New insights into the antifibrotic effects of sorafenib on hepatic stellate cells and liver fibrosis

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Background & Aims: During the process of liver fibrosis, hepatic stellate cells (HSCs) play a critical role in the excessive production of extracellular matrix (ECM). We evaluated the therapeutic effects of sorafenib, a multiple receptor tyrosine kinase inhibitor, targeting platelet-derived growth factor (PDGF) receptor and the Raf/extracellular-signal-regulated kinase (ERK) signaling pathway, on liver fibrosis and HSC proliferation.

Methods: The in vivo effects of sorafenib were monitored in the livers of rats with liver fibrosis, and simultaneously proliferation assays, apoptosis induction studies, and collagen synthesis measurement were conducted in vitro in rat and human HSCs and primary HSCs.

Results: Sorafenib treatment attenuated liver fibrosis and was associated with a significant decrease in intrahepatic fibrogenesis, hydroxyproline accumulation and collagen deposition. Sorafenib reduced HSC proliferation and resulted in significantly higher levels of apoptosis. Moreover, sorafenib downregulated Cyclin D1 and Cyclin-dependent kinase 4 (Cdk-4), simultaneously increased expression of Fas, Fas-L, and Caspase-3, and decreased the ratio of Bcl-2 to Bax. Sorafenib treatment increased the ratio of matrix metalloproteinases (MMPs) to tissue inhibitor of matrix metalloproteinases (TIMPs) and reduced collagen synthesis in HSCs. Sorafenib inhibited the phosphorylation of ERK, Akt and 70-kDa ribosomal S6 kinase (p70S6K), both in vitro and in vivo.

Conclusions: Sorafenib induces the suppression of collagen accumulation and HSC growth warranting the use of sorafenib as a potential therapeutic agent in the treatment of liver fibrosis. © 2010 European Association for the Study of the Liver. Published by Elsevier B.V. All rights reserved.

Introduction

Liver fibrosis is a common consequence of chronic liver injury that is induced by a variety of etiological factors that lead to liver cirrhosis [1]. This progressive pathological process is characterized by the accumulation of extracellular matrix (ECM) proteins. Hepatic stellate cells (HSCs), play a critical role in the excessive production and secretion of ECM, resulting in the deposition of fibrous tissue and scar formation [2].

The outcome of fibrosis is influenced by the synthesis of collagen and degradation mediated by matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs). The balance between MMPs and TIMPs plays a key role in collagen degradation [3,4]. HSC activation induces the release of platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF), which are important for the sustained activation and proliferation of HSCs [5,6]. PDGF-BB, a highly potent HSC mitogen, binds to PDGFR-β, activating Ras and sequentially propagating the stimulatory signal via the phosphorylation of mitogen-activated protein kinase (MAPK)/extracellular-signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K)/Akt/70-kDa ribosomal S6 kinase (p70S6K) signaling pathway. These two pathways regulate protein synthesis, the transcription of profibrogenic genes, proliferation, cell cycle control and apoptosis in HSCs [7]. Inhibition of PDGF-BB and blocking PI3K/Akt/p70S6K and ERK signaling pathways have been shown to induce HSC apoptosis and attenuate liver fibrosis [8–10].

Sorafenib is a multiple receptor tyrosine kinase inhibitor targeting the Raf/ERK signaling pathways, as well as the receptor tyrosine kinases VEGF receptor (VEGFR) and PDGFR-β [11]. It inhibits proliferation and induces apoptosis in various tumor cell lines. Cyclins and Cyclin-dependent kinases (Cdks), are associated with cellular proliferation and clinical outcome by sorafenib [12,13]. Previous studies indicate that sorafenib and sunitinib, another multiple receptor tyrosine kinase inhibitor, induce a significant decrease in portal pressure and angiogenesis in rats with liver fibrosis [14,15].

The purpose of the present study was to investigate the impact of sorafenib on liver fibrosis in two rat models and two HSC lines by investigating the effects of sorafenib on the proliferation, apoptosis and collagen expression of HSCs.
Materials and methods

Animals

Male Sprague-Dawley (SD) and Wistar rats weighing 200–300 g were provided by the Central Animal Care Facility of Hebei Medical University (Permission No.: SCXK 2008-1-003). All animals received humane care in compliance with the Chinese Animal Protection Act, which is in accordance with the National Research Council criteria. To induce biliary cirrhosis, SD rats underwent double ligation and sectioning of the common bile duct (BDL) as previously described [16]. Wistar rats had hepatic fibrosis induced via intraperitoneal injections of dimethylhydrazine (DMN; Sigma–Aldrich Corporation, St Louis, MO, USA) at a dose of 1 mL (diluted 1:100 with 0.15 M NaCl)/100 g body weight. The injections were given on the first three consecutive days of each week over a period of 28 days.

