Small-molecule-mediated OGG1 inhibition attenuates pulmonary inflammation and lung fibrosis

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Small-molecule-mediated OGG1 inhibition attenuates pulmonary inflammation and lung fibrosis

One Sentence Summary: TH5487, a novel OGG1 inhibitor, significantly decreases pulmonary fibrosis.

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Abstract: Interstitial lung diseases such as idiopathic pulmonary fibrosis (IPF) are caused by persistent micro-injuries to alveolar epithelial tissues accompanied by aberrant repair processes. Despite substantial advancement in our understanding of IPF progression, numerous questions remain concerning disease pathology. IPF is currently treated with pirfenidone and nintedanib, compounds which slow the rate of disease progression but fail to target underlying pathophysiological mechanisms. The DNA repair enzyme 8-oxoguanine DNA glycosylase-1 (OGG1) is upregulated following TGF- β 1 exposure in several fibrosis-associated cell types. Currently, no pharmaceutical solutions targeting OGG1 have been utilized in the treatment of IPF. In this study, administration of *Ogg1*-targeting siRNA, mitigated bleomycin-induced pulmonary fibrosis in mice, thereby highlighting OGG1 as a tractable target in lung fibrosis. The novel small molecule OGG1 inhibitor, TH5487, decreased myofibroblast transition and associated pro-fibrotic markers in fibroblast cells. In addition, TH5487 decreased pro-inflammatory cytokine production, inflammatory cell infiltration, and lung remodeling in a murine model of bleomycin-induced pulmonary fibrosis. OGG1 and SMAD7 interact to induce fibroblast proliferation and differentiation, with both increased in fibrotic murine and IPF patient lung tissue. Taken together, these data strongly suggest that TH5487 is a potent, specific, and clinically-relevant treatment for IPF.

Main Text:

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a disorder characterized by progressive lung scarring with a median survival time of three years post-diagnosis¹⁻³. The disease is associated with increasing cough and dyspnea, affecting approximately 3 million people worldwide, with incidence strongly correlated with increasing age⁴. IPF is defined on the histological and radiological basis of usual interstitial pneumonia⁵. Fibroblast and myofibroblast overactivation/overstimulation results in extracellular matrix (ECM) deposition in alveolar walls, reducing alveolar spaces⁶. Current IPF therapies focus on inhibiting collagen deposition by blocking myofibroblast activation. These approaches have shown limited success in achieving overall IPF resolution, highlighting the need for novel therapeutic strategies^{4,7}.

Injured IPF tissues produce reactive oxygen species (ROS), resulting in DNA damage and the upregulation of fibrotic-related pathways, ultimately leading to lung architecture collapse^{8,9}. The DNA base guanine is particularly prone to oxidation, forming 7,8-dihydro-8-oxoguanine (8-oxoG). 8-oxoG bases are recognized by the enzyme 8-oxoG DNA glycosylase 1 (OGG1), whereby OGG1 binding triggers DNA base excision processes. Furthermore, the increased expression of OGG1 is an important marker of inflammation and resultant fibrotic processes¹⁰⁻¹².

Following lung injury, fibroblasts transition to myofibroblasts through stimulatory factors such as TGF- β 1, which further induce the production of fibrotic markers including α -smooth muscle actin (α -SMA), collagen, and fibronectin^{13,14}. The fibroblast to myofibroblast transition (FMT) and migratory activities are well established hallmarks of IPF^{15,16}. Interestingly, siRNA-mediated *Ogg1* knockdown in murine embryonic fibroblast cells revealed decreased levels of

tissue-associated α -SMA, a well-defined marker of FMT¹⁷. Significant focus has been placed on OGG1's role during oxidative stress, particularly in various forms of cancer and Alzheimer's pathogenesis^{18–22}. Base excision due to OGG1 results in abasic sites that are cleaved by apurinic/apyrimidinic endonuclease 1 (APE1) generating a nucleotide gap. Polymerase β (POLB) subsequently fills nucleotide gaps, followed by sealing via DNA ligase III in a process mediated by X-ray repair cross-complementing 1 (XRCC1)^{23,24}. This process plays an extensively important role in host oxidative stress and inflammation homeostasis. OGG1's implication in fibrogenesis, combined with its role in inflammation, highlights this enzyme as a potential therapeutic target for IPF treatment^{25,26}.

In this regard, a novel small molecule inhibitor of OGG1-DNA interactions, TH5487, was shown to reduce *in vivo* levels of pro-inflammatory gene expression²⁵. More specifically, TH5487 reduced DNA occupancy of OGG1 at guanine-rich promoter regions, subsequently impeding tumor necrosis factor- α -induced OGG1-DNA interactions, reducing immune cell recruitment²⁵. In our study, a 21-day intratracheally bleomycin-challenged murine model was used to assess TH5487's anti-fibrotic efficacy. This model reproduces several phenotypic features of human IPF, including peripheral alveolar septal thickening and dysregulated cytokine production resolving in fibrosis^{27,28}. The novel small molecule OGG1 inhibitor reduced migratory and proliferative capacities of lung-derived epithelial and fibroblast cells *in vitro*. Intratracheal administration of *Ogg1*-targeting small interfering RNA (siRNA), mitigated distinct bleomycin-induced pulmonary immune cell recruitment and fibrotic lung damage, thereby validating OGG1's potential as a drug target for lung fibrosis. Therapeutic targeting of OGG1 was carried out *in vivo*, with TH5487 decreasing pro-inflammatory cytokine production, inflammatory cell infiltration, and lung remodeling in a murine model of bleomycin-induced pulmonary fibrosis. Comprehensive

proteomic analyses further elucidated the pathways by which TH5487 attenuates pulmonary fibrosis, confirming the involvement of nucleotide excision repair and Mothers against decapentaplegic homolog (SMAD) family of proteins. OGG1 and SMAD7 interact to induce fibroblast proliferation and differentiation^{26,29}, with both being increased in fibrotic murine and IPF patient lung tissue. Taken together, these data strongly suggest that TH5487 is a potent, specific, and clinically-relevant treatment for IPF.

RESULTS

TH5487 reduced migratory capacity of lung-resident cells

The migration of fibroblasts into the lung injury site is a key step in the pathogenesis of pulmonary fibrosis, with fibroblasts contributing the greatest generation of ECM components during disease³⁰. The human-derived HFL-1 and murine-derived MEF fibroblast cell lines were used for this analysis, alongside the human alveolar epithelium-derived cell line A549 and the human bronchial epithelium-derived cell line BEAS-2B. Cells were grown to form a confluent monolayer, scratched to form a wound, then stimulated with 10 ng/mL TGF- β 1 to induce cell migration and wound closure. The cells were additionally treated with TH5487 (10 μ M), vehicle, or dexamethasone (10 μ M) as a comparator drug. This assay showed the wound area percentage in all cell types was significantly reduced following TGF- β 1 addition, indicating the expected promigratory effects of TGF- β 1 stimulation (Fig. 1a). TGF- β 1-induced wound closure was inhibited by TH5487 treatment across all tested cell lines (Fig. 1a). Interestingly, dexamethasone only inhibited cell migration in BEAS-2B and HFL-1 cells and showed minimal effects in A549 and mouse lung fibroblast cells as shown by ANOVA testing with Dunnetts post-hoc analysis ($P < 0.05$). These results indicate that TH5487 is capable of inhibiting TGF- β 1-mediated migratory effects of

lung-derived cells, including both human- and mouse-derived fibroblasts, potentially limiting the severity of IPF disease.

