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Inhaled nintedanib is well-tolerated and delivers key pharmacokinetic parameters required to treat bleomycin-induced pulmonary fibrosis

Mark W. Surber [a], Steve Beck [a], Stephen Pham [a], Angela T. Marsden [b], Senthil K. Gandi [b], James Baily [c], Mary C. McElroy [b,*]

[a] Avalyn Pharma, Inc., 701 Pike Street, Suite 1500, Seattle, WA, 98101, USA
[b] Respiratory Pharmacology, Charles River Laboratories, Edinburgh, EH33 2NE, UK
[c] Pathology Department, Charles River Laboratories, Edinburgh, EH33 2NE, UK

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ABSTRACT

Oral nintedanib is marketed for the treatment of idiopathic pulmonary fibrosis (IPF), Systemic Sclerosis-Associated Interstitial Lung Disease and Chronic Fibrosing Interstitial Lung Diseases with a Progressive Phenotype. While effective at slowing fibrosis progression, as an oral medicine nintedanib has limitations. To reduce side effects and maximize efficacy, nintedanib was reformulated as a solution for nebulization and inhaled administration. To predict effectiveness treating IPF, inhalation was used as a tool to dissect the pharmacokinetic components required for nintedanib pulmonary anti-fibrotic activity. Following oral administration, nintedanib extensively partitioned into tissue and exhibited flip-flop pharmacokinetics, whereby resulting lung Cmax and AUC were substantially higher than plasma. By comparison, inhaled nintedanib was capable of delivering an oral-equivalent lung Cmax with lower local and systemic AUC. Using a multi-challenge bleomycin rat model, this distinct inhaled pharmacokinetic profile was dose responsive (0.05, 0.25 and 0.375 mg/kg), delivering oral-superior pulmonary anti-fibrotic activity with an equivalent delivered lung Cmax (QD inhaled 0.375 mg/kg versus BID oral 60 mg/kg). Possibly assisting this improvement, the infrequent high inhaled dose also improved bleomycin-challenged animal weight gain to levels equivalent to sham. By comparison, BID oral weight gain was substantially less than controls, suggesting a negative health impact on oral administered animals combating fibrosis. Both oral and inhaled administration exhibited anti-inflammatory activity, with oral achieving significance. In summary, inhalation (short-duration nintedanib lung Cmax without high local or systemic AUC) was well-tolerated and was effective reducing bleomycin-induced pulmonary fibrosis.

1. Introduction

Idiopathic Pulmonary Fibrosis (IPF) is a chronic lung disease resulting in progressive loss of lung function [1]. IPF is the most common form of interstitial lung disease with an estimate of 2.8–9.3 cases per 100,000 per year in Europe and North America [2]. IPF is widely considered the result of repetitive micro-injuries to the alveolar and peripheral airway epithelium leading to inadvertent activation of fibroblasts, destruction of the alveolar barrier and deposition of scar tissue [3].

In 2014, oral nintedanib (Ofev®) became one of two marketed drugs for the treatment of IPF. In 2019, Ofev was further approved for the treatment of Systemic Sclerosis-Associated Interstitial Lung Disease (SSc-ILD) and in 2020, Ofev was approved for Chronic Fibrosing Interstitial Lung Diseases with a Progressive Phenotype (Chronic Fibrosing ILD with a Progressive Phenotype). Nintedanib is a small molecule inhibitor of multiple tyrosine kinases including platelet-derived growth factor, vascular endothelial growth factor and fibroblast growth factor [4]. Inhibition of these kinases by nintedanib exerts a therapeutic benefit by interrupting different cellular processes that lead to lung fibrosis including the proliferation and transformation of fibroblasts to collagen producing myofibroblasts [5–7]. One phase 2 and two phase 3 IPF registration trials confirmed that oral nintedanib reduced annual forced vital capacity (FVC) decline [8,9]. The two additional phase 3 studies in SSc-ILD [10] and Chronic Fibrosing ILD with a Progressive Phenotype [11] showed similar results.

While oral nintedanib meaningfully slows IPF disease progression, nintedanib treatment induces well-described adverse effects (AE), most

* Corresponding author. Tranent, Charles River Laboratories, Elphinstone Research Centre, EH33 2NE, UK.
E-mail address: Mary.mcelroy@crl.com (M.C. McElroy).

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notably diarrhoea (55%, 62% and 63%, respectively for the phase 2 and two phase 3 studies) and increased aminotransferase levels at least three times the upper limit of the normal range (7.1%, 4.9% and 5.2%, respectively) [8,9]. Most of these adverse effects are managed with dose reduction, interruption or discontinuation protocols [Ofev Label]. In these studies, less than 5% of patients discontinued study medication. In 2017, the Ofev label was updated to include new risks including hyperbilirubinemia, jaundice, and the drug-induced liver injury. A similar adverse event profile was observed in the two phase 3 SSc-ILD [10] and Chronic Fibrosing ILD with a Progressive Phenotype [11] studies.

As demonstrated in the nintedanib phase 2 study (and administered in phase 3), only the highest oral dose was effective slowing annual FVC decline (150 mg BID). Given safety concerns, further oral dose escalation for potential additional efficacy was not possible. Complicating matters, first-pass metabolism and safety-driven dose-reduction/stoppage protocols further reduce the delivered lung dose and interrupt required maintenance therapy [Ofev label]. To address these shortcomings and maximize its potential, nintedanib was reformulated as a solution for nebulization and inhaled, direct-lung administration. Because inhalation broadly avoids the gastrointestinal tract and first-pass metabolism, this approach is anticipated to circumvent oral-associated side effects. Moreover, with reduced side effects, inhalation also holds promise to enable dose escalation for additional IPF efficacy while remaining below the oral systemic safety threshold.

From quick induction of pro-fibrotic pathways, bleomycin-induced lung fibrosis is one of the most common IPF models used [12,13]. Bleomycin causes direct epithelial injury and inflammation, and produces fibrotic lesions [14,15]. Clinically approved drugs such as nintedanib have been shown to reduce fibrosis in this model [6,7], and pathways driving bleomycin-induced fibrosis overlap with pathways in human IPF lungs [16,17]. Most studies are performed after a single bleomycin challenge (one injury event). However, this method produces a heterogeneous injury with uneven distribution of pulmonary fibrotic foci. Herein, we also describe a multi-challenge bleomycin approach to better model repetitive lung injury, fibrotic lesions in all lung lobes, and hence reduced bias during histology assessment [18,19].

Using this multi-challenge model, the objective of this study was to dissect the nintedanib properties required for anti-fibrotic activity in the lung and predict the effectiveness of inhaled therapy in treating IPF. Results herein indicate inhaled pharmacokinetics (short-duration nintedanib lung exposure in the absence of high local and systemic area under the curve; AUC) are well-tolerated and effectively reduce bleomycin-induced pulmonary fibrosis.

