Chapter 1
Introduction
1.1. Introduction

Lung cancer is the most common cancer in the world for several decades and the most common cause of death from cancer with 1.38 million deaths (18.2% of the total) [1]. Lung cancer is currently treated with intravenous administration of chemotherapeutic agents but is nonselective as it cannot differentiate between host cells and cancer cells leading to normal cell toxicity [2]. Further, the diagnostic tools available currently can inadequately detect the tumors and hence render the condition dejected [2]. This provides impetus to pursue the research for effectively treating the lung cancer.

Lung cancer is the most common cancer in developed and developing nations like India [3]. One million of the current 5 million deaths in world, and 2.41 million in developing countries is contributed by India and, in 2020, this figure is projected at 1.5 million [4, 5]. The most common etiological factor for the cause of lung cancer is smoking, which is on the rise in India. In India smoking is prevalent in 29% of adult males, 2.5% of adult females, 11.7% of male collegians and 8.1% among school children and adolescents [5]. Against this backdrop, the proposed project will significantly impact effective treatment of lung cancer.

Lung cancers are classified according to their histological type [6]. This classification has important implications for clinical management and prognosis of the disease. The vast majority of lung cancers are carcinomas—malignancies that arise from epithelial cells. The two most prevalent histological types of lung carcinoma, categorized by the size and appearance of the malignant cells seen by a histopathologist under a microscope: non-small cell and small-cell lung carcinoma.

The non-small cell lung carcinomas are grouped together because their prognosis and management are similar. There are three main sub-types. squamous cell lung carcinoma, adenocarcinoma, and large cell lung carcinoma. Accounting for 25% of lung cancers, squamous cell lung carcinoma usually starts near a central bronchus. A hollow cavity and associated necrosis are commonly found at the center of the tumor. Well-differentiated squamous cell lung cancers often grow more slowly than other cancer types. Adenocarcinoma accounts for 40% of non-small cell lung cancers. It usually originates in peripheral lung tissue. Most cases of adenocarcinoma are associated with smoking; however, among people who have never smoked ("never-smokers"), adenocarcinoma is the most common form of lung cancer. A subtype of adenocarcinoma, the bronchioloalveolar
carcinoma, is more common in female never-smokers, and may have different responses to treatment.

Small cell lung carcinoma is less common. It was formerly referred to as "oat cell" carcinoma. Most cases arise in the larger airways (primary and secondary bronchi) and grow rapidly, becoming quite large. The small cells contain dense neurosecretory granules (vesicles containing neuroendocrine hormones), which give this tumor an endocrine/paraneoplastic syndrome association. While initially more sensitive to chemotherapy and radiation, it is often metastatic at presentation, and ultimately carries a worse prognosis. Small cell lung cancers have long been dichotomously staged into limited and extensive stage disease. This type of lung cancer is strongly associated with smoking.

If investigations confirm lung cancer, CT scan and often positron emission tomography (PET) are used to determine whether the disease is localized and amenable to surgery or whether it has spread to the point where it cannot be cured surgically [2].

Surgery itself has an operative death rate of about 4.4%, depending on the patient's lung function and other risk factors [7]. Surgery is usually only an option in non-small cell lung carcinoma limited to one lung, up to stage IIIA. This is assessed with medical imaging (computed tomography, positron emission tomography). A sufficient preoperative respiratory reserve must be present to allow adequate lung function after the tissue is removed.

The combination regimen depends on the tumor type [6]. Non-small cell lung carcinoma is often treated with cisplatin or carboplatin, in combination with gemcitabine, paclitaxel, docetaxel, etoposide, or vinorelbine. In small cell lung carcinoma, cisplatin and etoposide are most commonly used. Combinations with carboplatin, gemcitabine, paclitaxel, vinorelbine, topotecan, and irinotecan are also used. In extensive-stage small-cell lung cancer celecoxib may safely be combined with etoposide, this combination showed improve outcomes.

Radiotherapy is often given together with chemotherapy, and may be used with curative intent in patients with non-small cell lung carcinoma who are not eligible for surgery. This form of high intensity radiotherapy is called radical radiotherapy. For both non-small cell lung carcinoma and small cell lung carcinoma patients, smaller doses of radiation to the chest may be used for symptom control (palliative radiotherapy).

In recent years, various molecular targeted therapies have been developed for the treatment of advanced lung cancer [2, 6]. Gefitinib (Iressa) is one such drug, which targets the tyrosine
kinase domain of the epidermal growth factor receptor (EGFR), expressed in many cases of non-small cell lung carcinoma. It was not shown to increase survival, although females, Asians, nonsmokers, and those with bronchioloalveolar carcinoma appear to derive the most benefit from gefitinib.

