Inhibition of PDGF VEGF and FGF signalling attenuates fibrosis

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Short title:
Inhibition of fibrosis by BIBF1000
Abstract

**Background:** BIBF1000 is a small molecule inhibitor targeting the receptor kinases of PDGF, bFGF and VEGF, which have known roles in the pathogenesis of pulmonary fibrosis.

**Methods:** The anti-fibrotic potential of BIBF1000 was determined in a rat model of bleomycin-induced lung fibrosis and in an *ex vivo* fibroblast differentiation assay. Rats exposed to a single intra-tracheal injection of bleomycin were treated with BIBF1000 starting 10 days after bleomycin administration. To gauge for anti-fibrotic activity, collagen deposition and pro-fibrotic growth factor gene expression was analyzed in isolated lungs. Furthermore, the activity of BIBF1000 was compared to imatinib mesylate (combined PDGFR, c-kit, c-abl kinase inhibitor) and SB-431542 (TGFβ receptor I kinase inhibitor) in an *ex vivo* TGFβ-driven fibroblast to myofibroblast differentiation assay, performed in primary human bronchial fibroblasts.

**Results:** Treatment of rats with BIBF1000 resulted in attenuation of fibrosis as assessed by the reduction of collagen deposition and the inhibition of pro-fibrotic gene expression. In the cellular assay both SB-431542 and BIBF1000 showed dose-dependent inhibition of TGFβ-induced differentiation whereas imatinib mesylate was inactive.

**Conclusions:** BIBF1000, or related small molecules with a similar kinase inhibition profile, may represent a novel therapeutic approach for the treatment of IPF.

**Key Words:** Bleomycin, Lung fibrosis, BIBF1000, imatinib mesylate
BACKGROUND

Fibrotic conditions can occur in all tissues but are especially prevalent in organs that have had frequent exposure to chemical and biological insults, for example the lung, skin, digestive tract, kidney and liver. These conditions often compromise the normal function(s) of the organ and many fibrotic diseases are at least severely debilitating, if not life-threatening.

Fibroses of the lung represent a set of pathological changes which accompany a wide range of inflammatory conditions of the conducting airways. For instance, in patients with chronic obstructive pulmonary disease a patchy alveolar wall fibrosis with peribronchiolar distribution is present, whereas in patients with chronic asthma fibrosis is predominantly localised to the lamina reticularis resulting in a thickening of the basement membrane. In both conditions a continuously on-going inflammation-repair cycle in the airways leads to permanent structural changes in the airway wall (remodelling) of which interstitial collagen fibrosis is the major component. Similar etiologies have been observed in the liver. In contrast to the fibrotic changes observed in COPD and asthma, in patients with diseases such as idiopathic pulmonary fibrosis (IPF) and acute respiratory distress syndrome (ARDS), the fibrotic changes are more severe and widely disseminated. In these diseases, fibrosis is associated with extreme morbidity and the clinical course is invariably one of gradual deterioration. Median length of survival from time of diagnosis varies between 2.5 and 3.5 yr.

Although the degree of pulmonary fibrosis differs between various lung diseases, there is evidence to suggest that the underlying pathophysiological mechanisms involved in the development may be similar across diseases. In all forms of pulmonary fibrosis, fibroblasts and myofibroblasts are the most predominant cells. Both cell types become activated by growth factors secreted by the airway epithelium after the inflammatory damage. Depending on the precise stimulatory milieu, fibroblasts transform to myofibroblasts or proliferate, resulting in areas of fibroblastic foci which are thought to be the sites of active extracellular matrix (ECM), collagen and fibronectin synthesis and which are regarded to be the leading edge of fibrosis.

