# 10 SUMMARY AND CONCLUSION

## 10.1 SUMMARY

Lung cancer is a malignant lung tumor which is identified by abnormal growth of cells in lung tissues. It begins when pulmonary cells become abnormal and start to grow rebellious. Docetaxel is an anti-neoplastic material belonging to the class of taxoids. It is mainly used as a single agent or with platinum agents for the treatment of breast, ovarian, prostate, and non-small cell lung cancer at a dosage of 75 mg/m2 every three weeks of treatment. It works by manipulating the microtubular framework that is important for cellular mitotic as well as interphase functions. It is only available on the market as an intravenous route infusion, which is followed by many drawbacks, including low dose reached at the site of action such as lungs, off target side effects including myelosuppression, nephrotoxicity, disruption of electrolytes, neurotoxicity, ototoxicity and many other tissue damaging consequences.

The current normal path to treatment of cancer is chemotherapy combined with radiotherapy and surgical resection. The occurrence of multidrug resistance (MDR) to chemotherapy, whereby cancer cells become immune to the cytotoxic effects of different structurally and mechanistically unrelated chemotherapeutic drugs, is a significant barrier to the effective treatment of malignant diseases. In over 90 percent of patients with metastatic disorder, this phenomenon leads to treatment failure. The tumor microenvironment selection pressures and inherent high expression by tumor cells of the ATP-binding cassette (ABC) transporters can lead to the emergence of intrinsic multi drug resistance (MDR). A drug stimulation that leads to the overexpression of ABC transporters and eventual efflux of anticancer drugs from cancer cell cytoplasm will lead to acquired resistance in cancers. Hence, developing an innovative multifunctional delivery system should be a priority in cancer chemotherapy to reverse MDR. MDR can also be managed by the gene knock-down strategy to suppress the expression of such proteins of the ABC family that are known for the chemotherapeutics' efflux and decreased therapeutic activity. Inhibition of P-gp is commonly used method hence gene silencing for P-gp is the most impactful technique of all approaches now-a-days by RNA interference technology.

RNA interference is a scientific principle that guides the degradation of complementary mRNA by a small dsRNA and performs sequence-dependent inhibition of a specific gene. Delivering shRNA pDNA for silencing multi drug resistant ABCB1 gene (MDR1) can impart

synergistic therapeutic activity by decreasing the resistance of cell to chemotherapeutic agents like docetaxel. To mask the multi drug resistance shRNA pDNA was complexed to pre-formed formulation of Docetaxel loaded polymer lipid hybrid nanocarriers (D-PLHNCs) which knockdown the protein responsible for efflux (P-gp) of docetaxel so that the drug can remain inside the cell for longer period of time and improving its efficacy as well as increase the amount of drug reaching the target site of action i.e., lungs. Formulating polymer lipid hybrid nanocarriers (PLHNCs), provides a unique drug delivery platform in which the biocompatibility and surface modifications, similar to liposomes can be achieved using lipid composition (DPPC:DOTAP:DSPE-PEG2000) and structural integrity with mechanical stability is provided by polymeric (PEG-PCL) core. Delivery of Docetaxel through PLHNCs dry powder inhaler (DPI) to the lung reduces the irritation caused by plain Drug DPI, as well as directs passive tumour targeting by Enhanced Permeability and Retention effect (EPR) owing to the nanosize of the particles (< 200 nm) and reduction in side effects due to off-targeted toxicity can be achieved by sustained release of small amount of drug at regular intervals at the site of action from the Nanoparticles. The current project aims to develop a novel combinatorial approach for treatment of lung cancer combining the use of chemotherapeutic agents such as docetaxel and shRNA pDNA of ABCB1 for combating multi drug resistance type lung cancer.