2. Materials and methods

2.1. Materials

Nintedanib esylate, provided by Fermion Oy (Espoo, Finland) and an alternative, proprietary nintedanib salt was provided by Avalyn Pharma. Bleomycin sulphate was obtained from Apollo Scientific Stockport, Cheshire, UK. Bleomycin was formulated in sterile saline. For oral dose solutions, nintedanib esylate was prepared on a weekly basis in Milli-Q ultra-pure water. Inhaled dosing solutions for intratracheal aerosol (IT) and oropharyngeal (OP) administration were prepared in 1.5% propylene glycol and 0.4% sodium chloride in water. IT dosing was performed using a Penn Century Aerosolizer® device (Wyndmoor, Pennsylvania). All other reagents were obtained from Sigma Aldrich. Trans-4-Hydroxy-L-proline (Hydroxyproline) and Trans-4-Hydroxy-L-proline-2,5,5-d3 (Hydroxyproline-d3) were obtained from Sigma-Aldrich (UK). Acetonitrile (HPLC grade) and Formic Acid (analytical grade) was obtained from Fisher Scientific (Loughborough). Deionised water was obtained in-house.

2.2. Animals, housing and dosing

This study was conducted in three phases; a pharmacokinetic study to establish lung exposure after nintedanib dosing by both IT and OP aspiration, a three-part tolerability study to establish critical inhaled tolerability parameters in bleomycin-challenged animals, and the main bleomycin efficacy study to compare the efficacy of inhaled and oral nintedanib. For all phases, rats were housed at 21 °C ± 2° and 40–70% humidity on a 12 h light-dark cycle and had free access to food and water according to standard operating procedures at CRL Edinburgh, UK. All animals were microchipped and in-life data collected using Provantis™ 10 (Instem LSS, Staffordshire, UK).

2.3. Nintedanib pharmacokinetic studies

To mimic inhalation, twenty-one Male SD rats with an average weight of 265 ± 16 g at start of dosing were administered nintedanib by IT and OP aspiration as described [20]. Briefly, OP administered rats were lightly anesthetised with isoflurane delivered in an inhalation chamber and nintedanib formulation (using pipette) was placed in the distal part of the oropharynx while the nose was gently closed [20]. For IT administration, rats were lightly anesthetised and a Penn Century Aerosolizer was used to deliver nintedanib formulation. Both OP and IT nintedanib were administered at a fixed volume (100 μL/rat) at approximately 0.1 and 0.5 mg/kg nintedanib. Lung and blood samples (for plasma) were taken at 2 min, 10 min, 20 min, 60 min and 240 min post dose. Samples were analysed by MicroConstants, Inc (San Diego, USA). IT and OP pharmacokinetic parameters were determined using the linear trapezoidal method. Oral pharmacokinetics were determined using Phoenix Winnonlin.

2.4. Tolerability studies

Tolerability studies were conducted in three parts to establish experimental conditions that would allow daily inhaled nintedanib administration for 20 consecutive days in the follow-on main efficacy study. These tolerability studies were considered necessary because the vehicle and test item were administered to an inflamed lung and the processes involved (rather the test item) may confound results. The tolerability studies were performed in a stepwise manner with a small number of bleomycin challenged animals (1 mg/kg in 100 μL). The final design of the efficacy study was based on data from the tolerability studies and consequently there were no premature inhaled decedents in the main efficacy study. Protocol details for the tolerability studies are summarised below and in the supplemental section.

Part 1 was designed to determine whether direct dosing to the lung was well-tolerated during the acute phase of bleomycin challenge. Vehicle or nintedanib formulations (0.25 mg/kg and 0.5 mg/kg) were administered by either OP aspiration or IT administration from Day 8 to Day 10 following bleomycin challenges (5 occasions, on Days 1, 2, 3, 6 and 7).

Part 2 was based upon Part 1 observations that OP was better tolerated than IT and 5 bleomycin challenge occasions was not well-tolerated. With this, Step 2 was designed to determine OP vehicle tolerability following 4 bleomycin challenges (Days 1, 2, 3 and 6) with vehicle administration starting after either one (Day 8 to Day 10) or two (Day 9 to Day 11) post-bleomycin recovery days.

Part 3 was based upon Part 2 observations that vehicle administration on Day 8 (i.e. one recovery day) following four bleomycin challenge occasions was well tolerated. With this, Part 3 was designed to determine OP nintedanib tolerability following bleomycin challenge (4 occasions on Days 1, 2, 3 and 6) with nintedanib administration starting after either one (Day 8 to Day 10) or two (Day 9 to Day 11) post-bleomycin recovery days.

All tolerability study groups consisted of three animals, with tolerability based on clinical signs and body weight changes.

Table 1
Main efficacy study design.

Group number	Route	Bleomycin (mg/kg)	Treatment	Regimen Day 8 to Day 27	Dose (mg/kg)
1	Oral	0	Vehicle	BID	0
2	OP	0	Vehicle	QD	0
3	Oral	1	Vehicle	BID	0
4	OP	1	Vehicle	QD	0
5	Oral	1	Nintedanib	BID	60
6	OP	1	Nintedanib	QD	0.05
7	OP	1	Nintedanib	QD	0.25
8	OP	1	Nintedanib	QD	0.375

Bleomycin and vehicle were administered by OP aspiration on Days 1, 2, 3 and 6. Drug treatments were initiated on Day 8 and continued daily by OP aspiration (inhaled) or twice-daily by oral until Day 27. BID: twice daily, QD: once daily, OP: oropharyngeal aspiration, Oral: oral gavage. 10 animals per group.

2.5. Main bleomycin efficacy study

The main efficacy study consisted of 7 treatment groups with 10 animals per group (Table 1). All animals were on-study for 28 days. There was one premature decedent in the oral nintedanib treatment group. Per the tolerability study results, bleomycin (1 mg/kg in 100 μL) or vehicle was administered to animals on four occasions by OP aspiration during Week 1 to induce lung fibrosis (Days 1, 2, 3 and 6). Treatments were started on Day 8 after a one-day bleomycin recovery period. Oral nintedanib was administered twice daily (BID) by oral gavage from Day 8 to Day 27 at 60 mg/kg. Inhaled nintedanib was administered once daily (QD) by OP aspiration from Day 8 to Day 27 at 0.05, 0.25 and 0.375 mg/kg. Target dose levels were based upon Day 1 animal weights. Body weights were recorded daily. Clinical signs were recorded before and after dosing on all study days.