The angiogenesis inhibitor bevacizumab, (in combination with paclitaxel and carboplatin), improves the survival of patients with advanced non-small cell lung carcinoma [2, 6]. Advances in cytotoxic drugs, pharmacogenetics and targeted drug design have showed promise in treatment of lung cancer. A number of targeted agents are at the early stages of clinical research, such as cyclo-oxygenase-2 inhibitors, the apoptosis promoter exisulind, proteasome inhibitors, bexarotene, the epidermal growth factor receptor inhibitor cetuximab, and vaccines.

Currently camptothecin, paclitaxil, carboplatin, cisplatin, docetaxel, topotecan, etoposide and gemcitabine are the most widely used anticancer agents in treatment of lung cancer with their known reported toxicities. The medications are available as injections for systemic use and result in hazardous side effects due to their non-specificity on the dividing cells in the body.

Intracellular transport of different biologically active molecules is one of the key problems in drug delivery in general. Currently the anticancer agents have poor intracellular concentration in the cancer cells.

Lung cancer prevalence in western countries and its treatment has drawn significant attention from NIH and other medical agencies. Prevalence of lung cancer in western countries has drawn attention of National Institute of Health and other medical agencies. As a result number of new drugs, formulations and techniques are being employed in research and clinical trials for therapy of lung cancer. Various drugs like camptothecin, docetaxel, paclitaxel, carboplatin, cisplatin, gemcitabine, etoposide, single or in combination with other drugs are in clinical trials for NSCLC and SCLC. These drugs are available in injection form while direct lung targeting through aerosolization may be a viable alternative. Recently Liposomal Camptothecin formulation has been tested clinically in Phase II clinical trials with successful results.

Recent research on targeted drug nanoparticles, liposomes, micellar formulations encapsulating these anticancer drugs after attaching with cancer cell over expressed receptor specific ligand is gaining high impetus owing to its very high selectivity and sensitivity.
towards cancer cells. Use of apoptotic genes like p53, mdm inhibitor genes and the siRNAs is also a topic of current research and yielding good outcomes. However the realities of marketing these targeted products is still a mile away. The recent success of CFTR gene delivery using liposomes has been a great impetus to the nanocarrier based gene delivery and it further improves the chance for viral and non-viral p53 gene delivery entering into the market.

Lung cancer research in India is comparatively in infancy compared to the research in western countries. Currently the focus is on drug encapsulating anticancer nanocarriers. Research is going on at laboratory scale by Misra et al. at M.S.University of baroda on pulmonary delivery and they have developed liposomal gene (p53) and drug (Etoposide and Docetaxel) formulations for their anticancer action in lung cancer and have obtained good results in lung cancer treatment in human lung cell lines (Unpublished data).

In spite of the recent developments in lung cancer research in India there is still a wide gap in research, diagnosis and therapy of lung cancer. The lung cancer targeted drug and gene therapy is still to be well explored and has lot of potential for betterment of lung cancer research and therapy.

RNA interference (RNAi) is the process of mRNA degradation that is induced by double-stranded RNA in a sequence-specific manner [8]. RNAi has been observed in all eukaryotes, from yeast to mammals. The power and utility of RNAi for specifically silencing the expression of any gene for which sequence is available has driven its incredibly rapid adoption as a tool for reverse genetics in eukaryotic systems.

The cell has a specific enzyme (in Drosophila; it is called Dicer) that recognizes the double stranded RNA and chops it up into small fragments between 21-25 base pairs in length. These short RNA fragments (called small interfering RNA, or siRNA) bind to the RNA-induced silencing complex (RISC). The RISC is activated when the siRNA unwinds and the activated complex binds to the corresponding mRNA using the antisense RNA. The RISC contains an enzyme to cleave the bound mRNA (called Slicer in Drosophila) and therefore cause gene suppression. Once the mRNA has been cleaved, it can no longer be translated into functional protein.
The structure of siRNA is highly specific to prevent erroneous gene silencing. siRNA molecules are 21-23 nucleotide double-stranded RNA (dsRNA) duplexes with symmetric 2-3 nucleotide 3’ overhangs and 5’ phosphate and 3’ hydroxyl groups [8].

RNA interference (RNAi) is a conserved cellular mechanism by which a small double-stranded RNA (dsRNA) directs the degradation of complementary mRNA and therefore inhibits the expression of a specific gene [8]. Since its discovery, RNAi has become a powerful tool to study gene functions in biological processes.[9-11] The ability to induce RNAi in mammalian cells using synthetic small interfering RNA (siRNA) has stimulated great interest in therapeutic applications of RNAi [12-14]. In numerous studies, siRNAs have shown promise for treating a variety of diseases, including influenza and HIV infection, cancer and genetic defects [15-17]. The double-stranded RNA-based molecule, siRNA, has a high potential as biopharmaceutical therapeutics. As RNAi interferes with translation, and not with DNA transcription, siRNA may not interact with chromosomal DNA. This lack of DNA interaction greatly reduces concerns about possible adverse gene alteration that might result from DNA-based gene therapy. The interaction of siRNA with mRNA, not protein, also makes it possible to reduce the production of harmful proteins before synthesis.

