NP603, a novel and potent inhibitor of FGFR1 tyrosine kinase, inhibits hepatic stellate cell proliferation and ameliorates hepatic fibrosis in rats

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Abstract:
Background/Aims: Fibroblast growth factor 2 (FGF-2) and its main receptor FGFR1 have been shown to promote hepatic stellate cell (HSC) activation and proliferation. However, scant information is available on the anti-fibrogenic activity of FGFR1 inhibitors. The aim of this study was to assess the impact of a selective FGFR1 tyrosine kinase inhibitor NP603 on HSC proliferation and hepatic fibrosis. We demonstrated that rat primary HSCs secreted significant amounts of FGF-2, and its tyrosine phosphorylation of FGFR1 was attenuated by NP603. NP603 inhibited HSC activation by measuring the expression of α-smooth muscle actin (α-SMA) and the production of type I collagen using ELISA. Furthermore, NP603 (25 μM) in vitro strongly suppressed HSC growth induced by FGF-2 (10 ng/mL) and FCS. This effect correlated with the suppression of ERK activity and its downstream targets cyclin D1 and p21. In addition, PO NP603 (20 mg/kg/day) administration significantly decreased hepatic collagen deposition and α-SMA expression in CCl4-treated rats. Collectively, these studies suggest that selective blocking of the FGFR1-mediated pathway could be a promising therapeutic approach for the treatment of hepatic fibrosis.

Keywords FGFR1; Hepatic stellate cell; Hepatic fibrosis; NP603; Proliferation

Introduction
HSCs, the primary source of extracellular matrix (ECM) proteins, play a crucial role in liver fibrosis (14, 27). In prolonged liver injury, HSCs undergo a remarkable transformation from quiescence to α-smooth muscle actin (α-SMA)-positive, proliferative, fibrogenic and contractile myofibroblasts (activated HSCs) (14). Increased production and/or activity of cytokines, especially autocrine cytokines including TGF-β1, platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF), are critical for sustaining HSC activation (13, 25). Additionally, enhanced HSC proliferation is also a dramatic feature of the pathogenesis of liver fibrosis. It is mainly triggered by autocrine/paracrine activation of the signaling for mitogenic PDGF, ET-1 and FGF (10, 15, 25).

FGFs are important modulators of cellular proliferation, migration, and differentiation depending on cell type and tissue context (20, 30). The external FGF signal is transduced by recruitment of signaling proteins to the tyrosine auto-phosphorylation sites on the activated FGF receptors (FGFRs) and to linked docking proteins creating additional binding sites for further signaling molecules (23). Hence, FGFRs are master mediators of a broad spectrum of cellular and developmental processes, including apoptosis, proliferation and angiogenesis. Moreover, substances targeting the FGF/FGFR system have been shown have promise for treatment in animal cancer models. Blockage of FGFR pathway by using FGFR small-molecule inhibitors significantly reduced the growth and survival of cancer cells (12). FGFR1, known as the principal FGF-2 receptor, is widely expressed and is involved in cell-specific functions and associated pathologies (3, 5). A main FGFR1 binding partner, FGF-2, is a mitogen for HSCs, and overexpression of its receptors has been reported in human liver myofibroblasts (26). There is
evidence that FGF-2 induces chemotaxis and chemoinvasion by HSCs and may participate in the recruitment and activation of HSCs in acute liver injury (7, 11). In light of these observations, FGF signaling pathway could be an ideal target for anti-fibrotic therapeutic approaches. Recent reports demonstrated that treatment of rats with an inhibitor targeting the receptor kinases of FGF-2 resulted in the attenuation of pulmonary fibrosis (8). NP603, a novel inhibitor of FGFR, has high binding affinity for FGFR1 and inhibits FGFR1 tyrosine kinase activity (19). There is no information about the role of FGFR1 inhibitors in the regulation of fibrogenic responses in HSCs. In this study, we investigated the influence of NP603 on FGF-2-mediated signaling in purified rat HSCs as well as the effect of NP603 on the development of liver fibrosis induced by CCl4.

