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Dovitinib sensitizes hepatocellular carcinoma cells to TRAIL and tigatuzumab, a novel anti-DR5 antibody, through SHP-1-dependent inhibition of STAT3

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ABSTRACT

Hepatocellular carcinoma (HCC) often displays resistance to recombinant tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis. Dovitinib, a multiple tyrosine kinase inhibitor, and tigatuzumab, a novel humanized anti-human death receptor 5 (DR5) agonistic antibody, are both under clinical investigations in HCC. Here, we report that dovitinib sensitizes resistant HCC cells to TRAIL- and tigatuzumab-induced apoptosis through inhibition of signal transducers and activators of transcription 3 (STAT3). Our data indicate that HCC cells showed significant resistance to TRAIL- and tigatuzumab-induced apoptosis. The combination of dovitinib and tigatuzumab restored the sensitivity of HCC cells to TRAIL- and tigatuzumab-induced apoptosis. Dovitinib down-regulated phospho-STAT3 (Tyr705) (p-STAT3) and subsequently reduced the protein levels of STAT3-regulated proteins, Mcl-1, survivin and cylcin D1, 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 sensitizing effect of dovitinib on TRAIL-induced apoptosis. Importantly, silencing SHP-1 by RNA-interference reduced the effects of dovitinib and TRAIL on p-STAT3 and apoptosis, whereas co-treatment of TRAIL and dovitinib increased the activity of SHP-1. Moreover, *in vivo* the combination of tigatuzumab and dovitinib inhibited Huh-7 xenograft tumor growth. In conclusion, dovitinib sensitizes resistant HCC cells to TRAIL- and tigatuzumab-induced apoptosis through a novel machinery: SHP-1 dependent STAT3 inhibition.

1. Introduction

Hepatocellular carcinoma (HCC), the fifth most common cancer in the world, remains a highly lethal malignancy and has a high recurrent rate despite surgical resection [1,2]. Advanced or recurrent HCC is frequently resistant to conventional chemotherapeutic agents and radiation; therefore, targeted agents with tolerable toxicity are mandatory to advance anti-HCC therapy [2]. Sorafenib, a multi-targeted receptor tyrosine kinase (RTK) inhibitor that targets Raf kinases, VEGFR1-3, PDGFR-β and other kinases such as FLT-3, and c-kit [3–6], has shown modest survival benefits in advance HCC in two randomized controlled phase III trials [7,8]. The success of sorafenib supports the use of molecularly targeted therapies in treatment of advanced HCC.

The inherent vascularity of HCC and the association of angiogenesis with HCC progression and prognosis make the application of small molecule anti-angiogenic agents a logical approach to block or interfere with HCC progression [9]. Dovitinib (TKI258; formerly CHIR-258) is a novel multiple RTKs inhibitor that targets VEGFR1-3; PDGFR-β; FGFR1-3; FLT-3; and cKIT, Ret, TrkA, and csf-1 RTKs [10]. Dovitinib has been shown to exert a dose- and exposure-dependent inhibition of RTKs expressed in tumor xenografts and stromal components in several preclinical models [10–12]. A phase I study showed a maximum tolerated dose of dovitinib was 125 mg/day, and dose limiting toxicities

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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were grade 3 hypertension, anorexia, and alkaline phosphatase elevation. Dovitinib is currently being tested for the treatment of solid tumors and hematological malignancies [13], such as in phase III development in renal cell carcinoma (NCT01223027), and in phase II development in advanced breast cancer (NCT00958971), relapsed multiple myeloma (NCT01058434) and urothelial cancer (NCT00790426). Many of the ongoing dovitinib trials are using sorafenib as a control arm, including a recent to-be-started phase II trial which will compare the safety and efficacy of dovitinib *versus* sorafenib as first-line treatment in advanced HCC (NCT01232296).

In addition to small molecule muti-kinase inhibitors, tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL) represents an attractive therapy targeting on receptor-mediated apoptosis [8]. TRAIL or TRAIL agonists bind to death domain-containing death receptors (DRs), DR4 (TRAIL-R1) or DR5 (TRAIL-R2) and form death-inducing signaling complex (DISC), which is a multi-protein complex consisting of an adaptor molecule, FADD and caspase-8 [14]. Activated caspase-8 initiates extrinsic apoptotic pathway in type I cells (through activating caspase-3, -6, and -7), and triggers intrinsic pathways in type II cells (through activating Bid) [14]. Interestingly, tigatuzumab (formerly CS-1008) is a novel TRAIL agonist that has shown *in vitro* and in *vivo* anti-tumor activity [15–18]. Tigatuzumab is a humanized anti-human DR5 antibody manufactured from a murine anti-human DR5 monoclonal antibody, TRA-8 [19]. Tigatuzumab has selective cytotoxicity toward tumor cells expressing DR5 [19] and an excellent safety profile in humans [20]. No dose limiting toxicity at doses of up to 8 mg/kg weekly was reported in a phase I trial [20,21]. Tigatuzumab is currently in clinical trials as a therapy for solid tumors [22], including HCC (NCT01033240).

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 [23]. Resistance to TRAIL may be induced by any step in the apoptosis signaling cascade, from receptor level (mutations or overexpression of DR4 or DR5) [24], or defects in DISC assembly, through dysfunctions of anti-apoptotic Bcl-2 family proteins (Bcl-2, Bcl-xl, Mcl-1, *etc.*) and pro-apoptotic proteins (Bax or Bak) or defects in mitochondria-derived activator of caspases (Smac/Diablo). Of particular note, Mcl-1, an anti-apoptotic Bcl-2 family protein, plays a critical role in conferring TRAIL resistance [25]. Data have shown that over-expression of Mcl-1 can neutralize TRAIL-induced signaling. Moreover, directly or indirectly destabilizing or disabling Mcl-1 may restore TRAIL sensitivity. Interestingly, as a highly regulated cell death and survival controllers that responds to various cytokines and growth factors, 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 [26].

