

# Inonotsuoxide B suppresses hepatic stellate cell activation and proliferation via the PI3K/AKT and ERK1/2 pathway

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**Abstract.** Hepatic stellate cells (HSCs) serve a pivotal role in the formation and degradation of the extracellular matrix during liver fibrosis. Inonotsuoxide B is a tetracyclic triterpenoid that can be extracted from *Inonotus obliquus* and has been previously reported to inhibit the growth of liver and gastric cancer cells. However, its effect on liver fibrosis remain poorly understood. Therefore, in the present study, the potential antiproliferative effects of inonotsuoxide B on HSCs was investigated. Initially, cells were divided into the following five groups: Control; platelet-derived growth factor (PDGF)-BB (10 ng/ml); and PDGF-BB + inonotsuoxide B (5, 10 and 20  $\mu$ g/ml) groups. Inonotsuoxide B treatment (5, 10 and 20  $\mu$ g/ml) was revealed to reverse PDGF-BB-induced HSC proliferation. Furthermore, the protein expression of  $\alpha$ -smooth-muscle actin ( $\alpha$ -SMA) and type I collagen was significantly decreased in the inonotsuoxide B (10 and

20  $\mu$ g/ml) groups compared with the PDGF-BB group. Inonotsuoxide B (5, 10 and 20  $\mu$ g/ml) was also revealed to suppress PDGF-BB-induced  $\alpha$ -SMA mRNA expression and activation of the PI3K/AKT and ERK signaling pathways in HSCs. These findings suggest that inonotsuoxide B suppresses the proliferation and activation of HSCs by inhibiting the PI3K/AKT and ERK1/2 signaling pathways.

## Introduction

Hepatic fibrosis (HF) is characterized by the abnormal secretion and degradation of the extracellular matrix (ECM) in the liver that may be caused by a number of different factors, including viral infection, alcohol consumption and drug abuse (1). HF is considered to be a healing reaction to liver injury, which if occurs in excess, can eventually develop into cirrhosis (2). The majority of pharmacological agents that are currently being investigated have been suggested to exert weak antifibrotic and also certain hepatoprotective effects (3). In particular, inhibition of hepatic stellate cell (HSC) proliferation is considered to be an important component for both preventing and subsequently treating HF, as in chronic liver injury HSCs are activated, proliferate and produce abundant ECM, thereby differentiating into myofibroblasts (4).

*Inonotus obliquus*, commonly known as Chaga, is a medicinal fungus (order, Hymenochaetales; division, Basidiomycota) that primarily grows on birch in the temperate regions or cold northern climates (5). *Inonotus obliquus* has been previously reported to exhibit anticancer, antiaging and hepatoprotective effects (6). In recent years, there has been an increasing number of studies exploring the clinical application of *Inonotus obliquus* and determining its biologically active ingredients (7). Inonotsuoxide B is considered to serve a role in mediating the effects of *Inonotus obliquus* and can be isolated by silica, octadecylsilane and Sephadex LH-20 column chromatography as white, needle-like crystals (8). The structure and composition of inonotsuoxide B have been previously confirmed by mass

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*Abbreviations:* HSCs, hepatic stellate cells; PDGF, platelet-derived growth factor; COL-I, type I collagen;  $\alpha$ -SMA,  $\alpha$ -smooth-muscle actin

*Key words:* liver fibrosis, hepatic stellate cells, *Inonotus obliquus*, inonotsuoxide B, platelet-derived growth factor

spectrometry,  $^1\text{H}$ - and  $^{13}\text{C}$ -nuclear magnetic resonance spectral data, where inonotsuoxide B has been referenced previously in published literature as a tetracyclic triterpenoid (8). Although tetracyclic triterpenoids have been previously suggested to potentially inhibit the proliferation of hepatocytes and gastric cancer cells, the potential antifibrotic effects of this compound remain poorly understood. Therefore, the present study examined the *in vitro* effects of inonotsuoxide B isolated from *Inonotus obliquus* on platelet-derived growth factor (PDGF)-BB-induced HSC proliferation and activation.

## Materials and methods

**Cells and reagents.** Inonotsuoxide B was provided by Dr. Wenming Cheng, Department of Natural Medicine and Chemistry, School of Pharmacy, Anhui Medical University (Hefei, China). The rat hepatic stellate cell line HSC-T6 was purchased from Jiangsu KeyGEN BioTECH Corp., Ltd. PDGF-BB was obtained from PeproTech China. TRIzol<sup>®</sup> reagent was purchased from Thermo Fisher Scientific, Inc. (cat. no. 14050) and TB Green<sup>™</sup> Premix Ex Taq<sup>™</sup> II kit was purchased from Takara Biotechnology Co., Ltd. (cat. no. AH80340A). The following antibodies were used: Anti- $\alpha$ -smooth-muscle actin ( $\alpha$ -SMA; cat. no. bs-10196R; lot. no. AG0734630), anti-collagen I (COL-I; cat. no. bs-10423R; lot no. AE120941; both from BIOSS), anti-AKT (cat. no. ESAP12208; Elabscience, Inc.), anti-phosphorylated (p)-AKT (cat. no. AF0016; lot no. 17q2826; Affinity Biosciences), anti-PI3K (cat. no. bsm-33219M; lot no. AH01181961; BIOSS), anti-phosphorylated (p)-PI3K (cat. no. ab138364; lot no. GR154304-8; Abcam), anti-p-ERK (cat. no. WL2201; lot no. I09051512), anti-ERK (cat. no. WL02371; lot no. I04102371; both from Wanlei Biological Technology Co., Ltd.), anti- $\beta$ -actin (cat. no. TA-09; lot no. 19C10511; OriGene Technologies, Inc.), horseradish peroxidase (HRP)-conjugated anti-rabbit (cat. no. S0001; lot no. 56j9958; Affinity Biosciences) and HRP-conjugated anti-mouse (cat. no. ZB-2305; lot no. 193701224, OriGene Technologies, Inc.).