On Day 28, animals were euthanized, and terminal lung and body weights were obtained. The right lung from each animal was inflated and then immersion fixed with 10% neutral buffered formalin and embedded in paraffin wax. Six sections were cut per lung for staining; three sections for the right caudal lobe and one section from the cranial, middle and accessory lobes. Sections from all six lung lobes were stained with either picrosirius red (PSR) for collagen evaluation or hematoxylin and eosin stain (H&E) for inflammation evaluation. Fibrosis was scored after complete lung section assessment using the Modified Ashcroft score (0–8 grades; [27]). Mixed cell inflammation, characterised by infiltrates of neutrophils, lymphocytes and macrophages, scored across the whole lung, was based upon a five-point scale (0 - absent, 1 -minimum, 2 - mild, 3 - moderate and 4 – severe; [21]. Both fibrosis and inflammation scoring were performed blind by a board-certified pathologist with no knowledge of the study conduct or other data. Each lung was assigned a final fibrosis or inflammation score based on the median of six values. Left lungs were snap frozen for hydroxyproline (HP) assessment.

2.6. Hydroxyproline analysis

Left lungs were finely chopped and homogenised in a 10x volume of distilled water using a hand-held homogeniser (IKA T10, Model T10 B S002). Duplicate samples were then hydrolysed in 0.1 M HCl at 120 °C for 6 h. Duplicate hydrolysed lungs samples per left lung lobe were assessed for HP concentration [22]. Recovery was confirmed using an internal standard and quality control standards were run with each assay and determined within the 20% acceptance criteria (accuracy). The HP value from each lung was assessed from two independent acid hydrolysate samples. HP data are expressed as μg hydroxyproline/mg lung.

2.7. Statistical analysis

Body weights, lung-to-body weight ratio and HP data are presented and means and standard error of the means (SEM). One-way analysis of variance (ANOVA) was performed for the following comparisons; vehicle control versus bleomycin/vehicle and bleomycin/nintedanib groups and bleomycin/vehicle versus bleomycin/nintedanib groups. Dunnett's multiple comparison were performed as the post-test analysis. Histology scores are presented as the median and 95% confidence interval and comparisons were performed using a non-parametric ANOVA or Mann-Whitney test. All statistical analyses were performed using Nevis or GraphPad Prism (Version 8).

Table 2
Nintedanib pharmacokinetics following oral and inhaled administration.

			Oral (mg/kg)	Inhaled (mg/kg)			
			Gavage	IT[a]		OP[b]	
			60	0.5	0.1	0.5	0.1
Plasma	$AUC_{0\text{-}t}$	mg·hr/L	0.876	0.009	0.002	0.003	0.001
	Cmax	μg/mL	0.340	0.053	0.012	0.006	0.002
	Tmax	Min	120	0	0	0	0
	$T_{1/2}\alpha$	Min	n/a	5.2	5.4	5.0	5.5
	$T_{1/2term}$	Min	100	96	83	170	174
Lung[c]	$AUC_{0\text{-}last}$	mg·hr/kg	175.0	48.1	9.7	16.3	3.1
	Cmax	μg/g	13.2	24.5	5.3	19.1	2.8
	Tmax	Min	120	0	0	0	0
	$T_{1/2}\alpha$	Min	n/a	22	27	14	42
	$T_{1/2term}$	Min	282	244	133	106	126

[a] IT: Intratracheal.
[b] OP: Oropharyngeal aspiration.
[c] Lung: Whole lung homogenate.

2.8. Ethics statement

Animal housing and care were in compliance with UK Home Office Code of Practice for the Housing and Care of Animals Bred, Supplied or Used for Scientific Purposes. All animal experiments were performed in accordance with the UK Animals (Scientific Procedures) Act, 1986, under the authority and controls of a project licence held at the Charles River Edinburgh facility. All protocols were also reviewed by an internal ethical committee for scientific rational and welfare management prior to study commencement.

3. Results

3.1. Inhalation delivers oral superior nintedanib lung Cmax with substantially lower systemic exposure

To choose the optimal inhaled delivery method, a pharmacokinetic study comparing IT and OP administration was first performed. Results indicate that both IT and OP efficiently delivered nintedanib to the rat lung (Table 2), with IT delivering an AUC and Cmax ~3-fold and ~1.5-fold greater than OP for 0.1 and 0.5 mg/kg. Focusing on OP delivery (selected as the most well-tolerated method to deliver inhaled nintedanib in the main efficacy study), nintedanib lung tissue AUC indicated that 0.1 and 0.5 mg/kg OP inhaled nintedanib delivered a 3.1 and 16.3 mg·h/kg lung tissue AUC, respectively, and a 2.8 and 19.1 μg/g lung tissue homogenate Cmax.

Compared to lung levels following the 60 mg/kg PO dose (Table 2 [18,19], data show that very small inhaled nintedanib dose levels delivered similar lung Cmax (Table 2). Plasma pharmacokinetic results show that the 0.1 and 0.5 mg/kg OP inhaled nintedanib delivered a 0.001 and 0.003 mg h/L plasma AUC and a 0.002 and 0.006 μg/mL plasma Cmax, respectively. Compared to 60 mg/kg dose delivered plasma levels, these inhaled nintedanib doses delivered very low systemic levels.

Table 3
Tolerability phase: Summary of objectives, design, and outcomes.

Tolerability Phase Objectives	Bleomycin Challenge (1 mg/kg)	Vehicle/ Nintedanib Administration	Main outcome
Part 1 To determine the tolerability of dosing nintedanib directly to the lung following bleomycin challenge. Nintedanib administered by either OP or IT administration	Days 1, 2, 3, 6 and 7 100 L by OP aspiration	OP and IT administration of vehicle, nintedanib at 0.25 mg/kg and 0.5 mg/kg, Day 8 to Day 11	Animals tolerated vehicle administration and low dose nintedanib while high dose nintedanib induced additional clinical signs not considered sustainable in the follow-on efficacy study with 20 days of consecutive dosing.
Part 2 To determine the tolerability of dosing nintedanib vehicle directly to the lung, by OP aspiration, in a four dose bleomycin challenge model. Evaluation of one or two day recovery period before the initiation of vehicle dosing	Days 1, 2, 3 and 6 100 µL by OP aspiration	OP aspiration, 100 µL/on Day 8 (one day recovery) or Day 9 (two days recovery) to Day 11.	Animals tolerated vehicle administration equally well with a one or two day recovery period.
Part 3 To determine the tolerability of lung nintedanib in the four challenge bleomycin model with one or two days recovery	Days 1, 2, 3 and 6 100 µL by OP aspiration	OP aspiration, 100 µL on Day 8–11, Nintedanib at 0.25 mg/kg and 0.5 mg/kg. High dose nintedanib (0.5 mg/kg) also evaluated with a two day recovery period.	Nintedanib at 0.25 mg/kg was well tolerated but there was some concern that 0.5 mg/kg would not be tolerated for 20 days dosing in the main efficacy study. Therefore, high dose reduced to 0.375 mg/kg.