Treatment regimens

Sorafenib (ALEXIS Biochemicals, San Diego, CA, USA) was dissolved in a vehicle composed of Cremophor EL (Sigma–Aldrich, Saint Louis, MO, USA), ethanol and water (1:1:6). Sorafenib 20 mg/kg (BDL, n = 6); 40 mg/kg (BDL, n = 6); 1 mg/kg (DMN, n = 6); 5 mg/kg (DMN, n = 6) or vehicle (BD, n = 6; DMN, n = 6) was administered orally by gavage once a day during the third and the fourth week. Animals were sacrificed during weeks 0, 1, 2, 3 and 4 of the experiment.

RNA isolation (Invitrogen, Carlsbad, CA, USA). RNA (2 μg) was reverse-transcribed using PrimeScript™ RT reagent kit (DRR037S, TaKaRa Bio, Japan), and the single-stranded cDNA was amplified by quantitative real-time RT-PCR using SYBR Green and the CFX96™ Real-Time PCR Detection System (Bio-Rad) for 40 cycles. Data were analyzed using the 2^-ΔΔCt method. The efficacy of sorafenib on collagen expression of the target gene was normalized to GAPDH.

Hydroxyproline determination

Hepatic hydroxyproline was measured using a hydroxyproline detection kit (Jiancheng Institute of Biotechnology, Nanjing, China) according to the manufacturer’s instructions. All experiments were performed in triplicates. Results are expressed as μg of hydroxyproline per g of wet liver tissue.

Collagen α1(I) mRNA analysis

The effects of sorafenib on collagen α1(I) mRNA expression of liver tissue from fibrotic animal models, rat T6, human L2X and primary rat HSCs were evaluated. RNA was extracted following the manufacturer’s protocols for Trizol isolation (Invitrogen, Carlsbad, CA, USA). RNA (2 μg) was reverse-transcribed using PrimerScript™ RT reagent kit (DRR041S, TaKaRa Bio, Japan), and the single-stranded cDNA was amplified by quantitative real-time RT-PCR using SYBR Green and the CFX96™ Real-Time PCR Detection System (Bio-Rad) for 40 cycles. Data were analyzed using the 2^-ΔΔCt method. The efficacy of sorafenib on collagen expression of the target gene was normalized to GAPDH.

Primary HSCs isolation and culture

Primary HSCs were isolated from normal male SD rats as described [17]. Cells were suspended in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Carlsbad, CA, USA) supplemented with 1% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA). Cells cultured for 8–10 days have activated phenotypes, which were microscopically evaluated by α-SMA staining with monoclonal antibody. After being serum-starved for 12 h, activated primary HSCs were used to evaluate cell viability and collagen synthesis by collagen α1(I) mRNA analysis.

Cell lines and treatment of HSCs

Rat T6 and human L2X are well-characterized HSC lines that recapitulate many features of the activated HSC phenotype, including the expression of the PDGFR-β subunit [18,19]. HSCs were cultured in DMEM supplemented with 10% FBS. Sorafenib was dissolved with 1% dimethyl sulfoxide (DMSO). HSCs were serum starved for 12 h and split into the following groups: the DMSO group, the PDGF-BB (Peptech, Rocky Hill, NJ, USA) (20 ng/mL) groups, the PDGF-BB (20 ng/mL) + sorafenib (2.5, 5, 10 μM) groups and the sorafenib (2.5, 5, 10 μM) groups. After being treated with the appropriate agent(s) for 24 h, HSCs were prepared for Caspase-3 activity assay, TUNEL, Western blot analysis, electron microscopy and proliferation, DNA synthesis assay and collagen synthesis analysis. The same set of cells was harvested at 6 h, 12 h, 24 h, and flow cytometric analyses were subsequently performed. For inhibition of the MAPK signaling pathway, the ERK inhibitor PD98059 (ALEXIS Biochemicals, San Diego, CA, USA) was used. For blockade of the Akt/p70S6K signaling pathway, the PI3K inhibitor LY294002 (ALEXIS Biochemicals, San Diego, CA, USA) was applied. T6 and L2X cells were serum starved for 12 h and split into the following eight groups: the DMSO group, the PDGF-BB (20 ng/mL) group, the sorafenib (10 μM) groups with or without PDGF-BB (20 ng/mL), the PD98059 (50 μM) groups with or without PDGF-BB (20 ng/mL), the LY294002 (25 μM) groups with or without PDGF-BB (20 ng/mL). After being treated for 24 h, cells were harvested for measuring the expressions of ERK1/2, phospho-ERK, Akt, phospho-Akt, p70S6K and phospho-p70S6K by Western blot analysis.

