Supplementary appendix

Section A: Determination of nintedanib binding mode

Methodology for FGFR-1 kinase domain preparation

The intracellular domain of human FGFR-1 aa456-765 triple mutant L457V, C488A, C584A was used for the crystallographic studies. The amino acid sequence (after His-tag cleavage with TEV protease) was as follows:

GAMVAGVSEYELPEDPRWELPRDRLVLGKPLGEGAFGQVVLAEAIGLDKDKPNRVTKVAVK
MLKSDATEKDLSDLISEMEMMKMIGKHKNIINLLGACTQDGPLYVIVEYASKGNLREYLQARR
PPGLEYAYNPSHNPEEQLSSKDLVSCAYQVARGMEYLASKKCIHRDLAARNVLVTEDNVMK
IADFGLARDIHHIDYYKKTTNGRLPVKWMAPEALFDRIYTHQSDVWSFGVLLWEIFTLGGSP
YPGVPVEELFKLLKEGHRMDKPSNCTNELYMMMRDCWHAVPSQRPTFKQLVEDLDRIVAL
TSNQE

FGFR-1 kinase was expressed in insect cells using Baculovirus infection. From a 3 L expression culture, FGFR-1 kinase was purified using a N-terminal His-tag via Ni-NTA affinity chromatography. The N-terminal His-tag was then removed by cleavage with TEV protease and the cleaved FGFR-1 kinase was found in the flow through a second Ni affinity purification. The protein was concentrated and further purified on size exclusion chromatography (Superdex 200, HiLoad 26/60) in a buffer containing 20 mM Tris pH 8.0, 100 mM NaCl, 2 mM DTT. Peak fractions were pooled and concentrated to 8-10 mg/mL. The purified FGFR-1 intracellular domain was >95% pure and the yield was 2.5 mg/L culture.

Methodology for FGFR-1 kinase domain crystallisation, nintedanib soaking and structure solution

The crystals were obtained using the hanging drop method by mixing 1 µL protein solution with equal amount of the reservoir solution containing 0.2 M (NH4)2SO4, 19% polyethylene glycol 3350, 0.1 M BIS-TRIS pH 6.0. The crystals appeared in 3-7 days and belonged to space group C2 with cell dimensions of a = 212.2 Å, b = 50.0 Å, c = 66.3 Å and β=107.3°. The nintedanib complex was generated by soaking crystals 16 h in reservoir solution supplemented with 1% of a nintedanib stock solution (nintedanib dissolved to 100 mM in dimethyl sulfoxide). For data collection, crystals were flash frozen in a 100 K nitrogen stream, with the mother liquor supplemented with 25% glycerol serving as cryoprotectant. Diffraction

data were collected at the SWISS LIGHT SOURCE (SLS, Villigen, Switzerland). Data were processed using the programmes XDS and XSCALE [1]. The structure was solved by molecular replacement using a published crystal structure of FGFR-1 kinase (PDB-ID 1FGK) as a search model. Model building and refinement were performed according to standard protocols with the software packages CCP4 [2], Buster (GlobalPhasing Ltd.) and COOT [3]. For the calculation of the free R-factor, a measure to cross-validate the correctness of the final model, about 5% of measured reflections were excluded from the refinement procedure. The structure was refined at a resolution of 2.07 Å to a final R-factor of 22.8% (Rfree=24.2%).

