# Smooth muscle cell proliferation and endothelin pathway in pulmonary arterial hypertension: Comparative effects of current and putative therapeutic agents

by

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# Declaration

I hereby declare that this thesis is my own work and effort and that it has not been submitted anywhere for any award. Where other sources of information have been used, they have been acknowledged.

I specifically confirm that the practical procedures resulting in the experimental data presented in this thesis were performed by me except for the following;

- Generation and characterisation of some of the human pulmonary arterial smooth muscle cell lines from pulmonary hypertensive patients were previously performed by Dr Susan Hall (Institute of child health).
- Fibroblast cell lines were generously provided by Professor David Abraham (Division of Medicine, Royal free hospital)
- Tail artery myography data was performed by Dr Nelson Orie.

For MUM, VIJAY and the rest of my FAMILY
(Love you loads!)

# Abstract

Pulmonary hypertension (PH) is a progressive and fatal haemodynamic state defined by a resting mean pulmonary arterial pressure close to or more than 25 mmHg. Pulmonary arterial hypertension (PAH), the first category in the classification of PH, is a rare and serious condition of unknown cause that is associated a huge variety of medical conditions. It has a poor prognosis and is characterised by a progressive increase in pulmonary vascular resistance (PVR) leading to right ventricular failure or a premature death. Its prevalence varies from 15-50 patients per million populations with an average age of 45 years.

Prostacyclin ($PGI_2$) analogues are used to treat PAH, but do not cure the condition. $PGI_2$ binds to prostacyclin receptors (IP) in the cell membrane stimulating adenylate cyclase to increase intracellular cyclic 3'5'-adenosine monophosphate (cAMP). Once elevated, cAMP is rapidly broken down by phosphodiesterases (PDEs), specifically 1, 3, 4 which appear responsible for regulating levels in the lungs. One of the reasons for the ineffectiveness of $PGI_2$ in PAH could be high activity of these PDE isoforms that specifically break down cAMP. So in chapter 3, I sought to evaluate the effect of prostacyclin analogues (e.g. treprostinil) on cAMP and cell proliferation in PAH. I was also interested to check whether phosphodiesterase inhibitors could potentiate the effects of $PGI_2$ analogues.

Elevated levels of endothelial ET-1 in idiopathic pulmonary arterial hypertension (IPAH) have been shown to be correlated with increased right atrial pressure,

pulmonary artery oxygen saturation and pulmonary vascular resistance (Cacoub *et al.*, 1993b). This has led to the use of ET-1 antagonists to treat PAH alone or in combination with other classical drugs. In chapter 4, I investigated the interaction between treprostinil, and endothelin antagonists on the ET-1 levels and growth characteristics of pulmonary smooth muscle cell cells (PASMCs) derived from IPAH patients.

Vascular remodelling has been considered a pseudo-malignant disorder and mediators from cancer research have been described as targets for therapeutic interventions for PAH (Paulin *et al.*, 2011). In chapter 5 I evaluated the antiproliferative effects of an already established anti-cancer drug ispinesib (Purcell *et al.*, 2010) and thus established a rationale for investigating this agent in PAH settings.

# ACKNOWLEDGEMENT

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# ABBREVIATIONS

| | |
|---|---|
| AC | Adenylyl cyclase |
| APS | Ammonium persulfate |
| ANOVA | Analysis of variance |
| ANP | Atrial natriuretic peptide |
| ALK-1 | Activin receptor-like kinase |
| αSMA | Alpha smooth muscle actin |
| $BH_4$ | Tetrahydrobiopterin |
| BMP | Bone morphogenetic protein |
| BMPR-II | BMP receptor II |
| BNP | Brain natriuretic peptide |
| BSA | Bovine serum albumin |
| CARBA | Carbacyclin |
| cAMP | Cyclic 3'5'-adenosine monophophate |
| cGMP | Cyclic 3'5'-guanosine monophosphate |
| COX-2 | Cyclo-oxygenase-2 |
| COPD | Chronic obstructive pulmonary disease |
| CREB | Cyclic-AMP-response element binding protein |
| DAB | Diaminobenzidine |
| DAPI | 4’,6-diamidino-2-phenylindole |
| DP | Prostaglandin D receptor |
| DMEM | Dulbecco's modified Eagle's medium |
| DMSO | Dimethyl sulfoxide |
| ECE | Endothelin-converting enzyme |
| eNOS | Endothelial nitric oxide synthase |
| ELISA | Enzyme-linked immunosorbent assay |
| $EP_2$ | Prostaglandin $E_2$ receptors |
| ET-1 | Endothelin-1 |
| $ET_A$ | Endothelin receptor A |
| $ET_B$ | Endothelin receptor B |
| FBS | Foetal bovine serum |
| FITC | Fluorescein-5-isothiocyanate |

| | |
|---|---|
| GC | Guanylate cyclase |
| GPCR | G-protein coupled receptor |
| HBSS | Hanks' balanced salt solution |
| HIV | Human immunodeficiency virus |
| 5-HT | 5-hydroxy tryptamine (serotonin) |
| 5-HTT | 5-hydroxy tryptamine transporter |
| IBMX | 3-isobutyl-1-methylxanthine |
| IP | Prostacyclin receptor |
| $IP_3$ | Phosphatidyl inositol triphosphate |
| IPAH | Idiopathic pulmonary arterial hypertension |
| IPRA | IP receptor antagonist |
| IL-6 | Interleukin-6 |
| KSP | Kinesin spindle protein |
| Kv | Voltage-gated potassium channels |
| MCT | Monocrotaline |
| MMP | Matrix metalloproteinase |
| NFAT | Nuclear factor of activated T cells |
| NF-κB | Nuclear factor-κB |
| NO | Nitric oxide |
| NOS | Nitric oxide synthase |
| NYHA | New York Heart Association |
| PA | Pulmonary artery |
| PAH | Pulmonary arterial hypertension |
| PAGE | Polyacrylamide gel electrophoresis |
| PAP | Pulmonary artery pressure |
| PASMCs | Pulmonary arterial smooth muscle cells |
| PBS | Phosphate-buffered saline |
| PCWP | Pulmonary capillary wedge pressure |
| PDGF-BB | Platelet-derived growth factor-BB |
| PDE | Phosphodiesterase |
| $PGI_2$ | Prostaglandin $I_2$ |
| PGIS | Prostacyclin synthase |
| PH | Pulmonary hypertension |

| | |
|---|---|
| PKA | Protein kinase A |
| PKG | Protein kinase G |
| PKA | Protein kinase C |
| PLC | Phospholipase C |
| PPAR | Peroxisome proliferator-activated receptor |
| P/S | Penicillin/streptomycin |
| PVR | Pulmonary vascular resistance |
| RO1138452 | [(4,5-dihydro-1H-imidazol-2-yl) - [4- (4-isopropoxybenzyl) phenyl] amine] |
| RVSA | Right ventricular systolic pressure |
| SEM | Standard error of the mean |
| sGC | Soluble guanylyl cyclase |
| SMC | Smooth muscle cell |
| TCA | Trichloroacetic acid |
| TEMED | N,N,N',N'-tetramethyl-ethane-1,2-diamine |
| TGF-β1 | Transforming growth factor beta 1 |
| TP | Thromboxane receptor |
| TREP | Treprostinil |
| Tris | Tris-(hydroxy methyl)-amino methane |
| $TXA_2$ | Thromboxane $A_2$ |
| VEGF | Vascular endothelial growth factor |
| VIP | Vasoactive intestinal peptide |
| VWF | Von-Willebrand factor |
| WHO | World Health Organization |
| WU | Wood units |

# PUBLICATIONS

1. Falcetti, E, Hall, SM, Phillips, PG, **Patel, J**, Morrell, NW, Haworth, SG & Clapp, LH. (2010). Smooth muscle proliferation and role of the prostacyclin (IP) receptor in idiopathic pulmonary arterial hypertension. *Am J Respir Crit Care Med*, **182**, 1161-1170.

2. **Jigisha Patel**, Nelson Orie, Lucie Clapp. (2010). Phosphodiesterase-type 3 inhibitor potentiates cAMP generation andantiproliferative effects of treprostinil in pulmonary arterial smooth muscle cells from patients with pulmonary hypertension. *European Respiratory Society.* #**6426**.

3. **Jigisha Patel**, Susan Hall, Lucie Clapp (2012) Inhibition Of Endothelin-1 Levels By Treprostinil Is Counteracted By Bostentan And Ambrisentan In Smooth Muscle Cells Derived From Patients With Pulmonary Arterial Hypertension. *American Thoracic Society* #**32943.**

4. Clapp, LH & **Patel, J.** (2010). The mechanistic basis for prostacyclin action in pulmonary hypertension. *International Journal of Respiratory Care*, **27-33.**

# 1 . Introduction

## 1.1 Introduction and classification

Pulmonary hypertension (PH) is a progressive and fatal haemodynamic state defined by a resting pulmonary arterial pressure of 25 mm Hg or greater. Pulmonary arterial hypertension (PAH) is the first category of PH characterised by vasoconstriction and remodelling of the small arteries of the pulmonary vasculature. PAH is frequently present in association with various cardiopulmonary diseases and can be characterised by sustained elevation of pulmonary artery pressure (PAP) and pulmonary vascular resistance (PVR) leading to impaired right-heart function and eventual failure (Barst *et al.*, 2004; Galie *et al.*, 2009). Unlike in most of the diseases where the left ventricle is affected, in PAH the right ventricle is the one which is primarily affected. The secondary right heart ventricular hypertrophy develops in response to the increased afterload and the subsequent heart failure is an important cause of mortality in patients, particularly those with congenital heart diseases. There are no treatments existing at present that cure PAH, however new therapeutic options have become available that helps to improve the life expectancy and quality of life of the patients (McLaughlin & McGoon, 2006; Diller & Gatzoulis, 2007).

In the early 1950s, Dresdale and colleagues were the first investigators to use the term “primary pulmonary hypertension” (PPH) to describe a disease distinguished from “secondary pulmonary hypertension” (SPH) by the absence of intrinsic heart or lung diseases in patients with unexplained PH (Dresdale *et al.*, 1951). Because of the diversity of the disease, a classification into various groups was needed. Therefore the World Health Organisation (WHO) held World Symposiums on PH

at Evian in 1998 and Venice in 2003 and from these classified PH into five clinical categories. Before 2003, PAH without any known cause was termed PPH. However, at the third WHO meeting held in 2003 a major modification was made replacing the term "primary PH" with "idiopathic PAH" (IPAH). IPAH is a rare condition with an incidence of approximately one to two per million (McLaughlin *et al.*, 2009). Likewise, the comprehensive register in France documents a prevalence and incidence of PAH, being 15 cases per million adult inhabitants and 2.4 cases per million adults each year, respectively (Humbert *et al.*, 2006). Moreover, females are affected twice as often as males. The National Institutes of Health Prospective Trial in the USA, which looked at patients with a diagnosis of PAH, found, more than 80% of cases to be idiopathic and 6% of that to be familial (Rich & Brundage, 1984; Rich *et al.*, 1987; Rich, 2000).

Further, the fourth world symposium on PH was held in Dana Point, CA in 2008 and provided slight modification in the classification table, in that the "Familial PAH" group was now termed "hereditable PAH" with further breakdown into genetic factors related to abnormalities in PH (Table 1 and 2).

The bone morphogenetic protein receptor type II (BMPRII) gene was identified in 2000 as the gene most likely causing familial/hereditable PAH and whose protein product was further recognised as belonging to the transforming growth factor β (TGF-β) receptor family. Surprisingly the loss of function of BMPRII was also found in about 26% of patients with IPAH, suggesting the receptor plays an important role in the disease (Deng *et al.*, 2000; Thomson *et al.*, 2000; Lane *et al.*,

2000). In rare cases of familial PAH mutations, genes that encode for 2 other cell surface receptors of the TGF-β superfamily found in endothelium, activin-like kinase-type I (ALK-1) and endoglin (ENG), were found to be associated with hereditary hemorrhagic telangiectasia (Abdalla *et al.*, 2004). There may be other genes, which are yet to be identified, that cause PAH. A point to be noted is that the presence of the mutation alone does not necessarily lead to PAH; suggesting onset of the disease requires a second hit and/or contribution from other risk factors (Morrell, 2006).

A number of risk factors involved in the development of PAH have been identified and are defined as any factor or condition that is suspected to play a predisposing or facilitating role in the development of the disease. Mutations in other signalling pathways have been also described. Serotonin (5-hydroxytriptamine, 5-HT) is a potent vasoconstrictor and when pulmonary arterial smooth muscle cells (PASMCs) of patients were exposed to this agent, cell proliferation increased extensively compared to normal cells (Eddahibi *et al.*, 2001; Marcos *et al.*, 2004). This abnormal response to 5-HT was thought to result from over expression of the serotonin transporter (5-HTT), as excessive proliferation was substantially reduced by 5-HTT inhibitors (Eddahibi *et al.*, 2001; Marcos *et al.*, 2004) .

Furthermore, in this study the homozygous presence of an L-allelic variant of the 5-HTT gene promoter was found in two thirds of patients with IPAH as opposed to 27% in control patients (Eddahibi *et al.*, 2001). Finally a miscellaneous

category of PH was expanded and includes the remaining classes which were originally listed under ‘other’ categories of PH.

With more understanding of the disease, the classification of PH has gone through a series of changes. The latest and most comprehensive classification published is the Dana Point Classification made at the 4th World Symposium on PH held in 2008 in Dana Point, California, which divided the PH into five groups (Table 1) that shared similar pathophysiologic mechanisms and clinical presentation as well as therapeutic approaches. The recommendations of the 5$^{th}$ World symposium have yet to be made available public. Untreated medial survival, following diagnosis rarely exceeds three years (D'Alonzo *et al.*, 1991).

| |
|---|
| **1. Pulmonary arterial hypertension (PAH)** |
| 1.1. Idiopathic PAH<br>1.2. Heritable<br>1.2.1. BMPR2<br>1.2.2. ALK1, endoglin (with or without hereditary hemorrhagic telangiectasia)<br>1.2.3. Unknown<br>1.3. Drug- and toxin-induced<br>1.4. Associated with:<br>1.4.1. Connective tissue diseases<br>1.4.2. HIV infection<br>1.4.3. Portal hypertension<br>1.4.4. Congenital heart diseases<br>1.4.5. Schistosomiasis<br>1.4.6. Chronic hemolytic anemia<br>1.5 Persistent pulmonary hypertension of the newborn<br>1.6 Pulmonary veno-occlusive disease (PVOD) and/or pulmonary capillary hemangiomatosis (PCH) |
| **2. Pulmonary hypertension owing to left heart disease** |
| 2.1. Systolic dysfunction<br>2.2. Diastolic dysfunction<br>2.3. Valvular disease |
| **3. Pulmonary hypertension owing to lung diseases and/or hypoxia** |
| 3.1. Chronic obstructive pulmonary disease<br>3.2. Interstitial lung disease<br>3.3. Other pulmonary diseases with mixed restrictive and obstructive pattern<br>3.4. Sleep-disordered breathing<br>3.5. Alveolar hypoventilation disorders<br>3.6. Chronic exposure to high altitude<br>3.7. Developmental abnormalities |
| **4. Chronic thromboembolic pulmonary hypertension (CTEPH)** |
| **5. Pulmonary hypertension with unclear multifactorial mechanisms** |
| 5.1. Hematologic disorders: myeloproliferative disorders, splenectomy<br>5.2. Systemic disorders: sarcoidosis, pulmonary Langerhans cell histiocytosis: lymphangioleiomyomatosis, neurofibromatosis, vasculitis<br>5.3. Metabolic disorders: glycogen storage disease, Gaucher disease, thyroid disorders<br>5.4. Others: tumoral obstruction, fibrosing mediastinitis, chronic renal failure on dialysis |

**Table 1 - Clinical Classification of Pulmonary Hypertension:** 4th World Health Organisation Symposium on Pulmonary Hypertension, Dana Point 2008 (Adapted from (Galie *et al.*, 2009)).

| |
|---|
| **Class I:** Patients with PH without limitation of usual activity. Ordinary physical activity does not cause increased dyspnoea, fatigue, chest pain or pre-syncope. |
| **Class II:** Patients with PH with slight limitation of usual physical activity, there is no discomfort at rest, but normal physical activity causes increased dyspnoea, fatigue, chest pain or pre-syncope. |
| **Class III:** Patients with PH with marked limitation of usual physical activity. There is no discomfort at rest, but less than ordinary activity causes increased dyspnoea, fatigue, chest pain or pre-syncope. |
| **Class IV:** Patients with PH with inability to perform any physical activity without symptoms and who may have signs of right ventricular failure. Dyspnoea and/or fatigue may be present at rest and symptoms are increased by almost any physical activity. |

**Table 2 - Current World Health Organisation & New York Heart Association (NYHA) classification of functional status in patients with pulmonary hypertension.** The classification refers to the impact of the disease on the functional capacity of the patient. The severity of PH in patients can be categorized using the NYHA (class I – IV) functional classification system. It is the physical limitations imposed on the patient by the disease which determines the functional classification. For example, patients with early-stage PAH are placed in class I and those most affected (late-stage PAH with right heart failure) are placed in class IV. The NYHA (class I – IV) system helps in making the choice of PAH therapy and also in an accurate predictor of patient mortality (Adapted from Galie *et al.*, 2009)).

## 1.2 Pathophysiology of PAH

### 1.2.1 Pulmonary arterial pressure (PAP) and pulmonary vascular resistance (PVR)

PAH is defined by sustained elevated PVR and PAP. This leads to impaired right-heart function, eventual failure and ultimately death (Barst *et al.*, 2004). PVR is the differentiating feature between the pulmonary and systemic circulation, in that the flow of blood in the pulmonary arteries of the lung has greater resistance compared to the systemic pulmonary arteries. PAP normally is low. The mean in the normal subject is about 15 mm Hg which rarely, if at all, exceeds a mean pressure of 20 mm Hg. In contrast patients with PAH have a PAP more than or equal to 25mm Hg at rest. PAH may also be defined as patients having a PVR ≥ 3 Woods Units (240 dynes/sec/cm5) and a trans-pulmonary gradient ≥ 12 mmHg (Humbert *et al.*, 2001).

The evolution of pulmonary vascular disease in children with congenital heart defects differs from person to person depending on the type of underlying cardiac lesions. However, the presence of increased pulmonary blood flow seems to be an essential trigger in the development of PAH. Children with extensive systemic-to-pulmonary shunts, whose pulmonary vascular bed is exposed to a combination of increased pulmonary arterial pressure and increased pulmonary blood flow, may develop PAH (Duffels *et al.*, 2007). More than half of these children develop irreversible pulmonary vascular disease in the first years of life (Rudolph & NADAS, 1962; Ikawa *et al.*, 1995), whilst in patients that have an isolated increased pulmonary blood flow, this is only 10 to 20%. Increases in PVR are associated with pulmonary vascular remodelling. The increased right ventricular work load leads to right ventricular hypertrophy and eventually to right ventricular

failure as is illustrated in figure 1.1. Due to an on-going pulmonary vascular remodelling, PVR rises to systemic levels resulting in bi-directional blood flow across the chambers. This eventually leads to a condition where the original left-to-right shunt is reversed, a phenomenon called the Eisenmenger syndrome.

### 1.2.2 Endothelial dysfunction in PAH

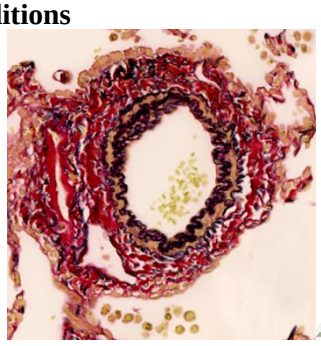
Endothelial dysfunction is common place in PAH and results from an imbalance in the production of vasodilators like prostacyclin ($PGI_2$), nitric oxide (NO) and vasoconstrictors like endothelin (ET-1), 5-HT and thromboxane $A_2$ ($TXA_2$). In PAH there is insufficiency of vasodilators and an over production of vasoconstrictors (Christman *et al.*, 1992). This leads to constriction of the pulmonary arteries causing the pressure to increase and the PVR to rise resulting in vascular remodelling.

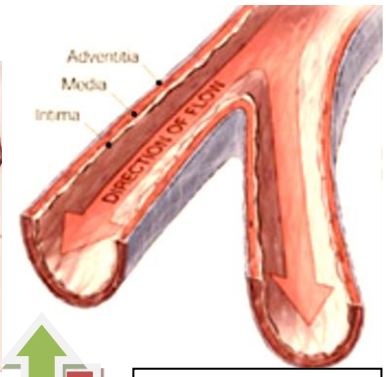
In PAH, $PGI_2$ levels have been found to be decreased in endothelial cells derived from patients due to a reduction in the expression of $PGI_2$ synthase, the enzyme which is responsible for its synthesis (Christman *et al.*, 1992; Tuder *et al.*, 1999).

Other endothelium-derived vasodilators are further decreased in PAH. A reduction of endothelial NO synthase (eNOS) expression has been reported in endothelial cells of patients with PAH (Giaid & Saleh, 1995) as has reduced serum levels of vasoactive interstitial peptide (VIP), a potent pulmonary vasodilator (Petkov *et al.*, 2003).

**1. Risk Factors and Associated Conditions**

- Collagen vascular disease
- Congenital heart disease
- Portal hypertension
- HIV infection
- Drugs and toxins
- Susceptibility
  - ✓ Abnormal BMPR2 gene
  - ✓ Other genetic factors

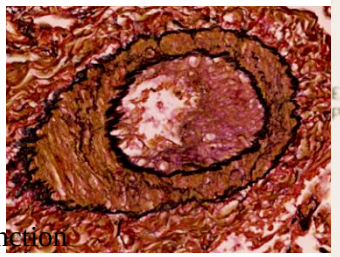


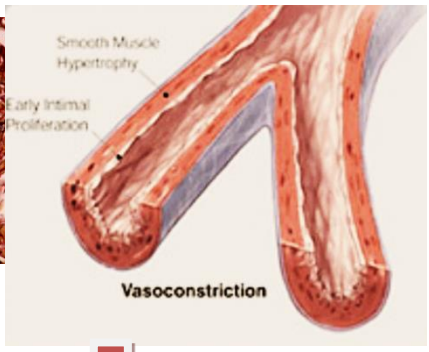



Early detection may be reversed with drugs

**2. Vascular Injury**

- Endothelial dysfunction
  - ➢ ↓ NOS, $PGI_2$,
  - ➢ ↑ $TXA_2$, ET-1
- Vascular smooth muscle dysfunction
  - ➢ Impaired $K_v1.5$ Channel.

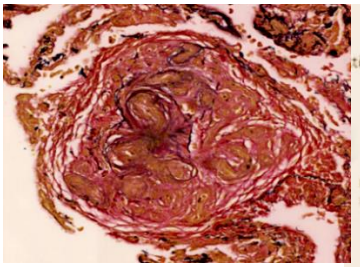





Irreversible damage

**3. Disease progression**

- Loss of response to short-Acting vasodilator trial



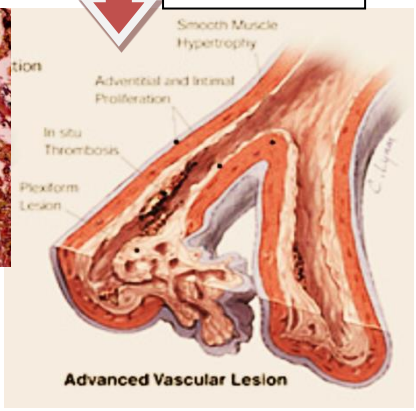



**Figure 1.1. Endothelial dysfunction and vascular remodelling in pulmonary arterial hypertension.** Pulmonary arterial hypertension occurs in susceptible patients as a result of an insult to the pulmonary vascular bed resulting in an injury that progresses to produce characteristic pathological features. Histology showing Van Gieson stain which stains the collagen in red and elastase in black are shown for normal (top left) and PAH lung (middle and bottom left) (Extreme right panels *adapted with prior permission from* (Gaine, 2000).

There is a further contribution to the pathology, as ET-1, a potent vasoconstrictor, is increased in both animal models and in PAH patients (Giaid *et al.*, 1993). ET-1 also acts as a growth factor and mitogen and plays a vital role in vascular remodelling in PAH (Yanagisawa, 1994). Thus endothelial dysfunction and imbalance in the vascular homoeostasis plays an important role in the pathophysiology of PAH (figure 1.1).

### 1.2.3 Pulmonary vascular remodelling

The common histological features of PAH are remodelling of all three layers of the pulmonary vasculature (intimal, medial and adventitia layers) as well as the formation of plexiform lesions (Gaine & Rubin, 1998). The abnormalities of the pulmonary vasculature comprise:

1) medial hypertrophy of large pulmonary arteries and muscularization of distal precapillary arteries.
2) Proliferation in the adventitia of small pulmonary arteries and arterioles.
3) Intimal hyperplasia that is particularly occlusive in vessels around 100–500 μm in diameter.
4) Plexiform lesions of arterial branches distal to a partially obstructed larger artery.
5) Loss of pre-capillary arteries (Stenmark & Mecham, 1997; Rabinovitch, 2007).

In a normal lung, the thickness of the arterial wall is maintained relatively constant by the balance between proliferation and apoptosis of the cells present in the vessel wall *i.e.* PASMCs, pulmonary artery endothelial cells (PAECs) and pulmonary

arterial fibroblasts (PAFs). A disruption in this balance towards a pro-proliferative phenotype of these different cell types may lead to vascular wall thickening and lumen occlusion, further increasing PVR (Mandegar *et al.*, 2004). Morphological analysis of pulmonary hypertensive lungs reveals excessive remodelling of smooth muscle layer from previously non muscularised thin vessels. In PAH, adventitial thickening with increased extracellular matrix deposition is prominent in the distal muscular pulmonary arteries (Das *et al.*, 1997; Frid *et al.*, 1997).

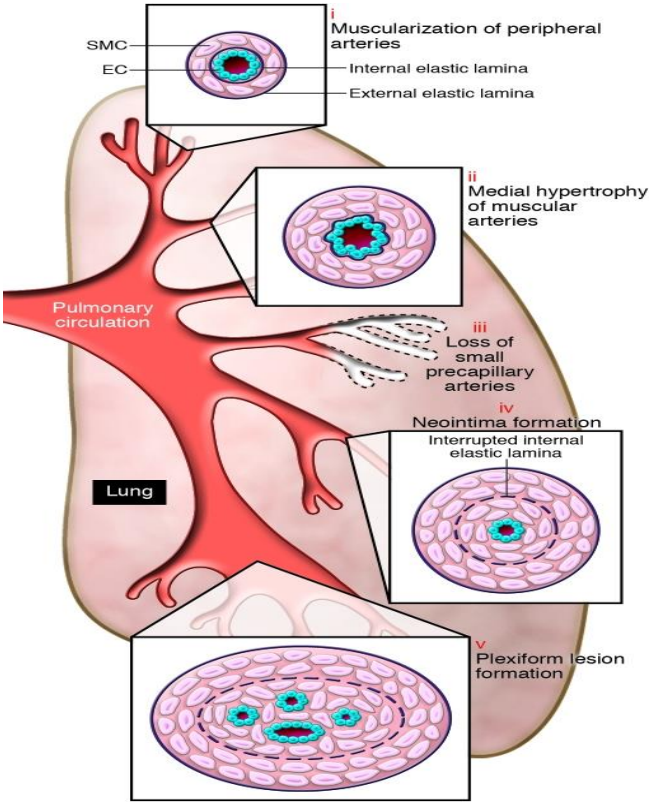



**Figure 1.2. Scheme of pathological abnormalities in PH throughout the pulmonary circulation.** Exposure of the pulmonary vasculature to increased pressure leads to vascular progressive remodelling (Reproduced with permission from Rabinovitch, 2008).

#### 1.2.3.1 Intimal thickening and plexiform lesions

Complex plexiform lesions which occur more frequently in severe PAH are associated with a poor prognosis for the patient (Strange *et al.*, 2002). Due to extensive intimal thickening there is a reduction in the lumen of the blood vessels (Chazova *et al.*, 1995). Intimal lesions are often associated with localised thrombi which might be responsible for the formation of more advanced lesions which can become fibrotic (Tuder *et al.*, 2007). Plexiform lesions are damaging as they obstruct normal blood flow, with a single lesion affecting and blocking blood flow along the rest of the length of the vessel (Cool *et al.*, 1999). The concentric intimal lesions show the presence and staining of endothelial cells and these can occur in association with plexiform lesions which will end up forming mixed lesions in the pulmonary hypertensive lung that share both concentric and plexiform lesion features (Cool *et al.*, 1997). It has been demonstrated that endothelial cells within plexiform lesions of IPAH patients expand in a monoclonal fashion, arising from a single cell, whereas those in secondary PAH they develop via a polyclonal expansion (Tuder *et al.*, 1998).

Although the cellular and molecular causes of the intimal lesions in PAH are not well understood, they are likely to be caused by a combination of injury to the vascular endothelium, increased proliferation and reduced apoptosis of PAECs, smooth muscle cell growth, migration, reduced apoptosis of PAECs, extracellular matrix (ECM) deposition and/or reorganised thrombi (Tuder *et al.*, 1998).

Formation of plexiform lesions is most likely due to abnormal proliferation. For example, there is evidence which suggests that loss of the tumour suppressor gene peroxisome proliferator-activated receptor-γ (PPAR-γ) in plexiform lesions of PAH patients may be responsible for apoptotic-resistant, highly proliferative phenotype of endothelial cells contained within these lesions (Ameshima *et al.*, 2003). PPAR-γ is an antiproliferative and anti-inflammatory mediator (Jiang *et al.*, 1998) and has been shown to mediate an important protective role in experimental PAH (Hansmann *et al.*, 2008). Restoring function of such apoptotic pathways would be predicted to inhibit progressive pulmonary vascular remodelling and is considered by investigators to likely be the most effective therapeutic strategy in PAH. The frequency of lesions is greater in smaller arteries (<200 μm) than in the arteries with bigger diameter (>400 μm) (Yi *et al.*, 2000). Yi and colleagues described concentric lesions to indicate a more severe pathology than eccentric lesions because they can completely obstruct the vessel lumen whereas eccentric lesions cause only partial luminal obstruction (Yi *et al.*, 2000). Plexiform lesions are a complex form of intimal lesion and are said to occur at the branching points of the smaller arteries (Chazova *et al.*, 1995). They are glomeruloid structures containing many small vessels with PAECs lining (Tuder *et al.*, 1994).

Myofibroblasts are also present in the core of plexiform lesions, but there is some debate about the source and origin of these cells. Some studies suggest that proliferative vascular smooth muscle cells migrating from the inner media into the intima lead to the pathogenesis of plexiform lesions (Heath *et al.*, 1990) while others suggest a role of endothelial dysfunction leading to PAEC growth following

vascular injury (Cool *et al.*, 1999). PAECs are present in both the concentric intimal and plexiform lesions (Cool *et al.*, 1997; Cool *et al.*, 1999). Similarly studies also suggest the presence of myofibroblasts in plexiform and concentric lesions (Smith *et al.*, 1990).

#### *1.2.3.2 Contribution of PASMC to vascular remodelling in PAH*

Pulmonary hypertension leads to enhanced muscularization and thickening of small arterioles, whose walls usually consist of a single elastic layer. Hypoxia in PAH is the suspected cause for remodelling of the vessels closest to the alveolar spaces (Heath & Williams, 1991). The arterial medial thickening due to this proliferation positively correlates with the pulmonary arterial pressure (Rich *et al.*, 1987). Due to this excessive proliferation, smooth muscle cells (SMCs) change their morphology and their cellular phenotype (Heath *et al.*, 1987). In normal circumstances SMCs are contractile, but in PAH, they change to become abnormally proliferative, migratory and synthetic in phenotype (Boudreau *et al.*, 1991). PASMCs show an increased proliferation and decreased apoptosis which can result and/or contribute to the thickening of the vessel wall and to vascular remodelling (Stenmark & Mecham, 1997). PASMCs from patients with PAH have been shown to be resistant to apoptotic inducers such as BMP 2, 5 and 7 (Zhang *et al.*, 2003). A reduction in Kv channel (Kv 1.5) expression and function in PASMCs decreases the programmed cell death process by affecting apoptotic volume decrease and inhibiting cytoplasmic caspases (Zhang et al., 2003). The anti-apoptotic protein Bcl-2 is also increased in the lungs of PAH patients (Postolow *et al.*, 2011; Tu *et al.*, 2011) .

## 1.3 Humoral regulators and therapeutic pathways targeted in PAH

To target the loss of NO and prostacyclin and to block the action of ET-1 in PAH, therapies directed towards three main pathways have been developed. Thus prostacyclin and stable derivatives, phosphodiesterase inhibitors and ET-1 receptor antagonists have been developed for the treatment of PAH.

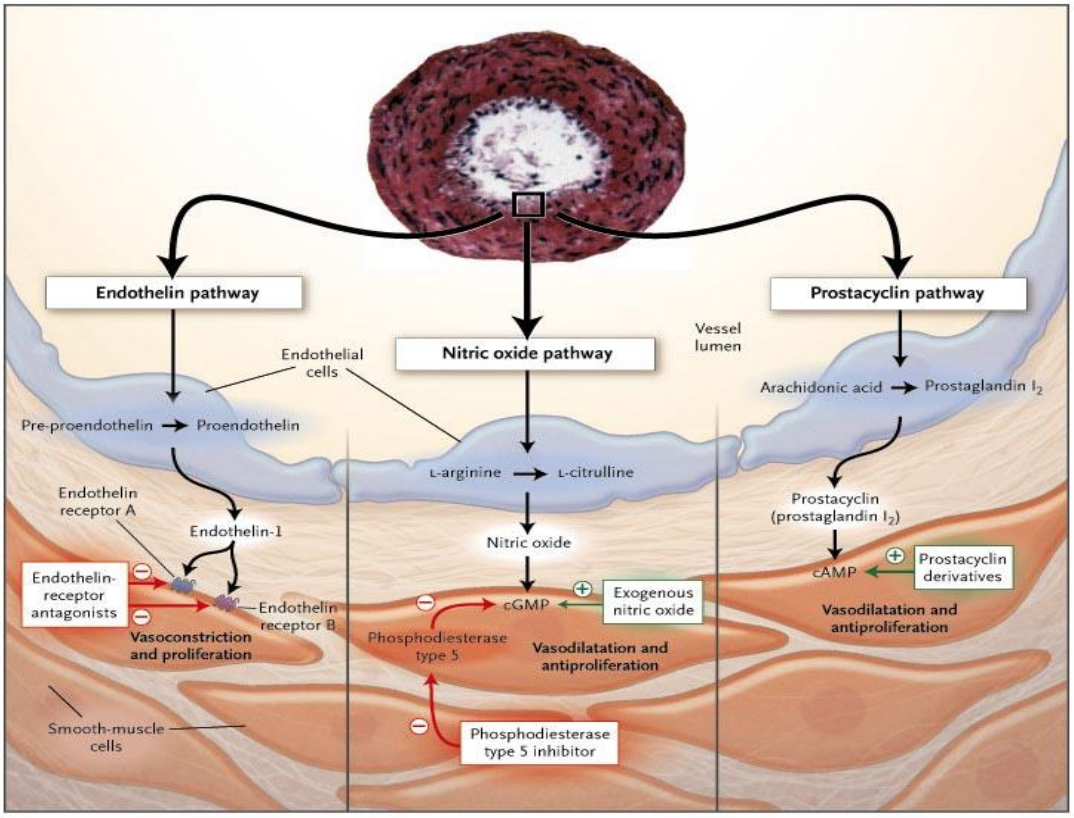



**Figure 1.3. Therapeutic pathways targeted in PAH**. The pulmonary vasculature is regulated by prostaglandins, nitric oxide, endothelin, serotonin and thromboxane. The first important pathway is prostacyclin. This agent is a powerful vasodilator and inhibitor of platelet aggregation but also a potent pulmonary vasodilator and inhibitor of smooth muscle proliferation. Second important pathway is the nitric oxide pathway. The biological action of nitric oxide (NO) is similar to that of prostacyclin. The third important pathway to be targeted is the endothelin pathway. Endothelin-1 is a potent mitogenic and vasoconstrictor peptide that plays an important role in the regulation of pulmonary vascular tone; both selective ($ET_A$) and mixed ($ET_A$ & $ET_B$) receptor antagonists have been developed. (Reproduced with permission from (Humbert, 2004b).

### 1.3.1 Nitric Oxide pathway.

Nitric oxide is a potent endothelial-derived vasodilator synthesised by eNOS utilising two substrates, the terminal guanidine nitrogen of L-arginine and molecular oxygen. The enzyme also requires a variety of co-factors including nicotinamide-adenine-dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and (6R-)5,6,7,8-tetrahydrobiopterin ($BH_4$) for NO synthesis (Zapol *et al.*, 1994). Nitric oxide causes vasodilation by binding to and activating soluble guanylate cyclase (sGC) which in turn increases intracellular cGMP and activates protein kinase G (Kwan *et al.*, 2000). Relaxation occurs through a number of routes, including reducing calcium entry by activation of plasma membrane potassium channels, by increasing calcium extrusion and/or calcium sequestering within the sarcoplasmic reticulum and by inhibition of inositol triphosphate ($IP_3$) turnover (Twort & van, 1988; Archer *et al.*, 1994; Bolotina *et al.*, 1994). A reduction in cytosolic calcium will lead to decreased calcium/calmodulin and stimulation of myosin light chain kinase. This, in turn, reduces the phosphorylation of myosin light chain, lowers smooth muscle tone, and results in vasodilation (Bode-Boger *et al.*, 2000; Galley & Webster, 2004).

Studies with knockout male mice have supported the significant impact of eNOS on pulmonary vascular homeostasis. Indeed, eNOS-deficient mice exhibit mild pulmonary hypertension under normobaric normoxia, and increased susceptibility to hypoxia induced pulmonary hypertension (Fagan *et al.*, 1999; Strange *et al.*, 2002). In contrast, overexpression of eNOS in transgenic mice prevents hypoxia-

induced vascular remodelling in the lung (Ozaki *et al.*, 2001). However, human studies suggested variable production of eNOS in patients with IPAH with reduced expression in pulmonary vessels (Giaid & Saleh, 1995) or increased expression in the endothelium of plexiform lesions, (Mason *et al.*, 1998), perhaps indicating a preferential regional distribution of the enzyme (Giaid & Saleh, 1995; Mason *et al.*, 1998; Demoncheaux *et al.*, 2005). Limitation of L-arginine might modulate NO concentrations in humans. Indeed, PAH patients with reported decreased NO levels have lower L-arginine content in their serum due to enhanced activity of arginase, an enzyme that converts L-arginine to ornithine and urea (Xu *et al.*, 2004). Oral L-arginine supplementation reduces pulmonary pressures in these patients, but does not halt the progression of the disease (Mori & Gotoh, 2000; Morris *et al.*, 2003). Besides exogenous NO, several other targets in the NO pathway have shown promising results as potential therapies (Coggins & Bloch, 2007). Firstly, inhibition of the enzyme, phosphodiesterase type 5 prevents the breakdown of cGMP, increasing the levels of cGMP and thus will potentiate the effects of NO and atrial natriuretic peptide (ANP), an activator of particulate guanylate cyclase (GC). Secondly, direct activation of soluble GC by compounds like BAY 41-2272 has been shown to reduce pulmonary vascular resistance in foetal and neonatal sheep with severe PH (Deruelle *et al.*, 2005a; Deruelle *et al.*, 2005b). Thirdly, the presence and availability of the NOS cofactor, $BH_4$ protects mice from hypoxia induced PH, decreasing pulmonary vascular tone and remodelling in this PH model (Khoo *et al.*, 2005).

### 1.3.2 Prostacyclin pathway:

Synthetic prostacyclin (epoprostenol) was one of the first FDA-approved drugs to be used in the treatment of PAH (Rubin *et al.*, 1990). To date, it appears to be the most efficacious drug for severe forms of PAH and the only agent shown to improve long term survival in randomised trials (Wozencraft *et al.*, 2012). Prostacyclin ($PGI_2$) is a 20 carbon prostanoid derivative of the arachidonic acid metabolic pathway. $PGI_2$ has for a long while been thought to be a cyclooxygenase-2 (COX 2) derived product in vascular endothelial cells and smooth muscle cells, although increasing evidence suggests that $PGI_2$ may be produced mainly from COX-1, at least under physiological conditions (Flavahan, 2007). $PGI_2$ is a potent vasodilator in the pulmonary circulation and exhibits anti-mitogenic and antithrombotic properties in experimental PAH (Hoshikawa *et al.*, 2001).

The half-life of $PGI_2$ is approximately 3 minutes when kept in a physiological buffer at 37 °C, the vinyl-ether linkage being susceptible to hydrolysis to 6-keto-$PGF_{1\alpha}$, a chemically stable product but weak inhibitor of platelet function and vascular tone (Figure 1.5). The metabolism is complex, but 6-keto-PGF1α can be further metabolised in the kidney to the 2,3-dinor derivative (Whittle & Moncada, 1984) Therefore, $PGI_2$ is typically monitored by measurement of 6-keto-PGF1α in plasma or its derivative in urine, 2,3-dinor-6-keto-$PGF_{1\alpha}$. Thromboxane $A_2$, another COX-derived prostaglandin produced by endothelial cells and platelets, is responsible for increasing vasoconstriction and activation of platelets and thus opposes the biological effects of $PGI_2$.

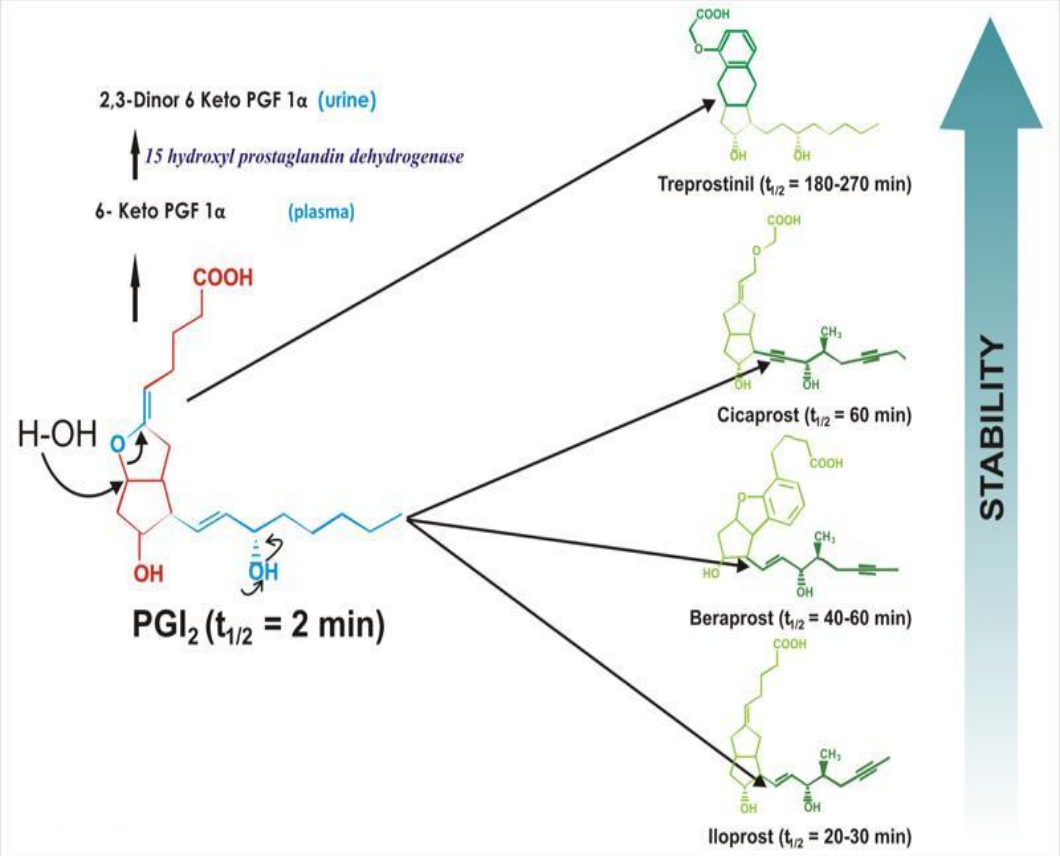



**Figure 1.4. Structures of prostacyclin and its analogues**. Prostacyclin is a chemically unstable prostaglandin which is readily hydrolysed in a physiological buffer to 6-keto- $PGF_{1\alpha}$. It is also subject to metabolism by 15 hydroxyl prostaglandin dehydrogenase and β-oxidation yielding the urinary metabolite 2,3-dinor-6-keto- $PGF_{1\alpha}$. To increase stability, a series of modifications in the structure of prostacyclin have been made, giving rise to chemically stable analogues with longer plasma half-lives (given as terminal). Stability comes from a hindered alcohol group at carbon 15 as well as an ether moiety replacing the methyl group at carbon 3 of the carboxylic acid side chain.

In patients with PAH, decreased urinary metabolites of prostacyclin was accompanied by increased levels of urinary metabolites of thromboxane $A_2$ (Christman et al., 1992). It is also reported that a deficiency in circulating $PGI_2$, due to decreased expression of $PGI_2$ synthase, contributes to the pathogenesis of PAH (Tuder *et al.*, 1999; Tuder *et al.*, 2007). Therefore the imbalance between

prostacyclin and thromboxane $A_2$ favours vasoconstriction, thrombosis, as well as vessel wall remodelling associated with PAH development.

Clinical trials confirm that patients receiving chronic administration of exogenous epoprostenol demonstrate a marked improvement in haemodynamic function, exercise capacity and survival time in NYHA class III and class IV PAH patients (Barst *et al.*, 1996; Sitbon *et al.*, 2002). However, the major limitation with epoprostenol is that it has to be given by chronic intravenous infusion due to its short half-life (< 3-6min), and additionally in some countries, requires an ice pack to maintain stability. Its side effects include flushing, jaw pain, headache and special mixing in *vivo* is required to prevent sepsis resulting from the indwelling catheter.

Treprostinil is a modern generation $PGI_2$ analogue with similar haemodynamic effects to epoprostenol, but with an improved half-life (~3 hours) and the ability to be administered subcutaneously (Sadushi-Kolici *et al.*, 2012). Iloprost, another prostacyclin analogue is typically given by inhalation, has been FDA approved for therapeutic use and this route of administration may reduce systemic side effects and minimise sepsis associated complications with IV administration. The main drawback of various prostacyclin analogues are their relatively short biological half-lives: epoprostenol (3-5min) < iloprost and beraprost (~ 30min) < treprostinil (4-5 h). The chemical stability order of various analogues is iloprost < beraprost < cicaprost << treprostinil. Treprostinil is the most stable amongst all these analogues, and has shown favourable effects in the treatment of PAH (Baskurt & Kucukoglu, 2010), including long-term survival (Sadushi-Kolici *et al.*, 2012).

Prostacyclin and its analogues have been the most successful treatments for improving the symptoms of PAH in humans to date. Epoprostenol (IV), beraprost (oral), iloprost (inhalation, IV) and treprostinil (subcutaneous infusion, inhalation, IV) have all been used with varying degrees of success in PAH patients in all clinical categories (Saji *et al.*, 1996; Nagaya *et al.*, 1999; Humbert *et al.*, 2011). Recently selexipag, an oral IP receptor agonist is in clinical development for the treatment of PAH (Simonneau *et al.*, 2012). Selexipag is highly selective for the IP receptor and does not bind to other prostanoid receptors and therefore can be distinguished from the above $PGI_2$ analogues currently used in the management of PAH (Kuwano *et al.*, 2007).

#### 1.3.2.1 Cellular targets for prostacyclins

The mode of action of $PGI_2$ (or its stable analogues) is thought to be through activation of plasma membrane prostacyclin (IP) receptors coupled via the Gs protein to adenylyl cyclase and cAMP production (Narumiya, 1994). Once elevated, cAMP is rapidly broken down by specific phosphodiesterases (PDE). In the normal lung PDE 1, 3, 4 appear responsible for regulating basal levels and analogue-induced elevation in cAMP (Phillips *et al.*, 2005; Murray *et al.*, 2007). However, $PGI_2$ synthase is also expressed in the perinuclear regions of cells, including in smooth muscle. Once formed, prostacyclin can then directly interact with the ligand-binding domain of a family of transcription factors called peroxisome proliferator-activated receptors (PPARs) to regulate a number of cellular processes including cell growth, insulin sensitivity and inflammation (Lim & Dey, 2002).

$PGI_2$ has poor selectivity for prostanoid receptors, binding to and activating $EP_1$, $EP_3$ and TP receptors, albeit at higher concentrations (15–45-fold for $EP_1$ and $EP_3$ and <100-fold for TP) compared with the corresponding natural ligand (Kennedy *et al.*, 1982; Coleman *et al.*, 1995; Alexander *et al.*, 2005). Activation of $EP_1$ and $EP_3$ receptors would tend to elevate $Ca^{2+}$ and/or lower cAMP through different G protein pathways, leading to vasoconstriction, thrombosis and cell proliferation.

While stable analogues also bind potently to the IP receptor with an affinity in a nanomolar range (Table 3), iloprost like prostacyclin, has poor selectivity for prostanoid receptors, being essentially equipotent at activating IP and $EP_1$ receptors (Narumiya *et al.*, 1999 ; Abramovitz *et al.*, 2000). Thus, it is not surprising that iloprost-induced vasorelaxation can be enhanced by an $EP_1$ receptor blockade in the isolated rabbit perfused lungs (Schermuly *et al.*, 2007a) or in guinea pig aorta (Clapp *et al.*, 1998). On the other hand, all analogue responses in rat tail artery are enhanced by blocking $G_i/G_o$ with pertussis toxin (PTX), presumed to relate to the $EP_3$ receptor, the main splice variant being negatively coupled to adenylyl cyclase via $G_i$ (Orie & Clapp, 2011). In addition, cicaprost has significant activity at the $EP_4$ receptor, as does iloprost for the human and rat but little for the mouse receptor (Table 3). Until very recently, far less was known about treprostinil binding affinity for different receptors.

A recent study by Whittle and his colleagues showed treprostinil had low nanomolar potency in human embryonic kidney 293 cells (HEK-293) expressing human $DP_1$, IP and $EP_2$ receptors, whereas it showed much lower activity at the

other prostanoid receptors (Whittle *et al.*, 2012). Thus, treprostinil seems to differ from iloprost in its pharmacological profile, and this may have therapeutic implications in the treatment of PAH.

