

Sorafenib Overcomes TRAIL Resistance of Hepatocellular Carcinoma Cells through the Inhibition of STAT3

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Abstract

Purpose: Recombinant tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) is a promising antitumor agent. However, many hepatocellular carcinoma (HCC) cells show resistance to TRAIL-induced apoptosis. Here, we report that sorafenib improves the antitumor effect of TRAIL-related agents in resistant HCC.

Experimental Design: HCC cell lines (PLC5, Huh-7, Hep3B, and Sk-Hep1) were treated with sorafenib and/or TRAIL-related agents (TRAIL or LBY135) and analyzed in terms of apoptosis and signal transduction. *In vivo* efficacy was determined in nude mice with PLC5 xenografts.

Results: Sorafenib, the only approved drug for HCC, sensitizes resistant HCC cells to an agonistic DR5 antibody (LBY135) and TRAIL-induced apoptosis in TRAIL-resistant HCC cells. We found that STAT3 played a significant role in mediating TRAIL sensitization. Our data showed that sorafenib downregulated phospho-STAT3 (pSTAT3) and subsequently reduced the expression levels of STAT3-related proteins (Mcl-1, survivin, and cyclin D1) in a dose- and time-dependent manner in TRAIL-treated HCC cells. Knockdown of STAT3 by RNA interference overcame apoptotic resistance to TRAIL in HCC cells, and ectopic expression of STAT3 in HCC cells abolished the TRAIL-sensitizing effect of sorafenib. Moreover, SHP-1 inhibitor reversed downregulation of pSTAT3 and apoptosis induced by sorafenib, and silencing of SHP-1 by RNA interference abolished the effects of sorafenib on pSTAT3. Notably, sorafenib increased SHP-1 activity in PLC5 cells. Finally, sorafenib plus LBY135 significantly suppressed PLC5 xenograft tumor growth.

Conclusions: Sorafenib sensitizes resistant HCC cells to TRAIL-induced apoptosis at clinically achievable concentrations, and this effect is mediated via the inhibition of STAT3. *Clin Cancer Res*; 16(21): 5189–99. ©2010 AACR.

Human hepatocellular carcinoma (HCC) is the fifth most prevalent solid tumor in the world and the fourth main inducer of cancer-related death (1). For patients with early HCC, surgery, local treatment, and liver transplantation may provide curative potential (2). However, many patients with advanced stage are not eligible for curative therapies. Importantly, previous clinical investigations have shown that traditional systemic chemotherapy could not provide survival benefits in patients with HCC (2). Recently, sorafenib (Nexavar), a multiple kinase inhibitor, has shown survival benefits in patients with advanced HCC in large

phase III studies and become the first clinically approved drug for HCC (3, 4). Sorafenib displays a remarkable inhibition of the serine-threonine kinase Raf of the Ras/Raf/mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) signaling pathway (5, 6) and also suppresses other receptor tyrosine kinases such as VEGFR2 (vascular endothelial growth factor receptor 2), VEGFR3, Flt-3, PDGFR (platelet-derived growth factor receptor), and FGFR-1 (fibroblast growth factor receptor-1; refs. 7, 8).

Tumor necrosis factor–related apoptosis-inducing ligand (TRAIL/Apo2L), a type II transmembrane protein from the tumor necrosis factor superfamily, has been shown to have a potent apoptosis effect suitable for cancer therapy (9–11). In addition to human recombinant TRAIL, monoclonal antibodies against death receptors have shown potential for cancer therapy. LBY135, a chimeric anti-DR5 agonistic antibody, is currently under clinical investigation (12). LBY135 binds to DR5 specifically with nanomolar affinity and mimics the ligand association for downstream apoptotic signaling. Although TRAIL selectively kills cancer cells and spares normal cells, some cancer cells are highly resistant to TRAIL-induced apoptosis. According to our previous study, we found that HCC cells were quite resistant to TRAIL and TRAIL alone was unable to induce apoptotic cell death even at the highest concentration of 1,000 ng/mL (13).

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Translational Relevance

Recombinant tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a promising antitumor agent. However, many hepatocellular carcinoma (HCC) cells show resistance to TRAIL-induced apoptosis. This current work shows that sorafenib, the only approved drug for the treatment of HCC, improves the antitumor effect of TRAIL and TRAIL-related antibody (LBY135) in TRAIL-resistant HCC. This study identifies the mechanism by which the combination of sorafenib and TRAIL overcomes the drug resistance in HCC and provides a mechanistic base for future clinical investigations.

Many factors may influence the sensitivity of these cancer cells to TRAIL. For example, overexpression of antiapoptotic proteins such as Mcl-1, a member of Bcl-2 family, may neutralize TRAIL-related signaling (14, 15). In addition, other reports have indicated that Mcl-1 is the critical gateway for TRAIL sensitization and Mcl-1 could confer TRAIL resistance by serving as a buffer for Bak, Bim, and Puma (16, 17).

STAT3 plays a critical role in transcriptional regulation of genes that are involved in cell proliferation and survival. STAT3 could be activated by tyrosine phosphorylation responding to stimulation by cytokines such as interleukin-6 and growth factors including epidermal growth factor receptor (EGFR), FGFR, and PDGFR (18). On tyrosine phosphorylation, STAT3 homodimerizes or heterodimerizes with STAT1, then translocates to the nucleus, and binds to consensus DNA sequences within promoters of its target genes. STAT3 regulates expression of numerous apoptosis-related proteins, including Bcl-2, Bcl-xL, Mcl-1, survivin, and cyclin D1. Notably, STAT3 signaling is down-regulated by protein tyrosine phosphatases, such as the SH2 domain-containing tyrosine phosphatase family (SHP-1 and SHP-2), and protein tyrosine phosphatase 1B (PTP-1B; ref. 19). These phosphatases reduce STAT3 activation directly by dephosphorylation of STAT3.

In this study, we show that sorafenib sensitizes resistant HCC cells to TRAIL-induced apoptosis through SHP-1-dependent STAT3 inhibition. The molecular mechanism by which sorafenib overcomes TRAIL resistance is unraveled, and STAT3 is identified as a novel target of sorafenib in HCC.

Materials and Methods

Reagents and antibodies

Sorafenib (Nexavar) and LBY135 were kindly provided by Bayer Pharmaceuticals and Novartis Pharmaceuticals, respectively. For *in vitro* studies, sorafenib at various concentrations was dissolved in DMSO and then added to the cells in 5% fetal bovine serum (FBS)-containing DMEM.

Recombinant TRAIL was purchased from Biomol. Vanadate and SHP-1 inhibitor were purchased from Cayman Chemical. Antibodies for immunoblotting such as Akt1, Bad, Bax, Mcl-1, and poly(ADP-ribose) polymerase (PARP) were purchased from Santa Cruz Biotechnology. Other antibodies such as anti-phospho-ERK1/2 (pERK1/2), ERK2, survivin, cyclin D1, Bcl-2, Bcl-xL, Bid, caspase-3, caspase-8, caspase-9, FADD (Fas-associated protein with death domain), c-FLIP (cellular FLICE-inhibitory protein), phospho-STAT3 (pSTAT3; Tyr⁷⁰⁵), STAT3, and phospho-Akt (pAkt; Ser⁴⁷³) were from Cell Signaling. DR4 and DR5 were from BioLegend (flow cytometry) and Diaclone (Western blot).

Cell culture

The Huh-7 HCC cell line was obtained from the Health Science Research Resources Bank (JCRB0403). The PLC/PRF/5 (PLC5), Sk-Hep1, and Hep3B cells were obtained from the American Type Culture Collection. HCC 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 under an atmosphere of 5% CO₂ in air (20).

Cell viability analysis

The effect of individual test agents on cell viability was assessed by using the MTT assay in six replicates. Cells (5,000 per well) were seeded and incubated in 96-well, flat-bottomed plates in DMEM with 10% FBS for 24 hours and were exposed to various concentrations of test agents in 5% FBS-supplemented DMEM for 24 hours. Controls received DMSO vehicle at a concentration equal to that of drug-treated cells. The medium was removed and replaced by 200 µL of 0.5 mg/mL MTT in 10% FBS-containing DMEM, and cells were incubated in the CO₂ incubator at 37°C for 2 hours. Supernatants were removed from the wells, and the reduced MTT dye was dissolved in 200 µL/well DMSO. Absorbance at 570 nm was determined on a plate reader.

