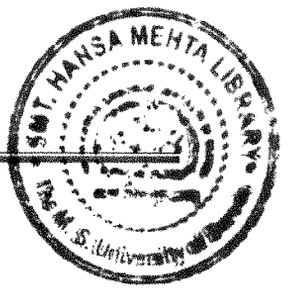

CHAPTER 7:

SUMMARY AND CONCLUSION

Chapter VII: Summary and Conclusion

7.1 Summary

Liposomes that are biodegradable and essentially non-toxic, can encapsulate both hydrophilic and hydrophobic materials, and are currently being used as carriers for drug delivery systems in research, clinical trials and clinical practice. Recent progresses in gene delivery (pDNA, siRNA, AS-ODN) provide a novel modality of therapy for various hereditary and acquired genetic diseases using a variety of novel liposomes. The success of any gene therapy strategy is in designing a vector which is able to serve as a safe and efficient gene delivery vehicle. This has encouraged the development of non viral DNA mediated gene transfer techniques such as liposomes. Many liposome based DNA delivery systems have been described including ligands for targeting the cell surface receptors for enhanced therapeutic action or for escaping from lysosomal compartment. Cationic liposomes have shown excellent *in vitro* transfection ability, however they suffer from the disadvantage of poor lipoplex stability. Further, *in vivo* lung delivery of the lipoplexes by intravenous route for pulmonary targeting is also an obstacle because of poor targeting to the alveolar region and poor formulation stability in parental form. Hence, our **objective** was focused on **preparing a stable lipoplex formulation with excellent transfection ability and targeting the formulation directly to the lung by developing dry powder inhaler (DPI) formulation of the lipoplex.**

In the current investigation, *p*53 was used as a therapeutic *p*DNA for treatment of NSCLC. The objective of the work was to develop the cationic liposomal formulations for efficient transfer and transfection of *p*53 to the lung cancer cells for enhanced cytotoxicity without dose dependant side-effects, as observed with chemotherapeutic agents. Development of ligand attached *p*53 lipoplexes for enhanced cancer cell uptake and transfection was studied. Also, role of *p*53 based gene delivery in treatment of resistant tumors requiring need of higher dose of cytotoxic agents was studied. The delivery of *p*53 as pretreatment approach and co-administration approach along with chemotherapeutic drugs as Etoposide and Docetaxel formulations was studied to determine their comparative effect on cytotoxicity in terms of sensitization and synergism towards cytotoxicity of NSCLC cells. Further, development of DPIs of *p*53 ligand attached lipoplexes and other cytotoxic formulations was performed and evaluated for direct lung deposition studies using Anderson Cascade Impactor.

7.1.1 Analytical Method Development:

The analytical methods employed during preparation and optimization of pDNA complexed lipoplexes and for estimating the marker β galactosidase transgene expression were developed. Further, analytical methods for estimating Etoposide and Docetaxel during their liposomal and lipoplex preparation, optimization and characterization are also developed.

The measurement of pDNA was generally carried out using UV spectrophotometer with the help of a generalized equation as Absorbance of 1 is equivalent to concentration of 50 µg /ml. The DNA solution in Tris buffer shows absorption maximum at 260 nm and the correlation coefficient of 0.9991, which indicates that the absorbance and concentration of DNA are linearly related. The slope of the regressed line 0.0205 indicates moderate sensitivity of the method. The method demonstrated linearity of the curve up to the range 2-60 µg / ml concentration.

β-galactosidase protein estimation was performed by the colorimetric assay for estimation of β-galactosidase gene expression by direct measurement of enzyme activity produced as a function of transfection. The expressed β-galactosidase protein hydrolyses the artificial chromogenic substrate ortho-nitrophenyl-β-D-galactopyranoside (ONPG) to release yellow colored orthonitrophenol which absorbs light at 405 nm. The intensity of the color developed directly correlates with the quantity of β galactosidase expressed in the cells. Using the synthesized β – galctosidase protein and ONPG, a calibration curve was prepared with range of 5-50 µg of the protein treated with constant quantity of ONPG to release yellow color. The correlation coefficient of 0.993 after 5 and 20 minutes of absorbance reading indicated that, the absorbance and concentration of ONP were linearly related. The absorbance of ONP after β gal transfection is converted to β galactosidase activity and is used for the estimation of transfection efficiency of various transecting agents *in vitro* on cell lines after cell lysis with lysis buffer.