The tolerability phase was conducted in a step-wise manner prior to the Main Efficacy study. Small number of animals were used per group (n = 3, supplemental). Tolerability was assessed on the bases of clinical signs and body weight changes; animals were humanely killed on Day 9 or Day 11.
OP – Oropharyngeal aspiration. IT – Intratracheal.

3.2. Multi-challenge bleomycin model is well-tolerated for inhaled nintedanib delivery

Part 1 clinical sign and body weight results indicate multi-challenge bleomycin animals were more tolerant to OP inhaled administration than IT (Table 3). However, neither route was well-tolerated over three consecutive dosing days, suggesting fewer bleomycin challenges may be required for animals to tolerate daily inhaled administration over the study period.

Part 2 reduced the number of bleomycin challenges from five (Days 1, 2, 3, 6 and 7) to four (Days 1, 2, 3 and 6) and tested if an additional post-bleomycin recovery day was necessary for OP vehicle tolerability (Table 3). Results demonstrated that both a one-day recovery (Day 7 recovery; dosed on Days 8 to Day 10) and a two-day recovery (Day 7 and Day 8 recovery; dosed on Days 9 to Day 11) were well-tolerated (no adverse clinical signs or body weight changes).

To assess techniques learned during Part 1 and Part 2, both 0.25 and 0.5 mg/kg OP nintedanib were administered following four bleomycin challenges (Days 1, 2, 3 and 6), and both a one-day recovery (Day 7 recovery; dosed on Days 8–10) and two-day recovery (Day 8 recovery; dosed on Days 9 to Day 11) were assessed. Results indicate that a one-day recovery was long enough to initiate OP nintedanib administration. Further, while 0.25 mg/kg OP nintedanib was well-tolerated, clinical signs and weight gain observatons suggested 0.5 mg/kg was less well-tolerated (Table 3). Based on this information, 0.25 mg/kg was selected as the mid-dose and as a precaution the high dose was reduced to 0.375 mg/kg; 0.05 mg/kg was selected as a low dose delivering both Cmax and AUC well-below PO delivered lung levels. Table 4 and Fig. 1 compare lung and plasma Cmax and AUC for these various delivered dose levels.

Table 4
Bleomycin study dose levels and comparative inhaled and oral nintedanib exposures.

		Lung Cmax		Lung AUC	
Route	Dose (mg/kg)	(µg/g)	Fold vs. Oral	(mg·hr/kg)	Fold vs. Oral
Oral	60	13.2	–	175.0	–
OP[a]	0.375	14.3	1.1	12.2	0.07
OP[b]	0.25	9.6	0.7	8.2	0.05
OP[b]	0.05	1.9	0.1	1.6	0.01
		Plasma Cmax (µg/mL)	Fold vs. Oral	Plasma AUC (mg·hr/L)	Fold vs. Oral
Oral	60	0.3400	–	0.8760	–
OP	0.375	0.0048	0.014	0.0030	0.0034
OP	0.25	0.0032	0.009	0.0015	0.0017
OP	0.05	0.0006	0.002	0.0003	0.0003

[a] Oropharyngeal (OP), PO-equivalent lung Cmax and lower AUC.
[b] Oropharyngeal (OP), Dose de-escalated, PO-inferior lung Cmax and AUC.

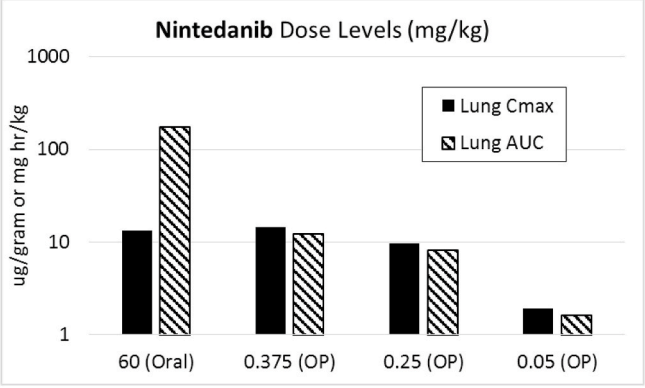

Fig. 1. Nintedanib study dose levels and comparative inhaled (OP) and oral lung exposures.

3.3. At oral-equivalent lung Cmax, inhalation delivered improved anti-fibrotic activity and animal health

3.3.1. Body weights

Compared to vehicle controls, group mean body weights were significantly reduced after bleomycin challenge (Fig. 2 and Supplement – group mean body weights demonstrate statistically significant differences between groups). This body weight decrease during week 1 was expected and due to a reduction in food consumption in bleomycin challenged animals (unpublished data). Administration of QD low- and mid-dose inhaled nintedanib did not affect body weight gain in comparison with bleomycin challenged animals treated with vehicle. However, QD high dose inhaled nintedanib animals exhibited improved body weight gain similar to vehicle sham controls (Fig. 2A and B). Administration of BID oral nintedanib at 60 mg/kg significantly reduced body