TH5487 reduced HFL-1 myofibroblast transition, whilst targeting OGG1 production

TGF- β 1 driven fibrosis results in the transition of fibroblasts to myofibroblasts resulting in the production of pro-fibrotic proteins. TH5487 treatment additionally reduced the production of collagen, fibronectin, and α -smooth muscle actin, as measured by immunofluorescence and qPCR (Fig. 1b and e). Compelling evidence of TH5487 involvement in OGG1 reduction is presented in Figure 1c, with significant decrease in OGG1 fluorescence in HFL-1 cells compared to TGF- β 1. To investigate myofibroblast transition, TGF- β 1-stimulated HFL-1 cells were analyzed for TH5487-mediated inhibition to myofibroblasts in a transwell assay (Fig. 1d). Interestingly, TH5487 significantly reduced myofibroblast transition (**** P <0.001) compared to TGF- β 1-stimulated HFL-1 cells as shown by a reduction in myofibroblasts in the bottom well.

***Ogg1* RNA interference protects against continued fibrotic lung damage**

Based on the effects of OGG1 inhibition *in vitro*, we tested whether nonselective administration of small interfering RNA (siRNA) targeting OGG1 was effective in mitigating *in vivo* experimental pulmonary fibrotic damage. Following bleomycin administration and subsequent fibrotic lung damage as denoted by mouse weight loss at day 14, *Ogg1*-targeting siRNA were intratracheally administered (Fig. 2a). Non-targeting siRNAs were included as negative controls for this study. To assess potential pharmacological interventions using small *Ogg1*-targeting molecules, TH5487 (40 mg/kg; intraperitoneal (i.p.) administered once daily) was

also included. *Ogg1*-targeting in this context, using either siRNA or TH5487, appeared to hinder the progression of weight loss (Fig. 2b) and subsequent lung damage, with doses of siRNA and TH5487 well-tolerated within this model (Fig. 2c). Flow cytometric analyses further elucidated significantly decreased neutrophil, alveolar macrophage, and inflammatory macrophage populations in bronchoalveolar fluid (BALF) of siRNA- and TH5487-treated mice as compared to the non-targeting siRNA/bleomycin control (Fig. 2d). Immune cell recruitment was reflected by cytokine analysis, with reductions in IL-4, IL-6, G-CSF, MCP-1, and TNF- α across BALF and lung tissue samples, indicative of reduced immune cell recruitment (Fig. 2e and Supp. Fig. 1-3).

Decreased OGG1 protein levels were reported following the use of either *Ogg1*-targeting siRNA or TH5487 (i.p.) compared to bleomycin/non-targeting siRNA controls (Fig. 2f). Interestingly, histological analyses of *Ogg1*-targeting siRNA and TH5487-treated murine lungs revealed reduced levels of collagen deposition and decreased structural deformation of the alveoli (Supp. Fig. 4). Given the initial success of this approach, we aimed to further elucidate the mechanisms by which TH5487 ameliorated pulmonary fibrosis.

Intraperitoneal TH5487 maintained murine body weight following bleomycin administration

We next investigated TH5487-mediated OGG1 inhibition *in vivo* using a murine model of pulmonary fibrosis. Mice received a once-off intratracheal administration of bleomycin (2.5 U/kg) and were dosed (i.p.) with TH5487 or dexamethasone 1h post-bleomycin administration, followed by additional dosing once per day, in five-day intervals, over a total period of 21 days (Fig. 3a). Body weights were recorded as a proxy for overall health status (Fig. 3b). Mice in the bleomycin/vehicle group lost 29.5 ± 4.7 % total bodyweight, whilst mice in remaining groups

either maintained their bodyweight or gained significant weight compared to the bleomycin/vehicle group over the experiment timeline. Representative lungs are shown in Fig. 3c alongside with total lung weight from each animal, where murine differences can be seen. Bleomycin-administered mice showed visual lung damage and significantly increased total lung weights compared to all other groups, but particularly in mice that received TH5487.

TH5487 treatment reduced cytokine levels and lung damage *in vivo*

The effect of TH5487 treatment on inflammatory cytokine levels was investigated in samples from the *in vivo* study using a 23-cytokine multiplex assay (Supp. Fig. 5-7). Cytokines were assessed in lung tissue homogenate, BALF, and plasma (Fig. 4 a-c). TH5487 treatment significantly reduced the levels of several bleomycin-induced inflammatory cytokines, including those in the BALF such as IL-9, eotaxin, MIP-1 α (* P <0.05), MIP-1 β and IL-5 (** P <0.01), and KC (*** P <0.005), whilst cytokines from the plasma displaying a reduction include IL-5 and MIP-1 β (* P <0.05), IL-17 and RANTES (** P <0.01), and eotaxin (**** P <0.001). Further, similar reductions in the lung homogenate were seen following TH5487 administration in particular with regards to levels of MIP-1 α , KC, and IL-9 (* P <0.05) and IL-2 (** P <0.01). Additionally, TGF- β 1 levels were measured in the BALF, plasma, and lung homogenate of murine samples (Fig. 4d). TH5487-treated samples displayed significantly less TGF- β 1 than in vehicle/bleomycin samples as compared by one-way ANOVA (* P <0.05; ** P <0.01; *** P <0.005). Plasma leakage into the BALF as a proxy for lung damage was assessed by BCA assay (Fig. 4e). TH5487-treated mice displayed significantly lower albumin levels compared to vehicle/bleomycin mice as assessed by one-way ANOVA (** P <0.01; *** P <0.005). Further, LDH was assessed as a marker for tissue damage in the lungs, with TH5487 and dexamethasone significantly reducing these levels. In

addition, hydroxyproline content (Fig. 4f) was assessed in murine lung tissue and found to be significantly reduced following TH5487 treatment but not following dexamethasone treatment, indicative of the distinct anti-fibrotic nature of TH5487.

TH5487 treatment reduced immune cell infiltration into the airways *in vivo*

We next investigated the effects of TH5487 treatment on immune cell infiltration into the airways by performing flow cytometry on BALF obtained from the *in vivo* bleomycin studies. Decreases in neutrophil count and inflammatory macrophages were seen following TH5487 administration compared to vehicle/bleomycin samples (Fig. 5a and b). Giemsa-Wright-stained BALF samples showed bleomycin-treated macrophages were enlarged, displaying an inflammatory phenotype not seen in mice treated with TH5487 (Fig. 5c). The inflammatory phenotype of these macrophages was confirmed using CD206/F4/80 immunofluorescence, with TH5487 treatment significantly reducing CD206 staining in cells obtained from both murine BALF (Fig. 5d) and lung tissue (Supp. Fig. 8), with decreased neutrophil-specific staining in murine BALF following TH-treatment (Fig. 5e).

Mass spectrometry reveals fibrotic-related protein decreases following TH5487 treatment

To further elucidate the mechanisms by which TH5487 decreases bleomycin induced fibrosis, differential proteomic analyses were conducted, comparing proteomic profiles in both, lung tissue and BALF fluids between treatment groups. Samples from different animals were considered biological replicates of the respective treatment condition. Protein isolates per animal

and compartment were analyzed via an optimized tissue label-free proteomic workflow based on nanoflow reversed phase chromatography and deep data-independent acquisition mass spectrometry. To support unbiased protein identification, a spectral-library free approach based on deep learning predicted peptide fragmentation and chromatographic retention time was employed³¹. Proteomic patterns were compared in all 5 treatment conditions in both BALF and lung tissue, comparing treatment schemes bleomycin combined with TH (BTH), dexamethasone (DEX), TH alone (TH) and vehicle control (V) relative to bleomycin alone (B) as control (Fig. 6a). A total of 9,872 protein groups were identified across lung and BALF samples, with 5,629 protein groups observed exclusively from lung (57 %), 495 protein groups identified exclusively in BALF and 3,748 protein groups observed across both compartments (Fig. 6b). To our knowledge, this represents the deepest single-shot proteomic map of murine lung and BALF proteomes reported to date and forms a comprehensive basis for the evaluation of proteomic alterations incurred by the investigated treatment schemes. Figure 6c provides an overview of protein intensities observed across the lung and BALF compartments across the cohort. Emphasis was placed on elucidating pathways associated with murine lung environment. Significant proteome alterations were determined from normalized precursor-level abundances via t-statistics, summary to protein level and multiple hypothesis testing correction (Significance criteria; fold-change ≥ 2 , Benjamini-Hochberg-corrected protein-level P -value ≤ 0.05 (Fig. 6d, Supp. Fig. 9-12 & Methods for details). Pathway analysis of the regulated protein sets using StringDB and PantherDB revealed a number of enriched pathways. Particular focus was placed on processes related to fibrotic changes as compared to the bleomycin control.

