CS-1008, an anti-human death receptor 5 antibody, and sorafenib induce apoptosis in hepatocellular carcinoma via SHP-1-dependent Stat3 inactivation.

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Abbreviations: HCC, hepatocellular carcinoma; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand ; STAT3, signal transducers and activators of transcription 3; FADD, Fas Associated protein with Death Domain ; c-FLIP, cellular FLICE-inhibitory protein; PARP, polypolymerase; DMEM, Dulbecco's modified Eagle's medium, FBS, fetal bovine serum.

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Abstract:

Recombinant tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is considered a promising agent for cancer therapy. However, many HCC cells show resistance to TRAIL-induced apoptosis. The tyrosine kinase inhibitor, sorafenib is capable of sensitizing hepatocellular carcinoma (HCC) to TRAIL-induced apoptosis and CS-1008 is a novel humanized anti-human death receptor 5 (DR5) agonistic antibody currently undergoing phase II trials for the treatment of advanced HCC. In this study, we demonstrated that signal transducers and activators of transcription 3 (Stat3) played a significant role in mediating the effect of sorafenib on CS-1008-induced apoptosis. Sorafenib down-regulated phospho-Stat3 (Tyr 705) and subsequently reduced the protein 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 effect of sorafenib on CS-1008-induced apoptosis, indicating that inhibition of Stat3 mediates the effects of the combination. Importantly, silencing SHP-1 by RNA-interference reduced the effects of sorafenib and CS-1008 on p-Stat3 and apoptosis, whereas co-treatment of CS-1008 and sorafenib increased the activity of SHP-1, suggesting that SHP-1 mediated the combinational effect of CS-1008 and sorafenib in HCC. Moreover, *in vivo* the combination of CS-1008 and sorafenib inhibited Huh7 xenograft tumor growth. In conclusion, CS-1008 and sorafenib induce apoptosis and inhibit tumor growth in HCC through SHP-1-dependent Stat3 inactivation.

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.[1, 2] Advanced or recurrent HCC is frequently resistant to current chemotherapeutic agents and radiation; therefore, the development of targeted agents with tolerable toxicity is mandatory to advance anti-HCC therapy.[2] Sorafenib, a multi-kinase inhibitor, has been approved for clinical use in advance HCC after significantly improving overall survival in two prospective randomized phase III trials for patients with advanced HCC {Cheng, 2009 #548}[3]. Sorafenib inhibits multiple kinases, including the Ras/Raf/MAPK/ERK signaling pathway, the angiogenic pathways VEGFR2, VEGFR3, as well as PDGFR-β and other kinases such as FLT-3 and FGFR-1.[4-7]

Among various targeted strategies for HCC treatment, tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL), which targets receptor-mediated apoptosis, represents an attractive option.[8-10] As a member of the TNF superfamily, TRAIL initiates apoptosis through binding to two important death domain-containing death receptors (DRs), DR4 (TRAIL-R1) and DR5 (TRAIL-R2).[11-13] TRAIL or TRAIL agonists bind to DR4 or DR5 and forms death-inducing signaling complex (DISC), which is a multi-protein complex consisting of an adaptor molecule, FADD and the initiator of extrinsic pathway caspase-8.[8, 11] Activated caspase-8 is capable of both initiating an extrinsic apoptotic pathway in type I cells (through activating caspase-3, -6, and -7), and triggering the intrinsic pathway in type II cells (through activating Bid).[8, 14]

CS-1008 is a novel DR5 agonist that exerts TRAIL-like activity. It is a humanized

anti-human DR5 antibody manufactured from a murine anti-human DR5 monoclonal antibody, TRA-8.[15] So far CS-1008 has shown selective cytotoxicity towards tumor cells expressing DR5[15] and an excellent safety profile in humans.[16] CS-1008 monotherapy induces apoptosis in various cancer cells and CS-1008 in combination with some chemotherapeutic agents (such as gemcitabine or docetaxel) also enhanced antitumor activity.[15] In a phase I trial, no dose limiting toxicity was reported fro CS-1008 at doses of up to 8 mg/kg weekly.[16]

Although TRAIL may be applied as anti-HCC strategy, more and more literature reports insufficient efficacy of TRAIL-induced apoptosis in HCC cells, often due to resistance to TRAIL or its agonists.[17-19] Resistance to TRAIL may be induced by any step in the apoptosis signaling cascade, from receptor level (mutations or overexpression of DR4 or DR5),[20] or defects in DISC assembly,[21,22] through dysfunctions of anti-apoptotic Bcl-2 family proteins (Bcl-2, Bcl-XL, Mcl-1, etc)[23,24] and pro-apoptotic proteins (Bax or Bak) or defects in mitochondria-derived activator of caspases (Smac/Diablo).[20,25] Of particular note, Mcl-1, an anti-apoptotic Bcl-2 family protein, plays a critical role in conferring TRAIL resistance.[23] Data have shown that over-expression of Mcl-1 can neutralize TRAIL-induced signaling.[26, 27] Moreover, directly or indirectly destabilizing or disabling Mcl-1 may restore TRAIL sensitivity.[28,29] Interestingly, as a highly regulated cell death and survival controllers that responds to various cytokines and growth factors,[30] Mcl-1 can be regulated by a number of transcription factors, including NF-kB targeting the cAMP response element (CRE-2) motif, and signal transducers and activators of transcription 3 (Stat3) targeting the sis-inducible element (SIE) motif of *mcl-1* promotor region.[23,30,31]

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.[32,33,34] 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 tumorigenesis, invasion, and metastasis.[34] Targeting Stat3 using antisense oligonucleotide reduces the growth and metastasis of HCC cells in vitro and in vivo.[32] Importantly, abrogation of constitutive STAT3 activity has been shown to sensitizes human hepatoma cells to TRAIL-mediated apoptosis.[33] Moreover, a number of protein tyrosine phosphatases have been shown to negatively regulate STAT3 signaling 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).[35-38] Therefore, activity of protein tyrosine phosphatases may be critical for the regulation of STAT3 phosphorylation in cancer cells.