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 [27]. 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 [27]. Targeting STAT3 using antisense oligonucleotide reduces the growth and metastasis of HCC cells *in vitro* and *in vivo* [28]. Abrogation of constitutive STAT3 activity by AG490, a Janus kinase 2 specific inhibitor, has been shown to sensitize human hepatoma cells to TRAIL-mediated apoptosis [29]. Interestingly, our recent work also found that sorafenib, through inhibition of p-STAT3, overcomes TRAIL-resistance of HCC cells [30]. Collectively these data implicated that targeting STAT3 signaling play a key role in sensitizing HCC cells to TRAIL-mediating cell killing. Moreover, a number of protein tyrosine phosphatases have been shown to negatively regulate STAT3 signaling through direct dephosphorylation of p-STAT3 (Tyr705); these include members of the SH2-domain containing tyrosine phosphatase family (SHP-1 and SHP-2), and protein tyrosine phosphatase 1B (PTP-1B). Activity of protein tyrosine phosphatases may be critical for the regulation of STAT3 phosphorylation in cancer cells.

In this study, we discovered a novel mechanism by which dovitinib sensitizes resistant HCC cells to TRAIL-induced apoptosis: through the SHP-1 dependent STAT3 inhibition. We demonstrated that combination of dovitinib and tigatuzumab restored the sensitivity of HCC cells to TRAIL- and tigatuzumab-induced apoptosis. We validated that combination of dovitinib and tigatuzumab down-regulates p-STAT3 and subsequently reduces the expressions of its downstream-regulated proteins, Mcl-1, survivin and cylcin D1. Furthermore, we showed that dovitinib, through increasing SHP-1 activity, inhibits STAT3 activity and enhances antitumor activity of tigatuzumab toward HCC cells. Importantly, this sensitizing effect was confirmed by *in vivo* nude mice model. Through the identification of STAT3 as a novel target mediating dovitinib's sensitization on TRAIL. The combination of dovitinib and tigatuzumab may constitute a novel anti-HCC treatment.

2. Materials and methods

2.1. Reagents and antibodies

Tigatuzumab and dovitinib and were kindly provided by Daiichi Sankyo Pharmaceuticals (Tokyo, Japan) and Novartis Pharmaceuticals (Basel, Switzerland), respectively. For *in vitro* studies, dovitinib 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-8, caspase-3, PARP phospho-STAT3 (Tyr705), STAT3 and phosphor-Akt (Ser473) were from Cell Signaling (Danvers, MA). Recombinant TRAIL was purchased from Biomol (Farmingdale, NY). SHP-1 inhibitor were purchased from Cayman Chemical (Ann Arbor, MI).

2.2. Cell culture and western blot analysis

The PLC5, Sk-Hep1 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). 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.

2.3. 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.

2.4. Gene knockdown using siRNA

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

2.5. PLC5 with ectopic expression of STAT3 and SHP-1

STAT3 cDNA (KIAA1524) and SHP-1 were purchased from Addgene plasmid repository (http://www.addgene.org/). Cells with stable expression of STAT3 were then treated with drugs, harvested, and processed for western blot analysis as described previously.

2.6. SHP-1 phosphatase activity

After treatment, 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).

2.7. 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 100–150 mm^3, mice received an intravenous injection of tigatuzumab (200 μg) three times a week, dovitinib (5 mg/kg) p.o. once daily, or a combination of tigatuzumab and dovitinib. Controls received vehicle. Tumors were measured weekly using calipers and their volumes calculated using the following standard formula: $width^2 \times length \times 0.52$.

2.8. 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).

3. Results

3.1. Dovitinib sensitizes resistant HCC cells to TRAIL- and tigatuzumab-induced apoptosis

To investigate the effects of dovitinib and TRAIL on HCC cells, we first examined the apoptotic effects of drugs on a panel of four human HCC cell lines Huh-7, Sk-Hep1, Hep3B, and PLC5. We found that adding dovitinib sensitizes HCC cells to TRAIL- and