The spectroscopic determination of Etoposide (ETP) in the liposomes is based on absorption spectrum of ETP in UV-Visible region after dissolving in Methanol. ETP after dissolving in methanol showed linear increment in absorbance with concentration at 289 nm in the range of 0 to 50 µg/ml. The regression coefficient of 0.9996 confirmed the linearity. The slope of the regressed line 0.0084 indicates high sensitivity of the method.

The spectroscopic determination of Docetaxel (DTX) in the liposomes is based on absorption spectrum of DTX in UV-Visible region after dissolving in Methanol. DTX after dissolving in methanol showed linear increment in absorbance with concentration at 229 nm in the range of 10

to 50 µg/ml. The regression coefficient of 0.9993 confirmed the linearity. The slope of the regressed line 0.020 indicates moderate sensitivity of the method.

7.1.2 Plasmid Transformation, Isolation and Purification:

β galactosidase and p53 plasmid DNAs were transformed into E. Coli DH 5 α strain by TransformAid™ kit. The transformed colonies were identified using antibiotic resistance achieved through plasmid transformation. From the transformed colonies, the pDNA was back isolated using alkaline lysis method and was confirmed for correct transformation by restriction endonuclease digestion. After confirmation, pDNA was isolated in bulk using maxi-precipitation technique using the principle of alkaline lysis. The isolated pDNA was purified using phenol chloroform extraction followed by PEG 8000-Lithium Chloride precipitation and was dissolved and stored in sterile double distilled water or Tris -EDTA buffer. The purity of the pDNA was assessed by calculating the ratio of UV absorbance at 260 and 280 nm. The ratio in between 1.8-2.0 indicates pure pDNA devoid of protein and RNA. Further, the supercoiled nature of pDNA, essential for excellent transfection was confirmed with Agarose gel electrophoresis.

7.1.3 Development of Ligand Attached p53 lipoplexes for Treatment of NSCLC:

The section described development of liposomal systems prepared by Ethanol Injection, Thin Film Hydration and Supercritical Fluid Technology for development of lipoplexes suitable for p53 transfection and excellent lung deposition. Further, attachment of Transferrin as a cancer cell targeting ligand was used as an approach to enhance cell uptake of the lipoplexes and enhancing the transfection of pDNA. Further, aerosolization of these systems after lyophillization to deliver these lipoplexes directly to the lungs was also developed.

The liposomes were prepared using three different methods as Thin Film Hydration, Ethanol Injection and Supercritical Fluid Technology. TFH and EI were traditional methods and were used widely as standard methods as per literature. However, SCF is a novel technique for developing liposomes, nanosized in range, but having higher stability than the other two methods. It forms the liposomes in dry form which may be reconstituted whenever required and freshly used.

The thin film hydration method formed multilammelar liposomes in the size range of 1-5 microns. The effect of process parameters and formulation parameters was studied on development of liposomes. HEPES buffer was used as a hydration medium because of better hydration properties of DOPE in alkaline pH. These optimized liposomes on extrusion through

0.45 and 0.22 micron PC membrane filter were extruded to give large unilammelar vesicles (LUV) in the size range of 200-300 nm. The cryoTEM studies of the liposomes showed LUVs with size near to 150 - 200 nm confirming the size distribution data. The LUVs showed the zeta potential of + 30 to + 60 mv because of presence of DOTAP as cationic agent.

The ethanol injection method used dilution of dissolved lipids in hydration media to precipitate lipids in nanosized range as a principle to formulate the liposomes. The liposomes were formed in the nanosized range and required no extrusion cycles for sizing. The size of the liposomes was optimized by changing ethanol and aqueous medium (HEPES buffer) volume, stirring speed and needle used for ethanol injection. The optimized liposomes showed particle size of 113.9 $\pm$ 12.1 nm. The liposomes formed by ethanol injection method were unilamellar in nature. The morphology and lamellarity of the liposomes was confirmed by CryoTEM images. Further, all the liposomes showed zeta potential in the range of 25-51 mv because of presence of cationic DOTAP lipid.