The polypeptide mediators and growth factors believed to be pivotal for the fibrotic process include transforming growth factor beta (TGFβ), vascular endothelial growth
factor (VEGF), basic fibroblast growth factor 2 (bFGF-2), platelet derived growth factor (PDGF), connective tissue growth factor (CTGF), insulin-like growth factor (IGF), epidermal growth factor (EGF), chemokine ligand-18 (CCL18) and endothelin-1 (ET-1) \(^{17-26}\). Amongst these, TGF\(\beta\) is believed to be a critical mediator of fibrogenesis, exerting immunologic actions, direct effects on structural cells involved in the synthesis of ECM, fibroblast proliferation, and the differentiation of fibroblasts into myofibroblasts \(^{27,28}\). Several preclinical studies have shown that inhibition of TGF\(\beta\)-signalling results in attenuation of fibrosis in rodents \(^{29-31}\), suggesting that drug-targeting of the TGF\(\beta\) pathway could provide a useful therapeutic intervention in human fibrotic diseases including IPF. Unfortunately, TGF\(\beta\) is a pleiotropic mediator and a number of reports have suggested that anti-TGF\(\beta\) therapy may result in a number of unacceptable adverse effects \(^{32,33}\), particularly, tumour promotion.

Another important fibrogenic mediator, PDGF, induces fibroblast chemotaxis, fibroblast proliferation, and promotes fibroblast-mediated tissue matrix contraction \(^{34}\). Furthermore, a number of fibrogenic mediators such as TGF\(\beta\), IL-1, TNF-\(\alpha\), bFGF and thrombin exhibit PDGF-dependent profibrotic activities \(^{35-39}\). Two isoforms of PDGF, namely PDGF-C and PDGF-D, are increased in expression during bleomycin-induced lung fibrosis and it has been shown that (PDGF) receptor tyrosine kinase inhibitors markedly attenuate radiation-induced pulmonary fibrosis \(^{35;40;41;45}\).

Fibroblasts isolated from patients with moderate to severe asthma have the ability to transform into myofibroblasts after \textit{in vitro} stimulation with TGF\(\beta\) resulting in the secretion of VEGF, FGF, and ET-1 \(^{42}\). ET-1 is a known potent mitogen for smooth muscle cells and is thought to be responsible for the increased smooth muscle mass in patients with chronic inflammation of the lungs. VEGF as well as bFGF-2 are elevated in patients with asthma and are associated with increased vascularity \(^{43,44}\). Transfection of the soluble VEGF receptor (\textit{sflt} -1) gene, resulted in attenuation of pulmonary fibrosis in a mouse model of bleomycin-induced pneumopathy, suggesting that an anti-VEGF approach might also offer a suitable anti-fibrotic therapy \(^{18}\).

We have recently shown in the bleomycin-induced lung fibrosis model in rats that an initial inflammatory phase is followed by subsequent fibrosis. Depending on the treatment scheme, anti-inflammatory and anti-fibrotic activities of test compounds can be discriminated in this model \(^{45}\). Using this model, we showed that a prototype anti-inflammatory treatment (the oral steroid prednisolone) attenuated lung fibrosis when commenced at day 1, but had no efficacy if administered from day 10 onwards. In
contrast, treatment with a prototype anti-fibrotic compound (oral imatinib mesylate, a c-abl / c-kit / PDGFR kinase inhibitor) was effective, even when administered beginning at day 10, post-bleomycin treatment 45. In this study, we used BIBF1000, a prototypical small molecule inhibitor selective for the family of VEGF, FGF, and PDGF receptor tyrosine kinases 46 and studied its activities in the aforementioned therapeutic bleomycin model and in an ex vivo assay of pulmonary fibrosis. We show that BIBF1000 attenuates fibrosis in vivo and inhibits the differentiation of fibroblasts to myofibroblasts in vitro, indicating that this class of compounds may be useful for the treatment of IPF while avoiding the possible adverse effects of direct TGFβ inhibition.
Materials and Methods

Compounds
Imatinib mesylate (Novartis, Basel, Switzerland) and bleomycin sulfate (HEXAL, Holzkirchen, Germany) were purchased from a local pharmacy. BIBF1000 was synthesized by the department of chemistry, Boehringer Ingelheim. SB-431542 is available from Sigma-Aldrich (Schnelldorf, Germany). Recombinant TGFβ1 (Serotec, Raleigh, North Carolina, USA) and TGFβ2 (Sigma-Aldrich, Schnelldorf, Germany) were diluted with sterile water and stored in siliconized tubes (Eppendorf, Hamburg, Germany).