It is difficult to obtain similar data to the above for $PGI_2$, because radioligand binding competition studies requires that binding reaches equilibrium, which can take at least 20 min. Over this period $PGI_2$ will be almost completely hydrolysed at physiological temperature and pH to a metabolite that has little or no affinity at the IP receptor. Thus binding experiments are not possible to do with $PGI_2$ except at very high pH (>10) and lower temperatures. If instead one uses historical dose-ratio studies and compare potency of biological effects with the natural ligand, this would predict a rank order potency for $PGI_2$ at various prostanoid receptors to be $IP>EP_3>EP_1\geq TP$. Iloprost, carbacyclin and beraprost all bind to peroxisome proliferator-activated receptors (nuclear transcription factors known as PPARs), cicaprost does not, thus highlighting another pharmacological difference. Prostacyclin will also bind to PPARs although other prostaglandin and lipoxygenase metabolites may be the natural ligand (Forman *et al.*, 1997).. There is no information available about whether treprostinil binds, but PPARs appear to underlie some of the biological effects of treprostinil (Falcetti *et al.*, 2010). None of the above receptor selectivity would be predicted based on the initial chemistry of the analogues in relation to $PGI_2$. Treprostinil does not intuitively look or resemble a prostacyclin-like agent to a pharmacologist/untrained chemist. The same could be said of beraprost. If we had the data as we know it now, treprostinil would be classed as a potent $DP_1$ and $EP_2$ agonist first and foremost (Table 3 and 4).

| Ligands | | IP | DP | TP | $EP_1$ | $EP_2$ | $EP_3$ | $EP_4$ | FP |
|---|---|---|---|---|---|---|---|---|---|
| **Carbacyclin** | Human | 275 | 132 | | 23 | 942 | 14 | 352 | 385 |
| | Mouse | 110 | | | | 1600 | 31 | 2300 | 1200 |
| **Cicaprost** | Human | 17 | >1340 | >1340 | >1340 | >1340 | 255 | 44 | >1340 |
| | Mouse | 10 | | | 1300 | | 170 | | |
| **Iloprost** | Human | 11 | 1035 | | 11 | 1870 | 56 | 284 | 619 |
| | Mouse | 11 | | | 21 | 1600 | 27 | 2300 | |
| **Beraprost** | Human | 39 | | | | | 680 | | |
| | Mouse | 16 | | | | | 110 | | |
| **Treprostinil** | Human | 31 | 4.4 | | 212 | 3.6 | 2505 | 826 | |
| | Mouse | YES | ND | ND | ND | YES | ND | NO | ND |
| **$PGE_2$** | Human | | 307 | | 9.1 | 4.9 | 0.3 | 0.8 | 119 |
| | Mouse | | | | 20 | 12 | 0.8 | 1.9 | 100 |
| **$PGE_1$** | Human | 318 | 53 | | 90-165 | 1 | 6-37 | 1.4 | ND |
| | Mouse | 33 | | | 36 | 10 | 1 | 2 | |
| **$PGD_2$** | Human | | 1.7 | | | 2973 | 421 | 1483 | 6.7 |
| | Mouse | | 21 | | | | 280 | | 47 |

**Table 3 - Distinct differences in prostanoid receptor binding affinities ($K_i$) for prostacyclin analogues compared with prostaglandin $E_2$ ($PGE_2$), $PGE_1$ and prostaglandin $D_2$ ($PGD_2$) at human and mouse prostanoid receptors.** Radio ligand binding data (Ki in nM) has been taken from original study references for prostacyclin analogues (Kiriyama *et al.*, 1997; Abramovitz *et al.*, 2000; Kuwano *et al.*, 2007; Whittle *et al.*, 2012) ,for $PGE_2$ & $PGD_2$ (Kiriyama *et al.*, 1997; Abramovitz *et al.*, 2000) and for $PGE_1$ from several individual studies (Funk *et al.*, 1993; Bastien *et al.*, 1994; Regan *et al.*, 1994; Wright *et al.*, 1998; Davis & Sharif, 2000; Sharif & Davis, 2002; Stitham *et al.*, 2007). The lower the number, the higher the affinity of the drug for the receptor. Blank means Ki value >3μM, ND means not done, YES indicates evidence for functional activity and NO means the opposite. Table modified from (Clapp & Patel, 2010) to include recently published data from (Whittle *et al.*, 2012) and $K_i$ for $PGE_1$. Some $K_i$'s have been calculated from $IC_{50}$ and $K_d$ reported in the actual paper.

| *LIGANDS* | *Interpretations from Table 3* |
|---|---|
| **Carbacyclin** | $EP_3=EP_1>DP\geq IP>EP_2$ **Equipotent $EP_3$ and $EP_1$ agonist; 10 fold lower potency at IP receptor** |
| **Cicaprost** | $IP>EP_4>EP_3$ **Potent IP agonist but with only two fold lower potency at $EP_4$ receptor** |
| **Iloprost** | $IP=EP_1>EP_3>DP_1$ **Equipotent IP and $EP_1$ receptor agonist** |
| **Beraprost** | $IP>>EP_3$ **3 fold lower potency at IP receptor compared to iloprost, some activity at $EP_3$ receptor** |
| **Treprostinil** | $EP_2=DP>IP>EP_1>EP_3$ **~10x more potent at activating $EP_2$ and DP than IP; Essentially equipotent as natural ligand $PGE_2$ and $PGD_2$ & 10 fold lower at activating IP receptor than Iloprost.** |
| **$PGE_1$** | $EP_2=EP_4>EP_3>DP>EP_1>IP$ **Equipotent at $EP_2$ and $EP_4$ receptors but 300 fold lower potency at IP receptor. Rank order of binding affinity bares no resemblance to any of the prostacyclin analogues.** |

**Table 4 - Pharmacological profiles of prostacyclin-Interpretation from table 3**

#### 1.3.2.1.1 Prostacyclin and vascular tone

Many studies suggest that $PGI_2$ analogues inhibit vascular tone almost exclusively through plasma membrane potassium channels, activation of which will inhibit calcium influx through voltage-dependent calcium channels (Tanaka *et al.*, 2004; Clapp & Tennant, 2005; Orie *et al.*, 2006). Potassium channels are able to influence vascular tone because of the very steep relationship between membrane potential and calcium influx, with a small change (< 5 mV) capable of enhancing calcium

entry by two fold (Clapp & Tennant, 2005). Depending on the vascular bed studied, ATP-sensitive ($K_{ATP}$), large conductance $Ca^{2+}$-activated ($BK_{Ca}$), inward rectifier have been implicated in the relaxation induced by iloprost, beraprost and treprostinil (Clapp *et al.*, 1998; Tanaka *et al.*, 2004; Orie *et al.*, 2006) . This can occur through direct coupling via Gs of the IP receptor to the channel, indirectly through cAMP-dependent activation of protein kinase A (PKA) or perhaps through other G-protein signalling pathways that might be activated by either IP or EP (2 & 4) receptors. In the lung, $K_{ATP}$ and $BK_{Ca}$ channels contribute to the reversal by iloprost of hypoxic-induced increases in perfusion pressure in the rat (Dumas *et al.*, 1997) while treprostinil activates a background potassium (TASK-1) current which is turned off by hypoxia and ET-1 in human PASMCs (Tang *et al.*, 2009). In addition, depressed activity and expression of voltage-gated (Kv) potassium channels (in particular Kv1.5) is consistently reported in response to hypoxia, serum, anorexic agents, ET-1 and in IPAH (Clapp & Tennant, 2005; Bonnet *et al.*, 2007; Morrell *et al.*, 2009). Serum and ET-1 will also inhibit $K_{ATP}$ channel function, though proliferating human PASMCs do still hyperpolarise to pharmacological openers of this channel (Quinn *et al.*, 2003). The combined loss of potassium function will lead to PASM depolarisation and a sustained rise in intracellular calcium, triggering not only vasoconstriction, but cell proliferation and a reduction in apoptosis (reviewed in (Clapp & Tennant, 2005; Hassoun *et al.*, 2009; Morrell *et al.*, 2009)). Thus likely underlying cause for some of the beneficial effects of $PGI_2$ therapy in IPAH could be the activation of potassium channel.

### 1.3.2.2 Impact of PAH on prostacyclin signalling

Decreased urinary levels of 2,3-dinor-6-keto-$PGF_{1\alpha}$ are found in patients with IPAH (Christman *et al.*, 1992) or pulmonary hypertension linked with congenital heart disease (Adatia *et al.*, 1993). In IPAH this is associated with a progressive loss of $PGI_2$ synthase expression from large to small pulmonary arterial vessels, with virtually no expression in plexiform lesions (Tuder *et al.*, 1999). Conversely overexpression of $PGI_2$ synthase is protective in monocrotaline- and hypoxia-induced models of PAH, reducing both medial thickening and pulmonary pressure (Geraci *et al.*, 1999; Nagaya *et al.*, 2000). A reduction in IP receptor expression has recently been reported in IPAH lungs and in rats following monocrotaline treatment (Lai *et al.*, 2008; Falcetti *et al.*, 2010). Such a loss is however unlikely to cause PAH as mice lacking the IP receptor gene do not spontaneously develop PH, although they are more susceptible to the hypertensive and remodelling effects of hypoxia (Hoshikawa *et al.*, 2001). Thus $PGI_2$ synthase and the IP receptor both appear protective in the context of PAH. Heightened activity of PDE1 (PDE1A & PDE1C) (Murray *et al.*, 2007; Schermuly *et al.*, 2007b) and to a lesser extent PDE3 has been reported in IPAH (Murray *et al.*, 2007), suggesting this may lead to reduced effectiveness of prostacyclin and its analogues.

#### 1.3.2.2.1 $PGI_2$ as regulators of cell proliferation

Previous studies in normal human PASMCs have shown that prostacyclin analogues can inhibit the mitogenic responses to PDGF and serum in a largely cAMP-dependent manner, with adenylyl cyclase inhibitors blocking around 75% of

the analogue responses in these cells (Wharton *et al.*, 2000; Clapp *et al.*, 2002; Falcetti *et al.*, 2010). The downstream mechanisms are not well understood, but prostacyclin analogues appear to inhibit smooth muscle cell proliferation by blocking progression from G1 to S phase (Kothapalli *et al.*, 2003; Ohtsubo *et al.*, 2007). This may occur through phosphorylation of the cAMP response element binding protein (CREB) inhibiting cyclin A expression (Kothapalli *et al.*, 2003) and up-regulating the inducible cAMP early repressor (Ohtsubo *et al.*, 2007), the latter pathway is thought to promote apoptosis as well. There are other mechanisms by which cAMP cascades can impede cell growth (figure 1.6). Treprostinil inhibits serum-induced cell growth in human PASMCs in part through cAMP-dependent activation of ATP-sensitive potassium $K^+$ channels (Clapp & Tennant, 2005). In aortic smooth muscle cells, cAMP elevating agents inhibit PDGF-mediated cell growth through inhibition of the calcineurin and nuclear factor of activated T-cells (NFAT) pathway (Jabr *et al.*, 2007). Such a mechanism may counteract the elevated NFAT activity reported in IPAH (Bonnet *et al.*, 2007), and for the expression of growth-promoting genes, including endothelin-1 (ET-1).

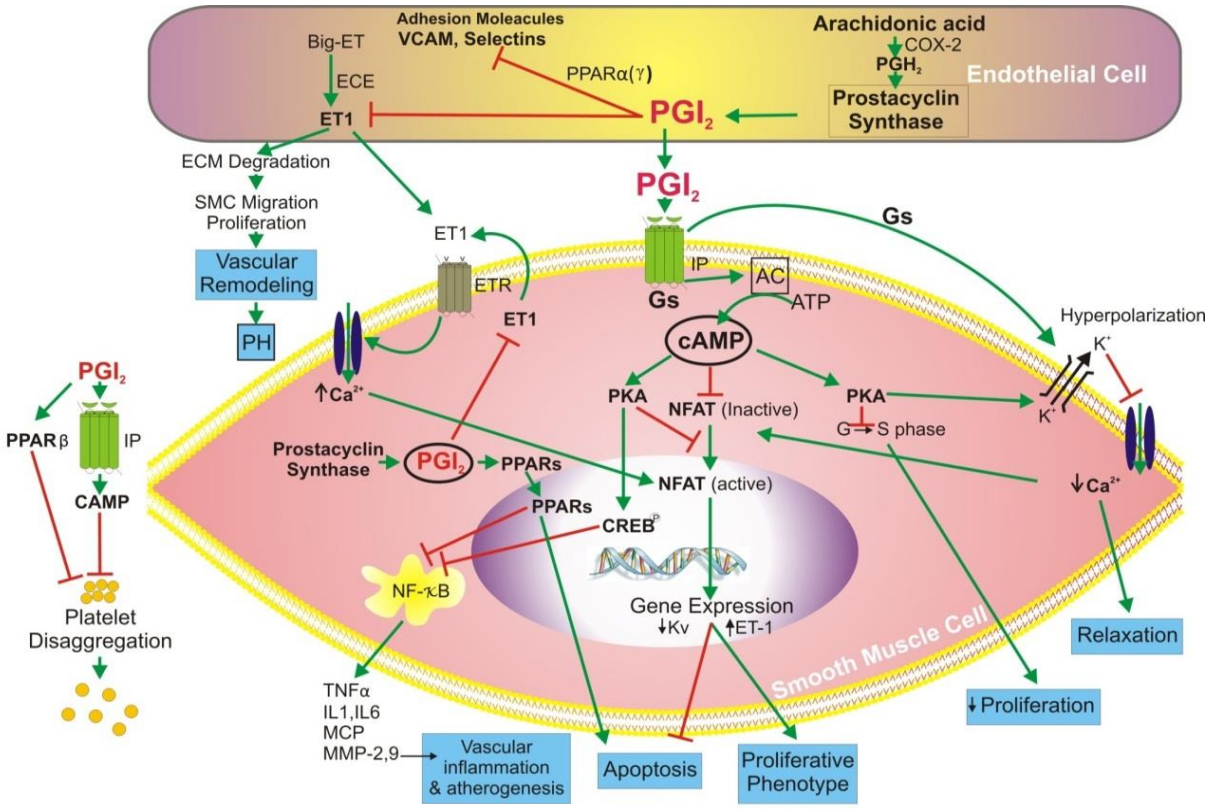



**Figure 1.5. Schematic representation of the cellular pathways by which prostacyclin inhibits vascular tone, cell proliferation, platelet activation.**

Recent studies have shown that PPARγ contributes to the IP receptor-dependent antiproliferative effects of treprostinil in HEK-293 cells (Falcetti *et al.*, 2007) and to iloprost-induced inhibition of lung tumourigenesis through an IP receptor-independent mechanism (Nemenoff *et al.*, 2008). In the latter study, pulmonary-specific over expression of either $PGI_2$ synthase or PPARγ resulted in suppression of tumour incidence and multiplicity in these lung models (Nemenoff *et al.*, 2008) while non-small cell lung cancer cells over-expressing PPARs exhibit significantly less invasiveness and metastases. PPARγ was shown to be largely responsible for

antiproliferative effects of treprostinil in human PASMCs derived from IPAH patients (Falcetti et al, 2010).

### 1.3.3 Endothelin pathway:

#### *1.3.3.1 Endothelin*

Endothelin, a 21-amino acid peptide secreted by the vascular endothelial cells was first discovered in 1988 and frequently reported as one of the most potent vasoconstrictors (Yanagisawa *et al.*, 1988). There are three different subtypes of endothelin, ET-1, ET-2 and ET-3 which are derived from different genes and produced by different cell types (Davenport & Kuc, 2002). All three endothelins, share structural similarities in that they all have two disulphide bonds, a hydrophobic C terminal and a polar side chains of amino acid residues. ET-2 shares 90% sequence homology with ET-1 whereas ET-3 shares about 71% homology (Nakajima *et al.*, 1989). ET-1 has been studied the most extensively and is a potent vasoconstrictor of many blood vessels throughout the body, including the lungs (Yanagisawa *et al.*, 1988). It is predominantly produced by vascular endothelium and to a lesser extent by pulmonary smooth muscle cells (Markewitz *et al.*, 2001), lung fibroblasts (Shi-Wen *et al.*, 2004) leucocytes, macrophage (Ehrenreich *et al.*, 1990) and circulating fibrocytes (Quan *et al.*, 2006). ET-1 is also produced in organs like heart, kidney, pituitary and the central nervous system, though production appears extremely low compared to the endothelium (Roux *et al.*, 1995).

#### 1.3.3.2 Endothelin Synthesis

Similar to other functional peptides in the body, ET-1 is synthesized from a large inactive precursor protein. This precursor preproendothelin-1 (PPET-1) is cleaved by different proteolytic enzymes, finally leading to the synthesis of ET-1 (Kimura *et al.*, 1988; Itoh *et al.*, 1988; Corder *et al.*, 1995). Human PPET-1 contains 212 amino acids (Yanagisawa et al. 1988) and cleavage occurs on the carboxyl side pairs of basic amino acids by the action of carboxypeptidases (Denault *et al.*, 1995b). First, a signal peptide is removed from the PPET-1 by a signal peptidase in the lumen of rough endoplasmic reticulum, yielding a precursor of 195 amino acids, proendothelin-1 (pro-ET-1) (Denault et al. 1995). Pro ET-1 is converted to a 38 amino acid peptide big ET-1, by the furin convertase (Denault *et al.*, 1995a). Conversion of big ET-1 into the biologically active ET-1 is believed to occur via specific metalloproteases called endothelin converting enzymes (ECE) (Levin, 1995a; Nakano *et al.*, 1997). Under normal culture conditions ET-1 is released continuously from endothelial cells by a constitutive secretion pathway. Both ET-1 and big ET-1 vesicles have been identified in endothelial cells, suggesting that the intracellular vesicles are a probable site for the conversion of big-ET-1 to ET-1 by ECE under physiological conditions (Harrison *et al.*, 1995). ECEs are membrane-bound proteases and have structural homology to neutral endopeptidase (NEP) (Opgenorth *et al.*, 1992; Turner, 1993). There are two isoforms, ECE-1 and ECE-2, though ECE-1 is the predominant one found in humans (Schmidt *et al.*, 1994; Xu *et al.*, 1994; Emoto & Yanagisawa, 1995)

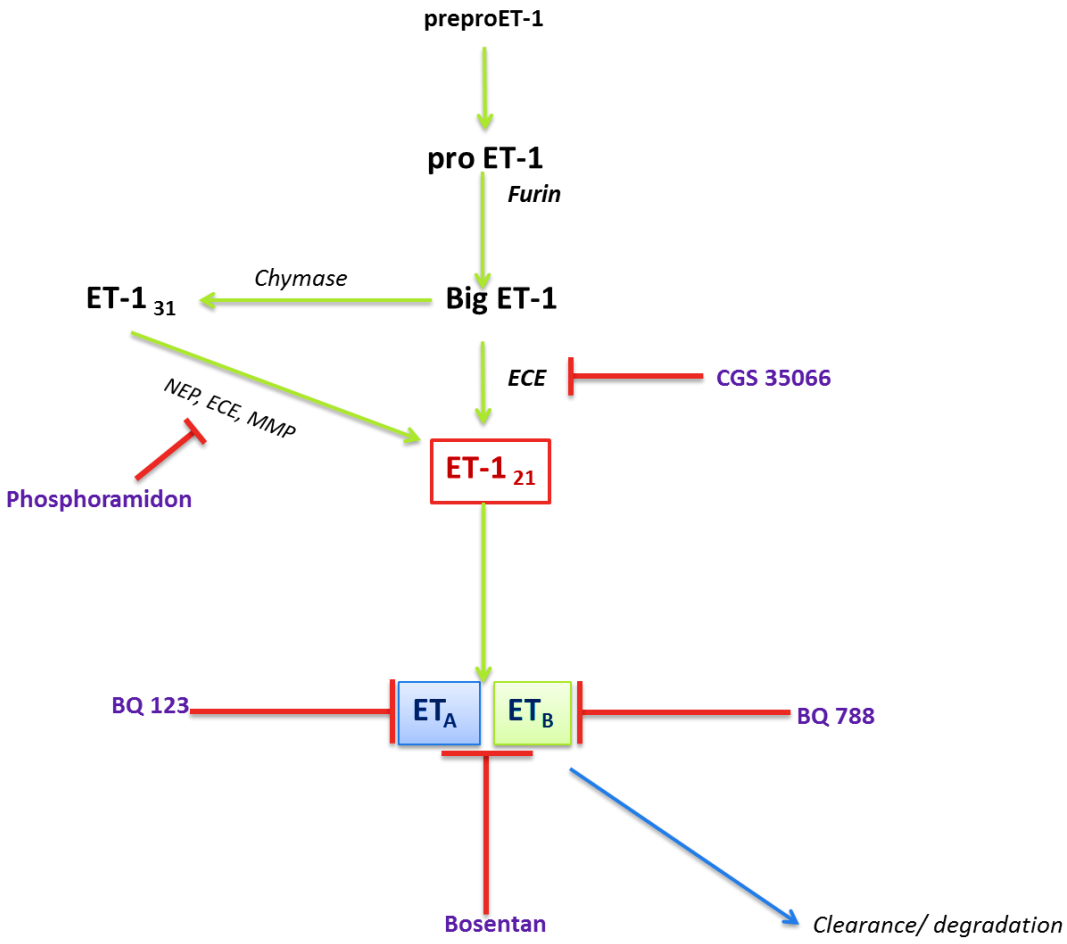



**Figure 1.6. Production and degradation of ET-1.** Prepro-ET-1 is regulated at the transcription level and is reduced to BigET-1 by a furin-like enzyme. The majority of BigET-1 is then reduced to mature ET-1(21-amino acids) by ECE, or to ET-1 (31 amino acids) by a chymase and subsequently cleaved by a metalloprotease (MMP) to yield active ET-1. ET-1 can then exert its biological effects through binding to G protein coupled receptors ($ET_A$, $ET_B$). The production of ET-1 can be inhibited at several steps of the signalling cascade. The use of ET receptor antagonism and ECE inhibition are extensively being studied in conditions with chronically high levels of ET-1.

ECE expression can been attributed to the activity of a converting enzyme located on the plasma membrane and intracellularly, and is particularly high in endothelial cells where it appears responsible for the conversion of big ET-1 to ET-1 (Corder *et*

*al.*, 1993; Xu *et al.*, 1994). Specific stimuli can rapidly induce the transcription of ET-1 mRNA (Xu et al. 1994). The synthesis and secretion of ET-1 can occur within minutes and thus regulation of ET-1 is primarily controlled through alterations in gene expression. While unbound, ET-1 has a half-life of less than two minutes in plasma; the effect of ET-1 in the body can last for many hours because of the irreversible nature of ET-1 binding to its receptor (Levin, 1995a).

There are two receptors that have been identified for ET-1, $ET_A$ and $ET_B$ and in smooth muscle both these receptors cause vasoconstriction mediated through increases in intracellular calcium levels (Takuwa *et al.*, 1990; Davenport & Kuc, 2002). $ET_A$ and $ET_B$ are G-protein coupled receptors, but the actions mediated by the binding at these two receptors are varied. Binding of ET-1 to smooth muscle cell $ET_A$ and $ET_B$ receptors activates phospholipase C (PLC), leading to an increase of inositol triphosphate ($IP_3$) and diacylglycerol (DAG) which in turn can release calcium from the SR or increase $Ca^{2+}$ flux through voltage gated $Ca^{2+}$ channels (Pollock *et al.*, 1995). This increase in DAG and calcium will also stimulate protein kinase C to further mediate the mitogenic action of ET-1 (Ohlstein *et al.*, 1992). The $ET_B$ receptor has additional actions, acting to release endothelium-dependent vasodilators, namely through increasing the production of NO and $PGI_2$ (Suzuki *et al.*, 1991).

Additionally, $ET_B$ receptors have been shown to be responsible for the clearance of plasma ET-1 (Dupuis *et al.*, 1996b; Schneider *et al.*, 2007). Under normal circumstances, the biological effect of ET-1 results from the balance between the

two receptor effects on the vasculature, though the overall result of combined $ET_A$ and $ET_B$ antagonism would be mild dilation of the vasculature (Haynes *et al.*, 1996; Shah, 2007). The $ET_A$ receptor is predominantly expressed in proximal pulmonary arteries, while $ET_B$ receptor expression is higher in vascular smooth muscle cells in distal resistance vessels (McCulloch *et al.*, 1996). Both endothelin receptors have a significant impact on vasoconstriction and proliferation (Bonvallet *et al.*, 1993; McCulloch *et al.*, 1996).

#### *1.3.3.3 Clearance of ET-1*

ET-1 elimination seems to be a rapid process happening within minutes, despite pressor effects lasting for about an hour (Sirvio *et al.*, 1990; Levin, 1995b; Vachiery & Davenport, 2009). There is a sustained increase in cytosolic calcium in SMCs coupled with an increase in the sensitivity of the contractile apparatus to calcium, resulting in prolonged vascular contraction. The majority (50-70%) of ET-1 uptake occurs in pulmonary circulation and therefore the lung is considered a major organ for clearance of plasma ET-1 (de *et al.*, 1988; Dupuis *et al.*, 1996b). A substantial amount of ET-1 synthesis is located in pulmonary tissue and this will lead to a neutral arterial-venous oxygen difference in the lungs (Dupuis *et al.*, 1996b). In animal experiments, it was shown that single pass extraction of ET-1 by the pulmonary circulation is completely abolished during selective blockade of the

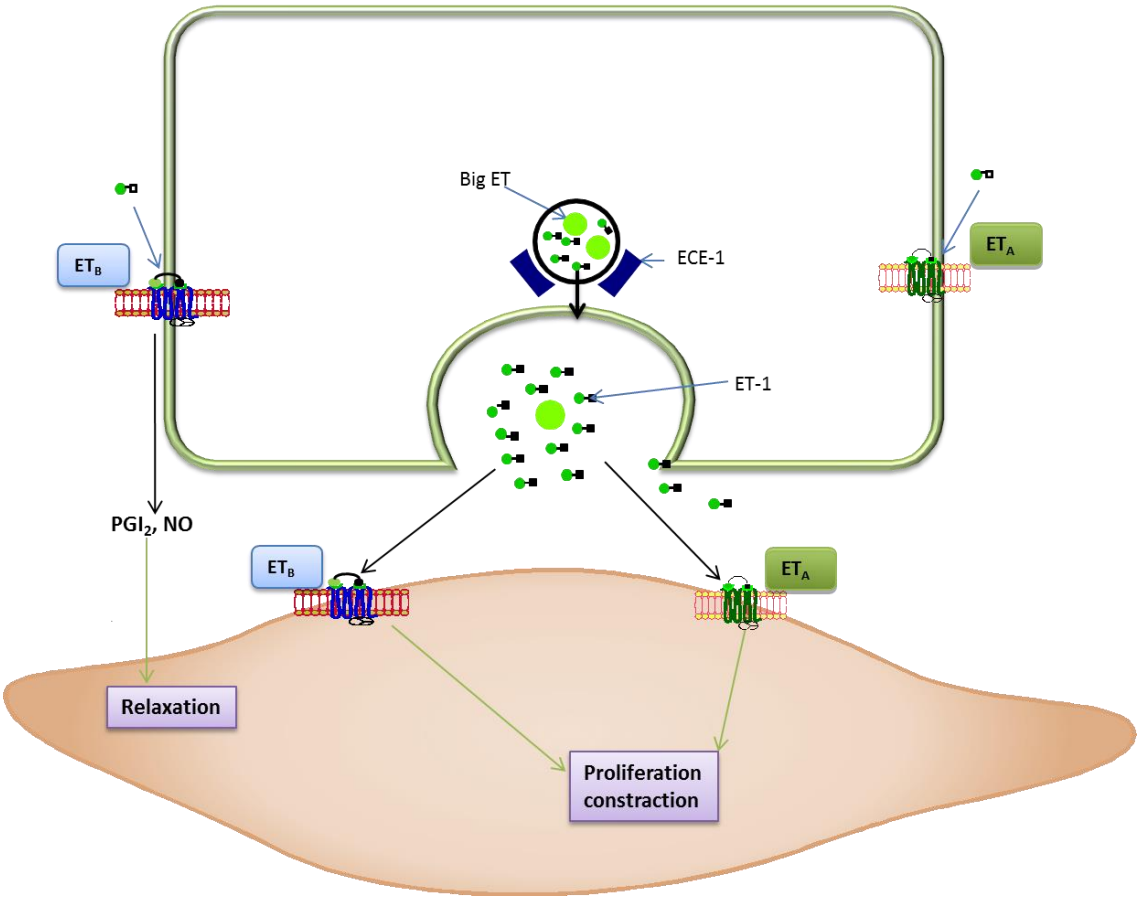



**Figure 1.7. Effect of ET-1 in the vasculature**. Endothelin-converting enzyme (ECE) catalyzes the conversion of ET-1 from its precursor Big ET-1 which then binds to its 2 main target receptors, $ET_A$ and $ET_B$. Binding of ET to $ET_A$ and $ET_B$ on VSMC leads to vasoconstriction, while binding of ET to $ET_B$ on endothelial cells can lead to vasodilation of VSMC. In fibroblasts and smooth muscle these receptors will also cause proliferation. In certain pathologies expression of the $ET_B$ receptor on SMC is up-regulated.

$ET_B$-receptor subtype (Dupuis *et al.*, 1996b). It was also demonstrated that the magnitude of increase in plasma levels of ET-1 in endothelial $ET_B$-receptor knockout mice (Bagnall *et al.*, 2006) is comparable to the change in ET-1 plasma levels after $ET_B$ blockade. Taken together, these results indicate that $ET_B$-receptors play an important role in the uptake of ET-1. Uptake of ET-1 also occurs in the

kidney, which as in the pulmonary circulation, appears to be mediated through the $ET_B$ receptor (Fukuroda *et al.*, 1994a). There is insufficient evidence to suggest significant uptake of ET-1 occurs in the liver in humans, although a study in dogs suggests that the $ET_B$ receptor in the liver is an important site for ET-1 clearance with close to 85 % extraction occurring in this organ (Dupuis *et al.*, 1999). The mechanism of $ET_B$ receptor-mediated clearance of ET-1 occurs through intracellular lysosomal degradation of the receptor complex (Bremnes *et al.*, 2000).

#### *1.3.3.4 Role of ET-1 in PAH*

ET-1 is frequently reported as a potent mitogen affecting the functions of endothelial cells, smooth muscle cells and fibroblasts and is thus likely to play a central role in the pathogenesis of PAH. In addition, ET-1 is also responsible for cytokine release. The role of the endothelin pathway in the pathogenesis of PAH was considered following several studies showing that plasma levels of ET-1 are three to four times the upper limit of normal in patients with most forms of PAH including IPAH, PAH related to connective tissue disease, heart failure and HIV infection (Yoshibayashi *et al.*, 1991; Vancheeswaran *et al.*, 1994; Rubens *et al.*, 2001; Sitbon *et al.*, 2004). Moreover ET-1-like immunoreactivity was abundant in patients with PAH, particularly in plexiform lesions (Gaid *et al*, 1995). ET-1 staining was predominantly in the endothelial cells of the pulmonary arteries which had medial thickening and there was a strong correlation between the intensity of the immunoreactivity and PVR in these patients. Also, elevated ET-1 levels have been shown to negatively correlate with haemodynamic variables, 6-min walk distance (6MWD) and survival (Rubens *et al.*, 2001).

PAH is a disease of excessive remodelling of the intimal layers of the vasculature and the ET-1 produced by the endothelial cells, is directed towards the underlying smooth muscle cells and fibroblasts, where it can interact with $ET_A$ and $ET_B$ receptors to promote vasoconstriction, proliferation and fibrosis (figure 1.8). A smaller amount of ET-1 is released into the lumen of the pulmonary vasculature where it can stimulate the endothelial $ET_B$ receptors, resulting in the production of vasodilatory molecules, such as NO and prostacyclin (McCulloch *et al.*, 1998; Davie *et al.*, 2002). Although there is clear evidence for ET-1 contributing to the adverse vasculopathy associated with PH, ET-1 is probably produced as a consequential effect of the disease and not a primary cause of PH. Chronic ET-1 infusion causes a reduction in pulmonary vascular reactivity to NO in rats, but does not result in PH (Migneault *et al.*, 2005). Furthermore, overexpression of ET-1 alone does not result in PH in transgenic animals (Hocher *et al.*, 2000) pointing to other mechanisms operating alongside to cause PH. Nonetheless, a genetic strain of rats deficient in the $ET_B$ receptor develop a more severe PAH phenotype associated with vessel occlusion in response to monocrotaline compared to treatment in control rats, where only moderate PAH and medial thickening takes occurs (Ivy *et al.*, 2005).

#### *1.3.3.5 ECE inhibition*

Most ECE inhibitors currently under development also inhibit neutral endopeptidase (NEP), resulting in the combined effect of inhibiting ET-1 production but at the same time having the potential to reduce the catabolism and hence clearance of ET-1 (Abassi *et al.*, 1992). In addition, by blocking ET-1

generation, ECE inhibitors would act as pseudo $ET_A/ET_B$ antagonists and perhaps importantly, would leave ET-1 clearance unaffected. However, so far, less progress has been made in the area of ECE inhibition than endothelin receptor antagonism (Doggrell, 2002; Kirkby *et al.*, 2008).

#### *1.3.3.6 Endothelin Receptor antagonists*

The role of ET-1 in PAH as described earlier along with other supporting evidences has led to a rationale therapeutic approach for antagonism of endothelin. At present, there are two commercially available endothelin receptor antagonists (ETRAs) for the treatment of PAH – bosentan and ambrisentan. The key differences between these ETRAs, are in their selectivity for $ET_A$ and $ET_B$ receptors. However, selectivity for $ET_A$ and $ET_B$ may vary according to the assay used for its determination and thus the distinction between their selectivity is poorly defined (Verhaar *et al.*, 1998). Different assays have revealed considerable variation in reported $ET_A$: $ET_B$ ratios. Those for ambrisentan range from 29:1 for ET-1-mediated contraction in the rat aorta up to 4000:1 in myocardial membranes (Bolli *et al.*, 2004) while $ET_A$:$ET_B$ ratio for sitaxentan and bosentan published so far are 6500:1 and 20:1, respectively (Davie *et al.*, 2009). In 2001, a randomised, placebo controlled trial showed beneficial effects of oral bosentan on exercise capacity and cardiopulmonary haemodynamics in PAH (Channick *et al.*, 2001). Similar effects were seen with 'BREATH-1' studies (Bosentan: Randomized Trial of Endothelin Receptor Antagonist THerapy), supporting the above findings (Rubin *et al.*, 2002). On the basis of these trials, bosentan was approved by WHO for the treatment of PAH class II patients. The most common

side effect is dose-dependent hepatotoxicity, especially seen in patients who are on high doses.

## 1.4 Additional therapeutic targets used in PAH

### 1.4.1 Calcium Channel blockers

Calcium channel blockers (CCBs) act to functionally reduce the conductance of voltage dependent calcium channel. In PASMCs, this results in the decrease in intracellular $Ca^{2+}$ concentration, resulting in pulmonary vascular smooth muscle relaxation. Clinical trials involving the use of CCBs in PAH have shown beneficial therapeutic effects, with an improvement in survival rate observed (Rich *et al.*, 1992), since initial observations, it was identified that CCB therapy is only effective as a treatment in patients who are vasoreactive *i.e.* their pulmonary vasculature still responds to local mediators via relaxation or contraction (Sitbon *et al.*, 2005). Less than 10% of PAH patients exhibit vasoreactivity, and of those, many do not have sustained effects of CCB therapy beyond a few months (Reeves *et al.*, 1986). Taken together, this indicates that CCBs have limited therapeutic use in the treatment of PAH. Moreover, significant adverse events were observed with these agents, including negative inotropic effects which resulted in fatal events, typically observed in NYHA class IV PAH patients with right ventricular failure (Packer *et al.*, 1984a; Packer *et al.*, 1984b).

## 1.4.2 Phosphodiesterase Inhibitors

### *1.4.2.1 Phosphodiesterase Type 5 Inhibitors*

NO released from PAECs acts in a paracrine fashion on the underlying PASMCs to activate the NO/cGMP pathway, resulting in increased levels of the second messenger cGMP which in turn decreases intracellular calcium ($[Ca^{2+}]_i$) to cause vasodilatation. Phosphodiesterases (PDEs) are a class of enzymes which mediate the hydrolysis of cGMP and cAMP. Phosphodiesterase type 5 (PDE5) is the most abundantly expressed PDE-isoform within the lung, and its activity and expression is also reported to be further increased in PAH (Black *et al.*, 2001). Sildenafil, the highly selective PDE5 inhibitor has been approved by the WHO for the treatment of PAH. It acts by inhibiting the hydrolysis of cGMP, therefore prolonging its half-life and causing prolonged cellular effects. PDE5 inhibition is reported to inhibit PASMC proliferation (Wharton *et al.*, 2005; Demoncheaux *et al.*, 2005) and attenuate hypoxia-induced PAH (Zhao *et al.*, 2001). Galie reported improvements in both NYHA functional class and exercise capacity in PAH patients when administered sildenafil (Galie *et al.*, 2005).

Other approved PDE5 inhibitors in the US are tadalafil and vardenafil (Schroll *et al.*, 2012). These newer agents have potential advantages over sildenafil such as a faster onset and longer duration of action, higher selectivity for PDE5, and increased absorption (Rosenkranz *et al.*, 2007). Out of the three, sildenafil and tadalafil are the most widely studied for their use in the treatment of PAH. A major difference between these two agents is that tadalafil is a more selective PDE5

inhibitor showing little or no affinity towards PDE1, whereas sildenafil may inhibit the latter at the higher end of the therapeutic dose range (Blount *et al.*, 2004).

### 1.4.2.2 Phosphodiesterase Type 3 inhibitors in PAH

Phosphodiesterases have a wide distribution in normal tissues. Mammalian PDEs are subdivided into 11 distinct families on the basis of substrate specificity, sensitivity and sequence homology (Essayan, 1999; Essayan, 2001). Phosphodiesterase type 3 (PDE3) enzymes are encoded by 2 different genes termed as PDE3A and PDE3B (He *et al.*, 1998) which specifically hydrolyses cAMP but can be competitively inhibited by cGMP (Lugnier, 2006) (figure 1.-8).

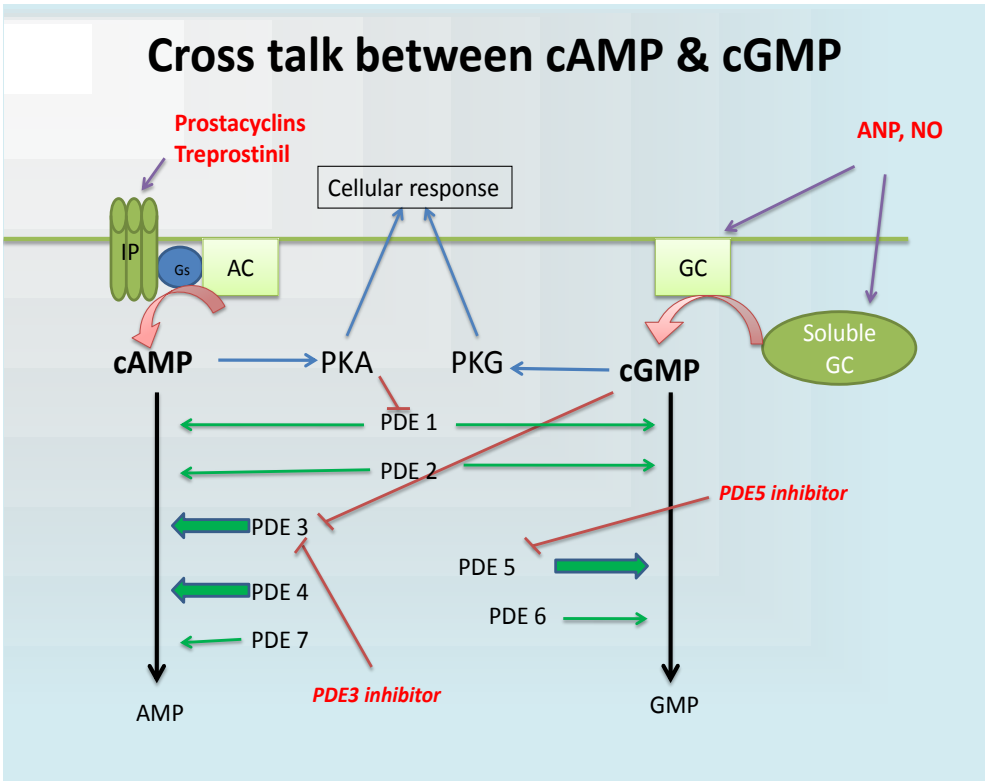



**Figure 1.8. Schematic diagram showing the potential cross talk between cAMP and cGMP**. One can increase cAMP by stimulating Gs coupled receptors like the prostacyclin (IP) receptor and/or by inhibiting its breakdown with a phosphodiesterase inhibitor such as a PDE3 inhibitor, milrinone or cilostazol. Cyclic GMP can be elevated by stimulating soluble or particulate guanylate cyclase with nitric oxide (NO) or atrial naturetic peptide (ANP) and/or by inhibiting its breakdown with PDE5 inhibitors such as sildenafil or tadalafil. So using a variety of interventions might enhance vasodilator and antiproliferative mechanisms in patients with PAH.

analogues are the most efficacious treatment for PAH to date; however the drawback is their short biological half-life, especially for epoprostenol and iloprost (Olschewski *et al.*, 1996; Schermuly *et al.*, 1999). These agents exert vasodilation via enhanced cAMP generation and various PDEs break it down to AMP; thus blocking the catabolism of this second messenger ought to prolong the pulmonary effects of these agents (Torphy, 1998). PDE 1, 3 4 and 5 are shown to be present in the lung parenchyma (Rabe *et al.*, 1994). However, the activity of PDE3 is increased in main, branch and intrapulmonary arteries from rats maintained under chronic hypoxic conditions and in PH (Maclean *et al.*, 1997). In the same study, PDE3 inhibitors also reversed the reduced responsiveness of pulmonary vessels to isoprenaline and forskolin in rats with PH.

Among the PDEs, PDE4, PDE7, and PDE8 selectively hydrolyse cAMP while PDE5, PDE6, and PDE9 are selective for cGMP. The other 5 subfamilies (PDE1, 2, 3, 10, and 11) hydrolyse both cyclic nucleotides with varying efficiency (Table 5) (Beavo, 1995; Soderling & Beavo, 2000; Lugnier, 2006; Conti & Beavo, 2007). Because of the crucial regulation role of cyclic nucleotides in signalling transduction, the concept that PDEs are involved in the pathological process of PAH is widely accepted (Theo *et al.*, 2005). Interestingly, expression and activities of PDEs were reported to be altered in both experimental and human PAH (Murray et al 2000). Expression profiling of single member of the PDE super family in healthy and remodelled pulmonary vasculature revealed that PDE1, PDE3 and PDE5 isoforms are differentially regulated (Maclean *et al.*, 1997; Schermuly *et al.*, 2007b).

| FAMILY | CHARACTERISTICS | TISSUE DISTRIBUTION | INHIBITORS |
|---|---|---|---|
| **PDE1** | $Ca^{2+}$/calmodulin-stimulated | Heart, brain, lung, smooth muscle | 8-methylmethoxy-IBMX, phenothiazines |
| **PDE2** | cGMP-stimulated | Adrenal gland, heart, lung, liver, platelets | EHNA |
| **PDE3** | cGMP-inhibited | Heart, lung, liver, platelets, adipose tissue, immunocytes | Cilostazol, milrinone |
| **PDE4** | cAMP-specific | Sertoli cells, kidney, brain, liver, lung, immunocytes | Rolipram, roflumilast |
| **PDE5** | cGMP-specific | Lung, platelets, smooth muscle | Zaprinast, sildenafil, Tadalafil |
| **PDE6** | cGMP-specific | Photoreceptors | Sildenafil, Tadalafil, Vardenafil, Zaprinast |
| **PDE7** | cAMP-high affinity | Skeletal muscle, heart, kidney, brain, pancreas, T lymphocytes | |
| **PDE8** | cAMP-high affinity | Testes, eye, liver, skeletal muscle, heart, kidney, ovary, brain, thyroid, pancreas | Dipyridamole |
| **PDE9** | cGMP-high affinity | Kidney, liver, lung, brain, testes, heart, thymus, spleen, intestinal smooth muscle | Sildenafil, Zaprinast |
| **PDE10** | Dual (cGMP and cAMP) | Testes, brain | Papaverine |
| **PDE11** | Dual (cGMP and cAMP) | Skeletal muscle, prostrate, kidney, liver, | Dipyridamole |

**Table 5 - Characteristics and distribution of PDEs**

In preclinical and clinical studies it has been shown that the inhibition of PDE1 by 8MM-IBMX (Schermuly *et al.*, 2007b) and PDE5 by sildenafil (Schermuly *et al.*, 2004), stabilizes the second messenger signalling and regulates vascular remodelling, vascular tone and optimization of gas exchange, which can improve the haemodynamics in PAH. Moreover, in monocrotaline-induced PH rats, inhibition of PDE3 and PDE4 isoforms was found to partly reverse the pathological inward remodelling in PH (Phillips *et al.*, 2005; Schermuly *et al.*, 2005a). Given that higher PDEs (PDE7-11) were defined more recently, further investigations should be performed to understand the possible involvements of higher PDEs in PAH and to improve the therapy of PAH by pharmacological PDE inhibitors.

### 1.4.3 Tyrosine kinase inhibitors:

Platelet derived growth factor (PDGF) is one of many growth factors and mitogens that contributes to the pathobiology of many vascular diseases including PAH (Raines, 2004) . PDGF is expressed by all major cell types from endothelial cells, smooth muscle cells, and platelets to macrophages where it behaves as a potent mitogen and chemokine. On vascular smooth muscle cells (VSMCs), PDGF can promote migration, proliferation and other cellular processes by activating specific cell surface receptors and causing their phosphorylation. In the early nineties a few research studies suggested a role for PDGF in PAH due to their observations showing that PDGF-A and -B were upregulated in pulmonary cells and lung tissue in PAH models (Arcot *et al.*, 1993; Katayose *et al.*, 1993). Lung tissue from patients with PAH showed similar findings and thus it was postulated that PDGF

had an important role in the pathogenesis of PAH (Humbert *et al.*, 1998). Studies by Lanner and his group showed that PDGF contributed to hypoxia induced proliferation of VSMCs (Lanner *et al.*, 2005). Likewise, inhibition of PDGF signalling in animal models of PH showed promising results in perinatal PH in the fetal lamb as well as in common PH animal models (Balasubramaniam *et al.*, 2003; Schermuly *et al.*, 2005a). Imatinib mesylate is a small molecule tyrosine kinase inhibitor capable of blocking the signalling of PDGF, and has been approved for the treatment of various malignant disorders such as specific chromosomal abnormality philadelphia chromosome-positive chronic myelogenous leukemia (CML), acute lymphoblastic leukemia, and gastrointestinal stromal tumours (Cohen *et al.*, 2005). Based on its mode of action, imatinib mesylate has been administered in PAH patients (Patterson *et al.*, 2006) and tested in animal models of PAH (Schermuly *et al.*, 2005a; Ghofrani *et al.*, 2006) with promising results. A phase III trial (IMPRES) has now been completed, with statistical improvements in 6MWD, PAP, PVR and cardiac output, though time to clinical worsening was unchanged (Reported to the European Respiratory Society Annual Congress, September 2011). Given the latter, it remains to be determined whether this agent will indeed be approved for treatment of PAH.

## 1.5 Approach towards combination therapy

Pulmonary arterial hypertension is a multifactorial disease, suggesting that if multiple pathways are targeted together, this ought to be the most effective treatment strategy (Benza *et al.*, 2007). Several clinical trials involving the use of multiple treatments have been designed and shown promising improvements in

both haemodynamic and exercise capacity (Benza, 2008; Ventetuolo *et al.*, 2008). The efficacy and safety of the combination of bosentan and epoprostenol was investigated using a small group of PAH patients (Humbert *et al.*, 2004a). Although hemodynamics and exercise capacity were improved, and data showed a trend for a greater improvement in all hemodynamic parameters in the combination-treatment group compared to the placebo-treatment group, though no statistical significance was demonstrated. Two other randomized controlled trials studying bosentan in combination with prostanoid therapy provided mixed results, with one study failing to show an improvement while the other indicating the combination is safe and possibly effective (Seyfarth *et al.*, 2005; McLaughlin *et al.*, 2006).

As in many other progressive diseases, patients with PAH may experience clinical and hemodynamic deterioration despite on-going initial effective monotherapy with the currently available disease-specific PAH drugs (*i.e.* PDE5 inhibitors, prostacyclins, endothelin receptor antagonists). Therefore it is reasonable to consider combination therapy. However, because of unknown risks, costs, drug interactions, combination therapy needs to be properly evaluated in randomized, controlled trials where safety and efficacy with the various possible combinations in the various PAH subgroups is demonstrated. The "Pulmonary Arterial Hypertension Combination Study of Epoprostenol and Sildenafil" (PACES) study is to my knowledge the only study that so far reached significance in terms of time to clinical worsening (Simonneau *et al.*, 2008). PACES was a 16-week, multinational, randomized, double-blind, placebo-controlled study involving 267 patients (257 completed the study) with WHO functional classes I–IV and with

PAH who were already receiving long-term intravenous epoprostenol therapy. In some patients with pulmonary arterial hypertension, the addition of sildenafil to long-term intravenous epoprostenol therapy improved exercise capacity, hemodynamic measurements, time to clinical worsening, and quality of life of PAH patients.

## 1.6 Aim and outline of the thesis

The aim of the studies presented in this thesis is to test potential treatment options in an in vitro model of PAH.

Phosphodiesterase 3 is one of the major phosphodiesterases to inactivate cAMP in the lung. Its heightened activity in PAH contributes to the loss of efficacy of beraprost in this disease (Murray *et al.*, 2007). In **chapter 3,** the effects of the prostacyclin analogue, treprostinil and its potentiation in the presence of phosphodiesterase 3 inhibitor (cilostazol) with respect to generation of cAMP, cell proliferation and vasorelaxation is assessed in experimental settings.

Lung endothelial $ET_B$ receptors promote ET-1 clearance, which can be improved in PAH patients by prostacyclin (epoprostenol) monotherapy (Langleben *et al.*, 1999). It is unclear if other prostacyclin analogues have similar effects or if smooth muscle $ET_B$ receptors can regulate ET-1 levels. In **chapter 4**, I investigated the interaction of prostacyclin with the endothelin pathway. In this chapter I have assessed the effect treprostinil and endothelin-1 receptor antagonists had on endothelin, endothelin receptors and the converting enzyme.

A concern with anti-cancer agents like tyrosine kinase receptor inhibitors is the high incidence of adverse events including nausea and diarrhoea as well as the potential for cardiac toxicity (Ghofrani *et al.*, 2010). Thus anti-cancer agents with a more selective mode of action need to be evaluated.

Mitotic kinesin inhibitors are a novel class of chemotherapy agents with low toxicity, whose mechanism in cancer involves disruption of various events in normal mitosis thereby promoting cell-cycle arrest and apoptosis. In **Chapter 5**, I assessed a newer anti-cancer agent, ispinesib with classical agents used to treat PAH and their effects on the proliferation of human PASMCs.

**Chapter 6** contains general conclusions regarding the results and the possible perspective studies for the future.

# 2. Materials and Methods

## 2.1 Patient Characteristics and Ethical considerations

Human PASMCs from normal and IPAH patients were used for the study. Peripheral PASMCs were isolated from children and adults with IPAH and from control adults. Lung tissue was taken with Ethics Committee approval from Great Ormond Street Hospital (ICH and GOSH REC 05/Q0508/45), Papworth Hospital (REC H00/531/T) and Brompton & Harefield Trust (NHLI REC 01-210) through Dr Wharton (Imperial College, London) after patient/relative consent was obtained. Cells were obtained from patients with IPAH undergoing transplantation after failed treatment (4 children, 5 adults). Treated children received epoprostenol for at least 1.3 years while adult patients were on varying prostacyclin therapy for an average of 1.2 years. For controls, cells were isolated from donor lungs found to be unsuitable for transplantation or from lung resection for suspected malignancy. Human endothelial cells (HECs) were obtained from TCS Cellworks (Buckingham,UK) and the fibroblast cell lines were generously provided by Professor David Abraham (Division of Medicine, Royal free Hospital).

## 2.2 Cell Culture

### 2.2.1 General materials and equipment used for cell culture

All materials and solutions used for cell culture were sterile unless otherwise used for terminating the experiments.

- *MATERIALS*

1. Growth Medium – Two different types of growth medium were used throughout this study.

- Growth medium - DMEM/F12; (PAA Laboratories, Munich, Germany) with 10% Foetal bovine serum (Gibco New Zealand breed, Invitrogen, Paisley UK) and 5% solution of penicillin/streptomycin (Invitrogen, UK).
- Growth Medium – Smooth muscle cell basal medium (ZHM-3933, TCS Cellworks, Buckingham, UK) with 10% Foetal bovine serum (FBS) (Gibco New Zealand breed, Invitrogen UK) and 5% solution of penicillin/streptomycin (Invitrogen, UK).
- Special growth medium to grow primary smooth muscle cells- Smooth muscle cell basal medium (ZHM-3933, TCS Cellworks, Buckingham UK) with human smooth muscle cell growth supplement (ZHS-8951, TCS Cellworks, Buckingham UK) and 5% solution of penicillin/streptomycin (Invitrogen, UK).

2. Phosphate-buffered saline (PBS), $Ca^{2+}$/$Mg^{2+}$ free (PBS) (PAA Laboratories, Munich, Germany)
3. Trypsin/EDTA solution (0.05% (w/w) tissue culture grade (Invitrogen, Paisley UK).

➢ *GLASSWARE AND PLASTICWARE*

4. Tissue culture plates – Different types and sizes of tissue culture plates were used depending upon the need of the experiment.
5. Flasks (75$cm^2$ and 125$cm^2$)

6. Chambered culture slides - used for staining and immunohistochemistry experiments (BD Falcon, Oxford, UK).

➢ *EQUIPMENT*

7. Culture incubator – all culture incubators are maintained to have a humidified atmosphere, temperature 37 °C and 5% $CO_2$. The incubators are cleaned and decontaminated every 2-3 months.
8. Working hoods - A clean functional biosafety hood which protects the culture by filtering the air which is coming out of the hood and maintains a laminar flow.
9. Water bath - Clean water bath maintained at 37°C to pre-warm the reagents.
10. An inverted phase contrast microscope - to view the live cell cultures.
11. A vacuum and suction setup for disposal of medium wastes.
12. Fridge/freezer – for storage of the reagents and stock solutions.
13. Centrifuges (Eppendorf, UK).

### 2.2.2 Isolation and characterisation of human PASMCs

Human PASMCs were isolated from explanted lungs of IPAH patients receiving a bilateral lung or heart and lung transplant. Normal cells were isolated from the lung tissue isolated from myeloma patients.

