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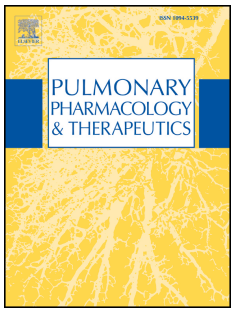

Use of animal models in IPF research

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Use of animal models in IPF research.

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Abstract

Idiopathic pulmonary fibrosis (IPF) is a fatal interstitial lung disease with a poor prognosis and limited treatment options. Many compounds have shown efficacy in pre-clinical models of this condition, but only Pirfenidone and Nintedanib have been approved for clinical use. It is widely accepted that the current animal models of IPF need to be improved and in this review we have critically evaluated the current state of play of preclinical models of IPF and discuss the challenges facing this field. The popular model of a single I.T. administration of bleomycin could be adapted to provide a more progressive fibrosis as is thought to occur in humans. Furthermore, currently the majority of new drugs are investigated in preclinical models of IPF are dosed using a prophylactic dosing regimen, whereas patients are almost always treated after the fibrosis is well established. Using a therapeutic dosing regimen in preclinical models would be a better way to establish potential efficacy of new drugs. The most popular endpoints examined in pre-clinical models of IPF are histological scoring and lung collagen content. However in IPF patients imaging and lung function tests are more commonly used as end points. We propose that examining more clinically relevant endpoints in pre-clinical models could also provide give a better indication of a compound's potential efficacy on endpoints measured in patients.

Keywords

Animal Models; IPF; Pre-clinical

Introduction

Idiopathic pulmonary fibrosis (IPF) is a progressive interstitial lung disease characterised by scarring of the lung tissue causing impaired gas exchange leading to symptoms such as dyspnoea, dry cough, and general fatigue. IPF is more prevalent in males than females and risk factors include increased age, a history of smoking and working in environments with poor air quality, such as mining [1-4].

There also appears to be a genetic component of IPF with variations of genes such as MUC5B and TOLLIP conferring a greater risk of developing IPF [3].

Despite the relatively recent approval of Pirfenidone and Nintedanib (approved in Europe in 2011 and 2015 respectively) which have both been shown to slow the progression of IPF in clinical trials [5,6] the prognosis of IPF is still very poor with approximately 50% of patients dying within 2-5 years of diagnosis [1, 2, 4]. Part of the reason for this poor prognosis is that the symptoms of IPF often don't present until the disease is at an advanced stage [2, 7], and also delays in diagnosis can occur as the symptoms of IPF are shared with several other respiratory disorders, and either a high resolution CT scan or lung biopsy are required to definitively diagnose IPF [8]. The fact that IPF can remain undetected for so long means that pharmacological intervention is often not introduced until the disease is already well established. In addition, the pathogenesis of IPF is not fully understood. However, it is currently thought that repeated lung injury or infection leads to an aberrant wound healing process which causes massive extra cellular matrix deposition and the scarring of lung tissue that is characteristic of IPF [9].

The incomplete understanding of the disease and lack of safe and effective treatment makes IPF a disease with considerable unmet need requiring novel approaches to treatment. To this end there has been a considerable amount of research in this area and a wide variety of different *in vivo* models have been used to investigate IPF over many years, but there remains no consensus on preclinical tests best used to model IPF and to have predictive value of clinical efficacy. Many different research groups have published work attempting to model IPF use different species, different fibrotic insults and different dosing routes and regimes to illicit a fibrotic response in the lungs of the animals. However, there is little consistency between laboratories regarding optimal protocols for these preclinical models.

The aim of this review is therefore to detail and compare the different *in vivo* models used to investigate IPF, to review the effects of different pharmacological classes of drugs evaluated in these

models and to critically discuss how these effects have translated into treatment for patients with IPF. Furthermore, we have suggested potential ways for improving the current preclinical models in IPF research.