In this study, we found that sorafenib sensitizes resistant HCC cells to TRAIL-induced apoptosis through SHP-1 dependent STAT3 inhibition. We demonstrated that a combination of sorafenib and CS-1008 down-regulates p-STAT3 and subsequently reduces the expression of its downstream-regulated proteins, Mcl-1, survivin and cylcin D1. Furthermore, we showed that sorafenib, through increasing SHP-1 activity, inhibits Stat3 activity and enhances the antitumor activity of CS-1008 toward HCC cells. Importantly, we were able to confirm this sensitizing effect *in vivo*. The combination of sorafenib and CS-1008 may constitute a novel anti–HCC treatment.

Materials and Methods:

Reagents and antibodies. CS-1008 and Sorafenib (Nexavar®) and were kindly provided by Daiichi Sankyo Pharmaceuticals (Tokyo, Japan) and Bayer Pharmaceuticals (West Haven, CT), 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). Other antibodies such as anti-pERK (1/2), ERK2, survivin, cylcin D1, Bcl-xL, Bid, caspase-3, caspase-8, caspase-9, phospho-STAT3 (Tyr705), STAT3 and phosphor-Akt (Ser473) were from Cell Signaling (Danvers, MA).

Cell culture and western blot analysis. The PLC5 and Hep3B cell lines were obtained from the American Type Culture Collection (Manassas, VA). The Huh-7 HCC cell line was obtained from the Health Science Research Resources Bank (Osaka, Japan; JCRB0403). Cells were maintained in DMEM supplemented with 10% FBS, 100 units/mL, penicillin G, 100 μg/mL streptomycin sulfate, and 25 μg/mL amphotericin B in a 37°C humidified incubator and 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.[36]

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), 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). The procedure has been described previously.[36]

Sk-Hep1 with ectopic expression of STAT3. STAT3 cDNA (KIAA1524) was purchased from Addgene plasmid repository (http://www.addgene.org/). Briefly, following transfection, cells were incubated in the presence of G418 (0.78 mg/mL). After 8 weeks of selection, surviving colonies, i.e., those arising from stably transfected cells, were selected and individually amplified. Sk-Hep1 cells with stable expression of STAT3 were then treated with drugs, harvested, and processed for western blot analysis as described previously.[36]

SHP-1 phosphatase activity. After sorafenib treatment, PLC5 protein extract was incubated with anti-SHP-1 antibody in immunoprecipitation buffer overnight. Protein G-Sepharose 4 Fast flow (GE Healthcare Bio-Science) was added to each sample, followed by incubation for 3 h at 4°C with rotation. A RediPlate 96 EnzChek® Tyrosine Phosphatase Assay Kit (R-22067) was used for SHP-1 activity assay (Molecular Probes, Invitrogen).

Xenograft tumor growth. Male NCr athymic nude mice (5-7 weeks of age) were obtained from the National Laboratory Animal Center (Taipei, Taiwan). 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 Huh7 cells suspended in 0.1 ml of serum-free medium containing 50% Matrigel (BD Biosciences, Bedford, MA). When tumors reached 200–300 mm^3, mice received an intravenous injection of CS-1008 (200 μg) three times a week, sorafenib tosylate (5 mg/kg) p.o. once daily, or a combination of CS-1008 and sorafenib. Controls received vehicle. Tumors were measured weekly using calipers and their volumes calculated using the following standard formula: $width^2 \times length \times 0.52$.

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).

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); however, the combination of sorafenib and CS-1008 overcame the resistance and induced apoptosis in all tested cell lines in a dose-dependent manner (Fig. 1A). Next, we examined the effect of sorafenib on CS-1008-induced apoptosis on DNA fragmentation in all HCC cell lines. DNA fragmentation was determined by cell death ELISA after 24 h of treatment. As shown in Figure 2, combining sorafenib at 7.5μM 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. 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