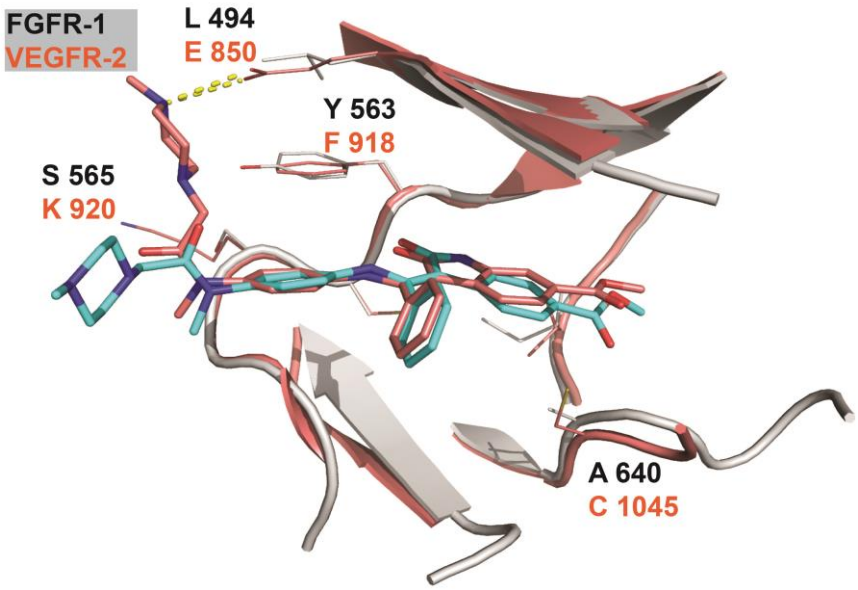
Results: nintedanib binding mode

Nintedanib binds to the adenosine triphosphate (ATP)-binding site in the cleft between the N- and C-terminal lobes of the kinase domain. The indolinone scaffold forms two hydrogen bonds to the backbone carbonyl oxygen of Glu562 and the backbone nitrogen of Ala564 in the hinge region. The carboxy methyl ether moiety points into the kinase specificity pocket where it forms hydrogen bonds to the backbone nitrogen of Asp641. The methyl piperacinyl-group points into the solvent region and is disordered in the crystal structure. A superimposition with the binding mode of nintedanib in the related angiokinase VEGFR-2 (Protein data bank entry 3C7Q) is shown in Figure S1. Overall kinase domain architecture and active site amino acid composition are largely conserved and the two binding modes are highly similar. Two differences in the respective binding modes can be explained on the basis of non-conserved amino acids. First, the ester moiety is stacked against the Ala640 side chain in FGFR-1. In VEGFR-2, its replacement by the larger Cys1045 results in a slight displacement of the ester moiety (and indolinone core). Second, in VEGFR-2 the methyl piperacinyl-group forms van der Waals interactions to Phe918 and a salt bridge to Glu850. In FGFR-1, Phe918 is replaced by the slightly larger Tyr563 and Glu850 is replaced by the lipophilic Leu494. Accordingly, the piperacinyl-group can no longer form productive interactions with the protein and is facing towards the solvent.

REFERENCES

1. Kabsch W. Integration, scaling, space-group assignment and post-refinement. *Acta Crystallogr (D)* 2010; 66: 133-144.
2. Collaborative Computational Project, Number 4 (CCP4). The CCP4 suite: programs for protein crystallography. *Acta Crystallogr (D)* 1994; 50: 760-763.
3. Emsley P, Lohkamp B, Scott WG, Cowtan K. Features and development of Coot. *Acta Crystallogr (D)* 2010; 66: 486-501.

FIGURE S1 A superimposition with the binding mode of nintedanib in the related angiokinase vascular endothelial growth factor receptor (VEGFR)-2



Section B: Effect of nintedanib on epithelial to mesenchymal transition (EMT)

Materials and methods

Human alveolar type II epithelial cells were isolated from lung parenchyma obtained by video-assisted thoracoscopic surgery from patients with IPF and from donors without fibrotic lung diseases. Cells were grown in epithelial growth medium. EMT was induced by stimulation with transforming growth factor (TGF)β (2 ng/mL) for 72 h. Cells were fixed by 2% paraformaldehyde and permeabilised. Fluorescein staining for α-SMA was used to assess the epithelial versus mesenchymal cell status.

Results

Nintedanib at a concentration of 300 nmol/L did not alter TGFβ-induced EMT.

