1 INTRODUCTION

Lung cancer or lung carcinoma in simple terms can be defined as any abnormal/uncontrolled growth of different types of cells in lungs. Lung cancer can be engendering from lung cells or due to metastasis from other organ cells which have extensive replicative potential. Lung cancer arises when healthy living cells present in the lung change their characteristic which results in uncontrolled growth of cells that form a mass called a tumor, a lesion, or a nodule (1). Any normal cell present in lung can produce a lung tumor. Tumors can be benign or cancerous in nature. Once tumor has been formed, some cells might leak from mass and be carried away by blood or extracellular fluid to lymph which are further drained in lymph nodes. When cancerous cells travels to lymph node and circulate to different parts of body it is called metastasis stage (2).

Several types of cancer, i.e., breast, kidney etc, can spread (also called metastasize) to the lung cells. When metastasis happenS, the resulting cancer is not considered lung cancer. This is because cancer is named on and treatment is based on the site of origin. For example, if breast cancer spreads to the lungs, it will be treated as metastatic breast cancer and not lung cancer (3).

1.1 TYPES OF LUNG CANCER

There are 2 main classifications of lung cancer: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). Both types of lung cancer are treated differently.

1.1.1 Small cell lung cancer (SCLC)

On an average one out of four malignancies involving the lungs are diagnosed as small cell lung cancer (SCLC). There are several types of SCLC (also called oat cell cancer) which includes a mixture of small cellS and other types of cells. SCLC grows aggressively as doubling time of cells in SCLC is approximately 30 days (4). SCLC can spread quickly to various lymph nodes (5) and other normal organs than another type. Out of total lung cancer cases, 10% to 15% of people are identified with SCLC (6).

1.1.2 Non-small cell lung cancer (NSCLC)

NSCLC arise from the lung epithelial cells. Near about 85 to 90 percentage of diagnosed lung cancer are NSCLC. NSCLC are divided in to three types based on the type of epithelial cells from where cancer starts (7):

- **Adenocarcinoma:** It is the most commonly diagnosed type of NSCLC. It is as common in non-smokers as often in smokers or former smokers. It tends to grow in the outer edges of the lungs (8). Growth of adenocarcinoma is slower than other types of cancer.
- **Squamous cell carcinoma (epidermoid carcinoma):** It is more frequent in chain smokers or former smokers. Epidermoid carcinoma is developed in the middle lung near to bronchi (9).
- **Large cell (undifferentiated) carcinoma:** It is the least common type of NSCLC. It grows very fast compared to other types of NSCLC and easily spread to other organs. This can make it difficult to treat (10).

It is very important for doctors to differentiate lung cancer that begins in the squamous cells of lungs and that begins in other cells of lungs to determine treatment regimen (11). Figure 1-1 shows tissue differentiation during lung cancer.

