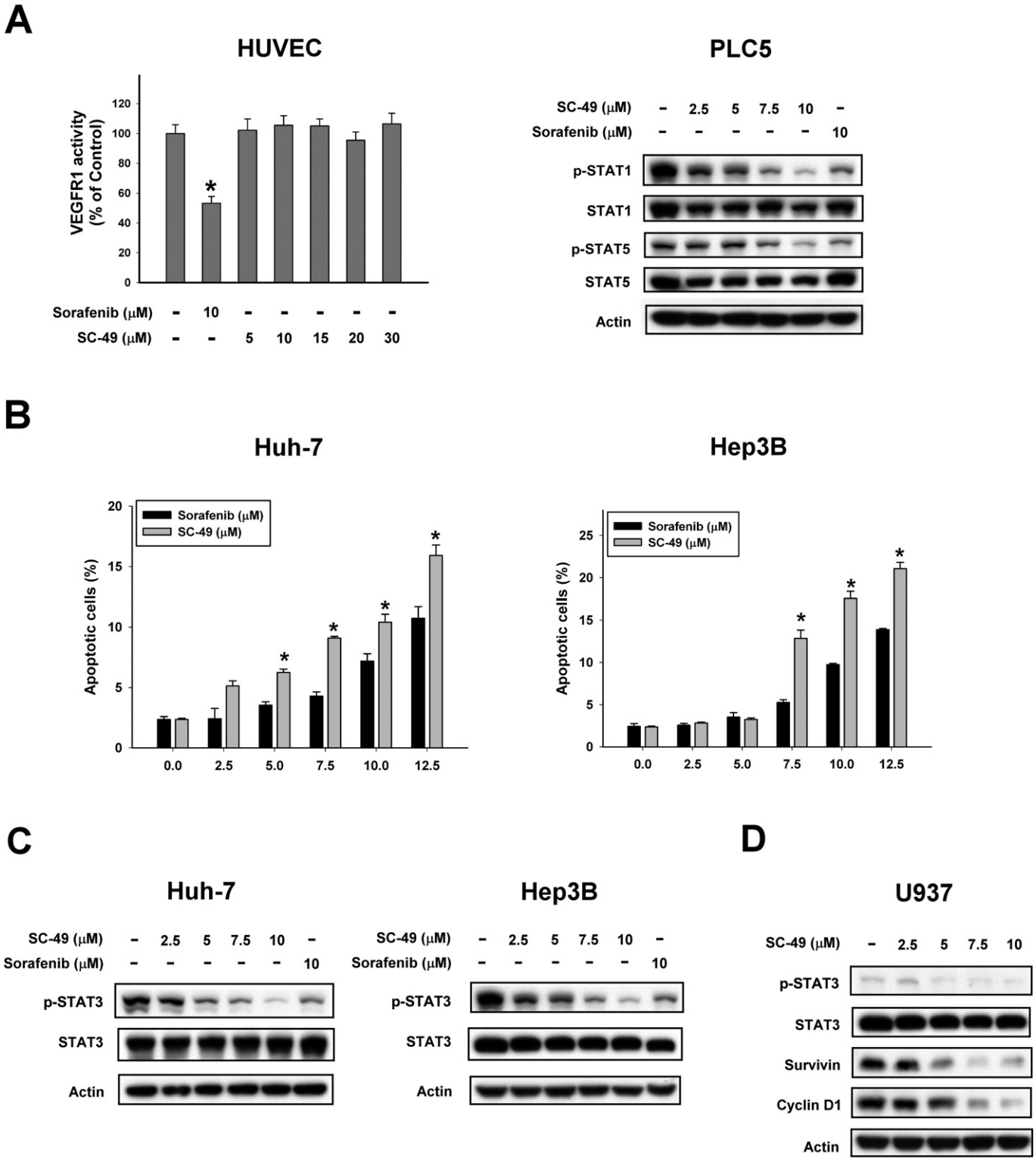

Figure 5

SC-49 showed better apoptotic effects than sorafenib in HCC. (A) *Left,* effect of sorafenib and SC-49 on VEGFR1 activity. *Right,* effect of sorafenib and SC-49 on p-STAT1 and p-STAT5. Cells were exposed to sorafenib or SC-49 at the indicated doses for 24 h. Points show means with bars representing SD ($n = 6$). (B) Dose escalation effects of sorafenib and SC-49 on apoptosis in HCC cells. Cells were exposed to sorafenib or SC-49 at the indicated doses for 24 h. Apoptotic cells were analysed by flow cytometry (sub-G1). (C) Effects of SC-49 on p-STAT3. Cells were treated with SC-49 or sorafenib at the indicated doses for 24 h. (D) Effects of SC-49 on p-STAT3 in U937 cells. Cells were treated with SC-49 at the indicated doses for 24 h.