Bleomycin administration and treatment protocol
All experiments were performed in accordance with German guidelines for animal welfare and were approved by the responsible authorities. A dose of 2.2 mg/kg bleomycin sulfate was determined to be efficacious in establishing interstitial pulmonary fibrosis. At day zero, male Wistar rats (10 per group) were intratracheally injected with bleomycin sulfate in 300 µl saline using a catheter (0.5 mm internal diameter, 1.0 mm external diameter) through the nasal passage. To determine the fully effective dose of BIBF1000 rats treated with 2.2 mg/kg bleomycin, were treated with BIBF1000 (10, 30, and 50 mg/kg in 1 ml 0.1% Natrosol) from day 0 to day 21 and fibrotic markers were analyzed in lungs isolated at day 21. 50 mg/kg was the most efficacious dose showing a complete inhibition of bleomycin-induced fibrosis. At none of the applied doses the animals showed any signs of toxicological side effects.
For the experiments described in this manuscript, BIBF1000 (50 mg/kg) was orally administered once daily from day 10 to day 21, after which the rats were sacrificed and the lungs excised. As controls, rats were treated on day 0 with saline only (= control group), or rats treated with bleomycin received vehicle alone from days 10 - 21 (= bleomycin group). The degree of fibrosis was analyzed again by gene expression profiling and histology of the excised lungs.

Histology
Histology was performed as described before. Collagen deposition was assessed using Masson’s Trichrome staining as previously described.
Total RNA extraction and synthesis of cDNA
The total RNA extraction and synthesis of cDNA was carried out using the methods we have previously published 45.

Investigation of gene expression using real time PCR
Gene expression was investigated using the methods we have previously published 45. Primers for the 18S endogenous control and TGFβ₁ were purchased as pre-developed assay reagent kits (PDAR; Applied Biosystems, California, USA), whereas primers and probes for pro-collagen I, connective tissue growth factor (CTGF) and fibronectin were designed using PrimerExpress™ (Applied Biosystems, California, USA). 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 following primer and probe sequences were used:


Gene expression investigation of primary fibroblast cultures from patients with fibrotic lung disease
CCD25 lung fibroblasts were purchased from ECACC European Collection of Cell Cultures (Porton Down, Salisbury, Wiltshire; UK). Fibroblasts were obtained from outgrowths of transbronchial biopsy material taken from patients with lung fibrosis at the University Hospital Freiburg (for patient information, see table 2). The study received ethics approval from the appropriate hospital authorities and all patients underwent a process of informed consent. Fresh bronchial biopsies were placed on a 15 cm Petri-dish pre-coated with collagen I (Sigma-Aldrich, Schnelldorf, Germany) in
culture medium (RPMI + 1 % Glutamine + 1 % penicillin / streptomycin + 15 % FCS; Invitrogen, Karlsruhe, Germany). After 21 days, cells were trypsinized and re-cultured in 75 cm² tissue culture flasks.

For the fibroblast differentiation assay cells were seeded at a density of 3 x 10⁵ cells. Serum-free medium was added 24 h before TGFβ2 (0.4 nM) and the inhibitors (used at concentrations of 30 nM, 100 nM, 300 nM, 1 µM and 3 µM). After 72 h cells were lysed with 500 µl of Trizol (Invitrogen, Karlsruhe, Germany) and the cell lysate was stored at -80 °C until further analysis.

**Immunofluorescent detection of α-SMA as a marker for myofibroblasts**

Fibroblasts seeded on 8 well chamber slides at a density of 5 x 10⁴ cells per well were incubated in serum free RPMI medium for 24 h. Inhibitors (3 µM) were added 30 min before addition of TGFβ2 (0.4 nM). After 72 h the medium was removed and the slides were fixed. Detection of αSMA was performed by incubation with a monoclonal anti-αSMA antibody (Sigma-Aldrich, Schnelldorf, Germany; diluted 1:100 with PBS) and a FITC conjugated rabbit anti-mouse antibody (DAKO, Glostrup, Denmark) (diluted 1:500 in PBS). The slides were cover-slipped using a mixture of propidium iodide (DAKO, Glostrup, Denmark) and mounted with MOWIOL (Calbiochem, San Diego, USA).

