Chapter 2

Literature Review
2.1. Lung Cancer

The lungs are located in the chest. They help you breathe. When you breathe, air goes through your nose, down your windpipe (trachea), and into the lungs, where it spreads through tubes called bronchi. Most lung cancer begins in the cells that line these tubes. Lung cancer, the most common cause of cancer-related death in men and women, is responsible for 1.3 million deaths worldwide annually, as of 2004 [1].

There are two main types of lung cancer:

- Non-small cell lung cancer (NSCLC) is the most common type of lung cancer.
- Small cell lung cancer makes up about 20% of all lung cancer cases.

If the lung cancer is made up of both types, it is called mixed small cell/large cell cancer. If the cancer started somewhere else in the body and spread to the lungs, it is called metastatic cancer to the lung.

2.1.1. Causes of the Lung Cancer

The most common cause of lung cancer is long-term exposure to tobacco smoke [1-3]. The occurrence of lung cancer in non-smokers, who account for as many as 15% of cases, is often attributed to a combination of genetic factors, radon gas [4], asbestos [5], and air pollution including secondhand smoke [7].

2.1.2. Symptoms that Suggest Lung Cancer Include [1, 2, 4]

- dyspnea (shortness of breath)
- hemoptysis (coughing up blood)
- chronic coughing or change in regular coughing pattern
- wheezing
- chest pain or pain in the abdomen
- cachexia (weight loss), fatigue, and loss of appetite
- dysphonia (hoarse voice)
- clubbing of the fingernails (uncommon)
2.1.3. Pathogenesis

Similar to many other cancers, lung cancer is initiated by activation of oncogenes or inactivation of tumor suppressor genes. Oncogenes are genes that are believed to make people more susceptible to cancer. Proto-oncogenes are believed to turn into oncogenes when exposed to particular carcinogens. Mutations in the K-ras proto-oncogene are responsible for 10–30% of lung adenocarcinomas. The epidermal growth factor receptor (EGFR) regulates cell proliferation, apoptosis, angiogenesis, and tumor invasion. Mutations and amplification of EGFR are common in non-small-cell lung cancer and provide the basis for treatment with EGFR-inhibitors. Her2/neu is affected less frequently. Chromosomal damage can lead to loss of heterozygosity. This can cause inactivation of tumor suppressor genes. Damage to chromosomes 3p, 5q, 13q, and 17p are particularly common in small-cell lung carcinoma. The p53 tumor suppressor gene, located on chromosome 17p, is affected in 60–75% of cases. Other genes that are often mutated or amplified are c-MET, NKX2-1, LKB1, PIK3CA, and BRAF.

Several genetic polymorphisms are associated with lung cancer. These include polymorphisms in genes coding for interleukin-1, cytochrome P450, apoptosis promoters such as caspase-8, and DNA repair molecules such as XRCC1. People with these polymorphisms are more likely to develop lung cancer after exposure to carcinogens. A recent study suggested that the MDM2 309G allele is a low-penetrant risk factor for developing lung cancer in Asians.

2.1.4. Prevention and Treatment

Eliminating tobacco smoking is a primary goal in the prevention of lung cancer, and smoking cessation is an important preventive tool in this process.

- **Surgery**: Positron emission tomography (PET) is 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. Video-assisted thoracoscopic surgery and VATS lobectomy have allowed
for minimally invasive approaches to lung cancer surgery that may have the advantages of quicker recovery,

- **Radiotherapy**: 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.[11] A refinement of this technique is continuous hyperfractionated accelerated radiotherapy (CHART), in which a high dose of radiotherapy is given in a short time period. For small-cell lung carcinoma cases that are potentially curable, chest radiation is often recommended in addition to chemotherapy. The use of adjuvant thoracic radiotherapy following curative intent surgery for non-small-cell lung carcinoma is not well established and is controversial. Benefits, if any, may only be limited to those in whom the tumor has spread to the mediastinal lymph nodes. 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). **Brachytherapy** (localized radiotherapy) may be given directly inside the airway when cancer affects a short section of bronchus. It is used when inoperable lung cancer causes blockage of a large airway. Patients with limited-stage small-cell lung carcinoma are usually given prophylactic cranial irradiation (PCI). This is a type of radiotherapy to the brain, used to reduce the risk of metastasis. More recently, PCI has also been shown to be beneficial in those with extensive small-cell lung cancer. In patients whose cancer has improved following a course of chemotherapy, PCI has been shown to reduce the cumulative risk of brain metastases within one year from 40.4% to 14.6%. Recent improvements in targeting and imaging have led to the development of extracranial stereotactic radiation in the treatment of early-stage lung cancer. In this form of radiation therapy, very high doses are delivered in a small number of sessions using stereotactic targeting techniques. Its use is primarily in patients who are not surgical candidates due to medical comorbidities.

- **Chemotherapy**: The chemotherapy regimen depends on the tumor type.
 Literature Review

- **Small-cell lung carcinoma**: Even if relatively early stage, small-cell lung carcinoma is treated primarily with chemotherapy and radiation. In small-cell lung carcinoma, cisplatin and etoposide are most commonly used. Combinations with carboplatin, gemcitabine, paclitaxel, vinorelbine, topotecan, and irinotecan are also used [1]. Celecoxib showed a potential signal of response in a small study [6].

- **Non-small-cell lung carcinoma**: Primary chemotherapy is also given in advanced and metastatic non-small-cell lung carcinoma. Testing for the molecular genetic subtype of non-small-cell lung cancer may be of assistance in selecting the most appropriate initial therapy. For example, mutation of the epidermal growth factor receptor gene may predict whether initial treatment with a specific inhibitor or with chemotherapy is more advantageous [7]. Advanced non-small-cell lung carcinoma is often treated with cisplatin or carboplatin, in combination with gemcitabine, paclitaxel, docetaxel, etoposide, or vinorelbine. Bevacizumab improves results in non-squamous cancers treated with paclitaxel and carboplatin in patients less than 70 years old who have reasonable general performance status. Pemetrexed has been studied extensively in non-small-cell lung cancer, with numerous studies since 1995. For adenocarcinoma and large-cell lung cancer, cisplatin with pemetrexed was more beneficial than cisplatin and gemcitabine; squamous cancer had the opposite results. As a consequence, subtyping of non-small lung cancer histology has become more important. Bronchoalveolar carcinoma is a subtype of non-small-cell lung carcinoma that may respond to gefitinib and erlotinib [8].

- **Maintenance therapy**: In advanced non-small-cell lung cancer there are several approaches for continuing treatment after an initial response to therapy. Switch maintenance changes to different medications than the initial therapy and can use pemetrexed, erlotinib, and docetaxel, although pemetrexed is only used in non-squamous NSCLC [9].

- **Adjuvant chemotherapy**: Adjuvant chemotherapy refers to the use of chemotherapy after apparently curative surgery to improve the outcome. In non-small-cell lung cancer, samples are taken during surgery of nearby lymph nodes. If these samples contain cancer, the patient has stage II or III disease. In this situation, adjuvant chemotherapy may improve survival by
up to 15%. Standard practice has often been to offer platinum-based chemotherapy (including either cisplatin or carboplatin). However, the benefit of platinum-based adjuvant chemotherapy was confined to patients who had tumors with low ERCC1 (excision repair cross-complementing 1) activity. Adjuvant chemotherapy for patients with stage IB cancer is controversial, as clinical trials have not clearly demonstrated a survival benefit [10]. Trials of preoperative chemotherapy (neoadjuvant chemotherapy) in resectable non-small-cell lung carcinoma have been inconclusive.
2.2. Drug Profile

Gemcitabine Hydrochloride: Gemcitabine Hydrochloride is a cytidine analogue. Gemcitabine (2,2 difluorodeoxycytidine; dFdC), a difluoro analog of deoxycytidine, has become an important drug for patients with metastatic pancreatic cancer, non-small cell lung cancer, ovarian, bladder, esophageal, and head and neck cancer.