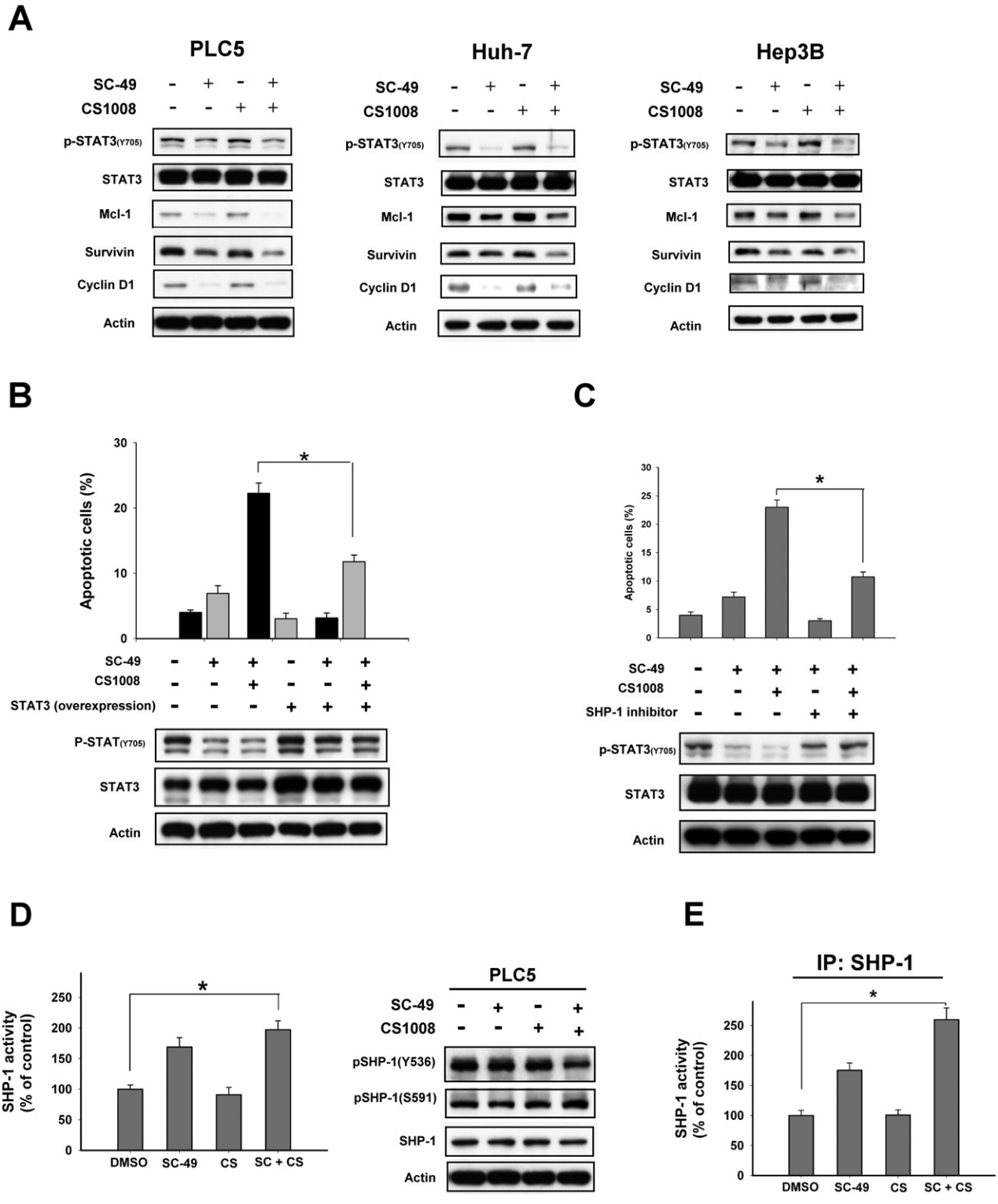

Figure 6

SHP-1 mediates the effect of SC-49 plus CS-1008 on p-STAT3 and apoptosis. (A) Effects of SC-49 (5 μM) and/or CS-1008 (1000 ng·mL^{-1}) on STAT3-related proteins. Cells were exposed to the drugs for 24 h. (B) Ectopic expression of STAT3 reduced apoptosis induced by SC-49 plus CS-1008 in PLC5 cells. (C) Inhibition of SHP-1 by adding a SHP-1 inhibitor reduced the effects of sorafenib on p-STAT3 in Hep3B cells. Columns show means with bars representing SD ($n = 3$). *$P < 0.05$. (D) *Left*, co-treatment of SC-49 (5 μM) and CS-1008 (1000 ng·mL^{-1}) enhanced the activity of SHP-1 in PLC5 cells. *Right*, effects of SC-49 (5 μM) and/or CS-1008 (1000 ng·mL^{-1}) on phospho-SHP-1 in Hep3B cells. (E) Effects of dovitinib on phosphatase activity in SHP-1-containing lysates. PLC5 cells were immunoprecipitated with anti-SHP-1 antibody. The lysates were incubated with dovitinib (10 nM) for 30 min then analysed by SHP-1 phosphatase activity.