➢ *MATERIALS*

1. Complete medium to grow primary smooth muscle cells- Smooth muscle cell basal medium (ZHM-3933, TCS Cellworks, Buckingham UK) with

human smooth muscle cell growth supplement (ZHS-8951, TCS Cellworks, Buckingham UK) and 5% solution of penicillin/streptomycin (Invitrogen, UK).

2. Hank's balanced salt solution (HBSS; Sigma/Aldrich, UK) – used for collection of the specimen.
3. Penicillin/streptomycin/gentamycin (3% solution) - prepared from a stock solution containing 5000 iu/ml of penicillin/streptomycin and 10,000 iu/ml gentamycin.
4. Enzymatic dissociation solution – A four times concentrated solution of the enzymatic dissociation cocktail was prepared and diluted with DMEM/F12 HEPES (Sigma/Aldrich, Poole, Dorset UK) buffer just before use. The cocktail was prepared by dissolving the following in 10 ml of DMEM/F12 HEPES (Sigma/Aldrich, Poole, Dorset UK)
    - 5 mg of elastase (Lorne Laboratories, Reading, UK.),
    - 10 mg of collagenase (Sigma/Aldrich, Poole, Dorset, UK),
    - 2.5 mg of trypsin inhibitor (Sigma/Aldrich, Poole, Dorset UK),
    - 150 mg of bovine serum albumin (BSA)
    - 100 µL of MEM vitamins.

    The dissociation cocktail was then sterilized by filtration and 1 ml aliquots were prepared and stored at - 20°C.
5. Confocal Microscopy reagents -
    - Antibodies to specific smooth muscle cells antigens.
    - Para-formaldehyde for fixation (Sigma/Aldrich, Dorset,UK).

- 0.1 % of Triton X-100 solution (Sigma/Aldrich, Dorset, UK).
- 3% of BSA solution prepared in 0.01% Triton X-100 solution
- 4’,6-diamidino-2-phenylindole (DAPI; Vectastain, VECTOR LABORATORIES, Peterborough UK)

The explanted lungs/lung tissue was washed with cold PBS and once with 3% of penicillin/streptomycin/gentamycin solution. Distal peripheral pulmonary arteries of approximately 250 µm in length were dissected out of the tissue block and placed in 3% penicillin/streptomycin/gentamycin sterile solution for 30 min at 4 °C. After the sterilisation procedure, the artery was pinned to the bottom of the dissecting gel plate and the adventitia gently pulled away from the medial layers leaving a smooth outer surface and then cut open longitudinally. The endothelium was gently scraped off with a sterile scalpel blade and the artery was then cut into transverse strips, of approximately 1- to 2-mm wide. These strips were then transferred to the pre-warmed (37°C) enzymatic dissociation cocktail and incubated for 30 minutes in a shaking water-bath. After 30 min, the cell suspension was strained through a 70 micron cell strainer and cells were sedimented by centrifugation at 350 g for 5 min. The supernatant was removed and the cell pellet was re-suspended in the complete growth medium. Cells were plated in T-25 $cm^2$ flasks and incubated at 37 °C in 5% $CO_2$ and media replaced every 3 days. After the cells reached confluence, they were washed with PBS (PAA Laboratories, Munich, Germany) and trypsinised for further passage. The primary cells were also characterised using immunofluorescent staining, confocal microscopy and western

blotting (all methods completely described in later chapters) to identify smooth muscle specific markers for example α-smooth muscle actin and smooth muscle cell marker SM-22. Endothelial cell specific markers, for example CD31 and Von Willebrand factor (VWf), were used as negative controls. Primary cultures were prepared in large batches and cells were frozen and preserved in liquid nitrogen with minimal loss of cell viability over several years. Cells between passages 3 and 10 were used for all experiments. A few vials of cells were frozen at each passage throughout each experiment to provide the possibility and advantage of repeating and studying any changes which might occur with increased passage.

### 2.2.3 Subculture of Human PASMCs from hypertensive and normal patients

#### *2.2.3.1 Reviving of cells*

In contrast to the optimal slow rate of freezing, cells were thawed as rapidly as possible so as to minimise ice crystal formation and cell damage. A vial of cells was removed from the liquid nitrogen tank and carefully immersed in a 37°C water bath with gentle agitation until thawed. The content of the vial was then aseptically plated in a T75 $cm^2$ flask and incubated at 37°C and 5% $CO_2$. The medium was replaced with fresh growth medium after 5-6 hours by which this time the smooth muscle cells had attached to the bottom of the flask. The medium was changed every three days till the culture reached confluency, after which the cells were tryspinised and plated according to the need of each particular experiment.

### 2.2.3.2 Subculturing cells

After reaching 80-90% confluence, cells were washed twice with ~5mls of warmed sterile PBS (PAA Laboratories, Munich, Germany) and detached using 5ml of trypsin/EDTA solution (0.05 % (w/w) Invitrogen, UK) previously warmed to $37^0$C. After the cells had become detached at least 10 mls DMEM/F12 (Invitrogen, UK) growth medium containing 10% FBS (Gibco New Zealand breed, Invitrogen UK) and 5 % penicillin/streptomycin (Invitrogen, UK) was added to neutralize the trypsin. The cell suspension was then centrifuged at 350g at room temperature for 5 min to obtain a cell pellet. The supernatant was then removed and the pellet resuspended in 2-3mls fresh growth media. Cells were counted using an automated cell counter (see the next paragraph) and plated at appropriate density in 6-well plates.

### 2.2.3.3 Freezing of cells

A minimum of 0.5 x $10^6$ cells /ml of freezing medium were always used to freeze down cells.

- *MATERIALS*

1. Freezing medium

   Two types of freezing mediums were used during this study

   - Freezing medium containing 90% of foetal bovine serum (FBS, Gibco, New Zealand breed, Invitrogen UK) and 10% dimethyl sulfoxide (DMSO) (Sigma/Alrich, UK) as a cryoprotective agent.

- Bambanker (Anachem Ltd, Luton, Bedfordshire) serum free cell freezing medium - In the later stage of experiments this medium was used to freeze down cells.

2. Freezing vials

   Sterile polypropylene freezing vials were used to freeze cells.

3. Freezing container

To maximize viability cells were frozen slowly, ideally with a steady decreasing rate of one degree per minute. The freezing container filled with iso-propyl alcohol helps to freeze cells at a steady rate and reduce ice crystal formation and damage, which is observed when snap freezing cells instantaneously. Before putting into the container, cells were washed with PBS (PAA Laboratories, Munich, Germany) and then detached from the bottom surface of culture flasks using trypsin/EDTA solution (0.05 % (w/w) Invitrogen, UK). After centrifugation at 350g for 5min, the supernatant was carefully removed without disturbing the cell pellet. The cells were then resuspended in the cell freezing medium at the desired cell density and aseptically pipetted into freezing vials.

## 2.3 Growth curves and proliferation assays

Cells were plated at a density of $1x10^4$/ml in 6-well plates giving a total of $2x10^4$ cells per well. The number of plates was dependent on each particular experiment. After plating, the cells were incubated at 37°C and 5 % $CO_2$ for 24 hours in growth medium. After this period, the wells were washed with 1 ml of PBS (twice) and media replaced with basal medium containing no serum to arrest the cells. 48 hours later, the cells were stimulated with 10% FBS with or without drug treatments and

incubated for 4 days. After 4 days the cells were counted using an automated cell counter.

### 2.3.1 Cell counters

#### *2.3.1.1 Sysmex F-520P*

For the first 18 months of my study period Sysmex F-520P (Malvern Instruments Ltd, Malvern, UK) was used to count cells, which due to technical problems had to be replaced by a new and more advanced cell counter (ADAM-MC counter, NanoEnTek Inc, Digital Bio, Korea). The cell count analyser, Sysmex F-520P uses the direct current electronic resistance method for particle (cell) counting and sizing. The counter contains a probe comprising a transducer with an aperture of an exact diameter (the standard being 100 μm) and an internal and external electrode. The probe is immersed in the cell suspension diluted into an electrically conductive diluent, which allows a direct current to pass through the aperture between the electrodes. Cells or particles in the suspension are non-conductive and when they pass through the aperture, they decrease the flow of current and increase the resistance. The Sysmex F-520P particle counter uses a non-mercury diluent-filled ballfloat manometer pump. Increased resistance due to cells in the aperture causes a change in the voltage between the electrodes which is proportional to the volume of the particle or cell. Since a constant current is maintained, the voltage change will be directly proportional to the change in resistance according to Ohm's Law:

$$E \text{ (voltage)} = I \text{ (current)} \times R\text{(resistance)}.$$

#### 2.3.1.2 ADAM-MC counter

The ADAM-MC (NanoEnTek Inc, Digital Bio, Korea) is a fast alternative to conventional manual counting methods using a haemocytometer. The principle of the technique used in the ADAM-MC is the well-known method of fluorescence microscopy, implemented in a small, highly advanced fluorescence microscope combined with a CCD camera and software-based image analysis. The excitation source is green LED. The emission filter removes all wavelengths from the light emitted from the sample except red fluorescence light. The red fluorescent light from the particles in the sample will then focused onto the detector (CCD camera). Then image analysis software counts the red particles to represent the cell number in the sample.

Adam-MC is based on staining the cell DNA with a fluorescent dye, in this case, propidium Iodide (PI). This dye does not enter cells with intact cell membranes, only entering cells with damaged membranes. As a result, the nuclei of non-viable cells will only be stained. The counter provides two kinds of staining solutions. AccuStain Solution 'T' is composed of the fluorescent dye (PI) and lysis solution which is used for obtaining total cell counts. The lysis buffer will disrupt the membrane and allows PI to enter the cells to stain them. AccuStain Solution 'N' cell is composed of the fluorescent dye and PBS for the counting of non-viable cells. With the later solution, live cells remain intact and are not stained; thus only the non-viable cells are stained and detected. After treatment with the staining solutions, the prepared cells are loaded into the Accu-chip. The viability is automatically calculated with installed software using the following equation,

**Viable cell count/ ml** = Total cell count (T1)/ml – Non viable cell count (N1)/ml

**Viablity (%)** = (Viable cell count / total cell count) x 100

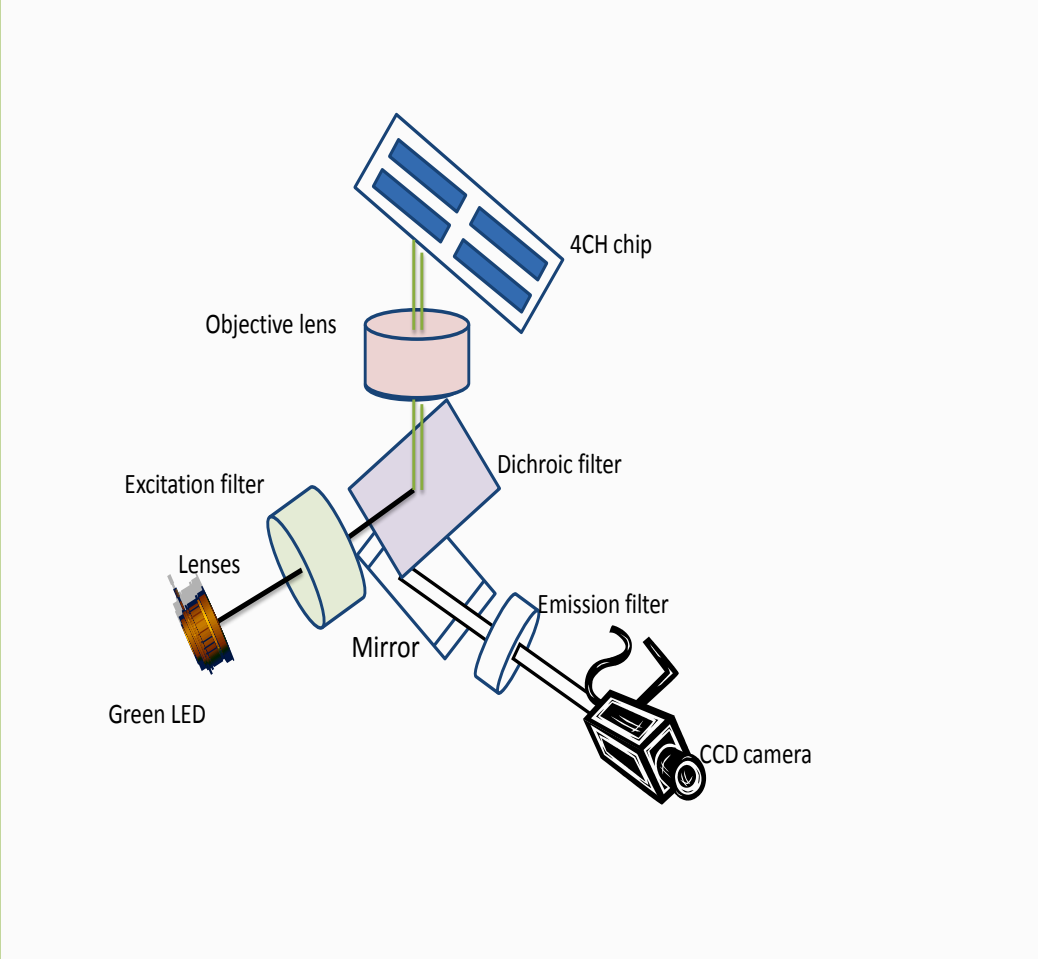



**Figure 2.1. Schematic of the ADAM-MC cell counter.** To count cells using ADAM-MC, cells are mixed with a Propidium Iodide (PI) stain and directly pipetted on to a disposable plastic chip. The chip is then loaded onto a stage in the counter. A 532nm green laser is automatically focused onto the chip and cells that have been stained are detected by a CCD camera.

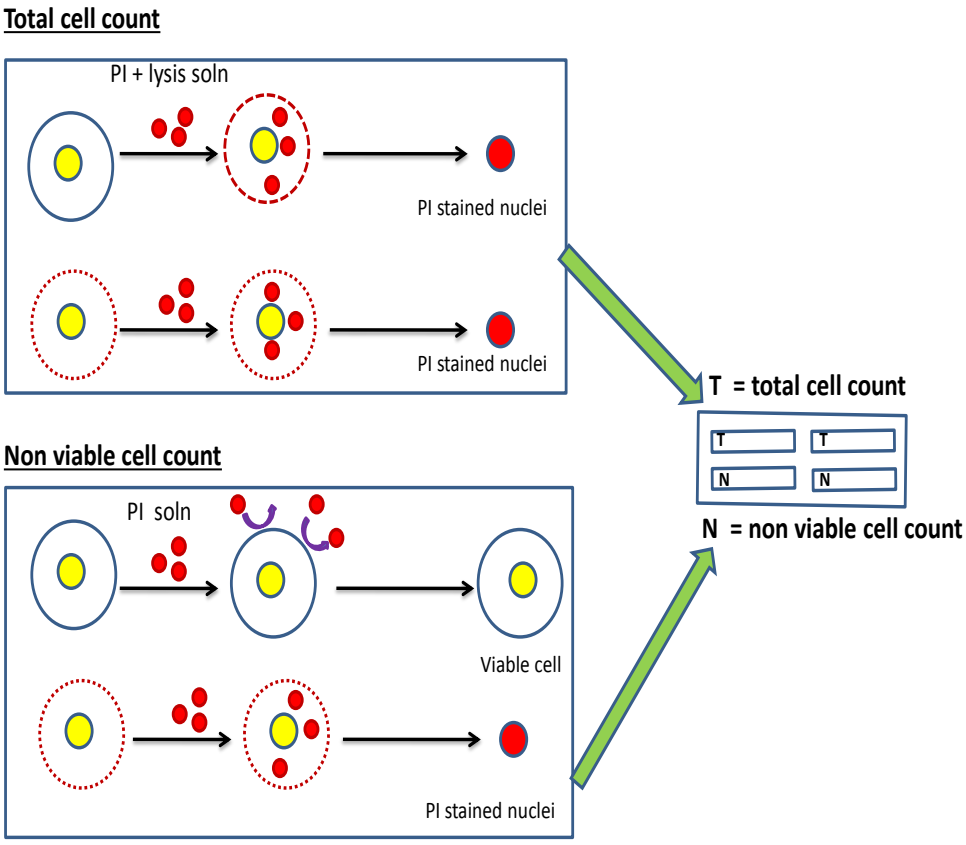



**Figure 2.2. Principle of cell counting by ADAM-MC counter.** For total cell count, the cell sample is mixed with an equal volume (1 to 1 dilution) of AccuStain Solution T and mixed thoroughly by vortexing. This cell lysate (15 µl) is then carefully loaded into the T1 or T2 channel of the Accu-chip 4X so as to avoid making bubbles. Similarly for non-viable cell count, the cell sample is mixed with an equal volume of AccuStain Solution N and mixed thoroughly by vortexing. This cell lysate (15 µl) is carefully loaded into the N1 or N2 channel of the Accu-chip 4X. Solution T' contains Propidium iodide (PI) along with a lysis solution and will give total cell count. The solution N' does not contain the lysis solution hence the dye will only stain those cells whose cell membranes are not intact, viz non-viable cells. Thus the solution N' will give the non-viable cell count.

## 2.4 Cyclic AMP enzyme linked immunoassay

Cyclic 3'5' adenosine monophosphate is a second messenger, signal transduction cascades of which involves cAMP regulating a variety of intracellular processes. A rise in cellular cAMP or activated PKA can promote pulmonary vasodilation (Haynes, Jr. *et al.*, 1992; Barman *et al.*, 2003) and exert antiproliferative and pro-apoptotic effects (Tantini *et al.*, 2005). ). Intracellular levels of cAMP depend on the balance between cAMP synthesis by adenylyl cyclase (AC) isoforms (Willoughby & Cooper, 2007) and cAMP hydrolysis by PDE enzymes (Baillie 2009).

### 2.4.1 Principle of the assay

The cAMP present in a sample competes in a competitive binding fashion with a fixed amount of horseradish peroxidase (HRP)-labelled cAMP for sites on a mouse monoclonal antibody. The incubation allows the monoclonal antibody to bind to the goat anti-mouse antibody coated onto the bottom of the microplate. Washes after incubation of the antibody helps to remove any non-specific binding. The substrate solution was used to determine the bound enzyme activity on the microplate and the absorbance was read at 450 nm where the intensity of the colour is inversely proportional to the concentration of cAMP in the sample.

### 2.4.2 Sample preparation and cyclic AMP measurement

Human PASMCs were lysed before assaying according to the following manufacturer's instructions. Cells were grown to ~80% confluence before drug

treatment and lysing. For sample preparation, cells were washed twice with cold PBS and resuspend in cell lysis buffer (R&D Systems Europe Ltd, UK) to a concentration of 1 x $10^7$ cells/ml. A freeze/thaw cycle was used once with gentle mixing to lyse all the cells properly. In initial experiments, trypan blue was used to confirm that the protocol used completely lysed cells, which it did as cell extracts were all stained blue. Lysed cells were then centrifuged at 600 x g for 10 min at 4° C to remove unwanted debris. Supernatants were stored at -80° C for cAMP estimation at a later stage. Cyclic AMP was measured according to the manufacturer's instructions (R & D Systems Europe Ltd, Abingdon, UK).

### 2.4.3 Protein measurement

- *MATERIALS*

1. BCA protein assay kit (Thermo Fisher Scientific, Northumberland, UK)
    - BCA solution which contains bicinchoninic acid, sodium carbonate, sodium tartarate and sodium bicarbonate in 0.1 M NaOH, pH 11.25.
    - 4% cupric sulphate.

    BCA working reagent was prepared by mixing 50 parts of BCA solution with 1 part of 4% cupric sulphate just before use.
2. Bovine serum albumin (BSA)

The standard colorimetric assay based on the Bradford assay (Bradford, 1976) was used on the cell extracts to measure protein. Bovine serum albumin (BSA, Sigma/Aldrich, Dorset, UK) was dissolved and diluted to generate a six point standard curve (0 to 2 mg/ml) using a clear 96 well plate. Dilutions of the standard solution were made in the same buffer as the samples. Specifically, 25 µl of

standard and protein sample (in duplicate) was pipetted into individual wells of a 96 well plate, followed by addition of 200 µl of BCA working reagent to each well. The plate was incubated at 37°C for 30 min and the absorbance read at 540 nm in a GENios absorbance plate reader (TECAN, Reading, UK). Protein concentration of samples was calculated from the standard curve, by first subtracting the background reading from the blank, and then finally multiplying by the dilution factor of the sample. An example of a standard curve generated during this study is shown in the figure 2.3.

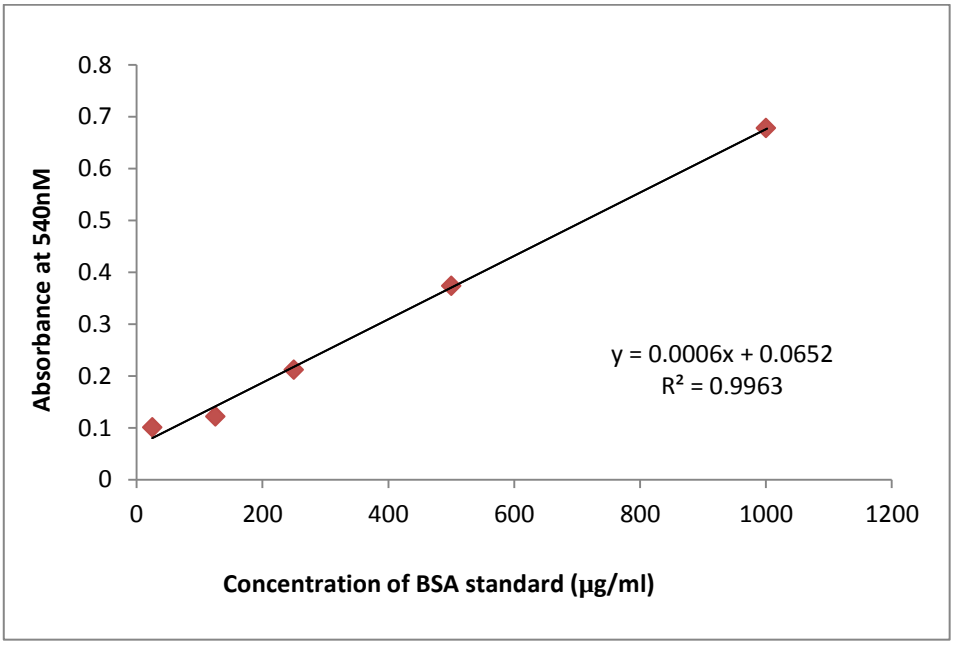



**Figure 2.3. A standard curve generated using a BCA protein assay kit.**

## 2.5 Endothelin pathway in IPAH

### 2.5.1 Measurement of Endothelin 1 (ET-1)

ET-1 exerts its biological actions through the activation of two receptor subtypes, $ET\text{-}_A$ and $ET\text{-}_B$ (Sakurai *et al.*, 1990). Both receptors belong to a large family of

transmembrane guanine nucleotide-binding protein-coupled receptors (GPCRs). The endothelin pathway seems to be involved in pathogenesis of many disease states including cancer, fibrosis, heart failure and pulmonary hypertension (Archer & Rich, 2000; Kedzierski & Yanagisawa, 2001). ET-1 is elevated in PH and thus is an important mitogen to study in the disease. In this study, I sought to elucidate the effect of different drugs currently used to treat pulmonary hypertension on ET-1 levels.

### 2.5.2 Sample preparation

- *MATERIALS*

1. Growth medium - Smooth muscle cell basal medium (ZHM-3933, TCS Cellworks, Buckinghamshire UK) with 10 % foetal bovine serum and 5% solution of penicillin/streptomycin (Invitrogen, UK).
2. Growth arrest medium - Smooth muscle cell basal medium (ZHM-3933, TCS Cellworks, Buckinghamshire UK) with 5% solution of penicillin/streptomycin (Invitrogen, UK).
3. RIPA lysis buffer (Sigma/Aldrich, Dorset, UK).

Cells were cultured in growth medium to ~80% confluence in 6-well plates and then placed in medium containing no serum for 48 hours. The growth arrested cells were then stimulated with growth medium with or without treatment with different drugs. Cell culture supernatants from each well were removed 24 hours later and aliquoted into 1 ml vials for storage at -80° C until assayed. Repeated freeze-thaw cycles of samples were avoided. Cells were lysed with RIPA lysis buffer to

measure protein content. The final amount of ET-1 were quantified to amount of protein per well.

### 2.5.3 Principle and protocol for the assay

ET-1 levels in cell culture supernatants were measured using the QuantiGlo Human ET-1 chemiluminescent immunoassay kit (R&D systems, Minneapolis, USA). It is a solid phase ELISA which employs the quantitative sandwich enzyme immunoassay technique for measuring ET-1 in the samples. Samples are incubated onto a microplate coated with monoclonal antibody specific for ET-1. Washes after the incubation of the antibody helps to remove any non-specific binding. An enhanced luminol/ peroxide substrate solution when added produces light in proportion to the amount of ET-1 bound in the initial step which was measured using the luminometer.

## 2.6 SDS-PAGE and western blot analysis

SDS-PAGE separates proteins migrating through a SDS -gel under the influence of an electric field. When coupled with antibody detection steps, the relative abundance of a particular protein in two or more protein extracts can be determined.

### 2.6.1 Sample preparation

- *MATERIAL*

1. Phosphate-buffered saline, $Ca^{2+}/Mg^{2+}$ free (PBS) (PAA Laboratories, Munich, Germany)

2. Lysis buffer-

- RIPA lysis buffer (Sigma/Aldrich, Dorset, UK).
- Phosphatase inhibitor (Sigma/Aldrich, Dorset, UK)

RIPA buffer was mixed with phosphatase inhibitor just before use (100:1).

Cells were washed with cold PBS twice followed by addition of lysis buffer and spread evenly over the cells using a cell scraper. After 2-3 min, cells were scrapped and the cell extracts placed in an eppendorf and centrifuged at 900 g for 15 min at 4°C. Supernatants were stored at - 80°C until use.

### 2.6.2 Principle of Western blotting

Heating of cell lysates with sample buffer and dithiothreitol (DTT) protects proteins by reducing disulphide bonds in the protein to thiol groups and covering the protein with SDS molecules, forming a negatively-charged complex. Under the influence of an electric field, proteins migrate through a gel whose pore size is determined by the concentration of acrylamide and cross-linker in the gel. Larger proteins are obstructed more than smaller proteins, resulting in a separation of proteins largely based on molecular size. During transfer, the protein/SDS complex is transferred out of the gel onto a protein-binding membrane under the influence of an electric field. For antibody detection, protein-binding sites on the membrane are blocked by incubating with 5% milk protein, and the protein of interest is then specifically bound with an antibody. This primary antibody is then detected with a secondary antibody conjugated to an enzyme. A liquid substrate is then added,

which in the presence of the peroxidase enzyme, emits light in a chemiluminescent reaction, and this light is detected using photographic film.

Western blotting is often described as a semi-quantitative technique, with the amount of protein originally present in the lysate proportional to the amount of peroxidase enzyme bound to protein on the membrane, and thus the amount of light generated and area and intensity of the band present on the film. For routine western blotting, samples were protein assayed before loading. Blotting with an antibody such as GAPDH, total ERK, α-tubulin or β-actin serves as a loading control and helps to visually confirm comparable protein loading.

### 2.6.3 Protocol of Western blotting

Protein content of the cell lysates was measured using the BCA protocol. 10 μg of protein extracted from cell lysates of smooth muscle cells subjected to different treatments was assayed for levels of endothelin receptor type B ($ET_B$) and endothelin converting enzyme -1 (ECE-1) by Western blotting. Each sample was reduced with sample reducing buffer (Life technologies, USA) by boiling at 100°C for 3 minutes. The reduced samples were run on a Bis-Tris Gel (either 5%, 7 % or 10%) in MOPS-SDS running buffer alongside a prestained molecular weight marker (Fermantas, Cambridge UK), at 200V for 45 to 60 minutes. The protein was then transferred to a PVDF membrane using a Wet Transfer apparatus in 1x Transfer buffer (Life technologies, Paisley, UK) containing 10% methanol. The transfer was performed at 100V for 1 hr or 25V overnight at 4°C. Following the transfer, the proteins on the membrane was blocked in PBS containing 5% skimmed milk and 0.1% Tween-20 (PBST) for one hour at room temperature. The

primary antibody (Table 2) was diluted in the blocking buffer to the required concentration. Following the blocking step, the membrane was incubated with the primary antibody overnight at 4°C on a shaking platform (Eppendorf, Stevenage, UK). Next morning, the membrane was washed three times with PBST then incubated with the appropriate secondary antibody (diluted in 5% milk in PBST) for one hour at room temperature. The membrane was washed again three times with PBST and then dried in air. The protein bands were visualized using the enhanced chemiluminescence plus reagent detection system (GE Healthcare, Buckinghamshire, UK) and recorded on hyperfilm (GE Healthcare Life Sciences, Buckinghamshire, UK).The exposed hyperfilm was scanned using the HP Deskscan system and bands were analysed using the software called 'Image-J' (Collins, 2007).

| Target antigen | Primary and secondary antibodies |
|---|---|
| **$ET_B$** | 1° = polyclonal rabbit anti-ETB (1:1000, ab39960, Abcam, UK)<br><br>2° = goat anti-rabbit IgG-horseradish peroxidase (1:1000, sc-2040, Santa Cruz, UK) |
| **ECE-1** | 1° = polyclonal rabbit anti-ECE-1 (1:1000, ab93117, Abcam, UK)<br><br>2° = goat anti-rabbit IgG-horseradish peroxidase (1:1000, sc-2040, Santa Cruz, Biotechnology, Heidelberg, Germany) |
| **β-Actin (loading control)** | 1° = monoclonal mouse anti-β Actin (Sigma/Aldrich, UK)<br><br>2° = goat anti-mouse IgG-horseradish peroxidase (1:5000, Santa Cruz Biotechnology, Heidelberg, Germany) |

**Table 6 - Antibodies used for western blotting.**

## 2.7 Lung Histology

Tissue blocks from distal portions of the lung tissue samples from diseased and control patients were fixed in neutral buffered formalin overnight, transferred to a 70% ethanol solution the next day and subsequently sent to the Queen Mary's laboratory, (St Bartholomew's, Barbican, London) for slide preparations. One slide of each section was used for Haematoxylin and Eosin (H & E) staining to look for gross pathological changes. Briefly H & E staining was performed by immersing the slide in hematoxylin solution (made up in PBS pH 7.2) for 5 minutes followed by washing 3 times in PBS before being immersed in 1% eosin counterstain solution (made up in PBS pH 7.2) for 5 seconds. After the slides were washed 3 times in PBS, sections were processed through an increasing alcohol gradient into xylene and then mounted in diethyl (phenyl) xanthine DPX mounting medium (Grale Scientific, Ringwood, Australia). A coverslip was placed over the section and the slide allowed drying. Slides were analysed under normal light microscopy with the assistance of Dr. Susan Hall, Institute of Child Health, University College London.

### 2.7.1 Principle of Immunohistochemistry

Immunohistochemistry (IHC) refers to the process of localizing antigens (proteins) in cells of a tissue section exploiting the fundamental principle of antibodies binding specifically to antigens in biological tissues. Visualising an antibody-antigen interaction is then accomplished by the most frequently used method in which the secondary antibody is conjugated to an enzyme, such as peroxidase, that can catalyse a colour-producing reaction upon addition of an appropriate substrate.

Alternatively, the secondary antibody is tagged to a fluorophore, such as fluorescein, rhodamine or alexafluor (this is known as immunofluorescent staining).

The advantage of IHC over techniques like enzyme-linked immunosorbent assay (ELISA) or Western blotting (which function on the same principle) is the ability to identify and locate specific proteins within the tissue structure, which adds a whole new dimension to the technique. The target immunogens may sometimes be physically "hidden" from the antibody, because of protein folding or formation of cross-linking methylene bridges during 'fixation'. An antigen retrieval step, where enzymes or heat are used to re-model the protein structure and break down methylene bridges, may be required to visualize some antigens.

### 2.7.2 Histology protocol

Paraffin sections on slides were dewaxed and hydrated, by sequential passage through the following steps: initially two washes with xylene for 5 min each were performed, followed by two washes with 100% ethanol for 3min and a wash each with 95% and 85% ethanol for 3min. Finally the racks were placed in distilled water for 5 min and the step repeated. Endogenous peroxidase activity was blocked by immersing the slide in 3% hydrogen peroxide (Sigma Aldrich) for 10 min. The slides were then rinsed in tap water. A boundary was made around the tissue section using an immunoedge pen (DAKO, Glostrup, Denmark). Antigen retrieval was then performed by trypsin digestion to unmask the antigen sites that may have become cross-linked during fixation. A fresh solution of 0.1% trypsin and 0.1% calcium chloride was prepared in 5mM Tris buffered saline at 37°C and

brought to pH 7.8 with 0.1M sodium hydroxide. Slides were immersed for 10 min and then rinsed in PBS three times.

A blocking step to prevent non-specific antibody binding to proteins in tissue preparations was performed by incubation with 5% non-immune goat serum for 30 min at room temperature. The serum being from the species from which the second layer antibody was derived. The primary antibodies were diluted in PBS with 0.1% BSA (PBS/BSA) at concentrations recommended in the data sheets for each antibody. They were carefully pipetted onto the tissue sections within the boundary (~200 µl). The slides were then incubated at 4°C overnight in a humidified chamber. For negative controls the serial sections were incubated without any primary antibody.

The next morning, the slides were washed with PBS/BSA and incubated with the secondary goat anti-rabbit with biotinylated IgG antibody which was diluted 1:200 for one hr at room temperature. During this time, the ABC Solution (PK4000, Vector Laboratories, Peterborough UK) was prepared as recommended by the manufacturer and allowed to stand at room temperature for at least 30 minutes before use. The slides were then washed with PBS/BSA once, followed by two washes with PBS. The ABC solution was then added onto the tissue sections after gently drying off the excess buffer and the slides were then incubated for an hr at room temperature. Meanwhile, the DAB substrate solution (D4168, Sigma Aldrich) was prepared by dissolving tablets in 1ml or 5ml of PBS and vortexed to prepare a homogeneous mixture. The slides were rinsed with PBS. The slides were incubated in the DAB solution for 1 to 5 minutes before the reaction was stopped by

transferring the slides into water. The stained sections were then counterstained with Harris's haematoxylin solution (HHS32, Sigma/Aldrich) for about 30 seconds and then rinsed under tap water until the colour stops leaching. Afterwards, sections were dehydrated by passing through an ascending alcohol gradient (95%, and two changes of 100% ethanol), followed by three rinses in xylene before finally mounting sections in DPX and covering with them with a cover slip. The slides were then left in the fume hood overnight to dry before viewing them under the microscope. The next day, the slides were analysed under normal light microscopy.

## 2.8 Immunofluorescent staining

In total, $2x10^4$ cells/ml were seeded in an 8-chambered slide (BD Falcon, Oxford, UK) and allowed to grow until ~70% confluent. The medium was aspirated and the cells fixed using 4% of paraformaldehyde (PFA) in PBS, which was added gently to each chamber and allowed to incubate for 20 min. After aspirating the PFA, the chambers containing fixed cells were then washed with PBS several times. The cell membranes were permeabilized by addition of 0.1% Triton X-100 (diluted in PBS) to each well. The solution was aspirated after 10 minutes and the cells were washed three times with PBS. This was followed by a10 min incubation with a solution containing 3% BSA in 0.01% Triton x100, used to block non-specific antibody binding to cells. Primary antibodies were prepared in 3% BSA in 0.01% Triton X-100. The antibodies used are outlined in Table 3. After the addition of the primary antibody, the plate was left overnight at 4°C on the shaking platform. Next morning, the cells were washed five times in PBS. The appropriate secondary

antibodies prepared in the same blocking solution and added to the permeabilised cells and incubated for one hr at room temperature. The wells were again washed five times with PBS. Each coverslip was then gently lifted up and flipped over, and a drop of 4’,6-diamidino-2-phenylindole (DAPI) solution added to the glass slide. Cells were then viewed and subsequently photographed using a confocal microscope. Negative controls were obtained by omission of the primary antibody.

| Target antigen for immuno-staining | Primary and secondary antibodies |
|---|---|
| **α-SMA** | 1° = Mouse monoclonal anti-α –smooth muscle actin (1:1000, A-2547, Sigma Aldrich, UK)<br><br>2° = Alexafluor-488 goat anti-mouse IgG (1:1000, A11001, Invitrogen, Paisley, UK) |
| **SM-22** | 1° = Rabbit polyclonal anti-SM22 (1:500, ab14106, Abcam, Cambridge UK )<br><br>2° = Alexafluor-488 donkey anti-rabbit IgG (1:1000, A21206, Invitrogen, Paisley, UK) |
| **vWF** | 1° = polyclonal rabbit anti-human vWF (1:400, A0082, Dako, Glostrup, Germany)<br><br>2° = Alexafluor-488 donkey anti-rabbit IgG (1:1000, A21206, Invitrogen, Paisley, UK) |
| **CD 31** | **1° = Mouse monoclonal anti-human CD-31 (1:400, 89C2, Cell Signaling , UK)**<br><br>**2° = Alexafluor-555 goat anti-mouse IgG (1:1000, A-21422, Invitrogen, Paisley, UK)** |

**Table 7 - Antibodies used for Immunofluorescence.**

## 2.9 Wire Myography

### 2.9.1 Vessel preparation

#### *2.9.1.1 Laboratory governance*

Animals used in this study were cared for in the central animal facility at University College London as approved by the UK home office department. All experiments were conducted according to the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Male Sprague–Dawley rats (180-250g) were sacrificed by stunning and cervical dislocation.

Lungs were removed from and placed in physiological salt solution (PSS) containing (in mM): 112 NaCl, 5, KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 25 $NaHCO_3$, 0.5 $KH_2PO_3$, 0.5 $NaH_2PO_3$, and 10 glucose (gassed with 95% $O_2$/5% $CO_2$ to pH 7.4). Third order pulmonary arteries were cleaned of connective tissue and cut into segments (2 mm long) and mounted on wires in an isometric myograph (510A JP Trading, Denmark).

### 2.9.2 Small vessel myography

Vessels were continuously aerated at 37 °C in PSS and pre-tensioned to an equivalent of ~22 mmHg (3K Pascal) for pulmonary and ~100mmHg (13.3K Pascal) for tail arteries. The normalized luminal diameter of each pressurised segment was obtained as described previously (Orie *et al.*, 2006) and averaged ~450 µm in pulmonary and ~750 µm in tail arteries. An equilibration period of at least 1 hr was allowed during which time tissues were contracted with a single

application of KCl (100 mM) followed by 2-3 washes and contracted with 100 nM of the thromboxane mimetic, U46619 or 10 µM phenylephrine (PE) for pulmonary and tail respectively. Concentration-response curves were constructed in arteries to treprostinil with or without pre-treatment with 10 µM cilostazol (for 1 hour). The presence of functional endothelium was assessed by examining responses to the endothelium-dependent vasorelaxant, acetylcholine (Ach).

### 2.9.3 Experimental Protocol

Two vessels were mounted each day and effects of treprostinil compared in separate vessels from the same animal. Contractions curve were allowed to plateau, before cumulative concentration–response curves (1–30,000 nM) were constructed for treprostinil. In all experiments, phosphodiesterase inhibitor was added at least 60 min before the addition of the contractile agonist. Changes in tone were expressed as the percentage of contractile response induced by U46619 or PE, just before the addition of the lowest concentration of treprostinil. Where possible, two concentration–response curves were obtained in the same preparation separated by a washout period of 60 min. With this protocol, there was no apparent time-dependent change in the response to any of treprostinil.

## 2.10 Statistical Analysis

Data are expressed as mean ± standard error of the mean (SEM). The $EC_{50}/IC_{50,}$ the concentration at which 50% of cell proliferation/contraction was inhibited and $E_{max}$ (maximal effect) was calculated using a variable slope sigmoidal curve fitting routine in GraphPad Prism (version 4.0, Jandel software, La Jolla, USA). For

myography, agonist log-concentration curves were constructed and fitted using the sigmodial fitting routine in Graph Pad Prism 4. Statistical analysis was performed between two groups using a non-paired student t-test or more than two groups using one / two ANOVA with Dunnett's or Bonferroni post-hoc tests. In all analyses a p value $< 0.05$ was considered statistically significant.

# 3. Stable prostacyclin analogues in PAH

## 3.1 Introduction

Once formed, $PGI_2$ binds to specific cell membrane receptors namely IP receptors (Hirata & Narumiya, 2011). These receptors are coupled to Gs which stimulates adenylate cyclase and increases cAMP levels and cAMP-dependent protein kinase A (PKA) activity (Torphy *et al.*, 1982; Kiriyama *et al.*, 1997). The latter mediates cAMP-induced decreases in intracellular calcium leading to vascular smooth muscle relaxation and vasodilation through a variety of mechanisms (Koike *et al.*, 2004; Maguire & Davenport, 2005). Cyclic AMP can also signal through exchange protein directly activated by cAMP (EPACs) and PKA can directly phosphorylate the transcription factor, CREB (Hirata & Narumiya, 2011) and together they can regulate a number of cellular functions, including cell proliferation (Hirata & Narumiya, 2011). $PGI_2$ acts via the IP receptor to mediate an acute potent pulmonary vasodilation and inhibition of platelet aggregation (Badesch et al., 2004). There is evidence that the lungs of PAH patients have decreased expression of the IP receptor (Badesch *et al.*, 2004; Falcetti *et al.*, 2010).

$PGI_2$ does, however, possess a very short biological half-life (2–3 min) at a physiological pH, and after inhalation of aerosolized $PGI_2$, the pulmonary vasodilatory effect is lost within 30 min both under experimental conditions and when tested in patients (Walmrath *et al.*, 1996). Because the vasodilatory effect of the prostanoids is exerted via enhanced cAMP generation, blocking the catabolism

of this second messenger might offer amplification of the pulmonary vasodilatory effects of these agents.

Not long after the discovery of $PGI_2$, this agent was given to patients with PAH and shown to be a potent dilator of both the systemic and pulmonary circulation (Rubin *et al.*, 1982), including in a subset of severely diseased patients not responding to traditional oral vasodilator therapy at all (Jones *et al.*, 1987). $PGI_2$ was amongst the first class of agent in the United States to receive Food and Drug Administration (FDA) approval for the treatment of PAH. Therapeutic application of $PGI_2$ results in the improvement of hemodynamics in PAH patients, and in randomized, controlled trials, has been shown to be of benefit in several clinical trials (Barst *et al.*, 1996; Walmrath *et al.*, 1997; McLaughlin *et al.*, 2003). Epoprostenol, being chemically unstable, can only be given as a continuous intravenous infusion through a central venous catheter. The freshly dissolved drug is inserted into the pump system and therefore pain and infection associated with the long-term presence of an indwelling intravenous catheter are common (Barst *et al.*, 1996). With respect to other $PGI_2$ analogues, these are chemically stable in solution and their plasma half-life is much longer: approximately 30 min with iloprost and about 3-4.5 h with treprostinil (Badesch *et al.*, 2004; Ivy, 2010). The metabolic stability order of various analogues is iloprost < beraprost < cicaprost < < treprostinil (Ruan *et al.*, 2010). Both iloprost and treprostinil have shown favourable effects in the treatment of PAH improving hemodynamics, exercise tolerance and clinical status. An impact on survival is assumed, though this has so far only been demonstrated

for epoprostenol in controlled trials (Wilkins & Wharton, 2001; Olschewski *et al.*, 2004; Barst *et al.*, 2009), though in open labelled or in retrospective long-term analysis of subcutaneous (SC) treprostinil, survival patterns do nonetheless appear similar to epoprostenol (Lang *et al.*, 2006; Barst *et al.*, 2006). Therefore, stable long-acting prostacyclin analogues can resolve some of the problems associated with IV epoprostenol. This can improve the prospects of long-term pulmonary vasodilator therapy.

Clinical studies are now being focused on the potential benefits that prostacyclin therapy could provide following the long-term supplementation of exogenous $PGI_2$. Until the recent study of Whittle and colleagues, little was known about the pharmacology of treprostinil (Whittle *et al.*, 2012; Stacher *et al.*, 2012). Its enhanced cAMP generation compared to other analogues in pulmonary smooth muscle cells and in mouse alveolar macrophages, suggests signalling through additional Gs coupled receptors, largely accounted for in the macrophage study by $EP_2$ receptors (Clapp *et al.*, 2002; Aronoff *et al.*, 2007). Treprostinil appeared to be more potent than iloprost and beraprost at inhibiting cell proliferation (Clapp *et al.*, 2002; Aronoff *et al.*, 2007), suggesting additional effects on other receptor or intracellular pathways. Both IP and non IP-receptor dependent effects involving activation of PPARs have been reported with $PGI_2$ analogues (Falcetti *et al.*, 2007) and such transcription factors may be involved in the antiproliferative effects of these agents (Hansmann & Zamanian, 2009; Falcetti *et al.*, 2010).

Phosphodiesterases have a wide distribution in normal tissues. Mammalian PDEs are subdivided into 11 distinct families on the basis of substrate specificity and sensitivity and sequence homology (Essayan, 1999; Essayan, 2001). The cGMP-inhibited cAMP PDE (PDE3) is expressed as two isoforms termed PDE3A and PDE3B. The activity of PDE3 is increased in main, branch and intrapulmonary arteries from rats maintained under chronic hypoxic conditions (Maclean *et al.*, 1997). PDE3 inhibitors reverse the reduced responsiveness of pulmonary vessels to isoprenaline and the adenylate cyclase activator, forskolin in rats with PH (Wagner *et al.*, 1997). It has been shown that cAMP and cGMP dependent PDEs (PDE3 and PDE5 respectively) activity is increased in pulmonary arteries from rats with chronic hypoxia-induced PH (Maclean *et al.*, 1997) which seemed to be correlated with decreased intracellular cAMP and cGMP levels (Sweeney *et al.*, 1995). Real time PCR and immunoblotting demonstrated that the expression of PDE1A, PDE1C, PDE3B, and PDE5A was enhanced in PASMCs from both IPAH and secondary pulmonary hypertension (SPH) patients compared with control PASMCs, suggesting that signalling through cAMP and cGMP may be impaired in PAH (Maclean *et al.*, 1997; Murray *et al.*, 2002; Schermuly *et al.*, 2007b). Indeed cAMP levels stimulated by beraprost were found to be more or less abolished in adult IPAH cells compared to normal (Murray *et al.*, 2002). Such increases in PDE activity might be responsible for increased proliferation in PAH (Phillips *et al.*, 2005).

The cGMP specific PDE5 is expressed as two isoforms termed PDE5A1 and PDE5A2. PDE5 activity is also increased in the branch and intrapulmonary artery from rats maintained under chronic hypoxic conditions and also elevated in an ovine model of perinatal PH (Maclean *et al.*, 1997; Hanson *et al.*, 1998a). PDE5 inhibitors cause vasodilatation of ovine pulmonary vessels (Ziegler *et al.*, 1995). There is also evidence showing the potential cross-talk between the cAMP and cGMP pathways. It has been reported that inhibition of PDE5 causes elevation of cGMP which further inhibits PDE3, thereby preventing the hydrolysis of cAMP and potentiating cAMP mediated effects (Zaccolo & Movsesian, 2007). The crosstalk also exists between the PKG and PKA signalling, whereby cAMP can also activate PKG increasing cGMP signalling and the later can further activate PKA (Zaccolo & Movsesian, 2007; Stangherlin *et al.*, 2011).

Smooth muscle cells undergo morphological and functional changes, where normally they are purely contractile, but in PH growth factors can convert the contractile arterial smooth muscle cells into abnormal proliferative, migratory and synthetic phenotype (Rabinovitch, 1997). Therefore in the first part of my study I focused on isolation, identification and characterization of the smooth muscle cells from distal pulmonary arteries and further used them to study different mechanisms involving cell proliferation.

The prostacyclin class of drugs are used to treat pulmonary arterial hypertension, and while evidence suggests they improve survival. But eventually as the disease progresses, dose escalation is required. Thus ways are being sort to improve their

clinical efficacy. I hypothesise that prostacyclin action could be enhanced by inhibition of phosphodiesterase type 3 (PDE3), a major regulator of cyclic AMP levels in the lung, whose activity appears increased in pulmonary hypertension (Murray *et al.*, 2007). I sought to investigate the action of the PDE3 inhibitor, cilostazol, an agent already in clinical use for the treatment of intermittent claudication (Liu *et al.*, 2011) where it manages muscle pain and increases exercise tolerance in peripheral vascular occlusive disease while favourably modifying the plasma lipid profile (Kambayashi *et al.*, 2003).

## 3.2 Results

### 3.2.1 Characterisation of lung tissue and pulmonary arterial smooth muscle cells

Lung tissue was obtained from the explanted lung of PAH patients receiving a bilateral lung or heart and lung transplant or from donor lungs found to be unsuitable for transplantation or from lung resection for suspected malignancy. Distal peripheral pulmonary arteries were dissected out and used for the isolation of PASMCs (figure 3.1A). Small preacinar and intra-acinar pulmonary arteries were thin walled in control lungs whereas in patients with PAH, these were partially or completely obstructed by a combination of medial thickening and intimal proliferation. Proliferation was also observed in the adventitial layer. In the examples shown, the thinly muscularised artery from a control myeloma patient stained for smooth muscle markers, α-smooth muscle actin (α-SMA) and SM-22

(figure 3.2A). Likewise strong staining for both markers was observed in the medial layer of partially obstructed arteries from a PAH patient (figure 3.2B), with the former also showing some staining in the adventitial layer. When PASMCs were isolated and subsequently cultured, they displayed hill-valley morphology, typical for smooth muscle cells (figure 3.3A). Further characterisation of these cells was performed using immunofluorescent staining to identify the same smooth muscle specific markers, which strongly stained human PASMCs derived from a PAH patient (figure 3.3B). Endothelial cell specific markers, like CD31 and VWf which are highly expressed in human endothelial cells (HEC), were used as negative controls. Both these endothelial cell markers strongly stained HEC but not human PASMCs (figure 3.4)

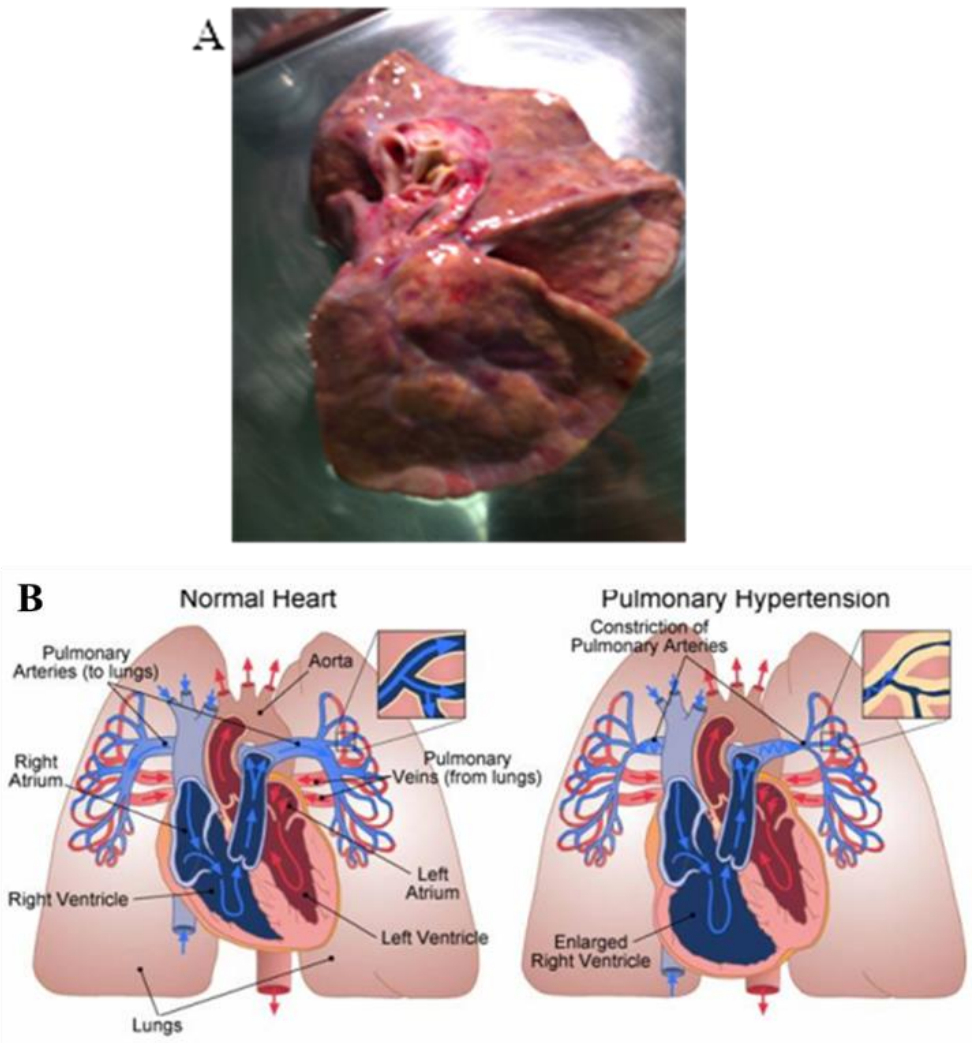



**Figure 3.1. Original picture and schematic representation of human lung**. A) Photograph of a lung lobe from a patient who had PAH with underlying venoocculsive disease. Distal pulmonary artery branches (as depicted in picture on the bottom B) were freshly dissected from the lung of control (normal) and PAH patients. Small arteries (less than 0.5 mm in diameter) in the square magnified in the right corner are used to enzymatically isolate pulmonary smooth muscle cells that are shown in culture from a PAH lung (Figure 3.1.B is reproduced with permission from (Nationwide Children's Hospital)).