Overview of current pre-clinical models of pulmonary fibrosis

Pulmonary fibrosis like features can be induced in experimental animals by very diverse agents, and these different agents can be administered via many different routes and using distinctive dosing regimens. We have summarised these different approaches in Table 1. Since the early 1970's when bleomycin was recognised as a drug able to induce pulmonary fibrosis as an unwanted effect in some patients, bleomycin has been the most widely used agent to induce pulmonary fibrosis in animals [10-12]. Bleomycin induces pulmonary fibrosis by production of DNA-cleaving superoxide and hydroxide free radicals which cause single and double stranded DNA breaks [13]. This damage preferentially occurs in the lungs because of low levels of bleomycin hydrolase, a bleomycin inactivating enzyme [14]. Bleomycin has been used in a variety of different species via a number of administration routes and regimes. Probably the most popular and best characterised animal model involves a single I.T. dose of bleomycin in rats or mice. This dosing regime leads to a neutrophil driven inflammatory response which lasts approximately 7-10 days, which then transforms into a fibrotic response from approximately Day 14 [15,16]. Terminal investigations are typically carried out on Day 21 or 28 after the initial bleomycin dose. This model has provided valuable insight into the process of pulmonary fibrosis, for example elucidation of the importance of the role of TGF-β in the development of IPF [17]. Furthermore, this model was used in the pre-clinical development of Nintedanib [18]. However, this bleomycin model does have several limitations with the most concerning being the fact that the fibrosis has been shown to spontaneously resolve beyond 28 days [15,19]. Since fibrosis does not resolve in most patients with pulmonary fibrosis, the use of this model is limited to the evaluation of the efficacy of potential anti-fibrotic compounds prophylactically. Many other bleomycin dose routes and regimes have been used to try and better model the progressive nature of IPF with repeated lower doses of bleomycin delivered both locally

to the lungs [20,21] or systemically [22,23]. For example, with a lower repeated I.T. dose of bleomycin, Peng et al [21] were able to show fibrotic like changes in the lungs up to 24 weeks after the initial bleomycin dose. However, the length of time that this model takes to develop and the significant mortality observed with this dosing regimen are limitations of this dosing regimen [21].

As well as bleomycin several other fibrotic agents have been used with varying degrees of success. Fluorescein isothiocyanate (FITC) has been shown to cause fibrosis like changes over a similar time scale to bleomycin. FITC is a fluorescent molecule with the advantage that molecular deposition in the lung can be easily visualised [2, 24, 25]. The disadvantages of this model are that certain histopathological features, such as fibroblast foci, are not observed, and there is a large amount of variation in the fibrotic response generated by different batches of FITC [2].

Administration of silica or asbestos to the lungs has also been shown to illicit persistent fibrosis like changes [18, 26-28]. However, as with FITC, there are histopathological features that are missing with respect to the histopathological features seen in patients with IPF. The pulmonary conditions that develops following exposure to silica or asbestos are more akin to the human disease of silicosis or asbestosis respectively rather than pulmonary fibrosis.

Systemic delivery of paraquat also produces fibrosis like changes in the lungs of animals [29-31]. However, paraquat is a broad spectrum herbicide that has been shown to cause necrosis in organs other than the lungs (such as kidney and liver) which can cause significant mortality and thus provides considerable challenges as a model associated with fibrosis-specific changes to the lungs [31].

Certain non-chemical *in vivo* approaches can induce pulmonary fibrosis. An exposure of the thorax of animals to radiation has been shown to result in persistent fibrosis like changes in the lungs [32,33]. However, as the fibrosis takes a long time to develop and the cost of the irradiating equipment can be high, this model is very expensive to run. The pulmonary response to radiation exposure also

lacks some of the histopathological features seen in patients with IPF such as complex fibroblast foci [1,2].

Intra-tracheal delivery of transgenes using viral vectors has shown some success, with delivery of genes for factors such as TGF-β1 and IL-1β eliciting fibrotic responses in the lungs of animals [34-36]. Although delivery of these transgenes leads to a progressive and persistent fibrosis (up to 9 weeks in certain studies [34,35], the downside of such models can be that the animals may have an immune response to the viral vector, and the expression of the transgenes is much higher than physiologically possible. Therefore, the pathways through which the transgene products work will be massively over activated which question the relevance of this approach as a model to test potential novel anti-fibrotic compounds.

Another animal model of pulmonary fibrosis that has been utilised is a humanized mouse model of IPF, where cells from human patients of IPF are injected into SCID mice. Infusion of human fibroblasts has been shown to lead to increases in fibrosis seen histologically and upregulation of pro-fibrotic genes such as TGF-β1 and surfactant proteins at 63 days post infusion [37]. However, the main issue with this model is the availability of cells from human patients with IPF, and also the length of time to generate fibrosis in this model.