A key challenge of RNAi-based therapeutic application is the efficient delivery of siRNA into target cells. Various challenges for siRNA deliveries are described below. siRNA is usually 21 nucleotides in length and highly charged and therefore cannot cross the cytoplasmic membrane by free diffusion. In the circulation and interstitial space, siRNA is vulnerable to degradation by nucleases [18]. Although siRNA can be delivered directly and
locally to the target sites in limited applications [19, 20], a carrier system is required in most applications to protect siRNA from degradation and to facilitate its uptake by target cells [21, 22]. The proposed carrier system contains a key cationic component, such as a cationic lipid, a cationic polymer or a cationic peptide, in order to bind siRNA effectively along with other neutral lipids.

So far, the most successful strategies against cancer have been the destructive ones. At the cellular level, this implies the elimination, as selectively as possible, of the neoplastic cells. However, not all oncogenes and oncosuppressors contribute equally to cancer development [23]. The plasticity of the cell with a network of signal-transducing pathways makes it difficult to pinpoint the key genes whose blockage would irreversibly lead to self-destruction. RNAi technology can help to discover genes essential for viability in cancer cells that can be then used as targets for suicide. Inhibiting overexpressed oncogenes, such as ras or myc, should block pathways that cancer cells depend on. In most cancers, however, it may be necessary to block pathways at several points, or even to target several pathways. Identifying the genes that are altered in the stepwise progression to malignancy has become one of the central goals of cancer research; automation of data generation (robotics) and computer analysis (bioinformatics) have significantly accelerated the process of discovering cancer-linked genes [23]. Once an oncogene that is highly represented in a particular type of cancer (melanoma or glioblastoma, for example) is identified, there is the hope that this will lead to clinically useful targeted therapies.

siRNA is of inherent potency because it exploits the endogenous RNAi pathway, allows specific reduction of disease associated genes, and is applicable to any gene with a complementary sequence [24]. As cancer belongs to the category of genetic diseases, many important genes associated with various cancers have been discovered, their mutations precisely identified, and the pathways through which they act characterized [25]. The genetic nature of cancer provides solid support for the rationale of siRNA-mediated gene therapy. Indeed, a number of siRNAs have been designed to target dominant oncogenes, malfunctionally regulated oncogenes, or viral oncogenes involved in carcinogenesis. Moreover, therapeutic siRNAs have been investigated for silencing target molecules crucial for tumor–host interactions and tumor resistance to chemo- or radiotherapy. The silencing of critical cancer-associated target proteins by siRNAs has resulted in significant antiproliferative and/or apoptotic effects [26].
Table 1.1 Some putative siRNA targets against cancer [23]

<table>
<thead>
<tr>
<th>Gene–protein target*</th>
<th>Cellular function</th>
<th>Type of cancer tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-raf</td>
<td>Serine/threonine kinase</td>
<td>Malignant melanoma</td>
</tr>
<tr>
<td>Nox1</td>
<td>Superoxide-generating oxidase</td>
<td>Transformed NRK cells**</td>
</tr>
<tr>
<td>FAS/Her2</td>
<td>Fatty acid synthase</td>
<td>Breast-MDA-MB-231</td>
</tr>
<tr>
<td>Cyclin E</td>
<td>Cell-cycle control</td>
<td>Hepatocarcinoma</td>
</tr>
<tr>
<td>Hec1</td>
<td>Chromosomal segregation</td>
<td>-</td>
</tr>
<tr>
<td>Gp210</td>
<td>Nuclear pore assembly</td>
<td>Adenocarcinoma (Hela cells)</td>
</tr>
<tr>
<td>c-Kit</td>
<td>Signal transduction</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>MDR</td>
<td>Multidrug resistance</td>
<td>Adenocarcinoma (Hela cells)</td>
</tr>
<tr>
<td>bcl-2</td>
<td>Antiapoptotic</td>
<td>Esophageal adenocarcinoma</td>
</tr>
<tr>
<td>livin</td>
<td>Antiapoptotic</td>
<td>Adenocarcinoma</td>
</tr>
<tr>
<td>survivin</td>
<td>Antiapoptotic</td>
<td>Adenocarcinoma (Hela cells)</td>
</tr>
<tr>
<td>Philadelphia chromosome</td>
<td></td>
<td>Chronic myeloid leukemia</td>
</tr>
<tr>
<td>Ribonucleotide reductase</td>
<td>Gemcitabine resistance</td>
<td>Hepatic metastasis</td>
</tr>
<tr>
<td>Rho C</td>
<td>Cell motility</td>
<td>Metastasis</td>
</tr>
</tbody>
</table>

**Normal rat kidney cells. *Genes are written in italics and lower case letters while proteins begin with a capital letter and are written in roman letters.