Apoptosis analysis

The following three methods were used to assess drug-induced apoptotic cell death: the measurement of apoptotic cells by flow cytometry (sub-G₁) and Western blot analysis of caspase activation and PARP cleavage.

Detection of surface TRAIL receptors

HCC cells were exposed to 7.5 µmol/L sorafenib for 24 hours and then incubated with DR4 or DR5 antibodies for 30 minutes. Antibodies were purchased from BioLegend. The procedure has been described previously (13).

Gene knockdown using small interfering RNA

SMARTpool small interfering RNAs (siRNA), including control, survivin, Mcl-1, STAT3, SHP-1, SHP-2, and PTP-1B, were all purchased from Dharmacon, Inc. The procedure has been described previously (13).

Transient transfection

The survivin and STAT3 plasmid DNA used in this study were obtained from OriGene and Addgene plasmid repository (<http://www.addgene.org/>), respectively. For transient transfection, 5×10^5 cells were seeded into 6-cm plates and transiently transfected with 3 μ g of survivin or STAT3 plasmids using Lipofectamine 2000 transfection reagent (Invitrogen).

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 hours at 4°C with rotation. A RediPlate 96 EnzChek Tyrosine Phosphatase Assay kit 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 done 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). When tumors reached 200 to 300 mm³, mice received an i.v. injection of LBY135 (200 μ g) three times a week, sorafenib tosylate (5 mg/kg) orally once daily, or the combination of LBY135 and sorafenib. Tumors were measured weekly using calipers, and their volumes were calculated using the following standard formula: width² \times length \times 0.52 (20).

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue sections were deparaffinized in xylene and rehydrated in a series of ethanol solutions with decreasing concentrations and finally in PBS. Antigen retrieval was done in 0.01 mol/L sodium citrate buffer (pH 6.0) at 100°C for 15 minutes. Frozen tissues were cut into 7- μ m sections and fixed in 4% paraformaldehyde for 15 minutes. Both paraffin-embedded tissue and cryostat sections were then treated with 3% hydrogen peroxide for 10 minutes, blocked with 3% bovine serum albumin (Sigma) and 10% FBS (Biological Industries) for 90 minutes, and incubated with rabbit monoclonal anti-human STAT3 (1:100) or anti-pSTAT3 (1:50) antibodies (Cell Signaling) overnight at 4°C. On the next day, the EnVision system (DAKO) and 3,3'-diaminobenzidine (Sigma) were used to detect specific signals. All sections were counterstained with hematoxylin (Merck). All pathologic samples of the patients in this study were obtained following written informed consent from patients. The Ethical Committee of the National Taiwan University Hospital has approved the protocol of this study.

Statistical analysis

Comparisons of mean values were done using the independent sample *t* test in SPSS for Windows 11.5 software (SPSS, Inc.; ref. 20).

Results

Sorafenib enhances TRAIL-induced apoptosis in resistant HCC cells

To investigate combinational effects of sorafenib and TRAIL on HCC cells, we first examined the effects of both drugs on cell viability in a panel of four human HCC cell lines (Hep3B, PLC5, Huh-7, and Sk-Hep1) at clinically relevant concentrations. Cell viability was measured by MTT assay. Our data showed that the combination of TRAIL and sorafenib enhanced the antitumor effects of sorafenib in a dose-dependent manner (Fig. 1A). We then examined the sensitizing effect of sorafenib on TRAIL-induced apoptosis in HCC cells. Apoptotic cells (sub-G₁) were determined by flow cytometry after 24 hours of treatment. Our previous results indicated that HCC cells were quite resistant to TRAIL and TRAIL alone was unable to induce apoptosis in any of the four types of cells even at the highest concentration of 1,000 ng/mL (13). However, combining sorafenib at 7.5 μ mol/L with TRAIL reversed the resistance in all four cell lines and induced significant apoptosis in a dose-dependent manner starting from TRAIL at the concentration of 25 ng/mL (Fig. 1B). In addition, we further examined whether sorafenib sensitized LBY135, an agonistic DR5 antibody, in HCC cells. We found that these HCC cells were also resistant to LBY135 and that combinations of LBY135 (500 ng/mL) and sorafenib (7.5 μ mol/L) enhanced LBY135-induced apoptosis significantly in all tested HCC cells, suggesting that synergistic interactions between sorafenib and TRAIL-related agents did exist in HCC cells (Fig. 1C).

To examine the apoptotic pathways, we did a dose-escalation analysis of sorafenib on TRAIL-induced apoptosis in Hep3B cells and found that a combination of sorafenib and TRAIL induced activation of caspase-8 and was followed by the cleavage of Bid and activation of caspase-9, caspase-3, and PARP cleavage in a dose-dependent manner. Our data showed that a combination of TRAIL (100 ng/mL) and sorafenib (7.5 μ mol/L) induced apoptosis in association with the cleavage of Bid and activation of caspase-9. These results indicate the importance of the intrinsic mitochondrial pathway in TRAIL-induced apoptosis (Fig. 1D).

Downregulation of pSTAT3 is associated with sensitizing effects of sorafenib in HCC cells

Previous reports have suggested that the sensitizing effect of sorafenib was mediated through affecting Mcl-1, an antiapoptotic Bcl-2 family protein (15, 16). Given the fact that STAT3 plays a significant role in regulating the expression of Mcl-1, we next investigated its related proteins, including pSTAT3 and STAT3, and STAT3-related proteins, including Mcl-1, survivin, and cyclin D1. We found that

