The effect of nintedanib versus mycophenolate mofetil in the Fra2 mouse model of systemic sclerosis-associated interstitial lung disease

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Abstract

Objective. Interstitial lung disease (ILD) is a key driver of mortality in patients with systemic sclerosis (SSc). A lack of approved treatments encompasses a high unmet medical need. Nintedanib has recently been approved for treatment in SSc-associated ILD (SSc-ILD) following SENSCIS®, a Phase III clinical trial showing that nintedanib slows the loss of pulmonary function in patients with SSc-ILD relative to placebo, as measured by annual rate of decline in forced vital capacity over 52 weeks. The aim of this study was to compare the activity of nintedanib and mycophenolate mofetil (MMF) in a transgenic Fra2 mouse model of SSc-ILD.

Methods. Fra2 transgenic mice were treated with MMF or nintedanib. Hae-matoxylin and Eosin and Sirius Red staining were used to identify pulmonary fibrosis and vascular remodelling in whole lung sections. Fibrosis was quantified by Ashcroft scoring, fold change in fibrotic area, and hydroxyproline. Ki67, SM22α, CD31, and caspase-3 staining were used to quantify proliferating vascular smooth muscle cells and apoptotic endothelial cells.

Results. Nintedanib effectively ameliorated pulmonary vascular remodelling and fibrosis in Fra2 transgenic mice. Pulmonary fibrotic and vascular remodelling parameter scores and the apoptosis of dermal endothelial cells were significantly reduced compared with vehicle-treated Fra2 transgenic mice. Treatment with MMF had only mild antifibrotic effects and no effect on pulmonary vascular remodelling.

Conclusion. In this model of SSc-ILD, nintedanib ameliorated pulmonary fibrosis, remodelling of pulmonary vasculature, and the apoptosis of endothelial cells. In contrast, MMF had minor effects on pulmonary fibrosis and no effects on vascular manifestations.

Introduction

Systemic sclerosis (SSc) is a systemic fibrosing orphan disease that can cause uncontrolled fibrosis of the skin and internal organs (1). A portion of patients go on to develop fibrosis of the lungs, or SSc-associated interstitial lung disease (SSc-ILD) (2-5). ILD is the leading cause of morbidity and mortality among SSc patients (3). SSc-ILD is driven by autoimmune, vascular, and fibrotic components, which represent the three hallmarks of the disease (3, 5). The autoimmune and vascular processes are thought to precede the accumulation of extracellular matrix proteins produced by pathologically activated fibroblasts (3). Latest insights in the complex pathogenic processes of SSc were reviewed by Orlandi et al. (6).

Currently, nintedanib is the only approved treatment for SSc-ILD (7), but other treatments are used off-label. Mycophenolate mofetil (MMF) is an immunosuppressant increasingly used in Europe in several countries and frequently used in the United States as an off-label treatment for SSc-ILD. MMF improved measures of lung function in SSc-ILD over 2 years from baseline in the Scleroderma Lung Study II (8) and may work through the inhibition of T and B lymphocyte proliferation, a key process in the autoimmune component of pathology (4, 9). Its effects on fibrosis and vascular remodelling in SSc-ILD have not been reported. Nintedanib is an antifibrotic drug that has recently been approved for use in SSc-ILD and is already being used to treat idiopathic pulmonary fibrosis (5, 7). It is a tyrosine kinase inhibitor that binds to platelet-derived growth factor (PDGF) receptors α and β, vascular endothelial growth factor 1-3 factor receptors, fibroblast growth factor receptors 1-3, transforming growth factor beta receptor 1, colony stimulating factor 1.
receptor, and SRC family kinases such as lymphocyte-specific protein tyrosine kinase Lck (5, 9, 10). Nintedanib inhibits the proliferation, migration, and contraction of lung fibroblasts, and attenuates the differentiation and migration of profibrotic fibrocytes and the transformation of lung fibroblasts to myofibroblasts (11). The antifibrotic effects of nintedanib were also associated with impaired M2 polarization of monocytes and reduced numbers of M2 macrophages (5). Nintedanib has also been shown to reduce inflammatory mediators such as tumour necrosis factor-alpha (TNF-α), interleukin (IL)-6, IL-1β, C-X-C motif ligand 1, C-C motif ligand 2, and macrophage chemotactic protein-1 (12-14). It has been suggested that the anti-inflammatory effects of nintedanib arise from its inhibition of fibroblasts, inducing paracrine inhibition on angiogenesis and macrophage migration and polarization (13). These pathways are thought to be involved in the pathogenesis of fibrosis, and thus could be potential targets for SSc-ILD treatment (3, 5).