**Cell culture and drug preparation.** The purchased frozen HSC-T6 cells were transferred to DMEM (Nanjing Wisent Biological Co., Ltd.) containing 5% FBS (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd.) and cultured at 37°C with 5% CO<sub>2</sub>. The culture medium was replaced every 2 days and cells were sub-cultured every 2-3 days. Following three passages, cells in a logarithmic growth phase were used for subsequent experiments.

Solid inonotsuoxide B crystals were dissolved in DMSO at a concentration of 1x10<sup>5</sup>  $\mu\text{g/ml}$ .

**Cell treatment.** HSC-T6 cells were collected and seeded into a 96-well culture plate at a concentration of 1x10<sup>4</sup> cells/well or HSC-T6 cells were seeded at a concentration of 1x10<sup>5</sup>/ml into a six-well culture plate and incubated in DMEM containing 5% FBS and cultured at 37°C with 5% CO<sub>2</sub> (9). The cells were then divided into the following five groups: Control, PDGF-BB (10 ng/ml) and PDGF-BB + inonotsuoxide B (5, 10 and 20  $\mu\text{g/ml}$ ). Following culture for 24 h at 37°C, the medium was changed to serum-free DMEM and appropriate concentrations

of inonotsuoxide B were added to the PDGF-BB + inonotsuoxide B groups. After 30 min, apart from the control group that did not receive any treatment, all other groups were treated with PDGF-BB.

The experimental groups used for treatment with the PI3K inhibitor LY294002 were as follows: Control, PDGF-BB (10 ng/ml), PDGF-BB + inonotsuoxide B (20  $\mu\text{g/ml}$ ) and PDGF-BB + LY294002 (20 nmol/l). HSC-T6 cells were seeded at a concentration of 1x10<sup>5</sup>/ml into a six-well culture plate and incubated at 37°C with 5% CO<sub>2</sub>. Following culture for 24 h at 37°C, the medium was changed to serum-free DMEM and inonotsuoxide B (20  $\mu\text{g/ml}$ ) and LY294002 (20 nmol/l) were added to PDGF-BB + inonotsuoxide B group and PDGF-BB + LY294002 group, respectively. Following incubation at 37°C for 30 min, all groups received PDGF-BB treatment except for the control group that did not receive any treatment. Following incubation for 24 h at 37°C, the cells were lysed in RIPA lysis buffer. The experimental groups used for treatment with the ERK inhibitor UO126 were as follows: Control, PDGF-BB (10 ng/ml), PDGF-BB + inonotsuoxide B (20  $\mu\text{g/ml}$ ) and PDGF-BB + UO126 (20 nmol/l). The aforementioned experimental method was followed.

**Cell cytotoxicity measurement.** HSC-T6 cells were collected and seeded into a 96-well culture plate at a concentration of 1x10<sup>4</sup> cells/well (8) and divided into seven groups: Control, inonotsuoxide B (5, 10, 20, 40 and 80  $\mu\text{g/ml}$ ) and DMSO (0.1  $\mu\text{l}$ ). A total of four duplicate wells were used for each group, with 100  $\mu\text{l}$  cell suspension per well. The outer wells were filled with 100  $\mu\text{l}$  PBS and the plate was incubated in an incubator at 37°C with 5% CO<sub>2</sub> for 8 h. Following attachment of the cells, the medium was replaced with serum-free DMEM. DMSO was added to the DMSO groups, whilst solutions of inonotsuoxide B at the stated concentrations were added to the cells designated to the inonotsuoxide B groups. Following 24 h, the culture medium was discarded and 20  $\mu\text{l}$  MTT solution (5 mg/ml) were added to each well. Following incubation at 37°C for 4 h, the supernatant was discarded and 150  $\mu\text{l}$  DMSO were added to each well and incubated on a shaker at 37°C for 10 min. The absorbance of each well was measured at 570 nm (10).

**Cell viability assay.** Before the effect of inonotsuoxide B on cell viability in the presence of PDGF-BB was determined using the MTT assay, HSC-T6 cells were collected. The cells were then divided into the following five groups: Control, PDGF-BB (10 ng/ml) and PDGF-BB + inonotsuoxide B (5, 10 and 20  $\mu\text{g/ml}$ ). The PDGF-BB + inonotsuoxide B groups were treated with corresponding concentrations of inonotsuoxide B for 30 min at 37°C. Thereafter, all groups except for the control were treated with 10 ng/ml PDGF-BB at 37°C for 24 h. The MTT assay experimental procedure was performed as aforementioned. Cell viability or cytotoxicity calculation formula: Cell viability=[(mean absorbance sample-mean absorbance blank)/(mean absorbance control-mean absorbance blank)]\*100.