Section C: Effect of nintedanib on bleomycin-induced pulmonary fibrosis in rats

Materials and methods

Eight- to ten-week-old male Wistar rats (Charles River, Kisslegg, Germany) were kept in groups of five. Animals had access to water and food *ad libitum*. To induce lung fibrosis the animals received a single dose of bleomycin intratracheally (2.2 mg/kg body weight in 300 µL saline) using a catheter (0.5 mm internal diameter, 1.0 mm external diameter) while being short-term anesthetised with Isofluorane 3-4%. Controls received the respective saline solution by intratracheal instillation. Nintedanib was administered once daily by oral gavage suspended in 0.1% Natrosol (10 mL/kg body weight). Control animals received vehicle only. In the preventive treatment regimen, animals were treated from the first day to day 20, in the therapeutic regiment treatment started at day 10 and lasted till day 20. In each treatment group, 10 animals were included. All animals were sacrificed at day 21. All experiments were performed in accordance with German guidelines for animal welfare, performed by persons certified to work with animals and approved by the responsible authorities.

Messenger RNA extraction and cDNA synthesis

A total of 100 mg of lung tissue was disrupted in 1.5 mL of Trizol (Invitrogen) by a Retsch MM300 tissue disruptor (Qiagen) at a frequency of 30.0 Hz for 8 min. Debris was removed by centrifuging at 12000 rpm for 10 min. The RNA was extracted using a modified version of the manufacturer's protocol supplied with Trizol. Briefly, 0.3 mL of chloroform was added to the tube and incubated at room temperature for 5 min while shaking. After centrifuging for 15 min at 12000 rpm at 4°C the aqueous phase was mixed with 750 µL isopropanol and stored at –80°C overnight. After centrifuging for 40 min at 12000 rpm at 4°C the supernatant was removed and 500 µL of 70% ethanol was added to wash the pellet. This wash step was repeated twice with 10 min centrifuging, after which the pellet was left to dry for 10–15 min. Finally the pellet was resuspended in 20 µL RNase free water and stored at –80°C. The concentration of each sample was measured using a spectrophotometer.

Using the Superscript™ III (Invitrogen, Paisley, UK) reverse transcriptase – first strand synthesis kit, 2 µg of each mRNA sample was reverse transcribed using a modified version of the manufacturer's protocol. Briefly, a mixture of 2 µg RNA, 1 µl random hexamer primers (50 ng/µL), 1 µl dNTP mix (10 mM) was made up to 10 µl with diethylpyrocarbonate (DEPC)-treated water and incubated at 65°C for 5 min, after which it was placed on ice for 5 min. Following this, to each reaction, 2 µL of reverse transcriptase buffer (10X), 4 µL of Magnesium chloride ($MgCl_2$) (25 mM), 2 µL of dithiothreitol (0.1 M), 1 µL of RNaseOUT™ (40 U/µL) and 1 µL of SuperScript™ III enzyme (200 U/µL) was added and the mixture placed in a thermal cycler (Applied Biosystems) under the following conditions: 25°C for 10 min, 50°C for 50 min and 85°C for 5 min, after which 1 µL of RNase H was added and incubated at 37°C for 20 min. The synthesised cDNA was diluted to 5 ng/uL using the assumption that the reverse transcriptase reaction fully transcribed all of the mRNA to cDNA and was at a concentration of 100 ng/µL.

Investigation of gene expression using real-time PCR (TaqMan)

Gene expression was investigated using the Applied Biosystems 7700 sequence detection system. Primers for the 18S endogenous control and TGFβ1 were purchased as pre-developed assay reagent kits, whereas primers and probes for pro-collagen I were designed using PrimerExpress™ (Applied Biosystems), ensuring that at least one of the primers or probes in each set overlapped an intron/exon junction, thus eliminating the possibility of amplifying any contaminating genomic DNA in the cDNA sample. The pre-developed assay reagents (PDARs) amplified only cDNA.