The Phase III SENSCIS® trial found that nintedanib slowed pulmonary function decline by 44% compared with placebo in patients with SSc-ILD, as measured by the annual rate of decline in forced vital capacity (FVC) over 52 weeks (2). In the trial, nearly half of the patients treated with nintedanib also received MMF (2); however, patients receiving MMF must have been on a stable dose for at least 6 months to continue into the trial. This has the potential to complicate the interpretation of the results in patients receiving MMF, as those receiving MMF who were not on a stable dose and were possibly more likely to worsen were excluded from the study (2).

The aim of this study was to compare the activity of nintedanib and MMF on vascular, fibrotic, and inflammatory features of SSc-ILD using the established fos-related antigen-2 (Fra2) transgenic (tg) mouse model of SSc-ILD. This model was chosen as it recreates key findings of SSc such as destructive and proliferative vasculopathy, inflammation, and widespread fibrosis (15).

Material and methods

**Compounds and formulation**

Nintedanib (batch number 67653, Boehringer Ingelheim, Ingelheim, Germany) was used at a dose of 50 mg/kg and administered in a volume of 0.1 mL ultra-pure H₂O orally (p.o.) twice daily (bid). MMF (order number: S1501, lot number: S150107, Selleck Chemicals, Munich, Germany) was administered p.o. in doses of 100 and 150 mg/kg in 0.1 mL once daily (qd) (16). MMF was dissolved in a vehicle containing: 0.861 mL benzyl alcohol, 0.376 mL Tween 80 and 0.5g carboxymethylcellulose in 100 mL distilled water at pH 3.5.

**Fra2 tg mice**

Fra2 tg mice were treated with nintedanib or MMF at 9 weeks of age, and mice were sacrificed at an age of 16 weeks. There were six mice in each group, consisting of four females and two males, and wild-type littermates were used as controls. Nintedanib and MMF were administered p.o. and control specimens received vehicle treatment. The study groups were as follows: control group (wild-type mice receiving vehicle solution); tg, sham-treated group (Fra2 tg mice also receiving vehicle solution); treatment group 1 (Fra2 tg mice treated with 100 mg/kg qd MMF); treatment group 2 (Fra2
tg mice treated with 150 mg/kg qd MMF); and treatment group 3 (Fra2 tg mice receiving nintedanib at 50 mg/kg bid). The current line of Fra2 tg mice, which was generated with new constructs and by a new embryo transfer in 2016, does not develop skin fibrosis as compared with wild-type littermates. Our current line develops progressive pulmonary fibrosis, and SSc-like vascular disease with the proliferation of pulmonary vascular smooth muscle cells (VSMCs) and apoptosis of microvascular endothelial cells (ECs).

**Histological evaluation of pulmonary fibrosis**

Whole lungs were excised, fixed in 4% formalin for 6 hours and embedded in paraffin. 5 μm sections were cut and stained with Sirius Red. Images were captured using a Nikon Eclipse 80i microscope (Nikon, Badhoevedorp, Netherlands). Histological changes of pulmonary fibrosis were quantified by Ashcroft scoring (17). In addition, whole lung sections were stained with Sirius Red (Sigma-Aldrich, Munich, Germany), and fibrotic area was determined as the percentage of Sirius Red-covered area per total area using ImageJ (V. 1.42q, National Institutes of Health, USA) as reported in previous studies (18-22).

**Hydroxyproline assay**

The amount of collagen protein was determined via hydroxyproline assay. After digestion of the right upper lobe in 6 M hydrogen chloride for 3 hours at 120°C, the pH of the samples was adjusted to 6 with 6 M sodium hydroxide. Afterwards, 0.06 M chloramine T was added to each sample and incubated for 20 minutes at room temperature. Following this, 3.15 M perchloric acid and 20% p-Dimethylaminobenzaldehyde were added and samples were incubated for an additional 20 minutes at 60°C. The reaction was quenched by putting the samples on ice for 4 minutes. The absorbance was determined at 557 nm with a Spectra MAX 190 microplate spectrophotometer.

**Evaluation of vascular remodelling in pulmonary arteries in Fra2 tg mice**

The degree of luminal occlusion of pulmonary arteries was examined by manually counting the numbers of arteries with occluded lumina as described previously (5, 23). The percentage of proliferating VSMCs was evaluated by triple-staining for 4′,6-diamidino-2-phenylindole (nuclear staining), Ki67 (proliferation marker), and SM22α (smooth muscle cell marker).