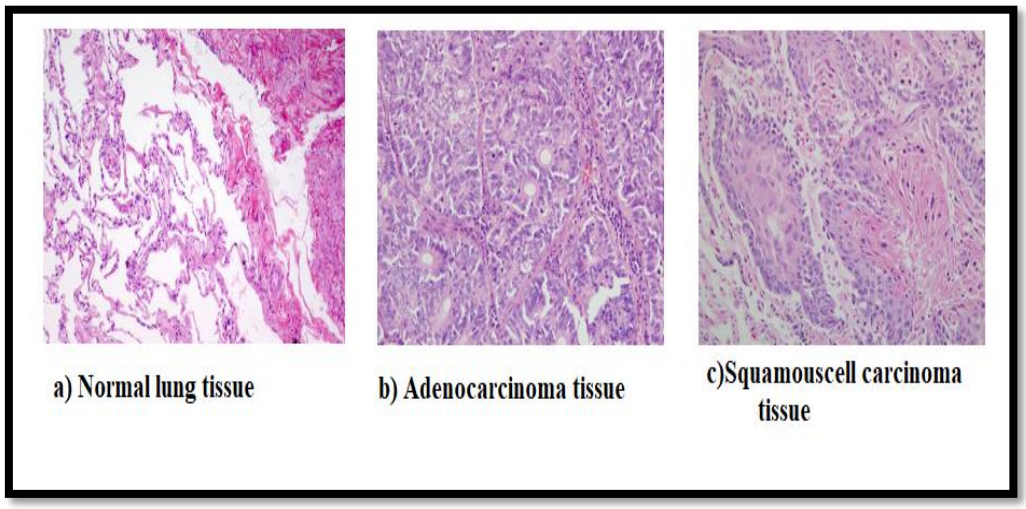

Figure 1-1 Tissue differentiation in normal lung, adenocarcinoma and squamous cell carcinoma. Reference: S.S.G hospital, Vadodara.

A few other subtypes of NSCLC, such as adeno squamous carcinoma and sarcomatoid carcinoma, are much less common. Along with the 2 main types of lung cancer, other tumors can occur in lungs. Carcinoid tumors of the lung account for fewer than 5% of lung tumors. Most of these grow slowly. Other types of lung cancer such as adenoid cystic carcinomas, lymphomas, and sarcomas, as well as benign lung tumors such as hamartomas are rare (12).

1.2 KEY STATISTICS

Lung cancer is the second most common type of cancer and the leading cause of cancer death, accounting for about one out of five reported malignancies case in men and one out of nine reported cases in women (13). In 1940 Approximately seven women in 100,000

developed lung cancer which rose up to 42 in 100,000 in 2001 and 1 in 17 in late 2020 (14, 15) . These statistics shows that lung cancer is rising alarmingly in women and all credits goes to evidence of increasing smoking in women. It is estimated that 135,720 (72,500 men and 63,220 women) deaths resulted from this disease in 2020 (15). The 5-year survival rate for people with any type of lung cancer is 19%. The 5-year survival rate for men is 16%and that for women is 23%. The 5-year survival rate for NSCLC is 24% and 6% for SCLC (16). For people with localized NSCLC, which means the cancer has not spread outside of the lung, the overall 5-year survival rate is 61%. For regional NSCLC, which means the cancer has spread outside of the lung to nearby areas, the 5-year survival rate is about 35%. For metastatic lung cancer, the 5-year survival rate is 6% due to ineffective treatments (17). These numbers are constantly changing.

1.3 LUNG CANCER STAGING

Lung cancer staging is an assessment of the degree of spread of cancer from its original source. It is one of the factors affecting the prognosis and potential treatment of lung cancer. The evaluation of NSCLC staging is done using the TNM (Tumor, lymph node, metastases). This is based on the size of the primary tumor, lymph node involvement and distant metastasis. Using the TNM descriptors, a group is assigned, ranging from occult cancer, through stages 0, IA, IB, IIA, IIB, IIIA, IIIB, and IV. This stage group assists with choice of treatment and estimation of prognosis (18). For both NSCLC and SCLC, the two general types of staging evaluation are clinical and surgical staging. Clinical staging is performed prior to definitive surgery. It is based on the results of imaging studies (Such as CT scans and PET scans), and biopsy results. Surgical staging is evaluated either during or after the operation, and is based on the combined results of surgical and clinical findings, including surgical sampling of thoracic lymph nodes (19).

1.4 DRUG RESISTANCE IN CANCER

Multidrug resistance (MDR) is a major obstacle in treating cancer in which cancer cells develops resistance towards ongoing chemotherapy. Mechanisms by which cells develops MDR can be broadly divided into cellular factors and physiological factors (20). Cellular factors involve over expressive efflux pump, reduced rate of cell apoptosis, genetic defects (i.e. gene deletion and polymorphism in gene), increased rate of drug metabolism, etc (21). Whereas physiological factors responsible for MDR includes interaction at cellular level, higher pressure of cell interstitial fluid, low pH environment around tumor, presence of hypoxic region at core of tumor, irregular nature of tumor vasculature, presence of cancer cells in areas that are difficult to penetrate (22).

As the MDR has been diagnosed in most of the patients, high doses are required to continue the treatment leading to increasing adverse effects. Adverse effects are responsible for the discontinuation of chemotherapy in more than 90% of the patients. Mechanism for the treatment of the MDR can vary depending on the types of lung cancer and stages of lung cancer.

