European Journal of Pharmacology 919 (2022) 174792

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Inhibition of CHIT1 as a novel therapeutic approach in idiopathic pulmonary fibrosis

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ARTICLE INFO

Chemical compounds studied in this article:
OATD-01 (PubChem ID 126642132)
Pirfenidone (PubChem ID 40632)

Keywords:
IPF
CHIT1
OATD-01
Pulmonary fibrosis
Anti-fibrotics

ABSTRACT

Idiopathic pulmonary fibrosis (IPF) is a progressive and eventually fatal lung disease with a complex etiology. Approved drugs, nintedanib and pirfenidone, modify disease progression, but IPF remains incurable and there is an urgent need for new therapies. We identified chitotriosidase (CHIT1) as new driver of fibrosis in IPF and a novel therapeutic target. We demonstrate that CHIT1 activity and expression are significantly increased in serum (3-fold) and induced sputum (4-fold) from IPF patients. In the lungs *CHIT1* is expressed in a distinct subpopulation of profibrotic, disease-specific macrophages, which are only present in patients with ILDs and *CHIT1* is one of the defining markers of this fibrosis-associated gene cluster. To define CHIT1 role in fibrosis, we used the therapeutic protocol of the bleomycin-induced pulmonary fibrosis mouse model. We demonstrate that in the context of chitinase induction and the macrophage-specific expression of CHIT1, this model recapitulates lung fibrosis in ILDs. Genetic inactivation of *Chit1* attenuated bleomycin-induced fibrosis (decreasing the Ashcroft scoring by 28%) and decreased expression of profibrotic factors in lung tissues. Pharmacological inhibition of chitinases by OATD-01 reduced fibrosis and soluble collagen concentration. OATD-01 exhibited anti-fibrotic activity comparable to pirfenidone resulting in the reduction of the Ashcroft score by 32% and 31%, respectively. These studies provide a preclinical proof-of-concept for the antifibrotic effects of OATD-01 and establish CHIT1 as a potential new therapeutic target for IPF.

1. Introduction

Idiopathic pulmonary fibrosis (IPF) is a progressive and eventually fatal lung disease with a complex and not fully understood etiology. The abnormal wound healing responses to a repetitive lung injury are the key mechanism initiating development of fibrosis (Richeldi et al., 2017). The common feature of IPF is the disordered accumulation of extracellular matrix (ECM) proteins within the interstitium and alveolar spaces that leads to chronic fibrosis with progressive destruction of lung architecture and a loss of function. The pathogenesis of IPF has not been fully elucidated, but the genetic complexity, variable pathology and heterogeneity of progression patterns suggest existence of multiple triggers and numerous pathogenic pathways. The intricate interplay between genetic, environmental, and ageing-associated factors in IPF has contributed to the limited availability of therapeutic options (Mora et al., 2017). Although two approved drugs, pirfenidone and nintedanib,

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https://doi.org/10.1016/j.ejphar.2022.174792
Received 2 September 2021; Received in revised form 25 January 2022; Accepted 26 January 2022
Available online 2 February 2022
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have been shown to slow down lung function decline in IPF patients, the condition remains incurable. Thus, there is an urgent need for new therapies (Somogyi et al., 2019).

Chitinases, enzymes that hydrolyze chitin, have been implicated in pathology of interstitial lung diseases (ILDs) and other pulmonary diseases (Lee et al., 2011). Although mammals do not synthetize chitin, they express two enzymatically active chitinases, chitotriosidase (CHIT1) and acidic mammalian chitinase (AMCase) (Di Rosa et al., 2016). In most mammals chitinases are thought to confer protection against chitin-containing pathogens. However, in humans chitinases evolved to play different roles, often associated with pathological inflammatory and fibrotic responses (Chang et al., 2020; Di Rosa et al., 2016; Przysucha et al., 2020). Expression of chitinases is highly induced in various fibrotic and inflammatory lung diseases, including IPF, chronic obstructive pulmonary disease, sarcoidosis and asthma (Bennett et al., 2020; Chang et al., 2020; Cho et al., 2015; James et al., 2016; Lee et al., 2012; Logue et al., 2019; Przysucha et al., 2020). Moreover, increased chitinolytic activity and chitinase expression have been correlated with disease stage and clinical prognosis (Agapov et al., 2009; Bennett et al., 2020; Popevic et al., 2016; Steinacker et al., 2018), implicating the contribution of chitinases in their pathogenesis. Several reports demonstrated that CHIT1 might be a dominant chitinase in human pulmonary diseases (Bargagli et al., 2007; Chang et al., 2020; Seibold et al., 2008). Furthermore, studies in animal models indicated a functional role of CHIT1 in ILDs. In *Chit1−/−* mice, lung fibrosis induced by either bleomycin or over-expression of IL-13 was markedly reduced compared to control animals (Lee et al., 2012). Additionally, enhanced lung fibrosis was observed in CHIT1 over-expressing transgenic mice after bleomycin administration. Together, data from clinical studies and preclinical models have implicated CHIT1 in pulmonary fibrosis.

Here, we report a comprehensive analysis of CHIT1 expression and activity in IPF patients. Furthermore, we demonstrate that pharmacological inactivation of CHIT1 results in attenuated lung fibrosis induced by bleomycin in mice, which is consistent with the effects of genetic deletion of Chit1. Collectively, the translational and preclinical data establish CHIT1 as a promising new therapeutic target for IPF.

2. Materials and methods

2.1. Human samples collection

Newly diagnosed and treatment-naïve IPF patients (11 male, 4 female) were recruited for the study in the Department of Internal Medicine, Pulmonary Diseases and Allergy of the Medical University of Warsaw, Poland between January 2016 and June 2018. The characteristics of healthy individuals and IPF patients is provided in Table 1. All included patients met the IPF diagnostic criteria according to the 2013 ERS/ATS guidelines for the diagnosis and management of IPF (Wells, 2013). The study was approved by the Local Bioethics Committee at the Medical University of Warsaw, Poland, No. of approval: KB/236/2015. Sputum induction, bronchoalveolar lavage fluid (BALF) collection and subsequent sample processing were performed as previously described (Paplinska-Goryca et al., 2020; Proboszcz et al., 2017). The BALF cells were evaluated and counted in May-Grünwald-Giemsa-stained smears (n = 19). IPF lung biopsy samples used for immunohistochemical (IHC) staining were obtained from the Pathology Research and Trial Support (PARTS) and the Erasmus MC Tissue Bank (normal lungs n = 4, IPF lungs n = 6). Procedures followed the local regulations that apply for using human biomaterials and data for medical scientific research.

Table 1
Characteristics of IPF and control populations.

	Healthy control	IPF
Number	29	15
Sex (female, male)	18, 11	4, 11
Age (year) median (minimum, maximum)	32 (22, 68)	73 (62, 84)
BMI median (minimum, maximum)	24.9 (17.6, 44.1)	27.3 (22.2, 33.6)
Race	29 Caucasian	15 Caucasian

2.2. Human CHIT1 concentration

The CHIT1 concentration in human serum samples was measured using CircuLex Human Chitotriosidase ELISA Kit (CycLex) according to the manufacturer's protocol.

2.3. Human BALF cells preparation and immunohistochemical CHIT1 staining

Smears were fixed in 10% neutral buffered formalin (NBF) and endogenous peroxidases were inactivated by a 3% hydrogen peroxide solution. Smears blocked in 1% bovine serum albumin (BSA)/10% normal goat serum (NGS) in TBST buffer were incubated with a primary antibody for CHIT1 followed by the secondary antibody conjugated with horseradish peroxidase (HRP) and visualized with the 3,3′-diaminobenzidine (DAB) reagent. Cells were counterstained with hematoxylin, dehydrated, cleared with xylene, and mounted with resin-based medium. CHIT1-positive cells were counted and the percentage of eosinophils, neutrophils, macrophages and lymphocytes in BALF was determined based on a microscopic examination of the morphology (the size of the cell and the ratio of the nucleus of the cell to the cytoplasm) of 300 cells from various fields. Antibodies: anti-CHIT1 (Biorbyt, orb377995; lot# CQ2228) used at 1:200; donkey anti-rabbit-HRP (Jackson ImmunoResearch, 711-035-152, lot# 126333), used at 1:500.

2.4. Expression of chitinases in IPF lungs

Gene expression analysis of *CHIT1* and *CHIA* in the human lungs of IPF patients as compared to control healthy lungs was performed using the IPF gene explorer software (montgomerylab.stanford.edu/resources.html) from the GSE32537 study (Idiopathic Interstitial Pneumonias n = 119; Control n = 50).