In SCF method, the dry liposomes were formed by precipitation of lipids from organic solvent in the extraction chamber. The solvents were driven away by the supercritical CO_2 because of their preferential miscibility over lipids at supercritical condition. Further, various process and formulation parameters as CO_2 automated back pressure, CO_2 flow, temperature of extraction chamber, flow of solvent containing dissolved lipids, % lipid solid content after dissolving in solvents, solvent composition, were studied for formation of liposomes with minimum particle size and maximum yield. Alone DOTAP and DOPE were unable to form the lipid particles because of their lower Tg. Hence, mixture of DPPC, Cholesterol, DOTAP and DOPE was used. Further, improved yield and lower particle size was achieved by increasing the CO_2 flow rate, automated back pressure of the system and reducing the solvent flow rate at a temperature of 38-40^0 C. The SCF system formed liposomes of particle size 321.9 nm and yield of 35.2 % at the optimized condition. These liposomes were cationic in nature and formed multilamellar liposomes in the range of 300-1000 nm as revealed in cryoTEM. Further, because of DOTAP, the liposomes were cationic in nature and showed zeta potential in the range of 20-35 mV.

The liposomes prepared by all the methods were complexed with β galactosidase pDNA and the lipoplexes were studied for particle size and zeta potential. The lipoplexes prepared by EI showed minimum particle size at a N/P ratio when compared with TFH and SCF technique. The lipoplexes showed complete pDNA retardation and DNAse I protection at N/P ratio of 2/1 for

TFH, 3/2 for EI and 3/1 for SCF technique. Further, the lipoplexes were studied for in vitro cell uptake in H 1299 cell line at the above N/P ratios and showed cell uptake n accordance with the particle size of the lipoplexes. EI method showed highest cell uptake as compared with TFH and SCF technique. These lipoplexes were studied for β galactosidase pDNA expression using ONPG assay. In all the methods, lipoplexes showed comparatively higher cell transfection at higher DOPE content because of its higher fusogenecity. The EI method showed highest pDNA transfection comparable to Lipofectamine at DOTAP:DOPE ratio of 1:1 and N/P ratio of 3/2. Further, attachment of Tf enhanced the cell uptake and transfection of the lipoplexes significantly by about 200 % because of its over-expression on lung cancer H 1299 cell line. These lipoplexes also showed significant cell death after p53 transfection with only 29 % cell viability after attachment of Tf to the EI developed p53-lipoplexes. The cytotoxicity was mediated by restoration of p53 mediated apoptotic

Further, the developed lipoplexes were converted to dry powder inhaler formulations using lyophillization technique with trehalose as a cryoprotectant at lipid:trehalose ratio of 1:3. These developed lipoplexes showed significant lung deposition of 37-42 % and mean mass aerodynamic diameter of 3-4 μm suggesting significant lung deposition in upper alveoli and respiratory tract.

7.1.4 *p*53 Mediated Chemo-sensitization of Anticancer Drugs:

The section described development of liposomal system for delivering anticancer agents as Etoposide (ETP) and Docetaxel (DTX) as cytotoxic agents along with *p*53 administration for lung cancer treatment *in vitro*. A multicomponent gene delivery system containing 1) Blank cationic liposomes without drug, 2) Etoposide or Docetaxel encapsulated in above liposomes (same lipid composition), 3) *p*53 lipoplex and 4) *p*53 complexed with ETP and DTX encapsulating liposomes to form ETP-*p*53 and DTX-*p*53 lipoplex was developed and these systems were investigated for studying the effect of pretreatment and co-administration of *p*53 on cytotoxicity of liposomally encapsulated ETP and DTX in *p*53 null H 1299 and *p*53 (wt) A-549 lung adenocarcinoma cell lines. These studied were based on the hypothesis that, *p*53 restoration in *p*53 deficient / mutated cancer cells would ameliorate the altered apoptotic pathway and also reduce the BCL-2 mediated antiapoptotic non pump resistance thus sensitizing the cells towards chemotherapeutic agent. The effect of pre-sensitization and co-administration on comparative cytotoxicity in two different cell lines with varying *p*53 character was

determined to demonstrate effect of time of *p*53 delivery and added advantage offered by multicomponent gene delivery system. The studies were also aimed to develop DPI formulations of these lipoplexes for studying the lung deposition pattern for direct lung delivery and targeting, thus enhancing the therapeutic efficiency, reducing unwanted side effects and lowering the dose of the drug.

