Novel $\alpha v \beta 6$ Inhibitor Reduces Fibrotic Progression in Idiopathic Pulmonary Fibrosis Murine Model

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Novel $\alpha\nu$6 Inhibitor Reduces Fibrotic Progression
in Idiopathic Pulmonary Fibrosis

Murine Model

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Master of Science

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ABSTRACT

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Idiopathic pulmonary fibrosis (IPF) is one of the most aggressive and severe interstitial lung diseases (ILDs) for which there is no cure. IPF is characterized by an excessive accumulation of fibroblasts which secrete an abundance of extracellular proteins such as collagen. These processes lead to repetitive tissue scarring and fibrosis in the lung parenchyma. As a result, lungs become rigid limiting oxygen intake and gas exchange. Once diagnosed, IPF is fatal within 2-3 years. There is no known cause or proven treatment that significantly improves outcomes.

Although the cause is unknown, the current model of IPF suggests that an overactive epithelial repair mechanism caused by genetic and epigenetic factors as well as environmental exposures is responsible for the chronic fibrosis and scarring characteristic of IPF. The transforming growth factor beta (TGF-β) signaling pathway has been implicated as a major contributor in activating this chronic fibrosis. An upstream activator of the TGF-β pathway, αvβ6, has been identified as a potential therapeutic target.

My collaborators in Dr. David Baker’s lab at the University of Washington have created a novel αvβ6 integrin inhibitor (BP2_disulf) whose efficacy in improving IPF outcomes has yet to be tested. In my study, I test the ability of BP2_disulf to combat IPF through the use of the standard IPF murine model and translatable end points like non-invasive µCT scans, pulmonary function tests, bronchoalveolar lavage fluid (BALF) profiles, and histology. With these methods, I demonstrate that intraperitoneal injection of BP2_disulf in bleomycin-injured mice has the ability to decrease rate of fibrotic progression and pulmonary function decline compared to mice treated with bleomycin alone. These results prove that BP2_disulf is a promising therapeutic not only for IPF but other ILDs as well. Further efficacy validation and investigation into an aerosolized delivery method will advance this drug to clinical trials and make it accessible to those in need.

Keywords: idiopathic pulmonary fibrosis, interstitial lung disease, IPF, αvβ6 integrin, TGF-β
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1. INTERSTITIAL LUNG DISEASES AND IDIOPATHIC PULMONARY FIBROSIS

1.1 Interstitial Lung Disease

Interstitial lung diseases (ILDs) are a group of over 200 heterogeneous lung abnormalities characterized by inflammation and fibrosis of the lung parenchyma. Diseased lung tissue around the air sacs or alveoli becomes inflexible causing difficulties in breathing and transportation of oxygen to the bloodstream. This loss of lung function leads to symptoms that include shortness of breath, cough, weight loss, chest pain, and fatigue. More severe ILDs can leave patients disabled frequently leading to anxiety and/or depression as quality of life deteriorates. These symptoms are often progressive and lung damage can be irreversible. This damage can be life threatening and lead to heart or respiratory failure. For some ILDs, there is no cure. Many suffer from ILDs worldwide. While ILD epidemiology data collection has its limitations due to the variability in past definitions and classifications, a study performed in New Mexico found disorder prevalence to be estimated at 80.9 in 100,000 men and 67.2 in 100,000 women.

Although related, each ILD is unique whether severe or mild, acute or chronic, and reversible or irreversible. ILDs can be categorized based on their etiology and other distinguishing features as seen in Figure 1-1. Many ILDs are induced by environmental or occupational exposures like asbestos, silica or wood dust, fumes, and long exposure to birds and livestock. Others can be brought on by medications such as amiodarone or chemotherapeutic
agents like cyclophosphamide and bleomycin. Infection and radiation treatment are other known causes. Idiopathic interstitial pneumonias (IIPs) are a subset of ILDs where the cause is unknown. The most common IIP is idiopathic pulmonary fibrosis (IPF).

Figure 1-1: Interstitial Lung Diseases. A categorization scheme for ILDs including examples for each category. Adapted from Meyer (2019). Copyright 2019 Springer Nature Switzerland AG.
With such a large variety of ILDs, correct diagnosis is key to patient recovery or appropriate palliative care. In order to diagnose, primary care physicians perform physical examinations, chest radiographs, high-resolution computed tomography (HRCT), and pulmonary function tests. ILD pulmonary function tests are commonly associated with reduction in total lung capacity (TLC), forced vital capacity (FVC), and diffusing lung capacity of carbon monoxide (DLco).\textsuperscript{11,12} Additionally, HRCT scans are used to differentiate between ILDs through the visualization of specific lung abnormalities such as honeycombing, ground glass attenuation, and bronchial wall thickening.\textsuperscript{10} In some cases, lung biopsies are performed but are limited due to their invasive nature and high risk in older and frail patients.\textsuperscript{10} ILD diagnoses should be confirmed with a pulmonologist because correct diagnosis can be delayed up to a year or more often being mistaken for other lung diseases like chronic obstructive pulmonary disease, bronchitis, or asthma.\textsuperscript{13}

After diagnosis has been successfully determined, an appropriate treatment is prescribed. Some treatments simply call to eliminate exposures causing the disease.\textsuperscript{3} In other cases, anti-inflammatory medication or corticosteroids are prescribed.\textsuperscript{3,14} Many patients have indicated that side effects from many of these medications are burdensome and better assistance in managing them is necessary.\textsuperscript{15} In more progressive ILDs, as with the case of IPF, there is no cure and treatments focus on relieving symptoms and slowing the progression of lung damage. Pulmonary rehabilitation can be used in these cases and involves disease education and personalized exercise.\textsuperscript{1} Patients are educated on disease mechanisms, available treatments, human nutrition, and stress management.\textsuperscript{3} Breathing and relaxation techniques, as well as strength, endurance, and respiratory training are also utilized.\textsuperscript{3} The use of pulmonary rehabilitation has been shown to reduce symptoms, improve physical fitness, and enhance quality of life for patients with ILDs.\textsuperscript{16}
However, limited funding, lack of knowledge, and shortage of rehabilitators leave this option unavailable for many. Lung transplantation is also a potential treatment for some patients, but insufficient number of donors and high risk of rejection and complications also leave this as a limited option. ILD patients often exhibit comorbidities such as gastroesophageal reflux disease (GERD), pulmonary hypertension, and emphysema that complicate treatment decisions and efficacy.

Limited and imperfect diagnoses and treatments leave ILD patients greatly disadvantaged. ILDs need to be studied more in depth to better determine specific causes and unique disease mechanisms. With this knowledge, personalized treatments and cures can be developed rather than relieving symptoms alone. This is especially critical for more severe ILDs like IPF whose cause is unknown and whose prognosis is fatal.

1.2 Idiopathic Pulmonary Fibrosis

Idiopathic pulmonary fibrosis (IPF) is one of the most common and aggressive interstitial lung diseases. IPF is characterized by repetitive tissue scarring and heterogeneous fibrosis in the lungs that is the result of excessive fibroblast activation and extracellular matrix deposition. Lungs become inelastic inhibiting lung function and limiting oxygen intake.

Lung function is highly dependent on lung structure. The lungs are made up of large and small airways called bronchi and bronchioles. During inhalation, air travels through these bronchi and bronchioles to capillary-rich air sacs called alveoli. As air moves through the alveolar walls, oxygen diffuses to red blood cells in the capillaries and is then transported throughout the body. Carbon dioxide exits the blood and is released on exhalation.
In IPF lungs, a dysfunctional epithelial repair system leads to development of scar tissue on the alveolar walls. This leaves lungs rigid making the ability to inhale difficult which decreases oxygen intake. Not only is oxygen intake decreased, but this scar tissue also creates a thick barrier between alveolar air space and capillaries inhibiting oxygen diffusion to the bloodstream. This leads to breathlessness, dry cough, weight loss, and fatigue. Lack of oxygen can also result in finger clubbing. Some patients may experience a steady decline over time while others experience rapid progression and acute exacerbations. Eventually this limited oxygen results in respiratory failure and death. Once diagnosed, IPF has a median survival time of only 2-3 years. There is no known cause and no treatments available that significantly improve patient outcomes. The plethora of pathways believed to contribute to the pathogenesis of IPF make understanding disease mechanisms and developing new treatments extremely difficult. While much is still unknown, epidemiology methods, diagnostic procedures, knowledge of disease mechanics, and treatment options have greatly improved over the last decade.

