

# Antifibrotic and Anti-inflammatory Activity of the Tyrosine Kinase Inhibitor Nintedanib in Experimental Models of Lung Fibrosis<sup>§</sup>

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## ABSTRACT

The tyrosine kinase inhibitor nintedanib (BIBF 1120) is in clinical development for the treatment of idiopathic pulmonary fibrosis. To explore its mode of action, nintedanib was tested in human lung fibroblasts and mouse models of lung fibrosis. Human lung fibroblasts expressing platelet-derived growth factor (PDGF) receptor- $\alpha$  and - $\beta$  were stimulated with platelet-derived growth factor BB (homodimer) (PDGF-BB). Receptor activation was assessed by autophosphorylation and cell proliferation by bromodeoxyuridine incorporation. Transforming growth factor  $\beta$  (TGF $\beta$ )-induced fibroblast to myofibroblast transformation was determined by  $\alpha$ -smooth muscle actin ( $\alpha$ SMA) mRNA analysis. Lung fibrosis was induced in mice by intratracheal bleomycin or silica particle administration. Nintedanib was administered every day by gavage at 30, 60, or 100 mg/kg. Preventive nintedanib treatment regimen started on the day that bleomycin was administered. Therapeutic treatment regimen started at various times after the induction of lung fibrosis. Bleomycin caused increased macrophages and lymphocytes in the bronchoalveolar lavage (BAL) and elevated interleukin-1 $\beta$

(IL-1 $\beta$ ), tissue inhibitor of metalloproteinase-1 (TIMP-1), and collagen in lung tissue. Histology revealed chronic inflammation and fibrosis. Silica-induced lung pathology additionally showed elevated BAL neutrophils, keratinocyte chemoattractant (KC) levels, and granuloma formation. Nintedanib inhibited PDGF receptor activation, fibroblast proliferation, and fibroblast to myofibroblast transformation. Nintedanib significantly reduced BAL lymphocytes and neutrophils but not macrophages. Furthermore, interleukin-1 $\beta$ , KC, TIMP-1, and lung collagen were significantly reduced. Histologic analysis showed significantly diminished lung inflammation, granuloma formation, and fibrosis. The therapeutic effect was dependent on treatment start and duration. Nintedanib inhibited receptor tyrosine kinase activation and the proliferation and transformation of human lung fibroblasts and showed antifibrotic and anti-inflammatory activity in two animal models of pulmonary fibrosis. These results suggest that nintedanib may impact the progressive course of fibrotic lung diseases such as idiopathic pulmonary fibrosis.

## Introduction

Idiopathic pulmonary fibrosis (IPF) is a progressive, severely debilitating disease with a high mortality rate (King et al., 2011). Mean survival after diagnosis ranges from 2 to 3 years (Raghu et al., 2011). Even though IPF is considered rare, it is the most common idiopathic interstitial lung disease (ATS and ERS, 2002). The pathomechanisms that result in IPF are not fully understood. It has been hypothesized that injuries of the lung lead to destruction of epithelial alveolar cells and that the resulting repair process is dysregulated, leading to the proliferation and migration of fibroblasts,

transformation to myofibroblasts, and excessive collagen deposition within the lung interstitium and alveolar space (Fernandez and Eickelberg, 2012). Progressive fibrosis with stiffening of the lungs leads to dyspnea and cough. The symptoms of IPF limit physical activity and reduce patients' quality of life and independence (De Vries et al., 2001; Swigris et al., 2005). Despite high medical need, the latest international guidelines for the management of IPF did not recommend any specific pharmacological treatments for the long-term treatment of IPF (Raghu et al., 2011).

Nintedanib (BIBF 1120) is a potent intracellular tyrosine kinase inhibitor targeting fibroblast growth factor receptor (FGFR) 1, 2, and 3, platelet-derived growth factor receptor (PDGFR)  $\alpha$  and  $\beta$ , and vascular endothelial growth factor receptor (VEGFR) 1, 2, and 3. Nintedanib also inhibits the Src

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**ABBREVIATIONS:** ANOVA, analysis of variance; BAL, bronchoalveolar lavage; BALF, bronchoalveolar lavage fluid; BIBF 1120, nintedanib, methyl (3Z)-3-[[[4-[N-methyl-2-(4-methylpiperazin-1-yl)acetamido]phenyl]amino]phenyl] methylidene]-2-oxo-2,3-dihydro-1H-indole-6-carboxylate ethane sulfonate salt; BrdU, 5-bromo-2-deoxyuridine; CAB, chromotrope aniline blue; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-regulated kinase; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL, interleukin; IL-1Ra, interleukin-1 receptor antagonist (protein); IPF, idiopathic pulmonary fibrosis; KC, keratinocyte chemoattractant; NHLF, normal human lung fibroblasts; PDGF, platelet-derived growth factor; PDGF BB, platelet-derived growth factor BB (homodimer); PDGFR, platelet-derived growth factor receptor; RTK, receptor tyrosine kinase; SMA, smooth muscle actin; TGF $\beta$ , transforming growth factor  $\beta$ ; TIMP-1, tissue inhibitor of metalloproteinase-1; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

family tyrosine kinases Lck, Lyn, and Flt-3 (Hilberg et al., 2008). The contribution of inhibition of specific kinases to the mode of action of nintedanib in IPF has not been clarified, but distinct functions that may affect IPF pathology have been described for specific tyrosine kinases. FGFR1 is expressed on epithelial cells, endothelial cells, smooth muscle cells, myofibroblast-like cells, and macrophages in the lungs of patients with IPF, and FGFR2 on smooth muscle cells, myofibroblast-like cells, and neutrophils (Inoue et al., 2002). Fibroblast growth factor 2 (FGF-2) stimulates proliferation of lung fibroblasts from patients with IPF (Hetzl et al., 2005). In vivo abrogation of FGF signaling reduces bleomycin-induced pulmonary fibrosis and improves survival in bleomycin-treated mice (Yu et al., 2012).

PDGF is produced by alveolar macrophages and epithelial cells (Antoniades et al., 1990; Bonner, 2004). PDGF is a potent mitogen for fibroblasts (Clark et al., 1993) and appears to play an essential role in the expansion of myofibroblasts by stimulating proliferation, migration, and survival. Elevated levels of PDGF have consistently been observed in the fibrotic lesions of various organs (Bonner, 2004). Myofibroblasts, when too active or too numerous, deposit excessive connective tissue products in the alveolar wall. The result is a distorted alveolar architecture with compromised gas exchange (Katzenstein and Myers, 1998; Kim et al., 2006; Raghu et al., 2011). PDGFR-specific tyrosine kinase inhibitors reduce pulmonary fibrosis in animal models of lung fibrosis (Rice et al., 1999; Abdollahi et al., 2005; Aono et al., 2005; Vuorinen et al., 2007; Li et al., 2009).

Vascular abnormalities are a common feature in interstitial lung diseases, but the roles of angiogenesis and vascular endothelial growth factor (VEGF) signaling in IPF are unclear. It is controversial whether angiogenesis plays a key role in abnormal extracellular matrix remodeling and fibrosis in the lung (Renzoni, 2004; King et al., 2011), and extensive temporal and spatial heterogeneity in angiogenesis has been observed in patients with IPF (Farkas and Kolb, 2011). However, experimental overexpression of VEGF in airways induces airway inflammation and remodeling, with mucus metaplasia and subepithelial fibrosis (Lee et al., 2011), and VEGF expression correlates with subepithelial fibrosis in patients with asthma (Chetta et al., 2005). Anti-VEGF gene therapy attenuates bleomycin-induced fibrosis in mice (Hamada et al., 2005).

The results of a phase II trial of nintedanib (the TOMORROW trial) suggest that 12 months' treatment with nintedanib at a dose of 150 mg twice a day slows decline in lung function, reduces short-term exacerbations, and preserves quality of life in patients with IPF and has an acceptable safety and tolerability profile (Richeldi et al., 2011). Two phase III trials of nintedanib in patients with IPF have recently been completed.

The rationale for the in vitro and in vivo studies presented here was to elucidate the potential mode of action of nintedanib in fibrotic lung diseases such as IPF. In this report, we describe the anti-inflammatory and antifibrotic properties of nintedanib. We provide the cellular kinase profile of nintedanib and assess effects on the proliferation of fibroblasts. Further, we show that nintedanib reduces inflammation and fibrosis in two animal models of lung fibrosis.

## Material and Methods

**Cellular BA/F3 Tyrosine Kinase Assay.** The cellular tyrosine kinase assay was performed by Carna Biosciences Inc. (Kobe, Japan). The assay principle builds on the work of Daley & Baltimore (Daley et al., 1987). In brief, the proliferation and survival of BA/F3 cells usually depends on interleukin-3 (IL-3). BA/F3 cells were transformed by inducing target kinase dimerization via viral vectors. Proliferation and survival were engineered to become dependent upon maintenance of activity of an introduced specific tyrosine kinase. Inhibition of this tyrosine kinase results in a directly proportional decrease in cell viability, which, as each cell is modified to produce a uniform quantity of luciferase, results in decreased luminescence. Nintedanib [methyl (3Z)-3-[(4-[N-methyl-2-(4-methylpiperazin-1-yl)acetamido]phenyl)amino](phenyl)methylidene]-2-oxo-2,3-dihydro-1H-indole-6-carboxylate ethane sulfonate salt] was provided by Boehringer Ingelheim Pharma GmbH & Co. KG (Biberach, Germany) and was tested at concentrations of 10–1000 nM on FGFR1–4, PDGFR $\alpha/\beta$ , VEGFR 1–3, Flt-3, Lck, Lyn, and Src.