2.2.1. Mechanism of Action

Gemcitabine enters cells via active nucleoside transporters [11]. Intracellularly, deoxycytidine kinase phosphorylates gemcitabine to produce difluorodeoxycytidine monophosphate (dFdCMP), from which point it is converted to difluorodeoxycytidine di- and triphosphate (dFdCDP and dFdCTP). While its anabolism and effects on DNA in general mimic those of cytarabine, there are differences in kinetics of inhibition, additional sites of action, effects of incorporation into DNA, and spectrum of clinical activity. Unlike cytarabine, the cytotoxicity of gemcitabine is not confined to the S phase of the cell cycle, and the drug is equally effective against confluent cells and cells in logarithmic growth phase. The cytotoxic activity may be a result of several actions on DNA synthesis: dFdCTP competes with dCTP as a weak inhibitor of DNA polymerase; dFdCDP is a potent inhibitor of ribonucleotide reductase, resulting in depletion of deoxyribonucleotide pools necessary for DNA synthesis; and dFdCTP is incorporated into DNA and after the incorporation of one more additional nucleotide leads to DNA strand termination. This "extra" nucleotide may be important in hiding the dFdCTP from DNA repair enzymes, as the incorporated dFdCMP appears to be resistant to repair. The ability of cells to incorporate dFdCTP into DNA is critical for gemcitabine-induced apoptosis [12].
Figure 2.1 Mechanism of action of Gemcitabine Hydrochloride

2.2.2. Absorption, Fate, and Elimination [13]

Gemcitabine is administered as an intravenous infusion. The pharmacokinetics of the parent compound are largely determined by deamination, and the predominant urinary elimination product is the inactive metabolite difluorodeoxyuridine (dFdU). Gemcitabine has a short plasma half-life of approximately 15 minutes, with women and elderly subjects having slower clearance. Clearance is dose-independent but can vary widely among individuals. Similar to that of cytarabine, conversion of gemcitabine to dFdCMP by deoxycytidine kinase is saturated at infusion rates of approximately 10 mg/m² per minute, which produce plasma drug concentrations in the range of 15 to 20 uM. In an attempt to increase dFdCTP formation, the duration of infusion at this maximum concentration has been extended to 150 minutes. In contrast to a fixed infusion duration of 30 minutes, the 150-minute infusion produces a higher level of dFdCTP within peripheral blood mononuclear cells, increases the degree of myelosuppression, but has uncertain effects on antitumor activity. The activity of dFdCTP on DNA repair mechanisms may allow for increased cytotoxicity of other chemotherapeutic agents, particularly platinum compounds. Preclinical studies of tumor cell lines show that cisplatin-DNA adducts are enhanced in the presence of gemcitabine, presumably through suppression of nuclear excision repair.
2.2.3. Therapeutic Uses

The standard dosing schedule for gemcitabine (GEMZAR) is a 30-minute intravenous infusion of 1 to 1.2 g/m² on days 1, 8, and 15 of each 28-day cycle [13].

2.2.4. Clinical Toxicities [13]

The principal toxicity of gemcitabine is myelosuppression. In general, the longer-duration infusions lead to greater myelosuppression. Nonhematologic toxicities including a flu-like syndrome, asthenia, and mild elevation in liver transaminases may occur in 40% or more of patients. Although severe nonhematologic toxicities are rare, interstitial pneumonitis may occur and is responsive to steroids. Rarely, patients on gemcitabine treatment for many months may develop a slowly progressive hemolytic uremic syndrome, necessitating drug discontinuation. Gemcitabine is a very potent radiosensitizer [14] and should not be used with radiotherapy except in closely monitored clinical trials.

Table 1 Drug Profile

<table>
<thead>
<tr>
<th>Name</th>
<th>Gemcitabine hydrochloride</th>
</tr>
</thead>
<tbody>
<tr>
<td>Category</td>
<td>Anticancer ( anti metabolite )</td>
</tr>
<tr>
<td>Chemical name (IUPAC)</td>
<td>4-amino-1-(2R,4R,5R)-3,3-difluoro-4-hydroxy-5-(hydroxymethyl)oxolan-2-yl</td>
</tr>
<tr>
<td>Proprietary name</td>
<td>DDFC , D&lt;sup&gt;®&lt;/sup&gt;D , GEO , Gemcin , Gemcitabina ( INN – Spanish ) , Gemcitabine HCl , Gemcitabinum ( INN – Latin ) , Gemtro , Gemzar</td>
</tr>
<tr>
<td>Molecular formula</td>
<td>C&lt;sub&gt;9&lt;/sub&gt;H&lt;sub&gt;11&lt;/sub&gt;F&lt;sub&gt;2&lt;/sub&gt;N&lt;sub&gt;3&lt;/sub&gt;O&lt;sub&gt;4&lt;/sub&gt;•HCl</td>
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<tr>
<td>Log P</td>
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</tr>
<tr>
<td>pKa value</td>
<td>3.6</td>
</tr>
</tbody>
</table>
2.2.5. Problem Associated with Gemcitabine Hydrochloride

- Major limit for the use of gemcitabine is represented by its rapid metabolic inactivation (deamination operated by deoxycytidine deaminase) responsible for its short half-life together with its low but still important systemic toxicity.
- The half-life and volume of distribution depends on age, gender and duration for infusion.
- The development of multidrug resistance in cells exposed to gemcitabine can limit its effectiveness. Gemcitabine hydrochloride is efflux by the Pgp (P glycol protein) and resistance is observed by MDR gene (multi drug resistance gene).
- Dose of Gemcitabine Hydrochloride 1000 to 1200 mg/m².

2.2.6. Chemoresistance with Gemcitabine Hydrochloride:

dCK is cellular enzyme required to metabolize Gemcitabine Hydrochloride to active metabolite. Decreased dCK (deoxycytidine kinase) expression is associated with acquired resistance to gemcitabine in NSCLC cells.

Several members of the ATP-binding cassette (ABC) transporter superfamily, such as multidrug resistance protein 1 (ABCC1), confer drug resistance to drug-sensitive cells by effluxing.
anticancer or antiviral agents or their metabolites from cells when expressed at high levels [15]. Recently, ABCC5, which lacks a transmembrane domain that is present in another family member, ABCC1, was shown to mediate the ATP-dependent transport of several anticancer agents and antiviral nucleosides and confer resistance to gemcitabine.