2.5. Web-based analysis of chitinases expression profile in lungs after single cell RNA sequencing

Single cell RNA sequencing (scRNAseq) analysis of chitinases expression profile in fibrotic vs. donor lungs was done retrospectively using the web-based tool: https://www.nupulmonary.org/resources, based on the published study (Reyfman et al., 2019).

2.6. Animal studies

All *in vivo* experiments were performed in accordance with protocols approved by the Institute for Animal Care and Use Committee and Local Ethics Committee for Animal Experimentation, Warsaw, Poland. The animal studies were approved by the First Local Ethics Committee for Animal Experimentation, Warsaw, Poland, No. of approval: 452/2017. C57BL/6 female mice were purchased from Charles River Laboratory (Germany), *Chit1−/−* and *Chia−/−* mice were from Taconic and the KOMP Repository of University of California, Davis (USA), respectively.

2.7. Bleomycin-induced pulmonary fibrosis mouse model

Lung fibrosis was induced by triple intranasal instillations of bleomycin (Sigma Aldrich) in saline to 8-week-old female C57BL/6 mice (Charles River Laboratories). OATD-01 and pirfenidone (Molekula SRL) prepared in 0.5% carboxymethylcelulose were administered twice a day, PO in the therapeutic regimen (day 7 to day 21) at a dose of 30 mg/kg and 250 mg/kg for OATD-01 and pirfenidone, respectively.

2.8. Enzymatic assays

IC_{50} determination for human and mouse AMCase and CHIT1 was performed as previously published by Mazur M et al. (Mazur et al., 2018).

2.9. Chitinolytic activity in human and murine samples

The chitinolytic activity in murine samples was measured as follows: 1 μl of plasma or appropriate volume of lung homogenate corresponding to 2 μg of protein and 196 μM 4-methylumbelliferyl B-D-N,N′ diacetylchitobioside hydrate (Sigma Aldrich) in the assay buffer (0.1 M citrate, 0.2 M dibasic phosphate, 1 mg/ml BSA pH 6.0) were incubated in the dark, at 37 °C for 60 min. Following addition of the stop solution (0.3 M glycine/NaOH Buffer, pH 10.5), the fluorescent reaction product, 4-methylumbelliferone, was measured (excitation 355 nm/emission 460 nm). The chitinase activity was calculated using a standard curve of 4-methlyumberlliferone. For human samples, 5 μl of serum or 20 μl of induced sputum and 46 μM 4-methylumbelliferyl B-D-N,N' diacetylchitobioside hydrate in the assay buffer (0.1 M citrate, 0.2 M dibasic phosphate, 1 mg/ml BSA pH 2.0 or pH 6.0) were used. The reaction was run as described for murine samples. The assay was run at pH 2 to determine the AMCase-specific activity.

2.10. Murine BALF collection and analysis

Briefly, lungs were washed via trachea with 1 ml of PBS. Average recovery of BALF was 0.8 ml. The BALF was centrifuged (10 min, 2000 rpm, 4 °C). The cell pellet was resuspended in 300 μl of PBS and used for FACS analysis. Total BALF cells were counted by flow cytometry (Guava, Merck Millipore) and analyzed using specific antibodies to discriminate leukocyte (CD45 positive: CD45.2-PercP-Cy5, eBioscience, cat. No. 45-0454-82) myeloid cells population.

2.11. Semi-quantitative scoring of fibrosis and inflammation in the bleomycin-induced mouse model

Fibrosis was assessed by a blinded pathologist on sections stained with picrosirius red (PSR) according to the modified Ashcroft scoring system adapted to laboratory rodents (Hubner et al., 2008). For each animal, the analysis was performed on 3 sections (every containing 3 cross-sected lobes from right lung) separated by 200 μm of tissue. Fibrosis was scored for every lobe and the average score per animal was calculated and used for statistical analysis.

2.12. Murine lung histology

Lungs were perfused with PBS via the right ventricle and fixed in situ using standard procedures. Briefly, lungs were instilled via catheter in trachea with 10% NBF under constant pressure of 25 cm of liquid for 1h. Lungs were then dissected, processed to paraffin blocks, cut into 5 μm sections and stained with hematoxylin and eosin (HE), PSR, Masson's trichrome or IHC. After staining, slides were dehydrated, cleared and mounted with synthetic resin-based medium. Analyses were performed under light microscope (PrimoStar, Zeiss) equipped with digital camera (Axiocam ERc5s, Zeiss).

2.13. Immunohistochemistry of CHIT1 and AMCase on murine and human FFPE slides

For mouse samples, after dewaxing, antigens were retrieved with HIER at 98 °C in citrate buffer pH 6 with 0.1% Tween 20 (for CHIT1 antibodies) or in Tris-EDTA pH 9 with 0.1% Tween 20 (for AMCase antibody). Endogenous tissue peroxidases were inactivated by 3% H_2O_2/H_2O and non-specific binding was blocked with 1% BSA/10% NGS in TBST buffer. Chitinases were detected by specific antibodies and visualized with secondary antibody conjugated to HRP and by DAB (DAKO kit, K3468, Lot#10111394). Sections were counterstained with hematoxylin. Immunohistochemistry on human material was performed with an automated, validated and accredited staining system (Ventana Benchmark ULTRA) using ultraView Universal DAB Detection Kit (Ventana Medical Systems, 760–500) and counterstaining with hematoxylin. Antibodies: anti-CHIT1 (Cloud Clone, PAJ374Mu01; lot# A20140410988, used at 1:50; anti-AMCase [clone EPR19984] (Abcam, ab207169; lot# GR284785-2, used at 1:5000; anti-CHIT1 (Biorbyt, orb377995; lot# CQ2228) used at 1:400; donkey anti-rabbit-HRP (Jackson ImmunoResearch, 711-035-152, lot# 126333), used at 1:500.

2.14. Soluble collagen levels

The levels of the soluble collagen in murine lungs were evaluated using Sicroll assay (Biocolor, S1000) according to the manufacturer's protocol.

2.15. OATD-01 pharmacokinetics/pharmacodynamics evaluation in mice

PK/PD profile of OATD-01 was evaluated in C57BL/6 female mice at the end of the bleomycin-induced pulmonary fibrosis model. OATD-01 was administered at day 21 in two doses separated by 8 h interval (30 mg/kg, PO, b.i.d.). Lungs were collected after 30 min, 2 h, 4 h, 6 h, 8 h, 12 h, 20 h and 32 h post the first dose and chitinolytic activity was analyzed as described. OATD-01 concentrations in lung homogenates were determined by liquid chromatography tandem-mass spectrometry (LC-MS/MS) technique.

2.16. Real-time PCR

Lungs collected from mice were stored in RNALater buffer (Invitrogen). Total RNA was isolated using RNeasy MiniKit (Qiagen). RT PCR was done with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to manufacturer's protocol. Gene-specific TaqMan Assays and TaqMan Gene Expression Master Mix (Applied Biosystems) were used for gene expression analysis.

2.17. Statistical analysis

Data was analyzed using GraphPad Prism v. 7.0. Parametric one-way ANOVA with the Dunnett's test for multiple comparisons was used to evaluate the differences between the groups with Gaussian distribution of results (verified with D'Agostino & Pearson omnibus normality test and Shapiro-Wilk normality test). In case of non-Gaussian distribution a non-parametric Kruskal-Wallis test was utilized with Dunn's multiple comparison test to evaluate the differences between the groups to determine p-values. P-values <0.05 were considered significant and noted with asterisks (* for $P < 0.05$, ** for $P < 0.01$, *** for $P < 0.001$, **** for $P < 0.0001$).

3. Results

3.1. CHIT1 is the dominant chitinase in IPF

Multiple reports demonstrated increased chitinolytic activity in the serum of patients with chronic lung diseases. However, as the expression and cellular localization of chitinases in lungs of patients with IPF remained to be elucidated, we first confirmed a significantly elevated chitinolytic activity at pH 6 and not at pH 2 in serum (Fig. 1A) and induced sputum (Fig. 1B), confirming CHIT1 and not AMCase as the dominant chitinase in IPF. The total chitinolytic activity in serum from IPF patients correlated with CHIT1 concentrations further supporting this conclusion (Fig. 1C). The characteristic of healthy control and IPF patients was presented in Table 1.