Cell viability analysis

Primary HSCs, T6 and L2X cells were seeded in a 96-well plate with 200 μL (5 × 10^4 cells/mL) per well. After incubation in serum-free media for 12 h, cells were pre-incubated with sorafenib (2.5, 5.0 and 10.0 μM) for 2 h and then stimulated with 20 ng/mL PDGF-BB for 24 h, 48 h and 72 h. Cell viability was evaluated using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma–Aldrich, Saint Louis, MO, USA) assay as previously described [17].

DNA synthesis assay

Cells were treated as above and during the last 6 h of incubation, 0.5 μCi of [Methyl-3H]-Thymidine (Tdr) (Perkin-Elmer, Waltham, MA, USA) was added to each well. HSCs were harvested in 96-well round-bottom sample plates, dried and mixed with scintillation fluid (Perkin-Elmer, Waltham, MA, USA). The incorporation of radioactivity was determined with a Wallac microbeta 1450 liquid scintillation counter (Turku, Finland).

Cell cycle analysis of HSCs

HSCs were fixed in ice-cold 75% ethanol, washed, and resuspended in 1 mL of PBS with 50 μg of propidium iodide and 0.25 mg of RNase A. Samples were assayed with an Epics-XL II flow cytometer of Beckman Coulter (Fullerton, CA, USA), and data were analyzed with the Multicycle AV software package of Phoenix Flow Systems (San Diego, CA, USA).

Apoptosis analysis of HSCs

Cells were treated as described above and harvested for morphological examination via transmission electron microscopy (TEM) evaluation as previously described [10].

The terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) technique using the In Situ Cell Death Detection Kit POD (Roche, Indianapolis, IN, USA) was performed to measure nuclear DNA fragmentation as previously described [17].
Both adherent and floating cells were harvested for the apoptosis assay by using the Annexin-V/propidium iodide double-labeled flow cytometry kit (Baosai, Beijing, China) as previously described[10]. Cells were analyzed by flow cytometry with a Becton Dickinson FACS-420 flow cytometer (Franklin Lakes, NJ, USA).

Caspase-3 activity assay

T6 and LX2 cells were seeded in 6-well culture plate and incubated in DMEM supplemented with 10% FBS. After being treated as described above, protein extracts were prepared following manufacturer’s instructions by using Bradford Protein Assay kit (Beyotime Institute of Biotechnology, Nantong, Jiangsu, China). Caspase-3 activity was measured using Caspase-3 Activity Assay kit (Beyotime Institute of Biotechnology, Nantong, Jiangsu, China) in which cell extracts were mixed with Ac-DEVD-pNA substrate for 2 h at 37 °C in 96-well plates prior to colorimetric measurement of p-nitroanilide product at 405 nm.

Collagen synthesis analysis of HSCs

During the last 24 h of incubation, 0.5 μCi of l-[2,3-3H]-proline (Perkin-Elmer, Waltham, MA, USA) was added to each well. The cell supernatants were harvested, and collagenase (Invitrogen, Carlsbad, CA, USA) was added at 37 °C for 90 min. Non-collagenous proteins were then precipitated using 50% trichloroacetic acid and incubated on ice for 1 h. The incorporation of radioactivity was determined with a Wallac microbeta 1450 liquid scintillation counter (Turku, Finland).

Western blot analysis

Primary antibodies used were ERK1/2, phospho-Tyr204/Thr202ERK, Akt, phospho-Ser727/Akt, p70S6K, phospho-Ser11p70S6K, Caspase-3, Bcl-2, Bax, Cyclin D1, Cdk-4, TIMP-1, type I collagen and gyceraldehyde phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology, Santa Cruz, CA, USA); MMP-1 and MMP-13 (Bioworld Technology, Atlanta, GA, USA). Western blotting was performed as described previously [17]. For protein quantification, bands were scanned and quantified with GAPDH as an internal control.