2.2.7. Chemosensitization

Despite a reasonable response rate after initial chemotherapy in patients with metastatic bladder cancer, 60–70% of responding patients relapse within the first year, with a median survival of 12–14 months drug-resistant phenotype during treatment. Experimental models have helped clarify mechanisms associated with acquisition of chemotherapeutic agents in cancer cells. However, no study has focused on the resistant phenotype of bladder cancer to gemcitabine; therefore, the application of gemcitabine-resistant bladder cancer cells to preclinical experimental model may uncover novel findings for elucidating molecular mechanism of drug-resistance resulting in the development of novel strategies for advanced bladder cancer. This limited efficacy may be due to de novo drug resistance and/or the development of cellular mechanisms of resistance. In gemcitabine metabolism, where 13 genes are involved, the first step in phosphorylation is catalyzed by dCK, 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’-difluorodeoxycytidine), an antimetabolite with activity in several malignancies including NSCLC [16]. 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 [17]. 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 [18]. These data were confirmed in a subcutaneous murine colon tumor model (Colon 26) where gemcitabine resistance had been generated through prolonged gemcitabine exposure and serial transplantation [18]. However, induction of drug resistance through continuous exposure results in alterations in multiple genes as demonstrated by these authors. Thus, these results in genetically modified lung cancer cell lines demonstrate directly that RRM1 is a major cellular determinant of cytotoxic efficacy of gemcitabine. In addition, data demonstrate that RRM1 is a minor determinant of platinum efficacy.
2.3. Liposomes

Liposomes are synthetic, single or multi-compartmental vesicles having lipid membranes enclosing aqueous chambers. Liposomes are vesicles composed of phospholipids bilayers surrounding aqueous compartments as described by Bangham et al [19]. They consist of one or more bilayers. The driving force for bilayer assembly is the amphiphilic nature of phospholipid molecules. Liposomes are composed of phospholipid/s or lipids or and glycerides with or without sterols. Phospholipid typically consists of a hydrophilic head group attached to two hydrophobic fatty acid chains. When suspended in an excess of aqueous solution, phospholipid molecules originate themselves in ordered bilayers so that the polar heads are hydrated and hydrophobic tails are excluded from the aqueous environment. Although suspended phospholipids may also assume other geometric(s) such as micelles and tubular aggregates in hexagonal phases, this can be controlled by several factors including lipid composition and method of preparation. Entrapment of compounds is highly influenced by their physiochemical properties. Generally hydrophobic molecules are incorporated into the lipid bilayers whereas hydrophilic compounds are entrapped in the internal aqueous volume [20].

2.3.1. Composition of Liposomes

2.3.1.1. Phospholipids

Glycerol containing phospholipids are by far, the most commonly used component of liposome formulations and represent more than 50% of the weight of lipid present in biological membranes [21]. As examples of potentially useful lipids can be mentioned natural lipids such as egg lecithin, soya lecithin, and synthetic lipids such as phosphoglycerolipids, sphingolipids, and digalactosylglycerolipids. Amongst natural lipids may be mentioned sphingolipids such as sphingomyelin, ceramide and cerebroside; galactosylglycerolipids such as digalactosyldiacylglycerol; phosphoglycerolipids such as egg-yolk phosphatidylcholin and soya-bean phosphatidylcholin; and lecithins such as egg-yolk lecithin and soya-bean lecithin. Amongst synthetic lipids may be mentioned dimyristoyl phosphatidylcholine, dipalmitoyl phosphatidylcholine (DPPC), distearoyl phosphatidylcholine, dilauryl phosphatidylcholine, 1-myristoyl-2-palmitoyl phosphatidylcholine, 1-palmitoyl-2-myristoyl phosphatidylcholine, dioleoyl phosphatidylcholine, hydrogenated soyaphosphatidylcholines (HSPC), and the like.
Some naturally occurring phospholipids include phosphatidylcholine (PC), phosphatidylinositol (PI) and phosphatidylglycerol (PG) while dipalmitoyl phosphatidylcholine (DPPC), dipalmitoyl phosphatidylserine (DPPS), dipalmitoyl phosphatidylethanolamine (DPPE), dipalmitoyl phosphatidic acid (DPPA), dipalmitoyl phosphatidylglycerol (DPPG), dioleoyl phosphatidylcholine (DOPC) and dioleoyl phosphatidylglycerol (DOPG) are some synthetic phospholipids.

2.3.1.2. Sterols

Sterols such as cholesterol, ergosterol, nanosterol, or its derivatives are often included as components of liposomal membrane. Cholesterol has been called the “mortar” of bilayer because by virtue of its molecular shape and solubility properties, it fills in empty spaces among the phospholipid molecules, anchoring them more strongly into the structure. Its inclusion in liposomal membranes has 3 effects (i) increasing the fluidity or microviscosity of the bilayer (ii) reducing the permeability of the membrane to water-soluble molecules and (iii) solubilizing the membrane in the presence of biological fluids such as plasma.

![Image of liposomes](image)

**Figure 2.2** The structure of multilamellar vesicles showing the organization of phospholipid bilayers and the encapsulation of lipophilic and hydrophilic compounds.

2.3.1.3. Other Non-Structural Components

Charge inducer materials which provides a negative charge, for example phosphatidic acid, dicetyl phosphate or beef brain ganglioside etc, or one which provides a positive charge for example stearylamine acetate or cetylpyridinium chloride etc. have been incorporated into liposomes so as to impart either a negative or a positive surface charge to these structures. Many
single chain surfactants of number of single and double chain lipids having fluorocarbon chains and also compounds like quaternary ammonium salts and dialkyl phosphates can also be used to form liposomes [22].

2.3.2. Types of Liposomes

Different types of liposomes can be prepared and are classified by the size and structure. Different types of liposomes are small unilamellar vesicles (SUV), large unilamellar vesicles (LUV), oligolamellar vesicles (OLV), and multi-lamellar vesicles (MLVs). MLVs consist of numerous concentric bilayers separated by aqueous spaces and range up to 15 μm in diameter. Vesicles consisting of a single bilayer encompassing a central aqueous compartment are referred to as small unilamellar vesicles (SUVs), which range up to 100 nm in diameter and large unilamellar vesicles (LUVs) ranging from 100 to 500 nm in diameter (Figure 2.2).

2.3.3. Methods of Preparation of Liposomes

Numerous procedures have been developed to prepare liposomes. There are at least fourteen major published methods for making liposomes. The seven, most commonly employed methods are, Lipid film hydration method [19], Ethanol injection method [23] Ether infusion method [24], Detergent dialysis method [25], French press method [26], Rehydration-dehydration techniques [27] and Reverse phase evaporation method [28].

2.3.4. Characterization of Liposomes

The behavior of liposomes in both physical and biological systems is determined to a large extent by factors such as physical size, chemical composition, quantity of entrapped solutes etc. Hence, liposomes are characterized with respect to the following parameters:

2.3.4.1. Size and Size Distribution

There are number of methods reported in the literature to determine size and its distribution of the vesicles [29, 30]. The most commonly used ones are light microscopy preferably using electron microscope, laser light scattering or cryoelectron microscopy.
2.3.4.2. Lamellarity

The lamellarity, the average number of bilayers present in liposomes, can be determined either by $^{31}$P-NMR spectroscopy or freeze fracture electron microscopy.

2.3.4.3. Determination of Percentage Capture

The quantity of material entrapped inside liposomes can be determined more commonly by mini-column centrifugation method, protamine aggregation method, dialysis technique or by gel chromatography.

2.3.5. Stability of Liposomes

A prerequisite for the successful introduction of liposomes in therapy is the long-term stability of the formulation. The stability of drug-laden liposome dispersions preferably should meet the standards of conventional pharmaceutical product. A 1-year shelf life is considered to be an absolute minimum. Both chemical and physical determines the shelf life of a product.

In the literature, on the physical stability of liposomes, attention has been focused on two processes affecting the quality and therefore acceptability of liposomes [31]. First, the encapsulated drug can leak from the vesicles into the extra-liposomal compartment (reduced retention). Second, liposomes can aggregate and/or fuse, forming larger particles. Both these processes change the disposition of the drug in vivo and thereby presumably affect the therapeutic index of the drug involved. Besides, other physical parameters may also change during storage. For instance, hydrolysis of phospholipids causes the formation of fatty acids and lysophospholipids. These compounds considerably affect the physical properties of the bilayer [31]. Apart from this, chemical degradation process may influence the safety of liposomes. Solid experimental data on the safety of partially hydrolyzed liposomes are not yet available; lysophospholipids alone have been reported to be toxic.