PARP cleavage (Fig. 1C). These data suggested that the intrinsic pathway played a role in mediating the combination 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 TRAIL in cancer cells [23, 26, 27]. As STAT3 regulates the expression of Mcl-1, we next examined its related proteins including phospho-STAT3 (p-STAT3), STAT3 and STAT3-regulated proteins including Mcl-1, survivin and cyclin D1. As shown in Figure 2A, co-treatment of sorafenib and CS-1008 down-regulated p-STAT3 (Tyr705) and related proteins, Mcl-1, survivin, and cyclin D1, in all tested cells without altering of total Stat3 protein levels. In addition, down-regulation of p-STAT3 was associated with the cleavage of PARP as shown by evidence of apoptosis induction in cells exposed to sorafenib and CS-1008 for 24 h (Fig. 2A). Furthermore, we found that the combination of sorafenib and CS-1008 down-regulated p-STAT3 in PLC5 cells in both dose- and time-dependent manners (Fig. 2B). Interestingly, co-treatment of sorafenib and CS-1008 did not affect phospho-Erk, suggesting that the sensitizing effect of sorafenib on CS-1008 is not be associated with its Raf-1 activity (Fig. 2C). Notably, the combination of sorafenib and CS-1008 did not alter the expression of Bax and Bcl-xl (Fig. 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. First, we knocked down protein expression of Mcl-1 and STAT3 by 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 24 h. Silencing Mcl-1 and STAT3 significantly sensitized PLC5 cells to CS-1008-induced apoptosis ($P < 0.05$) (Fig. 3A & 3B), suggesting that inhibition of the STAT3 signaling pathway is important for the sensitivity of HCC cells toward 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. Over-expression of Stat3 significantly reduced the combinational effects of sorafenib plus CS-1008 on p-STAT3 and apoptosis. ($P < 0.05$). Together, these results validate the importance of STAT3 inhibition in mediating the combination 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. First, we altered the expression of SHP-1 by siRNA in PLC5 cells and showed that silencing SHP-1 significantly reduced the effects of sorafenib plus CS-1008 on p-STAT3 and apoptosis (Fig. 4A *left)*. This result suggests that SHP-1 mediated effects of the 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 4A *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 tyrosine 536 may enhance its activity and phosphorylation at serine 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 (Fig. 4B *left*). In addition, we examined whether the combination of drugs affected the protein-protein interactions between SHP-1 and Stat3. Our data showed 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 SHP-1 and STAT3 protein interaction (Fig. 4B r*ight*). Finally we also examined other protein tyrosine phosphatases such as SHP-2 and PTP-1B that may also regulate STAT3 signaling pathway. However, neither knockdown of SHP-2 nor silencing PTP-1B affected the effect of sorafenib plus CS-1008 on p-STAT3 signaling and apoptosis (Fig. 4C & 4D). These data indicate that SHP-2 and PTP-1B do not play roles in mediating the effects of the drug on p-Stat3 and apoptosis.

In vivo effect of CS-1008 and sorafenib. To confirm whether the effect of CS-1008 and sorafenib in resistant cell lines has potentially relevant clinical implications, we assessed the *in vivo* effect of CS-1008 and sorafenib on the growth of HCC xenograft tumors. Tumor-bearing mice were treated with vehicle or CS-1008 i.v. at a dose of 200μg three times a week or sorafenib p.o. at a dose of 5 mg/kg/day or in combinations for the duration of the study. All animals tolerated the treatments well without observable signs of toxicity and had stable body weights throughout the course of study. No gross pathologic abnormalities were noted at necropsy.

Tumor growth was significantly inhibited by co-treatment with CS-1008 and sorafenib for two weeks (*versus* control, $P < 0.05$) and tumor size in the co-treatment group was only one third of that of the control group at the end of the study (Fig. 5A). Treatment with CS-1008 had no significant effect on Huh-7 tumor growth. Sorafenib

alone showed modest effects on tumor growth. As shown in Fig. 5B, co-treatment with sorafenib and CS-1008 enhanced SHP-1 activity significantly, indicating that SHP-1 plays a role in mediating the combinational effects of drugs in Huh-7 tumor. Together, these data indicate that a combination of CS-1008 and sorafenib exhibits good anti-tumor activity *in vivo*. Further clinical investigation is warranted.

Discussion:

In this study, we revealed that an important novel mechanism, SHP-1 dependent p-STAT3 inhibition, is responsible for sorafenib's sensitization of resistant HCC cells to TRAIL-induced apoptosis. Previous literature has consistently shown that sorafenib is capable of sensitizing various cancer cells, including HCC, to TRAIL-induced apoptosis. [26, 29, 39-41]. 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 tumors to evade TRAIL-induced apoptosis are heterogeneous [20], it has been suggested that Mcl-1 is the gateway to the sensitizing effect of sorafenib in cells (including HCC cells) that harbor defect in apoptosis mediated by the intrinsic pathway.[23, 27]. In addition, aberrantly activated antiapoptotic pathways such as phosphoinositol-3-kinase (PI3K)/Akt signaling, mitogen-activated protein (MAP) kinase pathway and the nuclear factor-kappa B (NF-kB) pathway may also contribute to the development of TRAIL resistance in HCC cells.[20,42,43] In particular, TRAIL treatment in resistant cells has been shown to induce mcl-1 expression through the Raf and NF-kB dependent pathway. Sorafenib, as a Raf kinase inhibitor, could, therefore, potentially block this TRAIL-induced

NF-kB–mediated transcriptional activation of Mcl-1, and NF-kB binding to the Mcl-1 promoter region.[23] Adding to previous data, here, we further showed that sorafenib suppresses other Stat3-regulated proteins, *i.e.* survivin and cyclin D1, in HCC. Our data confirm that the inhibition of Stat3 is the major mechanism by which sorafenib sensitizes TRAIL in HCC. It has previously been demonstrated that sorafenib inhibits Stat3 activity and enhances TRAIL-mediated apoptosis in other cancer cells, including pancreatic cancer cells,[41] medulloblastomas cells,[44] and cholangiocarcinoma cells.[45] Taken together, these data indicate that Stat3 represents a novel anti-cancer target of sorafenib. Furthermore, Stat3 is implicated as target for developing novel anti-cancer agents or TRAIL-sensitizers. For example, Kusaba and colleagues[33] showed that another targeted agent, Janus kinase 2 inhibitor AG490, inhibits Stat3 activation and also sensitize HCC cells to TRAIL. Sahu *et al.* also reported that a chemical compound, benzyl isothiocyanate (BITC), induces apoptosis in certain pancreatic cancer cells through reduced expression of activated/total Stat3 proteins and Stat3-DNA binding affinity.[46]