**Phospho-SMAD-2 ELISA**

HaCat cells (CLS Cell Lines Service; Eppelheim, Germany) seeded into a 96 well microtiter plate at a concentration of 3 x 10⁴ cell per well were incubated for two days. Following an incubation in serum-free medium for 3 h, the compounds, dissolved in medium containing 10 % dimethyl sulfoxide (DMSO), were added up to a final concentration of 50 µM and TGFβ1 (5 ng/ml) was added to the appropriate wells 15 min later. After incubation for 30 min cells were lysed with 120 µl 10 X lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na₃VO₄, 1 µg/ml leupeptin, 1 mM PMSF). Lysates were stored at -80 °C. To perform the phospho-SMAD-2 ELISA, a monoclonal anti-SMAD 2/3 antibody (Upstate, Dundee, UK; diluted 1:250) was coated on the surface of a 96 well plate (Nunc F8 Maxisorp) and incubated with the lysates at room temperature for 90 min. A rabbit polyclonal anti-phospho-Smad2 antibody (Upstate, Dundee, UK) was added to the bound material
and immunocomplexes were detected by addition of an alkaline phosphatase labeled mouse anti-rabbit antibody (Dako, Glostrup, Denmark) using p-Nitrophenyl Phosphate (pNPP; Upstate, Dundee, UK) as substrate. The plate was incubated in the dark at 37 °C and the optical density of the signal was measured at 406 nm with an ELISA plate reader (Tecan Genios Plus, Tecan, Männedorf, Switzerland).

**Determining the IC50 values for TGFβRI and TGFβRII kinase inhibition**

The inhibitory actions of SB-431542, imatinib mesylate and BIBF1000 on the kinase activity of TGFβRI and TGFβRII were determined using the Promega Kinase-Glo™ kit (Promega, Mannheim, Germany) according to the manufacturer's protocol.

**Statistics**

All statistical analyses were carried out using GraphPad Prism V 4.02 software (GraphPad, California, USA). Comparisons were made using a non-parametric T-test (Mann-Whitney U test) and a significant value was considered to be $p \leq 0.05$. On all graphs, * signifies a significance level of $p \leq 0.05$, ** signifies a significance level of $p \leq 0.01$ and *** signifies a significance level of $p \leq 0.001$. 
Results

The effect of BIBF1000 on the development of fibrosis in a therapeutic rat bleomycin model

BIBF1000 (figure 1) was identified as a selective inhibitor of the family of VEGF, PDGF, and FGF receptor tyrosine kinases. To test whether BIBF1000 would exert anti-fibrotic activity in lung fibrosis, the compound was tested in a rat bleomycin model. BIBF1000 was used at its fully effective dose (50 mg/kg) in a therapeutic setting with daily oral treatment from days 10 to day 21 post bleomycin administration. As controls, groups of rats received saline instead of bleomycin (saline group), or animals treated with bleomycin received vehicle only (bleomycin group). After 22 days, animals were sacrificed and the level of fibrosis was determined by gene expression profiling of TGFβ1, procollagen-I, fibronectin, and CTGF of isolated lung tissue. As shown in figure 2, the gene expression of these factors is very low in the saline-treated control group and is increased after bleomycin treatment. In rats exposed to bleomycin, treatment with 50 mg/kg BIBF1000 from day 10 to day 21 resulted in expression levels comparable to those observed in rats treated with saline alone.

To address the deposition of collagens at the protein level, lung sections obtained at day 22 were stained with Masson’s Trichrome. As shown in figure 3, collagen deposition, as indicated by blue staining, is weak in the saline-treated control group. In contrast, rats treated with bleomycin alone showed extensive pulmonary fibrosis in the interstitial spaces. Fibrosis was strongly attenuated when bleomycin-treated rats received 50 mg/kg BIBF1000, with collagen staining levels comparable to the rats treated with saline.