As well as many different agents, and dosing regimens, many different species have been used to model IPF. The most common are mice and rats due to the ease of handling, availability of reagents, their well characterised immune systems, and the possibility of utilising transgenic models in mice [38,39]. There are however, considerable differences in the structure and physiology of rat and mouse lungs compared to human lungs [40-42]. Furthermore, one particular aspect of human IPF that is impossible to model in rats and mice is cough. Although there is some controversy over whether mice and rats have the ability to cough it is clear that at best they have a greatly reduced sensitivity to common tussive agents such as citric acid or capsaicin that induce cough in patients with IPF [43,44] . In contrast, guinea pigs have a well characterised and generally robust cough

response and so are the animal of choice for studying cough [43,44]. As chronic cough is a major symptom of IPF affecting over 80% of patients [45,46], some groups have used induced pulmonary fibrosis in guinea pigs in order to be able to investigate the cough caused by pulmonary fibrosis. Another feature of rats and mice that may be a limiting factor in their usefulness for the study of pulmonary fibrosis is their small size. Using larger animals to model pulmonary fibrosis can have a number of advantages. For example the use of the sheep [16] has permitted the use of a fibre-optic bronchoscope to deliver bleomycin to a specific segment of lung. This specific delivery of bleomycin means that the overall burden on the animal is much lower as the remaining lung is easily able to compensate for the damage in the segment administered the bleomycin. Due to this compensation of the rest of the lung a higher relative dose of bleomycin can be used in the target segment to induce a more severe fibrosis that may be more akin to what is seen in advanced pulmonary fibrosis patients, a feature of the clinical disease that is difficult to model in animals where the whole lung is exposed to the fibrotic agent leading to significant levels of tissue damage raising a large number of animal welfare issues.

Table 1: An overview of *in vivo* animal models used to study IPF

Species	**Fibrotic Agent**	**Route and regime**	**Ref**
Mouse	Bleomycin	Single I.T. dose	18, 21,47-50
Mouse	Bleomycin	Repeat I.T. dose	20,21
Mouse	Bleomycin	Single I.N. dose	51
Mouse	Bleomycin	Repeat I.N. dose	52,53
Mouse	Bleomycin	Repeat I.P. dose	22,54
Mouse	Bleomycin	Repeat O.A. dose	53,55
Mouse	Bleomycin	Repeat I.V. dose	23
Mouse	Bleomycin	Single I.V. dose	56
Mouse	Bleomycin	S.C. osmotic mini pump	57,58
Rat	Bleomycin	Single I.T. dose	18,59-62
Rat	Bleomycin	Repeat I.N. dose	53
Rat	Bleomycin	Repeat O.A. dose	53
Sheep	Bleomycin	Segmental lung instillation	63
Dog	Bleomycin	Repeat I.V. dose	10
Guinea Pig	Bleomycin	Single I.T. dose	64
Mouse	Fluorescein isothiocyanate (FITC)	Single I.T. dose	24,25
Mouse	Silica	Single I.T. dose	18
Mouse	Silica	Repeat O.A. dose	26

Rat	Silica	Inhaled repeat dose	28
Mouse	Radiation	Single thorax exposure	32,33
Mouse	Viral vector delivery of TGF-β1 transgene	Single I.T. dose	36
Rat	Viral vector delivery of IL-1β transgene	Single I.T. dose	34
Rat	Viral vector delivery of TGF-β1 transgene	Single I.T. dose	35
Mouse	Asbestos	Single I.T. dose	27
Rat	Paraquat	Single O.G. dose	29,31
Mouse	Paraquat	Single I.P. dose	30
Mouse	Human IPF Cells	Single I.V. infusion	37

This table is not an exhaustive list of all animal models of IPF, rather it is a selection intended to give the reader an insight into the wide variety of models that have been used to investigate IPF.

Effect of drugs in pre-clinical models of pulmonary fibrosis

A wide variety of different compounds with different mechanisms of action have shown efficacy in animal models of pulmonary fibrosis (see Table 2). However, currently only 2 compounds have been approved for the treatment of IPF in humans. In addition to the choice of animal model another variable that is introduced when investigating novel potential anti fibrotic compounds in animal models of pulmonary fibrosis is the dosing regimen used for the drug. Generally, the administration of compounds falls into the categories of either prophylactic or therapeutic administration. Dosing of compounds that are tested prophylactically commences before or on the same day that the fibrosis is first induced. In contrast when drugs are tested therapeutically, this typically begins once the fibrosis is established (for example in the single IT bleomycin model, day 10-14 is often used as the start day for therapeutic dosing with a test compound).

