Nintedanib modulates surfactant protein-D expression in A549 human lung epithelial cells via the c-Jun N-terminal kinase-activator protein-1 pathway

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ABSTRACT

Idiopathic pulmonary fibrosis (IPF) is a progressive disease with a high mortality rate. Signalling pathways activated by several tyrosine kinase receptors are known to be involved in lung fibrosis, and this knowledge has led to the development of the triple tyrosine kinase inhibitor nintedanib, an inhibitor of vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor receptor (PDGFR), and fibroblast growth factor receptor (FGFR), for the treatment of IPF. Pulmonary surfactant protein D (SP-D), an important biomarker of IPF, reportedly attenuates bleomycin-induced pulmonary fibrosis in mice. In this study, we investigated whether nintedanib modulates SP-D expression in human lung epithelial (A549) cells using quantitative real-time reverse transcriptase polymerase chain reaction and western blotting. To investigate the mechanisms underlying the effects of nintedanib, we evaluated the phosphorylation of c-Jun N-terminal kinase (JNK) and its downstream target c-Jun. The effect of the JNK inhibitor SP600125 on c-Jun phosphorylation was also tested. Activation of activator protein-1 (AP-1) was examined using an enzyme-linked immunosorbent assay-based test, and cell proliferation assays were performed to estimate the effect of nintedanib on cell proliferation. Furthermore, we treated mice with nintedanib to examine its in vivo effect on SP-D levels in lungs. These experiments showed that nintedanib up-regulated SP-D messenger RNA expression in a dose-dependent manner at concentrations up to 5 μM, with significant SP-D induction observed at concentrations of 3 μM and 5 μM, in comparison with that observed in vehicle controls. Nintedanib stimulated a rapid increase in phosphorylated JNK in A549 cells within 30 min of treatment and stimulated c-Jun phosphorylation, which was inhibited by the JNK inhibitor SP600125. Additionally, nintedanib was found to activate AP-1. A549 cell proliferation was not affected by nintedanib at any of the tested concentrations. Moreover, blocking FGFR, PDGFR, and VEGFR function did not affect nintedanib-induced SP-D expression, suggesting that nintedanib mediates its effects through a mechanism that is distinct from its known role as a tyrosine kinase inhibitor. Nintedanib is also reported to inhibit Src kinase although pre-treatment of cells with a Src kinase inhibitor had no effect on nintedanib-induced SP-D expression. Increased expression of SFTPD mRNA and SP-D protein in the lungs of nintedanib-treated mice was also observed. In this work, we demonstrated that nintedanib up-regulated SP-D expression in A549 cells via the JNK-AP-1 pathway and did not affect cell proliferation. This is the first report describing SP-D induction by nintedanib.

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Abbreviations: IPF, idiopathic pulmonary fibrosis; SP-D, surfactant protein D; JNK, c-Jun N-terminal kinase; AP-1, activator protein-1; VEGFR, vascular endothelial growth factor receptor; PDGFR, platelet-derived growth factor receptor; FGFR, fibroblast growth factor receptor; SP-B, surfactant protein B; SP-A, surfactant protein A; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MAPK, mitogen-activated protein kinase.

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1. Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic disease that is characterised by progressive scarring of the lung parenchyma [1]. The course of IPF is difficult to predict; however, it generally involves progressive and inexorable lung deterioration, with a median survival time of 2.5–3.5 years after diagnosis [2]. Although the pathological processes underlying the progression of IPF are not fully understood, recent evidence implicates a degenerative process of alveolar epithelial cell injury and dysregulated repair that leads to myofibroblast proliferation and fibrotic scarring in the lungs. The anti-fibrotic drug pirfenidone has proven to be effective in ameliorating the decline of lung function in IPF patients [3,4] and is used in clinical settings; however, considerable efforts are underway to develop newer and more effective agents against IPF.

Nintedanib is a potent intracellular tyrosine kinase inhibitor that is in clinical development for the treatment of IPF and several types of cancer [5]. In enzymatic assays, nintedanib blocks the activity of receptor tyrosine kinases (RTKs), vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor receptor (PDGFR), and fibroblast growth factor receptor (FGFR) [6]. Moreover, nintedanib inhibited lung function decline in IPF patients in a recent phase III clinical trial [7].

