# Pharmacological Effect of Histone Deacetylase Inhibitors on Pulmonary Arterial Hypertension

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***I declare that this thesis was conducted and written by myself and the work included within is original and my own unless otherwise stated***

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## ABSTRACT

Pulmonary hypertension (PH) is characterized by structural remodelling of pulmonary arteries and arterioles, the result, at least in part, of excessive cell proliferation, resistance to cell death and hyperactive inflammatory reactions. Currently available treatments targeting the hyper-proliferative and pro-inflammatory pathology are limited. Epigenetic programming, dynamically regulated by histone acetylation, is an important mechanism for cell proliferation and survival. Aberrant changes of histone acetylation, modulated by histone deacetylase (HDAC), are shown in many proliferative and inflammatory disorders, especially cancer, and may contribute to the phenotypical changes in remodelling and overall to the development of PH. Hypothetically, HDAC inhibitors have therapeutic potential by reversing the imbalance of acetylation.

I examined the correlations between HDAC expression and PH development, followed by evaluation of the pharmacological effects and possible mechanisms of two HDAC inhibitors, class I inhibitor valproic acid (VPA) and pan-HDAC inhibitor suberoylanilide hydroxamic acid (SAHA), on animal models and cellular systems.

Altered HDAC expression, specifically increased HDAC1 and HDAC5 along with elevation of anti-apoptotic marker Bcl-2, were found in lungs from patients with idiopathic pulmonary arterial hypertension and chronically hypoxic rats. In *in vivo* studies, VPA and SAHA ameliorated the established PH in both hypoxia- and monocrotaline-induced PH rat models, by reducing pulmonary arterial pressure, right ventricular hypertrophy and pulmonary vascular muscularization, in parallel with increasing histone acetylation. In *in vitro* studies, VPA and SAHA inhibited stimulated cell growth of pulmonary artery smooth muscle cells and cytokine release from endothelial cells. Biochemical analysis indicated these two inhibitors exert anti-proliferative effects comprising cell cycle arrest by upregulation of p21 and apoptotic induction by downregulation of Bcl-2.

Collectively, this study shows the contribution of abnormal HDAC activity to vascular pathology of PH and provides a preclinical basis to further explore the therapeutic potential of HDAC inhibitors in human PH.

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| | | | |
|---|---|---|---|
| 5-HT | 5-hydroxytryptamine | cAMP | Cyclic adenosine monophospate |
| 5-HTT | 5-hydroxytryptamine transport | CBP | cAMP response element-binding binding protein |
| AA | Antibiotic-antimycotic | CCB | Calcium channel blockers |
| ADP | Adenosine diphosphate | CCL | Chemokine (C-C motif) ligand |
| ALK1 | Activin-like kinase type 1 | CCR | Chemokine (C-C motif) receptor |
| ALK5 | Activin-like kinase type 5 | CD | Cluster differentiation |
| AM | Adrenomedullin | CDK | Cyclin-dependent kinase |
| ANOVA | Analysis of variance | CDK9 | Cyclin-dependent kinase 9 |
| ANP | Atrial natriuretic peptide | cGMP | Cyclic guanosine monophosphate |
| Bcl-2 | B cell lymphoma 2 | CINC | Cytokine-induced neutrophil chemoattractant |
| Bcl-6 | B cell lymphoma 6 | CML | Chronic myelogenous leukemia |
| BH4 | Tetrahydrobiopterin | CMV | Cytomegalovirus |
| BMP | Bone morphogenetic protein | CNTF | Ciliary neurotrophic factor |
| BMPR2 | Bone morphogenetic protein receptor type 2 | CSA | Cross sectional area |
| BNP | Brain natriuretic peptide | Ct | Threshold cycle |
| BP | Blood pressure | CTCL | Cutaneous T cell lymphoma |
| BSA | Bovine serum albumin | CTEPH | Chronic thromboembolic pulmonary hypertension |
| C5/C5a | Complement factor 5/5a | CX3CL1 | Chemokine (C-X3-C motif) ligand 1 |

| | | | |
|---|---|---|---|
| CXCL | Chemokine (C-X-C motif) ligand | $ET_B$ | Endothelin B receptor |
| CXCR | Chemokine (C-X-C motif) receptor | FACS | Fluorescent-activated cell sorting |
| DC | Dendritic cell | FBS | Foetal bovine serum |
| DCA | Dichloroacetate | FC | Functional class |
| DEPC | Diethylpyrocarbonate | FDG | Fluorodeoxyglucose |
| DMEM | Dulbecco's modified eagle medium | FGF | Fibroblast growth factor |
| DMSO | Dimethylsulfoxide | FLIP | FLICE-like inhibitory protein |
| DNA | Deoxyribonucleic acid | FOXO | Forkhead box class O |
| DR-5 | Death receptor 5 | FOXP3 | Forkhead box P3 |
| dsRNA | Double-strand RNA | G-CSF | Granulocyte colony-stimulating factor |
| E2F | E2 promoter binding factor | GCN-5 | General control of amino acid synthesis protein 5 |
| EBV | Epstein–Barr virus | GIST | Gastrointestinal stromal tumours |
| EC | Endothelial cell | GM-CSF | Granulocyte-macrophage colony-stimulating factor |
| ECM | Extracellular matrix | GNAT | GCN5-related N-acetyltransferase |
| EDTA | Ethylenediaminetetraacetic acid | GR | Glucocorticoid receptor |
| EGF | Epidermal growth factor | GSK3 | Glycogen synthase kinase 3 |
| eNOS | Endothelial nitric oxide synthase | GTP | Guanosine triphosphate |
| ERA | Endothelin receptor antagonist | HAT | Histone acetyltransferase |
| ET-1 | Endothelin-1 | HDAC | Histone deacetylase |
| $ET_A$ | Endothelin A receptor | HDM | Histone demethylase |

| | | | |
|---|---|---|---|
| HE | Haematoxyline & eosin | IV | Intravenous |
| HHV-8 | Human herpes virus-8 | Kv | Voltage-dependet potassium |
| HIF | Hypoxia-inducible factor | LDH | Lactate dehydrogenase |
| HIF-1α | Hypoxia-inducible factor-1α | LIX | Lipopolysaccharide-induced chemokine (C-X-C motif) |
| HIV | Human immunodeficiency virus | LV | Left ventricle |
| HMT | Histone methyltransferase | LXR | Liver X receptor |
| HOP-β-CD | 2-hydroxypropyl-β-cyclodextrin | MCP-1 | Monocyte chemoattractant protein 1 |
| HP1 | Heterochromatin 1 | MCT | Monocrotaline |
| HPAEC | Human pulmonary artery endothelial cell | MDS | Myelodysplastic syndrome |
| HPSMC | Human pulmonary smooth muscle cell | MEF2D | Myocyte-specific enhancer factor 2D |
| HRP | Horseradish peroxidise | MIF | Macrophage migration inhibitory factor |
| Hsp90 | Heat shock protein 90 | MIG | Monokine induced by IFN-γ |
| ID | DNA-binding gene inhibitor | MIP | Macrophage inflammatory protein |
| IGF | Insulin-like growth factor | miRNA | MicroRNA |
| IL | Interleukin | MM | Multiple myeloma |
| INF | Interferon | MMP | Matrix metalloproteinase |
| INF-γ | Interferon-γ | MMP-2 | Matrix metalloproteinase 2 |
| INH | Inhaled | MMP-3 | Matrix metalloproteinase 3 |
| iNOS | Inducible nitric oxide synthase | MMP-9 | Matrix metalloproteinase 9 |
| IP-10 | Interferon-γ-induced protein 10 | MMP-10 | Matrix metalloproteinase 10 |
| IPAH | Idiopathic pulmonary arterial hypertension | MNC | Mononuclear cell |

| | | | |
|---|---|---|---|
| mRNA | Messenger RNA | PC | PDGF control or positive control |
| MTA1 | Metastatic tumour antigen 1 | PCAF | P300/CBP-associated factor |
| NAD | Nicotinamide adenine dinucleotide | PCH | Pulmonary capillary hemangiomatosis |
| NC | Normoxia control or negative control | PDE5 | Phosphodiesterase type 5 |
| NFAT | Nuclear factor activated T cells | PDE5-I | Phosphodiesterase type 5 inhibitors |
| NF-κB | Nuclear factor κ-light-chain-enhancer of activated B cells | PDGF | Platelet-derived growth factor |
| NK | Natural killer | PDH | Pyruvate dehydrogenase |
| NMDA | N-methyl-D-aspartate | PDK | Pyruvate dehydrogenase kinase |
| NO | Nitric oxide | PET | Positron emission tomography |
| NOS | Nitric oxide synthase | PGH2 | Prostaglandin H2 |
| NSCLC | Non small cell lung cancer | PH | Pulmonary hypertension |
| OPG | Osteoprotegerin | PI | Propidium iodide |
| PA | Pulmonary artery | PI3K | Phosphatidylinositol-3-kinase |
| PAB | Pulmonary artery banding | piRNA | Piwi-interacting RNA |
| PAH | Pulmonary arterial hypertension | PKB | Protein kinase B |
| PAP | Pulmonary arterial pressure | PPAR | Peroxisome proliferator-activated receptor |
| PARP | Poly ADP ribose polymerase | PPH | Primary pulmonary hypertension |
| PASMC | Pulmonary artery smooth muscle cell | pRb | Retinoblastoma protein |
| PBS | Phosphate buffered saline | PVOD | Pulmonary veno-occlusive disease |

| | | | |
|---|---|---|---|
| PVR | Pulmonary vascular resistance | sICAM-1 | Soluable intercellular adhesion molecule-1 |
| RANTES | Regulated by T cells, activation upon secretion | siRNA | Short interfering RNA |
| RANKL | Receptor activator of NFκB ligand | SLE | Systemic lupus erythematosus |
| RCC | Renal cell carcinoma | SSc | Scleroderma |
| RNA | Ribonucleic acid | SSRI | Selective serotonin reuptake inhibitor |
| ROS | Reactive oxygen species | STAT3 | Signal transducers and activators of transcription 3 |
| RT-PCR | Reverse transcription polymerase chain reaction | sTREM | Soluble triggering receptor expressed on myeloid cells-1 |
| RV | Right ventricle | TBP2 | Thioredoxin binding protein 2 |
| RVH | RV hypertrophy | TBS | Tris-buffered saline |
| RVSP | Right ventricular systolic pressure | TF | Transcription factor |
| SAHA | Suberoylanilide hydroxamic acid | TGF-β | Transforming growth factor-β |
| SBP | Systolic blood pressure | Th | T helper |
| SC | Subcutaneous | TIMP | Tissue inhibitors of metalloproteinase |
| SCLC | Small cell lung cancer | TIMP-1 | Tissue inhibitors of metalloproteinase 1 |
| SDF | Stromal cell-derived factor | TLR | Toll-like receptor |
| SDF-1 | Stromal cell-derived factor-1 | TNF | Tumour necrosis factor |
| SEM | The standard error of the mean | TNF-α | Tumour necrosis factor-α |
| sGC | Soluble guanylate cyclase | TRAIL | TNF-related apoptosis-inducing ligand |
| SHP | Small heterodimer partner | Treg | T regulatory |

| | |
|---|---|
| TRX | Thioredoxin |
| TSA | Trichostatin A |
| TXA2 | Thromboxane A2 |
| VEGF | Vascular endothelial growth factor |
| VEGFR-2 | Vascular endothelial growth factor receptor type 2 |
| vHL | Von Hippel-Lindau protein |
| VIP | Vasoactive intestinal peptide |
| VPA | Valproic acid |
| vWF | Von Willebrand factor |
| XIAP | X-linked inhibitor of apoptosis protein |

# Chapter 1: INTRODUCTION

# 1. Pulmonary Arterial Hypertension

## 1.1. Definition and Classification

Pulmonary hypertension (PH), an abnormal sustained elevation in pulmonary arterial pressure (PAP), is a progressive, intractable and lethal vascular disease. First described over 100 years ago in a patient with right heart failure [1], PH is recognized to arise from diverse causes, which in common lead to dysfunction in the pulmonary circulation. It may occur in isolation without demonstrable aetiology, when it is known as idiopathic pulmonary arterial hypertension (IPAH) or formerly primary pulmonary hypertension (PPH), or result from existent medical conditions including left heart failure, pulmonary parenchymal or vascular diseases, coagulation disorders, or a combination of different factors. On the basis of clinical and pathological criteria, PH is classified into five groups (Table 1.1) [2].

According to the classification, pulmonary arterial hypertension (PAH), group 1 of PH, includes IPAH, heritable PAH, drug- and toxin-induced PAH, PAH associated with other diseases (connective tissue diseases, HIV infection, portal hypertension, congenital heart diseases, schistosomiasis and chronic haemolytic anemia), persistent pulmonary hypertension of the newborn and pulmonary veno-occlusive disease (PVOD) and/or pulmonary capillary hemangiomatosis (PCH).

The pathogenesis of PAH is complicated but typically characterized by vasoconstriction, vascular remodelling and thrombosis in situ and increasingly inflammation. As the consequence of progressively increased pulmonary vascular resistance (PVR) to blood flow, right ventricle (RV) is compelled to compensate the excessive workload by raising right ventricular systolic pressure (RVSP) to preserve cardiac output until eventual failure, which accounts for the major cause of death in patients with PAH [3].

## Clinical Classification of Pulmonary hypertension (Dana Point, 2008)

**1. Pulmonary arterial hypertension (PAH)**

1.1 Idiopathic PAH

1.2 Heritable

- BMPR2
- ALK1, endoglin (with or without hereditary hemorrhagic telangiectasia)
- Unknown

1.3 Drug- and toxin-induced

1.4 Associated with

- Connective tissue diseases
- HIV infection
- Portal hypertension
- Congenital heart diseases
- Schistosomiasis
- Chronic hemolytic anemia

1.5 Persistent pulmonary hypertension of the newborn

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

2.2 Diastolic dysfunction

2.3 Valvular disease

**3. Pulmonary hypertension owing to lung diseases and/or hypoxia**

3.1 Chronic obstructive pulmonary disease

3.2 Interstitial lung disease

3.3 Other pulmonary diseases with mixed restrictive and obstructive pattern

3.4 Sleep-disordered breathing

3.5 Alveolar hypoventilation disorders

3.6 Chronic exposure to high altitude

3.7 Developmental abnormalities

**4. Chronic thromboembolic pulmonary hypertension (CTEPH)**

**5. Pulmonary hypertension with unclear multifactorial mechanisms**

5.1 Hematologic disorders: myeloproliferative disorders, splenectomy

5.2 Systemic disorders: sarcoidosis, pulmonary Langerhans cell histiocytosis: lymphangioleiomyomatosis, neurofibromatosis, vasculitis

5.3 Metabolic disorders: glycogen storage disease, Gaucher disease, thyroid disorders

5.4 Others: tumoural obstruction, fibrosing mediastinitis, chronic renal failure on dialysis

ALK1 = activin receptor-like kinase type 1; BMPR2 = bone morphogenetic protein receptor type 2; HIV = human immunodeficiency virus.

**Table 1.1**. Updated clinical classification of pulmonary hypertension[2].

The most common clinical manifestation of PAH is exertional dyspnoea, which is due to insufficient exercise tolerance and can be evaluated functionally through a six-minute walk test. The golden standard for diagnosis, however, is made by invasive haemodynamic assessment at right cardiac catherterization [4]. The clinical definition of PAH, by international agreement, is a mean PAP of more than 25 mmHg at rest or more than 30 mmHg during exertion [5].

Currently, PH in all its forms is estimated to affect up to 100 million people with an incidence of 1 in 500000, which is rare in comparison with systemic hypertension [2]; the general outcome is very poor. It was reported that the medical survival of IPAH patients was less than three years in the 1980s [6]. In recent years, spectacular advances have been made in treatment options of PAH, which have improved the prognosis to a certain degree. A recent report revealed 10-year and 3-year survival rates of 83% and 58% respectively [7]. Unfortunately, the mortality rate of PAH remains high. Once failing to respond to the current pharmaceutical therapies, patients have no choice but cardiopulmonary transplantation. Better understanding of the pathogenesis and new treatment options are eagerly required.

## 1.2. Pathogenesis of PAH

The pathogenesis of PAH is complex and involves vasoconstriction, vascular remodelling and thrombosis in situ, which suggest imbalances between vasodilators and vasoconstrictors, proliferative and apoptotic factors, coagulants and anticoagulants, involving all the vascular elements accompanied with circulating factors [8]. Increasingly inflammation is believed to play an important role. Elucidation of these underlying molecular mechanisms has provided current therapeutic targets and insights into innovation in the future (Figure 1.1).

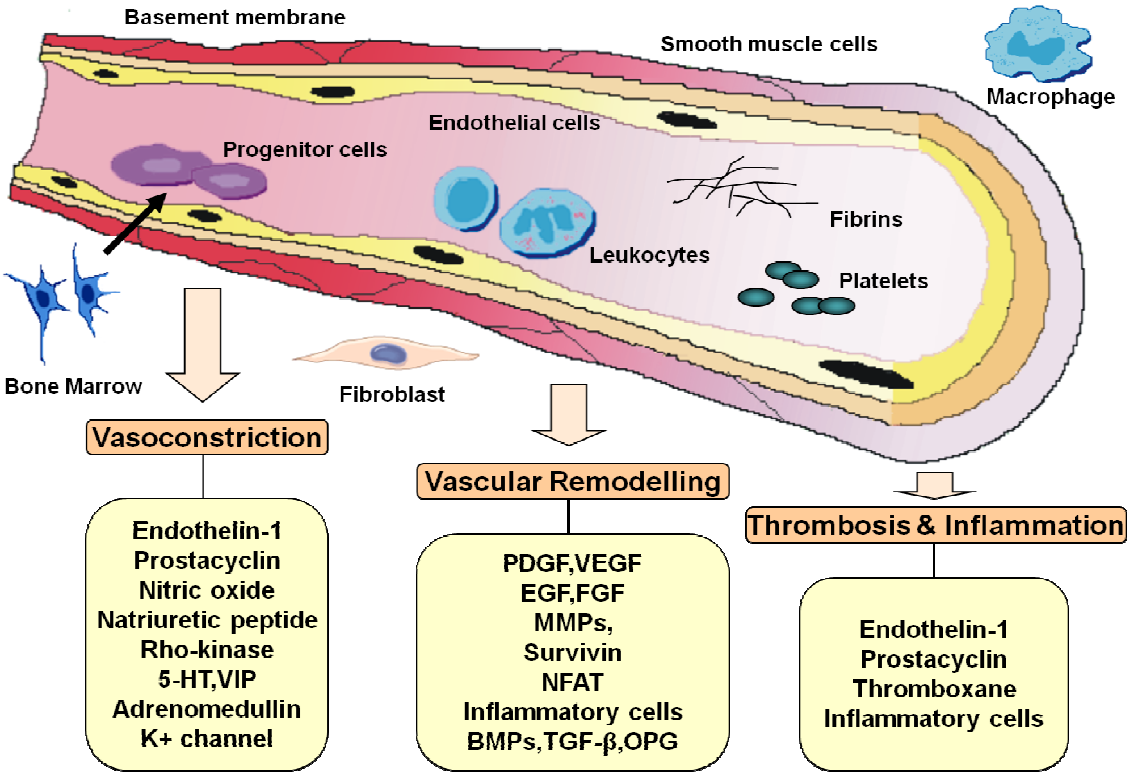



**Figure 1.1**. Schematic summary of pathogenesis of PAH, mainly featured as vasoconstriction, vascular remodelling and thrombosis [9].

### 1.2.1. Pulmonary vasoconstriction

Pulmonary vasoconstriction originates from abnormal regulation of vessel tone which is thought to be a result of endothelial dysfunction. Serving as the main source of various vasodilators, such as prostacyclin and nitric oxide, and vasoconstrictors, especially endothelin, the endothelium plays an essential role in maintaining pulmonary vascular homeostasis. Several related vasoactive mediators have been well studied in PAH and parts of them have been developed to be the targets of medical treatment currently in use [10].

- **Prostacyclin** is synthesized in endothelial cells from prostaglandin H2 (PGH2) by prostacyclin synthase. It induces vasodilation and inhibits platelet aggregation via cyclic adenosine monophospate (cAMP)-dependent pathway [11] in contrast with thromboxane A2 (TXA2), generated in active platelets, causing vasoconstriction and activating new platelets for aggregation [12]. In patients with PAH, prostacyclin synthase expression is decreased in pulmonary arteries of lungs [13] and urinary metabolites of prostacyclin are reduced while urinary metabolites of TXA2 are increased [14]. Furthermore, clinical studies show that intravenous infusion of prostacyclin (epoprostenol) significantly prolong survival in patients with PAH and also improve quality of life, exercise capacity and haemodynamics [15]. On the other hand, inhibition of TXA2 seems to be less convincing as a therapeutic choice in human [16].

- **Nitrix oxide (NO)** is a potent vasodilator, synthesized by nitric oxide synthase (NOS) in three isoforms, endothelial (eNOS), neuronal (nNOS) and inducible (iNOS), as well as an inflammatory mediator produced from phacocytes in response to inflammation. NO secreted from endothelial cells of vessels serves as signal transmitter to surrounding smooth muscle cells. Diffusing freely across membrane, NO activate soluble guanylate cyclase (sGC) in smooth muscle cells, converting guanosine triphosphate (GTP) to its active form cyclic guanosine monophosphate (cGMP), which is downregulated by phosphodiesterase type 5 (PDE5). Accumulation of cGMP leads to vasorelaxation and also inhibits cell growth, platelet aggregation and leukocyte adhesion to endothelium [17].

NO from eNOS is the principle mediator by which endothelium controls vessel tone in pulmonary vasculature. Multiple impairments have been proved in eNOS-NO-cGMP pathway in PAH, including decreased eNOS expression [18] and low NO bioactivity[19]. In animal models, deficency in eNOS increases susceptibilty to hypoxia-induced pulmonary hypertension while gene transfer of eNOS provides resistance [20]. Endothelial availability of tetrahydrobiopterin (BH4), a cofactor in eNOS synthesis, has been shown to regulate the pathophysiological response to hypoxia in the pulmonary circualtion [21]. Restoring eNOS-NO-cGMP signaling has been shown to an effective strategy underpinning currently available medical treatment in PAH, including oral PDE5 inhibitors and inhaled NO [22, 23].

- **Endothelin-1 (ET-1)**, a potent endothelium-derived vasoconstrictor and mitogen [24], regulate vessel tone via two receptors, $ET_A$ and $ET_B$. Binding to both receptors on pulmonary arterial smooth muscle cells leads to increased intracellular calcium and subsequent vasoconstriction, whereas binding to $ET_B$ on endothelial cells induces nitric oxide and prostacyclin release [25]. Circulating levels of ET-1 along with local production in pulmonary vasculature are significantly increased in patients with PAH [26, 27]. Strong correlations between expression levels of ET-1 and severity of disease as well as prevalence of $ET_A$ and pulmonary resistance arteries indicate $ET_A$ –dominant regulation in PAH and provide the foundation for treatment with endothelin receptor antagonists [28].

- **Natriuretic peptides**, atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP), are cardiac hormones secreted in response to excess myocardial stretch and causing diuretic and vasorelaxant effects. Binding to their receptors, NPR-A and NPR-B, which are membrane-bound guanylate cyclases, converts intacellular GTP to cGMP [29]. Circulating levels of BNP correlates to the extent of RV dysfunction and prognosis, and are elevated in patients with PAH [30].Genetic disruption of NPR-A augments PAH development while continuous infusion of ANP attenuate PAH phenotype in animal models [31, 32]. Although rapid degradation of natriuretic peptides may challenge long-term administration for treatment, alternatively, inhibition of neutral endopeptidase, responsible for metabolism of natriuretic peptides can be potential as a novel approach in PAH [33].

- **Rho-kinase** system regulates numerous cellular processes related to vasoconstriction and vascular remodelling in PAH including cell motility, cell survival, and cytokine release [34]. Controlling the myosin light chain dynamics in smooth muscle cells, Rho kinase contributes critically to refractory vasoconstriction and interacts with other vasoactive mediators, such as ET-1, TXA2 and serotonin [35]. The Rho kinase inhibitor, fasudil, ameliorates vasoconstriction in experimental PAH models [36] and causes acute vasodilator effects in patients with PAH [37]. However, inevitably systemic vasodilation accompanies this effect and reduces enthusiasm for the approach.

- **Serotonin (5-hydroxytryptamine, 5-HT)**, a monoamine neurotransmitter believed to be important in emotion control, was first implicated in PAH associated with chronic use of the appetite suppressant dexfenfluramine, which is serotoninergic, and increases the risk of PAH [38]. Synthesized in endothelial cells of pulmonary arteries, 5-HT stimulates pulmonary arterial smooth muscle cells and fibroblasts, leading to vasoconstriction and remodelling [39]. 5-HT transport, 5-HTT, is overexpressed in pulmonary arterial smooth muscle cells from patients with IPAH while treatment with 5-HT favours the development of hypoxic PAH in rats [40]. Administration with selective serotonin reuptake inhibitors (SSRIs) can protect against hypoxia-induced pulmonary hypertension in mice [41]and tends to be associated with a reduction in the risk of death in a cohort study of PAH patients [42].

- **Vasoactive intestinal peptide (VIP)**, a 28 amino-acid peptide belonging to the glucagon-growth hormone–releasing factor secretion superfamily, acts to increase cardiac contractility, vasodilation, glycogenolysis, scavenge reactive oxygen species (ROS), inhibit platelet activity and relax smooth muscle. Mice lacking VIP develop moderate PH [43]. The VIP receptor is highly expressed in human lungs and upregulated. Circulating VIP levels are reduced in patients with PAH [44]. VIP substitution selectively benefits pulmonary haemodynamics and improves cardiac function with minimal systemic effects both in animal models and patients [45, 46]. Similar to natriuretic peptide, the medical application of VIP is restricted by its short half-life. Modern dosing strategies for controlled release are under investigation for using VIP in PAH.

- **Adrenomedullin (AM)** is a ubiquitously expressed vasodilator with a variety of biological functions such as reduction of oxidative stress and endothelial cell apoptosis, via many signalling pathways including NO-cGMP and PI3K (phosphatidylinositol-3-kinase)/Akt [47]. It is elevated in hypoxic animals and patients with PAH and administration of AM demonstrates protective effects [48, 49]. Inhalation of AM attenuates PAH phenotypes in monocrotaline (MCT) induced PAH in rats [50]and humans, suggesting the therapeutic potential [51]. However, the effects of AM on systemic circulation are still undetermined.

- **Voltage-dependent potassium (Kv) channel**, a membrane-spanning protein forming potassium-selective pores, regulates vessel tone by maintaining resting membrane potential and gating voltage-gated calcium channels. Downregulation of Kv channels promotes intracellular calcium in smooth muscle cells, resulting in constriction. Additionally, Kv channels also control survival signals and dysfunction leads to cell proliferation and resistance to apoptosis, which contributes to vascular remodelling. Inhibition of Kv channel activity by stimuli such as hypoxia [52]or anorexigens [53]initiates pulmonary vasoconstriction and PAH development. Expression and activity of Kv channels, particularly Kv1.5, are decreased in human and experimental PAH [54, 55]. Restoration of Kv1.5 activity has been considered as a therapeutic strategy in experimental PAH models. Moreover, Kv channel downregulation is also found in other proliferative disorders, especially cancer, suggesting some shared properties between cancer and PAH [56].

### 1.2.2. Pulmonary vascular remodelling

Pulmonary histology provides strong evidence that vascular structural remodelling is extensively presented in almost all types of PAH, evident as intimal growth, medial thickening and deposition of extracellular matrix. This process involves all the vascular cells, including endothelial cells, smooth muscle cells, fibroblasts, platelets, inflammatory cells, progenitor cells as well as extracellular matrix (ECM) components such as collagen, elastin and fibronectin [8].

Injury to endothelial cells is believed to select apoptosis-resistant cells to proliferate, forming the complex plexiform lesions, which are characteristically found in PAH patients at the late stage [57]. The resultant exposure of smooth muscle cells to stimuli promotes proliferation and extends smooth muscle into distal peripheral arteries, which are normally nonmuscular [58]. Accumulation of extracellular matrix, attributed to imbalance between matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs), takes part in neointimal formation and smooth muscle cell growth, further thickening vascular wall [59]. Moreover, adventitia also participates in the remodelling process, that differentiating myofibroblasts from pericytes and intermediate cells in precapillary vessels marscularize small vessels. Adventitial fibrblasts are activated in response to injurious stimuli and undergo proliferation, differentiation, migration, secretion of paracrine factors, and collagen production, which profoundly increase adventitial thickness and jeopardize arterial capacitance [60].

The consequent occlusion of the vessel lumen along with the loss of vessel numbers results in obliterative pulmonary circulation. Vascular remodelling indicates an imbalance between proliferation and apoptosis, manifesting excessive and uncontrollable cell growth, which is analogous to cancer phenotypically [61].

Increasing attention has been concentrated on genetic and molecular mechanisms underlying the process of pulmonary vascular remodelling. A previous study showed a decrease in the pro-apoptotic/anti-apoptotic gene expression ratio in lungs from patients with PAH [62]. Several mediators have been implicated in the process of remodelling.

- **Platelet-derived growth factor (PDGF)**, a potent mitogen for cells of mesenchymal origin, acting via its receptor tyrosine kinase, has been implicated in recruitment of smooth muscle cells and endothelial dysfunction [63]. PDGF signalling is upregulated selectively in pulmonary vasculature in IPAH patients, and PDGF ligands and receptors are overexpressed in lungs patients with severe PAH [64], which is also observed in sheep with chronic intrauterine pulmonary hypertension [65]. The PDGF receptor antagonist, imatinib, has been shown to reverse the PH phenotype by attenuating vessel remodelling, in both hypoxic and MCT models [66]. Initial clinical studies are in favour of its potential as a novel therapy. Further investigation are ongoing [67].

- **Vascular endothelial growth factor (VEGF)**, the principle mediator in vascularization and angiogenesis, dominates endothelial cell survival [68]. Binding to its receptor type 2 (VEGFR-2) results in the downstream signalling including increased prostacyclin and NO synthesis through akt- and src-mediated pathways [69]. The role of VEGF in PAH is still controversial and complex. VEGF and VEGFR-2 are reported highly expressed in the plexiform lesions in patients with PAH [70]. VEGF is upregulated in chronically hypoxic rats but downregulated in MCT-induced PH rats [43, 71]. Blockade of VEGFR-2 with the antagonist SU-5416 combined with chronic hypoxia generates severe pulmonary hypertension associated with remarkable proliferative vasculopathy [72]. Overexpression of VEGF by gene transfer inhibits the development of experimental PH in animals [73, 74], but, conversely, treatment with VEGF in rats with established pulmonary hypertension worsens the disease [75]while VEGFR-2 blocker, sorafenib, attenuates experimental pulmonary hypertension in rats [76, 77]. Although the uncertainty of VEGF in PAH prevents its therapeutic application, better understanding of the role of VEGF is needed to help to unravel the pathogenesis.

- **Matrix metalloproteinases (MMPs)** are a family of proteolytic endopeptidases capable of degrading ECM, as well as various biological functions, such as cell proliferation, migration, differentiation, angiogenesis, apoptosis and cytokine activation. MMPs are categorized into several groups such as collagenases and gelatinases based on substrate specificity. Secreted as proenzymes, MMPs require extracellular activation and are regulated by TIMPs [78]. An imbalance between MMPs and TIMPs, containing excessive TIMP-1 and MMP-2 but decreased MMP-3, leads to ECM deposition in patients with IPAH [79]. Intervention in MMP activity exerts beneficial effects in MCT-induced PH rats [80]. The wide range of MMP functions in tissue homeostasis promises their therapeutic potential and investigation in more detail is deserved.

- **Survivin** is a member of the “inhibitor of apoptosis” family, selectively expressed in cells undergoing oncogenic transformation. Thought to be exclusive in cancer previously, survivin was found to be expressed in pulmonary arteries from patients with PAH and MCT-induced PH rats [81]. Gene therapy targeting survivin reverses

established PH and improves survival in the MCT rat model and inhibition of endogenous survivin induces vascular mitochondria-dependent apoptosis and prevents vascular remodelling. In contrast, gene transfer of wild type survivin results in the opposite effects [81]. The clinical value of using a survivin inhibitor is under evaluation in oncology currently and could be explored in PAH [82].

- **Nuclear factor activated T cells (NFAT)**, a transcription factor that regulates T cell activation, is expressed in most immune cells and also involved in cardiac development and hypertrophy. NFAT promotes proliferation of pulmonary arterial smooth muscle cells and reduces expression of Kv1.5. The activation of NFAT is shown in human and experimental PH, and seems to contribute to the hyperpolarization of mitochondria in smooth muscle cells. Inhibition of NFAT alleviates the PAH phenotype by upregulating Kv1.5 and downregulating Bcl-2 (B cell lymphoma 2), the anti-apoptotic protein, in both hypoxia- and MCT-induced PH models. Knockout of NFATc3 in mice protects against pulmonary vascular remodelling in response to chronic hypoxia [83].

- **Osteoprotegerin (OPG)** is a cytokine receptor and member of the tumour necrosis factor receptor superfamily [84]. OPG regulates osteoclast differentiation and induces apoptosis via binding to receptor activator of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) ligand (RANKL) or tumour necrosis factor-related apoptosis-inducing ligand (TRAIL), and plays an essential role in inflammation [85]. Overexpression of OPG in pulmonary vascular smooth muscle cells resulting from inflammatory stimuli evokes cell proliferation and migration. In patients with IPAH, increased OPG expression is evident in pulmonary lesions and circulating levels are elevated [86].

### 1.2.3. Inflammation

Accumulating evidence highlights the role of inflammation in the pathogenesis of PAH, involving monocytes/macrophages, lymphocytes (B and T cells), dendritic cells (DCs) and mast cells, and a variety of cytokines and chemokines. The contributions of inflammation to PAH are:

- **Perivascular accumulation of inflammatory cells** has been well-documented in both animals and humans with pulmonary hypertension. Histological examination shows mononuclear cells (MNCs), mostly T cells and macrophages, infiltrating around remodelled vessels, particularly plexiform lesions. Inflammatory cell infiltration is also observed in nearly all the animal models commonly in use, such as MCT-, chronic hypoxia-, SU5416-, and schistosomiasis-induced PH rats and mice deficient in bone morphogenetic protein (BMP) receptor type 2 (BMPR2). Moreover, serum level of DCs is increased in patients with IPAH [58, 87].

- **Circulating levels of certain cytokines/chemokines** are elevated in patients with IPAH and some of them predict the clinical outcome [88]. These include interleukin (IL)-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, tumour necrosis factor (TNF)-α, transforming growth factor (TGF)-β, RANTES (Regulated upon Activation, Normally T cell Expressed and Secreted)/CCL5, fractalkine/CX3CL1, interferon γ (INF-γ)-induced protein 10 (IP-10/CXCL10) and monocyte chemoattractant protein 1 (MCP-1/CCL2) [88-92]. The role of cytokines in the pathogenesis of PAH is further supported by increased IL-1β and IL-6 in the MCT model. Overexpression of IL-6 or injection with IL-6 to rats induces PH [93, 94], while IL-6 deficiency protects mice from developing PH in response to hypoxia [95]. In addition, IL-6 can modulate BMPR2, an identified genetic factor in human PAH, epigenetically through certain microRNA clusters [96]. On the other hand, excessive chemokines, such as RANTES, MCP-1 and fractalkine, are found in pulmonary endothelium or surrounding lymphocytes, which may contribute to recruitment and adherence of other inflammatory cells to endothelium [89, 90, 97]The cytokines/chemokines profiles implicated in pulmonary vascular inflammation are summarized in Table 1.2

| Cytokine Chemokine | Source | Target | Function |
|---|---|---|---|
| **IL-1β** | Macrophages, lymphocytes, EC, PASMC | Many cell types | Pro-inflammatory, EC and PASMC activation |
| **IL-4** | Th2 cells, mast cells | B cells, T cells, mast cells, macrophages, hemotopoietic progenitors | Proliferation and differentiation of B cells and Th2 cells |
| **IL-6** | Macrophages, T cells, EC, PASMC | B cells, T cells, EC, PASMC | Differentiation of myeloid cells, PASMC activation |
| **IL-8** | Monocytes, T cells, EC | Neutrophils, T cells, monocytes | Pro-inflammatory, leukocyte arrest |
| **IL-10** | Macrophages, B cells, Th2 cells, Treg cells, mast cells | Macrophages, B cells, T cells | Anti-inflammatory, differentiation of B cells |
| **IL-12** | Th1 cells | T cells, macrophages | Pro-inflammatory, cytotoxic activity |
| **IL-13** | Many cells, especially Th2 cells | Similar to IL-4 | Allergic and parasitic reactions |
| **TNF-α** | Macrophages, B cells, T cells, NK cells, PASMC | Many cell types | Pro-inflammatory, tumour necrosis, anticoagulant |
| **TGF-β** | Platelet, macrophages, B cells, Th2 cells, Treg cells, PASMC | Many cell types | Anti-inflammatory, profibrotic, angiogenesis Suppress Th1/Th2 responses |
| **MCP-1 CCL2** | Many cells including EC, PAMSC | Monocytes, memory T cells, DC, basophils | Monocyte migration to become macrophage |
| **RANTES CCL5** | T cells, EC | monocytes, eosinophils, basophils, T cells (any cells expressing CCR5 receptor) | Chemoattractant for monocytes and T cells, may induce ET-1 |
| **IP-10 CXCL10** | Monocytes, EC, fibroblasts in response to INF-γ | Monocytes, macrophages, T cells, NK cells, DC | Chemoattractant, promote T cell adhesion to EC |
| **Fractalkine CX3CL1** | Macrophages, T cells, EC, PASMC | $CD4^+$ and $CD8^+$ T cells, monocytes, activated EC | Chemoattractant, leukocyte adhesion to EC, growth factor of PASMC |

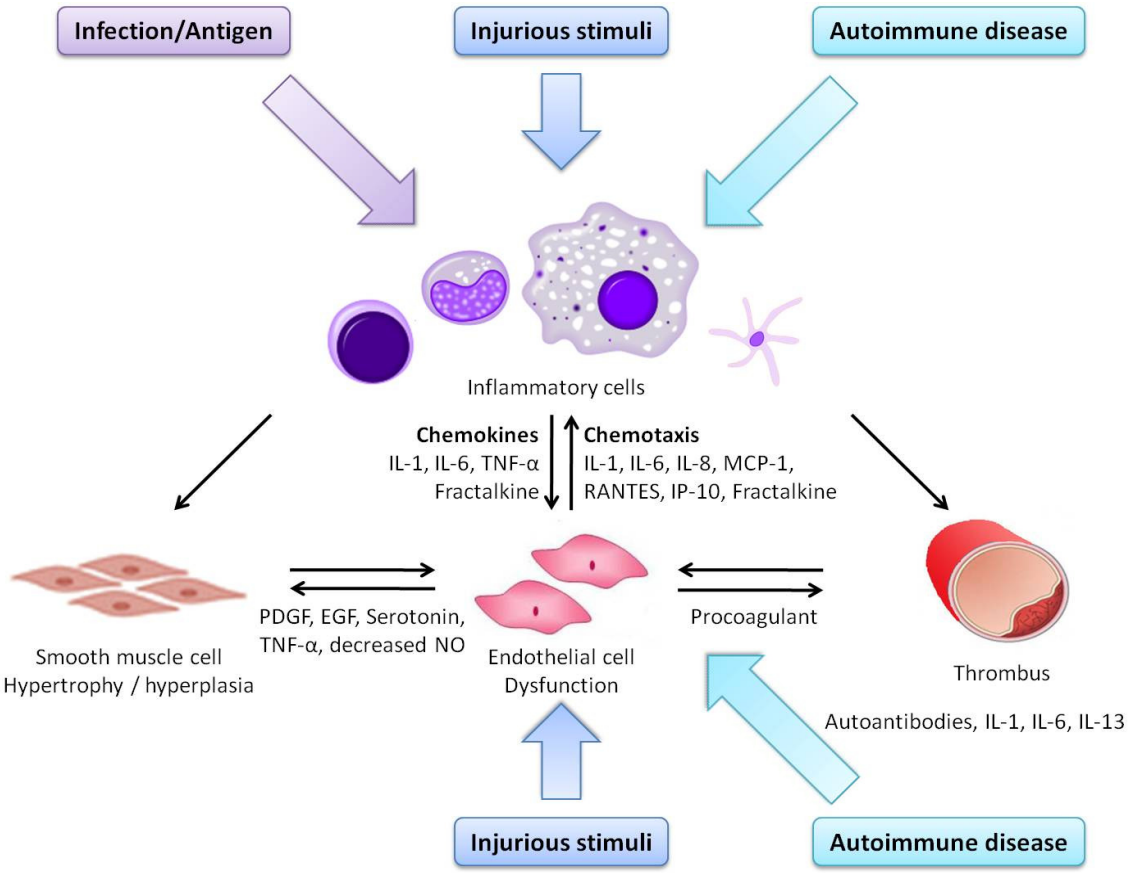
Abbreviations: CCL, chemokine (C-C motif) ligand; CCR, chemokine (C-C motif) receptor; CD, cluster differentiation; CX3CL1, chemokine (C-X3-C motif) ligand 1; CXCL, chemokine (C-X-C motif) ligand; CXCR, chemokine (C-X-C motif) receptor; DC, dendritic cell; EC, endothelial cell; ET-1, endothelin-1; IFN-γ, interferon-gamma; MCP-1, monocyte chemotactic protein 1; NK, natural killer; PASMC, pulmonary artery smooth muscle cell; RANTES, regulated by T cells, activation upon secretion; TGF-β, transforming growth factor beta; Th, T helper; TNF-α, tumour necrosis factor alpha ; Treg, T regulatory .

**Table 1.2**. Cytokines/chemokines implicated in pulmonary vascular inflammation [87].

- **Infection/inflammation/immune disorders can predispose** to the development of PH. Infectious pathogens can raise inflammation and luminal obliteration in pulmonary vessels. Latent viral infections with human immunodeficiency virus (HIV), human herpes virus-8 (HHV-8), Epstein–Barr virus (EBV) and cytomegalovirus (CMV), have been linked to PAH. Parasitical infection with schistosmiasis, affecting over 200 million people, is highly associated with PAH [98]. Furthermore, immune disorders, specifically autoimmune disease, covering scleroderma (SSc), systemic lupus erythematosus (SLE), Hashimoto disease and other connective tissue diseases are, with different frequencies, complicated by PAH, suggesting a role of immunopathology in PAH [99-102].

- **Anti-inflammatory therapies seem to benefit experimental PH.** Recent studies indicate that treatment with steroids has positive effects on MCT-induced PH rats and improves survival [103]. Immunosuppressants via other molecules like IL-1, MCP-1 and NFAT also prevent PH development [83, 104, 105]. Case studies report that PAH patients with mixed connective tissue disease or SLE respond to treatment with cyclophosphamide and glucocorticoids [106]. Noticeably, prostacyclin, which is one of current therapies in PAH, influences monocyte function and downregulates INF-γ-stimulated cytokines [107]. Another example of immunotherapy in PAH is the promising effects of imatinib, which causes immunosuppresion through PDGF inhibition [66, 67]. Although both the experimental and clinical findings make it logical to apply anti-inflammatory therapies, a formal trial with randomized controls in humans is still required.

