INHALABLE ANTIFIBROTIC COMPOUNDS

DEVELOPMENT OF A FORMULATION METHOD AND FORMULATION OF ANTIFIBROTIC SMALL MOLECULE TILORONE AS INHALABLE DRY POWDER

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Had to be me. Someone else might have gotten it wrong.

-Mordin Solus, A Salarian scientist
ABSTRACT

The purpose of this work was to develop a formulation method to produce high quality inhalable dry powder, to formulate an anti-fibrotic small molecule tilorone to a respirable form, and to assess its anti-fibrotic potential in pulmonary administration.

The aerosol flow reactor method was used to produce carrier-free formulations of water soluble and insoluble drugs as well as combination powders with drugs of opposing solubilities. L-leucine, L-valine and L-phenylalanine were studied for coating and encapsulation of drug particles. L-valine and L-phenylalanine were found unsuitable for formulation of inhalable dry powder, but the studies confirmed L-leucine’s high potential as an excipient.

The produced formulations were tested for their aerosolization properties, cytocompatibility and drug permeation properties. All leucine coated powders showed good aerosolization performance. They were shown to perform flow rate independently in terms of emitted dose and fine particle fraction. In addition the doses were shown to be repeatable with coefficient of variance of the emitted dose being ≤ 0.11 for every formulation at least with one of the tested inhalers. The fine particle fractions were 28-49% when mannitol was used as matrix former and 54-70% when cyclodextrin was used instead.

Water insoluble beclomethasone dipropionate in combination powder was found to have significantly faster permeation through a lung cell monolayer when compared to physical mixture of the formulation components. Similar difference was not seen with water soluble salbutamol sulphate. The effect was not observed with cyclodextrine based formulations of prednisolone and fludrocortisone-21-acetate.

The anti-fibrotic water soluble small molecule tilorone was formulated and its anti-fibrotic potential was studied in vivo.
The produced formulation showed flow rate independent emission and fine particle fraction when emitted from Easyhaler® at flow rates of 40 and 55 Lmin⁻¹. The emitted doses were 3.0 mg for both flow rates with fine particle fractions being 28% and 30% for 40 and 55 Lmin⁻¹ respectively. The formulation was shown to retain its biological activity *in vitro* after the processing despite high reactor temperatures.

Tilorone was studied in pulmonary administration in a silica model of pulmonary fibrosis in mice. It produced significant reduction in histological scoring of fibrosis when compared to the vehicle group. The effectiveness of tilorone in pulmonary administration could likely be increased with more refined dosing.

In this thesis we have shown that the aerosol flow reactor method is a versatile mean for formulating both water soluble and insoluble drugs. We have shown that tilorone can be formulated as inhalable dry powder and has high potential in treatment of fibrotic lung diseases.
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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications:

I  Drug permeation and cellular interaction of amino acid –coated drug combination powders for pulmonary delivery
   Vartiainen V, Bimbo LM, Hirvonen J, Kauppinen EI, Raula J

II Aerosolization, Drug Permeation and Cellular Interaction of Dry Powder Pulmonary Formulations of Corticosteroids with Hydroxypropyl-β-Cyclodextrin as a Solubilizer
   Vartiainen V, Bimbo LM, Hirvonen J, Kauppinen EI and Raula J

III Pulmonary administration of a dry powder formulation of the antifibrotic drug tilorone reduces silica-induced lung fibrosis in mice
   Int J Pharm. 2018 Jun, 544(1):121-128

The publications are referred to in the text by their roman numerals.
ABBREVIATIONS

ALAT  Latin American thoracic association
API   Active pharmaceutical ingredient
ATS   American thoracic society
bFGF  basif fibroblast growth factor
BLPI  Berner-type low pressure impactor
BMP   Bone morphogenic protein
COPD  Chronic obstructive pulmonary disease
CV    Coefficient of variation of the emitted dose
DPI   Dry powder inhaler
DPPC  Dipalmitoylphosphatidylcholine
ED    Emitted dose
EMA   European medicines agency
EMT   Epithelial to mesenchymal transformation
FBS   Fetal bovine serum
FDA   Food and drug administration, USA
FGF   Fibroblast growth factor
FPF   Fine particle fraction
FVC   Forced vital capacity
FXa   Coagulation factor X
GHE   Glutathione
HPLC  High performance liquid chromatography
IIP   Idiopathic interstitial pneumonia
IPF   Idiopathic pulmonary fibrosis
JRS   Japanese respiratory society
LC    Liquid chromatography
MDI   Metered dose inhaler
MS    Mass spectrometry
NAC   N-acetylcysteine
PBS   Phosphate buffered saline
PDGF  Platelet derived growth factor
PVD   Physical vapor deposition
ROS   Reactive oxygen species
SEM   Scanning electron microscopy
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>SMA</td>
<td>Smooth muscle actin</td>
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<tr>
<td>TGF-β</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor α</td>
</tr>
<tr>
<td>UIP</td>
<td>Usual interstitial pneumonia</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<tr>
<td>VC</td>
<td>Vital capacity</td>
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1 INTRODUCTION

The concept of inhaled therapy has been around at least for most of the written history of mankind. The oldest surviving document describing an inhalable medicine, the Ebers papyrus, dates back to the 1554 BC. The methods of delivery were mostly burning and boiling and breathing the resulting fumes.

In modern medicine pulmonary delivery route is mainly utilized in the treatment of asthma and COPD. Modern asthma treatment is a prime example of successful local treatment. Corticosteroids are the key stone of maintaining a symptom-free state of asthma patients as well as treatment of the acute exacerbations. However, in systemic dosing corticosteroids may exhibit serious adverse effects eg. osteoporosis and psychosis. Administering the drug by inhalation has essentially eliminated these risks for most asthma patients. This success in long term real life clinical setting serves as a proof of concept also for other drugs with a difficult adverse effect profile.

Currently there are two drugs in clinical use for treatment of idiopathic pulmonary fibrosis. While they have been shown to slow the disease progression in clinical trials, they have two major drawbacks. Firstly, they are very expensive. Clinicians are required to carefully select the patients that benefit most from the treatment. Secondly, over 90% of the patients that received the drugs in the clinical trials reported at least one adverse effect. Pulmonary administration could be the solution for both these problems. Smaller drug doses that are delivered locally are likely to be more affordable and cause less adverse effects.

While treatment of diseases of the lungs locally offers a vast field to research by itself, lungs offer a promising delivery route to systemic circulation as well. Firstly, the blood flow rate through the lungs is very high. In fact, it equals the blood flow of all other organs combined. Secondly, the lungs have evolved
to be extremely effective in diffusion. Therefore, they have high surface area with low diffusion length. Thirdly, when compared to the gastrointestinal tract the environment they offer is much less violent for exogenous substances. This is especially true for peptides, which have traditionally been delivered only by injection either intravenously or subcutaneously. While Exubera®, the first commercialized attempt to deliver systemic insulin by inhalation, was discontinued in 2007 only after year in the market, the next product, Afreeza®, was approved by FDA in 2014.

The purpose of this work was to develop a formulation method for dry powder inhalers, to formulate anti-fibrotic small molecule tilorone as inhalable dry powder, and to study the effect of tilorone in local delivery setting as a preclinical test.
2 REVIEW OF THE LITERATURE

2.1 INHALABLE DRY POWDER FORMULATIONS

Nebulizers, metered dose inhalers (MDI) and dry powder inhalers (DPI) are the main types of devices designed for pulmonary drug administration. Each of these comes with their advantages and disadvantages. Nebulizers operate by creating small drug droplets from a solution which can then be carried to the lungs of the patients during the normal breathing cycle. High doses can be achieved via nebulization but it’s time consuming and they require attention regarding bacterial contamination of both device and formulation. Characteristics of pressurized metered dose inhalers are defined by the device, the drug and the propellant. Chlorofluorocarbon propellants were originally used in the metered dose inhalers, but were subsequently phased away by the Montreal protocol because of their ozone depleting effect in the atmosphere. Chlorofluorocarbon propellants were originally used in the metered dose inhalers, but were subsequently phased away by the Montreal protocol because of their ozone depleting effect in the atmosphere. Currently, the drugs and excipients are propelled by environmentally safe hydrofluoroalkanes. Metered dose inhalers can reach high levels of pulmonary deposition, but require coordination of inhalation and actuation from the patient. Dry powder inhalers are breath-actuated and easy to operate. However, the energy for drug dispersion comes from inhalation and therefore sufficient inhalatory flow rate is required. Therefore, careful consideration is paramount when choosing the delivery method for given patient.