Several approaches have been developed to ensure the physical stability of liposomes on storage.

1. For storage of aqueous dispersions, the lipid composition of the bilayer and the aqueous solvent can be adjusted to induce optimum stability by reducing permeability/leakage. Phospholipids
with long and saturated alkyl chains (distearoyl phosphatidyl choline and dipalmitoyl phosphatidyl choline or saturated hydrogenated soyabean or egg phosphatidyl choline) provide rigid bilayers with low permeabilities for small, non-bilayer-interacting compounds [31]. The incorporation of the bovine serum albumin in the liposomal membrane and treatment with glutaraldehyde has been reported to prevent leakage of the entrapped contents [32]. Crommelin has reported the effect of bilayer composition on permeability of carboxyfluoresce [33].

To formulate drugs in liposomes it is necessary to reduce the leakage of an entrapped drug. The rate of leakage of a molecule from liposomes is governed by the physio-chemical properties of a molecule. Liposomes are freely permeable to water, but cations are released at a slower rate than anions [19], whereas aqueous hydrogen bonding may determine the leakage rate of non-electrolytes [34].

Phospholipids in the liquid-crystalline state are more permeable to entrapped material than when they are in the gel state. Thus, loss of entrapped material is temperature dependent, generally being greatest around the phospholipid phase transition temperature (Tc) [35]. The stability of liposomes in terms of retention of didexoyinosine triphosphate (ddITP) was measured by Betageri [36] at 4°C, 25°C, and 37°C. He observed that retention of ddITP in liposomes was maximum when stored at 4°C followed by 25°C and 37°C.

Another way to control stability is to incorporate cholesterol into the lipid structure, since it is known to reduce leakage of various solutes through the lipid bilayer when the membrane is in a fluid-like state [36, 37], or by polymerization of phospholipid molecules [38, 39]. The introduction of cholesterol in liposomes of 5,6-carboxyfluorescein (CF) has been reported to reduce the rate of leakage during storage [40]. He also observed that CF retention was greater in liposomes stored at 4°C in the presence of O₂ than those of room temperature, although liposomes stored at room temperature but in O₂-free atmosphere were more stable than those stored at room temperature in the presence of O₂.

2. Freezing the liposome dispersion is also an approach to achieve prolonged liposome shelf-life [41]. Lyophilization and rehydration, which include a freezing and thawing cycle, represent another method, used by many laboratories for better stability of liposomal formulations [42].
Several groups have published reports on freezing, drying [43] or freeze-drying of liposomes. Cryoprotectants play an important role in the physical stabilization of liposomes during freezing, drying or freeze-drying. The 100% CF retention could be found using cryoprotectant after a full freezing-thawing cycle [31]. Studies made on the stability of liposomes with time, when they were either freeze-dried or in solution have been reported [33].

3. In addition, two other techniques can solve the problem of drug leakage during storage, proliposomes and remote loading [31] that permit liposome dispersion preparation in situ. Several reports have been published in this context. Chemical analysis mainly concerns hydrolysis of the ester bonds in phospholipids and oxidation of their unsaturated acyl chains if present. Hydrolysis of phospholipid to free fatty acid and lysophospholipids can disturb the phospholipid bilayer structure and may disrupt it, leading to leakage of encapsulated products. Oxidation of unsaturated phospholipids and cholesterol may be initiated by the action of light and heavy metals [44]. According to Hernández-caselles [40], the presence of A-tocopherol decreased the breakdown of phosphatidyl choline to lysophosphatidyl choline and also reduced the level of peroxidation. Although the mechanism of the action of α-tocopherol is not clear, it is suggested that this may happen through specific binding to the phospholipid molecule [45]. α-tocopherol acetate was found to be much less effective than α-tocopherol in preventing lipid peroxidation [46]. Further information about chemical stability can be found in reviews of hydrolytic and oxidation reactions in phospholipids [31].

2.3.6. Liposomes as Drug Delivery Systems

In the recent past, controlled release concept and technology have received increasing attention in the face of growing awareness to toxicity and ineffectiveness of drugs when administered or applied by conventional methods. Liposomes as drug delivery systems are among research topics that are being vigorously investigated in both academic and industrial laboratories, with different outlooks and common goals and end products. The scientific literature is rich with comprehensive review of liposomes as drug delivery systems [47, 48].

Over the last twenty years, the liposome has changed its status from being a novel plaything for the laboratory worker to a powerful tool for an industrialist with the gap between the ideal
desired characteristics of liposomes and what is technically feasible becoming narrower all the time. Vastly improved technology in terms of drug capture, vesicle stability on storage, scale-up production and the design of formulations for special tasks has facilitated the application of a wide range of drugs in the treatment and prevention of diseases in experimental animals and clinically.

Liposomes may prove to be efficient carrier for targeting the drug to the site of action because of the following properties: Amphiphilic nature, flexibility in structural characteristics, localized drug effect, controllability of drug release rate, stability in vivo, direct cell liposome interaction, sterilizability, ability to protect drug and body from eachother, non-toxicity, non-immunogenicity, biocompatibility and biodegradability and accommodation of molecules with wide range of solubility and molecular weight. At the same time, there are certain problems associated with liposome as drug delivery such as difficulty in procuring pure phospholipids, difficulty in scale-up, poor stability over a long shelf-life, expensive, batch to batch variation in performance, low drug loading, difficulty in avoiding the reticulo-endothelial system and possibility of unwanted vascular obstruction caused by large liposomes [49]. However, research into the use of liposomes in drug delivery has led to vastly improved technology in terms of drug capture, vesicle stability, storage, scaled up production and the design of formulations for specialized tasks. **Table 2.2** shows the liposome application according to their mode of action.

Due to their high degree of biocompatibility, liposomes were initially considered as delivery systems for intravenous administration. The first parenterally applied formulation Ambisome (Vestar Inc., San Dimas, CA), a liposomal amphotericin formulation for the treatment of disseminated fungal infections that frequently occur in immunosuppressed patients, was launched in Ireland in 1990 that showed both high therapeutic activity and reduced toxicity as compared to the original product [50]. More recently in 1995, a sterically stabilized liposomal formulation containing the anticancer drug, doxorubicin has been launched in United States [51].

It has since become apparent that liposomes can also serve as an effective tool for other delivery systems that include oral [52], ophthalmic [53], aerosol [54], dermal/transdermal [55, 56] applications, as immunological adjuvants [57, 58], as carriers of antigens [57, 58], leishmaniasis,
lysosomal storage diseases, cell biological application [59] etc. The recent research is concentrated on the use of liposomes to deliver hemoglobin and act as red blood cell substitutes. The scientists are also engaged in designing of liposomal prodrug using principle of specific enzyme cleavage and facilitated spontaneous hydrolysis. Another field of liposomal research in producing sterically stabilized liposomes for prolonged circulation in blood stream. Liposomes are currently being studied as drug carriers for a variety of drugs that include recombinant proteins [60], gene transfer and immuno diagnostic applications [61]. Of these, non-invasive route of administration continuously demands significant efforts in designing the liposomes that will no doubt continue to contribute significantly to more efficient use of "old drugs" with better and established therapeutic index vis-à-vis minimum side effects.

