Translational pharmacology of an inhaled small molecule αvβ6 integrin inhibitor for idiopathic pulmonary fibrosis

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The αvβ6 integrin plays a key role in the activation of transforming growth factor-β (TGFβ), a pro-fibrotic mediator that is pivotal to the development of idiopathic pulmonary fibrosis (IPF). We identified a selective small molecule αvβ6 RGD-mimetic, GSK3008348, and profiled it in a range of disease relevant pre-clinical systems. To understand the relationship between target engagement and inhibition of fibrosis, we measured pharmacodynamic and disease-related end points. Here, we report, GSK3008348 binds to αvβ6 with high affinity in human IPF lung and reduces downstream pro-fibrotic TGFβ signaling to normal levels. In human lung epithelial cells, GSK3008348 induces rapid internalization and lysosomal degradation of the αvβ6 integrin. In the murine bleomycin-induced lung fibrosis model, GSK3008348 engages αvβ6, induces prolonged inhibition of TGFβ signaling and reduces lung collagen deposition and serum C3M, a marker of IPF disease progression. These studies highlight the potential of inhaled GSK3008348 as an anti-fibrotic therapy.
Fibrosis is the formation of scar tissue and occurs due to injury or long-term inflammation followed by abnormal wound healing. It is a leading cause of morbidity and mortality in a range of diseases. Fibrotic disorders include idiopathic pulmonary fibrosis (IPF), the most common of the idiopathic interstitial pneumonias, for which there are currently limited pharmacological therapeutic options. The global incidence of IPF is increasing and is conservatively estimated to be 3–9 people per 100,000 per year. Recently, there has been international approval of pirfenidone and nintedanib, drugs that slow disease progression in IPF. However, the failure of pirfenidone and nintedanib to halt or reverse disease progression, and their unclear mechanisms of action make them sub-optimal treatments. Furthermore, their side-effect profiles lead to considerable patient tolerability issues. Existing therapies are oral and one of the areas yet to be fully explored in IPF is the potential for the inhaled route of delivery directly to the fibrotic lung. By minimizing the drug dose and systemic exposure, topical delivery to the lungs could offer an improved safety profile compared with systemically bioavailable treatments.

The alpha-v beta-6 (αvβ6) integrin is a member of the arginyl-glycyl-aspartic acid (RGD) sub-family of the heterodimeric, transmembrane glycoprotein receptors. The primary function of the αvβ6 integrin is to activate the key pro-fibrotic mediator, transforming growth factor-β1 (TGFβ1). The αvβ6 integrin is upregulated in patients with IPF and the level of expression appears to be linked to prognosis, making this integrin not only a potential biomarker of disease progression, but also an attractive therapeutic target. The interest in αv integrins as therapeutic targets in fibrosis has increased significantly, with many pharmaceutical companies beginning multiple drug discovery initiatives within this space. This has been led by the development of the αvβ6 monoclonal antibody STX100 (known pre-clinically as 3G9 and clinically as BG000112) that has recently completed a phase II trial for IPF. The extensive target validation of αvβ6 within IPF, other types of organ fibrosis, and cancer, combined with the significant renewed interest in the RGD integrins by academic and industrial groups, has resulted in a renaissance for integrins as drug targets.

To improve the probability of success and enable robust testing of early pre-clinical efficacy, a bespoke, IPF-specific, inhaled drug discovery effort targeting the αvβ6 integrin was carried out. A clear path from pre-clinical to early clinical studies was designed to ensure target engagement could be measured and associated with functional, innovative pharmacodynamic (PD), and disease end points, including imaging and soluble biomarkers to increase the chance of successful translation into clinical trials. In this study, the pre-clinical characterization of an inhaled first in class clinical candidate, GSK3008348, is described. These studies demonstrate that GSK3008348 binds to the αvβ6 integrin with high affinity and selectivity in human fibrotic lung tissue and isolated lung epithelial cells. GSK3008348 inhibits TGFβ activation and reduces downstream pro-fibrotic signaling. In the murine bleomycin-induced model of lung fibrosis, GSK3008348 engages αvβ6 integrins, inhibits the activation of TGFβ with a prolonged duration of action, and reduces lung fibrotic end points including collagen deposition and serum C3M levels, a clinically relevant marker of IPF disease progression. These studies describe an exemplar pathway for the development of an inhaled αvβ6 integrin inhibitor, highlighting the potential of inhaled GSK3008348 as an anti-fibrotic therapy.

**Results**

**Physico-chemical and pharmacokinetic profile of GSK3008348.** GSK3008348 is an RGD-mimetic with the tetrahydronaphthyridine and arylobutanoic acid motifs replacing the arginine and aspartic acid residues, respectively (Fig. 1a). Molecular modeling was used to aid the structure activity relationships around GSK3008348 based on a homology model developed using the closed form of αvβ3 X-ray structure 1LSG as a template. Docked GSK3008348 (orange) predicts binding site electrostatic interactions between the 1,8-tetrahydronaphthyridine and α-Asp218, coordination of the carboxylic acid motif with a manganese ion in the metal ion dependent

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**Fig. 1 Structure, solubility, and pharmacokinetic properties of GSK3008348.** a The chemical structure of GSK3008348 with full chemical name: (S)-3-(3-(5,6,7,8-tetrahydro-1,8-naphthyridin-2-yl)ethyl)pyrrolidin-1-yl)butanoic acid. b Docked GSK3008348 (orange) in a homology model of αvβ6 based on αvβ3 X-ray (1LSG) composed of α-subunit (magenta) and β-subunit (gray). c Solubility and key pharmacokinetic properties for GSK3008348 are also shown. SLF, simulated lung fluid.
adhesion site and an H-bond with Ala143 (Fig. 1b). The dimethylpyrazolo phenyl moiety binds in proximity to β6 subunit residues Ala143, Lys187, Ser199, Ile200, Ala234, and Ile236 in the specificity binding loop (SDL) region.

Crystalline GSK3008348 hydrochloride was prepared in seven steps from commercially available starting materials with the key synthetic step featuring a stereoselective introduction of the pyrazoloaryl ring via a rhodium catalyzed boronic acid addition to a crotonate. The physico-chemical properties are commensurate with inhaled dosing with a measured moderate lipophilicity of chrom logD 2.77 and high solubility (>10 mg/ml) reflecting the presence of ionizable functionality. Pharmacokinetic (PK) studies show GSK3008348 has moderate permeability, a favorable distribution to the site of action, high clearance and low oral bioavailability, all appropriate for inhaled dosing (Fig. 1c) (see supplementary methods section for PK methods).