MDR tumors can be of two types i) intrinsic and ii) acquired. Intrinsic MDR tumors are developed by inherent increased expression of ABC (ATP Binding Cassette) transporter. On the contrary, acquired MDR tumors can be developed due to stimulation from drugs that leads to overexpression of ABC transporters. Over expressive ABC transporter leads to efflux of chemotherapeutic drug from cell cytoplasm (20).

ABC transporters are the most extensively studied mechanism for the treatment of MDR cancers. Different ABC transporters are belonging to ATP-binding cassettes superfamily. 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 adopted approaches and gene silencing through RNA interference technology is most impactful tool now-a-days amongst all approaches.

1.5 DOCETAXEL

Docetaxel is antineoplastic drug which acts by disrupting the microtubular network in cells that are essential for vital mitotic and interphase cellular functions. Docetaxel promotes the assembly of tubulin into stable microtubules while simultaneously inhibiting their disassembly (23). The binding of Docetaxel to microtubules does not alter the number of proto filaments in the bound microtubules; in fact, it differs from other spindle poisons. Docetaxel was found to be cytotoxic in in vitro against various murine and human tumor cell lines, and against freshly excised human tumor cells in clonogenic assays. In addition, Docetaxel was found to be active on a number of cell lines overexpressing the p- glycoprotein, which is encoded by the multidrug resistant gene.

- **Limitation of marketed formulation:**

Docetaxel (DTX) has been used as a primary agent for the treatment of solid tumors such as in lung, breast and pancreatic cancers. Currently available marketed formulation of Docetaxel face issues of resistance-development and in-vivo toxicities which are dose limiting (24). Taxotere is micellar formulation of DTX and used for intravenous therapy of breast, lung, prostate, gastric, head and neck, ovarian and pancreatic cancer. However, Docetaxel has dose dependent side effects like neutropenia, alopecia and anemia (25). Hence, a formulation

strategy is required that can overcome its toxicity while providing better alternative treatment approach in comparison to those available in the clinic.

1.6 RNAi MECHANISM

RNA interference (RNAi) is a basic conserved mechanism of cell by which a small double stranded RNA (dsRNA) directs the degradation mRNA which is eventually responsible for the inhibition of specific gene expression (26). Soon after its discovery, RNAi has been studied extensively for the role in gene function in the normal cellular biological process and protein synthesis (27).

RNA interference (RNAi) is the artificially induced cellular process for the degradation of particular mRNA and it is induced with the use of double stranded RNA which has a specific sequence related to target mRNA. RNAi mechanism has been observed in all eukaryotes, from yeast to mammals (28). The mammalian cell contains specific enzymes similar to dicer enzyme found in Drosophila which identifies the dsRNA (Double stranded RNA) and breaks it into smaller fragments having a base pair between length of 21-25. This double stranded RNA can be shRNA which binds to RISC (RNA induced silencing complex). This RISC complex detaches distal chain from the shRNA which then binds to mRNA (that is for the specific protein synthesis). After mRNA binds to the activated RISC complex, it is cleaved hence production of specific protein in the cells can be inhibited. Exact mechanism of the RNAi is shown in Figure 1-2.