The connection between inflammation and vascular remodelling can be attributed to a possible injurious hit to the vascular wall, followed by interactions among endothelial cells, smooth muscle cells, inflammatory cells and circulating cytokines/chemokines (Figure 1.2). The nature of the initiator has been unclear though; the impact of inflammation has to be taken into consideration in designing novel treatments.




Abbreviations: IL, interleukin; TNF-α, tumour necrosis factor alpha; MCP-1, monocyte chemotactic protein 1; RANTES, regulated on activation, normal T cell expressed and secreted; IP-10, interferon γ-induced protein 10; PDGF, platelet derived growth factor; EGF, epidermal growth factor.

**Figure 1.2**. The role of inflammation in the pathogenesis of PAH. Initial inflammatory stimuli can occur in the form of infection, autoimmune disease or other injurious hits, causing inflammatory cell activation, cytokine/chemokine release, and endothelial cell dysfunction. The resultant events form the characteristic complex of PAH, vascular remodelling and thrombus formation.

### 1.2.4. Thrombosis

Hypercoagulability is one of the major pathogenic features of PAH. Endothelial dysfunction is responsible for vascular thrombosis by secreting various coagulation factors such as thrombomodulin, tissue type plasminogen factor and von Willebrand factor (vWF) [108]. The coagulation initiator, tissue factor, is highly expressed in the pulmonary vasculature in humans and animals with PH [109]while circulating factors like fibrinopeptide, plasminogen activator inhibitor 1 and vWF are increased in patients with IPAH [110]. Hypercoagulable phenotype is also exhibited in patients with IPAH by calibrated

automated thrombography [111]. Platelet aggregation is enhanced in PAH which can be attributed to the interaction with endothelial cells and involvement of associated vasoactive mediators: prostacyclin, TXA2 and NO [14].

### 1.2.5. Genetics

Heterozygous mutations in BMPR2, a serine/threonine kinase receptor belonging to the TGF-β receptor superfamily, have been found in approximately 80% of cases of familial PAH, which accounts for around 10% of patients of IPAH, and 10-40% of sporadic IPAH cases [112]. Bound to its ligands BMPs, BMPR2 initiates intracellular smad-signalling gene transcriptions, mainly targeting DNA-binding gene inhibitors (IDs) [113]. The loss of BMPR2-smad signalling induces cell proliferation and resistance to apoptosis in pulmonary arterial endothelium. BMPR2 is found to be expressed in the plexiform lesions in humans and growth responses to TGF-β and BMPs of pulmonary arterial smooth muscle cells from patients with IPAH are altered [114]. Mice deficient in BMPR2 are predisposed to the development of PAH while mutants with overexpression of BMPR2 are resistant to vascular remodelling [115].

The low prevalence of BMPR2 mutations (10-20%) in nonfamilial PAH and even penetrance in familial PAH (25%) suggest that a "second hit" is required, which has been undetermined yet. Attempts at treatment by restoring BMPR2 function in experimental PH have been variably successful. Gene therapy with BMPR2 attenuates chronically hypoxic PH but fails to rescue MCT-induced PH [116]. Nevertheless, a recent study shows that inhibition of TGF-β signalling via activin-like kinase type 5 (ALK5) prevents development and progression of MCT-induced PH, which may be a consequence of inhibiting pulmonary arterial smooth muscle cell migration [117].

Other genetic mutations, such as ALK1, a member of the TGF-β receptor family, are reported in PAH [118]. Additionally, a polymorphic variant of the 5-HTT gene promoter has a high prevalence (65%) in IPAH compared with 27% in the control group [40]; notwithstanding, adequately powered cohorts are needed to verify this.

### 1.2.6. Right ventricular hypertrophy

RV function is a crucial determinant of survival in PAH. RV hypertrophy, as a result from compensatory response to increased pulmonary resistance, highly correlates to the morbidity and mortality of PAH [119]. Responding to the excessive workload, RV augments the cardiac mass through increasing protein synthesis and thus thickens the ventricular wall until decompensation, dilatation and failure. Along with enlargement in myocyte size, fibrosis can occur and further worsen myocardial performance. Preserving RV function has become an important consideration in treatment strategies. A recent study indicated that inhibition of PDE5, highly expressed in hypertrophied RV, improves cardiac contractility and suggests the potential of RV targeted therapy [120].

## 1.3. Treatment of PAH

It is only in the past 25 years that efficacious treatment options have become available. Current medical therapies are composed of three categories, namely prostanoids, endothelin receptor antagonists (ERA) and phosphodiesterase type 5 inhibitors (PDE5-I) [121](Figure 1.3). Unfortunately, despite delaying the progression, no definite cure exists still. Failure to respond to these therapies is unfortunately very common and the final therapeutic option, cardiopulmonary transplantation, is costly and limited by donor availability. Novel treatments are desperately in need.

### 1.3.1. Current available therapies

- **Conventional therapies** comprise oxygen supplementation, diuretic and inotropic agents. Since right heart failure usually signals the dying process in late-stage PAH, diuretics, reducing preload, and inotropic agents such as digoxin, enhancing cardiac contractility, are used to preserve RV function. These treatments offer symptomatic relief but evidence of long-term benefit is lacking. Oral anticoagulants are recommended empirically as an adjuvant therapy in most patients without contradictions [122].

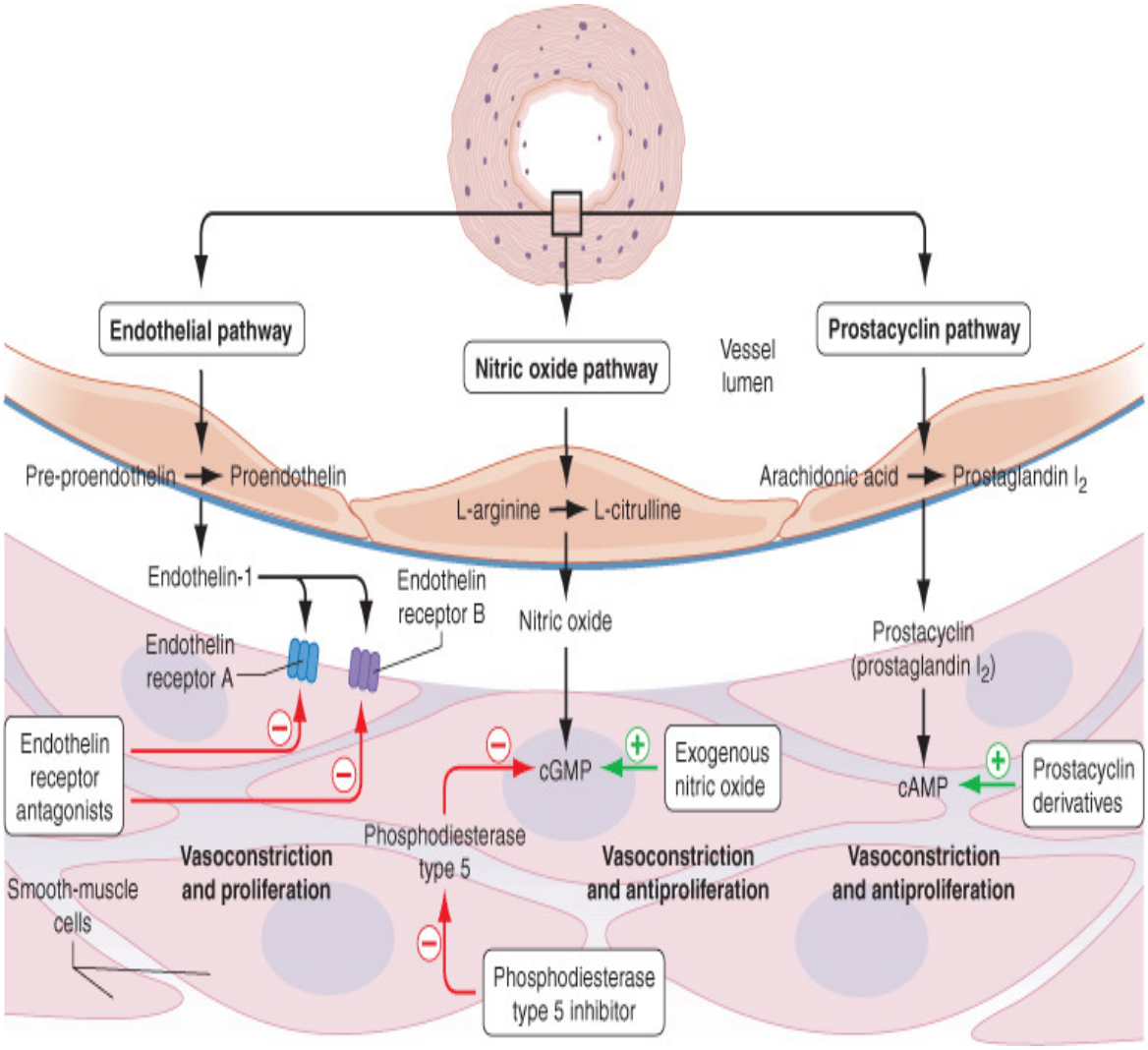



**Figure 1.3**. Targets of current therapies in PAH. The core of current therapy is composed of intervention in three major pathways, which are prostacyclin, phosphodiesterase type 5 and endothelin pathway, targeting the abnormal vasoconstriction and proliferation in PAH [3].

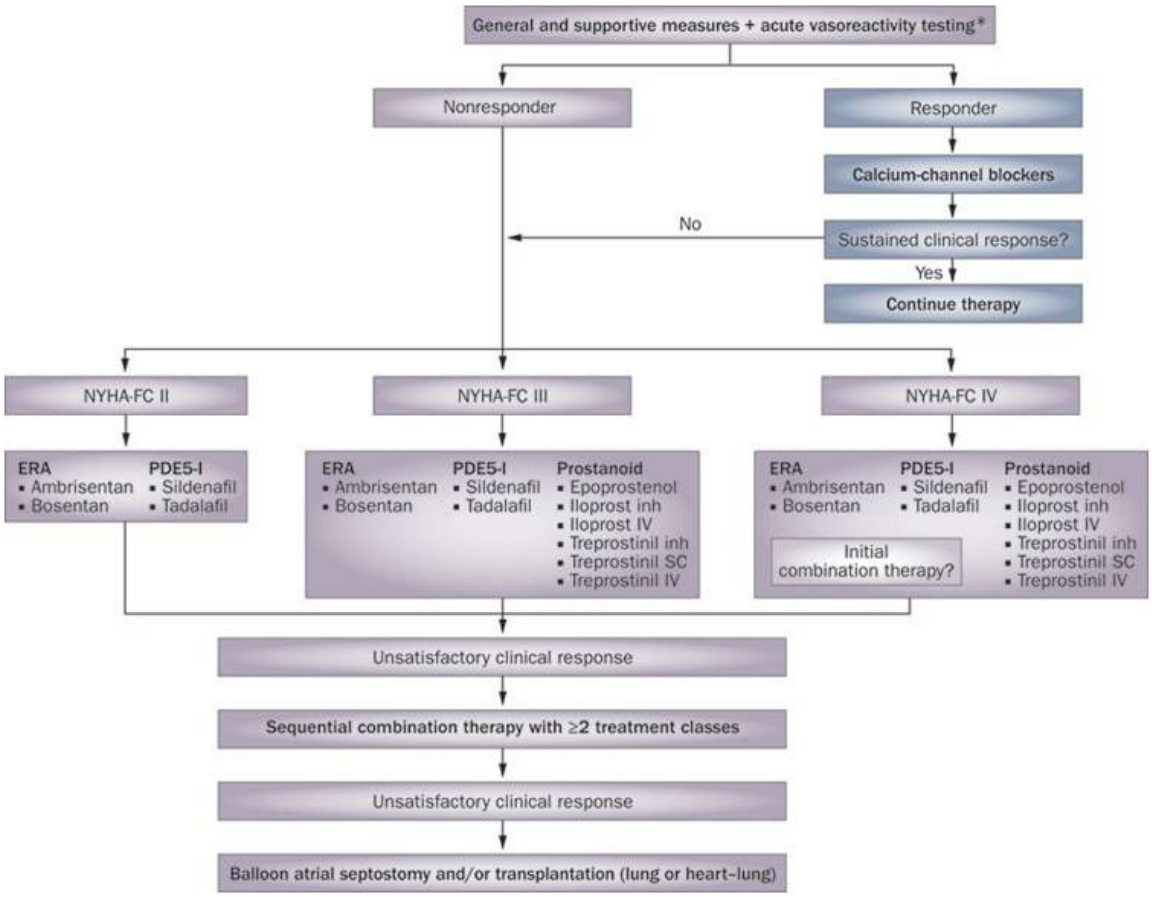
- **Calcium channel blockers (CCB)**, one of the standard treatments in systemic hypertension, act to relax muscle contraction in heart and vessels, resulting in decreased cardiac contractility and vasodilation. Long-term treatment with CCBs may prolong survival in a certain group of PAH patients (less than 10% of all patients). Acute vasodilator challenge during right heart catheterization with inhaled NO, adenosine or intravenous prostacylin, identifies patients who likely benefit from chronic CCB treatment [123].

- **Prostanoids**, a group of stable prostacyclin analogues, have proven a very important therapeutic option for PAH. By raising prostacyclin production, prostanoids exert potent vasodilation and simultaneously anti-thrombotic, anti-proliferative, anti-mitogenic, and immunomodulatory functions. Continuous intravenous infusion of epoprostenol was first used as a bridge to transplantation in PAH and found to prolong survival. New generation of compounds promise higher stability, perhaps less cost and alternative choices of administration, including subcutaneous (treprostinil), oral (beaprost) or by inhalation (iloprost) [121].

- **Phosphodiesterase type 5 inhibitors (PDE5-I)**, including sildenafil and tadalafil, result in vasodilation in pulmonary circulation by inhibiting cGMP metabolism. Randomized controlled studies show that treatment with PDE5-I has positive and durable effects on patients with PAH haemodynamically and functionally and is well tolerated [124].

- **Endothelin receptor antagonists (ERA)** inhibit vasoconstriction and proliferation through blockade of $ET_A$ and $ET_B$ on smooth muscle cells whereas lessen NO and prostacyclin production through inhibition of $ET_B$ on endothelial cells. Due to the predominance of $ET_A$ in pulmonary vasculature, the overall effects of ERA are against vasoconstriction and vascular remodelling. The non-selective ERA, Bosentan, was first approved for clinical use, which improves haemodynamics, exercise capacity, and functional class [125]. Selective blockers against $ET_A$ (ambrisentan, sitaxsentan) have been developed but whether they confer additional advantages have been not yet been demonstrated [126].

**Combination therapy** using two or more agents from prostanoids, PDE5-I and ERA is now common in patients with poor response to monotherapy. Since these three classes act on different pathways, synergic effects are expected. However, statistical significance that combination therapy offer long-term benefits over monotherapy and the optimal combination remain unclear. With this concern, few studies are undergoing to establish evidence-based treatment strategies. For example, a randomized controlled study, AMBITION, compares first-line combination therapy (ambrisentan and tadalafil) versus first-line monotherapy (ambrisentan or tadalafil) in patients with PAH [121]. Figure 1.4 shows an algorithm of treatment strategies currently adapted in clinical use (Figure 1.4).




Abbreviations: ERA, endothelin receptor antagonists; FC, functional class; INH, inhaled; IV, intravenous; PDE5-I, phosphodiesterase type 5 inhibitors; SC, subcutaneous.

**Figure 1.4**. Treatment algorithm for pulmonary arterial hypertension. *Licensed indications for individual agents with respect to functional class vary between countries. [121].

### 1.3.2. Emerging therapeutic options

Since the prognosis remains poor despite treatment, new therapeutic options are needed. Several candidates aiming at vasorelaxant mechanisms are under development, such as prostacyclin receptor agonists, sGC activators and VIP. One of the biggest challenges is to achieve pulmonary selectivity, avoiding systemic effects.

Recently, attention has been drawn to the fact that the three major treatment options predominantly intervene in the desregulation of vessel tone while influences on vascular remodelling are relatively indirect and secondary. In order to achieve maximal efficacy,

new approaches must target the pro-proliferative mechanisms that contribute to the remodelling. Several anti-proliferative drug types, mostly originally designed for cancer therapy, have been investigated for translational possibilities as PAH treatments [127].

**Tyrosine kinase inhibitor**, Imatinib (STI571 or Gleevec), which antagonizes the PDGF receptor, is currently licensed for the treatment of chronic myelogenous leukemia (CML) and malignant gastrointestinal stromal tumours (GIST). Upregulated PDGF signaling is seen in the lungs of patients with PAH and takes part in pro-proliferative abnormalities in PAH. Treatments that target PDGF signalling are expected to be beneficial. Imatinib treatment improves pulmonary haemodynamic and RV function with reversal of pulmonary vascular remodelling in experimental PH models [66]. Case studies report positive clinical responses to imatinib in patients with refractory IPAH [123]. A pilot study revealed that imatinib treatment in patients failing to respond to standard treatment improves haemodynamics but not exercise capacity [128]. Sorafenib, another tyrosine kinase inhibitor with a wider spectrum of tyrosine kinase inhibition, has been shown favourable effects on pulmonary vascular remodelling and haemodynamics in animal models [77]. In a phase Ib trial, oral sorafenib improves exercise capacity and RV function [129]. However, cardiotoxicity, particularly left ventricular dysfunction, resulting from prolonged use of tyrosine kinase inhibitor may undermine its clinical adaptability [130].

**Metabolic alternation** is another interesting focus of anti-proliferative therapy in PAH. In 1924, Otto Warburg described a shift of glucose metabolism from oxidative phosphorylation to glycolysis that might be responsible for the resistance to apoptosis in cancer cells, and is now known as the Warburg effect [131]. A paradoxically high glycolytic rate despite adequate oxygen supplement is widely found in cancer cells, resulting in extreme inefficiency in energy production and magnified glucose demand. Excessive metabolites from aerobic glycolysis increase mitochondrial membrane potential and intracellular potassium and thereby restrain mitochondria from releasing several pro-apoptotic mediators. Also, reduced oxidative phosphorylation inhibits the production of superoxide, which further reduces apoptosis. Several glycolytic enzymes, such as Akt and glycogen synthase kinase 3 (GSK3), have been linked to the regulation of apoptosis and interaction with oncoproteins [132]. Recent studies have indicated that aerobic glycolysis is not exclusive in tumour cells but in non-malignant proliferating cells, including

endothelial cells from IPAH patients. Increased glucose uptake, typically observable in cancer, is shown in patients with PAH by tracking with 18F-fluorodeoxyglucose positron emission tomography (18F-FDG-PET) [133]. Accordingly, correction of the metabolic alternation is expected to restore the apoptotic susceptibility and eventually reverse vascular remodelling in PAH.

Dichloroacetate (DCA), an orally available small molecule which has been used in treating lactic acidosis for decades, is under investigation as a treatment for cancer. DCA inhibits pyruvate dehydrogenase kinase (PDK) and subsequently activates pyruvate dehydrogenase (PDH), which functions as the gatekeeper for the entry of pyruvate into mitochondria. During glycolysis, inactivated PDH retains pyruvate in the cytoplasm where accumulative pyruvate is converted to lactate dehydrogenase (LDH). By inhibiting PDK, DCA boosts the conversion of pyruvate to acetyl-CoA, which enter the Krebs cycle to drive oxidative phosphorylation, and restore normal mitochondrial function [134]. DCA was found to activate mitochondria-dependent apoptosis in cancer cells and reduce tumour size in vivo and in vitro without affecting non-transforming tissues [56]. In animal PH models, DCA promotes apoptosis, reverses vascular remodelling, normalizes haemodynamics, attenuates RVH and improves survival [135, 136]. Biochemically, in these studies, DCA treatment increases oxidative phosphorylation, depolarizes mitochondrial membrane potential, increases ROS production, activates Kv channel expression and function, induces apoptosis and inhibits vascular proliferation. Noticeably, DCA does not affect normal vascular cells, suggesting the specificity for treatment. Moreover, DCA was found beneficial to patients with left heart failure by increasing ventricular mechanical efficiency and probably confers additional advantages to PH treatment [137].

# 2. Histone Deacetylase (HDAC)

## 2.1. Epigenetic modification

Classic genetics alone cannot explain the diversity of phenotypes within a population. Nor does classic genetics explain how, despite their identical DNA sequences, monozygotic twins [138]or cloned animals [139]can have different phenotypes and different susceptibilities to a disease. The concept of epigenetics offers a partial explanation of these phenomena. First introduced by C.H. Waddington in 1939 to name "*the causal interactions between genes and their products, which bring the phenotype into being*" [140], epigenetics was later defined as heritable changes in gene expression that are not due to any alteration in the DNA sequence [141]. As the genetic code is like a well-tuned piano, epigenetics plays the role of pianist who produces the tune. Epigenetic mechanisms are essential for normal development and maintenance of tissue-specific gene expression patterns in mammals. Disruption of epigenetic processes can lead to altered gene function and malignant cellular transformation. Global changes in the epigenetic landscape are a hallmark of different diseases. The initiation and progression of disease, traditionally seen as a genetic disease, is now realized to involve epigenetic abnormalities along with genetic alterations.

Epigenetic modulation is effected through several mechanisms, such as DNA methylation, nucleosome positioning, small RNA transcripts and histone modifications. These systems can act alone but more frequently in a multiple interacting complex.

### 2.1.1. DNA methylation

DNA methylation is a covalent modification formed by addition of a methyl group at the 5' carbon of cytosine in the sequence context CpG dinucleotides of the DNA molecule. DNA methylation is a common epigenetic modification existing in all types of cells. The methylation can silence DNA and hence downstream transcription. DNA methylation precludes detrimental expression such as viral genes or other adverse insertions into the genome as well as programs cells to be tissue specific during the development [142, 143]. Packaging DNA into nucleosome affects DNA accessibility by transcription factors (TFs): while some TFs can bind nucleosome-packed DNA, the majority of TFs cannot bind

nucleosome-packed DNA. Knowledge of nucleosome positioning is essential for understanding mechanisms of the regulation of gene expression in eukaryotic cells.

### 2.1.2. RNA transcripts

RNA transcripts primarily refer to three categories of small RNAs (~20-30 nucleotides), short interfering RNAs (siRNAs), microRNAs (miRNAs) and piwi-interacting RNAs (piRNAs), manipulating endogenous gene expression at post-transcriptional levels in both somatic and germline lineages. These noncoding RNAs originate from cleavage of double-strand RNAs (dsRNAs) and in most cases, silence specific genes by a mechanism known as RNA interference whereby binding to the messenger RNAs (mRNAs) prevents translation and protein synthesis. miRNA have been recognized as mediators of endogenous genes while siRNA protectors of invasive events like viral infection or transposon insertion, respectively. The small RNA-induced silencing has been recognized to be involved in diverse biological processes and widely employed as a powerful research tool due to the predictability and controllability [144].

### 2.1.3. Histone covalent modification

Histone modification is the predominant post-translational modification in gene regulation. In eukaryotes, an octamer of histones H2A, H2B, H3 and H4 is wrapped by 147 bp of DNA to form a nucleosome – the fundamental unit of chromatin [145]. As the core components of chromatins, histones act as spools around which DNA wind and wrap the whole genomes in the nuclei and maintain the chromosomal structure. Post-translational modifications of histones, specifically the states of amino-acid residues on histone tails, result in structural changes and consequent accessibilities of various transcriptional regulators that binds to specific DNA sequences to promote or repress certain physiological or pathological signallings. Therefore, histone modification governs widespread gene expression and generates effects on nearly all forms of biological events. Histones are subject to a wide variety of modifications including but not limited to, lysine acetylation, lysine and arginine methylation, serine and threonine phosphorylation, and lysine ubiquitination and sumoylation as well as adenosine diphosphate (ADP)-ribosylation and carbonylation [146]. These modifications occur primarily within the histone amino-terminal tails protruding from the surface of the nucleosome as well as on the

globular core region [147]. The combination of histone modifications is believed to form a unique well-established “histone code”, recognized by other proteins to lead to the distinct downstream pattern.

The histone-modifying enzymes affect histones either locally, through targeted recruitment by sequence-specific transcription factors [148], or globally throughout the genome in an untargeted manner affecting virtually all nucleosomes [149]. The best characterized histone modifications are acetylation, and methylation. In 'normal' cells, genomic regions that include the promoters of tumour-suppressor genes are enriched in histone-modification marks associated with active transcription, such as acetylation of H3 and H4 lysine (K) residues and trimethylation of K4 of H3 (*H3K4me3*) [150] while other heterochromatic regions are deacetylated but characterized by trimethylation of specific lysine residues, which function as repressive marks [151, 152]. However, many active and inactive genes have overlapping patterns of histone modifications. Nevertheless, it is clear that aberrant regulation of histone modification can affect gene activity and therefore oncogenic potential exists. Histone modifications are proposed to affect chromosome function through at least two distinct mechanisms. The first mechanism suggests modifications may alter the electrostatic charge of the histone resulting in a structural change in histones or their binding to DNA. The second mechanism proposes that these modifications are binding sites for protein recognition modules, such as the bromodomains or chromodomains, which recognize acetylated lysines or methylated lysine, respectively.

## 2.2. Histone Deacetylase

### 2.2.1. Histone-modifiying enzymes

For each modification, enzymes exist which either lay down the appropriate mark or remove it. Major players in this regulation are the histone acetyltransferases (HAT), which acetylate the histone tails and induce chromatin decondensation, histone deacetylases (HDAC), which remove the acetyl groups and promote a tighter binding of histones to DNA, histone methyltransferases (HMT) which promotes or inhibits transcription depending on the target histone residue and histone demethylases (HDM) which counteracts the HMTs.

### 2.2.2. Histone deacetylase (HDAC)

HDAC proteins occur in four groups depending on their sequence similarity and cofactor dependency. The first two groups are considered "classical" HDACs whereas the third group is a family of nicotinamide adenine dinucleotide (NAD)+-dependent proteins with distinct homology from other HDACs. The fourth group is considered an atypical category of its own, based solely on DNA sequence similarity to the others. HDACs are divided into the following classes: class I (HDAC1-3, 8), class II (HDAC4-7, 9, 10), class III (sirtuin1-7) and class IV (HDAC11). All class I, class II and class IV are zinc-containing in the catalytic sites. Based on phylogenetic analyses, these classes can be further divided into class Ia (HDAC1 and 2), Ib (HDAC3) and Ic (HDAC8); class IIa (HDAC4,5,7 and 9), IIb (HDAC6 and 10). Individual HDACs are found different in not only structure but also enzymatic function, cellular localization and tissue distribution. Class I HDACs are generally localized in the nucleus and ubiquitously expressed while class II HDACs shuttle between nucleus and cytoplasm (IIb are mostly cytoplasmic). Recent studies have gradually elucidated the roles of specific HDACs that class I HDACs are implicated in proliferation and cell survival and functions of class II HDACs are diverse and maybe tissue-specific. Sirtuins can be nuclear, cytoplasmic or mithochondrial in localization, depending on the subtype, and are linked to aging and metabolism (Table 1.3) [153, 154].

| Class | HDAC | Subcellular localization | Tissue distribution | Substrates | Knockout phenotype |
|---|---|---|---|---|---|
| I | HDAC1 | Nucleus | Ubiquitous | Androgen receptor, SHP, p53, MyoD, E2F1, STAT3 | Proliferation defects, increase in p21 and p27 |
| | HDAC2 | Nucleus | Ubiquitous | Glucocorticoid receptor, YY1, Bcl-6, STAT3 | Cardiac malformation |
| | HDAC3 | Nucleus | Ubiquitous | SHP, YY1, GATA1, RELA, STAT3, MEF2D, CDK9, SP1, PP4c | Gastrulation defects |
| | HDAC8 | Nucleus | Possibly ubiquitous | | Craniofacial defects |
| IIa | HDAC4 | Nucleus/cytoplasm | Heart, brain, smooth muscle | GCMA, GATA1, HP1 | Chondrocyte differentiation defect |
| | HDAC5 | Nucleus/cytoplasm | Heart, brain, smooth muscle | GCMA, SMAD7, HP1 | Exacerbated cardiac hypertrophy |
| | HDAC7 | Nucleus/cytoplasm | Heart, brain, pancreas, placenta | PLAG1, PLAG2 | Maintenance of vascular integrity, increase in MMP10 |
| | HDAC9 | Nucleus/cytoplasm | Brain, smooth muscle | | Exacerbated cardiac hypertrophy |
| IIb | HDAC6 | Mostly cytoplasm | Heart, liver, kidney, placenta | α-Tubulin, HsP90, SHP, SMAD7 | Increased tubulin acetylation |
| | HDAC10 | Mostly cytoplasm | Liver, spleen, kidney | | |
| III | Sirtuin1-7 | Nucleus, cytoplasm, mitochondria | | FOXO, Ku70, E2F1, TGF-β and NF-κB | Premature aging, reduced lifespan, cardiac hypertrophy |
| IV | HDAC11 | Nucleus/cytoplasm | Heart, brain, smooth muscle, kidney | | |

Abbreviations: HDAC, histone deacetylase; SHP, small heterodimer partner; Bcl-6, B cell lymphoma 6; STAT3, signal transducers and activators of transcription 3; MEF2D, myocyte-specific enhancer factor 2D; CDK9, cyclin-dependent kinase 9; HP1, heterochromatin 1; MMP10, matrix metalloproteinase 10; Hsp90, heat shock protein 90; HIF-1α, hypoxia-inducible factor-1α; FOXO, Forkhead box class O; TGF-β, transforming growth factor-β; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells.

**Table 1.3**. Identified HDAC characteristics [153-155]

### 2.2.3. Histone acetyltransferase (HAT)

In the contrast, HATs catalyze the acetylation of lysine residues on histone and non-histone proteins. Based on the cellular origin and functions, they are classified into nuclear HATs (type A) and cytoplasmatic HATs (type B) and further divided into to five families according to the structural homology. Three of them have been explored extensively, which are GNAT (GCN-5-related N-acetyltransferase) family, PCAF (p300/CBP associated factor) and the MYST family [156]. Relative to HDACs, the knowledge of the role of individual HATs in human diseases is less established. Functioning in opposition, HATs and HDACs achieve a dynamic balance, dominating the histone acetylation state.

### 2.2.4. Histone acetylation

Histone acetylation-deacetylation balance controls the higher-order structure of chromatins, acetylation of lysine residues within histone tails neutralizes the positive charges and thereby relaxes chromatin structure, allowing more accessibility of transcriptional factors to their target genes. By contrast, deacetylation of histones leads to compacted chromatin structures which incline to transcription repression. The balance between deacetylation and acetylation is maintained dynamically by HDACs and the opponent enzymes, HATs (Figure 1.5). Importantly, HATs and HDACs also act directly on non-histone proteins, mostly transcriptional factors such as NF-κB, hypoxia-inducible factor (HIF)-1α and tumour suppressor p53, and influence their activities. More than 50 non-histone substrates of known function have been identified, which may be acetylated by HDACs [153].

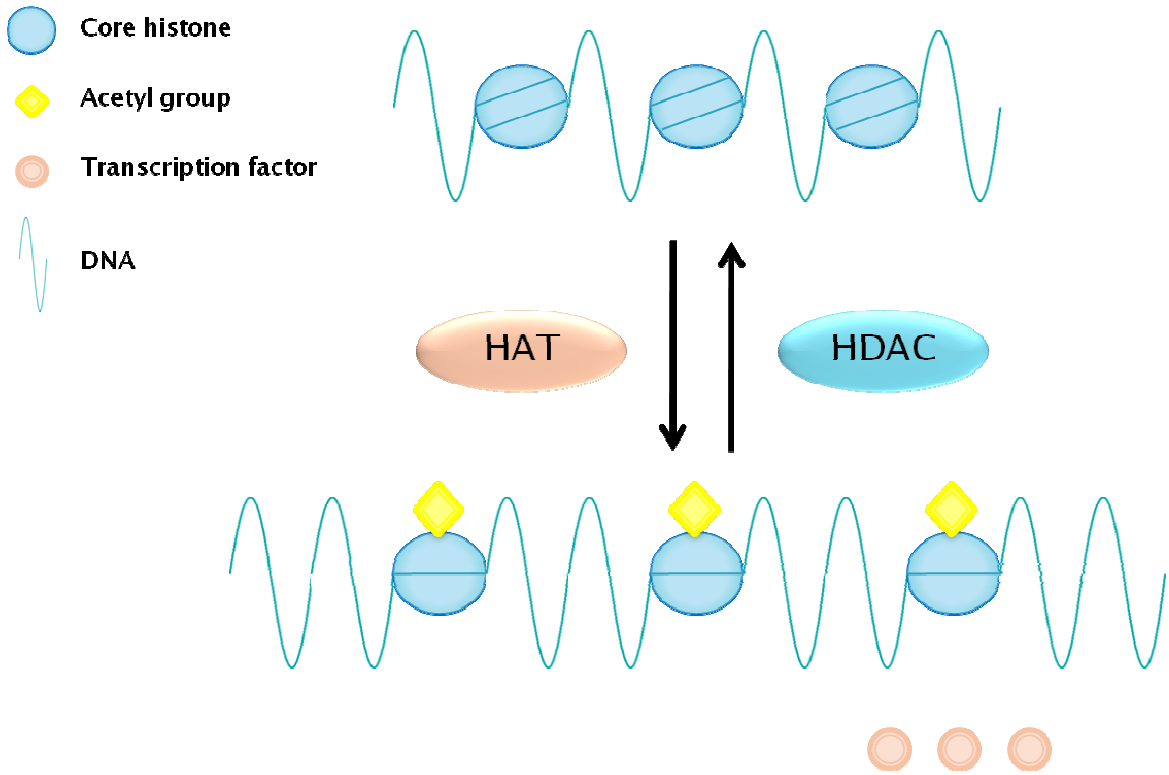



**Figure 1.5**. Schematic diagram of HDAC and HAT function. The chromatin structure is modulated by the counteraction between HDAC and HAT. This balance determines the accessibilities to the target genes of transcriptional factors and hence the following transcription. HDACi, HDAC inhibitor.

HDACs have attracted great attention due to their regulatory role in huge numbers of gene expression and protein activities. HDAC modifications are highly related to proliferative transformation, possibly through multiple mechanisms including cell-division cycle, intrinsic and extrinsic apoptosis, angiogenesis, senescence, ROS-facilitated reactions, mitotic autophagic cell death, DNA repair and inflammatory reactions [157]. Aberrant overexpression and the corresponding hypo-acetylation histone levels have been demonstrated commonly in a variety of cancer types, which is thought to be, at least partially, responsible for malignant transformation [158-160]. HDACs have been identified to be a new target for novel treatment of proliferative disorders, especially malignancy, and data to date indicate therapeutic potential.

## 2.3. Histone Deacetylase Inhibitor

### 2.3.1. Histone deacetylase inhibitor: general view

Owing to the extensive involvement of histone acetylation in biological functions, it is expected that global inhibition of HDAC would lead to universal depression of gene expression. Nevertheless, HDAC inhibition, specific or systemic, with certain inhibitors is well tolerated *in vivo* and seems to selectively shut down gene expression profiles associated with diseases. The phenomenon that transformed cells are more susceptible to HDAC inhibition-induced anti-proliferative effects is presumably attributed to distinctive signalling pathways or failure in defective rescue [161]. HDAC inhibitors exert anti-neoplastic capacities and are emerging as a new class of cancer treatment. The exact mechanisms underlying the effects of HDAC inhibitors on cancer are still to be defined. Several explanations have been introduced. First, HDAC inhibitors alter the acetylation status of gene promotors or repressor and subsequent gene expression [162]. Second, acetylation results in chromatin relaxation, allowing more transcription, which can be promotive or inhibitory [163]. Third, HDAC inhibitors jeopardize deacetylation of transcription factors and thus influence activities [164]. Furthermore, HDAC inhibitors can directly modify non-histone proteins, comprising transcriptional factors, hormone receptors, DNA repair enzymes, signalling and inflammatory mediators, structural and chaperone proteins, and manipulating their functions [157]. Finally, the inhibitors may break down the equilibrium among different HDACs [165]. Up to date, several HDAC inhibitors have been developed for evaluation in preclinical and clinical studies. Current available HDAC inhibitors can be grouped into four types based on chemical structure, which are hydroxamic acids, cyclic tetrapeptides, short-chain fatty acids and benzamide. More than ten of them have entered clinical trials, among which two compounds (Vorinostat and Isodax) have be approved for treatment of cutaneous T cell lymphoma (CTCL) in humans whereas valproic acid (VPA) had been clinically used for a long time in neurology and psychiatry (Table 1.4) [166].

| Group | Example | Specificity | Status | Indication in Monotherapy |
|---|---|---|---|---|
| **Hydroxamic acid** | Trichostatin | Pan-HDAC | Preclinical | |
| | Suberoylanilide hydroxamic acid (Vorinostat) | Pan-HDAC | Approved for cutaneous T cells lymphoma | NSCLC, soft tissue sarcoma, MDS, sickle cell disease |
| | Panobinostat (LBH589) | Pan-HDAC | Phase I > II > III | RCC, thyroid cancer, MM, Hodgkin's lymphoma, non-Hodgkin's lymphoma, SCLC, prostate cancer |
| | Belinostat | Pan-HDAC | Phase I > II | Lymphoma, MM, non-Hodgkin's lymphoma, peripheral T cell lymphoma, |
| **Cyclic tetrapeptide** | Romidepsin (FK228) | HDAC 1&2 | Approved for cutaneous T cells lymphoma | Non-Hodgkin's lymphoma, lymphoma, MDS, lung cancer, colorectal cancer, head and neck cancer, |
| **Short-chain fatty acid** | Valproic acid | Class I & IIa | Phase I > II > III | Chronic lymphocytic leukemia, head and neck cancer |
| | Phenyl butyrate | Class I & IIa | Phase I > II | Alpha 1-antitrypsin deficiency |
| | AN-9 | Class I & IIa | Phase I > II | Malignant melanoma, leukemia, lymphoma |
| **Benzamide** | Entinostat (MS-275) | HDAC 1&3 | Phase I > II | Hodgkin's lymphoma, MDS, breast cancer |
| | Mocetinostat | HDAC 1&3 | Phase I > II | Chronic lymphocytic leukemia, lymphoma, MDS, non-Hodgkin's lymphoma |
| | N-Acetyldinaline | HDAC 1&3 | Phase I > II > III | Advanced myeloma |

Abbreviations: HDAC, histone deacetylase; NSCLC, non small cell lung cancer; MDS, myelodysplastic syndrome; RCC, renal cell carcinoma; MM, multiple myeloma; SCLC, small cell lung cancer.

**Table 1.4**. Overview of HDAC inhibitors [166].

- **Trichostatin A (TSA)**, an antifungal antibiotic, is a potent, non-selective and reversible inhibitor of class I and II HDACs. TSA has been shown to increase histone acetylation and promote apoptosis in transformed cells in a seeming specific manner. The mechanisms are likely owing to cell cycle arrest, induction of differentiation and upregulation of apoptosis-related gene programs. Though TSA seems to be promising as an anti-cancer therapy in a series of preclinical studies, there have been few clinical trials [167, 168].

- **Valproic acid (VPA)** is one of the most widely prescribed anticonvulsants and mood-stabilizers worldwide, primarily used in epilepsy, bipolar disorder and some other psychiatric diseases. Several functional roles of VPA in neuropsychiatry have been established, such as GABA enhancer, N-methyl-D-aspartate (NMDA) inhibitor, sodium channel blocker [169]. VPA has been recently recognized as an effectve inhibitor of class I HDAC activity and emerged as potential therapeutic choice for cancer treatment. A series of the preclinical and early clinical trials have revealed encouraging results of VPA in treatment of several cancer types, including leukemia, glioma, multiple myeloma, melanoma and many solid tumours. Whole genome microarray of patients with cancer shows that VPA upregulates genes relevant to ribosomal proteins, oxidative phosphorylation, mitogen-activated protein kinase (MAPK) signaling; focal adhesion, cell cycle, antigen processing and presentation, proteasome, apoptosis, PI3K, Wnt signaling, calcium signaling, TGF-β signaling, and ubiquitin-mediated proteolysis among others [170]. VPA is oral available and generally well tolerated. The relatively easy administration and manageable side effects make it less challenging to translate to clinical application.

- **Suberoylanilide hydroxamic acid (SAHA, Vorinostat)**, structurally analogous to TSA, is the first drug approved by FDA in human cancer therapy for relapsed and refractory CTCL, under the trade name *Zolinza*. Similar to TSA, SAHA is a broad-spectrum (class I, II and IV) HDAC inhibitor but with less potency. It acts as a competitive antagonist occupying the binding sites of zinc containing domains of HDACs. SAHA-induced accumulation of acetylated histones and proteins influence cell proliferation, apoptosis, protein stability, cell motility, angiogenesis, ROS-facilitated cell death and inflammatory reactions [171]. The multiple targets of HDAC inhibition may account, in part, for the efficacy of SAHA as an anti-cancer agent. Currently, many clinical trials of SAHA are ongoing for various cancers, both haematological malignancies and solid tumours.

- **Romidepsin (depsipeptide, FK228)**, after SAHA, this agent is the second HDAC inhibitor approved as a treatment for CTCL in humans. With a relatively unique structure in all HDAC inhibitors, Romidepsin is a prodrug activated in cells to interact with zinc in the catalytic site of Zn-dependent HDAC. It preferentially acts

on HDAC1 and HDAC2 and is considered a more isoform-specific HDAC inhibitor [172]. In addition to CTCL, it is also linked to other cancers such as leukemia, melanoma and some solid tumours. Similar to SAHA, Romidepsin is believed to reprogram aberrant gene alterations, which are responsible for cell proliferation, differentiation and resistance to apoptosis in transformed cells. However, caution have been raised due to the risk of haematological disorders and cardiac toxicity [173].

### 2.3.2. Histone deacetylase inhibitor in cancer

HDAC inhibitors have been demonstrated to suppress cell proliferation and induce apoptosis both *in vivo* and *in vitro* cancer studies. Various molecular mechanisms have been unravelled that shows the anti-neoplastic effects of HDAC inhibitors are multifactorial via epigenetic and non-epigenetic modification. Some mechanisms have been elucidated (Figure 1.6).