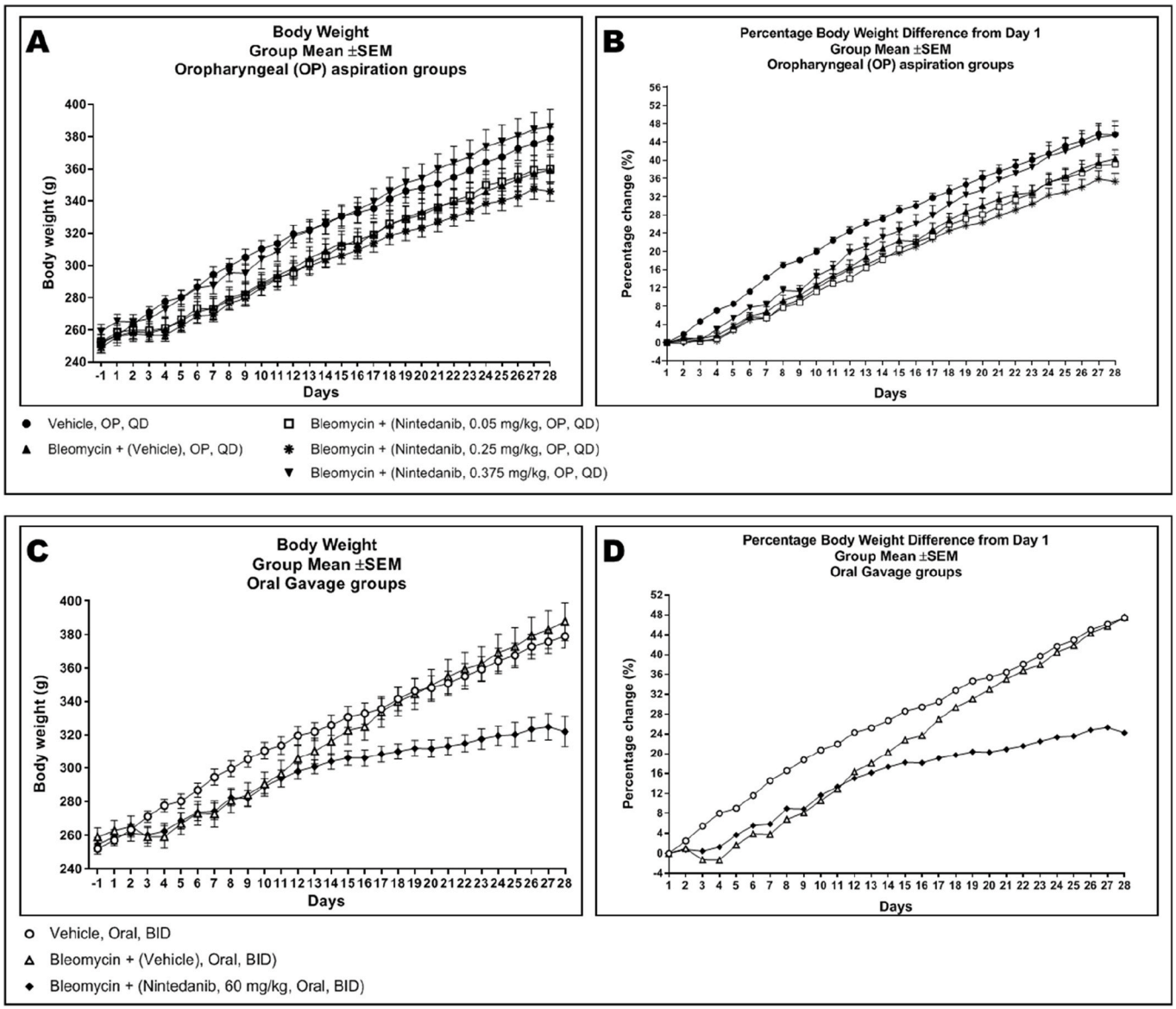

Fig. 2. Body weight changes (absolute and percentage from Day 1) for rats challenged with saline or bleomycin and treated with either inhaled nintedanib (A, B) or oral nintedanib (C, D) A & B: Bleomycin reduced the extent of body weight increase during dosing (Days 1–7) compared with vehicle controls and thereafter body weight increased at a similar rate to vehicle controls. Bleomycin/inhaled nintedanib groups followed a similar pattern to bleomycin/inhaled vehicle group although there was increased body weight observed with inhaled nintedanib at the high dose (0.375 mg/kg). C & D: Bleomycin/oral nintedanib reduced the extent of body weight gain for the study duration. Data presented as mean/SEM, data analysed by parametric ANOVA, Dunnett's post-test, n = 10 in all groups apart from oral nintedanib group where n = 9.

weight gain in comparison to bleomycin-exposed animals treated with vehicle (Fig. 2C and D).

3.3.2. *Lung weight-to-body weight ratios*

Group mean lung-to-body weight ratios at 28 days were significantly increased in bleomycin challenged animals treated with vehicle (Fig. 3). There was a dose-dependent reduction in lung-to-body weight ratio following inhaled nintedanib treatment. Oral nintedanib also reduced group mean lung-to-body weight ratio. While trends existed, neither inhaled nor oral results were significantly different than bleomycin challenged animals treated with vehicle.

3.3.3. *Fibrosis and inflammation score*

Group median fibrosis scores were increased in bleomycin challenged animals treated with vehicle (Figs. 4 and 5). There was a dose-dependent reduction in the median fibrosis score following inhaled nintedanib treatment (Fig. 5A). In particular, group median fibrosis score for bleomycin-challenged animals treated with vehicle was 2.5 while for OP nintedanib (0.375 mg/kg) the group median score was 1.5 ($p < 0.05$). Fibrosis score reduction in OP nintedanib-treated animals was observed microscopically as lower numbers and smaller individual sized fibrotic foci (Fig. 4B) compared with vehicle controls (Fig. 4A). Group median fibrosis score was also reduced in the oral nintedanib group, although the difference was not statistically significant (Fig. 5B). While not achieving significance, group median inflammation scores for inhalation treated animals exhibited a dose-responsive trend reducing the greatest inflammatory response to the upper range exhibited in the inhaled vehicle sham control (Fig. 6A). Group median inflammation score was significantly reduced in the oral nintedanib group (Fig. 6B).

3.3.4. *Hydroxyproline*

Group mean HP (μg/mg lung) was increased in bleomycin

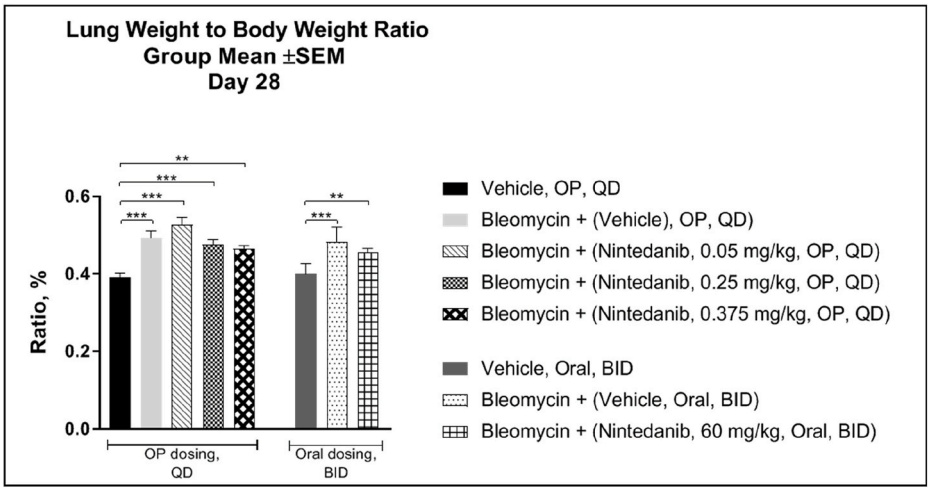

Fig. 3. Lung-to-body weight ratio for rats challenged with saline or bleomycin and treated with either inhaled or oral nintedanib. Group mean lung-to-body weight ratios were increased in all bleomycin/vehicle animals compared with sham/vehicle controls. There was a slight dose-dependent decrease in lung to body weight ratios in inhaled nintedanib groups (0.05 mg/kg to 0.375 mg/kg) (not statistically significantly). Group mean lung-to-body weight ratio of oral nintedanib (60 mg/kg, BID) was also non-significantly reduced compared with bleomycin/vehicle. **P < 0.01, ***P < 0.005, n = 10 in all groups apart from oral nintedanib group where n = 9.