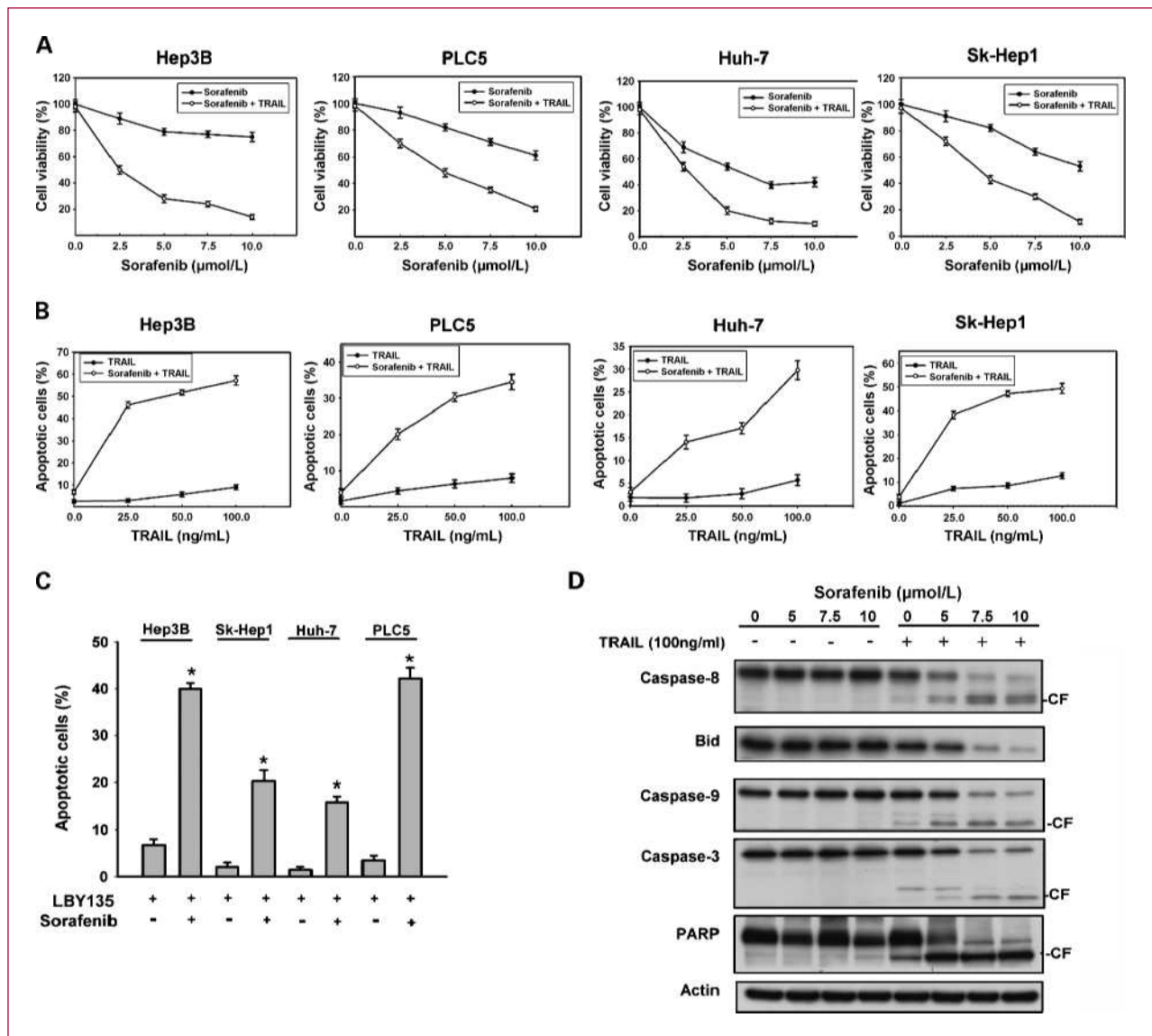


Fig. 1. Sorafenib enhances TRAIL-induced apoptosis in resistant HCC cells. **A**, dose-escalation effects of sorafenib with 100 ng/mL TRAIL on cell viability in four TRAIL-resistant HCC cells. HCC cells were exposed to drugs for 24 h and analyzed by MTT. Points, mean ($n = 6$); bars, SD. **B**, dose-escalation effects of TRAIL with 7.5 $\mu\text{mol/L}$ sorafenib on apoptosis in four TRAIL-resistant HCC cells. Cells were exposed to drugs for 24 h, and apoptotic cells were analyzed by flow cytometry. **C**, effects of 500 ng/mL LBY135 and 7.5 $\mu\text{mol/L}$ sorafenib on apoptosis in four TRAIL-resistant HCC cells. **D**, dose-dependent effects of sorafenib on TRAIL-induced apoptosis in Hep3B cells. Cells were exposed to drugs for 24 h. CF, cleaved form (activated form).

downregulation of pSTAT3 was associated with the sensitizing effect of sorafenib on TRAIL-induced apoptosis. As shown in Fig. 2A, sorafenib downregulated pSTAT3 (Tyr⁷⁰⁵) and related proteins, Mcl-1, survivin, and cyclin D1, in all types of HCC cells, whereas protein levels of total STAT3 were not altered (Fig. 2A). Furthermore, downregulation of pSTAT3 was associated with the cleavage of PARP as shown by evidence of apoptosis induction in cells exposed to sorafenib and TRAIL for 24 hours (Fig. 2A). Moreover, our data showed that sorafenib downregulated pSTAT3 in PLC5 cells in both dose- and time-dependent manners (Fig. 2B). In addition, both sorafenib alone and

with cotreatment with LBY135 showed downregulations of pSTAT3 and related proteins, including Mcl-1, survivin, and cyclin D1, in Sk-Hep1 cells (Fig. 2C), suggesting that inhibition of STAT3 may mediate sensitizing effects of sorafenib on TRAIL-related agents.

About other apoptosis-related molecules, protein levels of pAkt, Akt, Bcl-2, and Bcl-xL were not changed significantly in cells exposed to sorafenib. In comparison with pSTAT3, protein levels of proapoptotic proteins, including Bax and Bad, were not altered significantly in HCC cells after treatment with sorafenib (Fig. 2D, left). Notably, sorafenib did not downregulate pERK significantly, suggesting

that the effect of sorafenib on Raf-1 may not be important to its synergy with TRAIL (Fig. 2D, right).

No significant effects of sorafenib on TRAIL receptors

To understand the effect of sorafenib on TRAIL-induced apoptosis, we next examined TRAIL receptors and TRAIL DISC proteins, including FADD and c-FLIP, in sorafenib-treated cells. We examined the protein levels of DR4, DR5, FADD, and c-FLIP in HCC cells. Cells were exposed to sorafenib at various doses for 24 hours and analyzed by Western blot. We found that sorafenib did not alter the expression of these related proteins significantly in PLC5 and Sk-Hep1 cells (Fig. 3A). Moreover, FADD plays a key role in DISC formation and mediates TRAIL-induced apoptosis. We found that the expression of FADD was not much changed in the presence of sorafenib in HCC cells. Notably, the previous report has suggested that c-FLIP may play an important role in mediating the effect of sorafenib on TRAIL-induced apoptosis (21). In this regard, we found that sorafenib did not alter protein levels of c-FLIP (two isoforms) in HCC cells (Fig. 3A). In

addition, cotreatment of sorafenib and TRAIL did not affect protein levels of TRAIL-related molecules in PLC5 cells (Fig. 3B). Next, we examined the surface expression of TRAIL receptors in HCC cells. Cells were exposed to sorafenib at 7.5 $\mu\text{mol/L}$ for 24 hours and then harvested for analysis of surface expression of TRAIL receptors by immunofluorescent staining and subsequent flow cytometry. As shown in Fig. 3C, treatment of sorafenib did not change surface expressions of DR4 and DR5 significantly in any of the four types of HCC cells (Fig. 3C). These results suggest that sorafenib did not synergize TRAIL in HCC cells by affecting TRAIL receptors or DISC components.

Validation of STAT3

Several approaches were used to validate the inhibition of STAT3 signals responsible for the TRAIL-sensitizing effect of sorafenib on apoptosis in HCC cells. First, we knocked down protein expression of survivin and STAT3 by using siRNA. PLC5 cells were transfected with either control or survivin siRNA or STAT3 siRNA for 48 hours and then exposed to DMSO or TRAIL at the