ETP and DTX containing liposomes were developed using thin film hydration method. The effect of various process parameters as rotation RPM, vacuum, hydration temperature and formulation parameters as drug : lipid molar ratio, lipid composition, hydration medium and volume of hydration medium was optimized to formulate liposomes with highest entrapment and drug loading. The entrapment in all batches was determined after extrusion through 0.45 and 0.22 µm polycarbonate membrane filter paper (3 cycles). The liposomes were optimized at Drug : Lipid molar ratio of 1:10 for ETP and 1:20 for DTX. The lipid composition was optimized at DOTAP, DOPE, DPPC and cholesterol in the ratio of 0.5:0.5:7:2. The HEPES buffer, pH 8.0 was used as hydration medium with 1 ml/20 mg lipid in ETP liposomes and 1 ml/30 mg lipids in DTX liposomes as optimized volume. The optimized liposomes showed particle size of 212.7 ± 4.2 nm for ETP and 227.9 ± 3.1 nm for DTX respectively. These size reduced liposomes showed entrapment of 72.36 ± 1.34 % for ETP and 69.54 ± 2.87 % for DTX and drug loading of 6.073± 0.125 % for ETP and 4.34 ± 0.19 % for DTX. The CryoTEM studies further confirmed the size distribution near 200 nm and the unilamellar structure was also confirmed. The developed liposomes showed positive zeta potential because of presence of cationic lipid DOTAP. The optimized liposomal ETP and DTX formulations showed zeta potential of 24.6 ± 1.3 and 23 ± 2.1mV respectively. These liposomes were lyophilized with trehalose at lipid: sugar ratio of 1:3 to achieve a cake with easy and complete redispersion, near 100 % drug retention and unchanged particle size. The lyophilized formulation was further used for DPI development and in vitro lung deposition. These liposomes were stable upto 3 months at 2-8^0 C and showed similar physical properties. The drug loaded liposomes showed sustained release of drugs up to 24 hrs in pH 7.4 release media. ETP loaded liposomes showed 97.23 ± 1.67% drug release after 24 hrs and DTX loaded liposomes showed 99.32 ± 1.32% drug release after 24 hrs. However, when release is carried out in acidic conditions stimulating tumor environment, release was found to be comparatively faster with complete drug release in 16 hrs. ETP loaded liposomes showed 99.28 ± 2.98 % drug release after 16 hrs and DTX loaded liposomes showed 99.62 ±

2.24 % drug release after 16 hrs. The faster release in acidic pH was observed because of membrane disruption and lipid fusion property of DOPE observed at acidic pH.

The optimized ETP and DTX liposomes were further used for development of lipoplexes with *β galactosidase* and *p53 p*DNA at N/P ratios of 1/1 to 40/1. The lipoplexes showed higher particle size in the range of 200-500 nm and zeta potential in the range of 10-35mV. The size of drug loaded lipoplexes was comparatively higher than blank lipoplexes. The drug loaded lipoplexes showed comparatively slower drug release than their liposomal counterpart possibly because of presence of tightly condensed plasmid *p*DNA in the lipoplexes. ETP loaded lipoplexes showed 89.35 $\pm$ 3.12 % drug release after 24 hrs and DTX loaded lipoplexes showed 96.98 $\pm$ 2.17 % drug release after 24 hrs. In acetate buffer, drug release was found to be comparatively faster with complete drug release in 16 hrs. ETP loaded lipoplexes showed 98.69 $\pm$ 2.78 % drug release after 16 hrs and DTX loaded lipoplexes showed 99.40 $\pm$ 2.51 % drug release after 16 hrs. The lipoplexes when tested with agarose gel showed complete retardation of pDNA at N/P ratio of 2:1 and showed protection against DNAseI enzyme treatment.

The liposomes and lipoplexes were studied for cell uptake using 6-Coumarin as a fluoroscent marker in place of ETP or DTX. The formulation and process parameters along with lipid composition were kept constant as with ETP and the size was reduced by extrusion through 0.45 and 0.22 µm polycarbonate filter papers. 6-Coumarin liposomes and lipoplexes showed particle size of 230.6 nm and zeta potential of 26 mv. The lipoplex showed size of 409.9 nm and zeta potential of 19 mV. The liposomes and lipoplexes were studied for qualitative and quantitative cell uptake on H 1299 and A 549 cell lines, grown in complete DMEM medium with antibiotics at 37^0C in 5 % CO_2 containing humidified atmosphere. The quantitative uptake using FACS analysis showed higher uptake in H 1299 cell line after delivery in liposomes (89.9 $\pm$ 1.1 % cells) than with lipoplexes (81.8 $\pm$ 2.8% cells) and suspension (62.7 $\pm$ 2.12 cells). The uptake was comparatively higher in H 1299 cell line than in A 549 cell line, but with similar order. The higher uptake in liposomes was because of smaller size of liposomes than the comparative lipoplexes and suspension. In A 549 cell line, 86.3 $\pm$ 2.1 % of cells showed 6 Coumarin uptake in case of liposomal delivery, 79.7 $\pm$ 1.3 % of cells showed uptake after lipoplex delivery and 58.1 $\pm$ 1.88 % cells showed uptake after suspension delivery. Further, the intensity of cells for 6-Coumarin uptake was qualitatively compared using fluoroscent microscopy. The results indicated higher uptake in case of liposomes than in lipoplexes than in suspension.