**Assessment of microvascular changes in the skin of Fra2 tg mice**

The percentage of apoptotic ECs in the skin of mice was determined by double-staining for CD31 (EC marker) and active caspase-3 (5, 23).

**Determination of inflammation mediators**

Snap frozen lung tissue samples of about 100 mg were homogenized in 1 mL of tissue protein extraction reagent, T-PER™ buffer (Thermo Fisher Scientific, Schwerte, Germany) containing Pierce protease inhibitor (Thermo Fisher Scientific) using a Precellys® tissue homogenizer (Bertin Instruments, Montigny-le-Bretonneux, France). Homogenates were spun at 10,000 g for 5 minutes and the supernatant was used for the following analyses.

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**Fig. 2.** Representative images of haematoxylin and Eosin-stained lung sections at 40-fold magnification.

Wild-type mice (A) were compared with Fra2 tg mice (B) treated with mycophenolate mofetil (MMF) 100 mg/kg (C) or 150 mg/kg (D) once daily or nintedanib 50 mg/kg (E) twice daily. Representative images of haematoxylin and Eosin-stained sections of lungs at 40-fold magnification. MMF: mycophenolate mofetil; tg: transgenic.
natants were used to determine protein concentration by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Samples with 500 μg of protein were used to determine interferon-γ, interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, KC-GRO, and TNF-α by means of a multi-array ELISA assay (Meso Scale Discovery, Rockville, Maryland) according to the manufacturer’s instruction.

Statistics
All data are presented as median ± range, and differences between the groups were tested for their statistical significance by ANOVA and non-parametric Kruskal-Wallis test for multiple comparisons (GraphPad Prism Version 8.3 GraphPad Software, LLC). P-values of less than 0.05 were considered as statistically significant and are expressed as follows: *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. Experiments were conducted with full compliance with local, national, ethical, and regulatory principles and local licensing regulations. All experiments were approved by the governments of Mittelfranken and Unterfranken.

Results
Nintedanib ameliorates pulmonary fibrosis in Fra2 tg mice, whereas MMF has minimal effects
The wild-type control group had a median Ashcroft score of 0.3. The change in the fibrotic area and in the hydroxyproline concentration in the lung tissue was normalised to the baseline values of the wild-type control group. In the sham-treated Fra2 tg group the Ashcroft score was significantly (p<0.0001) increased to a median of 4.15, the fibrotic area increased 6-fold (p<0.0001), and the hydroxyproline content 2-fold (p<0.0001), altogether indicating profound lung fibrosis. Nintedanib (50 mg/kg bid) effectively ameliorated pulmonary fibrosis in Fra2 tg mice by halving the Ashcroft score (p=0.0074) and significantly reducing the fibrotic area (p=0.0066) and the hydroxyproline content (p=0.0049). Treatment of Fra2 tg mice with doses of 100 mg/kg qd or 150 mg/kg qd of MMF had only mild antifibrotic effects that did not yield statistical significance across all three outcomes (Fig. 1). These fibrotic changes are represented in Figure 2 (showing haematoxylin and Eosin staining of lung sections) and Figure 3 (showing Sirius Red staining of lung sections). In both sets of images, the fibrotic formations in the Fra2 tg mice compared with the wild type and how the histology is more preserved with nintedanib treatment compared with MMF treatment can be seen already macroscopically.

Nintedanib reduces vascular remodelling in lungs of Fra2 tg mouse
Compared with wild-type mice the proliferation of pulmonary VSMCs in Fra2 tg mice increased by <4-fold (p=0.0006) (Fig. 4A). The resulting thickening of the vascular walls led to a significant, <7-fold increase (p=0.0005) of occluded pulmonary vessels in Fra2 tg mouse lungs (Fig. 4B). Treatment of Fra2 tg mice with nintedanib significantly decreased the number of proliferating VSMCs (p=0.0073) close to the baseline level of wild-type animals (Fig. 4A). Consequently, nintedanib also significantly reduced the number of occluded pulmonary vessels in Fra2 tg mice as compared with vehicle-treated Fra2 tg mice (p=0.0316) (Fig. 4B). No effect on the
proliferation of pulmonary VSMCs and vascular occlusion was observed with MMF at doses of 100 mg/kg qd or 150 mg/kg qd (Fig. 4A and B).

Nintedanib reduces the number of apoptotic cells in skin of Fra2 tg mice

Figure 5 shows the immunohistochemistry of using nuclear staining with DAPI (blue), the EC marker, CD31 (red), and the apoptosis marker caspase-3 (green), with the double-staining indicating apoptotic microvascular ECs. Here, a >4-fold increase in apoptotic ECs in the skin of Fra2 tg mice was seen compared with the wild-type control group (p<0.0001) (Fig. 4C). Treatment of Fra2 tg mice with nintedanib significantly reduced apoptosis of microvascular ECs (p<0.0066), whereas MMF had no effect at either dose (Fig. 4C).