**Inhibition of Receptor Tyrosine Kinase Phosphorylation and Proliferation of Normal Human Lung Fibroblasts.** Normal human lung fibroblasts (NHLF) (no. AG CC-2512; Lonza, Basel, Switzerland) at passages 6 to 8 were stimulated with recombinant human platelet-derived growth factor BB (homodimer) (PDGF-BB, 220-BB; R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany), b-FGF (234-FSE; R&D Systems), or VEGF (293-VE; R&D Systems). Stimulation of the respective receptor phosphorylation was explored by Western blot analysis (data not shown) and enzyme-linked immunosorbent assay (ELISA). Fibroblast proliferation was assessed by 5-bromo-2-deoxyuridine (BrdU) incorporation. Because only PDGF-BB stimulation led to a statistically significant increase in receptor tyrosine kinase (RTK) phosphorylation of PDGFR $\alpha$  and PDGFR $\beta$  and fibroblast proliferation, the pharmacology of nintedanib was only explored on these two receptors.

For RTK phosphorylation, 24 hours after seeding 15,000 NHLF/well with 100  $\mu$ L/well fibroblast basal medium (no. CC-3131; Lonza), the cells were grown to ~90% confluence. Cells were starved for 24 hours and incubated with nintedanib at 0.128 nM–10  $\mu$ M for 30 minutes. Subsequently, the cells were stimulated with PDGF-BB (50 ng/ml) for 8 minutes. After lysis, the amount of phosphorylated receptor was determined by human phospho-PDGFR $\alpha$  and phospho-PDGFR $\beta$  ELISA (DuoSet IC, DYC2114-5 and DYC1767-5, respectively; R&D Systems).

For proliferation, 24 hours after seeding, 2000 NHLF/well cells were grown to ~50% confluence. Cells were starved in fibroblast basal medium containing insulin for 24 hours, then incubated with nintedanib at 0.3–1000 nM for 30 minutes and stimulated with PDGF-BB at 50 ng/ml for 72 hours. Subsequently, BrdU incorporation was determined after 18 hours according to the manufacturer's instructions (assay no. 11647229001; Roche, Basel, Switzerland) to determine the inhibition of proliferation.

**TGF $\beta$ -Stimulated Fibroblast to Myofibroblast Transformation.** The activity of nintedanib on transforming growth factor (TGF)- $\beta$ 2-induced  $\alpha$ -smooth muscle actin (SMA) gene expression was explored in primary fibroblast cell lines according to Chaudhary et al. (2007). In brief, human lung fibroblasts from three patients (mixed sex) with lung fibrosis were incubated with TGF $\beta$ 2 in the presence of nintedanib at concentrations ranging from 30 to 3000 nM. After incubation for 72 hours, the gene expression levels of  $\alpha$ -SMA were determined by quantitative real-time polymerase chain reaction and normalized relative to endogenous 18S RNA. Data are presented as a percentage of gene expression compared with vehicle alone.

**Inhibition of RTK Activation In Vivo.** Eight- to 10-week-old C57BL/6 mice (Charles River, Kisslegg, Germany) were housed under a 12-hour light/dark cycle and received food and water ad libitum. Animal experimentation was conducted in accordance with German national guidelines and regulations. An aqueous solution of nintedanib was prepared by heating to 50°C while stirring. Nintedanib was

administered by gavage at 3, 10, 30, and 100 mg/kg ( $n = 3$  per group); 110 minutes later, the animals were anesthetized with 60 mg/kg i.p. pentobarbital sodium and 2.5 mg/kg i.p. xylazine. Five minutes later, recombinant mouse PDGF-BB (50  $\mu$ g/animal, ProSpec-Tany; TechnoGene Ltd., Ness-Ziona, Israel) or vehicle was administered intratracheally, and then, 5 to 30 minutes after PDGF-BB stimulation, whole lungs were excised.

**Western Blot Analysis of Lung Tissue.** Frozen lungs were lysed in 1 ml of lysis buffer (50 mM Tris-HCl, pH 7.6, 137 mM sodium chloride, 10% glycerol, 0.1% Igepal, 0.1% SDS, 50 mM sodium fluoride, 1 mM sodium orthovanadate) containing protease inhibitor cocktail (Thermo Fisher Scientific, Rockford, IL) using an Ultrathurrax (IKA, Staufen, Germany). Lysates were cleared by centrifugation before the total protein determination with the BCA protein assay (Thermo Fisher Scientific). SDS PAGE and Western blotting were performed following a standard procedure using the Novex NuPAGE system (Life Technologies GmbH, Darmstadt, Germany). After this, the separation proteins were transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA) in a wet blotting system (Bio-Rad Laboratories, Hercules, CA). Afterward, membranes were blocked and then probed with antibodies directed against GAPDH, PDGFR $\beta$ , phosphorylated PDGFR $\alpha\beta$  (Tyr849/Tyr857), phosphorylated extracellular signal-regulated kinases 1/2 (pERK1/2, Thr202/Tyr204), phosphorylated AKT (pAKT, Ser473) (all Cell Signaling Technologies, Danvers, MA), and PDGFR $\alpha$  (Santa Cruz Biotechnology, Santa Cruz, CA). The membranes were then washed in Tris-buffered saline/Tween 20 followed by incubation with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). Immunoreactive bands were detected by addition of an enhanced chemiluminescence substrate (PerkinElmer Life and Analytical Sciences, Waltham, MA). The relative signal intensity of each band was determined with the AIDA image analysis software (Raytest Isotopenmessgeraete GmbH, Straubenhardt, Germany) and corrected to the signal intensity of the loading control GAPDH.

**Bleomycin- and Silica-Induced Lung Fibrosis in Mice.** Eight-week-old female C57BL/6 mice (Janvier, Le Genest Saint Isle, France) were kept in groups of five. Animals had access to water and food ad libitum. All animal experiments were conducted according to the French government's ethical and animal experiment regulations, and the protocols were approved by the regional ethics committee (CL2007-021). Nintedanib was administered each day by gavage at 30, 60, or 100 mg/kg per day. The administration volume was 10 ml/kg body weight. The control animals received vehicle only. Depending on the model, the animals received a single dose of bleomycin (1 mg = 1000 IU, clinical grade, Bleomycine Bellon; Sanofi-Aventis, France) at 3 mg/kg or silica particles at 2.5 mg/mouse by intranasal instillation, as previously described elsewhere (Gasse et al., 2007; Lo Re et al., 2010). The controls received the respective saline solution by intranasal instillation.

The study settings and drug treatment protocols are shown in Table 1. In brief, in the preventive studies, nintedanib treatment started on the day of bleomycin or silica administration. In the therapeutic studies, nintedanib treatment started after the induction of lung fibrosis when the initial lung injury and inflammation were

already abating. In each treatment group, 10 animals were included. All analyses were performed on the last day of the experiment. Lung function was analyzed in the bleomycin study only. For invasive measurement of airway resistance and dynamic lung compliance with a plethysmograph (Buxco, London, United Kingdom), the mice were anesthetized by intraperitoneal injection of a solution containing ketamine/xylazine. Lung function testing was performed with half of the animals ( $n = 5$  per group).

The mice were killed at the end of the experiment (Table 1). Total cell and differential cell counts were determined in the bronchoalveolar lavage fluid (BALF), total lung collagen was determined in lung tissue by means of the Sircol assay, and IL-1 $\beta$ , IL-6, keratinocyte chemoattractant (KC) and tissue inhibitor of metalloproteinase (TIMP-1) were determined in the lung homogenates. Details of the methods were according to Gasse et al. (2007).

After bronchoalveolar lavage (BAL) and lung perfusion, the large lobe was fixed in 4% buffered formaldehyde, and sections of 3  $\mu$ m were stained with H&E or chromotrope aniline blue (CAB). The severity of the morphologic changes (infiltration by neutrophils and mononuclear cells and destruction and thickening of the alveolar septae, and fibrosis and granuloma formation) were assessed semiquantitatively using a score of 0 to 5 by two independent observers blinded to the treatments.

**Statistics.** All data are presented as mean  $\pm$  S.E.M. of  $n$  animals. Statistical differences between groups were analyzed by one-way analysis of variance (ANOVA) with subsequent Dunnett's multiple comparison test for all parametric data and Kruskal-Wallis test followed by Dunn's multiple comparison test for nonparametric data (GraphPad Prism 5.04; GraphPad Software, Inc., La Jolla, CA).  $P < 0.05$  was considered statistically significant.

## Results

**Inhibitory Activity of Nintedanib on RTKs in a Cellular BA/F3 Assay.** Nintedanib inhibited FGFR1-4, PDGFR $\alpha/\beta$ , VEGFR 1-3, FLT-3, LCK, LYN, and SRC in a dose-dependent manner in BA/F3 cells engineered to be proliferation-dependent on a single RTK. The IC<sub>50</sub> values for nintedanib are shown in Table 2.

