1. INTRODUCTION

1.1 Background

Pulmonary hypertension is a syndrome in which pulmonary arterial obstruction increases vascular resistance to blood flow. It is estimated that this disease affects upto 100 million people worldwide, but it is difficult to state an accurate figure, as people with pulmonary hypertension in places without developed healthcare may die before diagnosis (1). Talking about Indian situation, it is estimated that every year more than 150,000 people are diagnosed with pulmonary hypertension in India alone (1). Mortality rate of PAH remains high (15%) even though treatment alternatives with prostacyclin, endothelin antagonists, and phosphodiesterase 5 inhibitors are available (2). Moreover, the mortality rate is much higher in new incidences of PAH rather than in pre-existing cases of PAH. It is not surprising that the prognosis of PAH varies depending on the associated co-morbid conditions (2).

Pulmonary hypertension arises due to multitude of disease processes involving both the cardiac and respiratory systems. Most common causes include congenital heart disease with increased pulmonary blood flow, diaphragmatic hernia with associated lung hypoplasia, broncho-pulmonary dysplasia, idiopathic persistent pulmonary hypertension and hereditary transfer. Regardless of the cause, chronic pulmonary hypertension is associated with maladaptive changes in pulmonary vascular structure and function (remodelling). These changes prompt further rise in pulmonary arterial pressure, limit responses to vasodilator therapies, and if persistent, lead to death (3).

Pathogenesis of pulmonary arterial hypertension (PAH) involves multiple and complex mechanisms activated by endothelial dysfunction in the pulmonary bed which results into both active vasoconstriction and structural changes in the pulmonary vascular wall that include cellular hyperplasia and increased production and deposition of extracellular matrix (**Figure 1.1**) (4). Endothelial dysfunction in PAH is reflected by reduced production of the vasodilators/growth inhibitors, nitric oxide (NO), prostaglandin I_2 (PGI_2), and increased production of the vasoconstrictors like endothelin-1 and thromboxane A_2 (5). Pulmonary arterial hypertension (PAH) is now considered to be a vasculopathy in which structural changes driven by excessive vascular cell growth and inflammation have a major role.

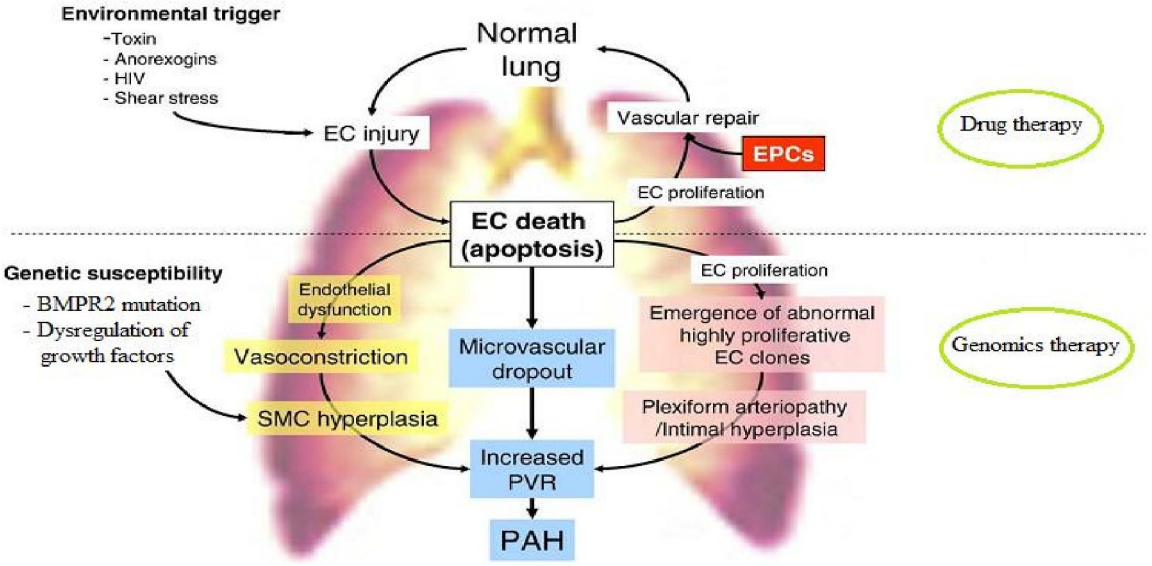

Figure 1.1 Pathophysiology of progression of PAH.