1.2.1 Epidemiology

Prevalence and incidence of IPF have been tracked over several studies. Results of these studies vary most likely due to differences in data collection methods as well as ambiguity and inconsistency of past IPF definitions. Recent clarification of IPF criteria will help to increase accuracy of epidemiological data. A review of several epidemiology studies found IPF to have a prevalence of 0.5-27/100,000 and an incidence of 0.22-8.8/100,000 inhabitants worldwide. IPF is more likely to occur in males and cigarette smokers. Prevalence is also found to be higher in North America and Europe. IPF is rare in younger individuals but

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increases with advancing age.\textsuperscript{10} For those older than 75 years the prevalence may exceed 175/100,000.\textsuperscript{29,30} The typical age of onset is between 50 and 70 with the mean age of diagnosis being 66.\textsuperscript{26,27}

1.2.2 Diagnosis

The process of diagnosing IPF can be challenging and lengthy due to the variety of disease manifestations. Physical examinations, radiology, and histology are all used to inform diagnosis. The diagnosis process starts with recognizing clinical manifestations of the disease. This includes chronic dyspnea, cough, bibasilar inspiratory crackles (Velcro-like crackles) and/or digital clubbing.\textsuperscript{18} A physiological examination that includes spirometry testing is performed to examine lung function. Reduction in FVC, TLC, and DLco are common in IPF patients and predicative of survival.\textsuperscript{23} IPF patients experience a decline in FVC over the progression of the disease with a mean rate of decline of 150-200mL/year.\textsuperscript{22}

Patients may also be subject to a 6-minute walk test measuring the distance a patient can quickly walk across a flat surface in 6 minutes.\textsuperscript{31} The 6-minute walk test is used to evaluate fitness capacity.\textsuperscript{31} Desaturation to less than 88% during a 6-minute walk test and 15% decline of DLco together correlate with increased risk of mortality.\textsuperscript{32} Along with pulmonary function testing, a detailed medical history is also examined to assess past hazardous exposures such as cigarette smoking or other risk factors like gastroesophageal reflux, chronic viral infections, or a family history of lung disease.\textsuperscript{27} An IPF diagnosis requires the exclusion of all other known causes of ILDs.\textsuperscript{27}

An IPF diagnosis also requires the presence of a usual interstitial pneumonia (UIP) pattern in HRCT or a specific combination of certain HRCT and histopathological patterns in
patients requiring lung tissue sampling. Diagnoses are highly reliant on radiological findings. With images acquired from HRCT, lung abnormalities are observed that allow radiologists to differentiate ILDs (Figure 1-2). In IPF lungs, UIP is the typical pattern observed through HRCT. A UIP pattern commonly consists of honeycomb cysts, traction bronchiectasis, and traction

**Figure 1-2: Chest HRCT images of ILD patterns.** Transverse, coronal and sagittal views (left to right) of UIP pattern, probable UIP pattern, and patterns associated with an alternative diagnosis. UIP pattern is distinguished by subpleural honeycombing (yellow arrows) with or without traction bronchiectasis (green arrows), ground glass opacification (black arrows), and fine reticulation (blue arrows) (A-C). A probable UIP pattern consists of reticular abnormalities without honeycombing (D-F). Patterns associated with an alternative diagnosis may include a predominance of ground glass opacities (black arrows) and reticular abnormalities in the lower lung (G-I). Figure adapted from Lederer and Martinez (2018). Copyright 2018 The New England Journal of Medicine.
bronchiolectasis. It can also be associated with ground glass opacification and fine reticulation.\textsuperscript{27} Honeycombing must be present for a “UIP pattern” diagnosis to be made.\textsuperscript{27} However, honeycombing diagnosis is prone to interobserver variability even among expert ILD radiologists.\textsuperscript{33}

In some cases, lung HRCT images will contain some characteristics of a UIP pattern but will not include honeycombing.\textsuperscript{27} This is classified as a “probable UIP pattern”.\textsuperscript{1} Further testing such as a surgical lung biopsy (SLB) may need to be performed to confirm an IPF diagnosis.\textsuperscript{27} SLB is often performed through a video-assisted thoracoscopic surgery if single-lung ventilation can be tolerated.\textsuperscript{27} Multiple biopsies must be obtained to confirm histological features and “UIP pattern” diagnosis as IPF affects the lung in heterogeneous ways.\textsuperscript{27} The criteria for a “UIP pattern” in SLB include the appearance of patchy dense fibrosis, remodeled lung architecture, and areas of honeycombing that alternate with areas of less-affected parenchyma.\textsuperscript{27} Subpleural and paraseptal areas are usually affected most severely.\textsuperscript{27} Dense areas of collagen along with proliferating fibroblasts and myofibroblasts are common.\textsuperscript{27} Even in the absence of honeycombing a “UIP pattern” diagnosis can be made if all other features are present.\textsuperscript{27} As with HRCT pattern diagnosis, diagnosis of histopathological patterns is also subject to interobserver variation.\textsuperscript{34}

While SLB is a powerful confirmation tool, costs often outweigh the benefits. SLB has been associated with an increased risk of mortality and acute exacerbations in elderly patients with comorbidities.\textsuperscript{18} The 30-day mortality rate following SLB has been described as high as 17%.\textsuperscript{35} SLB should therefore be considered on a patient-by-patient basis.\textsuperscript{27} A “probable UIP pattern” diagnosis in HRCT has been shown to have a high positive correlation with “UIP
pattern” diagnosis in SLB. As a result, some argue a “probable UIP” diagnosis in HRCT should be enough to have a working diagnosis of IPF, reducing the need for risky SLB.

With the risk of SLB complications, other methods, such as the analysis of bronchoalveolar lavage fluid (BALF), can be used to confirm IPF diagnosis. BALF is obtained through a procedure called bronchoscopy where fluid is flushed into and collected from the lungs. This fluid is analyzed and differential cell counts including relative percentages of neutrophils, macrophages, lymphocytes, and eosinophils are obtained. To aid clinicians in diagnosis, the American Thoracic Society (ATS) collected data from several studies to determine common BALF profiles for healthy individuals as well as IPF patients (Table 1-1). BALF can be used in narrowing differential diagnosis. However, its diagnostic abilities are limited as some ILDs have very similar BALF profiles to IPF. As a result of the limited capacity of BALF differential cell counts and the interobserver variability among both HRCT and SLB findings, multidisciplinary discussion among experts such as clinicians, radiologists, pathologists, and rheumatologists is recommended before final diagnosis is made.

<table>
<thead>
<tr>
<th>Cell analyzed in BALF</th>
<th>Healthy</th>
<th>IPF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophil</td>
<td>≤ 3%</td>
<td>5.9-22.08%</td>
</tr>
<tr>
<td>Macrophage</td>
<td>&gt;85%</td>
<td>49.18-83%</td>
</tr>
<tr>
<td>Eosinophil</td>
<td>≤1%</td>
<td>2.39-7.5%</td>
</tr>
<tr>
<td>Lymphocyte</td>
<td>10-15%</td>
<td>7.2-26.7%</td>
</tr>
</tbody>
</table>

As discussed, many methods such as HRCT, SLB, BALF, and multidisciplinary discussion are utilized to determine final IPF diagnosis. However, even with these variety of
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methods, the diagnosis process can be lengthy taking up to 5 years from first onset of symptoms.\textsuperscript{40} IPF is a battle against time and improvements to the lengthy diagnosis process should be considered. With faster and more accurate diagnosis, patients can receive better care. Clinical management can be optimized and the opportunity for lung transplantation may increase.\textsuperscript{41} Earlier diagnosis will help improve patient treatments, however lack of understanding regarding IPF pathogenesis remains a huge barrier to treatment development.

1.2.3 Pathogenesis

While the etiology of IPF remains elusive, significant research dedicated to this field has led to the proposed model of IPF we know today. First characterized as an inflammatory-based disease, IPF has since been redefined as an aberrant epithelial-dependent fibroblast-activated disease.\textsuperscript{42} Inflammatory pathways may still contribute to the onset of IPF in a subset of patients, however most patients remain unresponsive to anti-inflammatory therapies.\textsuperscript{42}

The current model for IPF proposes that genetic and epigenetic factors along with recurring epithelial injury lead to an aberrant repair mechanism causing fibroblast activation and differentiation as well as extracellular matrix deposition in the lung parenchyma.\textsuperscript{19} This creates the progressive fibrosis and distorted lung architecture characteristic of IPF.\textsuperscript{19}

Healthy lung architecture is composed of clusters of alveoli or air sacs covered in a net of capillaries. This is where gas exchange occurs. An alveolus is comprised of two continuous cell layers separated by interstitial space of varying thickness.\textsuperscript{43} The two cell layers consist of the epithelium lining facing the alveolar air space and the endothelium layer facing the capillaries.\textsuperscript{43} The epithelium is made up of two types of cells: type I alveolar epithelial cells (AECI) and type
II alveolar epithelial cells (AECII). AECIs primarily make up the lining of this layer while AECIIs are responsible for renewal and repair as well as secretion of surfactant surface film.