Pulmonary surfactant protein D (SP-D) is a member of the collectin subfamily of mammalian C-type lectins, which includes pulmonary surfactant protein A (SP-A) and mannose-binding lectin [8,9]. SP-D and SP-A are secreted into the distal airways and pulmonary alveoli by Clara cells and type II pneumocytes, and their expression by these cells increases following many forms of pulmonary injury [9]. SP-D has important innate immune properties such as regulating host immune defence and modulating inflammatory responses [10]. Because SP-D expression increases following lung injury, it is used as a biomarker for IPF [11].

Recent studies have shown that an increased serum SP-D level is an independent predictor of mortality in human IPF patients [12]. However, in mice, SP-D reportedly plays an important role in preventing bleomycin-induced pulmonary fibrosis by regulating the recruitment of macrophages or fibrocytes and the expression of profibrotic cytokines [13], and this in vivo observation suggests that SP-D protects the lungs from damage resulting from insults. However, the effect of nintedanib on SP-D expression in human alveolar epithelial cells has not been investigated. Therefore, in this study we investigated the effect of nintedanib on surfactant protein expression in human alveolar epithelial cells and mice. Findings from our preliminary studies were reported in abstract form at a meeting of the American Thoracic Society [14].

2. Materials and methods

2.1. Chemicals and reagents

A rabbit anti-human SP-D antibody (Ab), an anti-phosphorylated c-Jun N-terminal kinase (JNK) Ab, an anti-JNK Ab, an anti-phosphorylated c-Jun Ab, and a c-Jun Ab were purchased from Dako (Glostrup, Denmark). A mouse monoclonal anti-conjugated anti-rabbit immunoglobulin (IgG) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Biotin-an anti-phosphorylated c-Jun Ab, and a c-Jun Ab were purchased from R&D Systems (Minneapolis, MN, USA). A mouse anti-human SP-D antibody (Ab), an anti-SP-D Ab (clone AC-74) was purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Nintedanib was procured from LC Laboratories (Woburn, MA, USA), dissolved in dimethyl sulfoxide (DMSO) at 100 mM, aliquoted and stored at −20 °C. Alexa Fluor 488-conjugated streptavidin was purchased from Life Technologies (Carlsbad, CA, USA). VECTASHIELD Mounting Medium with 4′,6-diamidino-2-phenylindole (DAPI) was obtained from Vector Laboratories, Inc. (Burlingame, CA, USA). The selective JNK inhibitor SP600125 was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Radioimmunoprecipitation assay (RIPA) buffer was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Sunitinib, BGJ398, and dasatinib were purchased from Selleck Chemicals (Houston, TX, USA).

2.2. Cell culture

The A549 human lung epithelial cell line was obtained from the American Type Culture Collection (#CCL-185; Manassas, VA, USA). A549 cells were cultured in 100-mm tissue culture dishes (Falcon; Becton, Dickinson and Company, Lincoln Park, NJ, USA) with Roswell Park Memorial Institute (RPMI)-1640 medium, supplemented with 10% foetal calf serum, 100 μg/ml penicillin and 250 μg/ml streptomycin sulphate (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO₂. Subconfluent cells were serum-starved for 24 h before treatment with nintedanib. The concentration of nintedanib used (0.01–5 μM) was based on a previous study [15]. After 24 h incubation with nintedanib, cells were lysed, and target messenger RNA (mRNA) expression levels were assessed by performing quantitative real-time reverse-transcriptase polymerase chain reactions (RT-PCR). Western blot analysis, immunohistochemistry, and enzyme-linked immunosorbent assay (ELISA) were performed 72 h after nintedanib treatment.