TGFβ-stimulated myofibroblast formation is inhibited by BIBF1000 in vitro

It had been previously shown that stimulation of primary fibroblasts with TGFβ induces fibroblast proliferation and differentiation into myofibroblasts. To determine whether BIBF1000 would influence the TGFβ-mediated induction of myofibroblasts, primary fibroblasts obtained from outgrowths of transbronchial biopsies (table 1) were treated with 0.4 nM TGFβ2 for 72 h in the absence or presence of BIBF1000. Furthermore, SB-431542, reported to be a potent and selective inhibitor of the TGFβ superfamily of kinases, was included as a reference. The differentiation of fibroblasts to myofibroblasts by TGFβ2 was determined by assessing the expression...
of α smooth muscle actin (αSMA) and connective tissue growth factor (CTGF). As shown in figure 4 A and B, cells treated for 72 h with TGFβ₂ display a robust staining for αSMA, suggesting that differentiation into myofibroblasts had taken place. In contrast, both BIBF1000 and SB-431542 blocked the differentiation into myofibroblasts as seen by the absence of αSMA staining (figure 4 C and D). To quantify the effects of BIBF1000 and SB-431542, expression of αSMA was determined by real time PCR. As shown in figure 5, both BIBF1000 and SB-431542 inhibited αSMA gene expression (as well as CTGF gene expression, data not shown) in a concentration-dependent manner in three primary fibroblast cultures and in CCD25 lung fibroblasts.

Since BIBF1000 showed a weak inhibition of the isolated TGFβ-receptor I kinase (table 2), we asked whether the cellular activities mediated by BIBF1000 could be accounted for by direct inhibition of TGFβRI. We therefore established a quantitative ELISA assay for the detection of phospho-SMAD2 (an immediate downstream target of TGFβRI) as a marker for the intracellular activity of TGFβRI. HaCat cells were stimulated with TGFβ for 30 min in the presence or absence of BIBF1000 and the amount of phosphorylated SMAD2 was determined after lysis of the cells. Again, SB-431542 was used as a positive control. As shown in figure 5 B, treatment with BIBF1000, at concentrations exceeding those needed to inhibit TGFβ-mediated fibroblast differentiation, did not block the TGFβ-induced phosphorylation of SMAD2, whereas treatment with SB-431542 abrogated the phosphorylation of SMAD2 in a concentration-dependent manner (figure 5 B). Therefore, we surmise that BIBF1000 is blocking other cellular pathway(s) needed to induce and/or maintain the myofibroblast phenotype without directly interfering with SMAD-dependent TGFβ signalling.

Previously, it has been shown, that imatinib mesylate exerts anti-fibrotic activity in bleomycin-induced lung fibrosis model. We were therefore interested in studying the effects of imatinib mesylate on the TGFβ-mediated differentiation of primary fibroblasts to myofibroblasts and on the TGFβ-mediated phosphorylation of SMAD2. As shown in figure 4 E and 5 A, imatinib mesylate did not block TGFβ-induced αSMA expression at the protein or mRNA level in primary fibroblasts nor did it influence the TGFβ-induced phosphorylation of SMAD2 in HaCat cells (figure 5 B).
Discussion
The use of different treatment regimes in the bleomycin model may prove a valuable method by which drugs with true anti-fibrotic potential can be identified and investigated \(^{41,45}\). In the present study, we tested BIBF1000, previously identified as an inhibitor of the receptor tyrosine kinases for VEGF, FGF, and PDGF, and show that BIBF1000 is attenuating established lung fibrosis in an \textit{in vivo} setting. Furthermore, the compound blocked TGF\(\beta\)-mediated differentiation of human primary lung fibroblasts isolated from lung fibrosis patients.

Inhibition of the pathways regulated by CTGF, IGF-I, VEGF, FGF, PDGF and TGF\(\beta\) have been suggested to provide novel therapeutic approaches to the treatment of fibrosis associated with chronic lung diseases. As discussed earlier, each of these growth factors has distinctive roles in the pathophysiology of fibrosis and many are induced by TGF\(\beta\). However, the relative contribution of each of these pathways for the pathogenesis of lung fibrosis remains obscure and may furthermore depend on the specific stage and the type of the fibrotic disease. TGF\(\beta\) is the most potent pro-fibrotic growth factor known and it has been shown that interference with the TGF\(\beta\)-pathway will attenuate fibrosis of different origin \(^{30,51,53}\). However, direct inhibition of TGF\(\beta\)-signalling, e.g. via small-molecule inhibition of TGF\(\beta\) receptor kinases, may not offer a viable therapeutic option due to the pleiotropic functions of this growth factor which suggest that a number of side effects, including especially SMAD-dependent promotion of tumour formation, might be associated with a long-term anti-TGF\(\beta\)-treatment \(^{31-33}\). These concerns are particularly important in light of the dramatically increased lung cancer rates seen in IPF patients \(^{54,55}\). It was therefore interesting to note that BIBF1000 was able to block TGF\(\beta\)-mediated differentiation of primary fibroblasts isolated from normal lung and from patients with fibrotic lung diseases in the absence of inhibition of the TGF\(\beta\) receptor kinases. This suggests that fibroblasts transform to myofibroblasts through the actions of TGF\(\beta\) via downstream factor(s) which are inhibited by BIBF1000. Since differentiation of fibroblasts to myofibroblasts is a phenomenon seen in fibroblasts isolated from normal lung and from a number of different diseases including asthma \(^{13,56}\), liver cirrhosis \(^{57}\), renal fibrosis \(^{58}\), sarcoidosis, IPF, and UIP, BIBF1000 or related compounds may be of general utility in a number of fibrotic diseases.

