11. SUMMARY & CONCLUSION

Lung cancer is a malignant lung tumor characterized by uncontrolled cell growth in tissues of the lung. It starts when cells of the lung become abnormal and begin to grow out of control. Cisplatin is selected as model drug it shows non-cycle specific antitumor effects. It is used mainly for the treatment of breast, ovarian, prostate, and non-small cell lung cancer at the dose of 75 to 120 mg/m2 intravenously once every 3 to 6 weeks of the treatment as a single agent or along with other chemotherapeutic agents. The cytotoxicity of cisplatin is primarily ascribed to its interaction with nucleophilic N7-sites of purine bases in DNA to form DNA–protein and DNA–DNA interstrand and intrastrand crosslinks. It is available in the market only as infusion to be given by Intravenous Route, which is accompanied by many disadvantages which include low dose reaching at the site of action (i.e Lungs), off target side effects including myelosuppression, nephrotoxicity, electrolyte disturbances, neurotoxicity, ototoxicity and many more tissue damaging effects.

Chemotherapy combined with radiotherapy and surgical resection is the current standard approach to cancer treatment. A major obstacle to successfully treating malignant diseases is the emergence of multidrug resistance (MDR) to chemotherapy, whereby cancer cells become resistant to the cytotoxic effects of various structurally and mechanistically unrelated chemotherapeutic agents. This phenomenon contributes to treatment failure in over 90% of patients with metastatic disease. The selection pressures within a tumor microenvironment and inherent high expression of the ATP-binding cassette (ABC) transporters by tumor cells may result in the development of intrinsic MDR. An acquired resistance in cancers may come from a drug stimulus, which leads to the overexpression of ABC transporters and subsequent efflux of anticancer drugs from the cancer cell cytoplasm therefore designing an advanced multifunctional delivery system should be a priority to reverse MDR in cancer chemotherapy. Thus MDR can be treated by gene knock down approach to inhibit expression of these ABC family proteins which are responsible for efflux of oncological therapeutics and reduced therapeutic action. RNA interference, P-gp inhibitors and few peptides are extensively used approaches and gene silencing

through RNA interference technology is most impactful tool now-a-days amongst all approaches.

RNAi interference is a biological mechanism by which a small dsRNA directs the degradation of complementary mRNA and execute sequence-specific inhibition of a particular gene. Delivering siRNA for silencing multi drug resistant gene ABCC3 (MRP3) can impart synergistic therapeutic activity by decreasing the resistance of cell to chemotherapeutic agents like cisplatin. To mask the Multi Drug Resistance , siRNA was complexed to Pre-formed formulation of Cisplatin caprylate loaded Hybrid nanocarriers (HNCs) which knockdown the protein responsible for efflux of cisplatin so that the drug can remain inside the cell for longer period of time and improving its efficacy and and also to increase the amount of drug reaching the target site of action i.e lungs, Dry Powder Inhaler was formulated. Formulating Hybrid nanocarriers (HNCs), provides a unique drug delivery platform in which the biocompatibility and surface modifications, similar to liposomes can be achieved and structural integrity with mechanical stability is provided by PEG-PLA core. Delivery of cisplatin through HNCs 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 cisplatin and silencing RNA (siRNA) for combating multi drug resistance type lung cancer. As cisplatin display low loading efficiency, its caprylate conjugates were prepared to improve drug loading.

Analytical methods were developed to estimate pure Cisplatin, Cisplatin in HNCs, Dissolution media (Phosphate buffer Saline pH 7.4, Phosphate buffer 6.6, and Acetate buffer 5.5). Calibration curve of cisplatin was prepared by colorimetric estimation using OPDA derivatization method. The Calibration curve was plotted by measuring absorbance at 705 nm (λmax). The calibration plot of Cisplatin in DMF:water (7:3 % v/v) mixture was found to be linear in the concentration range from 0.5-3.5 ppm with high value of regression coefficient (R^2 =

0.9978) 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 cisplatin in biological samples, calibration curve of cisplatin was developed in NP-HPLC using L8 column (USP method; mobile phase - in ethyl acetate, methanol, dimethylformamide, and degassed water (25:16:5:5)) for plain drug, rat plasma, lung homogenate. The Calibration plot for drug was obtained in range of 4-12µg/ml. 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 siRNA (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 siRNA showed correlation between concentration and obtained band density. The RSD values for all the densitometry analysis were < 2.0%.