2.3. Quantitative real-time RT-PCR

Total RNA was extracted from cultured A549 cells using ISOGEN reagents with spin columns (Nippon Gene, Tokyo, Japan) and converted to complementary DNA as described previously [16]. Quantitative real-time RT-PCR was performed using the TaqMan method and an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems Japan, Ltd., Tokyo, Japan). TaqMan Gene Expression Assays for detecting human SP-D (SFTPD; Hs00358340_m1) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were purchased from Applied Biosystems Japan, Ltd., and THUNDERBIRD Probe qPCR Mix was purchased from Toyobo (Osaka, Japan). The relative amounts of SFTPD mRNA in the original samples were normalised to GAPDH mRNA expression levels. TaqMan Gene Expression Assays for detecting mouse Sftpd mRNA (Mm00485606_m1) and Gapdh mRNA were purchased from Applied Biosystems Japan, Ltd.

2.4. Western blot analysis

Cells from subconfluent cultures were washed twice with phosphate-buffered saline (PBS), scraped into PBS, and pelleted by centrifugation. Whole-cell lysates were prepared in RIPA buffer for immunoblotting experiments. Protein concentrations were determined using the BCA Protein Assay Kit (Thermo Fisher Scientific Inc.) using bovine serum albumin as a standard, according to the manufacturer’s protocol. Samples (8 μg protein/lane) were resolved by 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked in Tris-buffered saline (0.15 M NaCl, 0.05 M Tris–HCl (pH 8.0), and 0.05% (vol/vol) Tween 20) containing 5% skim milk and incubated with the indicated antibodies at the dilutions recommended by the manufacturer. An anti-β-actin Ab was used to confirm equal protein loading. After incubation with horseradish peroxidase-conjugated secondary antibodies, immunoreactive bands were detected with the
SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific Inc.), according to the manufacturer’s instructions, and an ImageQuant LAS4000 mini (GE Healthcare Life Sciences, Piscataway, NJ, USA). Membranes were re-probed after removing bound antibodies by incubation in Restore Western Blot Stripping Buffer (Thermo Fisher Scientific Inc.) according to the manufacturer’s protocol. Mice lung homogenates were also subjected to western blot analysis. Band intensity was quantified by densitometry using Image J 1.48 software (National Institutes of Health, Bethesda, MD, USA).

2.5. SP-D immunohistochemistry experiments

A549 cells were grown in a Chamber Slide System (Thermo Fisher Scientific Inc., Rockford, IL, USA) and treated for 72 h with nintedanib (5 μM) in growth medium. Cells were then briefly washed with PBS, fixed in 4% paraformaldehyde at room temperature for 30 min, blocked with 3% goat serum for 30 min at room temperature, incubated overnight at 4 °C with rabbit anti-human SP-D Ab in 3% goat serum, and subsequently incubated with a secondary biotin-conjugated anti-rabbit IgG Ab at room temperature for 1 h. Next, the cells were stained with Alexa Fluor 488-conjugated streptavidin at room temperature for 1 h. VECTASHIELD mounting medium was allowed to disperse over the entire section to counterstain nuclei with DAPI.

2.6. Quantification of SP-D using ELISA

Cells from subconfluent cultures were washed twice with PBS, scraped into PBS and pelleted by centrifugation. SP-D levels were measured in whole-cell lysates using an SP-D ELISA kit (R&D Systems Inc. Minneapolis, MN, USA) according to the manufacturer’s protocol.

2.7. Activator protein-1 (AP-1) binding assay

Nuclear proteins from A549 cells were extracted with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific Inc.). Nuclear pellets were resuspended in Active Motif Lysis Buffer (50 μl; Active Motif, Carlsbad, CA, USA). After incubation for 30 min on ice, samples were centrifuged and protein concentrations were measured. AP-1 consensus nucleotide-binding activity from nuclear extracts (10 μg) was measured with the TransAM AP-1 Family Colorimetric System (Active Motif), as recommended by the manufacturer. Briefly, nuclear extract was added to immobilised oligonucleotides, followed by the addition of a phospho-c-Jun Ab, and colorimetric values were measured at 450 nm and plotted.