**Effect of Nintedanib on PDGF-BB-Induced PDGFR $\alpha$  and - $\beta$  Phosphorylation and Proliferation of NHLF.** Nintedanib inhibited PDGF-BB-stimulated PDGFR $\alpha$  and - $\beta$  phosphorylation in a concentration-dependent manner with IC<sub>50</sub> values of 22 nM and 39 nM, respectively (Fig. 1).

Nintedanib inhibited proliferation of PDGF-BB-stimulated fibroblasts with an IC<sub>50</sub> value of 64 nM. Complete inhibition of PDGFR $\alpha$  and - $\beta$  phosphorylation led to 70% inhibition of fibroblast proliferation (Fig. 1).

**Effect of Nintedanib on TGF $\beta$ -Stimulated Fibroblast to Myofibroblast Differentiation.** Nintedanib inhibited TGF $\beta$ -stimulated fibroblast to myofibroblast differentiation as detected by  $\alpha$ SMA gene expression in human fibroblasts

TABLE 1  
Study settings and treatment protocols of the in vivo experiments

Study	Bleomycin-Induced Lung Fibrosis		Silica-Induced Lung Fibrosis	
	Preventive	Therapeutic	Preventive	Therapeutic
Dose (mg/kg)	30, 60	30, 60	30, 100	30, 100
Study duration (day)	14	21	30	30
Compound administration (day)	0–14	7–21	0–30	10–30 20–30

Posology, once daily, oral.

TABLE 2

Tyrosine kinase inhibition of nintedanib in a cellular BA/F3 assay

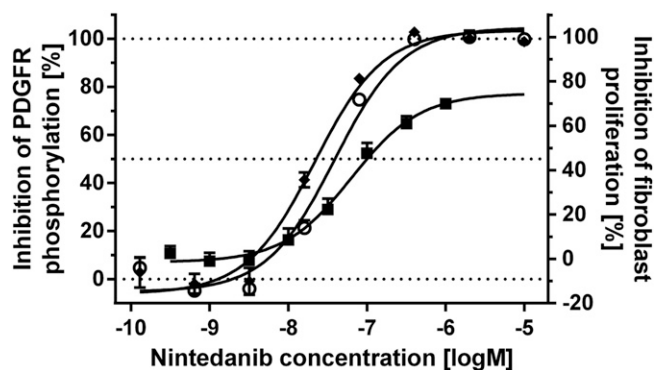
To compare the cellular activity of nintedanib on specific tyrosine kinase receptors, it was tested at 10–1000 nM in BA/F3 cells engineered to be proliferation-dependent on a single tyrosine kinase. IC<sub>50</sub> values for nintedanib to inhibit cell proliferation dependent on specific tyrosine kinases are listed in nanomoles.

Assay	IC <sub>50</sub>
	nM
FGFR1	300–1000
FGFR2	257
FGFR3	300–1000
FGFR4	300–1000
PDGFR $\alpha$	41
PDGFR $\beta$	58
VEGFR1	300–1000
VEGFR2	46
VEGFR3	33
LCK	22
LYN	300–1000
SRC	811
FLT-3	17

from patients with IPF with an IC<sub>50</sub> of 144 nM (data not shown).

**Effect of Nintedanib on PDGFR Phosphorylation in Mouse Lungs.** PDGFR $\alpha$  and - $\beta$  expression remained quite stable 5 to 30 minutes after PDGF stimulation (Supplemental Fig. 1). The maximum activation of the PDGFR detected by receptor phosphorylation was noted 5 minutes after stimulation with PDGF-BB (Supplemental Fig. 1), and the maximum downstream signaling of pAKT and pERK was detected at 15 minutes (Supplemental Fig. 1).

To explore the efficacy of nintedanib to inhibit PDGFR phosphorylation as a marker of receptor activation, nintedanib was dosed orally in mice, and 2 hours later PDGFR was stimulated by PDGF-BB. Nintedanib significantly inhibited PDGFR phosphorylation determined 5 minutes after PDGF stimulation in a dose-dependent manner (Fig. 2, A and B). Downstream signaling of pAKT and pERK was also diminished in a dose-dependent manner (Fig. 2A and Supplemental Fig. 2). The expression of PDGFR $\alpha$  and - $\beta$  was slightly



**Fig. 1.** Nintedanib inhibits PDGF-BB-stimulated PDGFR $\alpha$  and  $\beta$  phosphorylation and proliferation of human lung fibroblasts. Human lung fibroblasts were incubated with nintedanib at different concentrations and stimulated with PDGF-BB (50 ng/ml). PDGFR $\alpha$  and - $\beta$  phosphorylation was determined by ELISA specific for the phosphorylated receptors. Proliferation was determined by BrdU incorporation. Concentration-dependent inhibition of PDGFR $\alpha$  ( $\blacklozenge$ ) and - $\beta$  ( $\circ$ ) autophosphorylation and fibroblast proliferation ( $\blacksquare$ ) is presented as mean  $\pm$  S.E.M. ( $n = 3$  experiments).

elevated at higher doses of nintedanib 5 minutes after PDGF-BB stimulation (Supplemental Fig. 2).

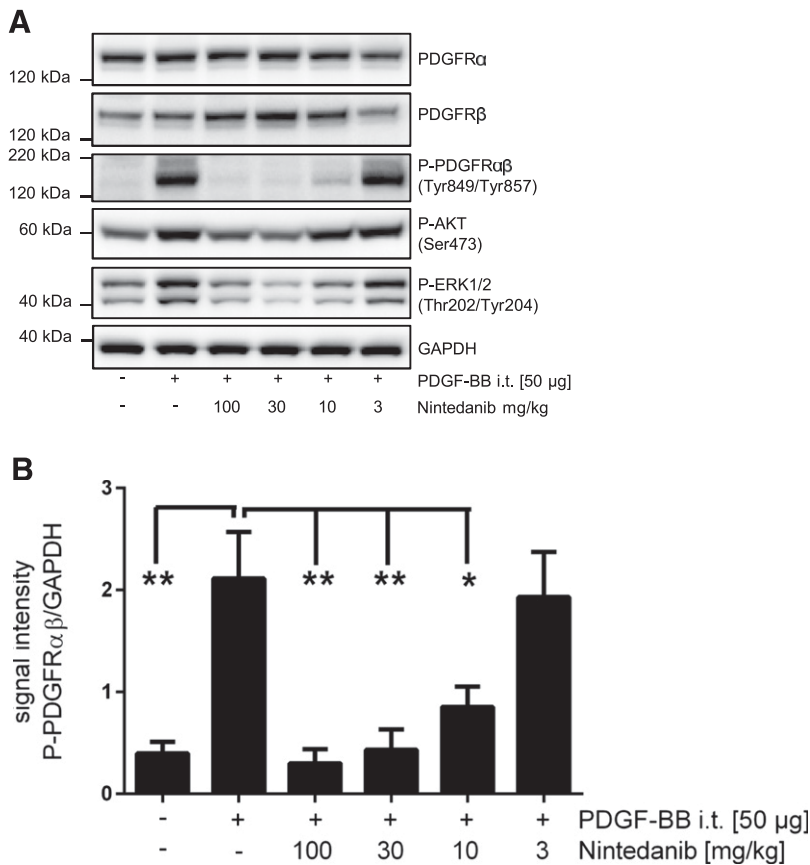
### Effect of Nintedanib on Pulmonary Inflammation and Fibrosis in a Mouse Model of Bleomycin-Induced Lung Injury and Fibrosis.

A single intranasal administration of bleomycin was well tolerated and not associated with clinical adverse effects, except for an initial loss of body weight in the preventive study of ~5%, and a more sustained loss in the therapeutic study of up to 10% of body weight reaching its maximum between days 9 and 11. Bleomycin administration caused a significant increase in lung weight at day 14 in the preventive study (vehicle control,  $0.24 \pm 0.010$  g compared with bleomycin control,  $0.32 \pm 0.013$  g,  $P < 0.01$ ) and at day 21 in the therapeutic study (vehicle control,  $0.28 \pm 0.014$  g compared with bleomycin control,  $0.41 \pm 0.023$  g,  $P < 0.001$ ). Neither airway resistance nor lung compliance was significantly changed by bleomycin administration at day 14 compared with the vehicle-treated controls (data not shown). In contrast, at day 21 bleomycin administration led to an increase in airway resistance ( $2.8 \pm 0.49$  cmH<sub>2</sub>O/ml\*s,  $P < 0.05$ ) and a decrease in lung compliance ( $0.013 \pm 0.0028$  ml/cmH<sub>2</sub>O,  $P < 0.05$ ) compared with vehicle-treated animals ( $1.39 \pm 0.059$  cmH<sub>2</sub>O/ml\*s and  $0.024 \pm 0.0012$  ml/cmH<sub>2</sub>O, respectively). Nintedanib treatment did not influence lung weight or lung function.