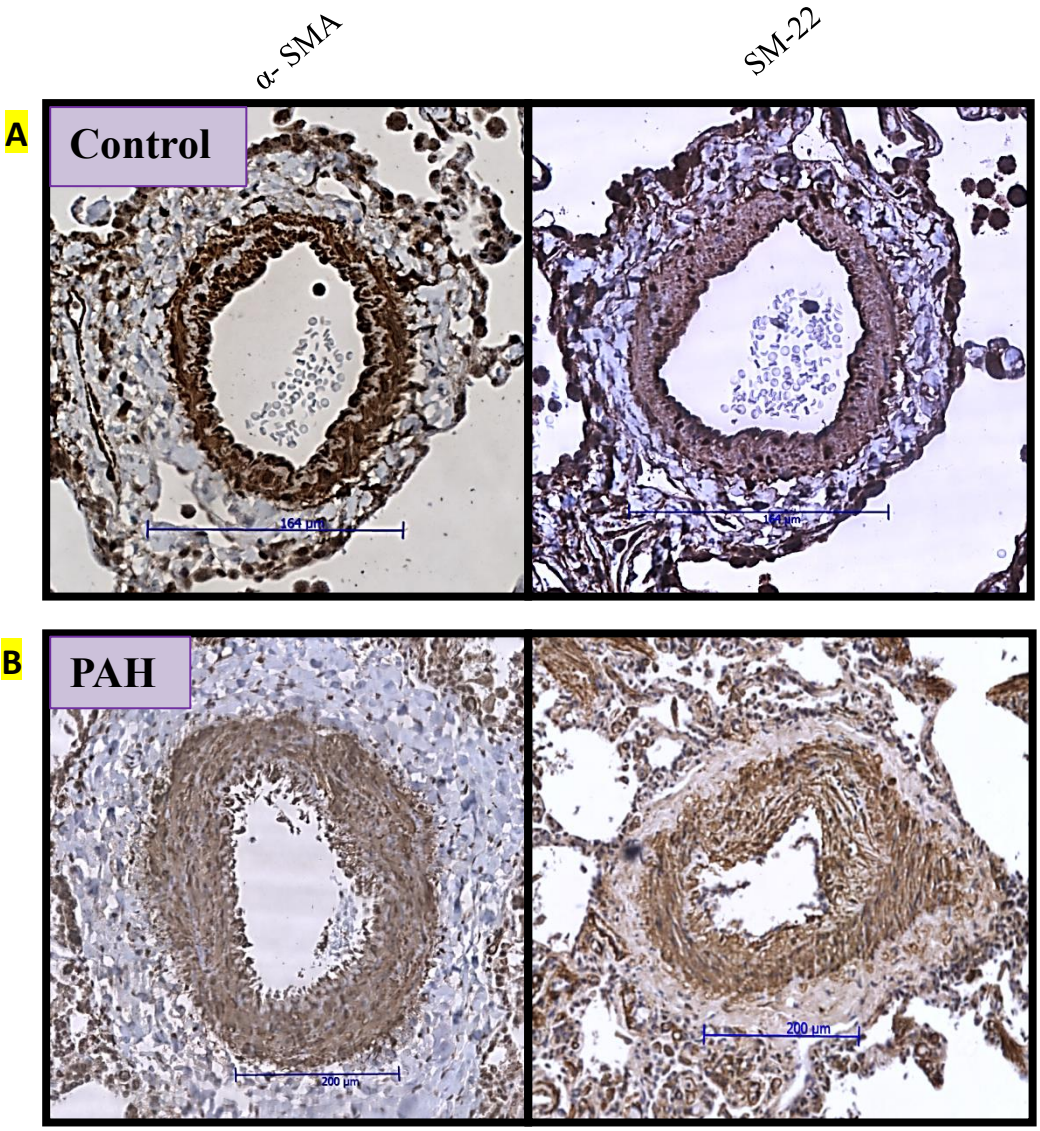



**Figure 3.2. Immunohistochemical staining of smooth muscle markers in small pulmonary blood vessels from a control and PAH patient.** Immunohistochemical staining of smooth muscle actin (α-SMA) and SM-22, visualised by diaminobenzidine (brown), in sections counterstained with haematoxylin. Sections of representative pulmonary artery of A) a normal lung and of B) a lung from pulmonary arterial hypertensive patient (PAH) stained with alpha smooth muscle actin (α-SMA) and SM-22 antibodies (staining the intimal-smooth muscle layer). Extensive medial thickening observed, showing the severity of disease in PAH versus minimal remodeling in normal lung.

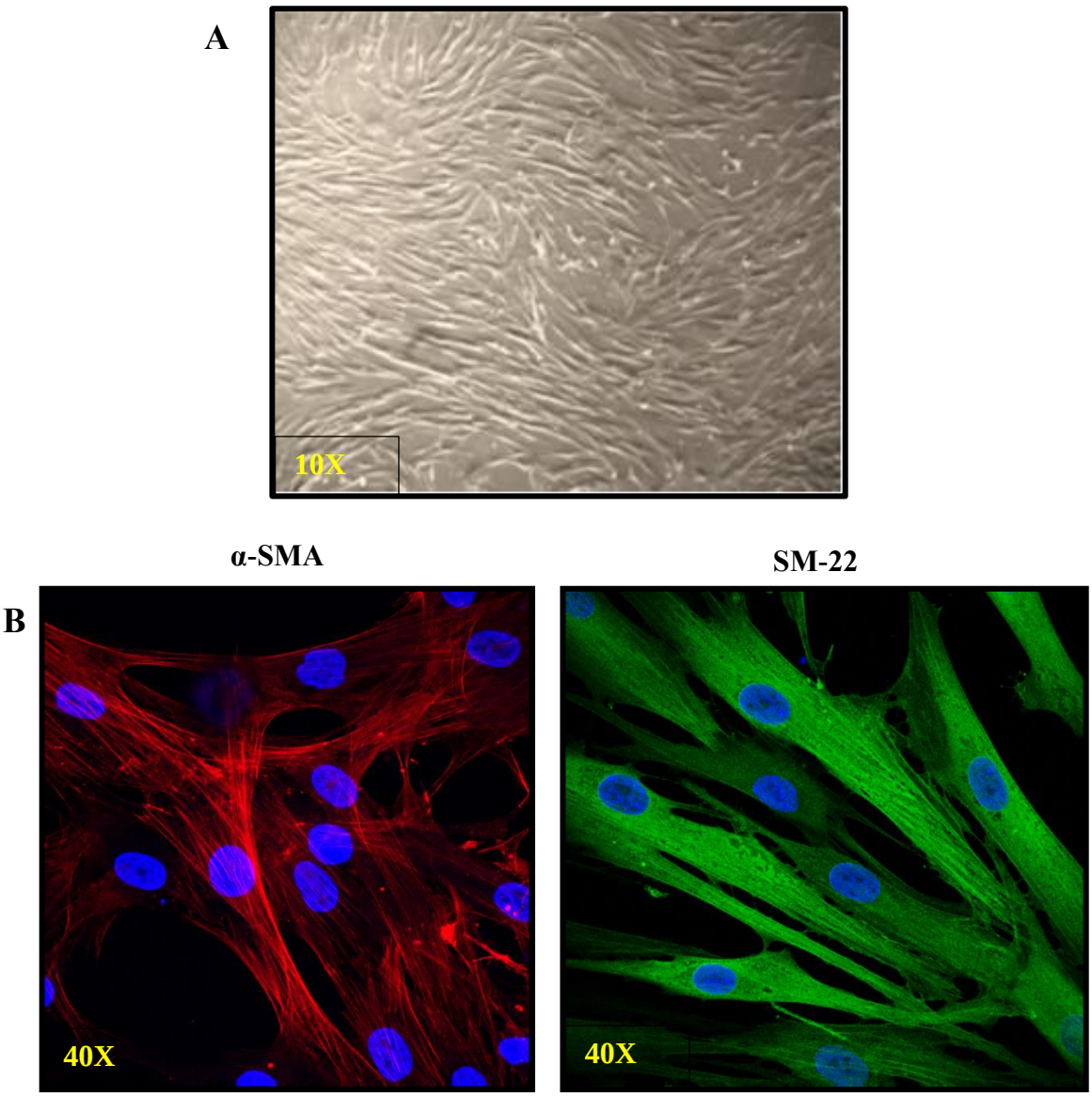



**Figure 3.3. Characterization of pulmonary arterial smooth muscle cells (PASMCs).** (A) Human PASMCs grown to 90% confluence in culture and observed with a phase contrast microscope showing classic hill and valley morphology. (B) Immunocytochemical staining of PASMCs isolated from PAH lung using antibodies against smooth muscle markers α-SMA and SM-22. Human PASMCs isolated from PAH lung in the left show positive staining of α-SMA fibers in red and in the right show positive staining of SM-22 fibers in green. In both cases the nucleus is stained blue with Dapi.

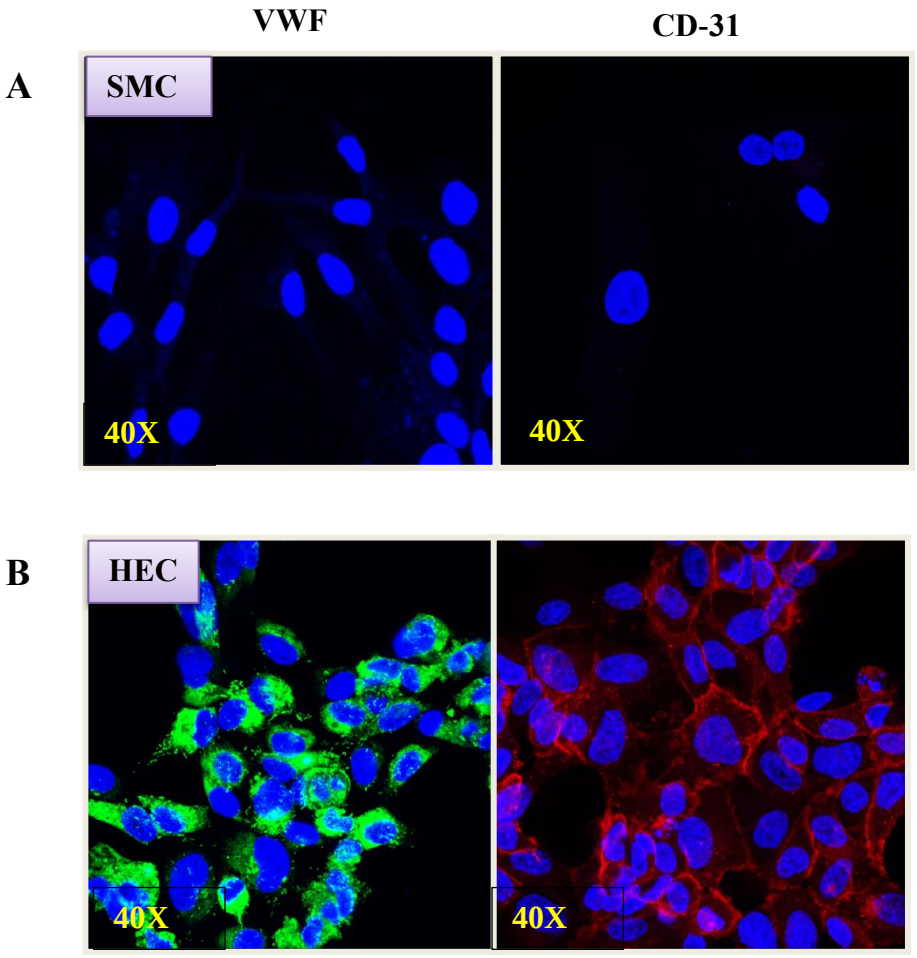



**Figure 3.4. Endothelial cell markers do not stain human smooth muscle cells (SMCs).** Immunocytochemistry staining of PASMCs isolated from PAH lung against the endothelial cell markers Von Willebrand factor (VWf) and CD-31 which are not expressed in SMCs. Human PASMCs isolated from PAH lung in (A) show no staining for VWf or CD-31, while human endothelial cells (HEC) shown in (B) showed positive staining for VWF (stained green) and CD31 (stained red). In all panels, the nucleus is stained blue with Dapi.

### 3.2.2 Effect of prostacyclin analogues on proliferation of human PASMCs

As part of the initial process to characterise human PASMCs from normal and diseased PAH patients, I assessed the effect of treprostinil on cell growth by performing cell proliferation assays with cells cultured for 4 days in DMEM/F12 containing 9% FBS (figure 3.5). In control PASMCs, serum increased cell number significantly (ANOVA, $P < 0.001$) compared to cells cultured in the absence of serum for the same length of time (basal). The growth rate of IPAH cells was however 2-3 fold higher than normal cells, indicating a more proliferative phenotype of smooth muscle cells obtained from PAH patients. Moreover, IPAH cells isolated from children appeared more proliferative than adult IPAH cells ($P<0.05$; Figure 3.5), consistent with the disease being more aggressive in children (Barst *et al.*, 2011). Incubation with treprostinil (0.1 nM to 10 µM) significantly inhibited human PASMC proliferation, reducing cell number in a concentration-dependent fashion, though in this set of experiments, treprostinil appeared least effective at inhibiting cell growth in control cells compared to previously published data (Falcetti *et al.*, 2010) and only inhibited growth by about 25% at 10 µM. The antiproliferative activity of treprostinil was also compared with another $PGI_2$ analogue, iloprost which was assessed under the same experimental conditions. Like treprostinil, iloprost also significantly reduced proliferation of human PASMC stimulated by FBS (9%). Both agonists displayed marked antiproliferative activity in a concentration-dependent fashion, inhibiting proliferation by 45-50% at the highest concentration of 10 µM in IPAH cells from children and adults. There was

however about a 10 fold difference in the potency of treprostinil to inhibit growth when compared with iloprost though this was not statistically significant. A point to note was that treprostinil started to significantly inhibit growth at 10 nM (one-way ANOVA, $P<0.05$) whereas for iloprost this occurred at 100 nM (ANOVA, $P<0.05$; Figure 3.6).

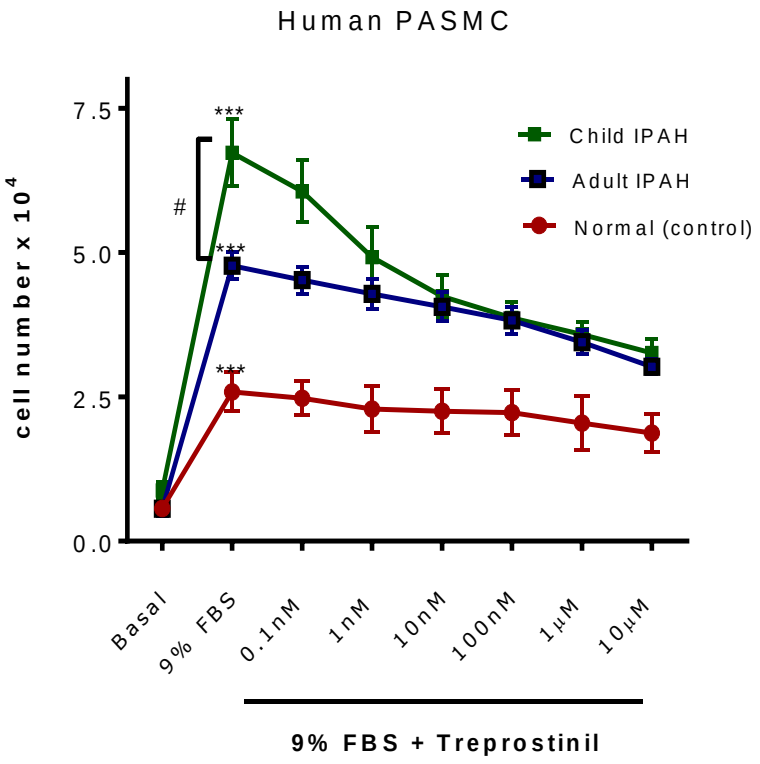



**Figure 3.5. Effect of treprostinil on proliferation of human PASMCs.** Cells were grown in culture for 24 hours in DMEM/F12 containing 9% FBS before they were growth arrested for 48 hours in DMEM alone. Cell counting was assessed after 4 days of stimulation with 9% FBS with or without treprostinil (concentration-range 0.1 nM to 10 µM) and compared with no FBS. *Error bars* represent mean ± SEM (n=4). Statistical significance was determined using 2-way ANOVA with Bonferroni correction. ***=$P<0.001$ when compared to the basal and #=$P<0.001$ when compared to response in normal (control) cells.

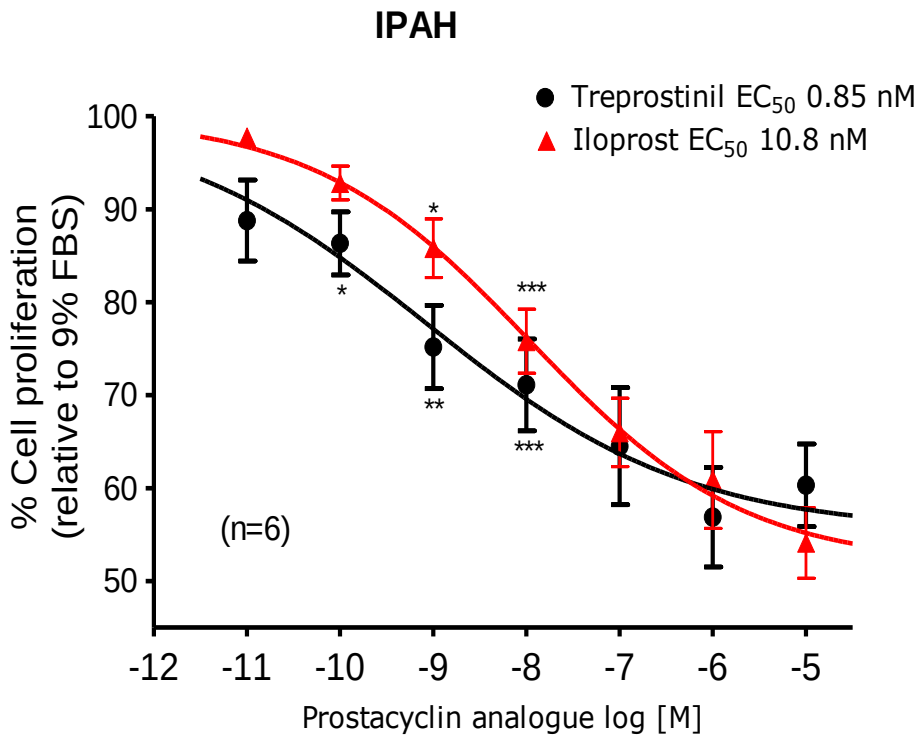



**Figure 3.6. Concentration–response curves to the $PGI_2$ analogues, treprostinil and iloprost.** Cells were grown in DMEM/F12 containing 9% FBS for 24 hours before they were growth arrested for 48 hours in media containing no FBS. Following this, cells were stimulated with 9% FBS in the absence and presence of increasing doses of analogues (0.1nM to 10 µM) and cell number counted at the end of 4 days. *Solid circles*, treprostinil and *solid triangles*, iloprost where data has been normalised to FBS response which was taken as 100% growth. Data, from the same batch of cells, is shown as mean ± SEM ($n$ =5 isolates), where each curve has been constructed using a variable slope sigmoidal fitting routine in GraphPad Prism. Statistical significance was determined using one-way ANOVA with Bonferroni correction. ***= $P$<0.001, **=$P$<0.01 and *=$P$<0.05 when compared to growth response with FBS alone.

### 3.2.3 Effects of treprostinil on cAMP

Cyclic AMP levels were assessed in IPAH cells at different time points after stimulation with treprostinil (where the analogue was added at time zero) and compared to levels obtained with just FBS treatment over the same period. Treprostinil produced an elevation in intracellular cAMP levels which increased at 15 min and peaked at 30 min as compared to growth-arrested (basal; t=0) or the equivalent time point with FBS alone. The maximum elevation of absolute cAMP levels noticed after a 30 min incubation with 1 µM treprostinil was 13 or 6 fold higher in magnitude as compared to either basal or the FBS time point, respectively (ANOVA, P<0.001). In contrast to previously published data in normal human PASMCs, (Clapp *et al.*, 2002), cAMP levels dropped sharply within an hour. Although there was still a trend for elevation in the cAMP levels at 1 to 24 hours compared to either basal or FBS time-control, this was not statistically significant (Figure 3.7).

Having established that the peak elevation of cAMP was achieved at 30 min with treprostinil, the nature of the receptor causing this increase in cAMP was assessed using the specific IP receptor antagonist, RO1183452 (Bley *et al.*, 2006). Cyclic AMP levels, which were again significantly elevated with a 30 min application of 1 µM treprostinil (one-way, ANOVA, *P*<0.001), were fully reversed with 1 µM of the IP receptor antagonist, RO1183452 which by itself did not affect basal cAMP levels. Similarly, 100 µM of the adenylate cyclase inhibitor 2, 5 dideoxyadenosine (DDA) also reversed this effect of treprostinil (Figure 3.8).

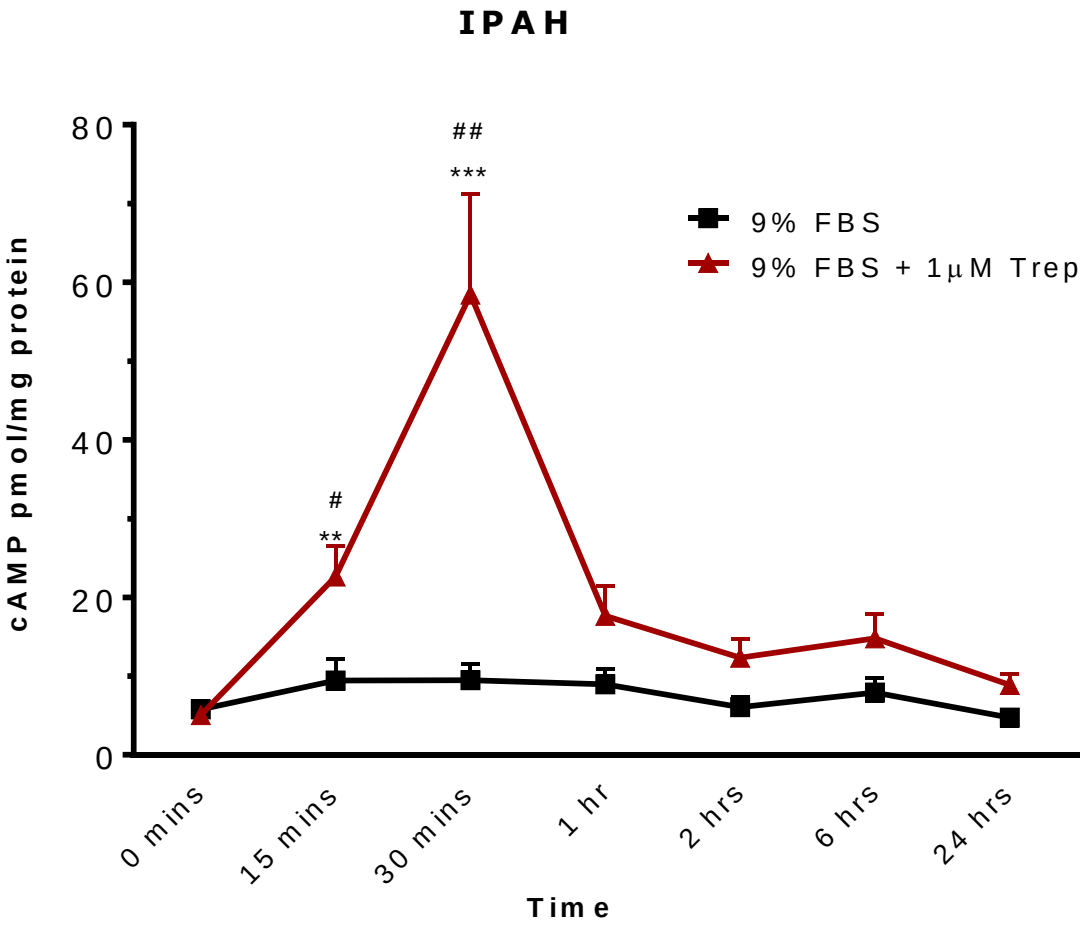



**Figure 3.7. Time-course of intracellular cAMP changes induced by treprostinil.** Human PASMCs obtained from IPAH patients and grown to 80-90% confluence, were growth arrested for 48 h and stimulated with 9 % FBS with and without 1µM treprostinil for up to 24 hours. Cells were then lysed for the estimation of cAMP levels. Results are expressed as pmol of cyclic AMP per mg of total protein. Data are shown as mean ± SEM ($n$ = 6). Statistical significance was determined using 2-way ANOVA with post-hoc test correction for multiple comparisons. ***=P<0.001,**=P<0.01 compared to time 0 value in treprostinil group. #=P<0.01and ##=P<0.001 compared to corresponding FBS value.

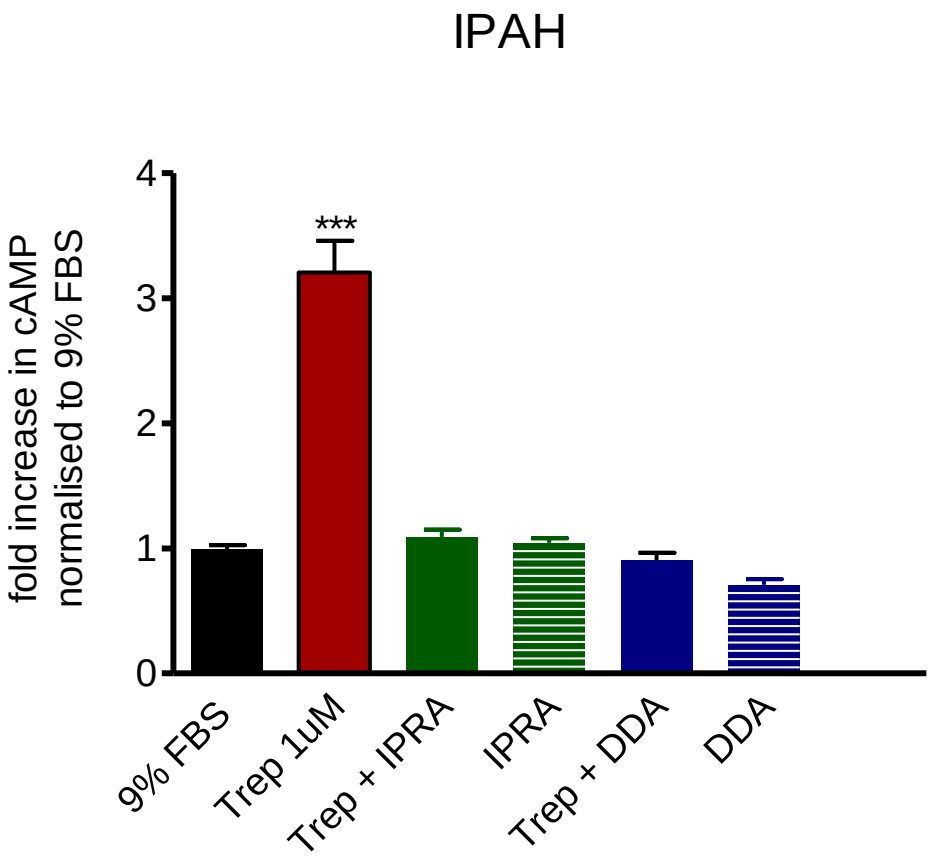



**Figure 3.8 Cyclic AMP elevation induced by treprostinil is mediated by the IP receptor.** Distal human PASMCs derived from IPAH patients were grown to 80-90 % confluence, growth arrested before being stimulated with 9% FBS with and without different drug treatments. For cyclic AMP measurement, cells were stimulated for 30 min with treprostinil (TREP, 1 µM), the IP receptor antagonist RO1183452 (IPRA; 1 µM), the adenylate cyclase inhibitor dideoxyadenosine (DDA; 1 µM) or a combination of treprostinil with inhibitor as shown above. RO1183452 or DDA was given 1 h prior to addition of treprostinil. Results are expressed as fold increase above basal and shown as mean ± SEM ($n$ = 5). Statistical significance was determined using one-way ANOVA with Bonferroni correction, where *** =$P$<0.001 compared to FBS (9%) alone.

### 3.2.4 Effect of phosphodiesterase 3 inhibitor, cilostazol on cAMP generated by treprostinil

To simply experiments, and given the lack of effect of FBS on cAMP over time, all subsequent experiments were done on growing cells. To test the hypothesis that high PDE3 activity is responsible for the short-lived cAMP levels induced by treprostinil in IPAH cells, I assessed the effect of the PDE3 inhibitor, cilostazol. In the absence of treprostinil, pre-treatment with 1 µM cilostazol for 1 hour caused about a 2 fold increase in the basal cAMP level, suggesting increased background PDE3 activity in these cells. With treprostinil (1 µM), I observed there was a time-dependent increase in cAMP level, which regardless of whether combined with 1 µM cilostazol, peaked at 30 min. Furthermore, in cells treated with treprostinil alone, cAMP levels dropped to almost control levels by one hour, but not with those pre-treated with cilostazol (Figure 3.9). Indeed, cyclic AMP levels were found significantly elevated even after 24 hours in cells treated with the analogue in combination with the PDE3 inhibitor (ANOVA, $P<0.05$) (figure 3.9).

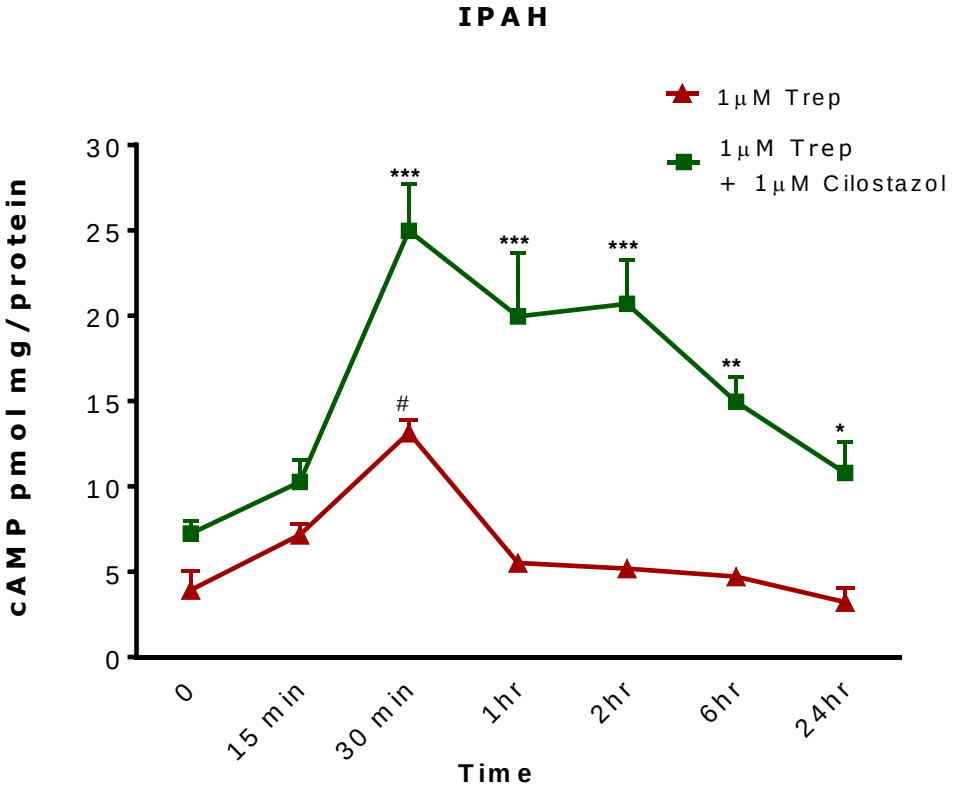



**Figure 3.9. Cilostazol potentiates the effect of treprostinil on intracellular cAMP levels.** For cyclic AMP measurement, growing cells were stimulated with Trep (1 µM) or in combination with 1 µM cilostazol (PDE3 inhibitor). Cells were pretreated with cilostazol for 1 hr prior to addition of treprostinil. Results are expressed as pmol of cyclic AMP per mg of protein. Data are shown as mean ± SEM ($n$ = 4) and statistical significance determined using 2-WAY ANOVA with post-hoc test correction for multiple comparisons. $^{\#=}P<0.001$ when compared to 9% FBS alone (time = 0), *=$P<0.05$, **=$P<0.01$, ***=$P<0.001$ when compared to 9% FBS and treprostinil.

When similar experiments were repeated using PASMCs isolated from normal (control) patients, I found that the cAMP levels produced after treatment with treprostinil in similar experimental conditions was significantly higher in normal compared to the diseased cells (compare figure 3.9 with figure 3.10). Also, the sudden drop observed at an hour with IPAH cells was less pronounced in normal cells, such that cAMP levels remained significantly elevated (~2 fold) compared to basal for up to 6 h (ANOVA, $P<0.05$). There was a trend for sustained elevation of cAMP levels at 6 h after treprostinil treatment in normal cells; however the difference was not statistically significant (figure 3.10). When combined with cilostazol, the peak cAMP levels were not significantly increased, suggesting that PDE3 activity may be lower in normal compared to PAH cells (figure 3.9 and 3.10). Nonetheless, cAMP levels remained substantially elevated at 24 hours.

I further assessed if cilostazol could potentiate the anti-growth responses of treprostinil. As shown, the antiproliferative effect of 1 µM treprostinil was enhanced when applied in combination with the PDE 3 inhibitor, cilostazol (1 µM or 10 µM) though this was only significant (ANOVA, $P<0.001$) at the higher concentration (Figure 3.11). Furthermore, cilostazol on its own significantly inhibited PASMC proliferation, with 10 µM cilostazol inhibiting growth by ~20% (one-way ANOVA, $P<0.01$) (figure 3.11).

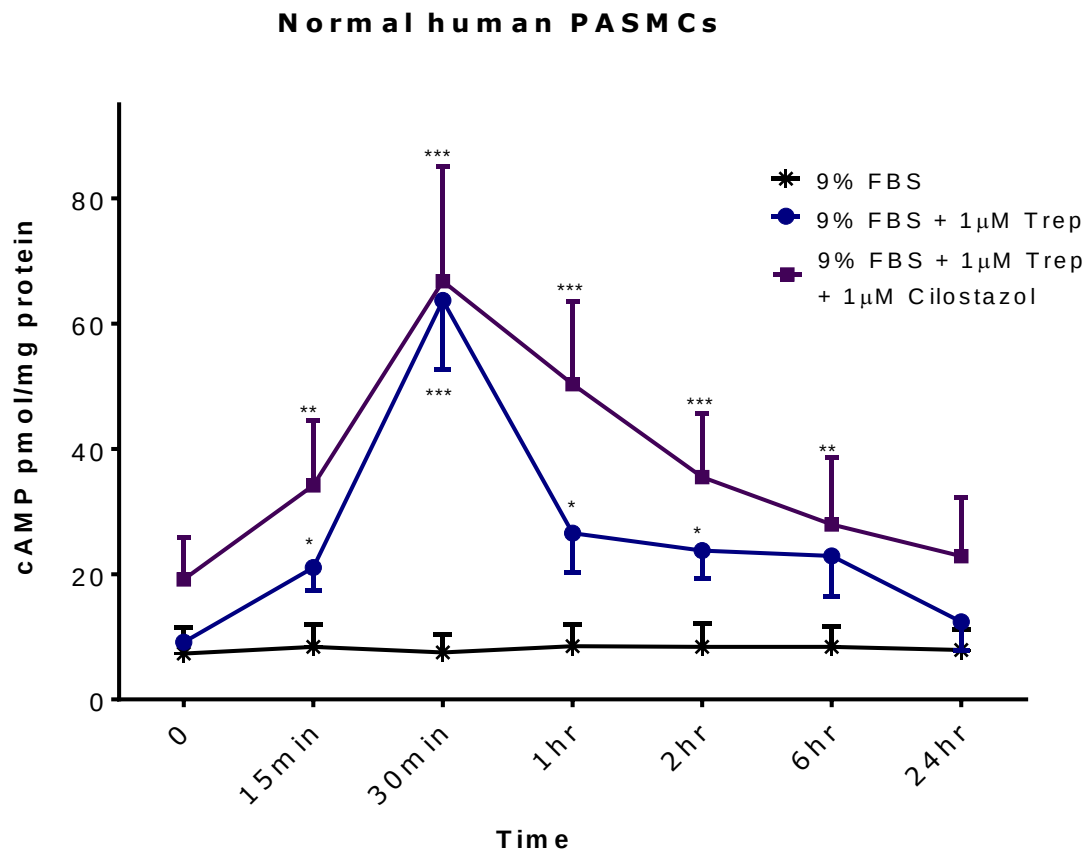



**Figure 3.10. Cyclic AMP generation in normal human PASMCs.** For cyclic AMP measurement, cells were stimulated with 9% FBS with or without treprostinil (TREP; 1μM) and or a combination of 1 μM cilostazol (PDE3 inhibitor) and treprostinil. Cells were pretreated with cilostazol for 1 hr prior to addition of treprostinil. Results are expressed as pmol of cyclic AMP per mg of total protein. Data are shown as mean ± SEM ($n$ = 4); Statistical significance was determined using 2-way ANOVA with post-hoc test correction for multiple comparisons.. *=p<0.05, **=p<0.01, ***=p<0.001 compared to 9% FBS alone (time =0).

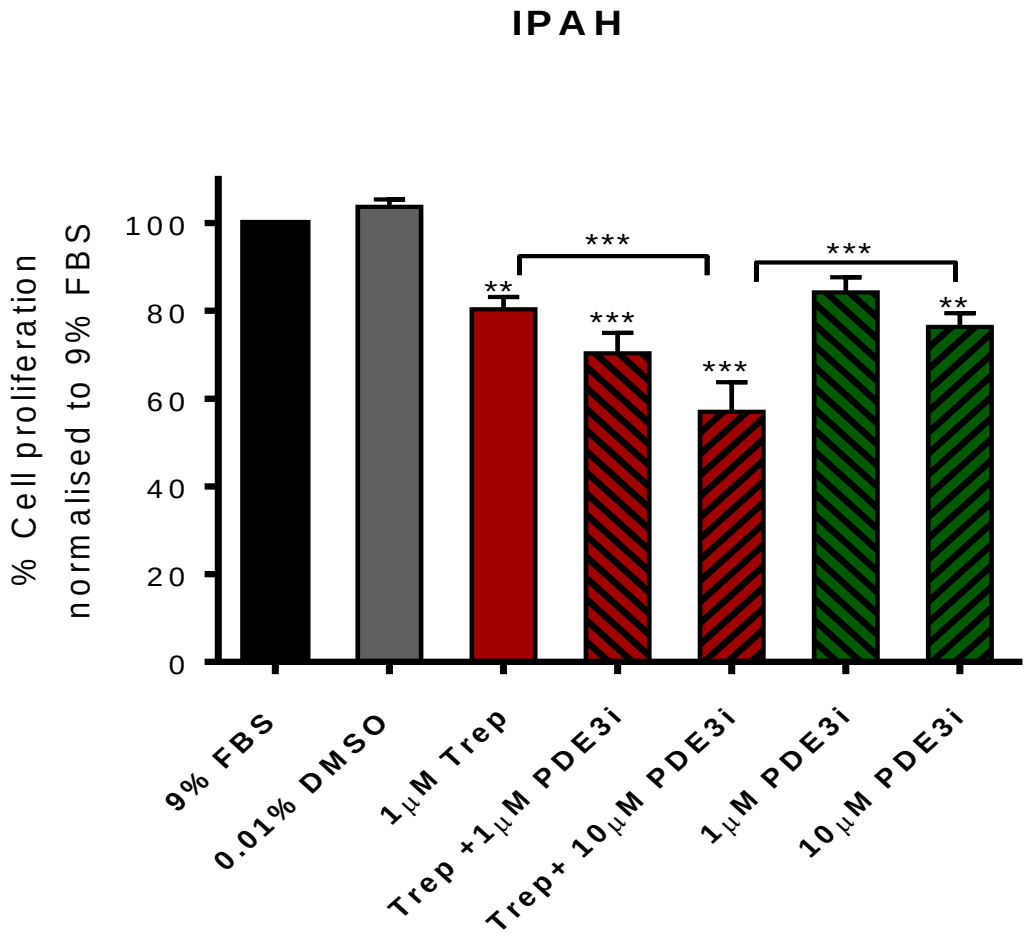



**Figure 3.11. Cilostazol potentiates antiproliferative effects of treprostinil.** Cells were grown in DMEM/F12 containing 9% FBS for 24 hours and then growth arrested for 48 hours in serum free DMEM/F12 before being stimulated with 9% FBS with or without treatment with treprostinil (1 µM), cilostazol (1 µM or 10µM) or a combination of both. Cells were counted after 4 days. Data are shown as mean ± SEM ($n$ = 4) and shown as % cell proliferation with respect to response to FBS alone. Statistical significance was determined by one-way ANOVA with Bonferroni correction. **=P<0.01 and ***P<0.001 when compared to the solvent DMSO.

### 3.2.5 Effects of treprostinil on vasorelaxation

Treprostinil has been shown to reduce pulmonary arterial pressure, as measured by right ventricular systolic pressure (RVSP) and total pulmonary vascular resistance in PAH and the efficacy of the treatment in PAH is demonstrated regardless of route of administration (Tapson *et al.*, 2006; McLaughlin *et al.*, 2010; Tapson *et al.*, 2012). In normal individuals prostacyclin causes potent acute vasorelaxation, but the contribution of dilatation of the pulmonary artery in PAH seemed to be compromised due to disturbance in vascular homeostasis, and upregulation of various factors like PDEs. Therefore I hypothesized that inhibiting PDE3 with cilostazol could potentiate the direct vasorelaxant efficacy of treprostinil in pulmonary artery. Distal pulmonary and tail arteries were isolated from a rat and mounted on a wire myograph and contracted with 100nM of the thromboxane mimetic, U46619 or 10µM phenylephrine (PE) respectively. The concentration-response curves were constructed to treprostinil with or without pre-treatment with 10 µM cilostazol. As expected, treprostinil significantly relaxed pulmonary artery contractions induced by 100 nM of the thromboxane mimetic, U46619 (figure 3.12). Treprostinil significantly relaxed pulmonary artery contractions, with a relaxation observed of ~25% at 10µM. However, pre-treatment with 10 µM cilostazol significantly potentiated the treprostinil response at concentrations at or above 100nM ($P<0.001$; n=4, two-way ANOVA) (figure 3.12). Similar effects were observed in rat tail arteries, where treprostinil significantly relaxed tail artery contractions induced by 10µM PE and this effect was significantly potentiated with 10 µM cilostazol ($P<0.001$; n=4, unpaired t-test) (figure 3.13).

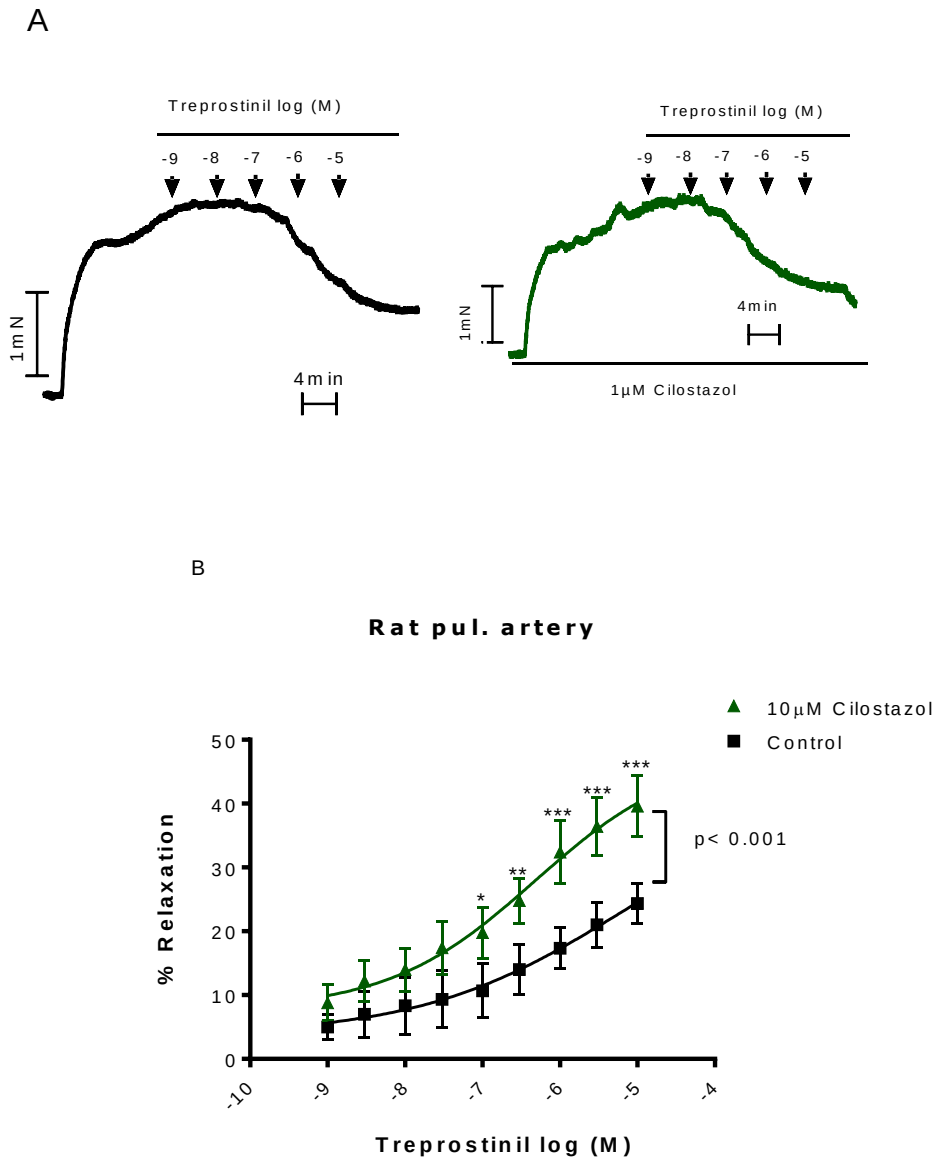



**Figure 3.12. Cilostazol potentiates the relaxant response to treprostinil in rat pulmonary artery.** A) After the normalisation procedure, distal vessels were contracted with 1 µM of the thromboxane analogue, U46619 followed by sequential application of increasing doses of treprostinil in the absence (left panel) or presence (right panel) of cilostazol (10 µM). B) Concentration-dependent relaxation by treprostinil in rat pulmonary arteries where effects were calculated as the % relaxation of the contraction produced with U46619 just before the addition of lowest dose to treprostinil. Data are shown as mean ± S.E.M. and curves generated by fitting data using a variable slope sigmoidal fitting routine in GraphPad. *=P<0.05, **=P<0.01, ***=P < 0.001, two-way ANOVA with post-hoc correction for multiple comparisons (n = 4) when compared to the appropriate control response.

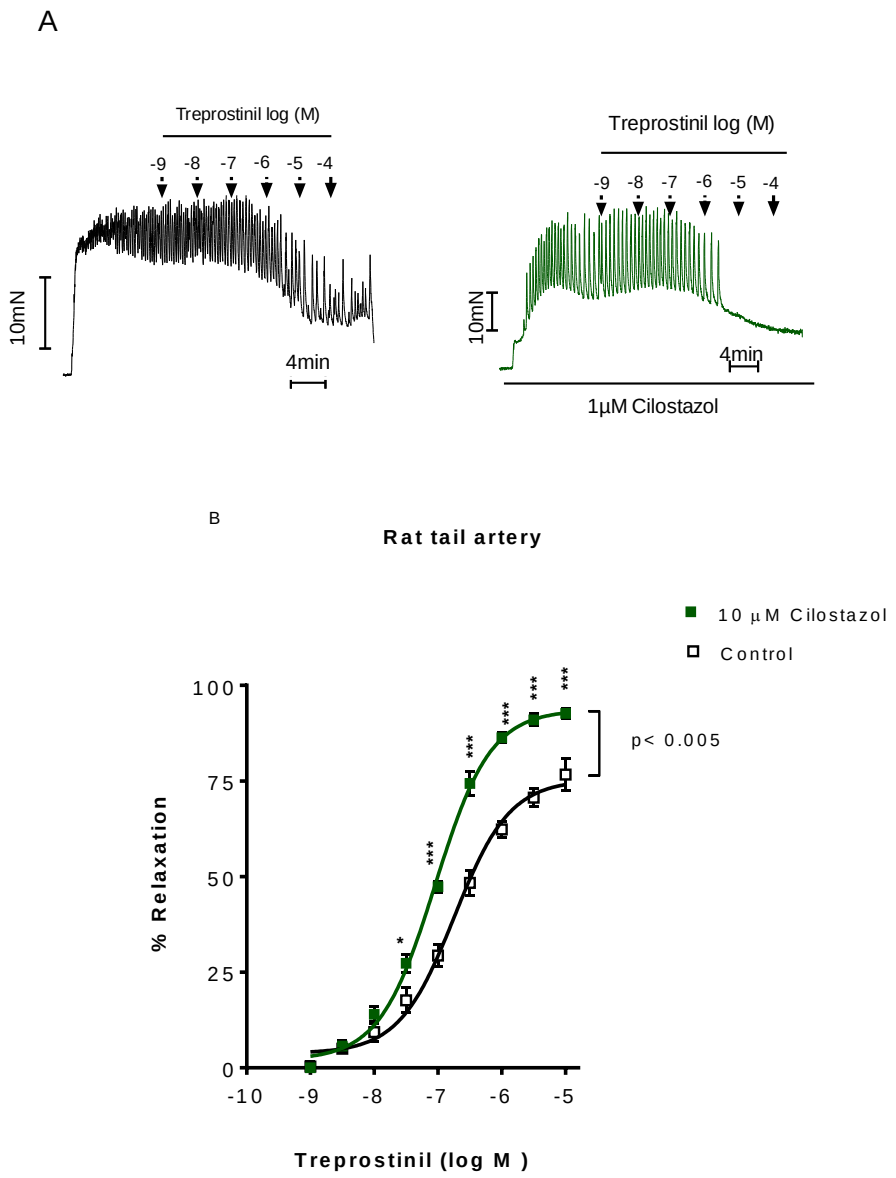



**Figure 3.13. Cilostazol potentiates the relaxation response of treprostinil in rat tail artery.** A) After a normalisation procedure, vessels were contracted with 10 µM phenylephrine followed by sequential application of increasing doses of treprostinil in the absence or presence of cilostazol (10 µM). Concentration-dependent relaxation induced by pre-contracted rat tail arteries was plotted as the % relaxation of the contraction which was measured just before the addition of the lowest drug concentration. Data shown as mean ± S.E.M and fitted using a unity slope sigmoid function in Graph-Pad Prism. *=P<0.05 and ***=P < 0.001 (two-way ANOVA with post-hoc correction for multiple comparison; n = 4).

## 3.3 Summary of key findings

- Cyclic AMP levels peaked within 30 min of treprostinil treatment in IPAH cells and dropped close to basal values within one hour. This is in contrast to normal human PASMCs, where cAMP levels remained elevated for at least 6 h (see also Clapp *et al.*, 2002).

- High activity of PDE3 was assumed as I observed that the peak cAMP level was higher with the combination of treprostinil and cilostazol than with treprostinil alone in IPAH cells and elevation was significantly sustained for up to 24 h.

- In this study I demonstrated that cilostazol could significantly potentiate cAMP levels, antiproliferative and relaxation effects of treprostinil.