2.8. Cell proliferation assay

The effects of nintedanib on cell proliferation were evaluated using Cell Counting Kit-8 (CCK8; Dojindo Laboratories, Kumamoto, Japan) following the manufacturer’s instructions. A549 cells were seeded into 96-well plates at a density of 2 × 10^4 cells per well in complete culture medium (190 μl). Cells containing culture medium only served as blanks. After adhesion for 4 h, various concentrations of nintedanib (0.1–5 μM) were added and the cells were incubated at 37 °C for an additional 72 h. Subsequently, CCK8 reagent (10 μl) was added to each well and the cells were incubated for 1 h at 37 °C. Finally, the absorbance at 450 nm, which reflected the amount of formazan dye generated by active dehydrogenases in living cells, was recorded using a microplate reader.

2.9. Animal studies

Nine-week-old female C57BL/6 mice (Charles River Laboratories Japan, Yokohama, Japan) were used. Nintedanib (50 mg kg^-1) suspended in DMSO (vehicle) was orally administered once daily for 5 or 10 days according to a previously published procedure [17]. After this time, the mice were sacrificed and the lungs were excised. Lung homogenates were then subjected to quantitative real-time RT-PCR (after 5 days treatment) and western blot analysis (after 10 days treatment) to measure the expression of Sftpd mRNA and SP-D protein, respectively. The experimental protocol was approved by the animal care and use committee of Nippon Medical School (Tokyo, Japan).

2.10. Statistical analysis

Data are expressed as means ± standard deviation. Experiments were conducted in triplicate with at least three independent cultures unless otherwise stated. Experiments with multiple comparisons were evaluated using one-way analysis of variance followed by Tukey–Kramer’s test to adjust for multiple comparisons. Student’s t-test was applied for the assessment of paired data. Differences were considered statistically significant if P-values were less than 0.05.

3. Results

3.1. Stimulation of SP-D gene expression by nintedanib in A549 human lung epithelial cells

Initially, we used quantitative real-time RT-PCR to examine the extent to which nintedanib stimulates SP-D (SFTPD) transcript expression in A549 human lung epithelial cells. A549 cells were treated with various concentrations of nintedanib (0.01–5 μM). Twenty-four hours after nintedanib treatment, total RNA was collected and SFTPD mRNA expression was analysed using quantitative real-time RT-PCR. As shown in Fig. 1, nintedanib induced SFTPD mRNA expression in A549 cells. Significant stimulation of SFTPD mRNA expression was observed after treatment with 3 and 5 μM of nintedanib (P < 0.05 and P < 0.01, respectively). These results demonstrated dose-dependent stimulation of SP-D gene expression by nintedanib.

![Fig. 1. Induction of surfactant protein-D (SP-D) messenger RNA (mRNA) expression by nintedanib in A549 human lung epithelial cells. A549 cells cultured in monolayers were serum starved for 24 h before being treated with various concentrations of nintedanib (0.01–5 μM). Twenty-four hours after nintedanib treatment, total cellular RNA was harvested, and SP-D (SFTPD) mRNA was analysed via quantitative real-time polymerase chain reaction and normalised to the expression of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Triplicate dishes were used for each experiment, and experiments were repeated 3 times with different cultures. Data are presented as means ± standard deviation. *P < 0.01, **P < 0.05, compared with vehicle-treated cells.](image-url)
expression by nintedanib of up to 2-fold in comparison with untreated cells, confirming that nintedanib potently stimulates SP-D gene expression in A549 cells.

3.2. Stimulation of SP-D production in A549 cells by nintedanib

To characterise further the stimulation of SP-D production at the protein level, we performed a western blot analysis with an anti-SP-D Ab. As shown in Fig. 2, nintedanib enhanced SP-D protein expression in a dose-dependent manner at concentrations of up to 5 μM (*P < 0.05 compared with vehicle control).

Immunofluorescence staining revealed low-level cytosolic expression of SP-D in A549 cells (Fig. 3A). Cytosolic SP-D expression was increased by 72 h of exposure to 5 μM nintedanib (Fig. 3B).

We further confirmed the effect of nintedanib using ELISA to quantify the amount of SP-D produced. As shown in Fig. 4, nintedanib up-regulated SP-D protein expression at concentrations of up to 5 μM; however, statistical significance was not achieved.

