9. SUMMARY AND CONCLUSION

Pulmonary hypertension has nowadays become a severe threat worldwide and is characterized by increased pulmonary vascular resistance due to pulmonary arterial obstruction. The number of patients affected with pulmonary hypertension has crossed exaggerating figure of 100 million and some of them die even before diagnosis resulting in high mortality rate of about 15%. The widespread occurrence of this disease can be estimated from the fact that such condition is diagnosed in more than 150,000 people every year and that too in India alone. Additionally, there is a great variation in prognosis of this disease as it depends on the associated co morbid conditions.

Aetiology of pulmonary arterial hypertension (PAH) mainly involves several defects of cardiac and respiratory system. Common aetiological factors 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. Such factors can lead to pathological changes involving pulmonary vascular remodelling which further cause augmentation of pulmonary artery pressure.

Vascular remodelling in pulmonary hypertension is governed by growth factors like platelet derived growth factor (PDGF), fibroblast growth factor 2 (FGF2), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF) etc. Amongst all the above mentioned growth factors, FGF2 is largely released from pulmonary endothelial cells during vascular remodelling.

The treatment of such devasting disease is further worsened by currently available treatments which are expensive and tend to be more palliative than curative. Intensive research in this field in understanding of pathophysiology has provided possible solution to this which is thought to lie at the genomic level. Gene therapy using RNA interference (RNAi) emerged as promising approach for the inhibition of gene expression and thus can be used effectively for diagnosis and treatment of several diseases. The use of novel therapeutic genomics based approaches acting directly on vascular structural changes (i.e. siRNA delivery, antisense oligonucleotides etc.) by targeting growth factors like FGF2 and PDGF involved in pathophysiology of PAH is a potential approach.

However, the delivery of therapeutic genomics like siRNA is not an easy task and lot of research has been focused in search of ideal delivery system. For RNAi therapy, non-viral

vectors, although having low transfection efficiency, are preferred over viral vectors due to various advantages including large siRNA loading capacity, no specific immune response, flexibility to design and potential for large-scale production. Various lipids, polymers and peptides are currently being used for this purpose. PEI-25k (a hyperbranched PEI with MW 25 kDa) is considered as the better choice for gene transfection as compared to other transfecting agent, however, its use is limited as it can cause cytotoxicity due to highly positive charge density. Therefore, in this study we modified branched PEI with Boc-amino acids (Boc-Alanine, Boc-Histidine and Boc-Leucine) to improve transfection and reduce toxicity.

The basic approach used for treating PAH in this study was to knockdown the up-regulated FGF-2 gene expression using siRNA delivery. siRNA is available in two purification grades i.e. desalted grade or HPLC grade either as predesigned siRNA molecules or as custom synthesized 21 mer or 27 mer duplexes. For this study siRNA was procured as a ready to use HPLC grade duplex with 1 mL 5x siMAX^{TM} buffer (30 mM HEPES, 100 mM KCl, 1mM $MgCl_2$, pH= 7.3). The obtained siRNA was analyzed by Matrix Assisted Laser Desorption Ionization- Time of Flight-Mass Spectrometry (MALDI-TOF) and capillary gel electrophoresis (CGE) which confirmed the molecular weight and purity of FGF2 siRNA respectively. UV spectrophotometric technique using the ratio of A260/A280 and A260/A230 was found to be a handy tool in daily laboratory analysis. A260/A280 and A260/A230 values suggested good purity of siRNA. Once purity was confirmed correlation curve was plotted to establish relationship between the results of NanoDrop and known siRNA concentration. For this, siRNA solutions of various concentrations were prepared in nuclease free water. Absorbances of these solutions were recorded at 260 nm on a NanoDrop UV spectrophotometer. The analytical method was partially validated to ensure good accuracy and precision as demanded for routine analysis. The results showed desired acuracy and reproducibility on NanoDrop UV spectrophotometer.