αvβ6 affinity and RGD integrin selectivity of GSK3008348. In recombinant soluble protein preparations, GSK3008348 exhibits a high affinity and a minimum of 26-fold selectivity for the αvβ6 integrin over the other RGD integrin family members (Fig. 2a and b). For the TGFB2-activating RGD integrins (αvβ1, αvβ3, αvβ5, and αvβ8), a minimum selectivity of 182-fold (αvβ8) and maximum selectivity of 3375-fold (αvβ3) was demonstrated (Fig. 2b). Of particular note, the inhibition constant for binding to the αvβ6 integrin was 190-fold higher than for the αvβ1 integrin and over a 1000-fold higher than for the αvβ3 or αvβ5 integrins (Fig. 2b). Plasma membranes prepared from normal and IPF human lung tissues were used to investigate the affinity of [3H]GSK3008348 for αvβ6 integrins in a disease relevant system as well as to quantify differences in the αvβ6 integrin expression between normal and diseased lung. [3H]GSK3008348 demonstrated a high affinity for the αvβ6 in normal (pKd 11.0 ± 0.06 (Kd 4.8 pM)) and IPF (pKd 11.1 ± 0.07 (Kd 4.8 pM)) human lung tissue membrane preparations (Fig. 2c). A significant increase in the amount of αvβ6 integrin was observed in IPF membranes (0.51 ± 0.11 pmol/mg) compared with normal membranes (0.18 ± 0.02 pmol/mg) (Fig. 2d).

GSK3008348-induced αvβ6 internalization and degradation. The surface (membrane) and total (membrane and intracellular pools) expression of αvβ6 integrins in normal human bronchial epithelial (NHBE) cells were measured using flow cytometric and imaging assays. GSK3008348 caused a significant reduction in surface expression of αvβ6 (Fig. 3a). When cells were permeabilized with saponin to allow antibodies intracellular access, there was no significant difference observed between anti-αβ6 antibody labeling with and without GSK3008348 (Fig. 3a). The internalization observed in the presence of GSK3008348 was inhibited when NHBE cells were pre-incubated with the clathrin-coated pit inhibitor chlorpromazine, whereas the lipid raft inhibitor filipin had no effect showing endocytosis was mediated via clathrin-coated pits (Fig. 3b). To determine the rates of ligand-induced internalization the surface expression of αvβ6 integrin was measured over time following the addition of a maximal concentration of GSK3008348. GSK3008348 caused a rapid internalization
of αβ6 integrins with a $t_{1/2}$ of 2.6 ± 0.5 min (Fig. 3c). Following washout of GSK3008348 after 1 h exposure, αβ6 integrin returned to the surface of NHBE cells in a time-dependent manner with a $t_{1/2}$ of 11.0 ± 1.9 h (Fig. 3c).

The internalization of cell surface αβ6 integrin was confirmed using confocal microscopy by immunofluorescence staining of the β6 subunit (Fig. 3d). Blocking internalization at 4°C revealed abundant cell surface αβ6 integrin expression (Fig. 3d panels a and b) and intracellular staining in cells permeabilized prior to staining with anti-β6 antibody (Fig. 3d panels c and d). Staining patterns were similar irrespective of the presence of GSK3008348. In contrast, although vehicle controls treated cells incubated at 37°C retained cell surface expression of αβ6 integrin (Fig. 3d panel e), those incubated with GSK3008348 for 1 h expressed no αβ6 integrin on the cell surface (Fig. 3d, f) and all the intracellular integrin showed juxtanuclear localization (Fig. 3d panel h).

To quantify the loss of αβ6 integrin membrane staining induced by GSK3008348 in confocal microscopy studies, the mean staining intensity in chamber slide wells for anti-β6 antibody in the four optical sections captured were calculated (Fig. 3e). In flow cytometric assays, GSK3008348 caused concentration-dependent ligand-induced αβ6 integrin internalization following 2 h incubation with NHBE cells with an pEC$_{50}$ value of 9.76 ± 0.25 (EC$_{50}$ 0.26 nM) (Fig. 3f). No αβ6 integrin internalization was observed with the TGFβR1 inhibitor SB-525334 (Fig. 3f). Further imaging studies using high-content screening were completed to measure total αβ6 integrin levels in the absence and presence of the lysosomal degradation inhibitor chloroquine. Following a 24 h incubation with GSK3008348, the levels of staining for αβ6 integrin was significantly reduced compared with control (Fig. 3g, h). The effect of GSK3008348 was reduced in the presence 10 µM chloroquine, demonstrating that GSK3008348-induced lysosomal degradation of αβ6 post internalization. A trend toward increased levels of αβ6 integrin in vehicle control treated cells exposed to the lysosomal inhibitor was also observed suggesting inhibition of endogenous turnover of the integrin in this system (Fig. 3g, h).

To understand whether αβ6 integrin internalization may be disrupted in pulmonary fibrosis, limiting efficacy GSK3008348, internalization studies were performed in small airway epithelial cells (SAECs) from patients with IPF (Fig. 3i) or non-diseased controls (Fig. 3j). In both diseased and non-diseased SAECs there was loss of cell surface αβ6 integrin 1 h following ligation with GSK3008348 to ~40–50% baseline levels. Whilst there was little difference in αβ6 integrin internalization between diseased and non-diseased SAECs, there was a trend toward reduced total αβ6
lung levels of drug were shown to be below the lower limit of
completion in C57BL/6 mice dosed with 1 mg/kg i.n. GSK3008348,
mg/kg GSK3008348 (Supplementary Fig. 1). In PK studies com-
radioligand binding was also observed 8 h post-i.n. dosing of 1
In an additional study in naive mice a signi
observed with 0.05 µg/kg and 50 µg/kg of GSK3008348 (Fig.4c).
radioligand binding 2 h post-intranasal (i.n.) dosing was also
SPECT/CT scans of thorax detected binding of [111In]A20FMDV2 in lungs of naive (saline treated) or bleomycin-treated mice receiving vehicle (i.n. saline)
Source data are provided as a Source Data

NanoSPECT-CT imaging of GSK3008348 engagement with αvβ6. We have previously shown upregulation of the αvβ6 integrin in bleomycin-induced pulmonary fibrosis using a non-invasive imaging (single photon electron computed tomography (SPECT)) of a highly selective radioligand for the αvβ6 integrin ([111In]-DTPA-A20FMDV2) [22]. Therefore, this system was used to measure the engagement of GSK3008348 with αvβ6 integrins in the lung in vivo. GSK3008348 caused complete inhibition of αvβ6 integrin specific radioligand binding in both saline control and bleomycin-treated mice 2 h following dosing (Fig. 4a, b). This confirmed that GSK3008348 could completely inhibit radioligand binding via inhaled delivery, even when αvβ6 integrins have been upregulated in fibrotic mouse lung. Dose-dependent inhibition of radioligand binding 2 h post-intranasal (i.n.) dosing was also observed with 0.05 µg/kg and 50 µg/kg of GSK3008348 (Fig. 4c).