The behavior of dry powders in the inhaler and during the inhalation is mainly governed by particle-particle interactions and fluid dynamics. The respirable size range is usually regarded to be between 1 to 5 μm, while aerodynamical diameter under 3 μm is required for deep lung deposition. As the volume of the particles is small, with the densities of typical organic materials importance of gravitational forces diminish.
In this size range electrostatic, capillary and van der Waals forces become increasingly important. In ambient temperatures where relative humidity is under 60%, van der Waals force tends to dominate and for solid spheres can be expressed as\(^7\)

\[
F_{vdW} = \frac{A d_g}{12 r^2}
\]

where \(d_g\) is the particle's geometric diameter, \(A\) is the material-dependent Hamaker parameter and \(r\) is the interparticle distance. While the assumption of homogenous solid spheres is unlikely to hold for any real-life formulation, equation 1 still gives qualitative understanding on the parameters involved. However, in real life morphological and material properties such as surface roughness, surface energy and Young’s modulus need to be considered.\(^8\)

Relative strength of cohesive forces is important in two aspects. Firstly, good flowability enables easy and precise metering during the manufacturing processes e.g. capsule filling.\(^9\) It is also important for the function reservoir type dry powder inhalers, as the powder needs to flow from the reservoir to the functional parts of the inhaler in a very precise and consistent manner. Any errors during this process inevitably lead to variations in the drug dosing. Flowability is determined by relative strength of gravitational force compared to cohesive forces. For solid spherical particles the gravitational force can be expressed as\(^7\)

\[
F_g = \frac{\pi}{6} \rho d_g^3 g
\]

where \(\rho\) is the particle’s density, \(d_g\) is the geometric diameter of the particle and \(g\) is the gravitational constant. For most organic materials gravitational forces dominate at a size range of 30 \(\mu m\)^10, which is far beyond the desirable 5 \(\mu m\).
Secondly, for efficient lung deposition the powder must be deagglomerated by the airflow when the patient is inhaling the drug. The drag and lift forces are main the interactions driving the deagglomeration and can be expressed as

\[ F_{D/L} = \frac{\pi}{8} C_{D/L} \rho_f (d_g U)^2 \]

where \( C_{D/L} \) is the drag or lift coefficient, \( \rho_f \) is the density of the fluid, \( U \) is the fluid velocity and \( d_g \) is the geometric diameter of the particle. Based on this model Weers and Miller give an estimate of powder fluidization when \( d_g \geq 12 \mu m \), which is again well over the respirable size range.

The problem of fluidization and flowability of respirable sized powders has been approached in multitude of ways. The current industrial standard is to use coarse carrier particles which give the formulation the desired flowability and the drug is released from carriers during emission from the inhaler. Using force control agents, increasing the surface roughness and engineering particles of low density have also been proposed.

### 2.1.1 COMMONLY USED FORMULATION METHODS

There are many formulation methods used in industry and research with the aim of producing optimal formulation for dry powder inhalers. Therefore, it is outside the scope of this review to address those in great detail. The methods presented here are chosen based on their wide use in industry or relevance for the work described in this dissertation.

In industry a common way of tackling flowability and dispersibility problems of drug micro particles has been to blend them with coarse carrier particles to an ordered mixture. Coarse particles provide flowability to the formulation, while the drug particles remain in the respirable flow range. This introduces two mutually exclusive goals for the
formulation technology. Firstly, the drug particle adherence to the carriers needs to be sufficient to avoid drug detachment from the carriers when good flowability is needed. Secondly, the drug particles must not adhere to the carrier particles too tightly to ensure drug detachment upon inhalation. In real-life the detachment of the drug particles from the carriers during inhalation is incomplete and has high interpatient variability.\textsuperscript{15,16} Low performance of the lactose carrier based formulations has been addressed by adding fine lactose particles to the formulation.\textsuperscript{17} Two mechanisms are usually considered to account for the improvement. Adding the lactose fines to the formulation mix before the drug particles allows them to occupy the high energy binding sites on the surface of the carrier particles producing more homogenous surface energy environment. Therefore, the micronized drug particles adhered on the carrier particles are located in the low energy binding sites. As it requires less energy to remove the drug particles from the carrier surface this improves the detachment and dispersion of drug particles upon inhalation.\textsuperscript{18,19} Added fines may also form agglomerates with drug particles. These agglomerates undergo increased drag and lift forces resulting in improved detachment from the carrier particles.\textsuperscript{20} Schematic representation of described situations is presented in figure 1. However, the strength of the interactions between blend components is material dependent which complicates the production of combination formulation as the number of different interactions between the components increases. While using coarse carriers is an established and straightforward method, the moderate performance and requirement for micronizable drugs leaves room for more sophisticated methods.
Particle engineering is a discipline that aims to overcome the problems of carrier formulations by tuning the properties of the individual particles rather than adding new ones. Spray drying is an old method developed for the food industry already in the 19th century. Although the discipline of particle engineering was founded after its invention, spray drying is the oldest method of particle engineering for DPIs. Dispersible fine powder with consistent size distribution is produced either from solution or colloidal media. In the spray drying process, the precursor solution is first dispersed by an atomizer and the resulting droplets are then dried by exposing them to a flow of heated gas. Typically air is chosen, but in case of combustible materials or solvents nitrogen or other inert gas can be used as well. Over the years, spray drying has proven to be simple, flexible and scalable technology. Both small- and macromolecules can be formulated and the method can be used with wide variety of solvents and even colloidal media can be used as feedstock.

A prime example of particle engineering is the concept of large porous particles. The idea was introduced twenty years ago by Edwards et al. The working principles of large porous particles can be understood from the equations (2) and (3). While the mean geometrical diameter of the large porous particles is around 20 μm, their low density results in small
aerodynamical diameter. As seen from the equation (2), the gravitational force is directly proportional to the density of the particle, but proportional to the cube of geometric diameter. Therefore to achieve sufficient flowability, one can afford to decrease the density if geometric diameter is sufficiently increased. The same holds for lift and drag forces that contribute to the dispersion of the powder. As seen from the equation (3), the lift and drag forces are only directly proportional to the density of the particles, but proportional to the square of the geometrical diameter. Increased aerosolization performance with decreasing density has not only been seen with large porous particles, but also with denser corrugated particles as well.24 Edwards et al. formed their large porous particles by double emulsification evaporation technique, where the drug of interest of was first emulsified with an excipient polymer and then the resulting emulsion was emulsified with another solvent. When the solvents were then evaporated by freeze drying, the remaining solid material formed large spherical porous particles.12

2.1.2 AEROSOL FLOW REACTOR METHOD

The aerosol flow reactor method is a novel method which focuses on the modification of particle surface morphology. L-leucine coating is formed on the particles to minimize the effect of drugs and other excipients on aerosol behavior of the powder. It is a one-step continuous process to produce respirable dry powder from a precursor solution.25,26 Figure 2 shows a schematic representation of the reactor used.

For the precursor solution either water or organic solvent may be used. In case of opposing solubilities, preformulation operations such as wet-milling may be used for insoluble material to produce a colloidal precursor solution as was shown in publication I. This produces nanos-in-micros type of particle structure, where surfactant covered drug nanoparticles are
embedded into the mannitol matrix of micro particles. As the coating material L-leucine is not readily soluble to organic solvents, similar preformulation is required if aqueous solution needs to be avoided. L-leucine can be solubilized to ethanol by wet-milling it with dipalmitoylphosphatidycholine (DPPC). During the formulation process L-leucine is sublimated from the particle core leaving DPPC to form the particle matrix alongside with the drug. Very high particle drug contents has be obtained with this method. (Vartiainen et al., unpublished results, Raula et al. unpublished results)

Figure 2: Basic structure of the aerosol flow reactor.
Droplets are produced from the precursor solution by an ultrasonic nebulizer or a jet atomizer. Depending on the choice of droplet generation technique, either nano or micro particles can be produced.\textsuperscript{26–28} The droplets are then lead by a carrier gas to the heated reactor tube, where surface modifications are initiated.

In the heated section of the reactor tube particles are carried in a laminar stream. The residence time in the reactor can be tuned by adjusting the carrier gas flow rate. The behavior of the coating agent L-leucine is determined by the reactor temperature. A schematic representation of the produced particle morphologies is given in figure 3. Below the sublimation point of L-leucine the formation of surface morphology is driven mainly by diffusion and drying. Due to its relative hydrophobicity L-leucine tends to accumulate in the air-liquid interface\textsuperscript{29,30} and upon drying it forms an encapsulating layer which has been shown to increase the particles physical stability against moisture and protect the compound from moisture-induced crystallization\textsuperscript{31}.