These results were further corroborated by a web-based analysis of

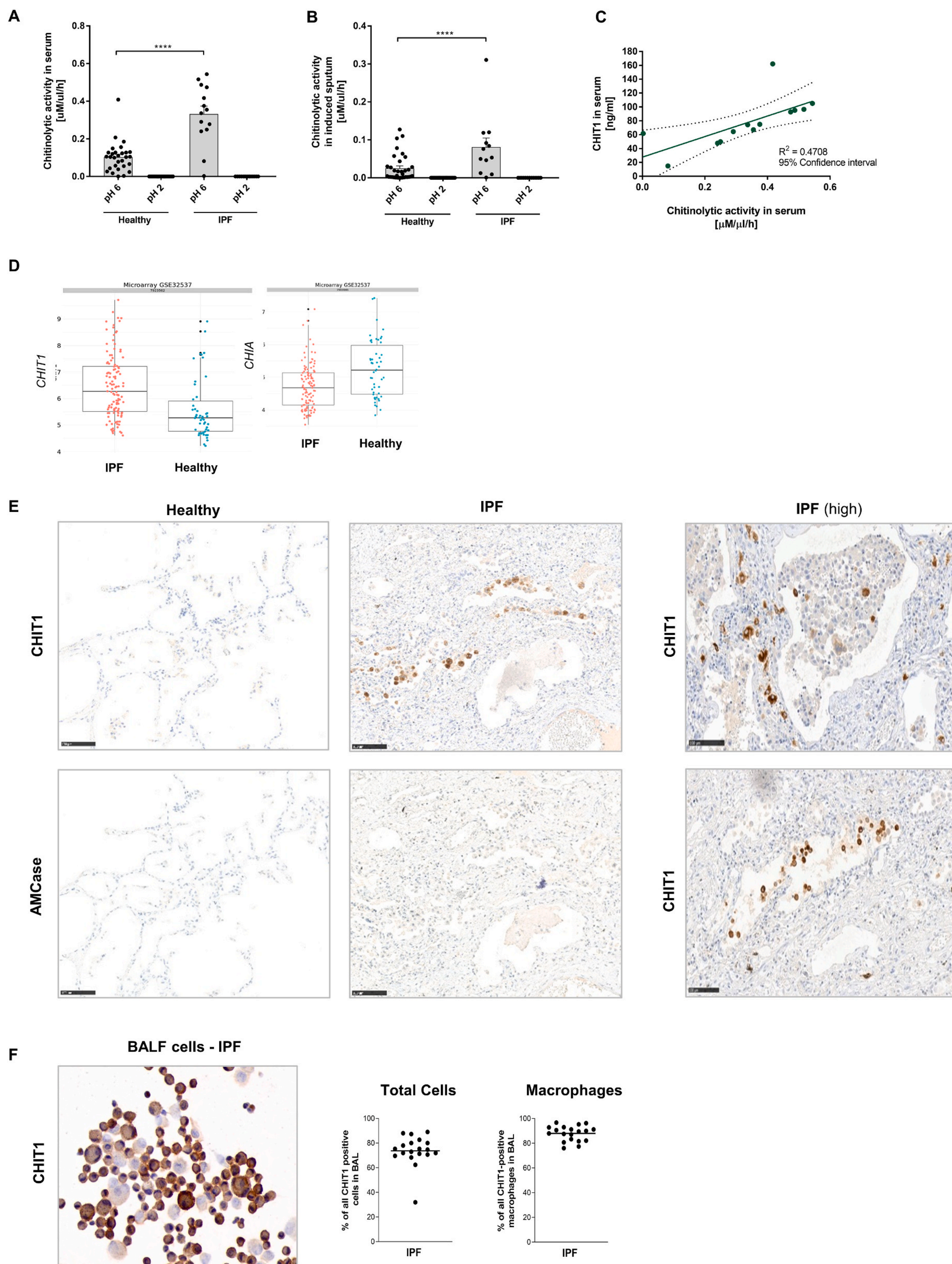

Fig. 1. Induction of chitinolytic activity and CHIT1 expression in IPF patients. **(A)** Chitinolytic activity in serum **(A)** and induced sputum **(B)** from healthy donors and IPF patients: total chitinolytic activity was determined at pH 6, and AMCase activity was determined at pH 2. **(C)** Correlation between CHIT1 concentration and the chitinolytic activity in serum of patients with IPF. (**D**) Gene expression analysis of *CHIT1* and *CHIA* in the lungs of IPF patients and healthy controls. Data were generated from the study GSE32537 (Idiopathic Interstitial Pneumonias n = 119; Control n = 50) using the IPF gene explorer software. **(E)** Representative images of immunohistochemical staining of CHIT1 and AMCase in explants from lungs of IPF patients (n = 6) and healthy controls (n = 4). Two images at higher magnification show CHIT1 staining in interstitial and alveolar macrophages **(F)** Immunohistochemical analysis of CHIT1 in BALF smears from IPF patients (n = 19); representative IHC for CHIT1 in BALF cells and quantitative analysis of CHIT1 positive total cells and macrophages in BALF of IPF patients. Data presented as mean ± s.e.m. ****P < 0.0001.

the relative gene expression of *CHIT1* and *CHIA* (AMCase coding gene) in lungs of patients with IPF. This analysis revealed a significant induction of *CHIT1* expression and downregulation of *CHIA* in patients with interstitial pneumonias (GSE32537) as compared to healthy individuals (Fig. 1D). These results demonstrated that CHIT1 is the main chitinase induced in fibrotic lungs of IPF patients.

Moreover, histological analysis of lung biopsies revealed highly upregulated, macrophage-specific CHIT1 expression localized to fibrotic areas (Fig. 1E). In contrast, we did not detect any AMCase expression in lungs of IPF patients (Fig. 1E). This result was further confirmed by the immunocytochemical staining of BALF cell smears from IPF patients demonstrating 70–90% CHIT1-positive cells, in particular macrophages (Fig. 1F).

To better understand macrophage expression profile of CHIT1 in fibrotic lungs, we interrogated a cell atlas of pulmonary fibrosis created by a single-cell RNA sequencing (scRNAseq) of fibrotic lungs from patients with ILDs and from healthy lungs (Reyfman et al., 2019). Our analysis revealed a strong expression of *CHIT1* in lungs of ILD patients, which was restricted to a subset of the macrophage cluster specific for fibrotic lungs and not present in the healthy tissue (Fig. 2AB). To further characterize this subset of macrophages, we analyzed expression of multiple profibrotic factors. While *TGFB1* was expressed in most macrophages, expression of *SPP1* and *CCL2* expression was most prominent in the sub-cluster of macrophages expressing *CHIT1* (Fig. 2). The strong association of *CHIT1* expression and activity with IPF suggests that it might play a pathological role in lung fibrosis. In contrast, we found that *CHIA* was moderately expressed in lungs, mostly in epithelial cells (Fig. 2A) and not in any type of macrophages (Fig. 2B), and with no correlation to fibrosis, further underscoring the disease-specific association of CHIT1 and pathologically activated macrophages. Together, our results indicate that macrophages are the primary source of CHIT1, which is the dominant chitinase induced in fibrotic lungs.

3.2. *Bleomycin-induced model of lung fibrosis recapitulates CHIT1 induction in IPF*

To evaluate contributions of chitinases to the development of pulmonary fibrosis in mice, we analyzed expression of CHIT1 and AMCase in the bleomycin-induced model, which recapitulates some features of IPF and is commonly used in preclinical studies (Mouratis and Aidinis, 2011). Firstly, we assessed activity of individual chitinases, AMCase and CHIT, in non-diseased mice. Study on knockout animals showed that in C57BL/6 mice about 60% of the chitinolytic activity in plasma was derived from AMCase and about 40% from CHIT1 confirming that in mice both chitinases are active (Fig. 3).