- **Cell cycle arrest** is one of the most common features of HDAC inhibition in most cell types. The cell cycle is controlled by several gatekeeper proteins, such as p21, p27 and p53, constituting checkpoints to protect cells against external stresses and internal errors. Dysfunction in these checkpoints is seen widely in transformed cells, which prevent cells with DNA damage from being repaired or undergoing apoptosis. HDAC inhibitors arrest cell cycle at G1 phase in low concentrations while G2/M phase in higher concentrations [174]. Disruption of cell cycle checkpoints by HDAC inhibitors contributes to selective cytotoxicity in transformed cells by driving cells to enter an abortive mitosis, culminating in apoptosis [175] . HDAC inhibitor-induced cell cycle arrest is commonly associated with upregulation of the cyclin-dependent kinase (CDK) inhibitor p21, leading to retinoblastoma protein (pRb) dephosphorylation and inhibition of E2F in a p53-independent manner [176]. On the other hand, p53 can be directly hyper-acetylated by HDAC inhibition and this increases p21 expression [177]. Other studies also suggest the role of p27, another CDK inhibitor and regulator of the cell cycle, in HDAC inhibitor-induced arrest [178, 179]. In addition to p21, HDAC inhibitors halt cell growth by suppressing DNA synthesis via related enzymes, such as thymidylate synthase and cytidine triphosphate synthatase [180].

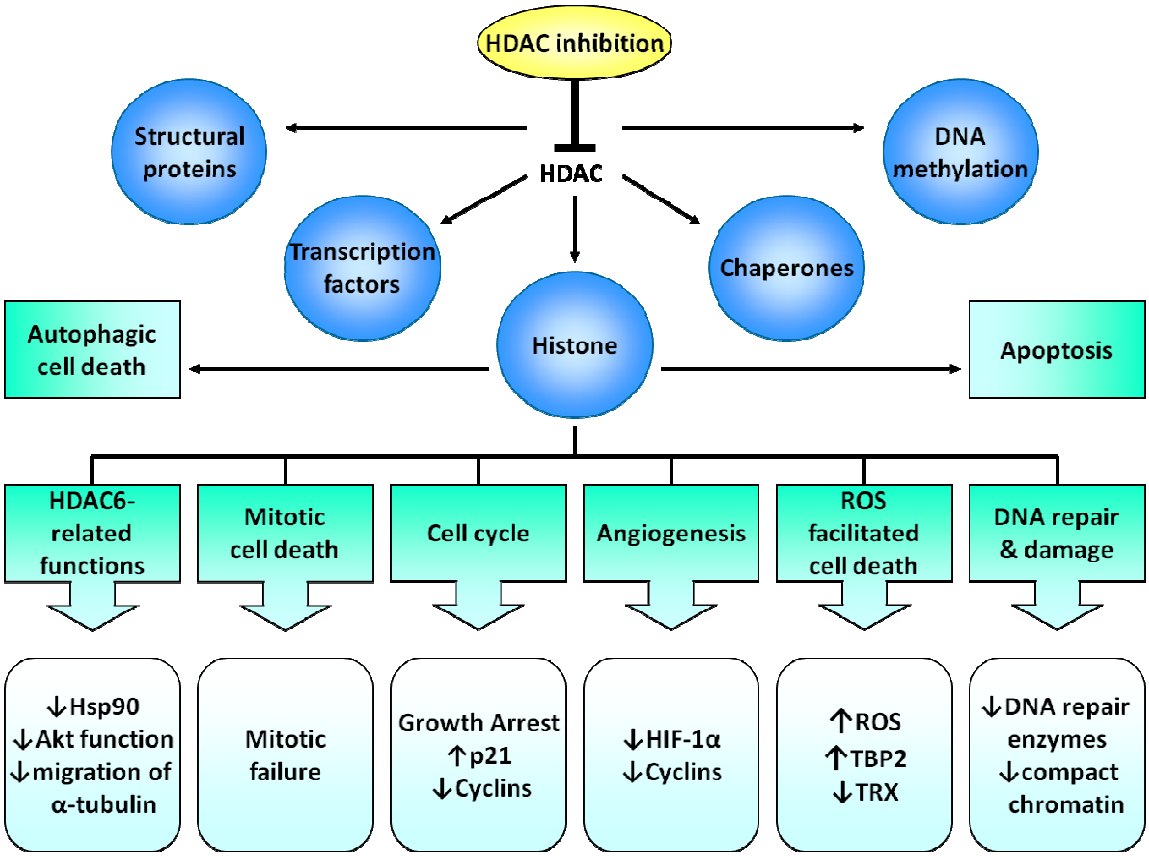
- **Apoptosis induction** is a major pathway in HDAC inhibitor-induced cell death. Apoptotsis comprises two forms, extrinsic or death-receptor and intrinsic or mitochondrial pathway, both of which are implicated in HDAC inhibitor-induced cell death. In the extrinsic pathway, HDAC inhibition increases the expression of death receptors and cognate ligands selectively in transformed cells but not normal ones, *in vitro* and *in vivo* [181]. These include Fas and its ligand, TRAIL and its receptor, death receptor (DR-5). Conversely, inhibitor of death receptor signalling is downregulated by HDAC inhibition, for example, c-FLICE-like inhibitory protein (FLIP) [182].

  The intrinsic apoptosis pathway is dominated by mitochondria, permeabilization of which releases of cytochrome c and initiates a caspase cascade. The mechanism is under control of pro- and anti-proapoptotic proteins of Bcl-2 family. Nearly all the HDAC inhibitors have been reported either of both upregulation of pro-apoptotic genes, Bim, Bmf, Bax, Bak and Bik [183-185], or downregulation of anti-apoptotic genes, Bcl-2, Bcl-xL, Bcl-w, Mcl-1 [183, 185], XIAP (X-linked inhibitor of apoptosis protein) and survivin [186-188]. These modifications undermine the characteristic resistance to apoptosis in transformed cells and shift the imbalance toward cell death. Additionally, some HDAC inhibitors can stimulate p53-mediated apoptosis by enhancing p53 stability and further activating pro-apoptotic transcriptors like Bax [181].

- **Angiogenesis** is a hallmark in cancer progression especially in the transition from hyperplasia to neoplasia and favours cancer cell expansion and invasion. In the past few decades, anti-angiogenesis has become a promising target in cancer therapy and a few angiogenesis inhibitors are available in cancer treatment currently [189]. For example, Bevacizumab, targeting VEGF, is shown effective in several solid tumours [190]. HDACs are linked to angiogenesis by their hypoxia-triggered repression of p53 and von Hippel-Lindau protein (vHL), both of which responsible for degradation of angiogenic transcription factor HIF-1 and HIF-2. Several HDACs (HDAC1,3,4,6 and 7) are involved in HIF-1α stability under hypoxic stimuli [191]. HDAC7 translocates to nucleus to activate HIF-1α under hypoxic conditions [192]. Early reports described that HDAC inhibitors promote p53 and vHL transcriptions and thereby degrade HIF and downregulate its downstream VEGF. Moreover, HDAC inhibition influences angiogenesis in a vHL-independent way, which is an acetylation-ubiquitination

proteasomal signalling as the result form direct acetylation of HIF-1α [193]. The fact that HDAC inhibitors prevent vascular endothelial cells from answering to VEGF makes it rational to be combined with VEGF inhibitors in anti-tumour treatment [194].

- **Reactive oxygen species (ROS)**. Apart from apoptosis, HDAC inhibitors induce cell death via other pathways, such as mitotic failure, autophagic cell death and ROS accumulation. HDAC inhibitors exert ROS-derived cell death by inhibiting thioredoxin (TRX) activity in transformed but not normal cells. TRX provides hydrogen to activate several proteins, including ribonucleotide reductase which is essential for DNA synthesis, and antioxidant NF-κB. Inactivation of TRX by HDAC inhibitors leads to ROS accumulation in cells and thus cell death [195]. However, downregulation of TRX can promote apoptosis through pro-apoptotic Bim, suggesting ROS acculumation may result from intrinsic apoptosis [196]. Furthermore, a study indicates that SAHA selectively enhances TRX function in non-transformed cells, supporting the increased sensitivity of transformed cells ROS-derived cell death [161]. Growing evidence has agreed that HDAC inhibitor-induced ROS generation may be important in the malignancy-specific cytotoxicity and therapeutically potential [161].




Abbreviations: ROS, reactive oxygen species; HIF-1α, hypoxia-inducible factor-1α; TBP2, thioredoxin binding protein 2; TRX, thioredoxin.

**Figure 1.6**. The mechanisms of HDAC inhibitors in cancer. Acetylation of histone and non-histone proteins affects a wide range of signalling pathways, such as cell cycle, angiogenesis and apoptosis. These abnormalities are generally exclusive in cancer cells and consequently can be therapeutic targets.

### 2.3.3. Histone deacetylase inhibitor in inflammation

Recent advances in the field of histone modifications have revealed that HDACs play a key role not only in oncogenic biology but also in immunological pathways. Current understanding concludes that protein acetylation is involved in innate and adaptive immune regulation, associated with numbers of inflammatory mediators, including cytokines, chemokines and MMPs, acting on different immune cells (Table 1.5). Some mechanisms have been identified, such as Toll-like receptor (TLR) and interferon signalling in innate immune system as well as lymphocyte development and function in acquired immunity. The role of HDACs in immune control can be dual and isoform-specific. For example, gene profiling reveals that HDACs promote TLR target genes encoding major inflammatory mediators, including IL-6, IL-12, TNFs, MCP-1, IP-10, MMP-9 and ET-1 [197, 198]. In contrast, HDACs can negatively regulate TLR-mediated inflammatory responses through NF-κB inactivation [199]. Moreover, HDAC5 is upregulated during differentiation of monocytes to macrophages [200]whereas HDAC3 downregulate myeloid cell differentiation [201].

HDAC inhibitors are shown beneficial in animal models of non-cancerous inflammatory disorders, such as arthritis, inflammatory bowel disease, ischemia-reperfusion injury, septic shock, airway inflammation, multiple sclerosis and neurodegenerative diseases [202]. The therapeutic effects are possibly attributed to mechanisms including suppressing the destructivity of innate immunity and intervening in the development of adaptive immunity. For example, SAHA is found to promote differentiation of antigen presenting cells along with reduction in cytokines, IL-6, IL-12 and TNF-α, as well as augment regulatory T cell in mice [203]. SAHA is also able to reduce circulating IL-1β, IL-6, TNF-α and INF-γ in experimental endotoxemia [204], suggesting the immunomodulatory activites of HDAC inhibitors. Furthermore, HDACs are presumed to be involved in the reverse transcription of HIV infection because they control the chromatin structure. Studies show that HDAC inhibitors can reactivate the viral expression in latent cellular reservoirs, supporting the promise of combination of HDAC inhibitors with current anti-retroviral agents for viral eradication [205]. Contradictorily, some studies indicated that HDAC inhibitors may compromise host immune [198]and worsen atherosclerosis [206]. The causes and effects of HDAC inhibition in immunomodulation remain to be investigated.

| Class | HDAC | Immune cell | Function |
|---|---|---|---|
| I | HDAC1 | B cells | Promote proliferation |
| | | T cells | Negative feedback regulator |
| | | Macrophages and non-immune cells | Enhance HIF-1α function, promote INF signalling, repress NF-κB function |
| | HDAC2 | B cells | Promote proliferation |
| | | Macrophages | Repress MTA1 function, promote GR-mediated repress NF-κB function |
| | HDAC3 | Macrophages | Enhance HIF-1α function, repress NF-κB function |
| | HDAC8 | Unknown | Unknown |
| IIa | HDAC4 | T cells | Repress IL-5 promoter |
| | | Macrophages | Enhance HIF-1α function, Control LXR-mediated repression of TLR-inducible genes |
| | HDAC5 | B cells | Unknown |
| | | T cells | Unknown |
| | | Monocytes/macrophasges | Unknown |
| | HDAC7 | $CD4^+CD8^+$ thymocyes | Control positive and negative selection |
| | | Unknown | Enhance HIF-1α function |
| | HDAC9 | $CD25^+$ regulatory T cells | Inhibit FOXP3 function |
| IIb | HDAC6 | T cells | Regulate immune synapse formation |
| | | Unknown | Promote Hsp90 chaperone function |
| | | $CD25^+$ regulatory T cells | Inhibit regulatory T cells function |
| | HDAC10 | Unknown | Unknown |
| IV | HDAC11 | Antigen presenting cells | Inhibit IL-10, promote expression of co-stimulatory molecules |

Abbreviations: HIF-1α, hypoxia-inducible factor-1α; INF, interferon; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; MTA1, metastatic tumour antigen 1; GR, glucocorticoid receptor; IL, interleukin; LXR, liver X receptor; TLR, toll-like receptor; Hsp 90, heat shock protein 90; FOXP3, forkhead box P3.

**Table 1.5**. Known functions of individual HDACs in the immune system [191].

### 2.3.4. Histone deacetylase inhibitor in cardiovascular system

In a series of knockout studies, HDACs have been shown to contribute to the development and differentiation of cardiac tissue. Deletion of HDAC2 causes multiple cardiac malformations while overexpression leads to cardiac hypertrophy through modulation of the Akt-GSK-3β signalling pathway [207]. HDAC1 and HDAC2 may cooperate to manage cardiac morphogenesis, growth and contractility [208]. HDAC3 serves a crucial role in maintaining cardiac energy metabolism through the nuclear receptor, peroxisome proliferator-activated receptor (PPAR)-α [209], and endothelial integrity through Akt activation [210]. However, overexpression of HDAC3 suppresses CDK inhibitors and promotes cardiac hyperplasia but not hypertrophy [211]. Cardiac-specific deletion of HDAC5 or HDAC9 leads to exacerbated cardiac hypertrophy and governs gene expression related to myocyte proliferation, differentiation and morphogenesis [212]. Although the global permanent absence of HDACs cannot reflect the real biological alternation in pharmacological manipulation, it indicates the essential involvement of HDACs in the developmental process.

In vascular biology, HDACs have been also implicated a key role maintaining the vascular cell homeostasis, specifically the proliferation, migration and apoptosis of endothelial and smooth muscle cells. For examples, HDAC7 is essential in vascular integrity and endothelial function [213]. HDAC7 can be mediated by VEGF signalling and performs as a switch of the downstream angiogenesis process [214]. Interacting with beta-catenin, HDAC7 can keep endothelial cells in a low proliferation stage while silencing HDAC7 inhibits endothelial cell migration via upregulation of PDGF signalling [215, 216]. Knockdown of HDAC5 promotes endothelial migration and sprouting whereas knockdown of HDAC7 and HDAC9 decreases [217]. HDAC1 was reported involving in oxidative-stress-induced apoptosis of smooth muscle cells via interaction with p53 [218]. Class III HDACs have been also described the importance in cardiovascular biology. Sirtuins, especially SIRT1, are suggested to regulate angiogenesis and vessel tone, contributing to endothelial homeostasis and function, and might be beneficial in vascular disease, such as atherosclerosis [219, 220]. Several underlying mechanisms have been explored, such as inhibition of proliferation and migration via cyclin D1-dependent cell cycle arrest [221], and vascular relaxation by activating eNOS [222].

Attempts have been made to evaluate the therapeutic potential of HDAC inhibitors in cardiovascular diseases. In an aorta-banding mice model, inhibition of class I HDAC prevents cardiac hypertrophy induced by angiotensin II [223]. Other studies also show that HDAC inhibitors partially recover pre-existing hypertrophy and improve cardiac function in animal models [224], suggesting potential in heart failure. In addition, as described in 2.2.2, HDAC modifications are highly connected to VEGF signalling pathway, and HDAC inhibitors have shown their potential as anti-angiogenesis agents. For example, SAHA, TSA and LBH589 are found to prevent human umbilical vein endothelial cells from sprouting and forming capillary-like structures by VEGF downregulation. This effect is specific in endothelial cells but in neither smooth muscle cells nor fibroblasts [194]. Histological evaluation of tumour blood vessel in xenograft models showed a significant reduction of angiogenesis in terms of both size and number [189]. Furthermore, TSA and another broad-spectrum inhibitor Scriptaid can inhibit vascular smooth muscle cell proliferation and neointima formation via induction of CDK inhibitors, such as p21, and subsequently arrest cell cycle [225, 226].

### 2.3.5. Histone deacetylase inhibitor in pulmonary hypertension

The present understanding of epigenetic modifications affecting phenotypic responses during pulmonary vascular remodelling is very limited but modern studies suggest epigenetic components in the pathogenesis of PAH. Archer et al have reported tissue-specific, epigenetic deficiency in mitochondrial superoxide dismutase-2 creates a heritable form of PAH with proliferative and apoptosis-resistant pulmonary artery smooth muscle cells [227].

Targeting the proliferative pathology underlying vascular remodelling is essential in PAH treatment. HDAC inhibitors may offer such efficacy. An early study showed that VPA inhibits right ventricular hypertrophy induced by pulmonary artery banding (PAB) and MCT [228]. Furthermore, the presence of pro-inflammatory fibroblasts with an epigenetically altered phenotype due to increased HDAC activity has been reported in severe hypoxic pulmonary hypertension [229]. However, TSA has been described to exacerbate right ventricular RV dysfunction in rat PAB models [230].

### 2.3.6. The limitation of histone deacetylase inhibitor

Although majority of evidence so far supports the therapeutic potential of HDAC inhibitors, many challenges have to be met and overcome. Most importantly, the understanding of individual HDAC function is still emerging. While some of them exert pathological events, others may offer protection. The fact that HDACs interact with one another seemingly provides a rationale to develop selective HDAC inhibitors. However, whether truly specific inhibitors can actually be developed is questionable because HDACs in cells usually locate in large protein complex containing other HDACs and the structural similarities may impede the specificities too. In addition, the exact mechanisms of sensitivity to HDAC inhibitors in transformed cells are not known, which raises concern with safety and long-term use. Better understanding of the nature of HDAC is urgently required to establish a more robust mechanistic conviction for clinical translation.

### 2.3.7. Epigenetics in pulmonary hypertension

Recently, possible epigenetic components in PAH have been described, especially DNA methylation and histone acetylation. These epigenetic abnormalities, also identified in cancer, may contribute to the development and phenotypic variability of PAH and could be therapeutically reversed [231]. This emerging recognition of the cross-talk between genetics and epigenetics provides a novel field for pharmacological intervention in PAH.

# 3. Hypothesis

Epigenetic modification, such as the alteration of histone acetylation status, may influence specific gene expression profiles leading to the abnormal, proliferative and dysfunctional cells contributing to the vascular pathology of pulmonary arterial hypertension. Histone deacetylase inhibitors, by modulating transcriptional factors to inhibit proliferation or induce apoptosis, have therapeutic potential in pulmonary arterial hypertension.

# 4. Objectives of study

- To clarify the role of HDAC-modulating histone acetylation in the pathogenesis of pulmonary hypertension.
- To evaluate the pharmacological effects of HDAC inhibition in experimental pulmonary hypertension models.

Specifically,

- To investigate HDAC expression in parallel with pathological changes during the development of PH.
- To investigate the pharmacological effects of HDAC inhibitors in chronic hypoxia-induced PH models.
- To investigate the pharmacological effects of HDAC inhibitors in monocrotaline-induced PH models.
- To investigate the pharmacological effects of HDAC inhibitors in proliferative human pulmonary endothelial cells and smooth muscle cells
- To identify the possible molecular mechanisms underlying the involvement of HDAC inhibition in pulmonary hypertension.

# Chapter 2:

# MATERIALS AND METHODS

# 1. Introduction

To test the hypothesis in a translational pre-clinical design, my study includes human samples, *in vivo* animal models and *in vitro* cell systems. This chapter contains all the materials and methods used in my study. Specific experimental designs are described in detail in the following chapters.

# 2. Materials

## 2.1. Human samples

Human lung tissues were obtained with the informed consent of each individual and the approval of the Brompton Harefield & NHLI and Hammersmith Hospitals Research Ethics committees. Human lung samples (lobectomy and IPAH) were obtained from the Imperial College Pulmonary Hypertension biorepository (ethics reference numbers: 01-210 & 2001/6003). The patients characteristics have been described previously [232]. Samples for histology were originally fixed in 10% formal-saline and embedded in wax, and sections were processed for immunohistochemistry.

## 2.2. Animals

All the animal experiments were conducted in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986 (London, UK).

### 2.2.1. Mice

The BMPR2-mutant mice (BMPR2) were introduced from Cambridge group and originally established by Beppu and colleagues via targeted gene disruption. Because homozygous BMPR2-mutant mice die in utero, breeding of BMPR2+/- mice was carried out by crossing BMPR2+/- mice with wild-type mice (C57/BL6 mice). Genotyping of the offspring was performed after weaning. The mutated BMPR2 allele in the mice was identified by a polymerase chain reaction with primer pairs 5_-GCTAA AGCGC ATGCT CCAGA CTGCC TTG-3_ and 5_-AGGTT GGCCT GGAAC CTGAG GAAAT C-3_.15 The amplification conditions were as follows: 1 cycle of 120 seconds at 95°C; 30 cycles

of 30 seconds at 95°C, 30 seconds at 63°C, and 90 seconds at 72°C; and 5 minutes at 72°C [233].

### 2.2.2. Rats

Adult male Sprague-Dawley rats (body weight = 200-250g) were from Charles River (Marget, UK), housed in plastic cages in a temperature-controlled room and used for all the rats experiments.

## 2.3. Cells

### 2.3.1. Human pulmonary smooth muscle cells (HPSMCs)

Human pulmonary smooth muscle cells (HPSMCs) were obtained from Cambridge group and maintained in Dulbecco's modified eagle medium (DMEM, high glucose, Invitrogen, Paisley, UK) with 10% foetal bovine serum (FBS, Invitrogen) and 1% antibiotic-antimycotic (AA, Invitrogen), referred to as SMC medium. The medium were changed every 3 days.

Cells in use for all experiments were from passage 5 up to 10. Cells were passaged at approximately 80% confluence and split in a 1 to 2 ratio. The passage involved harvest of cells by incubation with trypsin-Ethylenediaminetetraacetic acid (EDTA) (Invitrogen) at 37°C for 5 minutes. Cells were then neutralized in serum-containing medium and spun down in a centrifuge at 1200 rpm for 5 minutes, and the pellet were resuspended in DMEM with 10% FBS. Aliquots of cells were suspended in serum free cell freezing medium-Dimethyl sulfoxide (DMSO) (Sigmal-Aldrich, Dorset, UK) and kept at -80°C.

### 2.3.2. Human pulmonary artery endothelial cells (HPAECs)

Human pulmonary artery endothelial cells (HPAECs) were acquired from PromoCell (Heidelberg, Germany) and cultured in fibronectin-coated flasks at 37°C, 5% CO2 in Promocell Endothelial Cell Medium 2 with SupplementMix (referred to as EC medium), which ends up with 2% FBS, 5 ng/ml epidermal growth factor (EGF) 10 ng/ml basic fibroblast growth factor (FGF), 20 ng/ml insulin-like growth factor (IGF), 0.5 ng/ml

VEGF, 1 µg/ml ascorbic acid, 22.5 µg/ml heparin, 0.2 µg/ml hydrocortisone and 1% antibiotic-antimycotic. The medium were changed every other day.

Cells were passaged at approximately 80% confluence in a 1 to 3 ratio and used up until passage 8. The passage and storage were similar to HPSMC as previously described but resuspended in EC medium.

## 2.4. Drugs

### 2.4.1. Valproic acid (VPA)

VPA sodium salt was purchased from Sigma-Aldrich. For *in vivo* experiments, VPA was dissolved in tap water at aimed concentration and freshly prepared every other day; for *in vitro* experiments, VPA was dissolved in absolute ethanol to 1M as stock and kept at -20°C.

### 2.4.2. Suberoylanilide hydroxamic acid (SAHA)

SAHA was purchased from Chemos GmbH (Regenstauf, Germany). For *in vivo* experiments, SAHA was solubilized in 5 molar equivalents of 2-hydroxypropyl-β-cyclodextrin (HOP-β-CD, Sigma-Aldrich) in tap water, heated until fully dissolved and cooled to room temperature [234]. The solution was freshly prepared every other day. For *in vitro* experiments, SAHA was dissolved in DMSO (Sigma-Aldrich) to 100mM as stock and kept at -20°C.

# 3. Methods

## 3.1. In vivo pharmacological study

The pharmacological effects of histone deacetylase inhibitors, VPA and SAHA, and related molecular mechanisms were investigated based on two rat models of PAH, chronic hypoxia and MCT.

### 3.1.1. Reagents

- **VPA and SAHA** were prepared as previously described and administrated through drinking water. Animals were weighed every other day before making up treating solutions in order to adjust the concentration to the aimed dose according to daily drinking amount. Control group was given tap water with or without HOP-β-CD at the same concentration with SAHA-treated group.

- **Normal saline** was freshly prepared before every experiment as 0.9% sodium chloride in sterile water.

- **Haparin** was dilute in normal saline at 10 U/ml for catheter flushing and 1 U/ml for organ flushing.

- **Anesthesia:** Fentanyl/fluanisone (Hypnorm, VetaPharma, Leeds, UK) was used to anesthetize through intramuscular injection at 1 ml/kg body weight, followed by intraperitoneal injection with 0.8 ml/kg body weight midazolam (Hypnovel, Roche, Welwyn Garden City, UK).

### 3.1.2. Animal models

- **Chronic hypoxia-induced PAH model:** Sprague-Dawley rats with initial body weight 220-250g were kept in a normobaric hypoxia chamber ($FiO_2$ = 10%) (Figure 2.1). Excessive $CO_2$ accumulation was extracted by sodalime and maintained under 0.2%. The chamber was opened once daily for less than 30 minutes for drug administration and body weight measurement.

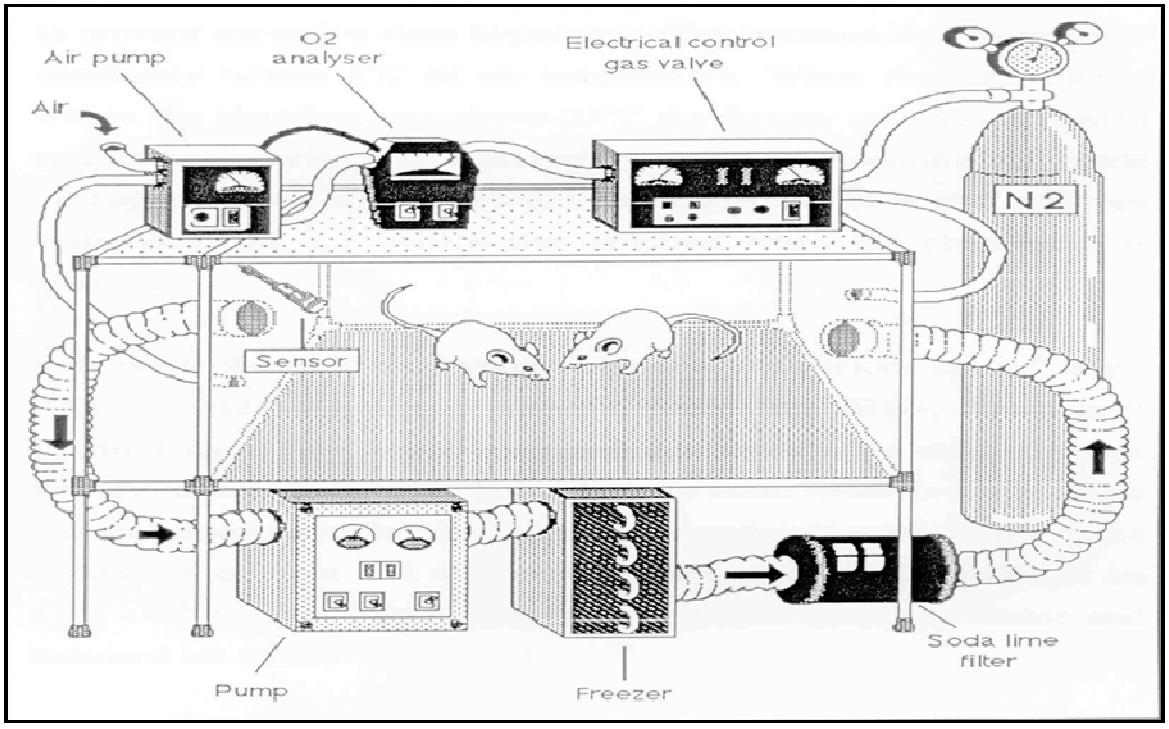



**Figure 2.1**. Hypoxia chamber schematic illustration.

- **MCT-induced PAH model:** MCT (Sigma-Aldrich) was fully dissolved in 1M HCl. Normal saline was added to reach the aimed concentration 40 mg/ml and the pH was adjusted to 7.4 using 10M NaOH. The MCT solution was injected subcutaneously to Sprague-Dawley rats weighing 220-250g. Control group was injected with normal saline. The body weight and health condition were followed daily according to the specific protocol.

### 3.1.3. Haemodynamic measurements

At the end of treatment course, animals were weighed, anaesthetized (Hypnorm 1 ml/kg *i.m*; Hypnovel 0.8 ml/kg *i.p*) and placed on a controlled warming pad to keep body temperature. A midline surgical incision was executed. Haemodynamic parameters, including PAP, RVSP and others, were measured via a pre-curved, catheter inserted through the right jugular vein, passed by the RV and into the pulmonary artery (PA). Systemic blood pressure (BP) was measured by cannulating the left carotid artery. These catheters were filled with heparinized saline (10 U/ml) during the whole measurement and

connected to the pressure transducer of a pressure transducer (PowerLab Data Acquisition system, ADInstruments Ltd, Chalgrove, UK) and pressure measurements were recorded from stable tracings. The whole surgery was managed to last for no more than 30 minutes.

### 3.1.4. Tissue collection

The animals were then sacrificed, plasma collected from inferior vena cava, heart harvested, individual chambers dissected carefully and weighed. The ratio of right ventricle to left ventricle plus the septum mass (LV+sep) was assessed as RV hypertrophy (RVH) index. Lungs were flushed gently with diluted heparinized saline (1 U/ml). The left lung and a piece of RV were fixed by inflation with 10% formalin in phosphate-buffered saline and then embedded in paraffin blocks for histology assessment. Additionaly, one of the right lung lobe was inflated and embedded in OCT mounting medium (VWR Intl Ltd) prior to freezing for later on laser capture microdissection. Collected tissues, including lungs, RV, LV, livers and kidneys, were snap frozen in liquid nitrogen and stored at -80°C for biochemical measurements.

### 3.1.5. Histological analysis

Vascular remodelling in lungs was assessed by counting every single vessel less than 100 µm under microscope (400X). Transverse lung sections were stained with van Gieson's elastic method. Vessels with double elastic laminae occupying more than 50% of the circumference were regarded as fully muscularized while less than 50% as partially muscularized (Figrue 2.2). The proportion of muscularized vessels was quantified in the whole lung section from each animal and expressed as a percentage of total vessels counted (% muscularized vessels). Counting was performed twice and blindly to the group of the animals.

Haematoxyline & eosin (HE) stained cardiac sections were used to assess cross sectional area (CSA) and minimal diameter of RV cardiomyocyte. The average CSA and minimal Feret's diameter were quantified with ImageJ 1.46b (National Institutes of Health, USA). At least 40 myocytes cut transversely at the level of nucleus from 10 different areas chosen randomly were measured in each animal.

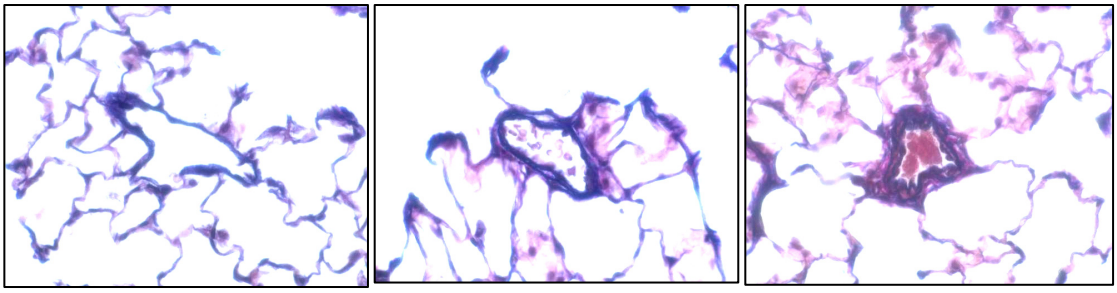



**Figure 2.2**. Transverse lung sections stained with van Gieson's elastic method showing muscularized vessles with double elastic lamina. Non-muscularized group is defined by single layer vessel wall. Paritally muscularized group is defined by double elastice lamina accounting for < 50% of the vessel wall while fully muscularized group > 50%.

### 3.1.6. Protein extraction

Frozen tissues were smashed into powder in a mortar, which was pre-cooled at -80°C and maintained at low temperature with liquid nitrogen. 200mg of tissue powder were added into 2ml self-lock micro test tubes (Eppendorf, Hamburg, Germany), which contained one 5mm stainless steel bead (Qiagen, Crawley, UK) in each and 1ml phosphate buffer (100mM $DO_4$ [$K_2HPO_4$:$KH_2PO_4$ = 3:1], 1mM EDTA, 1mM DTT) supplemented with protease inhibitor (1 tab in 5ml, Roche). Tissues were further homogenated by Tissue Lyser II (Qiagen) at speed 30Hz for 2 minute. After high-speed centrifuge for 5 minutes, supernatants were taken, aliquoted and the concentrations of protein homogenates were measured immediately with BCA protein assay (Thermo Scientific, Epsom, UK). Protein homogenates were kept at -80°C till next use and precipitated residues were kept at -80°C for histone extraction.

### 3.1.7. RNA isolation

100 mg of frozen tissue powder were transferred into 1ml of TriPure isolation reagent (Roche) at 4°C. After fully homogenated and centrifuged, supernatants were taken and left at room temperature for 5 minutes, and then separated into aqueous and organic phase by addition of 200µl of chloroform. DNA and proteins were removed from the aqueous phase containing RNA by subsequent centrifugation. Pure RNA was precipitated by addition of 500µl isopropanol, washed with 70% ethanol in diethylpyrocarbonate-treated water

(DEPC- $H_2O$, Invitrogen) and finally dissolved in appropriate volume of DEPC-$H_2O$. The concentration of RNA was determined by NanoDrop ND1000 (Thermo Scientific) and the purity was detected with agarose gel electrophoresis. The isolated RNA was kept at -80°C.

### 3.1.8. Histone extraction

Tissue residues from protein extraction were washed twice with ice-cold PBS containing 5mM sodium butyrate to retain levels of histone acetylation. Lysis buffer (10mM Tris, 50mM sodium bisulfate, 10mM $MgCl_2$, Sacarose 8.6%, 1% Triton X-100) was added to the tissues on ice for 15 minutes. After thorough homogenation, the supernatants were removed and the pellets were washed with lysis buffer and then Tris-EDTA (10mM Tris, 13mM EDTA). Precipitated nuclei were suspended in 50µl of ice-cold $dH_2O$. 50µl of 0.4M sulfuric acid was added to the samples, vortexed, and kept on ice overnight. Following centrifuge at 15000 rpm for another one hour, histone was precipitated as white pellets in 1ml acetone at -20°C overnight. Dry pellets were then resuspended in $dH_2O$ for measurement of histone concentration with Bradford assay (Bio-Rad, Hemel Hempstead, UK).

## 3.2. In vitro pharmacological study

### 3.2.1. Reagents

- **VPA and SAHA** stocks were prepared as previously described and diluted to target concentration in medium before treatment started. The treatment doses were determined on the basis of previous cancer studies[235] and tests of serial concentration, VPA 1-2mM and SAHA 2-10µM, for different time points, from 24 to 72 hours.

- **PDGF** (50 ng/ml) was used to stimulate proliferation of HPSMC (human PDGF-BB, eBioscience, Hatfield, UK) [64].

- **TNF-α** (10 ng/ml) was used to stimulate cytokine release from HPAEC (R&D Systems, Abingdon, UK) [236].

### 3.2.2. Treatment experiment in HPSMC

Harvested HPSMCs were seeded in sterile 6-well, 24-well tissue culture plates or T75 flasks at a density of 15000 cells per $cm^2$ and allowed to attach overnight. Cells were then deprived from serum for 24 hours before stimulation with PDGF (50 ng/ml) or exposure to hypoxia ($O_2$=2%) with/without HDAC inhibitors for 72 hours. At the end of exposure, cells were harvested for proliferation/cell death assessment, biochemical studies or fixed for immunostaining. All the experiments were executed in at least triplicate with separate cell preparation.

### 3.2.3. Proliferation assessment

Treated cells were harvested from 24-well plates after 72-hour treatment and counted with hemocytometer. Proliferation was further assessed with WST-1 cell proliferation assay (water soluble tetrazolium, Roche) and BrdU cell proliferation assay (bromodeoxyuridine, Millipore, Watford, UK). For WST-1 assay, cells were split at a density of $4x10^3$ cells per well in 100µl culture medium containing PDGF (50 ng/ml) with/without HDAC inhibitors. Cells were split into a 96-well plate and incubated for 72 hours. 4 hours before the end of treatment, 10µl of Cell Proliferation Reagent WST-1 was added into each well. The platet were read in a spectrophotometer microplate reader to measure absorbance at 450nm as reference wavelength was 600nm.

For BrdU assay, cells were plated at a density of $2x10^4$ cells per well in 100µl culture medium in a 96-well plate. 100µl of test reagents (PDGF and HDAC inhibitors), which had been diluted to twice the desired final concentration, were added into each well and cells were incubated for 72 hours. BrdU reagent was diluted according to the manufacture instruction 24 hours before the end of treatment. Cells were then fixed with supplied fixing solution and incubated with anti-BrdU monoclonal antibody (100µl per well) for one hour at room temperature. Washed thoroughly with washing buffer, they were incubated in peroxidise-conjugated goat anti-mouse IgG (1:2000) for 30 minutes at room temperature, followed by 30-minute incubation with TMB peroxidise substrate (tetramethylbenzidine, supplied) in the dark. The results were read immediately in a spectrophotometer microplate reader to measure absorbance at 450nm after stop solution was added to terminate the reaction.

### 3.2.4. Cell death analysis

Treated cells were harvested from 6-well plates after 72-hour treatment, washed in PBS and stained with 50 µg/ml of propidium iodide (PI, Sigma-Aldrich) in PBS for 30 minutes in the dark. Samples were analyzed immediately with Beckton-Dickinson Fluorescent-activated cell sorting (FACS) Calibur flow cytometer system with Cell Quest software (Beckton-Dickison, Oxford, UK) and Cyflogic version 1.2.1 (CyFlo Ltd, Turku, Finland), 488nm wavelength light used for excitation. The results were presented as the percentage of stained cells, representing necrotic or late apoptotic cells with penetrable plasma membrane to PI.

### 3.2.5. Cell cycle distribution

Treated cells were harvested from 6-well plates after 72-hour treatment and washed in PBS. 70% ethanol was added slowly to permeabilize cell membrane on vortexing and kept at least overnight at -20°C. Cells were stained with 50 µg/ml of PI in PBS for 30 minutes in the dark and analyzed immediately with FACS. The results were presented as the percentage of cells in G1, S and G2/M phase.

### 3.2.6. Protein expression analysis

Treated cells were harvested from T75 flasks after 72-hour treatment and centrifuged at 2500rmp for 8 minutes at 4°C. Cell pellets were washed with PBS, snap frozen in liquid nitrogen for few seconds, and resuspended in 100µl of TGN buffer (0.05M Tris, 0.15M NaCl, 10% (v/v) glycerol, 0.05M β-glycerophosphate, 1% (v/v) Tween 20, 0.2% (v/v) Nonidet P40) with protease inhibitor (1 tab in 5ml, Roche). Cells were left on ice for 30 minutes and vortexed every 10 minutes. After high speed centrifugation, supernatants were collected to measure protein concentrations. Protein expression levels were analyzed with Western Blotting as described in 3.3.1.

### 3.2.7. Inflammatory stimulation in HPAEC

Equal amount of HPAECs were seeded in each well of a 6-well plate. At 80% confluence, cells were treated with HDAC inhibitors, 2mM VPA or 10μM SAHA, for 24 hours. 8 hours after treatment started, TNF-α was added in at a final concentration of 10 ng/ml. At the end of treatment, supernatants were collected for cytokine array described in 3.3.4.

## 3.3. Biochemical analysis

### 3.3.1. Western blot analysis

Equal amounts of protein homogenates (50μg) were resolved by SDS-PAGE on a 4-12% or 12% bis-tris gel (Invitrogen), depending on the size of the target protein, and transferred to a nitrocellulose membrane (Amersham, GE Healthcare, UK). The membranes were blocked in 5 % non-fat milk in TBS-tween (TBS, 0.1% Tween 20) with agitation for one hour and then incubated with purified mouse polyclonal antibodies against human HDAC1-4 (1:1000, Cell Signalling Technology) and Bcl-2 (1:1000, BD Biosciences, Oxford, UK), purified rabbit polyclonal antibodies against human HDAC5, 7, PARP (poly ADP ribose polymerase), p21 (1:100, Abcam, Cambridge, UK) and survivin (1:1000, Novus Biologicals) and purified goat polyclonal antibodies against human TIMP-1 (1:2000, R&D Systems) in 5% bovine serum albumin (BSA, Sigma-Aldrich) in TBS-tween overnight at 4°C on a shaking platform. TBS was diluted from 10X TBS (24.2g Tris base, 80g NaCl in 1 liter $H_2O$, pH was adjusted to 7.6 with HCl). After washed in TBS-tween three times, 5 minutes each, they were incubated in horseradish peroxidise (HRP)-conjugated secondary antibodies against mouse (1:2500, GE Healthcare), rabbit (1:10000, Sigma-Aldrich) and goat (1:1000, R&D Systems) in 5% non-fat milk in TBS-tween for one hour at room temperature on a shaking platform. The membranes were then washed three times again, 5 minutes each, and the proteins were detected by Novex® ECL chemiluminescent substrate reagent kit (Invitrogen). Optical densities of individual bands were measured and protein expression was standardized to α-actin (mouse monoclonal, 1:5000, Sigma-Aldrich) for RV and β-actin (mouse monoclonal, 1:10000, Sigma-Aldrich) for other tissues.

### 3.3.2. RT-PCR

Total RNA (500ng) and the primer oligo$(dT)_{16}$ (250ng), mixed with DEPC-$H_2O$, were pre-heated to 70°C and then cooled on ice for cDNA synthesis. 20µl of reagents, containing ImProm-II™ reaction buffer, 3mM $MgCl_2$, 20U of Recombinant RNAsin® Ribonuclease Inhibitor (Promega Corp, Madison, WI, USA), 0.5mM of each deoxynucleoside triphosphate, and 1µl of ImProm-II™ Reverse Transcriptase (Promega Corp), were incubated at 25ºC for 5 min (anneal step), followed by an extension step at 42°C for one hour and a final step at 70ºC for 15 min (heat-inactivate).

mRNA expression of HIF-1α, BMPR2 and β-actin was quantified by TaqMan® real-time RT-PCR with a GeneAmp 7500 Fast System (Applied Biosystems, Carlsbad,CA, USA). A 10-fold dilution of RT reaction for each sample was analyzed using TaqMan® Gene Expression Assays (Applied Biosystems) with commercially available specific primer-probes: HIF-1α (Rn00577560_m1), BMPR2 (Rn01437208_m1) and β-actin (Rn01437208_m1) (Applied Biosystems). Real-time PCR reactions were set following the manufacture instructions: one initial step at 95ºC for 10 minutes for holding, followed by a 40-cycle step, which was 15 seconds at 95°C for denaturation and one minute at 60°C for annealing or extension. Threshold cycle (Ct) values obtained for each gene were converted to the linear form using the term $2^{-\Delta\Delta Ct}$ as a value directly proportional to the copy number of mRNA [237], where ΔΔCt was the Ct value normalized to β-actin and referenced to the values obtained for each gene under normoxic conditions.