Another important finding of the current study is that sorafenib inhibits Stat3 via increasing SHP-1 activity (Figure 4). 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-beta-boswellic acid and butein (3,4,2',4'-tetrahydroxychalcone), are thought to inhibit Stat3 by the induction of SHP-1 expression.[35, 37] 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.* suggested that sorafenib inhibited Stat3 in cholangiocarcinoma cells by influencing SHP-2 activity through down-regulation of phospho-SHP-2.[45] However, it is unclear whether sorafenib also affects SHP-1 in cholangiocarcinoma cells, as Blechacz and colleagues did not show the data of the SHP-1 in their report.[45] In contrast, in 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 (Fig. 4C). It is possible that sorafenib affects different protein tyrosine phosphases to inhibit STAT3 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 study revealed sorafenib overcomes TRAIL resistance in HCC through SHP-1 dependent Stat3 inhibition and indicates that the Stat3 signaling pathway may be a suitable target for the development of anti-HCC targeted agents. Future studies defining STAT3 as a useful therapeutic biomarker for HCC patients who receive sorafenib treatment, as well as the detailed mechanism by which sorafenib affects SHP-1 activity may lead to further progress in the development of molecular-targeted therapy for HCC.

Figure legends

Fig. 1 Sorafenib enhances CS-1008-induced apoptosis in resistant HCC cells. A, dose-escalation effects of a combination of CS-1008 and sorafenib (5 μM or 7.5 μM) on apoptosis in 3 TRAIL-resistant HCC cells. Cells were exposed to CS-1008 and/or sorafenib at the indicated doses for 24 h. Apoptotic cells were

determined by flowcytometry. B, effects of CS-1008 and sorafenib on DNA fragmentation in three HCC cell lines. Cells were treated with CS-1008 (500 ng/ml) and/or sorafenib (7.5 μM) for 24 h and DNA fragmentation was analyzed by using a cell death ELISA kit. C, effects of sorafenib on CS-1008-induced apoptosis in PLC5 cells. Cells were exposed to CS-1008 and/or sorafenib at the indicated doses for 24 h. Cell lysates were analyzed for caspase-8, bid, caspase-9, caspase-3, and PARP by western blot. Data are representative of three independent experiments. CF, cleaved form (activated form).

Fig. 2 Co-treatment of sorafenib and CS-1008 down-regulated p-Stat3 in HCC cells. A, effects of sorafenib (7.5μM) and/or CS-1008 (500 ng/ml) on STAT3-related proteins. Cells were exposed to the drugs for 24 h and cell lysates were analyzed by western blot. B, effects of sorafenib on phospho-STAT3 in PLC5 cells. Cells were exposed to the drugs for the indicated time and cell lysates were analyzed by western blot. C, effects of sorafenib and/or CS-1008 on p-Erk, Bax and Bcl-xl. Cells were treated with 500 ng/ml CS-1008 and/or sorafenib at 7.5μM for 24h. Cell lysates were analyzed by western blot.

Fig. 3 Validation of Stat3. A, down-regulation of mcl-1 by SiRNA overcame the resistance to CS-1008 in PLC5 cells. B, down-regulation of Stat3 by SiRNA overcomes resistance to CS-1008 in PLC5 cells. Cells were transfected with either control SiRNA or SiRNA (mcl-1 or STAT3) for 48 h and then exposed to CS-1008 and/or 7.5 μM sorafenib for 24 h. C, Ectopic expression of Stat3 reduced apoptosis induced by the combination of CS-1008 and sorafenib in

PLC5 cells. PLC5 Cells (wild type or Stat3-overexpression) were exposed to 7.5 μM sorafenib and/or CS-1008 500 ng/ml for 24 h. Apoptotic cells were analyzed by flow cytometry. *Columns*, mean; *bars*, SD ($n = 3$). *$P < 0.05$.

Fig. 4 SHP-1 plays a role in mediating the effects of CS-1008 plus sorafenib combination on p-Stat3 and apoptosis. A, *Left,* silencing SHP-1 by siRNA reduced the effects of sorafenib on p-STAT3 in HCC cells. PLC5 cells were transfected with control siRNA or SHP-1 siRNA for 48 h and then treated with 7.5 μM sorafenib and/or 500 ng/ml CS-1008 for 24 h. *Columns*, mean; *bars*, SD ($n = 3$). *$P < 0.05$. *Right,* co-treatment of CS-1008 and sorafenib enhanced the activity of SHP-1 in PLC5 cells. Cells were treated with sorafenib (7.5 μM) and/or CS-1008 (500ng/ml) for 24 h and cell lysates were analyzed for SHP-1 activity. B, *Left,* effects of CS-1008 and/or sorafenib on phospho-SHP-1 in PLC5 cells. Cells were treated with 500 ng/ml CS-1008 and/or 7.5μM sorafenib for 24 hours. *Columns*, mean; *bars*, SD ($n = 3$). *$P < 0.05$. *Right,* effects of CS-1008 and/or sorafenib on SHP-1 and STAT3 protein interactions. PLC5 cells were treated with sorafenib (7.5 μM) and/or CS-1008 (500ng/ml) for 24 h. Cell lysates were immunoprecipated with anti-SHP-1 then analyzed by western blot. 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. PLC5 cells were transfected with control siRNA or SHP-2 siRNA or PTP-1B siRNA for 48 h and then treated with the drugs for 24 h. Apoptotic cells were measured by flowcytometry. *Columns*, mean; *bars*, SD ($n = 6$). *$P < 0.05$.