**Western blot analysis.** HSC-T6 cells were seeded at a concentration of 1x10<sup>5</sup>/ml into a six-well culture plate and incubated at 37°C with 5% CO<sub>2</sub>. The cells were then divided

into the following five groups: Control, PDGF-BB (10 ng/ml) and PDGF-BB + inonotsuoxide B (5, 10 and 20  $\mu\text{g/ml}$ ). The processed cells were lysed in RIPA lysis buffer (Beyotime Institute of Biotechnology). Protein concentration was measured using the bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology) and the samples (50  $\mu\text{g}$ ) were subjected to 10% SDS-PAGE (11,12) and then transferred onto PVDF membranes, which were blocked with 5% skimmed milk at 25°C for 3 h. Subsequently, rabbit anti-AKT, rabbit anti-p-ERK, rabbit anti-p-PI3K, rabbit anti-p-AKT, rabbit anti-ERK, rabbit anti-PI3K, rabbit anti- $\alpha$ -SMA, rabbit anti-COL-I and mouse anti- $\beta$ -actin (all diluted 1:1,000) were added to the membranes and incubated at 4°C overnight. The membranes were then incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit or HRP-conjugated anti-mouse (all diluted 1:1,000) the following day for 1 h at room temperature, before the ECL chromogenic solution (Pierce; Thermo Fisher Scientific, Inc.) was added to develop the membrane (13). Finally, ImageJ software (version 1.8.0\_101; National Institutes of Health) was used for the quantification of densitometry.

**Reverse transcription-quantitative PCR (RT-qPCR).** HSC-T6 cells were divided into the following five groups: Control, PDGF-BB (10 ng/ml) and PDGF-BB + inonotsuoxide B (5, 10 and 20  $\mu\text{g/ml}$ ). Cells in each group were collected and total RNA was extracted using TRIzol<sup>®</sup> reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Briefly, 1 ml lysis buffer was added to the cells and incubated for 5 min at room temperature. Subsequently, 200  $\mu\text{l}$  CHCl<sub>3</sub> was added, mixed with the cell extract via vigorous shaking and the solution was incubated for 10 min at room temperature. The samples were centrifuged (14,800 x g) at 4°C for 30 min. The supernatant (400  $\mu\text{l}$ ) was collected and added to an equivalent volume of isopropyl alcohol. The mixture was stored at -20°C for 2 h and centrifuged (14,800 x g) at 4°C for 30 min. Subsequently, the supernatant was discarded and 1 ml of ethanol was added to each sample, which were centrifuged (14,800 x g) at 4°C for 15 min. RNA was collected following air-drying for 10 min before 20  $\mu\text{l}$  RNase-free water was added to dissolve the collected RNA, which was stored at -80°C until subsequent analysis (14). According to the manufacturer's protocol, cDNA was synthesized by reverse transcription using Takara PrimeScript RT Master Mix kit (Takara Bio, Inc.). The temperature protocol was as follows: 15 min at 37°C; 5 sec at 85°C; and 10 min at 4°C. qPCR was performed using SYBR<sup>®</sup>-Green Master Mix (Takara Bio, Inc.) according to the manufacturer's protocol. qPCR amplification was performed using  $\beta$ -actin as an internal reference. The following primer sequences were used:  $\beta$ -actin forward, 5'-CCGAGATCTCACCGACTACC-3' and reverse, 5'-TCCAGAGCGACA TAGCACAG-3' and  $\alpha$ -SMA forward, 5'-TCCTCCTGAGCG CAAGTACTCT-3' and reverse, 5'-GCTCAGTAACAGTCC GCCTAGAA-3'. Pre-denaturation was performed at 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 30 sec (15). Quantification of the mRNA expression was performed using the 2<sup>- $\Delta\Delta\text{C}_q$</sup>  method (16,17).

**Statistical analysis.** Data are presented as the mean  $\pm$  SEM. Statistical analysis was performed via one-way ANOVA

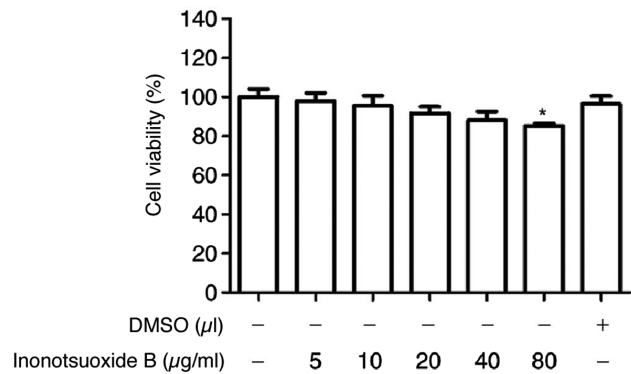


Figure 1. Effect of inonotsuoxide B on cell cytotoxicity in hepatic stellate cells. Hepatic stellate cells were treated with different concentrations of inonotsuoxide B for 24 h before cytotoxicity was measured using MTT assay. \*P<0.05 vs. the control group (untreated cells).

followed by Tukey's post hoc test using GraphPad Prism v5 software (GraphPad Software, Inc.). Each group of experiments was performed  $\geq 3$  times. P<0.05 was considered to indicate a statistically significant difference.

## Results

**Effect of inonotsuoxide B on cell cytotoxicity.** HSC-T6 cells were cultured with different concentrations of inonotsuoxide B dissolved in DMSO (5, 10, 20, 40 and 80  $\mu\text{g/ml}$ ) for 24 h. DMSO exhibited no effects on cell viability. Compared with that of the control group, inonotsuoxide B (5, 10, 20 and 40  $\mu\text{g/ml}$ ) exhibited only minor effects on cell viability (Fig. 1). However, cell viability was significantly reduced following incubation with 80  $\mu\text{g/ml}$  of inonotsuoxide B. As a result, inonotsuoxide B at concentrations of 5, 10 and 20  $\mu\text{g/ml}$  were used for subsequent experiments (Fig. 1).