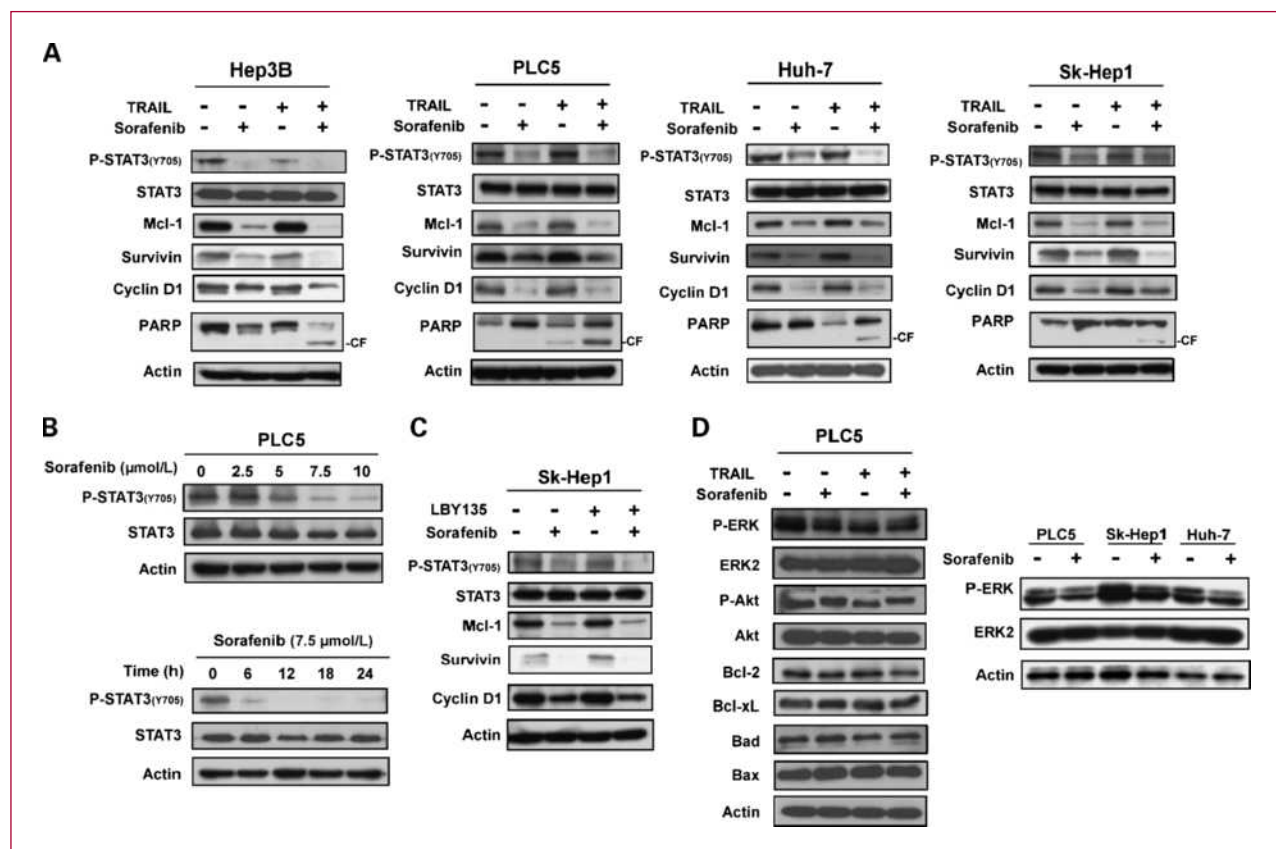


Fig. 2. Downregulation of pSTAT3 is associated with sensitizing effects of sorafenib in HCC cells. **A**, effects of sorafenib (7.5 $\mu\text{mol/L}$) and/or TRAIL (100 ng/mL) on STAT3-related proteins. **B**, top, dose-escalation effects of sorafenib on pSTAT3 in PLC5 cells; bottom, time-dependent analysis of sorafenib on pSTAT3 in PLC5 cells. PLC5 cells were exposed to the indicated drugs for 24 h (top) or the indicated time (bottom). **C**, effects of sorafenib and/or LBY135 on STAT3-related proteins. PLC5 cells were exposed to the indicated drugs for 24 h. **D**, left, effects of sorafenib (7.5 $\mu\text{mol/L}$) and/or TRAIL (100 ng/mL) on apoptosis-related proteins. PLC5 cells were exposed to the indicated drugs for 24 h. Right, effect of sorafenib (7.5 $\mu\text{mol/L}$) on pERK and ERK2. Cells were treated with sorafenib for 24 h.

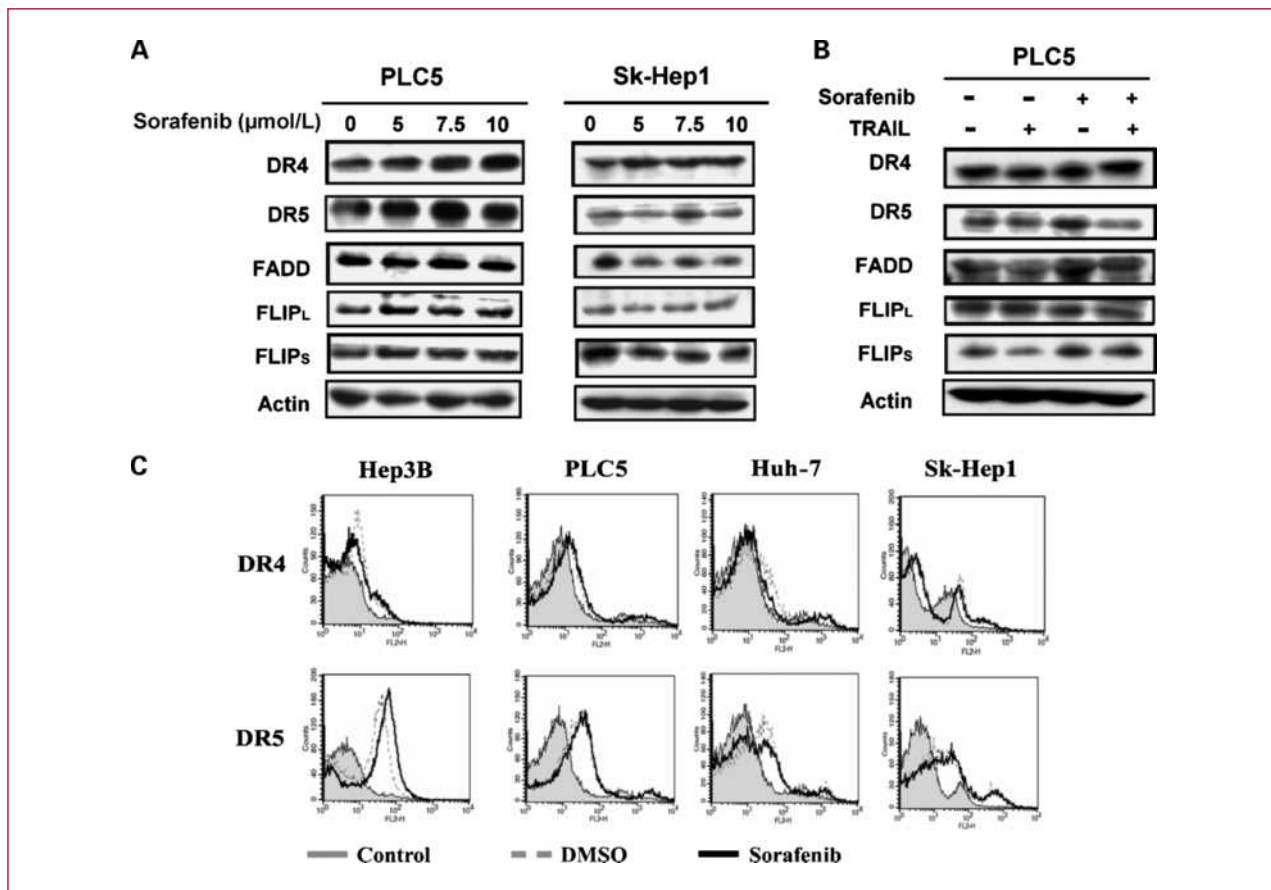


Fig. 3. No significant effects of sorafenib on TRAIL receptors. A, dose-dependent analysis of protein levels of TRAIL receptors and related components in HCC cells. Cells were exposed to sorafenib at the indicated concentrations for 24 h. B, analysis of protein levels of TRAIL receptor and related components in PLC5 cells. Cells were exposed to 7.5 $\mu\text{mol/L}$ sorafenib and/or 100 ng/mL TRAIL for 24 h. C, cell surface distributions of DR4 and DR5 in the presence of sorafenib. Cells were exposed to sorafenib at 7.5 $\mu\text{mol/L}$ for 24 h and then harvested for analysis.

indicated doses for another 24 hours. Our data showed that silencing STAT3 and survivin sensitized PLC5 cells to TRAIL-induced apoptosis significantly ($P < 0.05$; Fig. 4A and B), indicating that inhibition of STAT3 signaling pathway was critical to TRAIL sensitivity in HCC cells. Notably, downregulation of Mcl-1 also increased TRAIL-induced apoptosis in PLC5 cells, indicating that Mcl-1 plays a role in regulating the effects of TRAIL in HCC cells (Fig. 4A, right). Next, we upregulated expressions of survivin and STAT3 by transfections of both proteins transiently to investigate the TRAIL-sensitizing effect of sorafenib. As shown in Fig. 4C and D, overexpressions of survivin or STAT3 abolished the TRAIL-sensitizing effect of sorafenib on apoptosis with statistical significance ($P < 0.05$) as evidenced by activation of caspase-9. Together, these results validated the importance of STAT3 inhibition in mediating the effect of sorafenib on TRAIL sensitivity.