Regardless of whether it was analyzed after 14 days or 21 days, bleomycin administration caused a similar significant increase in total cells (Figs. 3A and 4A), macrophages (Figs. 3B and 4B), and lymphocytes (Figs. 3C and 4C) measured in the BALF, whereas neutrophils were not detectable in the BALF (data not shown). Nintedanib significantly reduced lymphocyte counts in the preventive study at doses of 30 and 60 mg/kg (Fig. 3C;  $P < 0.001$ ) and in the therapeutic study only at a dose of 60 mg/kg (Fig. 4C;  $P < 0.01$ ) but had no effect on total cell (Figs. 3A and 4A) or macrophage counts (Figs. 3B and 4B).

Bleomycin administration significantly increased IL-1 $\beta$  (Figs. 3D and 4D) and TIMP-1 concentrations (Figs. 3F and 4F) determined in lung tissue homogenates compared with controls. The increase in IL-1 $\beta$  was similar at days 14 (Fig. 3D) and 21 (Fig. 4D). TIMP-1 concentration further increased 1.9-fold between days 14 and 21 (Figs. 3F and 4F, respectively). IL-1 $\beta$  was normalized by nintedanib in the preventive study (Fig. 3D) and significantly reduced at a dose of 60 mg/kg in the therapeutic study ( $P < 0.05$ ; Fig. 4D). Nintedanib reduced TIMP-1 at doses of 30 and 60 mg/kg (Fig. 3F;  $P < 0.001$ ) in the preventive study and at a dose of 60 mg/kg ( $P < 0.05$ ) in the therapeutic study (Fig. 4F). KC concentrations were not changed (Figs. 3E and 4E).

Bleomycin administration significantly elevated the total collagen concentration in lung tissue compared with controls to a similar extent at days 14 and 21 (Figs. 3G and 4G). Significantly elevated semiquantitative histology scores indicated inflammation (Figs. 3H and 5B) and fibrosis (Figs. 3I and 6B) at day 14 after bleomycin administration, which was slightly increased at day 21 in the therapeutic study (Figs. 4, H and I, 5F, 6F). Nintedanib significantly reduced total lung collagen (Fig. 3G), inflammation (Figs. 3H and 5, C and D), and fibrosis (Figs. 3I and 6, C and D) in the preventive study ( $P < 0.01$ ,  $P < 0.01$ , and  $P < 0.05$ , respectively). In the therapeutic study, the efficacy of nintedanib was slightly reduced (Fig. 4, G and H), except for the significant reduction



**Fig. 2.** Nintedanib inhibits PDGF-BB stimulated PDGFR phosphorylation in mouse lung tissue. C57BL/6 mice received vehicle or 100, 30, 10, and 3 mg/kg nintedanib by gavage ( $n = 3$  per group). Two hours after compound dosing, PDGF-BB (50  $\mu$ g) was intratracheally instilled to induce PDGF receptor phosphorylation. Five minutes later, the animals were euthanized, and total lungs were excised, lysed, and used for Western blot analysis to detect total and phosphorylated PDGF receptors as well as phosphorylation of the downstream signaling molecules AKT and ERK1/2 by specific antibodies. GAPDH was used as a control. (A) One representative Western blot showing one animal from each group is depicted for each antibody analyzed. (B) The relative signal intensity of the phosphorylated PDGFR $\alpha\beta$  bands was evaluated by densitometry and corrected by the intensity of the GAPDH control. Data are presented as mean  $\pm$  S.E.M. of three animals per group. Statistical analysis was performed in GraphPad Prism using one-way ANOVA with subsequent Dunnett's multiple comparison test \* $P < 0.05$ ; \*\* $P < 0.01$ .

of the fibrotic score (Figs. 4I and 6, G and H) ( $P < 0.05$  and  $P < 0.01$ , respectively).

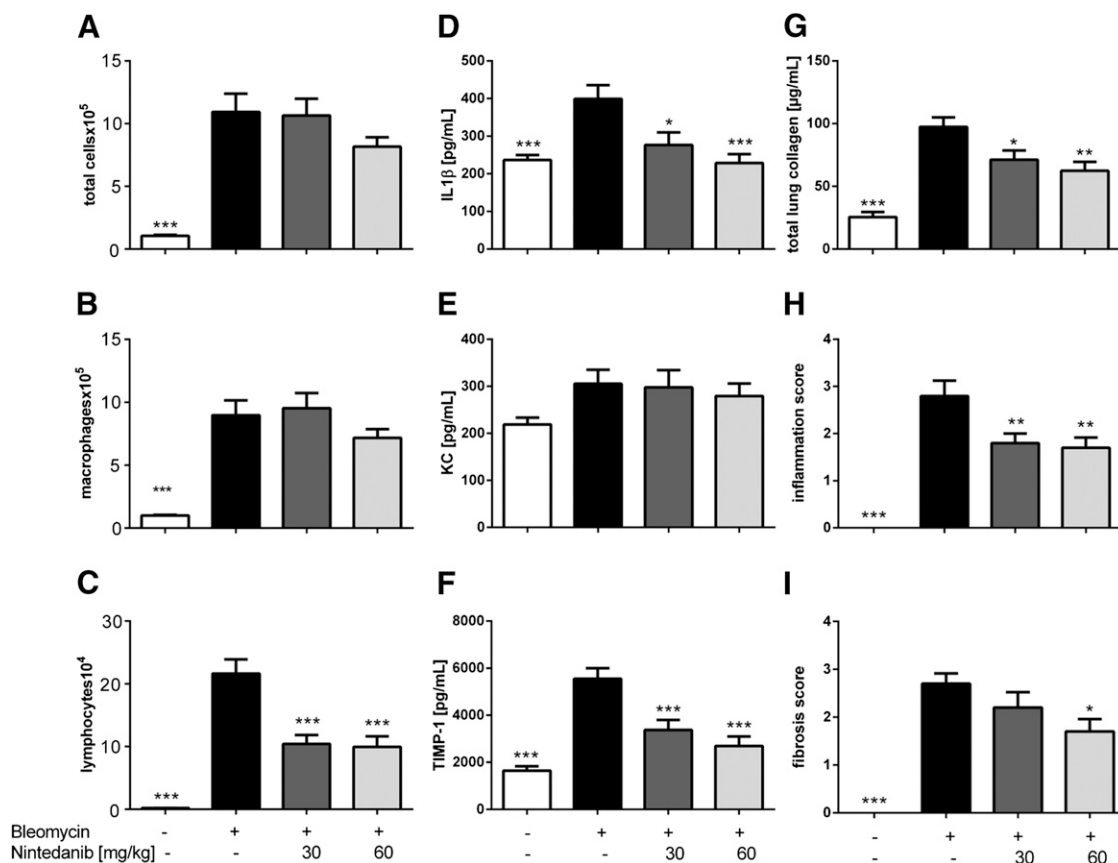
**Effect of Nintedanib on Pulmonary Inflammation, Granuloma Formation, and Fibrosis in a Mouse Model of Silica-Induced Lung Injury and Fibrosis.** Silica administration was also well tolerated and not associated with any clinical adverse effects in mice. Silica administration caused a similar significant increase in lung weight in the preventive study (vehicle control,  $0.30 \pm 0.037$  g compared with silica control,  $0.35 \pm 0.029$  g,  $P < 0.01$ ) and in the therapeutic study (vehicle control,  $0.29 \pm 0.034$  g compared with silica control,  $0.36 \pm 0.035$  g,  $P < 0.01$ ). In the preventive study, nintedanib at a dose of 100 mg/kg reduced the silica-induced increase in lung weight by 44%, but this did not reach statistical significance. However, in the therapeutic study, silica-induced lung weight increase was significantly reduced by 74% at a dose of 30 mg/kg ( $P < 0.01$ ) and by 86% at a dose of 100 mg/kg ( $P < 0.01$ ) if nintedanib treatment was started at day 10. If nintedanib treatment was started at day 20, reductions of 32% at a dose of 30 mg/kg and 43% at a dose of 100 mg/kg were detected (which did not reach statistical significance).

In both the preventive and the therapeutic study, silica administration caused significant increases in total cells (Figs. 7A and 8A), macrophages (Figs. 7B and 8B), lymphocytes (Figs. 7C and 8C), and neutrophils (Figs. 7D and 8D) measured in the BALF ( $P < 0.001$  for all cell types compared with negative control). Similar to the bleomycin study, nintedanib reduced silica-induced elevation in lymphocyte counts at a dose of 30 mg/kg (Fig. 7C;  $P < 0.01$ ) in the

preventive study, but not total cell counts (Fig. 7A) or macrophages (Fig. 7B). Neutrophil counts were reduced significantly by nintedanib at doses of 30 and 100 mg/kg (Fig. 7D;  $P < 0.01$ ). In the therapeutic study, the significant reduction by nintedanib of lymphocyte count was dependent on dose and start of treatment (Fig. 8C). Higher dose, earlier start of treatment, or longer treatment period provided better efficacy. Nintedanib reduced silica-induced neutrophil invasion, but only if the treatment was started at day 10 (Fig. 8D;  $P < 0.001$ ).