Near the sublimation temperature of L-leucine a second process affecting the surface morphology is introduced. With sufficient energy transfer from the ambient thermal energy, L-leucine will undergo phase transition via sublimation. This introduces a cloud of L-leucine in gas phase around the particles. Subsequent cooling with high gas flow (Reynolds number > 3000) in the next stage of the reactor leads to two nucleation processes. Firstly, there is homogenous nucleation where L-leucine particles start to condensate on each other resulting in L-leucine nanoparticles. Secondly, there is heterogeneous nucleation via physical vapor deposition (PVD) on top of the solid particles generated from the droplets by drying. Heterogeneous nucleation produces a rough coating layer of leaf like nanocrystals with preferred crystallographic orientations of (-110) and (001).\textsuperscript{32} These leaf like structures decrease the contact area and increase interparticle separation of the drug micro particles leading to increased flowability and dispersibility.\textsuperscript{25,26} In temperatures near the sublimation point
with sufficiently short residence time in the reactor not all L-leucine is evaporated from the particles and the surface morphology is generated by the combination of diffusion and PVD resulting in two qualitatively different layers on top of the drug particles i.e. the encapsulating and coating layers. When the temperature or residence time is further increased in the reactor stage, sublimation begins to dominate over diffusion as more thermal energy is transferred to the droplets and the morphology is mainly driven by PVD.

The device used to collect the resulting particles depends on their size range. Micro particles are collected with small-scale cyclones, which allow the nano particles formed by homogenous nucleation to pass through the collection stage. Variety of samples can be obtained directly from the particle stream before collection. These include particles deposited for transmission electron micrography, size distribution before the collection and measurements of electrostatic properties of the particles. The high flow rate of the cooling gas also prevents condensation of the solvent in the collection stage by gas volume dilution.

![Figure 3: Schematic representation of particle morphology between dilution and collection stages of the reactor with varying reactor temperature. A: Temperature is below sublimation point. Leucine diffuses to the surface and forms encapsulating crust. B: Temperature is near sublimation point. In addition to diffusion sublimation also takes place. Leucine nanocrystal coating is formed on top of encapsulation by PVD. Leucine nanoparticles are formed by homogenous nucleation. C: Temperature is above sublimation point. Nearly all leucine is sublimed and no encapsulation is formed. Leucine coating and nanoparticles are formed.](image-url)
2.2 IDIOPATHIC PULMONARY FIBROSIS

Idiopathic interstitial pneumonias (IIP) or interstitial lung diseases are a group of inflammatory and fibrotic diseases stemming from the lung parenchyma. Current ATS/ERS classification of IIPs is from 2002 and includes seven different diseases i.e. idiopathic pulmonary fibrosis (IPF), idiopathic nonspecific interstitial pneumonia, cryptogenic organizing pneumonia, acute interstitial pneumonia, respiratory bronchiolitis-associated interstitial lung disease, desquamative interstitial pneumonia, and lymphoid interstitial pneumonia. IPF is the most common IIP.

Idiopathic pulmonary fibrosis (IPF) is a progressive chronic lung disease with poor prognosis. In ATS/ERS/JRS/ALAT 2011 statement, suggest a median survival of 2-3 years, but stated that it is probably and underestimation. Also, during the past few years new anti-fibrotic drugs have entered the market and shown to be effective in slowing down disease progression. With the current treatment regimens being so young, we lack thorough longitudinal studies which describe progression and survival for patients with IPF. In systematic review by Kaunisto et al. prevalence of IPF was estimated to be 0.5-27.9 per 100,000 persons. According to a re-evaluated ATS/ERS/JRS/ALAT criteria filling registry study the prevalence of IPF in Finland is estimated to be 8.6 per 100,000.39

The condition, risk factors, and its diagnostic criteria are defined in the ATS/ERS/JRS/ALAT statement on diagnosis and management of IPF. The disease is characterized by histopathological or radiological pattern of usual interstitial pneumonia (UIP). Clinical manifestations may include signs hypoxemia, dyspnea, cough, and fine crackles bilaterally in the basal region of the lungs. Epidemiological studies have identified several risk factors for IPF. Cigarette smoking is regarded as a strong risk factor especially when smoking history exceeds 20 pack-years. Other risk factors include environmental exposures e.g. metal and wood dusts, microbial agents, gastroesophageal reflux and genetic factors.
The diagnosis of IPF requires exclusion of other causes of interstitial lung diseases and a typical radiological pattern of usual intestinal pneumonia (UIP). If the radiological diagnosis is possible UIP, further support is needed from a histological analysis of surgical lung biopsy. In this case histological diagnosis of UIP and probable UIP are sufficient for IPF diagnosis. In addition, diagnosis of IPF is possible in cases when both radiological and histological patterns are possible UIP or when histological pattern is UIP even when radiological analysis is inconsistent with UIP.

Acute exacerbation of IPF is defined as worsening of dyspnea over 30 days or less, worsened or severely impaired gas exchange, or new radiological alveolar infiltrates. As with diagnosis of IPF, the diagnosis of acute exacerbation requires that no other explaining factor can be identified. The acute deterioration may happen during any point in the natural course of the disease and may be the first manifestation of the disease. High dose of corticosteroids is generally used for treatment of acute exacerbation, but currently their efficacy has not been demonstrated in controlled clinical trials.41

### 2.2.1 MECHANISMS OF IDIOPATHIC PULMONARY FIBROSIS

In the end of last millennia, idiopathic pulmonary fibrosis was mainly considered to be an inflammatory disease.42 Although the pathogenesis of IPF is still incompletely understood, the current paradigm revolves around an impaired wound healing process after repeated damage to alveolar epithelium.43

In normal wound healing process the hemostasis is among the first mechanisms activated. The activation of coagulation cascade leads to formation of a clot that consists of collagen, platelets, thrombin, and fibronecting.44 The clot is in part responsible for the initiation of inflammatory response and it promotes accumulation of inflammatory cells and fibroblasts.45
Presence of basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), transforming growth factor β (TGF-β) and hemodialysate has been shown to increase number of fibroblasts in the wound area \textit{in vitro}. The fibroblasts undergo transformation to more contractile myofibroblasts induced by TGF-β. In normal lungs fibrotic tissue deposited during the healing process is cleared and normal lung architecture is restored. However, in IPF lungs sustained epithelial to mesenchymal transformation (EMT) is evident and fibroblasts have been shown to be resistant to apoptotic signaling. EMT is a process where epithelial cells lose their epithelial characteristics and begin to express mesenchymal markers e.g. α-smooth muscle actin (α-SMA) and fibronectin. The role of TGF-β in EMT has been reviewed in detail by Willis and Borok. TGF-β has been shown to induce EMT of Type II alveolar epithelial cells and it has been hypothesized to be an important source of myofibroblasts in fibrotic lesions. Changes are also observed in the normal function of epithelial cells as type II alveolar cells seem to have impaired ability to act as progenitors for type I alveolar cells.

Some susceptibility genes for IPF have been identified. In general, fibrotic lung disease in the family has been reported as an important risk factor for IPF. Also, subset of IPF cases come in familial form. A familial form of IPF, FPF, is estimated to account for 2-5% of all cases. Defects in telomerase enzyme encoding hTERT and hTR genes is often seen in familial forms of IPF and have been reported in sporadic cases as well. A defect leading to misfolded surfactant protein C leading to type II alveolar epithelial cell toxicity is also observed in some familial cases, but the result has not been reproduced in sporadic populations. In most familial cases the heritance pattern is autosomal dominant with reduced penetrance. Whether familial or sporadic, telomere shortening is seen both in lung epithelium and peripheral leucocytes and is associated with poorer prognosis. As telomere length is often used as marker of biological age of the patient, this finding is in line with the epidemiological fact that IPF is generally a disease
of elderly population. Armanios et al. hypothesized that the driving mechanism behind fibrosis in some cases could be loss of alveolar epithelial cells due to telomere shortening-initiated apoptosis. Loss of epithelial cells to apoptosis would induce recurrent damage to the barrier on the external surface of the body and could in principle trigger wound healing process.

At tissue level, IPF exhibits both spatial and temporal heterogeneity. Spatial heterogeneity means that not all parts of the lungs are equally compromised. IPF mostly affects basal regions of the lungs and forms patchy lesions centered on fibroblastic foci and are surrounded by healthy lung tissue. Fibroblastic foci are regarded to be the sites of active fibrinogenesis. Temporal heterogeneity means that even in single lung different sites are in different states in the natural progression of the disease. This suggests that IPF is a result of a continuous process instead of a single event and is currently thought to arise from genetic susceptibility to recurrent insult. Computer simulation on the mechanical properties of the lungs suggest that restrictive nature of the disease only arises when the fibrotic lesions are interconnected. Studies on interconnection of the lesions real-life setting in patients with radiological diagnosis of UIP has produced conflicting results. However, when interpreting the results one must consider that UIP pattern is a radiological finding does not equal to IPF.