### 3.3.3. Histone acetylation assessment

Histone acetylation was assessed with Western blotting. Equal amounts of histone extracts (10µg) were resolved by SDS-PAGE on a 12% bis-tris gel (Invitrogen) and transferred to a nitrocellulose membrane (Amersham). The membranes were blocked in 5 % non-fat milk in TBS-tween with agitation for one hour and then incubated with purified rabbit polyclonal antibodies against human acetyl-histone H3 (1:20000), acetyl-histone H3 lysine9 (acH3K9, 1:20000), acetyl-histone H4 (1:2000) and acetyl-histone H4 lysine12 (acH4K12, 1:2000, Millipore) in 5% BSA in TBS-tween overnight at 4°C on a shaking platform. After washed in TBS-tween three times, 5 minutes each, they were incubated in

HRP-conjugated secondary antibodies against rabbit (1:10000, Sigma-Aldrich) in 5% non-fat milk in TBS-tween for one hour at room temperature on a shaking platform. The membranes were then washed three times again, 5 minutes each, and the proteins were detected by chemiluminescent reagent (Invitrogen). Optical densities of individual bands were measured and acetyl-histone expression was standardized to total histone H3 (rabbit polyclonal, 1:10000, Millipore) or total histone H4 (rabbit polyclonal, 1:2000, Millipore), which were executed on parallel gels as loading control. The data were presented as ratio of acetyl-histone over total histone.

### 3.3.4. Cytokine array

Human and rabbit cytokine array kits were purchased from R&D Systems. For animal study, tissue powder from individual animals in the same group were poured together and homogenated in PBS with protease inhibitors (1 tab in 5ml, Roche), followed by addition of Triton X-100 to a final concentration of 1%. After centrifuged at 10000g for 5 minutes to remove cellular debris, BCA protein assay was performed to determine the concentration. Samples were stored at -80°C until being used. Equal amounts of tissue homogenates (250µg) were added to 0.5ml supplied blocking buffer and adjusted to a final volume of 1.5ml with supplied array buffer. For cell study, 1.6ml of culture supernatants were added directly to 0.8ml of blocking buffer and adjusted to a final volume of 3ml. with array buffer. Then samples were mixed with 15µl detection antibody cocktail and incubated at room temperature for one hour. At the same time, nitrocellulose membranes pre-coated with anti-cytokine antibodies (cytokine array panel A) were incubated in blocking buffer for one hour on a rocking platform. After incubation, sample/antibody mixtures were added to membranes and incubated overnight at 4°C on a rocking platform. After washed with supplied washing buffer three times, 10 minutes each, the membranes were incubated in Streptavidin-HRP-conjugated secondary antibodies (1:2000, supplied in the kit) in array buffer for 30 minutes at room temperature on a rocking platform. The membranes were then washed three times again, 10 minutes each, and exposed to chemiluminescent reagent (Invitrogen). Optical densities of individual dots were measured and cytokine expression was standardized to pre-coated positive control on each membrane. Corresponding signals on different arrays were compared to determine the relative change in cytokine levels between samples.

### 3.3.5. Immunohistochemistry

Dewaxes and rehydrated transverse lung sections were incubated in 3% hydrogen peroxide for 5 minutes and subjected to heat-induced antigen retrieval in 0.2% citric acid (pH was adjusted to 6 with 1M NaOH) at 80°C for 10 minutes. Sections were incubated in diluted normal goat serum (1 to 5 in PBS, Cell Signalling Technology) for 20 minutes and then immunostained with purified mouse antibody against HDAC1 (1:50, Cell Signalling Technology) and rabbit antibody against Ki67 (1:50, Thermo Scientific), HDAC5 (1:50, Cell Signalling Technology) in PBS for one hour at room temperature, followed by incubation in SignalStain® Boost IHC Detection Reagent (HRP) against mouse or rabbit (one drop, Cell Signalling Technology) for 30 minutes. Subsequently, sections were incubated in DAB (3,3'-diaminobenzidine) chromogenic substrate for 10 minutes (one DAB tablet in 10ml d $H_2O$ with 10µl $H_2O_2$, Sigma-Aldrich) before counterstain in filtered light green or HE for 3 seconds.

## 3.4. Statistics

All the results are presented as mean ± SEM and 'n' refers to the number of patients or animals per group. Statistical analysis was performed with GraphPad Prism version 4 (GraphPad Software, San Diego, CA, USA). One-way ANOVA was applied for data of multiple group comparisons whereas unpaired, nonparametric *t* test with Mann-Whitney modification was used between two groups. Statistical significance was regarded as p value<0.05.

# Chapter 3:

# HDAC EXPRESSION IN PH

# 1. Introduction

To investigate the role of HDACs in the pathogenesis of PAH and their clinical relevance, the first crucial question is whether histone acetylation status is altered in the diseased lung. Aberrant expression and recruitment of HDACs have been reported in many human cancers [154, 163, 238, 239]. Elevated expression levels of zinc-dependent HDACs are extensively found in different human cancer cell lines or tumour samples, relative to normal tissue. Some studies also indicate that the expression levels of HDACs positively correlate to prognosis of cancer patients [240]. Increased HDAC expression/activity results in imbalance between histone acetylation and deacetylaton, inducing abnormal transcription of key genes in charge of proliferation and apoptosis. PAH is now recognized as a proliferative disorder sharing cancer-like properties, but our knowledge of epigenetics in PAH is very limited. In this chapter, I examine HDAC protein expression in IPAH patient lungs and PH animal models.

The chronic hypoxic PH rat as well as the transgenic BMPR2 mouse were used. Alveolar hypoxia is a potent stimulus leading to pulmonary vasoconstriction and subsequently vessel remodelling [241]. Continuous exposure to hypoxia creates PH acutely in the first few days and reaches a plateau after 1 to 2 weeks. This effect can be reversed by exposing animals back to normal air. As previously described in chapter 1, mutations in *BMPR2* is the only identified genetic defect linked to human PAH. However, mutation in BMPR2 alone does not guarantee the development of PAH, but predisposes. Transgenic mice with BMPR2 deficiency have been increased susceptibility to hypoxia-induced pulmonary hypertension. The exact molecular mechanism is not clear yet, but endothelial dysfunction affecting the pulmonary vasculature was suggested [242].

Expression of the anti-apoptotic protein, Bcl-2, was also assessed along with HDACs. Governing mitochondrial outer membrane permeability, Bcl-2 controls the initiation of programmed cell death. Overexpression of Bcl-2 is responsible for resistance to apoptosis in proliferative disorders and its expression levels are associated with severity in a range of malignancies [243]. Therefore, Bcl-2 can be used as a proliferative indicator.

# 2. Objectives

- To examine protein expression levels of class I and II HDACs in lungs from IPAH patients.
- To examine protein expression levels of class I and II HDACs in tissues from rats exposed to chronic hypoxia for different time points.
- To examine protein expression levels of HDACs in lungs from transgenic mice deficient in BMPR2, under normoxic and hypoxic conditions.

# 3. Methods and Protocols

## 3.1. Chronically hypoxic rats

Male Spraque-Dawley rats (220-250 gram) were divided into four groups (n≥6):

1) NC: normoxia control

2) 2D: hypoxia for two days

3) 1W: hypoxia for one week

4) 2W: hypoxia for two weeks

## 3.2. Transgenic mice with BMPR2 deficiency

Mice with and without established BMPR2 deficiency were divided into four groups:

1) Wild type mice under normoxia condition

2) BMPR2-deficient mice under normoxia condition

3) Wild type mice expose to hypoxia for two weeks

4) BMPR2-deficient mice expose to hypoxia for two weeks

At the end of exposure, haemodynamic parameters were measured and tissues were collected for further biochemical and histological investigation immediately as described in *Methods.* PAP was measured only in rats but not in mice due to the accessibility to pulmonary artery.

# 4. Results

## 4.1. HDAC expression in humans and rats

### 4.1.1. HDAC1 and HDAC5 expression in human lungs

Increased protein expression levels of HDAC1 ($p<0.0005$) and HDAC5 ($p<0.005$) were observed in lungs from patients with IPAH, accompanied by the increase in anti-proapoptotic factor, Bcl-2 ($p<0.0005$), compared with control lung tissues (Figure 3.1).

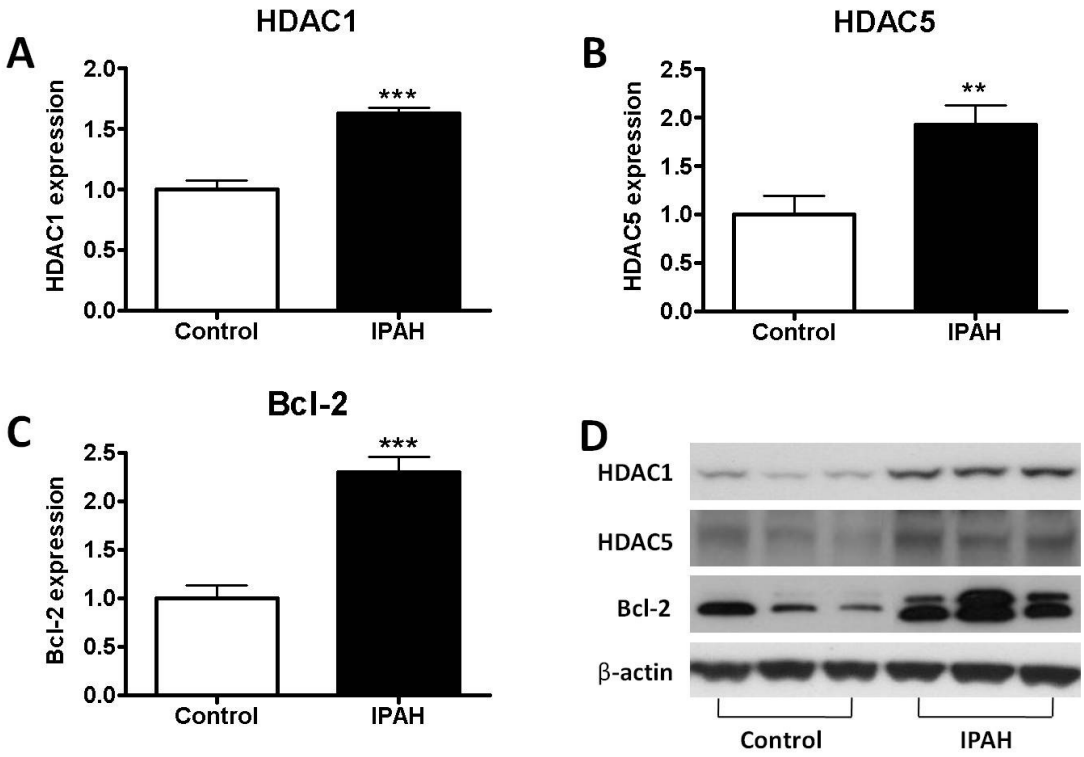



**Figure 3.1**. Protein expression levels in human lung extracts. (A) HDAC1, (B) HDAC5, (C) Bcl-2, (D) representative bands. Control: lobectomy; IPAH: IPAH patients. The data are presented as mean± SEM of fold change relative to control. n=21 in control and 12 in IPAH. ** $p<0.005$, *** $p<0.0001$ compared with control group.

### 4.1.2. HDAC1 and HDAC5 expression in hypoxic rat lungs

Exposing rats to chronic hypoxia induced PH gradually over two weeks, characterized by elevated mean PAP ($p<0.05$) and significant RV hypertrophy ($p<0.005$) along the two-week time course (Figure 3.2). Two-fold increases in both mean PAP and RV hypertrophy index were seen after two-week hypoxic exposure.

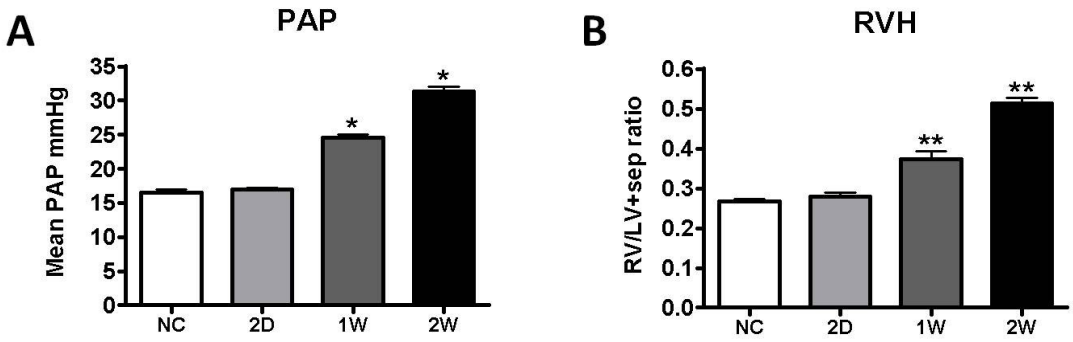



**Figure 3.2**. Haemodynamic phenotype of rats exposed to hypoxia for different time points. (A) PAP, mean pulmonary artery pressure, (B) RV/LV+sep, right ventricular hypertrophy. NC: normoxia control; 2D: hypoxia for two days; 1W: hypoxia for one week; 2W: hypoxia for two weeks. The data are presented as mean± SEM. $n\geq3$ in each group. * $p<0.05$, ** $p<0.01$ compared with NC.

HDAC1 protein expression in lungs from hypoxic rats was increased during hypoxia exposure ($p<0.05$) and reached 4-fold over normoxia control after two-week hypoxia whereas HDAC5 expression was strikingly increased by over 15-fold after one-week hypoxia and maintained relatively stable thereafter ($p<0.005$). Bcl-2 expression was increased up to 3-fold over control after two-day hypoxia and remained elevated during the two-week period ($p<0.05$, Figure 3.3).

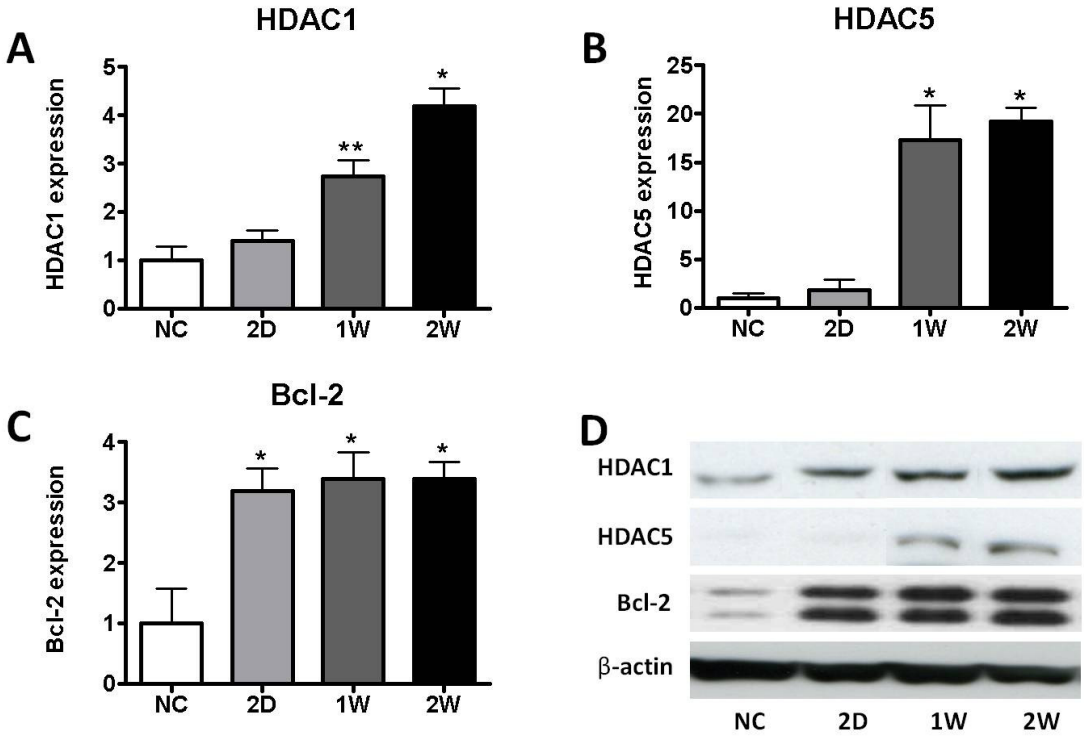



**Figure 3.3**. Protein expression levels in lung extracts from rats exposed to hypoxia for different time points. (A) HDAC1, (B) HDAC5, (C) Bcl-2, (D) representative bands. NC: normoxia control; 2D: hypoxia for two days; 1W: hypoxia for one week; 2W: hypoxia for two weeks. The data are presented as mean± SEM of fold change relative to NC. $n\geq3$ in each group. * $p<0.05$, ** $p<0.005$ compared with NC group.

### 4.1.3. HDAC2 and HDAC3 expression in human and rat lungs

In contrast to increases in HDAC1, protein expression of other two class I HDACs, HDAC2 and HDAC3, in lungs from IPAH patients and hypoxic rats were showed a gradual decline (Figure 3.4).

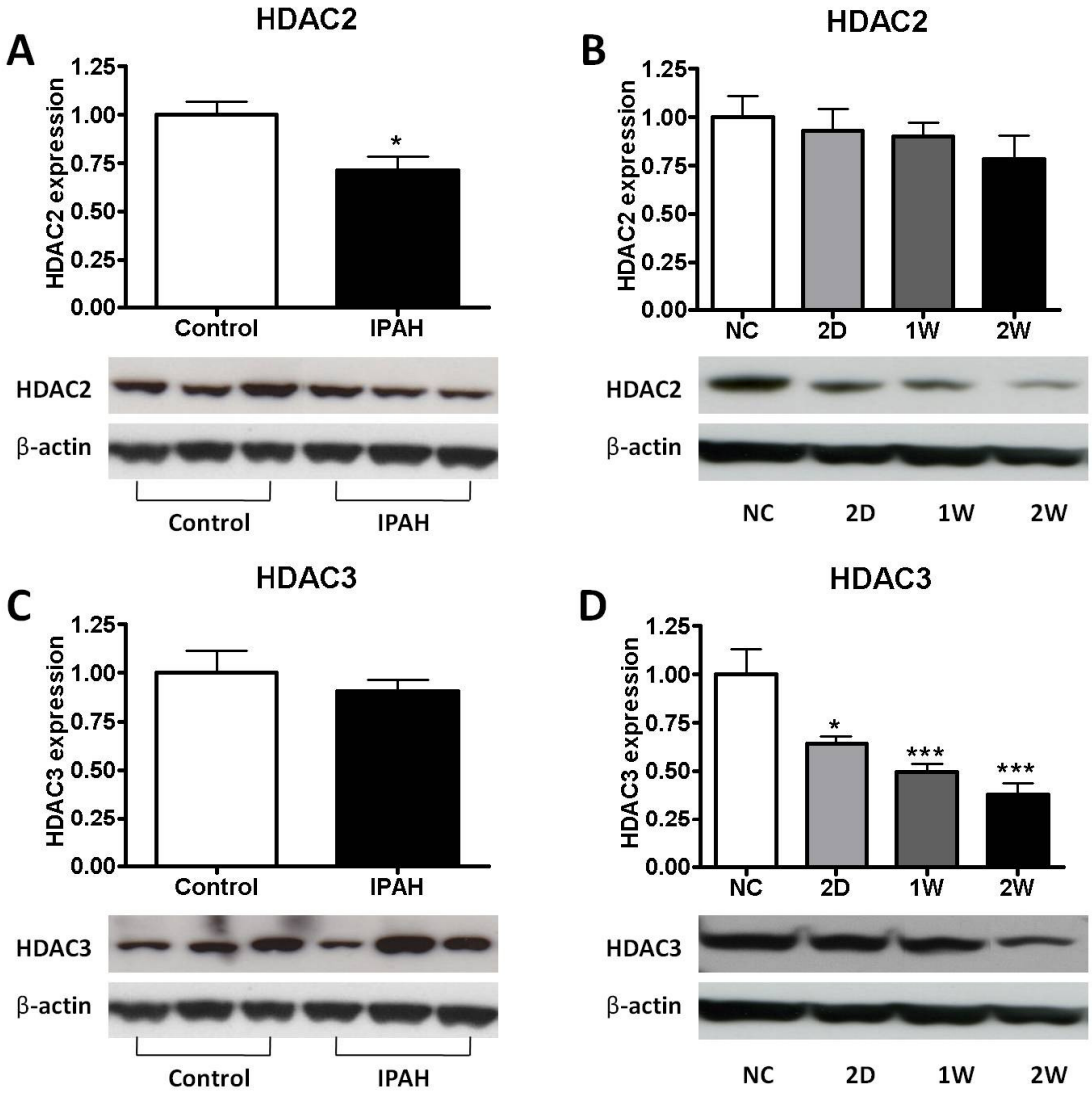



**Figure 3.4**. Protein expression levels in lung extracts from humans and rats exposed to hypoxia for different time points. (A) HDAC2 in humans, (B) HDAC2 in rats, (C) HDAC3 in humans, (D) HDAC3 in rats. Control: lobectomy; IPAH: IPAH patients; NC: normoxia control; 2D: hypoxia for two days; 1W: hypoxia for one week; 2W: hypoxia for two weeks. The data are presented as mean± SEM of fold change relative to control or NC. $n \geq 9$ in each group. * $p<0.05$, *** $p<0.0005$ compared with control or NC group.

### 4.1.4. HDAC4 and HDAC7 expression in human and rat lungs

Protein expression of other two class II HDACs, HDAC4 and HDAC7, in lungs from IPAH patients and hypoxic rats was measured. HDAC4 expression was significantly increased in patient lungs ($p<0.05$), but slightly increased in rats exposed to two-day hypoxia and decreased afterwards. HDAC7 expression in lungs increased in both IPAH patients and hypoxic rats (Figure 3.5).

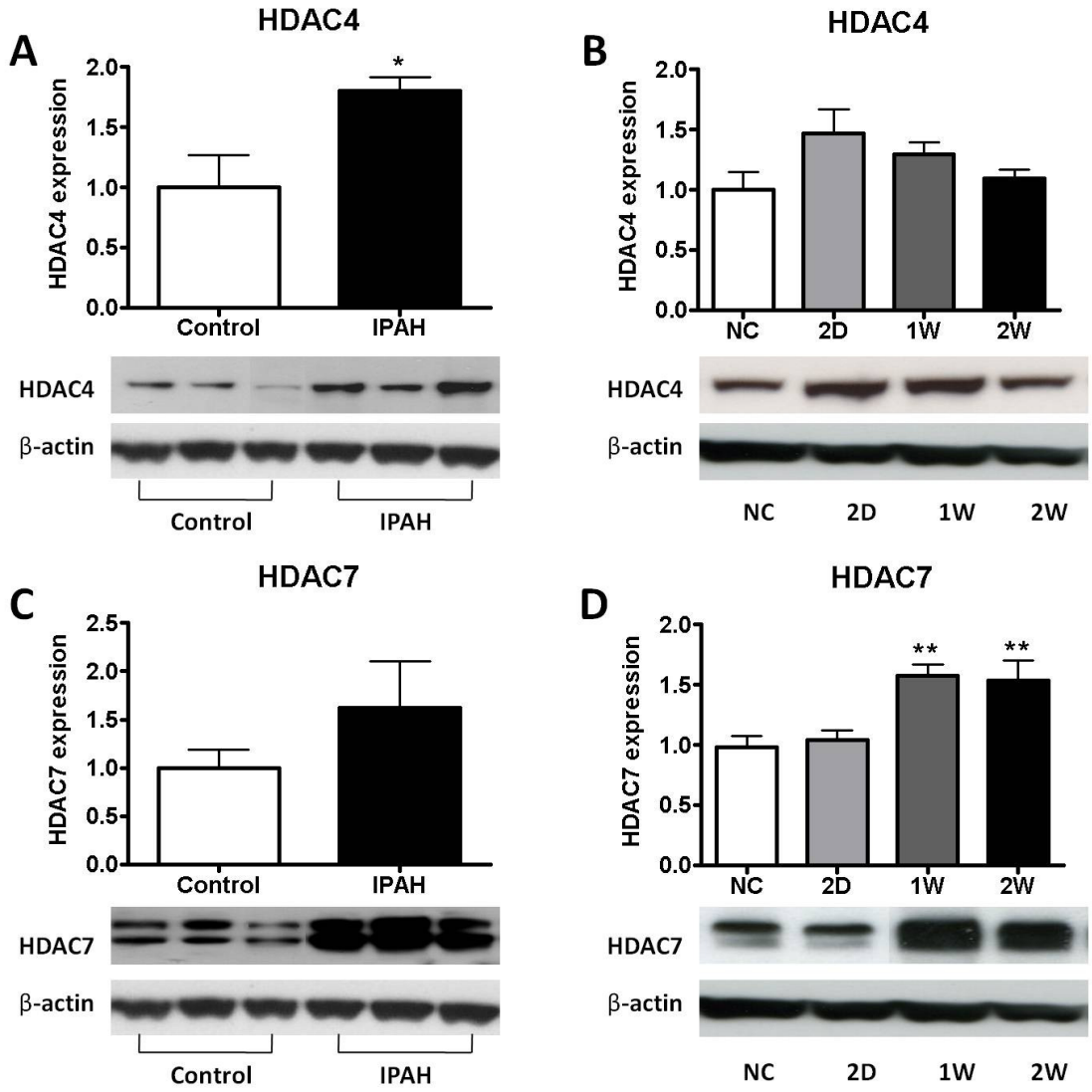



**Figure 3.5**. Protein expression levels in lung extracts from humans and rats exposed to hypoxia for different time points. (A) HDAC4 in humans, (B) HDAC4 in rats, (C) HDAC7 in humans, (D) HDAC7 in rats. Control: lobectomy; IPAH: IPAH patients; NC: normoxia control; 2D: hypoxia for two days; 1W: hypoxia for one week; 2W: hypoxia for two weeks. The data are presented as mean± SEM of fold change relative to control or NC. $n\geq7$ in each group. * $p<0.05$, ** $p<0.005$ compared with control or NC group.

### 4.1.5. HDAC expression in rat RV and kidney

HDAC1 and HDAC5 protein expression in RV from hypoxic rats was increased during hypoxia exposure as they were in lung tissues. Interestingly, no significant changes in HDAC1 expression were observed in kidney (Figure 3.6).

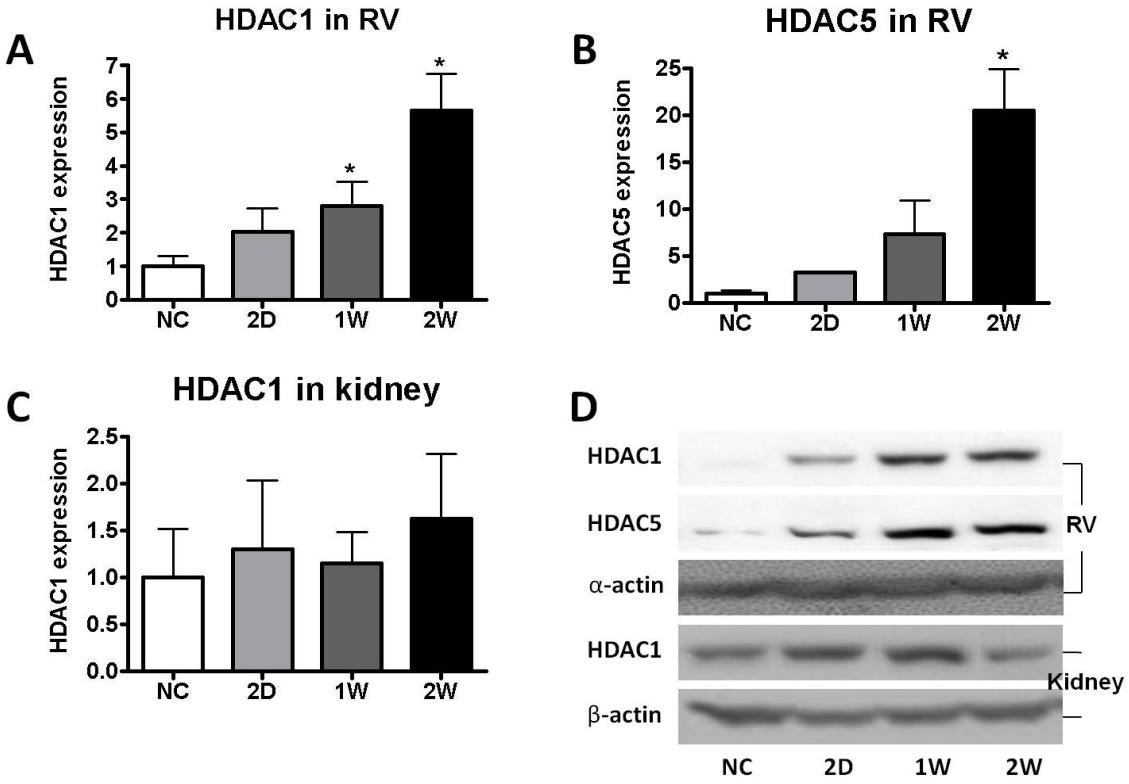



**Figure 3.6**. Protein expression levels in tissue extracts from rats exposed to hypoxia for different time points. (A) HDAC1 in RV, (B) HDAC5 in RV, (C) HDAC1 in kidney, (D) representative bands. NC: normoxia control; 2D: hypoxia for two days; 1W: hypoxia for one week; 2W: hypoxia for two weeks. The data are presented as mean± SEM of fold change relative to NC. n≥3 in each group. * $p<0.05$ compared with NC group.

## 4.2. Immunohistochemistry

HDAC1 immunoreactivity was predominantly localized to the nuclei of vascular cells and weakly to epithelium, while HDAC5 displayed a relatively specific distribution in cytoplasm of vascular cells in IPAH lung tissues (Figure 3.7B). Both of HDAC1 and HDAC5 exhibited weak expression in control lobectomy lung tissues (Figure. 3.7A)

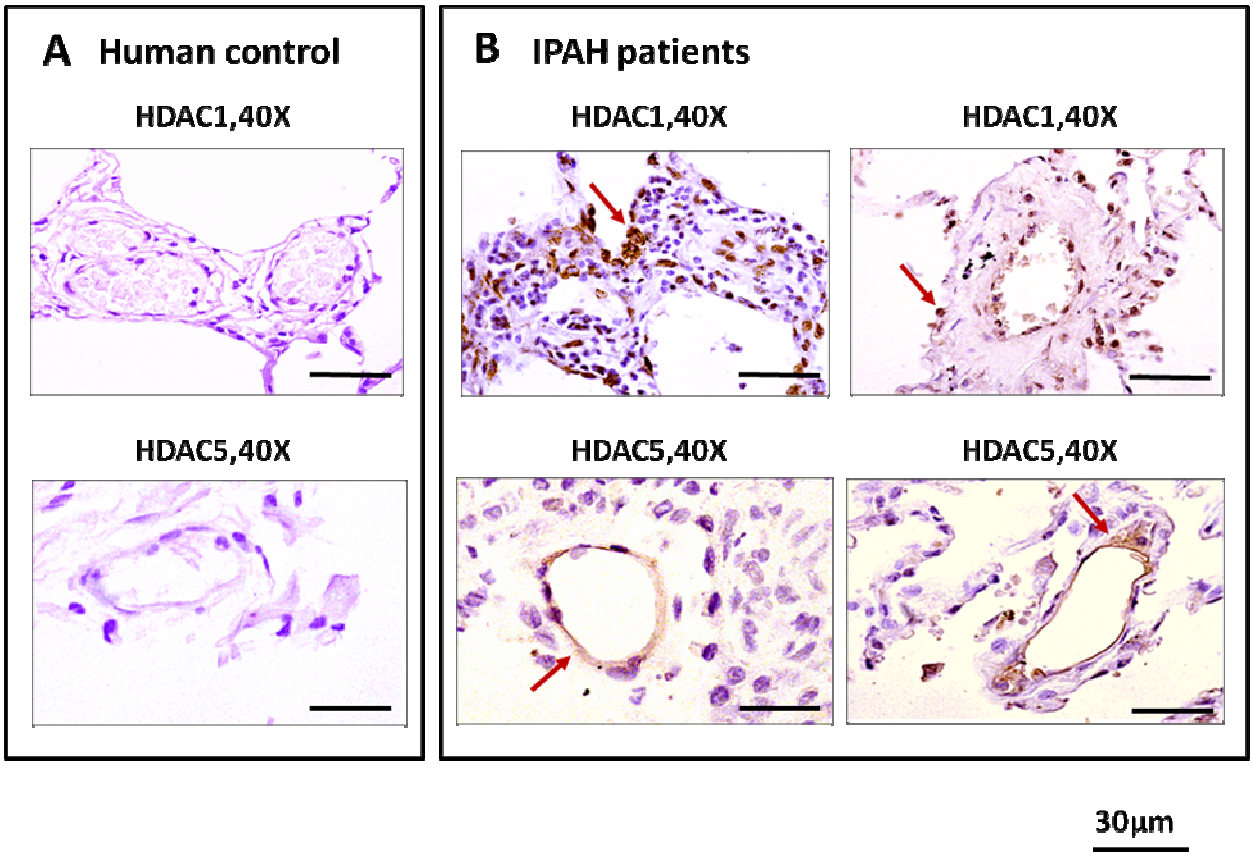



**Figure 3.7**. Distribution of HDAC1 and HDAC5 immunostaining in human lungs. (A) Sections from lobectomy, (B) Sections from patients with IPAH. Both HDAC1 and HDAC5 are stained in brown with HE counterstaining.

In lungs from chronically hypoxic rats, HDAC1 showed a similar distribution of expression to that seen in IPAH patients, prominent in the nuclei in the vascular cells of muscularized vessels. This co-localized to Ki67 staining. HDAC5 was principally expressed in the cytoplasm of vascular cells lining the vascular wall. However, nuclear staining of HDAC5 was observed as well. Immunoreactivity of both of HDAC1 and HDAC5 were weak in the epithelium and alveolar cells (Figure. 3.8).

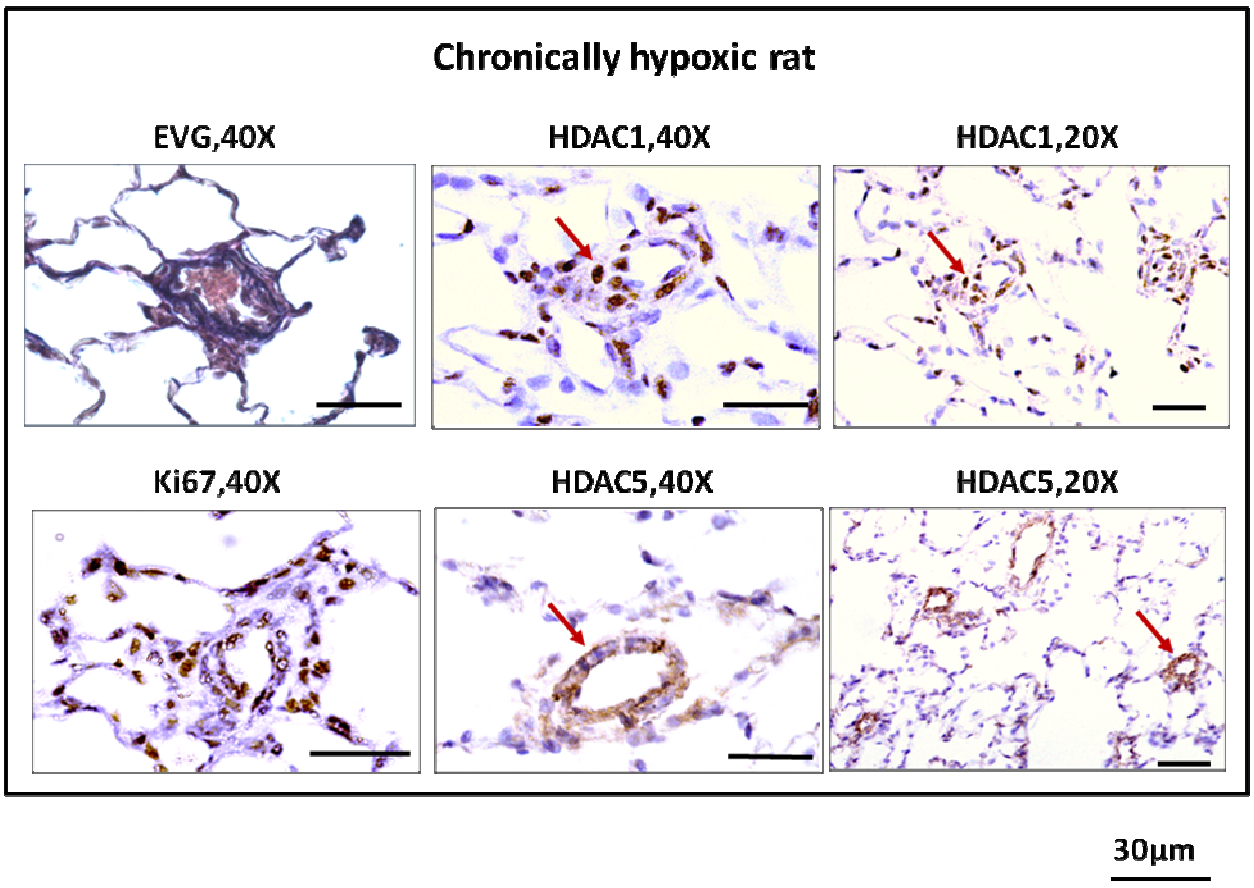



**Figure 3.8**. Distribution of HDAC1 and HDAC5 immunostaining, co-localized to van Gieson's elastic (EVG) and Ki67 stain, in lungs from rats exposed to chronic hypoxia for 4 weeks. HDAC1, HDAC5 and Ki67are stained in brown with HE counterstaining.

## 4.3. HDAC1 expression in BMPR2 deficient mice

Elevation of mean PAP and RV hypertrophy were noted in transgenic BMPR2 deficient mice in normal air. Exposure to chronic hypoxia induced further increase in mean PAP and RV hypertrophy in BMPR2 deficient mice (Figure 3.9).

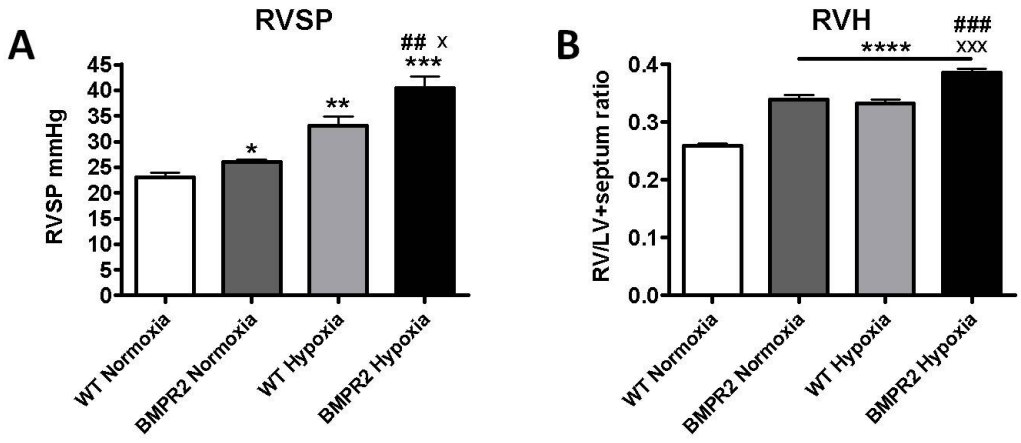



**Figure 3.9**. Haemodynamic phenotype of transgenic mice exposed to hypoxia for two weeks. (A) PAP, RVSP, right ventricular systolic pressure, (B) RV/LV+sep, right ventricular hypertrophy. WT: wild type; BMPR2: BMPR2 deficient. The data are presented as mean± SEM. n≥7 in each group. * p<0.05, ** p<0.005, *** p<0.0005, **** p<0.0001 compared with WT normoxia, ## p<0.005, ### p<0.0005 compared with BMPR2 normoxia, x p<0.05, xxx p<0.0005 compaed with BMPR2 hypoxia.

No significant differences in HDAC1 or Bcl-2 protein expression were found between lungs from wild type and BMPR2 deficient mice under normoxic condition (Figure 3.10). However, both were significantly increased in BMPR2 deficient mice after 2 week hypoxia exposure (HDAC: p< 0.001, Bcl-2: p<0.05, Figure 3.11).

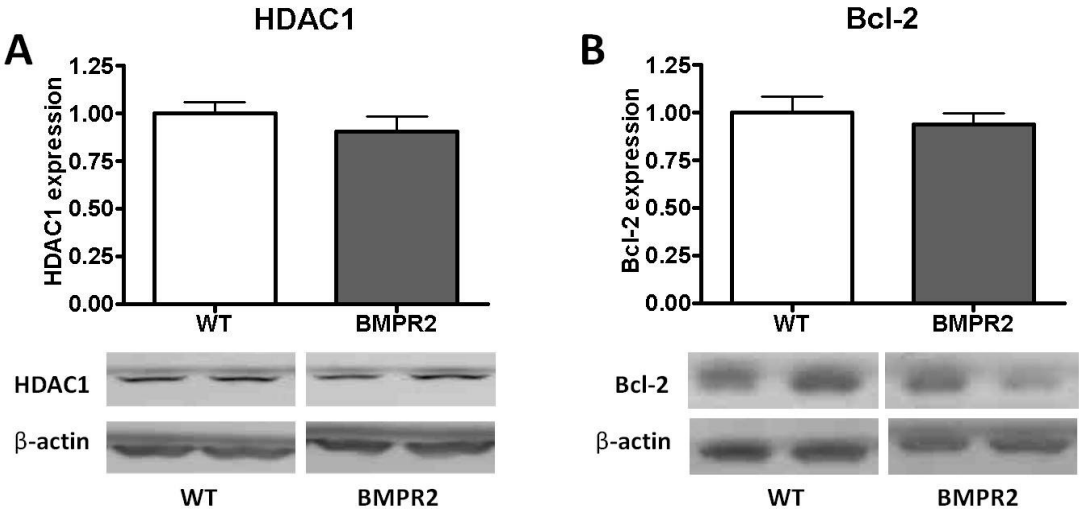



**Figure 3.10**. Protein expression levels in lung extracts from transgenic mice exposed to normal air. (A) HDAC1, (B) Bcl-2. WT: wild type; BMPR2: BMPR2 deficient. The data are presented as mean± SEM of fold change relative to WT. n=7 in each group.