Inhibition of SHP-1 reverses the effects of sorafenib on pSTAT3 and TRAIL-induced apoptosis

To answer the question of how sorafenib downregulates pSTAT3 in HCC cells, we investigated the role of protein

phosphatase on the effect of sorafenib on pSTAT3. Our data showed that vanadate, a nonspecific protein tyrosine phosphatase inhibitor, reversed the downregulation of pSTAT3 in PLC5 cells after treatment with sorafenib (Fig. 5A, left), suggesting that sorafenib may downregulate pSTAT3 via affecting protein tyrosine phosphatase. We then used the specific SHP-1 inhibitor to examine the role of SHP-1 in the effects of sorafenib on pSTAT3. Our data showed that SHP-1 inhibitor reversed the effects of sorafenib on pSTAT3 (Fig. 5A, middle). Next, we knocked down SHP-1 by siRNA in PLC5 cells and found that silencing SHP-1 abolished the effects of sorafenib on pSTAT3 (Fig. 5A, right), suggesting that sorafenib may downregulate pSTAT3 through influencing SHP-1. Notably, sorafenib did not affect the expression level of SHP-1 in HCC cells. In addition, the effect of sorafenib on SHP-1 phosphatase activity in PLC5 cells was measured. As shown in Fig. 5B (left), sorafenib elevated SHP-1 activity ($P < 0.05$). Moreover, we examined whether sorafenib upregulated SHP-1 activity by affecting the phosphorylation of SHP-1, as sorafenib is a kinase inhibitor. According to the previous report, phosphorylation of SHP-1 at Tyr⁵³⁶ may upregulate

its activity (22). Contrarily, phosphorylation of SHP-1 at Ser⁵⁹¹ might downregulate its activity (23). Our data showed that neither sorafenib alone nor with cotreatment with TRAIL altered phospho-SHP-1 (pSHP-1) at both sites (Fig. 5B, middle and right). Previous study has shown that other protein tyrosine phosphatases such as SHP-2 and PTP-1B may also regulate STAT3 signaling pathway. We then examined their roles in sorafenib-treated cells. We found that silencing SHP-2 did not influence the effects of sorafenib on pSTAT3, and neither did knockdown of PTP-1B in HCC cells (Fig. 5C and D, left), indicating that SHP-2 and PTP-1B are not crucial for mediating the effect

of sorafenib on pSTAT3. Notably, the interaction between STAT3 and SHP-1 was assessed by coimmunoprecipitation in drug-treated cells. Sorafenib did not alter the interaction between SHP-1 protein and STAT3 protein, suggesting that the effect is not mediated through protein interactions (data not shown). Furthermore, suppressors of cytokine signaling such as SOCS-1 and SOCS-3 play a key role in regulation of SHP-1 (24–27). SOCS-1 interacts with Janus-activated kinase (JAK) to suppress cytokine signaling (28, 29). The high prevalence of aberrant SOCS-1 hypermethylation is implicated in constitutive activation of JAK/STAT pathway to

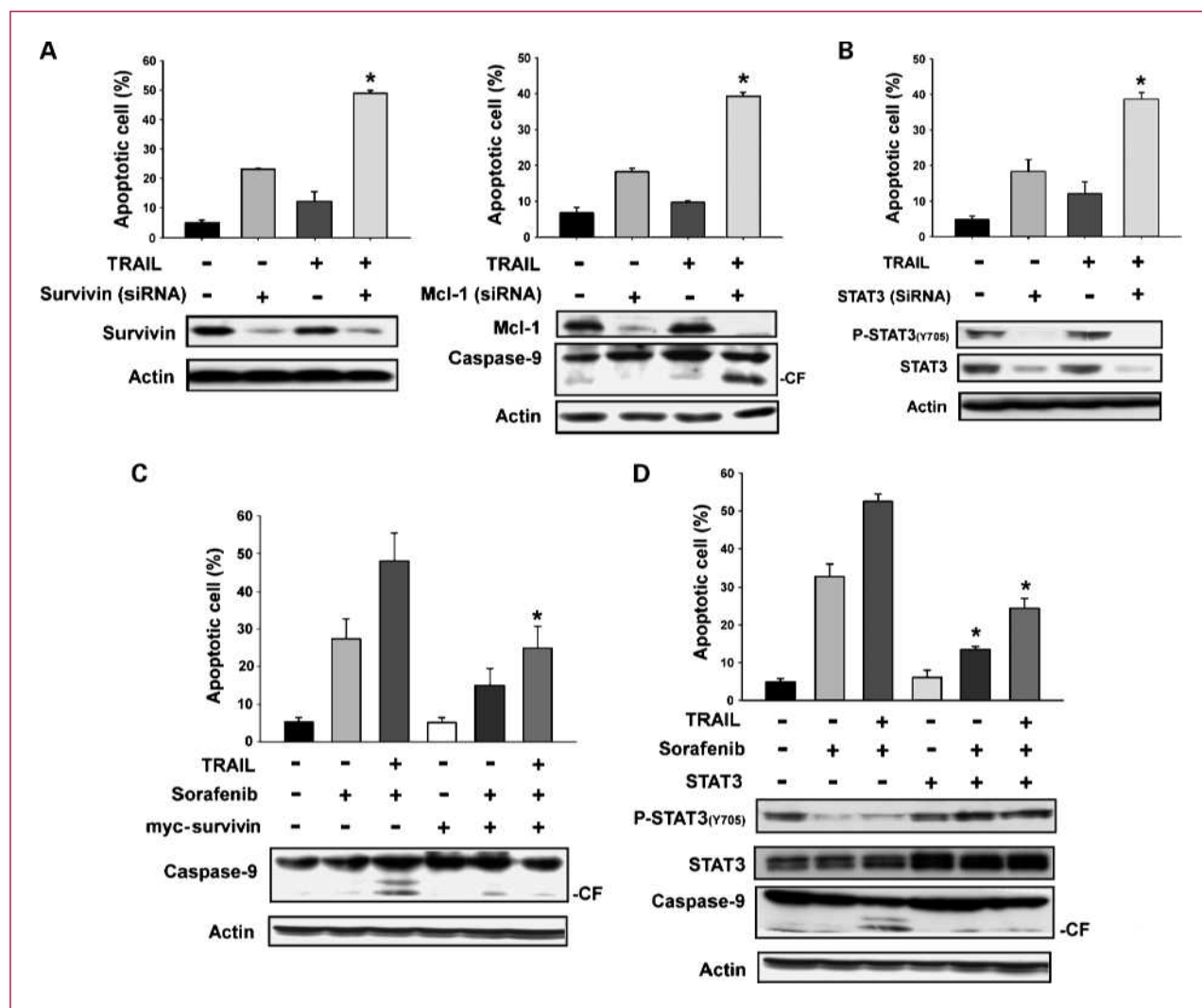


Fig. 4. Validation of STAT3. A, left, downregulation of survivin by siRNA overcomes the resistance to TRAIL in PLC5 cells; right, downregulation of survivin by siRNA increased TRAIL-induced apoptosis in PLC5 cells. B, downregulation of pSTAT3 by STAT3 siRNA overcomes the resistance to TRAIL in PLC5 cells. Cells were transfected with either control siRNA, survivin siRNA, or STAT3 siRNA for 48 h and then exposed to 100 ng/mL TRAIL for 24 h. C, protective effects of survivin on apoptosis induced by the combination of TRAIL and sorafenib in PLC5 cells. D, protective effects of STAT3 on apoptosis induced by the combination of TRAIL and sorafenib in PLC5 cells. Columns, mean ($n = 3$); bars, SD. *, $P < 0.05$. Cells were transfected with myc-survivin or STAT3 transiently and were exposed to the combination of 7.5 $\mu\text{mol/L}$ sorafenib and 100 ng/mL TRAIL for 24 h. Apoptotic cells were analyzed by flow cytometry. Columns, mean ($n = 3$); bars, SD. *, $P < 0.05$.