It has been shown that c-abl is a SMAD-independent signalling molecule downstream of TGF\(\beta\) required for morphological transformation and expression of extracellular
matrix\textsuperscript{59}. Although we and others have previously shown that imatinib mesylate (a PDGFR / c-abl / c-kit inhibitor) is efficacious in the bleomycin-induced lung fibrosis model\textsuperscript{41,45,59}, little effect on the differentiation of fibroblasts was observed following treatment with imatinib mesylate, indicating that neither PDGF nor c-abl (nor the combination) are the sole mediators of the differentiation process. As shown by global expression profiling\textsuperscript{60}, more than 100 genes play a role in TGF$\beta$-mediated fibroblast-myofibroblast differentiation. Future cell culture experiments comparing gene expression profiles with the inhibitors described here could provide important clues about the mechanism of TGF$\beta$-mediated fibroblast-myofibroblast differentiation.

Since BIBF1000 is an inhibitor of the receptor tyrosine kinases for PDGF, FGF, and VEGF it is tempting to speculate that the concerted inhibition of several pro-fibrotic factors is required for its anti-fibrotic activity. PDGF is believed to play a role in the pathogenesis of fibrotic disease by stimulating fibroblast chemotaxis, fibroblast proliferation, and by promoting fibroblast-mediated matrix contraction\textsuperscript{61}. Furthermore, PDGF is important in inducing the secretion of growth factors and ECM components in fibroblasts\textsuperscript{19} and it induces proliferation and the production of fibronectin of both normal and fibrotic lung fibroblasts. Interestingly, PDGF did not have any effect on the production of interstitial collagens, again, supporting the hypothesis that the concerted action of several factors may be required to induce all aspects of fibrosis. Basic FGF (bFGF or FGF-2), is released by activated fibroblasts and damaged epithelial cells during remodelling processes associated with bronchial asthma\textsuperscript{14,62-64} and it stimulates the proliferation and fibronectin production of human lung fibroblasts. Furthermore, TGF$\beta$-induced proliferation of fibroblasts is mediated through the release of extracellular FGF-2 since FGF-2-blocking antibodies inhibited the proliferation of fibroblasts\textsuperscript{19,62}. Finally, it has been shown that both PDGF and FGF-2 are important factors in the migration of myofibroblasts\textsuperscript{65}, suggesting that blockade of both pathways might be required to interfere with myofibroblasts.

The function of angiogenesis and of pro-angiogenic factors like VEGF for the pathophysiology of pulmonary fibrosis is currently not understood. Neovascularization with anastomoses between the systemic and pulmonary vasculature is apparent at sites of fibrosis\textsuperscript{44,66}. However, a regional heterogeneity of the vascularization in IPF patients has been reported and it has been proposed that this heterogeneity may on the one site support fibroproliferation but may block on the other site normal repair mechanisms\textsuperscript{66}. Although the exact site and mechanism of the neovascularization
remains controversial, it is tempting to speculate that angiogenesis may play a role in IPF, acute respiratory distress syndrome (ARDS) and other lung fibroses, and that the use of VEGF inhibitors might attenuate these processes. We presume that the combined VEGFR, FGFR and PDGFR inhibition of BIBF1000 is acting in a concerted manner to control fibrosis. Of course we cannot rule out the possibility that inhibitory effects of some, as yet, unidentified targets of BIBF1000 may also play a role in this process.