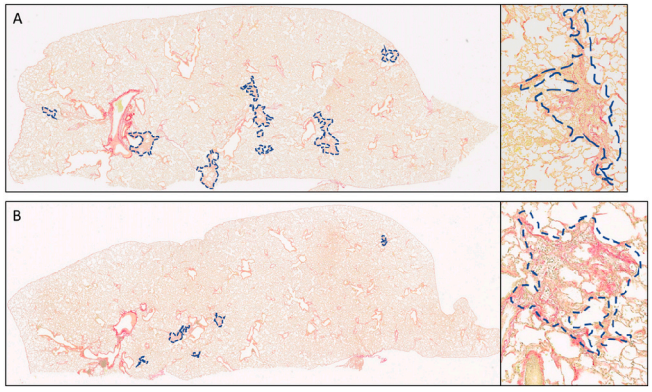

Fig. 4. Representative images of PSR stained lung sections from animals challenged with bleomycin and treated with inhaled vehicle (A) and inhaled nintedanib (0.375 mg/kg). Areas of fibrosis are identified by blue dashed line. Nintedanib was associated with lower numbers and smaller foci of fibrosis resulting in a lower median fibrosis score for animals in this group compared to inhaled vehicle treated controls.

challenged animals treated with vehicle following both inhaled and oral administration (Fig. 7a). Group mean HP content was also increased in bleomycin challenged animals treated with inhaled nintedanib, but there were no statistical differences or dose-related trends compared with bleomycin challenged animals treated with vehicle. In the oral nintedanib dose group, group mean HP was significantly decreased compared with bleomycin challenged animals treated with vehicle. In a separate experiment, HP was measured over the same duration in sham animals treated with oral vehicle. Group mean results showed 1.54 μg HP/mg lung at start of vehicle treatment compared to 2.54 μg HP/mg lung on Day 28, a 165% increase (Fig. 7b). This increase in HP illustrates the potential for baseline changes associated with growth in vehicle control animals and the importance of considering growth effects when interpreting HP levels between animals growing at different rates. In the light of these baseline changes and to provide insight into differential growth effects on the HP results, the HP data in Fig. 7a were corrected with each animal's respective lung-to-body weight ratio (Fig. 3). While the data remain insignificant, a dose-responsive trend emerged in inhaled nintedanib treated animals and the strong effect observed in oral treated animals was reduced (Fig. 7c).

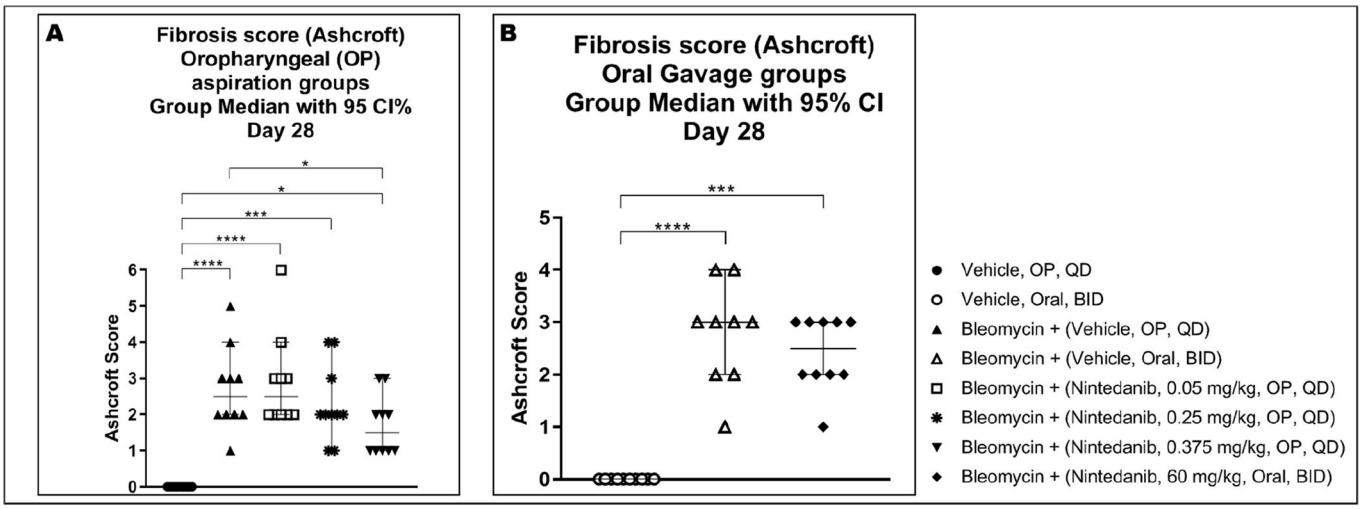

Fig. 5. Lung fibrosis at Day 28 for rats challenged with saline or bleomycin and treated with inhaled nintedanib at 0.05, 0.25 and 0.375 mg/kg (A) or oral nintedanib at 60 mg/kg (B). Inhaled nintedanib resulted in a dose-dependent reduction in median fibrosis score (A). High dose inhaled nintedanib (0.375 mg/kg) was significantly reduced compared with bleomycin/inhaled vehicle (P < 0.05). Median fibrosis scores were not significantly reduced in the oral nintedanib group compared with bleomycin/oral vehicle (B). Data presented as median/95% CI, n = 10 in all groups apart from oral bleomycin/vehicle group where n = 9. *P < 0.05, ***P < 0.001, ****P < 0.0001.