### Table 2.2 Major Modes of Liposomal Action and Related Applications

<table>
<thead>
<tr>
<th>Mode of action</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intracellular uptake (lysosomes, endosomes/cytoplasm)</td>
<td>Microbial disease, Metal storage disease, Gene manipulation, uptake by some tumour, cells, macrophage activation to a tumoricidal/microcidal state, efficient antigen presentation by antigen presenting cells (vaccines).</td>
</tr>
<tr>
<td>Slow release of drugs near the target area</td>
<td>Tumors near fixed macrophages.</td>
</tr>
<tr>
<td>Avoidance of tissue, sensitive to drugs</td>
<td>Cardio toxicity of doxorubicin</td>
</tr>
<tr>
<td>Circulating reservoirs</td>
<td>Blood surrogates</td>
</tr>
<tr>
<td>Facilitation of drug uptake by certain routes</td>
<td>Drug delivery to skin, lungs, eyes, mucosal tissues.</td>
</tr>
</tbody>
</table>

#### 2.4. Formulation Optimization

An experimental approach to Design of Experiment (DoE) optimization of drug delivery systems (DDS) comprises several phases [62-65]. Broadly, these phases can be sequentially summed up in seven salient steps. **Figure 2.3** delineates these steps pictographically.

The optimization study begins with **Step 1**, where an endeavor is made to ascertain the initial drug delivery objective(s) in an explicit manner. Various main response parameters, which closely and pragmatically epitomize the objective(s), are chosen for the purpose.
• In Step II, the experimenter has several potential independent product and/or process variables to choose from. By executing a set of suitable screening techniques and designs, the formulator selects the “vital few” influential factors among the possible “so many” input variables. Following selection of these factors, a factor influence study is carried out to quantitatively estimate the main effects and interactions. Before going to the more detailed study, experimental studies are undertaken to define the broad range of factor levels as well.

Figure 2.3 Seven-step Ladder for Optimizing Drug Delivery Systems

• During Step III, an opposite experimental design is worked out on the basis of the study objective(s), and the number and the type of factors, factor levels, and responses being explored. Working details on variegated vistas of the experimental designs, customarily required to implement DoE optimization of drug delivery, have been elucidated in the subsequent section.
Afterwards, response surface modeling (RSM) is characteristically employed to relate a response variable to the levels of input variables, and a design matrix is generated to guide the drug delivery scientist to choose optimal formulations.

- **Step IV** is the drug delivery formulations are experimentally prepared according to the approved experimental design, and the chosen responses are evaluated.

- Later in **Step V**, a suitable mathematical model for the objective(s) under exploration is proposed, the experimental data thus obtained are analyzed accordingly, and the statistical significance of the proposed model discerned. Optimal formulation compositions are searched within the experimental domain, employing graphical or numerical techniques. This entire exercise is invariably executed with the help of pertinent computer software.

- **Step VI** is the penultimate phase of the optimization exercise, involving validation of response prognostic ability of the model put forward. Drug delivery performance of some studies, taken as the checkpoints, is assessed vis-a-vis that predicted using RSM, and the results are critically compared.

- Finally, during **Step VII**, which is carried out in the industrial milieu, the process is scaled up and set forth ultimately for the production cycle.

The niceties of the significance and execution of each of these seven steps is discussed in greater detail below.

The foremost step while executing systematic DoE methodology is to understand the deliverables of the finished product. This step is not merely confined to understanding the process performance and the product composition, but it usually goes beyond to enfold the concepts of economics, quality control, packaging, market research, etc.

The term objective (also called criterion) has been used to indicate either the goal of an optimization experiment or the property of interest [64, 66]. The objectives for an experiment should be clearly determined after discussion among the project team members having sound expertise and empiricism on product development, optimization, production, and/or quality
control. The group of scientists contemplates the key objectives and identifies the trivial ones. Prioritizing the objectives helps in determining the direction to proceed with regard to the selection of the factors, the responses, and the particular design [62, 65]. This step can be very time consuming and may not furnish rapid results. However, unless the objectives are accurately defined, it may be necessary to repeat the entire work that is to follow. The response variables, selected with dexterity, should be such that they provide maximal information with the minimal experimental effort and time. Such response variables are usually the performance objectives, such as the extent and rate of drug release, or are occasionally related to the visual aesthetics, such as chipping, grittiness, or mottling [63].

The word ‘optimize’ simply means to make as perfect, effective, or functional as possible [66, 67]. The term optimized has been used in the past to suggest that a product has been improved to accomplish the objectives of a development scientist. However, today the term implies that DoE and computers have been used to achieve the objective(s). With respect to drug formulations or pharmaceutical processes, optimization is a phenomenon of finding the best possible composition or operating conditions [67, 68]. Accordingly, optimization has been defined as the implementation of systematic approaches to achieve the best combination of product and/or process characteristics under a given set of conditions [63].

2.4.1. Experimental Designs

The conduct of an experiment and the subsequent interpretation of its experimental outcome are the twin essential features of the general scientific methodology [67, 69]. This can be accomplished only if the experiments are carried out in a systematic way and the inferences are drawn accordingly.
An experimental design is the statistical strategy for organizing the experiments in such a manner that the required information is obtained as efficiently and precisely as possible [70-73]. Runs or trials are the experiments conducted according to the selected experimental design [64, 68]. Such DoE trials are arranged in the design space so that the reliable and consistent information is attainable with minimum experimentation. The layout of the experimental runs in a matrix form, according to the experimental design, is known as the design matrix [68]. The choice of design depends upon the proposed model, the shape of the domain, and the objective of the study. Primarily, the experimental (or statistical) designs are based on the principles of randomization (i.e., the manner of allocations of treatments to the experimental units), replication (i.e., the number of units employed for each treatment), and error control or local control (i.e., the grouping of specific types of experiments to increase the precision) [73-75]. DoE is an efficient procedure for planning experiments in such a way that the data obtained can be analyzed to yield valid and unbiased conclusions [76, 77]. An experimental design is a strategy for laying out a detailed experimental plan in advance to the conduct of the experimental studies [69, 71, 78]. Before the selection of experimental design, it is essential to demarcate the experimental domain within the factor space - i.e., the broad range of factor studies. To accomplish this task, first a
pragmatic range of experimental domain is embarked upon and the levels and their number are selected so that the optimum lies within its realm [79]. While selecting the levels, one must see that the increments between them should be realistic. Too wide increments may miss finding the useful information between the levels, while a too narrow range may not yield accurate results [63]. There are numerous types of experimental designs. Various commonly employed experimental designs for RSM, screening, and factor-influence studies in pharmaceutical product development are

a. factorial designs
b. fractional factorial designs
c. Plackett-Burman designs
d. star designs
e. central composite designs
f. Box-Behnken designs
g. center of gravity designs
h. equiradial designs
i. mixture designs
j. Taguchi designs
k. optimal designs
l. Rechtschaffner designs
m. Cotter designs

For a three-factor study, an experimental design can invariably be envisaged as a "cube," with the possible combinations of the factor levels (low or high) represented at its respective corners [77]. The cube thus can be the most appropriate representation of the experimental region being explored. Most design types discussed in the current article are, therefore, being depicted pictorially using this cubic model, with experimental points at the corners, centers of faces, centers of edges, and so forth. Such depiction facilitates easier comprehension of various designs and comparisons among them. For designs in which more than three factors are adjusted, the same concept is applicable except that a hypercube represents the experimental region. Such cubic designs are popular because they are symmetrical and straightforward for conceptualizing and envisioning the model.
2.4.1.1. Factorial Designs

Factorial designs (FDs) are very frequently used response surface designs [78, 80, 81]. A factorial experiment is one in which all levels of a given factor are combined with all levels of every other factor in the experiment [68, 81, 82]. These are generally based upon first-degree mathematical models. Full FDs involve studying the effect of all the factors (k) at various levels (x), including the interactions among them, with the total number of experiments being \( x^k \). FDs can be investigated at either two levels (\( 2^k \) FD) or more than two levels. If the number of levels is the same for each factor in the optimization study, the FDs are said to be symmetric, whereas in cases of a different number of levels for different factors, FDs are termed asymmetric [68].