On molecular level transforming growth factor \( \beta \) seems to be one of the key factors in the development of fibrosis. As discussed before, it is an important factor in both recruitment and differentiation of fibroblasts and myofibroblasts in normal wound healing process. In rodents, TGF-\( \beta \) pathway has been shown to increase net procollagen production while TGF-\( \beta \) induction of fibrosis can be inhibited by specifically blocking ALK5, a specific TGF-\( \beta \) receptor. As reviewed by Wakefield and Roberts, TGF-\( \beta \) pathway has been shown to inhibit the growth of most epithelial cells, which could play important role in impaired wound healing process. Bartram and Speer have shown TGF-\( \beta \) to be upregulated in fibrotic lung.
Several biomarkers indicate that oxidative stress may contribute to the development of IPF. It has been shown that glutathione (GHE) levels are decreased in BALF from IPF patients. Glutathione is an important antioxidant and has a versatile role in detoxification of external threats such as drugs and xenobiotics. Also, elevated levels of other molecules actively participating in oxidation reactions such as myeloperoxidase, eosinophil cationic protein and 8-isoprostane have been observed in IPF patients. Bocchino et al. demonstrated that fibroblasts derived from IPF patients contain excess reactive oxygen species (ROS) compared to those derived from healthy controls. TGF-β seems to play important role also in oxidative stress. Barcellos-Hoff and Dix showed that both irradiation and catalytically generated ROS induced release active TGF-β from its latent form. TGF-β has been shown to reduce cellular GHE content in murine embryo fibroblasts and transcription of rate limiting enzyme gamma-glutamylcysteine synthetase in GHE production. Reducing the levels of one of the most important antioxidants likely limits the capacity of the cell to respond to oxidative stress. TGF-β has also been shown to directly generate oxidative stress by activation hydrogen peroxide (H$_2$O$_2$) generating enzyme NADH oxidase. Stimulation by oxidative molecule H$_2$O$_2$ enhanced TGF-β mediated collagen synthesis while inhibition of ROS generation by N-acetylcysteine (NAC) blocked EMT and Smad3 phosphorylation in vitro, but exogenous addition of H$_2$O$_2$ was not sufficient to induce EMT. As phosphorylated Smad3 is one of the key signaling molecules in the TGF-β pathway, these results would indicate that oxidative stress is a profibrotic factor but requires TGF-β signaling to take effect. However, TGF-β induced generation of ROS and subsequent profibrotic events could in principle act as amplification loop for generation of fibrosis. Treatments based on controlling the oxidative stress in lungs of IPF patients have been proposed. Even tough NAC has been shown to increase GHE levels in IPF patient BALF, it failed to show benefit in clinical trial. However, Oldham et al. genotyped patients
from PANTHER-IPF and INSPIRE clinical trials and identified subpopulation of patients with single-nucleotide polymorphism within TOLLIP who seemed to benefit from NAC monotherapy in post hoc analysis.85

Bone morphogenic proteins (BMP) are group of proteins associated with lung development.86–90 They have been shown to counteract the effect of TGF-β and even trigger mesenchymal to epithelial transformation which may lead to regeneration of kidney fibrosis in mouse model.91 Figure 4 describes the relationship of BMP and TGF-β pathways. These observations indicate that modulation of BMP might lead to regeneration of alveoli and rescue of lung volume already lost to fibrotic lesions. Lung tissue of IPF patients show also increased levels of the BMP inhibitor gremlin-192 and exogenous administration of BMP-7 has been shown to reduce pulmonary fibrosis in in vivo mouse models.93,94 Gremlin mRNA levels have been shown to negatively correlate with alveolar volume corrected diffusion capacity and positive correlation was found between BMP-4 mRNA levels and forced vital capacity.95

Figure 4: Relationship of TGF-β and BMP pathways. Adapted from Selman et al.89 Gray indicates pro-fibrotic and white anti-fibrotic mediators.
One concern in targeting TGF-β pathway has been its dual role in oncogenesis. Reduction in TGF-β responsiveness has been shown to promote aggressiveness of certain breast cancer related cell lines in vitro. Since the molecular mechanism is poorly understood it is not known if the effect is dose related or changing property of the cells during oncogenic progression. TGF-β is also an important homeostatic factor in variety of tissues and targeted disruption of TGF-β signalling has been shown to multifocal inflammatory disease in mice.

Many of the signalling molecules associated with normal wound healing process have been shown to have an active role also in IPF. For example platelet derived growth factor (PDGF), tumor necrosis factor alpha (TNF-α) and TGF-β, have been shown to increase collagen synthesis in fibroblasts isolated from IPF patients and fibronectin synthesis was induced also by FGF. Levels of basic fibroblastic growth factor containing mast cells has been shown to be elevated in lung tissue from IPF patients when compared to healthy controls. Alveolar macrophages collected from the lungs of IPF patients spontaneously released significantly more PDGF than those from the normal controls. TNF-α levels have been shown to be elevated in IPF patients and are associated to patients with progressing disease. All of these factors are mediators or effectors of normal wound healing. The fact that they are elevated in and active in tissue material of the IPF patients supports the current paradigm where recurring epithelial insult and impaired wound healing play a central role.

Activation of coagulation cascade is one of the triggers of wound healing and has been tought to contribute to the development of fibrotic changes in IPF. There are several studies that show increased levels of molecules associated with coagulation cascade in IPF lungs. Scotton et al. demonstrated that expression of coagulation factor X (FXa) is increased in bronchial and alveolar epithelia of IPF patients and showed that it induced TGF-β mediated myofibroblast differentiation in human adult fibroblasts in vitro. Presence of fibrin or
fibrinogen in extracellular matrix of ex vivo cultured primary alveolar epithelial cells induce TGF-β mediated EMT. In IPF lungs tissue factor and fibrinogen antigens were associated especially with type II pneumocytes. In contrast to normal lung, tissue factor expression seems to be elevated in patients with IPF and has been shown to correlate with more advanced disease. Increased levels of thrombin have been observed in several conditions causing fibrotic changes in lung parenchyma and thrombin has been shown to trigger differentiation of lung fibroblasts to myofibroblasts.

As mechanical properties of the fibrotic lung are considerably different from normal lung tissue, changes in the physical properties of the extracellular matrix have been proposed to contribute to fibrotic processes. Using a bleomycin induced fibrosis in mice, Liu et al. demonstrated that the fibrosis induction resulted in six-fold increase in tissue stiffness. The change in tissue stiffness did not only affect the mechanical properties of the lungs, but also the behaviour of fibroblasts. They observe stiffness to suppress the expression of cyclo-oxygenase-2, prostaglandin E2 and matrix proteolytic genes while increasing matrix synthesis and proliferation. Culturing human fibroblast cells on a stiffness gradient also resulted in TGF-β independent accumulation of fibroblasts towards the greater shear modulus over the course of 120 hours. The stiffness of culturing substrate has also been shown to have effect on cell morphology and expression of α-smooth muscle acting, a protein largely responsible for myofibroblast contractility.

2.2.2 PHARMACOLOGICAL TREATMENT OF IDIOPATHIC PULMONARY FIBROSIS

For the last decade the treatment regimen of IPF has been in turmoil. In 2011 the ATS/ERS/JRS/ALAT consensus committee did not find suitable evidence to recommend any
pharmacological treatment for IPF management. The consensus committee found evidence supporting formerly used triple treatment of prednisone, azathioprine and N-acetylcysteine lacking and increased mortality was observed in triple treatment arm of PANTHER-IPF study in 2012. At the time of writing, two anti-fibrotic drugs, pirfenidone and nintedanib, were approved for clinical use by both European Medicines Agency (EMA) and US Food and Drug Administration (FDA), but there exists no study where the two drugs would have been compared head to head. As reviewed by Mylläriemi and Kaarteenaho, the differences in study design render comparison of the phase III trials impossible. Most notably, the INPULSIS trials included patients with a radiological diagnosis of possible UIP without surgical lung biopsy, which is in direct disagreement with the current diagnostic guidelines. This means that the study might include patients who do not have IPF as defined by ATS/ERS/JRS/ALAT guidelines. Even with the new anti-fibrotic medication we are unable to cure the disease. Respiratory symptoms, fatigue, pain, and depression disable patients and there is little evidence that recommended anti-fibrotics offer help to the symptoms. Currently there exists one non-placebo-controlled study where pirfenidone reduced 24 h cough count and improved subjective measures of cough.