Fig. 5 *In vivo* effect of sorafenib and CS-1008 on Huh-7 xeonograft nude mice A, the combination of sorafenib and CS-1008 showed significant anti-tumor effect on Huh7 tumors. B, analysis of SHP-1 activity. *Columns*, mean; *bars*, SD ($n = 6$). *$P < 0.05$ versus vehicle group.

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Fig. 1

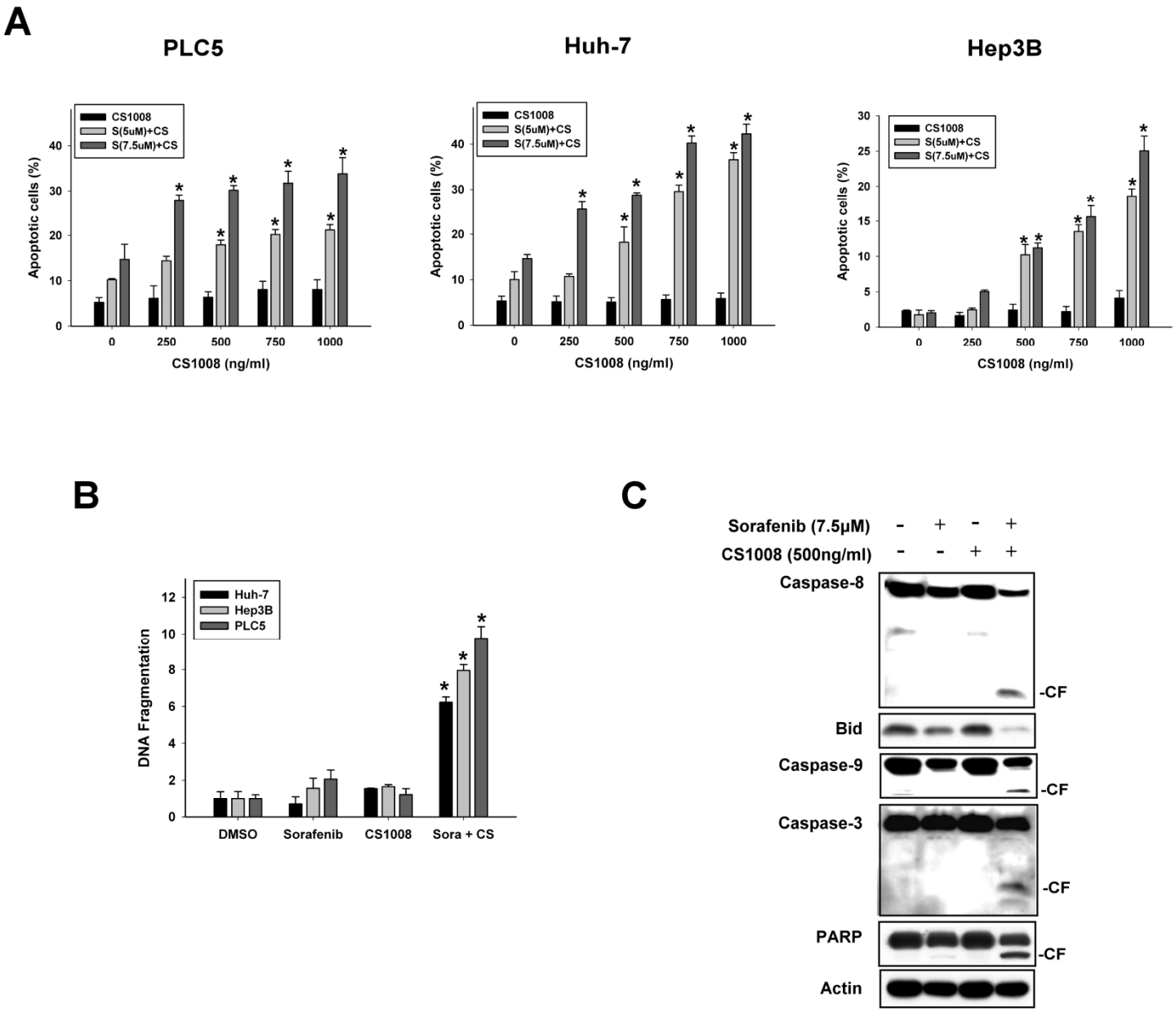

A
PLC5
Huh-7
Hep3B
CS1008
S(5uM)+CS
S(7.5uM)+CS
Apoptotic cells (%)
CS1008 (ng/ml)
0
250
500
750
1000
B
Huh-7
Hep3B
PLC5
DNA Fragmentation
DMSO
Sorafenib
CS1008
Sora + CS
C
Sorafenib (7.5μM)
CS1008 (500ng/ml)
Caspase-8
-CF
Bid
Caspase-9
Caspase-3
PARP
Actin