No effect on inflammatory mediators in this model

The levels of the inflammation markers IL-1β, -2, -4, -5, -6, -10, -12p70, KC-GRO, and TNF-α were determined in lung tissue homogenates of the lungs to get an impression about the contribution of inflammation in the outcome of the study. No significant increases in inflammatory markers were detected in Fra2 tg mice control compared with wild-type mice at the end of the experiment in Week 16. Consequently, both MMF and nintedanib treatment did not change the cytokine levels in the lung tissue of the Fra2 tg mice (Supplementary Fig. 1A and B).

Discussion

This study compared the effect of nintedanib and MMF on vascular and fibrotic features of SSc-ILD using an established Fra2 tg mouse model of the disease. Our results confirm that nintedanib was able to reduce or prevent fibrotic and vascular changes in Fra2 tg mice. In direct comparison to nintedanib, MMF treatment resulted in limited improvements of the pathogenic SSc-ILD features of the model. In patients with SSc, pulmonary fibrosis is reported to be a leading cause of death (24). Using semi-quantitative Ashcroft scoring for lung fibrosis, quantitative histological Sirius Red staining to detect collagen deposition, and hydroxyproline as a marker for collagen in lung tissue, we conclusively demonstrated strong antifibrotic activity for nintedanib but only very limited (non-significant) activity for MMF. In addition, in our previous study (5), a simultaneous dose-dependent reduction by nintedanib of the fibrotic area detected by Sirius Red staining, hydroxyproline content and myofibroblast count by alpha smooth muscle actin staining was demonstrated in lungs of Fra2 tg mice in a similar experimental setting. Hence, we assume a similar parallel reduction of the myofibroblast count by nintedanib in the lungs of the Fra2 tg mice in this study. A mechanistic explanation for the reduction by nintedanib of lung fibrosis in Fra2 tg mice is given by the explorations of Atanelishvili et al. (25) in lung fibroblasts from patients with SSc. Nintedanib reduced migration and proliferation of SSc lung fibroblasts. Nintedanib also blocked the differentiation of lung fibroblasts to myofibroblasts, reduced production of collagen and fibronectin, and decreased contractility.
The vascular pathology in SSc comprises hyperplasia of the vessel intima, fibrosis of the media and adventitia, and proliferation of smooth muscle cells in the vessel wall. In addition, EC apoptosis occurs in SSc and has been shown to trigger the fibrotic pathology (26).

The quantitative analysis of the proliferation of VSMC in the lung, as indicated by the reduced fold change in cells stained for SM22α (smooth muscle cell marker) and Ki67 (proliferation marker), showed a significant attenuation by nintedanib and consequently a significantly reduced number of occluded arteries in the lungs of Fra2 tg mice. In a previous study in Fra2 tg mice, we demonstrated elevated vascular endothelial growth factor levels in the serum of Fra2 tg mice, which were normalized by nintedanib treatment in a similar experimental setting (5). Cucina et al. (27) described that vascular endothelial growth factor increases the proliferation of VSMC coculture with ECs, which might explain the reduction of VSMC proliferation by nintedanib. In addition, nintedanib also significantly reduced the apoptosis of microvascular ECs in the skin of Fra2 tg mice, as indicated by CD31 (EC marker) and active caspase-3 (apoptosis marker) double-staining.

In contrast, MMF failed to have any significant effect on proliferative and destructive vasculopathy. Treatment with nintedanib initiated at the onset of vascular disease ameliorated vascular remodelling and may be beneficial in managing the vascular pathology in patients with SSc-ILD. In summary, these data suggest that nintedanib may have an antifibrotic and vascular remodeling impact in SSc-ILD, supporting the findings of a previous study where nintedanib proved to be effective in reducing dermal and pulmonary fibrosis in the same Fra2 mouse model (5).

