

TABLE OF CONTENTS

| | |
|---------------------------------------------------------------------------|-----------|
| 1. INTRODUCTION..... | 1 |
| 1.1 Introduction to lung cancer and key statistics..... | 1 |
| 1.2 Drug resistance in cancer | 2 |
| 1.3 Gene delivery vectors | 3 |
| 1.4 Chemotherapeutics and combination therapy in lung cancer | 5 |
| 1.5 Targeted delivery of siRNA therapeutics in lung cancer..... | 6 |
| 1.5.1 Gene targeting:..... | 7 |
| 1.6 Aim | 9 |
| 1.7 Objective of proposed work..... | 9 |
| 1.8 Rationale | 10 |
| 1.9 Hypothesis..... | 12 |
| 1.10 Research Plan..... | 12 |
| 1.11 References..... | 14 |
| 2. LITERATURE REVIEW | 16 |
| 2.1 Lung Cancer and its types..... | 16 |
| 2.1.1 Risk factors | 18 |
| 2.1.2 Signs and Symptoms & Pathogenesis of Non-Small cell lung cancer | |
| 20 | |
| 2.1.3 Treatment approaches for Non-Small cell lung cancer..... | 22 |
| 2.2 Mechanism of multidrug resistance and approaches used for reversal of | |
| resistance in cancer | 24 |
| 2.2.1 Multi drug resistance mechanism | 24 |
| 2.2.2 Various approaches used in reversal of resistance in cancer | 26 |
| 2.3 Gene therapy & RNA interference | 27 |

| | | |
|-----------|-----------------------------------------------------------------------------------------|-----------|
| 2.3.1 | Fundamental principles of gene therapy | 28 |
| 2.3.2 | Approaches for gene therapy | 31 |
| 2.4 | Delivery vector: Hybrid nanocarriers | 31 |
| 2.4.1 | Methods of preparation of HNCs..... | 34 |
| 2.4.2 | HNCs Reported in Literature with their applications | 34 |
| 2.4.3 | Combinatorial delivery of siRNA and anti-cancer drug therapeutics 35 | |
| 2.5 | Pulmonary Delivery of Nanocarriers | 36 |
| 2.6 | Drug Profile: Cisplatin..... | 37 |
| 2.7 | Excipients Profile..... | 40 |
| 2.8 | References..... | 42 |
| 3. | ANALYTICAL METHODS | 45 |
| 3.1 | introduction..... | 45 |
| 3.2 | Materials and instruments | 47 |
| 3.2.1 | Materials | 47 |
| 3.2.2 | Instruments..... | 47 |
| 3.3 | Methods..... | 48 |
| 3.3.1 | Estimation of Cisplatin using derivatization method by colorimetry | 48 |
| 3.3.2 | Estimation of Cisplatin using High Performance Liquid Chromatography (NP-HPLC) | 49 |
| 3.3.3 | Estimation of total phospholipid content by Stewart method..... | 52 |
| 3.3.4 | Analytical method development of siRNA..... | 53 |
| 3.4 | Results and Discussion | 56 |
| 3.4.1 | Estimation of Cisplatin using derivatization method by colorimetry | 56 |
| 3.4.2 | Estimation of Cisplatin using High Performance Liquid Chromatography (NP-HPLC) | 58 |

| | | |
|-----------|-----------------------------------------------------------------|-----------|
| 3.4.3 | Estimation of total phospholipid content by Stewart method..... | 63 |
| 3.5 | References..... | 69 |
| 4. | PRELIMINARY STUDIES | 70 |
| 4.1 | introduction..... | 70 |
| 4.2 | Materials and instruments..... | 70 |
| 4.2.1 | Materials | 70 |
| 4.2.2 | Instruments..... | 70 |
| 4.3 | Methods..... | 70 |
| 4.3.1 | Selection and procurement of siRNA | 70 |
| 4.3.2 | siRNA stability evaluation..... | 72 |
| 4.3.3 | Preparation of cisplatin caprylate complex..... | 72 |
| 4.3.4 | Drug-Excipient compatibility studies | 72 |
| 4.4 | Results and Discussion | 73 |
| 4.4.1 | Purity and concentration determination of siRNA..... | 73 |
| 4.4.2 | siRNA stability evaluation..... | 74 |
| 4.4.3 | Preparation of cisplatin caprylate complex..... | 75 |
| 4.4.4 | Drug-Excipient compatibility studies | 77 |
| 4.5 | References..... | 83 |
| 5. | FORMULATION & DEVELOPMENT OF HNCS | 84 |
| 5.1 | introduction..... | 84 |
| 5.2 | Experimental work..... | 85 |
| 5.2.1 | Materials | 85 |
| 5.2.2 | Instruments..... | 86 |
| 5.3 | Methods..... | 86 |
| 5.3.1 | Preliminary optimization and screening | 86 |