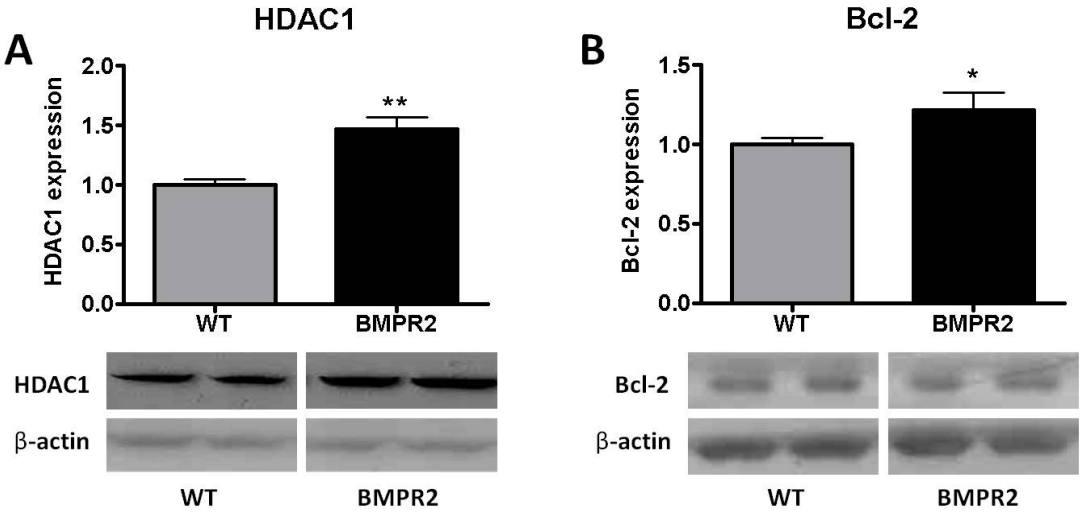



**Figure 3.11**. Protein expression levels in lung extracts from transgenic mice exposed to hypoxia for two weeks. (A) HDAC1, (B) Bcl-2. WT: wild type; BMPR2: BMPR2 deficient. The data are presented as mean± SEM of fold change relative to WT. n=7 in each group. * p<0.05, ** p<0.005 compared with WT group.

# 5. Discuission

Analysis of HDAC expression provided the following findings:

- Expression levels of HDAC1, HDAC5 and Bcl-2 were significantly elevated in lungs from patients with IPAH.
- Expression levels of HDAC1, HDAC5 and Bcl-2 were significantly elevated in lungs from chronically hypoxic rats.
- Expression levels of HDAC1 and HDAC5 were significantly elevated in RV from chronically hypoxic rats.
- HDAC1 and HDAC5 expression was prominent in remodelled pulmonary vessles.
- Expression levels of HDAC1 and Bcl-2 were significantly elevated in lungs of BMPR2-deficient mice exposed to hypoxia.

This study demonstrated for the first time alternations in the protein expression of class I and class II HDACs in lungs from IPAH patients. The pattern of HDAC expression was replicated in lungs from rats with hypoxia-induced pulmonary hypertension. The hypoxia model is characterised by the rapid development of structural changes in pulmonary arterioles, involving muscularization, proliferation of vascular cells and inflammatory cell infiltration [244].

Rats exposed to continuous hypoxia developed elevated mean PAP and RV hypertrophy in the first two weeks. Similar altered expression of all the examined HDACs, including class I, HDAC1, 2, 3, and class II, 4, 5, 7, in this model was shown in the rat model and human IPAH,. Among them, HDAC1 and HDAC5 were shown prominently increased in IPAH patients and in both lungs and RV from hypoxic rats, along with hypoxia-induced physiological changes. HDAC expression in the kidneys was unchanged in these animals, indicating that the observed HDAC changes in lungs and RV are related to the pathological events in pulmonary circulation induced by hypoxic stimulus, indicating tissue-specific involvement of HDACs.

Immunohistochemical assessment revealed strong nuclear overexpression of HDAC1 and increased cytoplasmic expression of HDAC5 lining at the remodelled vascular lesions, co-localizing to nuclear expression of the proliferative marker, Ki67 [245].

HDACs are normally expressed in all eukaryotic cells and regulate many genes engaged in cell proliferation, differentiation and survival. Aberrant expression and recruitment of HDACs to transcriptional regions repressing certain tumour suppressor genes have been implicated in cancerous transformation. A variety of specific alternations in individual HDAC expression has been described in different types of human malignancies, especially class I HDACs. For example, HDAC1 is overexpressed in gastric, pancreatic, colorectal, prostate and hepatocellular cancers, and correlates with poor prognosis [158, 160, 246, 247]. The increase in HDAC1 and HDAC5 in human IPAH and lungs from pulmonary hypertensive rats, together with the increased expression in survival marker Bcl-2, are consistent with the proliferative, apoptosis-resistant vascular pathology that characterizes pulmonary hypertension.

On the other hand, decreased expression of the other class I HDACs, HDAC2 and HDAC3, was observed in the diseased lungs. It has been known that individual HDACs may partially compensate for one another [248]. HDAC2, which is genetically homologous to HDAC1 with 86% identity at amino acid level, has considerable functional overlapping and redundancy with HDAC1 in the regulation of proliferation, apoptosis and cancerous transformation [249]. Many reports have shown up-regulation of one of them occurs with inactivation of the other [250-252]. However, more recent studies have indicated that HDAC1 has distinct and noncompensable functions in the regulation of cell proliferation and tumourigenesis [248]. HDAC3, crucial in lipid metabolism and endothelial integrity, may have more interactions with other HDACs by shuttling between the nucleus and cytoplasm [253]. In my study, the reduction of HDAC2 and HDAC3 may be explained a compensatory reaction to HDAC1 overexpression. In addition to HDAC5, expression of other class II HDACs, especially HDAC7, was also increased in the diseased lungs. HDAC7 has recognized involvement in endothelial function, smooth muscle cell differentiation, angiogenesis and vascular remodelling [213-215]. Increased expression of HDAC7 has been reported in cancer as well [254].

The consistent changes in expression in patients and hypoxic rats suggest a role for HDACs in the pathogenesis of PH. The predominant localization of HDAC1 and HDAC5 to distal muscularized vessels reveals that they appear to participate in the remodelling processes and supports these HDACs as drug targets.

While HDAC and Bcl-2 expression was no different between wild type mice and transgenic BMPR2-deficient mice under normoxic conditions, both were significantly increased in transgenic mice under hypoxic stimuli. Although a genetic linkage between BMPR2 mutations and human PAH has been identified, the typically low penetrance indicates that additional factors are required to initiate the disease. As a member of the TGB-β superfamily, inflammatory hits have been implicated in BMPR2-predisposing PH. This result suggests a possible role for HDAC1 in BMPR2 signalling during hypoxia-induced PH.

A complex range of downstream signalling of BMPR2 mutations have been proposed, among which variations within the smad family maybe potentially responsible for the infrequent cause to the disease [255]. However, the knowledge of epigenetic links to BMPR2 is less known. Previous studies revealed that smad-dependent recruitment of HDAC1 can modulate BMP transcriptional activity [256]. Further investigation is worthy to establish the epigenetic insight of BMPR2 in PH.

# Chapter 4:

# HDAC INHIBITORS PARTIALLY REVERSE HYPOXIA-INDUCED PH

# 1. Introduction

Animal models provide invaluable information regarding the pharmacological effects of drugs and mechanistic insights into disease development.. Several animal models have been developed to generate a PAH phenotype and resemble human pathology. Unfortunately, none of them perfectly recapitulates human PAH. The chronic hypoxic rat model is the most commonly used due to its predictability and reproducibility within a wide range of animal species. Following the findings of alternations in HDAC expression in the hypoxic model, pharmacological experiments were performed to understand its possible relationship to the PH phenotype. Two protocols typically conducted in pharmacological research were used, namely prevention and recovery. The former refers to treatment given before disease development while the latter means treatment given to groups with established PH.

In this chapter, the effect of two HDAC inhibitors, VPA and SAHA, were evaluated. The rationale of the investigation is that both VPA and SAHA have been approved for human use and are well tolerated clinically. Thus, both can be translated to treat human PAH. In the first part, the preventive effects of VPA were examined since increased HDAC 1 expression was seen in both rat and human lungs, and in the next part, the effects of both were assessed under the recovery protocol.

# 2. Objective

- To evaluate the pharmacological effects of VPA on the development of PH in the chronically hypoxic rat model.
- To evaluate the pharmacological effects of VPA and SAHA on established chronic hypoxia-induced PH in rats.

# 3. Methods and Protocols

## 3.1. Hypoxia prevention study:

Male Spraque-Dawley rats (220-250 gram) were divided into four groups (n=6 each group):

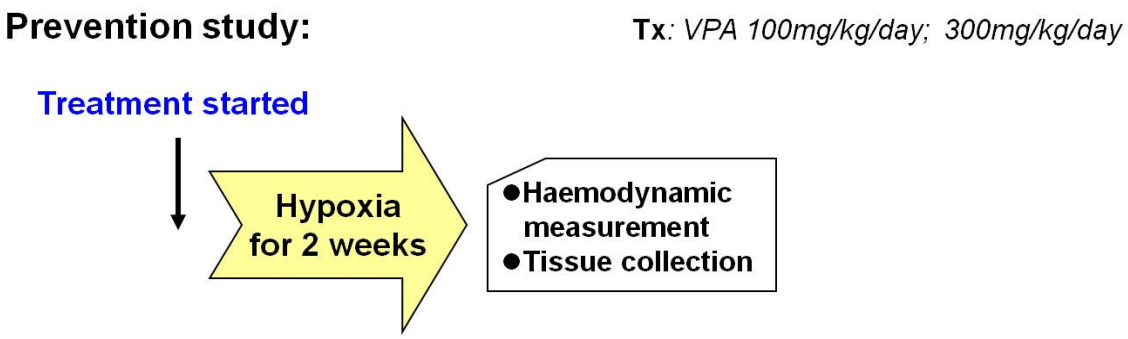
1) NC: normoxia control

2) HC: hypoxia control

3) low VPA: hypoxia with low dose VPA 100mg/kg/day *p.o.* (in drinking water)

4) high VPA: hypoxia with high dose VPA 300mg/kg/day *p.o.* [257]

Animals were weighed every other day. Treatment dose was adjusted by weighing drinking bottles daily and calculating intake. Animals were pre-treated for four days and then exposed to hypoxia in the chamber as previously described for 2 weeks (as the flowchart below). The treatment was continued during the exposure.



## 3.2. Hypoxia recovery study:

Male Spraque-Dawley rats (220-250 gram) were divided into five groups (n=9-12 each group):

1) NC: normoxia control

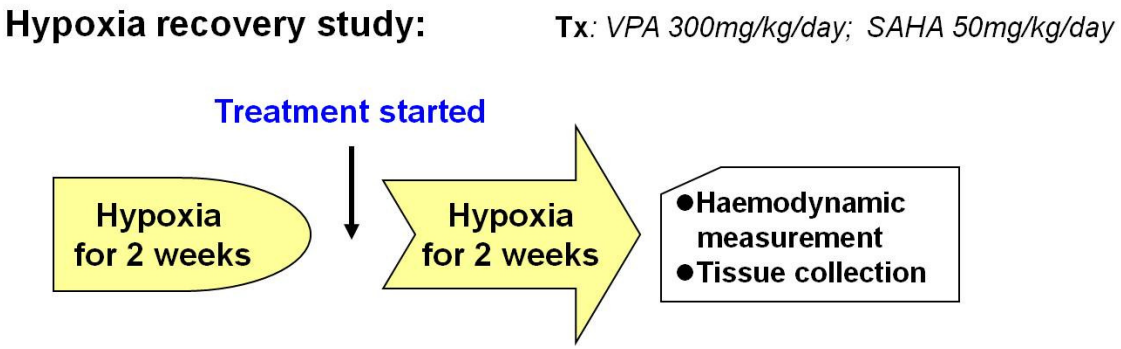
2) 2WH: two-week hypoxia control (starting point of treatment)

3) 4WH: four-week hypoxia control

4) VPA: hypoxia with VPA 300mg/kg/day *p.o*

5) SAHA: hypoxia with SAHA 50mg/kg/day *p.o.* (in drinking water with HOP-β-CD) [258]

Animals were weighed every other day. Treatment dose was adjusted by weighing drinking bottles daily and calculating intake. Animals were exposed to hypoxia for four weeks, and treatment was begun 2 weeks before the end of experiment (as the flowchart below).




At the end of exposure, haemodynamic parameters were measured and tissues were collected for further biochemical and histological investigation immediately as described in *Methods.*

# 4. Results

## 4.1. VPA prevents hypoxia-induced PH: haemodynamics

Two-week exposure to hypoxia resulted in a two-fold increase in mean PAP (HC: 33.6±0.7 mmHg v.s. NC: 16.7±0.5 mmHg, $p<0.01$, Figure 4.1A), RVSP (HC: 54.2±2.6 mmHg v.s. NC: 26.1±0.8 mmHg, $p<0.005$, Figure 4.1B) and RVH (HC: 0.58±0.02 v.s. NC: 0.26±0.01, $p<0.005$, Figure 4.1C) compared to the control group.

VPA treatment with two doses, 100mg/kg/day or 300mg/kg/day, significantly attenuated the hypoxia-induced rise in mean PAP (low VPA: 26.7±1.0 mmHg, high VPA: 24.1±1.2 mmHg v.s. HC: 33.6±0.7 mmHg, $p<0.01$, Figure 4.1A), but the mean PAP in VPA treatment group remained higher than the control group. VPA treatment, 100mg/kg/day and 300mg/kg/day, also attenuated the increased RVSP (low VPA: 44.1±3.6 mmHg, high VPA: 40.2±2.5 mmHg v.s. HC: 54.2±2.6 mmHg, Figure 4.1B). However, only the high dose VPA group showed significant reduction ($p<0.005$).

Hypoxia-induced RV hypertrophy was significantly attenuated by low dose VPA (-73%) and high dose (-89%) treatment (low VPA: 0.34±0.01, high VPA: 0.30±0.02, $p<0.005$, Figure 4.1C). A trend towards a dose-dependent effect was noted ($p=0.0645$).

No significant changes were found in heart rate and systemic mean BP between groups (Figure 4.1D). The physiological data from the hypoxia prevention study is presented in Table 4.1.

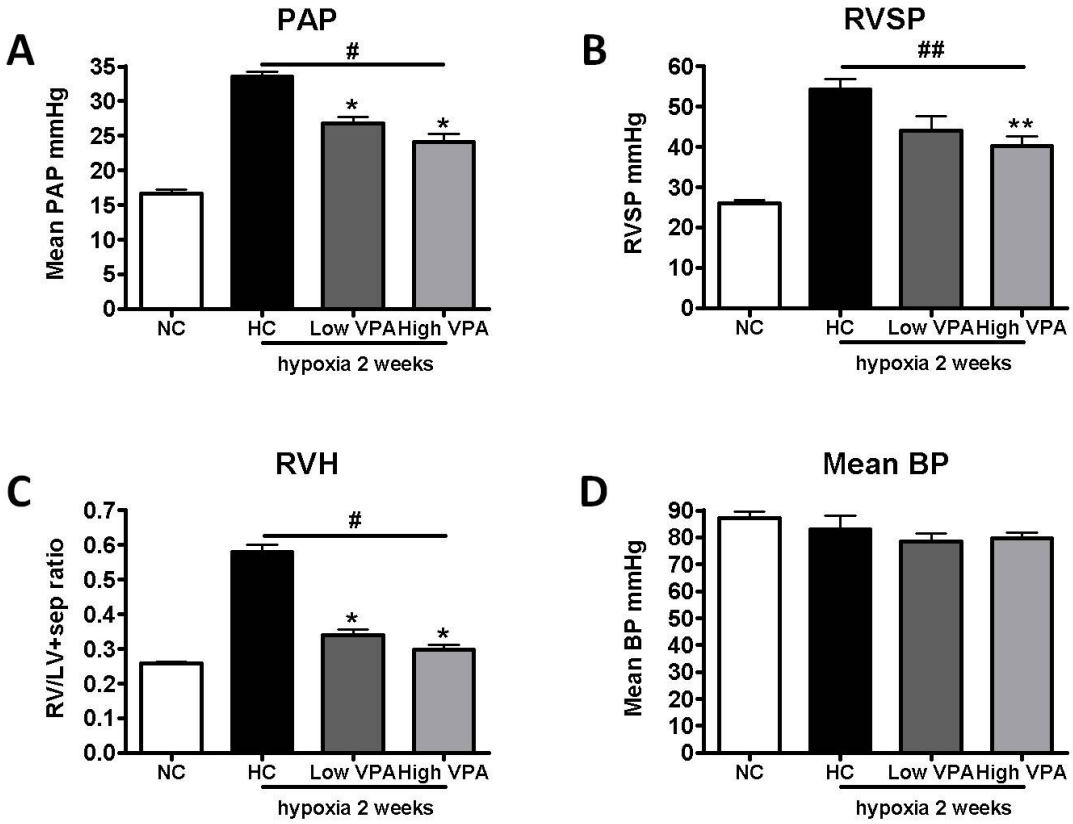



**Figure 4.1**. Prevention study: VPA was administered during 2 weeks hypoxia. (A) PAP, mean pulmonary artery pressure, (B) RVSP, right ventricular systolic pressure, (C) RV/LV+sep, right ventricular hypertrophy, (D) Mean BP, systemic mean blood pressure. NC: normoxia; HC: hypoxia for 2 weeks; low VPA: hypoxia with VPA 100 mg/kg/day; and high VPA: hypoxia with VPA 300 mg/kg/day. The data are presented as mean± SEM. n=6 in each group. * $p<0.01$, ** $p<0.05$ compared with HC, # $p<0.05$, ## $p<0.005$ compared with NC.

| Group | | NC | HC | Low VPA | High VPA |
|---|---|---|---|---|---|
| **N number** | | 6 | 6 | 6 | 6 |
| **RV** | Systolic (mmHg) | 26.1±0.8 | 54.2±2.6 | 44.1±3.6 | 40.2±2.5 |
| | Max dp/dt (mmHg/s) | 688±41 | 1429±55 | 966±138 | 879±99 |
| **Pulmonary** | Mean (mmHg) | 16.7±0.5 | 33.6±0.7 | 26.7±1.0 | 24.1±1.2 |
| | Pulse (mmHg) | 11.4±0.7 | 24.0±7.1 | 22.6±1.5 | 16.2±2.5 |
| **Systemic** | Pressure (mmHg) | 87.3±2.3 | 82.8±5.3 | 78.5±2.8 | 79.6±2.2 |
| | Heart rate | 308±21 | 383±15 | 340±17 | 299±26 |
| **General** | BW (gram) | 286±8 | 266±3 | 250±5 | 233±4 |
| | RV/LV+septum | 0.26±0.01 | 0.58±0.02 | 0.34±0.01 | 0.30±0.02 |
| | RV/BW | 0.61±0.03 | 1.09±0.03 | 0.74±0.02 | 0.67±0.04 |
| | Left lung/BW | 1.53±0.05 | 2.27±0.07 | 2.13±0.05 | 2.09±0.11 |

NC: normoxia control; HC: hypoxia control; Low VPA: VPA 100mg/kg/day; High VPA: VPA 300mg/kg/day; BW:body weight

**Table 4.1**. The physiological data of hypoxia prevention study.

## 4.2. VPA prevents hypoxia-induced PH: histology

Transverse lung sections stained with van Giessen's elastic method showed a substantial increase in the percentage of muscularized distal pulmonary vessels (HC: 59.2±3.6% v.s. NC: 5.7±1.2%, p<0.005, Figure 4.2). This was attenuated (-~20%) with VPA treatment (low VPA: 46.1±5.7%; high VPA: 47.9±3.8% v.s. HC: 59.2±3.6%, p<0.01, Figure 4,2). Lung mass was augmented with hypoxia exposure but no significant effect of VPA was seen in the study (Figure 4.2).

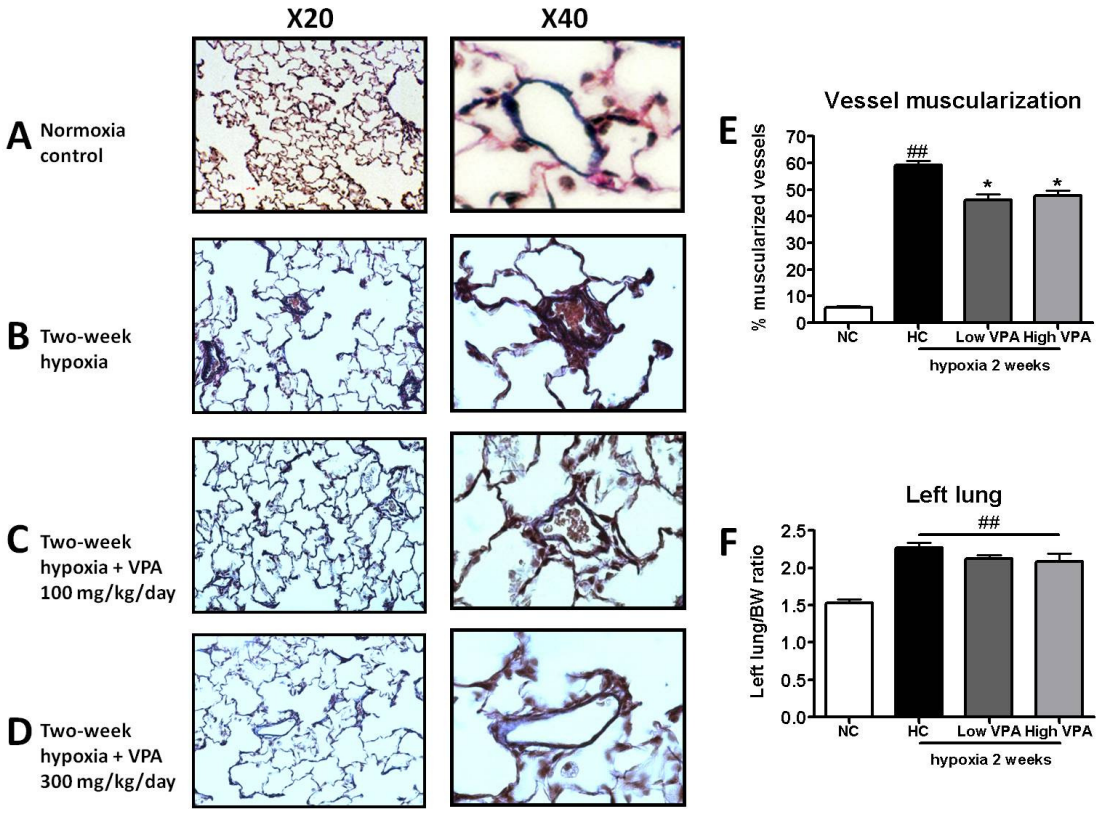



**Figure 4.2**. Effects of VPA on hypoxia-induced pulmonary vascular muscularization. (A,B,C,D) Histology figures with van Gieson's elastic stain, (E) Quantified by the percentage of vessels with double elastic lamina, (F) Left lung weight over body weigtht ratio. NC: normoxia; HC: hypoxia for 2 weeks; low VPA: hypoxia with VPA 100 mg/kg/day; and high VPA: hypoxia with VPA 300 mg/kg/day. The data are presented as mean± SEM. n=6 in each group. * p<0.01 compared with HC, ## p<0.005 compared with NC.

## 4.3. VPA and SAHA partially reverse hypoxia-induced PH: haemodynamics

To further examine the effects of HDAC inhibition, VPA and the pan-HDAC inhibitor, SAHA, were investigated after established PH in the rat. The increase in mean PAP, RVSP and RVH after two-week hypoxia were maintained for the following two weeks (PAP: 2WH: 30.0±1.3 mmHg, 4WH: 31.9±2.0 mmHg v.s. NC: 15.6±0.5 mmHg, $p<0.0001$; RVSP: 2WH: 47.0±3.2 mmHg, 4WH: 49.0±4.0 mmHg v.s. NC: 24.7±0.8 mmHg, $p<0.0001$; RVH: 2WH: 0.46±0.02, 4WH: 0.45±0.02 v.s. NC: 0.29±0.01, $p<0.0001$, Figure 4.3).

Treatment with either VPA or SAHA from the second to the fourth week significantly reduced mean PAP (VPA: 23.3±1.6 mmHg, SAHA: 23.2±1.8 mmHg v.s. 4WH: 31.9±2.0 mmHg, $p<0.005$, $p<0.01$, respectively, Figure 4.3A). Compared to baseline, these two inhibitors partially reversed the hypoxia-induced increased mean PAP (VPA: 23.3±1.6 mmHg; SAHA: 23.2±1.8 mmHg v.s. 2WH: 30.0±1.3 mmHg, $p<0.01$, Figure 4.3A). Similarly, the increased RVSP was significantly reduced by VPA and SAHA treatment, compared to both control group and baseline (VPA: 37.1±3.3 mmHg; SAHA: 32.1±2.4 mmHg v.s. 4WH: 49.0±4.0 mmHg, $p< 0.005$, $p<0.05$, respectively; v.s. 2WH: 47.0±3.2 mmHg, $p<0.05$, Figure 4.3B).

Consistent with the reduction in blood pressure, RVH was also attenuated by VPA (-56.5%) and SAHA (-59.7%) treatment (VPA: 0.36±0.01 and SAHA: 0.35±0.01 v.s. 4WHC: 0.45±0.02, $p<0.005$; Figure 4.3C). Compared to baseline, both HDAC inhibitors achieved partial reversal of RVH (VPA: -58.8% and SAHA -61.8%) (VPA: 0.36±0.01 and SAHA: 0.35±0.01 v.s. 2WH: 0.46±0.02, $p<0.005$; Figure 4.3C).

No significant changes in heart rate or systemic SBP were observed (Figure 4.3D). The physiological data from the hypoxia recovery study is shown in Table 4.2.

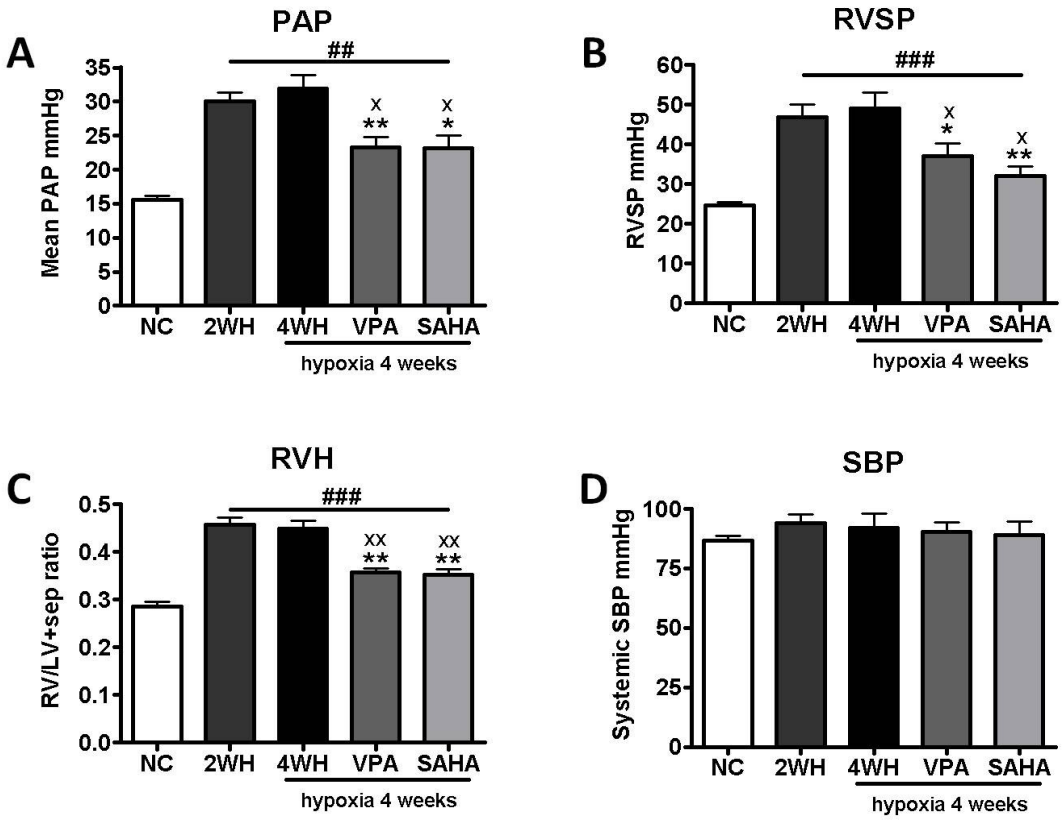



**Figure 4.3**. Recovery study: VPAand SAHA were administered during last 2 weeks of four-week hypoxia exposure. (A) PAP, mean pulmonary artery pressure, (B) RVSP, right ventricular systolic pressure, (C) RV/LV+sep, right ventricular hypertrophy, (D)SBP, systemic systolic blood pressure. NC: normoxia; 2WH: hypoxia for 2 weeks; 4WH: hypoxia for 4 weeks; VPA: hypoxia with VPA 300 mg/kg/day; SAHA: hypoxia with SAHA 50 mg/kg/day. The data are presented as mean± SEM. n≥9 in each group. * $p<0.05$, ** $p<0.005$ compared with 4WH, x <0.05, xx <0.005 compared with 2WH, ## $p<0.005$, ### $p<0.0005$ compared with NC.

| Group | | NC | 4WH | VPA | SVPA |
|---|---|---|---|---|---|
| N number | | 12 | 12 | 12 | 9 |
| RV | Systolic (mmHg) | 24.7±0.8 | 49.0±4.0 | 37.1±3.3 | 32.1±2.4 |
| | Max dp/dt (mmHg/s) | 783±90 | 1796±158 | 1433±160 | 1474±163 |
| Pulmonary | Mean (mmHg) | 15.6±0.5 | 31.9±2.0 | 23.3±1.6 | 23.2±1.8 |
| | Pulse (mmHg) | 9.7±1.4 | 18.8±1.8 | 17.6±2.9 | 15.3±3.5 |
| Systemic | Pressure (mmHg) | 86.8±1.9 | 92.0±6.0 | 90.3±4.1 | 89.0±5.7 |
| | Heart rate | 328±24 | 335±25 | 322±17 | 341±19 |
| General | BW (gram) | 379±13 | 391±11 | 391±8 | 292±15 |
| | RV/LV+septum | 0.29±0.01 | 0.45±0.02 | 0.36±0.01 | 0.35±0.01 |
| | RV/BW | 0.60±0.03 | 0.77±0.08 | 0.65±0.04 | 0.67±0.03 |
| | Left lung/BW | 1.13±0.03 | 1.34±0.02 | 1.43±0.04 | 1.58±0.06 |

NC: normoxia control; 4WH: hypoxia control; VPA: VPA 300mg/kg/day; SAHA: SAHA 50mg/kg/day; BW:body weight

**Table 4.2**. The physiological data of hypoxia recovery study.

## 4.4. VPA and SAHA partially reverse hypoxia-induced PH: histology

The percentage of muscularized peripheral distal pulmonary arteries was significantly increased after two-week hypoxia and maintained at a high level (2WH: 59.2±0.6%, 4WH: 58.4±2.5 v.s. NC: 5.7±0.5%, $p<0.001$, Figure 4.4). Treatment with VPA and SAHA showed a significant reduction in vascular muscularization compared to two-week and four-week hypoxia (VPA: 48.1±2.1%, SAHA: 47.0±2.6% v.s. 2WH: 59.2±0.6%, $p<0.005$, $p<0.05$, respectively; v.s. 4WH: 58.4±2.5, $p<0.05$, Figure 4.4).

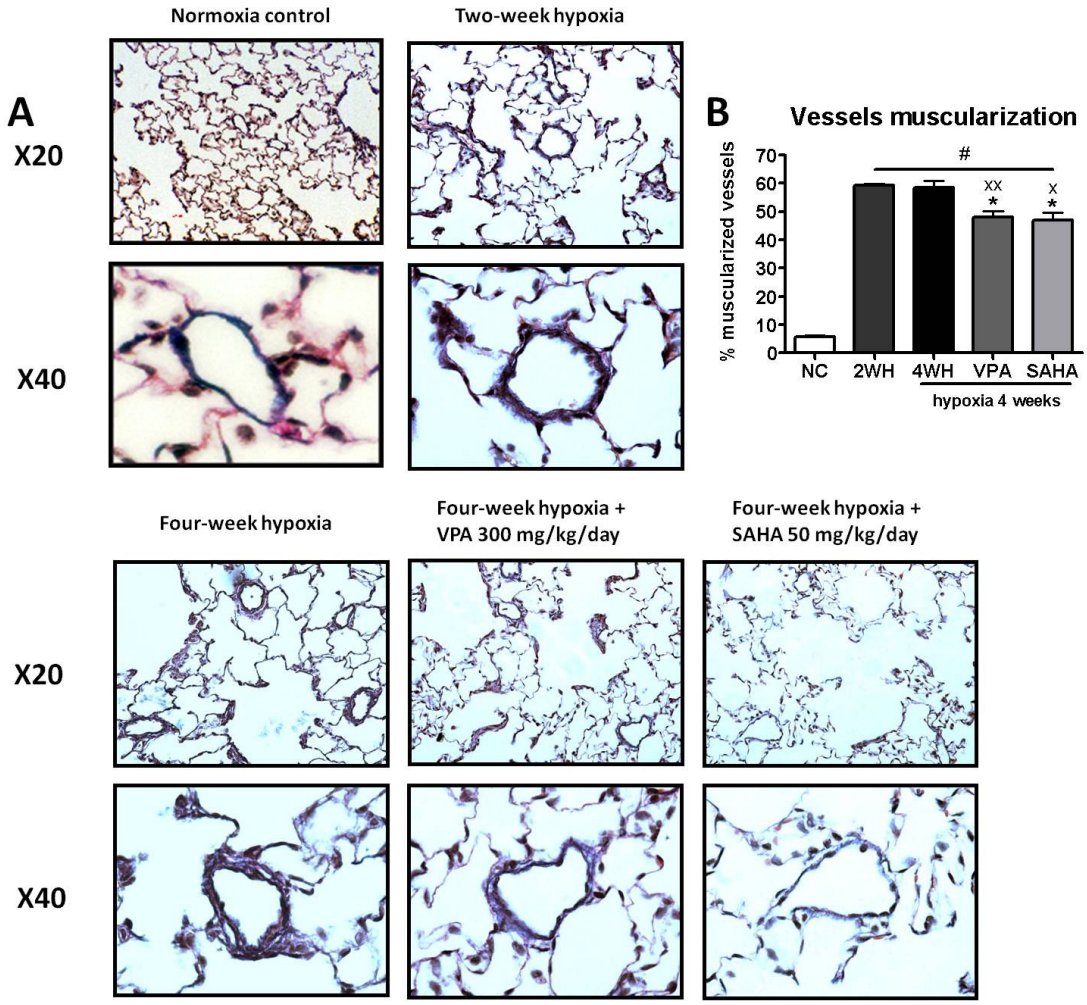



**Figure 4.4**. Effects of VPA and SAHA on hypoxia-induced pulmonary vascular muscularization. (A) Histology figures with van Gieson's elastic stain, (B) Quantified by the percentage of vessels with double elastic lamina. NC: normoxia; 2WH: hypoxia for 2 weeks; 4WH: hypoxia for 4 weeks; VPA: four-week hypoxia with VPA 300 mg/kg/day; and SAHA: four-week hypoxia with SAHA 50 mg/kg/day. The data are presented as mean± SEM. n≥6 in each group. * $p<0.01$ compared with 4WH, x $p<0.05$, xx $p<0.005$ compared with 2WH, # $p<0.05$ compared with NC.

## 4.5. VPA and SAHA partially reverse hypoxia-induced PH: biochemistry

### 4.5.1. Protein expression

Protein analysis of lung tissues from these animals revealed four-week exposure to hypoxia induced a 35% increase in Bcl-2 expression levels. Treatment with either VPA or SAHA reduced the Bcl-2 expression to near the normal range ($p<0.05$). In contrast, both treatments increased p21 expression levels compared to hypoxia control ($p<0.05$) (Figure 4.5).

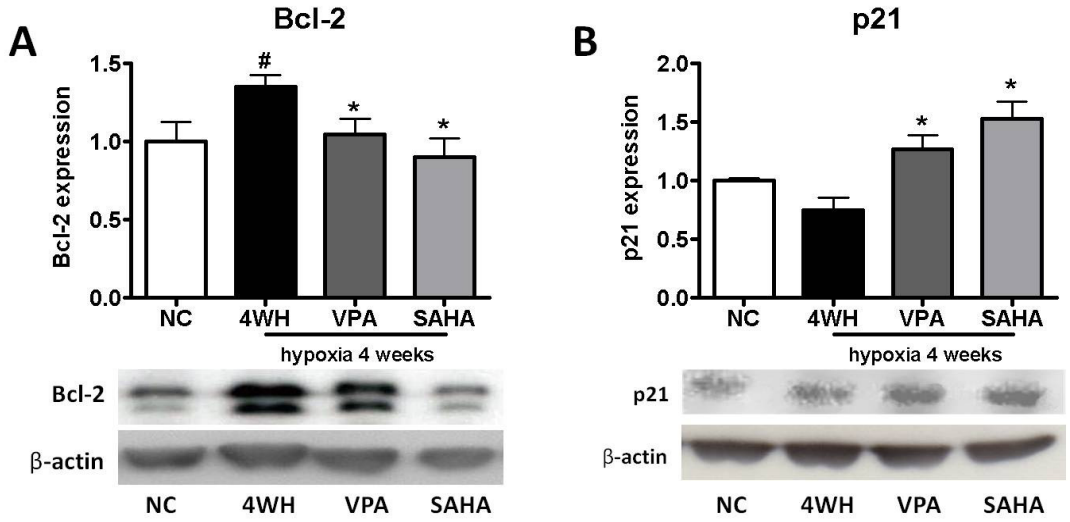



**Figure 4.5**. Protein expression levels in rat lung extracts from chronic hypoxia treatment study. (A) Bcl-2, (B) p21. NC: normoxia; 4WH: hypoxia for 4 weeks; VPA: hypoxia with VPA 300 mg/kg/day; SAHA: hypoxia with SAHA 50 mg/kg/day. Data are presented as mean ± SEM of fold change relative to NC. n=6. * $p<0.05$ compared with 4WH, # $p<0.05$ compared with NC.

### 4.5.2. Histone acetylation

Consistent with HDAC inhibition, H3 and H4 acetylation levels were both significantly increased by over 60% in SAHA-treated lungs ($p<0.05$). However, treatment with VPA resulted in slight but not statistically significant elevations in histone acetylation (Figure 4.6A-C).

Interestingly, total histone levels were also elevated in the rat lungs with hypoxia exposure, and this was reduced by VPA and SAHA treatment (Figure 4.6D). The total histone levels in lungs were observed to correlate with mean PAP ($r=0.35$, $p<0.05$).

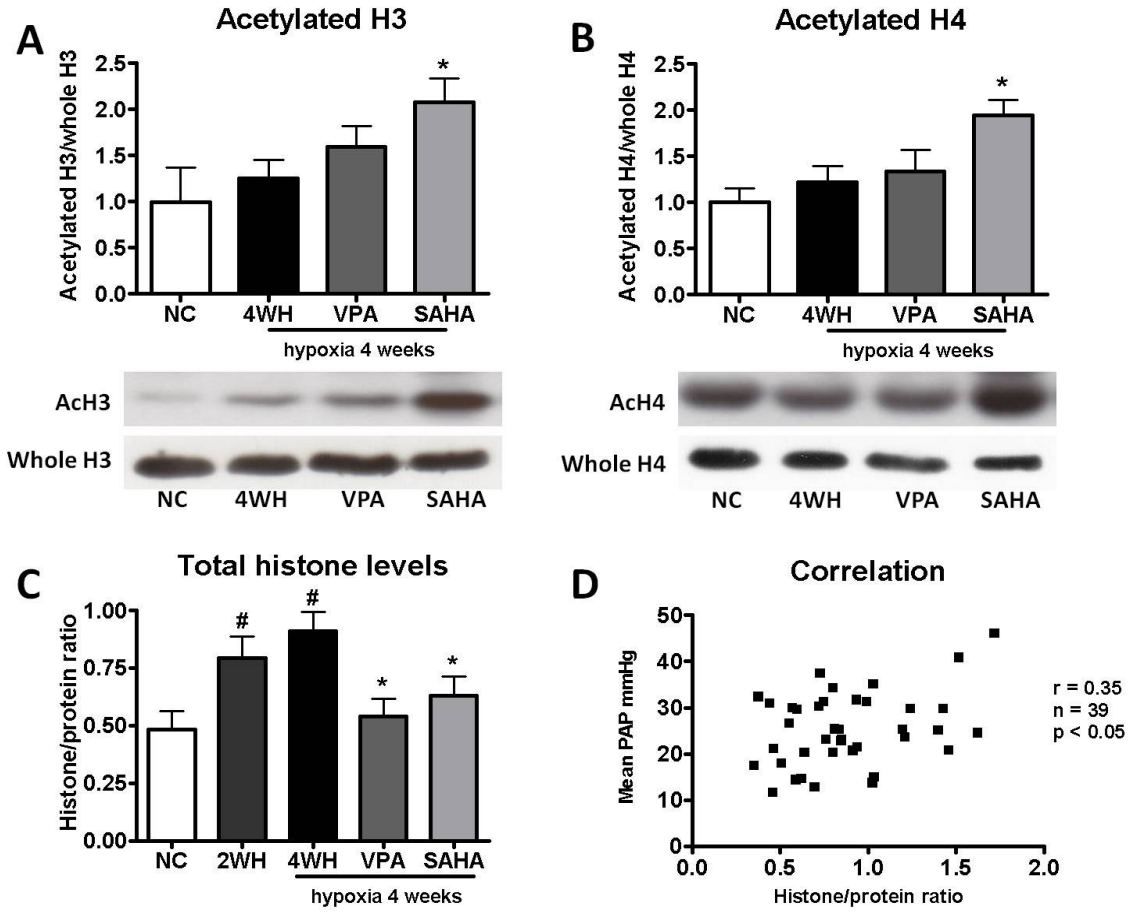



**Figure 4.6**. Histone expression levels in rat lung extracts from chronic hypoxia treatment study. (A) Acetylated H3, (B) Acetylated H4, (C) Total histone levels, (D) The correlation between total histone levels and mean PAP, $r=0.35$, $n=39$, $p<0.05$. NC: normoxia; 4WH: hypoxia for 4 weeks; VPA: hypoxia with VPA 300 mg/kg/day; SAHA: hypoxia with SAHA 50 mg/kg/day. Data are presented as mean ± SEM of fold change relative to NC. $n=6$. * $p<0.05$ compared with 4WH, # $p<0.05$ compared with NC.

Further analysis of the single lysine residue on histone tails, lysine 3 on H3 (H3K9) and lysine 12 on H4 (H4K12), revealed an increase in H3K9 acetylation under SAHA treatment ($p<0.05$). However, a discrepancy was observed in H4K12 acetylation in that VPA and SAHA treatment led to mild increases to the same extent (Figure 4.7).