Silica administration significantly increased IL-1 $\beta$  (Figs. 7E and 8E), KC (Figs. 7F and 8F), and TIMP-1 concentrations (Figs. 7G and 8H) determined in lung tissue homogenates to similar extents in both studies compared with saline-treated control lungs ( $P < 0.001$  for all mediators). IL-6 concentration was only significantly increased in the therapeutic study (Fig. 8G;  $P < 0.01$ ). In the preventive study, nintedanib significantly reduced IL-1 $\beta$  (Fig. 7E), KC (Fig. 7F), and TIMP-1 (Fig. 7G) concentrations independent of dose. In the preventive studies, nintedanib reduced IL-1 $\beta$  (Fig. 8E), KC (Fig. 8F), IL-6 (Fig. 8G), and TIMP-1 (Fig. 8H) if treatment was started at day 10, but not if treatment was started at day 20.

Silica administration slightly but significantly elevated the total collagen concentration in lung tissue compared with saline-treated controls in both studies (Figs. 7H and 8I). Semiquantitative analysis of the histology showed that silica administration led to elevated histology scores, indicating inflammation (Figs. 7I and 8J), granuloma formation (Figs. 7J and 8K), and fibrosis (Figs. 7K and 8L) in the lungs. Representative histology micrographs are shown as Supplemental



**Fig. 3.** Preventive nintedanib treatment reduces bleomycin-induced lung inflammation and fibrosis. C57Bl/6 mice received an intranasal instillation of 3 mg/kg NaCl or bleomycin. Nintedanib was administered each day by gavage at 30 and 60 mg/kg for 14 days. Analyses were performed at day 14. BAL cell differentiation is expressed as cells per mouse lung. (A) Total cell count. (B) Macrophages. (C) Lymphocytes. Mediator concentrations in whole lung homogenates were determined by ELISA. (D) IL-1 $\beta$ . (E) Chemokine (C-X-C motif) ligand 1/KC. (F) TIMP-1. Total lung collagen was determined by Sircol assay in lung homogenate (G). H&E and CAB trichrome stained slices of the left lung were semiquantitatively scored for inflammation (H) and fibrosis (I), respectively. All groups ( $n = 10$  per group) were compared versus bleomycin-treated control animals by ANOVA followed by Dunnett's multiple comparison test for parametric data and Kruskal-Wallis test followed by Dunn's multiple comparison test for nonparametric data. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . Values are presented as mean  $\pm$  S.E.M.

Figs. 3 and 4. Total lung collagen (Fig. 7H), lung inflammation (Fig. 7I), granuloma formation (Fig. 7J), and lung fibrosis (Fig. 7K) were significantly reduced by nintedanib in the preventive study. In the therapeutic study, significant reduction of the pathology by nintedanib was limited to total lung collagen at a dose of 30 mg/kg ( $P < 0.05$ ; Fig. 8I), granuloma formation at a dose of 100 mg/kg ( $P < 0.05$ ; Fig. 8K), and lung fibrosis at doses of 30 and 100 mg/kg ( $P < 0.05$ ; Fig. 8L) if treatment was started at day 10, but not if treatment was started at day 20.

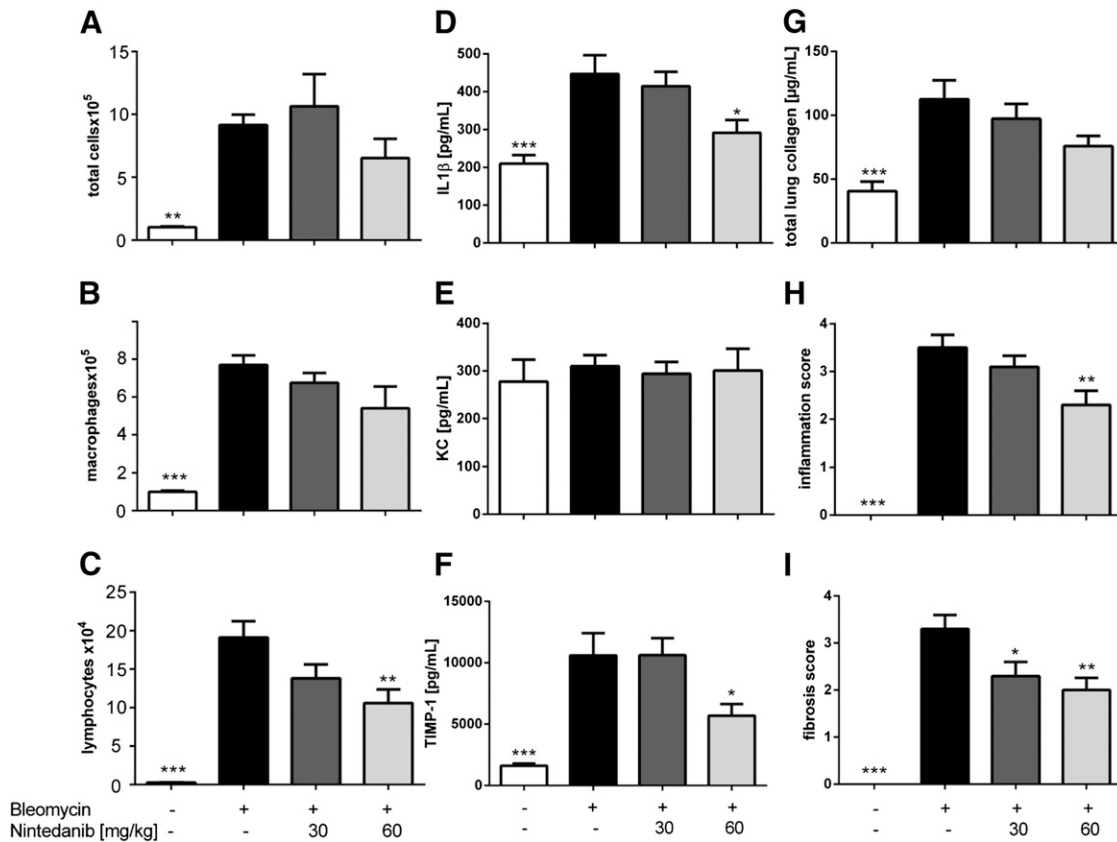
## Discussion

To improve understanding of the mode of action of nintedanib in fibrotic lung diseases such as IPF, this study explored the inhibitory activity of nintedanib in cellular assays specific for selected tyrosine kinases, in human primary fibroblasts, and in two animal models of pulmonary fibrosis.

A cellular BA/F3 assay confirmed the potent inhibitory activity of nintedanib on PDGFR $\alpha$  and PDGFR $\beta$ , VEGFR-2 and VEGFR-3, and Lck and Lyn and its lower potency on FGFR-3 and FGFR-4, as reported in in vitro kinase assays (Hilberg et al., 2008). Differences in IC<sub>50</sub> values between the cellular BA/F3 assay and the in vitro assay were found for

FGFR-1 and FGFR-2 and for VEGFR-1, with the cellular BA/F3 assay showing lower potency for nintedanib. Because BA/F3 cells are artificially engineered cells, these results can only be taken as a first indication of cellular activity. Compared with the cellular assays in endothelial cells, pericytes, and vascular smooth muscle cells described by Hilberg et al. (2008), the IC<sub>50</sub> values are in concordance.

More important insights were obtained with human lung fibroblasts stimulated with PDGF-BB. PDGF-BB stimulates PDGFR $\alpha$  and  $\beta$  causing  $\alpha\alpha$  and  $\beta\beta$  homodimerization or  $\alpha\beta$  heterodimerization (Heldin et al., 2002) and autophosphorylation of the receptor, which leads to stimulation of fibroblast proliferation (Hetzl et al., 2005). We demonstrated that nintedanib inhibited PDGFR $\alpha$  and  $\beta$  phosphorylation and proliferation of human lung fibroblasts, an observation that is relevant to the pathology of IPF (Gunther et al., 2012). The potency of nintedanib was quite similar on all the cellular systems tested, with IC<sub>50</sub> values ranging from 22 to 64 nM. However, complete inhibition of PDGFR $\alpha$  and  $\beta$  phosphorylation led to only 70% inhibition of fibroblast proliferation, indicating that a proportion of fibroblast proliferation is PDGFR independent. Nintedanib also inhibited TGF $\beta$ -induced fibroblast to myofibroblast differentiation, but only at higher concentrations (IC<sub>50</sub> = 144 nM).



**Fig. 4.** Therapeutic nintedanib treatment reduces bleomycin-induced lung inflammation and fibrosis. C57Bl/6 mice received an intranasal instillation of 3 mg/kg NaCl or bleomycin. Nintedanib was administered each day by gavage at 30 and 60 mg/kg starting at day 7. Analyses were performed at day 21. BAL cell differentiation is expressed as cells per mouse lung. (A) Total cell count. (B) Macrophages. (C) Lymphocytes. (D) IL-1 $\beta$ . (E) Chemokine (C-X-C motif) ligand 1/KC. (F) TIMP-1. Total lung collagen was determined by Sircol assay in lung homogenate (G). H&E and CAB trichrome stained slices of the left lung were semiquantitatively scored for inflammation (H) and fibrosis (I), respectively. All groups ( $n = 10$  per group) were compared versus bleomycin-treated control animals by ANOVA followed by Dunnett's multiple comparison test for parametric data and Kruskal-Wallis test followed by Dunn's multiple comparison test for nonparametric data. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . Values are presented as mean  $\pm$  S.E.M.