Interestingly, lung proteins related to the gene-ontology (GO) terms collagen biosynthesis process (GO:0032964), wound healing involved in inflammatory response (GO:0002246),

endothelial cell proliferation (GO:0001935), collagen metabolic process (GO:0032963), response to fibroblast growth factor (GO:0071774), regulation of cytokine production (GO:0001818), wound healing (GO:0042060), response to wounding (GO:0009611) were decreased following TH5487/bleomycin treatment, as compared to the bleomycin alone treatment condition (Fig. 6d).

Furthermore, proteins significantly downregulated following TH5487 treatment included tenascin-C (TNC; p value: 0.0374), carbamoyl phosphate synthetase 1 (CPS1; 0.03978), matrix metalloproteinase 19 (MMP19; 0.0402), tissue inhibitor of metalloproteinase 1 (TIMP1; 0.0460), Janus kinase 3 (JAK3; 0.0301), cAMP-dependent protein kinase catalytic subunit (PRKX; 0.0152), lysyl oxidase homolog 2 (LOXL2; 0.0208).

Other proteins involved with fibrotic pathways which experienced Log two-fold decreases following TH5487 treatment as compared to bleomycin control samples included hyaluronidase 1 (1.691), secreted frizzled-related protein 1 (SFRP1; 3.142), cathepsin S (CTSS; 1.830), A disintegrin and metalloproteinase with thrombospondin motifs 15 (ADAMTS15; 3.135), C-type lectin domain family 10 member A (CLEC10A; 2.132), heme oxygenase 2 (HMOX2; 2.991), collagen 1A1 (COL1A1; 2.241), arginase 1 (ARG1; 2.266), and other proteins (Fig. 6f).

Additionally, to explain the mechanism by which TH5487 reduces fibrotic damage, the SMAD family of proteins were compared to matching bleomycin control proteins (Fig. 6f). SMAD 1 and 5 were increased in response to TH5487 treatment (0.3082 and 0.9730, respectively) with SMAD 2/3 and 4 decreased following treatment (0.2830 and 0.4000, respectively). The mechanism by which TH5487 initiates base excision repair inhibition was further elucidated by analyzing proteins known to contribute to DNA base excision repair (Fig. 6f). This is shown by Log two-fold increases of greater than 1.5 in samples from mice treated with bleomycin alone versus mice treated with bleomycin/TH5487-treated for cyclin-H (CCNH), CDK-activating kinase

assembly factor MAT1 (MNAT1), DNA repair protein complementing XP-C cells (XPC), X-ray repair cross-complementing protein 1 (XRCC1), DNA polymerase delta subunit 2/3 (POLD 2/3), DNA ligase 1 (Lig1), and G/T mismatch-specific thymine DNA glycosylase (TDG).

To further elucidate the differences seen in BALF and lung tissues, cluster analysis was conducted using the gap statistics method, identifying 8 discrete clusters (Supp. Fig. 13). Using this analysis, proteins which were associated to the assigned clusters were assessed using the StringDB and PantherDB. These analyses revealed enriched reactome pathways in the lung samples associated with cluster 1 (IL-6 signaling), 3 (DNA-repair), 4 (degradation of extracellular matrix), and 5 (TCA cycle and respiratory and electron transport), indicative of a significant role for the lung proteome in this model.

TH5487 treatment decreased bleomycin-induced lung damage *in vivo*

Next, TH5487's impact on bleomycin-induced lung damage was investigated using histological analysis of whole lung sections obtained from *in vivo* studies (Supp. Fig. 14 and 15). Bleomycin-treated control mice displayed significant lung damage when compared to saline-treated controls (Fig. 7a and b). Bleomycin-treated mice that received bleomycin/TH5487 treatment showed reduced lung damage, with lesser degrees of alveolar structural collapse, less septal thickening of the alveoli, and less immune cell influx as indicated by the H&E stain (Fig. 7a). Picrosirius red staining revealed a reduced level of collagen deposition in bleomycin/TH5487-treated versus control-treated mice (Fig. 7b). Positive pixel analysis was used to quantify lung damage and showed significantly less H&E and picrosirius red staining in the TH5487-administered lungs, with no significant decreases in either staining reported for dexamethasone-treated lungs (Fig. 7a and b). Further, scanning electron microscopy analysis (SEM) revealed

collagen deposition surrounding the alveolar walls of bleomycin control mice was reduced in response to TH5487 treatment (Fig. 7c). Immunofluorescent staining of murine lung sections revealed decreased myeloperoxidase (MPO), fibronectin, COL1A1, and OGG1 staining compared to the vehicle bleomycin control group (Fig. 7d). To further support the involvement of OGG1 in fibrotic related lung damage, co-staining was carried out on murine lung sections, revealing increased COL1A1/OGG1 fluorescence in similar lung areas (Fig. 7e).

OGG1 and SMAD7 involvement in IPF patients

Given SMAD protein concentration measured in the bleomycin lung samples via proteomic mass spectrometry, confirmatory OGG1/SMAD7 immunofluorescence was measured in murine lung samples. Lungs treated with bleomycin appeared to co-stain in similar areas with both OGG1 and SMAD7, indicating potential interaction, with significantly reduced signal in the TH5487-treated samples (Fig. 8a). Confirmatory SDS-PAGE displayed lower levels of OGG1 and SMAD7 following TH5487 treatment as compared to untreated samples (Fig. 8b).

To further assess the translational viability of TH5487 for IPF treatment IPF and healthy lung controls were assessed for immunoreactivity measuring OGG1 and SMAD7. IPF patient lung immunoreactivity was significantly higher than healthy lung controls for both antibodies, indicating the potential role of OGG1 and SMAD7 in IPF disease (Fig. 8c and d; Supp. Fig. 16 and 17).

DISCUSSION

IPF is an interstitial lung disease characterized by dysregulated inflammation, progressive lung scarring, and eventual death due to respiratory complications^{1,32}. Ensuing TGF- β production following lung injury results in the upregulation of tissue repair genes, including the DNA base excision repair gene *OGG1*^{14,33,34}. Reactive oxygen species (ROS) generate localized increases of 8-oxoG, resulting in OGG1 recruitment, proinflammatory gene expression and recruitment of transcriptional effectors^{35–37}. Redox homeostasis imbalances have been implicated in several diseases including IPF, with reported deficiencies in glutathione and superoxide dismutase in the lower respiratory tracts of patients with IPF^{38–40}. However, therapeutic interventions targeting oxidative DNA repair have received relatively little attention for fibrosis treatment despite the implication of mitochondrial and NADPH oxidase-derived ROS in IPF progression⁴¹.

Current therapies, nintedanib and pirfenidone, significantly reduce lung function decline^{42,43}. However, no therapeutic solution exists which halts disease progression, necessitating the development of novel therapeutic interventions⁴⁴. The approach reported in this study utilized a potent and selective small molecule, TH5487, employing a novel and distinct mechanism of action, preventing OGG1 from binding damaged DNA, initiating transcription factors, and upregulating pro-inflammatory and pro-fibrotic pathways which directly inhibit pulmonary fibrosis²⁵. Herein, we report several arguments supporting OGG1 as an appealing target for IPF treatment.