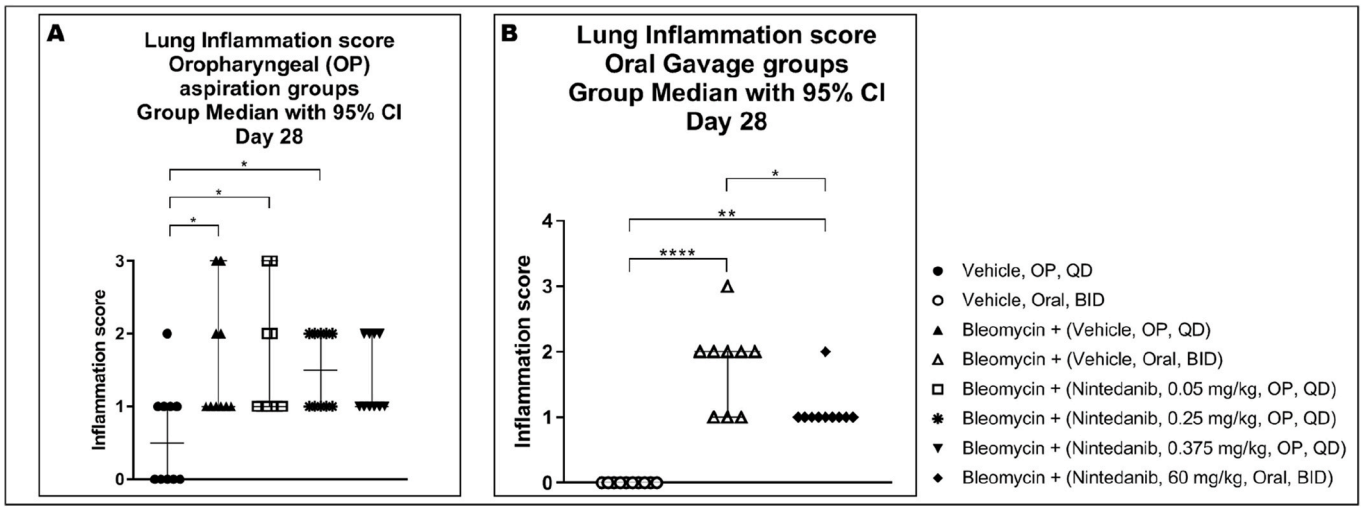

Fig. 6. Lung inflammation at Day 28 for rats challenged with saline or bleomycin and treated with inhaled nintedanib at 0.05, 0.25 and 0.375 mg/kg (A) or oral nintedanib at 60 mg/kg (B). Inhaled vehicle delivered to saline or bleomycin exposed animals exhibited a small increase in lung inflammation at Day 28 compared to corresponding oral controls. The extent of lung inflammation was slightly reduced in the mid- and high-dose inhaled nintedanib groups compared with bleomycin/inhaled vehicle controls (A). Administration of oral nintedanib significantly reduced lung inflammation in comparison with bleomycin oral vehicle controls (B). Data presented as median/95% CI, n = 10 in all groups apart from oral nintedanib group where n = 9. *P < 0.05, **P < 0.01, ****P < 0.0001.

4. Discussion

Oral nintedanib is one of two medicines approved for the treatment of IPF (the other being Esbriet®; oral pirfenidone). Similar between the two treatments is their need for large oral doses to deliver a pulmonary therapeutic effect, and their associated gastrointestinal and strong systemic side effects. While both medicines were approved to treat IPF, each approved dose was selected at their respective upper safety threshold, preventing demonstration of a maximum effect. Therefore, while oral efficacy has shown promise to extend life, continued side effects and a possible insufficient lung dose have limited their overall effectiveness. Given this promising start, a foundation has been established for development of alternative delivery methods reducing dose-limiting side effects and possibly improved therapeutic effect.

Oral administered nintedanib eliminates more quickly than it can accumulate in plasma (flip-flop pharmacokinetics) [23]. This phenomenon appears to be a combination of substantial nintedanib tissue partitioning and rapid metabolism. Such that, while intestinal absorption readily occurs, the combination of first-pass metabolism and rapid nintedanib partitioning into tissues results in low plasma levels that do not accurately represent oral bioavailability or systemic exposure. Moreover, it has been observed that nintedanib lung levels following oral administration are substantially higher than plasma levels [24], suggesting oral administration may be the preferred route for IPF efficacy. However, it has also been observed [30] that while oral nintedanib gains substantial access to the pulmonary compartment, achieved total lung levels may have limited access to the airway, alveolar epithelial surface and possibly underlying fibroblastic foci (key regions of IPF disease; [3]. From these observations, the data suggest that only a small portion oral nintedanib may be available for IPF efficacy and that this efficacy may be driven by short-duration nintedanib exposure, not time-over-AUC, per se.

To understand if oral delivered, lung-partitioned nintedanib is sufficient for anti-fibrotic activity in the bleomycin model, we utilized inhalation as a tool to dissect the partitioned lung dose into: 1. short-duration epithelial/underlying fibroblast exposure without large local or systemic AUC (inhaled; OP); and 2. longer-term, whole-body exposure capturing both this initial short-duration lung exposure and large local and systemic AUC (oral; PO).

Observations from the bleomycin model indicate QD inhaled nintedanib (0.05, 0.25 and 0.375 mg/kg) was dose responsive and significantly reduced pulmonary fibrosis [Fig. 5], with the highest dose improving animal weight gain to a level similar to sham animals treated with vehicle [Fig. 2B]. In contrast, BID oral nintedanib (60 mg/kg) was less efficacious and less well tolerated as assessed by the significant reduction in body weight gain [Fig. 2C and D]. Inhaled nintedanib treatment also showed a dose-responsive reduction in lung-to-body weight ratio while the single oral nintedanib dose effect was similar [Fig. 3]. These trends in lung-to-body weight ratio, while not statistically significant matched the histopathologic finding.

Hydroxyproline is a major component of collagen and is therefore used to quantitatively assess the extent of fibrosis in lung homogenates as an efficacy endpoint in preclinical fibrosis studies [13]. In this rat study, HP was increased in bleomycin challenged animals treated with vehicle. Interestingly, HP values from bleomycin animals treated with inhaled vehicle and nintedanib were similar; a result at apparent odds with histology and lung-to-body weight ratio trends. However, for interpretation it is important to consider that collagen (represented here as HP) is a structural component of the lung wherein the non-alveolar component has been estimated to be approximately 60% of total lung collagen [25]. Therefore, lung homogenate HP concentrations will be affected by changes in bleomycin-induced fibrosis and growth-induced structural changes. This point is particularly relevant in the rat bleomycin model where animals in different treatment and control groups grew at different rates. In fact, over the same duration, HP increased 165% in vehicle animals treated with oral vehicle (Fig. 7b). Because both histological findings (Figs. 4 and 5) and lung-to-body weight ratio trends (Fig. 3) support a positive inhaled treatment effect, we hypothesized that measured HP levels from these animals corresponded to a negating effect resulting from both treatment-reduced fibrosis and growth (Fig. 7c). Conversely, because oral treated animals grew poorly, we must ask if observed lung HP reduction was due to a treatment effect, reduced growth or both. To discern between these possible outcomes, we corrected the HP data for growth (Fig. 7c). Supporting this hypothesis, growth correction improved the inhaled treatment response correlating with histopathology and slightly reduced the oral response. Taken together, these results support HP as a specific marker for the extent of fibrosis. However, caution should be minded when interpreting data between study groups exhibiting differential growth.