To show that nintedanib exerts similar effects on PDGFR activation *in vitro* and *in vivo*, we demonstrated that nintedanib dose-dependently inhibited PDGFR phosphorylation and downstream signaling via pAKT and pERK in mouse lung tissue after oral dosing. Zhuo et al. (2004) demonstrated that PDGFR $\alpha$  phosphorylation was greater in bleomycin-treated mouse lungs compared with control lungs. Hence, it can be assumed that at least the proliferation of lung fibroblasts activated by PDGF/PDGFR interaction in mice is diminished *in vivo* by the nintedanib doses administered in the bleomycin- and probably in the silica-induced lung fibrosis studies.

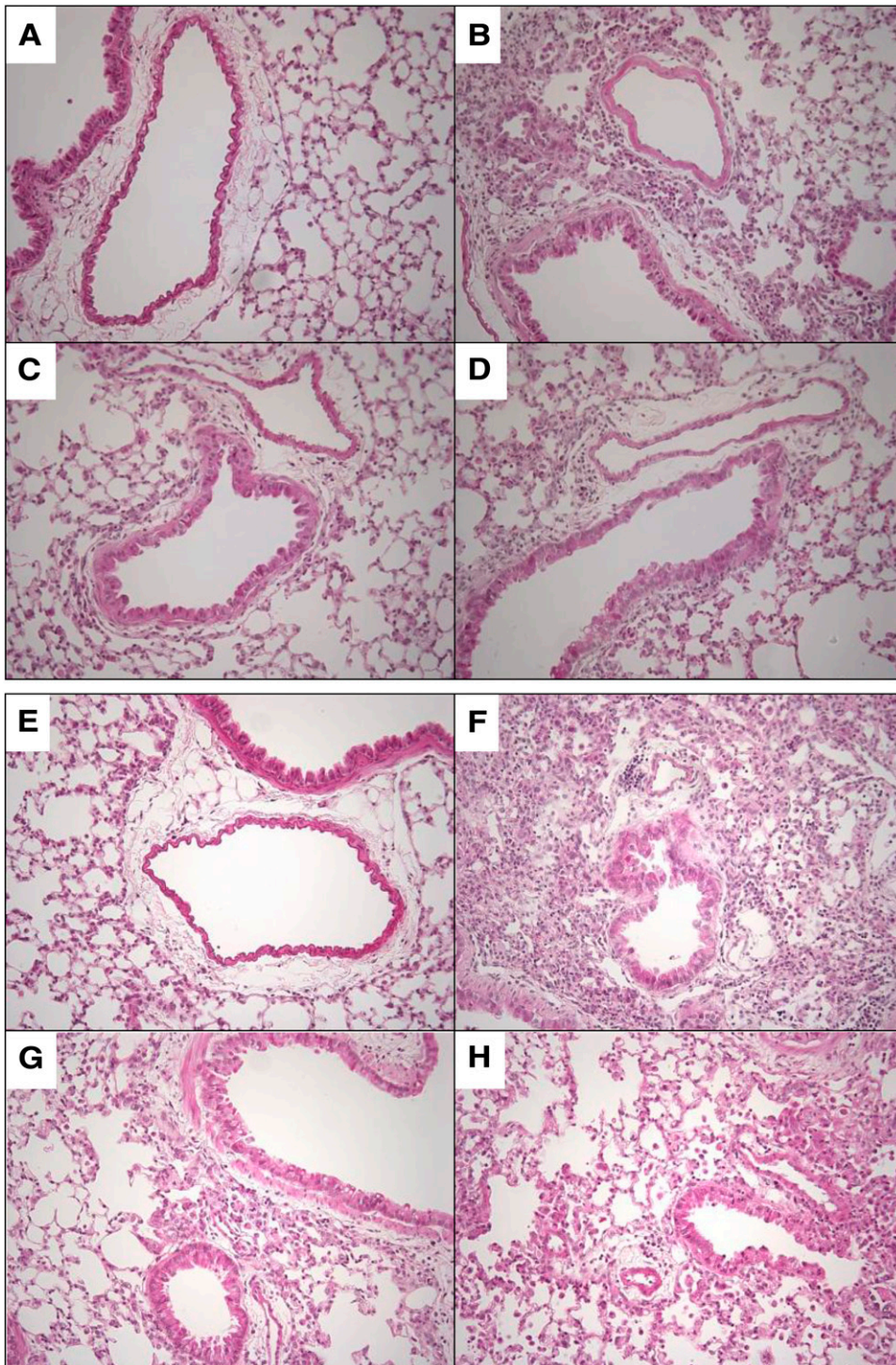
The *in vivo* experiments revealed that nintedanib exerted antifibrotic activity, as shown by reduced fibrosis in the histologic analysis and by diminished lung collagen, reflecting reduced extracellular matrix production and/or deposition. We also found that TIMP-1, a key factor in the fibrogenic response to bleomycin in mice (Manoury et al., 2006), was significantly reduced by nintedanib in mouse lung tissue. In general, the antifibrotic activity of nintedanib was similar in the preventive and therapeutic studies of the silica-induced and bleomycin-induced fibrosis models except that there was reduced inhibition of TIMP-1 and total lung collagen at a dose of 30 mg/kg in the therapeutic bleomycin study and no inhibitory activity in the therapeutic silica model if nintedanib

treatment was started at day 20. Either the late treatment start or the short treatment duration or both could be responsible for the observed reduction in antifibrotic activity.

Nintedanib exerted anti-inflammatory activity, demonstrated by reduced lymphocyte and neutrophil counts in the BALF, diminished IL-1 $\beta$  and KC concentrations in lung homogenates, and reduced inflammation and granuloma formation in the histology analysis. In general, the anti-inflammatory activity of nintedanib was weaker in the therapeutic studies than in the preventive studies. This might be related to the fact that, in the therapeutic studies, inflammation had peaked before nintedanib treatment was started. Reduction in the BALF lymphocyte count seems to be the parameter most sensitive to nintedanib treatment: even though treatment was started late (at day 20) in the therapeutic silica study, nintedanib significantly reduced BALF lymphocytes.

Bleomycin-induced deterioration in lung function was not influenced by nintedanib in the course of the experiments. This might be related to the relatively short treatment period of 14 days in the preventive as well as in the therapeutic part of the bleomycin study.

The strong and consistent inhibitory activity of nintedanib on IL-1 $\beta$  is an interesting finding. An imbalance of the IL-1 receptor antagonist (IL-1Ra)/IL-1 $\beta$  ratio, resulting in increased