Reported data indicate that OGG1 binds 8-oxoG at regulatory gene domains, mediating transcriptional activation of inflammatory responses^{11,45,54,46–53}. OGG1 is increased in lung epithelial cells and fibroblasts following TGF- β 1 addition²⁶, with IPF-derived fibroblasts generating higher levels of ROS⁵⁵. Furthermore, abnormal wound healing of the alveolar epithelium in response to micro-injuries plays a crucial role in IPF progression^{56–58}. Our study

found OGG1 inhibition by TH5487 significantly reduced migration and wound healing compared to TGF- β 1 controls in all tested cell types, suggesting OGG1 inhibition suppresses the initiation of wound healing. Whilst recent efforts have focused on removal of senescent or damaged fibroblasts^{59,60}, our approach advantageously suppresses pro-fibrotic mediators and mitigating aberrant myofibroblast and epithelial cell involvement.

Importantly, uncontrolled lung injury is a hallmark of IPF initiation and progression, resulting in pro-inflammatory and pro-fibrotic cytokine release driving further fibrosis-related immune cell influx and ECM remodeling^{16,61,62}. Our data demonstrate targeting OGG1 suppresses TGF- β 1 and several key immune modulatory cytokines and chemokines in murine BALF, lung, and plasma. Treatment with both TH5487 or OGG1 siRNA resulted in decreased profibrotic cytokine expression and diminished immune cell recruitment to the lung, both resulting in reduced lung collagen formation. Previous studies have described macrophage involvement in IPF^{63–65}, with particular emphasis on M2 macrophages^{66,67} due to their involvement in TGF- β production⁶⁸. Our study describes specific reduction of the M2 macrophage population following OGG1-targeting, supporting another beneficial aspect to this approach⁶⁹.

To elucidate the mechanism by which these effects were mediated, we examined murine and human lung samples. OGG1 has displayed a crucial role in the TGF- β /SMAD axis, modulating EMT/FMT transition through the phosphorylation of SMAD 2/3 by SMAD 7²⁶. This is supported in this study by proteomic analysis, immunostaining, and immunofluorescence which show that decreases in both SMAD 7 and OGG1, as well as several base excision repair-associated proteins, result in amelioration of fibrotic damage in both human and murine samples. Additionally, proteins with known roles in fibrosis development including ARG1, TIMP1, COL1A1, and others⁷⁰ were significantly decreased following TH5487 treatment, confirming OGG1 as a tractable target for

pharmaceutical intervention. In addition, 8-oxoG, OGG1, and base excision repair proteins may serve as biomarkers in patients with IPF, offering an alternative to clinical diagnoses.

Important limitations addressed in this study include, whether treatment using TH5487 can display utility in human IPF pathologies. Whilst the translational aspect of the study is suggested in our study using human lung sections, it is important to demonstrate that TH5487 treatment successfully reduces OGG1 levels and subsequent IPF lung damage in human clinical trials. Additional limitations in this study include the lack of monitoring of potential off-target effects induced by targeting OGG1. Whilst no obvious reductions in key murine health status measures were observed in this or other studies²⁵, any small-molecule utilization should be accompanied by long term monitoring of adverse effects to ensure safe therapeutic usage. Furthermore, Ogg1^{-/-} mice display no deleterious pathological changes⁷¹, suggesting specific OGG1 targeting may be safe. In any case, further efforts are required before progressing this compound to clinical trials.

Together, our findings demonstrate that TH5487 possesses a mechanism of action targeting OGG1 to suppress IPF, which is distinct from currently employed therapeutic interventions. This study further elucidates the downstream effects of this approach, decreasing myofibroblast transition, epithelial and fibroblast migration, inflammatory cell recruitment, and eventual inhibition of fibrotic-related lung remodelling. These data show pre-clinical proof-of-concept of TH5487 and motivate for its use in clinical trials for IPF.

MATERIALS AND METHODS

Study design

The goal of this study was to test a novel pharmaceutical approach to inhibit OGG1, ultimately leading to the inhibition of fibrosis-related progression. Initial *in vitro* experiments in bronchial epithelial and fibroblast cells displayed decreased fibrosis-related phenotypic features. Subsequent *in vivo* murine studies using intratracheally-administered bleomycin were chosen as well-established and relevant models of experimental lung fibrosis. Sample sizes were calculated by power analysis based on previous experience, feasibility, and to conform to the ARRIVE guidelines. For lung fibrosis experiments testing the viability of *Ogg1* as a drug target in siRNA experiments, $n \geq 4$ to 7 mice per group were used to achieve statistical significance, whilst for TH5487 experiments, $n \geq 8$ to 13 mice per group were used. Mice were randomly assigned to treatment groups, with sample sizes decided before commencing experiments. Downstream analyses were conducted with the investigator blinded to the treatment groups, and no animals were excluded as outliers from the reported dataset. All *in vitro* and *in vivo* experiments were performed in two to four technical replicates. Human resected lung sections were obtained with informed consent, with a statistically significant $n=4$ samples used for disease and healthy control samples.

Cell culture

Murine C57BL/6 embryonic lung fibroblasts (MEF's; ATCC, Manassa, VA) and HFL-1 (human lung fibroblast) cells were cultured in complete growth medium supplemented with L-glutamine, 100 U/mL penicillin, 100 μ g/mL streptomycin, and 10% FBS. A549 cells (derived from human alveolar epithelium) were cultured in RPMI 1640 with the addition of 10% FCS, and 100U/mL penicillin, 100 μ g/mL streptomycin. Finally, BEAS-2B cells (derived from human bronchial epithelium) were cultured in RPMI-glutamax and 10% FBS, with the addition of 100 U/mL penicillin and 100 μ g/mL streptomycin.

Wound healing assay

Wound healing assays were conducted using A549 cells, murine lung fibroblast (MEF), BEAS-2B, and HFL-1 cells grown to confluence in 24-well plates. Wounds were made in the confluent cell layer using sterile 200 uL pipette tips, followed by washing with PBS and incubation with complete culture medium with or without TGF- β 1 (10 ng/mL) at 37 °C in a 5 % CO₂ incubator for 48 h post-scratching. Cell images were monitored every 24 h using an Olympus CKX41 microscope with Olympus SC30 camera and cellSens Entry software (Olympus, Tokyo, Japan). Images were analyzed using the wound healing tool in ImageJ (<https://imagej.nih.gov/ij/>). Data are presented as percentages of the initial wound area.

Immunostaining of HFL-1 cells

HFL-1 cells were seeded (1×10^4 cells/mL) into 24 well plates containing rounded glass cover slips. TGF- β 1 (10 ng/mL) was added to each well, followed by the addition of medium containing TH5487, dexamethasone, medium only, or vehicle only. Following 96 h of treatment, cells were washed and fixed with ice cold methanol, containing 0.5% Triton-X100. Slides were blocked using Dako Protein Block (Agilent, CA, USA) for 1 h at room temperature and then stained with primary antibodies overnight, rabbit anti-COL I, rabbit anti- α SMA, rabbit anti-fibronectin (Abcam, Cambridge, UK), and rabbit anti-OGG1 (Invitrogen, Carlsbad, CA) antibodies were used. AlexaFluor 488-conjugated goat anti-rabbit antibody (Invitrogen) was used as secondary antibody. Glass cover slips were removed and mounted onto glass slides, with nuclei counterstained using DAPI-containing fluoroshield (Abcam). Images were visualized using a Nikon Confocal Microscope. Fluorescence was quantified using ImageJ software.