**Materials and Methods**

**Materials**

Cell culture medium, glutamine, and antibiotics were supplied by Gibco (Life Technologies, Cergy Pontoise, France). NP603 was from Merck (Darmstadt, Germany). Abs against α-SMA, cyclin D1, p21, p27, the phosphotyrosine Ab against FGF receptor-1 (Tyr766) and the corresponding non-phosphorylated types of total proteins were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Abs against p44/42 ERK1/2 and phospho-p44/42 ERK1/2 (Thr 200/Tyr 204) were from Upstate Biotechnology (Millipore, Bedford, MA, USA). FGF-2 and the MEK inhibitor PD98059 were purchased from R&D Systems (Minneapolis, MN).

**Isolation and culture of rat HSCs**

Primary HSCs were isolated from male Sprague–Dawley rats (200–250g) as previously described (18). The animal studies were approved by the local ethics committee of Sun Yat-Sen University. Cells in the upper cloudy layer were collected and were cultured in proline-free Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Assessment of activation was performed by α-SMA staining of cells. Semi-confluent activated HSCs with 2–4 passages were used for the experiments. In some of the experiments, cells were serum-starved for 24 h in DMEM with 0.2% FBS before treatment.

**Cell viability assay**

Cell viability analysis was assessed by 3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium (MTT) dye absorbance, using a commercially available kit (Roche) and executed according to the manufacturer’s instructions. Primary HSCs were plated at into 96-well plates at an initial density of 5 × 10^3, serum starved in 0.2% FBS-DMEM for 16 h followed by treatment under serum-free conditions, NP603 (5–100µm), or DMSO for 48 h. 125 µl of MTT solution (2 mg/mL in PBS) was added to each well and incubated at 37°C for 4h. Supernatant was removed and the cells were solubilized in DMSO (250 µl) for 30 min. The optical density was measured at 450 and 690 nm using a 96-well plate reader (Bio-Tek Instruments, NJ).

**Analysis of HSC proliferation and cell cycle**

Cells were seeded at a density of 5×10^3 cells per well in 24-well plates, incubated with serum-deprived DMEM for 24 h and then exposed to the desired experimental conditions. Control cultures received 0.2% FCS or 10% FCS. The proliferative capacity of HSCs was evaluated by
5-bromo-20-deoxyuridine (BrdU) assay according to the manufacturer's protocol (Amersham, Little Chalfont, England). Each treatment was given in triplicate. For cell cycle detection, HSCs were harvested, fixed in 70% ethanol for 30 min at -20°C and suspended in 500 μl PBS containing RNase A for 30 min at 4°C. Fixed cells were stained with PI (100 mg/l) before FACS (fluorescence-activated cell sorting) analysis as described elsewhere (24).

**Western blotting**

The cell lysates were centrifuged at 16,000 g for 5 min at 4°C, and aliquots containing 30 μg protein were used for Western blot analysis. Protein expression was quantified as previously described (28). Levels of target protein bands were densitometrically determined by using Quantity One® 4.6.2 (Bio-Rad). Variations in the density were expressed as arbitrary units (n=3) and β-actin was generally used as an internal control. Quantification with histograms is shown as the target protein/ internal control ratio.

**Immunoprecipitation**

Cells were washed twice with ice-cold PBS and then lysed with lysis buffer for 20 min at 4°C, and the protein content of the supernatant was determined by the bicinchoninic acid method (Pierce, Rockford, IL). Protein (500 μg) was immunoprecipitated at 4°C overnight using the polyclonal FGFR1 antibody (10 mg) and 40 ml of agarose-protein G (Roche Molecular Biochemicals). After being washed twice with lysis buffer, FGFR immune complexes were resolved by SDS-PAGE and immunoblotting was performed as described above.

**Enzyme-linked immunosorbent assay (ELISA)**

Freshly isolated HSCs were incubated in serum-starved DMEM overnight and the culture medium was replaced with new serum-starved DMEM and supernatant was harvested. FGF-2 concentrations were assayed using a sandwich ELISA Kit (R&D Systems, Wiesbaden-Nordenstadt, Germany). For measurement of secreted procollagen type I, after 24 h of serum starvation, HSCs were pre-incubated for one hour with or without NP603 before FGF-2 (10 ng/mL) was added. The secretion of procollagen type I by HSCs into the culture supernatant was assessed by ELISA using a commercially available kit (R&D Systems, Minneapolis, MN).