Inhibition of αvβ6-mediated TGFβ activation by GSK3008348. To demonstrate the functional activity of GSK3008348, NHBE cells were used to measure TGFβ activation. GSK3008348 inhibited Smad2 phosphorylation in a concentration-dependent manner, as did the TGFβ1 inhibitor SB-525334 and the αvβ6 integrin selective peptide A20FMDV2 (Fig. 5a). The maximal level of inhibition achieved for GSK3008348, SB-525334, and A20FMDV2 were comparable showing that TGFβ activation in this system was mediated via αvβ6 integrins. The pIC50 (IC50) values for inhibition of αvβ6-mediated TGFβ activation were 9.13 ± 0.04 (0.15 nM), 8.25 ± 0.14 (3.5 nM), and 7.28 ± 0.13 (26.8 nM) for GSK3008348, A20FMDV2, and SB-525334, respectively. To further investigate the mechanism by which GSK3008348 caused a prolonged inhibition of αvβ6-mediated TGFβ release from NHBE cells, washout studies were completed in the presence and absence of the lysosomal degradation inhibitor chloroquine. GSK3008348 caused inhibition of TGFβ activation in the presence and absence of chloroquine (Fig. 5b). In the absence of chloroquine, the inhibitory effect observed for GSK3008348 was only partially reversed following washout. The partial reversal of

 NanoSPECT-CT scans of thorax detected binding of [111In]A20FMDV2 in lungs of naive (saline treated) or bleomycin-treated mice receiving vehicle (i.n. saline) or GSK3008348 (1 mg/kg) from the study shown in panel a. c Dose-dependent effect of GSK3008348 on the binding of the selective αvβ6 integrin nanoSPECT-CT ligand [111In]A20FMDV2 in bleomycin-treated mice following i.n. dosing (mean ± SEM; n = 6–12 animals per group; ANOVA with Fisher’s LSD post test comparisons versus vehicle or IgG2A control or between active groups). Representative axial SPECT/CT scans of thorax detected binding of [111In]A20FMDV2 also shown. Source data are provided as a Source Data file.

 Fig. 4 In vivo engagement of αvβ6 via the inhaled route in a fibrotic lung. a Effect of GSK3008348 on the binding of the selective αvβ6 integrin nanoSPECT-CT ligand [111In]A20FMDV2 in naive (saline treated) and bleomycin-treated mice following i.n. dosing (mean ± SEM; n = 5–6 animals per group; ANOVA with Fisher’s LSD post test comparisons versus corresponding vehicle or IgG2A control or between active groups). b Representative axial SPECT/CT scans of thorax detected binding of [111In]A20FMDV2 in lungs of naive (saline treated) or bleomycin-treated mice receiving vehicle (i.n. saline) or GSK3008348 (1 mg/kg) from the study shown in panel a. c Dose-dependent effect of GSK3008348 on the binding of the selective αvβ6 integrin nanoSPECT-CT ligand [111In]A20FMDV2 in bleomycin-treated mice following i.n. dosing (mean ± SEM; n = 6–12 animals per group; ANOVA with Fisher’s LSD post test comparisons versus vehicle i.n. control). Representative axial SPECT/CT scans of thorax detected binding of [111In]A20FMDV2 also shown. Source data are provided as a Source Data file. 
In vitro human primary cells

Ex vivo Disease tissue

In vivo mouse model

Day 0
Saline or bleomycin 1 mg/kg o.p.

Saline i.n.
GSK3008348 1 mg/kg i.n.
SB-525334 30 mg/kg p.o.

Sal/Veh Bleo/Veh
Bleo/GSK3008348 4h
Bleo/GSK3008348 8h
Bleo/GSK3008348 24h
Bleo/SB-525334

0.0 0.1 0.2 0.3 0.4 0.5 0.6
Lung tissue pSMAD2/tSMAD2 ratio

P < 0.0001
P < 0.0001
P = 0.0039
P = 0.0002

Bleo/Veh
Bleo/GSK3008348 4h
Bleo/GSK3008348 8h
Bleo/GSK3008348 24h
Bleo/SB-525334

0 2 4 6 8 10 12 14
P = 0.0082

BAL cell pSmad2/tSMAD2 ratio

P < 0.0001

control
10 μM chloroquine

P = 0.0149

% Release of TGFβ (NHBE cells) -10 –9 –8 –7 –6 0 20 40 60 80 100
% pSMAD2 in IPF PCLS

Vehicle
1 μM GSK3008348
SB-525334
Log [GSK3008348] M

Vehicle
1 μM GSK3008348
SB-525334
Log [Compound] M

IPF PCLS pSmad2/tSmad2 ratio

p = 0.0009
p = 0.0003

–12 –11 –10 –9 –8 –7 –6 –5
–20 0 20 40 60 80 100 120

SB-525334
GSK3008348
A20FMDV2

Log [Compound] M

% of maximal pSmad2 (NHBE cells)

Fig. 5 Inhibition of αvβ6-mediated TGFβ activation in vitro and in vivo by GSK3008348. a Concentration-dependent inhibition of pSmad2 levels in NHBE cells by GSK3008348, SB-525334 (TGFβRI inhibitor), and A20FMDV2 (selective αvβ6 peptide) (mean ± SEM; n = 4). b Duration of action of the inhibition of GSK3008348 on TGFβ-activation, and the effect of the lysosomal degradation inhibitor chloroquine, measured in a NHBE cell and TMLC (expressing the firefly luciferase under the control of a TGFβ-sensitive portion of the PAI-1 promoter) co-culture system (mean ± SEM; n = 4–8; Student’s t test). Effect of GSK3008348 (i.n.) versus SB-525334 (p.o.) on the levels of pSmad2 in c lung tissue and d BAL cells from bleomycin-treated mice (mean ± SEM; n = 5–10 animals per group; ANOVA with Fisher’s LSD post test comparisons). e Concentration-dependent effect of GSK3008348 on pSmad2 levels in IPF PCLSs (mean ± SD; three PCLSs in a single donor representative of four donors). f Levels of pSmad2 in PCLS from an individual IPF donor in the presence of αv integrin tool inhibitors (mean ± SEM; three PCLSs in a single donor representative of four donors). Sal/Veh Saline/Vehicle, Bleo/Veh Bleomycin/Vehicle. Source data are provided as a Source Data file.
GSK3008348 inhibition caused by washout in the absence of chloroquine was significantly reduced in the presence of chloroquine (Fig. 5b).