- High activity of PDE3 might explain in part the ineffectiveness of prostacyclins in long-term therapy and I propose that a PDE3 inhibitor might potentiate prostacyclin signalling in PAH, possibly constituting a new therapeutic paradigm in IPAH.

## 3.4 Discussion

In this study I demonstrated that treprostinil and iloprost had anti-proliferative properties on distal pulmonary vascular smooth muscle cells derived from IPAH patients. These agents only produced ~50% inhibition of serum-induced growth, where previous work from this laboratory showed both these agents to produce full inhibition of growth in human PASMCs derived from a 15-year old control female (Clapp *et al.*, 2002). Thus it remains to be determined whether the disease impacts on the efficacy of these agents, either because the receptor is lost or more readily desensitised particularly. Indeed, both activation of thromboxane and endothelin receptors can promote IP receptor desensitization (Walsh *et al.*, 2000; Schermuly *et al.*, 2007a), and IP receptor expression is reduced in IPAH (Lai *et al.*, 2008; Falcetti *et al.*, 2010). However, the actions of $PGI_2$ and its analogues are not specific to the IP receptor as they can potently activate other prostanoid receptors including $EP_1$ for iloprost and $EP_3$ for all analogues (Narumiya, 1994; Orie & Clapp, 2011). Therefore if these pathways are up-regulated in PAH, then this may modulate the cellular effects of $PGI_2$ and its stable analogues. The $EP_1$ receptor, when activated increases intracellular calcium and produces vasoconstriction (Ungrin *et al.*, 2001) and blockade of this receptor can enhance analogue responses in aorta (Turcato & Clapp, 1999) and in the lung (Schermuly *et al.*, 2007a). The $EP_3$ receptor has various splice variants capable of coupling with Gi, Gq- and Gs-type G-proteins (Negishi *et al.*, 1993; Namba *et al.*, 1993). In general, $EP_3$ receptor activation leads to inhibition of adenylate cyclase, and activation of phospholipase C, and is

associated with vasoconstriction. Indeed blockade of $EP_3$ receptors potentiates the relaxation induced by cicaprost, iloprost and treprostinil in rat tail and pulmonary arteries (Kuwano *et al.*, 2008; Orie & Clapp, 2011; Morrison *et al.*, 2012). Moreover, contractile responses to $EP_3$ receptor agonists increase as the pulmonary vessel size decreases as well as after monocrotaline treatment (Kuwano et al., 2008; Morrison et al., 2012), suggesting $EP_3$ receptors may an important modulator of IP receptor function. Finally under certain circumstances, the IP receptor can couple with Gq and possibly Gi proteins which would promote vasoconstriction and thus limit relaxation to $PGI_2$ (Chow *et al.*, 2003).

There are other cellular pathways through which $PGI_2$ may act. $PGI_2$ synthase is heavily expressed in the perinuclear regions of cells, including in smooth muscle, and $PGI_2$ once formed, can directly interact with the ligand binding domain of a family of transcription factors known as PPARs. Some analogues, including beraprost and iloprost but not cicaprost, also bind to and transcriptionally activate PPARs (Lim & Dey, 2002; Nemenoff *et al.*, 2008). There is the potential for cross talk between this pathway and the IP receptor since both PPARα and PPARβ can be activated via protein kinase A (PKA) (Ali *et al.*, 2009). Work from our laboratory has shown a novel IP receptor-dependent activation of PPARγ by $PGI_2$ analogues that appears to be distinct from the known ligand binding effects of these analogues (Falcetti *et al.*, 2007). Such a mechanism contributes to the antiproliferative effects of treprostinil in HEK cells (Falcetti *et al.*, 2010) and in human PASMCs (Falcetti et al, 2012). Surprisingly, PPARγ activation does not

appear to necessitate cAMP elevation, nor does the IP receptor always appear to be a prerequisite for its involvement in inhibiting cell growth in mouse PASMCs or in PASMCs derived from patients with IPAH cells (Falcetti *et al.*, 2007; Falcetti *et al.*, 2010).

These antiproliferative effects of $PGI_2$ and its analogues may translate into a slowing of disease progression. In animals evidence suggests that iloprost can reverse vascular remodelling in a chronic experimental model of PAH in rats (Schermuly *et al.*, 2005b). Likewise, treprostinil can reverse remodelling when applied after the establishment of PAH with monocrotaline (Yang *et al.*, 2010). However, there is little evidence that $PGI_2$ can do this in IPAH. In paediatric patients treated with epoprostenol and bosentan, a doubling in the size of lesions compared to those without treatment was found when lungs were examined after transplant or postmortem (Hall *et al.*, 2011). In other studies, no difference in thickness of intima, media, or adventitia or in density of plexiform lesions occurred with epoprostenol treatment (Achcar *et al.*, 2006). Thus, the evidence to date suggests there is little impact on the remodelling process with PAH treatment.

In this study, I found that treprostinil (1 µM) elevated cAMP levels on average by 2-3 fold in cultured smooth muscle cells isolated from IPAH patients and that maximum elevation occurred at 30 min as compared to untreated cells. Cyclic AMP elevation was fully reversed with the IP receptor antagonist RO1183452 (1 µM), an agent seemingly specific for the IP receptor because it has no significant

binding affinity for prostanoid EP or TP receptors, although effects on DP receptors were not examined (Bley *et al.*, 2006). The IP receptor seems to be the major source of cAMP in human PASMCs for treprostinil. Whether this is the case at later time points, where the IP receptor antagonist was not specifically investigated, is not clear. The possibility remains however, that other receptors may be activated and contribute to cAMP generation. It is known that $PGI_2$ analogues can signal through other Gs-coupled prostanoid receptors, including $EP_4$ for iloprost (Lai *et al.*, 2008), and $EP_2$ for treprostinil (Aronoff *et al.*, 2007; Whittle *et al.*, 2012). Moreover, cAMP was reported to still be generated by these analogues in the absence of the IP receptor (Lai *et al.*, 2008; Falcetti *et al.*, 2010).

In IPAH cells, the peak in cAMP levels was not sustained and dropped close to basal levels within an hour. I hypothesize this was due to the higher activity of the phosphodiesterase enzymes which break down cAMP (PDE 1, 3, 4). This is in contrast to previous observations from our laboratory which demonstrated that treprostinil elevated cAMP for many hours in normal human PASMCs (Clapp *et al.*, 2002). Moreover, heightened PDE activity contributed to the complete loss of beraprost effects on cAMP generation in PAH and SPH which could be rescued by treatment with a non-selective PDE inhibitor (Murray *et al.*, 2007). High activity of PDE3 was assumed as I observed that the peak cAMP level was higher with the combination of treprostinil and the PDE3 inhibitor, cilostazol than with treprostinil alone and elevation was significantly sustained for up to 24 h compared. This would also be consistent with increases in PDE3 (specifically PDE3C) but not PDE4 activity in PASMCs isolated from both IPAH and secondary pulmonary

hypertension (SPH) patients (Murray *et al.*, 2007). I assume the effect of cilostazol is specific for PDE3, as it appears to have little effect on PDE4 activity even up to concentrations as high as 100 µM (Shakur *et al.*, 2002; Gibson *et al.*, 2006) One cannot however rule out that cilostazol may affect PDE1 activity, which is both increased in distal pulmonary arteries taken from IPAH and in experimental models (Murray *et al.*, 2007; Schermuly *et al.*, 2007b). To confirm high PDE3 activity in the disease, these experiments were also repeated using PASMCs isolated from normal (control) patients. In these cells, I found that the cAMP levels produced after treatment with treprostinil were significantly higher compared to diseased cells. Also, the sudden drop observed in an hour with IPAH cells was not as obvious in normal cells and the cAMP levels remained significantly elevated compared to basal for up to 6 hours. When combined with cilostazol, the peak cAMP levels were not increased in normal cells as it was in the IPAH cells, and breakdown was relatively less affected, suggesting that the PDE3 activity is probably lower in the normal human PASMCs, as compared to the IPAH cells.

How PDE3 activity might be elevated in PAH is largely unknown, but a number of possibilities exist. Phosphatidyl inositol 3 kinase (PI3 kinase), which is commonly activated by $G_i/G_o$ coupled receptors, is known to upregulate PDE3 as well as PDE4 activity leading to enhanced cAMP degradation (Kerfant *et al.*, 2006; Alfranca *et al.*, 2006). Indeed results from this laboratory have shown $PGI_2$ analogue relaxation to be enhanced by PI3 kinase inhibitors and also by $G_i/G_o$ inhibition with pertussis toxin (Orie & Clapp, 2011). Moreover, TP receptor activation impaired endothelium-independent relaxations to agents that elevated

cAMP levels via increasing the activity of PDE3, an effect reversed almost entirely by cilostazol with some contribution from PDE4 acknowledged (Liu *et al.*, 2010). I suggest that high circulating levels of thromboxane that occur in PAH (Christman *et al.*, 1992) may also contribute to an increase in PDE3 activity.

Indeed I observed that relaxation of U466191 contractions by treprostinil in small rat pulmonary was potentiated over the whole concentration range with cilostazol, whereas in tail arteries, relaxation of phenylephrine-induced contractions increased more at the higher concentrations of treprostinil, where $EP_3$ would become significantly activated (Whittle *et al.*, 2012; Morrison *et al.*, 2012). Moreover, another prostacyclin analogue, iloprost in addition to activating $EP_3$ receptors, is a potent activator of $EP_1$ receptors (Ungrin *et al.*, 2001), receptors which can in some instances be coupled to Gi (Hirata & Narumiya 2011) and may thus also potentiate PDE3 activity through PI3 kinase. Indeed, treprostinil evoked significantly higher (6 fold) and more prolonged elevation in cyclic AMP compared with iloprost in normal human pulmonary arterial smooth muscle cells (PASMCs) (Clapp *et al.*, 2002). I did not investigate whether cAMP enhanced by the PDE3 inhibitor cilostazol, is meditated by other prostanoid receptors that can be activated by treprostinil. Our lab has shown that there may be additional effects of treprostinil through other Gs-coupled receptors, including $EP_2$ and $DP_1$ (Whittle et al, 2012). In earlier studies, while the maximal antiproliferative effects of the $PGI_2$ analogues were more or less comparable, treprostinil was significantly more potent than iloprost and beraprost (Clapp *et al.*, 2002), despite all these analogues having similar affinities at the IP receptor (Ungrin *et al.*, 2001; Kuwano *et al*, 2007;

Whittle et al, 2012), suggesting additional effects on another receptor or intracellular pathway. I hypothesize it might be through other EP receptor subtypes coupled to Gs. Studies have shown that in alveolar macrophage of rats, $EP_2$ antagonist significantly blocked more than half of the increase in cAMP stimulated by treprostinil, whereas $EP_4$ antagonist had no significant effect (Lai *et al.*, 2008). These effects were also confirmed using $EP_2$ knock-out mice, where treprostinil stimulated cAMP rise in rat alveolar macrophage was significantly lower compared to the wild type (Aronoff *et al.*, 2007).

High activity of PDE3 (and possibly PDE1) might explain in part the ineffectiveness of $PGI_2$ in long-term therapy and I propose that a PDE3 inhibitor might potentiate $PGI_2$ effects in PAH. In this study I demonstrated that cilostazol could significantly potentiated not only the effect of treprostinil to generate cAMP but also potentiated the antiproliferative and vasodilatory effects of the analogue, suggesting that this combination could be clinically beneficial for treating remodelling in PAH. Cilostazol is already in clinical use for intermittent claudication (peripheral vascular occlusive disease) where it increases exercise tolerance and favourably modifies the plasma lipid profile, suggesting additional beneficial effects in diseases associated with atherosclerosis (Shakur et al, 2003). Studies also suggest that cGMP can inhibit PDE3, which can therefore elevate cAMP levels. PDE5 is the isoenzyme that selectively degrades cGMP into the inactive form. In PAH, lungs have abundant PDE5 and PDE3 expression/activity (Murray *et al.*, 2007). Pulmonary arteries have a large amount of PDE5 activity

compared to systemic arteries that further increases in experimental forms of pulmonary hypertension as well as in patients with PAH. In addition to vasodilatation, PDE5 inhibition also reduces DNA synthesis and cell proliferation in human PASMCs and stimulates apoptosis, suggesting that PDE5 inhibition also has anti-remodelling effects in the pulmonary vasculature (Rabe *et al.*, 1994). In a phenomenon referred to as "cross-talk", cGMP negatively feeds back on PDE3 and this slows the inactivation of cAMP (Omori & Kotera, 2007).

Experimental data indicate that treatment of PAH by manipulating multiple pathways concurrently may produce additive benefits. The current therapies for PAH are not curative, nor does it normalize pulmonary hemodynamics or functional capacity in the vast majority of cases. Thus, there is a possibility that combinations of prostacyclin analogues or PDE5 inhibitor (tadalafil, sildenafil) with agents that potentiate cAMP-prostacyclin pathway might offer more therapeutic efficacy. Preliminary studies suggest that combination of PDE5 inhibitor with prostacyclins, inhaled NO and natriuretic peptides improve pulmonary hemodynamics more than monotherapy (Ghofrani *et al.*, 2002; Ghofrani *et al.*, 2003).

## 3.5 Limitations of this chapter

The results contained within this chapter would be strengthened if I had undertaken the following:

1. Alternative techniques for measuring cell proliferation which could include measurement of DNA synthesis using:
   a) Radiolabeled $^{3}$H-thymidine (Life Technologies, UK).
   b) 5-bromo-2'-deoxyuridine (BrdU), which is available as a commercial cell proliferation kit (Merck Millipore, UK) and measures BrdU incorporation into cellular DNA.

2. Measurements of apoptosis to determine whether any treatments are pro-apoptotic. For example, Annexin V can be used to identify cells at an early stage of apoptosis. It binds to phosphatidylserine, which very early on in the apotopic process translocates to the membrane and becomes exposed at the plasma membrane of cells. This process can be determined through using fluorescently labelled Annexin V (Life Technologies, UK).

3. Measurement of PDE3 expression (mRNA) and activity (ELISA) to confirm the hypothesis that this PDE isoform is elevated in PAH and contributes to the reduced effectiveness of prostacyclin analogues to increase cyclic AMP in PASMCs obtained from end-stage disease patients.

4. Assessment of the characteristics of the lysis buffer to ensure that PDE activity was fully inhibited. Because the composition of the lysis buffer used in cyclic AMP experiments is proprietary information, and specifically the PDE inhibitors (specific or non-specific) that are included are not disclosed, their potential influence on the sensitivity and the magnitude of final cAMP measurements remains an important undetermined factor which could have been verified by supplementing with IBMX

5. Optimisation of a defined growth medium, where the identity of all the added growth factors are known, would have aided the investigation of the proliferation response and allowed a more specific characterisation of the interaction with prostacyclin.

# 4. Prostacyclin interaction with the endothelin pathway.

## 4.1 Introduction

Endothelin-1 (ET-1) is an endogenous peptide with potent vasoconstrictor, mitogenic and profibrotic properties and appears to be implicated as a mediator of increased vascular tone and vascular remodelling in PAH (Yoshibayashi *et al.*, 1991; Giaid *et al.*, 1993). Among all organ systems in the body, the lung has the greatest level of ET-1 production from pulmonary vascular endothelium, smooth muscle and airway epithelium suggesting that this peptide is an important autocrine and/or paracrine modulator of airway function (Blouquit *et al.*, 2003). Immunoreactivity for ET-1 can be detected in most cell types of developing human lung including pulmonary vascular endothelial cells, neuroendocrine cells, smooth muscle cells, platelets and alveolar macrophages (Fabregat & Rozengurt, 1990; Giaid *et al.*, 1991; Yap *et al.*, 2000). In humans ET-1 effects are mediated by binding to two receptors $ET_A$ and $ET_B$, both of which induce contraction in distal pulmonary resistance arteries, whereas the $ET_A$ receptor alone appears to mediate the response in proximal arteries (McCulloch & Maclean, 1995; McCulloch *et al.*, 1996). Activation of endothelial $ET_B$ receptors releases vasodilators which can then act on smooth muscle cells to cause vasodilation (Takayanagi *et al.*, 1991). Moreover evidence suggests that selective $ET_B$ receptor blockade increases the tissue accumulation of intravenously administered radiolabelled ET-1 indicating that $ET_B$ functions as a clearance receptor (Luscher & Barton, 2000; Kedzierski & Yanagisawa, 2001). An upregulation in vascular $ET_A$ and $ET_B$ expression has been

demonstrated in experimental models of systemic hypertension and is associated with augmented responses to ET-1.

The proliferative effects of ET-1 on PASMCs isolated from the main human pulmonary artery are also considered to be dependent on $ET_A$ receptors (Zamora *et al.*, 1993), but the contribution of $ET_A$ and $ET_B$ receptors in regulating the growth of cells from distal resistance vessels is more complex. ET-1 has an influence on DNA synthesis, cell proliferation and cellular hypertrophy. The mitogenesis caused by ET-1 involves activation of multiple transduction pathways, such as the production of second messengers, the release of intracellular calcium, and influx of extracellular calcium (Kawanabe & Nauli, 2011). ET-1 acts synergistically with various regulatory and growth factors to regulate cellular transformation and replication (Dube *et al.*, 2000). Several of these factors in turn stimulate the synthesis and release of endothelins. The synthesis and release of endothelins are also increased in acute and chronic pathological conditions such as atherosclerosis, hypertension, and carcinogenesis (Battistini *et al.*, 1993). The endothelin converting enzyme-1 (ECE-1) and endothelin degrading enzymes coexist in the airway tissue and levels of ECE-1 are elevated in patients with chronic rhinitis and idiopathic pulmonary fibrosis (Noguchi *et al.*, 1991; Saleh *et al.*, 1997). Elevation in plasma ET-1 levels in patients with various airway diseases and reduction in the symptoms when ET-1, ECE-1 and their receptors are antagonized provides evidence that these peptides are not only important in normal physiology but also in pathogenesis of lung diseases.

A striking increase in the expression of ET-1 mRNA and increased plasma levels of ET-1 are detected both in patients with most forms of PAH (Stewart *et al.*, 1991; Yoshibayashi *et al.*, 1991) and in experimental models of PAH (Stelzner *et al.*, 1992; Miyauchi *et al.*, 1993). Elevated levels of ET-1 in PAH have been shown to be correlated with increased right atrial pressure and pulmonary vascular resistance and decreased pulmonary artery oxygen saturation (Cacoub *et al.*, 1993a; Cacoub *et al.*, 1993b; Nootens *et al.*, 1995; Galie *et al.*, 2004). Also, immunoreactivity for ECE-1 is augmented in the endothelium of lung arteries of patients with pulmonary diseases (Saleh *et al.*, 1997; Giaid, 1998). Increased ET-1 is also associated with a reduction in expression of endothelial nitric oxide synthase (eNOS) (Giaid & Saleh, 1995). In patients with IPAH, levels of ET-1 are three to four times the upper limit seen in normal patients, and this correlates with worsening of haemodynamic parameters, 6MWD and survival (Rubens *et al.*, 2001). Despite a strong correlation of ET-1 levels with worsening of pulmonary haemodynamics and survival in humans, the overexpression of ET-1 alone does not result in PH in transgenic animals (Hocher *et al.*, 2000). This suggests that additional mechanisms must be driving the disease process although one cannot ignore that once the disease is established, higher levels of ET-1 gives an extremely poor prognosis in IPAH. Lung tissue and pulmonary arteries from human subjects with PAH showed increased ET-1-binding capacity (Davie *et al.*, 2002). ET-1 expression is increased in the lungs of patients with IPAH and secondary PAH, particularly in the small muscular pulmonary arteries (Giaid *et al.*, 1993).

The lung is considered to be a major organ for clearance of the plasma ET-1 (de Nucci *et al.*, 1988; Dupuis *et al.*, 1996a). In animal models, selective blockade of $ET_B$ receptor in the lung has been shown to abolish the uptake of ET-1 in the pulmonary circulation, suggesting that uptake in the lung is solely via the $ET_B$ receptor (Kelland *et al.*, 2010). The capacity to clear ET-1 from the circulation in animal models and in the human lung is shown to be reduced in various types of PAH suggesting that a reduction in $ET_B$ receptor activity occurs in PAH (Dupuis *et al.*, 2000; Dupuis *et al.*, 1998). This contrasts with another study where endothelial $ET_B$ receptor mediated clearance appears preserved in many PAH patients (Dupuis *et al.*, 1998). It has been reported that smooth muscle cells are an important source of ET-1 production in the pulmonary artery of the rat. The expression of big ET-1 and $ET_A$ receptors was markedly increased in the distal segments in hypoxic rats compared to normal conditions (Takahashi *et al.*, 2001). The cause of the modifications in the proportion of $ET_A$ and $ET_B$ receptors by the various pathological conditions and the crosstalk between them remains to be established.

The ET-1 system can be dampened by the inhibition of ECE-1 and by the blockade of ET-1 receptors. Blockade of the endothelin-receptors, has been shown to be an effective strategy in the management of PAH. Despite this, it is not clear whether the agents that selectively block $ET_A$ receptors are better than those blocking both $ET_A$ and $ET_B$ receptor subtypes (O'Callaghan *et al.*, 2011). Inhibition of ECE-1 reduces the production of ET-1 but the effectiveness of these drugs is limited by independent pathways contributing to ET-1 formation, such as chymase

and metalloproteases (Takahashi *et al.*, 1998). Therefore a more efficient approach is to antagonize the ET-1 system by using ET-1 receptor antagonists that can block either $ET_A$ alone (e.g. sitaxentan) or both $ET_A$ and $ET_B$ receptors (bosentan). Currently, several peptides and non-peptide compounds that block ET-1 receptors are available, and some have been tested in both animal models and clinical trials in patients with IPAH.

In clinical practice the combination of bosentan with prostacyclins is widespread but complicated by difficulties with administration of prostacyclin analogues (Wozencraft *et al.*, 2012). Thus, it is important to evaluate the impact of therapeutic agents on ET-1 levels either as a monotherapy or in combination. Earlier studies suggested that treatment of IPAH with chronic intravenous epoprostenol improves the abnormal net balance of pulmonary clearance and release of ET-1 (Langleben *et al.*, 1999). Curiously this is the only positive clinical report of prostacyclin use, although there was a similar trend shown with iloprost where treating PAH patients with inhaled iloprost lead to increased pulmonary clearance of big ET-1 (Wilkens *et al.*, 2003). This indicates the need for more studies focussing on monotherapy to learn their individual effects and to rule out the possible negative drug-drug interaction when used in combination. In view of the possibility that inhibition of ET-1 release may be a therapeutically important mechanism underlying the antiproliferative effects of prostacyclin analogues (Wort *et al.*, 2001), I sought to measure ET-1 production in primary human pulmonary arterial smooth muscle

cells (PASMCs). It is unclear if other prostacyclin analogues such as treprostinil have similar effects on ET-1 production and also if blockade of smooth muscle ET-1 receptors can regulate ET-1 levels. Therefore, I sought to assess the effect of treprostinil and ET-1 receptor antagonists (ETRAs) on ET-1 levels in adult and child PASMCs. Administration of either a non-selective ET-1 receptor antagonist (bosentan) or an $ET_A$ selective antagonist (ambrisentan) is able to improve haemodynamic indices in patients with PAH (Channick *et al.*, 2001). Although it remains unclear whether selective blockade of the receptors would be more beneficial than a non-selective blockade, this is now more difficult to assess since the highly selective $ET_A$ antagonist sitaxentan (Davie *et al.*, 2009) has now been withdrawn. I have endeavoured to assess the effects of $ET_A$ selective, $ET_B$ selective and non-selective $ET_A/ET_B$ receptor antagonism alone or in combination with treprostinil to study the scope of combination of these agents for IPAH treatment and to study the mechanistic pathways involved.

## 4.2 Results

### 4.2.1 Effect of treprostinil and endothelin receptor antagonists on ET-1 production

First, I wished to establish if treprostinil inhibited ET-1 production in PASMCs isolated from IPAH patients stimulated by serum. ET-1 levels were measured in the supernatants of cells from either growth arrested human PASMCs or those stimulated with FBS for 24 hours. In the absence of any growth factors, a basal

level of ~2pg/mg protein could be detected in the supernatants (figure 4.1). Following a 24 hour stimulation with FBS, levels rose 2 fold ($p < 0.001$), an effect completely abolished by the cell permeable ECE-1 inhibitor, CGS 35066 or by increasing doses of (1-1000nM) treprostinil (figure 4.1).

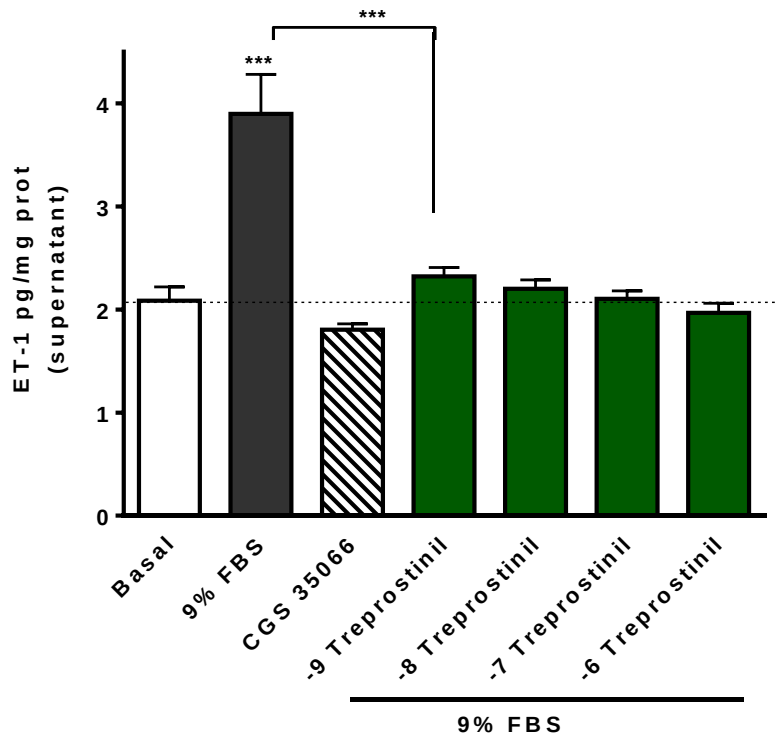



**Figure 4.1. Treprostinil potently inhibits serum-induced ET-1 levels in cultured human PASMCs from IPAH patients**. Measurement of ET-1 was made in the supernatant of distal PASMC isolates. Growth arrested PASMCs were treated for 24 hr with basal media or 9% serum (FBS) in the presence and absence of treprostinil (Trep; 1-1000 nM). CGS 35066 (1 μM), an endothelin-converting enzyme (ECE-1) inhibitor was used to validate the assay and confirm synthesis of mature ET-1. Data are mean ± SEM, from nine different experiments performed in duplicate using five PASMC isolates. ***=$p < 0.001$ when compared to basal or as shown (one-way ANOVA with Bonferroni post-hoc correction).

In addition, inhibition of serum induced ET-1 release by treprostinil occurred after 24 h of treatment at a concentration as low as 1 nM ($p < 0.001$; n=9), with a complete return to basal at a concentration between 100 and 1000 nM. Given the role of endothelial $ET_B$ receptors in clearing ET-1, next I wished to compare the effects of selective $ET_B$ and $ET_A$ receptor inhibition on ET-1 levels in smooth muscle cells. When used alone, the $ET_B$ receptor antagonists increased ET-1 levels significantly compared to

FBS alone (figure 4.2). The highly specific $ET_B$ antagonist BQ788 (100 nM) increased ET-1 levels by ~4 fold. Bosentan, the dual antagonist, increased levels by 3 fold over and above that increased by FBS alone ($p < 0.001$; n=6). No increase in ET-1 production was observed with highly specific $ET_A$ antagonist, BQ123 (figure 4.2). However, the supposedly $ET_A$ specific antagonist, ambrisentan also increased ET-1 levels to a similar degree to bosentan.

Next I wished to assess whether ETRAs interfered with treprostinil inhibition of ET-1 levels, particularly as bosentan and ambrisentan are commonly given clinically in combination with prostacyclin (epoprostenol) or an analogue. Surprisingly inhibition of serum induced ET-1 release from PASMCs by inhibition of ET-1 production with 1 nM treprostinil was not observed in the presence of 1 µM bosentan ($p< 0.001$) (figure 4.3). Moreover, bosentan when combined with 1 µM treprostinil, resulted in a significant reduction in the ability of treprostinil to inhibit ET-1, ($p< 0.001$, n=6) despite the higher analogue concentration (figure 4.3). To further investigate whether reversal of ET-1 inhibition with bosentan was

mediated via $ET_A$ or $ET_B$ receptors or both, inhibition of serum induced ET-1 release by treprostinil was assessed in combination with the relatively specific $ET_A$ receptor antagonists ambrisentan, BQ123 and the selective $ET_B$ antagonist, BQ788. This effect of bosentan seemed to be related to $ET_B$ blockade as combining treprostinil with a highly selective $ET_A$ receptor antagonist (BQ 123) meant that treprostinil could still fully inhibit FBS induced ET-1 levels in a similar manner to that without the antagonist. Furthermore the highly specific $ET_B$ antagonist (BQ 788) significantly increased ET-1 levels beyond that of FBS alone despite the presence of 1 µM treprostinil. However, ambrisentan which is classified as a highly specific $ET_A$ antagonist, behaved similarly to bosentan as opposed to behaving like BQ 123. This latter observation makes me question the specificity of this drug in our system (figure 4.4). Thus the ability of treprostinil to potently inhibit ET-1 levels in human PASMCs is adversely affected by ETRAs targeting $ET_B$ but not $ET_A$ receptors. The potential upshot clinically might be that when treprostinil is used in combination with bosentan and perhaps ambrisentan, this agent may end up as being less effective and/or higher concentrations required (close to 100 nM in this case) before treprostinil can begin to significantly inhibit serum-induced ET-1 levels in patients. In contrast, BQ 123 (selective $ET_A$ antagonist) did not affect the ability of treprostinil to inhibit ET-1 while BQ788, the highly selective $ET_B$ receptor blocker, showed the greatest antagonism to the treprostinil (figure 4.4).

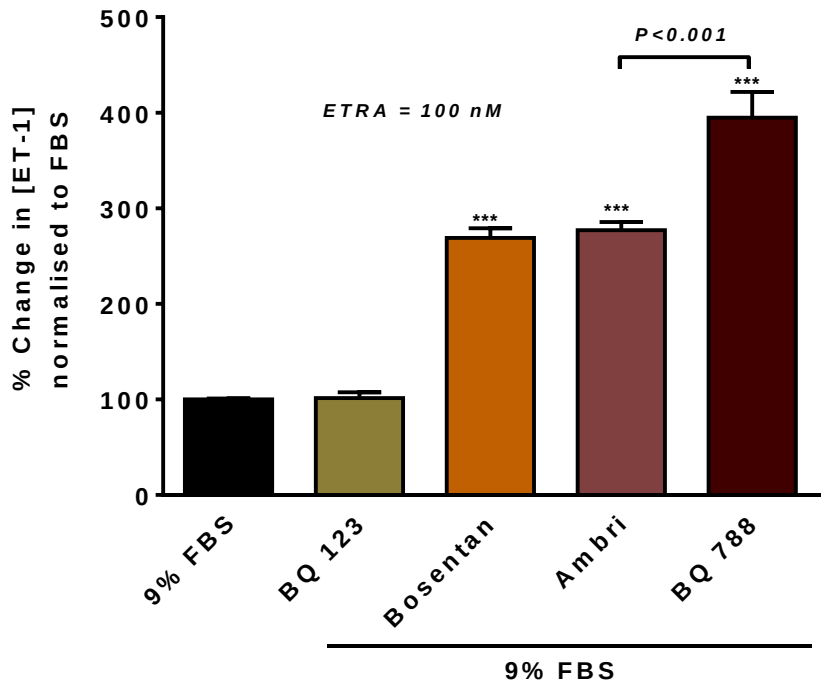



**Figure 4.2. $ET_B$ but not $ET_A$ selective antagonists increase ET-1 levels in cultured human PASMCs.** Measurement of ET-1 in the supernatants from distal PASMC isolates. The effect of selective inhibitors that block either $ET_A$ (BQ-123; Ambrisentan) or $ET_B$ (BQ-788) receptors or both (bosentan) on ET-1 levels measured in the supernatant of PASMCs isolated from IPAH patients.Growth arrested PASMCs were treated for 24 hr with 9% serum (FBS) in the presence and absence of clinically relevant doses (100 nM) of endothelin receptor antagonists. Data are mean ± SEM, of 6 experiments measured in duplicates from five PASMC isolates and normalised to increase in ET-1 induced by 9% FBS. ***=p< 0.001 versus 9% FBS (one-way ANOVA with Bonferroni post-hoc correction).

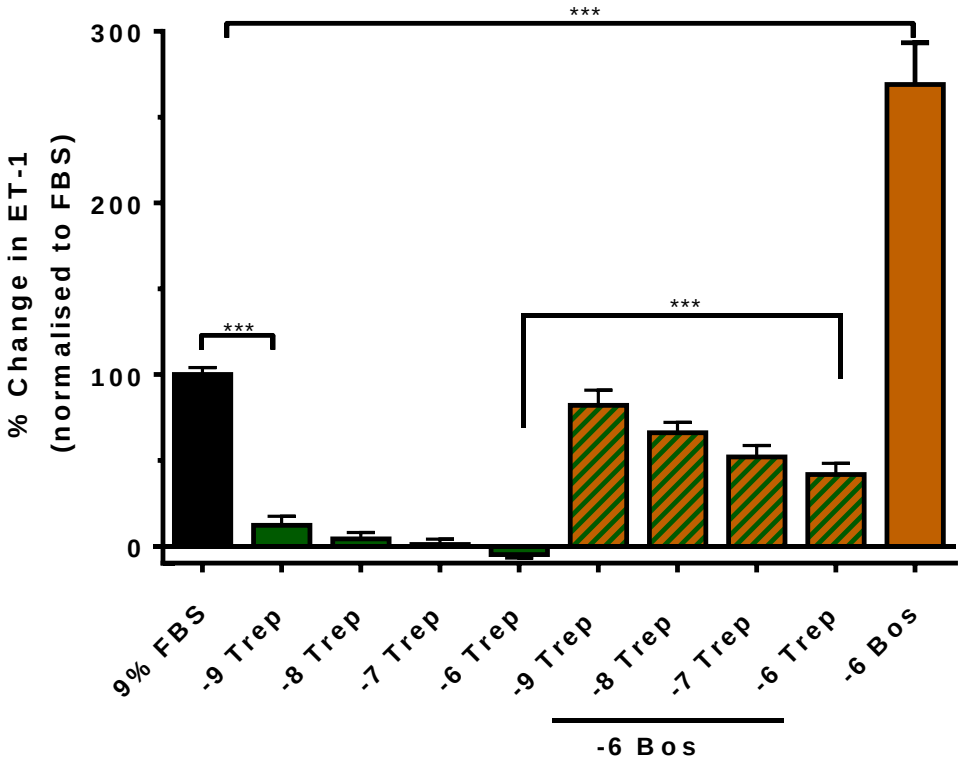



**Figure 4.3. Treprostinil inhibition of ET-1 production in IPAH cells is blunted by the mixed ET-1 receptor antagonist, bosentan.**. Growth arrested human PASMCs were treated for 24 hr with 9% serum (FBS) in the presence and absence of treprostinil (Trep; 1-1000nM), bosentan (1 µM) or in combination with bosentan (1 µM). Following treatment, ET-1 was measured in the supernatants of distal PASMC isolates grown in culture. Data are mean ± SEM, for nine experiments measured in duplicates from five PASMC isolates and normalised to increase in ET-1 induced by 9% FBS. ***=$p < 0.001$ as shown (one-way ANOVA with Bonferroni post-hoc correction).

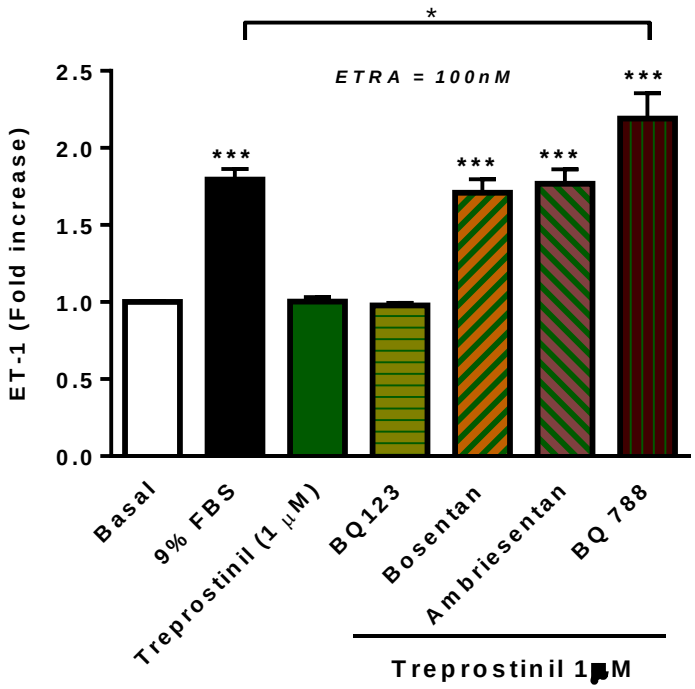



**Figure 4.4. Treprostinil inhibition of ET-1 production in IPAH cells is blunted by mixed ETRAs but not by the highly selective $ET_A$ receptor antagonist, BQ123.** Growth arrested PASMCs were treated for 24 hr with serum (9% FBS) alone or in the presence of treprostinil (1 μM) with or without endothelin receptor antagonists (ETRA; 100 nM). ET-1 released in the supernatants from distal human PASMC isolates was normalised to the ET-1 levels measured in growth-arrested cells at the same time point (basal). Data are expressed as mean ± SEM and are from nine separate experiments performed using five PASMC isolates. *=p < 0.05, ***=p < 0.001 versus basal or as indicated (one-way ANOVA with Bonferroni post-hoc correction).

### 4.2.2 Role of ECE-1 and $ET_B$ receptors

In order to assess the mechanism of mixed antagonists counteracting the effect of treprostinil, I examined the effect of these agents on both ECE-1 and $ET_B$ expression. It is possible that ET-1 levels may not only be regulated through $ET_B$

receptors, but also by the levels of ECE-1 expression and activity. In four separate experiments, ECE-1 protein levels in serum-stimulated human PASMCs were significantly higher with bosentan ($p < 0.001$, n=4) compared to either serum or treprostinil (figure 4.5). There was a trend to lower ECE-1 protein levels with treprostinil alone, though in the presence of bosentan these were similar to those seen with bosentan alone. Thus $ET_B$ receptors as well as promoting clearance may also regulate ET-1 levels through changes in ECE-1 protein expression. The ECE-1 protein levels in the presence of FBS were even more prominently increased with $ET_B$ antagonist BQ788 increasing by 3 fold. In contrast, ECE-1 expression was not significantly changed with either with BQ123 or ambrisentan (figure 4.6). Next I investigated if ECE-1 expression may be related to the ability of the ETRAs to increase ET-1 levels, as ET-1 itself significantly increased ECE-1 expression in a concentration-dependent fashion in these cells after 24 hours of treatment ($p< 0.05$, n=4) (figure 4.7).

Lung endothelial $ET_B$ receptors promote ET-1 clearance, which can be improved by $PGI_2$ in PAH patients (Langleben *et al.*, 1999). In view of this, I assessed if treatment with treprostinil could affect $ET_B$ expression on PASMCs and contribute to the mechanism of treprostinil induced inhibition of ET-1 in these cells. There was a trend showing increased $ET_B$ expression in these cells with treprostinil (1 µM), but when cells were treated with ETRAs, $ET_B$ expression was substantially decreased after 24 hours of treatment ($p < 0.05$, n=4). Surprisingly, $ET_B$ expression in human PASMCs was affected to more or less the same extent with both specific

and non-specific antagonists including with the specific $ET_A$ receptor antagonists (figure 4.8).

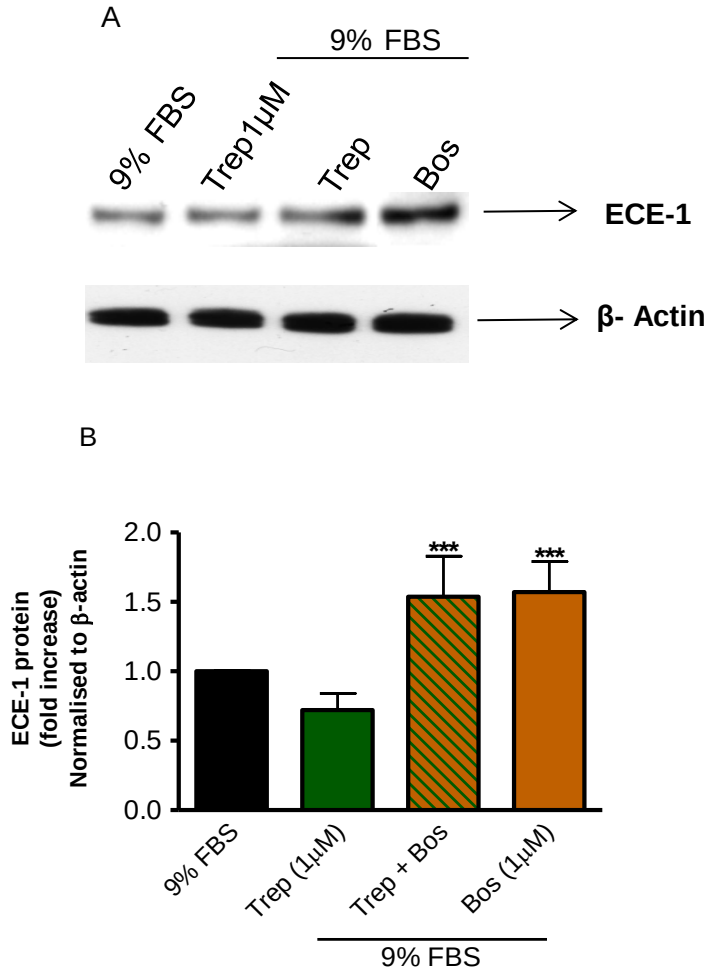



**Figure 4.5. Bosentan increases endothelin converting enzyme (ECE-1) protein levels in the absence and presence of treprostinil.** Growth-arrested cells were stimulated with 9% serum in the presence or absence of treprostinil (1 µM), bosentan (1 µM) or a combination of both drugs. Representative Western blots of crude homogenates of cultured human PASMCs derived from IPAH patients, where 10µg of protein was loaded in each lane is shown in (A). The blot was sequentially stained with an antibody against ECE-1 with a β-actin antibody used as a loading control. (B) Bottom figure shows relative protein expression as band intensity compared to β-actin. Results are mean ± SEM of 4 experiments. *** $p < 0.001$ versus 9 % FBS (one-way ANOVA with Bonferroni corrections).

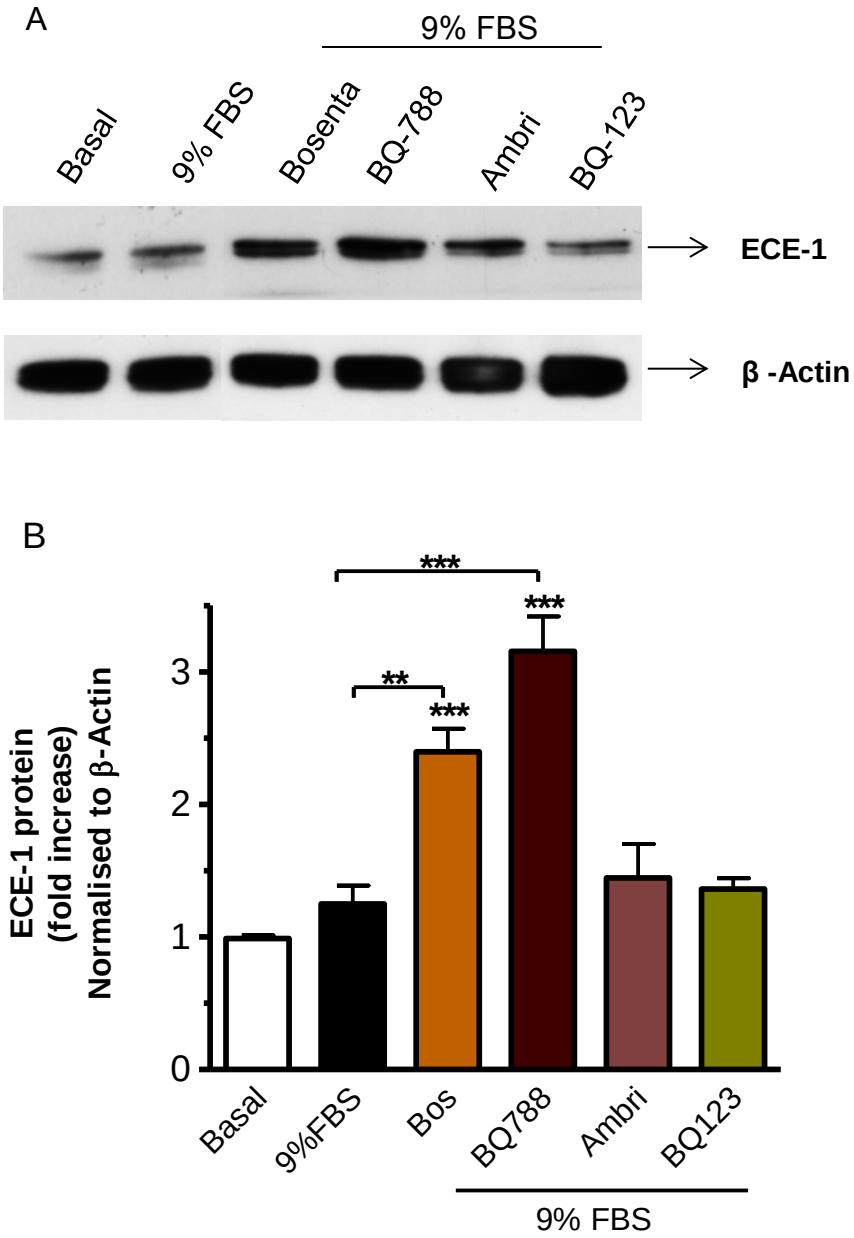



**Figure 4.6. $ET_B$ receptor antagonists increase ECE-1 protein levels in PASMCs from IPAH patients.** Cells were stimulated with 9% serum in the presence or absence of ETRAs (bosentan; BQ788; BQ123; ambrisentan), all administered at 1μM. (A) Representative Western blots of crude homogenates of cultured PASMCs where 10 μg of protein was loaded in each lane. The blot was sequentially stained with an ECE-1 antibody with a β-actin antibody used as a loading control. (B) ECE-1 protein levels relative to band intensity of β-actin and shown normalised to ECE-1protein levels measured in growth arrested cells. Results are mean ± SEM of 4 experiments. *** =$p < 0.001$ versus 9 % FBS (one-way ANOVA with Bonferroni post-hoc correction.

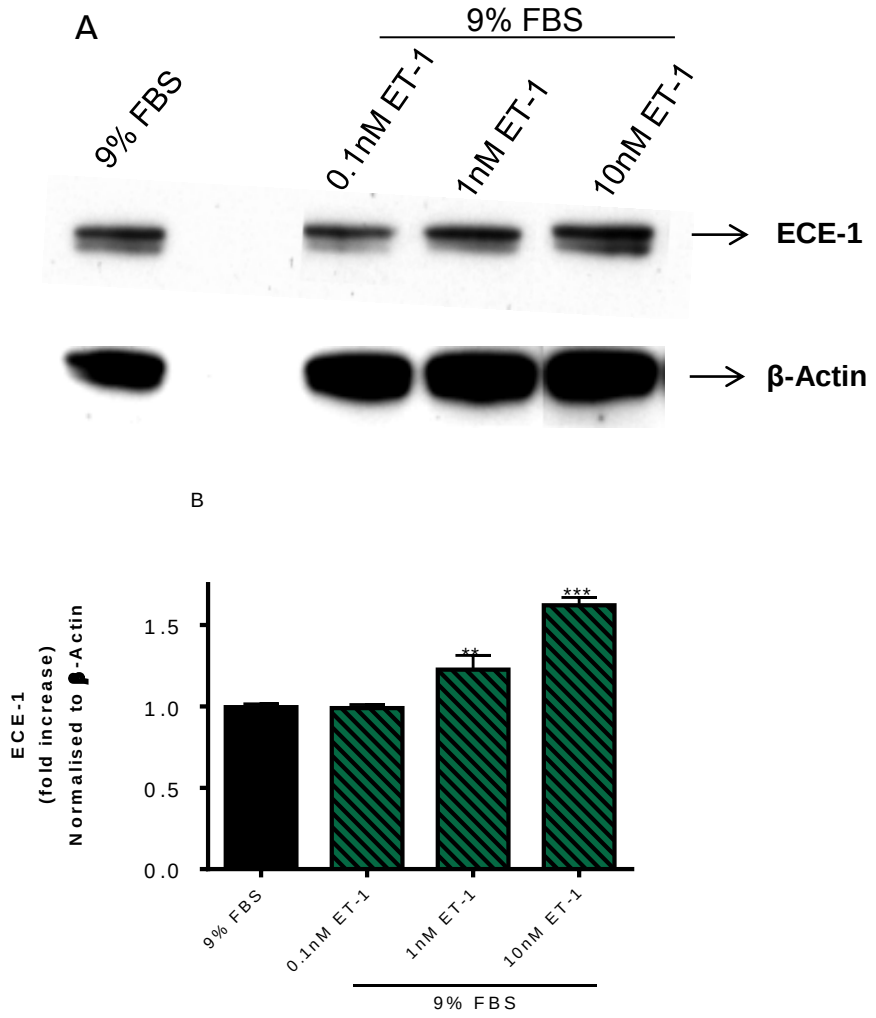



**Figure 4.7. Endothelin increases ECE-1 expression in a dose-dependent fashion in IPAH cultured pulmonary artery smooth muscle cells**. Changes in ECE-1 protein levels were measured after 24h in distal PASMC isolates in response to increasing doses ET-1 (0.1 – 10nM). (A) Representative Western blots of crude homogenates of cultured PASMCs where 10μg of protein was loaded in each lane. The blot was sequentially stained with an ECE-1 antibody with a β-actin antibody used as a loading control. (B) ECE-1 protein levels relative to band intensity of β-actin and shown normalised to ECE-1protein levels measured in cells treated with FBS alone. Results are mean ± SEM of 4 experiments. **=$p < 0.01$, ***=$p < 0.001$ versus 9% FBS (one-way ANOVA with Bonferroni post-hoc corrections).