**Real-time polymerase chain reaction (PCR)**

Total RNA was obtained from rat primary HSCs and hepatic tissue using TRIzol Reagent (Invitrogen, Carlsbad, CA). Real-time RT-PCR was performed with the iScript™ One-Step reverse transcription (RT)-PCR Kit with SYBR® Green (BioRad) as described previously (9). The housekeeping gene 18S served as an internal control. The primers used were as follows: rat α1(I) procollagen mRNA: (Fw) 5’-catgttcaagcttttgagggact-3’, (Rv) 5’-gcagctgacttacatgct-3’; rat α-SMA: (Fw) 5’-gcatagaaacgggtgctgctgct-3’, 5’-gcagctgacttacatgct-3’; rat MMP13: (Fw) 5’-ggagagcccttcctcctc-3’, (Rv) 5’-tcagacgacactacttacctgc-3’; rat TIMP-1: (Fw) 5’-agccgtaggtgcccccaaa-3’, (Rv) 5’-aactctcgtgcgggcttg-3’.

**Plasmids and cell transfection**

Hemagglutinin (HA) epitope-tagged WT rat MEK1 cDNA (Upstate, Charlottesville, VA, USA)
was subcloned into a pcDNA3.1 expression vector (Invitrogen, CA, USA). Expression constructs for HA-tagged MEK1 have been described (16). The MEK1 expression construct was transiently transfected into cells using Lipofectamine 2000 (Invitrogen, CA, USA). The transfected cells were incubated in fresh growth medium for 24 h. M199 supplemented with 10% FBS and NP603 was added to the cells and HSCs were collected 24 h later. In these experiments, cells transfected with empty vector served as a control.

**CCl<sub>4</sub>-induced rat model of liver fibrosis**

The study was performed in adult male Sprague-Dawley rats. The research protocol was approved by the Institutional Ethics Committee. Hepatic fibrosis was induced by peritoneal injection of CCl<sub>4</sub> as described previously (18). All fibrotic experimental groups were injected intraperitoneally with 0.2 ml/100 g sterile CCl<sub>4</sub> in a 1:1 ratio with olive oil twice weekly. For the *in vivo* study, one group of five rats was administered CCl<sub>4</sub> for two weeks and then administered 40 mg/kg NP603 (Merck, Inc., NY) via oral administration daily for six weeks together with CCl<sub>4</sub> injection. In parallel, another group of five rats received citrate-buffered solution as a vehicle by gavage and also CCl<sub>4</sub> by peritoneal injection for eight weeks. After animal sacrifice, specimens of liver tissue were obtained and fixed in 10% neutral buffered formalin for histological analysis. The levels of serum transaminases and alanine aminotransferase (ALT) were assessed using commercially available assays (Biotron Diagnostics; Hemet, CA).

**Immunohistochemistry and assessment of hepatic collagen content**

Deparaffinized and blocked 5-μm liver sections were incubated with anti-α-SMA antibody (Dako, Carpinteria, CA) at the concentration of 1:100 using the EnVision system (Dako). Diaminobenzidine tetrahydrochloride was used as a peroxidase substrate, and sections were counterstained with hematoxylin and mounted on a cover slip. Hepatic collagen content was assessed both by Masson’s trichrome staining of liver sections and by hydroxyproline concentration. The positive stained area was quantified using the morphometry program NIS-Elements Advanced Research (Nikon, Tokyo, Japan). Hepatic hydroxyproline content was determined by colorimetric analysis according to a described method (4).

**Statistical analysis**

Statistical analysis was performed using Microsoft EXCEL software. Unless otherwise specified, the results relative to the number of experiments indicated are expressed as means ± SD. The statistical significance of differences was evaluated using the unpaired Student’s *t*-test. *P* values less than 0.05 were considered significant.