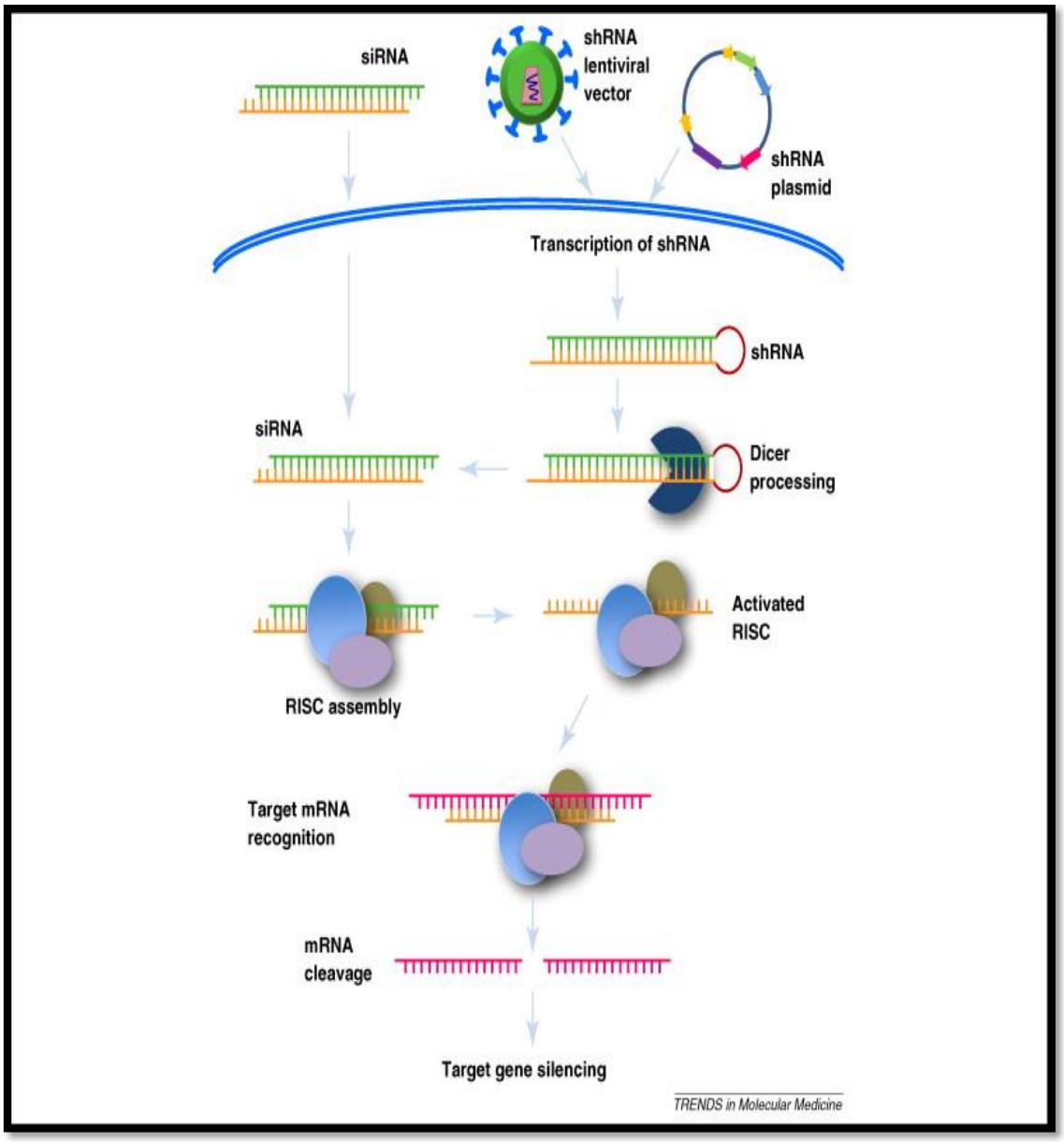

Figure 1-2 RNAi mechanism (29)

siRNA and shRNA are the powerful tools that are used to artificially induce RNAi in the mammalian cells. siRNA have been shown promising results in the treatment of influenza, HIV, cancer and few genetic defects (30). shRNA has been advantageous over siRNA as it has nuclear expression rather than siRNA which is artificially made. Expression of the siRNA has been limited up to 48 hrs or maximum three cell cycle whereas shRNA is expressed over the period of three years. Only 5 copies of shRNA are enough to produce therapeutic concentration. As it is nuclear expression, it has low stimulation of immune system and low cytotoxicity (31). Hence shRNA is a new tool for the RNAi which is proved to be better than siRNA in terms of efficiency, stability and duration of therapy along with safety. It has high potential to become future of biopharmaceutical medicines.

In spite of these recent studies on lung cancer there is still wide gap present with research, diagnosis and its industrial applicability. The targeted delivery and gene delivery for the lung cancer has enough potential for the betterment of the patients with lung cancer especially for those having multidrug resistance.