To measure the functional inhibition of αvβ6-mediated TGFβ over time in vivo, pSmad2 levels were measured in both lung tissue and cells present in bronchoalveolar lavage (BAL) following administration of GSK3008348. Mice challenged with 1 mg/kg (20IU) bleomycin showed an increase in the pSmad2 levels in the lungs after 14 days relative to saline controls. (Fig. 5c). Following a single therapeutic i.n. dose of GSK3008348 (1 mg/kg) in bleomycin-challenged animals, a significant reduction in pSmad2 was observed in lung tissue when compared with saline treated animals at 4 and 8 h post dosing (Fig. 5c) despite levels of drug being unmeasurable in the lung 2 h post dosing (Supplementary Table 1). By 24 h the pSmad2 in the bleomycin/GSK3008348-treated animals had returned to levels comparable with those observed in the lung tissue from bleomycin/vehicle-treated animals (Fig. 5c). The percentage inhibitions at 4, 8, and 24 h were calculated to be 75%, 62%, and 9%, respectively. These findings were also observed in the BAL cells of these same animals, where GSK3008348 caused a reduction in pSmad2 in BAL cells from bleomycin-treated animals compared with BAL cells from bleomycin/vehicle-treated control animals. By 24 h, the levels of pSmad2 in the BAL cells from bleomycin/GSK3008348-treated animals had returned to levels comparable with those observed in BAL cells from bleomycin/vehicle-treated animals (Fig. 5d). The TGFβR1 inhibitor, SB-525334 was shown to significantly reduce pSmad2 levels in both lung tissue and BAL cells 2 h post dosing (Fig. 5c, d). Levels of lung tissue pSmad2 in the bleomycin/TGFβR1 inhibitor group were significantly lower than in the saline/vehicle group, suggesting that under normal conditions there is a basal level of TGFβ activity in the lungs. However, GSK3008348, which reduces active TGFβ levels through αvβ6 inhibition, did not inhibit pSmad2 below this basal activity at the dose tested. The pattern of dose-dependent inhibition of pSmad2 in bleomycin-challenged animals with GSK3008348 was comparable to that observed in CT/SPECT studies (Fig. 4 and Supplementary Fig. 2).

To provide a link between pre-clinical and clinical studies, the effect of GSK3008348 was measured in precision cut lung slices (PCLS) from IPF patients. GSK3008348 caused a concentration-dependent reduction in pSmad2 phosphorylation (Fig. 5e) with an approximate IC50 of 3 nM. Levels of lung tissue pSmad2 following inhibition of TGFβR1 with SB-525334 in murine and human fibrosis was lower than inhibition by GSK3008348, suggesting that under normal conditions there is a basal level of TGFβ activity in the lungs which was not inhibited by GSK3008348 at the concentrations tested. It is possible that some of the residual TGFβ activity in the lung is due to activation by the αvβ1 integrin, therefore the αvβ1 inhibitor c8, and the αvβ3/αvβ5 inhibitor SB-267268, were assessed. Neither compound inhibited pSmad2 levels (Fig. 5f). These data suggest that in this system, the αvβ6 integrin is both necessary and sufficient for activating TGFβ in fibrotic lung.

GSK3008348 effects on PD and disease biomarkers. To further clarify the drug effects on PD and disease biomarkers, a number of analytes that are known to reflect fibrogenic end points, including analytes previously identified in the PROFILE (Prospective Observation of Fibrosis in the Lung Clinical End points) study23 were investigated in a murine pulmonary fibrosis model. Animals were exposed to 3 mg/kg (60IU) bleomycin for 14 days following subcutaneous (s.c.) implantation of osmotic pumps containing GSK3008348 or vehicle 3 days before. Bleomycin treatment increased both total lung collagen as measured by hydroxyproline levels (Fig. 6b) and serum levels of the matrix metalloproteinase (MMP)-degraded ECM protein neutropoetin C3M (Fig. 6c), which is known to reflect progressive IPF in patients. There was partial inhibition of hydroxyproline levels in the lungs of bleomycin-treated mice following prophylactic administration of GSK3008348 (Fig. 6b) but a substantial inhibition of serum C3M was detected in response to GSK3008348 (Fig. 6c).

TGFβ activation was also assessed in the lungs of IPF patients following collection of bronchoalveolar lavage fluid (BALF). Samples were obtained from healthy volunteers and IPF patients and the level of pSmad2 in BAL cells was measured along with TGFβ-induced gene h3 (βIG-H3) and plasminogen activator inhibitor-1 (PAI-1) within the BALF. All three biomarkers were significantly increased in IPF patients compared with healthy volunteers (Fig. 6d, e). To determine whether these markers could serve as PD biomarkers in a clinical study using GSK3008348 we also measured these analytes in samples collected from the study assessing the effects of prophylactic dosing with GSK3008348 in the bleomycin model of pulmonary fibrosis. There was a significant increase in lung tissue pSmad2 levels 14 days post instillation of bleomycin and phosphorylation of Smad2 was inhibited by GSK3008348 (Fig. 6f). Furthermore, GSK3008348 also inhibited bleomycin-induced increases in the level of βIG-H3 and PAI-1 detected in BALF (Fig. 6g). Finally, to determine whether local delivery of inhaled αvβ6 inhibitor could be used to ameliorate established fibrotic lung disease, therapeutic dosing of i.n. GSK3008348 was undertaken. Bleomycin-induced pulmonary fibrosis was induced with a 3 mg/kg oropharyngeal dose and after 14 days, at the time of established fibrosis, GSK3008348 was instilled twice daily i.n. for a further 14 days and fibrosis was assessed (Fig. 7a). Animals receiving vehicle control showed an increase in hydroxyproline (Fig. 7b) and areas of focal fibrosis assessed by histology (Fig. 7c) where those receiving twice daily i.n. αvβ6 inhibitor showed a significant reduction in fibrosis as measured by both hydroxyproline and histology (Fig. 7b, c).

Discussion

The small molecule RGD-mimetic αvβ6 inhibitor GSK3008348 represents a therapeutic agent for inhaled delivery to IPF patients and is the first in class inhaled integrin inhibitor to reach clinical development. GSK3008348 resides in a distinct niche, not only in the current IPF therapeutic pipeline space, but also as one of the highest affinity integrin inhibitors that has been developed in the field of small and large molecule drug discovery14. The aim of this study was to fully characterize GSK3008348 and generate translational drug discovery biomarkers that will facilitate the performance of future clinical trials. To date, GSK3008348 has been shown to be safe and well tolerated in phase I healthy volunteer studies24 and has completed a phase 1b study, demonstrating target engagement of the αvβ6 integrin in IPF patients25. It is hypothesized that the αvβ6 integrin is a key driver of TGFβ activation in IPF and therefore an attractive therapeutic target in this, and other, fibrotic diseases10–13. A highly selective αvβ6 integrin inhibitor was designed for topical delivery to the lungs to improve target engagement, reduce the dose, and minimize systemic exposure and potential adverse effects. A concern with this approach has been the risk of being unable to deliver drug to the peripheral regions of the lung where the fibrosis occurs in IPF, however, a recent study using 99mTcTechnetium-labeled salbutamol has shown that nebulized drug can be delivered to these fibrotic regions26. During development of GSK3008348, characterization of the pharmacology, the physico-chemical, and PK properties of the molecule were assessed to track suitability for inhaled delivery. Most of these properties were evident in the GSK3008348 molecule except for lung retention27. Therefore, the duration of
Fig. 6 Translational PD and disease biomarkers from pre-clinical to clinical samples. a Mice were treated with GSK3008348 prior to bleomycin challenge and the effect of GSK3008348 on the b collagen biomarkers hydroxyproline and c C3M in lung tissue (mean ± SEM; n = 6-10 animals per group). Levels of pSmad2 in d BAL cells and e βIG-H3, PAI-1 BAL fluid d in normal and IPF patient populations (mean ± SEM; 6-18 donors). The effect of GSK3008348 on PD biomarkers of TGFβ-activation in f lung tissue (pSmad2) and g BALF (βIG-H3 and PAI-1) in bleomycin-treated mice (mean ± SEM; n = 6-10 animals per group). All statistical analysis completed by ANOVA with Fisher’s LSD post test. Sal/Veh Saline/Vehicle, Bleo/Veh Bleomycin/Vehicle. Source data are provided as a Source Data file.
action of this inhaled molecule was designed to be driven by the PD of the interaction between GSK3008348 and the αvβ6 integrin.