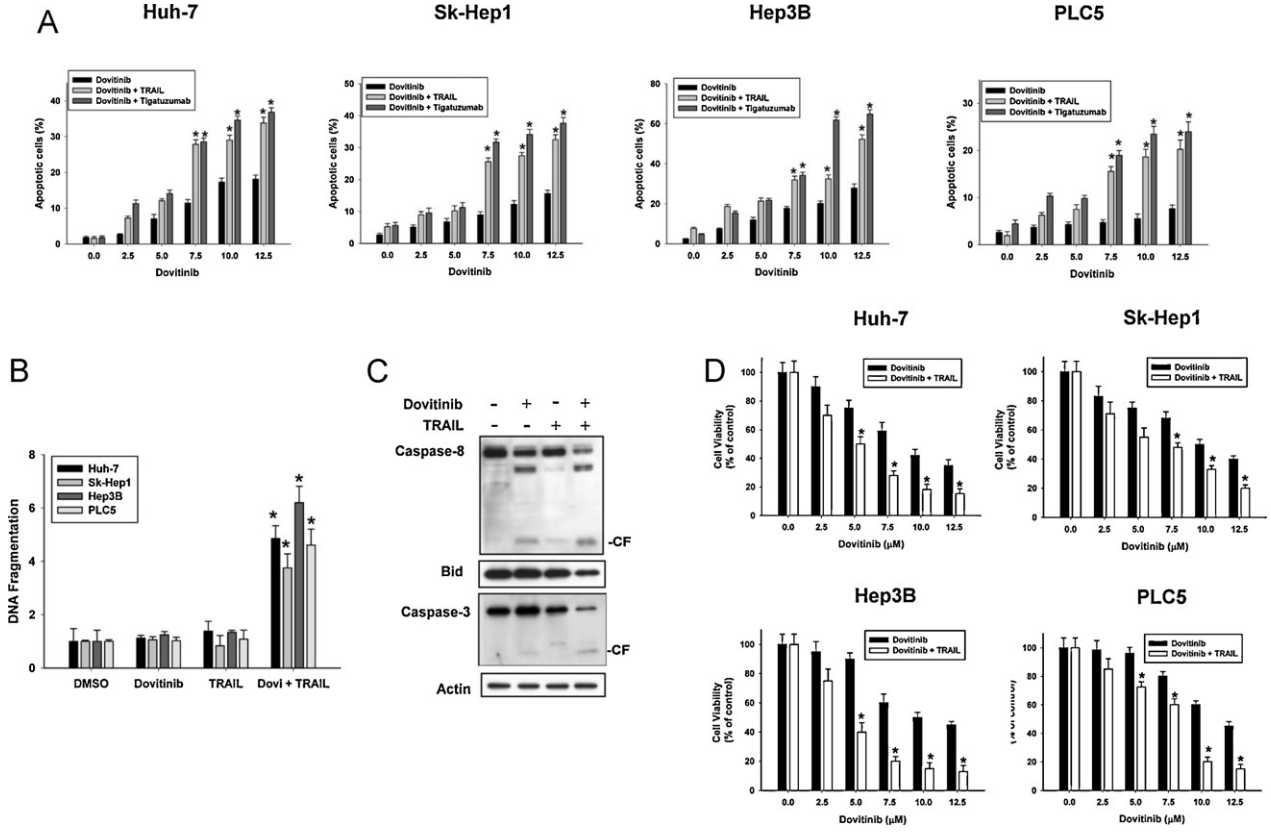

Fig. 1. Dovitinib sensitizes HCC cells to TRAIL- and tigatuzumab-induced apoptosis. (A) Dose-escalation effects of a combination of dovitinib (μM) and TRAIL (100 ng/ml) and tigatuzumab (1000 ng/ml) on apoptosis in four HCC cells. Cells were exposed to dovitinib and/or TRAIL or tigatuzumab at the indicated doses for 24 h. Apoptotic cells (sub-G1) were analyzed by flowcytometry. Comparisons of mean values were performed using the independent samples t test. Columns, mean; bars, SD ($n = 6$). *$P < 0.05$. (B) Effects of dovitinib and/or TRAIL on DNA fragmentation in four HCC cell lines. Cells were treated with dovitinib (7.5 μM) and/or TRAIL (100 ng/ml) for 24 h, and DNA fragmentation was analyzed by using a cell death ELISA kit. (C) Effects of dovitinib on TRAIL-induced apoptosis in Hep3B cells. CF, cleaved form (activated form). (D) Effects of dovitinib and/or TRAIL on cell viability in HCC cells. Cells were treated with dovitinib (7.5 μM) and/or TRAIL (100 ng/ml) for 24 h, and cell viability was analyzed by MTT. Columns, mean; bars, SD ($n = 6$). *$P < 0.05$.

tigatuzumab-induced apoptosis in all tested cell lines in a dose-dependent manner (Fig. 1A). Notably, as TRAIL has much better binding affinity to its receptors than the monoclonal antibody like tigatuzumab, the effective concentration of TRAIL is much lower than tigatuzumab (100 ng/ml *versus* 1000 ng/ml) in our study. Next, we examined the effect of dovitinib on TRAIL-induced DNA fragmentation in all HCC cell lines. DNA fragmentation was determined by cell death ELISA after 24 h of treatment. As shown in Fig. 1B, combining dovitinib (7.5 μM) with TRAIL (100 ng/ml) reversed the resistance in all four cell lines and induced significant apoptosis. Moreover, we further examined the apoptotic pathway by western blot. Our data indicated that co-treatment with dovitinib and TRAIL activated caspase-8 then induced cleavage of Bid and subsequently activated caspase-3 (Fig. 1C). Additionally, we examined the combinational effect of dovitinib and TRAIL on cell viability in our HCC cells. Our data indicated that adding dovitinib increased the effect of TRAIL on reducing cell viability significantly (Fig. 1D). These data suggested that the intrinsic pathway played a role in mediating the combination effect of dovitinib and TRAIL on apoptosis in HCC cells.

3.2. Combination of dovitinib and TRAIL down-regulates p-STAT3 in HCC cells

Previous studies have suggested that STAT3 may play a role in mediating the resistance to TRAIL in cancer cells including HCC. We next examined phospho-STAT3 (p-STAT3), STAT3 and STAT3-regulated proteins including Mcl-1, survivin and cyclin D1. As shown in Fig. 2A, co-treatment of dovitinib and TRAIL down-regulated p-STAT3 (Tyr705) and related proteins, Mcl-1, survivin, and cyclin D1, in all HCC 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 dovitinib and TRAIL (Fig. 2A). Furthermore, we found that the combination of dovitinib and TRAIL down-regulated p-STAT3 in Hep3B cells in a dose-dependent manner (Fig. 2B). In addition, we examined effects of drugs on other proteins which are related to cell survival, including p-Erk, Erk2, Bcl-xl, Bax, PTEN, PDK1, p-mTOR and mTOR; our data showed the combination of dovitinib and TRAIL did not alter the expression of these proteins (Fig. 2C). Notably, treatment of dovitinib alone did not induce PARP cleavage in Huh-7 and Hep3b. Therefore, we further investigated the dose-dependent effect of dovitinib on P-STAT3 and apoptosis in these cells. As shown in Fig. 2D, dovitinib down-regulated P-STAT3, Mcl-1, survivin, and cyclin D1 in a dose-dependent manner and induced significant cleavage of caspase-8 and PARP at 10 μM. These data suggest that STAT3 played a role in mediating the apoptotic effect of dovitinib in these HCC cells.