| | |
|--------------------------------------------------------------------------------------------------------------|-----|
| 5.3.2 One step method using Thin lipo-polymeric film formation followed by hydration and extrusion:..... | 87 |
| 5.4 Quality by design & design of experiment (QbD-DoE) approach for HNCs formulation and optimization | 87 |
| 5.4.1 Procedure for formulation of HNCs | 88 |
| 5.4.2 Dynamic light scattering (DLS) Nanocarrier size analysis..... | 89 |
| 5.4.3 Zeta potential analysis..... | 89 |
| 5.4.4 Entrapment efficiency and drug loading..... | 89 |
| 5.4.5 Transmission Electron Microscopy (TEM) | 90 |
| 5.4.6 Scanning Electron Microscopy (SEM) Analysis | 90 |
| 5.4.7 <i>In-vitro</i> drug release study and drug release kinetic | 90 |
| 5.4.8 Phospholipid content by Stewart method | 94 |
| 5.4.9 Estimation of residual solvent by HS-Gas Chromatography | 94 |
| 5.4.10 Small angle X Ray scattering (SAXS)..... | 94 |
| 5.5 Results and Discussion | 95 |
| 5.5.1 Optimization of process parameters..... | 95 |
| 5.5.2 Preliminary screening of formulation components..... | 96 |
| 5.5.3 Quality by design - design of experiment (QbD-DoE) approach for HNCs formulation and optimization..... | 98 |
| 5.5.4 Desirability plot and overaly plot for optimization..... | 124 |
| 5.5.5 Nanocarrier size analysis by Dynamic light scattering (DLS)..... | 128 |
| 5.5.6 Zeta potential analysis..... | 129 |
| 5.5.7 Entrapment efficiency and drug loading..... | 129 |
| 5.5.8 Transmission Electron Microscopy (TEM) | 129 |
| 5.5.9 Scanning Electron Microscopy (SEM) Analysis | 131 |
| 5.5.10 <i>In-vitro</i> drug release study and drug release kinetic | 131 |

| | | |
|-----------|---------------------------------------------------------------|------------|
| 5.5.11 | Phospholipid content by Stewart method | 133 |
| 5.5.12 | Estimation of residual solvent by HS-Gas Chromatography | 133 |
| 5.5.13 | Small angle X Ray scattering (SAXS)..... | 134 |
| 5.6 | References..... | 136 |
| 6. | DEVELOPMENT OF SIRNA ANCHORED CISPLATIN CAPRYLATE | |
| | LOADED HNCs | 139 |
| 6.1 | Formulation challenges and importance of N/P ratio | 139 |
| 6.2 | Materials and Equipments..... | 139 |
| 6.3 | Methods..... | 140 |
| 6.3.1 | Development of siRNA anchored Cisplatin caprylate loaded HNCs | |
| | 140 | |
| 6.3.2 | Size and Zeta potential measurement | 142 |
| 6.3.3 | Assay..... | 142 |
| 6.3.4 | Entrapment efficiency of siRNA in HNCs | 142 |
| 6.3.5 | Cryo TEM and Freeze fracture TEM studies..... | 143 |
| 6.3.6 | Serum stability study..... | 143 |
| 6.3.7 | Atomic force microscopy (AFM) analysis | 144 |
| 6.4 | Results and Discussions | 145 |
| 6.4.1 | Development of siRNA anchored Cisplatin caprylate loaded HNCs | |
| | 145 | |
| 6.4.2 | Nanocarrier Size and Zeta Potential | 146 |
| 6.4.3 | Cryo-Transmission Electron microscopy | 148 |
| 6.4.4 | Serum stability study..... | 149 |
| 6.4.5 | Atomic force microscopy (AFM) analysis | 151 |
| 6.5 | References..... | 153 |
| 7. | IN VITRO CELL LINES STUDIES | 154 |