PAH is characterised by a shift in the proliferative/apoptotic balance and enhanced glycolytic metabolism (6). A major component of the pulmonary vascular remodelling process that leads to development of PAH is the proliferation of pulmonary artery smooth muscle cells (PA-SMCs) (7). Pulmonary vascular remodeling is characterised by thickening of all three layers of the blood vessel wall. Thickening is due to hypertrophy (cell growth) and/or hyperplasia (proliferation) of the predominant cell type within each of the layers (i.e., fibroblasts, smooth muscle cells, and endothelial cells), as well as increased deposition of extracellular matrix components (e.g., collagen, elastin, and fibronectin). Thickening of the media occurs consistently in arteries. These alterations in vascular structure are seen in both human pulmonary hypertension and animal models of the disease, and take place more rapidly than the remodeling of systemic arteries in systemic hypertension.

Several growth factors, including platelet derived growth factor (PDGF), fibroblast growth factor 2 (FGF2), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF) and, more recently, the non-canonical Wnt pathway have been implicated in the abnormal proliferation in pulmonary hypertension (**Figure 1.2**) (7). Growth factors are known to participate in vessel development. Endothelial/pericyte interactions are involved in the abnormal crosstalk between pulmonary endothelial cells (P-ECs) and pulmonary artery smooth muscle cells (PA-SMCs) during the progression of PAH which results into proliferation of PA-SMCs and antiapoptosis of these cells. Among the main growth factors expressed by ECs (PDGF, transforming growth factor-β-TGF-β, EGF, and FGF2), FGF2 was

released in excessive amounts by P-ECs. FGF2, a member of a large family of heparin binding growth factors (8), is synthesized by several cell types including tumor cells, fibroblasts, ECs and macrophages (9).