Fibroblast transwell experiment

Fibroblast chemotaxis was measured using 24-well Nunc (8 μ m pore size) transwell inserts (ThermoFisher, MA, USA). HFL-1 cells were seeded (5×10^5 cells/ml) into the upper chamber in FBS-free medium, whilst the lower chamber contained complete medium with additional 10 % FBS as a chemoattractant. Medium containing TGF- β 1 (10 ng/mL) was added to each well and allowed to equilibrate for 24 h. Cells were washed with PBS, followed by the addition of medium containing TH5487, dexamethasone, medium only, or vehicle only (DMSO 2% and PBS pH 7.4), with the replacement of each every 24 h. Following 96 h, medium was removed and cells in the lower chamber were stained (crystal violet) and imaged using a Nikon microscope (Nikon, Tokyo, Japan) with a x10 objective.

mRNA analysis

HFL-1 cells were starved in culture medium with 0.5% BSA for 24 h. Cells were pretreated with TH5487, 5 μ M, 10 μ M or DMSO for one hour followed by TGF- β stimulation (10 ng/ml; Peprotech) for 48 hours. mRNA was extracted based on the RNA extraction kit protocol Direct-zol RNA MiniPrep (Zymo Research, Irvine, CA).

cDNA synthesis was performed using Quantitect Reverse Transcription kit (Qiagen) and quantified with the iTaqTM Universal SYBR[®] Green Supermix PCR Kit in Rotor Gene Q (Qiagen). Using the following pcr primers, Human Col1A1F, 5'GATTCCCTGGACCTAAAGGTGC3', Human Col1A1R, 5'AGCCTCTCCATCTTTGCCAGCA3', Human α -SMAF, 5'CTATGCCTCTGGACGCACAACT3', Human α -SMAR, 5'CAGATCCAGACGCATGATGGCA3', 18SrRNAF: 5'AGTCCCTGCCCTTTGTACACA 3', 18SrRNAR: 5'GATCCGAGGGCCTCACTAAAC 3'

The obtained Ct values of the samples were normalized to the Ct values of 18SRNA reference gene, $2^{-\Delta\Delta Ct}$ values were calculated using Microsoft Excel.

Ethical approval

All animal experiments were approved by the Malmö-Lund Animal Care Ethics Committee (M17009-18). Lung tissue was obtained after written informed consent, approval by the Regional Ethical Review Board in Lund (approval no. LU412-03), and performed in accordance with the Declaration of Helsinki as well as relevant guidelines and regulations.

Animal studies

10-12-week-old male C57Bl/6 mice (Janvier, Le Genest-Saint-Isle, France) were housed at least 2 weeks in the animal facility at the Biomedical Service Division at Lund University before initiating experiments and were provided with food and water *ad libitum* throughout the study. Mice were randomly allocated into five groups (n=8-14 animals per group): intratracheally (i.t.)-administered bleomycin (2.5 U/kg) + vehicle intraperitoneal (i.p.), bleomycin (i.t.) + TH5487 (40 mg/kg; i.p.), bleomycin (i.t.) + dexamethasone (10 mg/kg; i.p.), saline (i.t.) + vehicle (i.p.), and saline (i.t.) + TH5487 (40 mg/kg; i.p.).

For the siRNA experiments, lung fibrosis was induced by intratracheal introduction of bleomycin (2.5 U/kg) or saline as control. Following 14 days, siRNA targeting mouse *Ogg1* (L-048121-01-0050) or nontargeting control (D-001810-01-50) mRNA (Horizon Discovery, UK) was administered intratracheally at either 25 µg per mouse (*Ogg1* siRNA) or 50 µg of nontargeting siRNA. In addition, a TH5487 administration group involving daily i.p. administration (40 mg/kg) was included. On day 21, mice were euthanized followed by collection of blood, lung tissue, and BALF.

Blood collection

Blood was collected in 0.5 M EDTA tubes by cardiac puncture and centrifuged at 1,000 x g for 10 min. Plasma supernatant was used for the analysis of inflammatory mediators using a multiplex assay (Bio-plex assay; Bio-Rad, Hercules, CA).

Collection of lung tissue

Right lungs were collected in Eppendorf tubes on dry ice and stored at -80 °C. The snap-frozen lungs were thawed and homogenized in tissue protein extraction reagent (T-PER) solution (ThermoFisher) containing protease inhibitor (Pefabloc SC; Sigma-Aldrich) at a final concentration of 1 mM. Lung homogenates were centrifuged at 9,000 x g for 10 min at 4°C, and the supernatants were collected for multiplex analysis. Left lungs were collected in Histofix (Histolab, Göteborg, Sweden) and submerged in 4 % buffered paraformaldehyde solution.

Bronchoalveolar lavage fluid (BALF) collection

BAL was performed with a total volume of 1 ml PBS containing 100 µM EDTA. BALF was collected in Eppendorf tubes on ice, with aliquots made for flow cytometry, cytospin differential counts, and an aliquot transferred to -80°C for multiplex cytokine analysis. Cytospin preparations of cells were stained with modified Wright-Giemsa stain (Sigma-Aldrich, St. Louis, MO).

Bioplex cytokine analysis

For the detection of multiple cytokines in BALF, plasma, and lung homogenate, the Bio-Plex Pro mouse cytokine assay (23-Plex Group I; Bio-Rad) was used on a Luminex-xMAP/Bio-Plex 200 System with Bio-Plex Manager 6.2 software (Bio-Rad, Richmond, CA). A cytometric magnetic bead-based assay was used to measure cytokine levels, according to the manufacturer's instructions. The detection limits were as follows: Eotaxin (4524.58-1.23 pg/mL), GCSF (99318.6-

7.3 pg/mL), GMCSF (6310.48-3.91 pg/mL), IFN- γ (16114.01-0.87 pg/mL), IL-1 α (10055.54-0.54 pg/mL), IL-1 β (31512.04-1.75 pg/mL), IL-2 (19175.48-1.24 pg/mL), IL-3 (7514.5-0.44 pg/mL), IL-4 (5923.58-0.34 pg/mL), IL-5 (12619.59-0.78 pg/mL), IL-6 (9409.63-0.68 pg/mL), IL-9 (64684.09-2.41 pg/mL), IL-10 (77390.75-4.18 pg/mL), IL-12p40 (144560.15-18.62 pg/mL), IL-12p70 (78647.56-4.81 pg/mL), IL-13 (197828.67-11.16 pg/mL), IL-17 (8727.85-0.51 pg/mL), KC (23001.9-1.4 pg/mL), MCP-1 (393545.52-10.01 pg/mL), MIP-1 α (14566.62-0.63 pg/mL), MIP-1 β (7023.87-0.34 pg/mL), RANTES (19490.48-4.61 pg/mL), and TNF- α (74368.54-51.69 pg/mL). Cytokine measurements for lung homogenate samples were corrected for total protein concentration using a Pierce™ BCA Protein Assay Kit (ThermoFisher, Maltham, MA).

TGF- β 1 ELISA

The Quantikine ELISA kit targeting TGF- β 1 (R&D systems, UK) was used to assess TGF- β 1 levels in the BALF, plasma, and lung homogenate of murine samples according to the manufacturer's instructions. Optical density was measured at 450 nm using a VICTOR 1420 Multilabel plate reader (PerkinElmer, Waltham, MA).

Lactate Dehydrogenase H assay

LDH levels were determined in homogenized murine lung tissues using an LDH ELISA kit for murine samples according to the manufacturer's instructions (Cloud-Clone Corporation, Katy, TX). Optical density was measured at 450 nm using a VICTOR 1420 Multilabel plate reader (PerkinElmer).

Hydroxyproline assay

Hydroxyproline levels in murine lung tissues were determined using the QuickZyme Hydroxyproline Assay kit (Quickzyme Biosciences, Leiden, the Netherlands). Lung tissues were

homogenized as described above. Homogenates were diluted (1:1 vol:vol) with 12N HCl and hydrolyzed at 95 °C for 20 h. After centrifugation at 13,000 × g for 10 min, 200 µl from the supernatant was taken and diluted 1:2 with 4 N HCl. Hydroxyproline standard (6.25–300 µM) was prepared in 4 N HCl and transferred to the microtiter plate. Following addition of a chloramine T-containing assay buffer, samples were oxidized for 20 min at RT. Detection reagent containing p-dimethylaminobenzaldehyde was added and after incubation at 60 °C for 1 h, absorbance was read at 570 nm with a VICTOR 1420 Multilabel plate reader (Perkin Elmer, MA, USA). The hydroxyproline content in lung tissue is given as hydroxyproline (µg) per mg lung tissue, corrected using a Pierce™ BCA Protein Assay Kit (ThermoFisher).