1.7 COMBINATORIAL APPROACH

Treatment with combination therapy depends on the type of the tumor. Platinum derivatives along with taxens, etoposide, gemcitabine and vinorelbine has been used to treat non-small cell carcinoma. In worst cases of NSCLC, celecoxib is combined with etoposide which results in increased 5-year survival rate (32). Combination of drug with gene therapeutic were useful in the reversal of drug resistance in lung cancer.

As another approach, chemotherapy is often combined with radiotherapy for better disease management and increases the survival rate of patients especially for those patients who are not subjected for surgery. This modified high intensity radio therapy has been called radical therapy and it can also be used for the SCLC and NSCLC. Even smaller doses of radiation at chest are used at initial stages of cancer as palliative treatment (33).

1.8 TARGETED THERAPY

Nowadays, different molecular targets are being identified as novel approach for treating various cancers. This concept of targeted drug delivery proved to be very successful in treating lung malignancies. Higher Folate receptors are present on cancer cells, hence the ability of the folic acid is used to target with surface modified nanocarriers. Gefitinib (marketed as Iressa®) is drug from the class of epidermal growth factor receptor inhibitors (commonly known as EGFR) proved to be very useful against the NSCLC (34). Hence EGFR is distinctly present on tumor cells whose targeting efficiency can be combined with another anticancer drug for better targeting. Bevacizumab® is another great example for the targeted delivery. It is an angiogenesis inhibitors, which can be combined with carboplatin and cisplatin to improve the survival rate of the patients having NSCLC (35). Other targets i.e. COX-2 inhibitors, promoter of apoptosis (Exisulind®), Protease inhibitors (Bexarotene®) and another EGFR inhibitor (Cetuximabv®) along with few vaccines has been researched right now under clinical studies for the effective targeted therapy.

1.9 ROLE OF NANOCARRIERS AND GENE DELIVERY

Nanotechnology provides an innovative and promising alternative to conventional small molecule chemotherapeutics, circumventing MDR by encapsulating, attaching, and conjugating drugs or therapeutic biological products to nanocarriers. Nanocarriers can include small molecules such as lipids or polymer nanoparticles that target the therapeutic payload to

tumors or tumor cells. Simultaneously, multifunctional drug-loaded nanocarriers can also enhance particle penetration of physiological barriers and protect the labile drugs or therapeutic biological products.

Recent approaches for the targeted therapy are developing surface modified nanocarriers with specific ligand that bind only to cancer cells have been studied extensively for targeting approach. Variety of nanocarriers have been studied i.e., Liposomes, Polymeric nanoparticles, micellar solution for better delivery of anticancer therapeutics with surface modification with specific ligands. siRNA, p53 gene and MDM inhibitor genes have shown good results. Even success of the delivering CFTR gene with the help of liposomes has made great impact on the delivery of gene products using nanocarriers. Lot of viral and non-viral vectors has been studied extensively for the gene delivery.

Among all the nanocarriers, liposomes and polymeric nanoparticles have been most widely researched as novel strategy for delivery of variety of therapeutics including genetic materials due to their biocompatibility and in-vivo drug targeting. Polymeric nanoparticles possess key attributes i.e., long- term stability and tunability but generally lacking in inherent biocompatibility and potential toxicity of long-term accumulation of synthetic molecules in the body (36). On contrary, liposome is biocompatible, non-denaturing interface of liposomal capsules but unfortunately, the lack of long-term stability (37). PLHNCs has advantages of both liposomes and polymeric nanoparticles. Some extraordinary advantage performed by PLHNCs are listed here. The solid core made up of polymer acts as a cytoskeleton that provides mechanical stability, controlled released morphology, narrow size distribution, and higher availability of specific surface area. The outer lipid coat that encapsulates the polymeric core is biocompatible in nature and mimics the characteristic of cellular membranes. The lipid shell can interact with a huge variety of drugs and indigenous molecules and surface can be modified for efficient targeting.