The interstitial space between the two cell layers is made up of extracellular matrix, a network of elastic fibers and collagen bundles. The most abundant cells in the interstitium are fibroblasts and myofibroblasts that produce and maintain this extracellular matrix. The interstitium is made up of thicker areas concentrated with cell nuclei and fiber networks that contribute to structure and stability and thinner areas to maximize gas diffusion. The endothelial cell layer creates a structural barrier between the interstitium/epithelial layers and extravascular components. All components of alveoli are susceptible to injury as we inevitably breath in toxins, pathogens, and particulates every day.

Lung epithelial injury involves several inflammatory, repair, and reepithelization responses. After epithelial injury, an inflammatory response is activated, pro-inflammatory mediators are generated, and accumulation of neutrophils and macrophages occurs. Both neutrophils and macrophages are responsible for cleaning debris associated with injury. These debris may include matrix materials, surfactant components, capillary leakage, and apoptotic cells. This process of engulfing apoptotic cells is known as efferocytosis. During efferocytosis, macrophages undergo a change in phenotype from pro-inflammatory to anti-inflammatory and pro-repair. The release of pro-inflammatory cytokines decreases while more anti-inflammatory cytokines and growth factors that promote proliferation such as transforming growth factor beta (TGF-β), interleukin 10 (IL-10), vascular endothelial growth factor (VEGF), and hepatocyte growth factor (HGF) are generated.

If vasculature is breached in injury, a coagulation cascade and vascular remodeling will occur. Fibroblast and myofibroblast recruitment and epithelial-to-mesenchymal transition
(EMT) begins. Fibroblasts become activated leading to repair and buildup of extracellular matrix in the interstitium. During this time AECIIs will spread, proliferate and differentiate into AECIs which contribute to epithelial restoration. In IPF, this repair mechanism is defective. It is believed that repair mechanisms like EMT and fibroblast activation remain overactive causing an endless buildup of extracellular matrix. This creates thick barriers to the capillaries limiting oxygen exchange. The cause of this overactivation is still unknown, but many pathways and systems are suspected to be involved.

Cellular senescence, oxidative injury, proteostatic dysregulation, endoplasmic reticulum stress, and mitochondrial dysfunction have all been suspected to contribute to the profibrotic phenotype. Many other pathways have also been identified as mediators of progressive lung fibrosis such as those involving TGF-β, connective-tissue growth factor (CTGF), fibroblast growth factor 2 (FGF2), platelet-derived growth factor (PDGF), microRNAs, impaired autophagy, activation of developmental pathways, matrix metalloproteinase 7 (MMP-7), lysyl oxidase–like 2 (LOXL2), and lysophosphatidic acid (LPA).

Genetics may also play a role. Mutations and variations in genes involving maintenance of telomere length as well as cell adhesion, integrity and mechanotransduction are all associated with higher risk of IPF. A single-nucleotide polymorphism (rs35705950) in the promoter region of MUC5B is strongly associated with increased expression of MUC5B and development of IPF. MUC5B is a gel-forming mucin expressed in bronchial epithelial cells. Overexpression of MUC5B leads to mucus hypersecretion, impairs mucociliary clearance, and may interfere with alveolar repair suggesting a role in IPF pathogenesis. Lung bacterial burden especially that of streptococcal and staphylococcal species has also been thought to play a role in disease severity.
Recurring lung injury is also believed to contribute to the onset of IPF. Several environmental factors such as cigarette smoke, repetitive silent micro aspirations, and chronic viral infections may contribute to repeated lung injury and IPF pathogenesis. Increased understanding of IPF pathogenesis and the epigenetic, genetic, and environmental factors involved has led to the development of new IPF treatments.

1.2.4 Treatment

In the last two decades, significant progress has been made in the diagnosis, understanding, and therefore treatment of IPF. It was not until the year 2000 that an international consensus statement was published to define diagnostic criteria and standardized definitions of IPF. Previous to this, IPF was often referred to under several different names such as fibrosing alveolitis and chronic idiopathic pneumonia. Published clinical trials and research were difficult to interpret due to the vast variety of definitions of the disease. At the time, no pharmacological treatments existed and understanding of disease pathogenesis was extremely limited. With this incomplete knowledge, therapies targeting inflammatory pathways were suggested. One recommendation was a triple combination treatment of prednisone and azathioprine with cyclophosphamide or N-acetylcysteine. Many other anti-inflammatories were tested with most of them ending in failed clinical trials.

In 2011, new ATS guidelines for IPF were announced abandoning the idea that IPF was an inflammatory-based disease and rather a disease driven by epithelial and fibroblast morphologies. The ATS acknowledged that past aggressive immunosuppressive therapies failed to reduce the death rate and in some cases were harmful. In October 2014, the Food and Drug Administration approved two drugs against IPF, pirfenidone and nintedanib. Both
were approved for their antifibrotic effects.\textsuperscript{54} In 2015, ATS released a statement with updated treatment recommendations conditionally recommending both drugs.\textsuperscript{55}

Pirfenidone is an orally administered drug that has antifibrotic, anti-inflammatory and antioxidant effects.\textsuperscript{56} It has the ability to reduce fibroblast proliferation, collagen biosynthesis, and production of TGF-\(\beta\) and tumor necrosis factor alpha (TNF\(\alpha\)).\textsuperscript{54} In clinical studies, evidence suggests improved mortality and reduced rate of FVC decline in IPF patients with the use of this drug.\textsuperscript{55} Adverse effects were reported from mild to moderate in severity and included nausea, dyspnea, vomiting, anorexia, rash, photosensitivity, headache, dizziness, fatigue and elevated hepatic enzyme levels.\textsuperscript{54}

Nintedanib is a multiple tyrosine kinase inhibitor that targets receptors of FGF, VEGF, and PDGF.\textsuperscript{55} Studies have shown a reduced rate of decline in FVC, fewer acute exacerbations, and preserved quality of life with use of nintedanib.\textsuperscript{57,58} Common side effects included diarrhea and moderate liver toxicity.\textsuperscript{56-58} Both nintedanib and pirfenidone have been primarily tested on patients with mild to moderate symptoms.\textsuperscript{54} Evidence on efficacy of these drugs in patients with severe IPF is limited.\textsuperscript{56}

Many IPF patients experience other comorbidities that may be treated in conjunction with IPF symptoms. One of the most common comorbidities associated with IPF is GERD. Past studies have reported prevalence of GERD in IPF patients at about 90% commonly manifested as asymptomatic micro aspirations of stomach acid into the lungs.\textsuperscript{59-60} ATS recommends antacid therapy to treat GERD in a majority of patients.\textsuperscript{23}

ATS also recommends the use of nonpharmacological therapies in the treatment of IPF.\textsuperscript{23} A common nonpharmacological method used by IPF patients is long-term oxygen therapy. There is limited evidence to suggest that the use of oxygen therapy improves survival, however it may
improve exercise capacity which has been shown to improve patient quality of life.\textsuperscript{23} Despite uncertainty regarding benefits versus inconvenience and cost to patients, oxygen therapy is conditionally recommended by the ATS.\textsuperscript{23} This recommendation is heavily driven by ethical concern over withholding supplemental oxygen from patients experiencing significant resting hypoxia.\textsuperscript{23}

Along with oxygen therapy, the ATS also recommends the use of pulmonary rehabilitation programs.\textsuperscript{23} Pulmonary rehabilitation programs include disease education, individualized strength and aerobic exercise, and techniques to deal with stress, anxiety, and depression that often accompanies knowledge of IPF prognosis.\textsuperscript{3} These therapies have been shown to improve symptoms, six-minute walk distance, and quality of life in IPF patients.\textsuperscript{61,62}

Lung transplantation was also recommended for appropriate patients.\textsuperscript{23} Benefits of lung transplantation vary with five-year survival rates after lung transplantation estimated to be between 50-56\%.\textsuperscript{23} The survival benefit between single-versus-double lung transplant remains unclear.\textsuperscript{23,63} Limited donors, comorbidities, age, and IPF severity may leave this as a restricted option for many patients.\textsuperscript{3}

While major developments have been made in IPF therapies, there is still no cure. Disease prognosis is still short, and quality of life diminishes quickly. Many drugs and therapies are under investigation to solve this devastating problem.