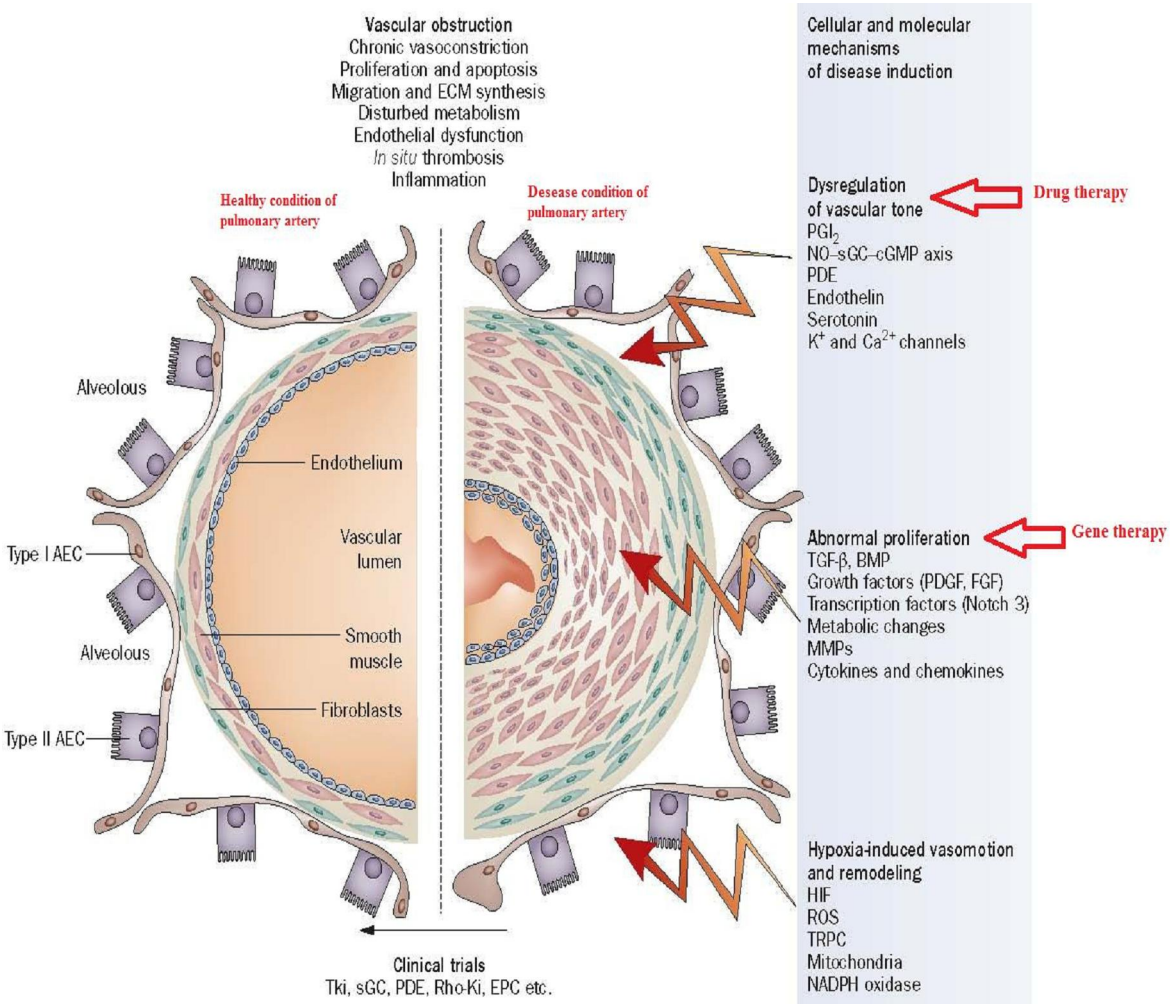

Figure 1.2 Role of vascular remodeling in the progression of PAH (10).

Currently there are three main classes of drugs are available for the treatment of this disease; prostanoids, endothelin-1 receptor antagonists, and phosphodiesterase-type 5 (PDE5) inhibitors. Epoprostenol was the first drug to be studied for treatment of PAH, and in addition to symptomatic improvement, it is the only treatment that has been shown to offer a survival advantage (10). However, its administration (intravenous, via an indwelling catheter) is complex and it carries the risk of line sepsis and rebound PAH from inadvertent interruption of infusion (11). Previous study reports of clinical trials suggested that other prostanoid analogues such as iloprost (inhalation), treprostinil (subcutaneous) and beraprost (oral) can provide only short-term benefits (12). Bosentan (endothelin receptor antagonist) is

also proved to be beneficial in patients with PAH, but its side effects like development of abnormal liver function, reduction of haemoglobin level require constant monitoring of liver function and haemoglobin level during therapy and its teratogenicity excludes its use in lactating women (13). Sildenafil is also associated with adverse effects like headache, flushing, upset stomach, nasal congestion, diarrhoea, urinary tract infection, rash or dizziness and decrease in systemic blood pressure (14). Unfortunately, there remains no cure for PAH and clinical worsening is unavoidable (15). Many patients with PAH also receive conventional therapy of warfarin, diuretics, digoxin and oxygen. Diuretics and digoxin provide symptomatic relief but are not thought to affect the course of the disease. Warfarin might provide a survival advantage but its contribution is difficult to estimate and its use is based on retrospective analysis (16). Calcium channel blockers, such as nifedipine, offer considerable benefit to the small number of patients that respond to them, although these account for only around 6% of patients (17).