Comparing delivered dose between the two regimens showed 60 mg/kg oral and 0.375 mg/kg inhaled (high dose) delivered an equivalent nintedanib lung Cmax. If only short-duration nintedanib exposure in the

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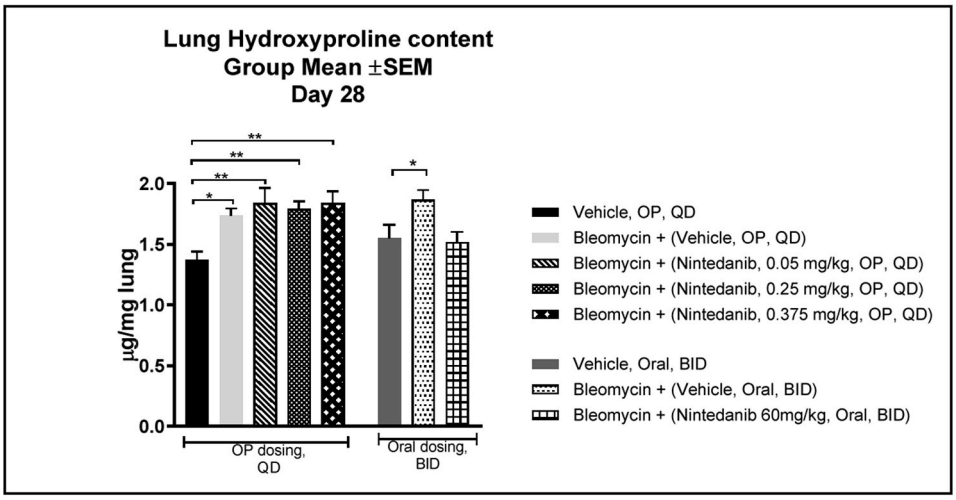

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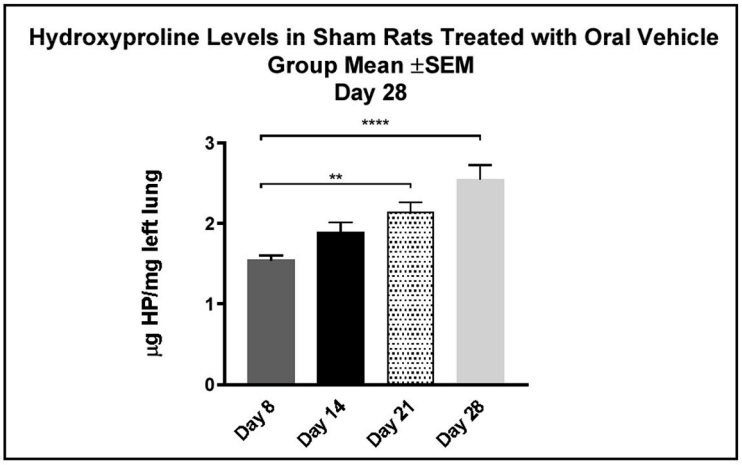

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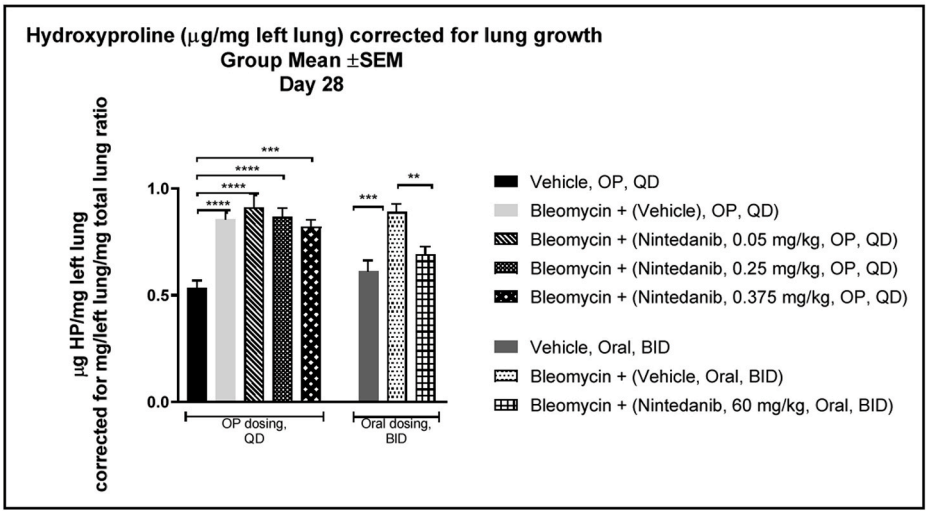

Fig. 7. (A) Group mean HP (µg/mg lung) was significantly increased in all bleomycin/vehicle animals compared with vehicle controls. µg HP/mg lung was not significantly different in bleomycin/inhaled nintedanib groups compared with bleomycin/inhaled vehicle. Oral nintedanib significantly reduced HP/left lung compared with bleomycin/vehicle controls. (B) HP levels in shame rats treated with oral vehicle as measured on Day 8, Day 14, Day 21 and Day 28 demonstrating a significant increase in group mean HP concentration between Day 8 versus Day 28 values. Data demonstrate baseline increase in lung HP associated with normal growth. (C) Group mean HP (µg/mg lung), corrected for lung growth indicated inhaled nintedanib reduced HP in a dose dependent manner compared with bleomycin/vehicle controls. In oral dosed animals, oral nintedanib significantly reduced HP/left lung compared with bleomycin/vehicle controls. Data shown as mean/SEM, (A and C) n = 10 in all groups apart from oral nintedanib group where n = 9, (B) n = 8–10 per group. *P < 0.05,**P < 0.01, ***P < 0.005, ****P < 0.001.

absence of a large local and systemic AUC was required for efficacy, then both groups were expected to show similar results. However, while both were effective, QD inhaled indicated a greater effect. Compared to BID oral, this observation may be explained by inhaled therapy exhibiting a greater body weight increase (i.e., better general animal health). Specifically, body weights of animals treated with inhaled nintedanib increased by approximately 44% (similar to sham animals treated with vehicle) compared to only 22% in oral treated animals (Fig. 2B vs Fig. 2D). In a related bleomycin study administering pirfenidone (another anti-fibrotic exhibiting similar gastrointestinal side effects; [26], oral administration also reduced animal health and therapeutic response. Together, these data suggest off-target effects or poor general health may reduce an animal's therapeutic response to bleomycin challenge.

Inhaled vehicle administration in sham control animals was associated with minor lung inflammation not seen in oral control animals. This low-level inflammation was not surprising given these twenty consecutive inhaled oropharyngeal doses may have caused irritation or deposited mouth and tracheal substances in the lung. This procedural inflammation may explain the observation that bleomycin exposed animals treated with vehicle exhibited a slightly greater inflammatory score compared with oral administered animals (Fig. 6A vs Fig. 6B). In both cases, inhaled and oral nintedanib administration reduced inflammation.

These data support that only infrequent, short-duration nintedanib lung exposure (in the absence of a large, oral-equivalent AUC) is required for anti-fibrotic effect. Because very small inhaled doses achieved this pharmacokinetic parameter while largely avoiding the GI tract, liver and systemic exposure, oral-observed side effects are predicted to be substantially reduced. With reduced side effects, the inhaled dose level may then be increased to test for additional efficacy. Although human studies are required to confirm whether these observations will translate to IPF, the promise for inhalation to reduce nintedanib side effects with possible treatment benefit should motivate such activities.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pupt.2020.101938.

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