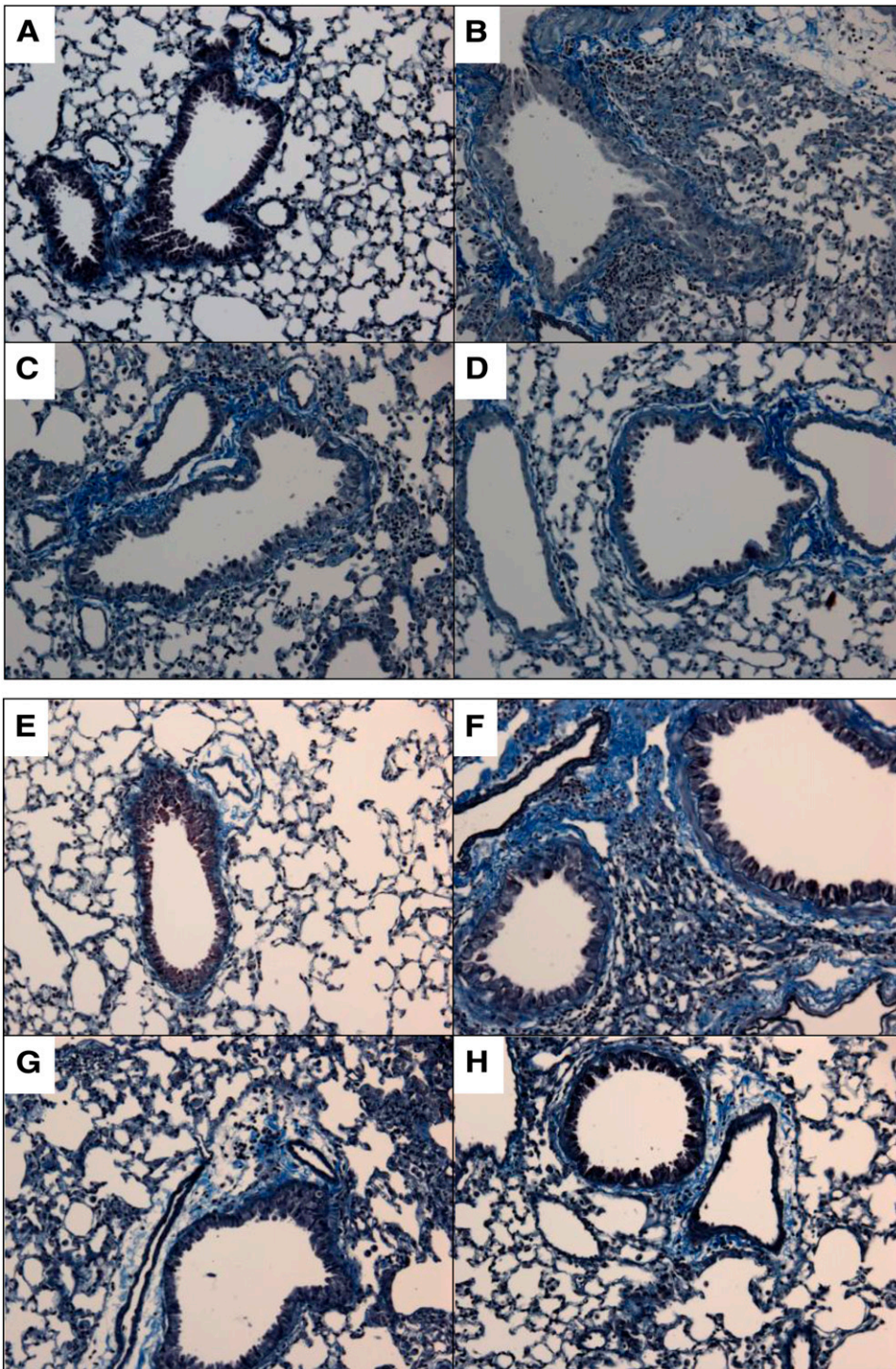


**Fig. 5.** Nintedanib treatment reduces bleomycin-induced lung inflammation and fibrosis. Representative micrographs of H&E-stained lung sections from mice of the treatment groups are shown. C57BL/6 mice that received an intranasal instillation of 3 mg/kg bleomycin (B and F) showed a prominent peribronchial and interalveolar inflammation that was absent in control animals (A and E). Nintedanib was administered each day by gavage at 30 mg/kg (C and G) and 60 mg/kg (D and H). Daily nintedanib treatment from days 0 to 14 in the preventive study (C and D) and from days 7 to 21 in the therapeutic study (G–H) reduced bleomycin-induced lung pathology. Analyses were performed on the last day of the study, which was day 14 (A–D) or day 21 (E–H).

IL-1 $\beta$  activity, has been reported in BALF macrophages from patients with IPF (Mikuniya et al., 1997). Polymorphisms in *IL1RN* influence IL-1Ra mRNA expression, suggesting that lower levels of IL-1Ra predispose to developing IPF (Korthagen et al., 2012). IL-1 $\beta$  is a well-known proinflammatory cytokine produced by the macrophages of patients with IPF (Zhang et al., 1993). IL-1 $\beta$  was shown to have an important role in driving the development of fibrosis (Wilson et al., 2010). The inhibition of IL-1 $\beta$  by nintedanib may help to dampen the profibrotic milieu in the lung.

A further interesting finding is the significant inhibition of TIMP-1 by nintedanib. TIMP-1 inhibits many matrix metalloproteinases (MMPs), including MMP-1 (also known as collagenase-1) which is capable of degrading type I and II fibrillar collagens (Pardo and Selman, 2012). TIMP-1 expression has been found to be elevated in interstitial macrophages (Selman et al., 2000), fibroblasts (Ramos et al., 2001), and sputum (Beeh et al., 2003) from patients with IPF. Thus, the reduction in lung collagen produced by nintedanib might be at least partly attributable to its inhibitory activity on TIMP-1.

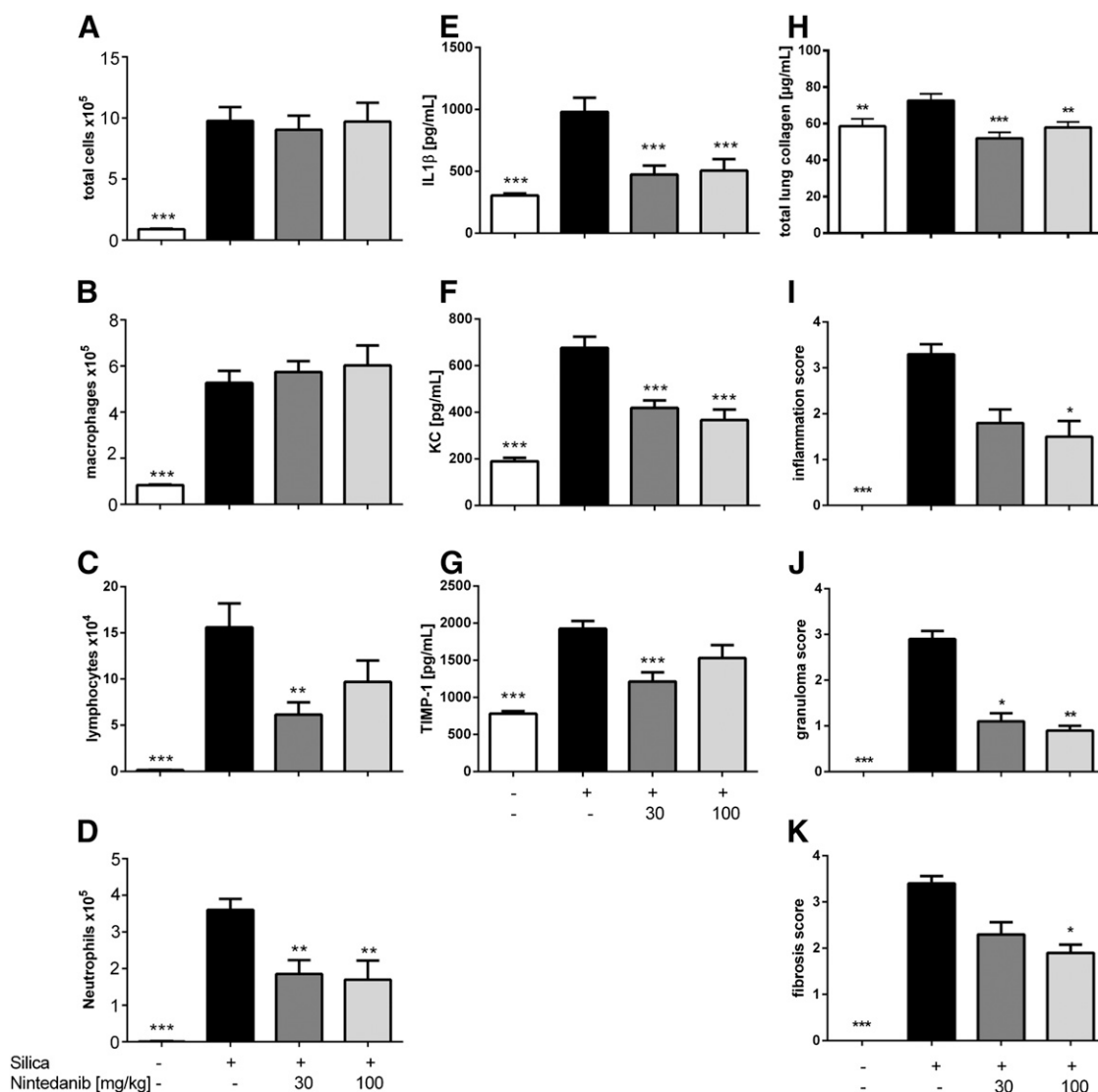




**Fig. 6.** Nintedanib treatment reduces bleomycin-induced lung inflammation and fibrosis. Representative micrographs of CAB-stained lung sections from mice of the treatment groups are shown. C57Bl/6 mice that received an intranasal instillation of 3 mg/kg bleomycin (B and F) showed a prominent peribronchial and perivascular collagen accumulation and peribronchial and interalveolar inflammation that was absent in control animals (A and E). Nintedanib was administered each day by gavage at 30 mg/kg (C, G) and 60 mg/kg (D and H). Daily nintedanib treatment from days 0 to 14 in the preventive study (C and D), and from days 7 to 21 in the therapeutic study (G and H) reduced bleomycin-induced lung pathology. Analyses were performed on the last day of the study, which was day 14 (A–D) or day 21 (E–H).