Pirfenidone was the first drug approved for the treatment of IPF. In the context of IPF its development span almost two decades from the initial discovery of its anti-fibrotic properties to the approval of EMA in 2011. It is a poorly soluble small molecule that is readily dissolved in both ethanol and chlorophorm. It is a pyridine analogue as seen from the chemical structure of pirfenidone in Figure 5. In in vivo models it has been shown to inhibit progression of fibrosis at least in lung, hepatic, kidney and cardiac models. While the mechanism of action of pirfenidone is incompletely understood, TGF-β signaling route seems to play an important role. Pirfenidone has been shown to decrease TGF-β induced proliferation and fibrogenic activity of primary human lung
fibroblasts and human Tenon's fibroblasts *in vitro*\(^{127,128}\). Regulation of interferon-γ and FGF2 levels have also been proposed to contribute to its anti-fibrotic effect.\(^{122}\)

Leading to the approval from FDA and EMA, four phase III clinical trials were conducted on pirfenidone. There was a marked difference between the endpoints of the trials. The first one conducted by Taniguchi *et al.*\(^{129}\) used percentual difference in vital capacity (VC) as primary endpoint while the CAPACITY\(^{130}\) replicate studies and ASCEND\(^{131}\) used forced vital capacity (FVC) instead of VC either at 72 weeks or 52 weeks, respectively. ASCEND also accepted death as primary end point. Three of the four studies showed benefit in their primary endpoints, CAPACITY 006 being the sole exception. However, analysis of pooled data from ASCEND and CAPACITY showed decline in both all cause and IPF related mortality.\(^{131}\)

![Chemical structure of pirfenidone](image)

**Figure 5: Chemical structure of pirfenidone**

In the modern anti-fibrotic treatment adverse effects are highly prevalent for both clinically used drugs. For example, in CAPACITY 004 study 98% of patients on pirfenidone reported at least one adverse effect. Most common was nausea, which was reported by 35% (compared to 18% in placebo group). Next were fatigue with 35% (18% in placebo group), diarrhea with 25% (17% in placebo group) and rash with 31% (10% in placebo group).
group). Other reported adverse effects were mainly GI-tract related including dyspepsia, vomiting and anorexia. There was increased photosensitivity reaction in the treatment group affecting 14% of the treatment group (1% in the placebo group). CAPACITY 006 was well in line with 004 showing only minor differences. RECAP study was a long-term open-label follow-up study which enrolled patients who had previously completed either one of the CAPACITY studies. In RECAP adverse effect profile of pirfenidone was consistent with CAPACITY in terms of both type and frequency. PASSPORT is a pirfenidone post-authorization safety registry, where safety data of patients on pirfenidone were recorded up to two years. In 2015 update adverse effect profile was mostly in agreement with the phase 3 trials and RECAP, although fraction of patients suffering from nausea was only 17%. There are also number of smaller studies on pirfenidone also in real-life setting, which are mostly in agreement with previously reported findings.

Nintedanib, formerly known as BIBF 1120, is a triple angiokinase inhibitor acting on vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF) receptors. Anti-fibrotic activity of nintedanib has been suspected to partly result from inhibition of fibrocytes. Its development was markedly different from pirfenidone’s. It is a product of focused development from the pharma industry and the first publications on nintedanib and its sister molecule BIBF1000 are only from around 2006. Chaudhary et al. showed that blockade of these three receptors by BIBF 1000 attenuates bleomycin induced fibrosis in rats and the results have been confirmed also with nintedanib in both bleomycin and silica models. However, the preclinical studies on nintedanib by Wollin et al. were only published 2014 during the same year that results from the phase III clinical trials IMPULSIS-1 and -2 were published and three years after the phase II clinical trial was conducted. It is a water soluble molecule producing increasingly viscous solution with rising concentration. Figure
3 shows the chemical structure of nintedanib. In addition to IPF, clinical trials on nintedanib against ovarian, endometrial, gastrointestinal, breast, and non-small-cell lung cancer have been conducted.

Prior to its approval by FDA and EMA, two replicate studies were conducted under the names INPULSIS-1 and INPULSIS-2. The primary end point was annual absolute decrease in FVC. In both studies treatment with nintedanib resulted in significant reduction of decline in FVC. However, the pooled analysis there was no benefit in mortality when compared to placebo.

As with pirfenidone, almost all patients suffer from adverse effects. In INPULSIS-1 study the two most common adverse effects reported were diarrhoea with 61.5% (versus 18.6% in placebo group) and nausea with 22.7% (against 5.9% in placebo group). 96.4 % of the patients in treatment group reported at least one adverse effects, while 88.7 % of patients reported at least one adverse effect in placebo group. Also in a recent study in real-life setting diarrhoea and nausea were found to be the most common adverse effects and large majority of patients had more than one adverse effect.

Tilorone is a water soluble small molecule with reflectional symmetry. Figure 4 shows the chemical structure of tilorone. It has been characterized as an oral interferon inducer in mice but not in humans. It has also been shown to have interferon independent antiviral effects such as prevention of reverse transcription. Tilorone is a DNA-intercalator and has been shown to bind to AT-rich regions of DNA. Tilorone inhibits TGF-β and induces BMP signalling pathway. As discussed before, TGF-β signalling has been shown to be upregulated in fibrotic lungs while BMP signalling is inhibited. This mechanism of action may provide a significant benefit when compared to current anti-fibrotic drugs. BMP signalling is an important regulator of distal airway and alveoli development and exogenous BMP administration has been shown to revert kidney fibrosis in mice. As the current medication only slows the progression of
fibrosis in IPF patients, tilorone might provide the first opportunity to improve the lung function and quality of life of the patients. Tilorone has been shown to have anti-fibrotic properties in vivo in a pulmonary model of lung fibrosis.\textsuperscript{159} In Russia and some of the neighbouring countries tilorone is used as anti-viral agent against influenza and hepatitis.\textsuperscript{160} Cummings et al. performed a phase II clinical trial on tilorone against advanced breast cancer. Tilorone arm of the study was discontinued due to lack of benefit and keratopathy was observed in two patients.\textsuperscript{161}

![Chemical structure of nintedanib](image)

**Figure 6: Chemical structure of nintedanib**

The formulation of antifibrotic drugs to an inhalable form is a field of ongoing research. Inhalable heparin was found to be safe and well tolerated in IPF patients in phase I trial\textsuperscript{162}, however, its efficacy in treatment of IPF has not been studied. The second inhalable drug for IPF to enter clinical studies was TD139 from Galecto Biotech. Authors have reported good tolerability in phase 1 and plans to proceed to phase 2.\textsuperscript{163} Pirfenidone has been formulated and studied both in vitro and
in vivo with promising results\textsuperscript{164–167}, but no clinical studies have been declared so far. To the authors knowledge, there has been very little activity regarding formulation of nintedanib in an inhalable form.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{tilorone.png}
\caption{Chemical structure of tilorone}
\end{figure}

\subsection*{2.2.3 IN VIVO MODELS OF PULMONARY FIBROSIS}

At the time of writing, there exists no inducable disease model for IPF. As pulmonary formulations in the past have mainly consisted of asthma and COPD medication, formulation of anti-fibrotic drugs is a relatively new field and no such formulations exist as a commercial products. Therefore, the relevance of preclinical models and their clinical correlation in terms of pulmonary administration of anti-fibrotics is still mostly unexplored field. Fibrotic changes in animal models of pulmonary fibrosis can be induced in numerous ways such as drugs, pulmonary administration of certain minerals or irradiation of the tissue. In this review, only those relevant for the study in hand, namely bleomycin, asbestos and silica models, are covered.