**Inonotsuoxide B reverses PDGF-BB-mediated increases in HSC viability.** Compared with the control group, PDGF-BB (10 ng/ml) significantly increased the viability of HSC-T6 cells, which was in turn inhibited by inonotsuoxide B (5, 10 and 20  $\mu\text{g/ml}$ ) in a concentration-dependent manner (Fig. 2).

**Inonotsuoxide B suppresses PDGF-BB-induced  $\alpha$ -SMA mRNA expression in HSCs.** The effect of inonotsuoxide B on the mRNA levels of  $\alpha$ -SMA in HSCs was examined by RT-qPCR. As presented in Fig. 3,  $\alpha$ -SMA mRNA expression in the PDGF-BB group was significantly increased compared with the control group. At concentrations of 5, 10 and 20  $\mu\text{g/ml}$ , inonotsuoxide B significantly reduced the mRNA expression of  $\alpha$ -SMA compared with the PDGF-BB group in a dose-dependent manner (Fig. 3).

**Inonotsuoxide B suppresses the PDGF-BB-induced increases in  $\alpha$ -SMA and COL-I protein expression in HSCs.** The protein expression of the HSC activation markers  $\alpha$ -SMA and COL-I was measured using western blotting. The protein expression levels of  $\alpha$ -SMA and COL-I were significantly increased in the PDGF-BB group compared the control group, but were significantly decreased following inonotsuoxide B (10 and 20  $\mu\text{g/ml}$ ) treatment. By contrast, 5  $\mu\text{g/ml}$  inonotsuoxide B did not exert

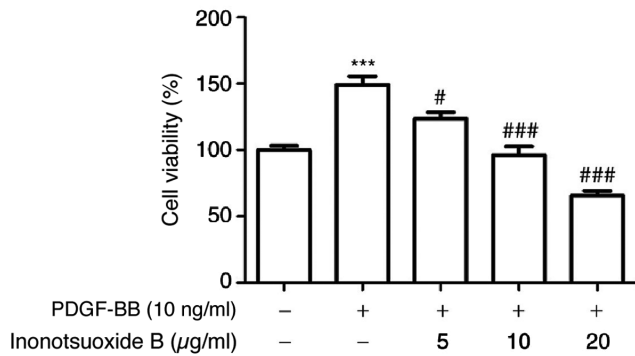


Figure 2. Effect of inonotsuoxide B on the PDGF-BB-induced potentiation of hepatic stellate cell viability. Cells were pretreated with different concentrations of inonotsuoxide B for 30 min before they were stimulated with PDGF-BB (10 ng/ml) for 24 h. \*\*\* $P < 0.001$  vs. the control group (untreated cells); # $P < 0.05$  and ### $P < 0.001$  vs. PDGF-BB. PDGF, platelet-derived growth factor.

significant effects on the protein expression levels of  $\alpha$ -SMA and COL-I compared with those in the PDGF-BB group (Fig. 4).

*Inonotsuoxide B suppresses PDGF-BB-induced AKT, ERK1/2 and PI3K phosphorylation in HSCs.* The phosphorylation of AKT, ERK1/2 and PI3K in each treatment group was measured via western blotting. As demonstrated in Fig. 5, the levels of AKT, ERK1/2 and PI3K phosphorylation were significantly increased in the PDGF-BB group compared with the control group. Treatment with inonotsuoxide B (5, 10 and 20  $\mu$ g/ml) markedly reduced the levels of AKT and PI3K phosphorylation compared with those in the PDGF-BB group in a dose-dependent manner. However, the protein expression level of p-ERK was not significantly altered following treatment with 5  $\mu$ g/ml inonotsuoxide B, while p-ERK was significantly decreased after treatment with inonotsuoxide B at 10 and 20  $\mu$ g/ml.

*PI3K inhibitor LY294002 suppresses PDGF-BB-induced  $\alpha$ -SMA and COL-I expression in addition to AKT phosphorylation in HSCs.* LY294002 is a specific blocker of the PI3K signaling pathway (18). The effects of LY294002 on the expression of the  $\alpha$ -SMA, COL-I, AKT and ERK1/2 phosphorylation was examined by western blotting. As indicated in Fig. 6, the expression levels of  $\alpha$ -SMA and COL-I proteins, in addition to AKT and ERK phosphorylation, were significantly increased in PDGF-BB-treated HSC-T6 cells compared with control cells. The expression levels of  $\alpha$ -SMA, COL-I and AKT phosphorylation were also significantly decreased in the presence of LY294002, whilst LY294002 did not exert significant effects on ERK1/2 phosphorylation compared with the PDGF-BB group. Compared with the PDGF-BB group, the protein expression levels of  $\alpha$ -SMA, COL-I, p-AKT and p-ERK1/2 in PDGF-BB + inonotsuoxide B (20  $\mu$ g/ml) group were significantly reduced.