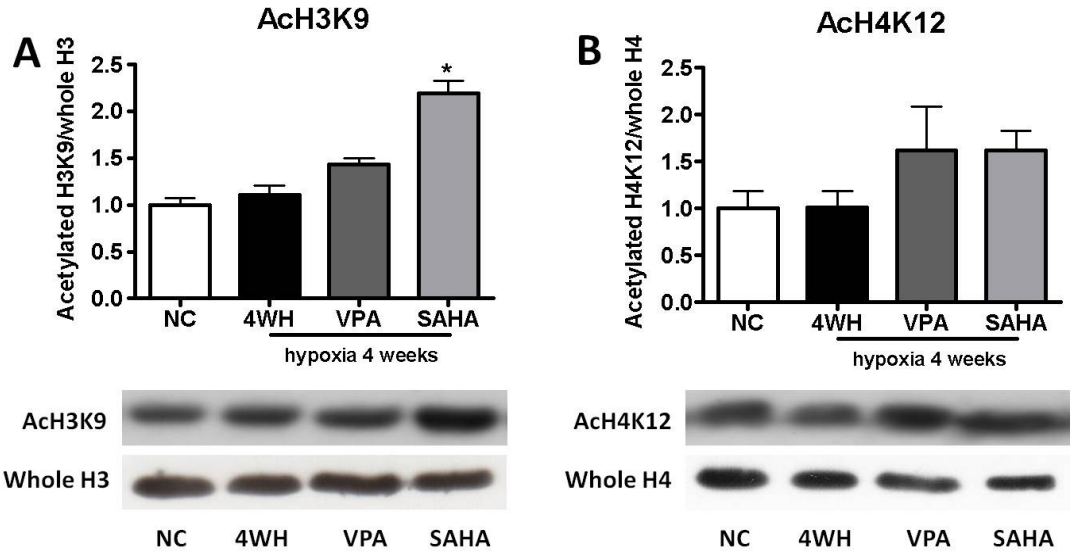



**Figure 4.7**. Histone expression levels in rat lung extracts from chronic hypoxia treatment study. (A) AcH3K9: acetylated H3 lysine 9, (B) AcH4K12: acetylated H4 lysine 12. NC: normoxia; 4WH: hypoxia for 4 weeks; VPA: hypoxia with VPA 300 mg/kg/day; SAHA: hypoxia with SAHA 50 mg/kg/day. Data are presented as mean ± SEM of fold change relative to NC. n=6. * $p<0.05$ compared with 4WH.

### 4.5.3. mRNA expression

The mRNA expression of HIF-1α was significantly increased by exposure to hypoxia, compared to normoxia control ($p<0.05$). Both VPA and SAHA treatment attenuated this rise ($p<0.05$, Figure 4.8A). There was no significant change in BMPR-2 expression in lungs during chronic hypoxia exposure. However, VPA but not SAHA treatment induced a significant increase in BMPR-2 expression in comparison to 2WH and 4WH group ($P<0.01$) (Figure 4.8B).

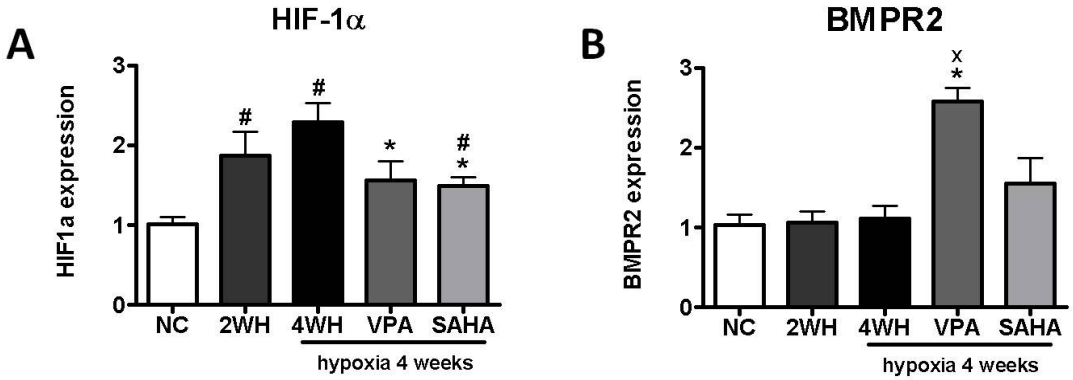



**Figure 4.8**. mRNA expression levels in rat lung extracts from chronic hypoxia treatment study. (A) HIF-1α, (B) BMPR2. NC: normoxia; 2WHL hypoxia for 2 weeks; 4WH: hypoxia for 4 weeks; VPA: hypoxia with VPA 300 mg/kg/day; SAHA: hypoxia with SAHA 50 mg/kg/day. Data are presented as mean ± SEM of fold change relative to NC. n=6. * $p<0.05$ compared with 4WH, x $p<0.05$ compared with 2WH, # $p<0.05$ compared with NC.

# 5. Discussion

## 5.1. VPA prevents hypoxia-induced PH

The VPA prevention study in the hypoxia-induced PH model provided the following findings:

- VPA treatment was well tolerated in the treated animals without perceptible adverse effects.
- VPA treatment significantly prevented elevation of pulmonary arterial and right ventricular pressure without influence on systemic blood pressure.
- VPA treatment significantly prevented right ventricular hypertrophy.
- VPA treatment significantly prevented pulmonary distal vascular remodelling.
- The pharmacological effects were in a dose-dependent trend, but not statistically significant.

These data suggest that VPA has protective effects against hypoxia-induced PH phenotype, physiologically and histologically. However, the direct contribution of HDAC inhibition cannot be determined from this study. VPA is a multi-functional agent with diverse molecular targets, especially GABAergic and sodium channel blocking effects, which might affect blood pressure [169]. Actually, in the animal experiment, a possible synergic effect of VPA with the anaesthesic midazolam, which can enhance GABA function on its receptor, was noted. A caution here is that the multiple non-HDAC-related functions of VPA may obscure its pharmalogical effects. In fact, even though VPA has been commonly prescribed for neuropsychiatric disorders, especially bipolar disorder, the mechanisms of action are still far from fully understood. Seemingly, GABAergic transmission plays a major role in mood-stabilization, but this alone is insufficient to explain the long-term effects of VPA. A variety of adjuvant mechanisms have been proposed and explored [257], e.g. inactivation of GSK3 [259], alternations in lipid metabolism [260]and promotion of neurotropic/neuroprotective factors[261]. However, their clinical relevance remains to be validated. In this study, the used dose was adopted according to

literature data which show to reach the therapeutic ranges in serum concentration of humans [257, 262]. Although, dose-dependent effects of VPA based on the two chosen doses were reported in previous studies, they were not statistically significant in the haemodynamics.

Nevertheless, the value of VPA is the fact that it has been widely prescribed for a long time and well tolerated according to the record. Its pharmacology and related side effects have been well documented that promise the translational accessibility to other clinical purposes. Additional advantages can be attributed to the cost and easy administration.

## 5.2. VPA and SAHA partially reverse hypoxia-induced PH

Treatment study in hypoxia-induced PH model provided the following findings:

- VPA and SAHA treatment was well tolerated in the treated animals without perceptible adverse effects, but decelerate the growth rate
- VPA and SAHA treatment significantly partially reversed elevation of pulmonary arterial and right ventricular pressure without influence on systemic blood pressure.
- VPA and SAHA treatment significantly partially reversed right ventricular hypertrophy.
- VPA and SAHA treatment significantly partially reversed pulmonary distal vascular remodelling.
- VPA and SAHA treatment significantly decreased Bcl-2 and increased p21 expression in lung tissues.
- VPA and SAHA treatment significantly increased histone acetylation in lung tissues.
- VPA and SAHA treatment significantly decreased HIF-1α expression in lung tissues.

Chronic treatment with VPA or SAHA had remarkable effects on hypoxia-induced PH in the rat. Both VPA and SAHA treatment attenuated the elevation in mean PAP and RVSP by around 50%, RV hypertrophy by over 60% and pulmonary vascular muscularization by 20%. VPA treatment seemed to have more potent effects on the RV than the pulmonary

vasculature. Quantification of vascular muscularisation is more challenging than measuring heart weight but this is not the only explanation.

The inhibitory effects of VPA and SAHA on HDAC activity is supported by increased lung histone H3 and H4 acetylation levels. Compared with VPA, SAHA had a more significant effect on histone acetylation. VPA inhibits the activity of class I HDACs, but SAHA is a broad-spectrum inhibitor covering all the zinc-dependent HDACs (class I, II and IV). This may explain the higher potency on total histone acetylation. HDAC inhibitor effects may be lysine-specific. Modification of specific lysines in histone tails have been linked to gene regulation in cancerous transformation, such as H3K9 and H4K16 [263, 264]. The analysis of lysine acetylation showed that acetylation of H3K9 highly correlated to acetylation of total H3 but the acetylation of H4K12 was paradoxical, VPA and SAHA seems to induce similar levels of acetylation. This finding suggests that the lysine-specificity of different HDAC inhibitors may contribute to the different pharmacological effects.

Biochemical analysis provides a clue that the therapeutic effects of VPA and SAHA on hypoxia-induced PH might be, at least in part, attributed to anti-proliferation. This hypothesis is evidenced by the following findings:

- The elevated protein expression of Bcl-2 in lungs induced by hypoxia was attenuated by VPA and SAHA. Increased levels of Bcl-2 prevents the release of cytochrome c from the mitochondrial intermembrane space and results in resistance to apoptosis. Inhibition of overexpressed Bcl-2 shifts back the imbalance and induces apoptosis [265]. Both VPA and SAHA have been reported to act on neoplastic disorders through downregulation of Bcl-2, and simultaneous activation of its counterparts, pro-apoptotic factors, Bid and Bim [184, 185], thereby increasing the ratio of pro- to anti-apoptotic proteins. In the hypoxia study, both of them exerted similar anti-proliferative effects on PH rats, suggesting pulmonary vascular remodelling was one of the targets.

- In parallel with downregulation of Bcl-2, VPA and SAHA upregulated the CDK inhibitor p21, leading to cell cycle arrest. P21 serves as a gatekeeper in cell cycle progression at G1/S phase and normally regulated by tumour suppressor protein p53

[266]. Induction of p21 arrests cell growth and inhibits proliferation. Growing evidence has indicated that most HDAC inhibitors promote p21 expression in both a p53-dependent and p53-independent manner [174, 176]. Furthermore, cell-growth-arrest induced by increased p21 expression has been associated with selective sensitivity to HDAC inhibitor-induced cell death in transformed cells [185]. The shifts in p21 and Bcl-2 expression give support to the view that HDAC inhibition participates in the restoration of balance between proliferation and apoptosis in pulmonary vascular remodelling.

- Total histone levels were increased in the chronic hypoxic rat lung and the rise was attenuated by treatment with VPA or SAHA. Total histone levels correlate with mean PAP. Upregulation of core histones may represent an increase in the chromatin content of cells compatible with a hyper-proliferative status. This observation also supports the anti-proliferative effects of VPA and SAHA.

Previous studies have revealed that HDACs regulate angiogenesis via HIF-VEGF transduction. HIFs are the principle mediators of the tisue response to decreased oxygen level. HIFs increase oxygen delivery through upregulating several angiogenesis genes, such as VEGF, and adjust oxygen utilization through metabolic shifts, both of which promote survival in hypoxic conditions. Overexpression of HADC1 upregulates HIF-1α and vascular endothelial growth factor (VEGF) indirectly, via suppression of p53 and von Hippel–Lindau (VHL) genes expression [267]. HDAC1 and HDAC3 have been shown to directly regulate HIF-1α stability and transcriptional activity via interaction with the oxygen-dependent degradation domain [268]. HIF-1α plays a key role in hypoxia-induced pulmonary hypertension but is also upregulated in human IPAH [269]. The increased HIF-1α mRNA levels in hypoxic rat lungs were reduced by both VPA and SAHA treatment, indicating a role for anti-angiogenesis in the therapeutic effects in addition to anti-proliferation.

Interestingly, BMPR2 mRNA expression in hypoxic rat lungs was upregulated by VPA but not SAHA treatment. Restoring BMPR2 function has been considered to be a therapeutic option in PAH but may be challenging and inadequate for reversing the pathological changes [116]. The dissociation between VPA and SAHA in BMPR2 expression suggests class-dependent functions of HDAC inhibition.

In conclusion, VPA and SAHA treatment prevents and ameliorates chronic hypoxia-induced PH as evident from improvement of pulmonary and cardiac haemodynamics, reversal of RV hypertrophy and reduction of pulmonary vascular muscularization. The underlying mechanisms can be attributed to induction of apoptosis and inhibition of proliferation.

The major limitation of this study is that chronic hypoxia can not fully reflect the pathology of PAH in humans. For example, hypoxia exposure does not lead to intimal fibrosis or plexiform lesions [270]. Whether hypoxia stimuli are obligatory for the initiation of PAH is controversial. This model is more relevant to PH from pre-existing lung diseases such as chronic obstructive pulmonary disease, pulmonary parenchymal diseases and living at high altitude (WHO class III). In addition, the HDAC doses used in the study were adopted from published studies in cancer, with moderate to high dose within the range of safe tolerated doses. A more exacting experimental design would explore a series of different doses.

# Chapter 5:

# HDAC INHIBITORS AMELIORATE MCT-INDUCED PH

# 1. Introduction

The limitation of animal experiments is the fact that no any existing animal model completely reproduces human diseases. Using more than one model to validate the pharmacological effects raises more confidence and rationale towards clinical trials. Following the findings from hypoxia experiments that both VPA and SAHA seem to alleviate the PH phenotype, it is worthwhile to further testify the pharmacological effects on other models. As chronic hypoxia model is regarded as a model for more reversible and less severe PH, MCT-induced PH is relatively irreversible and more severe, especially in terms of vascular remodelling and inflammation.

MCT is a toxic pyrrolizidine alkaloid, activated in the liver to become active form and resulting in direct endothelial injury in pulmonary vessels. A single subcutaneous or intraperitoneal injection of MCT can simply develop PH with striking vascular remodelling, neointimal thickening, dramatic perivascular inflammatory cells infiltration and eventually lethality. MCT model is also significantly associated with advanced RV hypertrophy and dysfunction [271]. Therefore, MCT model offers a useful platform to investigate the pharmaceutical effects on pulmonary vascular remodelling and inflammatory reactions more specifically.

In this chapter, the effects of chronic treatment of VPA and SAHA were tested in established MCT-induced PH. To avoid animal mortality from rapid progression of PH and right heart failure, end point haemodynamic, histological and biochemical assessment were performed in four weeks after MCT injection.

# 2. Objectives

- To evaluate the pharmacological effects of VPA and SAHA on established MCT-induced PAH in rats.

# 3. Methods and Protocols

Male Spraque-Dawley rats (220-250 gram) were divided into five groups (n=6-12 each group):

1) Ctrl: healthy control

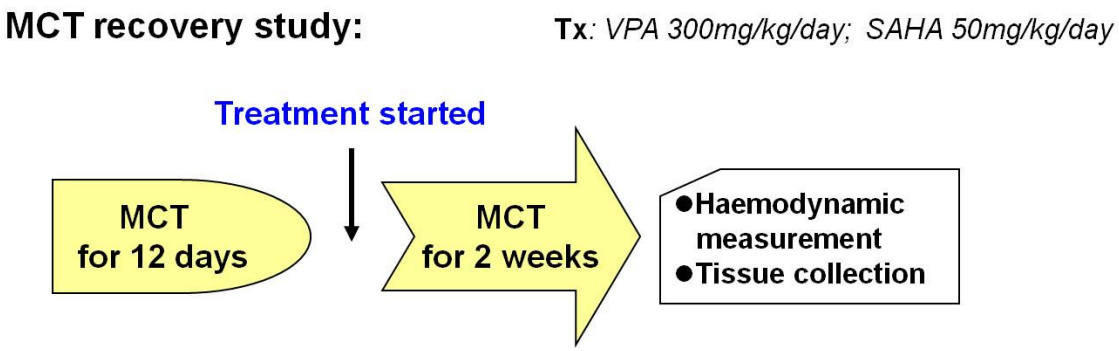
2) M12: MCT for 12 days

3) M26: MCT for 26 days

4) VPA: MCT-injected with VPA 300mg/kg/day *p.o*

5) SAHA: MCT-injected with SAHA 50mg/kg/day *p.o.* (in drinking water with HOP-β-CD) [258]

MCT was injected subcutaneously into rats on Day 0. End point measurement of M12 group was performed on Day 12 as starting point of treatment. Treatment with VPA or SAHA was administrated through drinking water as previously described for the following two weeks. Animals were weighed every other day. Treatment dose was adjusted accordingly by weighing drinking bottles daily. End point measurement of other groups was done on Day 26 (as the flowchart below).



# 4. Result

## 4.1. VPA and SAHA ameliorate MCT-induced PH: haemodynamics

MCT led to a minimal elevation in mean PAP 12 days after injection and rapidly reached up to over 30mmmHg in 26 days (M12: 17.9±1.6 mmHg, M26: 31.1±2.0 mmHg v.s. Ctrl: 16.1±0.6 mmHg, $p<0.0001$, Figure 5.1A). MCT did not cause changes in RVSP as well as RVH in 12 days but increased significantly both in 26 days (RVSP: M12: 26.1±2.2 mmHg, M26: 54.9±4.5mmHg v.s. Ctrl: 25.9±0.8 mmHg, $p<0.005$; RVH: M12: 0.28±0.01, M26: 0.46±0.04 v.s. Ctrl: 0.27±0.01, $p<0.005$, Figure 5.1B,C).

Treatment with SAHA for two weeks significantly reduced mean PAP while VPA only cause slight reduction without statistical significance due to higher difference within group (VPA: 27.6±2.4 mmHg, SAHA: 21.3±1.4 mmHg v.s. M26: 31.1±2.0mmHg, $p<0.05$, Figure 5.1A). Similarly, SAHA reduced MCT-induced elevation of RVSP significantly while VPA tended to reduce but not significantly (VPA: 46.7±4.7 mmHg, SAHA: 38.2±2,1 mmHg v.s. M26: 54.9±4.5mmHg, $p<0.05$, Figure 5.1B).

MCT for 26 days also induced remarkable RVH (M12: 0.28±0.01, M26: 0.46±0.04 v.s. Ctrl: 0.28±0.01, $p<0.005$, Figure 5.1C), which was significantly attenuated by SAHA treatment (-73.4%) and mildly improved by VPA (-29.1%) (VPA: 0.41±0.03, SAHA: 0.32±0.00 v.s. M26: 0.45±0.02, $p<0.005$, Figure 5.1C).

No significant changes in heart rate or systemic SBP were observed in SAHA-treated group. However M26 and VPA seemed to cause a slight drop in systemic SBP (Figure 5.1D). The physiological data of MCT recovery study is demonstrated in Table 5.1.

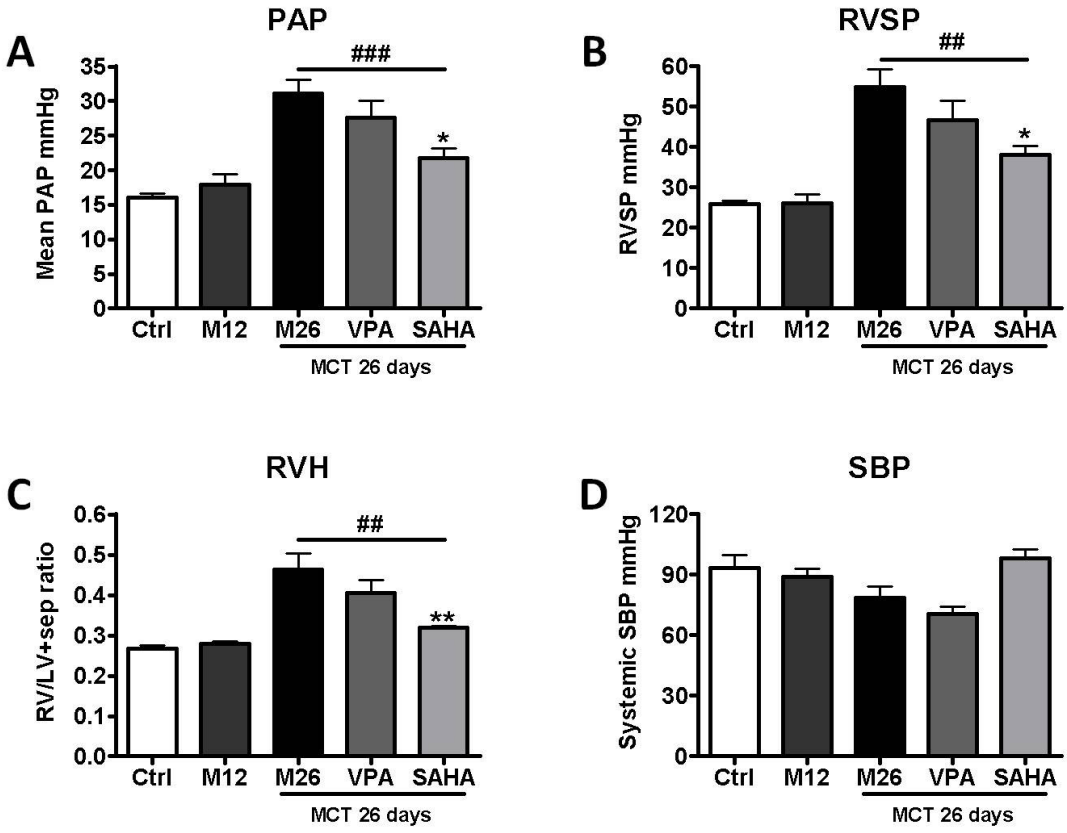



**Figure 5.1**. Recovery study: treatment with VPA and SAHA were started 12 days after MCT injection and continued for another two weeks. (A) PAP, mean pulmonary artery pressure, (B) RVSP, right ventricular systolic pressure, (C) RV/LV+sep, right ventricular hypertrophy, (D)SBP, systemic systolic blood pressure. Ctrl: healthy control; M12: 12-day MCT; M26: 26-day MCT; VPA: MCT with VPA 300 mg/kg/day; SAHA: MCT with SAHA 50 mg/kg/day. The data are presented as mean± SEM. n≥6 in each group. * $p<0.05$, ** $p<0.005$ compared with M26, ## $p<0.005$, ### $p<0.0005$ compared with Ctrl.

| Group | | Control | MCT26 | VPA | SVPA |
|---|---|---|---|---|---|
| N number | | 6 | 11 | 6 | 6 |
| RV | Systolic (mmHg) | 25.9±0.8 | 54.9±4.5 | 46.7±4.7 | 38.2±2.1 |
| | Max dp/dt (mmHg/s) | 783±48 | 1605±104 | 1006±115 | 1012±78 |
| Pulmonary | Mean (mmHg) | 16.1±0.6 | 31.1±2.0 | 27.6±2.4 | 21.7±1.4 |
| | Pulse (mmHg) | 11.0±0.9 | 42.8±9.5 | 23.1±5.0 | 20.2±3.8 |
| Systemic | Pressure (mmHg) | 93.4±6.3 | 78.6±5.7 | 70.7±3.7 | 98.1±4.5 |
| | Heart rate | 306±14 | 288±17 | 298±20 | 322±18 |
| General | BW (gram) | 322±4 | 297±15 | 302±9 | 278±7 |
| | RV/LV+septum | 0.27±0.01 | 0.46±0.04 | 0.41±0.03 | 0.32±0.00 |
| | RV/BW | 0.55±0.01 | 0.90±0.09 | 0.79±0.06 | 0.64±0.01 |
| | Left lung/BW | 1.34±0.03 | 2.46±0.28 | 1.99±0.08 | 2.12±0.08 |

MCT26: MCT control; VPA: VPA 300mg/kg/day; SAHA: SAHA 50mg/kg/day; BW:body weight

**Table 5.1**. The physiological data of MCT recovery study.

## 4.2. VPA and SAHA ameliorate MCT-induced PH: histology

MCT for 12 days raised significantly peripheral distal pulmonary arteriolar muscularization and MCT for 26 days further augmented this phenotype (M12: 58.0±1.7%, M26: 79.8±1.0 v.s. NC: 5.7±0.5%, $p<0.005$, Figure 5.2). Both VPA and SAHA treatment showed significant reduction in vascular muscularization compared to 26-day MCT exposure (VPA: 72.0±1.1%, SAHA: 69.2±2.1% v.s. M26: 79.8±1.0%, $p<0.005$, Figure 5.2) and partial reversal compared to 12-day MCT exposure (VPA: 72.0±1.1%, SAHA: 69.2±2.1% v.s. M12: 58.0±1.7%, $p<0.005$, Figure 5.2).

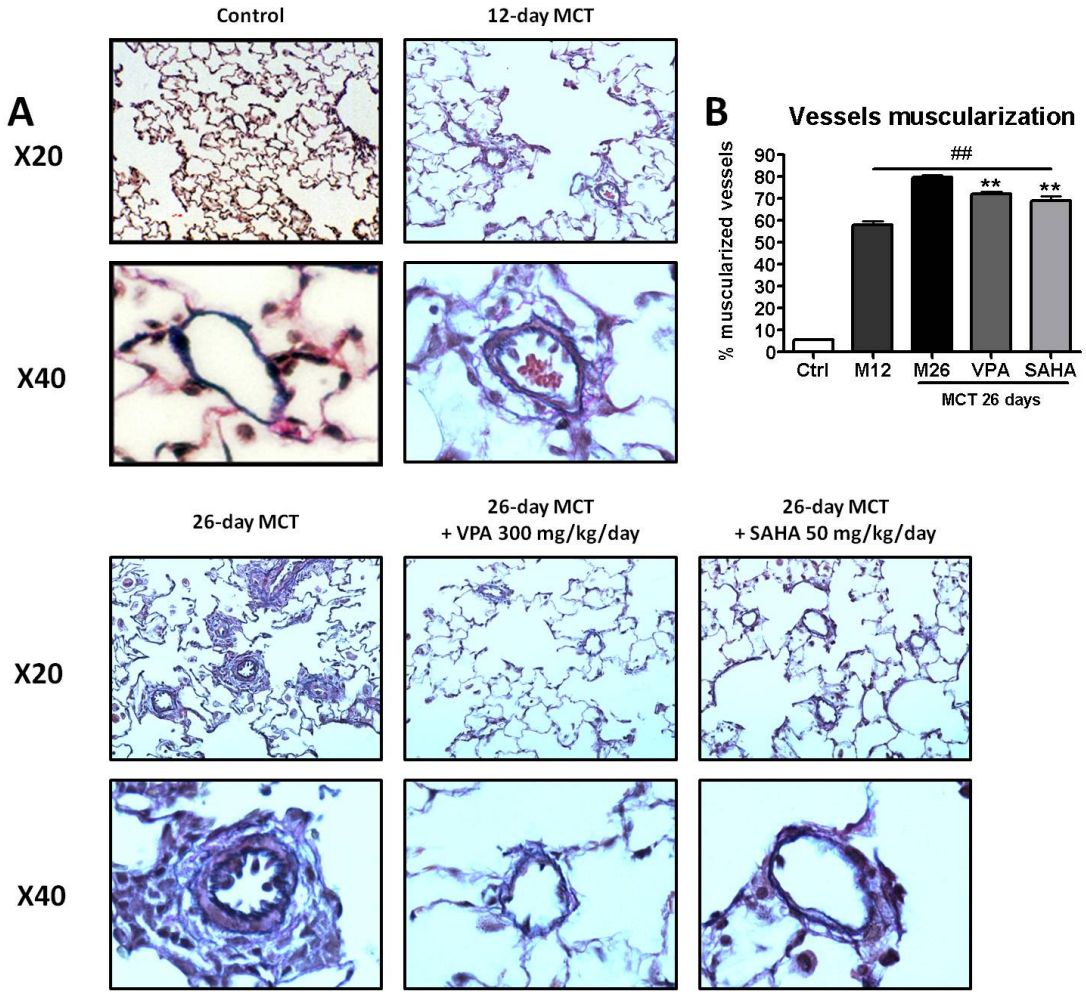



**Figure 5.2**. Effects of VPA and SAHA on MCT-induced pulmonary vascular muscularization. (A) Histology figures with van Gieson's elastic stain, (B) Quantified by the percentage of vessels with double elastic lamina. Ctrl: healthy control; M12: 12-day MCT; M26: 26-day MCT; VPA: MCT with VPA 300 mg/kg/day; SAHA: MCT with SAHA 50 mg/kg/day. The data are presented as mean± SEM. n=6 in each group. ** $p<0.005$ compared with M26, ## $p<0.005$ compared with Ctrl.

To assess the myocyte hypertrophy at histological level, cross sectional area (CSA) and minimal Feret's diameter were measured. Both of these two indexes were significantly increased at 26-day MCT group ($p<0.005$, Figure 5.3). Treatment with VPA reduced the rise in CSA significantly ($p<0.05$, Figure 5.3A) while SAHA had a more potent reduction in both CSA and myocyte diameter ($p<0.005$, Figure 5.3).

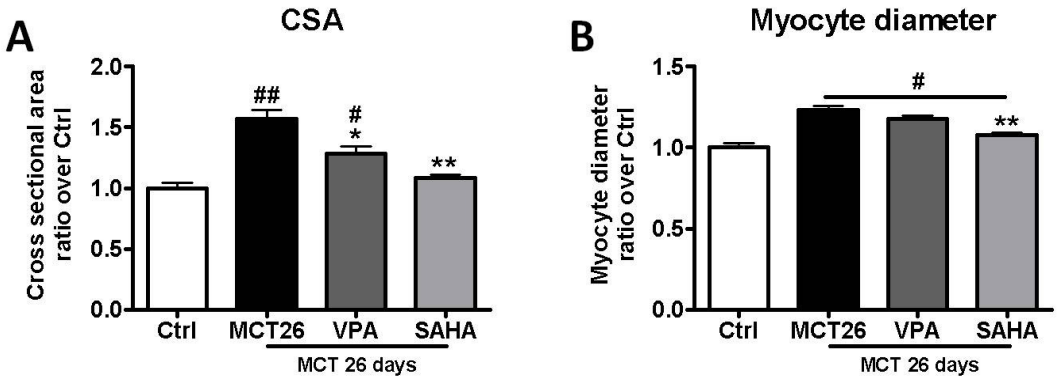



**Figure 5.3**. Effects of VPA and SAHA on MCT-induced pulmonary RV myocyte hypertrophy. (A) Cross sectional area, (B) Myocyte diameter. Ctrl: healthy control; M26: 26-day MCT; VPA: MCT with VPA 300 mg/kg/day; SAHA: MCT with SAHA 50 mg/kg/day. The data are presented as mean± SEM of fold change relative to Ctrl. n=6 in each group. * $p<0.05$, ** $p<0.005$ compared with M26,# $p<0.05$, ## $p<0.005$ compared with Ctrl.

## 4.3. VPA and SAHA ameliorate MCT-induced PH: biochemistry

### 4.3.1. Protein expression

Similar to the findings in hypoxia study, protein expression levels of Bcl-2 in the lungs from the animals exposed to 26-day MCT were increased by 50% ($p<0.05$, Figure 5.4A). Treatment with SAHA brought back the Bcl-2 expression to a relatively lower level than control significantly ($p<0.01$), while VPA seemingly caused a moderate reduction without statistical significance (Figure 5.4A). Nevertheless, both VPA and SAHA significantly promoted p21 expression levels compared to 26-day MCT, with relatively higher levels in SAHA ($p<0.05$) (Figure 5.4B).

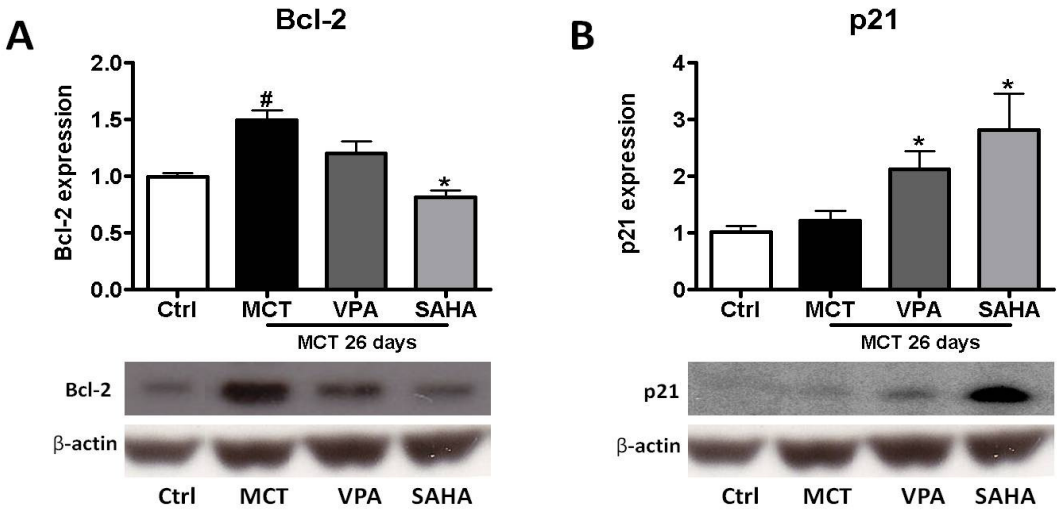



**Figure 5.4**. Protein expression levels in rat lung extracts from MCT treatment study. (A) Bcl-2, (B) p21. Ctrl: healthy control; MCT: 26-day MCT; VPA: MCT with VPA 300 mg/kg/day; SAHA: MCT with SAHA 50 mg/kg/day. The data are presented as mean± SEM of fold change relative to Ctrl. n=6 in each group. * $p<0.05$ compared with MCT, # $p<0.05$ compared with Ctrl.

To further confirm the hyper-proliferative phenotype induced by MCT, expression levels of another marker, survivin, were analyzed, which revealed survivin was highly expressed in 26-day MCT group by a nearly 10-fold increase over healthy control ($p<0.05$). Treatment with VPA and SAHA both dramatically decreased survivin expression levels by around 50% ($p<0.05$, Figure 5.5).

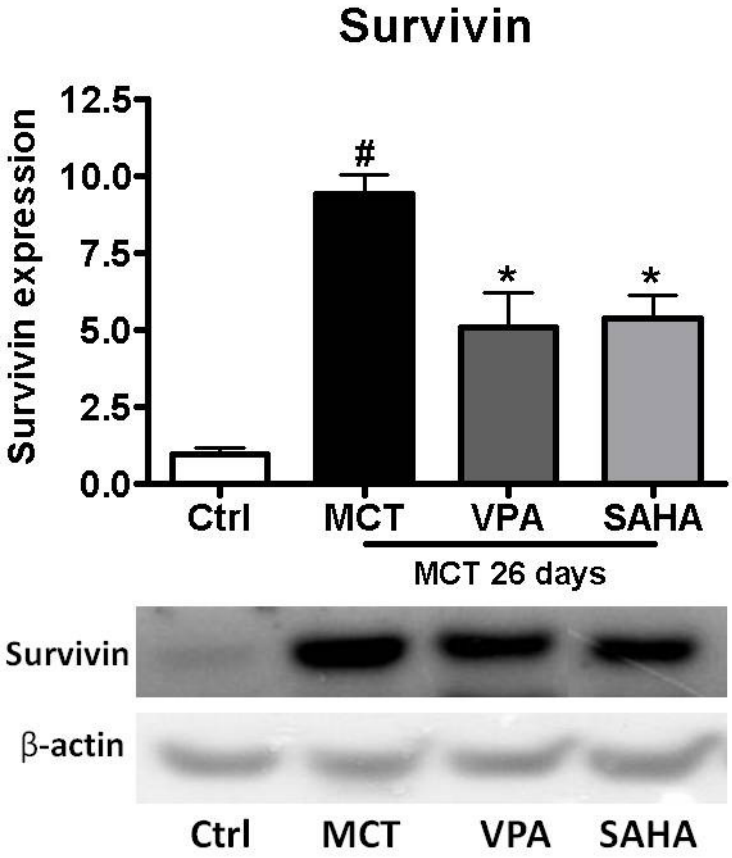



**Figure 5.5**. Protein expression levels of survivin in rat lung extracts from MCT treatment study. Ctrl: healthy control; MCT: 26-day MCT; VPA: MCT with VPA 300 mg/kg/day; SAHA: MCT with SAHA 50 mg/kg/day. The data are presented as mean± SEM of fold change relative to Ctrl. n=6 in each group. * $p<0.05$ compared with MCT, # $p<0.05$ compared with Ctrl.

### 4.3.2. Histone acetylation

The effects on HDAC inhibition were assessed by the acetylation levels of H3 and H4. The acetylation level of H3 was slightly decreased in 26-day MCT ($p<0.05$) which was reversed mildly by VPA ($p=0.067$) and strongly by SAHA (+34%, $p<0.05$, Figure 5.6A). On the other hand, the acetylation level of H4 was significantly increased by both VPA (+100%) and SAHA (+50%) compared to 26-day MCT ($p<0.0001$, Figure 5.6B).

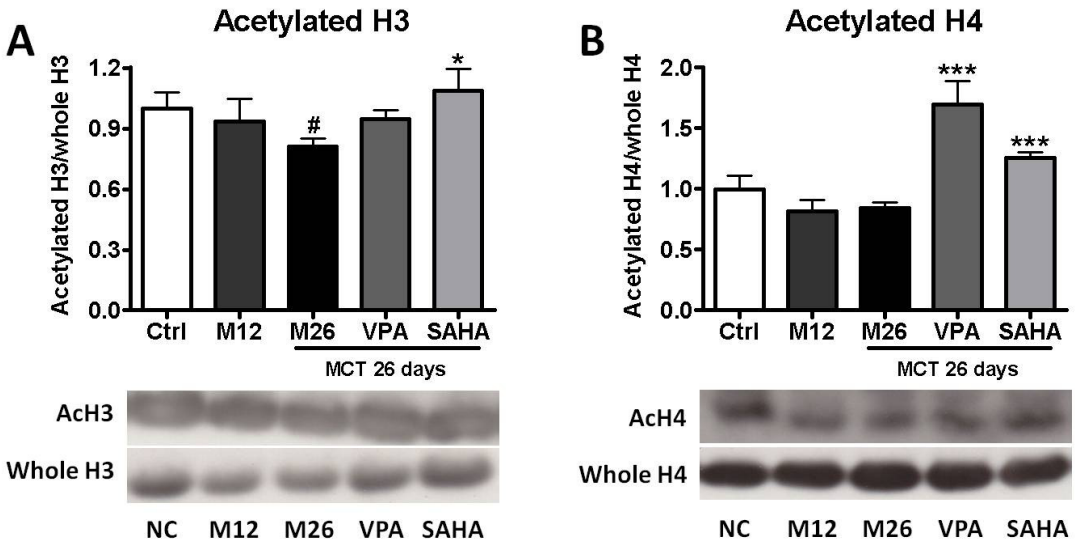
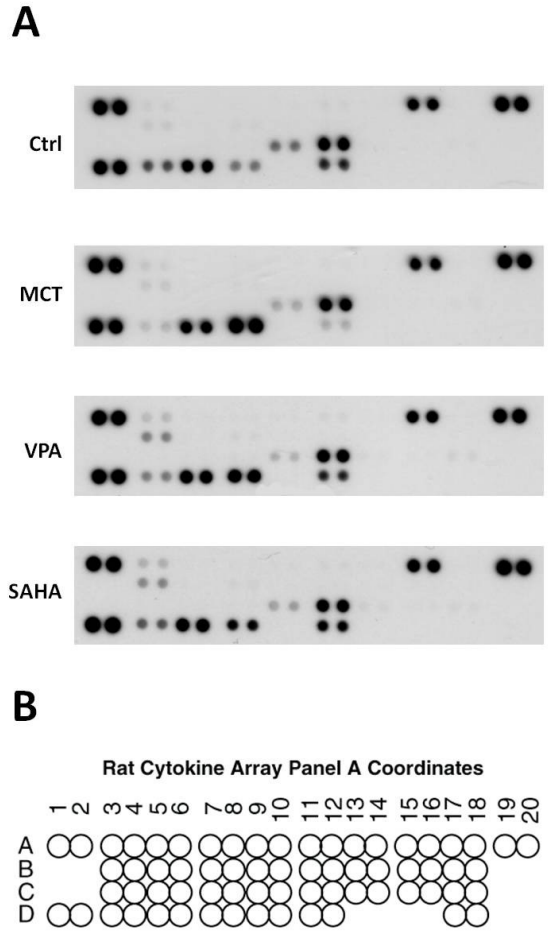



**Figure 5.6**. Histone expression levels in rat lung extracts from MCT treatment study. (A) Acetylated H3, (B) Acetylated H4. Ctrl: healthy control; MCT: 26-day MCT; VPA: MCT with VPA 300 mg/kg/day; SAHA: MCT with SAHA 50 mg/kg/day. The data are presented as mean± SEM of fold change relative to Ctrl. n=6 in each group. * $p<0.05$, $p<0.0001$ compared with M26, # $p<0.05$ compared with Ctrl.

### 4.3.3. Cytokine assay and expression

To examine the effects of VPA and SAHA on inflammatory responses to MCT-induced PH in the lung, selected cytokines and chemokines were screened by R&D system rat cytokine array panel A. The original results are displayed in Figure 5.7.




C

| Coordinate | Target | Coordinate | Target |
|---|---|---|---|
| A1,A2 | PC | B17,B18 | IL-10 |
| A3,A4 | CINC-1 | C3,C4 | IL-13 |
| A5,A6 | CINC-2α/β | C5,C6 | IL-17 |
| A7,A8 | CINC-3 | C7,C8 | IP-10 |
| A9,A10 | CNTF | C9,C10 | LIX |
| A11,A12 | Fractalkine | C11,C12 | L-Selectin |
| A13,A14 | GM-CSF | C13,C14 | MIG |
| A15,A16 | sICAM-1 | C15,C16 | MIP-1α |
| A17,A18 | IFN-γ | C17,C18 | MIP-3α |
| A19,A20 | PC | D1,D2 | PC |
| B3,B4 | IL-1α | D3,D4 | RNATES |
| B5,B6 | IL-1β | D5,D6 | CXCL7 |
| B7,B8 | IL-1ra | D7,D8 | TIMP-1 |
| B9,B10 | IL-2 | D9,D10 | TNF-α |
| B11,B12 | IL-3 | D11,D12 | VEGF |
| B13,B14 | IL-4 | D17,D18 | NC |
| B15,B16 | IL-6 | | |

Abbreviations: PC, positive control; CINC, cytokine-induced neutrophil chemoattractant; CNTF, ciliary neurotrophic factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; sICAM-1, soluable intercellular adhesion molecule-1; IFN-γ, interferon-gamma; IL, interleukin; IP-10, IFN-γ-induced protein 10; LIX, lipopolysaccharide-induced chemokine (C-X-C motif); MIG, monokine induced by IFN-γ; MIP, macrophage inflammatory protein; RANTES, regulated by T cells, activation upon secretion; CXCL, chemokine (C-X-C motif) ligand; TIMP-1, tissue inhibitor of metalloproteinases; TNF-α, tumour necrosis factor alpha; VEGF, vascular endothelial growth factor; NC, negative control.