3.3. SHP-1 mediated STAT3 inhibition

We next validated the finding that inhibition of STAT3 signals is responsible for the sensitizing effect of dovitinib on TRAIL- induced apoptosis in HCC cells. First, we knocked down protein expression of STAT3 by small interference RNA (siRNA). Hep3B cells were transfected with either control, or STAT3 siRNA for 48 h then exposed to DMSO or TRAIL at 100 ng/ml for additional 24 h. Silencing STAT3 significantly sensitized Hep3B cells to TRAIL-induced apoptosis ($P < 0.05$) (Fig. 3A, left), suggesting that inhibition of the STAT3 signaling pathway is important for the sensitivity of HCC cells toward TRAIL. Next, we examined the

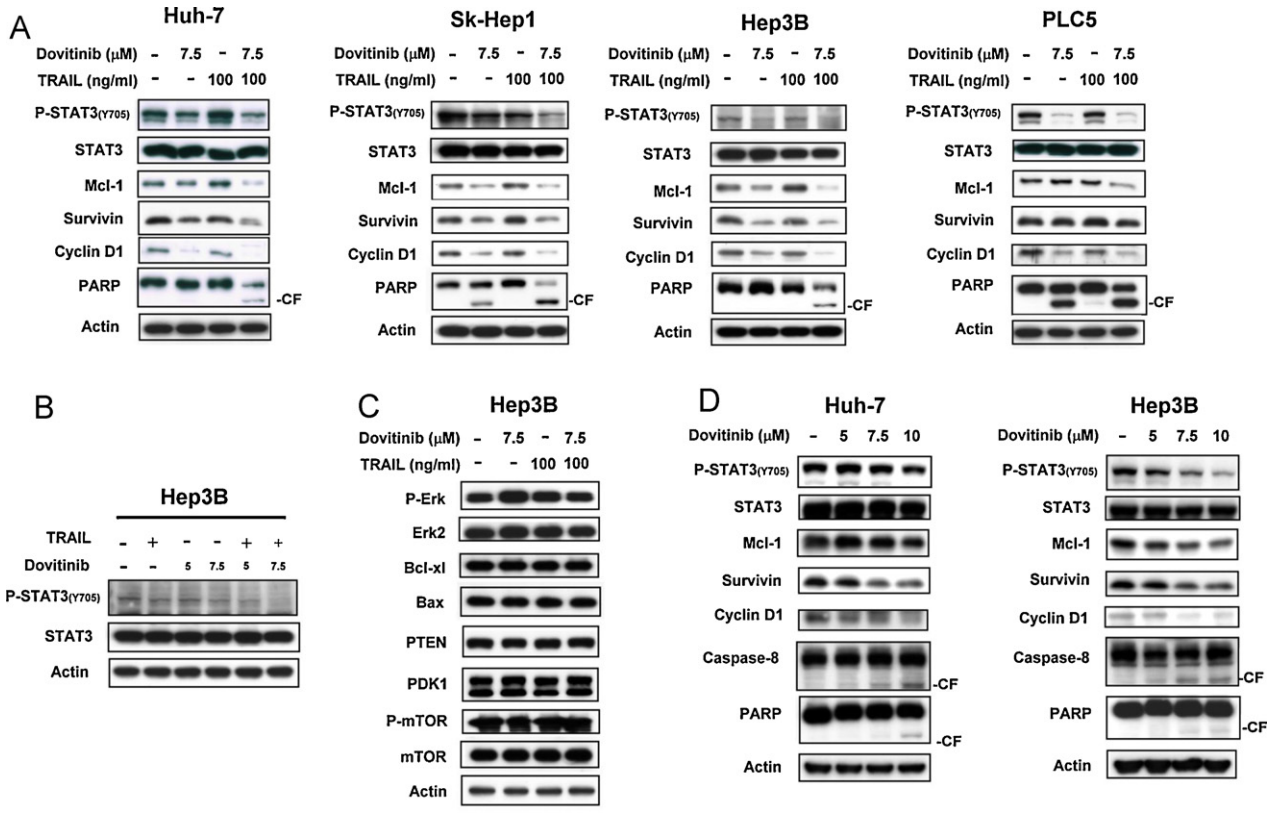

Fig. 2. Co-treatment of dovitinib and TRAIL down-regulated p-STAT3 in HCC cells. (A) Effects of dovitinib (7.5 μM) and/or TRAIL (100 ng/ml) on STAT3-related proteins. Cells were exposed to the drugs for 24 h (B) dose-dependent analysis of dovitinib (μM) and/or TRAIL (100 ng/ml) on p-STAT3 in Hep3B cells. Cells were exposed to the drugs for 24 h. (C) Effects of dovitinib and/or TRAIL on apoptosis-related molecules. Hep3B cells were treated with 100 ng/ml TRAIL and/or dovitinib at 7.5 μM for 24 h. (D) Effects of dovitinib on STAT3 and apoptosis. (Left) Huh-7. (Right) Hep3B. Cells were treated with dovitinib at the indicated doses for 24 h.