Statistical analysis

Data are presented as means ± SD. The statistical analyses that were performed included one-way ANOVA and, when the F value was significant, the Student–Newman–Keuls test. p-values less than 0.05 were considered statistically significant.

Results

Effect of sorafenib on liver fibrosis

We used a series of experimental approaches to evaluate whether sorafenib inhibited hepatic fibrosis. The liver tissues of BDL rats
apparent to exhibit a distortion of the normal architecture, with a marked increase in the number of bile ductules and an extensive deposition of collagen (Fig. 1A and B). In contrast to the liver tissue of BDL rats, the liver tissue of rats that had undergone DMN-induced liver injury and fibrosis exhibited extensive hemorrhagic necrosis and lobular architecture with thin bands of reticulin joining central areas (Fig. 1C and D). In the Masson-stained sections, histological evidence of liver fibrosis appeared in samples

![Fig. 2. Sorafenib reduces hydroxyproline content and downregulates type I collagen expression in BDL and DMN rats. (A) Sorafenib reduces hydroxyproline content in rat livers. (B) Sorafenib downregulates type I collagen protein expression in the livers of BDL and DMN rats, as shown by Western blot analysis. (C) Real-time PCR analysis for collagen $\alpha_1$ (I) mRNA expression. *p < 0.05 compared to the SHAM group or the 0-week group. **p < 0.05 compared to the vehicle-treated groups.](https://example.com/fig2)
collected during the third and fourth weeks after liver injury. Sorafenib attenuated the collagen deposition in a dose-dependent fashion. Sorafenib (40 mg/kg/day in BDL rats and 5 mg/kg/day in DMN rats) significantly decreased collagen deposition, with a 70.07% and a 58.31% reduction as compared to vehicle-treated BDL and DMN rats, respectively (Fig. 1E).

Hepatic hydroxyproline content increased in BDL- and DMN-treated rats gradually (Fig. 2A). In BDL rats, treatment with sorafenib reduced hydroxyproline content in a dose-dependent fashion by 54.81% and 66.78% at doses of 20 mg/kg and 40 mg/kg. In DMN-treated rats, sorafenib inhibited the secretion of hydroxyproline by 34.52% at the 1 mg/kg dose, which was less than the effect of sorafenib at 5 mg/kg. Assessments of type I collagen protein expression via Western blot and collagen α1 (I) mRNA via real-time PCR revealed a gradual increase in its expression, reaching the maximum value in both models during the fourth week. Sorafenib attenuated the upregulation of type I collagen in a dose-dependent manner in both BDL and DMN rats (Fig. 2B and C).

BDL- and DMN-treated rats developed hepatic injury as evidenced by significantly higher plasma concentrations of AST, ALT, total bilirubin and a lower concentration of ALB, compared with normal control rats. Sorafenib treatment did not aggravate these changes of blood chemistry in BDL rats (Table 1). In DMN-treated rats, sorafenib of lower concentration (1 mg/kg) ameliorated the increase of ALT, BIL, and the decrease of ALB, while higher concentration of sorafenib (5 mg/kg) aggravated the increase of AST and ALT levels significantly (Table 2).

Sorafenib remarkably suppressed the levels of phospho-ERK, phospho-Akt and phospho-p70S6K in a dose-dependent fashion by Western blot (Fig. 3A).

Immunostaining of liver sections to explore the distribution of phospho-ERK, phospho-Akt, and phospho-p70S6K revealed that they localized to vascular endothelial and perisinusoidal cells. With the development of hepatic fibrosis, the positive cells increased and were mainly distributed in hepatic stellate cells around portal ducts, fiber septa and the bile ducts, as well as in hepatocytes. After treatment with sorafenib the increased expressions of phospho-ERK, phospho-Akt and phospho-p70S6K were markedly decreased both in perisinusoidal cells and hepatocytes (Fig. 3B). These results suggested that the antifibrogenesis effects of sorafenib may be mediated by the inhibition of those two signaling pathways, which play a major role in the regulation of hepatic fibrogenesis [7,20].