A recent meta-analysis has been critical of the short-term nature of the studies that have investigated the efficacy of pharmacological treatments for PAH, calling into question the true benefit of the current treatments (18). Although they offer some hope for patients, they are not devoid of adverse effects and do not offer a cure as well. Whereas the time to clinical worsening of symptoms is increased, the progression of the disease is inevitable and improved treatments must be developed. Definitely, in clinical practice, combination therapy has become the default position even though trial evidence to support this strategy is limited. Small scale clinical evaluation of combinations of prostanoids, Endothelin-1 receptor antagonists and PDE5 inhibitors have been tried with some success (19), with additional studies currently recruiting [e.g. COMPASS-2 (sildenafil plus bosentan), STEP (iloprost plus bosentan)]; however, validation of these combination therapies will require further larger scale trials.

Current treatments available in the market for PAH are expensive and/or difficult to deliver and more palliative than curative as described earlier. These treatments may slow the progression of the disease but do not afford a cure. Advances in understanding of cellular pathways implicated in PAH pathophysiology has revealed various targets that can be used in treatment of PAH. Platelet derived growth factor (PDGF) and basic fibroblast growth factor (FGF2) has been shown to play a very significant role in development of pulmonary arterial hypertension. Thus, endothelial FGF2 and PDGF are identified as promising targets for new

treatments against PAH (7). This has dramatically changed the current approach for PAH treatment to more advanced and novel cell based approaches (i.e. siRNA delivery, antisense oligonucleotides etc.) that may target these factors and more directly act on the vascular changes like proliferation, antiapoptosis, immune mechanism and inflammation that impair blood flow through the pulmonary circulation and would treat pulmonary arterial hypertension in significant way.

RNA interference (RNAi) has been recognized as a general endogenous mechanism in many organisms to silence the expression of genes that control various events in the cell, as well as defend the cell from viral replication (20). Post-transcriptional gene silencing by RNA interference (RNAi) appears to be a promising new approach for the targeted inhibition of gene expression in cell culture and *in vivo*. It is also a cost-effective molecular biology tool for the determination of gene function, signalling pathway analysis and target validation shows tremendous potential for diagnostics and therapeutics. RNAi represents a promising new approach for producing gene-specific inhibition and knockouts, producing transgenic animal models, and designing new therapeutics for treatment of various diseases. This field is progressing at a very rapid pace and showing very promising results in treatment of number of diseases.

The expression of a specific gene can be regulated using different mediators like short hairpin RNA (shRNA), micro-RNA (miRNA), and synthetic or endogenous small interfering RNA (siRNA). Gene silencing by siRNA) includes its binding to corresponding mRNA and reversible knockdown of target mRNA (21). As an intracellular gene inhibitor, siRNA can degrade complementary mRNA with high specificity and potency (22). Numerous studies have demonstrated that introduction of siRNA into cultured cells can trigger highly efficient gene silencing through degradation of the endogenous mRNA. The mechanism of siRNA for gene silencing consists of three steps such as initiation step, effector step and inhibition step (23). The *in vivo* reversible knockdown of a specific gene by siRNA makes it a potential therapeutic agent in treating many diseases (24). Such siRNA-mediated degradation at the mRNA level substantially expands the “druggable target classes”, which have been traditionally limited to cell surface receptors, ion channels, or any cellular protein (25).

Since the discovery of RNAi, there has been increased interest from both industry and academia in siRNAs as potential new therapeutics (26). However, the less number of reports on *in vivo* siRNA delivery in literature is an outcome of lack of successful siRNA delivery.

Success of siRNA delivery necessitates formulation approaches to deliver siRNA more safely and efficiently with high robustness, convenience and patient compliance, regulation of pharmacokinetic and pharmacodynamic properties of administered siRNA, as well as adequate cost and risk–benefit ratio (23).