1.3 Conclusion

IPF is a harsh, progressive interstitial lung disease usually fatal within 2-3 years after diagnosis.\textsuperscript{23} Patients often experience dyspnea, dry cough, and fatigue brought on by excessive scar tissue and fibrosis in the lung parenchyma.\textsuperscript{21} Knowledge of IPF etiology remains limited.
Although the cause is unknown, the current IPF model suggests that genetic and epigenetic factors lead to dysfunction in the epithelial repair process in IPF lungs. As a result of defective repair mechanisms, environmental exposures lead to exacerbated micro-injuries in the lungs that cause chronic and progressive fibrosis. Limited treatments are available to IPF patients and do little to substantially elongate survival. More work needs to be done in pursuit of improved therapies and a better future for suffering IPF patients.
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2. A NOVEL αvβ6 INHIBITOR TO COMBAT IPF

2.1 The link between IPF and the TGF-β pathway

Many pathways are being investigated and many drugs are being developed in hopes of finding the key to discovering a cure for IPF. One of these pathways is the transforming growth factor beta (TGF-β) cell signaling cascade. TGF-β is known to have roles in embryogenesis, cellular development and differentiation, immunologic system development, inflammatory response, and wound repair. High expression of TGF-β and TGF-β target genes have been found in IPF patients. As a result, the TGF-β pathway has been implicated as a key driver in the pathogenesis of IPF and has become a major focus in the development of IPF therapeutics.

2.1.1 TGF-β pathway and activation

TGF-β is a multifunctional cytokine that is initially produced as a homodimeric peptide with a C-terminal mature TGF-β and N-terminal propeptide known as latency-associated peptide (LAP). LAP is eventually cleaved from the mature TGF-β by a furin convertase. After cleavage, LAP and TGF-β remain associated through noncovalent interactions. This is known as the small latent complex (SLC). LAP then becomes linked with a TGF-β binding protein
(LTBP) through a pair of disulfide bonds.\textsuperscript{4,5} This is known as the large latent complex (LLC).\textsuperscript{4,5} TGF-β is secreted by cells as either an SLC or LLC.\textsuperscript{6}

Once activated, TGF-β is released from the complex and binds to a transmembrane receptor kinase known as TGF-β receptor II (TβRII).\textsuperscript{1} The TβRII then recruits and phosphorylates TGF-β receptor 1 (TβRI) creating a stable heteromeric complex.\textsuperscript{5} Activin receptor-like kinase 5 (ALK-5) is a TβRI that plays a critical role in TGF-β-mediated tissue fibrosis.\textsuperscript{5} Signaling from phosphorylated ALK-5 occurs through a Smad-mediated cascade to the nucleus where the modulation of expression of target genes associated with fibrotic tissue formation occurs.\textsuperscript{5} TGF-β stimulates type 1 collagen gene transcription and plays a role in fibroblast proliferation, myoblast differentiation, and extracellular matrix deposition.\textsuperscript{1,4} Evidence suggests that other TGF-β signaling pathways like those initiated by ALK-1 may play a role in fibrotic phenotypes, however, ALK-5 Smad-mediated pathways have shown to be critical in the pathogenesis of IPF.\textsuperscript{4,5}

For these important signaling cascades to occur, TGF-β must first be activated. TGF-β can be activated through the action of proteases, pH changes, reactive oxygen species, and integrin compounds.\textsuperscript{5,7} The most common TGF-β activation compound associated with fibrotic lung diseases is the integrin αvβ6. The αvβ6 integrin is a heterodimeric transmembrane protein whose expression is restricted to the epithelium.\textsuperscript{7} It binds ligands through an arginine-glycine-aspartate (RGD) sequence.\textsuperscript{7} Much is still unknown about the precise mechanisms of αvβ6-mediated TGF-β activation. However, it has been identified that αvβ6 binds to the RGD motif on LAP in the LLC to release an active TGF-β (Figure 2-1).\textsuperscript{8}

Alpha-vβ6 has been identified as a potential therapeutic target against TGF-β activation and thus IPF.\textsuperscript{9} This is due to its high expression levels and contribution to fibrotic diseases.\textsuperscript{10}
Figure 2-1: TGF-β activation and Smad-mediated signaling cascade. TGF-β is produced as a homodimeric peptide consisting of a mature TGF-β unit and a latency-associated peptide (LAP). LAP is cleaved by furin convertase. After cleavage, TGF-β and LAP still associate through noncovalent bonds. This is known as the small latent complex (SLC). TGF-β binding protein (LTBP) binds to the complex and is known as the large latent complex (LLC). A transmembrane protein, αvβ6, binds to the LAP on the LLC to release active TGF-β. Once active, TGF-β binds to a transmembrane receptor kinase known as TGF-β receptor II (TβRII). TβRII recruits and phosphorylates TGF-β receptor 1 (TβRI). The most common TβRIs associated with fibrotic diseases are ALK-5 and ALK-1. Signaling progresses through a Smad-mediated cascade to the nucleus where transcription of critical genes associated with fibrotic development occurs. Examples represented in this figure are connective tissue growth factor (CTGF) and COL1A1 and COL1A2 associated with production of type 1 collagen. TGF-β cell signaling is associated with fibroblast proliferation, myofibroblast differentiation, and extracellular matrix (ECM) deposition. Illustration is adapted from Biernacka et. al (2011). Copyright 2011 Department of Health and Human Services.
Alpha-\(\alpha\beta6\) is expressed at low levels in normal adult tissues but has seen to be overexpressed during epithelial injury.\(^7\) Studies have also shown that \(\alpha\beta6\) null mice do not develop lung fibrosis despite bleomycin injury.\(^10\) The lack of lung fibrosis was shown to be at least in part due to decreases in TGF-\(\beta\) activation implicating the critical role of \(\alpha\beta6\)-mediated TGF-\(\beta\) pathways in the pathogenesis of IPF.\(^10\)

Many challenges arise with drug development of targeted inhibition. In this context, total TGF-\(\beta\) inhibition therapeutic methods are problematic since TGF-\(\beta\) has several other important homeostatic roles in inflammation, immune tolerance, and cancer biology.\(^11\) Since \(\alpha\beta6\) is restricted to epithelial cells, \(\alpha\beta6\) is a promising target to reduce TGF-\(\beta\) activation in diseased lung tissue without adverse effects due to TGF-\(\beta\) total loss of function.\(^11\) As IPF is suspected to be an epithelium-driven disease targeting an epithelium-restricted activator is an excellent avenue to combat IPF.

### 2.1.2 Alpha-\(\alpha\beta6\) as a therapeutic target in IPF

Alpha-\(\alpha\beta6\) has been recognized as a useful biomarker and target to track development and inhibit progression in a variety of cancers and fibrotic diseases like IPF.\(^10,12,13\) Many studies have been performed to test the specificity of \(\alpha\beta6\) binders and their ability to bar TGF-\(\beta\) activation and prevent advancement of these diseases.

One strategy used to create \(\alpha\beta6\) binders utilizes the use of cysteine-rich knottins. Knottins, also known as inhibitor cystine-knots, are miniproteins characterized by interwoven disulfide bridges forming intramolecular knots.\(^14\) Their high stability and resistance to temperature, enzymatic degradation, and extreme pH have led to their use as scaffolds in drug engineering and medical imaging.\(^14\) Alpha-\(\alpha\beta6\) knottin binders have been developed with single-
digit nanomolar affinity and high long-term storage stability. However, production of these knottins requires complicated synthetic or biosynthetic procedures and subsequent refolding.

Much research has also been dedicated to developing antibodies against αvβ6 with therapeutic relevance. 264RAD is a human monoclonal antibody against αvβ6. It has been observed to prevent αvβ6-mediated dermal skin fibroblast activation through localized TGF-β inhibition as well as reduce tumor growth in vivo. While 264RAD has shown promising results in its affinity and inhibition of αvβ6 activity, it lacks total specificity as it is known to bind to another αv integrin variant, αvβ8. BG00011, formerly known as STX-100, is another monoclonal antibody against αvβ6 that initially showed promising results in its potential to inhibit collagen accumulation and other fibrotic phenotypes in an IPF murine model. BG00011 proceeded to clinical trials but was terminated due to safety concerns. GSK3008348 is another small protein αvβ6 inhibitor that has proceeded to clinical trials, however long-term influence against IPF has yet to be determined. Lack of specificity and stability, complicated manufacturing procedures, and adverse side effects have left αvβ6 inhibition an unsuccessful arena for IPF treatment. Despite these complications more can be done to improve αvβ6 inhibitors and their potential to combat IPF.