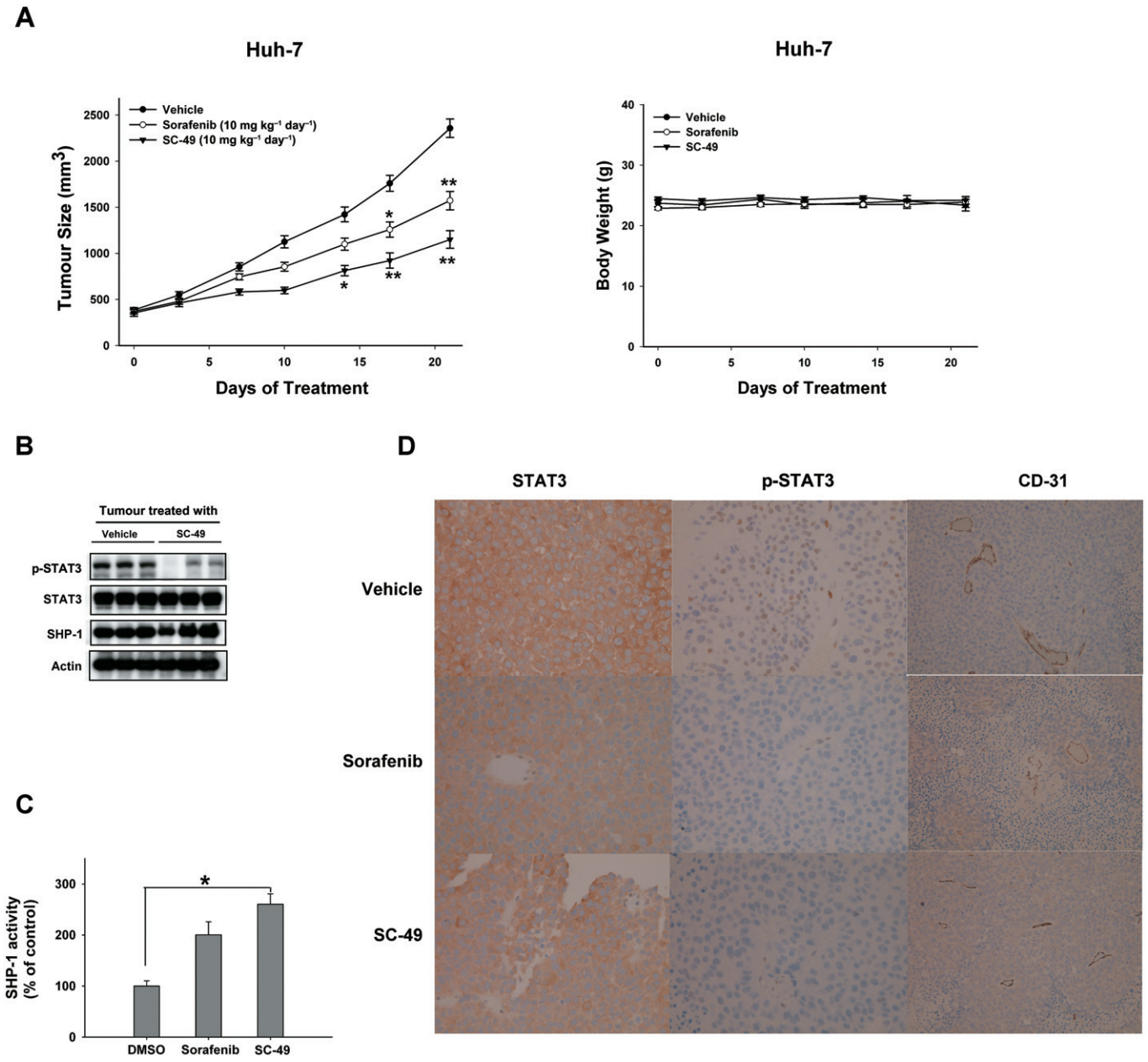

Figure 7

In vivo effects of SC-49 in Huh-7 xenograft tumour. (A) *Left*, SC-49 showed significant anti-tumour effect on Huh-7 tumours. *Right,* body weight. Points show means with bars representing SEM ($n = 6$). *, $P < 0.05$; **, $P < 0.01$. (B) Western blot analysis of p-STAT3, STAT3, and SHP-1 in Huh7 tumours. (C) Analysis of SHP-1 activity. Columns show means with bars representing SD ($n = 6$). *$P < 0.05$ versus vehicle group. (D) Immunohistochemical staining for tumours. Slides were then stained using the Leica Microsystems BONDMAX autostainer according to the manufacturer's protocol (400 folds).

(Zhang and Fang, 2005), it has been suggested that Mcl-1 is the gateway to the sensitizing effect of sorafenib in cells (including HCC cells) that harbour defects in apoptosis mediated by the intrinsic pathway (Meng *et al.*, 2007; Kim *et al.*, 2008). In addition, aberrant activation of anti-apoptotic pathways, such as PI3K/Akt signalling, MAPK pathway and the NF-κB pathway, may also contribute to the development of TNFSF10 resistance in HCC cells (Bortul *et al.*, 2003; Ehrhardt *et al.*, 2003; Zhang and Fang, 2005). In particular, TNFSF10 treatment in resistant cells has been shown to induce Mcl-1 expression through the Raf and NF-κB-dependent pathway. Sorafenib, as a Raf kinase inhibitor, could, therefore, potentially block this TNFSF10-induced NF-κB–mediated transcriptional activation of Mcl-1, and NF-κB binding to the Mcl-1 promoter region (Kim *et al.*, 2008). In the present study, we added to previous data by further showing that sorafenib suppresses other STAT3-regulated proteins (i.e. survivin and cyclin D1) in HCC. Our data also confirmed that the inhibi-