**Conclusion**

In summary, our data suggest that BIBF1000, or a molecule with a similar kinase inhibition profile, may present a novel therapeutic opportunity to treat IPF. Its distinctive inhibitory profile is uniquely capable of preventing fibroblast-myofibroblast differentiation, a crucial step in the establishment of fibrosis, without directly affecting SMAD signalling. Ultimately, only clinical trials in IPF and other fibrotic diseases will show whether such compounds can stop or slow the inexorable course of this invariably fatal disease.
Competing interests

The authors declare that have no competing interests.

Authors contributions
First author: NIC

Acknowledgements
The excellent technical assistance of Erika Mueller, Susanne Mueller, Margit Ried, and Melanie Trojan is acknowledged.
Reference List


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Figures

Figure 1
Chemical structure of BIBF1000

Figure 2:
**BIBF1000 inhibits the gene expression of pro-fibrotic marker genes in the rat bleomycin model.** Rats (10 animals per group) were treated either with saline or Bleomycin on day 0. Treatment with vehicle or BIBF1000 (p.o.; 50 mg/kg) commenced at day 10 and was continued daily until day 21. On day 22, rats were sacrificed and a part of the left lung lobe was processed for RNA extraction. The gene expression levels of TGFβ1, Procollagen I, Fibronectin, and CTGF were determined by quantitative RT-PCR. The gene expression for each gene is indicated relative to endogenous 18S RNA control. Values are given as fold induction. The bars represent median values. Statistics were determined by use of a Mann-Whitney U test ( *** p = 0.001).
Figure 3

Collagen staining of representative lung sections. Rats (10 animals per group) were treated either with saline or Bleomycin on day 0. Treatment with vehicle or BIBF1000 (p. o.; 50 mg/kg) commenced on day 10 and was continued daily until day 21. On day 22, rats were sacrificed and the lungs were fixed with paraformaldehyde,
prior to paraffin embedding. Sections (4 µM) were stained with Masson's Trichrome stain. Muscle and cells are stained red, nuclei are stained black and collagens are stained blue. Three representative photomicrographs are shown for each of the groups. Magnification x 250.

Figure 4

**BIBF1000 blocks TGFβ-mediated differentiation of fibroblasts.**

Fibroblasts obtained from from biopsies of patients with fibrotic lung disease were cultured on collagen I coated chamber slides for 72 h in (A) serum free medium (SFM) alone, (B) SFM + 0.4 nM TGFβ_2_, (C) SFM + 0.4 nM TGFβ_2_ + 5 µM SB-431542, (D) SFM + 0.4 nM TGFβ_2_ + 5 µM BIBF1000, or (E) SFM + 0.4 nM TGFβ_2_ + 5 µM imatinib mesylate. αSMA filaments (green) were detected with a monoclonal antibody and visualised with a fluorescein conjugated rabbit anti-mouse antibody. Magnification x 400.
Figure 5
Activity of BIBF1000, imatinib mesylate, and SB-431542 on TGFβ2-induced α-smooth muscle gene expression and on TGFβ-mediated SMAD phosphorylation. (A) Primary fibroblasts lines isolated from bronchial biopsies of three patients with lung fibrosis (# 2217, 2272, 2278) and the primary lung fibroblast line CCD25 were incubated with TGFβ2 in the presence of SB-431542, BIBF1000 and imatinib mesylate at concentrations ranging from 0 - 30 µM. After for 72 h the gene expression levels of αSMA were determined by quantitative RT-PCR, normalised relative to endogenous 18 S RNA. Data are presented as % of gene expression compared to DMSO alone. (B) HaCat cells were incubated in serum free medium with the respective inhibitors to final concentrations ranging from 0 to 50 µM. After 15 min, 5 ng/ml TGFβ1 was added and incubation was continued for 30 min before the cells were lysed. The amount of phosphorylated Smad2 was determined by ELISA. 100% corresponds to the phosphorylation of Smad2 after stimulation with 5 ng/ml TGFβ1.
Figure 5
Tables

Table 1: Patient information from cultured primary bronchial fibroblasts

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Table 2: The IC50 values for BIBF1000, Imatinib Mesylate and SB-431542 against TGFβ receptors I & II

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