Sorafenib inhibits HSC proliferation and regulates the cell cycle distribution

After treatment with sorafenib, the cell viabilities of both HSC cell lines (including T6 and LX2 cells) and primary HSCs were significantly reduced in a dose- and time-dependent manner (Fig. 4A). Both T6 and LX2 cell DNA synthesis was significantly reduced in a dose-dependent manner after treatment with sorafenib (Fig. 4B). The inhibitory effect experienced by the HSCs that received sorafenib at a concentration of 10 μM was more potent than that experienced by rats treated with sorafenib at concentrations of 2.5 and 5 μM. This inhibition effect remained pronounced when compared with those observed after pre-stimulation by PDGF-BB, indicating that sorafenib inhibited the proliferative activity induced by PDGF-BB.

Cell cycle analysis of T6 cells showed a decrease in G1 and M phase cells and an increase in S phase cells after sorafenib treatment for 24 h, with dose-dependent increases in the S population and decreases in G1 and M populations. A less noticeable effect on cell cycle distribution was observed in LX2 cells, with only an increase in the S phase in rats treated with 10.0 μM sorafenib (Fig. 4C).

Sorafenib causes progressive HSC apoptosis

TEM revealed that HSCs in the 10 μM sorafenib groups exhibited chromatin that appeared to be shrunken, aggregated and condensed along the inside of the nuclear membrane. The morphologies of the cell nuclei were crescent-shaped or spherical. Dilated endoplasmic reticulums were observed in apoptotic cells (Fig. 5A).

Sorafenib caused progressive cell apoptosis in both T6 and LX2 cells over a period of 24 h in a dose- and time-dependent fashion, as evaluated by flow cytometry (Fig. 5B). Similarly, the apoptotic rates of LX2 cells in groups treated with the same concentrations of sorafenib were also all found to be significantly elevated as compared with the apoptosis rate of the control group. LX2 cells

| Table 1. Impact of sorafenib on ALT, AST, ALB, and BIL in BDL rats (mean ± SD). |
|--------------------------|--------------------------|---------------------------|---------------------------|
| Group                   | ALT (U/L)                | AST (U/L)                 | ALB (g/L)                 | BIL (μmol/L) |
| SHAM                    | 35.09 ± 9.34             | 40.71 ± 3.94              | 33.22 ± 7.85              | 15.37 ± 0.93 |
| Four-week vehicle       | 126.72 ± 12.74           | 157.55 ± 18.97            | 23.09 ± 2.12              | 132.43 ± 2.60 |
| Sorafenib 20 mg/kg      | 135.30 ± 3.53            | 155.00 ± 13.78            | 25.76 ± 3.47              | 130.07 ± 3.08 |
| Sorafenib 40 mg/kg      | 133.02 ± 15.89           | 166.34 ± 5.72             | 25.18 ± 5.03              | 132.90 ± 5.41 |

Sorafenib treatment does not aggravate the changes in blood chemistry in BDL rats. ALT, alanine transaminase; AST, aspartate transaminase; ALB, albumin; BIL, bilirubin.

* p <0.05 compared with the SHAM group.

| Table 2. Impact of sorafenib on ALT, AST, ALB and BIL in DMN rats (mean ± SD). |
|--------------------------|--------------------------|---------------------------|---------------------------|
| Group                   | ALT (U/L)                | AST (U/L)                 | ALB (g/L)                 | BIL (μmol/L) |
| Zero week               | 4.45 ± 1.03              | 25.98 ± 4.08              | 30.39 ± 10.24             | 15.39 ± 1.10 |
| Four-week vehicle       | 77.80 ± 37.93            | 57.25 ± 4.45              | 23.09 ± 1.02              | 28.20 ± 6.44 |
| Sorafenib 1 mg/kg       | 56.89 ± 15.46            | 66.52 ± 1.49              | 27.98 ± 3.26              | 21.07 ± 5.3  |
| Sorafenib 5 mg/kg       | 93.18 ± 62.23           * | 115.13 ± 38.99           * | 22.52 ± 6.57              | 24.02 ± 11.5 |

In DMN-treated rats, 1 mg/kg sorafenib ameliorates the increase of ALT, BIL, and the decrease of ALB, while 5 mg/kg sorafenib aggravates the increase of AST and ALT levels significantly. ALT, alanine transaminase; AST, aspartate transaminase; ALB, albumin; BIL, bilirubin.