GSK3008348 demonstrates a high affinity for the αvβ6 integrin in soluble protein preparations but more importantly in plasma membranes generated from fibrotic regions of human IPF lung. The use of a radiolabeled version of GSK3008348 has enabled expression levels of αvβ6 integrins to be accurately quantified in normal and IPF lung. First, this has confirmed and quantified the upregulation of αvβ6 integrins in IPF previously observed semi-quantitatively using immunohistochemistry12. Second, the amount of integrin present in disease tissue can be incorporated into more accurate models to predict the therapeutic dose of αvβ6 integrin inhibitors. NanoSPECT-CT imaging studies using A20FMDV2 in saline and bleomycin-treated mice demonstrated the dose- and time-dependent engagement of GSK3008348 with αvβ6 in an in vivo model of lung fibrosis. The quantification of αvβ6 integrin upregulation in IPF and the profiling of GSK3008348 binding to the integrin in diseased tissue will allow this pre-clinical work to be translated to clinical studies in IPF25,28.

It has been demonstrated for RGD-peptides that binding to αvβ6 integrin induces internalization, followed by a delay in the return of the integrin to the cell surface29,30. In this study, GSK3008348-induced rapid internalization of the αvβ6 integrin that was concentration-, time-, and clathrin-dependent, in agreement with published data31. Following washout of GSK3008348, the return of αvβ6 integrins to the cell surface was markedly slower compared with endogenous turnover31 as well as post internalization via RGD-peptides30. Furthermore, in NHBE cells in the constant presence of GSK3008348 over 24 h, a complete loss of αvβ6 integrins was also observed at both the cell surface and intracellularly. Both these observations suggest that the internalized GSK3008348–αvβ6 complex is degraded and that returning αvβ6 integrins in washout studies are newly synthesized. Furthermore, in washout experiments investigating αvβ6-mediated inhibition of TGFβ activation, the sustained inhibition observed with GSK3008348 was prevented in the presence of chloroquine. These observations suggest that GSK3008348 induces internalization of αvβ6 integrins that are subsequently sorted for degradation in lysosomes. The high affinity of GSK3008348 for the αvβ6 integrin combined with its previously described fast association and slow dissociation profile32 likely results in a prolonged activation of the integrin in intracellular vesicles. We hypothesize that the longer the integrin is engaged at the RGD-site post internalization, the more likely it is to be intracellularly designated for degradation. This effect has previously been observed with other small molecule/protein interactions33,34 and would ultimately result in the prolonged duration of inhibition of TGFβ activation in vivo demonstrated in this study.

One of the current challenges of clinical study design within the IPF field is the size and duration of studies required to measure meaningful changes in lung function or mortality, especially in addition to standard of care1–7. Therefore, there have been significant efforts to identify IPF biomarkers detectable in blood or BALF samples that reflect disease biology and which could be used as early surrogates for long-term decline in forced vital capacity or mortality, predict disease progression and allow enrichment of patient cohorts for clinical studies as well as facilitating potentially shorter and smaller studies.

The purpose of the bleomycin model of fibrosis in these studies was twofold. Initial studies were designed primarily to assess target engagement and effects on TGFβ signaling and other PD biomarkers rather than to predict clinical efficacy. Therefore, these studies used a low bleomycin dose (1 mg/kg) to induce a mild bleomycin injury and minimize distress to animals where possible. A higher dose of bleomycin (3 mg/kg) was used in studies in which fibrotic end points (hydroxyproline) were assessed including both the prophylactic and therapeutic dosing studies with GSK3008348. We demonstrated that prophylactic treatment with GSK3008348 led to a modest inhibition of lung hydroxyproline similar to values reported following prophylactic dosing with nintedanib in bleomycin-treated mice35. Subsequent studies were used to assess the feasibility of inhaled small molecules to penetrate and ameliorate established lung fibrosis, and indeed 2 weeks inhaled GSK3008348 lead to a substantial reduction in established fibrosis as measured by total lung collagen and histological assessment. However, there are known limitations of the bleomycin model of pulmonary fibrosis that mean it cannot reliably predict the clinical effectiveness of an anti-fibrotic therapy36. Therefore, to ensure the translational
efficacy of GSK3008348 could be determined, we also measured the effects of GSK3008348 on established and recently identified biomarkers each of which could be translated rapidly into clinical practice. Initial studies evaluated the effects of GSK3008348 on TGFβ activation pathways. These data established that levels of pSmad2 measured in BAL cells recovered from the fibrotic lungs of bleomycin-treated mice were reduced following administration of GSK3008348 in comparison with vehicle control treated mice, thus demonstrating engagement of the mechanism in the lung. Furthermore, proteins known to be regulated by active TGFβ including PAI-1 and βIG-H3, were also inhibited by GSK3008348 in mouse BALF confirming the effects of GSK3008348 on the TGFβ activation pathway and offering further options for measuring a PD effect on this signaling pathway in clinical studies. Moreover, we demonstrated that GSK3008348 could inhibit pSmad2 in human diseased samples using PCLS from IPF patients confirming that GSK3008348 can inhibit TGFβ activation pathways both in human fibrotic tissue as well as a murine model of lung fibrosis. These data suggest that measuring levels of pSmad2 in BAL cells obtained from clinical studies in the presence of GSK3008348 could be a good surrogate measure of the effects of this αvβ6 inhibitor on TGFβ activation in IPF lung. One promising and recently identified serum biomarker in IPF is the MMP-degraded ECM protein neoepitope C3M, which is also promising and recently identified as a biomarker for disease progression and mortality in patients with IPF. We demonstrated that GSK3008348 was able to inhibit serum C3M in the murine model of bleomycin-induced fibrosis and may therefore be useful as a mechanism-specific biomarker in future IPF clinical trials.