Several Methods available in literature were tried including Two Step Method, Double Emulsion Solvent Evaporation and Single Step Nanoprecipitation Method To Formulate and Develop of Hybrid nanocarriers (HNCs). From this, Thin lipo-polymeric film formation followed by hydration, and extrusion was found to formulate HNCs of desired characteristics. Folate functionalized PEGylated DSPE was used to avoid RES recognition and increase blood circulation time of nanocarriers with target specific delivery. DPPC (glass transition temperature 41 C) was a primary lipid to form hybrid nanocarriers and it also assist to release its cargo in cancerous tissues having temperature (39-40 C) higher than normal body temperature. pH sensitive characteristics is due to the DOPE which helps nanocarriers for endosomal escape. Fatty acid carboxyl ions of DOPE makes nanocarrier stable at lamellar phase in neutral pH due to electrostatic repulsion but at acidic pH, these groups are protonated, converting them into the unstable hexagonal phase which in turn fuse, aggregate and release the cargo into the cytosol easily. Furthermore, DOTAP was added to impart cationic charge to nanocarriers for effective complexation of negatively charged siRNA. In this method, all lipids, polymer and drug were dissolved in chloroform and film were produced using Rotary Flask evaporator and hydrated using Preheated distilled water (40°C). Process parameters like solvent

evaporation temperature, vacuum condition, rotation speed, extrusion cycle Several Parameters were optimized then QbD approach by applying Box-behnken Design was implemented by taking Lipid Concentration (1-5 mM), Polymer Concentration (0.5-0.83 mg/ml) and Lipid molar ratio (1.5-4.0) as independent variables and particle size (nm), % entrapment efficiency as dependent variables. QbD enabled design expert software suggested an optimized batch having composition of Lipid Concentration (4.24 mM), Polymer Concentration 0.83 mg/ml) and Lipid molar ratio (3.36) and predicted size of 159.99 nm and 72.77 % entrapment efficiency and the same batch was formulated to validate the results and the particle size was found to be 162.2± 3.45 nm with the PDI of 0.12 and Zeta Potential was found to be +43.18±1.83 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 71.53±2.12 % (n=3) in the optimized formulation. The drug loading was found to be 0.072 w/w and 7.2 % w/w (n=3).

The transmission electron microscopy (TEM) was performed to characterize HNCs 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-PLA core. The thickness of ring is less than 20 nm. It confirms the morphology and architecture of HNCs i.e. formation of solid matrix core of polymeric unimers region inside lipid bilayer. Polymeric core showed distinct PEG-PLA particles inside cavity of HNCs with embedded matrix formation as well which strengthen the architecture of HNCs in comparison to liposomes alone. Surface visualization and shape of vesicle were conformed through SEM and HNCs were found to be spherical in shape with size of 175 nm approximately. The average size was found to be 149 nm through TEM. Surface visualization and shape of vesicle were confirmed by SEM and HNCs vesicles were found to be spherical in shape with size of 170 nm approximately. The morphological studies performed by AFM showed uniform and spherical shaped discrete particles without aggregation with approx. Size of 150 nm. The height histogram shows fairly narrow distribution of peak heights from 5 to 20 nm corresponding to lipid bilayer thickness and PEGylation layer. The average roughness (Ra) was observed to be 10.2 nm. These values indicated a slightly rougher surface. The increase in roughness could be attributed to the presence of

PEGylation layer due to DSPE-PEG-2000 in HNCs which is necessary for stability and long circulation time in-vivo. HNCs shape and architecture in three dimensions were analyzed through SAXS. The results revealed nanocarrier size in the range of 142 nm of maximum dimension and 71 nm of Radius of gyration thus confirming spherical shape of HNCs. Moreover, sharp peaks obtained in scattering patterns indicated formation of complex matrix core of PEG-PLA inside HNCs.