* p <0.05 compared to the 0-week group.
* p <0.05 compared to the vehicle-treated groups.
Fig. 3. Sorafenib inhibits the phosphorylation of ERK, Akt, and p70S6K in BDL- and DMN-treated rats. (A) Western blot analysis of the total- or phospho-ERK, Akt and p70S6K in BDL and DMN rats. The expression levels of phospho-ERK, Akt, and p70S6K are measured relative to the total-Akt, total-ERK and total-p70S6K, respectively. (B) Phospho-ERK, phospho-Akt, and phospho-p70S6K are stained by immunohistochemistry in liver tissue (magnification 200×). *p < 0.05 compared to the SHAM group or the 0-week group. #p < 0.05 compared to the vehicle-treated groups.
exhibited a higher apoptosis rate than that of T6 cells at the same time point (12 h). Sorafenib increased TUNEL staining in a dose-dependent manner in both cell lines (Fig. 5C). Sorafenib (10.0 μM) increased the percentage of TUNEL-positive cells from 9.44% and 15.96% in the controls to 59.29% and 67.66% in the treated T6 and LX2 cells, respectively.

**Sorafenib mediates cell cycle and apoptosis regulatory protein levels and Caspase-3 activity in HSCs**

Sorafenib reduced Cyclin D1 and Cdk-4 protein levels in both T6 and LX2 cells (Fig. 6A). Recent studies suggest that cell apoptosis induced by sorafenib involves the downregulation of anti-apoptotic Bcl-2 family proteins, and the upregulation of the pro-apoptotic proteins Fas, Caspases [21]. The exposure of HSCs to sorafenib significantly increased the levels of Fas, Fas-L and Caspase-3 and downregulated the ratio of Bcl-2 to Bax in both cell lines (Fig. 6B). Strikingly, the treatments with sorafenib (10 μM) significantly increased Caspase-3 activities suppressed by PDGF-BB in both T6 and LX2 cells (Fig. 6C).

**Sorafenib inhibits collagen synthesis and induces collagen degradation in HSCs**

Treatment with sorafenib markedly decreased collagen synthesis by [3H]-proline incorporation assay (Fig. 7A). Stimulation with PDGF-BB induced an increase of collagen synthesis, while sorafenib resulted in dose-dependent decreases in collagen synthesis in T6 and LX2 cells in the absence or presence of PDGF-BB. Sorafenib inhibited collagen α1 (I) mRNA expression in both cell lines and primary HSCs dose-dependently (Fig. 7B). Sorafenib significantly reduced intracellular type I collagen protein levels that had initially been increased by PDGF administration as compared to the levels observed in T6 and LX2 cells of DMSO groups (Fig. 7C).

Increased TIMP-1 expression was inhibited by sorafenib, with a significant reduction noted at the 10.0 μM dose level in T6 cells as well as at the 5.0 μM dose level in LX2 cells. Sorafenib had no effect on MMP-13 expression, but it increased the ratio of MMP-13/TIMP-1 in T6 cells (Fig. 7D). In contrast to the T6 cells, the expression of MMP-1 in LX2 cells was upregulated by different concentrations of sorafenib, and especially by the 5.0 μM dose. The ratio of MMP-1 to TIMP-1 was also increased in LX2 cells.

**Sorafenib inhibits ERK and Akt/p70S6K signaling pathways in HSCs**

In both of the PDGF-BB-stimulated cell lines, sorafenib inhibited ERK, Akt and p70S6K phosphorylation at concentrations ranging from 2.5 to 10 μM in T6 and LX2 cells. Total ERK, p70S6K and Akt levels were unchanged (Fig. 8A). Sorafenib, ERK inhibitor PD98059 and the PI3K inhibitor LY294002 in inhibiting their target pathways were verified by Western blot analysis for total and phosphorylated ERK, Akt and p70S6K. PD98059 and LY294002 were effective in inhibiting the phosphorylation of ERK and Akt/p70S6K, respectively. Sorafenib inhibited both the phosphorylated ERK and Akt/p70S6K (Fig. 8B).
Discussion