Immunohistochemical analysis of the fibrotic lung tissues demonstrated that CHIT1 was localized predominantly to macrophages present in fibrotic areas, with a strong induction observed from day 7 that

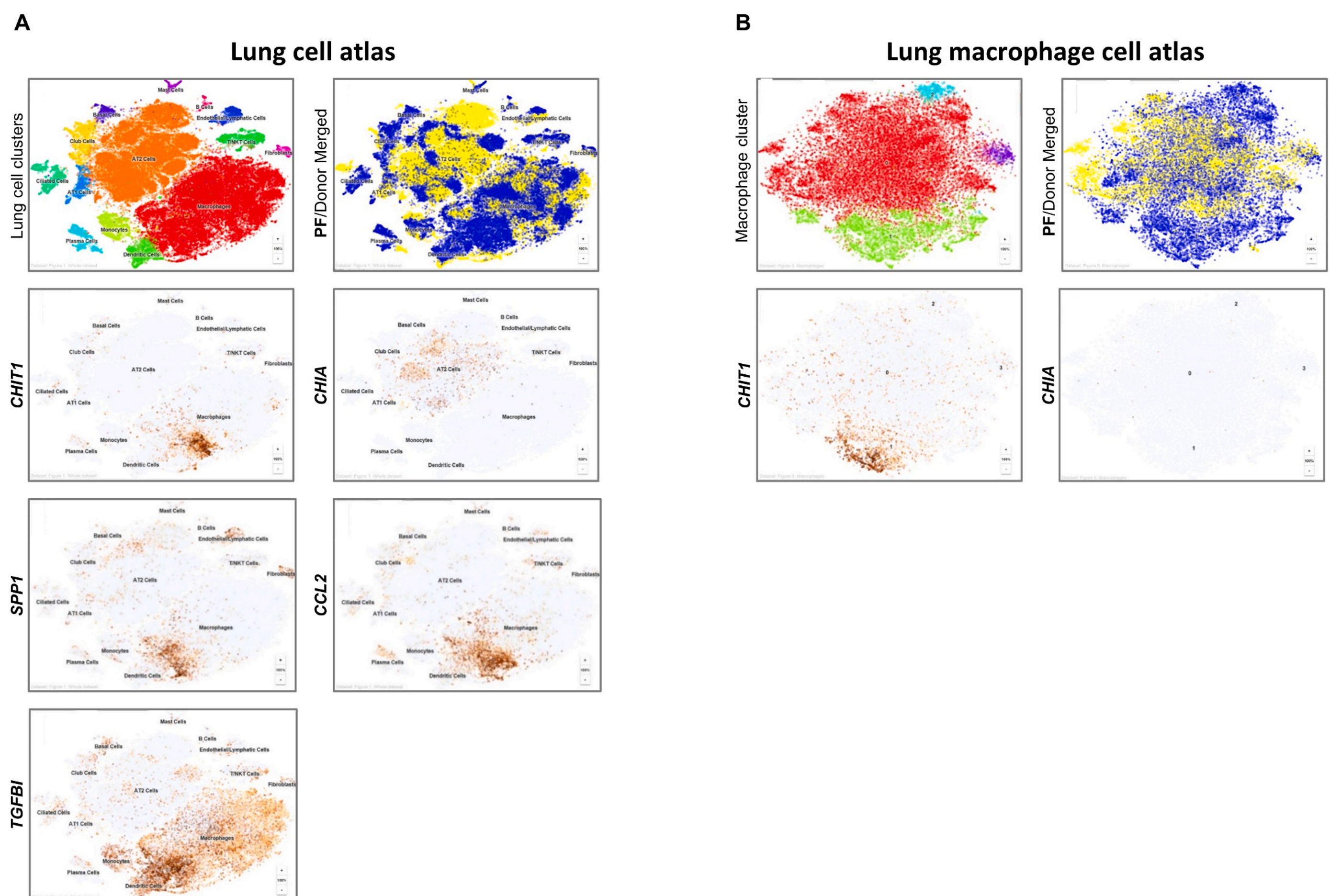

Fig. 2. CHIT1 is expressed exclusively in a subset of emerging fibrosis-specific macrophages the lungs of IPF patients. ScRNAseq lung atlas was used to analyze chitinases expression profile within all cellular populations identified in healthy and fibrotic lungs **(A)** and within a population of alveolar macrophages emerging during pulmonary fibrosis **(B)**. **(A)** All major cell types identified in lungs are indicated. Cell populations derived from healthy donors (yellow) and fibrotic lungs (blue) are shown. ScRNAseq analysis of expression of *CHIT1* and *CHIA* in lungs and of expression of profibrotic markers *SPP1*, *CCL2* and *TGFB1*. AT1 and AT2; alveolar type 1 and type 2 cells; T/NKT: T and natural killer T cells. **(B)** Analysis of macrophages in lungs identifies 4 major clusters. Macrophages derived from healthy donors (yellow) and fibrotic lungs (blue) are shown. Expression of *CHIT1* and *CHIA* in pulmonary macrophages. Expression of *CHIT1* is restricted to a fibrosis-specific cluster of macrophages.

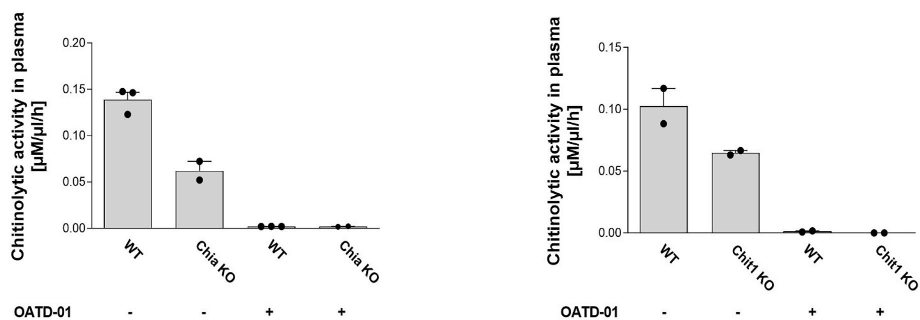

Fig. 3. Chitinolytic activity in mouse is derived from CHIT1 and AMCase. Total chitynolytic activity was measured in plasma of *Chia−/−* mice and *Chit1−/−* mice, and their corresponding WT control animals (n = 2 or 3 per group). AMCase- and CHIT1-specific chitinolytic activity in plasma was suppressed by a chitinase inhibitor OATD-01 *ex vivo* at the concentration 1 µM.

Fig. 4. Induction and localization of CHIT1 and AMCase during bleomycin-induced lung fibrosis in mice. The kinetics of expression of chitinases and markers of pulmonary fibrosis in the bleomycin-induced model of lung fibrosis was evaluated in lungs at day 3, 7, 14 and 21 post bleomycin administration (number of animals n = 8 per timepoint per group). **(A)** Representative images of immunohistochemical staining of CHIT1 and AMCase in sections from murine lungs (one section per animal, n = 5 per timepoint per group) during progression of bleomycin-induced pulmonary fibrosis as determined by a picrosirius red staining (PSR) of fibrotic lesions. **(B)** Analysis of pulmonary fibrosis by Ashcroft scoring **(C)** Kinetics of mRNA expression of *Chit1* and *Chia* in lungs during fibrosis development **(D)** Kinetics of mRNA expression of profibrotic genes: *Col1a1*, *Col3a1*, *Fn1* and *Timp1* in lungs as compared to vehicle (saline) treated controls. Data presented as mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001.

correlated with the onset and duration of the fibrotic phase (Fig. 4A). AMCase expression was continuous throughout the study and was limited to bronchial epithelial cells (Fig. 4A). Downregulation of AMCase mRNA and robust upregulation of CHIT1 mRNA in the lungs of mice with bleomycin-induced pulmonary fibrosis was observed (Fig. 4C) recapitulating the gene expression data obtained from lungs of IPF patients (Fig. 1B). Moreover, upregulation of *Chit1* correlated with the expression of fibrosis-associated genes (Fig. 4D): *Col1a1*, *Col3a1*, *Fn1* and *Timp1*. Together, these results demonstrate that in the context of chitinase induction and macrophage-specific expression of CHIT1, the bleomycin-induced model recapitulates lung fibrosis in patients with fibrosing ILDs, indicating that it represents a rational approach to evaluate therapeutic efficacy of chitinase inhibitors.

3.3. Genetic inactivation of Chit1 ameliorates pulmonary fibrosis in the bleomycin-induced model

The effects of genetic inactivation of CHIT1 on pulmonary fibrosis development were evaluated in the bleomycin-induced murine model. *Chit1−/−* mice did not show any overt phenotype, no visual lung pathologies and no effects on expression of pro-fibrotic markers in non-diseased lungs (Fig. 5A). The lack of *Chit1* expression in knockout animals instilled with bleomycin correlated with significant attenuation of the pulmonary fibrosis score in the model (Fig. 5BC). *Chit1−/−* mice also exhibited significantly reduced pulmonary inflammation as measured by the total number of $CD45^+$ leukocytes in BALF (Fig. 5D).