Charge based migration of free siRNA on agarose gel using gel electrophoresis combined with sensitive densitometric detection system can be used for quantification of siRNA. Hence, agarose gel electrophoresis method was developed to determine siRNA complexation efficiencies of various formulations and for various *in vitro* tests. Limit of quantitation (≥ 30 pmole concentrations of siRNA) was determined and all measurements were performed above this limit. Calibration curve was generated by plotting relative band

densities vs. different concentrations of siRNA. Curve was found to follow a linear equation $y = 0.0207x + 0.034$ with correlation coefficient of 0.9977.

Among the various non-viral vectors available, PEI was chosen because of its better nucleic acid condensation capacity and tremendous transfection efficiency. However, most prominent disadvantages associated with PEI i.e. cytotoxicity has limited its use. Hence, modification of native PEI using Boc protected amino acids was proposed in order to serve several purposes i.e. to provide biocompatibility, to reduce cytotoxicity without affecting its transfection potential. Modifications of PEI were performed by using different mole ratios of Boc-amino acids resulting in different degree of amino acid substitution on PEI. Synthesized polymers were characterized by infrared spectroscopy, nuclear magnetic resonance spectroscopy, trinitrobenzene sulfonic acid (TNBS) assay and gel permeation chromatography. The FTIR spectrum confirmed grafting of amino acids on PEI chains by giving strong absorption around 1644 cm^{-1}, 1649 cm^{-1} and 1646 cm^{-1} in the spectra of Boc-alanine, Boc-histidine, Boc-leucine grafted copolymers respectively corresponding to –C=O of amide group formed when compared with the spectrum of polyethylenimine. ^{1}H-NMR spectra was further used to support the results of FTIR spectra in which characteristic peaks of PEI (–$NHCH_2CH_2$ –) appeared at δ 2.2 ppm to 3.2 ppm and those of tertiary carbon (≡CH) appeared at δ1.2 ppm to δ 1.4 ppm for all synthesized polymers. The results of TNBS assay showed increasing substitution percentage of primary amines on the polymer with increasing molar ratios of the Boc-amino acids used (notified as AXP, BXP and CXP with high (40-45%) conjugation, medium (30-35%) conjugation and low conjugation (20-25%) conjugation, X represents amino acid type i.e. A for alanine, H for histidine and L for leucine). All the three amino acids substituted on primary amine of PEI at almost same level as the molar ratio of all Boc-amino acid to PEI was same. Additionally, gel permeation chromatography confirmed the molecular weight of modified PEI.

Endosomal escape of the delivered vectors is the primary requisite for efficient delivery of gene delivery system. Thus synthesized polymers were evaluated for their buffering capacity by using acid base titration. The titration curves of Boc-alanine-PEI and Boc-leucine-PEI were very close to PEI and thus have almost equal buffering capacity to that of PEI. Degree of substitution and substituting moiety (Boc-alanine and Boc-leucine) didn't improve the proton sponge effect of modified PEIs as the amine groups of both Boc-amino acid do not take part in protonation due to resonance effect of carbonyl group. However,

titration curves of Boc-histidine-PEI with increasing degree of substitution showed higher buffering capacity as compared to native PEI. As the degree of substitution of Boc-histidine on PEI was increased, consumption of acid was also increased. The results indicated that ionisable amines (pKa ~ 6.5) of imidazole ring of Boc-histidine took part in improvement of proton sponge effect of modified PEI and it will help in endosomal escape of siRNA polyplexes .

For *in vivo* application, it is very important that it should be compatible with blood cells. This was checked by performing hemolysis study. Results showed that there was concentration dependent rise in hemolytic potential of all the polymers suggesting the increased amounts of primary cationic amines that would confer higher blood cell destruction. However, synthesized polymers showed reduced hemolytic potential as compared to that obtained with PEI. All modified polymers exhibited less than 6% hemolysis at all concentrations, while non-modified PEI showed ~7% hemolysis at lowest concentration of 10 μg/mL which increased to about 20% at 1000 μg/mL concentration.