3.3. Effect of nintedanib on JNK activation in A549 cells

SP-D gene expression is positively regulated at the transcriptional level by AP-1 [18,19]. Because c-Jun proteins can stably heterodimerise with c-Fos to form the transcription factor AP-1, which can bind to AP-1 DNA recognition elements following activation by JNK [20], we investigated whether the JNK-AP-1 pathway is involved in nintedanib-induced SP-D up-regulation. To assess this possibility, we performed western blotting to detect phosphorylated, and thus activated JNK. Nintedanib strongly stimulated rapid and transient increases in JNK activity in whole cell lysates, which peaked at 30 min and declined to basal levels thereafter (Fig. 5).

Fig. 2. Effects of nintedanib on surfactant protein-D (SP-D) production in A549 human lung epithelial cells. (A) A549 cells cultured in monolayers were serum starved for 24 h before being treated with various concentrations of nintedanib (1–5 μM). Seventy-two hours after nintedanib treatment, whole-cell lysates were prepared and total protein concentrations were determined. Equal amounts of cell extracts (8 μg protein/lane) were separated via sodium dodecyl sulphate-polyacrylamide gel electrophoresis and western blot analysis was performed using an antibody against SP-D. β-actin was used as an internal control for loading and transfer. A representative result is presented from three independent experiments. (B) The intensity of each band was assessed using densitometry. Relative intensity was calculated by dividing the intensity of each SP-D band by the intensity of the appropriate β-actin band. Data are presented as means ± standard deviation. *P < 0.05 compared with vehicle control by Student’s t-test.

Fig. 3. Immunofluorescence image of surfactant protein-D (SP-D) expression (green) in A549 human lung epithelial cells. A549 cells cultured in monolayers were serum starved for 24 h before being treated with nintedanib (5 μM). Seventy-two hours after nintedanib treatment, A549 cells were stained with an anti-SP-D antibody. Nuclei were counterstained with 4',6-diamidino-2-phenylindole. Cytosolic SP-D expression in A549 cells was increased by nintedanib treatment. A representative figure from two independent experiments is presented. Original magnification, 40×. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Nintedanib-dependent c-Jun activation and its inhibition by a selective JNK inhibitor in A549 cells

Activated JNK activates the downstream target c-Jun by phosphorylation. Nintedanib-mediated JNK activation was further confirmed by examining c-Jun phosphorylation via western blotting. As shown in Fig. 6, basal c-Jun phosphorylation was observed in quiescent A549 cells. Nintedanib stimulated c-Jun phosphorylation, and this effect was blocked by JNK inhibitor SP600125.

Fig. 4. Quantification of nintedanib-induced surfactant protein-D (SP-D) using enzyme-linked immunosorbent assay (ELISA). A549 cells cultured in monolayers were serum starved for 24 h before being treated with various concentrations of nintedanib (1–5 μM). Seventy-two hours after nintedanib treatment, whole-cell lysates were prepared and subjected to ELISA. Nintedanib up-regulated SP-D protein expression at concentrations of up to 5 μM. Duplicate dishes were used for each experiment, and experiments were repeated three times with different cultures. Data are presented as means ± standard deviation.

3.5. Effect of nintedanib on transcription factor activation

c-Jun proteins can stably heterodimerise with c-Fos to form the transcription factor AP-1, which binds AP-1 DNA recognition elements. We investigated whether the potential stimulatory effect of nintedanib on SP-D expression is mediated by affecting the binding of AP-1 to a consensus sequence in the promoter region of the SP-D gene. AP-1 activation was increased by up to 2.29-fold after 30 min of nintedanib stimulation (Fig. 7).
3.6. Effect of nintedanib on proliferation of A549 cells

To investigate whether nintedanib modulates A549 cell growth, cell proliferation was measured after nintedanib treatment. The proliferation of A549 cells, determined using the Cell Counting Kit-8, was not affected by nintedanib treatment (0.1–5 μM) (Fig. 8).