To gain more insight into molecular mechanisms of fibrosis driven by CHIT1, expression of a wide array of fibrosis markers in lungs was analyzed by qtPCR. Expression of *Col1a1*, *Col3a1*, *Fn1*, *Tnc* and *Acta2*

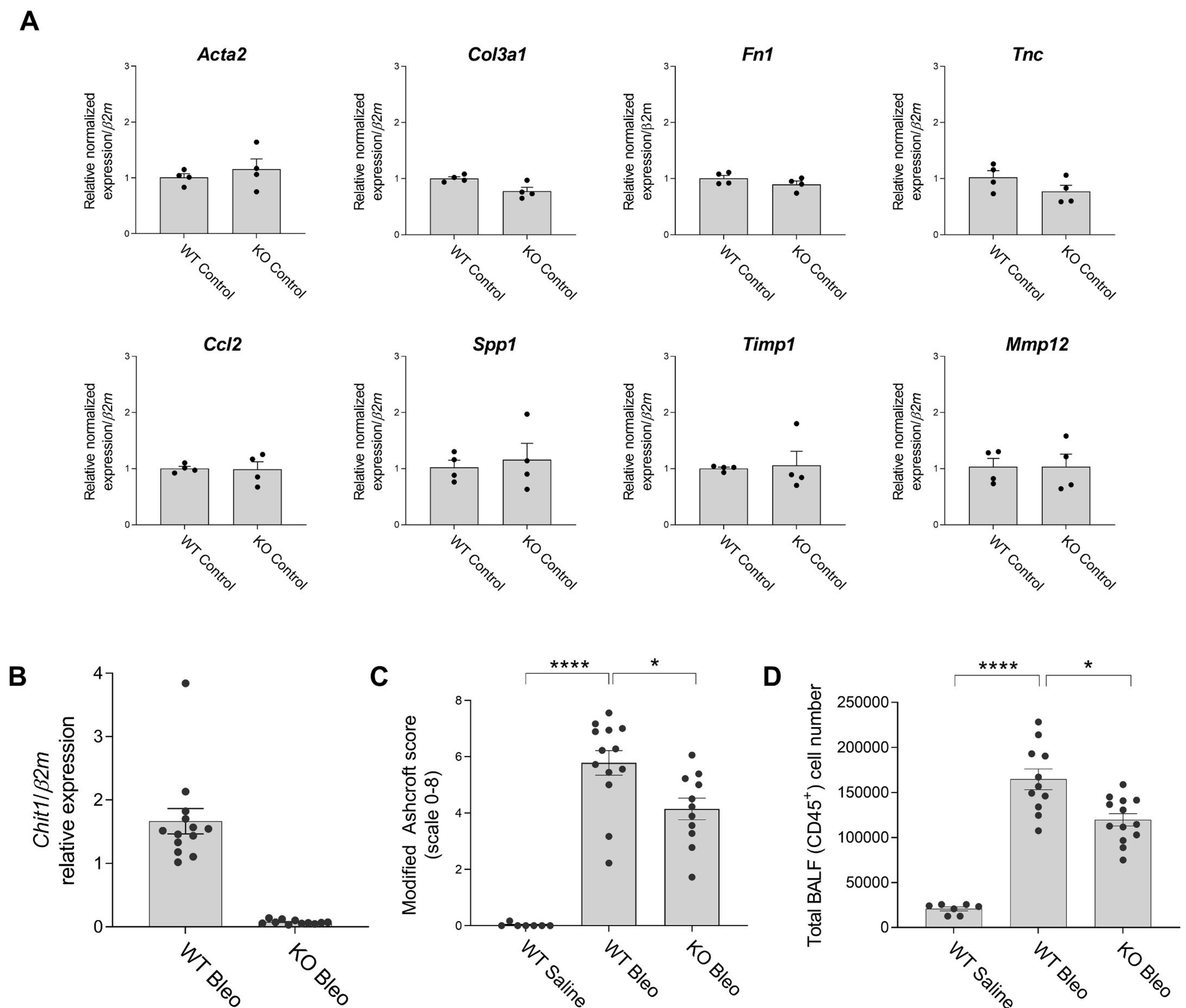

Fig. 5. Genetic CHIT1 inactivation reduces bleomycin-induced pulmonary fibrosis and inflammation. The expression of markers of fibrosis in non-challenged WT and *Chit1−/−* mice (number of animals n = 4 per group). The pulmonary fibrosis and inflammation in WT and *Chit1−/−* mice in the bleomycin-induced model of lung fibrosis were evaluated at day 21 post bleomycin administration (number of animals: n = 7 in control group and n = 13 in bleomycin-challenged groups). **(A)** mRNA expression of selected fibrosis markers: *Col3a1*, *Fn1*, *Tnc*, *Timp1*, *Mmp12*, *Spp1* and *Ccl2* in normal lungs of WT and *Chit1−/−* mice. Expression was normalized to *B2m* gene expression. **(B)** *Chit1* expression in lungs of WT and *Chit1−/−* mice **(C)** Analysis of pulmonary fibrosis by the Ashcroft scoring in WT and *Chit1−/−* mice 21 days after bleomycin administration. **(D)** Analysis of $CD45^+$ leukocytes in BALF from WT and *Chit1−/−* mice 21 days after bleomycin administration. Gene expression was evaluated by qtPCR. Data shows means ± s.e.m. *$P < 0.05$, ****$P < 0.0001$.

was consistently and significantly reduced in knockout compared to the wild-type animals (Fig. 6A). Additionally, expression of genes coding for fibrosis-associated proteins MMP12 and TIMP1 and profibrotic mediators CCL2 and SPP1 was strongly suppressed in *Chit1−/−* mice compared to wild-type (WT) animals (Fig. 6BC). Together, these data demonstrate that genetic ablation of CHIT1 attenuates development of fibrosis in the bleomycin-induced model, indicating a pathologic role of CHIT1.

3.4. OATD-01: a potent chitinase inhibitor

The results of translational and preclinical studies prompted us to develop a chitinase inhibitor to evaluate the therapeutic potential of inhibition of chitinolytic activity in murine models of pulmonary fibrosis. In this context, we developed OATD-01, a potent inhibitor of CHIT1 with a low nanomolar activity against human and murine chitinases (Fig. 7A). The chemical properties, pharmacokinetics in rodents and dogs and preclinical safety of OATD-01 were previously established by our group (Koralewski et al., 2020). Further evaluation of pharmacokinetic and pharmacodynamic properties demonstrated that PO administration of OATD-01 at 30 mg/kg b.i.d. fully suppressed the chitinolytic activity in fibrotic lungs (Fig. 7B).

Importantly, in *ex vivo* studies with human samples, OATD-01 fully inhibited the highly elevated chitinolytic activity in serum, BALF and induced sputum from patients with IPF with $IC_{50} < 20$ nM (Fig. 7C), suggesting its therapeutic potential in humans. Moreover, OATD-01 also reduced the levels of IL-1 receptor antagonist, IL-15 and IFNγ - modulators of immune and inflammatory responses, secreted by BALF macrophages isolated from IPF patients (Fig. 7D). Thus, OATD-01 demonstrated a potent inhibition of chitinolytic activity in both, patient-derived samples and in murine lungs *in vivo* and showed direct effects on the expression of immune response modulators by IPF macrophages.

3.5. OATD-01 attenuated bleomycin-induced pulmonary fibrosis

Having established that the bleomycin-induced model recapitulates disease-associated induction of chitinases, we evaluated the anti-fibrotic efficacy of OATD-01 in a head-to-head comparison with pirfenidone applying therapeutic treatment scheme. Histological analysis of lung slices using the Ashcroft scoring scale (Hubner et al., 2008) confirmed a significant suppression of fibrosis by OATD-01 (30 mg/kg; PO; b.i.d.), with anti-fibrotic efficacy comparable to pirfenidone (250 mg/kg; PO; b.i.d.) (Fig. 8AB). Moreover, the soluble collagen concentration in lungs was reduced by OATD-01, comparably to pirfenidone (Fig. 8C). A decrease of the lung/body weight ratio, a measure of lung injury, further confirmed therapeutic efficacy of OATD-01 (Fig. 8D). The antifibrotic efficacy of the compound was associated with a full suppression of the chitinolytic activity in plasma (Fig. 8E), confirming OATD-01 target engagement. These data provide a pharmacological proof-of-concept for the profibrotic function of CHIT1 and demonstrate the therapeutic potential of OATD-01.