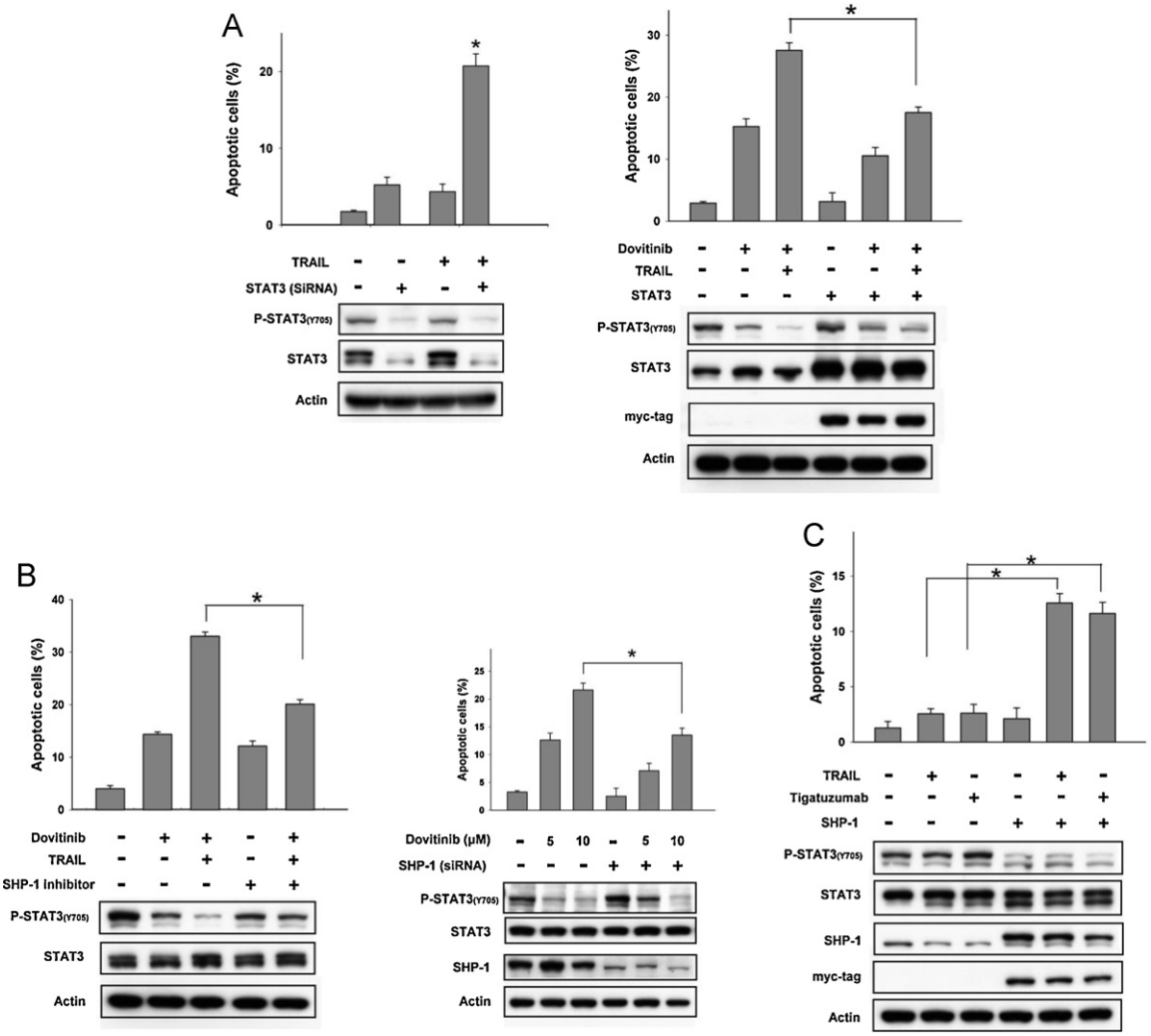

Fig. 3. SHP-1 mediated STAT3 inhibition. (A, left) down-regulation of STAT3 by siRNA overcame the resistance to TRAIL in Hep3B cells. (Right) Ectopic expression of STAT3 reduced apoptosis induced by the combination of dovitinib and TRAIL in Huh-7 cells. Apoptotic cells (sub-G1) were analyzed by flowcytometry. (B, left) SHP-1 inhibitor reduced the combinational effect of dovitinib and TRAIL on p-STAT3 and apoptosis in Huh-7 cells. (Right) Silencing SHP-1 by siRNA reduced the effects of dovitinib on p-STAT3 in HCC cells. Hep3B cells were transfected with control or SHP-1 siRNA for 48 h then were treated with dovitinib (5 or 10 μM) for 24 h. (C) Over-expression of SHP-1 sensitizes HCC cells to TRAIL and tigatuzumab. PLC5 cells (wild type or SHP-1) were treated with 100 ng/ml TRAIL or 1000 ng/ml tigatuzumab for 48 h. Columns, mean; bars, SD ($n = 3$). *$P < 0.05$. C.

effects of dovitinib in combination with TRAIL in both wild-type Huh-7 cells and Huh-7 cells with ectopic expression of STAT3. Over-expression of STAT3 significantly reduced the combinational effects of dovitinib plus TRAIL on p-STAT3 and apoptosis ($P < 0.05$) (Fig. 3A, right). Together, these results validate the importance of STAT3 inhibition in mediating the combination effect of dovitinib and TRAIL. To elucidate the mechanism by which dovitinib plus TRAIL down-regulated p-STAT3 in HCC cells, we investigated the roles of several protein phosphatases on the effect of dovitinib and TRAIL on p-STAT3 and apoptosis. First, we employed a specific SHP-1 inhibitor and our data showed that adding SHP-1 inhibitor reduced the effect of dovitinib plus TRAIL on p-STAT3 and apoptosis (Fig. 3B, left). Next, we altered the expression of SHP-1 by siRNA in Hep3B cells and showed that silencing SHP-1 significantly reduced the effects of dovitinib plus TRAIL on p-STAT3 and apoptosis (Fig. 3B, right). Notably, co-treatment with dovitinib and TRAIL did not affect the expression level of SHP-1 in HCC cells. To investigate the effect of SHP-1 on TRAIL sensitivity in HCC cells, we tested the apoptotic effect on TRAIL on two different PLC5 cell lines (wild type or ectopic expression of SHP-1). Ectopic SHP-1 increased the sensitivity of PLC5 cells to TRAIL- and tigatuzumab-induced apoptosis significantly, suggesting that targeting SHP-1 may later the sensitivity of HCC cells to TRAIL (Fig. 3C). Together, our results suggest that SHP-1 mediated effects of the drugs on p-STAT3 and apoptosis.