Among various non-viral gene delivery approaches, cationic polymer and lipid based complexes (referred to as nanoplexes herein) are most widely used. Complexes of PEI or modified PEIs with siRNA with a net positive charge were prepared through electrostatic interaction. The net cationic charge aids its cellular uptake and transfection. Several other polyplex properties including N/P ratio, concentration of siRNA, size and zeta potential also affect transfection efficiency. Apart from the polyplexes, development of lipoplex formulation of FGF2 siRNA containing DPPC, Cholesterol, DOTAP, DOPE and DSPE-mPEG2000 was also carried out using our past experience with liposomes (DL). Such lipoplex formulation was developed in order to obtain a stable reference to evaluate the *in vivo* performance of polyplexes. However, in vitro performance of various polyplexes was also evaluated in comparison to commercial lipid based transfection agent Lipofectamine-2000 based lipoplexes (L2KL).

Polyplexes were prepared by incubating siRNA with various dilutions of polymer solution for 30 min at 37^{o}C after vortexing it for 10 mins. Optimization was done by taking various w/w ratios of polymer/siRNA ranging from 0-4. Liposomes were prepared by thin film hydration method by using combination of lipids. siRNA was incubated with these preformed liposomes at different N/P ratios ranging from 0 to 2.0, for 30 min at 37ºC.

Following incubation, siRNA nanoplexes were subjected to gel electrophoresis to assess complexation of siRNA as amino acid conjugation on PEI might affect the polymer's ability to form a complex with siRNA. Optimized formulations were further confirmed by NanoDrop UV spectroscopic determination for siRNA complexation efficiency. All polyplexes showed complete complexation between 0.75-2.0 w/w ration and above, while lipoplexes showed N/P ratio of 2.0 and above. All the synthesized polymers were able to condense more than 95% of siRNA at their optimized polymer-to-siRNA weight ratios.

Particle sizes (hydrodynamic diameter) and zeta potentials of the developed nanoplexes were determined by using Malvern Zetasizer. Mean particle size of PEI polyplexes was 101.2 nm with low polydispersity index of about 0.15 suggesting uniform particle size distribution within the formulation. In case of Boc-histidine-PEI, the charge compensation provided by imidazole ring showed low particle size increase as compared to other Boc-amino acid-PEIs at each conjugation level. However, particle sizes observed for all the type of polyplexes were below 170 nm with low polydispersity index (<0.2). Lipoplex formulation showed mean particle size of 120.2 nm, which was 109.3 nm before siRNA complexation. Increase in size can be attributed to the siRNA adsorption on the surface of preformed cationic liposomes.

At optimized w/w ratio of Boc-amino acid-PEI complexes, a strongly positive zeta potential (about +15–35 mV) was observed due to amines that are not involved in binding with siRNA. The net positive charge remaining on polyplexes formed with modified PEIs would cause electrostatic repulsion between complexes that would prevent aggregation and in turn improve stability.

The cytotoxicity study of the prepared polyplexes and lipoplexes was carried out by MTT colorimetric assay using CPA-47 cell line. Viability of PBS treated cells was used as negative control. Polyplexes of unmodified PEI reduced the viability to 85.84 ± 1.68% at w/w ratio of 1, while L2KL reduced viability to 88.05 ± 0.84% following 48 h of incubation. It was seen that at all w/w ratio, polyplexes developed from synthesized polymers were significantly less toxic on CPA-47 cells than unmodified PEI as well as L2KL. From the results of MTT assay, it was seen that polyplexes developed by all synthesized polymers were significantly ($p < 0.05$) less toxic than L2KL even at w/w ratio of 5. Additionally, developed lipoplexes (DL) had less toxicity as compared to polyplexes developed by using

PEI PP) which may be attributed to neutralization of positive charge by combination of lipids.