Analytical techniques were developed to estimate pure Docetaxel, Docetaxel in PLHNCs, Dissolution media (Phosphate buffer Saline pH 7.4, Phosphate buffer 6.6, and Acetate buffer 5.5). Calibration curve of Docetaxel was prepared by Direct UV estimation The Calibration curve was plotted by measuring absorbance at 229 nm (λmax) Calibration curve was prepared for Docetaxel in Acetonitrile, Phosphate buffer Saline pH 7.4, Phosphate buffer 6.6, and Acetate buffer 5.5 separately and the methods were validated for accuracy, precision, LOD and LOQ. Similarly, for estimation of Phospholipid content in formulation by Stewart method, calibration of total Phospholipid was prepared in chloroform. For estimation of docetaxel in biological samples, calibration curve of Docetaxel was developed in HPLC for plain drug, rat plasma, lung homogenate. For checking purity and estimation of siRNA, calibration curve was developed by using Gel Electrophoresis and nanodrop and methods were validated. The Correlation of known concentration of shRNA pDNA (pmol/µl) Vs. obtained concentration (ng/µl) by Nanodrop results were determined and the method was found to be linear. Further gel retardation assay (Gel electrophoresis) for quantification of shRNA pDNA showed correlation between concentration and obtained band density. The RSD values for all the densitometry analysis were < 2.0%..

Preformulation studies were performed to evaluate the compatibility of the Docetaxel with various polymers and lipids used in the formulation development. Melting point prediction and IR spectra of obtained Docetaxel was compared with standard Docetaxel. The results confirm the purity and identification of Docetaxel. Compatibility study was performed with help of DSC. The comparative evaluation of DSC thermogram shows that there is negligible change in the endothermic peak of Docetaxel after mixing with excipients and confirms compatibility. To optimize the working condition for the shRNA pDNA, samples of shRNA pDNA were kept at different temperature ranging from 30$^{o}$ C to 70 $^{o}$ C and pH 6.8 to pH 75 in TRIS buffer. Results of band intensity after gel electrophoresis confirmed that as the temperature increase to 70$^{o}$ C approx. 20 % of the shRNA pDNA were degraded. pH modified samples shows stability over pH range of 6.8 to 7.5.

Several Methods available in literature were tried including two step method, double emulsion solvent evaporation and single step nanoprecipitation method to formulate PLHNCs. From this, single step nanoprecipitation with little modification followed by extrusion was found to formulate PLHNCs of desired characteristics. Preliminary studies were performed to define the ranges of formulation as well as process parameters. Preliminary studies also played significant role in determination of amount of cationic lipid needed to achieve maximum entrapment efficiency of shRNA pDNA. It was confirmed that 60:25:15 ratio of DPPC: DOTAP: DSPE-$PEG_{2000}$ has enough cationic lipid (DOTAP) to complexed 96.2 ± 1.4 % of shRNA pDNA. Further increase in the cationic lipid doesn't show any increment in shRNA pDNA complexation. Total seven factors (polymer concentration (mg/mL), lipid/polymer percentage (%), drug input percentage (%), stirring speed (RPM), stirring time (h), sonication time (S) and extrusion cycle (Nos) were selected for Placket-Burman screening study. From that three factors (i.e., concentration of polymer, lipid to polymer ratio and drug input) were selected to further optimize the design space using Box-Behnken design.

QbD enabled design expert software suggested an optimized batch having composition of polymer concentration (10 mg/ml), lipid to polymer ratio (25.26 %) and Drug input (5 %) which possessed predicted size of 91.36 nm and 81.56 % entrapment efficiency and the same batch was formulated to validate the results and the particle size was found to be 126.3 ± 1.6 nm with the PDI of 0.108 ± 0.11 and Zeta Potential was found to be 22.4 ± 1.4 mV which is due to presence of Cationic Lipid i.e., DOTAP. The entrapment efficiency was determined using Ultracentrifuge to separate entrapped and unentrapped drug. The % Entrapment efficiency was found to be **91.5** ± 1.4 (n=3) in the optimized formulation.