4. Discussion

Chitinases have been implicated in multiple ILDs (Chang et al., 2020;

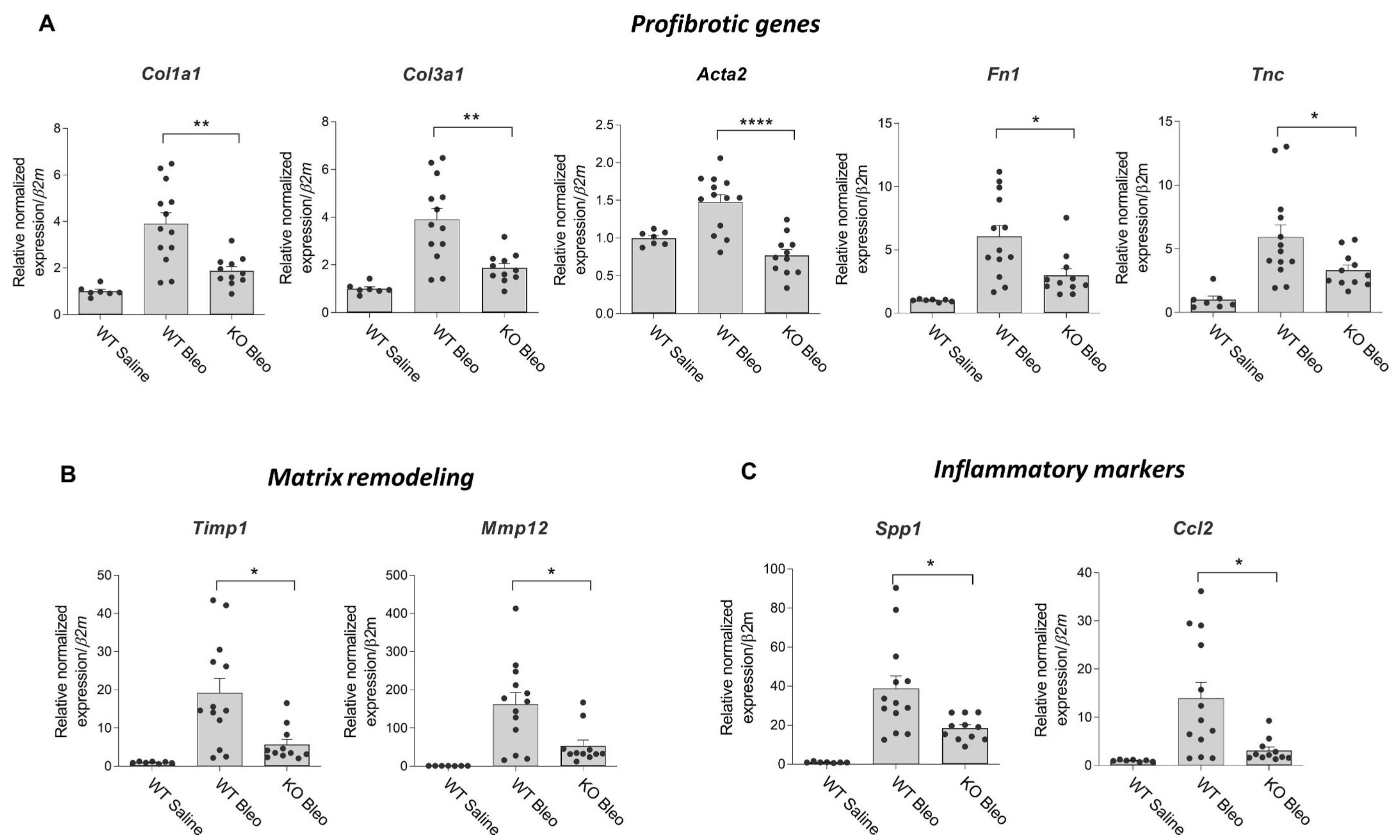

Fig. 6. CHIT1 deficiency attenuates profibrotic gene signature in the lungs in the bleomycin-induced model of pulmonary fibrosis. The expression of markers of pulmonary fibrosis in WT and *Chit1−/−* mice in the bleomycin-induced model of lung fibrosis was evaluated in lungs 21 days post bleomycin administration (number of animals number of animals: n = 7 in control group and n = 13 in bleomycin-challenged groups). The expression was evaluated by qtPCR and normalized to *B2m* gene expression. **(A)** Relative expression of fibrotic genes: *Col1a1*, *Col3a1*, *Acta2*, *Fn1* and *Tnc* **(B)** Relative expression of fibrosis-associated matrix remodeling genes: *Timp1* and *Mmp12*. **(C)** Relative expression of fibrosis-associated inflammatory genes: *Spp1* and *Ccl2*. Data shows means ± s.e.m. *P < 0.05, **P < 0.01, ****P < 0.0001.

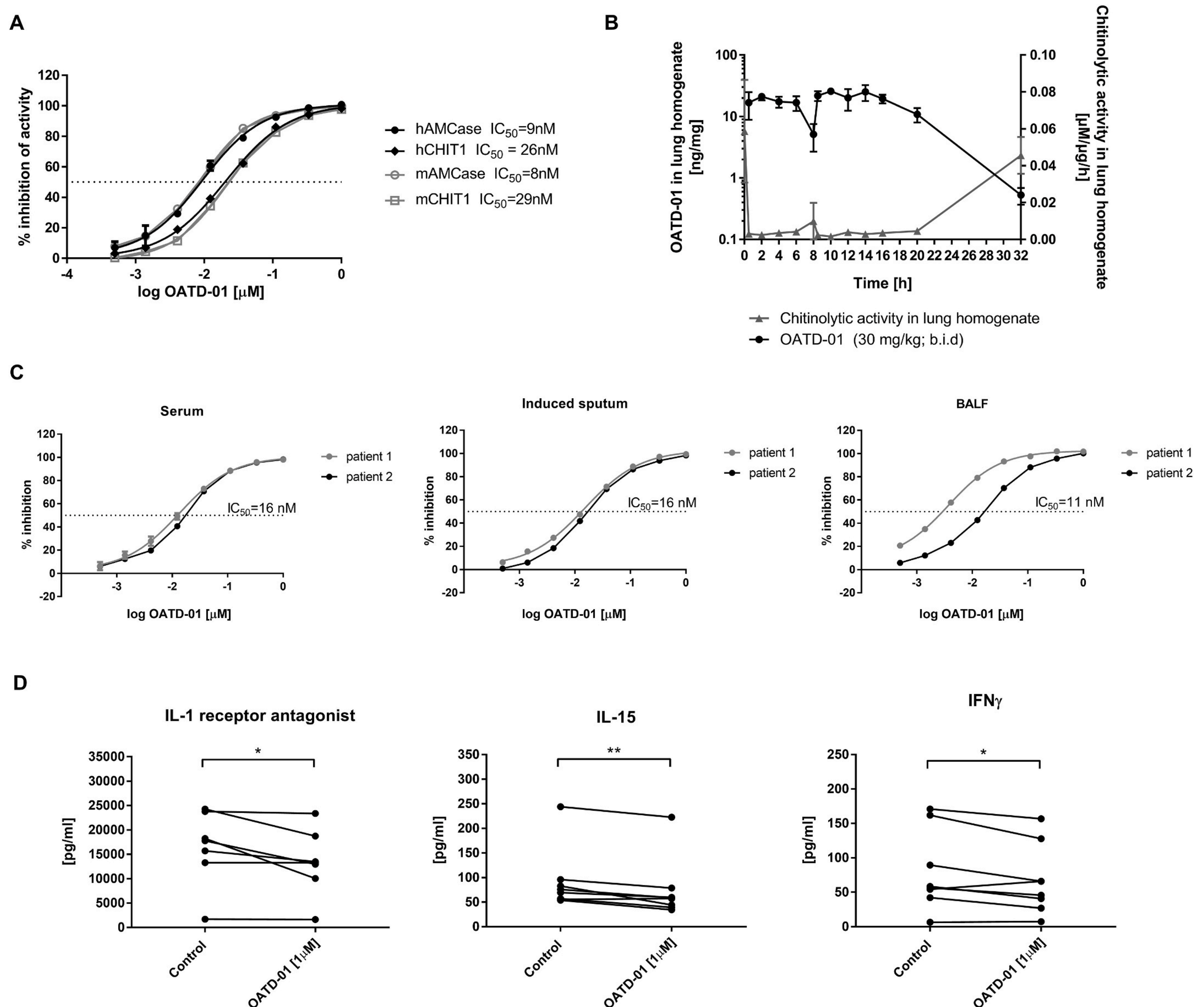

Fig. 7. OATD-01, a potent chitinase inhibitor, suppressesed chitinolytic activity in fibrotic lungs in mice, and in serum, BALF and induced sputum from patients with IPF. (A) IC_{50} determination of OATD-01 against human and mouse AMCase and CHIT1 by the enzymatic assay **(B)** PK/PD profile of OATD-01 in fibrotic lungs from mice 21 days post bleomycin administration (3x intranasally, 1 U/kg). OATD-01 was administered on day 21 in two doses (30 mg/kg, PO, b.i.d.) at 0 h and 8 h. PK/PD was determined at indicated time points. **(C)** Inhibition of the chitinolytic activity in serum, BALF and induced sputum from patients with IPF (n = 2) by OATD-01 determined by the *ex vivo* enzymatic assay. (D) The levels of modulators of immune and inflammatory responses (IL-1 receptor antagonist, IL-15, IFNγ) in IPF macrophages supernatants (n = 7) following OATD-01 treatment (at 1 μM concentration for 24 h). *$P < 0.05$, **$P < 0.01$.