Research in the lung cancer in India has still not reached up to the mark compared to research in western countries. On the lab scale, a pioneer research has been done by Prof. Misra and group on gene delivery using various vectors for Lung cancer. They have developed formulation for anticancer drugs i.e. etoposide and docetaxel for lung cancer along with delivery of p53 gene liposomes for lung targeting (38). Another RGD grafted modified siRNA liposomes formulated by Prof. Misra et. al. has shown promising results in the preclinical studies (39) .

1.9.1 PLHNCs (Polymeric Lipid Hybrid Nanocarriers)

In attempt to mitigate certain drawbacks associated with liposomes and polymeric nanoparticles, novel, integrated structures known as Polymeric Lipid Hybrid Nanocarriers (PLHNCs) was introduced. Briefly, to create a potentially superior delivery system, the biomimetic properties of lipids and the architectural benefit of the polymer structure are combined. PLHNCs are solid, submicron-sized particles composed of minimum two components: the polymer and the lipid (40). In the developed hybrid system, various bioactive molecules such as drugs, genes, proteins, and targeting ligands may be entangled, adsorbed, or covalently bound. Polylactic-co-glycolic acid (PLGA), polycaprolactone (PCL), dextran, or albumin are likely choices for biodegradable polymers because of their biocompatibility, biodegradability, non-toxicity and prior use in licensed products (41-43). Zwitterionic, cationic, anionic, and neutral phospholipids such as lecithin, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-3-trimethylammonium-propane (DPTAP), 1,2-dipalmitoyl-3-trimethylammonium-propane (DOTAP), or 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DOPE) are commonly used lipids (40, 42, 44-46).

1.10 DRY POWDER INHALER TECHNOLOGY

DPI has been formulated as a coarse powder mixture of particles with micronized drug particles having combined aerodynamic diameters of 1–5 μm. Carriers in the DPI are important to improve the dosage flowability to ensure the accurate dosing and minimum variability. Carriers are also effective in manufacturing and filling of the dosage form in to DPI capsules. Carriers particles are also effective for the emission of the dose from the device after punctuation (47). Therefore, the design of the carrier particle is important for the development of dry powder inhalations. Carrier particles should have several characteristics such as physico-chemical stability, biocompatibility and biodegradability, compatible with the drug substance and must be inert, available and economical. During insufflation, the drug particles are detached from the surface of the carrier particles by the energy of the inspired air flow that overcomes the adhesion forces between drug and carrier. Consequently, it has been stated that the efficiency of a DPI formulation is extremely dependent on the carrier characteristics and the selection of carrier is a crucial determinant of the overall DPI performance.

1.11 OBJECTIVE OF WORK

The present research was aimed at evaluating effectiveness of novel nanocarriers for silencing of gene ABCB1 (MDR1) that confers resistance to chemotherapeutic agents in treatment of lung cancer. Novel nanocarriers: PLHNCs (Polymeric-lipid hybrid nanocarriers)

comprising of lipids & polymers were formulated for pulmonary route as Dry Powder Inhaler (DPI).

Objectives:

- ✓ To develop Novel Nanocarriers (PLHNCs) for simultaneous delivery of drug and gene therapeutics.
- ✓ To conjugate developed PLHNCs with folate targeting ligand to impart selectivity towards cancer cells.
- ✓ To mask the drug resistance in chemotherapy of lung cancer by silencing drug efflux transporter through delivery of plasmid for shRNA pDNA against ABCB1.
- ✓ To potentiate drug efficacy and treatment in cases of drug resistant lung cancer by formulation & development of PLHNCs that can deliver plasmid for shRNA against ABCB1 gene along with anti-neoplastic agent Docetaxel.

1.12 RATIONALE

The developed PLHNCs would effectively deliver chemotherapeutic agent; Docetaxel & Plasmid-shRNA for ABCB1 by attachment with tumor targeted ligand.