Compounds such as prednisolone or melatonin which are anti-inflammatory and anti-oxidant compounds respectively, and other compounds having similar mechanisms of action have shown some efficacy in pre-clinical models when dosed prophylactically [23, 30, 58, 62 ,65 ,66]. However, anti-inflammatory and anti-oxidant therapy have only shown very weak benefit, if any, in the treatment of pulmonary fibrosis in the clinic [67,68], and their use is currently not recommended in the treatment of IPF [69]. A recent phase 2 clinical trial explored the possibility of combining an anti-

192 inflammatory and anti-oxidant compound, N-acetylcysteine, with Pirfenidone treatment.
193 Unfortunately this study concluded that the minor treatment related change in forced vital capacity
194 (FVC) in patients receiving this combination therapy suggested that marginal clinical benefit, but
195 potentially increased unwanted effects compared with treating with Pirfenidone alone [70].

196 Another distinct class of compound that has shown promise pre-clinically are anti-coagulants [49, 71,
197 72]. However, clinical trials with anticoagulants in patients with IPF have not yielded any positive
198 results, with warfarin showing a negative effect on mortality [73]. Warfarin depletes vitamin K,
199 which affects a wide range of clotting factors [74]. It has been suggested by groups such as
200 Chambers et al [75] that a more narrowly targeted local anti-coagulant approach may still have merit
201 in the treatment of IPF. For example, targeting of PAR 1 in a mouse single I.T. bleomycin dose model
202 [76] and clotting factor Xa in a mouse single O.A. bleomycin dose model [72] in vivo have been
203 shown to reduce levels of fibrosis.

204 Other classes of compound such as certain classes of antibiotics [33, 48], angiogenesis inhibitors [29,
205 60] and mucolytics [61] have shown similar efficacy in pre-clinical models, but this has not translated
206 to the clinic [77-79].

207 However, there are emerging targets that have shown promise pre-clinically that have not yet been
208 fully investigated clinically such as inhibition of the autotaxin pathway [80, 81] and there are
209 currently on-going clinical trials investigating the use of an autotaxin inhibitor in patients with IPF
210 [82].

211

Table 2: Effect of drugs in preclinical models of IPF

Model used	Compound	Timing of dose administration	Mechanism of action	Summary of pre-clinical effect	Ref
Mouse repeat I.V. bleomycin	Pirfenidone	Prophylactic	Broad spectrum anti-fibrotic and anti-inflammatory	Reduction in lung hydroxyproline and histological fibrosis scores	23
Mouse and rat single I.T.	Nintedanib	Prophylactic and therapeutic	Broad spectrum tyrosine kinase	Reduction in histological fibrosis	18

bleomycin, Mouse single I.T. silica			inhibitor	scores	
Mouse S.C. bleomycin osmotic mini pump	Imatinib mesylate	Prophylactic and therapeutic (effect only seen with prophylactic)	Tyrosine kinase inhibitor	Reduction in histological fibrosis scores. Decrease collagen content in lungs	57
Mouse repeat I.V. bleomycin	Prednisolone	Prophylactic	Corticosteroid	Reduction of pro-inflammatory cytokines in early bleomycin response	23
Mouse S.C. bleomycin osmotic mini pump	IMD-0354	Prophylactic and therapeutic	IκB kinase-β inhibitor	Decreases in histological fibrosis scoring, body weight loss and lung collagen content. Improved survival	58
Rat single I.T. bleomycin	Naja naja atra venom	Prophylactic	Anti-inflammatory and free radical scavenger	Decrease in lung hydroxyproline content. Increase in PO_2 and SO_2 in arterial blood	66
Mouse single I.P. paraquat	Rapamycin	Prophylactic	Immunosuppressant	Decrease in TGF-β1 expression, and lung hydroxyproline content	30
Rat single I.T. bleomycin	Ginkgo Biloba	Prophylactic	Antioxidant	Decreases in histological fibrosis scoring and lung hydroxyproline.	62
Rat single I.T. bleomycin	Melatonin	Prophylactic	Hormone, Antioxidant	Decreases in histological fibrosis scoring and lung hydroxyproline	65
Mouse single I.T. bleomycin	Dabigatran	Prophylactic and therapeutic	Direct thrombin inhibitor	Decreases in histological fibrosis scoring lung hydroxyproline content and BAL TGF-β1 levels	71
Mouse single O.A. bleomycin	ZK 807834	Prophylactic	Clotting factor Xa inhibitor	Decreases in lung collagen content and α-SMA expression	72
Mouse single I.T. bleomycin	ONO-1301	Prophylactic	Prostacyclin agonist with thromboxane synthase inhibitory	Decreases in histological fibrosis scoring and lung	49