3.7. Effect of inhibition of FGFR, PDGFR, and VEGFR function on SP-D gene expression in A549 cells

Because nintedanib potentially inhibits signal transduction by FGFR, PDGFR, and VEGFR, we assessed whether inhibition of these receptors modulated SP-D induction by nintedanib. Sunitinib (100 nM) was used to block PDGFR and VEGFR function, and BGJ398 (50 nM) was used to block FGFR function. Both drugs were added 1 h before the addition of nintedanib. As shown in Fig. 9, co-treatment of A549 cells with sunitinib and BGJ398 did not affect SFTPD mRNA expression. Nintedanib stimulated SFTPD expression despite the blockade of FGFR, PDGFR, and VEGFR, suggesting that nintedanib mediates its effects through a mechanism that is distinct from its known role as a tyrosine kinase inhibitor.

3.8. Effect of Src kinase inhibition on SP-D gene expression in A549 cells

Src kinases, a family of non-receptor tyrosine kinases, are important mediators of pro-fibrotic signalling pathways [21,22]. Nintedanib has the potential to inhibit Src kinases [5], therefore, we investigated if this was the mechanism by which nintedanib modulated SP-D expression. Dasatinib (50 nM) was used to block Src kinase activity and was added 1 h before the addition of nintedanib. As shown in Fig. 10, treatment of A549 cells with dasatinib did not affect SFTPD mRNA expression. Nintedanib also stimulated SFTPD expression following treatment with a Src kinase inhibitor.

3.9. Effect of nintedanib on SP-B gene expression in A549 human lung epithelial cells

Because the 5’-flanking region of the SFTPB gene harbours AP-1 recognition sequences [23], we investigated the effect of nintedanib on SP-B (SFTPB) transcript expression in A549 human lung cells.

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Fig. 5. Effect of nintedanib on c-Jun N-terminal kinase (JNK) activation. A549 cells were pre-incubated for 24 h in serum-free medium, followed by the addition of nintedanib (5 μM) for the indicated period. Whole cell lysates were prepared and subjected to western blot analysis with antibodies specific to the JNK and phosphorylated form of JNK. Nintedanib treatment induced JNK phosphorylation within 30 min, which declined to basal levels thereafter. β-actin was used as an internal control for loading and transfer. A representative figure from 2 independent experiments is presented.

Fig. 6. Effect of nintedanib on c-Jun activation and its inhibition by the selective c-Jun N-terminal kinase (JNK) inhibitor SP600125. A549 cells were pre-incubated for 24 h in serum-free medium, followed by the addition of nintedanib (5 μM) for 30 min. SP600125 (50 μM) was added 1 h before the addition of nintedanib. Western blot analysis of c-Jun demonstrated that nintedanib stimulated c-Jun phosphorylation, and SP600125 blocked c-Jun phosphorylation. β-actin was used as an internal control for loading and transfer. A representative figure from two independent experiments is presented.

Fig. 7. Nintedanib-dependent activator protein-1 (AP-1) activation. Nuclear AP-1 content in the presence of 5 μM nintedanib was measured by the enzyme-linked immunosorbent assay-based Trans AM™ kit. AP-1 activation increased by 2.29-fold after 30 min of nintedanib stimulation. Data are expressed as mean absorbance values at 450 nm ± standard deviation measured in three independent experiments.

Fig. 8. Effect of nintedanib on cell proliferation. A549 cells were allowed to adhere to 96-well plates and treated with various concentrations of nintedanib (0.1–5 μM) for 72 h, after which Cell Counting Kit-8 reagent (10 μL) was added. The optical density, which reflected the amount of formazan dye generated by dehydrogenase activity in the living cells, was measured at 450 nm. Nintedanib did not affect cell proliferation.
epithelial cells by quantitative real-time RT-PCR. A549 cells were treated with nintedanib (5 μM). Twenty-four hours after nintedanib treatment, total RNA was collected and SFTPB mRNA expression was analysed by quantitative real-time RT-PCR. As shown in Fig. 11, nintedanib did not affect SFTPB mRNA expression in A549 cells.