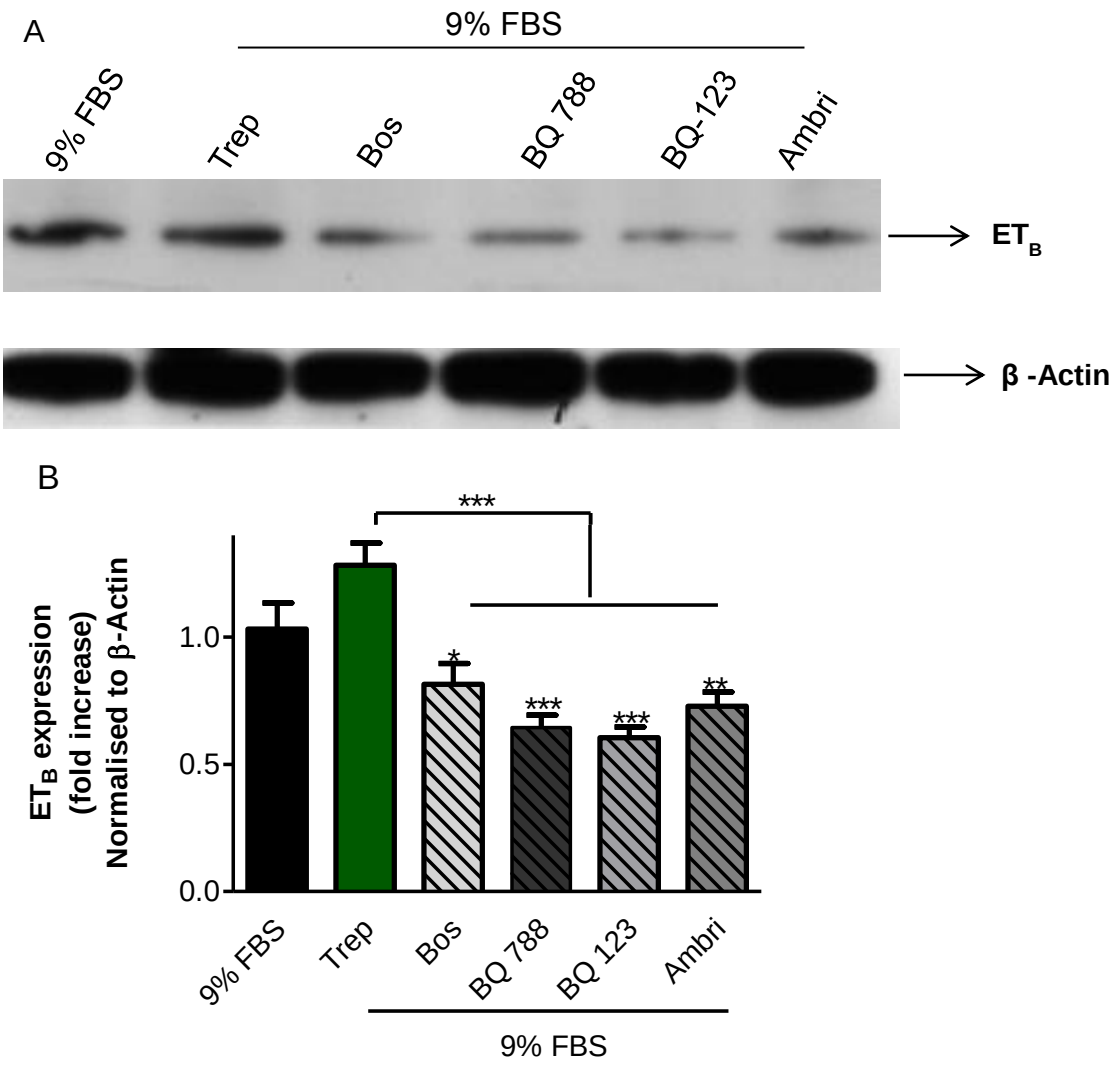



**Figure 4.8. Endothelin receptor antagonism decreases the expression of $ET_B$ receptors in distal pulmonary artery smooth muscle cells from IPAH patients.** Changes in $ET_B$ receptor protein levels were measured in response to treatment with treprostinil or ETRAs. Growth arrested cells were stimulated for 24 hours with 9% FBS in the presence or absence of treprostinil (100nM) or ETRAs (bosentan, BQ788, BQ123, ambrisentan) all administered at 1 µM to cells. (A) Representative Western blots of crude homogenates of cultured PASMCs where 10µg of protein was loaded in each lane. The blot was sequentially stained with an $ET_B$ antibody with a β-actin antibody used as a loading control. (B) $ET_B$ protein levels relative to band intensity of β-actin are shown normalised to $ET_B$ protein levels measured in cells treated with FBS alone. Results are mean ± SEM of 4 experiments. *=$p < 0.05$, **=$p < 0.01$ and ***=$p < 0.001$ (one-way ANOVA with Bonferroni post-hoc correction).

### 4.2.3 Effect of ETRAs versus treprostinil on cell proliferation.

After assessing the effects of these agents on ET-1 levels, I sought to assess their ability to inhibit proliferation of human PASMCs. Treprostinil, as described in the earlier chapter, concentration-dependently inhibited proliferation of human PASMCs, significantly inhibiting proliferation induced by FBS at concentrations ≥ 1nM (figure 3.5 and 3.6). Next proliferation assays were performed using ETRAs, bosentan, ambrisentan and BQ788. ETRAs only inhibited proliferation in these cells at higher doses, with bosentan significantly inhibiting proliferation compared to solvent control (DMSO) at 100 nM ($p < 0.01$, n=5) (figure 4. 9) and ambrisentan ≥10nM ($p < 0.01$, n=5) (figure 4.10). Ambrisentan was slightly more effective than bosentan, significantly inhibiting proliferation from 10 nM compared to solvent control. The selective $ET_B$ antagonist, BQ788 appeared the least effective amongst the three antagonists, only inhibiting proliferation by ~20% at 1μM ($p < 0.01$, n=5) (figure 4.11). Further when bosentan was assessed in combination with treprostinil, no additive/synergistic effect on proliferation of human PASMCs was observed. On the contrary there was trend at doses above 0.1nM for these two agents to be slightly less effective at inhibiting proliferation compared to treprostinil on its own (n=5; figure 4.12).

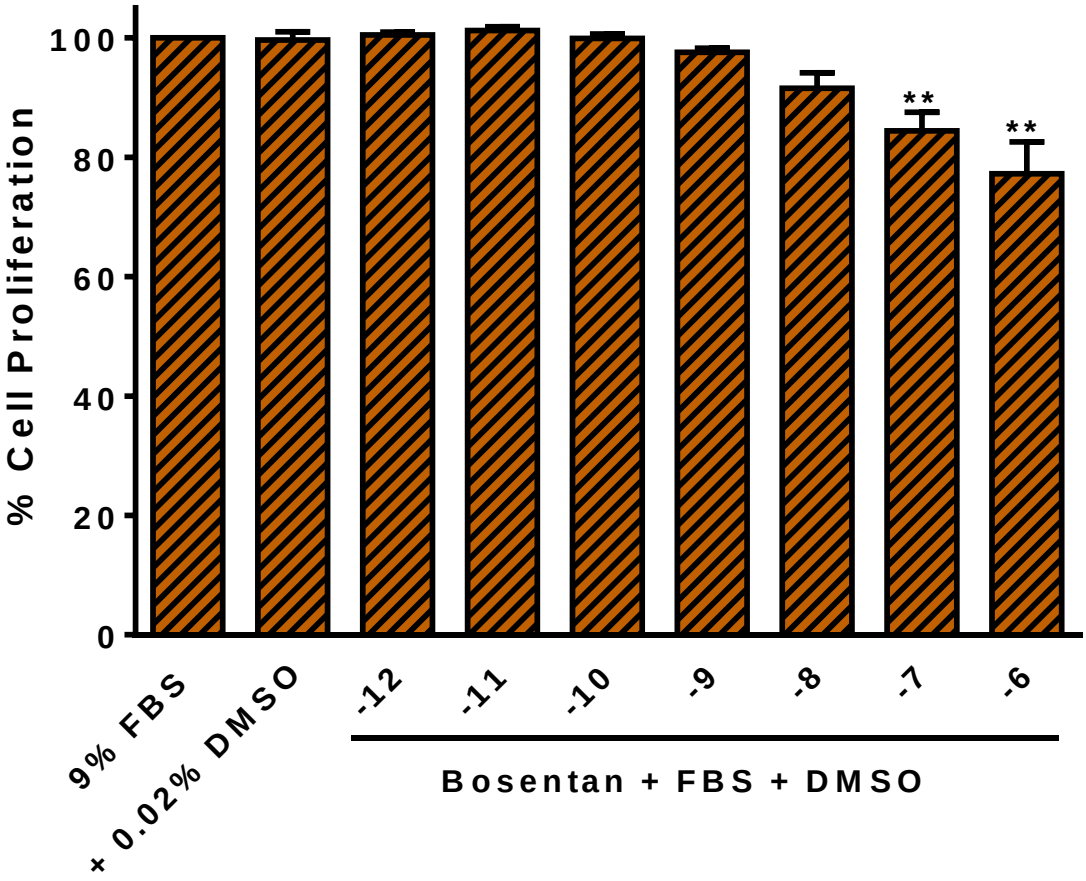



**Figure 4.9. Effect of bosentan on FBS-induced growth in human PASMCs derived from IPAH patients.** Human PASMCs were grown in 9% FBS for 4 days in the absence and presence of increasing concentrations of bosentan (0.001-1000 nM). Data have been normalised to the growth response induced by FBS and expressed as % cell proliferation. * =$p < 0.05$ and **=$p < 0.01$ when compared to solvent (DMSO) control (One-way ANOVA, Dunnett's post-hoc, n=5).

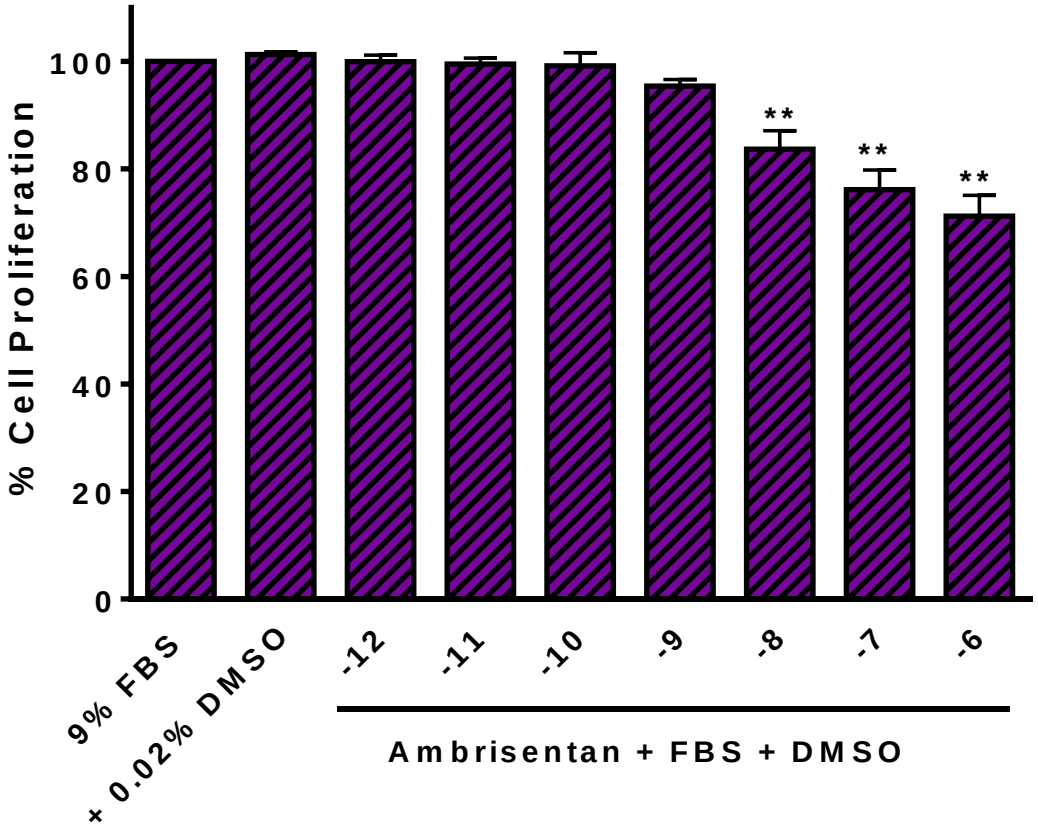



**Figure 4.10. Effect of ambrisentan on FBS-induced growth in human PASMCs derived from IPAH patients.** Human PASMCs were grown in 9% FBS for 4 days in the absence and presence of increasing concentrations of ambrisentan (0.001-1000nM). Data have been normalised to the growth response induced by FBS and expressed as % cell proliferation. *= $p < 0.05$ and **=$p < 0.01$ when compared to solvent (DMSO) control (One-way ANOVA, Dunnett's post-hoc, n=5).

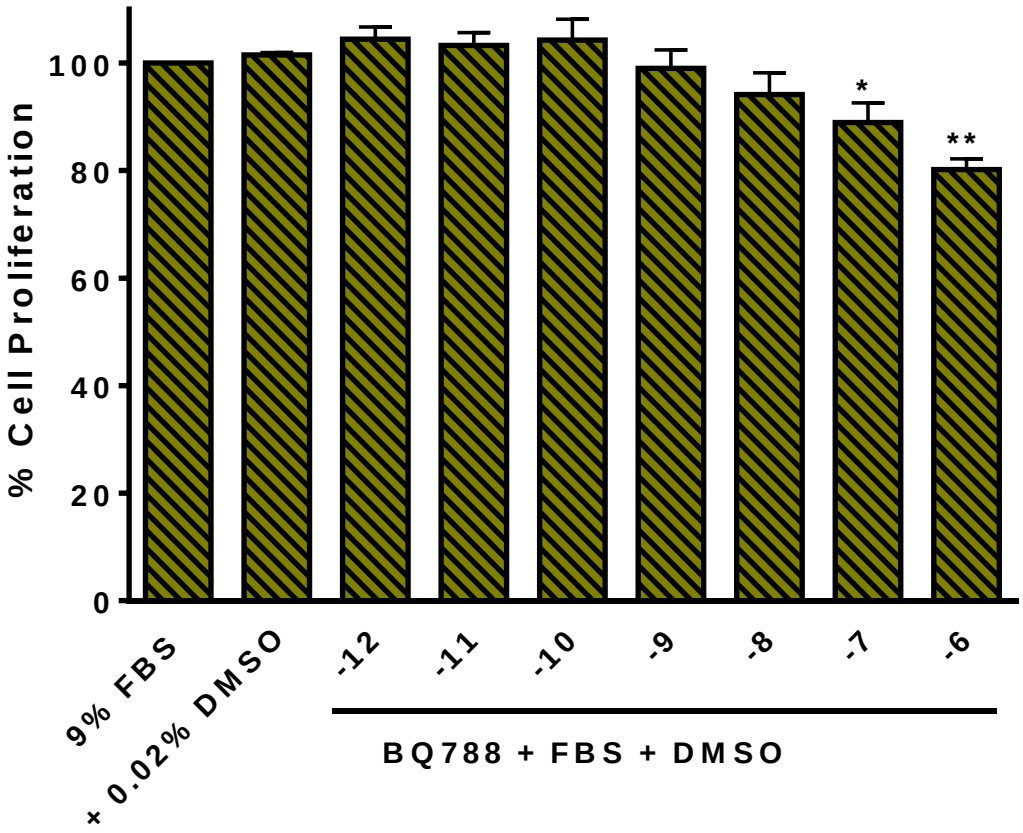



**Figure 4.11. Effect of $ET_B$ selective antagonist, BQ-788 on FBS-induced growth in human PASMCs derived from IPAH patients.** Human PASMCs derived from IPAH patients were grown in 9% FBS for 4 days in the absence and presence of increasing concentrations of BQ 788 (0.001-1000nM). Data have been normalised to the growth response induced by FBS and expressed as % cell proliferation. *= $p < 0.05$ and **=$p < 0.01$ when compared to solvent (DMSO) control (One-way ANOVA, Dunnett's post-hoc, n=5).

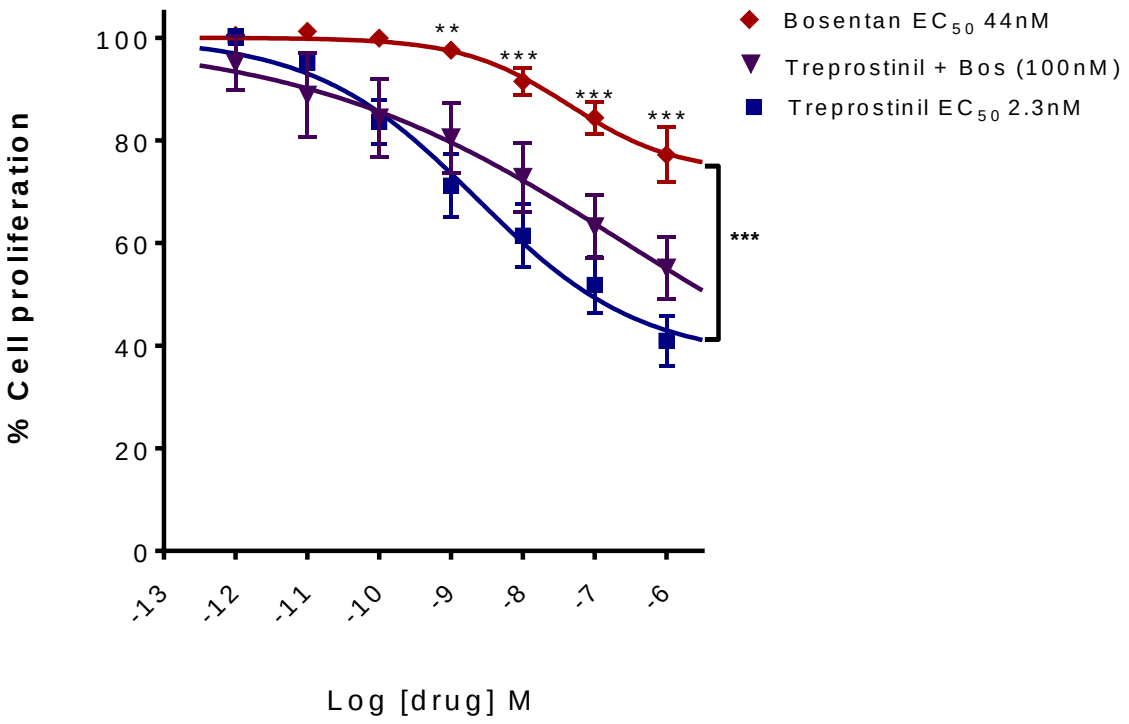



**Figure 4.12. No additive/synergistic effect of bosentan and treprostinil treatment on proliferation of human pulmonary arterial smooth muscle cells derived from IPAH patients.** Human PASMCs were grown in 9% FBS for 4 days in the absence and presence of increasing concentrations of treprostinil (0.001-1000 nM), bosentan (0.001-1000 nM) or a combination of treprostinil (0.001-1000 nM) with bosentan (100 nM). Data have been normalised to the growth response induced by FBS and expressed as % cell proliferation. Mean data are fitted using a variable slope, sigmoidal fitting routine in GraphPad Prism. **=$p < 0.01$ and ***=$p < 0.001$ when compared to treprostinil (two-way ANOVA, with Bonferroni post-hoc correction; n=5).

### 4.2.4 Expression of ECE-1 and $ET_B$ in normal versus IPAH lung.

In PAH, the cells from the arterial wall undergo a series of morphological changes, including changes in the cellular and matrix components of the three layers - intima, media, and adventitia (Tuder *et al.*, 2007). Expansion of the intimal layer of arteries due to cellular proliferation and extracellular matrix deposition and these proliferating cells are phenotypically smooth muscle cells expressing alpha smooth muscle actin (α-SMA) (Graham & Tuder, 2009). Also endothelial dysfunction or the loss of proper endothelial function, is a hallmark for PAH (Budhiraja *et al.*, 2004) and therefore the changes in the intima were studied by staining the cells with the endothelial cell specific adhesion molecule, also known as cluster of differentiation 31 (CD-31). Using immunostaining techniques, localisation of ECE-1 staining with the endothelial marker protein CD31, confirmed ECE-1 expression in the endothelial cells of both normal (figure 4.13A) as well as in lung sections form IPAH patients (figure 4.13B). When localisation of ECE-1 staining was compared with the smooth muscle cell marker protein, alpha smooth muscle actin (α-SMA), ECE-1 expression appeared far more strongly expressed in the medial smooth muscle layer of the IPAH lung compared to that of the normal lung, where the ECE-1 stain was very faint (Figure 4.13). When comparison of $ET_B$ expression in normal and IPAH lungs were performed using similar technique, the differential staining intensities in both the intimal layers and in medial smooth muscle was significantly increased in IPAH versus normal pulmonary arteries. In IPAH,

$ET_B$ expression in both the medial smooth muscle and endothelium of pulmonary arteries was markedly increased compared with control lung (figure 4.14).

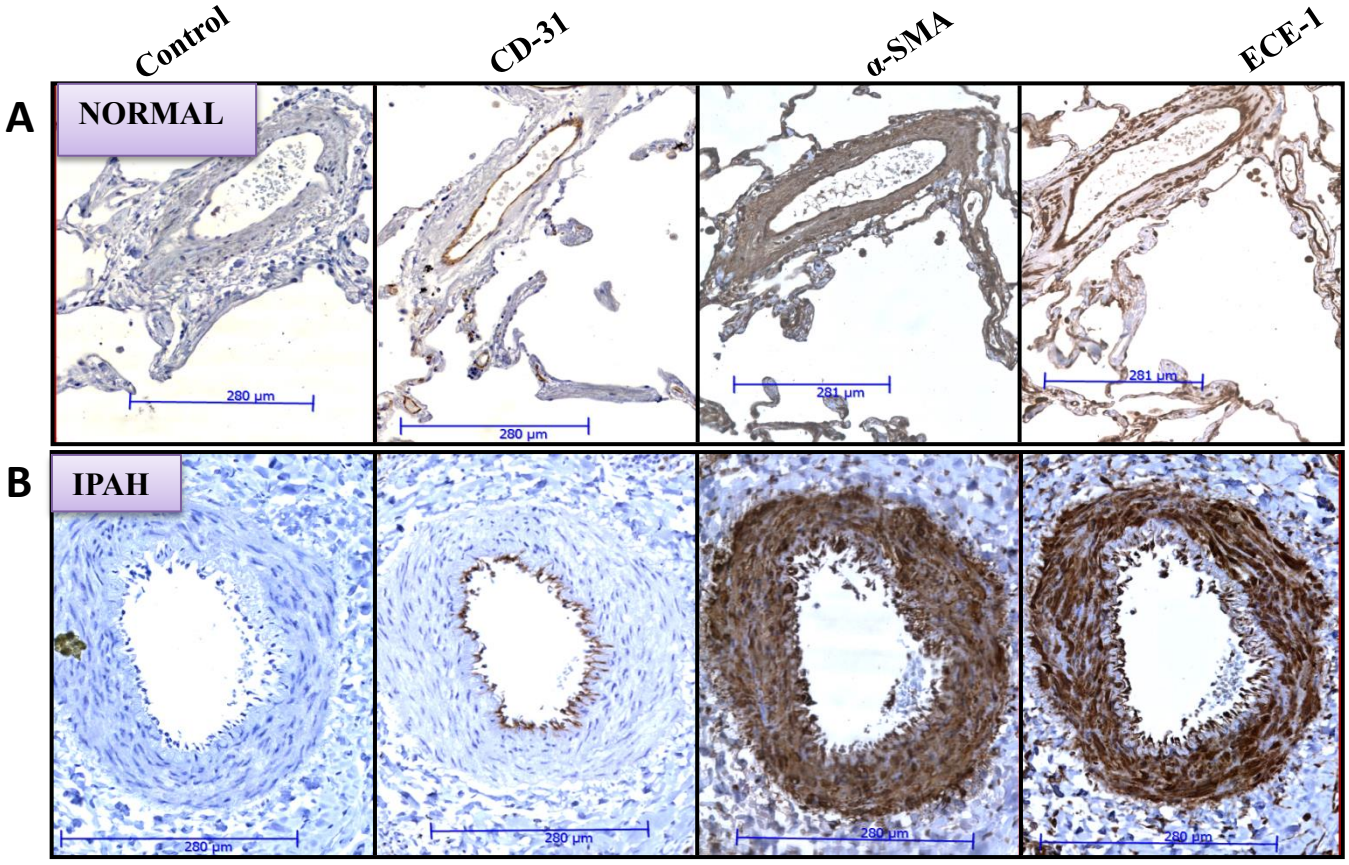



**Figure 4.13. Increased expression of endothelin converting enzyme-1 (ECE-1) in a PAH compared to a control lung.** Immunohistochemical staining for the endothelin converting enzyme 1 (ECE-1) visualised by diaminobenzidine (brown) in sections counterstained with haematoxylin. Adjacent sections of representative pulmonary artery of A) a control lung from a 55 year old male and B) an 18 year old female patient with idiopathic pulmonary arterial hypertension (IPAH) stained with a CD-31 antibody (staining the endothelium), an alpha smooth muscle actin (α-SMA) (staining the intimal-smooth muscle layer) and ECE-1 antibody.

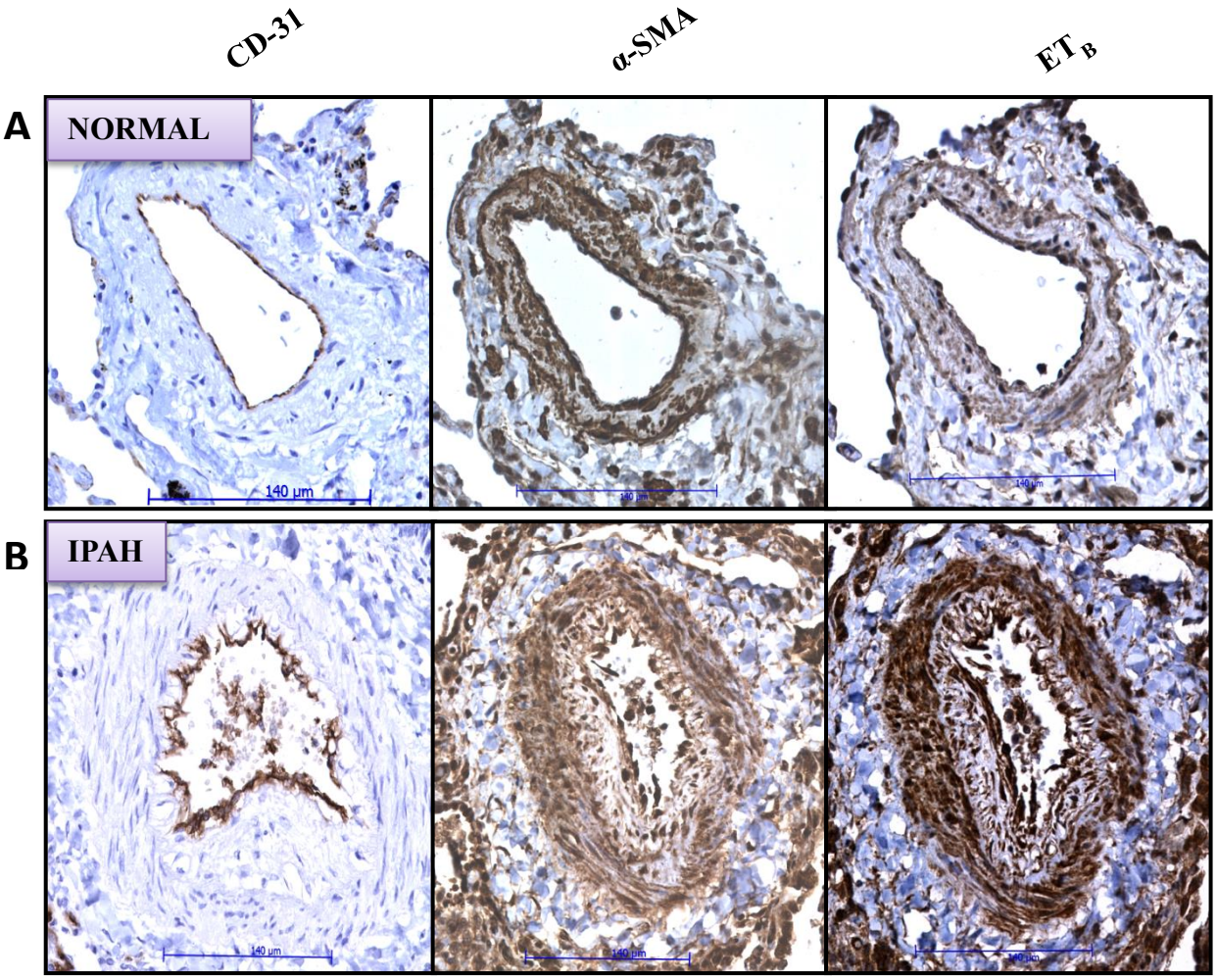



**Figure 4.14. Increased expression of $ET_B$ receptor observed in a PAH lung compared to a control lung.** Immunohistochemical staining for the $ET_B$ receptor visualised by diaminobenzidine (brown), in sections counterstained with haematoxylin. Adjacent sections of representative pulmonary artery of A) a normal lung from a 62 year old male and B) 18 year old female patient with idiopathic pulmonary arterial hypertension (IPAH) stained with CD-31 antibody (staining the endothelium), alpha smooth muscle actin (α-SMA) (staining the intimal-smooth muscle layer) and $ET_B$ antibody.

## 4.3 Summary of key findings

- Treprostinil potently inhibited serum induced ET-1 levels in PASMCs isolated from IPAH patients with end stage disease. In contrast, bosentan and ambrisentan significantly elevated ET-l levels above that seen with serum alone.
- Inhibition of ET-1 levels by treprostinil was significantly impaired by bosentan and ambrisentan, but not by the highly selective $ET_A$ antagonism, BQ 123.
- Bosentan, the highly selective $ET_B$ antagonist BQ 788, and possibly ambrisentan, all increased ECE-1 expression in human PASMCs from IPAH patients, while BQ 123 and treprostinil did not.
- $ET_B$ expression was downregulated by all ETRAs, but not by treprostinil.
- Ambrisentan shows characteristics of mixed antagonism and may reflect previously reported variable $ET_A/ET_B$ binding affinities (29-2000:1) (reviewed in (Davie *et al.*, 2009).
- ECE-1 expression is dramatically increased in the pulmonary arterial medial smooth muscle layer of IPAH compared to normal lungs.
- $ET_B$ expression appeared to be markedly increased in both endothelium and medial smooth muscle layers of IPAH lungs compared to normal lungs.

- Smooth muscle cells appear important in regulating ET-1 levels in two ways, one associated with the $ET_B$ receptor being linked to ET-1 clearance the other through up regulation of synthesis via increased ECE-1 expression.

- The potential implication of my work clinically is that higher concentrations of prostacyclin agents might be required in order to overcome the increased levels of ET-1 associated with current ETRA treatment.

- My data suggest that selective $ET_A$ antagonists may possibly be a better choice of treatment in PAH when combined with prostacyclin agents such as treprostinil. This does however remain to be further investigated.

## 4.4 Discussion

In this chapter, I have demonstrated that treprostinil could significantly inhibit serum induced ET-1 release from PASMCs of IPAH patients and this confirms other studies showing inhibition with the prostacyclin analogue, cicaprost. Neil Davie and colleagues showed that cicaprost inhibited serum and TGF-β stimulated ET-1 production in distal PASMCs isolated from normal patients by approximately 50% at a concentration of 100nM. This effect was mimicked by forskolin and IBMX, suggesting a cAMP-dependent mechanism was bringing about a reduction in ET-1 (Davie *et al.*, 2002).

On the other hand, prostacyclins like treprostinil can activate non-cAMP dependent pathways like PPARγ (Falcetti *et al.*, 2007), which in different studies have been shown to inhibit ET-1 levels (Satoh *et al.*, 1999). PPARγ activation can attenuate hypoxia-induced activation of HIF-1α, NF-κB and ET-1 signalling pathway components (Kang *et al.*, 2011). The cross-talk between PPARγ and nuclear factor of activated T-cells (NFAT) was reported in T lymphocytes (Chung *et al.*, 2003). Common stimuli of PAH such as hypoxia cause inflammatory responses in the pulmonary vasculature and NFAT is a potent activator of T cells regulating the expression of many inflammatory genes. This transcription factor is upregulated in the pulmonary artery walls and circulating inflammatory cells of PAH patients (Bonnet *et al.*, 2007). In PAH, increases in NFAT appear largely responsible for the down regulation of voltage-gated (Kv) current and the decreased Kv1.5 expression reported in human PASMCs from these patients, as well as the expression of pro-inflammatory cytokines and growth promoting genes (including ET-1) in a number of different cell types (Bonnet *et al.*, 2007). Endothelin induced cardiac hypertrophy is inhibited by activation of PPARγ, which is at least partly due to cross-talk between PPARγ and calcineurin/NFAT (Bao *et al.*, 2008). In several rat models of hypertension, treatment with PPARγ ligands, reduced ET-1 expression vasculature (Iglarz *et al.*, 2003). Collectively, these findings suggest that PPARγ activation can attenuate expression of ET-1 in cardiovascular tissues in response to a variety of stimuli and can attenuate ET-1-mediated signalling in selected models. These strategies could be applied to inhibit ET-1 signalling by targeting PPARγ in IPAH (Green *et al.*, 2011). Nonetheless, it seems reasonable to

postulate that treprostinil might be acting to inhibit multiple steps in the ET-1 signalling pathway by activating PPARγ and/or inhibiting NFAT. Thus clinically, treprostinil might provide both more effective endothelin signalling inhibition and simultaneously reduce other pathogenic pathways, to attenuate IPAH pathogenesis more effectively then ETRAs (Kang *et al.*, 2011). Activation of PPARγ by treprostinil may represent a previously unrecognised target to inhibit enhanced ET-1 signalling in the pathogenesis of IPAH. Inhibition of ET-1 release may also contribute, in part, to the anti-proliferative effects of $PGI_2$ analogues on distal human PASMCs (Wharton *et al.*, 2000; Falcetti *et al.*, 2010).

Next, I demonstrated that inhibition of ET-1 levels by treprostinil was also impaired by bosentan and ambrisentan, but not by the highly selective $ET_A$ antagonist, BQ 123. I also demonstrated that selective $ET_A$ receptor blockade in human PASMCs might be a better choice of therapeutic agent as this approach did not affect ET-1 release *in vitro*. By contrast selective $ET_B$ receptor blockade or non-specific blockade of the endothelin receptor might cause adverse effects associated with a rise in ET-1 concentrations. Consistent with this notion, plasma ET-1 concentrations were elevated upon administration of either bosentan or a highly selective $ET_B$ receptor antagonist either in heart failure (Cowburn *et al.*, 2005) or PAH (Hiramoto *et al.*, 2007; Hiramoto *et al.*, 2009) patients. In the former study, addition of an $ET_A$ selective antagonist on top of established $ET_B$ receptor blockade, led to pulmonary and systemic vasodilation without further increases in plasma ET-1 concentrations. These data suggest that the $ET_B$ receptor has a role in

the clearance of ET-1 and that blockade of the $ET_B$ receptor may not be beneficial for patients with heart failure (Cowburn & Cleland, 2001; Cowburn *et al.*, 2005).

Similar conclusions may be postulated for clinical trials which did not achieve significance and success. Long-term studies in patients with symptomatic heart failure have been conducted (Research on Endothelin Antagonism in Chronic Heart Failure (REACH)) and Endothelin Antagonist Bosentan for Lowering Cardiac Events in Heart Failure (ENABLE). Both trials were stopped prematurely, due to unexpected increases of adverse events without improvements in clinical status, possibly due to fluid retention (Mylona & Cleland, 1999; Kalra *et al.*, 2002; Packer *et al.*, 2005). Thus one can say that use of endothelin antagonists in heart failure have shown disappointing results, the reasons for which are still being studied. In another trial, Dr. Talmadge E and co-authors said that the findings of their study BUILD-3: A Randomized, Controlled Trial of Bosentan in Idiopathic Pulmonary Fibrosis, were "disappointing." They started the trial after a previous study of bosentan suggested it extended the time to disease progression or death in Idiopathic Pulmonary Fibrosis - although it failed to improve 6-minute walk distance (King, Jr. *et al.*, 2008; King, Jr. *et al.*, 2011). One possibility is that the effectiveness of the drug is being counteracted by too much ET-1 release from different cell types, including fibroblasts. This in turn would have the effect of allowing ET-1 to bind tightly to the $ET_A$ receptor in a non-competitive fashion. ETRAs can prevent ET-1 binding, but do not reverse established agonist–receptor complexes in membrane preparations (Hilal-Dandan *et al.*, 1997). As a result of

the tight nature of the agonist–receptor complexes, certain ETRAs cannot influence long lasting $ET_A$ receptor activation initiated by the peptide. This effect could be due to the fact that endothelin binds to its receptor in an allosteric fashion which makes it very difficult to be reversed by ETRAs; the latter seemingly can only bind to one site on the receptor unlike ET-1 which is able to bind to both high and low affinity sites (Christopoulos & Kenakin, 2002).

In this chapter, I also demonstrated that antagonism of endothelin receptors using the non-selective antagonist (bosentan), ambrisentan or a highly specific $ET_B$ antagonist, adversely elevated the ET-1 levels compared to FBS control in PASMCs isolated from IPAH patients. IPAH is a multifactorial disease and while monotherapy using oral agents can is effective as an initial therapy, it clearly does not control the disease in the long term where ultimately lung transplant becomes the only option. However, prostacyclin is rarely the upfront monotherapy, and is often added on top of bosentan treatment, it is possible this could be a confounding factor in the decline of this therapy as the disease progresses. The combined use of drugs targeting different pathways is becoming more widespread, although the evidence to support such a strategy remains limited (Olsson & Hoeper, 2009; Levinson & Klinger, 2011). On the basis of a great number of studies describing the role of ET-1 in pathogenesis of many diseases, including PAH (Cacoub *et al.*, 1993a; Cacoub *et al.*, 1993b; Saleh *et al.*, 1997; Veyssier-Belot & Cacoub, 1999), drugs such as bosentan, an oral non-selective endothelin receptor antagonist, were developed to reduce the pathophysiological effects of the endothelin system. This

provides a rationale for the use of ETRAs for treatment of various cardiovascular and pulmonary diseases. Deletion of the $ET_B$ receptor gene, produces salt-sensitive hypertension, vascular oxidative stress and impaired endothelium-dependent vasodilatation (Quaschning *et al.*, 2005) while systemic $ET_B$ receptor antagonism in healthy men leads to increased PVR and increased plasma ET-1 concentration (Strachan *et al.*, 1999). Such studies indicate the lack of potential clinical applications for specific $ET_B$ receptor antagonists in cardiovascular disease.

The adverse events observed with ETRA treatment include headache, dizziness and oedema but the abnormalities of liver function are of concern in IPAH patients (Fattinger *et al.*, 2001). Although use of bosentan requires careful monitoring due to this dose-dependent liver toxicity, and is also contraindicated in pregnancy because of teratogenicity, nonetheless, outside of the UK, bosentan remains the first-line treatment for many IPAH patients, in particularly those in Class II of the WHO classification. Although liver toxicity was first reported with bosentan it was later also observed with both mixed and selective $ET_A$ receptor antagonists (Barst *et al.*, 2002). Despite the adverse effects, ETRAs are still widely used in IPAH.

It has been shown that chronic intrauterine pulmonary hypertension causes the loss of $ET_B$-mediated vasodilation, progressive $ET_A$-mediated vasoconstriction, and increased lung ET-1 protein content (Giaid *et al.*, 1993). Thus it is important to study changes in expression of ET-1 and its receptors as these could contribute to the hypertensive and arterial remodelling changes in the lungs after chronic treatment for pulmonary hypertension in patients. To examine this, I measured

levels of ET-1, ECE-1 and $ET_B$-receptor in lungs and PASMCs isolated from IPAH patients. These findings are pertinent because not much is known about the effect of different treatments on levels of ET-1 and its receptors in pulmonary hypertension, nor have their anti-proliferative properties been compared. An imbalance in the NO-cGMP system and ET-1 system, favouring reduced NO bioavailability and over production of ET-1 may also contribute to pulmonary hypertensive states (Maclean *et al.*, 1997; Hanson *et al.*, 1998b). Indeed, a decrease in NO production may lead to increased ET-1 production (Kourembanas *et al.*, 1993). In addition, increased ET-1 activity is also likely to stimulate pulmonary smooth muscle proliferation (Davie *et al.*, 2002), which may further increase PVR through increased wall thickness (Zamora *et al.*, 1993). Inhaled nitric oxide treatment in PAH is reported to be associated with ET-1 induced reactive oxygen species (ROS) production (Wedgwood *et al.*, 2001b). Furthermore, $ET_A$ receptor stimulation tends to promote cell proliferation and fibrosis mediated by ROS generation (Wedgwood *et al.*, 2001a) and this mitogenic effect of ET-1 on PASMC can be blocked by antioxidants treatment (Wedgwood et al 2001).

Little is known about the role of ECE-1 in pulmonary hypertension. I compared the ECE-1 expression in histological sections of IPAH and normal lung tissue to see if expression of the endothelin receptors and ECE-1 change in pulmonary hypertension. When compared with normal lung tissue, ECE-1 expression in medial smooth muscle of the IPAH lung was substantially increased and so was $ET_B$ receptor expression. One may speculate that an imbalance of ET-1 production

and ET-1 receptor expression contributes to the pathophysiological changes in pulmonary hypertension by favouring increased vasoconstriction and smooth muscle proliferation. The main drawback of this study was that it could only be repeated twice and so could not be quantified statistically. However, observations were similar to that reported in PAH patients, who showed immunoreactivity for ECE- 1 was augmented in the endothelium of diseased pulmonary arteries (Giaid, 1998). With respect to $ET_B$ receptors, I observed increased expression in the media as well as intima in pulmonary artery of IPAH versus control patient, a result similar to previous studies that showed $ET_B$-receptor upregulation in distal pulmonary blood vessels (Bauer *et al.*, 2002; Davie *et al.*, 2002; Hall *et al.*, 2011). Also human PASMCs express both $ET_A$ and $ET_B$ receptors which couple to contraction and proliferation; in endothelium, $ET_B$ is linked to vasodilation, though $ET_A$, recently detected in the lung endothelium (Hall *et al.*, 2011) may have this role, but this is unknown. This suggests a dual beneficial role for $ET_B$ receptor in PAH, where endothelial dysfunction could be combated through enhanced ET-1 clearance and nitric oxide release (Wilson *et al.*, 2012). Loss of $ET_B$ signalling in cultured rat pulmonary endothelial cells has been reported to increase ECE-1 mRNA expression in (Naomi *et al.*, 1998), which may further contribute to an increase in ET-1. In other studies, there is a suggestion of cross talk between ET-1 receptors which may allow the $ET_B$ receptor to adopt functions of the $ET_A$ receptor when the latter is under selective blockade (Okada *et al.*, 1994; Fukuroda *et al.*, 1994b). Such studies raise the controversial issue regarding use of selective $ET_A$ versus mixed ET-1 receptor antagonism in IPAH. Since with some agents such as

ambrisentan, selectivity is highly dependent on dose used, future strategies ought to employ agents that retain $ET_A$ receptor selectivity at the doses used (Battistini *et al.*, 2006). In animal models the role of $ET_B$ receptor in the context of PAH is also complex. In rats increased ET-1 activity led to an up-regulation in the endothelium of $ET_B$-dependent vasodilatory, clearance and anti-proliferative pathways whereas in animal models $ET_B$ deficiency in rats (Ivy *et al.*, 2002) or $ET_B$ antagonism in sheep (Ivy *et al.*, 2000), results in a severe PAH phenotype following monocrotaline treatment.

I also demonstrated that $ET_B$ blockade with either a mixed (bosentan) or a specific $ET_B$ antagonist (BQ 788) was not very effective at inhibiting proliferation of human PASMCs isolated from IPAH patients. $ET_B$ receptors on vascular smooth muscle cells mediate ET-1 induced remodelling and hypertrophy (Nishida *et al.*, 2004). Even, studies performed on hypoxic rats suggested a role for $ET_B$ in right ventricular hypertrophy (Motte *et al.*, 2006). The importance of ET-1 receptor mediated pathways in human PAH remains unclear and thus bosentan as well as ambrisentan are still widely used for the treatment of this disease due to the rationale of significantly elevated ET-1 levels contributing to the pathology in this disease.

However, it is likely that additional mechanisms are involved in mediating the effects of ET-1 on distal PASMC proliferation. The desensitization or down-regulation of pulmonary $ET_B$ receptors has been postulated to contribute to the development of human pulmonary hypertension (Dupuis *et al.*, 1996a). Recently

Kirkby and his group reported that $ET_B$ receptor activation may play an important role in limiting neointimal lesion formation following acute vascular injury and provided evidence that $ET_B$ receptors expressed on smooth muscle but not endothelial cells were involved in preventing intimal proliferation (Kirkby *et al.*, 2012). The authors concluded that blockade of selective $ET_A$ receptors may be preferable to mixed $ET_A/ET_B$ antagonism for treating the vascular injury caused in PAH.

The first placebo-controlled trial where a combination of bosentan and epoprostenol was studied raised questions about their combined efficacy. The trial, BREATHE-2 was a 16-week, double-blinded, randomized, placebo-controlled prospective trial examining the efficacy of adding oral bosentan at the initiation of intravenous epoprostenol therapy (Humbert *et al.*, 2004b). In patients receiving the combination, hemodynamic improvement was not significant when compared with placebo and epoprostenol, nor was there significant improvement in functional class or exercise capacity. In another multicenter trial, COMBI, Hoeper and co-workers studied patients with IPAH who were stable on bosentan and to whose regimen iloprost or placebo was added (Hoeper *et al.*, 2006). The investigators planned to enroll 72 patients, but the study was terminated early because the interim analysis of 40 patients did not demonstrate efficacy. Interestingly, as in the STEP trial, the 3 patients whose condition showed substantial deterioration in all objective outcomes were in group receiving combination therapy (McLaughlin *et al.*, 2006).

A recent study, FREEDOM-C trial, hinted that combining ETRAs with prostacyclin analogues might affect the efficacy of the latter (Tapson *et al.*, 2012). Patients participating were already on therapy with an ETRA and/or sildenafil when oral treprostinil was added on. The study failed to meet clinical outcomes, despite the initial trial meeting such endpoints when given as a monotherapy (Simonneau *et al.*, 2002). In the TRIUMPH study, patients on treatment with sildenafil or bosentan showed a marked improvement in 6MWD with the addition of inhaled treprostinil for 12 weeks (McLaughlin *et al.*, 2010). Notably, in this study, the improvement in 6MWD was greater in patients receiving sildenafil than those receiving an ETRA. Looking at all these studies, one could speculate that perhaps prostacyclin monotherapy could be clinically superior, though proper crossover trials would be required to confirm this.. Two more phase 3 trials, a large-scale trial of oral treprostinil monotherapy (FREEDOM-M) and a second combination study (FREEDOM-C2) are currently underway. Results of these studies and larger clinical trials with prostacyclin analogue monotherapy might throw further light on how to better approach treatment of PAH patients. Interestingly, in an open labelled or in retrospective long-term analysis of subcutaneous treprostinil, patterns of observed survival versus predicted appear to be similar or possibly slightly better (69% at 3 years) than those seen with *IV* epoprostenol involving patients with similar baseline characteristics (McLaughlin et al., 2002; Lang et al., 2006; Barst et al., 2006; Skoro-Sajer et al., 2008; Sadushi-Kolici et al., 2012). In the latter study, this occurred despite a high proportion (44%) of patients in WHO functional class IV. Moreover, outcomes were beyond

expectations in a group of patients who could tolerate up-titration of treprostinil beyond 6 months, with survival being 57% at 9 years, though overall the rate in the whole patient group at this time was 35% (Sadushi-Kolici et al., 2012).

## 4.5 Limitations of data contained in this chapter

1. One of potential limitations of the work presented in this chapter was that the levels of ET-1 were normalised to cellular protein and not cell numbers. The main reason for this approach was that cells were lysed for western blot experiments so as to be able to assess the effects of different treatment on $ET_B$ and ECE-1 expression and correlate this directly with ET-1 levels.
2. Another limitation of the current work is that I did not confirm if any of the drugs used had cytotoxic effects on cells. Thus measurement of ET-1 mRNA by qRT-PCR should be assessed in order to confirm results obtained in assays and from westerns blots.
3. To confirm that the ECE-1 inhibitor was actually preventing conversion of big ET-1 to ET-1, big ET-1 should have been measured in parallel to confirm the pharmacological mechanism of CGS35066.
4. To confirm that $ET_B$ receptors are regulating ET-1 levels through ECE-1, I should measure mRNA levels of both ECE-1 and pre-pro ET-1 and ET-1 and assess the effect of an $ET_B$ selective agonist (sarafotoxin 6c) together with a selective $ET_B$ antagonist.

5. In the ET-1 assays, I have tended to use a single dose of each ETRAs. Keeping in mind the complexity with regard to specificity of different ETRAs against $ET_A$ & $ET_B$, it would have been preferable to assess concentration-dependent effects so as to gauge whether the antagonist was working selectively or non-selectively on the specific ET receptor subtypes.
6. For immunohistochemistry experiments, controls should have been performed to demonstrate that pre-incubation of the antisera/antibody against ECE-1 and ETB with antigen, blocked the immunostaining.
7. Use of IP receptor and PPARγ antagonists and/or adenylyl cyclase inhibitors could have been used to establish whether the effects of treprostinil on ET-1 synthesis (measured by ELISA/mRNA) is dependent upon the IP receptor, PPARγ and/or cAMP elevation.

# 5. Ispinesib as a potential anti-proliferative agent in PAH

## 5.1 Introduction

Pulmonary vascular remodelling is characterised by thickening of all three layers of the blood vessel wall i.e., fibroblasts, smooth muscle cells, and endothelial cells, as well as increased collagen deposition, elastin, and fibronectin (Ishizaki *et al.*, 1995). In addition, there is extension of new smooth muscle into the partially muscular and non-muscular peripheral arteries. This is due to the differentiation of precursor cells into smooth muscle cells (Meyrick & Reid, 1980) and is termed neomuscularisation. These various alterations in vascular structure are seen in both human pulmonary hypertension and animal models of the disease, and take place more rapidly than the remodelling of systemic arteries in systemic hypertension (Zhao *et al.*, 1996). The increased production of mitogenic factors by hypoxia can cause induction of various transcription factors (e.g., hypoxia-inducible transcription factor-1, activating protein-1, and nuclear factor-kB) that control the transcriptional activation of genes encoding for growth factors and other mediators of mitogenesis.

Extensive remodelling and proliferation appears to be the main factor in PAH and thus needs to be targeted to treat the disease. Despite advances in the treatment of PAH, dose escalation is required for all of the current medications as the disease becomes progressively severe and eventually these therapies are no longer effective. Lung transplantation remains the only option for a small group of patients, but as few can benefit from such procedures improvements in treatment

are urgently needed. Earlier intervention and the combined use of drugs targeting different pathways is becoming more widespread. The rationale being that a multifactoral disease like PAH requires the attack of multiple pathways to control the disease (Levinson & Klinger, 2011; Vachiery, 2011). Given that vascular remodelling results from suppressed apoptosis and uncontrolled smooth muscle, myofibroblast and endothelial cell proliferation, key features in the pathology of PAH, this disease could be viewed as form of cancer. Recently vascular remodelling has been considered a pseudo-malignant disorder and so mediators from cancer research have been described as potential targets for therapeutic interventions in PAH (Paulin *et al.*, 2011).

### 5.1.1 Tyrosine kinase pathway in PAH

Several growth factors, including platelet derived growth factor (PDGF), fibroblast growth factor 2 (FGF), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF) have been implicated in the abnormal proliferation in PAH (McMurtry *et al.*, 2003; Hassoun *et al.*, 2009). Levels of PDGF and its tyrosine kinase receptor PDGFR, are elevated in PAH patient lung samples (Merklinger *et al.*, 2005; Schermuly *et al.*, 2005a; Perros *et al.*, 2008) as are the levels of VEGF and KDR (VEGF receptor) in plexiform lesions (Cool *et al.*, 1997). These two growth factors act as potent mitogens and chemo-attractants, and through their transmembrane tyrosine kinase receptor pathways, activate major proliferative signalling pathways resulting in proliferation, migration and resistance to apoptosis

(Hassoun *et al.*, 2009). Reversal of lung vascular remodelling rather than prolonged vasodilation is the concept underlying the use of tyrosine kinase inhibitors in PAH. One of the most promising drugs in this class of agents to reverse vascular remodelling in PAH is imatinib mesylate (Gleevec), an anti-cancer drug already approved to treat chronic myelogenous leukemia (CML) and gastrointestinal stromal tumors (Deininger *et al.*, 2005). Imatinib is thought to inhibit the PDGFR and thus improve experimental PH induced by monocrotaline (Schermuly *et al.*, 2005a; Klein *et al.*, 2008), though other targets such as c-kit may play a role (Buchdunger *et al.*, 2000). Mainly in the end stage of PAH, when the disease is highly proliferative and drugs fail to cause any significant improvements, treatment with imatinib might provide benefit, particularly in reducing PVR (Ghofrani *et al.*, 2006; Patterson *et al.*, 2006). In a more recent and larger study, where patients who had a baseline PVR of greater than or equal to 1,000 dynes · s/cm$^5$, improvements in 6MWD, PVR, and CO after imatinib treatment compared with placebo were documented, though overall 6MWD was not significantly improved in the whole patient group (Ghofrani *et al.*, 2010). Despite these positive results, serious adverse events including nausea and diarrhea may restrict the widespread use of this drug, and cardiac toxicity still remains a concern with this class of agents (Ghofrani *et al.*, 2010). Thus anti-cancer agents with a more selective mode of action need to be evaluated.

### 5.1.2 Kinesin Inhibitors

Mitotic kinesin inhibitors are a novel class of antiproliferative agents with low toxicity. Their mode of action involves disruption of a specific event in normal mitosis thereby promoting cell-cycle arrest and driving cells into apoptosis (Purcell et al., 2010). In this study I sought to evaluate a novel anticancer compound, ispinesib that has already been advanced to clinical trials. This compound is an allosteric small-molecule quinazolinone inhibitor of the kinesin spindle protein (KSP). Ispinesib affects the mechanochemical cycle of KSP by altering the interactions of KSP and microtubules, thereby preventing KSP-driven mitotic spindle pole separation (Lad *et al.*, 2008). The main objective of these experiments was to evaluate the anti-growth properties of ispinesib in PASMCs derived from IPAH patients and establish a rationale for investigating this agent in PAH settings.