Although it is believed that gene-based therapies hold tremendous potential for the treatment, its use is hindered by failure to deliver therapeutic genes safely and conveniently in naked form. The site of action of siRNA therapeutics is the cytosol. There are multiple barriers to siRNA delivery which depend on the targeted organs and the administration routes. Following administration, the first biological barrier is the nuclease activity in blood and tissues (27). Naked siRNA does not readily cross the anionic cell membrane through passive diffusion due to the high molecular weight, large size, negative charge and hydrophilicity of the phosphate backbone (27). The success of genomics therapy is highly dependent on the delivery vectors that protect the therapeutic genomics from enzymatic attack, assist cellular internalization and deliver siRNA at the site of action where transcription takes place (**Figure 1.3**) (26). The endosomal entrapment and lysozomal degradation of siRNA–carrier contributes to the low transfection efficiency and is a major obstacle of siRNA delivery (27).

Although, viral vectors (Adenovirus, Adeno associated virus, Lentivirus) have emerged as attractive scientific strategy for exploiting natural mechanism (28), such systems suffer from inherent problems of effective pharmaceutical processing, oncogenicity, immunogenicity, scale up and possibility of reversion of engineered virus to wild type. Due to these safety and processing concerns with viral-vectors, many siRNA delivery studies focus on the development of non-viral vectors. Non-viral carrier systems have ability to target gene to specific cell type, to overcome extracellular and intracellular barriers and to provide high transfection efficiency and minimum toxicity, which is the prime goal in the field of gene therapy (29). These Non-viral vectors have greater control of their molecular composition for simplified manufacturing and analysis, flexibility in size of transgene to be delivered and relatively low immunogenicity (30). Various vectors for siRNA delivery have been developed, like monoclonal antibodies, cell-penetrating peptides, liposome (lipoplexes), polycations (polyplexes), dendrimers etc., but cationic lipoplex and polyplex systems has been widely used as transfection agents for *in vitro* as well as *in vivo* application (31).