2.1.3 BP2_disulf: an improved αvβ6 binder therapeutic

My collaborators in Dr. David Baker’s lab at the University of Washington (UW) sought to create a de novo αvβ6 inhibitor that addressed the weaknesses of past αvβ6 binders. They developed a novel αvβ6 inhibitor protein referred to as BP2_disulf with sub-nanomolar affinity and >2000x specificity over other RGD-binding integrins. This high affinity and specificity are obtained by BP2_disulf’s unique structure. As αvβ6 is a protein that binds to ligands with
RGD motifs, BP2_disulf contains an RGD loop.\textsuperscript{17} It also contains a second and third loop that makes contacts with the $\alpha v$ subunit and the $\beta 6$ subunit on $\alpha v \beta 6$ allowing for increased affinity and specificity.\textsuperscript{17}

Not only does BP2_disulf have high affinity and specificity, it is also easy to manufacture.\textsuperscript{17} This novel protein is easily expressed and produces high yields in \textit{E.Coli}, and crude cell lysate is easily purified in one simple heating step.\textsuperscript{17} BP2_disulf was also shown to have high thermal stability during nebulization, and a short serum half-life of under 2 hours.\textsuperscript{17} This short half-life reduces systemic exposure and the possibility of adverse side effects.\textsuperscript{17} High affinity, specificity, thermal stability, short serum half-life, and manufacturing ease makes BP2_disulf an excellent candidate for further research in its ability to inhibit TGF-\beta signaling and IPF progression.

My UW collaborators provide evidence that BP2_disulf blocks $\alpha v \beta 6$-mediated TGF-\beta signaling \textit{in vitro}.\textsuperscript{17} However, BP2_disulf's ability to reduce fibrotic progression and improve IPF outcomes had yet to be tested. I hypothesized that BP2_disulf’s inhibition of $\alpha v \beta 6$ would reduce the rate of fibrotic progression and lung function decline in IPF. I sought to test this hypothesis in the standard IPF murine model. I measured fibrotic progression and lung function of bleomycin-injured mice treated with BP2_disulf through the analysis of measured weight loss, $\mu$CT scans, pulmonary function tests, BALF profiles, and histology patterns. I observed decreases in fibrotic burden and fibrotic cellular responses as well as improvements in lung compliance and capacity in bleomycin-injured mice treated with BP2_disulf compared to mice treated with bleomycin alone. The quality of BP2_disulf design and positive results from this study have important implications for the future of IPF treatment.
2.2 Results

2.2.1 Experimental Design

In preparing an experimental design to test the efficacy of the novel αvβ6 inhibitor, BP2_disulf, I followed ATS recommendations regarding the use of animal models for preclinical assessment of potential therapies for IPF.\textsuperscript{23} The ATS recommends use of a murine intratracheal bleomycin model suggesting the use of male mice in initial studies.\textsuperscript{23} C57BL/6 mice are commonly used in IPF research due to their relative sensitivity to bleomycin.\textsuperscript{24} The ATS also recommends the administration of experimental therapeutics at or after 7 days from initial bleomycin injury to allow time for full fibrotic phenotype to develop.\textsuperscript{23}

In this study, twelve-week-old C57BL/6 mice were split into three groups: non-treated (NT), bleomycin (BLM), and bleomycin plus BP2_disulf (BLM+BP2). Bleomycin was administered through intratracheal instillation at day 0 (Figure 2-2). BP2_disulf was administered through intraperitoneal injection every other day starting at day 7. Mouse weights were recorded daily. Micro-CT scans were collected weekly. At day 21, flexiVent machinery

\textbf{Figure 2-2: Experimental Design Schematic.} ATS recommended murine model for IPF. 21-day study with bleomycin (BLM) instillation at day 0 to induce fibrosis and BP2_disulf (BP2) treatment every other day (q.o.d.) starting at day 7.
was used to perform lung function tests. At that time, BALF was collected and tissue was harvested for histology.

2.2.2 BLM+BP2 mice recapture weight loss due to bleomycin injury

IPF patients often experience weight loss as the disease progresses, therefore mice were weighed daily to examine weight fluctuations. As seen in Figure 2-3, BLM and BLM+BP2 mice have a steep decline in weight starting at day 1. Throughout the study, BLM mice lost significantly more weight than NT mice. BLM+BP2 weight loss was significantly lower than NT at the beginning of the study. However, as the study progressed, BLM+BP2 weight was slowly regained with no significance from NT at day 21.

![Figure 2-3: Percent weight change over 21-day study. Mouse body weight was recorded daily. Data are shown as mean±SEM. Significance between NT and BLM is indicated using asterisks (*). Significance between NT and BLM+BP2 is indicated using pound signs (#). (NT: n=24; BLM: n=7; BLM+BP2: n=5)](image)
2.2.3 BP2_disulf decreases fibrotic burden quantified in µCT scans

HRCT scans are often used as a non-invasive way to diagnose and visualize lung abnormality in IPF patients. Likewise, bleomycin-induced fibrosis in mice can be visualized in µCT scans. In this study, mice were scanned weekly to track progression of fibrosis. Representative raw scan images and 3-D object maps can be seen in Figure 2-4 (a-b). Both raw scan images and 3-D object maps show the progression of fibrotic development from day 0 to day 21. NT mice show little change while BLM mice show severe injury progression throughout the study. BLM+BP2 mice have minor injury as expected but visually less severe than BLM. Fibrotic lesions are less frequent and smaller compared to BLM upon viewing cross sectional lung scans (Figure 2-4 b).

To give greater weight to qualitative fibrotic marker observations, all scans were quantified using lung segmentation methods. A histogram of intensities of captured lung segments at day 21 was plotted (Figure 2-4 c). Intensities were calibrated to Hounsfield units (HU). Lighter intensities (higher HU) represent denser tissue and darker intensities (lower HU) represent less dense tissue. A rightward shift of the BLM curve compared to the NT curve is observed. This is expected as BLM object maps and images displayed more dense tissue qualitative markers than NT. Healthy tissue in BLM mice had been replaced with dense, fibrotic tissue. The BLM+BP2 curve appears comparable to the NT curve as far as peak intensity, indicating less fibrotic burden than BLM. Mean intensity at day 21 was also calculated (Figure 2-4 d). BLM mean intensity was significantly higher than both NT and BLM+BP2 further demonstrating the ability of BP2_disulf to prevent fibrotic development.

Dense tissue over total captured volume was also quantified for all scans (Figure 2-4 e). Dense tissue is defined as any intensity above the intersection of NT and BLM day 21 histogram.
**Figure 2-4: Micro-CT Scan Analysis.** Micro-CT scans were collected weekly. Representative 3-D object maps (for day 0, day 7, day 14, and day 21) obtained through lung segmentation(a). Representative day 21 raw µCT scan images with asterisks indicating fibrotic lesions (b). Day 21 histogram analysis of captured lung volumes by frequency of intensities measured in Hounsfield units (HU) (c). Day 21 mean intensity calculated from day 21 histogram analysis (d). Quantifications of dense tissue over 21-day study: dense tissue volume over total captured volume with dense tissue being defined as any intensity above intersection of NT and BLM day 21 histogram curves(e). Data are shown as mean (c), min to max box and violin plot (d), and mean±SEM (e). (NT: n=29; BLM: n=14; BLM+BP2: n=6)
curves. At day 14, BLM+BP2 mice tended to have less dense tissue than BLM mice. By day 21, BLM+BP2 had significantly less dense tissue than BLM comparable to NT. Through observation and analysis of µCT scans, we see BLM+BP2 mice have significantly less fibrotic burden than BLM mice at day 21.

2.2.4 Pulmonary function is retained with BP2_disulf treatment

Pulmonary function tests are commonly used in initial assessment in the IPF diagnosis process. While these tests are limited in their ability for differential diagnosing, they are a useful tool for disease management, assessing disease severity, and defining prognosis. IPF is commonly characterized by decrease in lung compliance, forced vital capacity (FVC), and diffusing capacity of carbon monoxide (DLco) in patients. FlexiVent machinery mimics these tests in mice through forced oscillation maneuvers. At day 21, mice were subject to pulmonary function tests using this machinery. Lung function data regarding lung elastance, resistance, and capacity was collected. Pressure-volume (PV) curves show the behavior of the lungs during inflation and deflation and can be indicative of lung

Figure 2-5: FlexiVent Lung Mechanics. Pulmonary function testing was performed at day 21 using flexiVent machinery. Pressure-volume curves (a), static compliance (Cst) (b), and forced vital capacity (FVC) (c) were calculated. Data are shown as mean ± SEM. (NT: n=17; BLM: n=7; BLM+BP2: n=5)
elastance. The BLM PV curve is shifted lower compared to the NT curve with the BLM+BP2 PV curve very similar to NT (Figure 2-5 a). BLM+BP2 also has significantly higher static compliance (Cst) and FVC than BLM and is comparable to NT (Figure 2-5 b-c). These results are reflective of BLM+BP2 mice retaining lung distensibility, capacity, and function using NT as a baseline.