**Results**

**NP603 Attenuates tyrosine phosphorylation of FGFR1 in cultured HSCs**

Rat primary HSCs retained a fibroblastic morphology after 7 days cultured with FBS (Fig. 1A). Additionally, expression of α-SMA protein (Fig. 1B) and lost of retinol stores (Fig. 1C), widely accepted as reliable indicators of HSC activation, became apparent in plated HSCs. As shown in Fig. 1D-E, activated HSCs also express cell surface FGFR1 and secreted significant amounts of biologically active FGF-2, as it was detected at 6 h and continually increased for up to 48 h (Fig.
Before observing the effect of an FGFR1 tyrosine kinase inhibitor on HSC, we first assessed the cytotoxicity of NP603 in activated HSCs by MTT assay. NP603 at concentrations of less than 50μM and DMSO induced minimal cell death (Fig. 1F), whereas NP603 at concentrations of 75μM and 100μM significantly affected cell viability. Next the tyrosine kinase activity of FGFR1 and the expression level of FGFR1 protein were detected. The results shown in Fig. 1G clearly indicate that isolated HSCs displayed constitutive activation of FGFR1, whereas NP603 efficiently down-regulated phosphorylation of FGFR1 in a dose-dependent manner. This suggested that NP603 has a potential inhibitory effect on the phosphorylation of FGFR1 in HSCs.

NP603 inhibits HSC activation and FGF-2-induced proliferation in HSCs

The effects of FGFR1 inhibition on the protein expression of α-SMA were investigated in HSCs to measure HSC activation. The results revealed that the expression of α-SMA was almost negligible in quiescent HSCs (day 0) but markedly increased after 7-16 days of culturing, and this increase was impeded by NP603 (Fig. 2A).

To test the FGF-2-regulated proliferative activity of HSCs, FCS was used as a positive control. FGF-2 (10 ng/mL) significantly increased the proliferation of rat HSCs, and this was reduced by pre-treatment with NP603, as expected (Fig. 2B). We next examined the effects of FGFR1 inhibition on the activity of ERK. Western blot analysis clearly indicated FBS-induced activation of ERK, and NP603 dose-dependently reduced the level of phosphorylated ERK, which increased significantly following the addition of FGF-2 (Fig. 2C). These results suggest that NP603 might block FGF-2-induced activation of the ERK signaling pathway, and they confirmed the ability of NP603 to interrupt FGF-2 signaling in activated HSCs.

Influence of NP603 on the cell cycle and the activity of cell cycle-signaling proteins in cultured HSCs

Flow cytometric analysis of DNA content was performed to investigate whether NP603 blocks the cell-cycle progression of HSCs. The proportion of cells in either S-phase or G2/M-phase was reduced by NP603 compared with the proportion of HSCs maintained in FGF-2-induced conditions (Fig. 3B). Western blot analyses suggested that the abundance of cyclin D1 at 12, 24 and 48 hours treated with NP603 was markedly reduced by 49%, 81% and 78% as compared with that at 0h, whereas p21 was increased by 65%, 73% and 240% at 12, 24 and 48 hours (Fig. 3C). But NP603 had no evident effect on p27. Together, these results indicate that NP603 inhibits the growth of activated HSCs by regulating cell cycle signaling proteins.

Involvement of ERK in the growth-inhibitory effect of NP603 on activated HSCs

To evaluate the role of the ERK signaling pathway in the inhibition of HSC growth by NP603, semi-confluent HSCs were pretreated with or without the specific MEK inhibitor PD98059 (PD) before the addition of NP603. As demonstrated in Fig. 4A, NP603 with PD pretreatment at different doses caused a further significant reduction in cell proliferation via BrdU incorporation assay. Furthermore, it was observed that the treatment of HSCs with PD similarly led to the down-regulation of cyclin D1 and the up-regulation of p21 in a dose-dependent pattern (Fig. 4B).