A recent official American Thoracic Society (ATS) workshop decided to recommend that a murine bleomycin model is best characterized for preclinical testing of anti-fibrotic compounds
for IPF.\textsuperscript{168} Bleomycin is an antibiotic drug isolated from a strain of gram-positive bacteria \textit{Streptomyces verticillus} already in 1966. Since then, it has been used in variety of neoplastic diseases and has been observed to inflict significant pulmonary toxicity most commonly leading to fibrotic changes.\textsuperscript{169}

There have been numerous studies on bleomycin models of pulmonary fibrosis in mice, hamsters and rats since 90s making it well characterized fibrosis model for both systemic\textsuperscript{118,119,122,170–178} and pulmonary\textsuperscript{179–181} administration. As expertly reviewed by Moore and Hogaboam\textsuperscript{182}, the fibrosis is likely caused by DNA breakage and oxidative stress and relies on CCL1 and CCL12 mediated recruitment of inflammatory cells as well as upregulation of TGF-\(\beta\) signalling. Decreased expression of BMP and increased expression of BMP antagonist Gremlin 1 on mRNA level has been observed in bleomycin challenged mice, indicating reduced BMP signaling.\textsuperscript{183} Another important factor in development of bleomycin induced fibrotic lesions is TNF-\(\alpha\). Upregulation of TNF receptor p75 has been observed in bleomycin challenged mice and TNF receptor knock out mice were shown to be resistant to bleomycin induced fibrosis despite the increased TNF levels.\textsuperscript{184} Expression of PDGF has also shown to be increased on mRNA level.\textsuperscript{185} A substantial estrogen mediated difference in reaction to bleomycin has been observed between male and female rats.\textsuperscript{186} The anti-fibrotic effect of both pirfenidone and nintedanib has been shown in the bleomycin model.\textsuperscript{118,141}

However, unlike in IPF the fibrosis in bleomycin model has been shown to be self-limiting and the fibrosis is shown to resolve without intervention.\textsuperscript{187,188} To counteract this spontaneous healing and to more closely mimic the condition in humans some authors have suggested introducing a repeated bleomycin exposure model.\textsuperscript{189,190} This view was challenged by Peng \textit{et al.} who observed no benefit in terms of pathogenic molecular changes when compare to single bleomycin insult.\textsuperscript{191} As reviewed by the ATS workshop most of the authors failed to report mortality during the experiments.
The two studies in which the mortality was reported 40-60% of the mice in the control groups died during the experiment. Peng et al. performed a dose escalation study where dose of 3 U/kg was associated with 19% mortality and 5 U/kg caused 50% mortality. Although dose of 2 U/kg has been reported to cause no mortalities, others have suggested mortality of 15% to be accepted in bleomycin experiments. In conclusion, mortality resulting from fibrosis induction remains as downside of the bleomycin model. As reduction and refinement are demanded from any modern in vivo study, one must question the ethical basis of the bleomycin model.

Asbestos is a group of natural silicate minerals with varying morphologies. The different minerals of asbestos are divided into two groups. Serpentine group consists of only one mineral chrysotile. It differs from the other by having layered instead of chained structure. The other five asbestos minerals belong to the amphibole group and are named crocidolite, amosite, tremolite, actinolite and anthophyllite. Aside from the macroscopic differences, main difference between the types of asbestos is that different metallic elements complete the silicate lattice. Therefore, asbestos is not a single entity, but different types have distinct chemical and physical properties. Differences in inhaled concentrations and fibrosis patterns have been observed between different asbestos types. For example, increased retention of crocidolite fibers compared to chrysotile fibers have been observed while mesothelial cells have been proposed to be more sensitive to crocidolite than chrysotile fibers.

Asbestos is known of its pulmonary toxicity (see eg. Wagner) and is one of the compounds that can be used to induce pulmonary fibrosis in experimental models. Seen as a benefit of the model, both IPF and asbestosis share histopathology of UIP in humans although similar histological appearance does not necessarily indicate common pathological processes. King et al. experimented on relationship of the asbestos fiber length to fibrosis and found longer fibers to induce more severe insult and the finding has
been confirmed in numerous studies afterwards (see eg. Davis et al.\textsuperscript{198} and Lee et al.\textsuperscript{199}). Wagner et al. showed already in 1974 that exposure to asbestos induces progressive fibrosis that continues to develop even after removal of the exposure.\textsuperscript{200} Fibrosis has been shown to develop in variety of animals including mice\textsuperscript{201,202}, rat\textsuperscript{203,204}, guineapig\textsuperscript{205} and sheep\textsuperscript{206,207}. As in human IPF, Gremlin mediated reduction of BMP and resulting imbalance between BMP and TGF-\(\beta\) signalling pathways have been observed in asbestos exposed mice.\textsuperscript{93} Asbestos is also shown to induce ROS generation in mice models.\textsuperscript{208} Pirfenidone has been shown to reduce asbestos induced fibrosis.\textsuperscript{209} However, no studies on pulmonary administration of anti-fibrotics have been performed in asbestos models.

Silica, formally known as silicon dioxide, is a material extensively found from nature and used in industry. Upon inhalation it causes progressive fibrosis in both humans (see eg. Wagner)\textsuperscript{195} and other mammals. In response to pulmonary exposure to silica TGF-\(\beta\) signalling has been shown to be upregulated in fibrosis prone mouse strain C57BL/6, while significant increase in TGF-\(\beta\) levels was not seen in fibrosis resistant BALB/c strain suggesting that it is one of the key factors in development of fibrosis in this model.\textsuperscript{210} However, there fibrosis induction by silica has been reported also in BALB/c strain.\textsuperscript{184} Substantial differences have been demonstrated also between other inbred strains of mice in terms of both qualitative and quantitative response.\textsuperscript{211} BMP inhibitor gremlin is overexpressed in silica induced pulmonary fibrosis in mice\textsuperscript{92} and intraperitoneal administration of BMP-7 has been shown to be anti-fibrotic in silica-induced model of pulmonary fibrosis in rats.\textsuperscript{212} Even when fibrosis is developed, the response has been shown to differ between different models. Barbarin et al.\textsuperscript{213} demonstrated that in response to silicia exposure Sprague-Dawley rats developed fibrosis and chronic inflammation with upregulation of TNF-\(\alpha\) and the development of fibrosis was prevented with anti-inflammatory medication. However, in NMRI mice only transient
Inflammation response with no TNF-α overproduction and the fibrosis was not prevented by anti-inflammatory drugs. As with bleomycin, upregulation of TNF receptor p75 has been shown to contribute to silica induced fibrosis. Expression of p75 has been shown to be elevated in C57BL/6, BALB/c and 129/J mice after silica exposure and double TNF receptor knock out mice were protected from the silica induced fibrosis. Anti-fibrotic effect of nintedanib has been shown in the silica model in C57BL/6 mice. To the author’s knowledge, silica model has not been used to study pulmonary administration of anti-fibrotic drugs before.

In conclusion, there are multiple ways to induce fibrosis in relevant preclinical models. The preclinical models of fibrosis show significant variation. Even confining oneself to one inductor, marked differences are seen between species and strains. This highlights the fact that when choosing a model for experiments or interpreting results, a careful consideration of characteristics of the specific insult, species and strain is required. Even so, many of the central signalling pathways are shared between the mural models and they seem to be in agreement with the observations from IPF patient samples.
3 AIMS AND HYPOTHESES

The aims of this study were to
1) develop a method to formulate carrier-free combination powders and poorly soluble drugs
2) formulate the antifibrotic small molecule tilorone as an inhalable dry powder
3) show the antifibrotic effect of tilorone in pulmonary administration

The hypotheses were
1) With the aerosol flow reactor method poorly water-soluble drugs can be formulated from aqueous solution with correct choice of excipients
2) Combination powders of drugs with opposing solubilities can be formulated using the aerosol flow reactor method
3) Tilorone is anti-fibrotic in pulmonary administration
4 MATERIALS AND METHODS

4.1 ANALYSIS OF POWDER COMPOSITION

The compositions were determined with proton nuclear magnetic resonance spectrometry (1H-NMR) (Bruker AVANCE 400 MHz) in D2O. Chemical shifts used were for tilorone: $\delta = 1.2$ ppm (6H), L-Leucine: $\delta = 1.6$ ppm (3H), D-Mannitol $\delta = 4.5$ ppm (2H), HP-$\beta$-CD: $\delta = 4.95$ ppm (7H), Prednisolone: $\delta = 7.49$ (1H) and Fludrocortisone-21-acetate: $\delta = 5.84$ (1H).

4.2 PARTICLE MORPHOLOGY

The particles were imaged with field-emission scanning electron microscopes (SEM, JEOL JSM-7500FA and Zeiss Sigma VP) at an acceleration voltage of 2–5 kV. The samples were coated with sputtered platinum or gold. The Calu-3 cell monolayers with the particles were dried at ambient conditions prior to the sputtering.

4.3 INHALATION SIMULATOR

An in-house developed inhalation simulator was used to characterize the aerosolization properties of the produced formulations. The method was designed to overcome difficulties presented by unstable aerosol sources for accurate sampling. The design has been described in detail elsewhere and only the key features and their rationale will be briefly outlined here as described by the patent.

Impactors, e.g. Berner-type low pressure impactor, next generation impactor and electrical low pressure impactor, are widely used to sort aerosols by their aerodynamical diameter.
However, they require a steady flow rate to function accurately. During the inhaler actuation process most of the particles are released in the very beginning of the inhalation for both DPI and MDI allowing insufficient time for the flow to reach steady state within the impactor. Additionally, collisions with the device tubing and turbulence developed within the device may break particle agglomerates biasing the results towards smaller particle size.