The drug release at pH 7.4 will give idea about Cisplatin caprylate release from HNCs in blood and inside the normal tissue. Here, Lipid coating around HNCs and polymeric core matrix of PEG-PLA retard the drug release kinetics by keeping the external dissolution fluid medium away from the polymeric matrix core thereby drug release kinetic is governed by both lipid as well as polymer's characteristics. At pH 7.4, its release was very slow showing only 15 % release within initial 10 hr time period and reached to a maximum of 27 % in the span of 3 days. This again infers that Cisplatin caprylate release in the blood as well as in normal tissues will be very low thereby a low toxicity might be conferred by the HNCs. At pH 6.6, release was found to be somewhat faster, showing 25 % release within 10 hr but then after it was slowed down. Cisplatin caprylate release from HNCs at pH 5.5 showed a drastic release pattern with 38 % release within first 10 hr and it went on increasing to around 48 % in 24 hr which indicated that 48 % Cispatincparyalte could be released inside the cells within one day once HNCs gets internalized by cancer cells. Endosomes and lysozomes of cancer cells (pH 5-6) would be playing important role in inducing release of cisplatin caprylate from HNCs. From the Kinetic model fitting analysis, it was concluded that for Cisplatin caprylate loaded HNCs the best fit was Higuchi model with R^2 value of 0.9844. This shows that the drug release is matrix diffusion controlled release process HNCs. Estimation of Residual solvent was checked by GC-FID. The USP guidelines suggest that Chloroform is CLASS II solvent and the limit for PDE (Permitted Daily Exposure) is 0.6 mg/day equivalent to 60 ppm. From the data of the residual solvent, it was confirmed that chloroform present in the final

optimized batch of HNCs is within the limits as per USP guidelines for residual solvents.

Cisplatin caprylate loaded siRNA anchored HNCs were prepared and optimized with N/P ratio having 1.5 with % siRNA Complexation 95.83 ± 0.192. The particle size after lyophilization was found to be 171.2±2.74 nm. Zeta potential was found to be decreased from +43.18±1.83 to 25.39±0.183 after siRNA complexation and further confirms the surface interaction between positively HNCs and negatively charged siRNA. All vesicles were unilamellar in structure and having particle size below 200 nm. These ranges assist in EPR effect for tumor internalization of nano materials and also avoiding RES uptake. Further confirmation of HNCs structure was done by Cryo-TEM which revealed unilamellarity in structure and having particle size below 200 nm. Formations of distinct PEG-PLA particles along with matrix formation inside cavity of HNCs were also confirmed by Cryo-TEM. Further insight into the HNCs cavity was captured by freeze fractured Cryo-TEM and it revealed solid polymeric matrix core inside HNCs. From the results of band density of gel electrophoresis in serum stability assay, it was conformed that HNCs retained integrity of siRNA thereby protecting it from nucleases of external environment. Additional protection might be provided by post insertion technique of PEGlyation employed after attachment of siRNA with HNCs.

Cell line studies were performed to assess cytotoxicity, cellular uptake of formulation along with chemo sensitization of A549 lung adenocarcinoma cells. MTT assays of placebo formulation of HNCs and siRNA-HNCs complexes were performed to assess effect of lipids, polymer and siRNA complexation on cell cytotoxicity. Both formulations were non-cytotoxic to cells. From the images of confocal microscope obtained for cellular uptake studies it can be concluded that HNCs could successfully carry and deliver siRNA and cisplatin into the cytosol i.e. transfection efficiency is improved with HNCs. Fluorescence intensity of HNCs is very much near to transfection standard lipofactamine-2000. Successful transfection of the cells using HNCs depict that they are easily uptaken by the cells through endocytosis due to their cationic characteristic. Chemo sensitization effect was proved by the fold change in IC50 values when siRNA was complexed with cisplatin caprylate loaded HNCs. All Cisplatin loaded siRNA complexed HNCs showed

significantly less IC50 value as compared to Cisplatin loaded HNCs. The Fold change in IC50 values of siRNA complexed HNCs are 1.52, 1.92 and 3.25 for 24 hr, 48 hr and 72 hr respectively. Highest chemo-sensitization was observed at 72 hr. Further, To examine the cell uptake efficiency of folate ligand (DSPE-PEG 2000-folate) anchored HNCs, H1299 and A549 cells were transfected with HNCs containing FITC labelled siRNA. It revealed significant more uptake and delivery of DSPE PEG-2000-folate anchored HNCs in folate overexpressing H1299 cells than low or no folate expressing A549 cells. Cell cycle analysis of cisplatin caprylate treated A549 cells was performed using FACS to know mechanism of action of cisplatin & cell cycle progression. It revealed that cell populations had been able to move on to S+G2+M phase of cell cycle and all phases showed similar amount of cell populations therefore it was established that cisplatin is cell cycle non-specific and it exerted its action on all phases of cell populations. Gene knock down study revealed significant knock down of ABCC3 mRNA.