Further experiments were aimed at the role of the ERK pathway in the regulation of the cell cycle and the activity of cycle-related proteins by NP603. We transfected cDNA encoding MEK1 into
HSCs, and there was a marked increase in the protein expression of p-ERK in MEK1-transfected cells as shown in Fig. 5A. Activation of the MEK/ERK pathway is required for cell cycle progression through regulation of cyclin D1 and p21 in some cell lines (6, 17). We confirmed that the influence of NP603 on the protein expression of cyclin D1 and p21 in HSCs was dramatically abrogated by MEK1 overexpression (Fig. 5C), which lead to a reversal of the NP603-mediated DNA synthesis decrease (Fig. 5B). Thus, these results implied that inhibition of activated HSC growth by NP603 might be partially mediated through blockade of ERK activity and its downstream targets cyclin D1 and p21.

**Effect of NP603 on collagen type I accumulation in the culture medium and α1(I) procollagen mRNA expression**

To examine whether NP603 has an anti-fibrotic effect on HSCs, we assessed the impact of NP603 on the synthesis and mRNA expression of collagen type I. As shown in Fig. 6A, the levels of α1(I) procollagen mRNA expression were inhibited by NP603 compared to the control, with a maximal effect at 50 μM. Pretreatment with NP603 significantly reduced FGF-2-induced synthesis of procollagen type I in cell supernatant, but NP603 did not affect collagen secretion from HSCs incubated in DMEM with 10% FCS (Fig. 6B).

**NP603 mitigates CCl₄-mediated liver fibrogenesis**

Given the above findings, we next asked whether NP603 could reverse liver fibrogenesis in vivo following CCl₄ administration. Serum ALT concentrations were elevated by CCl₄ challenge, but they decreased after treatment with NP603 (Fig. 7A). Moreover, determination of the hydroxyproline content in the liver revealed a significant decrease in collagen accumulation in CCl₄-treated rats that received NP603 compared to rats challenged with CCl₄ alone (Fig. 7B). This result was further confirmed by the detection of liver fibrosis using Masson’s trichrome stain. Liver tissue morphometry after trichrome staining demonstrated that NP603 (40 mg/kg/day) treatment significantly decreased hepatic ECM deposition (Fig. 7C-D). CCl₄ resulted in a marked increase of collagen Iα (I) and MMP13 mRNA, which were suppressed in NP603-treated animals (Fig. 7E). However, TIMP1 was not reduced by NP603. Taken together, these data show the therapeutic effect of NP603 on CCl₄-induced hepatocellular damage.

**NP603 inhibits activation of HSCs in a CCl₄-induced liver fibrosis rat model**

As demonstrated in Fig. 8A, sustained CCl₄ administration induced a strong upregulation of hepatic α-SMA protein and mRNA in vivo as compared with untreated groups. NP603 treatment reduced α-SMA immunoreactivity and mRNA expression by 67% and 70%, respectively (Fig. 8B-C). Thus, these findings indicate that NP603 efficiently reduces the number of activated HSCs in vivo.

**Discussion**

FGFs stably bind a family of four receptor tyrosine kinases (RTKs) designated the high affinity FGF-receptors FGFR1–FGFR4. Previous studies suggested that several RTKs, which mediate many of the stellate cell’s responses to cytokines, are broadly up-regulated during liver injury (2). NP603, similar to the multi-targeted RTK inhibitor SU6668 in structure, exhibits potent inhibition of FGF-2-induced proliferation of endothelial cells through FGFR1 binding (19). In this study, we
found that NP603 inhibits FGFR1 tyrosine kinase activity by reducing the phosphorylation levels of FGFR1. We did not evaluate the potential effect of NP603 on the other three FGF-receptors. Although FGFR inhibitors as promising therapeutic agents exert a direct effect on tumor cell growth and survival (21, 22), their role in the regulation of HSC biology and their fibrogenic potential are largely unknown. Here we found that NP603 displays a strong suppressive effect on HSC activation, proliferation and ECM production both in vitro and in vivo.