Although αvβ6 integrins are considered to drive the majority of TGFβ activation in IPF, there are data suggesting a role for αvβ5 and, more recently, αvβ1. However, data generated in this study utilizing PCLS from diseased human IPF tissue has compared the effects of GSK3008348 with highly selective αvβ1 (c8) and αvβ5 inhibitors and shown that of these the αvβ6 integrin appears to be the major contributor to TGFβ activation and downstream phosphorylation of Smad2 in this system. The comparable potency for inhibition of pSmad2 in primary cells and in human lung tissue suggests that the epithelial αvβ6 integrin is the main driver of TGFβ activation in human IPF tissue. However, this does not contain a potential role of other integrins in the pathogenesis of pulmonary fibrosis. Although epithelial cell injury may be a driver of pulmonary fibrosis, myofibroblasts are a key effector cell and fibroblast to myofibroblast differentiation is a major step in this process. Active TGFβ is well known to induce myofibroblast differentiation, which is mediated in part through Smad signaling, but also by non-canonical activation of focal adhesion kinase (FAK) through interactions with integrins. Therefore, even if epithelial cells do generate sufficient active TGFβ to lead to downstream TGFβ signaling in neighboring fibroblasts, as these data suggest, it is possible that myofibroblasts develop autonomous, non-canonical, pro-fibrotic pathways through TGFβ-induced αvβ1 integrin and FAK activation that will not be inhibited by GSK3008348. Therefore, further studies are required to determine the relative contribution of αvβ1 and αvβ6 integrins on myofibroblast differentiation.

This study has some strengths and limitations that will only be fully addressed in clinical studies with GSK3008348. A key strength of this study is the demonstration that twice daily (b.i.d.) inhaled dosing can ameliorate established bleomycin-induced pulmonary fibrosis. This is especially important in light of the recent early termination of the STX100 study owing to safety concerns. Although it is not known at the current time whether the adverse effects of STX100 were due to on-target effects, the difference in drug delivery (i.n. versus s.c.), dosing schedule (b.i.d. versus weekly) and TGFβ inhibition (reduced rather than blocked completely) offer considerable advantages for GSK3008348 compared with the αvβ6 antibody blocking approach. The additional strengths of this study are the comprehensive assessment of both the anti-fibrotic potential and target engagement of the molecule using both human and murine model systems in addition to measuring both traditional biochemical and clinically translatable end points. Limitations include use of the bleomycin mouse model of lung fibrosis that is recognized to be a poor surrogate for clinical efficacy in IPF. However, the use of the bleomycin model in this study has been to demonstrate that inhaled delivery of an integrin inhibitor can mitigate established fibrosis and to establish PD and mechanism-specific biomarkers. These data demonstrate that inhalation of a small molecule can penetrate fibrotic lung and the biomarkers studies demonstrate proof of mechanism that can be further assessed in future IPF clinical trials. Thus, these data have been used to create a clear clinical translational pathway and to generate and test a hypothesis, rather than to raise unrealistic hopes for clinical efficacy at this stage of pre-clinical development.

All the data generated as part of this study have contributed to the design and clinical dose prediction used for GSK3008348 in phase Ib studies where the ability of this molecule to engage the αvβ6 integrin in IPF patients is currently under investigation. Based on this translational data package the hypothesized mechanism of action of GSK3008348 post administration into an IPF patient’s lung is as follows: (1) nebulized GSK3008348 would enter the lung and bind to αvβ6 with high affinity and fast association kinetics. (2) The GSK3008348/αvβ6 complex would then be rapidly internalized in minutes and the integrin degraded in lysosomes. (3) The αvβ6-mediated TGFβ activation in the IPF lung would be inhibited reducing pro-fibrotic mediators and extracellular matrix deposition. These studies therefore represent a different approach to pre-clinical drug development that will reduce the chances of failed translation to early phase clinical studies and increase the prospect of generating effective therapies for IPF.

Methods

Study design. The objective of this study was to develop a clear path for a proposed IPF therapeutic from pre-clinical to clinical studies that linked target engagement through to functional inhibition of PD and disease end points. In order to achieve this, studies focused on human primary cells and IPF disease tissue in vitro and ex vivo studies to characterize the pharmacology of an αvβ6 RGD-mimetic inhibitor investigating binding, receptor internalization and inhibition of TGFβ. Studies investigating these end points in vivo were then completed using a mouse model of pulmonary fibrosis, with disease end point relevance and validation determined in ex vivo BAL cell and fluid from IPF patients that could be readily profiled in future clinical experiments.

All animal studies were approved by the University of Nottingham Animal Welfare and Ethical Review Board and carried out in accordance with Animals (Scientific Procedures) Act 1986, the GSK Policy on the Care, Welfare and Treatment of Animals and the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. Mice were randomized into treatment groups and for Experiments) guidelines. Mice were randomized into treatment groups and for
All chemicals and reagents were purchased from Sigma-Aldrich Co. Ltd. (Gillingham, Kent, UK) and antibodies from R&D Systems (Minneapolis, MN, USA) unless otherwise stated. All tissue culture flask, membranes, and 96-well plates were purchased from Greiner Bio-One (Frenkendorf, Germany). All in vitro studies were conducted at least three times and at 37 °C, unless otherwise stated. Cellular assays were conducted in an atmosphere of 5% CO2 with a relative humidity of 95% at 37 °C. Synthesis of inhibitors. The small molecule compounds GSK3008348, c8 (avp1 inhibitor18), SB-523534 (TOG/FR1 inhibitor19), and SB-267268 (avp3/avp5 inhibitor20) used in this study were synthesized by the Fibrosis DPU Medicinal Chemistry group at GlaxoSmithKline Medicines Research Centre (Stevenage, Hertfordshire, UK). The avp6 selective peptide A20FMDV24 was synthesized by Cambridge Research Biochemicals (Cleveland, UK).

Radioligand binding assays. Radioligand binding and platelet aggregation (allhþ3 only) assays were conducted against the RGD integrins. All radioligand binding experiments were performed in 96-deep well plates at 37 °C in binding buffer (25 mM HEPES, 5 mM CaCl2, 150 mM NaCl, 1 mM MgCl2, and 1 mM 3-[3-cholamidopropyl]dimethylammonio]–1-propane sulfonate at pH 7.4 (NaOH)) in a total volume of either 0.5 ml or 1.5 ml (1.5 ml for saturation binding studies with lung parenchyma membranes) consisting of 50 µl of either unlabeled compound at varying concentrations or vehicle (1% dimethyl sulfoxide; DMSO), 50 µl of [3H]RGD ligand32 or [3H]GSK3008348 and either 0.4 or 1.4 µl/ml of purified integrin or membranes (concentration dependent on the number of binding sites of individual soluble protein or membrane fragment preparations). Non-specific binding (NSB) was determined with 10 µM SC-68448 (pan-av RG small molecule32) except for saturation binding experiments with lung parenchyma membranes where 10 µM AMD3100 (2-selective beta3 integrin-specific ligand Fig 3c) radioligand was used. Specific binding was measured by subtracting the NSB from the total radioligand binding in the presence of vehicle (1% DMSO). Binding was terminated by rapid vacuum filtration and the amount of specific radioligand bound was measured by L5 spectroscopy using a TriCarb 2900 TR LS counter (PerkinElmer LAS UK Ltd., Beaconsfield, UK). Binding assays were conducted in the presence of 2 mM Mg2+ to standardize integrin activation state.