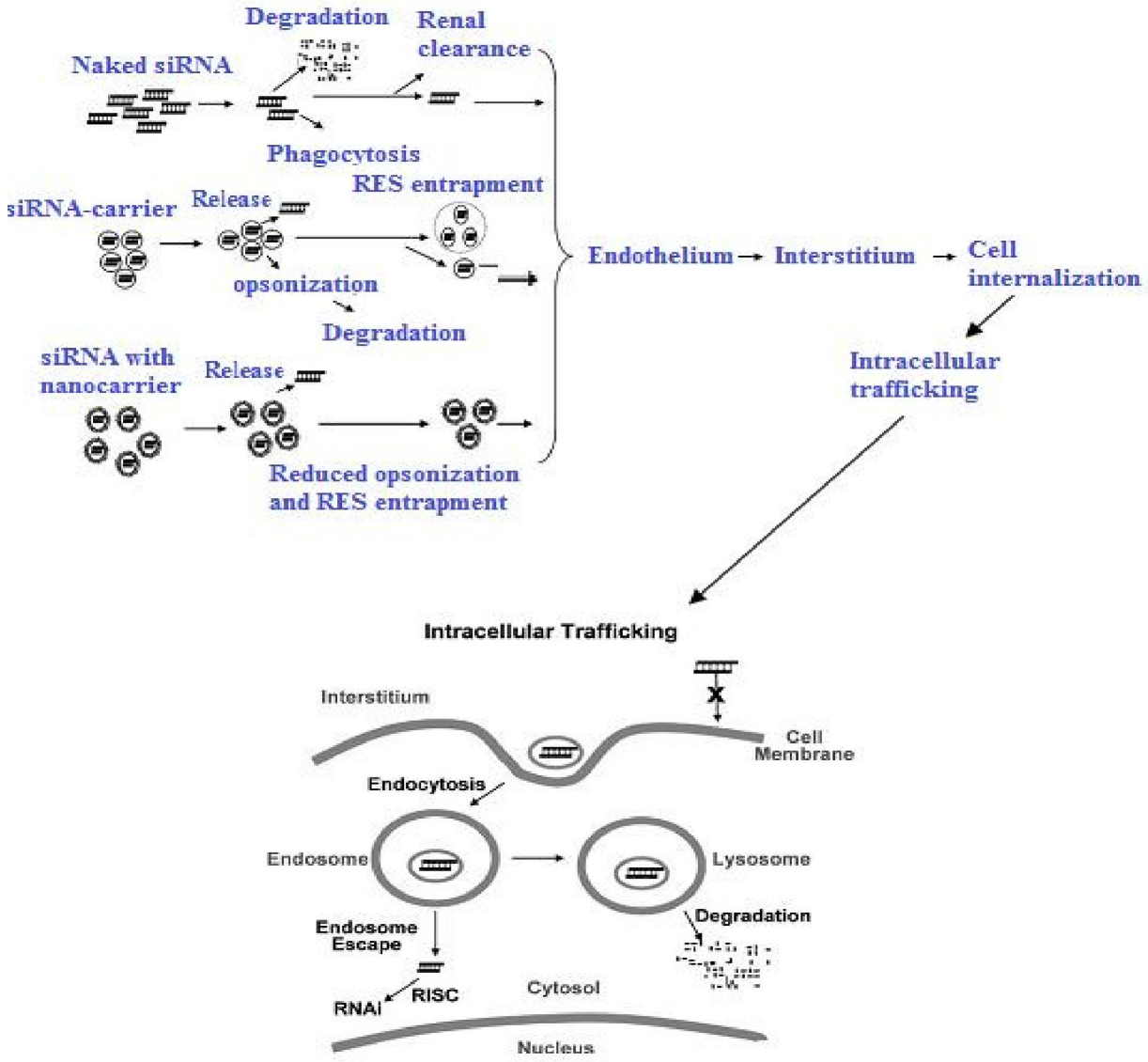

Figure 1.3 Mechanism of action of siRNA therapy.

Commonly used non-viral siRNA delivery vectors include lipids, polymers and peptides. Various lipids like *N*-(1-(2,3-dioleoyloxy)propyl)-*N,N,N*-trimethyl ammonium chloride (DOTAP); 1,2-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine (DOPE); 2′-(1″,2″-dioleoyloxypropyl dimethyl-ammonium bromide)-*N*-ethyl-6-amidospermine tetratrifluoro acetic acid salt (DOSPA); 1,2-dihexadecanoyl-sn-glycero-3-phosphocholine (DPPC); lipofectamine, and biodegradable polymers like chitosan, polyethylenimine (PEI), poly(D,L-lactide-co-glycolide) (PLGA) and poly-L-lysine (PLL) are proved to useful carriers for delivery of siRNA (29). These nano-carriers used for siRNA delivery are advantageous over viral vectors in many ways that they (i) condense siRNA into nanosized particles; (ii) protect siRNA from enzymatic degradation; (iii) facilitate cellular uptake; (iv) promote endosomal escape; (v) release siRNA into the cytoplasm where the RISC is located. However, Non-viral

gene carriers' exhibit reduced transfection efficiency because they are hindered by various extra- and intracellular obstacles. Nevertheless, they are still proposed as safer gene vectors and investigated frequently because of their advantages such as large siRNA loading capacity, no specific immune response, flexibility to design and potential for large-scale production (32, 33). Among the non-viral delivery vectors, PEI-25k (a hyperbranched PEI with molecular weight of 25 kDa) has been regarded as the ''golden standard'' for gene transfection both *in vitro* and *in vivo* (34). Even so, the usage of PEI in gene delivering area is limited by the cytotoxicity effects resulting from its high positive charge density. Several studies have attempted to decrease the cytotoxicity of PEI and to improve the transfection efficiency. We have also modified branched PEI with Boc-amino acids (Boc-Alanine, Boc-Histidine and Boc-Leucine) at different degrees of substitution with the aim of improved transfection and reduced toxicity due to reduced surface charge, increased hydrophobicity and good buffering capacity.

However, optimal delivery should be developed without compromising the gene silencing activity and specificity of siRNA. **Table 1.1** contains some siRNA therapies which are in clinical trials.