3.10. Administration of nintedanib to mice

To investigate whether our in vitro observations were reproducible in vivo, C57BL/6 mice were orally administered nintedanib (50 mg·kg⁻¹) once daily for up to ten days. Mice were sacrificed and lung homogenates were subjected to quantitative real-time RT-PCR on day 5 and western blotting on day 10 following administration of nintedanib. As shown in Fig. 12A, nintedanib administration increased the expression of SFTP D mRNA and appeared to downregulate the expression of SFTPB. Western blot analysis revealed increased SP-D protein expression in the lungs of nintedanib-treated mice (Fig. 12B and C).

4. Discussion

In this study, we demonstrated for the first time that nintedanib induces SP-D expression in A549 human lung epithelial cells at the mRNA and protein levels. This effect was mediated via the JNK-AP-1 pathway as evidenced by the loss of this effect following pre-treatment of cells with a selective JNK inhibitor. Proliferation and death of A549 cells were unaffected by nintedanib at any tested concentration. Moreover, our data show that nintedanib-induced SP-D expression occurs even in the presence of tyrosine kinase inhibitors of FGFR, PDGFR, and VEGFR. Additionally, nintedanib-treated mice were also found to have increased levels of SP-D expression in the lungs.

Nintedanib was originally developed as a potent intracellular inhibitor of tyrosine kinases; however, we found that nintedanib exerted a stimulatory effect on SP-D in A549 cells, which was mediated via signalling through JNK, a mitogen-activated protein kinase (MAPK). Although MAPK-mediated up-regulation of SP-D expression in human corneal epithelial cells has been reported [24], such an effect has yet to be investigated in human lung epithelial cells. MAPKs are activated in response to a variety of cellular and environmental stresses such as changes in osmolarity or metabolism, DNA damage, heat shock, ischemia, inflammatory cytokines, shear stress, UV irradiation, ceramide, and oxidative stress [25]. In this study, nintedanib was found to activate the JNK-AP-1 pathway, resulting in up-regulation of SP-D. This effect appeared to be independent of tyrosine kinase inhibition, because blockade of FGFR, VEGFR, and PDGFR did not affect nintedanib-induced SP-D expression. This observation suggests that nintedanib exerted its effect through a mechanism that is distinct from its known role as a tyrosine kinase inhibitor. Additionally, nintedanib up-regulated the expression of SFTP D mRNA expression in the presence of the Src kinase inhibitor, dasatinib. This demonstrated that the mechanism by which nintedanib up-regulates SP-D expression is distinct from its role as a Src kinase inhibitor.

Although nintedanib stimulated MAPK signalling in A549 cells in the current study, it has also been reported to inhibit MAPK and Akt signalling pathways, which contributes to angiogenesis in endothelial cells, pericytes, and smooth muscle cells, resulting in the inhibition of cell proliferation and apoptosis [5]. In this study,
however, nintedanib had the opposite effect on MAPK in A549 cells. Hence, the regulation of MAPK activity by nintedanib appears to be cell-type specific.

Although SP-D is now widely used as an IPF biomarker, recent reports have suggested that SP-D has a beneficial effect on various pathological conditions in addition to its role as a molecule of the innate immune system. Considerable attention is being focused on clarifying the pathogenic mechanisms underlying IPF, and apoptosis of alveolar epithelial cells in the lungs of IPF patients following injuries has been shown to accelerate the progression of the condition [26]. SP-D reduced the rate of alveolar macrophage apoptosis in mice, but this result might not be related to the effect of SP-D in A549 human lung epithelial cells [27]. We did not investigate the direct effect of nintedanib on apoptosis in the current study; however, it is likely that nintedanib-induced SP-D expression could inhibit apoptosis of epithelial cells, and future studies should examine this possibility.