In addition to the growth inhibition of HSCs by NP603, our results demonstrated that ERK1/2 activity was upregulated in response to FGF-2 and that this effect was clearly reduced by NP603. Prior reports indicated that suppressive effects on HSC proliferation are mediated partially through cyclin D1 and the cyclin-dependent kinase inhibitors p27 and p21 (1, 28). Consistent with these data, the impact of NP603 on cyclin D1 and p21 expression might make an important contribution to the inhibition of cell proliferation. However, additional studies are necessary to elucidate the mechanisms by which FGFR1 inhibitors exert an inhibitory effect on HSC growth. We also explored whether ERK1/2 activated by FGF-2 is required for the regulation of p21 and cyclin D1.

Our data showed that over-expression of MEK1 abolished the NP603-stimulated alteration of cyclin D1 and p21 as well as DNA synthesis in HSCs. These results indicate that the inhibition of HSC proliferation by NP603 of partly involves the ERK signaling pathway and its regulation of cell cycle-related proteins. Another important finding of this study is the depressive effect of NP603 on α-SMA expression, a marker of HSC activation, both in vitro and in vivo. These results conflict with a study that showed FGF1(-/-)FGF2(-/-) mice exhibited a normal increase in α-SMA and desmin associated with activation and migration of HSCs to damage (31).

In this set of experiments, additional potential anti-fibrogenic properties of NP603 were evaluated via both in vitro studies and in vivo animal models. Although serum-free medium did not affect collagen synthesis and secretion in HSCs, we observed that in vitro, NP603 suppressed type I collagen mRNA and protein production by HSCs induced with FGF-2. This effect is consistent with the in vivo observation showing that NP603 PO administration can significantly attenuate CCl4-induced hepatic fibrosis in rats. This observation is supported by the finding that chronic hepatic fibrosis is markedly reduced in FGF1/FGF2-deficient mice (31). Interestingly, NP603 at even higher concentrations (50 μM) had no effect on the synthesis of collagen type I induced by FCS in vitro. Several studies have also suggested that autocrine FGF-2 is the principal cytokine responsible for fibronectin and plays a central role in the pathogenesis of bone marrow fibrosis and pulmonary fibrosis (3, 8). However, a contribution from other cell types in the liver such as hepatocytes cannot be excluded in the net anti-fibrotic effect of NP603. Some reports suggest that FGFR signaling pathways in hepatocytes are crucial regulators of liver regeneration (29). They also showed that blocking secretion of FGF-2 may inhibit survival pathways of hepatocytes and promote liver injury. Therefore, the exact mechanisms of effective anti-fibrotic agents that target the FGF-2 signaling pathway merit further investigation.

In summary, the present study demonstrates that the FGFR1 inhibitor NP603 effectively attenuates phosphorylation of FGFR1 in HSCs and prevents HSC activation. Moreover, it inhibits proliferation of activated HSCs induced by FGF-2, in part by blocking ERK activity and its targets cyclin D1 and p21. Our in vivo experiments demonstrated that NP603 significantly ameliorates CCl4-induced hepatic fibrosis in a rat model. Although further research in this area is needed, our findings position selective FGFR1 inhibitors as potential HSC-targeted drug candidates for the treatment of liver fibrosis.
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References:


13. Friedman SL. Cytokines and fibrogenesis. 


**Figure Legends**

**Fig. 1.** (A-C) Characterization of isolated rat HSCs (Cultured for 7 days). (A) Phase-contrast micrographs (with a ×10 objective), (B) α-SMA expression (red) and (C) UV autofluorescence of retinoid (with a ×20 objective). Light and fluorescence micrographs are representative of 3 independent experiments. (D) Immunohistochemical staining for FGFR1 (indicated by arrows, with a ×20 objective) was demonstrated in culture-activated HSCs. (E) FGF-2 is secreted by HSCs during *in vitro* activation. Freshly isolated HSCs were cultured on 6-well plates, and supernatants were harvested at the indicated time points. FGF-2 accumulation was evaluated by ELISA. Data represent the mean ± SD (n=3). (F) Cytotoxicity by NP603 were tested in activated HSCs. HSCs were treated with various concentrations (0-100 μM) of NP603 for 24 h in a serum-free condition. Cytotoxicity was assessed by MTT assay. Data represent the mean ± SD of 3 independent experiments performed in duplicate. (G) Effect of an FGFR1-targeted inhibitor on FGFR1 phosphorylation in passaged HSCs. HSCs were treated with NP603 for 30 min. Cell lysates were immunoprecipitated for FGFR1. After immunoblotting with a phosphotyrosine-specific antibody (PTyr), the membranes were re-probed to control for FGFR1 protein levels. Representative blots were from three independent experiments. *P < 0.05 vs. NP603 0 μM.