For platelet aggregation, platelets were resuspended in assay buffer (5 mM HEPES, 140 mM NaCl, 3 mM CaCl2, 12 mM NaHCO3, 7 mM KH2PO4, and 60 mM d-glucose at pH 7.4 (NaOH)), and resuspended to 4 × 108 cells/ml. Cells were then incubated in 95%:5% air:CO2 at 37 °C for varying times up to 1 h and then immediately on to ice to stop any further internalization. In all, 10 µl [3H]RGD ligand in the presence of 2 mM Mg2+ or [3H]GSK3008348 were added to 96-well polypyrrole microplates containing 5 µl/well compound (at a concentration that caused maximal internalization) or vehicle (0.1% DMSO). Plates were incubated in 95%:5% air:CO2 at 37 °C for varying times up to 1 h and then immediately on to ice to stop any further internalization. In all, 10 µl [3H]RGD ligand in the presence of 2 mM Mg2+ or [3H]GSK3008348 were added to 96-well polypyrrole microplates containing 5 µl/well compound (at a concentration that caused maximal internalization) or vehicle (0.1% DMSO). Plates were incubated in 95%:5% air:CO2 at 37 °C for 1 h then centrifuged at 500 × g for 5 min, supernatant removed and cell pellets resuspended in PBS (150 µl/ml). This process was repeated prior to re-suspension of cells in cell medium (150 µl/ml) and incubation for varying times up to 48 h. Plates were then transferred on to ice and 10 µl Igg2B PE or IgP6 PE antibody added. All samples were then processed and read on the FACS Canto II as detailed above.

Flow cytometry and imaging assays. The measurement of surface and intracellular avp6 in NHBE cells and SAEC cells was determined via flow cytometry. NHBE cells were harvested and 96-well plates were coated with NHBE cells suspended in flow cytometry buffer (45 µl/well with 70,000 cells/well) in the presence of appropriate concentrations of drug or vehicle (0.1% DMSO) as required. Where stated, cells were permeabilised by incubating with 0.2% w/v saponin for 5 min at ambient temperature (20–22 °C) prior to permeabilization/aid addition. Inhibition of clathrin or lipid raft mediated endocytosis by their forward and side-scatter characteristics and a single population gated with cell debris excluded (Supplementary Fig 3) and the mean fluorescence intensity (MFI) of antibody conjugated conjugated cells measured. The fluorescence was quantified on at least 5000 cells and following acquisition all data was exported as flow cytometry standard format 3.0 files with raw data values captured as MFI. Fluorescence-activated cell analyses history and flow data was plotted using FlowJo software (Tree Star Inc., USA).

For internalization, concentration–response curves NHBE cells were added to 96-well polypyrrole microplates containing 5 µl/well compound at varying concentrations or vehicle (0.1% DMSO). Plates were incubated for 2 h in 95%:5% air:CO2 at 37 °C prior to addition of 10 µl Igg2B PE or IgP6 PE antibody. For determination of the rate of ligand-induced integrin avp6 internalization, NHBE cells were added to 96-well polypyrrole microplates containing 5 µl/well compound (at a concentration that caused maximal internalization) or vehicle (0.1% DMSO). Plates were incubated in 95%:5% air:CO2 at 37 °C for 30 min, 1 µl/well compound (at a rate of 100 µM) were added to 5% air:CO2 at 37 °C for 1 h then centrifuged at 500 × g for 5 min, supernatant removed and cell pellets resuspended in PBS (150 µl/ml). This process was repeated prior to re-suspension of cells in cell medium (150 µl/ml) and incubation for varying times up to 48 h. Plates were then transferred on to ice and 10 µl Igg2B PE or IgP6 PE antibody added. All samples were then processed and read on the FACS Canto II as detailed above.

For cell imaging studies, NHBE cells were adhered in glass chamber slides and incubated with vehicle (0.1% DMSO) or GSK3008348 in the absence or presence of 10 µM chloroquine for 2 h prior to [6 integrin staining with sheep anti-human integrin [6 antibody then donkey anti-sheep IgG Alexa Fluor® 488 antibody (Invitrogen Ltd., Renfrewshire, UK). Chamber slides were then washed with a Leica TCS SP5 confocal microscope (Leica Microsystems Inc., Buckinghamshire, UK) to produce a 3D cell z-stack. Quantitative analysis of the intensity of [6 staining was determined using Columbia Image Data Storage and Analysis System (PerkinElmer LAS UK Ltd., Buckinghamshire, UK). For high-content screening (HCS) studies, supplement starved NHBE cells were plated in collagen I coated 96-well imaging plates and treated with vehicle (0.1% DMSO) or GSK3008348 in the absence or presence of 10 µM chloroquine for 24 h prior to [6 integrin staining with mouse anti-human integrin [6 then goat anti-mouse IgG Alexa Fluor® 488 antibody (Invitrogen Ltd., Renfrewshire, UK). Image acquisition and analysis of staining were performed using the ArrayScan VTI (Thermo Fisher Scientific, MA, USA), applying biosimilations to quantify total [6 immunofluorescence. For all imaging studies, nuclear staining was completed with Hoechst 33342 dye (Invitrogen Ltd., Renfrewshire, UK) and if required cells were permeabilised with 0.2% w/v saponin (flow cytometry) or Triton X-100 (HCS) to determine total avp6 staining (membrane and intracellular).

TGFβ1 activation and signalling assays. To determine activation of TGFβ1 via avp6 in NHBE cells following washout, a TMLC co-culture system was used. Cultured NHBE cells were harvested, resuspended in NHBE cell medium and 25,000 cells (100 µl/well) were plated on 1 collagen I coated plates then left for 24 h to adhere in 95%-5% air:CO2 at 37 °C before removal of medium. Cultured TMLCs were harvested, resuspended in TMLC co-culture medium containing 20 µM l-lysophosphatidic acid (LPA) and 25,000 cells (100 µl/well) seeded onto the NHBE cells. Vehicle (0.1% DMSO) or test compounds were then added (10 µl/ml) and plates incubated for 24 h in 5% air:CO2 at 37 °C. Cells were then removed from the NHBE cell/TMLC co-culture and 100 µl/well PBS (containing 1 mM CaCl2 and 1 mM MgCl2) and 100 µl/well Steady-Glo® Reagent (Promega
Corporation, Madison, WI, USA) added. Plates were then incubated at ambient temperature (20–22 °C) for 5 min before supernatants were transferred to 96-well white, flat-bottom, 96-well (PerkinElmer LAS UK Ltd., Beaconsfield, UK). For washout studies, vehicle (0.1% DMSO) or GS3K008348 in the absence or presence of 10 μM chloroquine were incubated with the NHBE cell/TMLC co-culture for 1 h prior to washout. Washout studies were also completed in the presence of chloroquine (10 μM) made up in TMLC co-culture medium to ensure comparability of results. Under experimental conditions to readings obtained from a standard curve derived from increasing concentrations of active TGFβ added to the co-culture under identical conditions.