Cho et al., 2015; Przysucha et al., 2020), including IPF and might represent a novel therapeutic target. While series of contradictory data have been reported on expression and pathological role of AMCase in pulmonary diseases (Fitz et al., 2012; Mackinnon et al., 2012; Mazur et al., 2018, 2019; Van Dyken et al., 2017; Vannella et al., 2016; Zhu et al., 2004), CHIT1 has been consistently described in the literature as the primary active chitinase in humans with profibrotic potential *in vitro* and *in vivo* (Bargagli et al., 2007; Lee et al., 2012, 2019; Reyfman et al., 2019). Therefore, to evaluate the role of CHIT1 in lung fibrosis, it was critical to establish expression and activity profiles for chitinases in lungs of IPF patients and further to assess the therapeutic potential of CHIT1 inactivation applying mouse model of lung fibrosis.

In the current study we report a comprehensive analysis of both chitinases in patients with IPF. We demonstrated a significantly elevated serum chitinolytic activity in > 90% patients with IPF suggesting that it represents a common and early event in IPF pathology. In accordance, CHIT1 concentration in serum correlated with total chitinolytic activity. The major limitation of the obtained data is the lack of age-matching of the IPF and healthy individual groups. Importantly, the conclusion concerning CHIT1 expression in IPF was further corroborated by the immunocytochemical analysis, which demonstrated a robust, mostly macrophage-derived expression of CHIT1 in lungs and BALF cells from IPF patients. In contrast, we were not able to obtain any evidence of AMCase expression in these studies. Our results are in accordance with several reports, which indicated that CHIT1 is the main source of the elevated chitinolytic activity (Bargagli et al., 2007; Chang et al., 2020; Seibold et al., 2008). In accordance with these results, the analysis of a single cell lung atlas (Reyfman et al., 2019) generated by scRNAseq of fibrotic and healthy lungs revealed a strong induction of *CHIT1* in the lungs of patients with pulmonary fibrosis, which was restricted to a macrophage cluster specific to fibrotic lungs and not present in healthy lungs. In contrast, *CHIA* showed modest expression in the epithelial cell

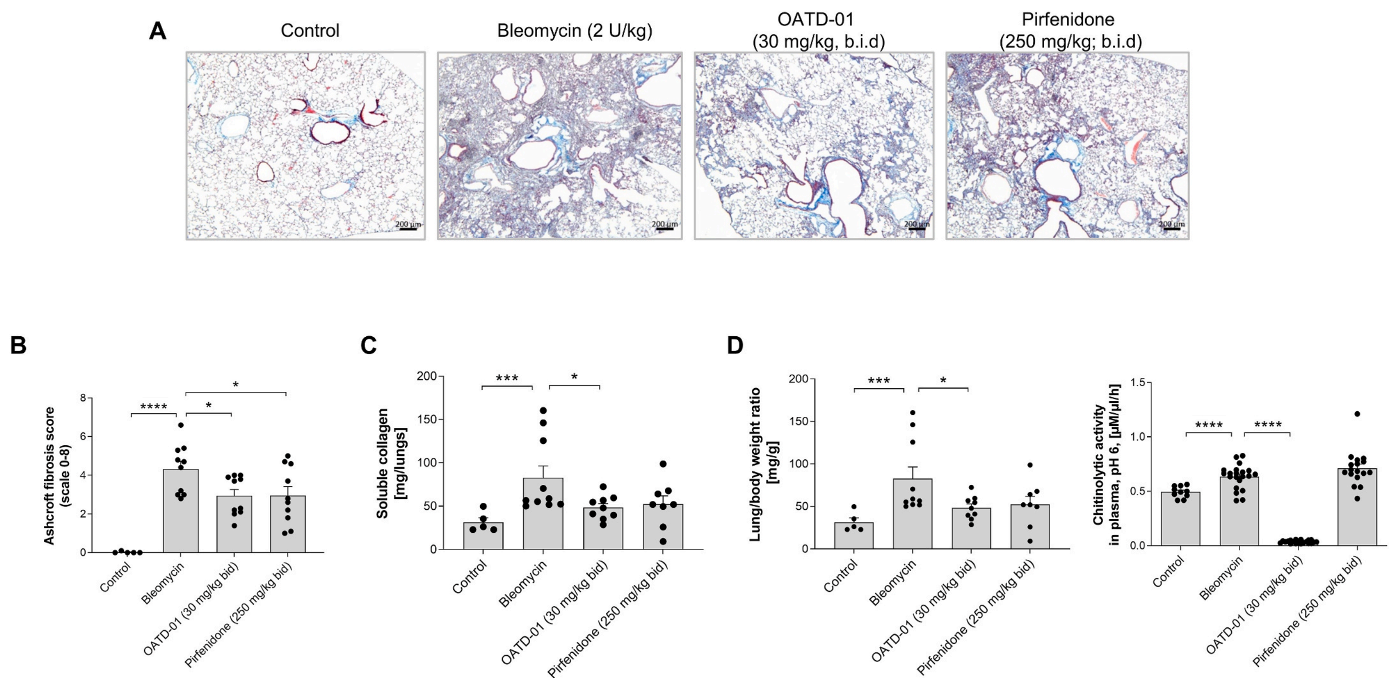

Fig. 8. Chitinase inhibitor OATD-01 attenuates fibrosis in bleomycin-induced lung fibrosis model with anti-fibrotic efficacy comparable to pirfenidone. Head-to-head comparison of anti-fibrotic activity of OATD-01 (30 mg/kg, b.i.d.) vs. pirfenidone (250 mg/kg, b.i.d.) in the bleomycin-induced model of pulmonary fibrosis in mice (n = 10 in control group and n = 20 in bleomycin-challenged groups; each group was radomly divided into two at day 21 for analysis of lung fibrosis by Ashcroft scoring (n = 11) and analysis of collagen levels in the lungs (n = 10). Both compounds were administered by oral gavage from day 7 until day 21. **(A)** Representative images of lung fibrosis detected by the trichrome staining in control animals, and mice administered bleomycin alone, and after treatment with OATD-01 and pirfenidone. **(B)** Analysis of pulmonary fibrosis by the modified Ashcroft scoring system in the lung sections (n = 3 per animal) in animals after OATD-01 and pirfenidone as compared to the vehicle treated controls. **(C)** Soluble collagen levels (Sircoll assay) in lungs at day 21 post bleomycin administration **(D)** Lung weight to body weight ratio analysis at day 21 post bleomycin administration **(E)** Chitinolytic activity in plasma after OATD-01 and pirfenidone treatments as compared to the vehicle treated controls. Data are representative for three independent experiments. Data presented as mean ± s.e.m. *P < 0.05, ***P < 0.001, ****P < 0.0001.

cluster with no correlation to fibrosis. In summary, the published reports and our own comprehensive studies demonstrate that CHIT1 is the predominant chitinase in patients with pulmonary fibrosis, of which expression is limited to disease-associated macrophages and strongly correlates with pulmonary fibrosis. Together, these data suggest that CHIT1 might be a novel therapeutic target for IPF.

To confirm this notion, we evaluated the expression profile of chitinases in the bleomycin-induced model of pulmonary fibrosis, which is the most commonly used murine model with an established translational value. While induction of CHIT1 in the model has been reported (Lee et al., 2012), our study provides a detailed analysis of kinetics and localization of CHIT1 and AMCase expression following bleomycin instillation. We confirmed macrophage specific CHIT1 induction in bleomycin-injured lungs. In addition, we observed that early on in development of fibrosis, *Chia* expression is downregulated while *Chit1* expression correlates with fibrotic markers, indicating and further confirming that CHIT1 represents a rational approach to evaluate therapeutic efficacy of chitinase inhibitors.

To better understand the pathological role of CHIT1, which is the dominant chitinase in human IPF, the effects of a genetic inactivation of CHIT1 on fibrosis development were evaluated in the bleomycin-induced model. In the model, knock-out of *Chit1* strongly attenuated pulmonary fibrosis and inflammation. This conclusion was further supported by the observation that CHIT1 deficiency strongly suppressed bleomycin-mediated induction of a wide array of fibrosis-associated genes, as well as inflammatory mediators *Ccl2* (Kolahian et al., 2016) and *Spp1* (Lenga et al., 2008; Morse et al., 2019) with a documented profibrotic activity in pulmonary fibrosis. Our results expand the published data demonstrating a profibrotic activity of CHIT1 (Lee et al., 2012), and provide a detailed analysis of the fibrosis-attenuating phenotype of C*hit1*−/− mice.