Real-time PCR was carried out in 25 µL reactions, using 25 ng (5 µL) of cDNA per reaction. A quantitative PCR core kit was purchased (Eurogentec) and a master-mix was made up as follows for 100 reactions: 500 µL 10× reaction buffer, 500 µL $MgCl_2$ (50 mM), 200 µL deoxyribonucleotide triphosphate mix solution (5 mM), 25 µL Hot Goldstar enzyme, 75 µL

18S PDAR, 22.5 µL forward primer, 22.5 µL reverse primer, 15 µL probe and 640 µL DEPC treated water. 20 µL of this master-mix was then added to 25 ng (5 µL) target cDNA. Each analysis was carried out in triplicate. In order to quantify the gene expression, a standard curve was constructed for each primer set and was included on each plate. The standards were made up of a mix of all the cDNAs under investigation; this mix of cDNAs was serially diluted 10, 20, 50, 100 and 1000 times. A standard curve was constructed of the obtained CT (Cycle at which amplification reaches a set Threshold) against the log10 of the dilution factor. Curves were drawn for the target gene and the 18S rRNA endogenous control. The CT value for both targets was then converted to a fold dilution using the standard curve and the target gene value was normalised to the 18S gene value.

Histology

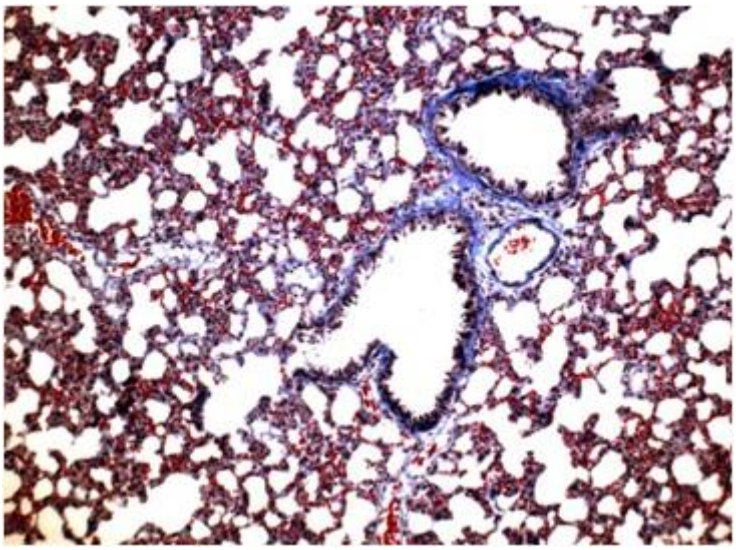
The lung tissues were fixed in 4% formalin, embedded in paraffin and 5 µm sections were cut. Collagen deposition was assessed using Masson's Trichrome staining. Pathology was scored in a semi-quantitative, blinded manner by two independent observers.

Statistics

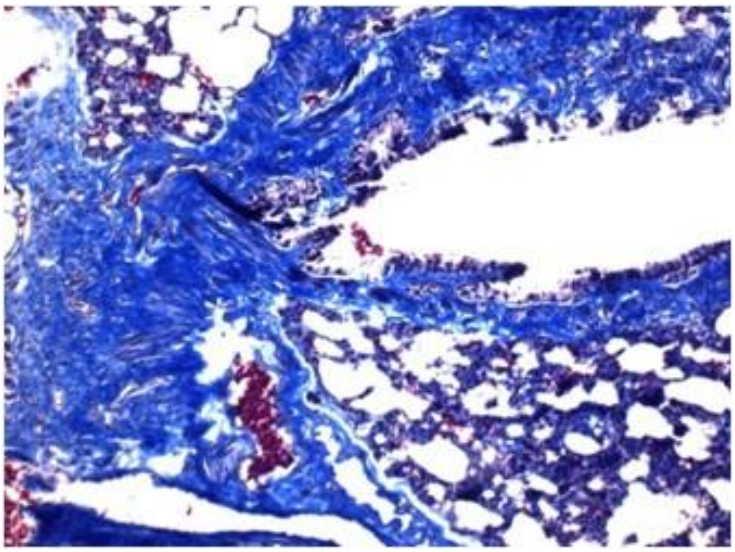
All statistical analyses were carried out using GraphPad Prism V 4.02 software (GraphPad Software Inc., La Jolla, CA). Comparisons were made using a non-parametric t-test (Mann-Whitney U test). Statistical significance was accepted at $p<0.05$.