Liver fibrosis is believed to be reversible through the apoptosis of activated HSCs and degradation of ECM proteins [22]. Mejias et al. found that sorafenib treatment resulted in a reduction in portal pressure and angiogenesis in BDL rats [15]. Liver fibrosis induced by DMN is more similar to the mechanism involved in human liver fibrosis [23]. Our study showed that sorafenib had beneficial effects on intrahepatic fibrogenesis in two different experimental models of liver fibrosis: biliary fibrosis induced by BDL and toxic fibrosis induced by DMN. Interestingly, administration of sorafenib substantially decreased the extent of fibrosis, indicating that sorafenib exhibited in vivo antifibrotic effects that mitigated the liver fibrogenesis induced by BDL and DMN. Hydroxyproline, which is a major constituent of collagen, is known to be a good marker of ECM accumulation [24]. In our study, we showed that sorafenib markedly reduced the hydroxyproline content and downregulated the expression of type I collagen in the livers of fibrotic rats. The data demonstrate that sorafenib treatment inhibited the accumulation of ECM components in the livers of rats with liver fibrosis induced by BDL and DMN. In the present study, we found that serum markers of liver damage, such as ALT, AST and total bilirubin were not affected by sorafenib in BDL rats. In DMN-treated rats, lower...
Fig. 6. Sorafenib regulates Cyclin D1, Cdk-4, Bcl-2, Bax, Fas, Fas-L, and Caspase-3 levels in HSCs. (A and B) Western blot analysis of Cyclin D1, Cdk-4, Bcl-2, Bax, Fas, Fas-L, and Caspase-3 in HSCs. Control was treated with vehicle. GAPDH was used as a loading control. (C) Sorafenib increases Caspase-3 activities of T6 and LX2 cells. *p < 0.05 compared to vehicle. #p < 0.05 compared to PDGF group.
concentration of sorafenib (1 mg/kg) ameliorated the liver damage, while higher concentrations of sorafenib (5 mg/kg) aggravated the injury. Distinct from that in BDL rats, the process of liver fibrosis induced by DMN exhibited extensive hemorrhagic necrosis and lobular architecture with thin bands of reticulin joining central areas. DMN itself is a potent hepatotoxin, and lower doses of this chemical cause subacute and chronic liver injury with varying degree of hepatocyte necrosis and nodular degeneration. Therefore, we used lower doses of sorafenib rather than the doses used in BDL rats. We found that higher dosage of sorafenib (5 mg/kg) aggravated the liver damage induced by DMN. Our data also suggest a note of caution for the use of sorafenib in the treatment of liver fibrosis.

Many studies have demonstrated that sorafenib inhibits the proliferative activity of various tumor cells and induces their apoptosis by targeting the tyrosine kinase associated with PDGFR-β [25–27]. Similar to the mechanism above, in the present study, sorafenib is capable of inhibiting cellular proliferation activated by PDGF-BB in a dose-dependent manner in both cell lines. Suppression of proliferation and induction of apoptosis are accompanied by a downregulation of Cyclins and Cdk's [19,28]. We also found that sorafenib caused T6 and LX2 cells to

Fig. 7. Sorafenib reduces collagen synthesis in HSC T6 and LX2 cells. (A) Sorafenib reduces collagen synthesis as determined by [3H]-proline incorporation. (B) Sorafenib inhibits collagen α1 (I) mRNA expression increased by PDGF-BB in T6, LX2, and primary HSCs, as measured by real-time PCR analysis. (C) Sorafenib regulates the expressions of TIMP-1 and MMP-13 (MMP-1), as shown by Western blots analysis. GAPDH was used as a loading control. *p<0.05 compared to vehicle. #p<0.05 compared to PDGF group.
undergo arrested growth in the S phase of the cell cycle. This inhibition was also accompanied by a decrease in Cyclin D1 and Cdk-4, which may represent important downstream targets of sorafenib. These effects were similar to those reported previously in human esophageal adenocarcinoma and glioma cells [13,29]. The controlled activation of the kinase complexes at various intervals of the cell cycle is regulated by the availability of the Cyclins. The expression and stability of the regulatory subunit fluctuates depending on the stage of the cell cycle. In the present study, a more rapid entry into the S phase was accompanied by a downregulation of Cyclin D1 and Cdk-4, unlike the G1 arrest reported previously in HSCs [7]. One possible explanation could be that other cell cycle regulatory proteins, such as Cyclin D2, D3 or E, might be involved in regulating the cell cycle in HSCs during sorafenib treatment. With the inhibited proliferation and increased apoptosis, the total number of the HSCs decreases, and the increased percentage of S phase cells is compensatory. Nevertheless this compensatory raise of S phase cells is limited and the total DNA synthesis is decreased.