The transfection of blank cationic liposomes with β gal pDNA was performed at varying N/P ratio from 1:1 to 40:1 keeping the quantity of pDNA constant at 300 ng. The gene expression was quantitatively estimated at 405 nm using microplate-reader by ONPG - β galactosidase enzyme colorimetric assay. Significant transfection efficiency was observed at N/P ratio 2:1 because of their zeta potential near neutrality and lower particle size which causes lower interaction with serum proteins followed by transfection efficiency at N/P ratio above 20:1 which shows significantly lower particle size theirby enhancing cellular uptake. Transfection with Lipofectamine showed maximum transfection and was kept as positive control and transfection with β gal naked pDNA and blank liposomes was kept as negative control. Further, the lipoplexes encapsulating ETP and DTX were tested for transfection. These lipoplexes showed comparatively lower transfection than their counterpart blank lipoplexes, probably because of their higher size and cytotoxicity caused by ETP and DTX. Maximum transfection in presence of ETP and DTX was achieved at N/P ratio of 25/1. These lipoplexes showed higher transfection in H 1299 cell line than A 549 cell line. At this N/P ratio, p53 was used to form lipoplexes for cytotoxicity, p53 transfection and Annexin V FITC assay.

The p53 mediated enhancement in cytotoxicity was studied by modified MTT assay. p53 lipoplexes, ETP and DTX liposomes, ETP-p53 and DTX-p53 lipoplexes and drug solutions were tested for cytotoxicity with pretreatment and co-administration approach. Cytotoxicity studies were performed in two cell lines as H 1299 (p53 null) and A 549 (p53 wt), which mimics the deleted / mutated / normal but inactive *p*53 character in majority of lung cancers *in vivo.* The results demonstrated enhanced cytotoxicity of ETP and DTX formulations when administered along with p53 in both the approaches. Cationic liposomes and *p*53 solution showed non-significant toxicity and were considered as controls. ETP and DTX as liposomal formulations showed higher cytotoxicity than their drug solutions, because of higher cell uptake of nanosized drug loaded cationic liposomes than their solutions. The ETP liposomes showed 69.90 ± 1.26 % in H 1299 and 72.11 ± 2.15% cell viability in A 549 cell line, where as DTX liposomes showed 63.19 ± 2.71% in H 1299 and 69.56 ± 2.63% cell viability in A 549 cell line. Further, p53 lipoplexes (N/P-25/1) themselves showed comparatively low cytotoxicity with 79.17 ± 1.54 % viability in H 1299 and 91.29 ± 4.12% viability in A 549 cell line, (Single factor ANOVA, $p < 0.05$) consistent with the transfection studies performed earlier. The effect of p53 was more

pronounced in H 1299 because of initial absence of p53, where as lower effect of p53 was observed in p53 wt A 549 cell line.

The co-administration approach involved delivery of p53 solution or lipoplex along with drug solution or liposomes as well as delivering p53-ETP or p53-DTX lipoplex for synergistic cytotoxic action of p53 and chemotherapeutic agents. The p53 solution did not show any significant cytotoxicity enhancement, because of low cytotoxic action of p53 solution itself, however, p53 lipoplex showed 15-20% enhanced cytotoxicity when administered along with EPT liposomes and 20-25 % enhanced cytotoxicity when administered along with DTX liposomes. The enhancement in cytotoxicity may be attributed to simultaneous transfection of *p*53 resulting in regaining normal apoptosis function theirby causing cytotoxicity as well as suppressing the level of BCL-2 anti-apoptotic cellular defense resistance protein, thus sensitizing the cells towards the action of chemotherapeutic agent. Further, when *p*53 complexed drug liposomes (*p*53- drug lipoplexes) were treated with the cells, further enhancement by 10-15 % of the drug cytotoxicity was observed than simultaneous administration of drug liposomes and *p*53 lipoplexes treated together ($p<0.05$). The enhancement was more potent in case of DTX (38.83 ± 3.06 % in H 1299 and 49.12 ± 2.89% in A 549) than in case of ETP (50.67 ± 2.11% in H 1299 and 58.63 ± 1.82 % A 549 cell line) . This enhancement may be attributed to the uptake of drug and the DNA in appropriate concentrations in the same cell theirby enhancing the effect.