Flow cytometry

Flow cytometry was carried out using a BD Accuri C6 Plus (BD Biosciences, Franklin Lakes, NJ). The washed cells were incubated with Fixable Viability Stain 510 (FVS510; BD #564406) to differentiate live and dead cells. Cells were washed with Stain buffer 1x (BD #554656) and incubated with Lyse Fix 1x (BD #558049 (5x)). Fixed cells were washed with stain buffer and aliquoted into two samples incubated with either anti-CD11b (BD553312), anti-CD11c (BD558079), anti-Ly6G (BD551461), or anti-CD11c, anti-MHCII (BD558593), anti-SiglecF (BD562680) antibodies.

H&E and picrosirius red staining of lung tissue

Mouse left lungs were fixed in Histofix (Histolab Products AB, Askim, Sweden), paraffin-embedded and sectioned at 3 µm. The tissue sections were placed on slides (Superfrost Plus; Fisher Scientific) and deparaffinized in serial baths of xylene and ethanol followed by staining using Mayer hematoxylin and 0.2% eosin (Histolab Products AB, Askim, Sweden) or picrosirius red staining kit (Abcam). The stained slides were imaged using an Aperio CS2 image capture device.

SEM analysis of lung histology

Lung tissue sections were fixed as reported above. After fixation, samples were washed and dehydrated in alcohol at increasing concentrations, critical point dried, mounted on aluminium holders, and covered with 20 nm of gold. Samples were examined in a Philips XL30 FEG scanning electron microscope (Eindhoven, The Netherlands) operated at an acceleration voltage of 5 kV.

Immunostaining of murine lung sections

Lung tissue sections were fixed as reported above. Lung samples underwent antigen retrieval (pH9 buffer) using a Dako PT Link pre-treatment module (Agilent, CA, USA). Samples were washed and blocked for 10 min (Dako protein block; Agilent, Santa Clara, CA) before being treated with primary antibodies overnight. Mouse anti-COL1A1, rabbit anti-fibronectin, mouse anti-ly6G, rabbit anti-MPO, rabbit anti-mannose receptor, rat anti-F4/80, and mouse anti-SMAD7 (Abcam, CAM, UK), and rabbit anti-OGG1 (Invitrogen, Carlsbad, CA) antibodies were used. Alexa Fluor 488-conjugated goat/anti-mouse and Alexa Fluor 647 goat/anti-rabbit (Invitrogen, CA, USA) were used as secondary antibodies. Glass cover slips were placed onto slides and mounted with DAPI-containing fluoroshield (Abcam). Images were visualized using a Nikon Confocal Microscope and fluorescence was quantified using ImageJ software.

Immunohistochemistry of human lung samples

Macroscopically normal, tumor-free lung tissue samples were obtained during transplantation or resection from patients undergoing cancer surgery. Immediately after collection, samples were placed in 4% buffered formaldehyde. Following dehydration and embedding in paraffin, sections (3 μ m) were produced. A single staining protocol (EnVision™ Detection system, K5007, Dako, Glostrup, Denmark) was used for visualization of OGG1 and SMAD7. Briefly, after

antigen retrieval, OGG1 and SMAD7 were detected using rabbit anti-Ogg1 (Abcam) and rabbit-SMAD7 (Sigma-Aldrich) antibodies (1:1000) and visualized using secondary goat anti-rabbit antibodies conjugated with peroxidase polymers (Dako). IHC protocols were performed using an automated IHC robot (Autostainer Plus, Dako). Sections were counter-stained with Mayer's hematoxylin for visualization of background tissue, dehydrated in alcohol/xylene, and mounted on Pertex (Histolab, Göteborg, Sweden). The stained slides were imaged using an Aperio CS2 image capture device. Positive staining was quantified manually (number of cells/area of tissue) or as positivity (positive brown pixels divided by all stained pixels) using computerized image analysis on blinded sections using ImageScope (Aperio).

Sample preparation for LCMS

Tissue homogenization

Freshly harvested lungs in PBS were homogenized in MagNA Lyser (Roche) with silica beads (VWR) using 2 x 2minute cycles. Protein estimation in the homogenates was performed using BCA kit (Thermo Fisher Scientific). 50 micrograms of protein from organs and 1 microliter of plasma were utilized for digestion.

Protein denaturation, tryptic digest & peptide solid-phase-extraction

Proteins were resuspended in 8M urea in 100 mM ammonium bicarbonate, reduced with 5mM TCEP, pH 7.0 for 45 min at 37 °C, and alkylated with 25 mM iodoacetamide (Sigma, USA) for 30 min followed by dilution with 100 mM ammonium bicarbonate to a final urea concentration below 1.5 M. Proteins were digested by incubation with trypsin (1/100, w/w, Promega) for at least 9h at 37 °C. Digestion was stopped using 5% trifluoroacetic acid (Sigma) to pH 2-3. The peptides were cleaned up by C18 reversed-phase spin columns as per the manufacturer's instructions

(Harvard Apparatus, USA). Samples were resuspended in 30 µl HPLC-water (Fisher Chemical) with 2% acetonitrile, 0.2% formic acid (Sigma). Samples were spiked with iRT peptides (Biognosys AG, Switzerland).

LC- Data-independent acquisition MS analysis

All peptide analyses were performed on a Q Exactive HF-X mass spectrometer (Thermo Fisher Scientific) connected to an EASY-nLC 1200 ultra-high-performance liquid chromatography system (Thermo Fisher Scientific). Peptides were trapped on pre-column (PepMap100 C18 3 µm; 75µm x 2cm, Thermo Fisher Scientific) and separated on an EASY-Spray C-18 reversed phase column with integrated ESI emitter (250 mm, ES902, column temperature 45 °C, Thermo Fisher Scientific). Equilibrations of columns and sample loading were performed per manufacturer's guidelines. Solvent A was used as stationary phase (0.1 % formic acid), and solvent B (mobile phase; 0.1 % formic acid, 80% acetonitrile) was used to run a linear gradient from 5 % to 38 % over 120 min at a flow rate of 350 nl/min. The 44 variable windows data-independent acquisition (DIA) acquisition method is derived from Bruderer *et al.*(1). The mass range for MS1 was 350-1,650 m/z with a resolution of 120,000 and a resolution of 30,000 for MS2 with a stepped normalized collision energy (NCE) of 25.5, 27 and 30. The 44 variably sized MS2 windows were 350-371, 370-387, 386-403, 402-416, 415-427, 426-439, 438-451, 450-462, 461-472, 471-483, 482-494, 493-505, 504-515, 514-525, 524-537, 536-548, 547-557, 556-568, 567-580, 579-591, 590-603, 602-614, 613-626, 625-638, 637-651, 650-664, 663-677, 676-690, 689-704, 703-719, 718-735, 734-753, 752-771, 770-790, 789-811, 810-832, 831-857, 856-884, 883-916, 915-955, 954-997, 996-1057, 1056-1135 and 1134-1650 m/z, resulting in a total cycle time of ~3.3s and 6-8 peaks per chromatographic peak on average.