2.4.1.2. Design Augmentation

In the whole DoE endeavor, a situation sometimes arrives in which a study, conducted at some stage, is found to be inadequate and needs to be investigated further, or when the study carried out during the initial stages needs to be “reused” [63]. In either situation, more design points can be added systematically to the erstwhile design. Thus, the erstwhile primitive design can be enhanced to a more advanced design furnishing more information, better reliability, and higher resolution. This process of extension of a statistical design, by adding some more rational design points, is known as design augmentation [83]. For instance, a design involving study at two levels can be augmented to a three-level design by adding some more design points. A design can be augmented in a number of ways, such as by replicating, adding center points to two-level designs, adding axial points (i.e., design points at various axes of the experimental domain), or by folding over.

2.4.2. Response Surfaces

During this crucial stage in DoE, one or more selected experimental responses are recorded for a set of experiments carried out in a systematic way to develop a mathematical model [70, 71, 74, 78, 81, 84]. These approaches comprise the postulation of an empirical mathematical model for each response, which adequately represents change in the response within the zone of interest. Rather than estimating the effects of each variable directly, response surface modeling (RSM) involves fitting the coefficients into the model equation of a particular response variable and
mapping the response over the whole of the experimental domain in the form of a surface [62, 64, 68, 79].

Principally, RSM is a group of statistical techniques for empirical model building and model exploitation [62, 85]. By careful design and analysis of experiments, it seeks to relate a response to a number of predictors affecting it by generating a response surface, which is an area of space defined within upper and lower limits of the independent variables depicting the relationship of these variables to the measured response.

Experimental designs, which allow the estimation of main effects, interaction effects, and even quadratic effects, and, hence, provide an idea of the (local) shape of the response surface being investigated, are termed response surface designs [64, 68, 70, 86]. Under some circumstances, a model involving only main effects and interactions may be appropriate to describe a response surface. Such circumstances arise when analysis of the results reveals no evidence of "pure quadratic" curvature in the response of interest - i.e., the response at the center approximately equals the average of the responses at the two extreme levels, +1 and - 1.

In each part of Figure 2.5, the value of the response increases from the bottom of the figure to the top and those of the factor settings increase from left to right. If a response behaves as in Figure 2.5, the design matrix to quantify that behavior needs only to contain factors with two levels - low and high. This model is a basic assumption of simple two-level screening or factor-influence designs. If a response behaves as in Figure 2.5(b), the minimum number of levels required for a factor to quantify that behavior is three.
Addition of center points to a two-level design appears to be a logical step at this point, but the arrangement of the treatments in such a matrix may confound all the quadratic effects with each other [85,86]. A two-level design with center points can only detect the quadratic nature of the response, but not estimate the individual pure quadratic effects. Generally, the quadratic models are proposed for optimization of drug delivery devices [67-69]. Therefore, response surface designs involving studies at three or more than three levels are employed for DoE optimization purposes. These response surface designs are used to find improved or optimal process settings, troubleshoot the process problems and weak points, and make a formulation or process more robust (i.e., less variable) against external and non-controllable influences [86]. Relatively more complicated cubic responses (Figure 2.5(c)) are quite infrequent in pharmaceutical practice [68,69].

The prediction ability of response surface designs can be determined by prediction variance, which is a function of experimental variance ($\sigma^2$) and variance function ($d$) as described by Eq. (1) [68,75,86].

$$var(\hat{y}) = d.\sigma^2$$ (1)

where var ($\hat{y}$) is the prediction variance. The variance function ($d$) further depends upon the levels of a factor and the experimental design. When the prediction variance of a response is
constant in all the directions at a given distance from the center point of the domain, the design is termed rotatable [75, 78]. Ideally, all response surface designs should possess the characteristic of rotatability - i.e., the ability of a design to be run in any direction without any change in response prediction variance.

Conduct of DoE trials, according to the chosen statistical design, yields a series of data on the response variables explored. Such data can be suitably modeled to generate mathematical relationships between the independent variables and the dependent variables. Graphical depletion of the mathematical relationship is known as a response surface [70, 79, 86]. A response surface plot is a 3-D graphical representation of a response plotted between two independent variables and one response variable. The use of 3-D response surface plots allows us to understand the behavior of the system by demonstrating the contribution of the independent variables.

**Figure 2.6 (a) A Typical Response Surface Plotted Between A Response Variable, Release Exponent, and Two Factors, HPMC And Sodium CMC, In Case of Mucoadhesive Compressed Matrices; (b) The Corresponding Contour Plot**

The geometric illustration of a response obtained by plotting one independent variable against another, while holding the magnitude of response and other variables as constant, is known as a contour plot [64]. Such contour plots represent the 2-D slices of the corresponding 3-D response surfaces. The resulting curves are called contour lines. **Figure 2.6** depicts a typical response surface and contour plot for a diffusional release exponent (proposed by Korsemeyer et al. [87] as the response variable, reported with mucoadhesive compressed matrices of atenolol[88]. For complete response depiction among k independent variables, a total of $^kC_2$ number of response surfaces and contour plots may be required. In other words, 1, 3, 6, or 10 number of 3-D and 2-D
plots are needed to provide depiction of each response for 2, 3, 4, or 5 number of variables, respectively [63].

2.4.3. Mathematical Models

The mathematical model, simply referred to as the model, is an algebraic expression defining the dependence of a response variable on the independent variable(s) [89, 90]. Mathematical models can either be empirical or theoretical [64]. An empirical model provides a way to describe the factor/response relationship. It is most frequently, but not invariably, a set of polynomial equations of a given order [75]. Most commonly used linear models are shown in Eqs. (2)-(4):

\[ Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \ldots + \varepsilon \]  
\[ Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \ldots + \varepsilon \]  
\[ Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \ldots + \varepsilon \]

where \( Y \) represents the estimated response, sometimes also denoted as \( E(y) \). The symbols \( X \), represent the value of the factors, and \( \beta_0, \beta_1, \beta_{ii} \) and \( \beta_{ij} \) are the constants representing the intercept, coefficients of first-order (first-degree) terms, coefficients of second-order quadratic terms, and coefficients of second-order interaction terms, respectively. The symbol implies pure error. Equations (2) and (3) are linear in variables, representing a flat surface and a twisted plane in 3-D space, respectively. Equation (4) represents a linear second-order model that describes a twisted plane with curvature, arising from the quadratic terms. A theoretical model or mechanistic model may also exist or be proposed. It is most often a nonlinear model, where transformation to a linear function is not usually possible [64]. Such theoretical relationships are, however, rarely employed in pharmaceutical product development.
2.5. RNA Interference