These problems are addressed by the inhaler simulator in several ways. When not actuating, the flow control section of the inhalation simulator is adjusted to give zero net flow to the mouth piece of the inhaler while feeding bypass flow with appropriate flow rate for the analyzing instruments. Upon actuation, part of the gas flow is lead through the mouthpiece of inhaler directed by computer generated inhalation profile. This ensures constant total flow rate that can be adjusted to the requirements of the analyzing instruments. A particle free film is formed on the edge of the flow by leading the bypass flow to the device through porous metal tube. This prevents particle collisions with the tubing and with a sufficiently large tube diameter to reduce turbulent flow, it ensures that particle agglomerates are not broken after the particle flow leaves the inhaler.

Emitted dose was measured gravimetrically by weighing the inhaler device before and after each inhalation. To determine the fine particle fraction a Berner-type low pressure impactor was used with stage aerodynamic cut-off diameters ranging from 0.03 to 15.61 μm. After collection in the stages, the mass fraction of the particles on each stage was gravimetrically detected to find the fine particle fraction for each formulation and inhaler device combination.
4.4 IN VITRO ASSAYS

Cytocompatibility of the formulations was studied in publications I and II. In publication I we assessed cell viability with human adenocarcinoma cell lines A549, Calu-3, and BEAS-2B and human monocytic cell line THP-1 differentiated to macrophages. Leucine coated powder was dissolved at concentrations of 200, 100, 50 and 15 μg/mL and cell viability assessed after 1, 3 and 6 h incubation. In publication II we performed similar toxicity assays with A549 and Calu-3 cell lines. We also determined the formulation induced generation of reactive oxygen species with similar protocol in said cell lines.

In all publications the drug permeation was studied across a differentiated monolayer of Calu-3 cells. The Calu-3 cell line differentiates into an epithelial-like monolayer with similar lung barrier properties.215 Drug was dispersed on the apical side of the monolayer and samples were collected from the basolateral chamber at predetermined time points. Drug concentration was then quantified by high performance liquid chromatography (HPLC).

In publication III in vitro activity of the formulation was assessed before proceeding to in vivo studies. TGF-β [(CAGA)_{12-luc}] and BMP [(Bre)_{2-luc}] pathway reporter cell lines were used as described by Leppäranta et al.159 Stimulation media was supplemented with varying concentrations of either pristine tilorone or formulated dry powder. After the incubation, concentration matched reporter activity was compared between the pristine drug and formulated dry powder.

4.5 IN VIVO STUDY

In publication III we performed an in vivo –study to assess the anti-fibrotic potential of tilorone in pulmonary administration. The study was approved by the Finnish national animal experiment board.
(ESAVI/10418/04.10.07/2016) and carried out in accordance with institutional guidelines, which fulfill the requirements defined in regulations of the Finnish Act on the Protection of Animals used for Scientific or Educational Purposes (497/2013) and were performed according to the 3R. Male C57b6/J mice were purchased from Enivgo.

The anti-fibrotic activity of tilorone in pulmonary administration was studied using silica induced lung fibrosis as model. To induce fibrosis, a modified version of the method described by Lakatos et al. was used.\textsuperscript{216} SiO\textsubscript{2} was suspended in sterile PBS and administered at a dose of 50 mg/kg in volume of 50μl. Anaesthesia was inducted with 3% isoflurane vapour in oxygen.

The mice were euthanized in a CO\textsubscript{2} chamber on experiment day 22. Right lung tissues were prefixed in a 4% paraformaldehyde solution and embedded in paraffin. They were sectioned with a 3 μm thickness and stained with hematoxylin and eosin. The degree of pulmonary fibrosis and inflammation was evaluated using a semi-quantitative scale from 0-3 allowing half steps. The scoring was done by two blinded researchers and in case of disagreement the scoring was discussed until consensus was found. The left lung was snap frozen in liquid nitrogen and homogenized. The homogenized powder was then used for mRNA and tissue concentration analyses.

Total tissue RNA was extracted and RNA integrity was analyzed with TapeStation at the Biomedicum Imaging Unit. Complementary DNA was synthesized with the iScript cDNA Synthesis Kit (BioRad) and quantitative RT-PCR performed using Taqman primers. TATA binding protein was used for reference. For tissue concentration tilorone was extracted from the lung samples as described by Zhang et al.\textsuperscript{217} and measured with a liquid chromatography system coupled to a tandem mass spectrometer (LC-MS).
5 RESULTS AND DISCUSSION

To successfully treat a patient it is never enough to come up with a potent drug. Leaving aside patient related problems and treatment adherence, active pharmaceutical ingredient (API) is still not sufficient in real life treatment. Each API needs to be formulated and it is the properties of the formulation as whole that determine pharmacokinetics and –dynamics of the API. With inhalable medicines drug delivery becomes even more complex, because the formulation is interacting not only with the patient, but with the inhaler as well. Also, all drug molecules interact differently with both excipients and device materials. With combination powders the complexity is increased further, as the number of different interactions increases exponentially as the number of compounds increases. No drug enters the market if it’s not deemed commercially profitable. Scalability of the formulation method and sufficient shelf life of the produced powder are therefore paramount for any attempted delivery platform. An ideal formulation method for inhalable molecules would therefore be easily scalable from laboratory to industrial scale and yield an easily storable powder, which could be administered independently from the clinical state of the patient, molecules used in the formulation, and inhaler used for the delivery at an affordable price.

5.1 COATING MATERIALS AND PARTICLE MORPHOLOGY

In publication I we produced a carrier-free combination powder of beclomethasone dipropionate and salbutamol sulphate. These are drugs of opposing solubilities and need highly different processing to produce an acceptable precursor for aerosol flow reactor processing. As the two drugs in clinical
use for treatment of IPF, pirfenidone and nintedanib, are also of opposing solubilities, the technology presented could allow combination treatment of IPF with single product.

In publication I we examined the use of three different amino acids, L-leucine, L-valine, and L-phenylalanine, as encapsulation and coating materials. Because the used aminoacids are amphiphilic and surface active, they have a tendency to accumulate the surface of the particles. They are therefore correctly positioned to form the coating and encapsulation layers at the heated stage of the reactor. There was extensive sintering in L-valine and L-phenylalanine coated particles. We hypothesized that the mannitol fraction of the particles is initially in a solid amorphous phase. Upon cooling it starts to crystallize and the particle integrity is largely determined by the coating materials ability to resist particle deformation upon crystallization. According to this hypothesis, L-leucine would be best suited to prevent the formation of large crystals within the particles, while L-phenylalanine would be the worst.

In publication III our first attempt to formulation of tilorone was to use L-leucine as the sole excipient. However, we found that tilorone is readily crystallized during the processing and L-leucine alone does not prevent the crystallization. This resulted in rhombohedral morphology seen in SEM micrograph in Figure 8. This morphology allows high contact area between the individual particles and is likely to lead poor aerosolization properties. Addition of mannitol to the formulation resulted in spherical particles with rough L-leucine coating as presented in publication III and this was hypothesized to result from prevention of tilorone crystallization by dilution.
Another way to prevent crystallization would be faster cooling. Mannitol has been shown to stay in amorphous form when rapidly cooled in aqueous solution\textsuperscript{219,220} and in general crystal sizes in rapidly cooled materials are smaller, since the molecules have less time and thermal energy to search for nucleation sites. In the current reactor configuration the dilution and cooling gas is injected into the particle stream at room temperature. If the dilution gas temperature was considerably lower, it could allow prevent sintering upon collecting even with the weaker coating materials. However, without further studies it is impossible to predict how the particles would behave upon storage. Phase changes upon storage could affect the pharmacokinetic profile of the powder lowering the shelf life of the end product.

Figure 8: SEM micrograph of tilorone crystals formed during the formulation process. Addition of 2.5 m-% of L-Leucine was not sufficient to prevent the crystallization and dilution by adding mannitol was needed.
5.2 AEROSOLIZATION AND DISSOLUTION

None of the coating materials produced inhaler-independent results in aerosolization experiments indicating that coating and encapsulation are not sufficient to block the interactions between inhaler materials and formulation components other than the coating material. These conclusions are further supported by results from publications II and III. In publication II we produced leucine-coated particles using cyclodextrine as matrix former with either prednisolone or fludrocortisone-21-acetate as API. In the aerosolization experiments there were differences between inhalers in coefficient of variance and fine particle fractions for both formulations and these interinhaler differences were again confirmed in publication III for tilorone dry powder. There was also significant difference in emitted dose between prednisolone and fludrocortisone powder when the same inhaler was used.