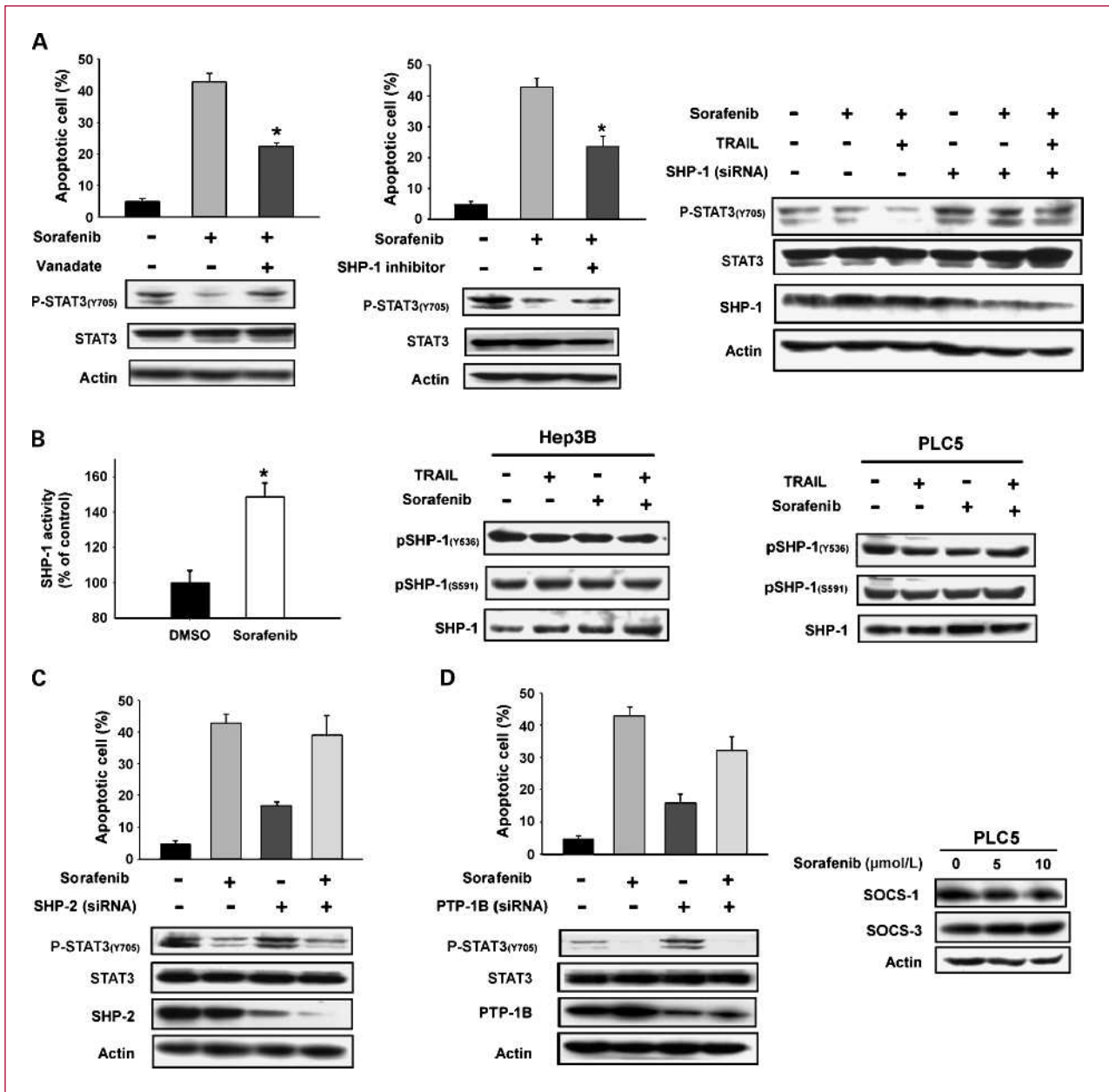


Fig. 5. Inhibition of SHP-1 reverses the effects of sorafenib on pSTAT3 and apoptosis. **A**, left, vanadate, a nonspecific SHP-1 inhibitor; middle, specific SHP-1 inhibitor. Both inhibitors abolish downregulation of pSTAT3 in sorafenib-treated cells. Columns, mean ($n = 3$); bars, SD. *, $P < 0.05$. Right, silencing SHP-1 by siRNA reduces the effects of sorafenib on pSTAT3 in HCC cells. PLC5 cells were transfected with control siRNA or SHP-1 siRNA for 48 h and then treated with sorafenib and/or TRAIL for 24 h. **B**, sorafenib enhances the activity of SHP-1 in PLC5 cells. Left, activity of SHP-1; middle and right, effects of sorafenib on pSHP-1 in HCC cells. Cells were treated with drugs for 24 h. **C**, knockdown of SHP-2 does not affect the effects of sorafenib on pSTAT3. **D**, left, knockdown of PTP-1B does not affect the effects of sorafenib on pSTAT3. PLC5 cells were transfected with control siRNA, SHP-2 siRNA, or PTP-1B siRNA for 48 h and then treated with sorafenib for 24 h. Right, effects of sorafenib on the expression of SOCS-1 and SOCS-3 in PLC5 cells. Cells were treated with sorafenib at the indicated doses for 24 h.

promote HCC oncogenesis (30). We examined the effects of sorafenib on the expression of SOCS-1 and SOCS-3. Our data showed that sorafenib did not alter the protein level of SOCS-1 and SOCS-3 significantly in PLC5 cells, suggesting that the SOCS family may not mediate the effect of sorafenib on SHP-1.

Effect of sorafenib and LBY135 on PLC5 xenograft tumor growth *in vivo*

To confirm whether the synergistic effect of sorafenib and TRAIL-related agents in resistant cell lines has potentially relevant clinical implications, we assessed the *in vivo* effect of sorafenib plus LBY135 on the growth of HCC

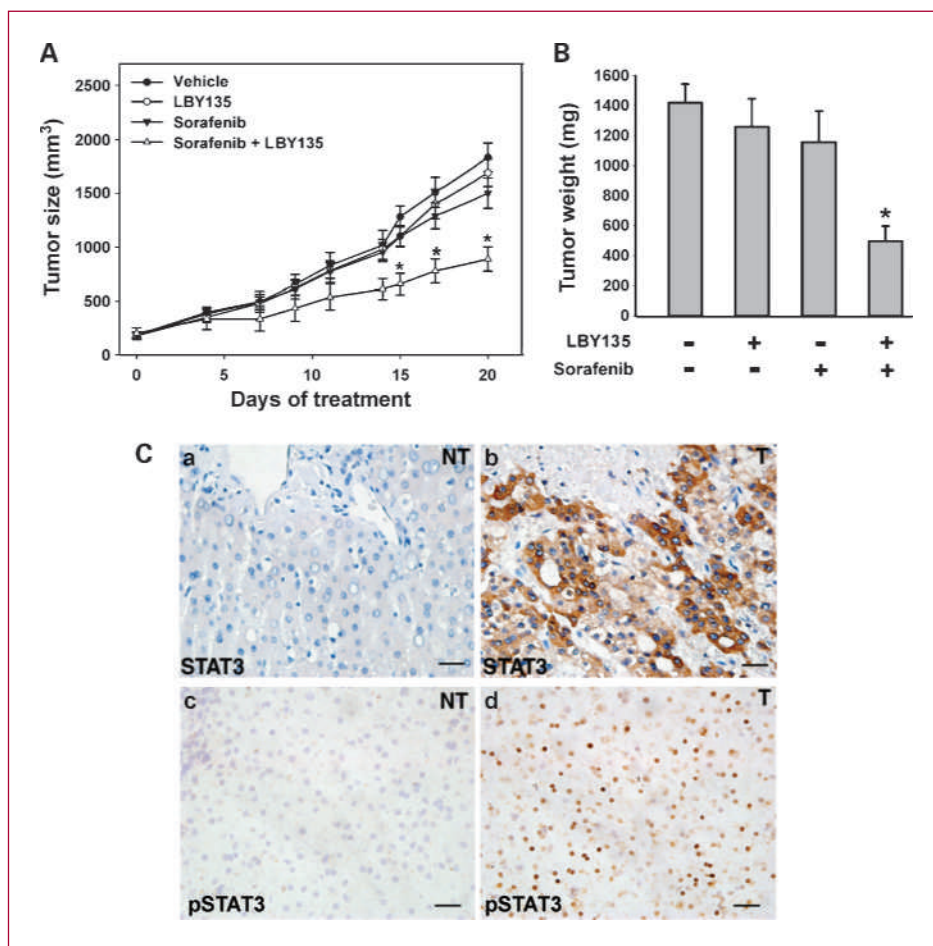
xenograft tumors. Several studies including ours indicated that sorafenib showed good antitumor activity in PLC5 xenograft (6, 31). In addition, our *in vitro* data indicated that sorafenib downregulated pSTAT3 in PLC5 cells significantly (Fig. 2B). Therefore, we used PLC5 cells to test our hypothesis *in vivo*. According to Liu et al.'s study (6), sorafenib inhibited PLC5 tumor growth significantly at the dose of 10 mg/kg/d. As we have shown that the sub-effective dose of sorafenib (5 mg/kg/d) enhanced the effects of bortezomib on PLC5 tumor growth previously (31), we then decreased the dose to 5 mg/kg/d to test the combinational effect with LBY135. As sorafenib shows significant dose-related adverse effects in patients with higher doses, lower dose of sorafenib might be more feasible in the combinational therapy. Tumor-bearing mice were treated with vehicle or sorafenib orally at 5 mg/kg/d or LBY135 i.v. at 200 μ g three times a week for 3 weeks. 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.