The transmission electron microscopy (TEM) was performed to characterize PLHNCs structure with negative staining by uranyl acetate which stains DPPC and the lipids conjugated with PEG to enhance their electron density, resulting in a dim ring surrounding the PEG-PCL core. The thickness of the ring is less than 20 nm, which equals the thickness of DPPC monolayer plus a DSPE-$PEG_{2000}$ shell. PEG-PCL core was found to be dense indicating its presence inside vesicles The average size was found to be 128 nm through TEM. Surface visualization and shape of vesicle were confirmed by SEM and PLHNCs vesicles were found to be spherical in shape with size of 130 nm approximately. The morphological studies performed by AFM showed uniform and spherical shaped discrete particles without aggregation. The histogram shows fairly narrow distribution of peak heights from 15 to 30 nm corresponding to lipid bilayer thickness and PEGylation layer. The average roughness (Ra) was calculated to be 10.2 nm and the root mean square roughness (Rq) was found to be 12.0 nm. These values indicate a slightly rougher surface.

In vitro drug release studies was performed in phosphate buffer pH buffer 7.4, phosphate buffer pH buffer 6.6 and Acetate buffer 5.5 for Docetaxel and shRNA loaded PLHNCs and it showed sustained release pattern. Docetaxel shows pH dependent release at pH 5.5 it shows highest drug release as compared to Phosphate buffer 7.4.The order of release from Docetaxel LPHNPs at different pH media after 24 hours starting from pH 7.4 to pH 5.5 : 24.65% < 45.65% < 51.26%. However, within 24 hr, 50 % release was observed which indicates that approximately 50 % Docetaxel shRNA PLHNCs would be released inside the cells within one day once docetaxel gets internalized by cancer cells. Endosomes and lysozomes of cancer cells (pH 5-6) would be playing important role in inducing release of docetaxel from PLHNCs. Release of shRNA was performed using Tris buffer pH 7.4 and it shows the sustained release profile of shRNA from the PLHNCs. Estimation of Residual solvent was checked by GC-FID. The USP guidelines suggest that acetonitrile is CLASS II solvent and the limit for PDE (Permitted Daily Exposure) is 4.1 mg/day equivalent to 410 ppm. From the data of the residual solvent it was confirmed that acetonitrile present in the final optimized batch of PLHNCs is within the limits as per ICH guidelines for residual solvents.

Dry powder for inhalation of Docetaxel loaded shRNA PLHNCs was formulated by using lyophilization method. The ratio of PLHNCs: Trehalose (Cryoprotectant) 1:6 was found to be optimum for producing DPI with good flow properties and low residual water content. Addition of albumin at ratio of 1:1 was found to retained more drug (94.6 ± 1.8 %) in to PLHNCs compare to trehalose alone. The Characterization of Aerosol Performance was done

through Anderson Cascade Impactor by addition of Lactohale LH 300 and Respitose SV003. The addition of 15 % fine lactose (Lactohale LH 300) with Respitose SV003 has increased the fine particle fraction up to ~68%, which was maximum. The FPF ratios of the developed PLHNCs DPI were 2.25 and 2.40 and the emitted dose ratios were 1.84 and 1.88 for PLHNC1 and PLHNC2 DPI respectively. The optimized batch was having MMAD of 3.56 ± 1.5 µm, Fine Particle Fraction (FPF) of **68.3 ± 2.5** %, and effective index of 78.66 ± 1.8. All these characteristics are considered sufficient to achieve deep lung delivery of nanoparticles. The SEM were performed to study the microscopic features of the dry powder. It can be observed in that fines were adsorbed on the surface of carrier particles. The small particles adhere on the energy rich active sites present on the surface of carrier. The carrier particles are easily entrained in the inspiratory air flow from which the adhered particles are then stripped off due to turbulent shear stress and inertial separation mechanisms. The pXRD results showed that the dry powder formulation was crystalline in nature, which could be due to trehalose during lyophilization and powder processing with Respitose SV003 and Lactohale LH 300. Amorphous regions have higher surface adhesion energy than crystalline regions which leads to poor de-aggregation after fluidization in air stream. In contrast, crystalline regions interact weakly with and are easily overcome by turbulent shear during inspiration by patient.