Cell uptake study was performed using confocal microscopy and flow cytometry using fluorescence activated cell sorter. In confocal microscopy, naked siRNA showed negligible cellular uptake, while PEI polyplexes showed marked cellular uptake. Low uptake of naked siRNA can be attributed to its high molecular weight and high hydrophilicity hindering its passive diffusion through cell membrane. PEI can condense siRNA by electrostatic interactions and the residual positive charge of polyplexes improves its cellular uptake by interacting with negatively charged cell membrane which would lead to endocytosis. Conjugation of Boc-amino acids increases hydrophobicity of polyplexes and resulted in higher uptake than that of native PEI polyplexes. . Additionally, lipoplex formulations showed much higher cellular uptake than that of naked siRNA which can be attributed to enhanced interaction between cationically charged lipoplexes and negatively charged cell surface and subsequent endocytosis.

Results of flow cytometry corroborated results obtained using confocal microscopy in quantitative terms and to compare trend within individual amino acid group. It was found that, as the conjugation level of amino acid on PEI increased, there was increase in cellular uptake i.e. regardless of the amino acid type, cellular uptake of PEI and modified PEIs exhibited following pattern: AXP≥BXP>CXP>PP (where X represents the amino acid type i.e. A-alanine, H-histidine, L-leucine. Among the amino acid types, at each conjugation level, the cellular uptake exhibited following pattern: *Boc-histidine-PEI>Boc-leucine-PEI>Boc-alanine-PEI* which can be attributed to hydrophobicity and cationic charge. Both lipoplex formulations showed more than 65% cellular uptake. Such higher cellular uptake of lipoplexes can be ascribed to high surface charge density mediated endocytosis and DOPE mediated membrane fusion.

From the results of cytotoxicity study and cellular uptake studies, three polyplex formulations i.e. AAP, AHP and ALP and both lipoplex formulations i.e. DL and L2KLwere further evaluated for gene silencing efficacy. In case of polyplexes, AHP was the obvious choice for gene transfection due to higher cellular uptake as compared to other formulations. However, in case of polyplexes made from Boc-alanine and Boc-leucine modified PEIs, though cellular uptake was not increased when conjugation level was increased from BAP to

AAP and from BLP to ALP, AAP and ALP were chosen for transfection studies due to their low cytotoxicity. mRNA expression levels were estimated as a percent FGF-2 mRNA expression of PBS treated control. Naked siRNA reduced this expression, however, at very low extent (<10%).

Comparing different formulations, at each siRNA concentration evaluated, the gene expression was in concordance with the cellular uptake studies. Among the screened polyplexes, AHP showed maximum gene silencing, followed by ALP, AAP and native PEI polyplexes. In case of lipoplex formulations, although, optimized lipoplex formulations showed similar transfection efficiency as that of L2KL the gene silencing efficiencies of both lipoplexes were lower than polyplexes due to absence of proton sponge effect. Additionally, it was confirmed that observed gene silencing of various formulations was due to FGF-2 siRNA only and not due to any off-target effects of siRNA by evaluating the effect of scrambled sequence siRNA. Since, scrambled sequence siRNA did not cause any suppression of FGF-2 mRNA, the sequence specificity of siRNA for target mRNA degradation was confirmed.

Hypoxia is the leading cause of hypertension. In order to evaluate the effects of developed formulations under hypoxic conditions, pulmonary arterial endothelial cells grown under hypoxic conditions were used for *in vitro* studies. Such study gave exact idea about the cellular uptake, cytotoxicity and gene expression under hypoxic conditions prevailing *in vivo* and help correlate the results obtained in normoxic and hypoxic conditions as well as to establish an extrapolation for animal studies. As it can be seen, cytotoxicity followed similar pattern that was observed in normoxic cell line. PEI showed highest cytotoxicity as compared to all other polyplexes as well as lipoplexes. Further, the cellular uptake in hypoxic cells showed that cellular changes that might have taken place in hypoxic pulmonary arterial endothelial cells did not cause any changes in the uptake of PEI and modified PEI polyplexes as well as lipoplexes. The same was also confirmed by gene expression studies.