TGFβ levels in supernatants harvested from human IPF PCLSs were determined by incubation with TMLC cells for 18 h. For the measurement of concentration dependent effects of avp6 and TGFβ1 inhibitors on TGFβ activation and signaling in NHBE cells, pSmad2 (normalized to GAPDH) was measured. pSmad2 and GAPDH was quantified using the Milliplex TGFβ Signaling Magnetic Bean Panel 6 plex (Merck Millipore, Billerica, MA, USA) luminescent assay according to the manufacturer’s instructions. pSmad2 was determined using the same method for in vivo and ex vivo tissue and BAL samples, however data were normalized to tSmad2. tSmad2 was measured in the Pathscan Total Smad2 ELISA kit (Cell Signaling Technologies, London, UK), according to the manufacturer’s instructions with standard curves generated using recombinant GST-Smad2. tSmad2 was determined in the Pathscan Total Smad2 ELISA kit (R&D Systems, Abingdon, USA) respectively, according to the manufacturer’s instructions. PAI-1 was determined in mouse and human BAL fluid samples using the mouse or human βG-H3 DuoSet ELISA kit (R&D Systems, Abingdon, USA) respectively, according to the manufacturer’s instructions. PAI-1 was measured in mouse and human BAL fluid samples using the mouse or human PAI-1 Total antigen ELISA kit (Molecular Innovations, Novi, MI, USA) according to the manufacturer’s instructions.

In vivo mouse studies. Male C57BL/6 mice (6–12 weeks old from Charles River, Kent, UK) were acclimatized for 5–7 days before undergoing procedures and ranged from 16 to 28 g at the time of study. Animal welfare followed the requirements of the Home Office Code of Practice for the Housing and Care of Animals used in Scientific Procedures. In brief, mice were housed in individually ventilated cages in groups of three to four in a temperature and humidity controlled environment, with a 12 h light/dark cycle with food and water available ad libitum. Play tunnels and bedding material were used to provide environmental enrichment. In addition to the standard weight and health checks performed by researchers, animals were monitored at least once daily by a trained animal technician to ensure that they were in good health. Mice were randomized among the different treatment groups for each experiment and were observed regularly during the study. Mice used were either naive or treated with a single oropharyngeal 50 µl dose (1 mg/kg or 3 mg/kg) of bleomycin sulfate (Bleo-Kyowa; Aesica Pharmaceuticals) 7 days before undergoing procedures and exposed to isoflurane for the duration of the scan (45 min). CT images were obtained using a four-parametric mathematical fit model and data were analyzed using the Softmax Pro (version 6.3) software. Levels of the biomarkers were measured in duplicates. Lung histology. Histological sections of murine lung were cut at three microns and dewaxed in xylene prior to rehydration in decreasing concentrations of ethanol. The sections were embedded in either Mayer’s haematoxylin, and eosin or Sirius red and Weigert’s haematoxylin. Tissue staining was imaged using Nikon Eclipse 950 microscope and NIS Elements AR3.2 software (Nikon).

Ex vivo human studies. Human fibrotic lung tissue was collected from human explants after transplantation. Tissue which the pathologist deemed suitable for research was infused with 2–3% low boiling point agarose, with the agarose being allowed to set at 4 °C. PCLS were then cut at 400 µm on a vibrating microtome and cultured in DMEM media. PCLS were rested for 48 h prior to treatment. PCLS were equilibrated to room temperature (20 °C) prior to anoxic flow rate of 1 L/min. Whole-body helical CT and SPECT scans were performed with a nanoSPECT-CT imaging system (Biocan Inc, Washington, DC) fitted with four tungsten collimators and nine 1.4 mm diameter pinholes. SPECT images were obtained 2 h (saline/GSK3008348) or 24 h (IgG isotype control/1015 anti-avp6 antibody intra-peritoneal administration in PBS at 2.5 mg/kg (Merck Millipore, Billerica, MA, USA)) post dosing of test agents with a time per view of 60 s resulting in a scan time of 30–40 min. CT images were obtained using a tube voltage of 45 kVp and an exposure time of 500 ms per view.

Lung histology. Histological sections of murine lung were cut at three microns and dewaxed in xylene prior to rehydration in decreasing concentrations of ethanol. The sections were embedded in either Mayer’s haematoxylin, and eosin or Sirius red and Weigert’s haematoxylin. Tissue staining was imaged using Nikon Eclipse 950 microscope and NIS Elements AR3.2 software (Nikon).

Statistical analysis. Statistical analyses were completed using GraphPad Prism 7.0 (GraphPad Software, San Diego, CA, USA) for in vitro studies and R version 3.4.0 for in vivo/ex vivo studies. Statistical significance between two data sets was tested using a Student’s unpaired t test. One-way analysis of variance was used for comparison of more than two data sets and, where significance was observed, an appropriate post test completed. Unless otherwise indicated data shown graphs as mean ± standard error of the mean.
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Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The authors declare that the data supporting the findings of this study are available within the paper and its supplementary information files. Source data are provided with this paper.

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Author contributions

R.J.S. wrote the paper and contributed to overall study design and data interpretation; A.E.I., R.G.I., S.F.M., and P.T.L. contributed to the writing of the paper. A.E.I. performed and contributed to in vivo study design and data interpretation; G.V., J.L.M., R.H.G., and J.W.B. performed or supervised DMPK studies; R.J.S., A.E.I., R.H.G., K.T.P., E.J.F., P.F.M., R.F.R., M.H., L.I.B., E.G., V.S.M., Y.M., J.A.K., J.C.L., L.A.B., B.S.B., R.A.B., following a single dose of a novel inhaled αvβ6 integrin inhibitor. Respir. Res. 21, 75 (2020).

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R.B., J.L., D.J.F., S.P., A.H., L.A.O., C.J., R.C.E.P., N.S.G., D.J.L., R.C.C., and R.G.J. designed, performed, and/or analyzed in vitro and/or in vivo studies, or the supervision thereof; J.L. performed homology modeling; T.M.M. and A.J.F. provided human IPF BAL and lung tissue, respectively, and contributed to data interpretation. R.G.J., P.T.L., and R.P.M. contributed to overall study design and data interpretation.

Competing interests
T.M.M., A.J.F., R.C.C., and R.G.J. are or have been paid consultants to GlaxoSmithKline. The remaining authors declare no competing interests.

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