Fig. 2

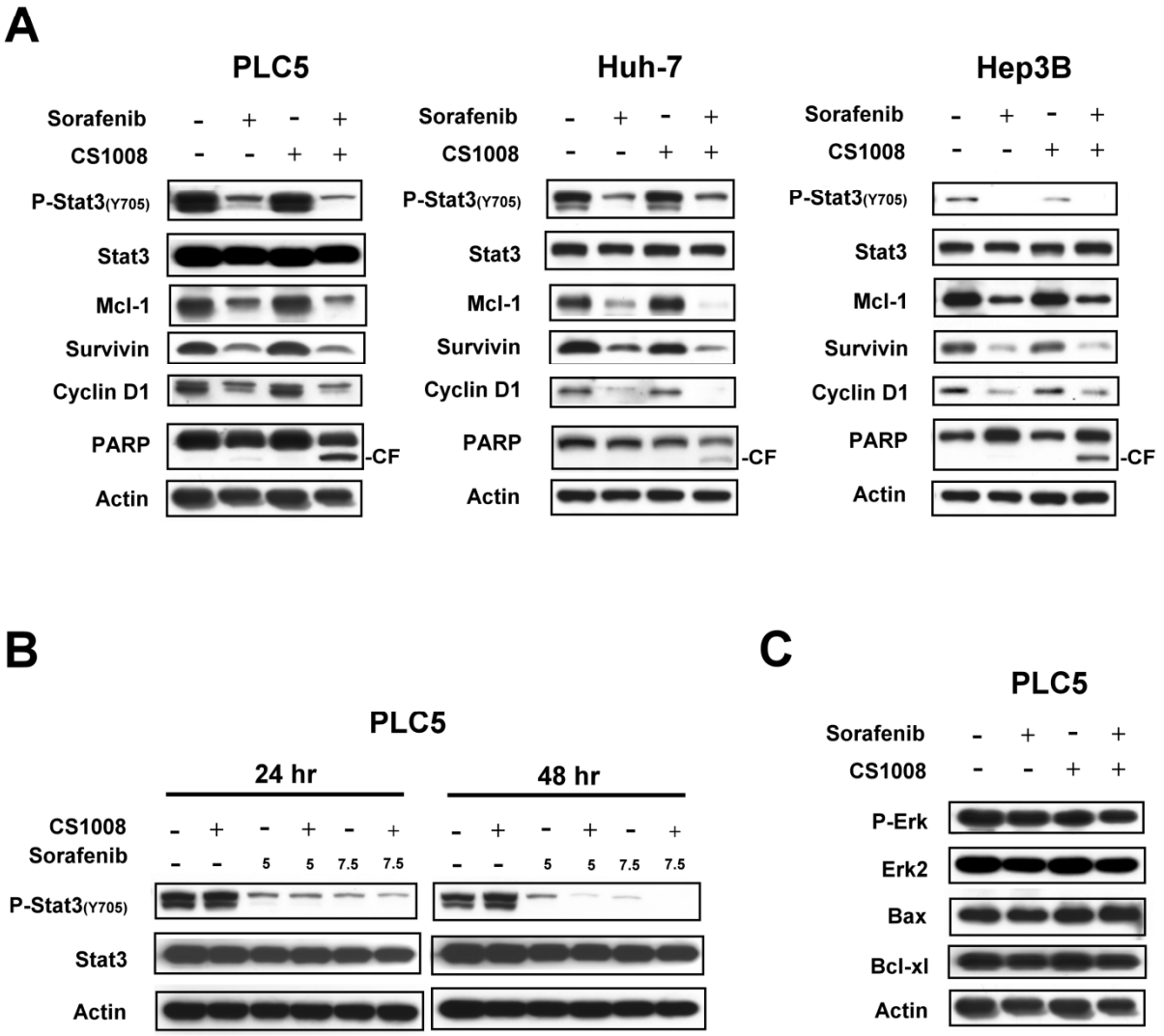

A
PLC5
Huh-7
Hep3B
Sorafenib
CS1008
P-Stat3(Y705)
Stat3
Mcl-1
Survivin
Cyclin D1
PARP
-CF
Actin
B
PLC5
24 hr
48 hr
CS1008
Sorafenib
5
7.5
P-Stat3(Y705)
Stat3
Actin
C
PLC5
Sorafenib
CS1008
P-Erk
Erk2
Bax
Bcl-xl
Actin