3.4. Dovitinib increases SHP-1 activity

To assess the effect of dovitinib plus TRAIL on SHP-1, we measured SHP-1 phosphatase activity in Hep3B cells that were treated with dovitinib plus TRAIL. As shown in Fig. 4A (left), dovitinib alone or in combination with TRAIL significantly increased the activity of SHP-1 ($P < 0.05$). To examine whether drugs bind to SHP-1 directly, we next immunoprecipitated SHP-1 then SHP-1-containing cell lysates were incubated with dovitinib and/or TRAIL. Treatment of dovitinib did enhance the activity of SHP-1, suggesting that dovitinib may have direct interactions with SHP-1. Moreover, as dovitinib is a kinase inhibitor, we examined whether dovitinib plus TRAIL enhanced SHP-1 activity by affecting the phosphorylation of SHP-1. Notably, phosphorylation of SHP-1 at tyrosine 536 may increase its activity and phosphorylation at serine 591 may decrease its activity [30]. We found that neither dovitinib alone nor co-treatment with TRAIL altered phospho-SHP-1 at either site (Fig. 4B). Moreover, we examined other protein

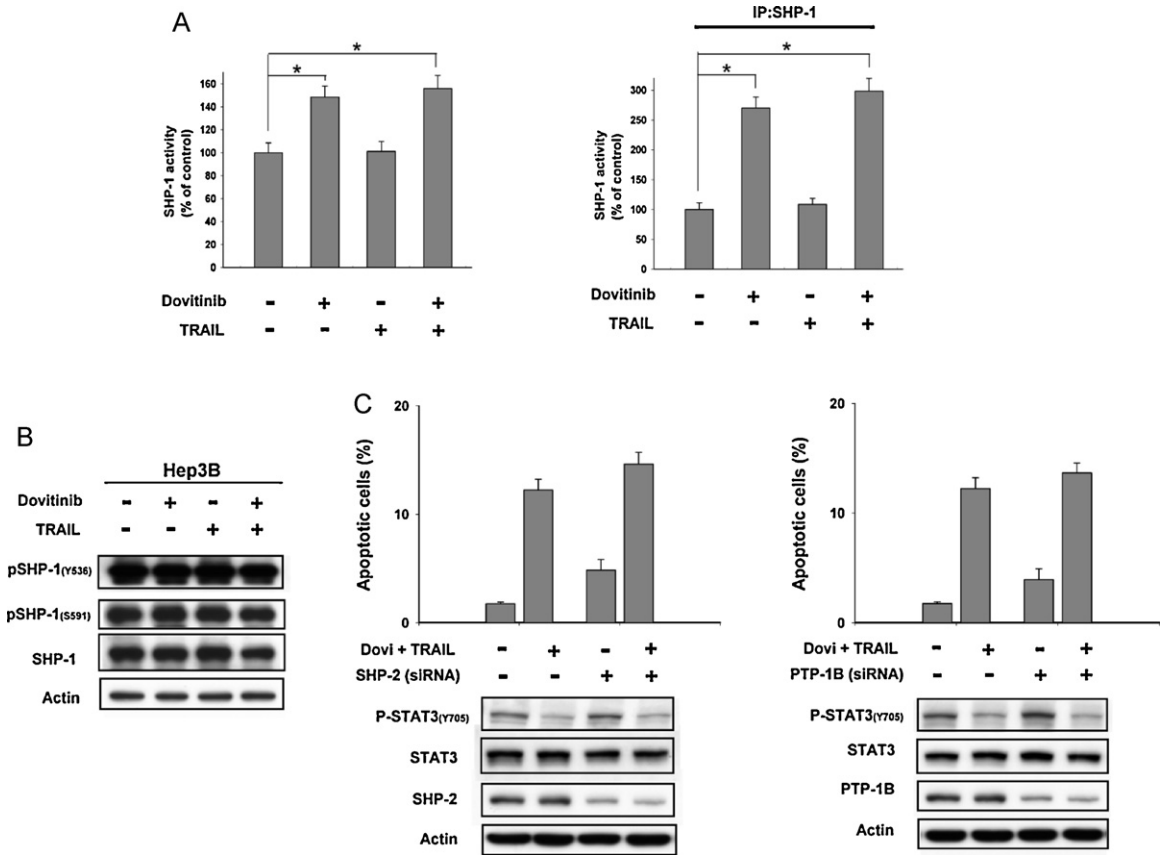

Fig. 4. Dovitinib increases SHP-1 activity. (A, left) Co-treatment of dovitinib and TRAIL enhanced the activity of SHP-1 in Hep3B cells. (Right) Effects of dovitinib and/or TRAIL on SHP-1 activity in SHP-1-containing lysates. Hep3B cells were immunoprecipitated with anti-SHP-1 then were incubated with drugs for 24 h. Columns, mean; bars, SD ($n = 3$). $^*P < 0.05$. (B) Effects of dovitinib and/or TRAIL on phospho-SHP-1 in Hep3B cells. (C, left) Knock down of SHP-2 did not affect effects of co-treatment of dovitinib and TRAIL on p-STAT3 and apoptosis. (Right) Knock down of PTP-1B did not affect effects of dovitinib and TRAIL on p-STAT3 and apoptosis. Columns, mean; bars, SD ($n = 3$). $^*P < 0.05$.

tyrosine phosphatases such as SHP-2 and PTP-1B that may also regulate phosphorylation of STAT3. Our data showed that neither knockdown of SHP-2 nor silencing PTP-1B affected the effect of dovitinib plus tigatuzumab on p-STAT3 signaling and apoptosis (Fig. 4C). These data suggest that SHP-2 and PTP-1B do not mediate the effects of the drug on p-STAT3 and apoptosis.