As shown in Fig. 6A, tumor growth was significantly inhibited by treatment with sorafenib plus LBY135 for

2 weeks (versus control, $P < 0.05$), and tumor size in the treatment group was only half that of the control group at the end of the study. Treatment with sorafenib or LBY135 had no significant effect on PLC5 tumor growth. Tumor weight was measured at the end of the study, and our data showed that cotreatment of sorafenib and LBY135 reduced tumor weight significantly (versus control, $P < 0.05$; Fig. 6B). Together, these data indicate that combinations of sorafenib and LBY135 exhibited better antitumor activity *in vivo*. Further clinical investigation is warranted.

To examine the status of STAT3 activation in human, we did the analysis of immunohistochemistry for STAT3 and pSTAT3 (Tyr⁷⁰⁵, activated STAT3) on HCC tissues and normal liver tissues to determine whether STAT3 expression is related to HCC. We stained 29 pairs of HCCs and the nontumor part. We found that 72.4% (21 of 29) of our samples showed overexpression of STAT3 in the tumor part compared with the nontumor part in terms of the combinatory scoring of the percentage of STAT3-positive cells and the staining intensity. In these 21 cases, we detected nuclear staining pattern of STAT3 in the majority of cells, which was suggestive of active STAT3, in 14 cases (48.3%). Our data indicated that STAT3 and pSTAT3 ex-

Fig. 6. *In vivo* effect of sorafenib and LBY135 on PLC5 xenograft nude mice. A, the combination of sorafenib and LBY135 shows significant antitumor effect on PLC5 tumors. B, analysis of tumor weight. Columns, mean ($n = 6$); bars, SE. *, $P < 0.05$ versus vehicle group. C, immunohistochemistry showed overexpression of STAT3 in HCC tissues. a, nontumor liver tissues were negative for STAT3. b, HCC tissues in the same patient as (a) were strongly positive for STAT3. Both (a) and (b) were paraffin-embedded tissues. c, nontumor liver tissues were negative for pSTAT3. d, positive staining (brown) for pSTAT3 was found in nuclei of HCC tumor cells in the same patient as (c). Both (c) and (d) were cryostat sections. NT, nontumor; T, tumor. Scale bar, 20 μ m.



pression was significantly increased in HCC tissues when compared with normal liver samples (Fig. 6C), which is consistent with the previous report (32). Our data suggest that the activation of STAT3 signaling pathway is present in human HCC.

Discussion

Several previous studies have shown the cytotoxic synergy between sorafenib and TRAIL in various types of cancer cells (15, 16, 21). According to these reports, the inhibition of Mcl-1 is a target of sorafenib on TRAIL sensitivity. Consistent with previous data, we further showed that sorafenib suppressed not only Mcl-1 but also other STAT3-related proteins, survivin and cyclin D1, in HCC. Our data confirm that the inhibition of STAT3 represents the major mechanism for the sensitizing effect of sorafenib on TRAIL in HCC. Notably, Yang et al. (33) reported that sorafenib inhibits STAT3 signaling in medulloblastomas cells, consistent with our current findings in HCC cells. In addition to Yang et al.'s report, a recent study from Blechacz et al. (34) suggested that sorafenib inhibited STAT3 by influencing SHP-2 through downregulation of pSHP-2 in cholangiocarcinoma. However, Blechacz et al. did not show the data of SHP-1 in their report. In this regard, our present study indicated that knockdown of SHP-2 did not alter the effects of sorafenib on apoptosis and STAT3 phosphorylation in HCC cells (Fig. 5C), and our data showed that SHP-1, but not SHP-2, is the primary target of sorafenib in HCC (Fig. 5A and B). Although it is still unclear why sorafenib affected different protein phosphatase among various types of cancer, our study provides the important information that SHP-1, but not SHP-2, is the target for HCC, which might be crucial clinically. Certainly, more efforts need to be done to understand the reason why sorafenib affect different protein tyrosine phosphatases among HCC and cholangiocarcinoma.

Previous reports have suggested that STAT3 would act as a therapeutic target for tumor suppression. Kusaba and colleagues (35) showed that Janus kinase 2 inhibitor, AG490, could abrogate the activation of constitutive STAT3 to sensitize human HCC cells to TRAIL-mediated apoptosis in Huh-1, Huh-7, and HepG2, indicating that the inhibition of STAT3 confers TRAIL sensitivity in HCC. Several studies have also shown that tumors with

JAK/STAT3 signaling activation become more aggressive and are associated with poor prognosis in HCC patients, suggesting that STAT3 inhibitors may serve as therapeutic modality for human hepatocellular cancer. Several chemicals have shown antitumor activity; for example, benzyl isothiocyanate induces apoptosis in some types of pancreatic cancer cells through reduced expression of activated/total STAT3 proteins and STAT3-DNA binding affinity (36).

Several reports have shown that compounds may inhibit STAT3 by the induction of SHP-1. For example, acety-11-keto- β -boswellic acid and butein (3,4,2',4'-tetrahydrochalcone) exhibited tumor inhibition through downregulation of STAT3 in myeloma cells via the induction of SHP-1 (37, 38). Unlike previous studies, our data indicate that sorafenib increased SHP-1 activity without affecting expression levels of SHP-1. Moreover, phosphorylated forms of SHP-1 (Tyr⁵³⁶ and Ser⁵⁹¹) were not affected in sorafenib-treated cells, indicating that sorafenib may not upregulate SHP-1 activity by affecting its phosphorylation. However, the mechanism by which sorafenib influences SHP-1 activity remains unclear and needs to be further studied.

Collectively, we have shown that sorafenib overcomes TRAIL resistance in HCC and identified that STAT3 signaling pathway plays a significant role in mediating the effect of sorafenib on TRAIL sensitivity. This study suggests that STAT3 may be a useful therapeutic biomarker for HCC patients with sorafenib treatment.

Disclosure of Potential Conflicts of Interest

A-L. Cheng is a consultant for and a member of the speaker's bureau of Bayer-Schering. Other authors have nothing relevant to this manuscript to disclose.

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References

1. Kao JH, Chen DS. Changing disease burden of hepatocellular carcinoma in the Far East and Southeast Asia. *Liver Int* 2005; 25:696-703.
2. Verslype C, Van Cutsem E, Dicato M, et al. The management of hepatocellular carcinoma. Current expert opinion and recommendations derived from the 10th World Congress on Gastrointestinal Cancer, Barcelona, 2008. *Ann Oncol* 2009;20 Suppl 7: vii1-6.
3. Llovet JM, Ricci S, Mazzaferro V, et al. Sorafenib in advanced hepatocellular carcinoma. *N Engl J Med* 2008;359:378-90.
4. Cheng AL, Kang YK, Chen Z, et al. Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomised, double-blind, placebo-controlled trial. *Lancet Oncol* 2009;10:25-34.
5. Adnane L, Trail PA, Taylor I, Wilhelm SM. Sorafenib (BAY 43-9006, Nexavar), a dual-action inhibitor that targets RAF/MEK/ERK pathway in tumor cells and tyrosine kinases VEGFR/PDGFR in tumor vasculature. *Methods Enzymol* 2006;407:597-612.
6. Liu L, Cao Y, Chen C, et al. Sorafenib blocks the RAF/MEK/ERK pathway, inhibits tumor angiogenesis, and induces tumor cell apoptosis in hepatocellular carcinoma model PLC/PRF/5. *Cancer Res* 2006;66:11851-8.
7. Auclair D, Miller D, Yatsula V, et al. Antitumor activity of sorafenib in FLT3-driven leukemic cells. *Leukemia* 2007;21:439-45.