*ERK inhibitor UO126 suppresses PDGF-BB-induced increases in  $\alpha$ -SMA and COL-I expression in addition to ERK phosphorylation in HSCs.* UO126 is a specific blocker of the ERK signaling pathway (19). The effect of UO126 on the expression of the  $\alpha$ -SMA and COL-I in addition to AKT and ERK1/2 phosphorylation was examined via western blotting. As demonstrated in Fig. 7, the expression levels of  $\alpha$ -SMA, COL-I, AKT phosphorylation and ERK phosphorylation were significantly

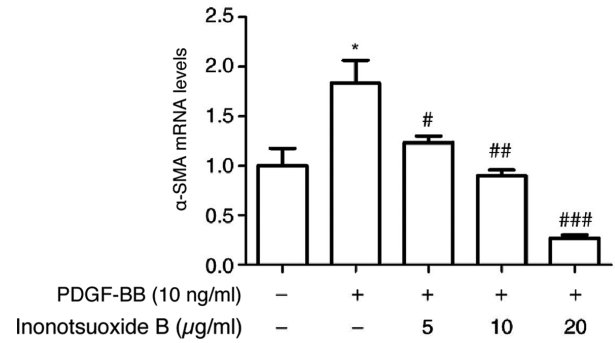


Figure 3. Effect of inonotsuoxide B on the PDGF-BB-induced increase of  $\alpha$ -SMA mRNA expression in hepatic stellate cells. Cells were first treated with PDGF-BB (10 ng/ml) in serum-free DMEM for 24 h in the presence of inonotsuoxide B (5, 10 or 20  $\mu$ g/ml). The expression of  $\alpha$ -SMA was then determined using reverse transcription-quantitative PCR. \* $P < 0.05$  vs. the control group (untreated cells); # $P < 0.05$ , ## $P < 0.01$  and ### $P < 0.001$  vs. PDGF-BB. PDGF, platelet-derived growth factor;  $\alpha$ -SMA,  $\alpha$ -smooth muscle actin.

increased in PDGF-BB-treated HSC-T6 cells compared with the control cells. The expression levels of  $\alpha$ -SMA, COL-I and ERK phosphorylation were found to be significantly reduced in the UO126-treated group compared with those in the PDGF-BB group. By contrast, UO126 treatment did not result in significant effects on AKT phosphorylation compared with that in the PDGF-BB group. In addition, compared with the PDGF-BB group, the protein expression levels of  $\alpha$ -SMA, COL-I, p-AKT and p-ERK1/2 in PDGF-BB + inonotsuoxide B (20  $\mu$ g/ml) group were significantly reduced.

## Discussion

The mechanism of liver fibrosis is complex (20). The proliferation and activation of HSCs and associated signaling pathways have been the focus of studies on liver fibrosis (21). However, research on potential treatments against liver fibrosis has primarily focused on singular targets and pathways. Consequently, treatments that are currently available for liver fibrosis have not always been effective (22). Previous studies have suggested that the chemical constituents of *Inonotus obliquus* (polysaccharide, inonot and betulin) exhibited anticancer, antiaging, blood sugar and blood pressure reducing pharmacological effects (23,24). Inonotsuoxide B is a tetracyclic triterpenoid that can be extracted from *Inonotus obliquus* and has been previously demonstrated to inhibit the growth of liver and gastric cancer cells (24). However, its effects on liver fibrosis remain poorly understood. The present study primarily focused on the effects of inonotsuoxide B treatment on HSC viability and activation with respect to the PI3K/AKT and ERK1/2 signaling pathways.

PDGF is an important mitogenic factor in HSC-T6 cells (2). During liver fibrosis, PDGF has been demonstrated to promote HSC proliferation and collagen expression (2). A previous study has reported that 10 ng/ml PDGF-BB stimulated the activation of HSCs (25). Therefore, the effect of inonotsuoxide B on HSC-T6 cells following treatment with PDGF-BB (10 ng/ml) was examined in the present study. Compared with those in the untreated cell group, PDGF-BB significantly increased the cell viability and activation of HSC-T6 cells, in addition to potentiating the expression of  $\alpha$ -SMA and COL-I. Furthermore,

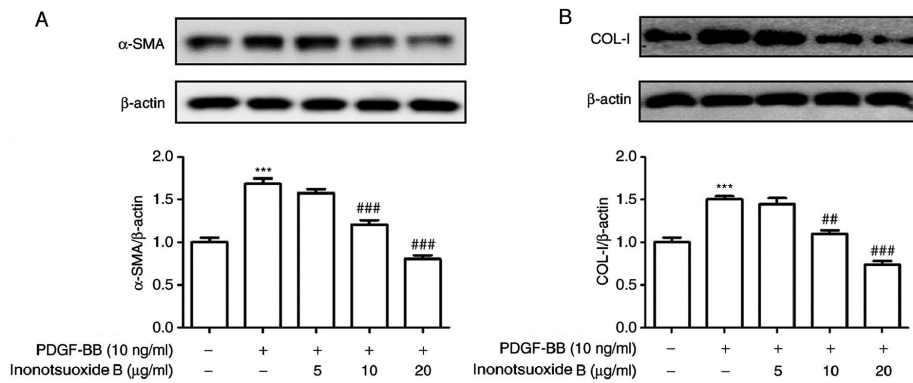


Figure 4. Effect of inonotsuoxide B on the PDGF-BB-induced increase of  $\alpha$ -SMA and COL-1 protein expression in hepatic stellate cells. Cells were treated with PDGF-BB (10 ng/ml) in serum-free DMEM for 24 h in the presence of inonotsuoxide B (5, 10 or 20 mg/ml). The expression levels of (A)  $\alpha$ -SMA and (B) COL-1 were measured by western blotting. \*\*\* $P < 0.001$  vs. the control group (untreated cells); # $P < 0.01$  and ### $P < 0.001$  vs. PDGF-BB. PDGF, platelet-derived growth factor;  $\alpha$ -SMA,  $\alpha$ -smooth muscle actin; COL-1, collagen I.