Dry powder for inhalation of Cisplatin loaded siRNA HNCs was formulated by using lyophilization method. Anderson cascade impactor (ACI) was used to assess the aerodynamic properties of the processed lyophilized bulk containing cisplatin. All these characteristics are considered sufficient to achieve deep lung delivery of nanoparticles. When the data of aerodynamic behaviour was compared it revealed the critical role played by coarse carriers in development of DPI. At lower carrier mass ratios, in case of both respitose SV003 and inhalac 230, there was low emitted dose i.e. 52.30% and 51.35% for inhalac 230 and respitose SV003, respectively. This indicates that bulk properties were dominated by lyophilized bulk with sticky/cohesive nature and was poorly fluidized. The incorporation of coarse carrier at higher mass ratio, led to improvement in emitted dose up to 75.10% and 78.90 % for inhalac 230 and respitose SV003, respectively due to improved fluidity. However, the FPF and consequently the MMAD observation revealed differences in inter-particulate forces when using two different carrier. Respitose showed higher FPF than the Inhalac 230 i.e. 37.48 % and 27.87% respectively at carrier mass ratio of 1:3. This clearly indicated the obvious choice of respitose SV003 as carrier for present formulation. 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. 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 crystalline drug material in the DPI samples. This shows the crystallinity of the drug has been reduced significantly in the nanoparticles. Hence it could be concluded that in the prepared DPI, the drug was present in the amorphous phase. All these peaks are considered characteristic to Cisplatin caprylate and are prominently observed. Whereas, in case of DPI all characteristic pertaining to Cisplatin caprylate are absent, indicating the enhanced encapsulation of drug in the HNCs and only a diffused peaks throughout the spectra at wavenumber higher than 1400 cm^{-1} are observed.

The pulmonary pharmacokinetic parameters were calculated and are represented in Table 9.2. A maximum t½ value of 8.06 hours was observed with Cisplatin HNCs compared to 2.20 hours with marketed formulation. Eventually, there was an increase in AUC for HNCs compared with the AUC of Marketed formulation. HNCs showed 2.44 times higher AUC values than marketed formulation. The Tmax values for HNCs was 4hours compared to marketed formulation was 2 hours, thereby confirming the maintenance of effective drug concentration with HNCs in lung tissue for prolonged period compared to marketed formulation. L/B ratio, LDH & ALP estimation were carried out as lung function evaluation tests which showed satisfactory results. Haemolysis was measured in using above mentioned method. From the results one can see that haemolytic properties of optimized formulation are below marketed Cisplatin injection. Comparative graphs of the haemolytic study are shown in figure. Even optical method confirms that the shape and concentration of the RBCs in the HNC R4 and marketed formulation are almost same

The stability studies were carried out, for optimized formulations, in accordance with the ICH guidelines to evaluate the suitability of the storage condition for the developed formulations. Short term stability study was carried out for 3 months at 2-8°C and Room Temperature25 ± 2°C /60 ± 5% RHfor the HNCs and DPI.). There was no significant difference in assay and complexation efficiency of formulations at different storage time points in real time condition. However, at accelerated condition there was a drop of around 10% for assay, though it was within specification (80-120%). The water content showed a gradual increase with time and this cloud have been responsible for a minor drop in complexation efficiency at accelerated storage conditions. The increase in water content may influence complexation efficiency as siRNA are prone to hydrolytic degradation. However, water content up to3% has been reported to be non-detrimental from aerosolization point of view. Therefore, it is recommended that water content of such formulations be strictly controlled < 3%.Stability studies at accelerated and refrigerated conditions demonstrate that the product was stable at both conditions for a period of 3 months and suggest that the product will be stable for longer periods at refrigerated conditions.

Conclusion

In current investigation, Hybrid nanocarriers were developed for combinatorial delivery of anti-neoplastic drug and siRNA. It established potential to treat cancer using combinatorial therapy involving chemotherapy and genomic approach with improved therapeutic index and reduced dose dependent toxicity of the Cisplatin. Delivery of ABCC3 siRNA grafted onto HNCs targeting ABCC3 protein exerted its knock-down followed by sensitization of cancer cells. Formulated cisplatin caprylate loaded siRNA anchored HNCs are stable in presence of serum and delivered the siRNA and cisplatin caprylate inside the cells having significant transfection efficiency. Selective targeting to cancer cells were achieved by anchoring HNCs with PEGylation. The core idea of masking resistance to chemotherapy in lung cancer was thus fulfilled and this targeted combinatorial delivery of drug and gene in a single formulation established a potential route to enhance efficacy and effectiveness of anti-neoplastic drugs in cancer while diminishing off targeted toxicity issues due to reduced dosing profile of anti-neoplastic drug.