Fra2 belongs to the AP-1 family of transcription factors, which is activated by prominent profibrotic mediators such as TGFβ and PDGF (28). Transgenic overexpression of Fra2 in mice results in the development of microvascular disease and lesions characteristic of pulmonary arterial hypertension at an age between 9 and 12 weeks (15). The lesions are followed by the development of fulminant fibrosis of the lungs, skin, and heart (5). In diseased Fra2 tg lungs, peribronchial and perivascular inflammatory infiltrates were described (29). Although mRNA expression of inflammatory mediators IL-4, -5, -6, -13, and eotaxin were reported to be increased in the bronchoalveolar lavage of Fra2 tg mice compared with wild-type animals, only IL-6, -13 and eotaxin protein levels were significantly elevated in the lung tissue of these animals (30). In our study the Fra2 tg mouse model does not show a prominent inflammation pathology in the lung at Week 16, which is reflected by comparable levels of inflammatory cytokines in the lungs of control wild-type and Fra2 tg mice. The cytokine levels detected in the lung tissues in this study are in good agreement with previous findings except for IL-6 and IL-13 (29, 30). Hence, anti-inflammatory activity by MMF or nintedanib could not be determined in this animal model of SSc-ILD.

The results of the recent SENSCIS trial indicate the clinical potential of nintedanib in patients with SSc-ILD (2). In SENSCIS, treating patients with
SSc-ILD with nintedanib (about half of them receiving background MMF) resulted in a reduction of the adjusted rate change in FVC by 41 mL (p=0.04) compared with placebo. Based on our own findings, we postulate that this change in patient outcome may be a result of the impact of nintedanib on fibrotic and vascular changes seen in SSc-ILD.

MMF was explored in patients with SSc-ILD in the Scleroderma Lung Study (SLS) II (8); however, no prospective placebo-controlled study was conducted with MMF. A similar trial (SLS I) compared cyclophosphamide against placebo. Although there are methodological limitations in comparing participants from different trials, treatment with MMF was associated with improvements in FVC% predicted, predicted diffusing capacity of the lungs for carbon monoxide, and dyspnoea over 2 years, when comparing placebo from SLS I with the active MMF treatment group of SLS II (31). This potential clinical efficacy could not be described by the Fra2 mouse model. There are a number of explanations for the limited effects of MMF in this model compared with its effects in SSc-ILD patients. It might be because there are differences in the type of inflammation seen in this model compared with SSc itself; for instance, there is evidence of macrophage-dependent fibrosis in the mouse model (5, 32) and MMF is not thought to directly target macrophages. The model may also have a partially inflammation-independent mechanism (33). MMF, which is assumed to exert immunosuppressive activity on B cells and T cells (34), seems to have only marginal direct anti-fibrotic activity and inhibitory activity on vascular remodelling.

Our study was conducted on a Fra2 tg mouse model of SSc-ILD and may not accurately represent the effects of both drugs on their targets when compared with the human disease. Another potential limitation is that the model did not show a prominent T- and B-cell contribution and inflammation pathology, thus we were not able to determine the effects of nintedanib and MMF on immune and inflammatory pathways. We may expect MMF to have some positive effect in regulating inflammation and B-cell biology. The SENSCIS trial showed some beneficial outcomes of MMF in patients with SSc-ILD; however, our model suggests MMF is not effective in slowing fibrosis. This discrepancy may be accounted for by anti-inflammatory effects unable to be shown here, or through the mechanism-related dependency of Fra2 tg mice on alternative macrophage activation (32), which is not targeted by MMF, but is inhibited by nintedanib (5). The Fra2 tg mouse line used for this study did not show pronounced enough skin fibrosis to evaluate treatment responses for nintedanib and MMF. This is different to our previous study (5), where a significant reduction of skin fibrosis by nintedanib was demonstrated. Nintedanib also improved skin fibrosis in three other mouse models reflecting features of SSc (21). Similar explorations to assess the activity of MMF on skin fibrosis and in models of SSc with a prominent inflammatory component would be interesting for follow-up studies. The focus of this study was to unravel the differences in the modes of action between nintedanib and MMF. It was not feasible to combine nintedanib and MMF treatment because large animal numbers would be necessary to demonstrate significant differences between three treatment groups. Recent clinical results demonstrated that nintedanib reduced the rate of decline in FVC both in patients who had not taken mycophenolate and in patients who had taken a stable dose of mycophenolate for ≥6 months prior to randomization (35).

The improved vascular effects of nintedanib compared with MMF seen in this study shows that it can be considered to be disease-modifying in this model. Now that nintedanib is approved for use in SSc-ILD, it will be interesting to confirm that the effects shown in preclinical and clinical models are able to help patients in the real world.

Conclusion
Nintedanib ameliorates pulmonary fibrosis, proliferation of pulmonary VSMCs, and apoptosis of microvascular ECs. In contrast, MMF has minor effects on pulmonary fibrosis and no effects on vascular manifestations. Overall, we have shown that nintedanib and MMF may have different effects on vascular manifestations of SSc.

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