Our lab has previously demonstrated that PASMCs these end-stage disease cells have an abnormal proliferative phenotype (Falcetti *et al.*, 2010) and thus might be particularly responsive to an anti-mitotic agent. In addition, I wished to ascertain if established agents used to treat PAH patients might affect the efficacy of ispinesib, either positively or negatively, to inhibit smooth muscle growth. Lastly, I wished to evaluate anti-growth effects of ispinesib in lung fibroblasts derived from patients with fibrotic disease and secondary PAH. Effective therapies are urgently needed given the recent failure of bosentan to meet the primary outcome in the recent clinical trial (BUILD-3) conducted in patients with idiopathic pulmonary fibrosis (King, Jr. *et al.*, 2011).

## 5.2 Results

### 5.2.1 Effect of ispinesib on cell proliferation

The effect of ispinesib (developed and supplied by Cytokinetics, San Francisco, USA) on cell proliferation stimulated by FBS (9%) was investigated in human PASMCs isolated cells grown in culture for 4 days (figure 5.1). Under control conditions, FBS (with or without 0.02% DMSO) significantly (one way ANOVA, $P < 0.001$) increased cell number from basal on average by 6 fold in 5 different patient isolates (Figure 5.1A). Ispinesib started to inhibit growth at concentrations of 0.01 nM, though cell number was only significantly inhibited at or above 1 nM (one way ANOVA, $P<0.01$). Inhibition by ispinesib of the growth promoting effects of FBS was concentration-dependent and this agent appeared to have little to no effect on cell viability (up to 1 µM) as measured by propidium idodide (figure 5. 1A). A higher concentration (10 µM) did however cause cells to lift off the bottom of the dish and was thus deemed to be cytotoxic at this concentration (not shown).

Given that mitotic kinesins appear to be highly expressed only in proliferating cells (Sakowicz *et al.*, 2004), I then investigated whether ispinesib responds in a similar or less effective manner in normal distal human PASMCs. In this way, I might be able to establish a dose-range that effectively targets cells responding abnormally to growth factors (e.g. PDGF), a hallmark of PAH disease (Rhodes *et al.*, 2009).

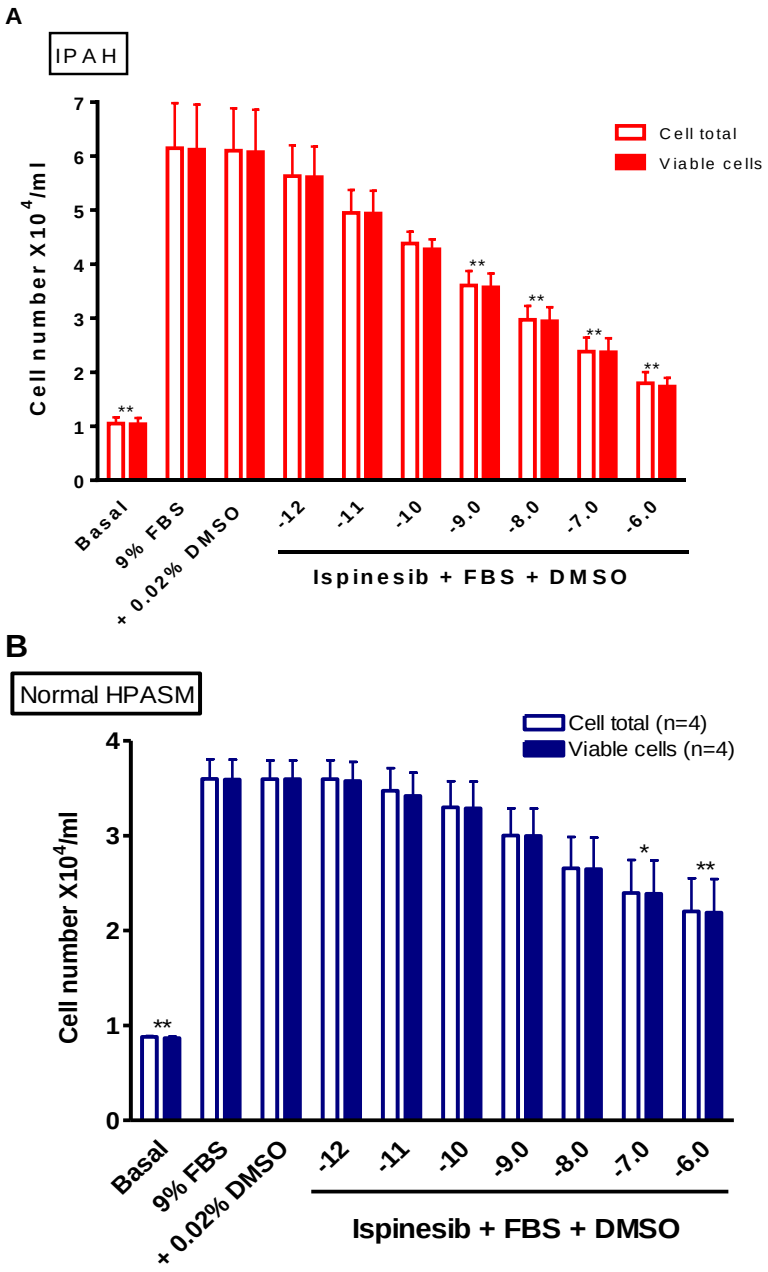



**Figure 5.1. Effect of ispinesib on cell number and viability in distal human PASMCs derived from IPAH (A) and control (B) patients.** Cells were grown in 9% FBS for 4 days in the absence and presence of increasing concentrations of ispinesib. Graphs show total and viable cell count as assessed by propidium iodide in an automated cell counter (ADAM). *=P<0.05, **=P<0.01 compared to appropriate DMSO control (One-way ANOVA, Dunnett's post-hoc, n=4-5).

Using the same protocols described above (page 77), I found that ispinesib only began to significantly inhibit growth at 100 fold higher concentrations in normal compared to IPAH cells. A concentration of 1 nM was the lowest concentration showing inhibition of growth though significant inhibition occurred only at the 100 nM concentration and above (figure 5.1B).

Given the inherent variability in cell proliferation rates, particularly between adult and child PAH (Falcetti *et al.*, 2010), a generally accepted way to present cell proliferation data are to normalise data to % change relative to the growth response induced by FBS alone (set at 100%). As cell isolates from different patients grow at different rates, this increases data variability potentially masking significant antiproliferative effects at the lower concentrations in any given patient cell isolate. When such analysis is done, ispinesib was found to significantly inhibit growth at concentrations as low as 0.01 nM in IPAH cells (figure 5. 2A), while in normal cells this occurred at 1 nM (figure 5.2B). When data were fitted using a variable slope sigmoidal fitting routine within GraphPad, the extrapolated $EC_{50}$ and $E_{Max}$ value for ispinesib was -8.78 ± 0.65 and 2.1 ± 15.5% n=5, respectively in IPAH cells (Figure 5.2C). In contrast, at the highest dose examined (1 µM), ispinesib was only able to inhibit cell growth by ~50% in normal human PASMCs as opposed to 85% (2-way ANOVA $P<0.01$) in IPAH cells. The lower efficacy in normal cells occurred across the whole concentration-response range (figure 5.2C) despite a similar potency of ispinesib (extrapolated $EC_{50}$ value being -8.58 ± 0.53, n=4). This is presumed to occur because of the lower expression of KSP in normal PASMCs cells rather than a decrease in binding of ispinesib to this protein.

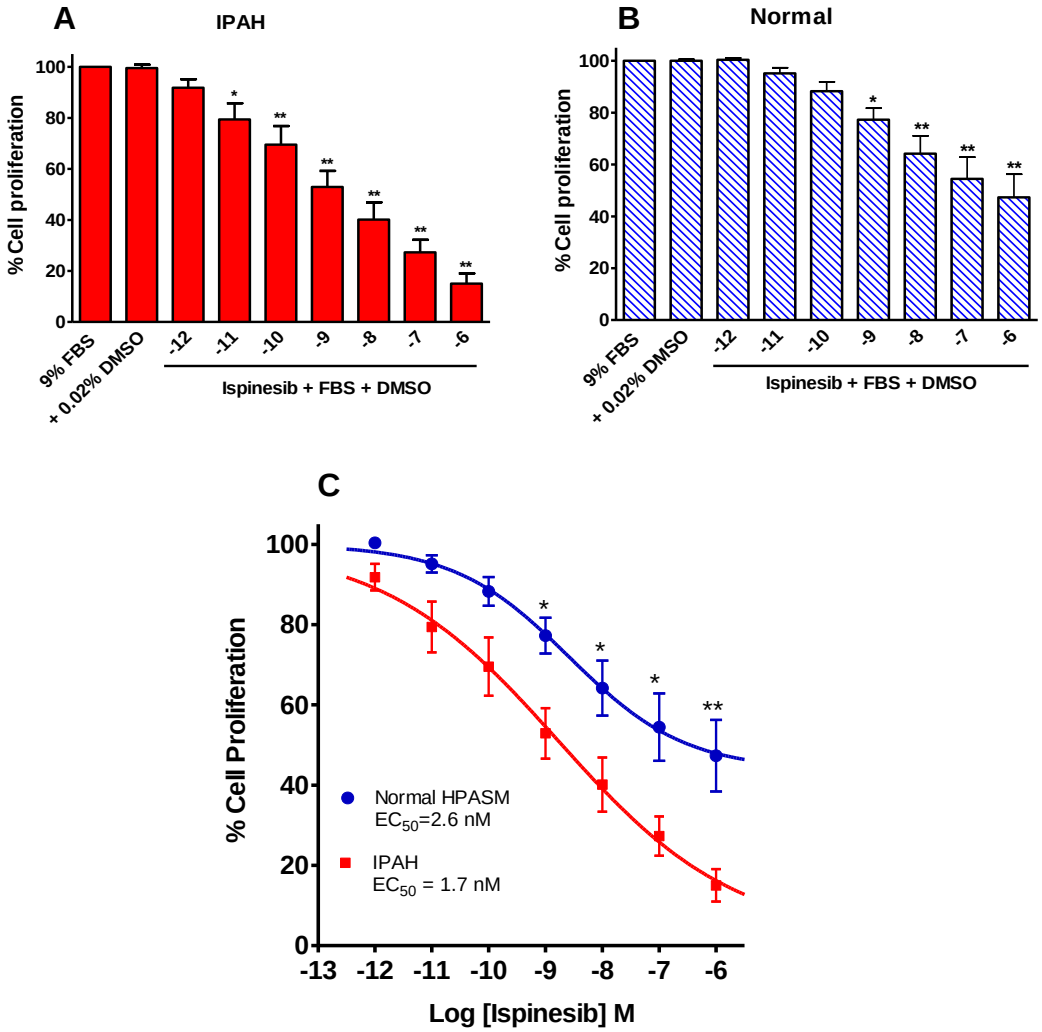



**Figure 5.2. Ispinesib inhibits cell proliferation induced by FBS in distal human PASMCs derived from IPAH and control patients.** Cells were grown in 9% FBS for 4 days in the absence and presence of increasing concentrations of ispinesib. Effect of ispinesib on FBS-induced growth in human PASMCs derived from IPAH (A) and normal patients (B) where data are expressed as a % change in cell proliferation relative to the growth response induced by 9% FBS. In C, data from A & B have been fit using a variable slope sigmoidal fitting routine in GraphPad Prism. Significant difference was determined using one-way ANOVA, Dunnett's post-hoc (n=4-5) in A & B and using 2-way ANOVA with Bonferoni corrections (n=4-5) in C. *=$P<0.05$ *and* **=$P<0.01$ when compared to solvent (DMSO) control or the appropriate ispinesib concentration.

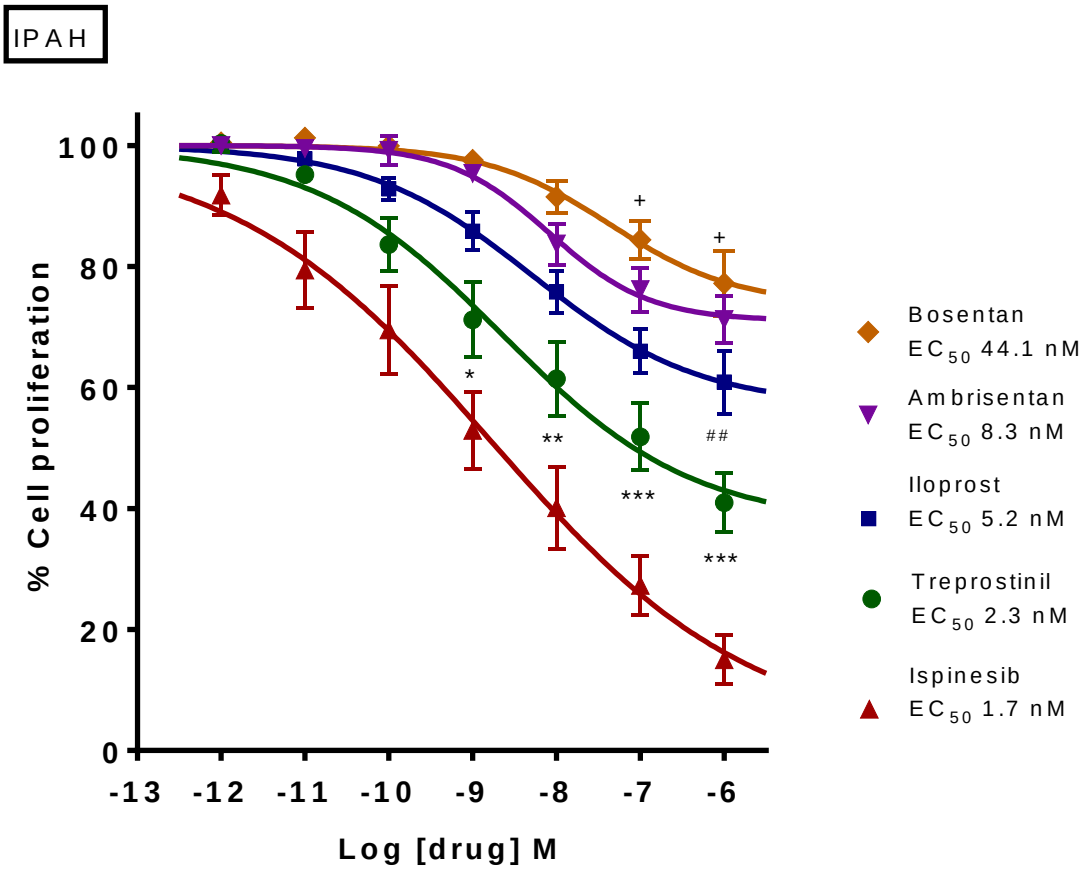



**Figure 5.3. Comparison of antiproliferative effects of current therapeutic agents compared with ispinesib in human PASMCs derived from IPAH patients.** Data are expressed as % change in cell proliferation relative to the growth response induced by 9% FBS. Extrapolated $EC_{50}$ values were obtained from fits to data using a variable slope sigmoidal-curve fitting routine in GraphPad Prism. += P<0.05 compared to iloprost; ##= P<0.01 compared to treprostinil and *P<0.05, **P<0.01, ***P<0.001 compared to ispinesib. Statistical comparisons made using 2-way ANOVA, with posthoc Bonferroni correction (n=5 from same patient isolates).

Although both prostacyclin analogues and ET-1 receptors antagonists are reported to be antiproliferative in the context of PAH, so far there has been no direct comparison between these groups of drugs in the same experimental setting. Comparing these agents along with ispinesib, the extrapolated maximal response ($E_{Max}$) in IPAH cells was in the order: Ispinesib > treprostinil > iloprost > ambrisentan ≥ bosentan. Bosentan had the lowest potency ($EC_{50}$ 44 nM) and like ambrisentan, had a lower maximal inhibitory effect ($E_{Max}$ 71.0 ± 3.2%) on growth. Both were also significantly less effective than treprostinil or iloprost (table 1 & figure 5.3). Furthermore, iloprost was weaker at inhibiting cell growth than treprostinil giving a significantly lower $E_{Max}$ (56.5 ± 6.8% versus 37.4 ± 8.7%).

### 5.2.2 Ispinesib combined with other drugs used to treat PAH

Treatment of IPAH patients with ispinesib is likely to be given with an existing background therapy. Thus I evaluated the concentration-response of ispinesib in combination with two standard therapies, treprostinil and bosentan. Doses of these agents were chosen on the basis of measured plasma concentrations likely to be achieved in patients although bosentan levels may actually be closer to ~0.75 µM (Burgess *et al.*, 2008) and treprostinil may reach 50 nM in some patients (McSwain *et al.*, 2008). Combined treatment with treprostinil (10 nM) shifted the ispinesib concentration-response to the left by 3 log units, such that significant antiproliferative effects were now observed at 1 pM, regardless of whether cell

number or % cell proliferation was used to determine statistical difference (compare figure 5.4 B & C).

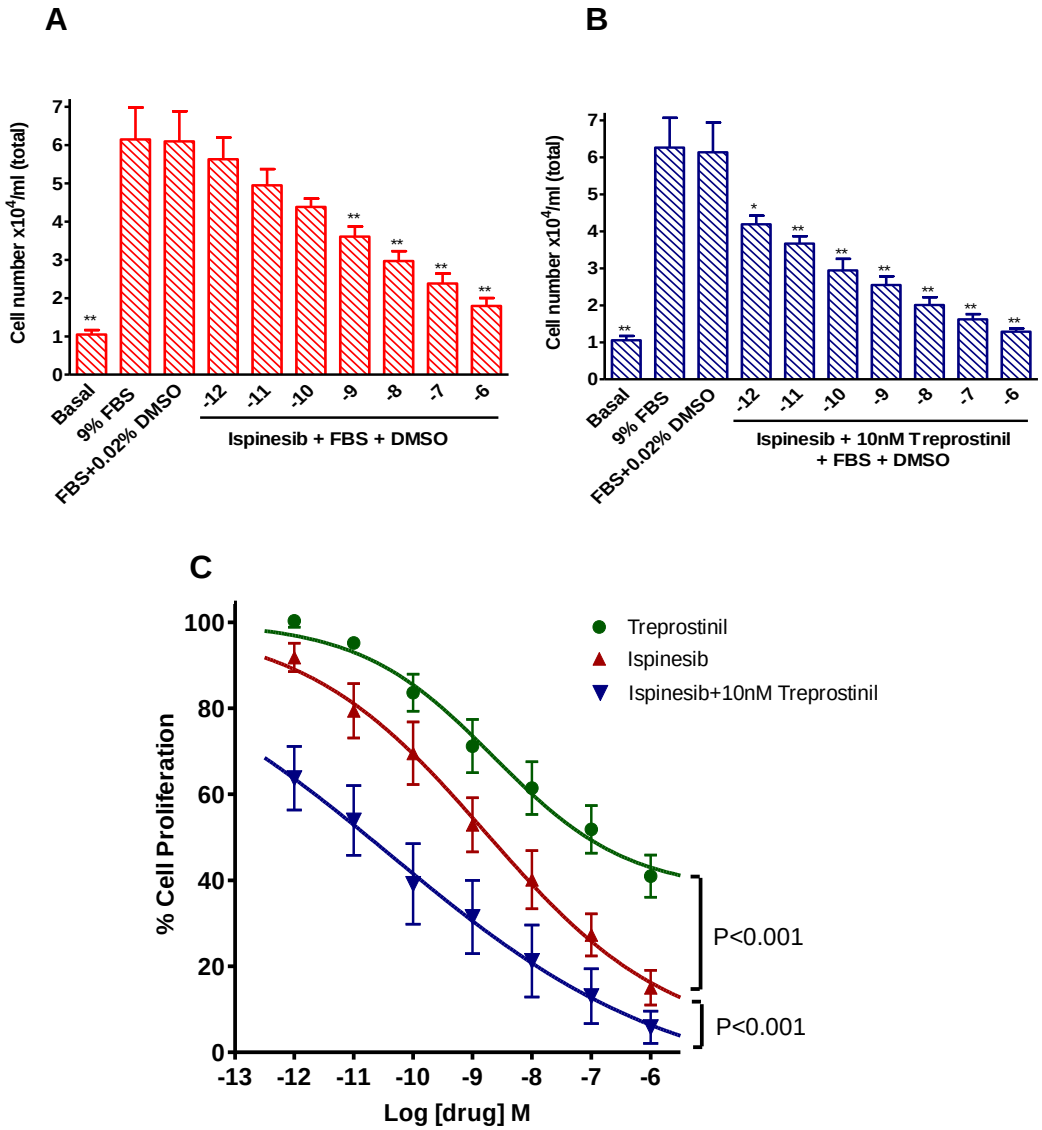



**Figure 5.4. Enhancement of the antiproliferative effects of ispinesib by treprostinil in human PASMCs derived from IPAH patients.** Cells were grown in 9% FBS for 4 days in the absence and presence of increasing concentrations of ispinesib, treprostinil or ispinesib in combination with 10 nM treprostinil (B). Data are shown as cell number in A & B and % cell proliferation in C where data from A & B and figure 5.3 have been used. *=P<0.05, **=P<0.01 compared to appropriate DMSO control (One-way ANOVA, Dunnett's post-hoc, n=5) in A & B. In C data are fit using a variable slope sigmoidal function in GraphPad Prism and statistical analysis performed using 2 way ANOVA.

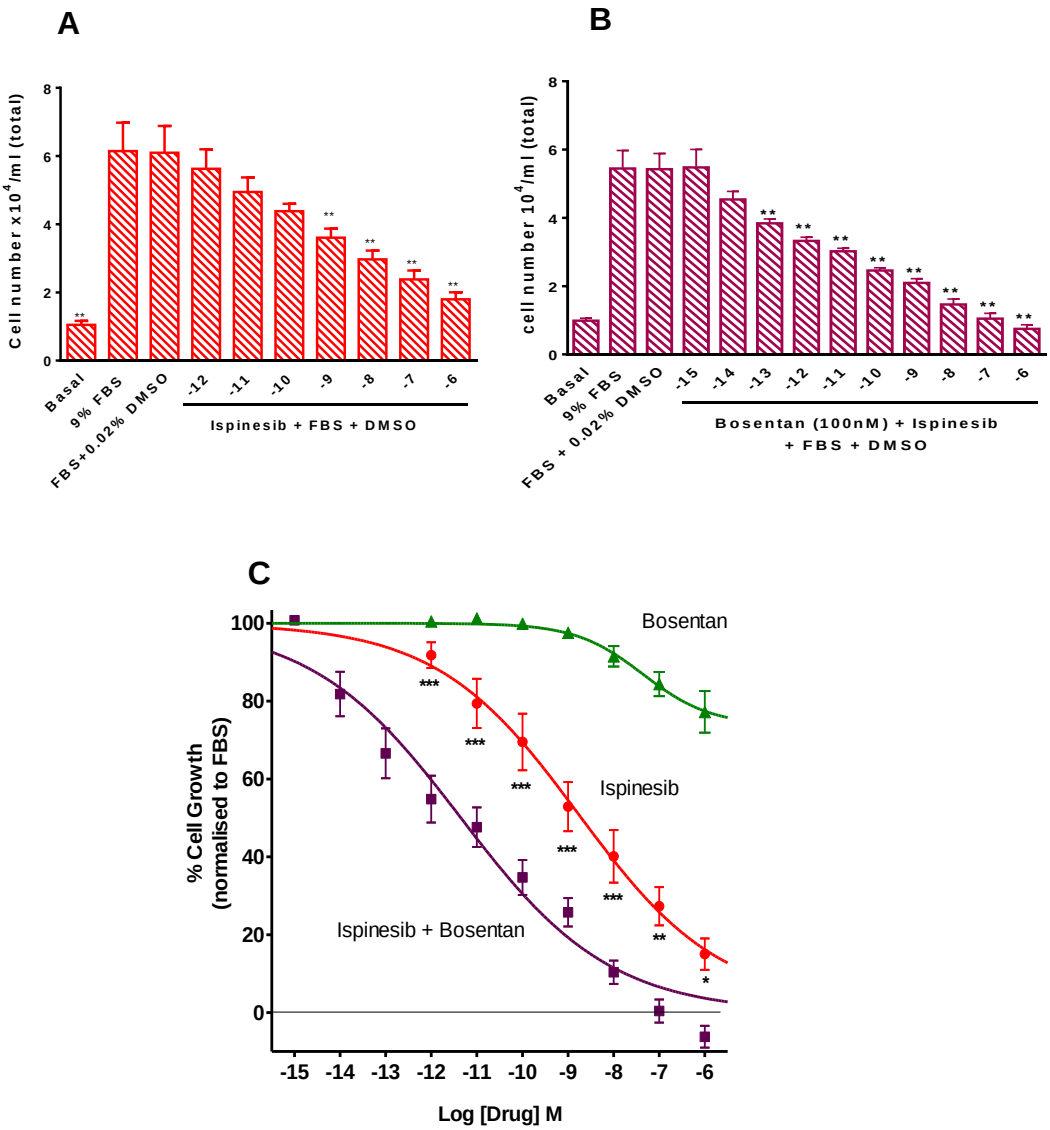



**Figure 5.5. Treatment with bosentan at a therapeutic dose synergistically enhances the antiproliferative of ispinesib across a wide concentration range.** Human PASMCs derived from IPAH patients were grown in 9% FBS for 4 days in the absence and presence of increasing concentrations of ispinesib, bosentan or a combination of ispinesib and 100 nM bosentan. Data in A & B are shown as actual cell number while in C this has been normalized to the FBS growth response and fitted using a variable slope sigmoidal fitting routine. *P<0.05, **P<0.01, ***P<0.001 compared to FBS and DMSO in A & B (One-way ANOVA with Dunnetts posthoc, n=5) or compared to combination of ispinesib and bosentan (Two-way ANOVA with posthoc Bonferroni conrrection, n=5) in C. Bosentan data used for comparison is taken from figure 5.3.

The potentiating effect was seen over the whole concentrating range (0.001 - 1000 nM) and appeared additive at concentrations <1 nM but converged at higher concentrations. The effect on ispinesib responses was even more marked with bosentan. A significant antiproliferative effect was now observed well below 1 pM (figure 5. 5B). This was associated with a 400-fold increase in potency ($EC_{50}$ 4.5 pM) compared with ispinesib alone (table 2). Moreover, the potentiating effects of bosentan were synergistic at <100 nM, and the combination produced some cell death at 1 µM ispinesib. Thus both treprostinil and bosentan at clinically relevant doses are able to potentiate the antiproliferative effects of ispinesib in human PASMCs from IPAH patients.

Next I wished to investigate the time course over which ispinesib significantly inhibited proliferation of IPAH cells. This was performed to serve as a preliminary guide for what dose and duration of exposure in patients might be required with ispinesib in order to have a significant inhibitory effect on growing smooth muscle cells. For these studies, we used cells in the growing phase and then treated them with a near maximal anti-proliferative dose of ispinesib (100 nM) over a time range of between 0-96 h both in the absence and presence of bosentan (figure 5.6). Under these conditions, ispinesib (100 nM) inhibited cell proliferation as early as 10 hr with complete growth inhibition occurring at 24 hr. Inhibition was maintained for more or less up to 2 days, with less cell growth seen between 2-4 days, even though growth was still significantly ($P<0.001$) depressed.

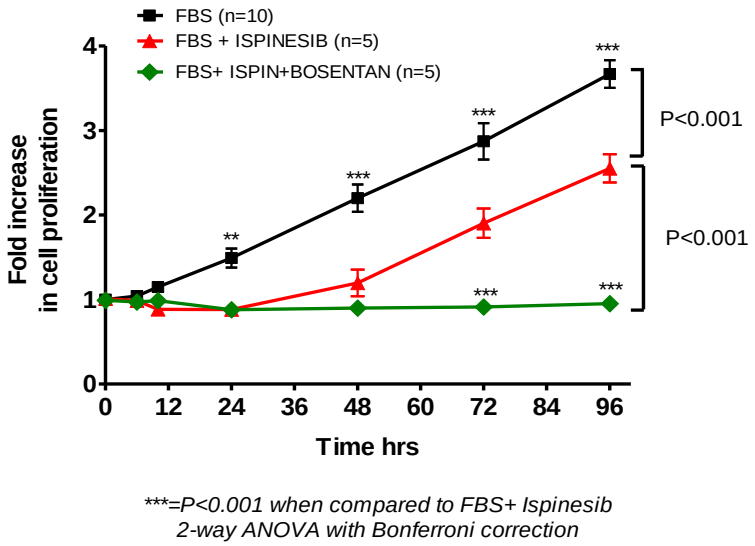



**Figure 5.6. In the presence of bosentan, ispinesib growth arrests human PASMCs for a significantly longer time.** Human PASMCs derived from IPAH patients were grown in 9% FBS for 8 days with ispinesib (100 nM) or in combination with bosentan (100 nM) added at day 4 which is depicted as t=0 on the graph. Data are expressed as fold change in proliferation normalised to growth in FBS alone at t=0. **P<0.01, ***P<0.001 compared to FBS and ispinesib in combination with bostentan. Two-way ANOVA with post-hoc Bonferroni correction at corresponding time point.

Next I wished to establish (a) if incubation of cells with bosentan and ispinesib prevented the waning effect over time compared to ispinesib alone and (b) if the time taken for this agent to have an inhibitory effect on cell growth occurred more quickly. Ispinesib (100 nM) in combination with bosentan (100 nM) completely inhibited growth with a similar time course (figure 5.6), though in contrast to treatment with ispinesib alone, this growth arrest was maintained for up to 4 days in culture. Thus combination therapy is likely to produce growth inhibition that is better maintained.

### 5.2.3 Role of $ET_A$ & $ET_B$ receptors in mediating bosentan synergy with ispinesib

In order to establish whether the synergy observed with bosentan was due to specific inhibition of either the $ET_A$ or $ET_B$ receptor or both, cells were treated with ambrisentan, a relatively selective $ET_A$ receptor antagonist (Davie et al, 2009), BQ788, a highly selective $ET_B$ antagonist or a combination of both these drugs with ispinesib. Co-incubation of either antagonist with ispinesib produced no significant potentiation and there was even a trend towards a lower maximal response with each (figure 5.7). It is likely that potentiation of ispinesib effects on cell growth requires $ET_A$ & $ET_B$ to be blocked simultaneously, as a triple combination with ambrisentan and BQ788 did cause a significant shift in the concentration-response curve compared to ispinesib alone (figure 5.8). However, bosentan was still significantly more effective than even this combination (figure 5.8) over the concentration range of 1 pmol to 1000 nM. The reason for this is unclear but may relate to incomplete blockade of $ET_{A/B}$ receptors with the ambrisentan and BQ788 combination.

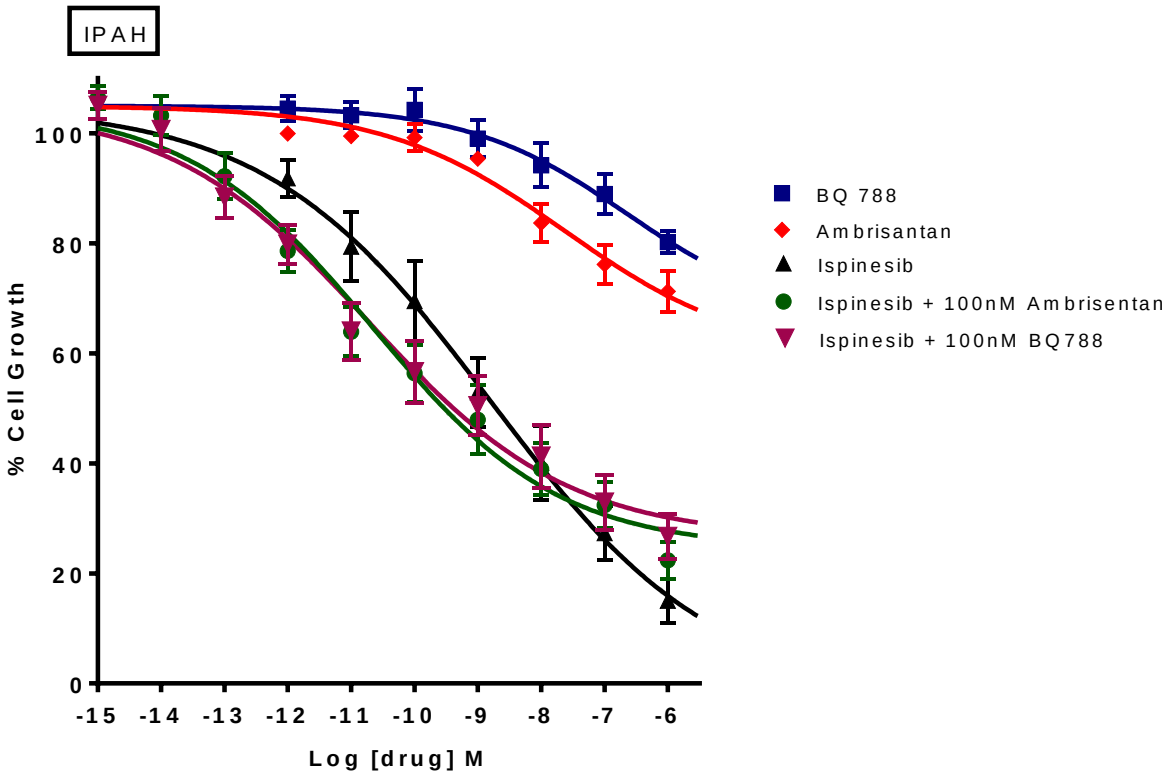



**Figure 5.7. Potentiation of anti-growth effects of ispenesib is not observed with either selective $ET_A$ or $ET_B$ receptor blockade**. Human PASMCs derived from IPAH patients were grown in 9% FBS for 4 days in the absence and presence of increasing concentrations of BQ788 (an $ET_B$ antagonist), ambrisentan (a relative selective $ET_A$ receptor antagonist), ispinesib or a combination of ispinesib with either ET-1 antagonist (100 nM). Data have been normalised to the growth response induced by FBS and expressed as % cell proliferation. Mean data are fitted using a variable slope sigmoidal fitting routine in GraphPad Prism.

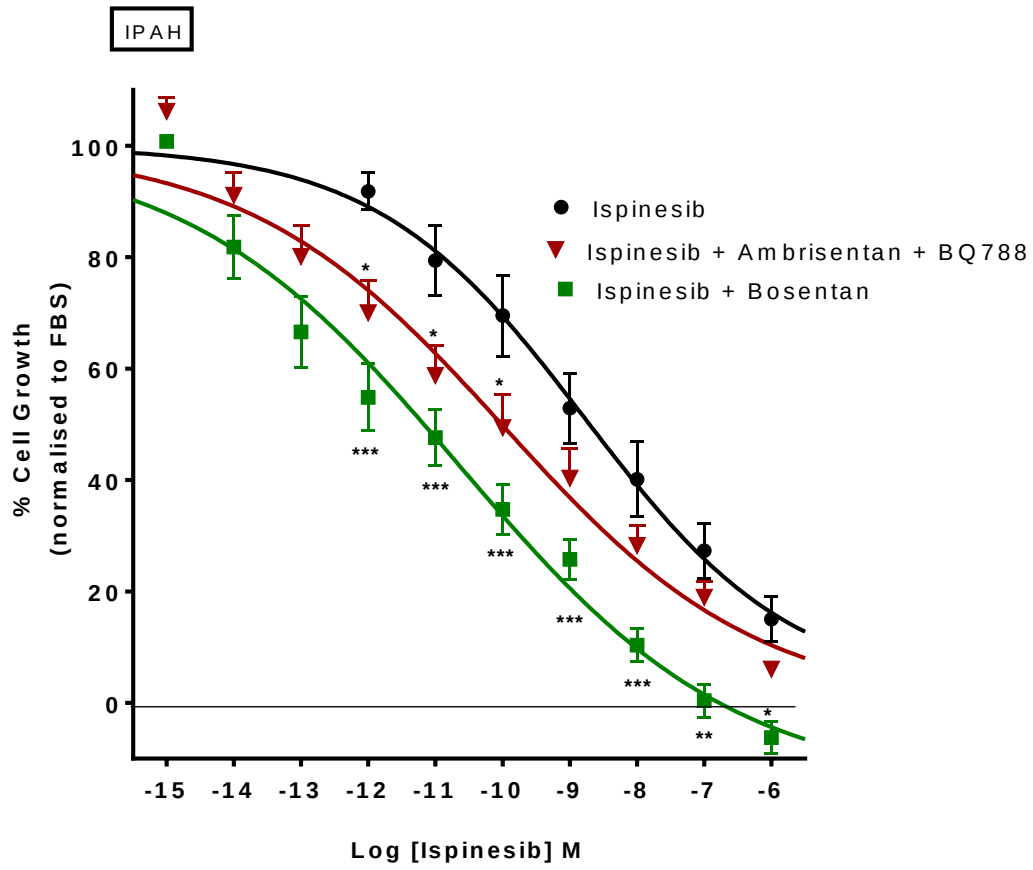



**Figure 5.8. Blockade of both $ET_A$ & $ET_B$ receptors is required to potentiate the effects of ispinesib.** Human PASMCs derived from IPAH patients were grown in 9% FBS for 4 days in the absence and presence of increasing concentrations of ispinesib, a combination of ispinesib and 100 nM bosentan (data from figure 5.5) or a triple combination of ispinesib, ambrisentan and BQ788. Data have been normalised to the growth response induced by FBS and expressed as % cell proliferation. Mean data are fitted using a variable slope sigmoidal fitting routine in GraphPad Prism. *P<0.05, **P<0.01, ***P<0.001 when triple combination compared to ispinesib and bosentan or ispinesib alone (Two-way ANOVA with posthoc Bonferroni correction; n=5).

### 5.2.4 Antiproliferative effects of ispinesib on lung fibroblasts

Pulmonary fibrosis can often be associated with PAH. The incidence of PAH increases with severity of fibrotic disease and ranges between 28% and 62% in patients diagnosed with pulmonary fibrosis (Mura *et al.*, 2012) or connective tissue disease (Yaqub & Chung, 2013). Prognosis and response to therapies is considerably worse in these patients compared to IPAH patients. It would therefore be of interest to assess the effects of ispinesib in lung fibroblasts derived from patients with PAH and also compare it with agents used to treat IPAH. In cells stimulated with 9% FBS, ispinesib significantly inhibited cell growth at concentrations as low as 0.01 nM in lung fibroblasts from patients with idiopathic pulmonary fibrosis (IPF) and secondary PAH (figure 5. 9A). With respect to treprostinil, a 100 fold higher concentration was required to begin to significantly inhibit growth (figure 5. 9B). However neither agent affected cell viability. Furthermore, ispinesib was a 100 fold more potent than treprostinil and was significantly more antiproliferative, inhibiting growth with an $EC_{50}$ value of 0.03 nM and 4 nM, respectively (figure 5.10). Compared with effects of ispinesib in human PASMCs from IPAH patients, there was also a trend towards a lower maximal inhibitory effect in fibroblasts ($E_{Max}$ 25% versus 2%; n=4).

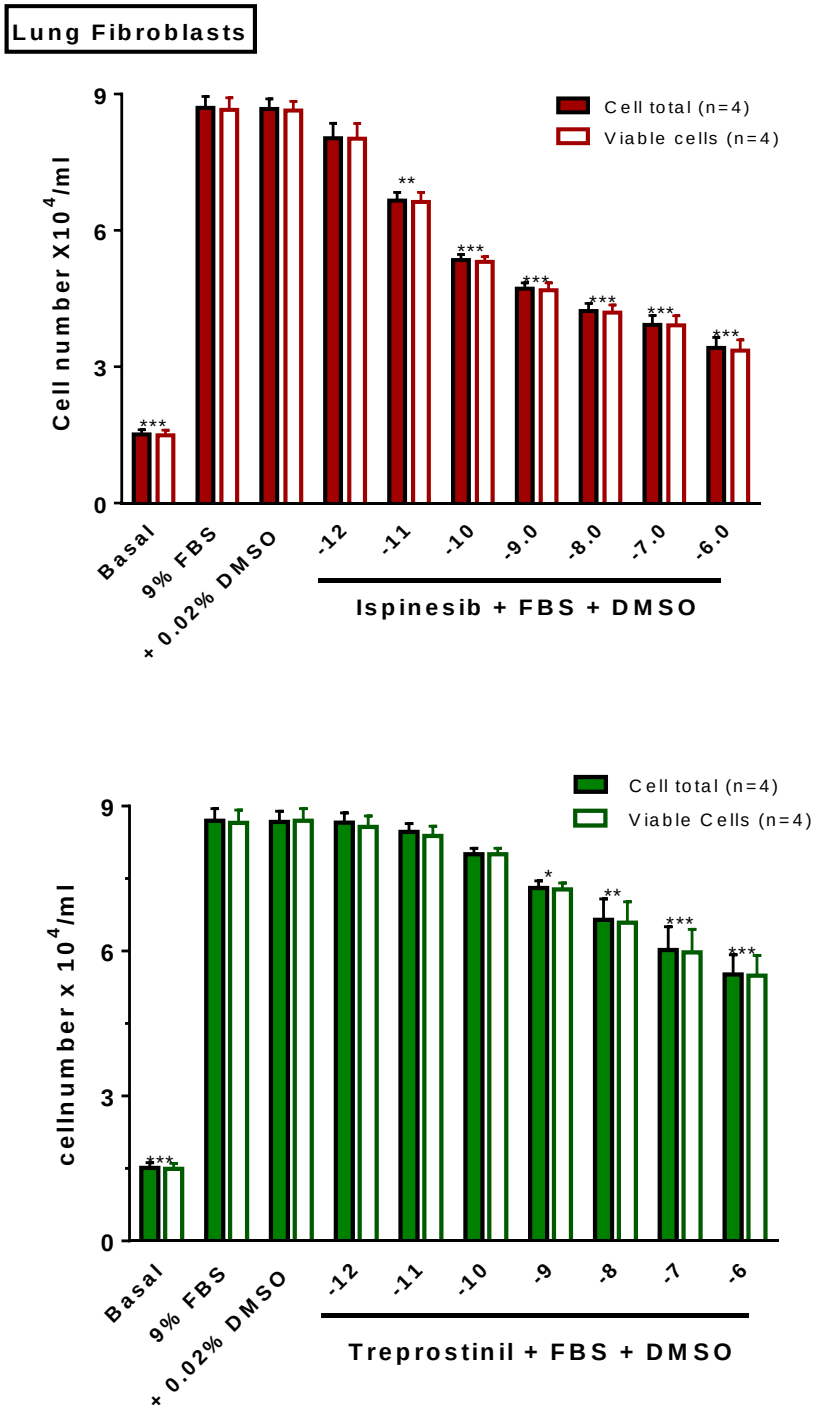



**Figure 5.9. Effect of ispinesib and treprostinil on cell proliferation induced by FBS in human lung fibroblasts derived from patients with pulmonary fibrosis and secondary PAH.** Cells were grown in 9% FBS for 4 days in the absence and presence of increasing concentrations of ispinesib or treprostinil. Graphs show total and viable cell count as assessed by propidium iodide. *=P<0.05, **=P<0.01, ***P<0.001 compared to appropriate solvent (DMSO) control (One-way ANOVA, Dunnett's post-hoc, n=5).

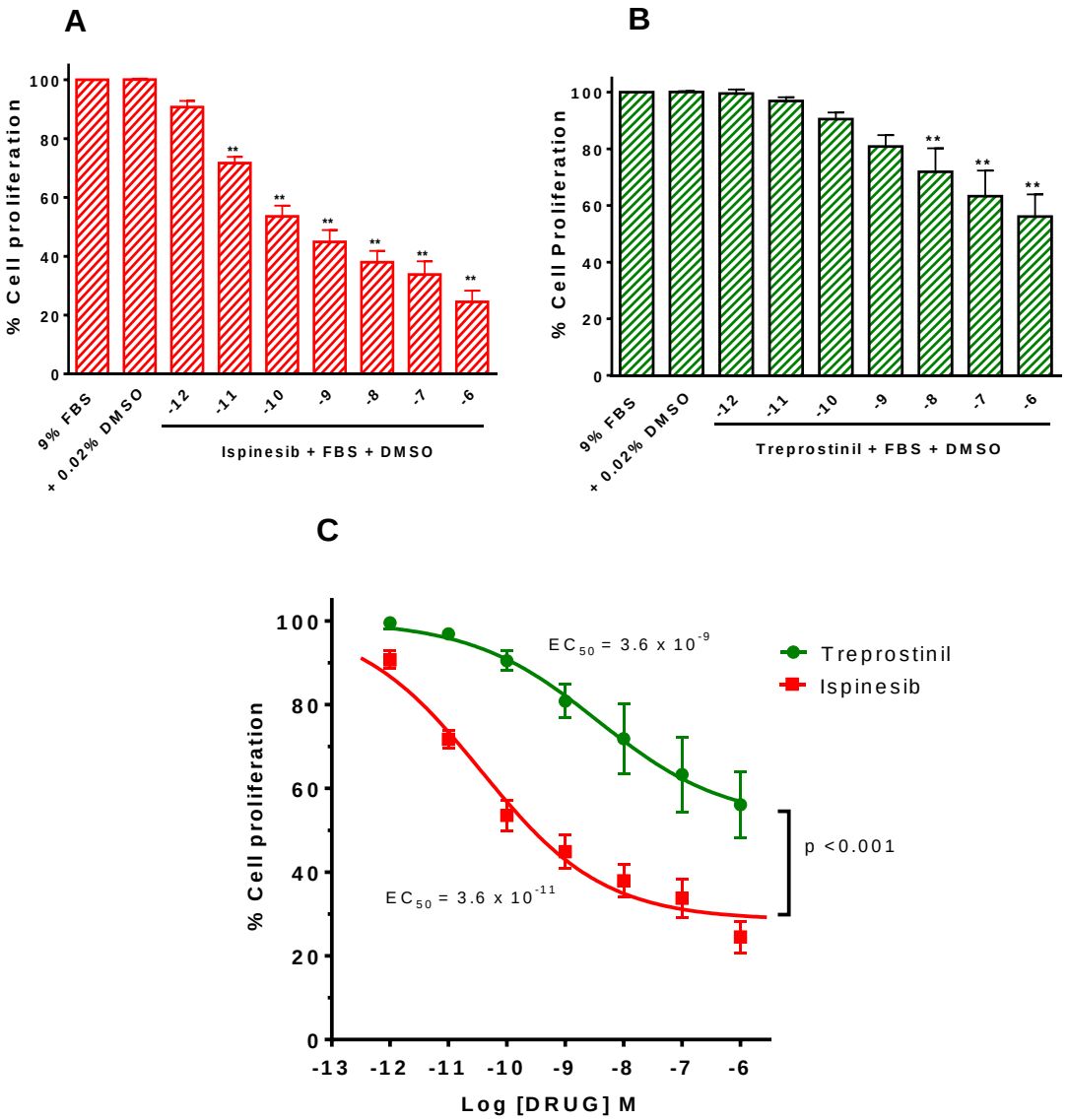



**Figure 5.10. Effect of ispinesib and treprostinil on cell proliferation induced by FBS in human lung fibroblasts derived from patients with pulmonary fibrosis and secondary PAH.** Cells were grown in 9% FBS for 4 days in the absence and presence of increasing concentrations of ispinesib or treprostinil and data expressed as % cell proliferation normalized to growth response induced by FBS alone. In A & B **=P<0.01 compared to appropriate DMSO control (One-way ANOVA, Dunnett's post-hoc, n=4). In C the $EC_{50}$ for both agents was derived by fitting data with a variable slope sigmoidal-curve using GraphPad Prism and statistical difference assessed using 2-way ANOVA (n=4).

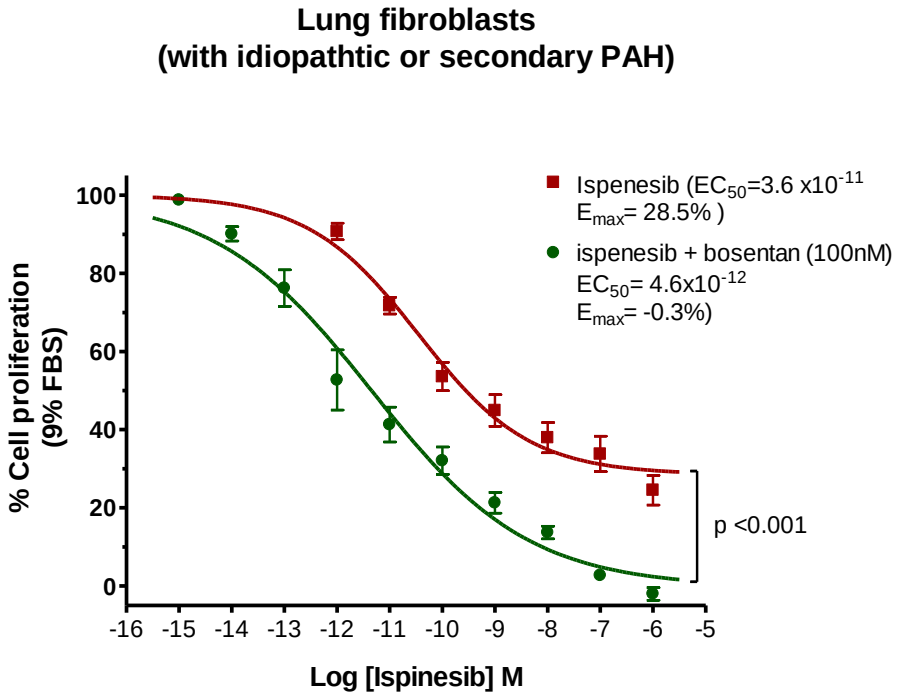



**Figure 5.11. Bosentan potentiates the anti-proliferative effects of ispinesib on cell proliferation induced by FBS in human lung fibroblasts derived from patients with pulmonary fibrosis and secondary PAH.** Cells were grown in 9% FBS for 4 days in the absence and presence of increasing concentrations of ispinesib with or without bosentan (100nM) and data normalized to the growth response induced by FBS. Data are expressed as mean ±S.E.M (n=4) and $EC_{50}$ derived by fitting data with a variable slope sigmoidal-curve using GraphPad Prism. Statistical difference was assessed using 2-way ANOVA (n=4).

To ascertain whether there was synergy between ispinesib and bosentan, I assessed the antiproliferative effects of ispinesib in combination with bosentan in pulmonary fibroblasts isolated from PAH patients. As observed in PASMCs, this combination was significantly more potent at inhibiting proliferation in fibroblasts compared to ispinesib alone (figure 5.11). The combination of agents shifted the dose response curve to the left by 1 log unit and now full inhibition of growth was observed at 1 µM ispinesib.

| Drug (s) | $pEC_{50}$ | $E_{Max}$ (%) |
|---|---|---|
| **IPAH (n=5)** | | |
| Bosentan | -7.35 ± 0.59 | **73.8 ± 7.3$^{***+}$** |
| Ambrisentan | -8.08 ± 0.25 | **71.0 ± 3.2$^{***+}$** |
| Iloprost | -8.28 ± 0.41 | **56.5 ± 6.8$^{**}$** |
| Treprostinil | -8.64 ± 0.47 | **37.4 ± 8.7$^{**}$** |
| Ispinesib | -8.77 ± 0.60 | **2.1 ± 15.5** |
| **Con PASMC (n=4)** | | |
| Treprostinil | -8.42 ± 0.27 | **70.2 ± 3.1** |
| Ispinesib | -8.58 ± 0.53 | **43.8 ± 9.34** |
| **Lung Fibroblasts (n=4)** | | |
| Treprostinil | -8.43 ± 0.74$^{\#}$ | **52.3 ± 11.1** |
| Ispinesib | -10.45 ± 0.19 | **25.1 ± 3.3** |

**Table 8 - $EC_{50}$ and $E_{max}$ values for ispinesib, ET-1 antagonists and prostacyclin analogues on cell growth in human PASMCs and lung fibroblasts.** PASMCs were derived from control (Con) or IPAH patients and fibroblasts from patients with either idiopathic pulmonary fibrosis and secondary PAH or IPAH. Values extrapolated from data presented in figures 5.2, 5.3, 5.10. *=P<0.05, ***=P<0.001 and +=P<0.05 when compared to ispinesib and treprostinil, respectively in IPAH group (one way ANOVA with Bonferroni correction for multiple comparisons). #=P<0.05 when compared to ispinesib in control PASMCs or lung fibroblasts (unpaired Student t-test).

| Drug (s) | $pEC_{50}$ | $E_{Max}$ (%) |
|---|---|---|
| Ispinesib | -8.78 ± 0.65 | **2.1 ± 15.5** |
| Ispinesib + Bosentan | -11.34 ± 0.29** | **0 ± 5.4** |
| Ispinesib + BQ788 | -10.55 ± 0.34 | **28.0 ± 5.1** |
| Ispinesib + Ambrisentan | -10.49 ± 0.30 | **25.8 ± 4.8** |
| Ispinesib + Ambrisentan + BQ788 | -10.02 ± 0.49 | **0 ± 9.4** |

**Table 9 - Potency and efficacy of ispinesib in combination with various ET-1 receptor antagonists at inhibiting proliferation in IPAH cells.** Values extrapolated from data presented in figures 5.7, & 5.8. **=P<0.01 when compared to ispinesib alone (one-way ANOVA with Bonferroni correction for multiple comparisons.