**Figure 5.7**. Cytokine and chemokine array in rat lung extracts from MCT treatment study. (A) Array data on developed X-ray films, (B) Array coordincates, (C) Reference to represented cytokine and chemokine. Ctrl: healthy control; MCT: 26-day MCT; VPA: MCT with VPA 300 mg/kg/day; SAHA: MCT with SAHA 50 mg/kg/day.

Noticeable elevation induced by 26-day MCT was observed in TIMP-1, IP-10, IL-4, IL-17, CINC-2α/β and MIP-3α. The elevation was attenuated under VPA or SAHA treatment, as SAHA led to greater reduction in nearly all of these cytokines (Figure 5.8).

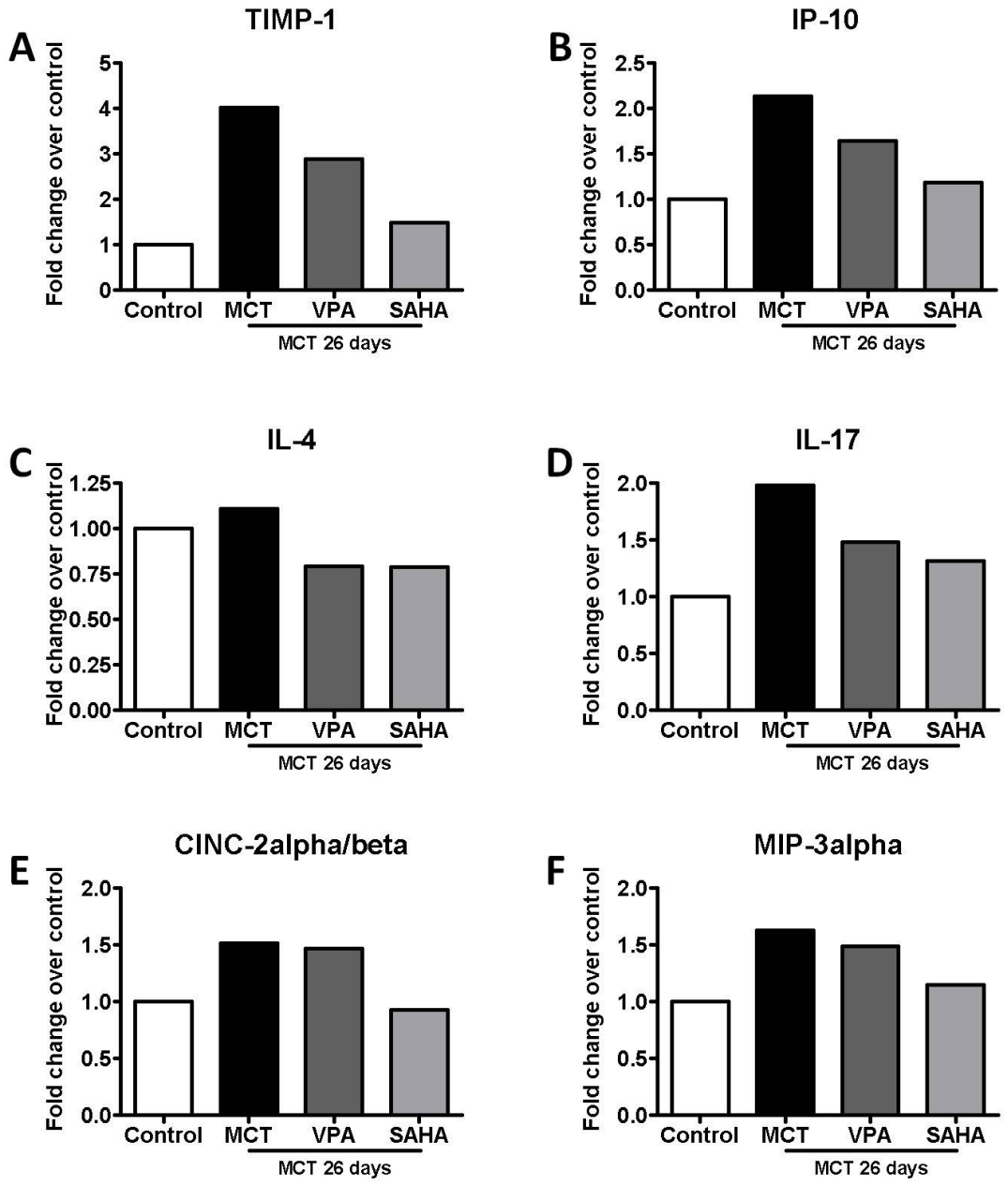



**Figure 5.8**. Cytokine levels detected by cytokine array in rat lung extracts from MCT treatment study. (A) TIMP-1, (B) IP-10, (C) IL-4, (D) IL-10, (E) CINC-2α/β, (F) MIP-3α. Data are presented as fold change relative to Ctrl. Samples from each group (n=6) were poured together with eqaul amounts. Ctrl: healthy control; MCT: 26-day MCT; VPA: MCT with VPA 300 mg/kg/day; SAHA: MCT with SAHA 50 mg/kg/day.

To further confirm the findings from cytokine array, protein expression levels of TIMP-1 were analyzed by Western blotting. MCT for 26 days induced a six-fold increase in TIMP-1 ($p<0.005$) compared to healthy control and VPA reduced the elevation by 50% while SAHA almost brought the levels back to the normal range ($p<0.005$, Figure 5.9).

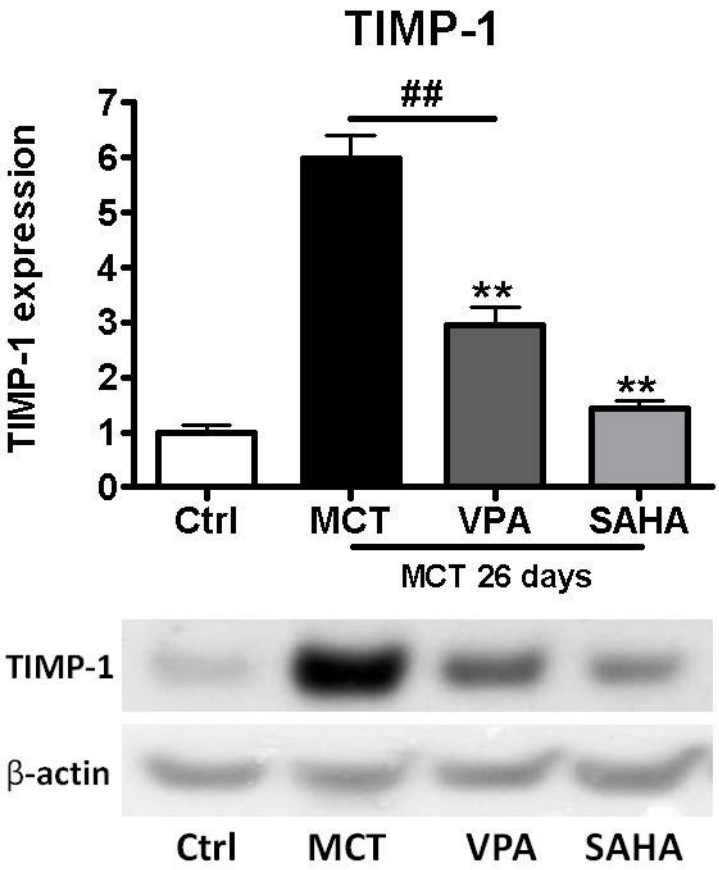



**Figure 5.9**. Protein expression levels of TIMP-1 in rat lung extracts from MCT treatment study. Ctrl: healthy control; MCT: 26-day MCT; VPA: MCT with VPA 300 mg/kg/day; SAHA: MCT with SAHA 50 mg/kg/day. The data are presented as mean± SEM of fold change relative to Ctrl. n=6 in each group. ** $p<0.005$ compared with MCT, ## $p<0.005$ compared with Ctrl.

# 5. Discussion

Treatment study in MCT-induced PH model provided the following findings:

- VPA and SAHA treatment was well tolerated in the treated animals without perceptible adverse effects, but decelerate the growth rate
- VPA and SAHA treatment significantly ameliorated elevation of pulmonary arterial and right ventricular pressure without influence on systemic blood pressure.
- VPA and SAHA treatment significantly ameliorated right ventricular hypertrophy.
- VPA and SAHA treatment significantly ameliorated pulmonary distal vascular remodelling.
- VPA and SAHA treatment significantly decreased Bcl-2 and survivin, and increased p21 expression in lung tissues.
- VPA and SAHA treatment significantly increased histone acetylation in lung tissues.
- VPA and SAHA treatment inhibited the release of several inflammatory mediators in lung tissues, such as TIMP-1 and IP-10.

Similar to the findings from hypoxia study, VPA or SAHA treatment ameliorated established PH induced by MCT. SAHA reduced the elevated mean PAP and RVSP by around 60% and hypertrophied RV by over 70% compared with MCT control. Histological assessment of RV hypertrophy by measuring myocyte diameter and area demonstrated a 70% reduction, which is consistent with the haemodynamic measurement. Animals were well tolerated with VPA and SAHA during the whole period of treatment. However, a mild deceleration in growth was noted in SAHA treated group. The therapeutic effects of VPA treatment appeared to be less convincing on MCT model. The failure in gaining the statistical significance is due to a higher within-group difference in VPA treated group, suggesting the susceptibility to VPA is relatively diverse in MCT model. Nevertheless, histological assessment revealed VPA treatment attenuated pulmonary vascular muscularization to a similar degree to SAHA.

The effects on HDAC inhibition were evidenced by increased histone H3 and H4 acetylation as they were in hypoxia study. Interestingly, VPA exhibited an even higher increase in H4 acetylation than SAHA but not in H3 acetylation. The inconsistence with hypoxia study may be due to different pathological mechanisms between two models and implies that "histone codes" can be composed and adjusted in response to different conditions.

MCT-induced model has been recognized a more severe model owing to more profoundly pulmonary vascular remodelling, involving medial hypertrophy, extracellular matrix secretion, arteriolar muscularization, thrombi formation, increased endothelial permeability, perivascular inflammatory cells infiltration and inflammatory mediators release [93, 272]. Protein analysis showed the compatible alternations with hypoxia study which are downregulation of Bcl-2 and upregulation of p21 by VPA and SAHA treatment. The anti-proliferative effects of these two inhbitors can be further confirmed by the changes in survivin expression. As an anti-apoptotic protein as Bcl-2, survivin has been linked to human PAH more specifically [81]. Treatment with VPA and SAHA both reduced the elevated survivin expression, suggesting a prominent anti-proliferative effect on MCT model.

Accumulating evidence has implicated the role of inflammation in the pathogenesis of PAH. The cytokine screening in rat lungs revealed that several inflammatory mediators were association with attenuation of MCT-induced PH phenotype by VPA and SAHA treatment. One with the strongest significance is TIMP-1. TIMP-1 acts to inhibit most of the known MMPs and control ECM remodelling. It also promotes cell proliferation, inhibits apoptosis and involves in tissue inflammation and fibrotic formation. Aberrant activity of TIMP-1 along with its downstream MMPs has been reported in smooth muscle cells from patients with IPAH [79]. Imbalance between TIMP-1 and MMPs featured by augmentation of TIMP-1 and ECM deposition plays a crucial role in vascular remodelling [273]. Rebuilding the balance between TIMP-1 and MMPs by suppressing overactive TIMP-1 may reverse severe PAH by ameliorating vascular remodelling and repairing dysfunctional endothelium [274]. Biochemical analysis in MCT treatment study showed a humongous elevation in TIMP-1 induced by MCT, which was significantly attenuated by VPA and SAHA, implicating the potential targets of HDAC inhibition.

Both VPA and SAHA treatment ameliorated the rise of IP-10, which was elevated in lungs from MCT-treated rats. IP-10, secreted mainly by monocytes, endothelial cells and fibroblasts in response to INF-γ, is a chemoattractant for monocytes, macrophages, T cells, natural killer cells and dendritic cells [275]. It is important for T cell adhesion to endothelial cells. Serum IP-10 level is reported elevated in patients with PAH and associated with survival [92]. A previous study indicated the activation of IP-10 is paralleled with deacetylation of histone and HDAC activity is required for INF-γ stimulation [276]. Consistently, VPA and SAHA might block the HDAC-dependent transcription of IP-10, leading to impaired recruitment of immune cells and exerting anti-inflammatory effects in MCT-induced PH model.

# Chapter 6:

# HDAC INHIBITORS IN HPSMC AND HPAEC

# 1. Introduction

Based on the results from *in vivo* experiments, HDAC inhibitors VPA and SAHA are shown encouraging effects on improving PH pathology, possibly via anti-proliferation and anti-inflammation. In order to clarify the cellular mechanisms, especially to identify whether HDAC inhibition can target pulmonary vascular remodelling, the pharmacological effects of these two are testified in *in vitro* system.

Pulmonary vascular remodelling is a multi-cell-dependent process involving nearly all elements of vascular structure, among which endothelial cells and smooth muscle cells are recognized to take crucial parts in the whole event. Endothelial dysfunction has been identified as a possible initiator of vascular remodelling [57]. Endothelial cells are also acting as a main source of many growth factors like PDGF and VEGF, vasoactive mediators, such as eNOS and endothelin, and inflammatory mediators, including IL-6, IL-8, IP-10, RANTES and fractalkine. Secondary to endothelial injury, underlying smooth muscle cells undergo both hypertrophy and hyperplasia and become resistance to apoptosis. The overgrowth of smooth muscle cells, accompanied with over-activated fibroblasts and deposition of ECM as well as recruited immune cells, results in restriction of pulmonary circulation and eventual occlusion. Pharmacological experiments in primary cell culture of endothelial cells and smooth muscle cells provide the information of cell-specific effects and help to unravel the molecular mechanisms.

Hyper-proliferative smooth muscle cells and pro-inflammatory endothelial cells are established to mimic the cellular system in human PAH. Stimulated proliferation of smooth muscle cells are achieved by PDGF-BB. Growing evidence has suggested a vital role of PDGF in the pathogenesis of PAH. Overexpression of PDGF and the receptors has been identified in PAH patients [64, 64] PDGF induces a higher growth rate in HPSMC from patients with IPAH than cells from normal control [277]. PDGF receptor antagonist, imatinib, has been shown potential efficacy in treatment of PAH in pre-clinical and clinical studies.

To create a pro-inflammatory cell system, endothelial cells are stimulated by TNF-α. TNF-α is the primary regulator in systemic inflammatory reactions, inducing other inflammatory mediators release. Elevation of circulating TNF-α has been reported in IPAH patients. This elevation correlates to cardiac index and functional performance [88].

In this chapter, VPA and SAHA are tested in these two stimulated cell systems to assess their effects on proliferation and inflammation.

# 2. Objectives

- To evaluate the pharmacological effects of VPA and SAHA on PDGF-stimulated HPSMC
- To evaluate the pharmacological effects of VPA and SAHA on TNF-α-stimulated HPAEC

# 3. Methods and Protocols

## 3.1. Human pulmonary smooth muscle cell (HPSMCs)

Harvested HPSMCs were seeded and allowed to attach overnight before 24-hour serum deprivation. PDGF-BB (50 ng/ml) stimulation or exposure to hypoxia ($O_2$=2%) with or without VPA (1mM, 2mM) or SAHA (2.5μM, 10μM) for 72 hours is conducted subsequently (Figure 6.1). At the end of exposure, cells were harvested to assess proliferation by cell counting, WST-1 assay and BrdU incorporation assay; cell death by viability staining with PI (50μg/ml), cell cycle analysis by FACS, protein analysis by Western blot and immunostaining. All the experiments were executed in at least three times with separate cell preparations.

## 3.2. Human pulmonary artery endothelial cells (HPAECs)

Confluent HPAECs in equal amount are treated with 2mM VPA or 10μM SAHA for 24 hours. 10 ng/ml TNF-α was added 8 hours after treatment starts. At the end of treatment, cell culture supernatants are collected for cytokine array (Figure 6.2).

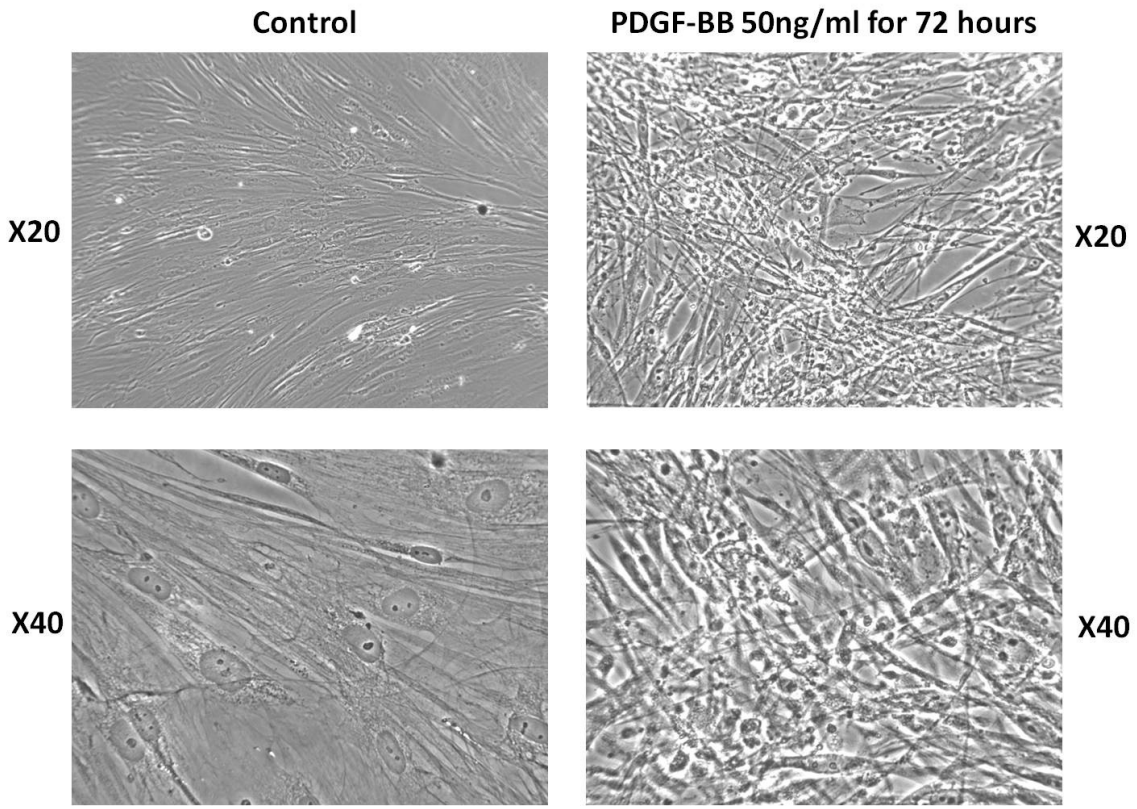



**Figure 6.1**. Morphology of human pulmonary smooth muscle cells with and without PDGF-BB stimulation.

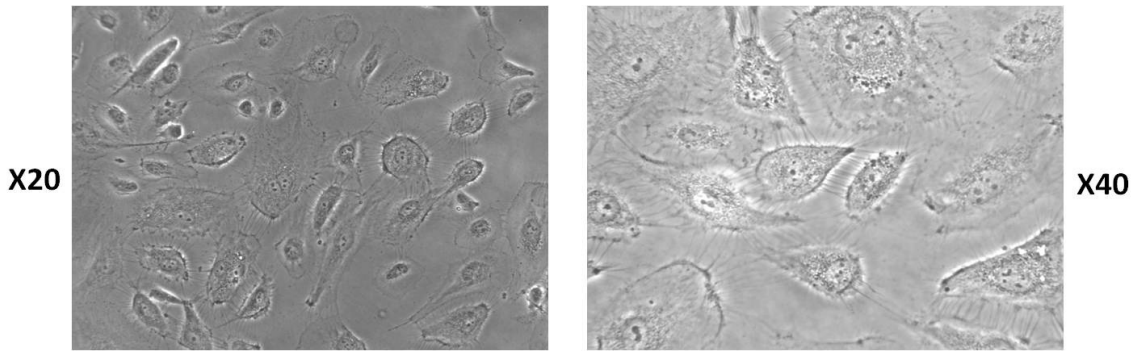



**Figure 6.2**. Morphology of human pulmonary arterial endothelial cells.

# 4. Result

## 4.1. Human pulmonary smooth muscle cell (HPSMCs)

### 4.1.1. VPA and SAHA inhibit stimulated proliferation

Exposure to hypoxia for 72 hours stimulated HPSMC proliferation by over 30% ($p<0.05$) and this rise was inhibited by both VPA and SAHA in a dose-dependent trend based on cell counts. The low doses of both bought cell counts back to the baseline (VPA, $p<0.005$; SAHA, $p<0.05$) while high doses seemed to further reduced proliferation ($p<0.005$, Figure 6.3A).

Stimulated cell proliferation with PDGF-BB (50ng/ml) for 72 hours lead to a 80% increase in cell counts ($p<0.05$). The increase was attenuated by VPA or low dose SAHA while high dose SAHA further reduced the proliferation ($p<0.005$, Figure 6.3B).

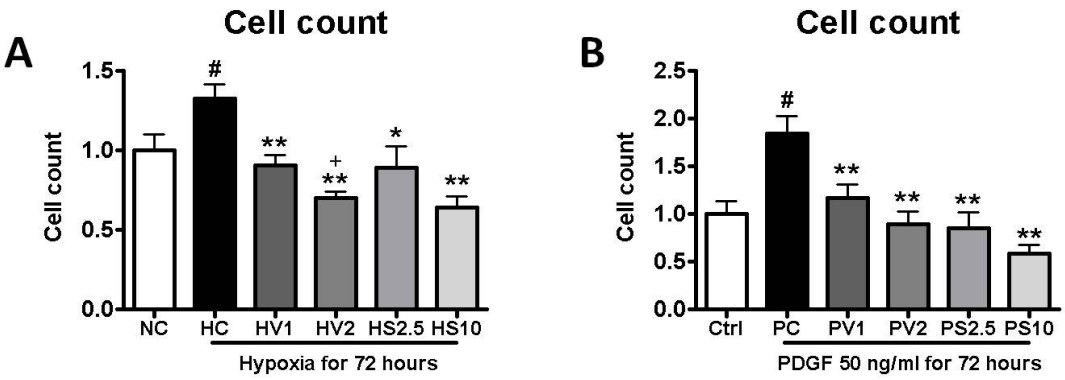



**Figure 6.3**. Effect of histone deacetylase inhibition on stimulated proliferation of human pulmonary smooth muscle cells based on cell counts. (A) hypoxia-induced proliferation, (B) PDGF-induced proliferation. NC: normoxia control; HC: hypoxia control; HV1: hypoxia+VPA 1mM; HV2: hypoxia+VPA 2mM; HS2.5: hypoxia+SAHA 2.5µM; HS10: hypoxia+SAHA 10µM; Ctrl: control; PC: PDGF control; PV1: PDGF+VPA 1mM; PV2: PDGF+VPA 2mM; PS2.5: PDGF+SAHA 2.5µM; PS10: PDGF+SAHA 10µM. The data are presented as mean± SEM of fold change relative to NC or Ctrl. $n\geq4$. * $p<0.05$, ** $p<0.005$ compared with HC or PC, + $p<0.05$ compared with HV1, # $p<0.05$ compared with NC or Ctrl.

To further confirm the effects of these two HDAC inhibitors on stimulated HPSMC proliferation, WST-1 and BrdU incorporation assay were employed. WST-1 assay showed the PDGF-induced proliferation ($p<0.05$) was moderately inhibited by VPA or SAHA, no significantly dose-dependent effects in VPA ($p<0.005$, Figure 6.4A). BrdU assay revealed a two-fold increase induced by PDGF, which was mildly attenuated by VPA ($p<0.05$) and SAHA arrested cell growth at the baseline compared with control group, both in a dose-dependent manner ($p<0.05$, Figure 6.4B).

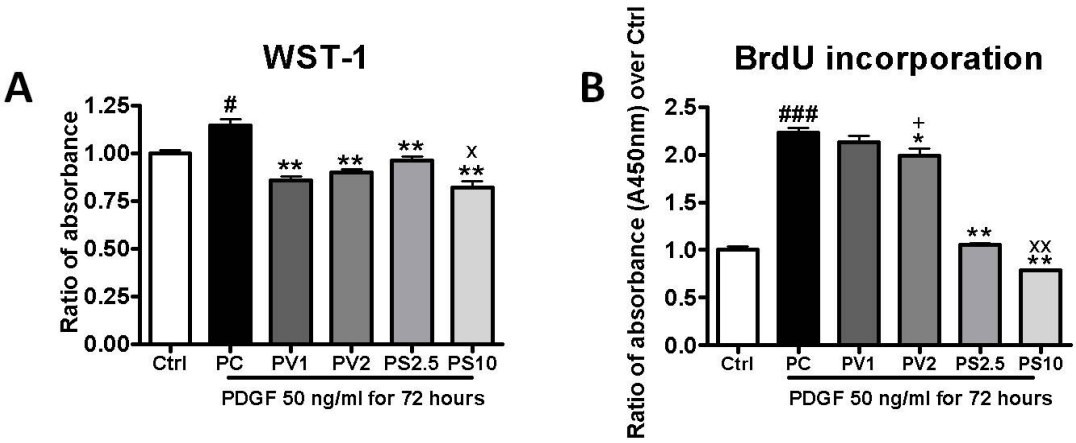



**Figure 6.4**. Effect of histone deacetylase inhibition on PDGF-stimulated human pulmonary smooth muscle cells in culture. (A) WST-1 assay, (B) BudU incorporation. Ctrl: control; PC: PDGF control; PV1: PDGF+VPA 1mM; PV2: PDGF+VPA 2mM; PS2.5: PDGF+SAHA 2.5μM; PS10: PDGF+SAHA 10μM. The data are presented as mean± SEM of fold change relative to Ctrl. n=8. * $p<0.05$, ** $p<0.005$ compared with PC, + $p<0.05$ compared with PV1, x $p<0.01$ compared with PS2.5, # $p<0.05$, ### $p<0.0005$ compared with Ctrl.

### 4.1.2. SAHA leads to cell death

Viability staining indicated that exposure to SAHA led to an increase in proportion of PI stained cells (6.9~8.8%) representing dead or dying cells, while no significant change was observed in the VPA treated group.

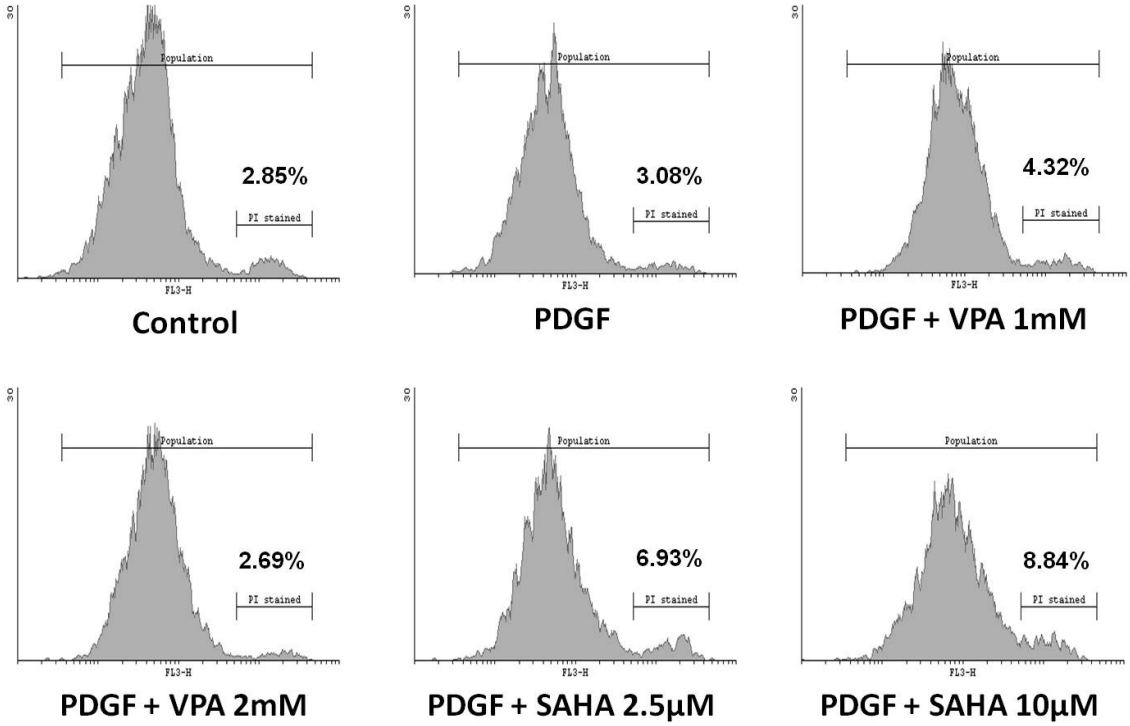



**Figure 6.5**. FACS analysis of viability staining with PI in HPSMC stimulated with PDGF 50 ng/ml for 72 hours, with or without VPA or SAHA treatment. The calculations of defined areas were done by software Cyflogic v1.2.1. Each experiment was repeated at least 3 time3 with separate cell preparations.

### 4.1.3. VPA and SAHA arrest cell cycle at G1/S phase

FACS analysis revealed that both VPA and SAHA arrested cell growth at the G1-S phase, reversing the shift caused by PDGF stimulation. The percentage of cells in G1 (PDGF: 55.6%) was higher in VPA (VPA 1mM: 63.4%; VPA 2mM: 69.0%, $p<0.05$) and SAHA treated cells (SAHA 2.5µM: 71.5%; SAHA 10µM: 71.4%, $p<0.05$), along with lower percentage of cells in G2/M phase in both VPA and SAHA treated groups (Figure 6.6).

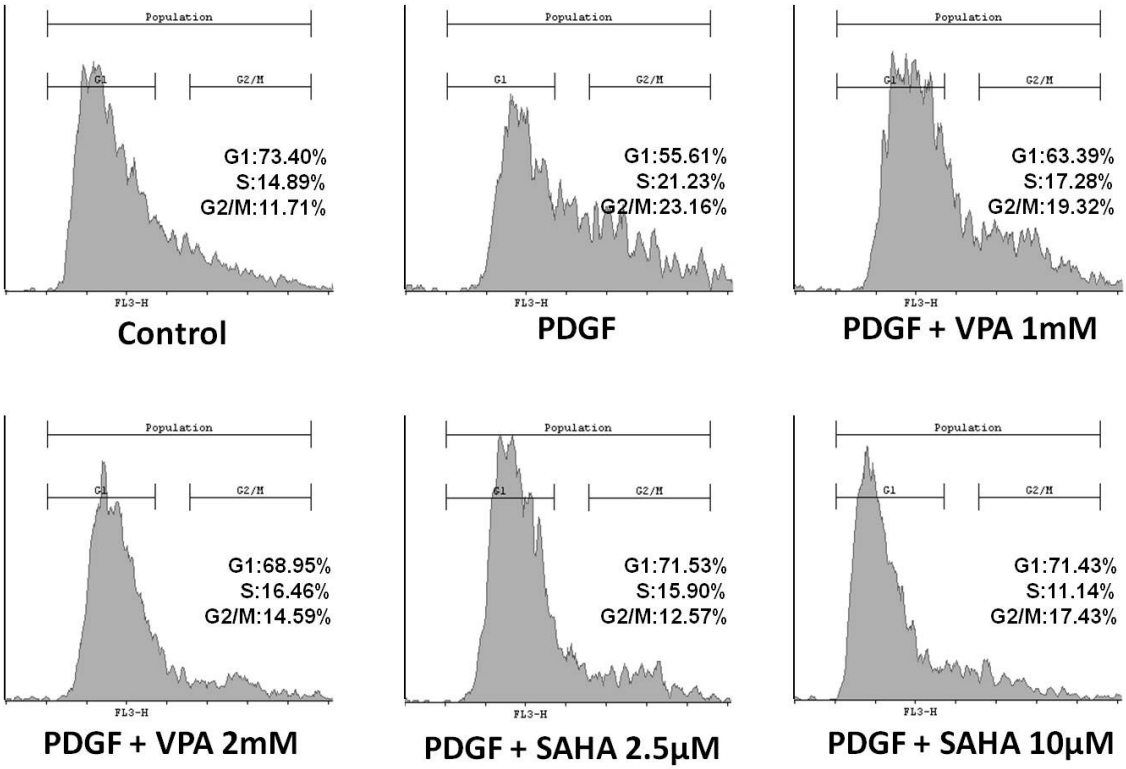



**Figure 6.6**. FACS analysis of cell cycle distribution in HPSMC stimulated with PDGF 50 ng/ml for 72 hours, with or without VPA or SAHA treatment. The calculations of defined areas were done by software Cyflogic v1.2.1. Each experiment was repeated at least 3 time3 with separate cell preparations.

### 4.1.4. Biochemistry

Biochemical analysis of cell lysates showed that Bcl-2 expression was induced by PDGF stimulation which was reduced by high dose of SAHA treatment (Figure 6.7A). Both VPA and SAHA led to increased p21 expression in cells in a seemingly dose-dependent manner (Figure 6.7B). Furthermore, SAHA treatment at 10 μM resulted in PARP cleavage to an 85kDa fragment, suggesting induction of apoptosis (Figure 6.7C).

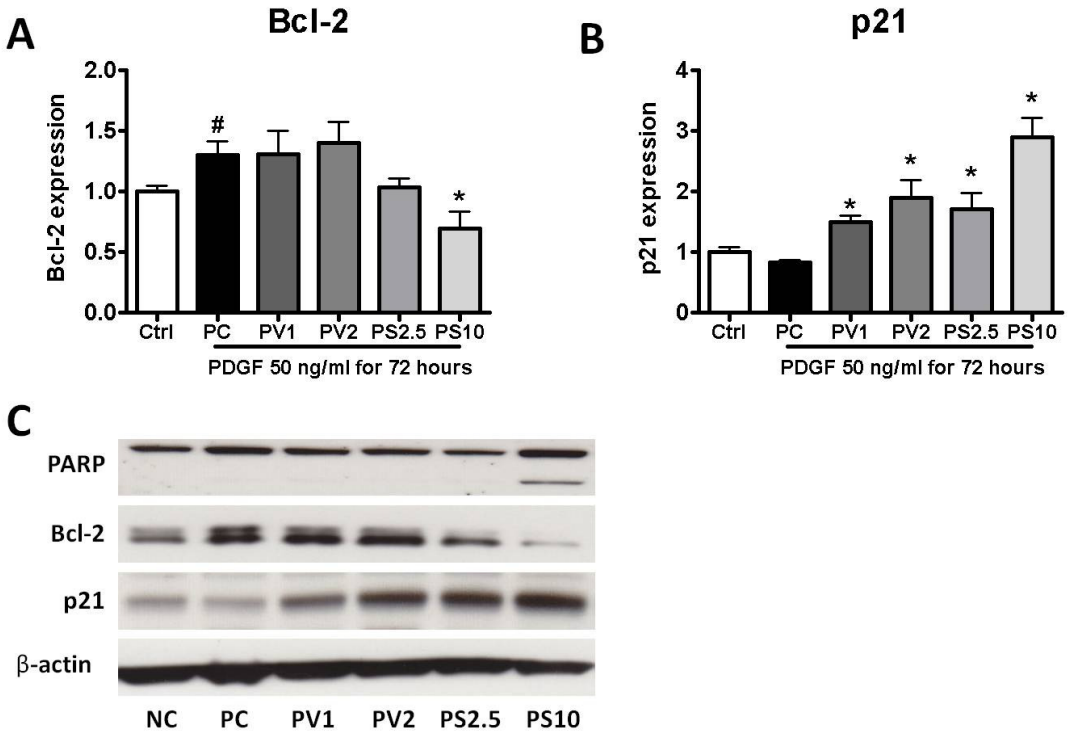
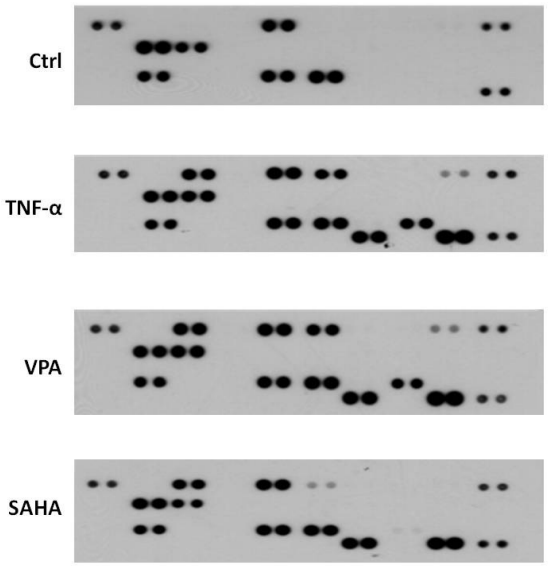



**Figure 6.7**. Protein expression levels in HPSMC lysates from PDGF stimulation study. (A) Bcl-2, (B) p21, (C) PARP and representative bands. Ctrl: control; PC: PDGF control; PV1: PDGF+VPA 1mM; PV2: PDGF+VPA 2mM; PS2.5: PDGF+SAHA 2.5μM; PS10: PDGF+SAHA 10μM. The data are presented as mean± SEM of fold change relative to Ctrl. Each experiment was repeated at least 3 times with separate cell preparations. * $p<0.05$ compared with PC, # $p<0.05$ compared with Ctrl.

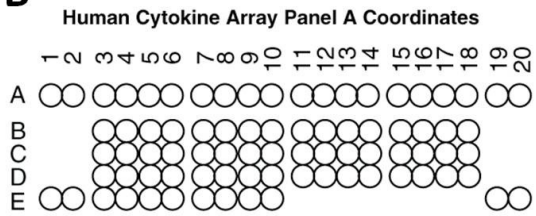
## 4.2. Human pulmonary artery endothelial cells (HPAECs)

Human cytokine array panel A was used to examine the effects of VPA and SAHA on inflammatory mediators release from endothelial cells in response to TNF-α (Figure 6.8).

A




B




C

| Coordinate | Target | Coordinate | Target |
|---|---|---|---|
| A1,A2 | PC | C7,C8 | IL-13 |
| A3,A4 | C5/C5a | C9,C10 | IL-16 |
| A5,A6 | CD40 ligand | C11,C12 | IL-17 |
| A7,A8 | G-CSF | C13,C14 | IL-17E |
| A9,A10 | GM-CSF | C15,C16 | IL-23 |
| A11,A12 | CXCL1 | C17,C18 | IL-27 |
| A13,A14 | CCL1 | D3,D4 | IL-32α |
| A15,A16 | sICAM-1 | D5,D6 | IP-10 |
| A17,A18 | IFN-γ | D7,D8 | CXCL11 |
| A19,A20 | PC | D9,D10 | MCP-1 |
| B3,B4 | IL-1$^{\alpha}$ | D11,D12 | MIF |
| B5,B6 | IL-1β | D13,D14 | MIP-1α |
| B7,B8 | IL-1ra | D15,D16 | MIP-1β |
| B9,B10 | IL-2 | D17,D18 | Serpin E1 |
| B11,B12 | IL-4 | E1,E2 | PC |
| B13,B14 | IL-5 | E3,E4 | RANTES |
| B15,B16 | IL-6 | E5,E6 | SDF-1 |
| B17,B18 | IL-8 | E7,E8 | TNF-α |
| C3,C4 | IL-10 | E9,E10 | sTREM-1 |
| C5,C6 | IL-12 p70 | E19,E20 | NC |

Abbreviations: PC, positive control; C5/C5a, complement factor 5/5a; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; CXCL, chemokine (C-X-C motif) ligand; CCL, chemokine (C-C motif) ligand; sICAM-1, soluable intercellular adhesion molecule-1; IFN-γ, interferon-gamma; IL, interleukin; IP-10, IFN-γ-induced protein 10; MCP-1, monocyte chemotactic protein-1; MIF, macrophage migration inhibitory factor; MIP, macrophage inflammatory protein; RANTES, regulated by T cells, activation upon secretion; SDF, stromal cell-derived factor; TNF-α, tumour necrosis factor alpha; sTREM-1, soluble triggering receptor expressed on myeloid cells-1; NC, negative control.

**Figure 6.8**. Cytokine levels detected by cytokine array in culture supernatants of TNF-α-stimulated human pulmonary arterial endothelial cells. (A) Array data on developed X-ray films, (B) Array coordincates, (C) Reference to represented cytokine and chemokine. The experiment was duplicated with separate cell preparations. Ctrl: control; TNF-α: TNF-α without HDAC inhibitors; VPA: TNF-α with VPA 2mM; SAHA: TNF-α with SAHA 10μM.

Several inflammatory mediators were induced by TNF-α, including IP-10, IL-2, IL-6, IL-10, IL-12 p70, IL-13, IL-16, IL-17E, IL-23, IL-27, RANTES, CCL1, CXCL11, sICAM-1, G-CSF, GM-CSF, INF-γ, C5/C5a and GROα. The most eminent elevations were shown IP-10 and RANTES with ~45-fold increase. Exposure to SAHA led to a broad-spectrum inhibition of most of these mediators, especially in IL-2, IL-6, IL-16, IL-12 p70, IL-17E, IL-23, IL-27, CCL1, INF-γ and C5/C5a, in which SAHA nearly reversed the TNF-α-induced elevations to the baseline level compared with control group. SAHA also led to a prominent reduction in IP-10 (from 45-fold to 7-fold) and moderated reduction in others like RANTES, sICAM-1, GM-CSF, CXCL11, IL-10 and IL-13 (selected results shown in Figure 6.9 and 6.10).

Interestingly, effects of VPA seemed to be more diverse. Treatment with VPA prevented the elevation of IP-10, IL-2, IL-6, IL-10, IL-17E, IL-23, IL-27, RANTES, CCL1, and CXCL11. In contrast, VPA leads to further increase in G-CSF, GM-CSF, IL-12 p70, IL-13, INF-γ and C5/C5a (selected results shown in Figure 6.9 and 6.10).