As the formulation is to be delivered in the lungs, formulation was designed in the form of dry powder for inhalation (DPI). Due to sensitivity of siRNA to heat and other processing conditions, lyophilization method was employed for preparation of DPI using different cryoprotectants like sucrose, lactose and trehalose. All DPIs were evaluated for siRNA integrity and were found to retain intact siRNA on lyophilization. DPIs developed

were further evaluated for moisture content and aerosolization properties like Emitted Dose, mass median aerodynamic diameter, geometric standard deviation and fine particle fraction. Among the DPIs developed, lactose based DPI showed higher fine powder fraction and hence was further analysed by X-ray diffraction which confirmed the amorphous nature of the DPI. This optimized lactose based DPIs was analysed for its *in vivo* activity.

In vivo performance of lactose based DPI of each nanoplex formulation was evaluated by carrying out pharmacodynamics studies of nanoplexes in rat model of monocrotaline induced pulmonary arterial hypertension and acute toxicity studies of carrier polymers and placebo liposomes in female rats through intratracheal administration. In vivo studies showed that intratracheal administration of formulations led to marked reduction in FGF2 mRNA levels in lung homogenates. This in turn extrapolated to significant reduction in the study parameters like right ventricular systolic pressure and right ventricular hypertrophy with respect to positive control without affecting mean systolic arterial pressure. In acute toxicity studies both carriers were found to be nontoxic up to a level 5 times higher than that required for therapeutic siRNA delivery as confirmed from the visual observation of animals over a period of 14 days for any signs of toxicity as well as signs of inflammation observed from the histopathological examination of the lung tissue and polymorphonuclear cells counts in bronchoalveolar lavage fluid.

Consistent *in vivo* performance can be achieved only if formulation retains its physicochemical characteristics on long term storage. Hence, storage stability studies of nanoplex DPIs were performed at accelerated (25°C ± 2°C, 60% RH ± 5% RH) and at long term conditions (5°C ± 3°C) as required for products stored under refrigeration . Both lipoplex and nanoplex based DPIs were found to be stable at both storage conditions. There were changes in particle size of formulations and some loss of siRNA activity at accelerated conditions; however, moisture content was maintained well below 3% ensuring that there will be no change in aerosolization performance of all the DPIs. Based on the stability studies, all DPI formulations were found to be stable under accelerated as well as refrigeration conditions. However, to ensure better retention of siRNA activity and other characteristics DPIs should be stored under dry conditions in refrigerator.

Conclusions

Novel modification of PEI has been proposed with Boc-amino acids which showed better safety profile *in vitro* as compared to native PEI. Polymers were synthesized with different degree of conjugation and were used for development of polyplexes of FGF2 siRNA. A stable lipoplex formulation has also been developed using DOTAP, DOPE, DPPC, Cholesterol and DSPE-mPEG-2000. *In vitro* screening studies in normoxic pulmonary endothelial cell line showed that polyplexes of all synthesized polymers and developed lipoplexes had better cytotoxicity profile, good cellular uptake and better gene silencing activity, with optimum results for polyplexes of polymers with highest conjugation level (40-45%) and developed lipoplexes. Screened nanoplexes were further evaluated for cytotoxicity and transfection characteristics under hypoxic conditions that is at the root of pulmonary arterial hypertension development. Studies showed similar trends in results as obtained under normoxic conditions, however cellular uptake and gene transfection were found to be higher under hypoxic conditions demonstrating high FGF2 mRNA level available and acidic microenvironment of hypoxic cells leading to higher uptake and high gene silencing. In addition, through various *in vitro* studies, developed nanoplexes have been demonstrated to be less haemolytic and stable in presence of polyanions, serum components, electrolytes and bronchoalveolar fluid. Nanoplexes were developed into dry powders for inhalation by lyophilization with different cryoprotectant cum carrier agents, lactose, sucrose and trehalose. DPIs developed with lactose were found to show optimum aerosolization characteristics in terms of MMAD and FPF. Developed DPIs showed disease alleviating action along with better safety profile through *in vivo* pharmacodynamics studies and toxicity studies respectively. Long term storage stability studies showed, lyophilized DPI formulations were stable under accelerated as well as refrigeration conditions with better retention of siRNA activity at refrigeration conditions. All studies carried out prove that developed formulations will serve as a good alternative for pulmonary hypertension treatment after clinical evaluation.