The pretreatment of *p*53 also enhanced the cell sensitization towards the action of ETP and DTX by 40-60 % as compared to drug solution and liposomes. The cytotoxic action was 25-40 % higher than the *p*53- ETP / DTX co-administration with 20 to 35 % of cell viability after drug treatment. The cells showed viability of 31.91 and 19.78 % after *p*53 followed by ETP / DTX treatment in H 1299 cells and 42.25 and 33.65 % in A 549 cell line. The cells especially H 1299 showed high chemosensitization than in A 549 which may be attributed to higher *p*53 transfection in H 1299 than A 549. DTX showed comparatively higher cytotoxicity than ETP formulation thereby showing higher chemosensitization and potency of the molecule. Further, when *p*53 is treated alone followed by drug treatment as in pretreatment approach, the *p*DNA has shown higher transfection than when treated along with the drug solution or liposome. The *p*53 transfection in turn makes the cells more responsive towards chemotherapy by restoring the normal cell apoptotic function and reducing BCL 2 mediated cellular antiapoptotic defense. Further, the bystandard action of *p*53 protein after expression in lung cancer cell lines has also

reported to play a significant role in enhancing the action of *p*53 throughout the complete cells even after possibly getting transected in fewer cells thereby enhancing the cytotoxic action of the drug.

The transfection of p53 by liposomal systems in presence of chemotherapeutic agents was studied quantitatively by flow cytometry using human p53 specific primary mouse monoclonal IgG2a antibody and Alexafluorr 488 goat antimouse primary antibody. The results observed were similar to transfection with β galactosidase transfection and cytotoxicity results after p53 pre and p53 co-administration with ETP and DTX formulation treatment.

The treatment of H 1299 with p53 lipoplex showed significant p53 transfection evidenced by fluoroscence shift of cells in the histogram. At N/P ratio of 25/1, the cells showed lower transfection with 51.5 % of cells showing fluoroscence. Further, treatment with *p*53 - drug lipoplex at N/P ratio 25/1 showed lower transfection possible because of cytotoxic effect of the drug. The *p*53-DTX and *p*53-ETP lipoplex showed *p*53 expression of 17.49 % and 28.26 % respectively in H 1299 cells. This lowered transfection was consistent with the transfection and cytotoxicity data provided earlier. However, the pretreatment approach showed significantly higher *p*53 expression irrespective of the drug treated later. *p*53 followed by ETP and DTX liposomes, both showed *p*53 transfection in 44-45 % cells. This higher transfection followed by significant drug action was responsible for higher cytotoxicity of the cells.

Further, the action caused by p53 i.e. restoration of apoptosis function and the action caused by chemotherapeutic agents, i.e. necrosis and apoptosis was recorded with Annexin V FITC assay, which differentiates the cell death by apoptosis and necrosis. The results showed significant increase in Annexin V positive cells and Annexin V and PI positive cells after pretreatment and co-administration of *p*53 and ETP / DTX when compared with plain ETP and DTX liposomes ($p < 0.001$). The enhancement was significant in H 1299 cell line and in presence of DTX ($p<0.001$). After *p*53 pretreatment, followed by ETP and DTX delivery, higher Annexin V positive – early apoptotic cells were observed along with necrotic cells demonstrating chemo-sensitization of the cells. When *p*53- ETP / DTX lipoplex were co-administered, synergistic action of *p*53 and chemotherapeutic agent was observed. However, % early apoptotic cells were lower than the pretreatment approach. Higher Etoposide apoptosis was observed in A 549 cells (p53 wt), as compared to H 1299 probably because of property of ETP to induce apoptosis inside cells in presence of *p*53 as reported previously.