Fig. 3

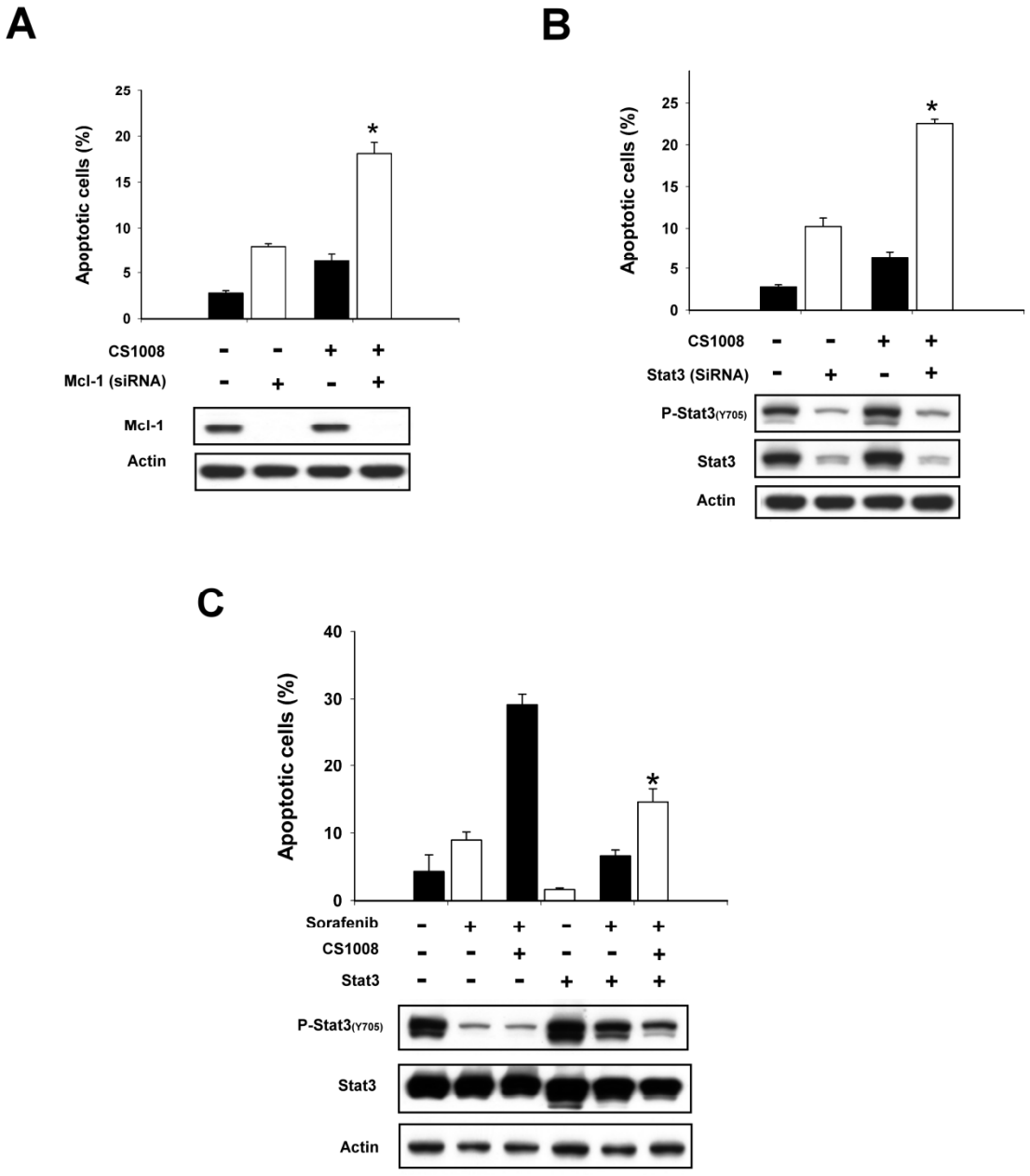

A
Apoptotic cells (%)
25
20
15
10
5
0
*
CS1008 - - + +
Mcl-1 (siRNA) - + - +
Mcl-1
Actin
B
Apoptotic cells (%)
25
20
15
10
5
0
*
CS1008 - - + +
Stat3 (SiRNA) - + - +
P-Stat3(Y705)
Stat3
Actin
C
Apoptotic cells (%)
40
30
20
10
0
*
Sorafenib - + + - + +
CS1008 - - + - - +
Stat3 - - - + + +
P-Stat3(Y705)
Stat3
Actin