| | | |
|-----------|-------------------------------------------------------|------------|
| 7.1 | introduction | 154 |
| 7.2 | Materials and Instruments | 155 |
| 7.3 | Methods..... | 156 |
| 7.3.1 | Cytotoxicity studies by MTT assay | 157 |
| 7.3.2 | Cellular uptake studies | 163 |
| 7.3.3 | Chemosensitization studies | 166 |
| 7.3.4 | Wound scratch assay method..... | 167 |
| 7.3.5 | Apoptosis detection and cell cycle analysis..... | 168 |
| 7.3.6 | Gene knockdown efficiency | 168 |
| 7.4 | Results and Discussion | 175 |
| 7.4.1 | Cytotoxicity studies by MTT assay | 175 |
| 7.4.2 | Cellular uptake studies of HNCs..... | 176 |
| 7.4.3 | Chemo-sensitization study | 179 |
| 7.4.4 | Wound scratch assay | 182 |
| 7.4.5 | Apoptosis detection and cell cycle analysis..... | 184 |
| 7.4.6 | Gene knock-down by RT PCR | 186 |
| 7.5 | References:..... | 187 |
| 8. | DEVELOPMENT OF DRY POWDER INHALER OF HNCS..... | 189 |
| 8.1 | introduction | 189 |
| 8.2 | Materials and Equipements:..... | 191 |
| 8.3 | Preparation and characterization of DPI | 192 |
| 8.3.1 | Optimization of lyophilization..... | 192 |
| 8.3.2 | Moisture content analysis | 193 |
| 8.3.3 | Powder processing and preparation of DPI | 193 |
| 8.3.4 | In-vitro deposition studies..... | 194 |
| 8.3.5 | Aerodynamic particle size..... | 194 |

| | | |
|-----------|---------------------------------------------------------------|------------|
| 8.3.6 | Scanning electron microscopy | 196 |
| 8.3.7 | Powder X-ray diffraction (PXRD)..... | 196 |
| 8.3.8 | DSC and FTIR | 196 |
| 8.3.9 | Integrity of siRNA | 196 |
| 8.4 | Results and Discussion | 196 |
| 8.4.1 | Optimization of lyophilization..... | 196 |
| 8.4.2 | Moisture content analysis | 199 |
| 8.4.3 | Powder processing and preparation of DPI | 200 |
| 8.4.4 | Aerosolization performance of dry powder for inhalation | 201 |
| 8.4.5 | Scanning electron microscopy | 203 |
| 8.4.6 | FTIR..... | 206 |
| 8.4.7 | Integrity of siRNA | 207 |
| 8.5 | References..... | 208 |
| 9. | IN VIVO STUDIES..... | 210 |
| 9.1 | Introduction..... | 210 |
| 9.2 | Materials and methods | 210 |
| 9.2.1 | Intratracheal Instillation | 210 |
| 9.2.2 | Broncho alveolar lavage (BAL) and Lung homogenate (LH) | 211 |
| 9.2.3 | L/B ratio, LDH and ALP estimation..... | 212 |
| 9.2.4 | Histopathological examination of lung..... | 212 |
| 9.2.5 | Acute Toxicity Study | 212 |
| 9.2.6 | Haemolytic study | 214 |
| 9.3 | Results and discussion | 215 |
| 9.3.1 | Concentration of drug in LH and BAL..... | 215 |
| 9.3.2 | L/B ratio, LDH and ALP estimation..... | 218 |
| 9.3.3 | Histopathological images..... | 219 |

| | |
|------------------------------------------|------------|
| 9.3.4 MTD Estimation | 220 |
| 9.3.5 Haemolytic study | 221 |
| 9.4 References | 224 |
| 10. STABILITY STUDIES..... | 225 |
| 10.1 introduction | 225 |
| 10.2 materials and methods..... | 225 |
| 10.3 Result and Discussion | 226 |
| 10.4 References | 227 |
| 11. SUMMARY & CONCLUSION..... | 229 |