It was confirmed evident from DSC studies that there was no free crystalline drug material in the DPI samples. Hence it could be concluded that in the prepared D-PLHNCs DPI, the drug was present inside the polymeric matrix. All characteristic pertaining to docetaxel were absent in FT-IR, indicating the encapsulation of drug in the LPHNPs and only a diffused peak throughout the spectra at wave number higher than 1400 $cm^{-1}$ are observed. From results of band density obtained by gel electrophoresis; initially as 1.054 ± 0.039 and after powder processing; it was found to be 1.032 ± 0.042 it can be concluded that the developed DPI formulation of D-PLHNCs retained the integrity of the shRNA after the lyophilization and powder processing.

Cell line studies were performed to assess cytotoxicity, cellular uptake of formulation along with p-gp inhibition of DR-A549 lung adenocarcinoma cells. MTT assays of Docetaxel solution, blank PLHNCs, D-PLHNCs and shRNA pDNA-PLHNCs complexes were performed to assess effect of lipids, polymer and shRNA pDNA complexation on cell cytotoxicity. Both formulations and bank formulation were found non-cytotoxic to cells. From the images of confocal microscope obtained for cellular uptake studies it can be concluded that sh-PLHNCs could successfully carry and deliver shRNA and docetaxel into the cytosol i.e. transfection

efficiency is improved with PLHNCs. Fluorescence intensity of sh-PLHNCs is very much near to transfection standard lipofactamine-2000. Successful transfection in to cells using PLHNCs depict that they are easily uptaken by the cells through endocytosis due to their cationic characteristic. Chemosensitization of A549 cells was confirmed by performing and comparing MTT assays of Docetaxel solution, D-PLHNCs and D-sh-PLHNCs and it was found that D-sh-PLHNCs is having $IC_{50}$ 0.316 μM after 72 hr which indicates approximately 1.55-fold reduction as compared to D-PLHNCs and 2.2-fold reduction in compare to Docetaxel solution. From cell migration assay it was concluded that both D-PLHNCs and D-sh- PLHNCs have enhanced the anti-migratory effect of the Docetaxel, which could prove a great tool for the shrinkage of the tumors and regression of tumor metastasis. RT-PCR studies have suggested that production of the mRNA responsible for p-gp synthesis has been reduced greatly after treatment with 40 ng shRNA pDNA containing PLHNC.

The pulmonary pharmacokinetic parameters were calculated and it was found that maximum t½ value of 6.05±1.23 hours were observed with D-sh-PLHNCs DPI compared to 1.98±0.54 hours with docetaxel solution after intratracheal administration. Eventually, there was an increment in AUC for PLHNCs compared with the AUC of Docetaxel solution. D-sh-PLHNC DPI showed 3.4 times higher AUC values than docetaxel solution after intratracheal administration. The Tmax values for D-sh-PLHNC DPI was 14.2 ± 1.8 hours compared to docetaxel solution which has only 2.1 ±0.85 hours, thereby confirming the maintenance of effective drug concentration with D-sh-PLHNCs in lung tissue for prolonged period compared to Docetaxel solution. Even when compare to intravenous route the developed D-sh-PLHNCs suspension shows better pharmacokinetic profile compare to Docetaxel solution but unable to target lung.

Comparative stability studies were carried out of the potential lyophilized PLHNCs and dry powder inhaler formulations at accelerated condition (25°C ± 2°C, 60% RH ± 5% RH) for three months and at long-term conditions (2-8°C) up to three months. During stability monitoring, no significant differences ($p > 0.05$) were found in particle size, zeta potential and assay of PLHNCs as well PLHNCs dry powder formulations. Lyophilized PLHNC formulations maintained their physical integrity and were observed as white porous cakes. Assay of the formulations at each time point was between the range of 95-105% of initial levels which was acceptable. There was no significant change ($p>0.05$) in particle size and zeta potential after storage period.