Clinical progress of RNAi therapy in asthma and Respiratory Syncytical Virus (RSV) infection shows that siRNA has tremendous potential in the treatment of lung diseases. Many studies have been carried out in the past few years for delivering siRNA to the lungs for the treatment of various lung diseases. However, the majority of these investigations focus on the design of siRNA molecules to target a specific disease instead of looking into the delivery system and route of administration perspectives. Apart from the cellular barriers described already, a number of other lung delivery specific physical and immunological barriers play important role in gene transfer across the surface of the epithelial cells. The respiratory tract, being in direct contact with the external environment, possesses a series of defences against inhaled materials. The mucociliary escalator, coughing, and alveolar clearance are the three major physical ways of removing deposited particles. In the conducting airways, deposited particles are rapidly cleared by the mucociliary clearance into the pharynx. In the terminal airways (alveoli), absorptive or non-absorptive processes remove deposited particles. The absorptive process may involve either direct penetration into the epithelial cells or uptake and clearance by the alveolar macrophages. The non-absorptive process

involves transport of particles to the ciliated region (conducting airways) followed by clearance by the mucociliary escalator (35).

Table 1.1 siRNA therapy in clinical trials

Delivery Agent	Delivery Approach	Target Disease	Company	Name of Active Component	Stage of Clinical Trial
Naked siRNA	Inhalation	Asthma	Excellair	SYK kinase	II
	Intranasal	Respiratory Syncytial Virus (RSV)	Alnylam Pharmaceuticals	ALN-RSV01	II
	Intravenous	Acute kidney injury	Quark Pharmaceuticals	QPI-1002	II
	Intraocular/ Intravitreal	Ocular hypertension & glaucoma	Sylentis	SYL04001 2	I/II
		Glaucoma or acute eye injury	Quark	QPI-1007	I
		Age-related Macular Degeneration (AMD)	Quark/Pfizer	PF-04523655	II
	Intradermal	Pachyonychia Congenita	TransDerm	TD101	I
Non-viral	Intravenous (CD nanoparticles)	Solid state tumors	Calando Pharmaceuticals	CALAA01	I
	Intravenous (lipid nanoparticles)	Solid cancers with liver Involvement	Alnylam Pharmaceuticals	ALN-VS P02	I
		Transthyretin mediated amyloidasis (ATTR)	Alnylam Pharmaceuticals	ALN-TTR 01	I
		Advanced solid cancer	Silence Therapeutics	Atu027	I

Use of a dry powder inhalation system is thought to result in a local targeted delivery of therapeutics with improved lung deposition, reduced dosing frequency, higher patient acceptance and adherence to long term therapy and an improvement of the quality of life of patients. These systems can be broadly classified into immediate release (e. g. lactose-drug mixtures) and controlled release systems (e.g. liposomes, micelles, nanoparticles, microparticles and lipopolyplex). Particulate nanocarriers can be used to improve the transfection efficiency of siRNA, reduce nuclease based degradation of siRNA, assist cellular internalization, reduce toxicity and provide better targeting and delivery to lung (36).

Conclusively, current treatments available to treat PAH are not able to cure the disease completely, rather provide symptomatic alleviation only. Additionally, the repeated administration of currently available marketed formulations leads to severe toxic effects. This raises the need for new targets for the treatment of PAH. FGF2 is one of the significantly involved factors which play key roles in the vascular remodeling and progression of PAH. Use of the RNAi therapy using siRNA is a good option to target this growth factor to cure the disease. For RNAi therapy to be effective in patients with pulmonary arterial hypertension, the specific siRNA to suppress the overexpression of FGF2 mRNA must be delivered effectively to endothelial cells of lungs. Specific delivery of siRNA will be a promising approach to treat PAH completely and will be helpful to patients suffering from it. Talking about the delivery vectors, viral vectors such as adenovirus, adeno-associated virus etc. are unsuited to repeated dosing as the immune response reduces the effectiveness of each subsequent dose as well as can cause fatal adverse immunological and carcinogenic reactions. Non-viral approaches, such as cationic liposomes (lipoplex) and polymeric nanoparticles (polyplex) appear more suited to repeated dosing [39]. Additionally, to efficiently deliver siRNA to target cells, nanocarrier systems for pulmonary delivery would be advantageous providing targeted delivery along with higher transfection efficiency.