Chapter VII: Summary and Conclusion

From the transfection, cytotoxicity and Annexin V FITC assay results, conclusively, p53 pretreatment and p53- ETP/DTX co-administration demonstrated enhanced cytotoxicity with increased cell apoptosis and reduced antiapoptotic defense mechanism.

The optimized lipoplexes were lyophilized for enhanced stability of the formulations and DPI development for direct lung deposition. The lyophillization was optimized with trehalose, (lipid:trahalose ratio 1:3) to achieve same physical properties of the lipoplexes without non-significant changes in particle size and zeta potential and near 100 % drug retention when they come in contact with aqueous medium to release nanosized lipoplexes..

The developed trehalose based lyophilized formulations on testing for transfection and cytotoxicity showed statistically non-significant changes in results as compared to the freshly prepared formulations theirby offering the more stable formulations for DPI development and *in vivo* drug delivery. All the formulations with their similar nanosized particles in range of 200-500 nm and same lipid:trehalose ratio showed similar deposition pattern with FPF in range of 33-37 % and MMAD of 3-4 μm. The MMAD of 3-4 μm indicates lung distribution in trachea, upper respiratory area and upper alveoli. When the powder comes in contact with lung fluids, the liposomes get hydrated and after getting detached from sugar moiety. These liposomes then can be internalized to give intracellular cytotoxic effect as well as pDNA transfection. The powder on impactor plates when collected and purified for DNA extraction showed supercoiled DNA on agarose gel indicating pure and stable cDNA with efficient transfection capacity.

Thus, the direct lung delivery as dry powder inhaler helps in opening a new strategy to treat lung cancer in patients having drug resistance. Although DPI formulations favors, the co-delivery approach because of simultaneous entry of cDNA and drug in the same cell and tissue, but the pretreatment approach may also show significant enhancement in cytotoxicity, probably because of the p53 bystandard effect observed in vitro and in vivo, theirby making the direct lung delivery approach a suitable treatment strategy to treat NSCLC especially in cases of drug resistance with enhanced target specificity

7.2 Conclusion:

Overall, the p53 based therapy proved to be viable option for treatment of NSCLC when considered in terms of its in vitro performance on cell lines. From the present work, it can be concluded that, Ethanol injection proves to be the best method for p53 transfection in the NSCLC cell lines. The method demonstrated lowest particle size and highest cell uptake resulting in highest transfection. SCF proved to be a novel method for developing liposomes with enhanced stability and comparable transfection of a pDNA in the H 1299 lung adenocarcinoma cell line. Further, attachment of cancer cell selective ligand as Transferrin, helped to enhance the cell uptake and transfection of a marker pDNA in optimized formulations. Amongst all the developed formulations, p53-Tf-Lipid formulations prepared by EI method showed highest cytotoxicity in H 1299 lung cancer cell line. The higher cytotoxicity was observed because of restoration of p53 leading to apoptosis of the cancer cells. Further, development of these lipoplexes as freeze dried systems significantly improved the stability of the lipoplexes and showed significant *in vitro* lung deposition. Overall, the developed p53 based formulation conclusively demonstrated significant anticancer action with potential lung deposition and may be useful for restoring the normal condition of a patient after multiple dosing in cancer therapy.

Further, the results of chemosensitization also conclusively demonstrated that, pre-treatment and co-administration of p53 tumor suppressor gene lead to increase in cytotoxicity of Etoposide and Docetaxel by enhanced apoptosis leading to reduced BCL-2 mediated antiapoptotic defense of the cells. Increased apoptosis suggests restored p53 function and reduced antiapoptotic drug resistance. It causes cell sensitization and synergism towards drug cytotoxicity. This enhancement in cytotoxicity may finally help in reducing drug dose, resistance and side-effects and may also help in remission of the tumor completely by correcting the genetic malfunction. Further, direct lung delivery as dry powder inhaler will help in opening a new strategy to treat lung cancer in patients having drug resistance. Although DPI formulations favors, the co-delivery approach because of simultaneous entry of pDNA and drug in the same cell and tissue, but the pretreatment approach may also show significant enhancement in cytotoxicity, probably because of the p53 bystandard effect observed in vitro and in vivo, theirby making the direct lung delivery approach a suitable treatment strategy to treat NSCLC especially in cases of drug resistance with enhanced target specificity.

7.3 Future Scope:

Although the work shows excellent in vitro cell line cytotoxicity, however, the preclinical and clinical testing must be performed for actual performance of the formulation in the in vivo condition.