FIGURE S2: Representative Masson's Trichrome-stained histology slides of lungs of rats treated with saline, bleomycin, and bleomycin plus nintedanib. Saline group (controls) received saline intratracheally on day 0 followed by oral treatment with vehicle from day 10 till day 21 (A). The bleomycin group received bleomycin intratracheally on day 0 followed by oral treatment with vehicle from day 10 till day 21 (B). The nintedanib group was treated with bleomycin intratracheally at day 0 followed by once daily administration of nintedanib (50 mg/kg) from day 10 till day 21 (C).

A



B



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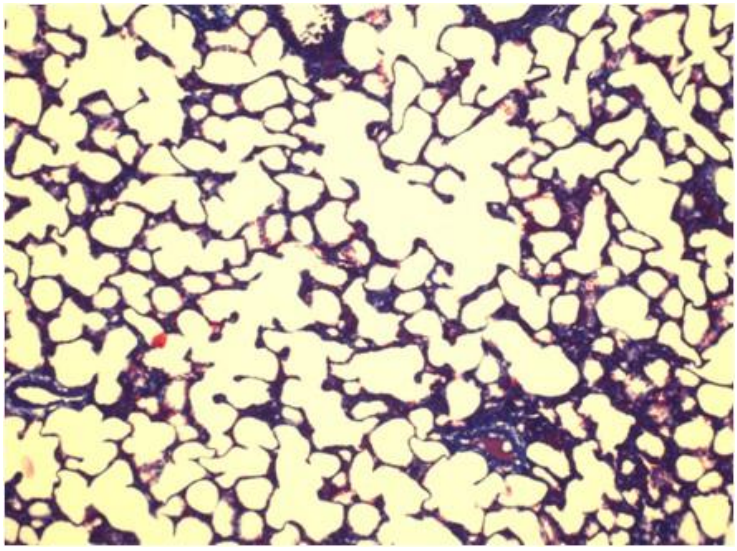
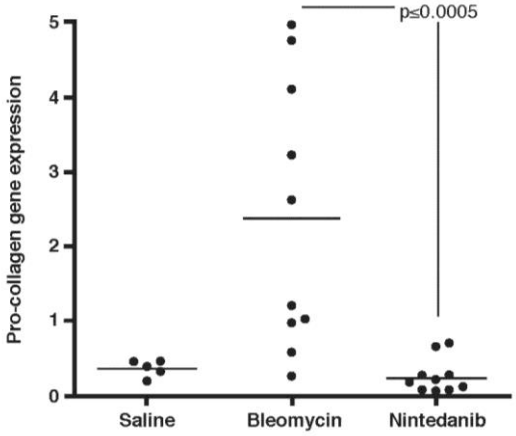


FIGURE S3: Real-time PCR analysis of procollagen-1 (A) and TGFβ1 (B) gene expression in lungs of rats treated with saline, bleomycin, and bleomycin plus nintedanib. Saline group (controls) received saline intratracheally on day 0 followed by oral treatment with vehicle from day 10 till day 21. The bleomycin group received bleomycin intratracheally on day 0 followed by oral treatment with vehicle from day 10 till day 21. The nintedanib group was treated with bleomycin intratracheally at day 0 followed by once daily administration of nintedanib (50 mg/kg) from day 10 till day 21.

A



B