**Fig. 2.** Impact of NP603 on HSC activation and proliferation. (A) Primary rat HSCs were freshly isolated and were cultured for 7-16 days. Then the primary cells at day 16 were incubated with various doses of NP603 for 24 h. The expression of activated HSC marker α-SMA was analyzed by Western blot. The results of a representative study are shown (n=3). *P <0.05 vs. 0d; &P <0.05 vs. 16d. (B) Serum-deprived primary HSCs were cultured with or without NP603 (25 μM) in the presence of FGF-2 (10 ng/mL) or FCS (10%) for 48 hours. DNA synthesis was evaluated as incorporation of BrdU in 60% confluent cells. Data are presented as fold-increase (mean ± SD, n=5) over controls (non-stimulated cells). (C) NP603 inhibits FGF-2-induced ERK activation. Serum-deprived HSCs were treated with various concentrations of NP603 for 10 minutes in the presence of FGF-2 (10 ng/mL) or not. Cell extracts were analyzed by Western blots for p-ERK and ERK. Results are representative of three independent experiments (n=3). In (B) and (C), *P <0.05 vs. controls or none; ^P <0.05 vs. FGF-2; #P <0.05 vs. FCS.

**Fig. 3.** NP603 induces cell cycle arrest at G1 in HSCs. (A) Serum-starved cells were exposed to FGF-2 (10 ng/mL) or FCS (10%) with or without NP603. After 24 h, the distribution of HSCs in the cell cycle was determined by flow cytometry using PI staining (n=3). FACS plots are representative of 1 of 3 experiments of identical design. (B) An illustration of the FACS data, values are means±SD of three independent experiments. (C) The effects of NP603 treatment on
cyclin D1, p21 and p27 protein expression in HSCs. Whole cell protein extracts were prepared from pre-confluent HSCs treated with or without NP603 for 24 h for Western blotting analyses. β-actin was used as an internal control and the results of a representative study are shown (n=3). *P < 0.05 vs. 0h.

**Fig. 4.** Involvement of the ERK pathway in the inhibition of activated HSCs by NP603. (A) Blocking the ERK pathway enhanced the growth-inhibitory effect of NP603 on activated HSCs. Serum-starved HSCs were pretreated with or without PD98059 (10-40 μM) for 30 min before the addition of NP603 (10 μM) to the medium with FBS (10%) for an additional 24 h. Cell proliferation was evaluated by BrdU incorporation into cDNA of cultured HSCs (n=3). (B) Western blot analysis of the effects of PD98059 treatment on the regulation of cyclin D1 and p21. Values of BrdU incorporation were presented as fold changes (mean ± SD). Representative blots were from three independent experiments. *P < 0.05 and **P <0.01 vs. controls or 0 μM.

**Fig. 5.** Overexpression of MEK1 enhances the proliferation of NP603-induced HSC growth. (A) Phosphorylated ERK was overexpressed in HSCs transfected with a cDNA encoding MEK1. HSCs were transiently transfected with a control vector (pcDNA 3.1) or a vector coding for constitutively active MEK1 followed by NP603 (25 μM) treatment for 24 h. Over-expression of MEK1 abrogated the NP603-induced decrease of BrdU incorporation (B) apparently by reversing the NP603-induced changes in cyclin D1 and p21 (C). Representative blots were from three independent experiments. $P < 0.05 vs. controls or pcDNA; **P < 0.05 vs. NP603.