In the last decade, ‘RNA Interference’ (RNAi) has been termed as one of the most important innovations in the field of biology and it is utilized to manipulate gene expression within cells for the treatment of number of diseases and as powerful tool to study gene function. Various carrier systems were utilized in this approach to fight against diseases by regulating expression of a specific factor or gene, which actually responsible for a particular disease.\[91\] RNAi was principally demonstrated by Fire et al. in 1998 for potent target-specific gene silencing in the nematode *Caenorhabditis elegans*.\[92\] However, in the last decade endogenous small RNAs were identified and found to modulate gene expression with control over accurate cell function. MicroRNAs (miRNA) (endogenous) short interfering RNAs (siRNAs, endo-siRNAs) and piwi interacting RNAs (piRNAs) are identified as basic controllers of various endogenous processes such as apoptosis, stem cell self-renewal, differentiation and maintenance of cell integrity.\[93, 94\] In recent time, microRNA (miRNA) has come out as a new approach for the treatment of cancer and various neurodegenerative diseases. Therapy by miRNA involves re-introduction of a synthetic version of a natural miRNA which gets depleted in the diseased tissue.\[95\] Proper knowledge of target mRNA sequence and designing of its complementary anti sense sequence may lead effective silencing of a specific gene responsible for disease conditions. Hence, these strategies being extensively investigated for personalized therapy of cancer, HIV and other mutate viral diseases.\[96-99\] Resistance of a chemotherapeutic can also be modified using these approaches.\[100\] miRNA technique controls the production disease specific protein or gene by down-regulating its expression rather than relieving the symptoms of the disease. This approach can be differentiated from other genetic approaches by its action on the mRNA, expressing the disease producing protein, rather than acting on a particular faulty gene. The success of this approach depends on the understanding of the correct sequence of mRNA carrying the message of protein responsible for the disease. Due to colossal number of completed human genome projects lots of information on target genes for the rational design of antisense drugs is available within hours for research and clinical trials. Currently, different RNAi therapeutics are under clinical trials and many others at preclinical stage are in queue to enter the clinics for various applications like cancer, HIV, age-related macular degeneration, respiratory syncytial virus as
well as rare diseases like pachyonychia congenita. Unfortunately, Sirna-027 from Sirna Therapeutics has recently been terminated which were in phase II respectively of clinical trials. Constraints in regulatory approval of these molecules is inefficient delivery to target site but this can be resolved by better understanding of barriers encountered from the site of administration to the site of action. These barriers can be overcome by designing the delivery systems involving either chemical modifications like structural changes or using nanocarriers or surface modification by specific ligand attachment targeting at particular receptors. Combinations of these methods may also prove beneficial.[101, 102]

But for all its potential, RNAi therapy is a long way from entering the clinic. In this review, we concisely describe how RNAi is accomplished, with a focus on various carrier systems for both modified and unmodified RNA molecules like siRNA, ShRNA and miRNA and their potential therapeutic applications in various human diseases.

2.5.1. Development of RNAi Technology:

Since its discovery, the constant development in the RNAi therapy and its applications as a therapeutic agent has been attained. In 1992, the unfolded mechanism of action of antisense therapeutics was first elucidated by Fire et al. In 1998, he identified the RNAi a key factor for the knock down of targeted mRNA responsible for the target protein synthesis [103]. A major breakthrough in RNAi technology was an identification of the dsRNA processing enzyme Dicer [104] and the RNA induced silencing complex (RISC) [105], which trigger RNAi by using the small dsRNA species generated by Dicer as assistant fragments to target mRNA for degradation [106-108]. In 2001, Thomas Tuscola et al defined the basic conditions for siRNA that initiates RNAi in mammalian cells, which involves between 19 and 23 nucleotides (19 to 23mer) with 2 nucleotide overhangs on each 3’ end [106, 109]. With the above findings the invasion of siRNA, a 20-23 nucleotide was investigated for its antiviral activity to inhibit disease related gene expression. Currently, based on the knowledge gained and continuous development in the area of RNAi therapy it became a central target to the scientist and pharmaceutical industries. At present, research is not merely confined to the development of an RNAi therapeutics having target orientation, affinity and *in vitro*, *in vivo* activities in different cell lines and animal models.
but also to formulate this technology for its therapeutic applications considering its pharmacological and toxicological profile and to get through in all stages of clinical trials is most significant. A lot can be done in design, development and optimization of transfection carriers, its formulations for RNAi agents and to evaluate their doses and dosage frequency for therapeutic activity both at preclinical and clinical levels [110-112]. Presently, gene silencing is accomplished by using small molecules such as dsRNA (double stranded RNA), shRNA (short hairpin RNA), siRNA (small interfering RNA), microRNAs (miRNA) and piwi interacting RNAs (piRNAs). Furthermore, the therapeutic and biopharmaceutical profile of this therapeutics is improved by modifying physical and chemical properties like sugars, bases or by conjugating with different novel carriers [101, 102, 110-112]. The amount of antisense agent, concentration of mRNA produced, production and degradation rate of mRNA and the type of knock down mechanism involved were identified as crucial factors in monitoring therapeutic and biopharmaceutical parameters of these therapeutics. The challenges in delivery of RNAi therapeutics is becoming quite uncomplicated with development of novel RNAi agents and their efficient carrier systems which knock down the targeted mRNA in cytoplasm itself instead of the nucleus [113]. Since, discovery of RNAi technology, its development and conceptual understanding are achieving newer heights day by day which make use of these agents at therapeutic level very easy and such a progress also helps to achieve an improved success rate later at all stages of clinical trials.

2.5.2. Targets of RNAi

The different approaches available for gene silencing are as follows:

1) Blocking transcription process (i.e. synthesis of complementary mRNA from the targeted DNA molecule). This is done by two different strategies - strand invasion and triple-strand formation. Out of these, strategies triple-strand formation was used most commonly which includes formation hydrogen bonds between the third strand and the complementary strand of dsDNA molecule [114] e.g. Homopyrimidine oligonucleotides.[115-120]
2) Blocking of post transcriptional gene silencing (PTGS) phenomenon which includes the knock down or knock-out of transcribed target mRNA to inhibit the protein synthesis.

Gene therapy targets a particular gene which gets either knock-out or knock down by antisense molecules such as RNAi but to achieve effective knock-down or knock-out has always remained a huge challenge in development and formulation aspects of RNAi technology. Better clinical and therapeutic profile of RNAi agents can be achieved with more knowledge and better understanding of different pharmaceutical and pharmacological parameters [110, 111].

2.5.3. Challenges to RNAi Delivery

The objective of RNAi therapy is to bring out the therapeutic outcome by reaching at the target site in amount greater than minimum effective concentration. The path from the site of administration to the target site comprises of many hurdles like physiological, cellular and immunological barriers. In addition, large size and ionic nature of RNAi nanoconstructions affect the transfection capacity of these molecules [110]. Here, the focus of this review is to understand structure, function and physiological role of these barriers in therapy and to reflect the probable approaches for effective RNAi therapy.

2.5.3.1. Physiological Barriers

This is the first barrier coming across the effective delivery of RNAi molecules. This barrier comprises of many check points like glomerular filtration, hepatic metabolism, RES uptake, endothelial barrier and degradation by nucleases. Nucleases degrade the RNAi molecules within a minute after their admiration and lower the potency and therapeutic profile of these molecules by 70% [121]. To overcome this hurdle, approaches like chemical modification or use of non-viral carriers were used to deliver and to prevent the cleavage of the RNAi agents [113, 122-125]. The probable approaches which will improve the stability of RNAi agents towards nucleases are stated below:

1) Alteration can be possible in pentose sugars at the 2’-OH position and 3’ half of the siRNA molecule.[123]
2) Formation of phosphorothioate oligonucleotides by replacing the oxygen with sulphur.[123]

3) Hexitol nucleic acids (HNAs), morpholino compounds, locked nucleic acids (LNAs) and peptide nucleic acids (PNA3) can also be modified at 2’-OH position.