3.5. *In vivo* effect of dovitinib and tigatuzumab

To confirm whether the effect of dovitinib and TRAIL in resistant cell lines has potentially relevant clinical implications, we assessed the *in vivo* effect of dovitinib and tigatuzumab on the growth of Huh-7 xenograft tumors. Tumor-bearing mice were treated with vehicle or dovitinib p.o. at a dose of 5 mg/kg/day or tigatuzumab i.v. at a dose of 200 μg three times a week 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 dovitinib and tigatuzumab (*versus* control, $P < 0.05$) and tumor size in the co-treatment group was only one fourth of that of the control group at the end of the study (Fig. 5A). Treatment with low dose dovitinib had not significant effect on Huh-7 tumor growth. Tigatuzumab alone showed modest effects on tumor growth. As shown in Fig. 5B, co-treatment with dovitinib and tigatuzumab down-regulated p-STAT3. In addition, the combination of dovitinib and tigatuzumab enhanced SHP-1 activity significantly (Fig. 5C), 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 dovitinib and tigatuzumab exhibits good anti-tumor activity *in vivo*. Further clinical investigation is warranted.

4. Discussion

Although sorafenib is clinically approval, most of HCC patients benefits from sorafenib by stabilizing the disease progression instead of tumor regression. Accordingly, only 2–3% of patients have significant tumor remission after the treatment of sorafenib [7,8]. Therefore, more potent VEGFR inhibitors like dovitinib which holds additional effects on other oncogenic signaling receptor such FGFR may show better effects than sorafenib clinically. In this study, we revealed that an important novel mechanism of dovitinib, SHP-1 dependent p-STAT3 inhibition, is responsible for its sensitization of resistant HCC cells to TRAIL-induced apoptosis. Our data have several important implications. First, our results strengthen the evidence that STAT3 may be a key mediator in HCC targeted therapy, particularly for TRAIL-induced cytotoxic killing. HCC is well-known for its complicity and heterogeneity in genomic alterations [31,32]. Aberrant signaling cascades have been found in HCC including EGFR, Ras/raf/ERK, PI3K/mTOR, HGF/MET (hepatocyte growth factor/mesenchymal-epithelial transition factor), Wnt, Hedgehog, and apoptotic signaling [32]. However, no specific oncogene addictions are yet identified critical to HCC progression [31]. Interestingly, a growing number of reports have demonstrated that constitutively activated JAK/STAT signaling play a significant role in oncogenesis of HCC. For example, loss of or suppressed function of SOCS family

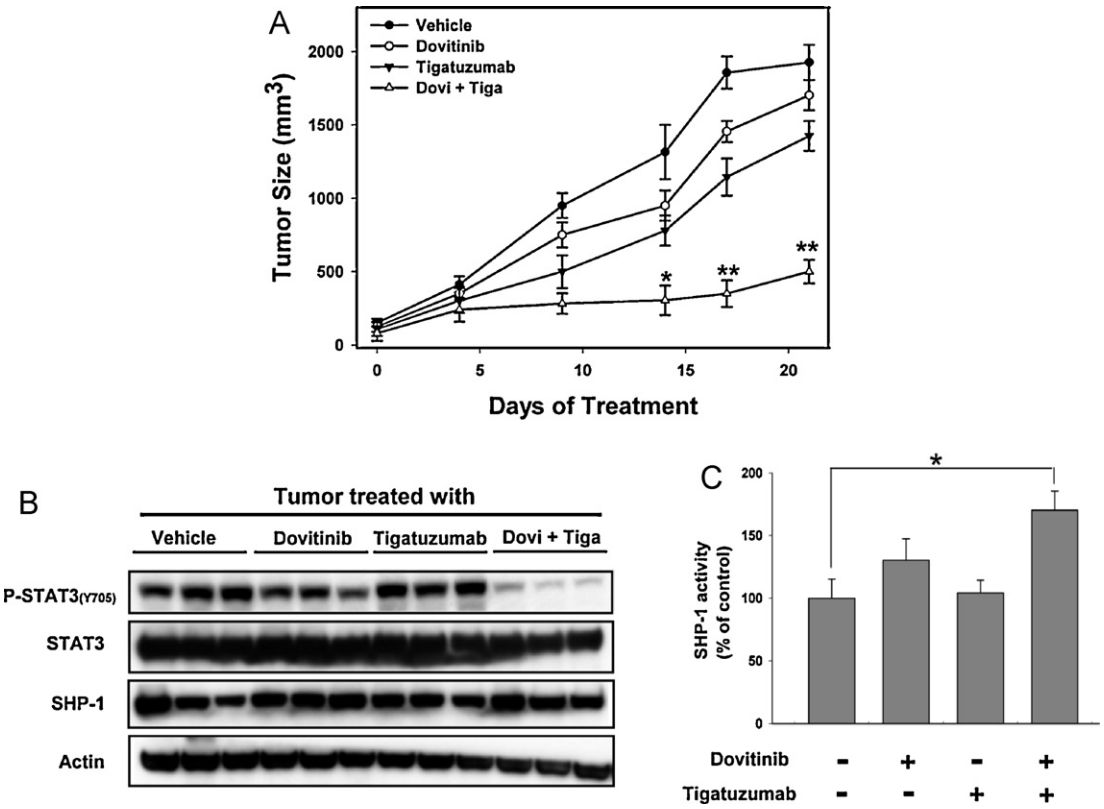

Fig. 5. *In vivo* effect of dovitinib and tigatuzumab on Huh-7 xeonograft nude mice (A) the combination of dovitinib and tigatuzumab showed significant anti-tumor effect on Huh-7 tumors. Points, mean ($n = 6$); bars, SE. $^{*}P < 0.05$; $^{**}P < 0.01$. (B) Analysis of p-STAT3, STAT3 and SHP-1. (C) Analysis of SHP-1 activity. Columns, mean; bars, SD ($n = 6$). $^{*}P < 0.05$ *versus* vehicle group.