**Fig. 6.** Impact of NP603 on type I collagen accumulation from HSCs. Serum-deprived HSCs were stimulated with FGF-2 (10 ng/ml) or 10% FCS for 24 h in the absence or presence of NP603 (10-50 μM). (A) The de novo synthesis of procollagen type I by activated rat HSCs was determined in rat HSC supernatants by ELISA (n=3). (B) Quantification of α1(I) procollagen mRNA levels. HSCs grown in medium containing 10% FBS were treated with or without NP603 for 24 h. α1(I) procollagen gene expression was normalized to 18S mRNA values. Values were presented as fold change (mean ± SD, n=3). *P <0.05 vs. control groups; $P <0.05 vs. DMEM+FGF-2.

**Fig. 7.** NP603 attenuates CCl₄-induced liver fibrosis. CCl₄-treated rats with hepatic fibrosis were treated either with vehicle or NP603 (40 mg/kg/d) for six weeks. We determined (A) serum ALT levels and (B) hepatic hydroxyproline content in liver samples. (C-D) Measurement of liver fibrosis with Masson’s trichrome staining. The area of positive fibrosis was quantified by morphometry in ten high-power fields. (E) Levels of mRNA for collagen 1α (I), MMP13 and TIMP1 in rat livers were measured by real-time PCR (n=5). Data represent the mean±SD from five slides (animals). $P <0.05 vs. untreated; *P < 0.05 vs. CCl₄+vehicle.

**Fig. 8.** Effects of NP603 on HSC activation in CCl₄-treated rats. (A) Liver sections from representative rats were immunostained for α-SMA. (B) Quantification of α-SMA stained area
was done by morphometry in ten high-power fields. *$P < 0.05$ vs. CCl$_4$+vehicle. (C) $\alpha$-SMA mRNA levels in rat livers were assessed by real-time PCR (n=5). Data represent the mean±SD from five slides (animals). *$P < 0.05$ vs. CCl$_4$+vehicle.
Figure 1

A, B, C, and D: Photos showing different cellular or tissue samples.

E: Graph showing FGF-2 secretion (ng/10^6 cells) over hours (0h, 6h, 12h, 24h, 48h, 72h).

F: Graph showing viability (%) of cells treated with DMSO and NP-603 over concentration (0-100 μM).

G: Western blot analysis showing PTyr and FGFR1 expression levels with NP603 treatment (0, 10, 25, 50 μM).
Figure 2

A

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**α-SMA**

**β-Actin**

B

BrdU incorporation (% of control)

C

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**p-ERK/ERK**

**Protein expression (arbitrary units)**
Figure 3

A

10% FBS

0 μM

10 μM

25 μM

50 μM

B

Percent Cells in Phase of Cell Cycle

FBS

0

10

25

50

NP603(μM)

FGF-2(10ng/ml)

C

NP603(25μM)

Time

0h

12h

24h

48h

Cyclin D1

p21

p27

β-Actin

Protein expression (arbitrary units)

NP603

0h

12h

24h

48h

CyclinD1/β-Actin

p21/β-Actin

p27/β-Actin
Figure 4

A

BrdU incorporation (% of control)

control 0 10 20 40 PD98059 (μM)
NP603 (10 μM)

B

Protein expression (arbitrary units)

0 10 20 40 PD98059 (μM)
Cyclin D1
p21
β-Actin

PD98059

CyclinD1/β-Actin
p21/β-Actin

* *
Figure 5

A

Parent pcDNA HA-MEK1

p-ERK

ERK

B

BrdU incorporation (% of control)

control pcDNA NP603 HA-MEK1+NP603

0 20 40 60 80 100 120

C

Control pcDNA NP603 HA-MEK1+NP603

Cyclin D1

p21

β-Actin

Protein expression (arbitrary units)

Cyclin D1/β-Actin p21/β-Actin

Control pcDNA NP603 HA-MEK1+NP603

$**$
Figure 7

A

ALT (U/l)

untreated  CCl4+vehicle  CCl4+NP603

B

Hydroxyproline (μg/mg liver)

untreated  CCl4+vehicle  CCl4+NP603

C

Vehicle  CCl4+Vehicle  CCl4+NP603

D

Fibrotic positive area (%)

untreated  CCl4+vehicle  CCl4+NP603

E

mRNA fold change vs. 18S

untreated  CCl4+vehicle  CCl4+NP603

α1(I) procollagen  MMP13  TIMP1