Peptide and precursor identification and quantification from DIA-MS data

Protein sequences employed for proteomics data interpretation were downloaded from the UniProt database (1 sequence per gene, 17.09.2020). Peptide-centric analysis of DIA data was carried out using an *in silico* predicted spectral library using the DIANN Toolset (v1.7.12) and a two-pass analysis procedure. In the first pass, an empirically corrected, *in silico* predicted spectral library was created from the sample set of interest by querying the full proteome predicted library (with parameters Trypsin/P, up to 1 missed cleavage, excision of N-terminal M and carbamidomethylation of cysteine residues enabled as variable modification). The predicted library and initial search space thereby spanned 22,159 proteins, 33,323 protein groups and 4,141,742 precursors of 1,357,505 peptide species in distinct elution groups. DIANN was instructed to optimize mass accuracy and RT extraction windows separately for each MS run to maximize identification sensitivity and to then write out a new, constrained and empirically corrected spectral library (First-pass FDR was set to 1 %. Protein inference was disabled to maintain protein grouping information and the option to reduce RAM usage was enabled). The produced libraries for lung/BALF/plasma datasets contained 10,121/5,017/941 protein groups, 97,775/33,857/6,025 precursors and 78,267/28,672/4,335 peptides in distinct elution groups, respectively. In the second pass of the analysis, the three corrected libraries were then applied to the datasets of the respective tissue type to derive quantitative information (FDR set to 1 % and with high accuracy quantification strategy selected).

Downstream analysis of DIA proteomics data

DIA quantitative data from DIANN (Pass2.tsv) were imported and further processed in the R environment for statistical computing for visualization and analysis (R version 4.0.2) employing packages including data.table, pheatmap, fviz, ggplot2, ggrepel and limma.

To detect protein expression differences, quantitative data were scaled on precursor level, tested for statistically significant differences and summarized to protein level via a customized script. Specifically, raw peptide intensities ("Precursor.Quantity") were log2-transformed, scaled by quantile normalization per MS run, excluded from analysis if observed in ≤ 4 MS runs, and then pair-wise student's t-tests performed per precursor and pair of conditions/cohorts. Since DIANN 1.7.12 does not provide an alignment or background quantification module, prior to statistical tests, missing values were imputed, sampling from a standard distribution centered on the 0.1%-ile of the observed data points, with a standard deviation of 0.2, parameters chosen upon visual inspection of the observed data. Protein level metrics were derived from the mean of precursor-level metrics (both, log2 fold-change and p-value). Multiple hypothesis testing correction via the Benjamini Hochberg method was applied.

To infer groups of co-regulated proteins, protein-level log2 fold-change profile similarity across the comparisons of interest BALF and lung tissue datasets was calculated (1- Euclidean distance), including only proteins that were regulated in at least one of the comparisons ($|\log_2 \text{fold-change}| \geq 1$ and corrected protein level p-value ≤ 0.05). The number of clusters in the accordingly sub-setted data was determined via the Gap statistic method as implemented in the package fviz.

Functional annotation over- and under- representation testing among protein sets of interest (regulated in a particular comparison, members of a specific co-regulation cluster, etc.) was performed in the PANTHER classification system ((2) (<http://pantherdb.org/>, Release 15.0) using the Statistical over representation test (Fisher's exact test), testing against the background of all proteins covered by the MS analysis.

Statistical analysis

In this study, groups of three or more mice were compared using one-way analysis of variance (ANOVA) with Dunnett's post hoc test. In experiments using two groups, results were compared using unpaired *t* test with Welch's correction. Results in this study are displayed throughout as mean $\pm$ SEM. Statistical testing was carried out using GraphPad Prism 9.1.1 with statistical significance defined as $P < 0.05$.

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Writing – review & editing: LT, ABS, AE, CK, MH

Competing interests:

T.H. is listed as inventor on a provisional U.S. patent application no. 62/636983, covering OGG1 inhibitors. The patent is fully owned by a nonprofit public foundation, the Hellday

Foundation, and T.H. is a member of the foundation board developing OGG1 inhibitors toward the clinic. An inventor reward scheme is under discussion. The remaining authors declare no competing financial interests.

Data and materials availability:

All data are available in the main text or the supplementary materials.

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Figures and figure legends:

Figure 1: *In vitro* wound healing assay (a), immunofluorescence and myofibroblast transition assay using

human lung fibroblast (HFL-1) cells (b-d), and qPCR of HFL-1 cells following TGF-β1 stimulation. (a)

Migration of human alveolar epithelial (A549), mouse lung fibroblast (MEF), human bronchial epithelial (BEAS-2B),

and human lung fibroblast (HFL-1) cells was measured at 0, 24, and 48 h post-wound induction. Cells were treated

with 10 ng/mL of TGF-β1 or vehicle (with an equivalent concentration of DMSO) in control samples. Wound area

percentage was compared to the control samples using a one-way ANOVA followed by a Dunnett's post-hoc test:

* $P < 0.05$; *** $P < 0.0005$ **** $P < 0.0001$. Representative images are shown from 3 independent experiments containing

4 biological replicates (scale bar=100 μm). Immunostaining of myofibroblast cells (b and c) following 96 h of TGF-

β1 treatment, with TH5487 treatment displaying visually reduced levels of collagen (COL1A1), fibronectin, and α-

smooth muscle actin (α-SMA) in (b). Similarly reduced levels of OGG1 immunofluorescence (c) compared to no

treatment/TGF-β1 control were observed (green fluorescence; scale bar=50 μm). (d) Myofibroblast transition of

human lung fibroblast cells was measured over 96 h. HFL-1 cells in the basal wells were stained and counted, with

significantly more myofibroblast cells appearing in the untreated control wells. Drug-treated samples were compared

to untreated control samples using a one-way ANOVA followed by a Dunnett's post-hoc test: **** $P < 0.0001$. Data is

representative of 3 independent experiments containing 4 biological replicates (scale bar=100 μ m). (e) The effects of TH5487 on transcription of α -SMA and Col1A1 in TGF- β 1 stimulated HFL-1 cells were investigated by qPCR. TH5487 (10 μ M), significantly reduced transcription of Col1A1 and α -SMA, with compiled data representative of 3 independent experiments.

Figure 2: *In vivo* bleomycin model highlighting OGG1 as a therapeutic target. (a) Bleomycin (2.5 U/kg) was intratracheally administered to wild-type C57Bl/6J mice, with mice left to develop fibrosis over a period of 14 days. Subsequent *Ogg1*-targetting or non-targetting siRNA administration occurred at Day14, with the inclusion of a TH5487 (40 mg/kg; i.p.; 5 daily doses) treatment group. (b) Murine weights and percentage total weight loss for the bleomycin administered groups displayed similar trends until day 14, thereafter the *Ogg1* siRNA and TH5487-treated groups displayed maintenance of weight until the end of the study. (c) Representative images of murine lungs and murine total lung weight. Significant differences were seen between TH5487 and *Ogg1* siRNA- treated groups compared to the non-targetting group (**** P <0.0001). (d) Flow cytometry conducted on murine BALF indicates a significant reduction in murine inflammatory cell recruitment following *Ogg1* siRNA or TH5487 treatment (**** P <0.0001). (e) Murine cytokine expression (day 21) following bleomycin administration and subsequent treatment. (f) Lung homogenate samples were analyzed by SDS-PAGE, followed by immunoblotting using rabbit

antisera specific to OGG1. (g) Hydroxyproline levels display significant reductions in both siRNA and TH5487-treated groups, indicative of reduced levels of collagen in these lungs.

Figure 3: TH5487 murine dosing strategy and weights. (a) Mice received intratracheally administered bleomycin (Bleo; 2.5 U/kg) and were subsequently dosed intraperitoneally (i.p.) with TH5487 (TH) or dexamethasone (Dex) 1h post-bleomycin administration. TH5487 or dexamethasone administration occurred five times per week, over the course of 21 days, followed by euthanasia and removal of BALF, plasma, and lung tissues. (b) Mice receiving bleomycin showed weight loss up until day 10, where after those dosed with TH5487 picked up significant amounts of weight compared to the vehicle/bleomycin group. (c) Representative images of murine lungs (right lobes) removed after 21 days, with lung weights shown inset.