2.2.5 BP2_disulf treatment reduces fibrotic cellular response

Bronchoalveolar lavage fluid (BALF) and differential cell counting is conditionally used in the diagnosis of IPF to differentiate the disease from other ILDs. BALF is also used as a common end point in IPF animal models to track alveolar cellular responses. Previous studies have shown that patients with IPF tend to have lower macrophage and higher polymorphonucleocyte (PMN) populations as compared to healthy adults. Lymphocyte populations in IPF patients were comparable to those in healthy individuals, but in some cases were higher.

At day 21, BALF was collected, processed and stained. Differential counting of cell types was performed and relative percentages of macrophages, lymphocytes, and PMNs were accessed. BLM+BP2 and NT mice had a significantly higher percentage of macrophages and significantly lower percentage of lymphocytes compared to BLM mice (Figure 2-6 a-c). Although not significant, BLM+BP2 and NT mice are seen to have lower amounts of PMNs compared to BLM (Figure 2-6 d). This data suggests BP2_disulf is inhibiting fibrotic cellular responses brought on by bleomycin injury.
2.2.6 Lung morphology is preserved with BP2_disulf treatment

After animal sacrifice at day 21, lung tissue was harvested, paraffin embedded, and sectioned. Masson-trichrome staining was performed to observe collagen and lung morphology differences. As seen in representative sections in Figure 2-7 a, NT and BLM+BP2 have healthy
lung morphology with open spaces for airways and gas exchange. BLM mice have denser tissue and more collagen as expected. Blinded Ashcroft scoring was performed on histological slides according to previous guidelines with a higher score corresponding to denser tissue. BLM+BP2 and NT sections scored significantly lower compared to BLM mice indicating healthy lung morphology and validating healthy lung function and μCT analysis described previously (Figure 2-7 b).

2.3 Discussion

IPF is a relentless and progressive interstitial lung disease for which there is no cure. Limited IPF treatments leave patients with a devastating prognosis of usually only 2-3 years. Much research is being conducted to unravel IPF pathogenesis, determine major contributors and therapeutic targets, and develop novel IPF treatments in hopes of improving patient outcomes.
The TGF-β signaling pathway has been recognized as a major contributor to the pathogenesis of IPF involved in fibroblast accumulation, myofibroblast differentiation, and extracellular matrix deposition.⁴ An upstream activator of this pathway, αvβ6, is overexpressed in fibrotic diseases and has been identified as a potential therapeutic target for IPF.¹⁰,¹³ Alpha-vβ6 inhibitors have been studied in the context of combating IPF and a variety of other fibrotic diseases and cancers.¹⁰,¹²,¹³ However, lack of affinity, specificity, and manufacturing ease along with side effect complications have left these αvβ6 inhibitors ineffective in treatment of IPF.¹⁵, ¹⁶,¹⁸,¹⁹

My collaborators at the University of Washington designed a novel, highly specific αvβ6 inhibitor, BP2_disulf, in hopes of overcoming weaknesses of previously studied αvβ6 inhibitors.¹⁷ This study seeks to determine the efficacy of BP2_disulf by evaluating its ability to combat the fibrotic phenotype observed in IPF. Using the standard IPF murine model, I show that the injection of BP2_disulf in bleomycin-injured mice reduces rate of fibrosis progression and lung function decline compared to mice treated with bleomycin alone. These results suggest that BP2_disulf is a promising therapeutic to improve outcomes of IPF patients.

Several translational end points were used to draw these conclusions. Decrease in weight is a common symptom of IPF patients and is reflective of disease progression and deteriorating quality of life. Thus, weight loss was observed in this study as an indicator of fibrosis severity. After bleomycin injury in mice, weight loss is expected during the first seven days post injury as the lungs experience inflammatory responses. After this, weight loss may continue as lungs begin to develop fibrosis. In this study, BLM and BLM+BP2 mice initially lost weight due to bleomycin injury at day 0 (Figure 2-3). However, starting at day 9, shortly after BP2_disulf treatments began, BLM+BP2 weight was recuperated and was comparable to NT at day 21.
These initial results showed promise that treatment of BP2_disulf was decreasing symptoms caused by lung fibrosis in bleomycin-injured mice.

Although recovered weight was indicative of a decreased fibrotic response in BLM+BP2 mice, results needed to be confirmed through visualization of decreased fibrosis in lung tissue. Fibrotic progression was visualized through the use of μCT scans and lung histology. Micro-CT scans of mouse lungs conducted weekly offered a non-invasive way to observe fibrotic progression. HRCT scans are one of the major methods used to diagnose and assess IPF and its progression through visualization of lung abnormalities. In IPF patients, a usual interstitial pneumonia (UIP) pattern is often observed and generally consists of honeycomb cysts, traction bronchiectasis and ground glass opacification. Similarly, bleomycin-induced fibrosis in mice can be visualized in μCT scans through fibrotic nodules and lesions represented by light gray patches.

Observation of raw scan images and 3-D object maps allowed for qualitative evidence of limited fibrosis in BLM+BP2 mice. Light grey patches in raw scan images and “absent” tissue in 3-D object maps compared to NT represent regions of denser tissue. These dense tissue areas are suggestive of excessive extracellular matrix deposition due to bleomycin injury. After visual observation of scan images and object maps collected in this study, it was clear that BLM+BP2 mice often had less fibrotic lesions and extracellular matrix accumulation as compared to mice treated with bleomycin alone (Figure 2-4 a-b). In IPF patient HRCT scans, images are observed and patterns are assessed by experienced pulmonologists and radiologists. In murine models, many studies have been performed in attempt to quantify fibrotic tissue in μCT scan images. Currently there is no gold standard for such quantification. However, through adaptation of previous methods described, I quantified fibrotic tissue in lung scan images through lung
segmentation and analysis of intensities present.\textsuperscript{35-39} Intensities were reported in Hounsfield units (HU) as calibrating \(\mu\)CT scans intensities to HU is standard practice.\textsuperscript{28}

Air and less dense tissue appear darker in \(\mu\)CT scans, therefore lower HU represent less dense tissue and higher HU represent denser tissue.\textsuperscript{28} I plotted a histogram of intensity frequencies present in segmented lungs at day 21 in order to examine density differences (Figure 2-4 c). There was a clear rightward shift of the BLM curve compared to the NT control. This shift represents healthy tissue being replaced by dense tissue as expected with bleomycin injury. The BLM+BP2 curve has only a slight shift compared to NT suggesting less buildup of dense tissue. After further quantification, dense tissue volume appeared to be lower in BLM+BP2 mice compared to BLM at day 14 (Figure 2-4 e). This reduction was statistically significant at day 21. While not statistically significant, dense tissue in BLM+BP2 appears to lessen from day 14 to day 21. This may imply the potential of BP2\_disulf treatment to not only slow the progression of fibrosis but also heal and reverse fibrotic damage. However, further testing should be done to investigate this theory. Regardless, through \(\mu\)CT scans, I was able to visually observe and quantify the severe fibrotic progression in bleomycin-injured mice and the lack thereof in mice treated with BP2\_disulf.

To further validate results gathered through \(\mu\)CT scan analysis, lung fibrosis was also visualized through lung histology. Lung histology is commonly used to observe abnormal lung patterns including UIP during the diagnostic process of IPF.\textsuperscript{27} Histological UIP pattern usually consists of patchy dense fibrosis, honeycombing, and remodeled architecture. Conversely, healthy lung histological patterns consist of a fine lace arrangement of alveolar air spaces and thin alveolar walls and fibers. This healthy lung morphology was observed in BLM+BP2 and NT sections compared to the dense, fibrous architecture lacking air spaces observed in BLM sections
(Figure 2-7 a). Ashcroft scoring is the standard method to quantify differences in lung section morphologies with higher scores representing fibrotic obliteration. As expected, BLM+BP2 scores were significantly lower than BLM and comparable to NT representing the healthy lung tissue retained in mice treated with BP2_disulf (Figure 2-7 b). Lack of dense scar tissue in BLM+BP2 lung architecture further suggests BP2_disulf’s ability to inhibit fibrotic progression.

To confirm the lack of fibrotic development in BLM+BP2 mice, I observed differential cell counts in BALF. BALF collection is commonly used to diagnosis and differentiate IPF among other ILDs. It is also a common method used in IPF animal models to observe alveolar cell profiles. Healthy individuals usually have a specific BALF profile: ≤3% neutrophils, ≤1% eosinophils, >85% macrophages, and 10-15% lymphocytes. IPF patients have been observed to have more neutrophils (5.9% to 22.08%) and eosinophils (2.39% to 7.5%) and less macrophages (49.18% to 83%). Lymphocyte levels appear to overlap between healthy individuals and IPF patients but in some cases can be higher with IPF(7.2% to 26.7%). These differences are similar to differences seen in bleomycin-injured mice compared to control. In BLM+BP2 mice, BALF profiles consisted of significantly more macrophages and significantly less lymphocytes compared to BLM BALF profiles (Figure 2-6 b,c). Differences in BALF differential cell counts allow a glimpse into the cellular processes at work in alveolar spaces. The changes in BALF profiles as a result of BP2_disulf treatment represent a decreased fibroproliferative response as compared to mice treated with bleomycin alone. BLM+BP2 BALF features confirm BP2_disulf’s ability to hinder fibrotic progression.