Fig. 4

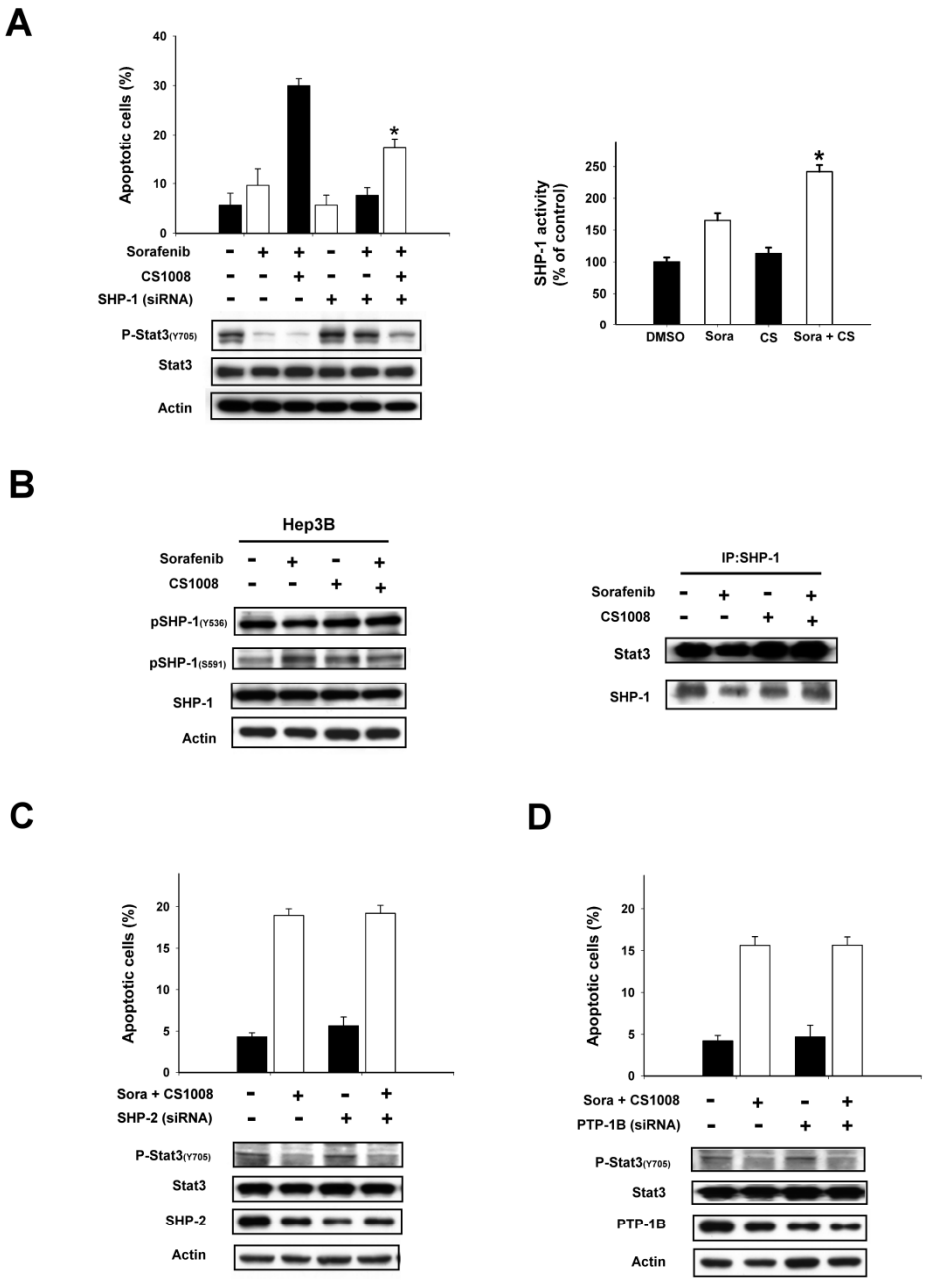
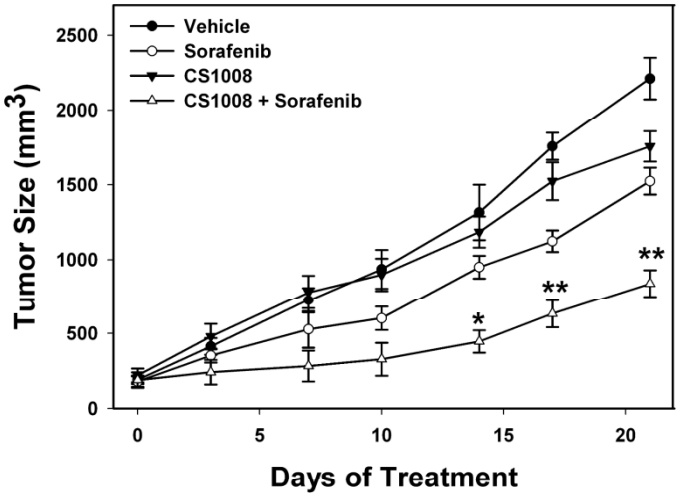
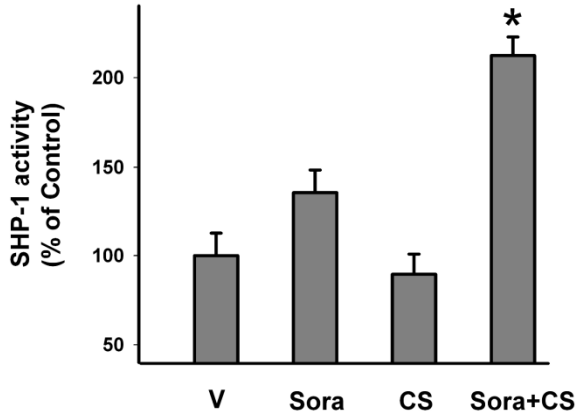

A
Apoptotic cells (%)
40
30
20
10
0
*
Sorafenib - + + - + +
CS1008 - - + - - +
SHP-1 (siRNA) - - - + + +
P-Stat3(Y705)
Stat3
Actin
SHP-1 activity (% of control)
250
200
150
100
50
0
*
DMSO
Sora
CS
Sora + CS
B
Hep3B
Sorafenib - + - +
CS1008 - - + +
pSHP-1(Y536)
pSHP-1(S591)
SHP-1
Actin
IP:SHP-1
Sorafenib - + - +
CS1008 - - + +
Stat3
SHP-1
C
Apoptotic cells (%)
20
15
10
5
0
Sora + CS1008 - + - +
SHP-2 (siRNA) - - + +
P-Stat3(Y705)
Stat3
SHP-2
Actin
D
Apoptotic cells (%)
20
15
10
5
0
Sora + CS1008 - + - +
PTP-1B (siRNA) - - + +
P-Stat3(Y705)
Stat3
PTP-1B
Actin

Fig. 5

A


Vehicle
Sorafenib
CS1008
CS1008 + Sorafenib
Tumor Size (mm3)
2500
2000
1500
1000
500
0
0
5
10
15
20
Days of Treatment
*
**
**

B


SHP-1 activity
(% of Control)
200
150
100
50
*
V
Sora
CS
Sora+CS