## 5.3 Mechanism of action of KSP inhibitors

Having established that ispinesib was an effective antiproliferative agent, I sought to ascertain if the mechanism of action of ispinesib is through its known inhibitory effects on the kinesin pathway rather than through a novel mechanism in this experimental setting in a non-cancerous, but highly proliferative cellular phenotype. In order to assess this, we compared a compound SB-743921 (CK0929866) with an identical mechanism of KSP inhibition and a similar pharmacophore to ispinesib with its less active enantiomer (CK0929867), compounds developed and supplied by Cytokinetics (San Francisco, USA). The biochemical $IC_{50}$ for KSP inhibition for the more active enantiomer was recently

measured by Cytokinetics as 2 nM while that of its less active enantiomer to be 176 nM (Cytokinetics, published conference proceedings; http://aacrmeetingabstracts.org/cgi/content/abstract/2006/2/B11). Thus if the mechanism of antiproliferative action is by inhibiting KSP in our experimental setting then an 80 fold difference in potency should be reflected in a shift in the potency/effectiveness of the two compounds against cell proliferation. Indeed, CK0929867 was significantly less effective at inhibiting cell proliferation, and depending on whether $E_{max}$ was freely fitted or constrained to zero, the decrease in potency ($EC_{50}$) was either 4.5 or 57 fold, respectively (figure 5.12). We suspect it to be closer to the latter given that 10 µM CK0929867 appeared to be cytotoxic/ apoptotic at this dose, suggesting that an intermediate dose (e.g. 3 µM) may have caused complete inhibition of cell growth.

The function of KSP at the earliest stage of mitosis is to mediate centrosome separation with the formation of a bipolar mitotic spindle, thus facilitating chromosome alignment and segregation during cell-division. Failure of KSP to function normally leads to cell cycle arrest in mitosis with a classic monopolar mitotic spindle (Blangy *et al.*, 1995; Kapoor *et al.*, 2000). In order to confirm that this is indeed the mechanism of action of ispinesib, immunostaining was performed using antibodies directed towards alpha-tubulin which should show whether the organization of microtubules is affected due to KSP inhibition.

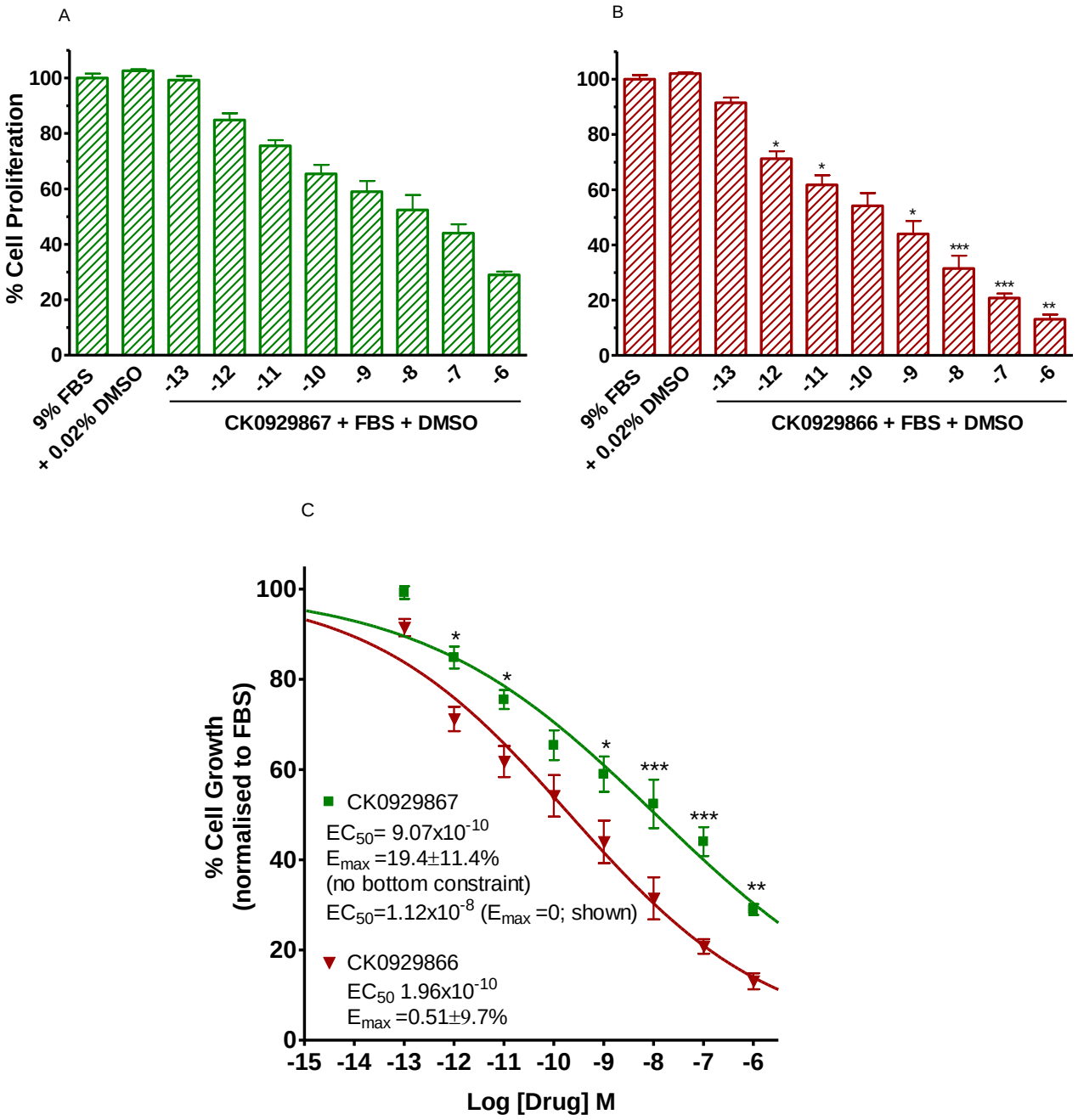



**Figure 5.12. Comparison between *SB-743921* (pharmacophore of ispinesib) & CK0929867 (less active enantiomer) on cell proliferation.** Cells were grown in 9% FBS for 4 days in the absence and presence of increasing concentrations of CK0929867 (A) or SB-743921 (CK0929866) (B) and data normalized to growth response to FBS. *=P<0.05, **=P<0.01, ***=P<0.001 when compared to CK0929867 (two-way ANOVA, Bonferroni post-hoc, n=5). $EC_{50}$ and $E_{max}$ given in C were derived by fitting data with a variable slope sigmoidal-curve using GraphPad Prism.

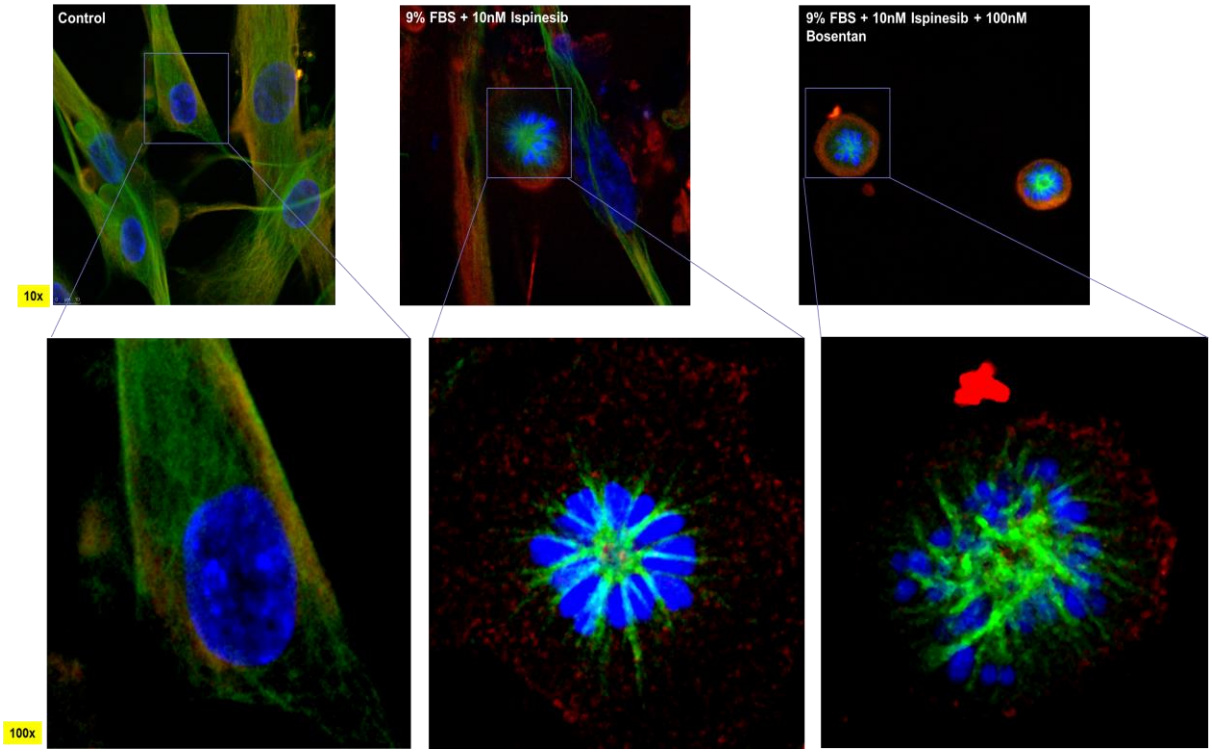



**Figure 5.13. Immunofluorescence confirming the mechanism of inhibition of cell division by ispinesib.** Human PASMCs isolated from IPAH patients were treated for 24 hours with 9% FBS in the absence or presence of 10 nM ispinesib with or without 100 nM Bosentan, followed by immunofluorescence staining as per methods. Confocal images taken at 10 and 100 x magnification showing immunofluorescence staining in cells using antibodies against the microtubule marker alpha-tubulin (stained in green), the centromere marker gamma-tubulin (stained red) and the chromosome marker dapi (blue).

This was indeed the case, as incubation of cells with 10 nM ispinesib in the absence or presence of 100nM bosentan for 24 hr produced the expected formation with a monopolar arrangement of the chromosomes clearly observed (figure 5.13, middle panel) which were also visibly disordered (figure 5.13, right panel). If KSP is not affected, then the chromosomes would be expected to proceed to arrange themselves in a bi-polar fashion on the formed spindle, a pre-requisite for effective cell-division. It was not possible to capture this normal phenotype in growing cells at our 24 hr time frame, either because the mitotic phase is short lived and hard to capture, or it occurs in a different time window to mitotic arrest with ispinesib.

## 5.4 Summary of key findings

- The mitotic kinesin inhibitor, ispinesib, is a potent ($EC_{50}$ ~2 nM) and effective inhibitor of serum-induced proliferation in human pulmonary arterial smooth muscle cells (PASMCs) derived from patients with idiopathic pulmonary arterial hypertension (IPAH).
- Under the same experimental conditions, ispinesib was significantly more efficacious (but not more potent) than prostacyclin analogues (iloprost or treprostinil) and even more effective than endothelin receptor antagonists, bosentan or ambrisentan.
- In contrast, ispinesib was 100 fold more potent ($EC_{50}$ 0.036 nM) than treprostinil at inhibiting cell proliferation in lung fibroblasts derived from patients with primary or secondary PAH. The enhanced potency may relate

in part to the higher proliferative capacity of fibroblasts compared to hypertensive PASMCs, though there was a trend for less maximal growth inhibition with the former.

- Ispinesib was significantly less efficacious in normal human PASMCs over a wide concentration range, which suggests that this agent may be more effective against a cell type with a more proliferative phenotype.
- The mixed ET-1 receptor antagonist bosentan synergistically enhanced the potency of ispinesib by 400 fold in human PASMCs from IPAH patients, and by 10 fold in fibroblasts isolated from patients with IPF and secondary PAH. The stable prostacyclin analogue treprostinil also showed enhancement in human PASMCs, though effects on cell proliferation with ispinesib appeared additive.
- Blockade of $ET_A$ receptor with ambrisentan or the specific $ET_B$ with BQ788 did not enhance the effect of ispinesib in IPAH cells, but combining the two did show synergy. Thus blockade of both ET-1 receptors appears required to potentiate the effect of ispinesib.
- When ispinesib was combined with a clinically relevant concentration of bosentan, complete growth arrest was maintained for at least 4 days after the original application in IPAH cells. I suggest this could slow disease progression by inhibiting the exaggerated proliferation of vascular smooth muscle cells in later stages of the disease. Patients on background therapy with bosentan or treprostinil could therefore derive greater benefit than

those treated with ispinesib alone due to enhanced efficacy and duration of response or both.

- As in tumour cells, ispinesib appeared to inhibit cell proliferation of PASMCs from IPAH patients by inhibiting the kinesin spindle motor protein. A comparison between the KSP inhibitor SB-743921 (an identical mechanism of action, closely related analog of ispinesib) and CK0929867 (its less active enantiomer) showed the latter to be less effective in cell proliferation assays, while immunofluorescence showed that ispinesib produced the expected phenotype of disordered chromosomes.

## 5.5 Discussion

Pulmonary arterial hypertension and cancer share certain features, in that both diseases involve abnormal cellular growth. In the case of PH, these abnormal cells are those that line the blood vessels which undergo abnormal phenotypic changes and stimulate growth allowing further proliferation, and thickening of the vessel walls. It involves hypertrophy and/or hyperplasia leading to thickening of all three layers of the blood vessel wall, as well as extracellular matrix deposition and formation of plexiform and neointimal lesions (Rabinovitch, 2008). Evidence suggests that a number of interleukins and cytokines which could serve as chemoattractants, proangiogenic and proproliferative effectors (Coscoy, 2007) are also found in human plexogenic lesions in PAH (Cool *et al.*, 2003; Coscoy, 2007). Shear stress, hypoxia and/or various mediators like angiotensin II, ET-1, 5-HT,

growth factors, and inflammatory cytokines, increased serine elastase activity, carcinogens and others (Rabinovitch, 2007; Rabinovitch, 2008) are involved in the remodelling of the pulmonary vasculature. Tissue hypoxia, a key feature of both PAH and cancer, inhibits the release of anti-mitogenic factors (Madden *et al.*, 1986) and increases the production and/or release of mitogenic factors e.g., interleukin-1 from pulmonary artery smooth muscle cells (Cooper & Beasley, 1999) and growth factors (ET-1 and PDGF) from endothelial cells (Dawes *et al.*, 1994; Gong *et al.*, 2011). Sustained hypoxia can cause the recruitment of cells with enhanced growth, migratory and pro-mitogenic features in the wall of distal pulmonary arteries (Frid *et al.*, 2009; Burke *et al.*, 2009). Factors that appear to drive these phenotypic changes, include platelet-derived growth factor, and other mitogens such as the chemokine stromal cell derived factor SDF-1/CXCL12 and interestingly the calcium binding protein and a mediator of metastasis, S100A4 (Frid *et al.*, 2009). Thus PAH and cancer share many elements of pathophysiology.

In my experiments using the anticancer drug candidate, ispinesib, I have shown it to have potent and efficacious antiproliferative effects in PASMCs isolated from IPAH patients at the end-stage disease. It inhibited growth with an $EC_{50}$ of ~2 nM thus having a similar potency ($K_i$ 1.7 nM) to that found for inhibiting KSP motor domain ATPase activity (Lad *et al.*, 2008). The potency of ispinesib to inhibit proliferation of IPAH cells, was higher than when tested in a panel of 50 breast cancer cell lines, where ispinesib exhibited $EC_{50}$ sensitivities between 7.4 and 600 nM, although sensitivity mostly fell within a 10-fold range, between 7.4 and 80 nM

(Purcell *et al.*, 2010). Ispinesib was however 100 fold less effective at inhibiting proliferation of slower growing normal cell lines suggesting that the main target for this agent is likely to be the cells with fast uncontrolled growth rate. Indeed, KSP was found to be most abundant in proliferating human tissues (e.g. thymus, tonsils, testis, esophageal epithelium, and bone marrow) and was absent from cultured postmitotic human central nervous system neurons (Sakowicz *et al.*, 2004). Mitosis, being an important process for cell division of tumour cells, has long been identified as a key target in cancer chemotherapy. The mitotic spindle mediates the mechanical aspects of mitosis and KSP functions exclusively to form it in proliferating cells. KSP has been reported to be overexpressed in tumour tissue compared to normal tissues, making it an attractive target for proliferative diseases (Moores *et al.*, 2002; Sakowicz *et al.*, 2004). Drugs inhibiting KSP affect chromosome segregation by arresting cells in the G2/M phase of the cell-cycle and causing apoptosis (Sakowicz *et al.*, 2004; Marcus *et al.*, 2005; Miglarese & Carlson, 2006). Ispinesib alters the ability of KSP to bind to microtubules and inhibits its movement by preventing the release of ADP without stopping the release of the KSP−ADP complex from the microtubule (figure 5.14B). Thus, this agent works exclusively in mitosis and does not affect non-dividing cells, displaying a unique phenotype known as the monopolar spindle initially shown with monoastrol in tumour cells (Kashina *et al.*, 1996; Kapitein *et al.*, 2005; Peters *et al.*, 2006; Lad *et al.*, 2008). This phenotype was also observed following treatment of human PASMCs with ispinesib for 24 h. In addition, ispinesib is highly selective for KSP, since other mitotic kinesins are not inhibited by this

agent, even up to concentrations as high as 20 µM (Lad *et al.*, 2008), which may explain in part the lower side-effect profile of this agent compared to other cancer drugs that are anti-mitotic.

Instead of checking a single dose of the drug, I assessed the full concentration-response range in order to obtain a reliable $EC_{50}$ for antiproliferative effect of ispinesib on PAH cells. When compared with current treatments under the same experimental conditions, it was significantly more efficacious (but not more potent) than prostacyclin analogues (iloprost or treprostinil) or endothelin receptor antagonists (ambrisentan), agents currently approved to treat PAH. Interestingly when ispinesib was combined with the mixed ET-1 receptor antagonist, bosentan, the potency ($EC_{50}$ 4 pM) and ability of ispinesib to inhibit growth in IPAH cells was synergistically enhanced. Likewise, ispinesib has been shown to synergise with other types of anti-mitotic agents as well as inhibitors of the epidermal growth factor receptor, HER-2 (Purcell *et al,* 2010), suggesting certain combinations may be particularly effective at growth causing  arrest in otherwise resistant cells.

When I look into the literature for a possible explanation for this synergy between ispinesib and bosentan, there are a few parallels to the situation here. Endothelin-1 is one of the major mitogens which is elevated in cancer, where $ET_A$ receptors appear to play a major role in driving carcinomas through multiple cell growth and survival pathways, involving amongst other things, Bcl-2 and the EDGF receptor (Lalich et al., 2007).

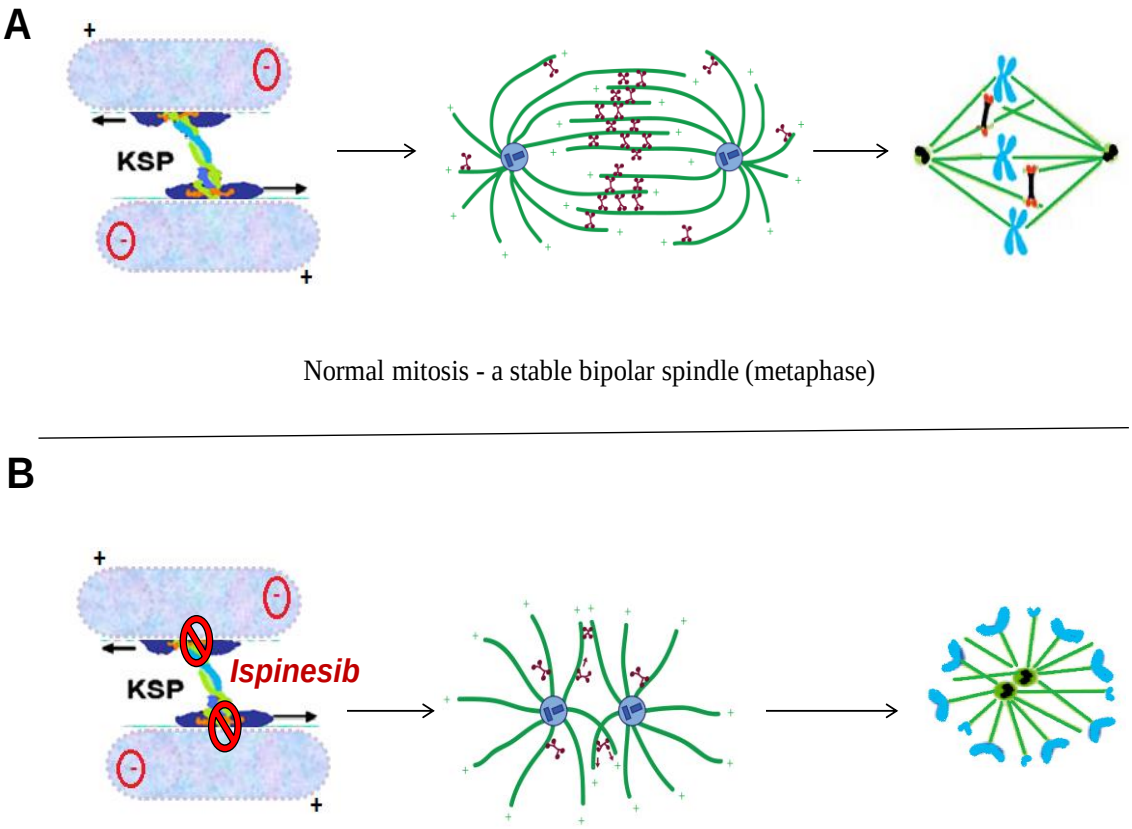



**Figure 5.14. Schematic for mechanism of inhibition of cell division by ispinesib** A) Kinesin spindle protein 5 (KSP) plays a vital role in the formation of a bipolar mitotic spindle which is required for cell cycle progression through mitosis. Thus normal mitosis when progressed will have a stable bipolar spindle formation in the metaphase. B) Ispinesib is an anticancer agent that has been shown to cause mitotic arrest and growth inhibition in several human tumour cell lines. Ispinesib alters the ability of KSP to bind to microtubules and inhibits its movement by preventing the release of ADP without preventing the release of the KSP−ADP complex from the microtubule. This causes cell cycle arrest by forming a monopolar spindle.

Although I found bosentan alone to have limited efficacy to inhibit proliferation of PASMCs of IPAH patients, there was synergy when combined with. Likewise, in the study performed by Berger and colleagues, showed that exogenous ET-1 did not act as a growth factor for human melanoma cells, but blockade of ET-1 receptors potentiated the effects of anti-cancer agents and helped decrease proliferation and induce apoptosis in defined melanoma cells (Berger et al., 2006). This suggests that combination therapy of ET-receptor antagonists with alkylating agents that promote DNA damage may be more effective in inhibiting cell proliferation. Antagonists of the ET receptors sensitised human tumour cells to Fas ligand-mediated apoptosis, thus helping the anti-cancer drug to penetrate a more resistant cellular phenotype (Peduto *et al.*, 1999). Lastly in a rat model of pancreatic fibrosis and cancer, bosentan exerted both antifibrotic and antitumor affects in vitro (Fitzner *et al.*, 2009; Jonitz *et al.*, 2009), demonstrating that this agent can inhibit proliferative processes of different origins.

However, when I evaluated the onset and time-course of ispinesib effects in growing cells, I found that while ispinesib was able to completely growth arrest cells, its effects began to significantly wane at 72 h. The reason for this is unknown, but may involve either drug resistance or a re-entry into the cell cycle as previously shown for KSP inhibitors in some circumstances (Gascoigne & Taylor, 2008; Shi *et al.*, 2008). In contrast, the same concentration of ispinesib combined with a clinically relevant concentration of bosentan, was able to maintain complete growth arrest, for at least 4 days after the original application. Taken together, this

may suggest that ispinesib could be of benefit in slowing disease progression by inhibiting the exaggerated proliferation of vascular smooth muscle cells in later stages of the disease particularly in those patients already on background therapy with bosentan. One possibility is that both compounds work in concert to reduce the expression of the anti-apoptotic protein, Bcl2. Previous experiments have shown that ispinesib to reduce phospho-Bcl2 and Bcl-XL consistent with increased induction of apoptosis (Purcell et al., 2010). In the same study, ispinesib treatment was also associated with increased cleavage of caspase-3 (a marker of apoptosis), decreased staining for Ki67 (a marker of active proliferation), and decrease in tumour cellularity. Likewise synergy could be found with ispinesib and bosentan in pulmonary fibroblasts isolated from patients with secondary PAH. The shift in the concentration-response curve was however only 10-fold, presumably because ispinesib was already particularly potent ($EC_{50}$ 36 pM) on its own in these cells which I found to grow at a faster rate than PASMCs. Recently bosentan failed the trial of IPF, possibly due to adverse events associated with high doses of the drug (Gunther et al., 2007). It is tempting to speculate that this combination of ispinesib with low dose of bosentan could potentially work in IPF or when associated with PAH.

The stable prostacyclin analogue treprostinil also boosted the action of ispinesib, though effects on cell proliferation appeared additive. Higher levels of ET-1 are associated with PAH and this potent mitogen could be responsible for making the cells more proliferative (Davie *et al.*, 2009). In my previous chapter I demonstrated that treprostinil was a potent inhibitor of ET-1 levels in the nano-molar range in

IPAH cells and this effect could possibly make the cells less resistant and more sensitive to ispinesib in a manner similar to bosentan. Alternatively, both drugs could inhibit proliferation by affecting different individual pathways. A major mechanism whereby prostacyclin or its stable analogues inhibit smooth muscle growth is to block G1 phase progression by blocking the degradation of p27 and the activation of cyclin E-cdk2 (Kothapalli *et al.*, 2005) and such a mechanism could act in concert with the anti-mitotic effects of ispinesib. Like in PAH, a lower expression of prostacyclin synthase (PGIS) has been found in malignant cells compared to normal cells (McLemore *et al.*, 1988; Hubbard *et al.*, 1989), and so its loss may contribute to tumour genesis. Indeed, pulmonary-specific over expression of prostacyclin synthase was reported to result in suppression of tumour incidence and multiplicity in lung cancer models (Nemenoff *et al.*, 2008). Due to its cardio-protective effects, prostacyclins have also been considered for use in combination with anticancer drugs to take care of the cardiac damage that many of these drugs can cause (Wang *et al.*, 2011). Thus, the same theory could be applied using anticancer therapies in combination with cardio-protective prostacyclin analogues in PAH (O'Callaghan & Gaine, 2007). Adding to the increasing body of evidence that prostacyclins are also vasoprotective and have regenerative effects on the endothelium (Hamblin *et al.*, 2009; Katusic *et al.*, 2012), points to another potential benefit of combining these two class of agents. Thus patients on background therapy with bosentan or treprostinil could derive greater benefit than those treated with ispinesib alone due to enhanced efficacy, duration of response or both. Cardiovascular protection with treprostinil may also be of benefit.

Drugs in current use for the treatment of PAH are based around their properties as vasodilators, including $Ca^{2+}$-channel antagonists, prostacyclin and its stable analogues and NO or agents to increase NO bioavailability (Homer & Wanstall, 1998; Wanstall & Jeffery, 1998; Rubin & Morrell, 2011). As vasodilators, each of these drug types acts by opposing any abnormal vasoconstriction. Since abnormal vasoconstriction becomes progressively less important and vascular remodelling progressively becomes more important as the disease advances (Reeves *et al.*, 1986), an alternative, and possibly more fruitful approach is to target pulmonary vascular remodelling. Indeed <15% of PAH patients are responsive to any type of vasodilator therapy at all, and of those, only ~6% respond to long-term oral calcium channel blocker therapy (Sitbon et al., 2005), suggesting therapeutic benefit comes from mechanisms unrelated to hemodynamic effects. Even though prostacyclins are shown to be antiproliferative (Falcetti *et al.*, 2010) as well as anti-remodelling in experimental PAH (Schermuly *et al.*, 2004; Yang *et al.*, 2010), they do lose their efficacy as the disease progresses. This perhaps could be due to desensitisation of the receptor and/or phenotypic changes of those cells which might develop some kind of resistance towards less aggressive treatments. Patients in functional class III or IV who fail to improve or deteriorate with monotherapy can be treated with combination therapy; atrial septostomy; or transplantation (lung or heart-lung). However, to date, only lung transplantation offers a cure for PAH, which in itself comes with its own risks and chances of organ rejection. Current rationale for using chemotherapeutic drugs in PAH has been on the basis that they

inhibit growth factors induced or upregulated in the disease. Initial experimental studies on pulmonary hypertension demonstrated positive effects of epidermal growth factor and PDGF receptor inhibition on hemodynamics, remodelling and survival (Merklinger *et al.*, 2005; Schermuly *et al.*, 2005a). Imatinib, which is an inhibitor of the tyrosine kinases PDGF receptor, BCR-ABL, and c-kit, indicated beneficial results in individual clinical case reports from patients with pulmonary arterial hypertension (Ghofrani *et al.*, 2009). This and other studies have led to a Phase 3 randomized, placebo-controlled clinical trial of imatinib in PAH known as IMPRES (ClinicalTrials.gov identifier: NCT01179737). The results reported at the annual European respiratory society meeting, September, 2011, found that treated patients had a placebo-adjusted mean increase in 6MWT which was associated with statistically significant improvements in pulmonary arterial pressure, cardiac output and pulmonary vascular resistance, though not time to clinical worsening. Nonetheless, despite some encouraging results, there is still some concern that long-term treatment of imatinib could be associated with left ventricular dysfunction and heart failure (Kerkela *et al.*, 2006), although this did not seem to be a major problem in a recent clinical trial (Ghofrani et al., 2010).

Most of the chemotherapeutic agents so far being seriously considered in PAH are targeted towards specific growth factor or mitogen pathways which are known to be elevated in the disease, but PAH is a multifactorial disease and inhibition of a single mitogen or selective growth factors might not necessarily demonstrate the most desired effect on remodelling. To this end the multi-kinase inhibitor sorafenib

has recently been assessed and appears to have a greater effect on RV systolic pressure and cardiac hypertrophy compared to imatinib in a rat monocrotaline model of PAH, the superior effect being attributed to blocking of the serine/threonine kinase, Raf-1 (Klein *et al.*, 2008). This drug is now being evaluated clinically (Gomberg-Maitland et al., 2010). However, another broad spectrum tyrosine kinase inhibitor, dasatinib, while decreasing PDGF-induced proliferation in addition to multiple growth factor-induced PASMC proliferation through inhibition of Src phosphorylation in experimental PAH, is nonetheless associated with severe precapillary pulmonary arterial hypertension cell in patients treated for chronic myelogenous leukemia (Montani et al., 2012). Several other molecules, which were originally developed as anti-cancer agents, have also been investigated in animal models of PH and have shown positive outcomes, and thus are likely to lead to clinical evaluation in patients with PAH, particularly those molecules that are already licensed medicines. Some examples of such molecules are cell cycle inhibitors rapamycin (Paddenberg *et al.*, 2007), anti-apoptotic drugs which are survivin inhibitors (McMurtry *et al.*, 2005) and elastase inhibitors (Cowan *et al.*, 2000). Thus several cancer drugs are in the pipeline to be further evaluated in PAH, though it remains to be determined whether side effects outweigh clinical improvement and survival and the potential to cause PAH with some agents is a concern. In terms of KSP inhibitors, their major advantage could be their low side-effect profile with neutropenia being the commonest side effect with none of the documented neuropathy, mucositis, or alopecia that is observed with many anti-cancer therapies (Gomez et al., 2012).

All class of agents used to treat PAH are expected to promote inhibition of vascular smooth muscle cell growth (Rabinovitch, 2008; Rhodes et al., 2009) and the antiproliferative effects of prostacyclins and endothelin antagonists are recognised as an important property of these agents in ACCF/AHA 2009 Expert Consensus Document on Pulmonary Hypertension (McLaughlin et al., 2009). When agents from these two classes of agents were compared against their ability to inhibiteserum-derived growth of human PASMCs derived from IPAH patients with endstage disease, the mixed ETRA, bosentan or the selective $ET_A$ antagonist ambrisentan, only weakly inhibited growth compared to either iloprost or treprostinil. Significant inhibition with bostentan or ambrisentan only occurred at concentrations at or above 10nM (ambrisentan) or 100 nM (bosentan) compared to 1 nM with the two prostacyclin analogues. Thus, while ET-1 is a potent smooth muscle mitogen and driver of vascular smooth muscle proliferation and may contribute to enhanced proliferation in PAH through increased smooth muscle receptor expression (Davie et al, 2009), a number of pro-proliferative signalling pathways involving growth factors, cytokines, metabolic signalling, and matrix remodelling have been identified in the pathobiology of PAH (Rabinovitch, 2008; Hassoun et al., 2009) which may be more sensitive to inhibition by prostacyclin. I speculate that prostacyclins have a much stronger inhibitory effect on cell proliferation, because they are able to inhibit growth through multiple pathways, including through both membrane and nuclear receptors (Clapp & Patel 2010). Clinical observations support the idea that endothelin antagonists are less effective

than prostacyclins, where time to clinical worsening with endothelin antagonists appears much shorter than for the prostacyclin class of drug, with many patients requiring prostacyclin therapy after 1-2 years (McLaughlin et al., 2009). Moreover, significant effects on long term survival have only really been shown for the prostacyclin class of drugs (McLaughlin *et al*., 2009; Rhodes *et al*., 2009; (Sadushi-Kolici *et al.*, 2012).

I found that treprostinil was a significantly more effective inhibitor of cell growth compared to iloprost in IPAH cells. This concurs with a previous study from our laboratory, where the rank order of prostacyclin analogues to inhibit normal human PASMC proliferation was treprostinil (UT-15) >iloprost>beraprost (Clapp et al., 2002). Indeed examining the potency and efficacy of these prostacyclin analogues in this cell type using a similar in vitro assay to here, has been able to predict clinical efficacy in PAH (Gomberg-Maitland & Olschewski, 2008). Moreover, our laboratory has previously demonstrated that compared with treprostinil, iloprost is a less effective agent at increasing intracellular cyclic AMP (Clapp et al., 2002) despite having an 8 fold higher affinity ($K_i$ 32 versus 3.6 nM) for the human prostacyclin (IP) receptor (Whittle et al., 2012). This can best be explained by the differential pharmacology now recently appreciated, with treprostinil unexpectedly having a higher affinity at the prostaglandin $E_2$ ($EP_2$) and $D_2$ (DP) receptor (Ki 3.6 and 4.4 nM, respectively) than even the IP receptor (Whittle et al., 2012). Given the plasma concentrations achieved with intravenous or subcutaneous treprostinil in

patients range between 2.5 to 25 nM (McSwain et al., 2008), suggests these prostanoid receptors will be activated within the therapeutic window. Moreover, the ability of treprostinil over iloprost to inhibit smooth muscle proliferation in *vivo* may be even more marked, as the upper plasma concentration that is achieved with iloprost is >1 nM (Krause & Krais, 1986).

In summary, I described the successful antiproliferative effect of KSP inhibitor ispinesib in experimental settings of pulmonary arterial hypertension. The combined use of ispinesib with existing treatments in PAH to inhibit the mitotic pathway could be beneficial in tackling pulmonary vascular remodelling in PAH and may provide an option to treat pulmonary arterial hypertension and the associated right heart remodelling.

## 5.6 Limitations of this chapter

1. As with every cancer drug, cytotoxicity and apoptosis of different cell types should be considered as potential unwanted-side effects. Therefore experiments are needed to assess cytotoxicity of ispinesib, including whether this agent causes apoptosis of smooth muscle and endothelial cells.
2. In future work, I will explore how bosentan is sensitising and potentiating the effects of ispinesib. As a first step, I plan to use FACs analysis to identify if bosentan is causing cells to growth arrest in a different or similar phase of the cell cycle, or if it is preventing cells from re-entering the cell cycle.

# 6. Conclusions and future perspectives

Most of the targets described in my thesis have been implicated in the proliferative processes of the PASMCs. However, establishing which of these targets and potential treatment options is the most relevant and potentially the most efficacious in the setting of PAH requires further investigation. The purpose of my experiments was to explore the anti-proliferative pathway and the interaction of two or more mediators that might interfere with proliferation in human PASMCs isolated from patients with IPAH. Specifically, I aimed to investigate mechanisms in which multiple proliferation pathways might be linked or affected and thus might serve targets for future drug development for PAH.

## 6.1 Stable prostacyclin analogues in PAH

In the first chapter I have assessed the stable prostacyclin analogue treprostinil with respect to its ability to generate cAMP and inhibit proliferation of PASMCs. I demonstrated that despite being a chemically stable prostacyclin analogue, cyclic AMP levels were not well sustained after treprostinil treatment in human PASMCs isolated from IPAH patients compared to control human PASMCs, where cAMP levels remained elevated for several hours (Finney et al, 2002). Phosphodiesterase 3 enzymes hydrolyse the second messenger cyclic 3’5’ AMP to 5’-AMP inactivating this critical second messenger, thus playing an important role in cell signalling. In the work described in this thesis I have investigated the potential of the phosphodiesterase 3 inhibitor, cilostazol in combination with treprostinil and showed that cAMP levels were not only higher, but that elevation was also

significantly sustained for up to 24 h, indicating high activity of PDE3 may play a vital role in prostacyclin analogues signalling in PAH. Cilostazol potentiated the effects of treprostinil, whether it was generation of cAMP, inhibition of SMC proliferation or vasodilation of arteries. Thus PDE-3 seems integral for regulating the many different cellular effects of prostacyclins.

While prostacyclin appears to be important in the normal pulmonary vascular transition, dysregulation of the prostacyclin pathway has also been reported in PAH (Konduri, 2004; Lakshminrusimha *et al.*, 2009). Pre-treatment with milrinone, another PDE3 inhibitor, restores relaxation to prostacyclin to levels similar to that seen in control by restoring the expression of the prostacyclin synthase and the prostacyclin IP receptor in a sheep model of PAH (Chen *et al.*, 2009; Lakshminrusimha *et al.*, 2009). Use of cilostazol has shown very encouraging outcomes in animal models of PAH (Chang *et al.*, 2008; Sun *et al.*, 2009). Combination therapies of existing drugs with these emerging agents could be one interesting option to improve the efficacy and decrease side effects of these drugs in PAH patients. It is important to note that PAH is a multifactorial disease and so most promising agents should ideally combine regression of vascular remodelling with vasodilatator and anti-inflammatory effects, specifically within the pulmonary circulation. Cilostazol has been reported as a successful anti-inflammatory agent in different diseases including pulmonary disorders (Deree *et al.*, 2008; Osawa *et al.*, 2012). Cilostazol is approved for the treatment of intermittent claudication (Liu *et al.*, 2011), significantly reducing muscle pain. As we know, one of the main side-

effects associated with prostacyclin is pain, thus cilostazol, if combined with this agent, may help manage pain associated with the treatment. Cilostazol is also used clinically for peripheral vascular occlusive disease where it increases exercise tolerance and favourably modifies the plasma lipid profile (Kambayashi *et al.*, 2003). Cilostazol is more selective at inhibiting PDE3 whereas milrinone can inhibit PDE4 in addition to PDE3. This has been demonstrated in normal rabbit heart where inhibition of PDE4 by milrinone partly contributes to the greater cardiotonic effect of milrinone when compared to cilostazol. However, the lower level of PDE4 activity in failing human heart suggests that factors other than inhibition of PDE4 by milrinone may even contribute to differences in cardiotonic action when compared to cilostazol. Cilostazol appears to preferentially act on vascular elements like platelets and coronary blood flow, consistent with its beneficial and safe clinical outcomes in patients with intermittent claudication (Shakur *et al.*, 2002; Kunz *et al.*, 2006). This should be tempered with the observation in a trial of PDE3 inhibitors, where treatment with milrinone significantly increased mortality rates in heart failure (Amsallem et al., 2005, Cochrane Database Syst Rev, CD002230.). It could be argued that PDE3 inhibitors are unlikely to translate into clinical practice, accepting that with milrinone, failure may relate to the fact that it inhibits both PDE3 and PDE4 together (Shakur *et al.*, 2002), exacerbating heart failure potentially because of reduced systemic pressure. In this chapter I have provided evidence suggesting use of cilostazol in combination with prostacyclins as a possible new therapeutic regime in IPAH which should be explored.

***Future studies:***

- The therapeutic mechanism, leading to the beneficial effects of prostacyclin and its analogues in patients with PAH, is largely unknown. The exact mechanisms, by which cell proliferation is inhibited, still needs to be determined, although recent work from this laboratory suggests involvement of PPARγ (Falcetti *et al.*, 2007). In my future research I would like to explore the mechanistic pathway of prostacyclin further evaluating different possible targets for treprostinil using receptor binding studies or immunofluorescence techniques to tease out the role of membrane versus nuclear receptors as specially detailed below.
- I also wish to explore more on how different phosphodiesterases may play a role in PAH. I have demonstrated that the acute effect of prostacyclin on cAMP is mediated mainly through IP receptor. I hope to test the hypothesis that other Gs coupled receptor pathways, for example the $EP_2$ or $DP_1$ receptor pathway (Whittle et al, 2012) may play a role particularly in the presence of PDE inhibitor in cells where treprostinil is given chronically. It is also possible that there is an up regulation of phosphodiesterase 3 or other phosphodiesterase enzymes (PDE1, PDE4, and PDE7) which might be responsible for reducing the effectiveness of prostacyclin.

Other opposing receptor pathways like thromboxane A2 or $EP_3$ receptor, which act through $G_i$ inhibiting cAMP, might be responsible for the increasing ineffectiveness of prostacyclins as the disease progresses in PAH. Given also that activators of PKC are known to cause receptor desensitization, could be one possible mechanism for the desensitization or down regulation of IP receptor in long term therapy. $EP_3$ and/or $TXA_2$ receptors can also increase PDE3 activity through activation of PI3 kinase, a well-known target for receptors coupled to $G_i$. Thus I wish to assess the effects of specific $EP_3$ and/or $TXA_2$ antagonists along with treprostinil on cAMP generation, phosphodiesterase activity and on proliferations of human PASMCs. In addition, I wish to test this combination in vivo, particularly with an advanced model of PAH with heart failure.

## 6.2 Prostacyclin interaction with the endothelin pathway

The observation that circulating plasma levels of the vasoactive peptide ET-1 are raised in patients with PAH, and that ET-1 production is increased in the pulmonary tissue of affected individuals, makes it a particularly interesting target for a therapeutic intervention in PAH. Clinical trials with ETRAs showed that they provide symptomatic benefit in patients with PAH, but time to clinical worsening is relatively short with this class of drugs (Attina *et al.*, 2005). However, the role of ETRAs in the treatment of pulmonary hypertension whether used alone or

combined with other classical drugs like prostacyclins or PDE inhibitors remains incompletely understood. In chapter 4, I have demonstrated that the prostacyclin analogue, treprostinil, at a therapeutically relevant dose, could inhibit release of ET-1 induced by serum. In contrast, ETRAs significantly increased ET-1 levels over and above that induced by serum alone. This mirrors what happens clinically when giving these compounds, although it is not clear whether up-regulation of ET-1 levels is maintained with chronic treatment of these agents. Paradoxically, treprostinil in combination with ETRAs was less effective at inhibiting ET-1 levels. The other important observation was that this increase in the ET-1 levels with the ETRAs treatment seems to be an effect relating to agents interacting with the $ET_B$ receptor as the ET-1 levels were not increased with specific $ET_A$ receptor blockade. This study suggests that the potential deleterious effects of $ET_B$ receptor blockade in the treatment of pulmonary hypertensive disorders is an important point to be considered during treatment of PAH patients. One can speculate that ET-1 concentration at 24 h after a single bolus of bosentan depends on ET-1 clearance through $ET_B$ receptor, similar to that shown in the earlier studies (Dupuis *et al.*, 1996a). I have also demonstrated that this sustained elevation of ET-1 further affects the expression of $ET_B$ receptors in PASMCs. The level of expression of $ET_B$ receptors in pulmonary vessels in PAH patients may thus affect responsiveness to ETRAs. We can conclude that the $ET_B$ receptor contributes to chronic regulation of pulmonary vascular tone and therefore loss of $ET_B$ receptor activity may adversely affect patients in PAH. Also the measurement of ET-1 concentration after ETRA administration might predict responsiveness to chronic treatment and will be useful

in determining the effectiveness of various conventional combination therapies in PAH.

***Future Studies:***

- I wish to repeat similar studies in endothelial cells to assess whether the ET-1 levels and the expression of its receptors are affected with the conventional treatments used in PAH.
- I would also like to assess the mechanism of inhibition of endothelin, whether this is through NFAT/calcineurin, HIF-1α and/or PPARs and if such pathways are translated in animal models of PAH.
- Lastly, studies using $ET_B$ knock-out mice may provide some more insight into the importance of this receptor in PAH.

## 6.3 Ispinesib as a potential anti-proliferative agent in PAH

Vascular remodelling which is characterised as smooth muscle cell proliferation and migration, contributes to the pathology of pulmonary hypertension. This can be driven by growth factors, vasoconstrictors, inflammatory mediators, and /or vascular changes such as increased shear stress or injury (Mandegar *et al.*, 2004). Based on therapies used in other proliferative diseases such as in cancer, new agents tested in PAH patients aim to inhibit excessive proliferation and/or to induce apoptosis in PASMCs, thus contributing to the reversal of pulmonary vascular remodelling and

subsequently decreasing pulmonary artery pressure (Schermuly *et al.*, 2005a; Klein *et al.*, 2008). In the final chapter, I determined the antiproliferative effect of a newer anti-cancer drug, ispinesib which acts by inhibiting mitotic kinesins. Ispinesib proved to be a very potent inhibitor of human PASMCs isolated from IPAH patients. I also demonstrated that under the same experimental conditions, it was significantly more efficacious than currently approved agents such as prostacyclin analogues (iloprost or treprostinil) and even more effective than ETRAs (bosentan or ambrisentan), used to treat PAH. Ispinesib, when assessed in normal cells, was significantly less efficacious at inhibiting cell proliferation, indicating that the drug is more likely to target a cell type with a more proliferative phenotype. I also demonstrated that the effectiveness of ispinesib was improved significantly when combined with treprostinil or bosentan, which were given at doses close to the upper limit of those measured in the plasma of patients. The synergy observed with bosentan seemed drug related at first since neither specific $ET_A$ nor $ET_B$ blockade produced such synergy unless ispinesib was combined with ambrisentan and BQ788 so as to presumably block both receptors. I further confirmed that ispinesib appeared to inhibit cell proliferation of PASMCs from IPAH patients by inhibiting the kinesin spindle motor protein, as it does in tumour cells. Whether this single mechanism accounts for the synergy with bosentan remains to be determined. This contrasts the synergy I observed with treprostinil, which based on Western blot experiments, is likely to occur despite the up-regulation of the $ET_B$ receptor and may simply synergise because it is a good inhibitor of ET-1 production. In summary, my data suggest that ispinesib is an effective inhibitor of smooth muscle and fibroblast

proliferation in cells derived from patients with primary or secondary pulmonary arterial hypertension. Given, its superior effects on cell growth to current therapies, its enhancement when combined with bosentan and treprostinil, and its low incidence of side effects, strongly suggests that ispinesib ought to have beneficial effects in pulmonary arterial hypertension and may even be superior to imatinib, based not only a better selectivity profile, but on a reduced side-effect profile. Given that treatments for lung and systemic fibrosis are largely ineffectual, this agent may well have a wider clinical application that should be explored in the future. To this end, Cytokinetics have applied to the FDA to obtain approval for a small-scale clinical to be conducted, based on the data presented in chapter 5.

Reversal of lung vascular remodelling rather than prolonged vasodilation is the concept underlying the use of tyrosine kinase inhibitors in PAH (Klein *et al.*, 2008). Long term effects of imatinib are complex despite the potential beneficial effects, probably because it targets multiple tyrosine kinases, such as PDGF receptors and c-kit (Souza *et al.*, 2006; Ghofrani *et al.*, 2009). However, another broad spectrum tyrosine kinase inhibitor, dasatinib, while decreasing PDGF-induced proliferation in addition to multiple growth factor-induced PASMC proliferation in experimental PAH, causes severe precapillary pulmonary arterial hypertension cell in patients treated with this agent for chronic myelogenous leukemia (Montani *et al.*, 2012). Indeed broad spectrum agents will actually cause PAH suggesting the balance between inhibiting cell growth and allowing for endothelial regeneration is finely balanced.

***Future Studies:***

- I wish to assess the mechanism behind synergy of ispinesib with current therapeutic agents like treprostinil and bosentan.
- It is reported that ispinesib inhibits proliferation by inhibiting/ arresting cells in G2/M phase of the cell cycle, I wish to further assess if the drug does the same in experimental settings of PAH and also whether combining with agents like bosentan facilitates this process of ispinesib.
- In future, I also wish to assess this agent in animal models in order to determine its safety and adverse effect profile in an in vivo setting of PAH.

## 6.4 Clinical Perspective:

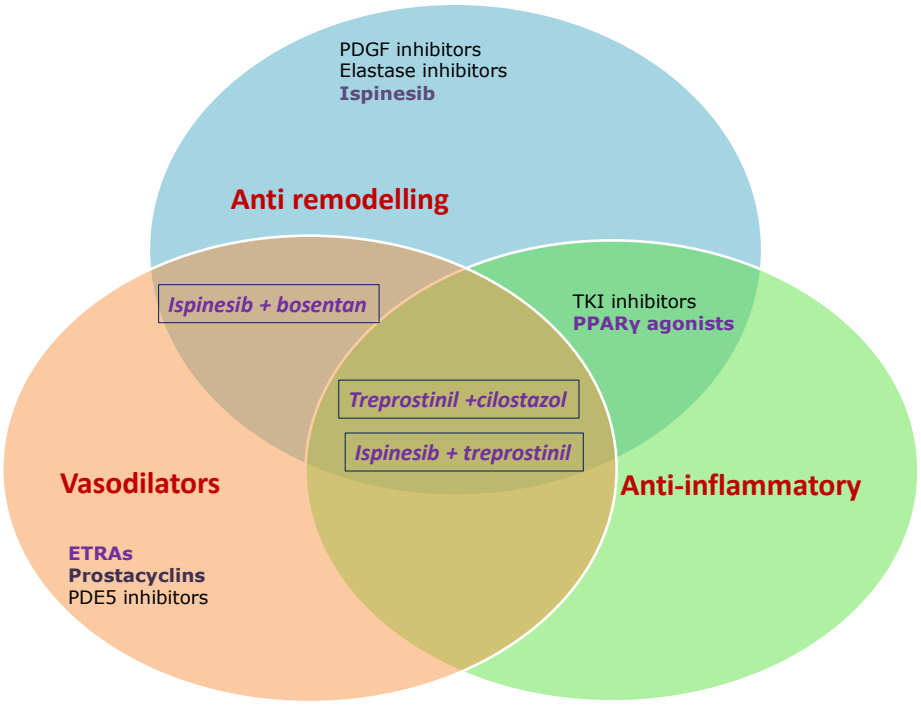



**Figure 6.1 Schematic representation of the summary of the possible therapeutic interventions I studied in this thesis with regard to anti-remodelling, vasodilator and anti-inflammatory targets for pulmonary arterial hypertension.**

All in all we can summarise that PAH, being a multifactorial disease, needs to be managed with agents that can target multiple pathways, without interfering or counteracting the respective roles of individual agents. With respect to this thesis I can propose the following:

1 Treprostinil, if used in combination with cilostazol, can potentially not only target proliferation and vasodilation (treprostinil effects), but might also control inflammation and pain (cilostazol effects). Thus this might be a potential combination for the treatment of PAH to be explored.

2 Our data, accepting that it comes from in vitro experiments, does not support the use of bosentan combined with treprostinil as bosentan not only counteracted ET-1 inhibition by treprostinil but also did not potentiate the antiproliferative effects of the drug.

3 Ispinesib in combination with bosentan may be a potent inhibitor of remodelling in PAH.

4 Ispinesib if used in combination with treprostinil may control remodelling, vasoconstriction as well as inflammation, possibly through activation of PPARγ.

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