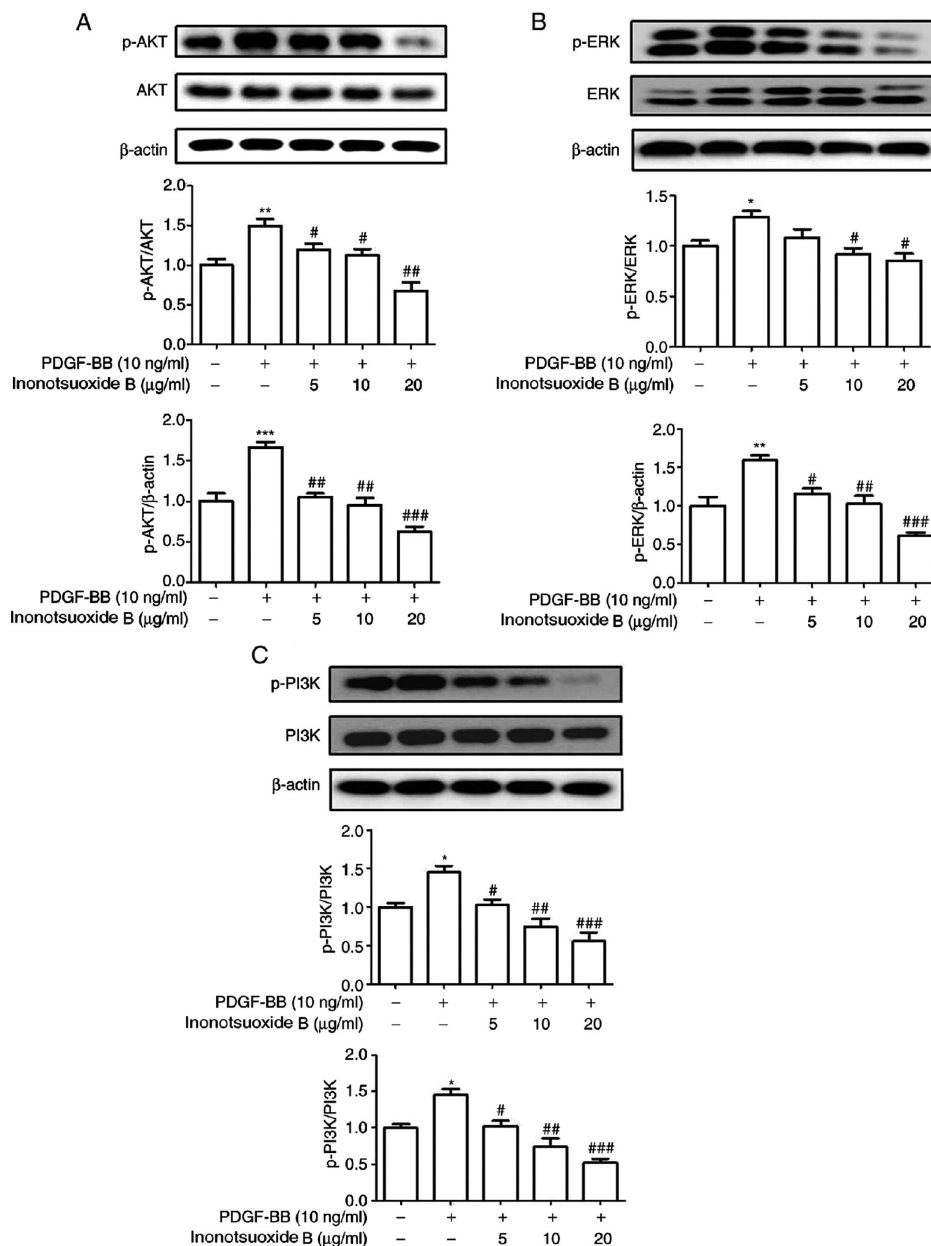


Figure 5. Effect of inonotsuoxide B on PDGF-BB-induced AKT, ERK1/2 and PI3K phosphorylation in hepatic stellate cells. The phosphorylation levels of (A) AKT, (B) ERK1/2 and (C) PI3K were measured by western blotting. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  vs. the control group (untreated cells); # $P < 0.05$ , ## $P < 0.01$  and ### $P < 0.001$  vs. the PDGF-BB group. PDGF, platelet-derived growth factor; p, phosphorylated.