In gemcitabine metabolism, where 13 genes are involved, the first step in phosphorylation is catalyzed by dCK (deoxyxycytosine kinase), which is the rate-limiting step for further phosphorylation to active metabolites, and thus is essential for the activation of gemcitabine. Alternatively, gemcitabine is inactivated by DCTD into its inactive form. RRM1 is the rate-limiting step of DNA synthesis and is inhibited by diphosphorylated gemcitabine (dFdCDP). The RRM1 gene encodes the regulatory subunit of ribonucleotide reductase, an essential enzyme that catalyses the reduction of ribonucleotide di-phosphates to the corresponding deoxyribonucleotides. It is the molecular target of gemcitabine (2’, 2’-difluorodeoxyxycytidine), an antimetabolite with activity in several malignancies including NSCLC [27]. dCK deficiency, increased DCTD, and increased RRM1 activity are the main mechanisms of gemcitabine resistance. Earlier work had suggested that patients with low as compared with high levels of tumoral RRM1 expression had improved survival when treated with
gemcitabine-based therapy [28]. In addition, continuous exposure of lung cancer cell lines to increasing amounts of gemcitabine resulted in increased RRM1 expression. A recent report suggested that gemcitabine resistance, generated in vitro through exposure of two NSCLC cell lines (H358 and H460) to increasing concentrations of the drug, was primarily a function of increased expression of RRM1 [29]. Thus, RRM1 is a major cellular determinant of cytotoxic efficacy of gemcitabine. Therefore, the rate limiting step involving RRM1 was chosen as an siRNA target for improving therapy of lung cancer using gemcitabine.

RGD-targeted nanocarriers may specifically address drugs to angiogenic endothelial cells and/or cancer cells by the binding of the RGD peptide to αvβ3 overexpressed by these cells, allowing the “active targeting” of the tumors [30]. RGD-targeted nanocarriers can be internalized via receptor-mediated endocytosis, which is not possible with single peptide constructs or with non-targeted nanocarriers; this is particularly interesting for the intracellular delivery of drugs to cancer cells [31]. RGD-targeted nanocarriers have recently proven advantageous in delivering chemotherapeutics, peptides and proteins, nucleic acids, and irradiation. The rationale behind the design of RGD-targeted nanocarriers is the delivery of various pharmacological agents to the α,β3-expressing tumor vasculature. The cytotoxic drug destroys the tumor vasculature, resulting in the indirect killing of tumor cells induced by the lack of oxygen and nutrients. The tumor growth might be inhibited by preventing tumors from recruiting new blood vessels. α,β3 integrin is up regulated in angiogenic endothelial cells but also in several tumor cells, leading RGD-targeted nanocarriers to a potential double targeting.

1.2. Objective of the Proposed Work

The objective of the proposed investigation was to enhance the chemo sensitization effect of the anticancer agent by pre exposure of lung cancer cells with siRNA encapsulated in a liposomal forms.

1.3. Rationale

To achieve success rate in cure of lung cancer having second highest incidence and mortality rate in India. The current cure chemotherapy for lung cancer has limitation being non-selective and manifests in dose related toxicity.
1.4. Hypothesis

It is hypothesized that the pre exposure to nanoconstructs encapsulating siRNA will enhance the chemosensitization effect of the anticancer drugs.

1.5. Research Design and Method

2. Development of siRNA liposomal formulations using different lipid excipients.
3. In vitro characterization of developed formulations by cell line studies and to assess chemosensitization potential.
4. In vivo toxicity studies for developed liposomal formulations.

1.6. Expected Results

The scientific literature refers to the enhanced chemosensitization effects of anticancer agents after initial siRNA exposure. The exposure of the tumor cells sensitive to siRNA may show anticancer effect at lower doses of the drug after their exposure to siRNA containing liposomes.

1.7. Work Plan

1. Development of liposomes encapsulating siRNA.
2. Characterization of liposomes encapsulating siRNA to find out particle size, zeta potential, % siRNA encapsulated, stability etc.
3. Cell line studies including intracellular uptake studies, cytotoxicity study, transfection study, cell cycle analysis in lung cancer cell lines.
4. Further in vitro characterization of developed formulations for serum stability of siRNA in liposomal form, hemolytic potential and electrolyte induced flocculation.
5. In vivo toxicity studies to assess safety profile of developed liposomal carriers.
6. Stability studies of developed formulations at storage and accelerated conditions.
7. Statistical Analyses of data.
1.8. References