While it was clear the fibrotic phenotype was reduced in BLM+BP2 mice, I wanted to determine if these results corresponded with pulmonary function including lung elastance and capacity. Pulmonary function tests are another standard tool used by clinicians to diagnose and
track IPF progression in human patients. Decreases in FVC and DLco are often observed as fibrosis progresses. Eventually this decline leads to total respiratory failure and death. Therefore, lung function improvement can be used as an indicator of prolonged survival.

Pulmonary function tests are also often used as an end point in IPF murine models with FVC and Cst as common markers of lung health. FVC is the measure of the total air volume exhaled after complete inhalation and maximal exhalated force. This measure reflects the lung’s ability to inhale and exhale. Cst measures the change in lung volume with change in pressure characterizing the elastic properties of the lung. In this study, both FVC and Cst are statistically greater in BLM+BP2 mice than in BLM mice and are comparable to NT (Figure 2-5 b,c). BLM+BP2 mice maintain lung elasticity and breathing ability that is usually lost with bleomycin injury. Through these results, I confirm that healthy lung function in BLM+BP2 mice mimics lack of fibrosis observed in μCT scans and histology. Retained lung elasticity and function with BP2_disulf treatment reveal the potential of BP2_dsulf to improve IPF outcomes in human patients.

These end points were chosen not only because they are often used in IPF animal models but because they are translatable to methods used in IPF diagnosis and management. This data repeatedly confirms that injection of BP2_disulf does in fact reduce the rate of fibrotic progression and pulmonary function decline in bleomycin-injured mice. These findings have major implications for the future of IPF treatment and prognosis.

2.4 Conclusion

Weight change, μCT scan analysis, pulmonary function tests, BALF differential cell counts, and lung histology were all used as endpoints to measure the differences of fibrotic
progression and lung function in NT, BLM and BLM+BP2 mice. Weight was recuperated and reduced fibrosis was observed in µCT scans and lung morphology in mice treated with BP2_disulf compared to mice treated with bleomycin alone. Significantly more macrophages and less lymphocytes in BLM+BP2 BALF profiles compared to BLM further validated evidence of reduced fibrotic responses with BP2_disulf treatment. Lung function tests indicated retained lung elasticity and health in BLM+BP2 mice. According to these results, injection of BP2_disulf successfully reduced fibrotic progression in bleomycin-injured mice providing a new potential treatment for IPF patients.

2.5 Materials and Methods

2.5.1 Study Design

The standard IPF murine model was used for this study. Body weight was recorded daily. Micro-CT scans were conducted to visualize progression of fibrosis and flexiVent machinery was used to measure lung mechanics. After euthanization, BALF was collected, and lung tissue was harvested to further observe fibrotic markers and lung morphology.

2.5.2 Animals

C57BL/6 male mice were obtained from Jackson lab and allowed to acclimate to vivarium conditions for at least one week before starting the study. Mice were separated into three groups: non-treated, bleomycin treated, and bleomycin-plus-BP2_disulf treated. Mice had free access to standard rodent chow and water and were subject to the following conditions: room temperature - 22-24 degrees Celsius; relative humidity - 40-70%; 12-hour light-dark cycle. All mice were housed in Brigham Young University’s pathogen free facility and all
experimentation was done in accordance to the protocol approved by the IACUC of Brigham Young University.

2.5.3 Bleomycin Instillation and BP2_disulf Treatment

12-week-old C57BL/6 mice were intratracheally instilled with 3U/ml bleomycin sulfate in 0.9% sterile saline (50ul/animal) as previously described. Mice were anesthetized using ketamine/xylazine (100mg:10mg/kg body weight). When anesthetic had taken full effect as tested by reflex to toe pinch, mice were hung on wire by their upper incisors. Using padded forceps, the tongue was pulled out, and an IV catheter was inserted into the trachea. Bleomycin was administered through the catheter along with 300ml of air to ensure complete dispensation. Mice were observed until full recovery from anesthesia. BP2_disulf was administered to bleomycin-plus-BP2_disulf mice every other day starting at day 7 with a dose of 100 ug/kg body weight and a volume of 10uL/g body weight per intraperitoneal injection.

2.5.4 Lung Imaging and Analysis

Micro-CT scans were collected weekly, at day 0, day 7, day 14, and day 21 using the Quantum GXII Micro-CT scanner (Perkin Elmer, Waltham, MA). Mice were anesthetized with 1.5-2.5% isoflurane. Scans were performed under the following settings: 90kV, 88uA, Al 0.5 mm + Cu 0.06 mm filter, 36mm/60mm/72mm FOV, standard and high resolution, acquisition time 2-4 minutes. Mice received a maximum of 996 mGy radiation exposure per scan. A past study has shown that similar amounts of radiation do not affect findings. Scan images were calibrated to Hounsfield Units (HU).
Collected scan images were analyzed using Caliper Analyze Software (AnalyzeDirect, Inc., Overland Park, KS). Scan images were filtered using a median filter to reduce noise. Whole lungs were isolated from surrounding tissue through a semi-automatic segmentation technique. A lung tissue threshold was determined by sample histogram analysis of the frequency of Hounsfield Unit (HU) intensities in scan images. Total lung volume, dense tissue volume and mean intensity was calculated for scans. Dense tissue was defined as any captured intensity above the point of intersection between non-treated and bleomycin day 21 histograms.

2.5.5 Pulmonary Function Tests

FlexiVent FX system (SCIREQ Inc., Montreal Qx, Canada) was used to record lung mechanics prior to euthanization. The flexiVent FX system is equipped with a FX1 module and a Negative Pressure-Driven Forced Expiration (NPFE) extension for mice run by flexiWare 8.0 software.

Mice were anesthetized through an intraperitoneal injection of ketamine/xylazine (100mg:10mg/kg body weight). When fully anesthetized, mice were placed in the supine position. The trachea was exposed, and a cannula was inserted. Once the cannula was securely tied in place, the mice were coupled to flexiVent machinery. Vital signs were monitored, and a ventilator was used to assist breathing (tidal volume: 10 mL/kg, frequency: 150 breaths/min).

Mice were then intraperitoneally injected with pancuronium bromide (0.8mg/kg body weight). This was used to paralyze mice and stop spontaneous breathing to ensure consistent results measured by the ventilation system. Vitals were recorded. The baseline breathing was recorded, and the following scripts were run three times: Deep Inflation, Snapshot-150, Quick
Prime, Negative Pressure-Driven Forced Expiration (NPFE). After these measurements were recorded, mice were euthanized in accordance with BYU protocols.

2.5.6 BALF collection

BALF procedure was performed immediately after euthanization with the cannula still inserted. PBS (800ul) was collected into a 1 ml syringe and then inserted into the lungs through the attached cannula. The PBS was flushed into the lungs three times before being collected. BALF samples were then centrifuged at 1000 rpm for 10 minutes at 4 degrees Celsius. The supernatant was recovered, and the remaining pellet was resuspended in 500 ul of PBS. The cell suspension was spun down onto microscope slides using the Cytospin™ 3 Centrifuge for 3 minutes at 800 rpm. Wright stain was applied, and slides were imaged at 1000x on the Olympus BX51 Microscope. About 200 cells were differentially counted for macrophages, lymphocytes, and PMNs. Relative percentages of these cells were calculated.

2.5.7 Histology

After mice had been sacrificed and BALF had been collected, tissue was harvested. Lungs were perfused with 4% paraformaldehyde and were left in 4% paraformaldehyde overnight. After 24 hours, lungs were washed in PBS, 30% ethanol, 50% ethanol and 70% ethanol. Lungs were further processed and paraffinized overnight using Shandon Citadel 1000 tissue processor. Following paraffinization, lungs were embedded in paraffin blocks and sectioned into 5-7 um slices using a Microm 325 microtome. Tissue was deparaffinized using standard protocol (IHC Deparaffinization Protocol, Abcam). Masson-Trichrome stains were
performed according to manufacturer’s protocol (Sigma Aldrich, HT15-1KT). Lung sections were imaged and blindly scored using the standard Ashcroft scoring method.\textsuperscript{40,41}

2.5.8 Statistical Analysis

All values were reported as mean or mean ± SEM as described in figure descriptions. Statistically significant differences were assessed using one-way ANOVA followed by Tukey’s post-hoc test for multiple comparisons. Analysis and graphs were prepared with GraphPad Prism 8.0 (GraphPad 8.0; GraphPad Software, Inc. La Jolla, CA) and a p-value <0.05 was considered statistically significant.