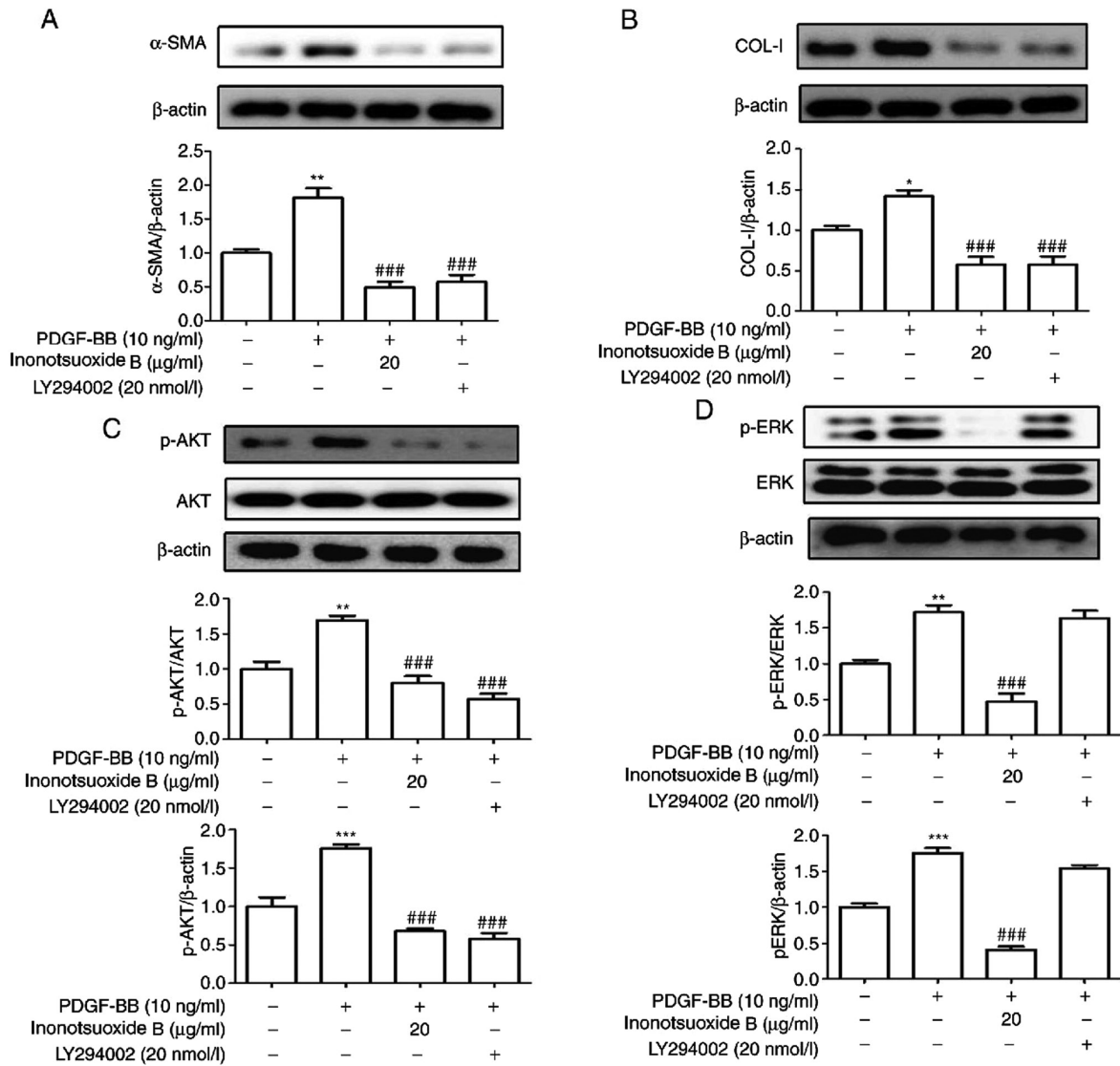


Figure 6. Effects of the PI3K inhibitor on PDGF-BB-induced increases of  $\alpha$ -SMA and COL-I expression and AKT and ERK1/2 phosphorylation in hepatic stellate cells. The cells were treated with PDGF-BB (10 ng/ml) in the presence of LY294002 or with PDGF-BB (10 ng/ml) in the presence of inonotsuoxide B (20  $\mu$ g/ml) in serum-free DMEM for 24 h. The expression levels of (A)  $\alpha$ -SMA and (B) COL-I, and the phosphorylation levels of (C) AKT and (D) ERK1/2 were subsequently measured using western blotting. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  vs. the control group (untreated cells); ### $P < 0.001$  vs. the PDGF-BB group. PDGF, platelet-derived growth factor;  $\alpha$ -SMA,  $\alpha$ -smooth muscle actin; COL-I, collagen I; p, phosphorylated.

all of these aforementioned effects were reversed following treatment of the cells with inonotsuoxide B. These findings indicated that inonotsuoxide B reduced the viability and activation of PDGF-BB-stimulated HSC-T6 cells.

A previous study demonstrated that the PI3K/AKT and ERK signaling pathways primarily mediated PDGF-induced signaling (26). PI3K regulates the phosphorylation of AKT, where the subsequent PI3K/AKT downstream signaling pathway has been suggested to serve a regulatory role in a number of cell proliferation and activation processes (27). By contrast, the ERK signaling pathway has been reported to serve a key role in transducing signals from cell-surface receptors to the nucleus, thereby regulating the activation and proliferation of cells (28). Therefore, the phosphorylation of PI3K, AKT and ERK1/2 was examined in the present study. It was observed that the PI3K/AKT and ERK1/2 signaling pathways were associated with the inhibitory effects of inonotsuoxide B on the viability and activation of PDGF-BB-stimulated HSC-T6 cells.

Compared with the untreated cell group, PDGF-BB treatment significantly upregulated the phosphorylation of PI3K, AKT and ERK in HSC-T6 cells, all of which were revealed to be reversed by inonotsuoxide B treatment. UO126 and LY294002 have been demonstrated to be specific blockers of the ERK and PI3K/AKT signaling pathways, respectively (29). Previous studies have reported that UO126 and LY294002 inhibited the proliferation of HSCs at concentrations  $< 20$  nmol/l, in a concentration-dependent manner (30,31). Therefore, in the present study, UO126 and LY294002 at 20 nmol/l were used to investigate their potential effects on PDGF-BB-induced HSC proliferation and activation. The results indicated that the expression levels of the  $\alpha$ -SMA and COL-I proteins in PDGF-BB-stimulated HSCs were decreased by both UO126 and LY294002 treatments.