Fig. 8. Sorafenib reduces phosphorylation of ERK, Akt, and p70S6K in HSCs. (A) Western blot analysis of the total- or phospho-ERK, Akt, and p70S6K. Sorafenib abolishes the PDGF-BB-stimulated expression of phospho-ERK, phospho-Akt, and phospho-p70S6K. The expression levels of phospho-ERK, Akt, and p70S6K are determined relative to the total-Akt, total-ERK, and total-p70S6K, respectively. (B) Effects of treatment of HSCs with the ERK inhibitor PD98059, the PI3K inhibitor LY294002, and sorafenib on phosphorylated and total ERK, Akt, and p70S6K as measured by Western blot analysis. *p < 0.05 compared to vehicle. #p < 0.05 compared to PDGF group.
In addition to inhibiting cell proliferation, sorafenib was also associated with the induction of apoptosis in HSCs. In fact, augmentation of HSC apoptosis is known to promote the resolution of fibrosis [30–32]. The apoptosis rate of LX2 cells was higher than that of T6 cells at the same time point and sorafenib concentration, indicating that sorafenib had a more potent effect on LX2 cells than on T6 cells. We also observed that sorafenib-induced apoptosis might be dependent on increases in Caspase-3, Fas and Fas-L, and a decrease in the ratio of Bcl-2 to Bax via deregulation of the Akt and ERK pathways. Indeed, a decrease in HSC apoptosis is associated with a reduction in Caspase-3 and an increased ratio of Bcl-2 to Bax [9,33]. The induction of apoptosis by sorafenib through Caspases- and Bcl-2/Bax-dependent pathways has been reported previously in many tumor cells [13,34,35]. Fas and Fas-L were increasingly expressed during the course of HSC activation and spontaneous apoptosis [36]. A Fas-dependent pathway contributes to sorafenib-induced apoptosis in colon carcinoma and other tumors, which is in agreement with the results found in the present study [35].

In addition to expressing matrix proteins, particularly type I collagen, activated HSCs also express MMPs and TIMPs. Intact type I collagen promotes persistence of activated HSCs and the degradation of extracellular matrix appears to be critical to the induction of HSC apoptosis [37]. Our study shows that sorafenib significantly inhibits the expressions of type I collagen and TIMP-1, and consequently induces HSC apoptosis. Although interstitial collagenase MMP-13 activity was not modulated by sorafenib, the balance between MMP-13 and TIMP-1 was altered in T6 cells. In contrast to T6 cells, both interstitial collagenase MMP-1 and the ratio of MMP-1/TIMP-1 were elevated in LX2 cells. Our results show that sorafenib mediates a reduction in collagen deposition both directly, by reducing type I collagen protein synthesis, and indirectly, by decreasing TIMP-1 expression, thereby potentially enhancing extracellular matrix degradation.

PDGF stimulates the Raf/ERK and Akt/p70S6K signaling pathways during liver fibrogenesis [7,38]. As an inhibitor of PDGFR and Raf, sorafenib may be beneficial in the treatment of many human malignancies because it can inhibit the PDGF signaling cascade, including the ERK and Akt pathways [27,39]. The reduction in intrahepatic fibrosis induced by sorafenib was associated with the efficacious inhibition of the PDGFR cascade as well as the Raf/ERK and Akt/p70S6K signaling pathways in vivo, all of which are vital to collagen deposition and HSC activation. These results are similar to those found previously in rats exposed to CCl4 inhalation and treated with sunitinib [14]. The specific inhibitors of ERK, PD98059 and the inhibitor of Akt/p70S6K, LY294002, reduce the PDGFR-induced activation of these respective target pathways, and are associated with complete inhibition of HSC proliferation, apoptosis and collagen expression [9,40,41]. Sorafenib, by inhibiting the phosphorylation of ERK, Akt/p70S6K in vitro, had effects on the proliferation, collagen synthesis of HSC similar to those two inhibitors.

Sorafenib treatment is able to improve the histological changes and reduce ECM accumulation during liver fibrosis in vivo. Simultaneously, sorafenib ultimately alters the balance between collagen synthesis and degradation in HSCs in vitro. Furthermore, sorafenib inhibits HSC proliferation and induces HSC apoptosis. The relevant mechanism involves inhibiting the phosphorylations of ERK and Akt/p70S6K, blocking cell cycle regulatory proteins and anti-apoptotic proteins. These results provide evidence that sorafenib may be an attractive agent for the treatment of liver fibrosis.

Conflict of interest
The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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References

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