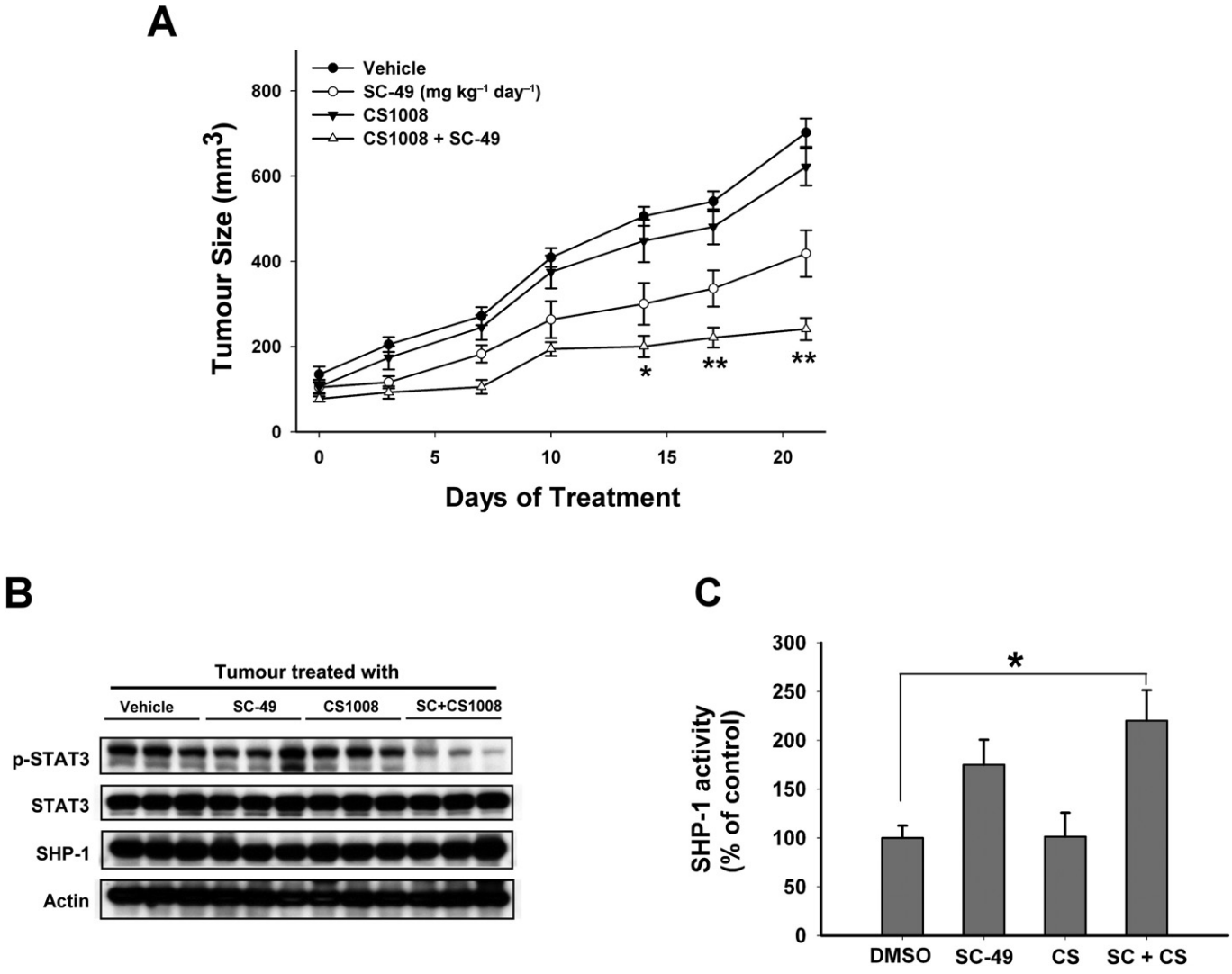

Figure 8

The effect of the combination of SC-49 and CS-1008 *in vivo*. (A) The combination of SC-49 and CS-1008 showed significant anti-tumour effect on PLC5 tumours. Columns show means with bars representing SEM ($n = 6$). *, $P < 0.05$; **, $P < 0.01$. (B) Western blot analysis of p-STAT3, STAT3, and SHP-1 in PLC5 tumours. (C) Analysis of SHP-1 activity. Columns show means with bars representing SD ($n = 6$). *$P < 0.05$ versus vehicle group.

tion of STAT3 is the major mechanism by which sorafenib sensitizes TNFSF10 in HCC. It has previously been demonstrated that sorafenib inhibits STAT3 activity and enhances TNFSF10-mediated apoptosis in other cancer cells, including pancreatic cancer cells (Huang and Sinicrope, 2010), medulloblastomas cells (Yang *et al.*, 2008) and cholangiocarcinoma cells (Blechacz *et al.*, 2009). Taken together, these data indicate that STAT3 represents a novel anti-cancer target of sorafenib.

Another important finding in the current study is that sorafenib inhibits STAT3 by increasing SHP-1 activity (Figure 3). Our results showed that sorafenib increased SHP-1 activity but did not alter SHP-1 protein expression level and, despite being a kinase inhibitor, did not alter the phosphorylation of SHP-1 at either the Y-536 or S-591 sites, both known to change SHP-1 activity upon phosphorylation. Moreover, sorafenib did not influence the SHP-1 and STAT3 protein–protein interactions. In contrast, several chemical compounds such as acety-11-keto-β-boswellic acid and butein (3,4,2′,4′-tetrahydroxychalcone), are thought to inhibit STAT3 by the induction of SHP-1 expression (Kunnumakkara *et al.*, 2009; Pandey *et al.