However, in all publications the particles coated with L-leucine performed well in terms of flow rate independence for at least one of the tested inhalers. Flow rate independence is probably the most important property of the powder, because it reflects the independence of patients inspiratory effort. It is relatively easy to normalize doses in respect to inhaler and specific drug, but nigh impossible to normalize it in respect to the clinical state of the patient. As discussed before, the encapsulation layer provides protection from moisture up to 65% relative humidity\textsuperscript{31}, which greatly increases the powders´ storability. While we have shown that the aerosol flow reactor method falls short from the described ideal formulation method, it still produces premium quality powders from both medical and commercial point of view.

For poorly water-soluble drugs, such as corticosteroids used in publications I and II, dissolution is a significant bottleneck en route to the site of action. However, currently European pharmacopoeia describes no standardized \textit{in vitro} methods to measure dry powder dissolution.\textsuperscript{221} In the work described by
this thesis we have used differentiated Calu-3 cells to model pulmonary epithelium\textsuperscript{215} to provide data on drug behavior after the aerosolization and deposition. However, \textit{in vivo} – \textit{in vitro} –correlation of the model remains to be established and therefore caution is required when these results are interpreted. Despite their limitations, the permeation experiments allow us to compare different formulations in terms of rate and repeatability of the dissolution. It has been shown that mucociliary clearance removes large beclomethasone particles before they have been absorbed into the lungs.\textsuperscript{222} Mucociliary clearance transfers the drug to the GI-tract where they are prone to cause systemic side effects. As seen from permeation of salbutamol sulphate in publication I and tilorone dihydrochloride in publication III, the formulation process doesn’t seem to have significant effect on the dissolution rate of water soluble drugs but it does produce more repeatable permeation profiles. However, the poorly soluble drugs beclomethasone dipropinoate from publication I, and fludrocortisone-21-aceta te and prednisolon from publication II seemed to benefit from the formulation mainly in terms of dissolution rate.

5.3 FORMULATION OF THE ANTI-FIBROTIC DRUGS

The currently used anti-fibrotic drugs have a difficult adverse effect profile. In the clinical trials for both nintedanib and pirfenidone almost all patients reported at least one adverse effect and majority of patients reported two or more. Altough the real-life studies indicate that discontinuation of the treatment can be managed with careful follow up and counseling, the reported adverse effects e.g. diarrhea, nausea, rash and photosensitivity are bound to lower the quality of life of the patients. Corticosteroids are a prime example how difficult adverse effect profile can be managed with a careful choice of administration route. Aside from oral and
intravenous forms, they also come in inhaled and topical formulations. Despite the drastic adverse effects of systemic corticosteroid medication, they can be used quite freely locally in treatment of asthma and many types of eczemas.

We have formulated the currently used anti-fibrotic drugs to inhalable form using the aerosol flow reactor method and shown that high quality dry powder can be formulated also from pirfenidone and nintedanib. (Vartiainen et al., unpublished data) The aerosolization results are presented in table 1. Pirfenidone is emitted from Easyhaler in a consistent manner. Coefficient of variance of emitted dose (CV) is under < 10% and fine particle fraction doesn’t change as function of applied pressure drop. However, the formulation is emitted from Twister very inconsistently as described by CV. This could result from particle interaction with the capsule or inhaler materials, but it has not been explored further. In contrast, nintedanib powder seems to work very well with the Twister. As seen from table 1, ED shows virtually no pressure dependence and FPF is also very stable. Even tough there is more pressure dependence in ED when emitted from the Easyhaler, the powder shows low CV at all of the tested pressure drops. Both of the drugs were shown to retain their biological activity in vitro when compared to their unprocessed counterparts.

While anti-fibrotic activity of these formulations is yet to be tested, viability of pirfenidone in pulmonary administration has been demonstrated in preclinical models by others.164–167 As pirfenidone and nintedanib are two drugs of opposing solubilites and pirfenidone is readily dissolved in ethanol, the combination particles of the two would likely be very similar to those presented in publication I and in principle there is no apparent reason why tilorone could not be added to the same particles. Production of combination particles with several drugs could even render addition of mannitol unnecessary, since chrystallization of tilorone could be prevented by dilution by the other two drugs.
Since diagnostic criteria of IPF are mainly descriptive, the condition is likely to consist of several different entities. As demonstrated in the works by Thomas et al.\(^6^1\) and Oldham et al.\(^8^5\) the disease may arise from different genetic backgrounds and personalized medication is therefore required for effective treatment and minimal side effects. In the work described in this thesis, we have shown that the aerosol flow reactor method can be used to formulate drug molecules with vastly different chemical properties. It could be used to combine wide range of molecules to best serve the needs of both clinicians and patients alike. Two clinical trials on combination of nintedanib with add-on pirfenidone have been published.\(^{223,224}\) While both showed that patients tolerated the combination therapy, the adverse effects were more common in patients on combination therapy than in patients on nintedanib only. Also the two studies were contradicting in their findings on pirfenidone’s effect on nintedanib’s pharmacokinetics. As pulmonary administration is a parenteral route, it avoids the first pass metabolism by liver. This is especially important for nintedanib, since its oral bioavailability was shown to be less than 5 % compared to intra venous dosing.\(^{225}\) Extensive first pass metabolism means relatively high GI-tract exposure when sufficient systemic drug concentrations are maintained. It also makes the drug prone to interpatient variation in dosing and

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<td>A 2 kPa EH</td>
<td>1.9</td>
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<td>2.2</td>
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<tr>
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<td>49</td>
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Table 1: Aerosolization results for pirfenidone (A) and nintedanib (B) formulations. Actuation from Easyhaler (EH) and Twister (Tw) at two pressure drops and inhalation flow rates. For Easyhaler 2 kPa = 43 L/min and 4 kPa = 55 L/min, and for Twister 2 kPa = 40 L/min and 4 kPa = 55 L/min. ED is average emitted dose (n=10); CV is coefficient of variation of emitted dose; FPF is fine particle fraction (geometric mean diameter <5μm)
slight changes in metabolism lead to large relative changes in drug exposure. Therefore, nintedanib treatment would likely to be safer and better tolerated in pulmonary administration for both nintedanib alone and in combination with other medications. It would also mean more affordable treatment, since less API would be needed.

As shown by Leppäranta et al\textsuperscript{159} and confirmed in publication III, tilorone is a potent antifibrotic agent. However, since TGF-\(\beta\) signaling is widely involved in homeostatic regulation and tumor suppression, systemic blockade could result in difficult adverse effect profile in humans. In publication III we have shown that pulmonary administration resulted in reduction of fibrosis and did not show any signs of acute toxicity. The difference in effectiveness between the systemic and pulmonary administration is most likely dose related.

The \textit{in vivo} experiment described in publication III was not designed to study or show adverse effects of tilorone in pulmonary administration and to the authors knowledge no such work has been conducted elsewhere. However, Seto et al. studied the pharmacokinetics of spray dried pirfenidone in rat and showed that pulmonary administration led to 600-fold lower systemic exposure when compared to oral dosing.\textsuperscript{165} In subsequent study by the same group they showed skin and eye exposure to be 90-130-fold lower in pulmonary administration.\textsuperscript{167} While the pharmacokinetics of pirfenidone and tilorone are not likely to be equivalent due to their difference in solubilities, the results are still encouraging as tilorone was suggested to cause keratopathy in the clinical trial by Cummings et al.\textsuperscript{161}

In the field of anti-fibrotics, tilorone’s mechanism of action is intriguing. The currently approved drugs do not cure the disease and only slow the progression at best. As described before, tilorone upregulates BMP signalling which is also promotes the formation of distal airways and alveoli during development.\textsuperscript{88} As has been shown in a \textit{in vivo} model of kidney fibrosis\textsuperscript{91}, upregulation of BMP signaling in fibrotic lesions
could in principle lead to rescue and regeneration of already
lost alveolar regions. To the authors knowledge, no experiment
has been performed to follow the response of individual lesions
to induction BMP signalling.

5.4 CONCLUSIONS

In this thesis we have shown that the aerosol flow reactor
method is a versatile method to produce inhalable carrier free
dry powders. As a proof of concept, we have formulated both
water soluble and insoluble drugs as well as their combinations
using multiple different excipients. We have shown that
antifibrotic small molecule tilorone can be formulated to
inhalable form and is effective in pulmonary administration in
a murine model of silica induced fibrosis. The next step in this
work is to commercialize the formulation method and proceed
to phase 1 clinical safety trials with inhalable tilorone.
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