Having established the fibrogenic activity of CHIT1, we developed OATD-01, a highly potent and selective chitinase inhibitor, and confirmed its ability to suppress the chitinolytic activity in fibrotic lungs of mice after oral administration. OATD-01 resulted in attenuation of fibrosis induced by bleomycin, with anti-fibrotic efficacy comparable to pirfenidone that did not alter chitinolytic activity, indicating a novel mechanism of action of CHIT1 inhibitor. IPF is a heterogenous, multifactorial disease and our data indicate that CHIT1 may be one of several drivers of pulmonary fibrosis.

Our study has demonstrated that CHIT1 is a primary chitinase in human lung and AMCase does not play a role in IPF. While in the lungs of human IPF subjects AMCase activity and expression was not detected, in a mouse bleomycin-induced pulmonary fibrosis model AMCase expression was present, but decreased after fibrosis induction. OATD-01 is a dual AMCase/CHIT1 inhibitor with pharmacodynamic effect on both chitinases, therefore we cannot rule out the role of AMCase in the bleomycin-induced lung injury model. However, studies with genetic inactivation of CHIT1 demonstrated a similar phenotype and comparable level of the antifibrotic effect to the pharmacological inhibition of this enzyme. Taken together we conclude that inhibition of CHIT1 has antifibrotic effects in murine fibrosis model and an increased CHIT1 activity may be one of the drives of fibrosis in IPF.

Since CHIT1 is predominantly expressed in pulmonary macrophages, both in patients with pulmonary fibrosis and in murine models of IPF, it is relevant to consider its expression in the broader context of the role of macrophages in development of lung fibrosis. Although there is ample evidence implicating inflammation in the pathogenesis of lung fibrosis (Heukels et al., 2019; Wynn, 2011), the role of excessive inflammation as an important driver of IPF pathology remains controversial, mostly based on the failed clinical trials of anti-inflammatory drugs like steroids in IPF patients. However, since then the role of macrophages in the

pathogenesis of pulmonary fibrosis has been redefined (Byrne et al., 2016; Wynn and Vannella, 2016). New data revealed a remarkable plasticity of macrophages, which can acquire a wide spectrum of distinct phenotypes with different effects on fibroproliferative responses to the injury (Wynn and Vannella et al., 2016). Lineage tracing and scRNAseq facilitated identification of several specific subsets of macrophages with a profibrotic phenotype in murine models of pulmonary fibrosis (Aran et al., 2019; McCubbrey et al., 2018; Misharin et al., 2017; pJoshi et al., 2020; Satoh et al., 2017; Ucero et al., 2019). These macrophages expressed multiple factors associated with fibrosis, like TGFβ1, CCL2 and MMPs. While various approaches were used, subtype-specific deletions and adaptive transfers of fibrotic macrophages confirmed their ability to drive pulmonary fibrosis. This concept has been further supported by scRNAseq studies, which confirmed existence of distinct populations of macrophages associated with pulmonary fibrosis in ILD patients (Morse et al., 2019; Reyfman et al., 2019). In this context, our retrospective analysis demonstrating that CHIT1 is highly and exclusively expressed by a distinct subset of macrophages specific to fibrotic lungs and co-expressing fibrotic factors like TGFB1, SPP1 and CCL2, directly links CHIT1 to pulmonary fibrosis. CHIT1 was one of the most abundantly expressed genes in this macrophage cluster and part of the subtype-defining genetic signature. Decreased expression of Spp1, Ccl2 and other profibrotic factors induced by bleomycin in Chit1−/− mice suggests that CHIT1 might promote acquisition and/or maintenance of the profibrotic phenotype by macrophages.

It is noteworthy that in the preclinical models, pirfenidone and nintedanib demonstrated anti-fibrotic and anti-inflammatory activity similar to CHIT1 inhibition, including reduction of collagen deposition, decreased infiltration of inflammatory cells to the lungs, inhibition of expression of metalloproteases and TIMPs, as well as CCL2 and other proinflammatory cytokines and chemokines (Kolb et al., 2017; Wollin et al., 2015). In particular, nintedanib has been shown to exert anti-inflammatory properties via modulation of the activation state and the function of macrophages (Bellamri et al., 2019). Both approved drugs were reported to inhibit TGFβ-mediated proliferation of lung fibroblasts, their differentiation to myofibroblasts and collagen synthesis (Conte et al., 2014; Wollin et al., 2015). In this context, several studies demonstrated that CHIT1 enhanced TGFβ1-induced fibrosis by various mechanisms, including stimulation of fibroblast proliferation, myofibroblast transformation (Lee et al., 2019), and TGFβ1 signaling by increasing expression of its receptors (Lee et al., 2012) and by suppressing induction of TGFβ feedback inhibitor SMAD7 through interaction with TGFBRAP1 and FOXO3 (Lee et al., 2019). Increased CHIT1 levels correlated inversely with SMAD7 expression in patients with IPF. Thus, inhibition of CHIT1 by OATD-01 might lead to the antifibrotic responses, which overlap with those induced by pirfenidone and nintedanib, further indicating a clinical potential of CHIT1 inhibition.

Together, this report demonstrates a strong clinical association of CHIT1 with pulmonary fibrosis and provides a preclinical proof-of-concept for its fibrogenic activity. Results of the pharmacological inhibition and genetic inactivation in animal models combined with translational data clearly establish CHIT1 as a promising novel therapeutic target for IPF. This provides a robust rationale for developing OATD-01, a potent chitinase inhibitor as a novel therapy for IPF.

Sources of funding

Studies were supported by two projects: (1) "Development of a first-in-class small molecule drug candidate for treatment of idiopathic pulmonary fibrosis through chitotriosidase inhibition" (POIR.01.01.01-00-0551/15), acronym IPF and (2) "Preclinical research and clinical trials of a first-in class development candidate in therapy of asthma and inflammatory bowel disease" (POIR.01.01.01- 00–0168/15), acronym IBD – both co-funded by the National Center for Research and Development, Poland in the framework of European Funds Smart Growth. BAD was supported by the Ministry of Science and Higher Education, Poland (50//DW/2017/01/1).

Declaration of competing interests

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: At the time of the study PS, BAD, MM, AMS, RoK, MiM, SK, NCG, AZ, AR, RMK, AG, KD, and PD were employees and shareholders of OncoArendi Therapeutics SA, which develops inhibitors of CHIT1. AG, MM and RoK are co-inventors on the patent application related to this work (US Patent No. US 9,944,624 B2 Substituted amino triazoles useful as human chitinase inhibitors). JG is a shareholder and consultant for OncoArendi Therapeutics. JvdT received research funding from OncoArendi Therapeutics, BMS and Roche, and consulting fees from MSD, Boehringer, and Roche. RKr received fees for lectures and/or travel expenses from Boehringer Ingelheim, AstraZeneca, Roche, Chiesi and Polpharma. KG received fees for lectures and travel expenses from Boehringer Ingelheim and Roche. PN-G, MP, MP-G, ŁC declare no conflicts of interests.

CRediT authorship contribution statement

Piotr Sklepkiewicz: Investigation, Formal analysis, Methodology, Writing – original draft. **Barbara A. Dymek:** Investigation, Formal analysis, Methodology, Writing – original draft. **Michal Mlacki:** Investigation, Formal analysis. **Robert Koralewski:** Resources. **Marzena Mazur:** Resources. **Patrycja Nejman-Gryz:** Investigation, Resources. **Serdar Korur:** Investigation, Formal analysis. **Agnieszka Zagozdzon:** Investigation. **Aleksandra Rymaszewska:** Investigation. **Jan H. von der Thüsen:** Investigation, Resources. **Anna M. Siwińska:** Investigation. **Nazan Cemre Güner:** Investigation. **Łukasz Cheda:** Investigation. **Magdalena Paplinska-Goryca:** Investigation, Resources. **Małgorzata Proboszcz:** Investigation, Resources. **Thierry P.P. van den Bosch:** Investigation, Resources. **Katarzyna Górska:** Resources. **Jakub Golab:** Conceptualization. **Rafał M. Kamiński:** Conceptualization, Writing – review & editing. **Rafał Krenke:** Resources, Supervision, Writing – review & editing. **Adam Golebiowski:** Supervision. **Karolina Dzwonek:** Conceptualization, Supervision. **Pawel Dobrzanski:** Conceptualization, Supervision, Writing – original draft.

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