*, 2009). Nevertheless, the mechanism by which sorafenib influences SHP-1 activity remains to be elucidated and further studies are needed to address this issue. Interestingly, Blechacz *et al.* (2009) suggested that sorafenib inhibits STAT3 in cholangiocarcinoma cells by influencing SHP-2 activity through the down-regulation of phospho-SHP-2. However,Blechacz *et al.* (2009) did not show whether sorafenib also affects SHP-1 in cholangiocarcinoma cells. In contrast, in the present work we have shown that knockdown of SHP-2 did not alter the sensitizing effect of sorafenib on apoptosis and STAT3 phosphorylation in HCC cells (Figure 3C). However, it is possible that sorafenib inhibits STAT3 by affecting different protein tyrosine phosphatases

in various cancer cells. More effort is needed to fully understand why sorafenib affects different protein tyrosine phosphatases in HCC and cholangiocarcinoma, and perhaps other cancer cells.

In conclusion, our results revealed that sorafenib as well as SC-49 have a synergistic effect with CS-1008 on HCC through SHP-1-dependent inhibition of STAT3 and indicate that the STAT3 signalling pathway may be a suitable target for the development of anti-HCC targeted agents. Sorafenib may serve as a lead compound for the development of more potent STAT3 inhibitors.

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Disclosure of potential conflict of interest

A-LC is a consultant for and a member of the speaker's bureau of Bayer-Schering and a consultant of Daiichi Sankyo. K I is an employee of Daiichi Sankyo. Other authors have nothing relevant to this manuscript to disclose.

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