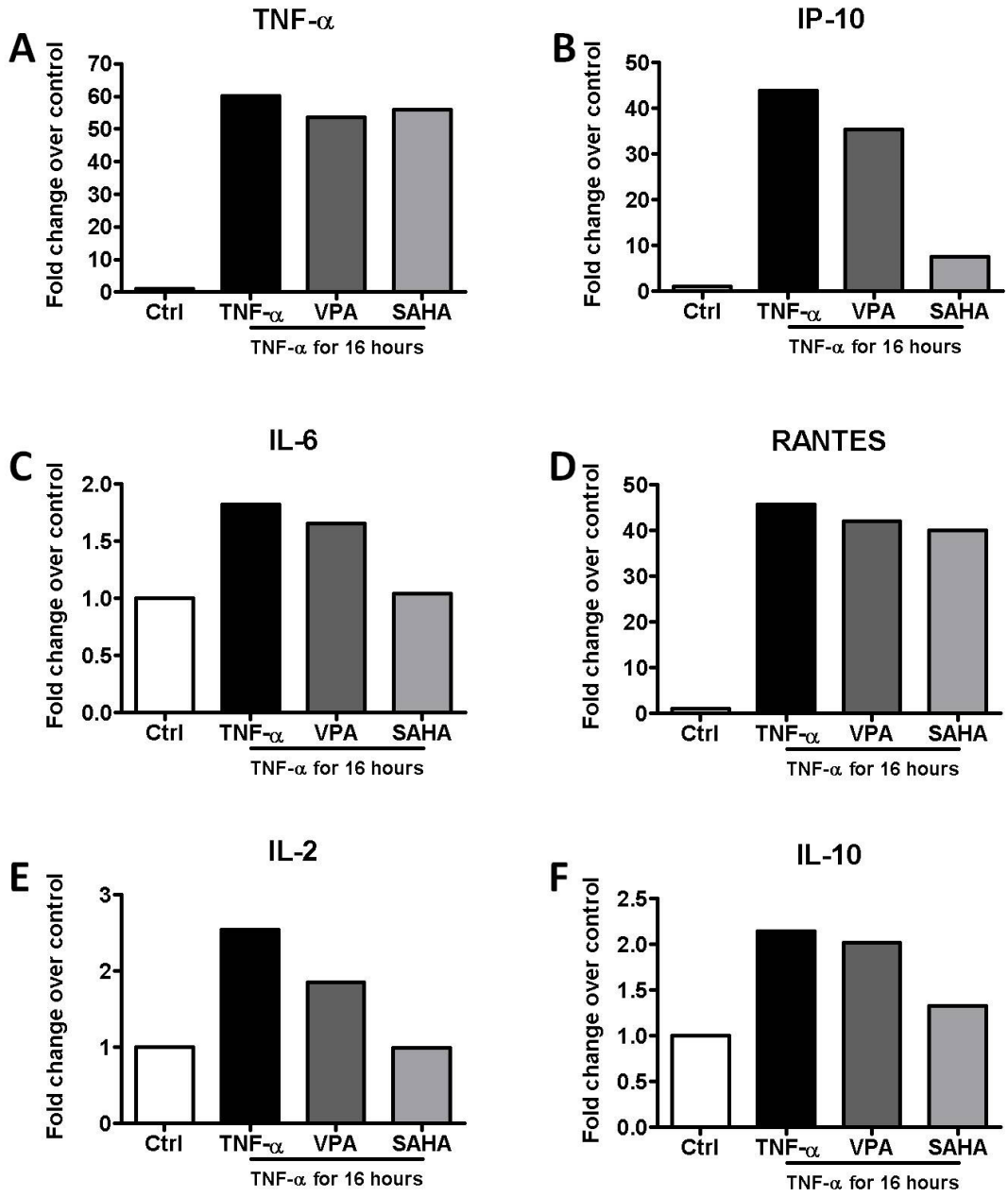



**Figure 6.9**. Cytokine levels detected by cytokine array in culture supernatants of TNF-α-stimulated human pulmonary arterial endothelial cells. (A) TNF-α, (B) IP-10, (C) IL-6, (D) RANTES, (E) IL-2, (F) IL-10. Data are presented as fold change relative to Ctrl. The experiment was duplicated with separate cell preparations. Ctrl: control; TNF-α: TNF-α without HDAC inhibitors; VPA: TNF-α with VPA 2mM; SAHA: TNF-α with SAHA 10μM.

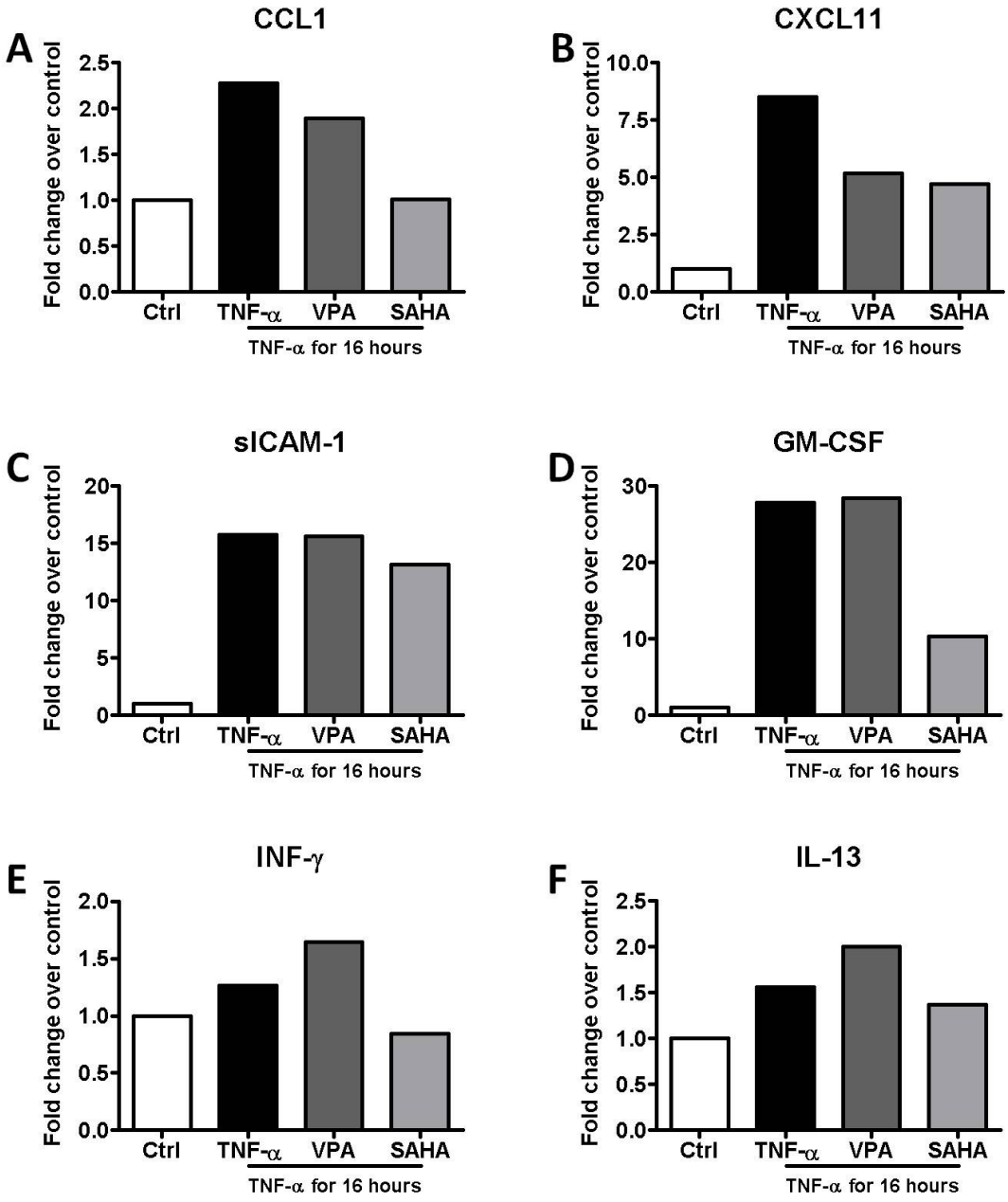



**Figure 6.10**. Cytokine levels detected by cytokine array in culture supernatants of TNF-α-stimulated human pulmonary arterial endothelial cells. (A) CCL1, (B) CXCL11, (C) sICAM-1, (D) GM-CSF, (E) INF-γ, (F) IL-13. Data are presented as fold change relative to Ctrl. The experiment was duplicated with separate cell preparations. Ctrl: control; TNF-α: TNF-α without HDAC inhibitors; VPA: TNF-α with VPA 2mM; SAHA: TNF-α with SAHA 10μM.

# 5. Discuission

The *in vitro* experiments using HDAC inhibitors in HPSMC and HPAEC provided the following findings:

- VPA and SAHA treatment inhibited stimulated HPSMC proliferation.
- SAHA treatment induced apoptosis in HPSMC.
- VPA and SAHA treatment arrest cell cycle at G1/S phase in HPSMC.
- VPA and SAHA treatment significantly decreased Bcl-2 and increased p21 expression in HPSMC.
- VPA and SAHA treatment inhibited the release of several inflammatory mediators from HPAEC stimulated by TNF-α.

## 5.1. VPA and SAHA inhibit stimulated proliferation of HPSMCs

Hyper-proliferation of pulmonary artery smooth muscle cells is a fundamental character in vascular remodelling. PDGF-stimulated smooth muscle cell growth is believed to take part in the remodelling process of PAH [278]. On the other hand, hypoxia also promotes HPSMC growth in cultured system, but the role of hypoxia-induced smooth muscle cell proliferation is relatively controversial in PAH since whether hypoxic stimuli are required for the development of human PAH is undetermined. In my study, VPA and SAHA inhibited both PDGF- and hypoxia-induced HPSMC proliferation in a dose-dependent trend. In BrdU assay, exposure to SAHA nearly blocked PDGF-stimulated cell growth in low dose and further reduced cell number in high dose while VPA only caused mild inhibition (-10%) of proliferation. In contrast, WST-1 assay indicated no visual difference between VPA and SAHA as both of them led to mild to moderate (~25%) inhibition compared with PDGF control. This discrepancy could be explained by the fact that BrdU incorporation directly measures DNA synthesis in proliferating cells while WST-1 evaluates viability based on metabolic activity of mitochondrial enzymes [279]. This finding also suggested different behaviours in regulation of proliferation between VPA and SAHA.

The differences remained in the viability staining with PI which showed that SAHA resulted in an increased cell death whereas VPA did not. The cytotoxic effect of high dose SAHA was furthered confirmed by protein analysis that showed only SAHA in high dose significantly decreased Bcl-2 expression and resulted in PARP cleavage. Unexpectedly, VPA did not influence Bcl-2 expression in HPSMCs as it did in hypoxic rat lungs, suggesting other alternative targets of VPA in pulmonary vascular remodelling.

A possible explanation is the induction of p21 in HPSMCs. Consistent with the results from animal studies, p21 expression was significantly increased by VPA and SAHA in dose-dependent trend. FACS analysis of cell cycle distribution showed that both of them arrested cell cycle at G1/S phase and prevented cells from entering mitosis. Collectively, the anti-proliferative effects on HPSMC derived from SAHA can be attributed to inhibition of cell growth by p21-regulated cell cycle arrest and simultaneously induction of apoptosis. On the other hand, VPA may inhibit cell proliferation by p21-regulated cell cycle arrest only but not induction of apoptosis. This conclusion agrees with previous studies that HDAC inhibition strongly activates the expression of p21 and therefore arrests cell proliferation in many cancer cell lines [280]as well as other studies proved this HDAC-p21-growth arrest signalling in other cells, including vascular smooth muscle cells in neointimal formation [226]and fibroblasts in rheumatoid arthritis [281]. This finding also fits in with the results from animal experiments, consolidating the role of p21 in response to HDAC inhibition in pulmonary vascular remodelling.

Concern may be arisen that HDAC inhibitors commonly have selective cytotoxicity in most cancer cells, which was not strongly demonstrated in the PDGF-stimulated cell systems. Induction of apoptosis is believed a major part of the anti-neoplastic capacities of HDAC inhbitors. Nevertheless, the challenge is that PDGF stimulation in the in vitro setting is not equivalent to cancerous transformation. Caution has to be taken in interpreting information gained from these cellular systems.

## 5.2. VPA and SAHA inhibit inflammatory mediators release from HPAECs

Consistent with the results that VPA and SAHA inhibited inflammatory mediators release in MCT model, in TNF-α-stimulated endothelial cells, these two HDAC inhibitors generated a broard-spectrum inhibition of pre-inflammatory transduction. HPAEC stimulated by TNF-α released a wide range of cytokines and chemokines, among which IL-6, IL-2, IL-10, IL-12, IL-13, IP-10 and RANTES have been previously shown to be associated with human PAH. Especially, overexpression of IL-6 has been repeatedly reported in IPAH patients, animal models and cellular systems [94-96] and thought to have a role in the pathogenesis. Overexpressed IL-6 can induce smooth muscle cell proliferation and endothelial cell apoptosis through multiple pathways, such as VEGF up-regulation, BMPR2 downregulation and TGF-β signalling. Furthermore, IL-6 signalling leads to recruit inflammatory cells, such as lymphocytes and monocytes, by stimulating release of other cytokines like fractalkine. The inflammatory infiltration provides a major source of sustained IL-6 and further induces IL-6 release from vascular cells [282]. Lowering IL-6 levels has been shown clinical relevance in PAH patients [88]and considered as a novel therapeutic target. In my study, treatment with SAHA efficiently prevented the release of IL-6 while VPA did mildly that indicates HDAC inhibition may have anti-inflammatory effects on stimulated endothelium. Actually, HDAC inhibition has been reported to disrupt IL-6 production in autoimmune disease.

Another interesting target is RANTES, a chemokine attracting T cells, monocytes, basophils, eosinophils and natural killer (NK) cells. Elevated expression of RANTES mRNA, originated from pulmonary endothelium, is demonstrated in patients with severe PAH [90]. In my study, VPA and SAHA both reduced the rise of RANTES released from endothelial cells, suggesting RANTES might be the therapeutic target connected to HDAC inhibition.

The manipulation in IP-10 release observed in MCT model was supported by the finding that VPA and SAHA spectacularly blocked the induced-release from endothelial cells. The TNF-α-induced secretion of soluble intercellular adhesion molecule-1 (sICAM-1), also known as CD54, was attenuated by SAHA treatment. This downregulation may

contribute to prevent leukocytes from binding to endothelial cells and transmigrating into lung tissues [283]. Collectively, the result implicates a role of anti-inflammatory effects in pharmacological HDAC inhibition.

Noticeably, while SAHA was revealed consistently prominent inhibition of the release of most cytokines and chemokines in the study, the effect of VPA was less potent and relatively diverse. In some inflammatory mediators, including IL-6, IL-2, IL-10, IP-10 and RANTES, VPA treatment was displayed inhibitory effects, to a less degree compared with SAHA. However, in others, VPA had no effects, such as sICAM-1, or even reversely induced their release, which were INF-γ, G-CSF, GM-CSF, IL-12, IL-13 and C5/C5a. The probabilities can be interpreted by that, firstly, VPA owns multiple functions in addition to HDAC inhibition. Despite of the fact that VPA has been shown beneficial in clinical neurology and psychiatry, the current identified mechanisms of its action cannot provide satisfactory explanations and unknown underlying remains to be explored. Secondly, the class-specific of HDAC inhibition may vary the pharmacological effects. Recently, great efforts have been engaged into the development of isoform-specific HDAC inhibitors with expectation of more potency and less side effects.

The effects of VPA and SAHA on endothelial proliferation were not evaluated in this thesis. Hyper-proliferative endothelial cells are manifested in PH in a hypothetical complex process, which an initial loss of endothelial cells occurs before the following exuberant proliferation of the surviving cells [284]. This process ends up selecting apoptosis-resistant proliferative vascular cells, forming the plexiform lesions. Experimental induction of endothelial apoptosis by VEGF receptor blocker SU5416 combined with chronic hypoxia generates severe PH [72]. Accordingly, using the cellular system to examine the pharmacological effects on endothelial proliferation may be insufficient and has to be interpreted carefully.

# Chapter 7:

# CONCLUSIONS AND FUTURE PLANS

# 1. Conclusions

## 1.1. Pulmonary hypertension (PH)

PH remains a life threatening disease without a cure, characterised by vasoconstriction, vascular remodelling, thrombosis and inflammation. New thinking argues that pulmonary vascular remodelling resembles a "neoplastic-like" vascular pathology, and may originate from an imbalance between proliferation and apoptosis, in favour of a pro-proliferative property in PH. Currently approved therapeutic options for PH are limited and primarily target the dysregulation of vessel tone. Although these treatments succeed in functional improvement and may affect the natural course of the condition, termination or reversal of the disease progression is still not achieved. New strategies aimed directly at the structurally disorganized remodelling are urgently required.

## 1.2. Histone deacetylase (HDAC)

Epigenetic modifications regulate gene expression and affect a variety of physiological and pathological events. Histone acetylation, manipulating chromatin structure and transcriptional activity, plays a key role in proliferative and inflammatory disorders, especially cancer. Aberrant recruitment of HDACs to certain genomic regions is responsible for cancerous transformation by repressing tumour suppressor genes and enhancing oncogenic transcription. Apart from modifying histone on chromatin, HDACs can act directly on non-histone proteins and dominate downstream signalling transduction. HDAC inhibitors offer therapeutic benefit in cancer and suggest potential in other proliferative disorders, including PH.

## 1.3. HDAC inhibitors in PH

This thesis commenced with the finding of altered HDAC expression in lung tissue from PH patients and animal models. Specifically, the expression of HDAC1 and HDAC5 were increased along with pro-survival factor Bcl-2 in lung tissues from patients with IPAH. Elevated expression of these three enzymes was also found in lung and RV tissues from rats with chronic hypoxia-induced PH. Histological assessment revealed that

overexpressed HDAC1 and HDAC5 were localized to the vascular walls and associated with vascular muscularization in humans and rats. The levels of HDACs in rats correlated with the disease development, as measured by elevated PAP and hypertrophied RV. In chronic *in vivo* experiments, inhibition of HDACs by VPA and SAHA ameliorated and partially reversed the PH phenotype in two animal models, hypoxia and MCT. The molecular mechanisms accounting for the anti-vascular remodelling effects were partially related to anti-proliferation in vascular cells, evidenced by downregulation of Bcl-2, upregulation of p21 and downregulation of survivin, both Bcl-2 and p21 as survival markers and therapeutic targets in cancer [243, 285, 286]. Increased histone acetylation support the claim that administration of these two inhibitors in animals decreased HDAC activity. These findings are consistent with observations in cancer and suggest that the pathology of PH can be modified by HDAC inhibitors. The *in vitro* experiments showed that VPA and SAHA modulated Bcl-2 and p21 in human pulmonary artery smooth muscle cells and inhibited stimulated cell growth, which suggest that VPA and SAHA can target directly pulmonary vascular remodelling. Furthermore, these two inhibitors also inhibited inflammatory mediator release in MCT models and human pulmonary artery endothelial cells, suggesting anti-inflammatory effects might be another contributor to their pharmacological effects. Collectively, these results provide a preclinical basis to support the therapeutic potential of HDAC inhibitors as a PH treatment.

# 2. Discussions

In this study, biochemical observations suggest that the therapeutic effects of VPA and SAHA are associated with anti-proliferation and anti-inflammation. These findings are consistent with current understanding of HDAC inhibition in cancer and inflammatory disorders [202, 287].

## 2.1. HDAC inhibitors: anti-proliferative effects

The therapeutic effects of HDAC inhibitors in malignant disease are based on their potential to inhibit cell proliferation and induce apoptosis. The anti-proliferative effects are conducted through multiple interacting pathways. Among them, p21 is one of the most studied factors, commonly upregulated in association with HDAC inhibitor-mediated cell

cycle arrest. Treatment with HDAC inhibitors increases p21 expression in parallel with hyperacetylation of histone H3 and H4 in its promoter region in various cancer cell types [174]. This upregulaion of p21 is generally independent of p53. In the normal situations, p21 is an essential guardian to maintain genetic stability by arresting cell cycle to allow damaged DNA to undergo repair and initiate programmed cell death when damaged. Among all the HDACs, HDAC1 is believed the most important in the regulation of proliferation [248]. Deficiency of HDAC1 leads to lethal growth retardation in mice embryo and reduced proliferation rates in embryo stem cells, coinciding with elevated expression of CDK inbhitors, not only p21 but also p27. The loss of HDAC1 induces concomitant increased expression of other class I HDACs (HDAC2 and HDAC3), but the compensation is inadequate [248, 288]. This indicates that HDAC1 has a pivotal function in regulating proliferation by its effects on cell cycle gatekeepers. The HDAC inhibitor-cell cycle arrest is not exclusive to cancer cells but is also observed in vascular smooth muscle cells in *in vivo* models of vascular injury [226]. Consistently, in our PH models, HDAC1 expression increases in line with disease development and VPA and SAHA, both of which inhibit HDAC1, upregulate p21 and attenuate proliferative vascular remodelling.

During the course of this thesis, Stenmark et al have described the effect of HDAC inhibition in cellular models based on adventitial fibroblasts (PH-Fibs) and morphologically distinct cells of hematopoetic origin (R-cells), which were isolated from the adventitial and the medial layer of distal pulmonary arteries of chronically hypoxic claves [289]. Both cell types display a stable hyper-proliferative / apoptosis resistant phenotype, even under serum deprived ex-vivo conditions [237]. Class I HDAC catalytic activity is significantly elevated in PH-Fibs compared to fibroblasts from control group [229]. Recent unpublished data (in collaboration with our group) revealed that HDAC1 mRNA expression levels, but not HDAC2 or HDAC3 mRNA levels, are significantly increased in R-cells compared to smooth muscle cells from control animals. VPA and SAHA exert greater inhibitory effects on proliferation in both PH-Fibs and R-cells compared to control cells under serum starved condition, suggesting specific modulation of epigenetically altered signalling pathways in these cells. Simultaneously, these cells express significantly lower levels of p21 and FOXO3 (forkhead box O3) and higher levels of Bcl-2 and survivin compared to cells from control animals. Exposure to VPA or SAHA results in elevation of p21 and FOXO3 expression as well as reduced Bcl-2 and survivin in these

cells, further supporting the hypothesis that VPA and SAHA can target apoptosis-resistant remodelled cells in PAH.

On the other hand, my study suggests involvement of mitochondrial apoptotic factors in HDAC inhibitor-mediated effects on pulmonary vascular remodelling [185]. Retrieval of apoptotic function, evidenced by VPA and SAHA downregulation of Bcl-2 in lung tissue, would be expected to induce cell death and contribute to reversing vascular remodelling. Noticeably, the effects of VPA on induction of apoptosis seem to be less significant in the *in vitro* study, reflecting class- or isoform-specific impacts of HDAC inhibition on molecular mechanisms.

In addition to indirect manipulation of transcription by chromatin modification, HDAC inhibitors can directly affect the acetylation status of several proteins. For example, Romidepsin can activate tumour suppressor gene p53 and thereby enhance p21 signalling. The acetylation status of p53 is highly related to its transcriptional activity [177]. This was not examined in this thesis.

Another interesting pathway which might take part in HDAC inhibitor-mediated anti-proliferation is the inactivation of Akt. Akt, a serine/threonine protein kinase, also referred to as protein kinase B (PKB), has been extensively studied as a key pro-survival factor [290, 291] and implicated in many proliferative disorders [292]. Activation of Akt by phosphorylation boosts cells to overcome the checkpoints in the cell cycle by suppressing p21 [293, 294] and is also associated with upregulation of Bcl-2 [295]. Overactivity of Akt signalling causes a shift of vascular smooth muscle cells from a contractile to synthetic phenotype [296], leading to proliferation, migration and fibrosis, and thereby play a role in vascular remodelling and myocardial hypertrophy. Recent studies report that HDAC inhibition can downregulate Akt signalling through a histone acetylation-independent route [297]. Preliminary data from our lab has shown that VPA and SAHA inhibit Akt activity in lungs from chronically hypoxic rats (Huang, unpublished data). These data suggested a possible interplay of Akt/Bcl-2/p21 with HDAC inhibition, leading to anti-proliferative effects (Figure 7.1).

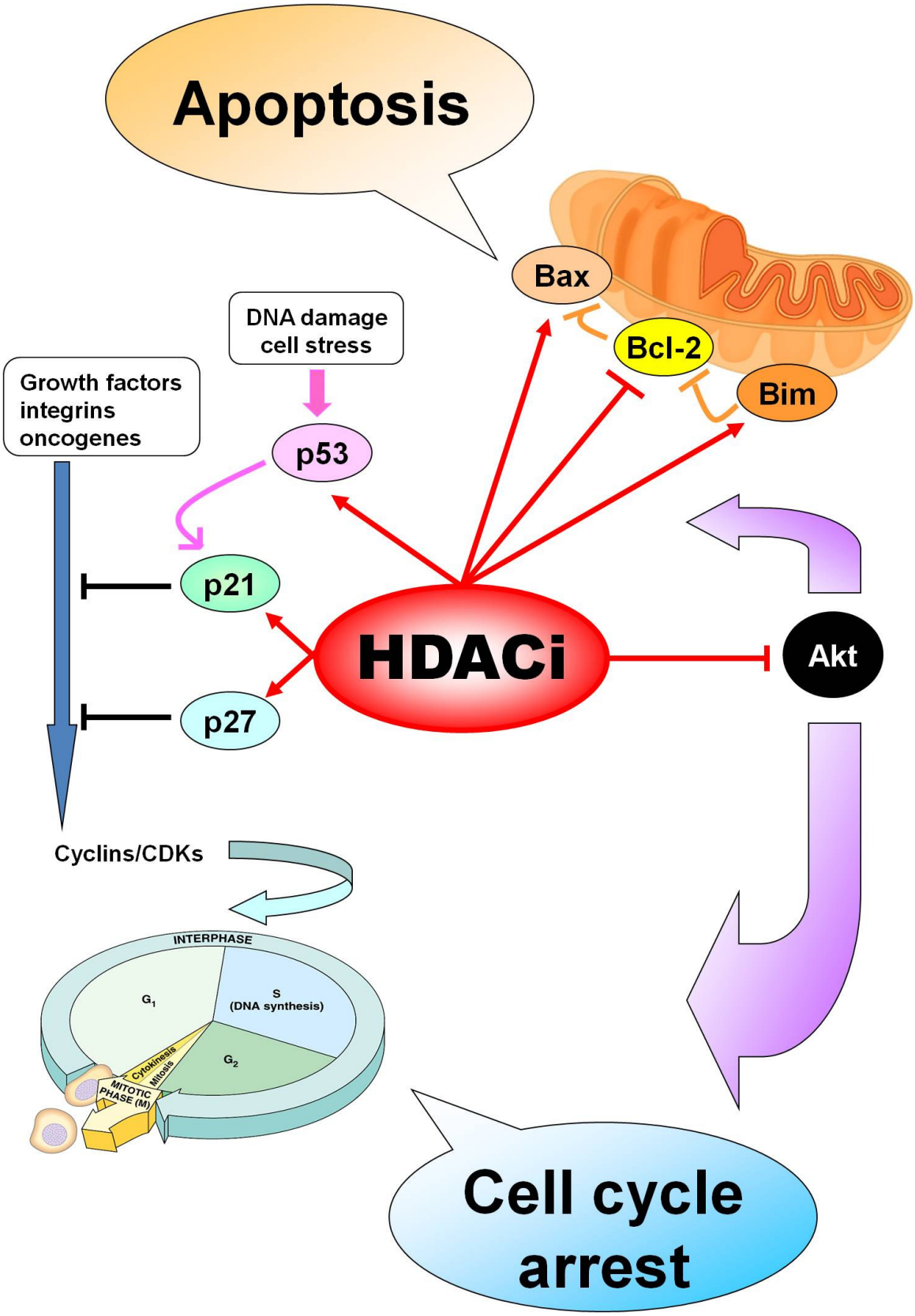



**Figure 7.1**. HDAC inibition exerts anti-proliferative effects via cell cycle arrest and apoptotic induction. HDACi: HDAC inhibitor [157, 294, 295, 297].

## 2.2. HDAC inhibitors: transformation-selectivity

HDACs are expressed in almost all the cell types and control the expression of a number of genes. This gives concern that global inhibition of HDACs may result in universal transcriptional activation and cytotoxic effects on normal cells. Surprisingly, experience to date suggests this is not a major issue.

First of all, HDAC inhibitors generally only alter a relatively small percentage of genes and proteins. Gene profiling analyses point out that HDAC inhibitors only affect 2-17 % of total genes, depending on the nature of HDAC inhibitors, concentration, time course, and most importantly, the cell types [298]. Global analysis of acetylated proteins in human cells using mass spectrometry revealed that less than 10 % out of 3600 lysine acetylation sites on 1750 proteins are changed in response to SAHA and MS-275 [299].

Second, transformed cells are more sensitive to HDAC inhibitor-induced anti-proliferative effects [161, 300]. HDAC inhibitor-upregulation of p21 arrests cell cycle at G1/S phase in transformed and non-transformed cells; however, normal cells are insensitive whereas transformed cells are susceptible to HDAC inhibitor-mediated apoptosis. The exact mechanisms are not determined yet, but might be hypothetically explained by exclusive signalling pathways or failure of defective rescue in cancer. Several molecular pathways specifically aberrant in transformed cells are targeted by HDAC inhibition, such as IGF/Akt, IL-6, TNF/ NF-κB and survivin anti-apoptotic pathways. Moreover, the cell cycle checkpoints may play a role in the selective cytotoxicity of HDAC inhibitors. Cells with checkpoint defects enter deviant mitosis and subsequent death through a compensatory bypass of mitotic spindle checkpoint when exposed to HDAC inhibitors, while cells with intact function of checkpoints are exempt from this lethal mitosis. Loss of checkpoint function may be the weak point of transformed cells in HDAC inhibitor-mediated cytotoxicity.

Third, normal cells seem to have higher capacity for recovery from HDAC inhibition during non-exposure intervals because the binding of HDAC inhibitors to target sites are rapidly reversible in most cases. A supportive observation is that patients receiving SAHA were found only a transient accumulation of acetylated histones in normal peripheral mononuclear cells without effect on leukocyte count [301].

Finally, although HDACs generally lead to compact chromatin, preventing transcriptional factors from accessing target genes, inhibition of HDACs does not necessarily promote global transcription. While activating transcription from certain genomic regions, other transcriptions can be repressed. For example, knoct-out of HDAC1 in mice embryonic stem cells upregulates 5% of total genes but downregulate 3% simultaneously [154]. This suggests HDAC inhibitors reform specific gene programmes rather than simply activate all promoters

## 2.3. HDAC inhibitors: anti-inflammatory effects

Anti-inflammatory properties have emerged as an important element of the therapeutic benefits of HDAC inhibitors. Their pleiotropic effects on angiogenesis and the immune system may not only be beneficial in inflammatory disorders but also contribute to their anti-neoplastic activity. In *in vivo* systems, HDAC inhibitors influence differentiation, survival and proliferation of distinct non-cancerous immune cell populations, both of innate and acquired immune systems [302]. This impact is relevant to pulmonary vascular remodelling in PAH. In my study, treatment with VPA or SAHA altered the release of several PH-relevant cytokines and chemokines, including IL-6, IL-2, IL-4, RANTES and IP-10. Similarly, the PH-Fibs from Stenmark et al displayed an epigenetically altered pro-inflammatory phenotype, defined by elevated expression of IL-1β, IL-6, MCP-1, stromal cell-derived factor-1 (SDF-1), and RANTES, which may contribute to chronic inflammatory reactions in the pulmonary vasculature [229]. Consistently, R-cells expressed higher levels of several pro-inflammatory factors such as MCP-1, IL-6 and SDF-1 [289], which were significantly reduced by VPA and SAHA treatment (unpublished data). Collectively, these results implicate the extensive involvement of HDACs in regulating pro-inflammatory activation which could be targeted therapeutically in PH. However, although HDAC inhibitors have been shown to be promising in preclinical studies and more and more compounds have entered clinical trials, currently approved agents, SAHA and Romidepsin, are only licenced for cutaneous T cell lymphoma. The fact that CTCL appears to be more sensitive to HDAC inhibitors than other solid tumours, including other cutaneous malignancies such as melanoma and skin squamous cell carcinoma [303], implicates a cell-specific effect and extrapolation to other diseases has to be carefully tested. But growing evidence suggests that anti-inflammation might have a more powerful

and critical role in therapeutic effects of HDAC inhibitors than what they have been thought.

## 2.4. HDAC inhibitors: class or isoform specificity

As the first attempt of using HDAC inhibitors in PH in this thesis, I chose VPA and SAHA, mainly because of their accessibility and clinical relevance. Covering different classes HDACs, these two inhibitors can provide a preliminary view of class-dependent differences. With the emerging roles of individual HDAC isoforms in cancer and other diseases, the importance of the development of isoform-specific HDAC inhibitors has attracted growing attention.

The functional pleomorphism in HDACs provides a plausible rationale for using selective HDAC inhibitors to target specific HDACs involved in pathological events and spare those responsible for protective effects. Ideally, selectively inhibition of a specific class or isoform of HDAC is expected to achieve higher efficacy and lower toxicity. However, clinical evidence supporting the concept that selective HDAC inhibitors are superior to pan-HDAC inhibitors is quite limited. One challenge is that HDACs work in a multiple interacting complex. Selective inhibition of asingle subtype of HDAC inevitably leads to compensatory reactions in other HDACs, which could be detrimental. Better understanding of the individual functions and interactions among HDCAs is required for the development of selective inhibitors. On the other hand, using selective inhibitors in preclinical studies helps to clarify these mysteries and optimize the choice for clinical use.

## 2.5. Novelty and value

The value of this study is that it is the first attempt to tackle PH from epigenetic insights. This study provides a new concept that epigenetics may have a role in the pathogenesis and help to unravel some important questions in PH. It also, for the first time, shows that two HDAC inhibitors currently in clinical use, VPA and SAHA, have promising effects in PH animal models. This treatment targeting pulmonary vascular remodelling may generate synergic effects when combined with conventional vasoactive therapies. More and more HDAC inhibitors with different specificity have been designated, and they can be a new category worthwhile taking into consideration as novel treatments for PH.

# 3. Limitations

A few limitations to this study have to be evaluated with caution. First, although the chronic hypoxia- and MCT-induced PH rat models have been shown to have practical value in evaluating treatments for PH, neither of them replicates the full spectrum of pathologies in human PAH. For example, these two models do not develop the human PAH form of plexifom lesions. Obviously, preventing or reversing the elevated PAP, hypertrophied RV and increased muscularization of pulmonary arteries in these models is not equivalent to "dissolving" the obstructive vascular lesions and plexogenic arteriopathy in human PAH.

Second, there is no evidence that the maximal pharmacological effects of these two inhibitors have been achieved. The relationship between dosage and effect were not well established in this study. The doses used here were based on cancer studies, which may not be appropriate for PH. On the other hand, side effects as well as morbidity are always major concerns in translational pharmacology. Although VPA and SAHA did not cause any appreciable adverse effects in the studies reported here, mild growth retardation in the treated groups was noted. Furthermore, VPA has multiple functions, which can mask the real effects of HDAC inhibition.

Third, HDAC activity is a central indicator of the efficacy of HDAC inhibitors, which is important for determining whether the phenotypical improvements truly result from HDAC inhibition. In this study, HDAC activity was assessed indirectly by acetylation status of total histone. Although this provides a high-level signal of HDAC activity, it does not reflect catalytic changes in individual HDACs. As a sum of total acetylated histone, it alone cannot present the lysine-specific modification, which may differ upon exposure to different inhibitors. Most importantly, increased histone acetylation is not necessarily the result of HDAC inhibition, but can be attributed to activation of HATs.

Moreover, cardiac function was not formally assessed. RV hypertrophy has conventionally been thought secondary to increased pulmonary resistance, but RV function can conversely affect the pulmonary circulation. In the study, seemingly more profound impacts on RV hypertrophy than PA pressure or vascular remodelling in treated groups suggest possible direct effects on RV.

The current experience of HDAC inhibitors in cancer treatment primarily refers to zinc-dependent HDACs (classes I, II and IV). Nevertheless, growing evidence has implicated the role of class III HDACs, sirtuins, which is exclusively of different homology to the other HDACs [304]. Sirtuins are linked to longevity, ageing processes, metabolism and vascular homeostasis, and have the potential in cancer [219, 305]. For example, Resveratrol, an activator of SIRT1, exerts anti-inflammatory, anti-oxidant and anti-proliferative effects in pulmonary vasculature and prevents MCT-induced PH in rats [306]. However, the functions of sirtuins were not assessed in this thesis.

Finally, evaluation of inflammatory responses in lung tissues and endothelial cells is inadequate to consolidate the regulatory function of VPA and SAHA on the immune system. Immune cells have to be involved.

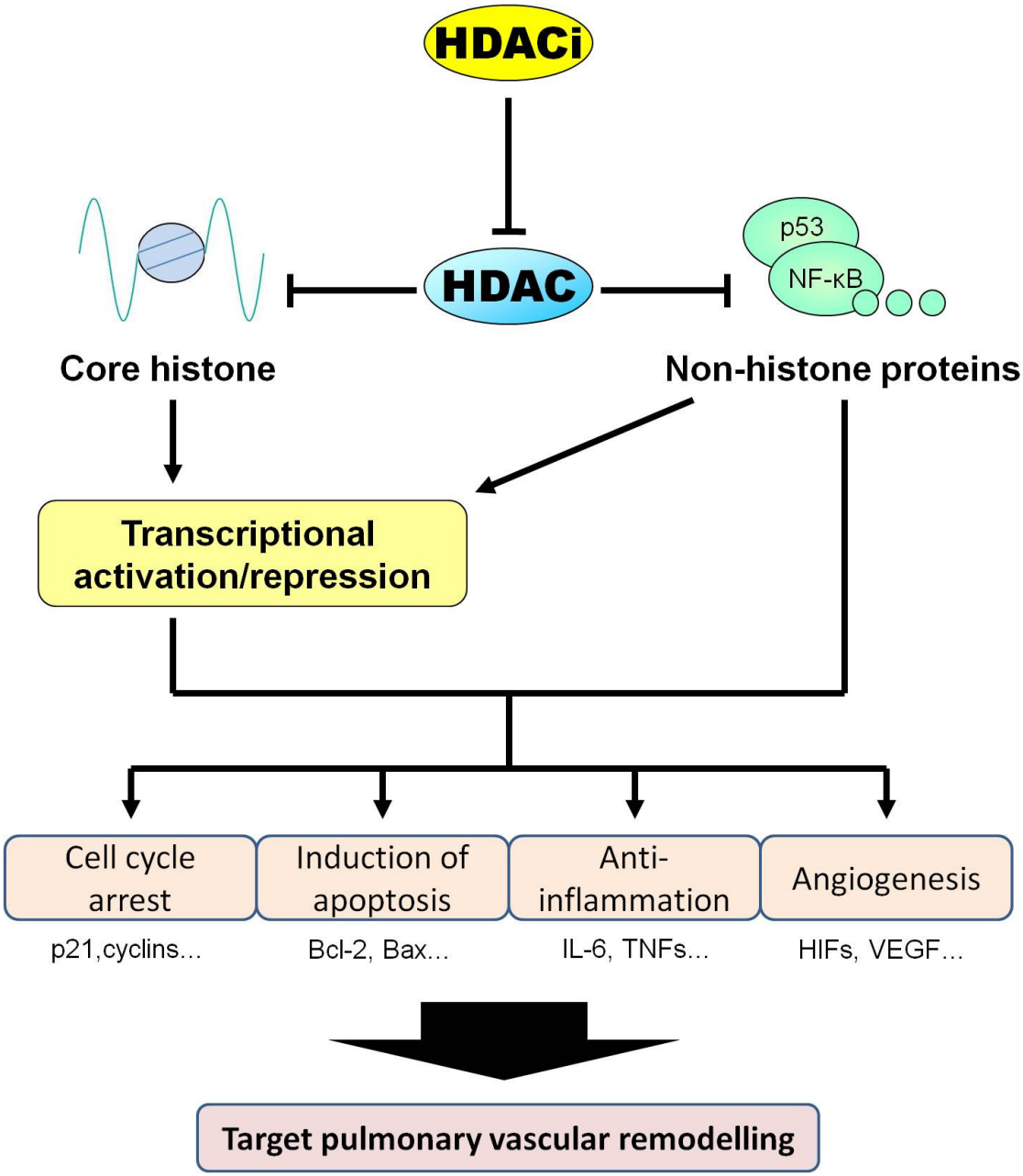



**Figure 7.2**. Summary of the study demonstrating that HDAC inhibitors target vascular remodelling. HDACi: HDAC inhibitor.

# 4. Future plans

- The possible molecular mechanisms are demonstrated by the downstream read-outs Bcl-2, p21 and survivin. A more precise connection between these pathways and histone acetylation can be established by certain techniques such as mass spectrometry, RNA array and chromatin immunoprecipitation. Insights into the genetic and molecular basis of HDAC inhibition will help to understand the pathogenesis of PH and guide to development of optimal therapeutic strategies.

- The class- and isoform-specificity of HDACs needs to be explored. As the functional roles of individual HDACs are clarified, more and more selective inhibitors are becoming available. Comparison of the pharmacological effects between HDAC inhibitors with different specificity as well as manipulation of certain subtype of HDACs, such as sirtuins, will also provide information on mechanistic insights of HDAC modifications.

- The impressive effects of VPA and SAHA on RV hypertrophy give a good rationale for examining RV function in detail, which can be achieved by isolated perfused heart system (Langendorff), cardiac output measurement and imaging approaches such as echocardiography.

- To further identify the link between HDAC inhibitors, PH and inflammatory reactions, cellular systems including immune cells and fibroblasts should be studied. Human peripheral leukocytes, which are relatively easy to be obtained, can be a useful tool to assess the immune responses in PH.

- Following the encouraging findings of this study and the fact that few HDAC inhibitors are safely used in clinic, the ultimate goal is to conduct a proper clinical trial with double blind design and randomized controls to investigate the therapeutic potential in human PH.

# PUBLICATIONS

**Papers:**

- L Zhao, **CN Chen**, N Hajji, E Oliver, TJ Huang, D Wang, M Li, T McKinsey, KR Stenmark, MR Wilkins. Histone deacetylation inhibition in pulmonary hypertension: therapeutic potential of valproic acid (VPA) and suberoylanilide hydroxamic acid (SAHA). *Circulation under revision*.
- **CN Chen**, MR Wilkins, L Zhao. Inhibition of histone deacetylation attenuates monocrotaline-induced pulmonary arterial hypertension in rats through immune modulation. (*drafted*)

**Book Chapter:**

- L Zhao, **CN Chen**, ZG Zhai, MR Wilkins. Pathobiology of pulmonary arterial hypertension. Book Chapter in Pulmonary Arterial Hypertension OCL Oxford Cardiology Library 2011

**Conferences:**

- **CN Chen**, MR Wilkins, N Hajji, L Zhao. Histone deacetylase inhibitor, valproic acid, prevents chronic hypoxia induced pulmonary arterial hypertension. CIMPP Annual Symposium, Imperial College London, London, UK, May 2010.
- **CN Chen**, MR Wilkins, N Hajji, L Zhao. Histone deacetylase inhibitor, valproic acid, attenuates chronic hypoxia induced pulmonary arterial hypertension. American Thoracic Society International Conference, Denver, USA, May 2011.
- **CN Chen**, MR Wilkins, N Hajji, D Wang, M Li, T McKinsey, KR Stenmark, L Zhao. Histone deacetylase inhibition pulmonary arterial hypertension: therapeutic potential of valproic acid (VPA) and suberoylanilide hydroxamic acid (SAHA). American Thoracic Society International Conference, San Francisco, USA, May 2012.

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