members which are known negative regulators of JAK/STAT signaling, such as aberrant epigenetic silencing of SOCS1 or SOCS-3 [33,34], have been shown to be crucial events in the development of HCC. Moreover, accumulating evidence indicates that STAT3 activity in tumor cells may drive the immune response in cancer toward tumor progression [35]. For example, Yu et al. showed that STAT3 activity in tumor cells promotes the synthesis of cytokines (IL-6, IL-10) and growth factors (VEGF) that impair the maturation of dendritic cells and compromise their ability to stimulate the antitumor effects of CD8+T cells and natural killer (NK) cells [36]. These findings highlight the fundamental role of STAT3 in carcinogenesis, cell survival and tumor progression in HCC. Importantly, several novel or investigational agents have been shown to have anti-HCC activity through targeting STAT3 [29,30,37–39]. Furthermore, some of them have been shown to sensitize HCC cells to TRAIL-mediated killing [29,30]. Similarly, it has previously been demonstrated that inhibiting STAT3 activity enhances TRAIL-mediated apoptosis in other cancer cells, including pancreatic cancer cells [40], medulloblastomas cells [41], and cholangiocarcinoma cells [42]. Taken together, these data implicated STAT3 as an ideal target for developing novel anti-cancer agents or TRAIL-sensitizers.

Second, the novel SHP-1 dependent p-STAT3 inhibition by dovitinib highlights a new drug-mechanism of this multiple-targeted TKI and a new strategy targeting on the interactions of protein tyrosine phosphatases and tyrosine kinases. Previously sorafenib has been shown to overcome TRAIL resistance of HCC cells through a similar SHP-1 dependent-inhibition of p-STAT3 [30]. Interestingly, β-escin and gamma-tocotrienol have been demonstrated to induce the expression of SHP-1 that correlates with the down-regulation of constitutive STAT3 activation and their anti-HCC activity [37,38]. Similarly, acety-11-keto-beta-boswellic acid and butein (3,4,2′,4′-tetrahydroxychalcone) have also been shown to induce SHP-1 expression that inhibits p-STAT3, correlating to their apoptotic and anti-proliferative effects on multiple myeloma cells [43]. Taken together these structurally unrelated agents that show a common drug machinery suggest that targeting on the interactions of phosphatases and oncokinases could be a novel anti-HCC or anti-cancer strategy.

Last but not the least, several combination strategies may be implicated to enhance the antitumor activity of TRAIL and its agonists through cross-talk between the intrinsic and extrinsic apoptotic pathways [22,23]. Among the numerous novel agents with potentials to sensitize or overcome TRAIL resistance, the combination of dovitinib with tigatuzumab may be a promising and safe regimen that warrants further clinical trials. Data from our *in vivo* experiment demonstrated no gross toxic effects on xenografted mice. Dovitinib has been shown to be safe and well tolerated even at dose up to 500 mg/day [13]. Whereas tigatuzumab has also shown excellent safety profile that is feasible to patients with HCC [13,19,20]. Moreover, tigatuzumab-mediated killing may not be affected by DR4 mutations or dysfunction, given its specificity toward DR5 and that DR5 mutations have been reported less frequent in HCC cells [44].

The further mechanism by which dovitinib activates SHP-1 phosphatase activity; however, remains unknown and further study is warranted. STAT3 activity can be triggered by IL-6 through receptor-associated JAKs; by growth factor RTKs such as the EGFR and VEFGR; or by non-receptor tyrosine kinases like SRC [36]. STAT3 activity is also regulated through negative feedback mechanism by the SOCS family [45]. In addition, several PTPs have been implicated in STAT3 signaling including SHP-1, SHP-2, PTP-1B [43]. It has been found that loss of SHP-1 may contribute to the activation of JAK or STAT proteins in cancer and that SHP-1 has been implicated as a tumor suppressor [46,47]. Although dovitinib could also inhibit p-STAT3 through its TKI effect, such as the

inhibiting effect on VEGFR or IL-6/JAKs signaling, it is also possible that dovitinib inhibits p-STAT3 through a kinase-independent mechanism [48]. Nevertheless, we validated that (Figs. 3B and C and 4) SHP-1 stimulation by dovitinib correlated with the down-regulation of constitutive STAT3 phosphorylation and its sensitizing effect on TRAIL-mediated apoptosis. Our results showed that dovitinib 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, dovitinib did not influence the SHP-1 and STAT3 protein–protein interactions. Whether dovitinib also affects other putative regulators as mentioned above requires further investigation.

In conclusion, dovitinib sensitizes HCC cells to TRAIL or tigatuzumab-mediated killing through a novel phosphatase–kinase interactive mechanism, the SHP-1-dependent inhibition of p-STAT3. Our study indicates that the STAT3 signaling pathway may be a suitable target for the development of targeted agents or TRAIL-sensitizers in HCC and that the combination of dovitinib and tigatuzumab may constitute a novel anti-HCC treatment. Future studies defining STAT3 as a useful therapeutic biomarker for HCC patients who receive dovitinib treatment, as well as the detailed mechanism by which dovitinib affects SHP-1 activity may lead to further progress in the development of molecular-targeted therapy for HCC.

Disclosure of potential conflict of interest

Dr. Ann-Lii Cheng is a consultant for Novartis and Daiichi Sankyo. Dr. Kimihisa Ichikawa is an employee of Daiichi Sankyo. Other authors have nothing relevant to this manuscript to disclose.

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