Although in this study only the inhibitory activity of nintedanib on PDGFR was explored *in vitro*, it is likely that inhibition of other tyrosine kinases contributes to the efficacy of nintedanib *in vivo*. According to Hilberg et al. (2008), exposure in mice after oral administration of 60 and 100 mg/kg nintedanib is sufficient to inhibit FGFRs, VEGFRs, Lck, Src, and Flt-3 in the animal experiments presented here. The integrated antifibrotic and anti-inflammatory activity of nintedanib might be dependent on its inhibitory activity on multiple kinases. It is well documented that the PDGF/

PDGFR signaling cascade is implicated in the development of pulmonary fibrosis (Bonner, 2004), but inhibition of FGFRs might also have a role in the *in vivo* efficacy of nintedanib. Enhanced FGF levels as well as increased expression of FGFR1 on epithelial, endothelial, and smooth muscle cell/myofibroblast-like cells and increased expression of FGFR2 on interstitial cells have been detected in the lungs of patients with IPF (Inoue et al., 2002). *In vivo* abrogation of FGF signaling has been shown to reduce bleomycin-induced pulmonary fibrosis and improve survival in bleomycin-treated



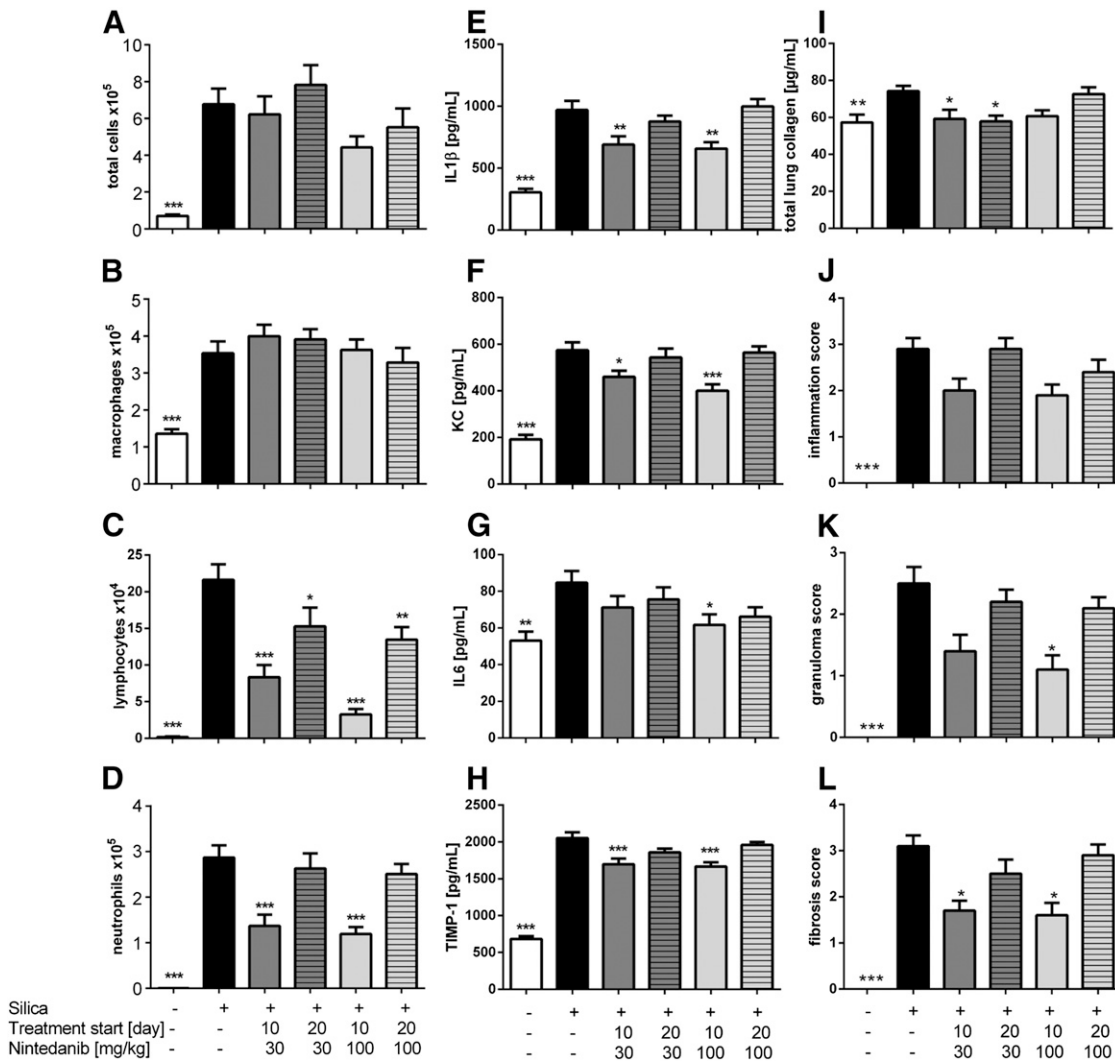
**Fig. 7.** Preventive nintedanib treatment reduces silica-induced lung inflammation and fibrosis. C57Bl/6 mice received an intranasal instillation of NaCl or silica particles at 2.5 mg/animal. Nintedanib was administered each day by gavage at 30 and 100 mg/kg for 30 days. Analyses were performed at day 30. BAL cell differentiation is expressed as cells per mouse lung. (A) Total cell count. (B) Macrophages. (C) Lymphocytes. (D) Neutrophils. The mediator concentrations in whole-lung homogenates were determined by ELISA. (E) IL-1 $\beta$ . (F) Chemokine (C-X-C motif) ligand 1/KC. (G) TIMP-1. Total lung collagen was determined by Sircol assay in lung homogenate (H). H&E and CAB trichrome stained slices of the left lung were semiquantitatively scored for inflammation (I), granuloma formation (J), and fibrosis (K). All groups ( $n = 10$  per group) were compared versus silica-treated control animals by ANOVA followed by Dunnett's multiple comparison test for parametric data and Kruskal-Wallis test followed by Dunn's multiple comparison test for nonparametric data. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . Values are presented as mean  $\pm$  S.E.M.

mice (Yu et al., 2012). The role of VEGF in IPF remains to be further explored, but experimental evidence in rats suggests that inhibition of VEGFR may reduce fibrosis (Hamada et al., 2005). No specific role in the pathology of IPF has been described for Lck, Src, and Flt-3, but the anti-inflammatory effects of nintedanib could potentially be partly attributable to its inhibitory activity on these tyrosine kinases (Das et al., 2006).

No animal model is available resembling the exact pathology and chronicity of human IPF. In our attempt to elucidate the mode of action of nintedanib, inhibitory activity was explored in two well-described animal models of lung inflammation and fibrosis: the bleomycin-induced (Gasse et al., 2007) and the silica-induced model (Lo Re et al., 2010). Both these models reflect at least some aspects of the pathology seen in patients with IPF. When designing our

studies, we followed the recommendations of Moeller et al. (2008) in that all potential antifibrotic compounds should be evaluated in the phase of established fibrosis, rather than in the early period of bleomycin-induced inflammation, for assessment of antifibrotic properties.

Both models show similarities but also differences in lung pathology. The bleomycin model is reported to show initial lung injury, with strong but transient neutrophilic inflammation subsequently leading to lung fibrosis (Izbicki et al., 2002). Bleomycin is metabolized in the lung, leading to a decrease in neutrophilic inflammation and a fibrotic response that spontaneously resolves after 2 to 3 months (Walters and Kleeberger, 2008). In the silica model, the stimulating crystals are not cleared from the lung, leading to ongoing inflammation, granuloma formation, and progressive fibrosis (Callis et al., 1985; Brass et al., 2012). In our study,



**Fig. 8.** Therapeutic nintedanib treatment reduces silica-induced lung inflammation and fibrosis. C57Bl/6 mice received an intranasal instillation of NaCl or silica particles at 2.5 mg/animal. Nintedanib was administered each day by gavage at 30 and 100 mg/kg starting at day 10 (clear bars) or day 20 (hatched bars). Analyses were performed at day 30. BAL cell differentiation is expressed as cells per mouse lung. (A) Total cell count. (B) Macrophages. (C) Lymphocytes. (D) Neutrophils. The mediator concentrations in whole-lung homogenates were determined by ELISA. (E) IL-1 $\beta$ . (F) Chemokine (C-X-C motif) ligand 1/KC. (G) IL-6. (H) TIMP-1. Total lung collagen was determined by Sircol assay in lung homogenate (I). H&E and CAB trichrome-stained slices of the left lung were semiquantitatively scored for inflammation (J), granuloma formation (K), and fibrosis (L), respectively. All groups ( $n = 10$  per group) were compared versus silica-treated control animals by ANOVA followed by Dunnett's multiple comparison test for parametric data and Kruskal-Wallis test followed by Dunn's multiple comparison test for nonparametric data. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . Values are presented as mean  $\pm$  S.E.M.

lung inflammation and fibrotic score were quite similar in both models except for the strong neutrophilic inflammation and granuloma formation only detected in the silica-treated lungs and the stronger increase in lung weight in the bleomycin-treated lungs, which might be related to stronger matrix deposition indicated by the higher lung collagen increase. The consistent inhibitory effects shown by nintedanib in both animal models suggest that the antifibrotic activity demonstrated in this study is an intrinsic feature of nintedanib and not only an indirect activity caused by suppressing the initial inflammatory response.

In summary, we have shown that nintedanib inhibits RTK activation and the proliferation and transformation of human lung fibroblasts. In vivo nintedanib showed consistent antifibrotic and anti-inflammatory activity in two animal models that reflect aspects of pulmonary fibrotic diseases. We propose that the antifibrotic and anti-inflammatory activities demonstrated

in this study are likely aspects of the mode of action of nintedanib in fibrotic lung diseases. The inhibitory activity of nintedanib on lung fibrosis in the therapeutic studies raises the hope that nintedanib might reduce disease progression in patients with lung fibrosis. The antifibrotic and anti-inflammatory activity of nintedanib may impact the clinical course of pulmonary fibrotic diseases such as IPF.

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## Authorship Contributions

Participated in research design: Wollin, Quesniaux, Holweg, Ryffel.

Conducted experiments: Wollin, Maillet, Holweg, Ryffel.

Performed data analysis: Wollin, Maillet, Quesniaux, Holweg, Ryffel.

Wrote or contributed to the writing of the manuscript: Wollin, Maillet, Quesniaux, Holweg, Ryffel.

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