8. Wilhelm S, Carter C, Lynch M. Discovery and development of sorafenib: a multikinase inhibitor for treating cancer. *Nat Rev Drug Discov* 2006;5:835–44.
9. Takeda K, Stagg J, Yagita H, Okumura K, Smyth MJ. Targeting death-inducing receptors in cancer therapy. *Oncogene* 2007;26:3745–57.
10. Wang S. The promise of cancer therapeutics targeting the TNF-related apoptosis-inducing ligand and TRAIL receptor pathway. *Oncogene* 2008;27:6207–15.
11. Falschlehner C, Ganten TM, Koschny R, Schaefer U, Walczak H. TRAIL and other TRAIL Receptor agonists as novel cancer therapeutics. *Adv Exp Med Biol* 2009;647:195–206.
12. Natori A, MacFarlane M, Inoue S, et al. TRAIL signals to apoptosis in chronic lymphocytic leukaemia cells primarily through TRAIL-R1 whereas cross-linked agonistic TRAIL-R2 antibodies facilitate signalling via TRAIL-R2. *Br J Haematol* 2007;139:568–77.
13. Chen KF, Yeh PY, Hsu C, et al. Bortezomib overcomes tumor necrosis factor-related apoptosis-inducing ligand resistance in hepatocellular carcinoma cells in part through the inhibition of the phosphatidylinositol 3-kinase/Akt pathway. *J Biol Chem* 2009;284:11121–33.
14. Taniai M, Grambihler A, Higuchi H, et al. Mcl-1 mediates tumor necrosis factor-related apoptosis-inducing ligand resistance in human cholangiocarcinoma cells. *Cancer Res* 2004;64:3517–24.
15. Meng XW, Lee SH, Dai H, et al. Mcl-1 as a buffer for proapoptotic Bcl-2 family members during TRAIL-induced apoptosis: a mechanistic basis for sorafenib (Bay 43-9006)-induced TRAIL sensitization. *J Biol Chem* 2007;282:29831–46.
16. Ricci MS, Kim SH, Ogi K, et al. Reduction of TRAIL-induced Mcl-1 and cIAP2 by c-Myc or sorafenib sensitizes resistant human cancer cells to TRAIL-induced death. *Cancer Cell* 2007;12:66–80.
17. Hall MA, Cleveland JL. Clearing the TRAIL for cancer therapy. *Cancer Cell* 2007;12:4–6.
18. To KF, Chan MW, Leung WK, et al. Constitutional activation of IL-6-mediated JAK/STAT pathway through hypermethylation of SOCS-1 in human gastric cancer cell line. *Br J Cancer* 2004;91:1335–41.
19. Gu F, Dube N, Kim JW, et al. Protein tyrosine phosphatase 1B attenuates growth hormone-mediated JAK2-STAT signaling. *Mol Cell Biol* 2003;23:3753–62.
20. Chen KF, Yeh PY, Yeh KH, Lu YS, Huang SY, Cheng AL. Down-regulation of phospho-Akt is a major molecular determinant of bortezomib-induced apoptosis in hepatocellular carcinoma cells. *Cancer Res* 2008;68:6698–707.
21. Rosato RR, Almenara JA, Coe S, Grant S. The multikinase inhibitor sorafenib potentiates TRAIL lethality in human leukemia cells in association with Mcl-1 and cFLIPL down-regulation. *Cancer Res* 2007;67:9490–500.
22. Zhang Z, Shen K, Lu W, Cole PA. The role of C-terminal tyrosine phosphorylation in the regulation of SHP-1 explored via expressed protein ligation. *J Biol Chem* 2003;278:4668–74.
23. Liu Y, Kruhlak MJ, Hao JJ, Shaw S. Rapid T cell receptor-mediated SHP-1 S591 phosphorylation regulates SHP-1 cellular localization and phosphatase activity. *J Leukoc Biol* 2007;82:742–51.
24. Minoo P, Zadeh MM, Rottapel R, Lebrun JJ, Ali S. A novel SHP-1/Grb2-dependent mechanism of negative regulation of cytokine-receptor signaling: contribution of SHP-1 C-terminal tyrosines in cytokine signaling. *Blood* 2004;103:1398–407.
25. Johan MF, Bowen DT, Frew ME, Goodeve AC, Reilly JT. Aberrant methylation of the negative regulators RASSF1A, SHP-1 and SOCS-1 in myelodysplastic syndromes and acute myeloid leukaemia. *Br J Haematol* 2005;129:60–5.
26. Xu SB, Liu XH, Li BH, et al. DNA methylation regulates constitutive expression of Stat6 regulatory genes SOCS-1 and SHP-1 in colon cancer cells. *J Cancer Res Clin Oncol* 2009;135:1791–8.
27. Yuan Q, Li PD, Li BH, et al. Differential IL-4/Stat6 activities correlate with differential expression of regulatory genes SOCS-1, SHP-1, and PP2A in colon cancer cells. *J Cancer Res Clin Oncol* 2009;135:131–40.
28. Waiboci LW, Ahmed CM, Mujtaba MG, et al. Both the suppressor of cytokine signaling 1 (SOCS-1) kinase inhibitory region and SOCS-1 mimetic bind to JAK2 autophosphorylation site: implications for the development of a SOCS-1 antagonist. *J Immunol* 2007;178:5058–68.
29. Rottapel R, Ilangumaran S, Neale C, et al. The tumor suppressor activity of SOCS-1. *Oncogene* 2002;21:4351–62.
30. Yoshikawa H, Matsubara K, Qian GS, et al. SOCS-1, a negative regulator of the JAK/STAT pathway, is silenced by methylation in human hepatocellular carcinoma and shows growth-suppression activity. *Nat Genet* 2001;28:29–35.
31. Chen KF, Yu HC, Liu TH, Lee SS, Chen PJ, Cheng AL. Synergistic interactions between sorafenib and bortezomib in hepatocellular carcinoma involve PP2A-dependent Akt inactivation. *J Hepatol* 2010;52:88–95.
32. Lin L, Amin R, Gallicano GI, et al. The STAT3 inhibitor NSC 74859 is effective in hepatocellular cancers with disrupted TGF- β signaling. *Oncogene* 2009;28:961–72.
33. Yang F, Van Meter TE, Buettner R, et al. Sorafenib inhibits signal transducer and activator of transcription 3 signaling associated with growth arrest and apoptosis of medulloblastomas. *Mol Cancer Ther* 2008;7:3519–26.
34. Blechacz BR, Smoot RL, Bronk SF, Werneburg NW, Sirica AE, Gores GJ. Sorafenib inhibits signal transducer and activator of transcription-3 signaling in cholangiocarcinoma cells by activating the phosphatase shatterproof 2. *Hepatology* 2009;50:1861–70.
35. Kusaba M, Nakao K, Goto T, et al. Abrogation of constitutive STAT3 activity sensitizes human hepatoma cells to TRAIL-mediated apoptosis. *J Hepatol* 2007;47:546–55.
36. Sahu RP, Srivastava SK. The role of STAT-3 in the induction of apoptosis in pancreatic cancer cells by benzyl isothiocyanate. *J Natl Cancer Inst* 2009;101:176–93.
37. Pandey MK, Sung B, Ahn KS, Aggarwal BB. Butein suppresses constitutive and inducible signal transducer and activator of transcription (STAT) 3 activation and STAT3-regulated gene products through the induction of a protein tyrosine phosphatase SHP-1. *Mol Pharmacol* 2009;75:525–33.
38. Kunnumakkara AB, Nair AS, Sung B, Pandey MK, Aggarwal BB. Boswellic acid blocks signal transducers and activators of transcription 3 signaling, proliferation, and survival of multiple myeloma via the protein tyrosine phosphatase SHP-1. *Mol Cancer Res* 2009;7:118–28.

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