Figure 4: Significantly decreased murine cytokines following intraperitoneal TH5487 administration, with accompanying reduction in markers of lung damage. Heatmaps (a-c) showing the differences in cytokine levels, as measured by multiplex assay, in murine BALF, plasma, and lung homogenate (Red indicating high value; Green indicating low value) with corresponding significantly decreased or increased cytokine levels alongside. Cytokine values were compared to the vehicle/bleomycin group using a one-way ANOVA (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$; **** $P < 0.001$). TGF- β 1 ELISA (d) conducted on murine BALF, plasma, and lung homogenate revealed significantly decreased TGF- β 1 levels in all three murine sample types with values compared to the vehicle/bleomycin group using a one-way ANOVA (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$). Murine albumin content (e) and Lactate dehydrogenase (f) were measured in BALF samples as markers for lung damage and plasma leakage, with TH5487 treatment significantly decreasing both albumin content and LDH levels in the BALF compared to the vehicle/bleomycin group. (g) Murine lung collagen content was measured using a hydroxyproline assay with TH5487 significantly reducing collagen production compared to bleomycin control lungs.

Figure 5: Inflammatory cell influx measured in murine BALF. Murine BALF was assessed for neutrophils, alveolar macrophages, and inflammatory macrophages (a) using flow cytometry, with the representative gating strategy depicted alongside (b). Decreased numbers of neutrophils and inflammatory macrophages were detected in response to TH5487 (i.p.) treatment, with no significant difference reported between the neutrophils and inflammatory macrophages of mice treated with dexamethasone. No significant differences were reported for alveolar macrophage numbers. Inflammatory cell numbers were compared to the vehicle/bleomycin group using a one-way ANOVA (* $P < 0.05$). (c) Giemsa-Wright stained cytopsin slides showing vehicle/bleomycin BALF samples containing enlarged inflammatory macrophages, with TH5487 treatment reducing the presence of inflammatory macrophages, whilst the corticosteroid dexamethasone similarly reduced inflammatory macrophage influx comparable to vehicle treated control samples. Scale bar=20 μ m. Immunofluorescent staining of murine BALF samples measured inflammatory macrophage (d) and neutrophil (e) content using CD206/F4/80 and LY6G/MPO, respectively.

Figure 6: Mass spectrometry of lung and BALF proteome. (a) Experimental workflow showing biological fluids analyzed by mass spectrometry. (b) Protein identifications subdivided by treatment condition and compartmentalized by biological fluid. (c) Response profile heatmaps displaying protein expression across treatment conditions in each biological fluid. (d) Volcano plots depicting differentially expressed proteins in BALF (top panel) and lung (bottom panel) with proteins associated with significant gene ontology (GO) terms highlighted in each plot. (e and f) Specific lung proteins downregulated following bleomycin/TH5487 treatment compared to the bleomycin alone condition. Proteins highlighted in these plots are specifically associated with (e) fibrotic-related changes or (f) SMAD (top panel) and nucleotide base repair (bottom panel). (g) Network analysis (StringDB) comparing the B/BTH treatment condition displaying significant biological GO terms, protein counts in the network, strength, and FDR.

Figure 8: Lower expression of OGG1 and SMAD7 are seen in both TH5487-treated mice and human healthy control lungs. (a) Murine lung immunofluorescence analysis showed decreased levels of immunoreactivity for OGG1/SMAD7 in TH5487 (TH)-treated mice compared to bleomycin (Bleo) controls (scale bar=50 μ m), (b) with mouse lung homogenate samples analyzed by SDS-PAGE, followed by immunoblotting using rabbit antisera specific to OGG1 and SMAD7 (protein levels quantified inset; statistical analyses were conducted using a one-way ANOVA (** P <0.005)). (c) This result translated into human patient samples, with healthy control lung tissue displaying significantly decreased levels of OGG1 and SMAD7 immunoreactivity (* P <0.01 and ** P <0.05), as compared using an unpaired t test with Welch's correction. Scale bar= 6mm and 100 μ m for inset images. (d) Excessive OGG1 production facilitates pro-inflammatory gene expression, promoting inflammatory cell recruitment, leading to further exacerbation of the fibrotic lung environment. In addition, OGG1 activates TGF- β , thereby promoting the phosphorylation of SMAD 2/3 by SMAD7, promoting the FMT and EMT and excessive ECM deposition. Therefore, TH5487 has been shown to inhibit OGG1 DNA binding, inhibiting downstream production of pro-inflammatory gene expression and pulmonary fibrosis. In this study, OGG1 and SMAD7 were both reduced after the administration of TH5487, as shown by SDS-page/immunoblotting.

Supplementary Figure legends:

Supplementary Figure 1: Murine BALF cytokine levels from siRNA experiments. Cytokine values were compared to the vehicle/bleomycin group using a one-way ANOVA ($*P<0.05$; $**P<0.01$; $***P<0.005$; $*****P<0.001$).

Supplementary Figure 2: Murine plasma cytokine levels from siRNA experiments. Cytokine values were compared to the vehicle/bleomycin group using a one-way ANOVA ($*P<0.05$; $**P<0.01$; $***P<0.005$; $*****P<0.001$).

Supplementary Figure 3: Murine lung homogenate cytokine levels from siRNA experiments. Cytokine values were compared to the vehicle/bleomycin group using a one-way ANOVA ($*P<0.05$; $**P<0.01$; $***P<0.005$; $*****P<0.001$).

Supplementary Figure 4: Whole lung scans of murine lungs from siRNA experiments following H&E and picrosirius red staining (scale bar=2 mm).

Supplementary Figure 5: Murine BALF cytokine levels. Cytokine values were compared to the vehicle/bleomycin group using a one-way ANOVA ($*P<0.05$; $**P<0.01$; $***P<0.005$; $*****P<0.001$).

Supplementary Figure 6: Murine plasma cytokine levels. Cytokine values were compared to the vehicle/bleomycin group using a one-way ANOVA ($*P<0.05$; $**P<0.01$; $***P<0.005$; $*****P<0.001$).

Supplementary Figure 7: Murine lung homogenate cytokine levels. Cytokine values were compared to the vehicle/bleomycin group using a one-way ANOVA ($*P<0.05$; $**P<0.01$; $***P<0.005$; $*****P<0.001$).

Supplementary Figure 8: Macrophage staining in (A) BALF and (B) lung sections. F4/80 and CD206 immunofluorescence staining, with quantification inset.

Supplementary Figure 9: Proteomic DIA analysis. (A) Proteomic analysis workflow, (B) Representative chromatograms, (C) Volcano plot displaying condition comparisons, (D) PCA plot showing treatment condition groupings.

Supplementary Figure 10: PANTHER gene ontology (GO) biological processes from BALF and lung homogenates.

Supplementary Figure 11: STRINGDB GO terms from lung homogenate samples. (A)

Bleomycin/Bleo/TH5487, (B) Bleomycin/dexamethasone treatment conditions; false discovery rate (FDR).

Supplementary Figure 12: STRINGDB GO terms from lung homogenate samples. (A)

Bleomycin/vehicle, (B) Bleomycin/TH5487 treatment conditions; false discovery rate (FDR).

Supplementary Figure 13: STRINGDB GO terms from lung homogenate samples analyzed using

cluster analysis.

Supplementary Figure 14: Whole lung scans of murine lungs following H&E staining (scale bar=2

mm).

Supplementary Figure 15: Whole lung scans of murine lungs following picrosirius red staining (scale

bar=2 mm).

Supplementary Figure 16: Lung scans of human lung explants following immunostaining for OGG1

(scale bar=6 mm).

Supplementary Figure 17: Lung scans of human lung explants following immunostaining for SMAD7

(scale bar=6 mm).

Supplementary Files

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