			activity	hydroxyproline. Increase in survival rate	
Mouse single I.T. bleomycin	Doxycycline	Prophylactic	Tetracycline (antibiotic)	Decreases in histological fibrosis scoring and lung hydroxyproline	48
Mouse Radiation	Clarithromycin	Prophylactic	Macrolide (antibiotic)	Reduction in histological fibrosis scores, TGF-β1 gene expression, and lung collagen content	33
Rat Single I.T. bleomycin	Endostatin	Prophylactic and therapeutic (effect only seen with prophylactic)	Angiogenesis inhibitor	Decrease in expression of VEGF and TGF-β1, decrease in inflammation in early bleomycin response	60
Rat single O.G. paraquat	Losartan	Prophylactic	Angiotensin II receptor antagonist	Decrease in TGF-β1 expression, and lung hydroxyproline content	29
Rat single I.T. bleomycin	Erdosteine	Prophylactic	Mucolytic/Anti-oxidant	Decreases in histological fibrosis scoring	61
Mouse single I.T. bleomycin	GWJ-A-23	Prophylactic	Autotaxin inhibitor	Decrease in lung collagen content, BAL collagen content and BAL TGF-β1 content	80

This table is not an exhaustive list of all compounds that have shown efficacy in pre-clinical models of IPF; rather it is a selection intended to give the reader an insight into the wide variety of compounds and different mechanisms of action that have been evaluated pre-clinically.

A way forward for pre-clinical models of pulmonary fibrosis?

It can be seen from the above discussion that the current preclinical models are a poor predictor of clinical efficacy. This may be due to deficiencies in the design of both pre-clinical and clinical studies, but here we focus only on improvements that could be made to the design of pre-clinical studies in IPF.

The majority of the compounds tested pre-clinically for the treatment of IPF are tested in models where a single fibrotic insult is used. In humans the development of IPF is thought to be progressive

and due to repeated micro-injuries [59]. Models that use repeated smaller insults that result in a progressive development of fibrosis such described elsewhere [3-6] may provide a better model of pulmonary fibrosis.

Perhaps one of the biggest issues questioning the relevance of many of the existing preclinical models of IPF to evaluate new drugs is that the majority of studies test compounds by using a prophylactic dosing regimen. It is possible that the pre-clinical compounds may be able to interfere with the mechanisms by which bleomycin or other fibrotic insults cause fibrosis, but have little effect on established fibrosis that would be present in patients with IPF who enter clinical trials. It would seem sensible therefore to only evaluate novel drugs destined for the treatment of IPF in animal models of established fibrosis. This should therefore reduce the number of "false positives" arising from preclinical work and clearly reduce the number of animals used in the assessment of drugs for the treatment of IPF.

Another major issue is the choice of species and thus the endpoints that are most commonly examined in pre-clinical models of IPF which are almost always histological scores (commonly modified Ashcroft scoring [83, 84]), lung collagen or hydroxyproline (a major component of collagen) content, and occasionally TGF-β1 expression or levels in the lungs, or levels recovered in BAL fluid in mice or rats. Although these endpoints give valuable information about the levels of fibrosis in the lungs of animals they may not provide the whole picture and most models rarely measure any functional readouts such as lung function decline or impairment in gas exchange, changes which are the hallmark of IPF clinically, in part this is because it is difficult to measure such changes robustly in mice. Clinically repeated lung biopsies to examine histopathological changes and measure the collagen contents are limited. However, high resolution CT imaging and functional endpoints such as forced vital capacity, forced expiratory volume in one second, diffusing capacity of the lungs for carbon monoxide or 6 minute walking distance are used to diagnose and monitor patients with pulmonary fibrosis. It has previously been shown that imaging techniques such as MRI or SPECT/CT

[52, 85, 86] can be used in animal models to assess levels of pulmonary fibrosis. These techniques, as well as being more clinically relevant, provide researchers with the opportunity to longitudinally examine the progression of pulmonary fibrosis in a single animal, rather than having to sacrifice numerous animals to examine the fibrosis. There are also systems available to measure many different clinically relevant lung function end points in animals. However, neither imaging nor lung function is commonly assessed in mice, possibly due to the large cost and time implication that can be involved when assessing these endpoints. Despite the cost and time that these techniques may take the assessment of more clinically relevant endpoints in pre-clinical models of pulmonary fibrosis may give a better indication of translation of the potential treatment from the *in vivo* models to the clinic.

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