2.5.9 Illustrations

Illustrations in Figure 2-1 and Figure 2-2 were created using BioRender.com (2020).
2.6 REFERENCES


17. Roy, Anindya; Shi, Lei; Chang, Ashley; Dong, Xianchi; Li, Jing; Viazzo Winegar, Rebecca; et.al *De novo* design of a highly specific inhibitor of integrin αvβ6. to be submitted for publication.


3. **FUTURE WORK**

The results of this study confirm that BP2_disulf, a novel, highly specific αvβ6 inhibitor, reduces fibrotic progression and rate of pulmonary function decline in bleomycin-injured mice. The preliminary success of this novel inhibitor provides a promising new avenue for future IPF drug development. Despite this success, much work still needs to be done to improve IPF outcomes. Improved IPF outcomes are reliant on quicker and more reliable diagnostic methods, better understanding of disease mechanisms and more comprehensive and accessible treatment options.

### 3.1 Quicker and more reliable diagnosis

Even if improved therapies like BP2_disulf are developed, enhanced recovery is dependent on quicker and more accurate diagnosis. As the disease progresses and lung damage continually increases in scope and severity, IPF becomes harder to treat. Therefore, early diagnosis is critical for optimizing clinical management and improving quality of life.\(^1\) Slow, drawn-out testing and sometimes inconclusive findings are a weakness in the IPF diagnosis process. Definite diagnosis can take up to 5 years after initial onset of symptoms.\(^2\) Many studies have sought to identify easily accessible and measurable gene expression and biomarkers unique to IPF in hopes of using these identifiers to facilitate diagnosis.\(^3,4\) Elevated levels of circulating
blood biomarkers such as surfactant protein A (SP-A), matrix metalloproteinase-1 (MMP1), and matrix metalloproteinase-7 (MMP7) have been observed in IPF patients and could be used to discriminate IPF among other ILDs. Others have studied common comorbidities observed among IPF patients hoping to identify trends that could lead to earlier clinical suspicion. The most common IPF comorbidities are cardiovascular and thromboembolic disorders, gastroesophageal reflux disease (GERD), pulmonary hypertension, emphysema, diabetes, and lung cancer. By investigating the pathological links between these diseases and IPF, clinical features can better be defined. As a result, prompt investigation and earlier diagnosis can occur.

IPF diagnosis is often reliant on HRCT scan findings. Qualitative findings are limited as they can be inconclusive and are subject to interobserver variation even among pulmonary experts. Work to improve quantitative approaches to HRCT scan analysis is being performed and offers rapid, reproducible, objective measurements. Many quantitative tools have been studied such as histogram kurtosis/density measures, computer-aided lung informatics for pathology evaluation and rating (CALIPER), and data-driven textural analysis (DTA). The study of quantitative methods for μCT scan analysis in IPF animal models is also crucial. The lack of a gold standard fibrosis quantification method for μCT makes comparing quantified fibrosis in μCT scans across studies difficult.

Along with HRCT qualitative analysis, histopathological analysis is also subject to interobserver variation during diagnosis. As with HRCT, quantitative methods are being studied to improve histopathological conclusions. A recent study developed an automated histological image analysis software to quantify bleomycin-induced fibrotic alterations in mice. With further development of quantitative procedures for both HRCT scans and histopathological
findings, IPF diagnosis can be made quicker and more reliably. While early and improved
diagnostic methods are important, they are insufficient alone to improve IPF drug development.

3.2 Future approaches to disease modeling

Another major obstacle to improving IPF treatments and outcomes is limited knowledge
of IPF pathogenesis. The key to better understanding this complex disease is better disease
modeling. While bleomycin instillation is the most commonly used method to induce fibrosis in
animal models for IPF, it is not a perfect model. It does not capture important aspects of IPF
disease manifestation. IPF is a progressive disease characterized by irreversible fibrosis while the
bleomycin model involves rapid injury and spontaneous resolution as early as 21 days post
injury.11 In this way, the bleomycin model resembles acute lung injury rather than chronic
fibrosis.12 The bleomycin model also fails to capture traditional UIP patterns, a defining feature
of IPF lungs.12 Significant UIP patterns in IPF such as fibroblastic foci, hyperplastic epithelium,
and temporal heterogeneity are lacking in bleomycin-injured mice.12 Bleomycin model
limitations create a barrier to understanding disease mechanisms ongoing in IPF patients. These
limitations also make transition from pre-clinical studies to clinical trials difficult as results are
not always translational.

Modifications to the current standard IPF model are being explored to better reflect
human pulmonary fibrosis. While the use of 8-to-12-week-old mice is common in current IPF
animal models, some studies have used older mice (18-24 months) to better reflect typical age of
diagnosis and examine the effects that aging and cellular senescence may have in IPF
etiology.13,14 The advantages of using older mice in these studies is still under investigation.13
As stated previously, another approach to better understanding IPF and creating better pre-clinical models is the study of common comorbidities. One of the most common comorbidities found in IPF patients is GERD with a prevalence of about 90\%.\textsuperscript{15} While the relationship between IPF and GERD remains unclear, some suspect that repetitive, subclinical micro-aspirations of stomach acid are a major contributor to IPF pathogenesis.\textsuperscript{16,17} Much research has been dedicated to stomach acid contents and its contribution to lung injury. One study instilled gastric contents into the lungs of F344 rats over the course of 16 weeks.\textsuperscript{18} Histological and BALF specimens were obtained and increases in TGF-\(\beta\) expression and lung fibrosis were observed.\textsuperscript{18} Further development of this animal model would be useful not only in defining the relationship between GERD and IPF but also in potentially mimicking aspects of IPF that the bleomycin mouse model fails to capture. As better models are created, we can better comprehend disease mechanics and develop better therapies.

### 3.3 BP2\_disulf moving forward

Despite the limitations of the standard IPF mouse model used in this study, evidence strongly suggests that BP2\_disulf treatment will be useful in combating IPF. However, more can be done to improve BP2\_disulf administration and further confirm its efficacy. In this study, BP2\_disulf was administered through intraperitoneal injection, though aerosol administration may prove to be a superior alternative. Not only would aerosol-driven delivery increase ease of use for patients, but it would also provide direct lung exposure and systemic drug availability.\textsuperscript{19} An inhaled route has the potential to maximize drug exposure to all areas of the lung with lower doses compared to administration by injection.\textsuperscript{19} Direct exposure would also decrease possibility of off-target effects caused by administration into the bloodstream.\textsuperscript{19}
My collaborators at the University of Washington have already confirmed BP2_disulf’s stability with nebulization. Studies are already underway examining the ability of inhaled BP2_disulf in bleomycin-injured mice. Preliminary results look promising. Furthermore, proteomic analysis and PCR array could be utilized to track changes in expression of TGF-β, ανβ6 and other fibrotic markers with treatment of BP2_disulf. Results from these experiments would lead to better understanding of drug mechanism of action and allow anticipation of hurdles that may lie ahead for the drug development of BP2_disulf.

Preliminary efficacy of BP2_disulf has been proven, but superior results may be obtained with its use in a combination therapy. Many combination therapies for IPF are already being investigated. This has been recognized as an attractive approach to IPF drug development because of the complex interplay of genetic and epigenetic factors, environmental exposures, and profibrotic pathways involved in the pathogenesis of IPF. Synergetic efficacy of combined therapies may lead to improvements in IPF treatment. However, drug-drug interactions and combined adverse effects still need to be considered. As the mechanisms of BP2_disulf are better understood, synergistic drug combinations can be identified for maximized efficacy.

With development of aerosolized administration methods and confirmation of efficacy, BP2_disulf can advance to clinical trials and become a standard therapy for suffering IPF patients. Evidence from this study suggests BP2_disulf has the potential to inhibit fibrosis progression and combat IPF. The scope of BP2_disulf may not be limited to IPF alone. As many interstitial lung diseases (ILDs) involve lung fibrosis, BP2_disulf could also have major health benefits for other ILDs as well. The results of this research provide a bright future for IPF research, drug development, and the lives of many suffering worldwide.
3.4 REFERENCES


20. Roy, Anindya; Shi, Lei; Chang, Ashley; Dong, Xianchi; Li, Jing; Viazzo Winegar, Rebecca; et.al *De novo* design of a highly specific inhibitor of integrin αvβ6. to be submitted for publication.