Liver fibrosis has been indicated to be reversible, as effective drug therapy can inhibit the activation and proliferation of HSCs and reduce the degree of hepatic fibrosis (32). The results of the present study demonstrated that inonotsuoxide B inhibited the

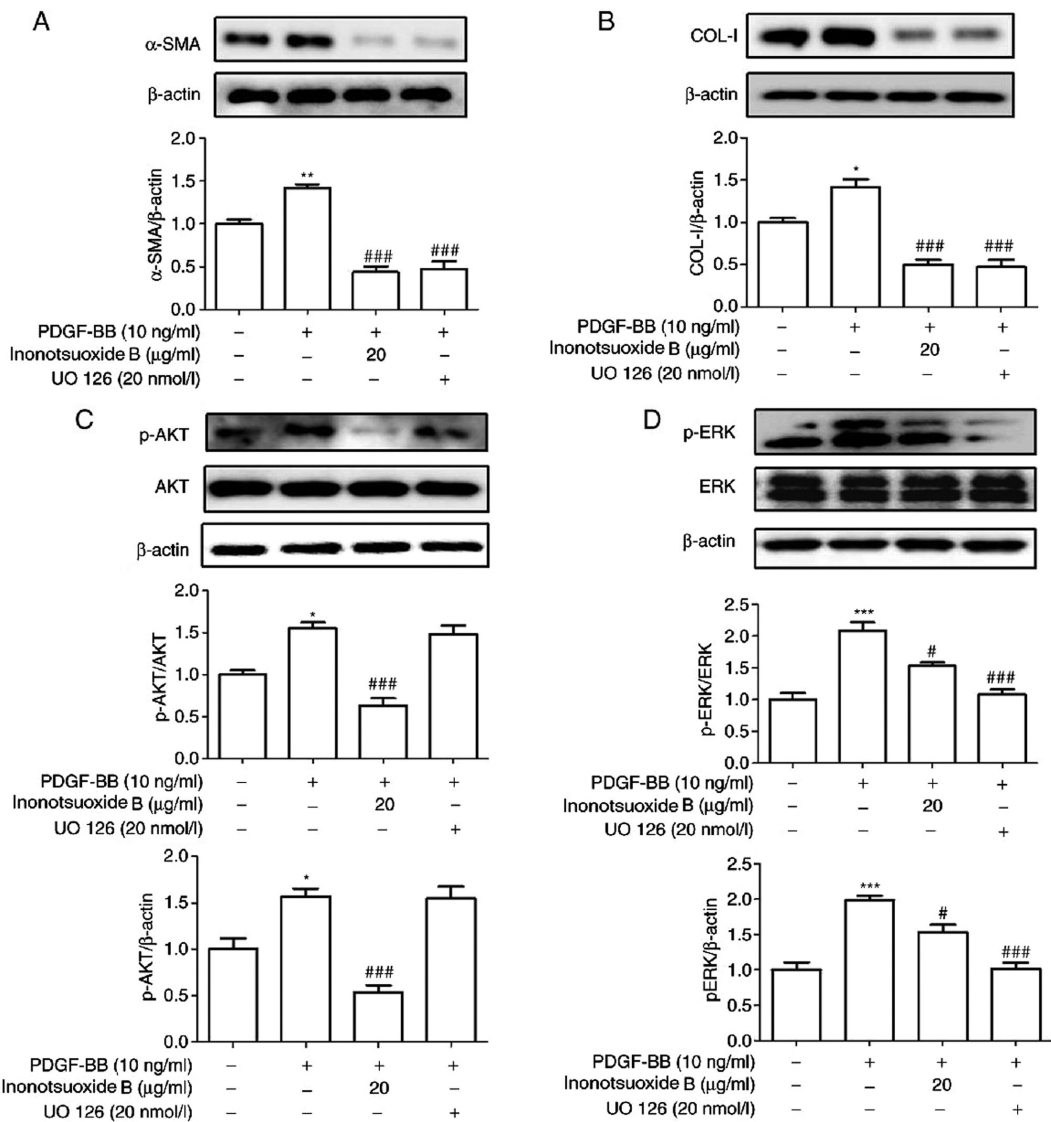


Figure 7. Effects of the ERK inhibitor on the PDGF-BB-induced increases of  $\alpha$ -SMA and COL-1 expression, in addition to AKT and ERK1/2 phosphorylation, in hepatic stellate cells. The cells were treated with PDGF-BB (10 ng/ml) in the presence of UO126 (20 nmol/l) or with PDGF-BB (10 ng/ml) in the presence of inonotsuoxide B (20  $\mu$ g/ml) in serum-free DMEM for 24 h. The expression levels of (A)  $\alpha$ -SMA and (B) COL-1, and the phosphorylation levels of (C) AKT and (D) ERK1/2, were measured using western blotting. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  vs. the control group (untreated cells); # $P < 0.05$  and ### $P < 0.001$  vs. the PDGF-BB group. PDGF, platelet-derived growth factor;  $\alpha$ -SMA,  $\alpha$ -smooth muscle actin; COL-1, collagen I; p, phosphorylated.

viability and activation of PDGF-BB-stimulated HSC-T6 cells. The underlying mechanism of action of inonotsuoxide B may be associated with the inhibition of the PI3K/AKT and ERK signaling pathways. These observations are clinically relevant and may enable the development of an efficient treatment strategy against liver fibrosis.

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**Availability of data and materials**

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

### Authors' contributions

YH and WC designed the current study. JJ and HY performed the experiments. LH and YW analyzed the data. CH drafted the manuscript and analyzed data. KW and ZW interpreted data and revised the final manuscript. WW performed the experiments and wrote the manuscript. JJ and YH confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

### Ethics approval and consent to participate

Not applicable.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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