Among the MAPKs, it has been proposed that activation of JNK triggers apoptosis in response to many types of stimuli. For instance, the DNA-damaging anticancer drugs etoposide and camptothecin have been linked to apoptosis induction through the activation of the JNK-c-Jun pathway [28]. However, the c-Jun protein has been implicated in both the induction and prevention of apoptosis, and whether c-Jun activation promotes or prevents programmed cell death is highly dependent on the cell type studied [29]. Moreover, it is likely that the gene expression patterns manifested via MAPK pathway activation are largely divergent (even in the same cell type) and dependent on the stimuli to which the cells are exposed. Because A549 cells were originally derived from a lung cancer specimen, there is a concern that nintedanib-induced MAPK signalling could lead to abnormal proliferation and growth or the induction of apoptosis of A549 cells through JNK phosphorylation. However, according to the results of the cell proliferation assay, nintedanib had no effect on cell proliferation or apoptosis at the concentrations tested.

In the current study, we observed that nintedanib did not affect the expression level of SP-B in A549 cells. In human cells, NO donors decrease SP-B promoter activity, which is mediated via increased AP-1 binding, and a reduction of SP-B also results from c-Jun over-expression [23]. In primary rat type II pneumocytes NO was found to decrease SP-D gene expression [30]. Therefore, these data suggest that distinct mechanisms of action regulate the effect of AP-1 on SP-B and SP-D gene expression. Our observations appear to be consistent with these reports because nintedanib did not stimulate SFTPb mRNA expression regardless of Jun protein phosphorylation, implying that the nintedanib-induced expression of SFTPb is relatively specific—at least in A549 human lung epithelial cells.

To examine the effect of nintedanib in vivo, we administered nintedanib to C57BL/6 mice and examined the expression of SP-D in the lungs using quantitative real-time RT-PCR and western blot analysis. We observed up-regulation of Sftpd mRNA expression in the lungs of nintedanib-treated mice. Because mice also harbour AP-1 recognition sequences in the 5′-flanking region of the Sftp gene [31], we analysed the expression of Sftp in the lungs of nintedanib-treated mice and the results suggested that nintedanib inhibited Sftp mRNA expression. Despite the presence of a consensus AP-1 site in the Sftp promoter region, Jun-dependent inhibition of Sftp promoter activity has been reported, suggesting different mechanisms of Jun-dependent gene regulation [32,33]. Our observations regarding the effect of nintedanib on Sftp mRNA expression appear to be consistent with a previous study, which suggests that AP-1 mediates Sftp inhibition.

Although Sftpd mRNA expression appeared to be up-regulated in the lungs of nintedanib-treated mice, the results were not statistically significant. This result may be attributable to the fact that whole lung homogenates were used and this may have reduced the sensitivity of the experiments. The dosage used in the in vivo study was comparable to a previous study [17]. Western blot analysis of the lungs of nintedanib-treated mice suggested a correlation between nintedanib treatment and SP-D protein up-regulation, and these in vivo observations could make our in vitro findings physiologically relevant.

This study has two principal limitations. First, we used A549 cells as a type II pneumocyte model. However, A549 is a continuous cell line derived from a human pulmonary adenocarcinoma and therefore A549 cells possess biological characteristics that are different from those of normal lung epithelial cells. Second, the concentration of nintedanib used in this study appears to be higher than the Cmax of nintedanib achieved when it is administered to humans at 300 mg/day. However, such discrepancies are common when compounds are tested in vitro, and the concentration used in this study was comparable to that used in a previous study [15]. To address this issue, we will need to explore the effect of nintedanib on SP-D expression in clinical trials.
5. Conclusions

Nintedanib was shown to increase SP-D expression in A549 cells, and this was mediated via the JNK-AP-1 pathway without affecting cell proliferation. Further study is required to clarify the relevance of this finding to the treatment of IPF.

Competing interests

The authors declare that they have no competing interests.

Author's contributions

KK, JU, KM, and AA participated in the study design, conducted data analysis and interpretation, performed the statistical analysis, drafted the manuscript, revised the manuscript critically with respect to important intellectual content, and (in the case of AA) acquired funding. TI, MI, HH, NK, KY, and TM revised the manuscript critically with respect to important intellectual content. AG participated in the design of the study, revised the manuscript critically with respect to important intellectual content, and (in the case of AA) acquired funding. All authors read and approved the final manuscript.

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