Recently role of ABCB1 as multidrug resistant gene and having high level of upregulation in resistant groups has been identified (48). ABCB1 is a member of ATP binding cassette (ABC) transporter family. Among all ABC resistance causing transporter genes, ABCB1 transporter genes are highly expressed in tumor cells where they actively efflux a broad spectrum of anti-cancer drugs and thus contribute to multi-drug resistance (MDR) (49). To date, platinum-based doublet chemotherapy is first-line therapy in NSCLC. However, these agents have limited use in patients who have relapsed or have metastatic disease or drug resistance. Therefore, combinatorial approach is required to improve the clinical outcome which includes RNAi mechanism (shRNA) to achieve chemo sensitization. RNAi is a biological mechanism by which a small double stranded RNA (dsRNA) directs the degradation of complementary mRNA and therefore inhibits expression of specific gene. So shRNA were used for silencing gene imparting multidrug resistance which enhances efficiency of chemotherapy by decreasing the resistance of cancer cells to chemotherapeutic agent (50).

1.13 HYPOTHESIS

Preparation of PLHNCs of therapeutic gene will provide a better and safe delivery vector for gene delivery as compared to viral delivery vectors. In addition, this will provide better stability to therapeutic gene (ABCB1 shRNA pDNA) from DNase mediated degradation and targeted delivery will also ensure followings:

- This will reduce the exposure of other cells to gene & chemotherapeutic agent.
- Targeting shRNA for silencing multi drug resistant gene ABCB1(MDR1) can impart synergistic therapeutic activity by decreasing the resistance of cell to chemotherapeutic agents.
- Combination delivery will enhance efficiency of chemotherapy to a level that cannot be achieved by applying its components separately
- Anticipating highly selective & targeted anti-tumor activity and low adverse side effects in healthy organs.

1.14 EXPECTED OUTCOMES

This research is expected to result in development of a novel non-viral vector PLHNCs for gene therapy.

i. Targeting shRNA for silencing multi drug resistant gene ABCB1 (MDR1) will enhance efficiency of chemotherapy by decreasing the resistance of cancer cells to chemotherapeutic agents & will bridge the gap in the treatment of chemotherapy resistant lung cancer.

ii. It will enhance efficiency of chemotherapy to a level that cannot be achieved by applying its components separately in treatment of lung cancer. Moreover, highly selective & targeted PLHNCs for clinical evaluation as a novel alternative to currently researched chemotherapeutic strategies is anticipated.

iii. It is also emphasized that the biologicals will be the future drugs or the future therapeutics for either increasing the efficacy of available treatments or providing superior treatment for otherwise untreatable diseases such as lung cancer.

1.15 WORK PLAN

1. Literature review covering various aspects of cancer, Docetaxel, Lipid Polymer Hybrid Nanoparticle delivery System, Dry Powder Inhalers.
2. Procurement of Drug (Docetaxel), gene therapeutics (ABCB1 shRNA pDNA) and excipients (PEG-PCL, DPPC, DSPE-PEG_{2000}, DOTAP etc.)
3. Preformulation study including DSC and FTIR analysis.
4. Analytical Method development of Docetaxel and shRNA pDNA estimation.

5. Preliminary studies for development of Docetaxel Polymeric Lipid Hybrid Nanocarriers (PLHNCs).

6. Optimization of Docetaxel loaded Polymeric Lipid Hybrid Nanocarriers (PLHNCs) by Quality by design – design of experiment (QbD-DoE) approach through Placket-Burman design and Box-behnken design. Formulation of Docetaxel and shRNA pDNA loaded Polymeric Lipid Hybrid Nanocarriers (PLHNCs).

7. Formulation and optimization of Dry Powder Inhaler of Optimized PLHNCs by lyophilisation technique.
8. Characterization of formulated PLHNCs:
    a) Entrapment efficiency
    b) Particle size and ζ potential
    c) SEM, Cryo-TEM & freeze fracture TEM to study internal structures
    d) In-vitro drug release study
    e) In-vitro endothelial binding capacity of formulation
9. Solid State Characterization and Powder Performance study of DPI
10. *In-vitro* cytotoxicity study by MTT assay using suitable lung cancer cell line and cellular uptake studies of formulation on lung adenocarcinoma A549 cell lines through Flow cytometry and confocal microscopy
11. Evaluation of *In-vivo* efficacy of the formulation in suitable animal model.
12. Stability study of final DPI Formulation.

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