1.2 Objective of the Proposed Work

The objective of the proposed investigation was to develop and characterize the delivery system for siRNA to achieve success rate in treatment of PAH.

1.3 Rationale

Current therapy for pulmonary hypertension has limitation of being non-selective, toxic and more palliative than curative. FGF2 is an important growth factor implicated in pathology of PAH which could be used as a potential target for its treatment. Selective targeting of FGF2 which is implicated in pathophysiology of PAH through siRNA therapeutic will help to cure the patients with PAH.

1.4 Hypothesis

It is hypothesized that the delivery of therapeutic genomics will improve the quality of therapy for PAH patients.

1. 5 Research Design and Method

1. Synthesis and characterization of amino acid modified polyethylenimine
2. Development and characterization of polyplexes and lipoplexes (both referred herein as nanoplexes) using different polymers as well as lipid excipients
3. *In vitro* cell line studies of developed formulations to assess the effect of developed carriers on cell lines viz. toxicity, cellular uptake and gene expression
4. Development and characterization of Dry Powder for Inhalation formulation
5. *In vivo* animal studies

1.6 Expected Results

The scientific literature shows that the therapeutic genomics, siRNA in particular, may be the most powerful resources for the treatment of PAH. In preclinical evaluation, therapy with FGF2 targeted siRNA formulations in PAH animal models will possibly help cure the PAH. Favorable results from the present study would be a platform for clinical study and commercialization of dry powder for inhalation of nanoplexes of siRNA for effective treatment of PAH. The designed formulation of siRNA will help to reduce the associated side effects of current marketed therapy and provide other simple route for administration.

Development and optimization of a delivery system would establish a Platform Technology for the lung targeted delivery of siRNA. The outcome of this study will provide base for researchers working on siRNA delivery for the treatment of PAH. Thus, an effective formulation approach will be available for different types of siRNA for the treatment of most deadly diseases.

1.7 Work Plan

- Literature survey
- Selection & procurement of siRNA, polymer, Boc-amino acids and other excipients
- Synthesis of Boc-amino acid modified PEI
- Characterization of synthesized polymers by IR, NMR, GPC, TNBS Assay, proton sponge effect etc.
- Method development for detection of siRNA by UV (NanoDrop) and gel electrophoresis

- Development and optimization of polyplexes
- Development and optimization of lipoplexes
- *In vitro* characterization and evaluation
    - siRNA complexation
    - Particle size analysis and physical characterization of formulation
    - Determination of zeta potential
- *In vitro* cell line studies

  In vitro cell line studies in two cell lines i.e. pulmonary arterial endothelial cells and hypoxia induced pulmonary arterial endothelial cells by
    - Cytotoxicity study
    - Intracellular uptake by confocal microscopy and flow cytometry
    - Gene expression study
- *In vitro c*haracterization of optimized nanoplexes by
    - Heparin polyanion competition Assay
    - Serum stability study
    - Salt induced aggregation study
    - Stability study in bronchoalveolar lavage fluid
    - Transmission Electron Microscopy (TEM)
- Development and optimization of dry powder for inhalation by freeze drying and using various cryoprotectants. Characterization of dry powder for inhalation for
    - Emitted dose
    - Mean mass aerodynamic diameter (MMAD)
    - Geometric standard deviation (GSD)
    - Fine particle fraction (FPF)
    - X Ray diffraction
    - Differential scanning colorimetry
- *In vivo* evaluation by pulmonary route to study the efficacy of siRNA delivery in monocrotaline induced PAH in rats.
- Stability studies

1.8 References

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