4) Substituting 6-carbon sugar for ribose, 2’-F and 2’-OMe group along with the gapmers helps to sustain the therapeutic activity of these molecules.[113, 123]

5) Formulating the anionic molecules into cationic nanoparticles, liposomes, lipoplex or polyplex prevents the cleavage from nucleases by virtue of the electrostatic interaction.[126, 127]

Delivery of various formulation of RNAi molecules like nanoparticles, liposomes, lipoplex or polyplex having particle size (PS) more than 200nm are prone to the phagocytosis by reticuloendothelial system (RES) system [128] whereas, PS less than 100nm get caught at the hepatic Kupffer cells. The rate and extent of clearance of these nanoconstructs from the systemic circulation is depends on the size and charge of the complex formed between RNAi nanoconstructs and serum proteins [129-132]. The clearance can be lowered by coating theses nanoconstructs with hydrophilic agents like polyethylene glycol, which compensate the surface charge of these nanoconstructs and make them long circulating [133, 134]. Hence, control on PS and charge of the final formulation may help to improve its therapeutic activity. Development of the delivery system targeting to the tissues of liver, spleen is beneficial due to opsonisation of RNAi nanoconstructs at these organs [113].

To elicit the pharmacological action antisense molecules has to reach at parenchymal cells which are highly protected by the layer of endothelial cells. These endothelial cells hold their position at the extracellular matrix in association with various adhering molecules like integrins. Only the small molecules can get through this paracellular route [135]. In certain organs like liver and spleen the space at the junction allows the larger molecule to travel across the barrier. In addition, the RNAi molecules may travel to target site via clavolin based transcytosis [136]. Due to flexibility in entry of various size particles the molecules such as cell penetrating peptides, targeting ligands or molecular conjugates can be used to deliver the RNAi therapeutics [113].
2.5.3.2. Cellular Barriers

Next to physiological barrier, the antisense molecule has to overcome cellular barrier which comprise of different check points like cell entry, endosomal escape, nuclear localization and knock-down of protein expression. Rigorous toxicity is an outcome of non-viral carriers which non-specifically get inside the non-targeting cells. The non-specific uptake is an outcome of interaction between negatively charged cell membranes and cationic carriers [137] which can be minimized by coating with hydrophilic molecules like polyethylene glycol or conjugating with ligand motif such as transferrin [138], folate [139], surface receptor-specific antibodies [140], etc which will increase their cellular entry [141] and reduce RES uptake. The transfection efficacy of carrier system is based on rate of cellular internalization and endosomal escape [142, 143]. The endosomal degradation is achieved using various approaches like use of fusogenic lipids or peptides to rupture lysosomal membranes and by forming pores in membranes [144, 145]. Literature reveals that lipofectin and DOPE (1, 2-dioleoyl-sn-glycero-3-phosphoethanolamine) were used to formulate a pH-sensitive liposomes encapsulating antisense agent for delivering active at low pH surroundings [146]. DOPE present inside the liposomes destabilized the endosomal membrane by forming pores inside it [147, 148]. The structure of endosomal membrane can be disturbed using high buffering capacity polymers like polyethyleneimine which prevent acidification of endosomes [149, 150]. In addition, the endosomal membrane can be osmolysed by using osmotic agents like glycerol, sucrose, PVP etc.

Nuclear localization of RNAi molecules is required for knock down of the related protein synthesis. It has been reported in literature that the anionic lipid competes for the anionic RNAi molecules and displace it from the complex of cationic lipid/polymer-antisense molecules [151, 152]. Nuclear localization of RNAi molecules can be enhanced by conjugating them with cationic polymers like polyethyleneimine (PEI) or poly-l-lysine (PLL). The duration of action of RNAi agents is depends on many steps such as cellular uptake, intracellular trafficking, endosomal release and nuclear entry. The molecules like cell penetrating signal peptides, endosomal release signal peptides or nuclear localization signal peptides along with antisense agent has been reported to direct all these steps.[119, 153] Inhibition or down regulation of the protein or gene expression is an outcome of an interaction between RNAi agent and its
complementary mRNA. Transfection efficiency of antisense carrier governs strength of interaction between them. The lipid/polymer to oligonucleotide ratio is a key factor in deciding the transfection efficiency of the carrier [142]. Hence, knowledge and conceptual understanding of these barriers and carriers leads to the efficient delivery of RNAi molecules at the target site. Moreover, the better understanding of bio-distribution and physiochemical properties of RNAi molecules also helps to enhance the success of RNAi therapy.

2.5.4. Cellular Mechanisms of RNAi

The main objective of RNAi therapy is knocking down or knocking out target gene or mRNA to elicit it pharmacological effect. Matured complementary target mRNA produced from DNA undergoes the process of protein synthesis i.e. translation or PTGS which can be a potential target for RNAi molecules to exert their therapeutic action (Figure 2.7). Translation comprise of three steps such as initiation, elongation and termination.

![Figure 2.7 Approaches for Knockdown of Target Gene Or mRNA: (A) Transcription Inhibition: DNA Targeting (B) Preventing mRNA Formation: Pre-mRNA Targeting (C) Translation Inhibition: Protein Targeting](image-url)
RNA interference (RNAi), monitor activity and potency of genes within mortal cells. RNA interference was also called as co-suppression, post transcriptional gene silencing (PTGS), and quelling. The RNAi pathway is divided into two phases such as initiation phase and execution phase. The initiation phase is triggered in the presence of dsDNA precursor which subsequently gets cleaved at 11 nucleotide interval by the enzyme dicer with C-terminal dsRNA binding domain, an N-terminal RNA helicase as well as two RNaseIII-like domains,[104] into short fragments of 20-23 nucleotides with over-hanging 3’ends that are known as siRNA.[154] In execution phase each siRNA so formed is uncoiled into two single strands i.e. passenger strand and guide strand. Out of these two strands of uncoiled siRNA the passenger strand get degraded and guide strand couples with RNA induced silencing complex (RISC) forming a large multiprotein complex which brings out the post transcriptional gene silencing (PTGS). PTGS implicates sequence specific base coupling between the guide strand of the siRNA and the target mRNA followed by endonucleolytic cleavage of the mRNA strand across the middle of the siRNA strand[106, 155] and later degradation of the targeted unprotected mRNA. Due to the potency, maximal effectiveness, duration of action, and sequence specificity of small interfering RNA (siRNA) it becomes an important tool of RNAi therapy both in-vitro and in-vivo [119, 156, 157]. The cellular mechanism of RNAi involves several complicated steps which are depicted in (Figure 2.8).

Figure 2.8 Cellular mechanisms of RNAi
2.5.5. Small Interfering RNA (siRNA)

Knockdown or silencing of targeted genes in most of the cells can be done by small interfering RNA (siRNA) which belongs to a class of double stranded RNA. siRNA is a double stranded RNA molecules which are 19–23 base pair (bp) in length with the molecular weight of about 13 to 15 kd and have 38 to 46 negative charges. The structure of siRNA is well defined, which contains a two-nucleotide overhang on the 3' end of both strands, phosphate group on the 5' end and a hydroxyl group on the 3' end [106]. siRNAs are double-stranded duplexes which need to be unwound before they assembled into a RISC. siRNAs is divided into two classes and is depend on the thermodynamic stabilities at the two ends,: symmetric siRNAs and asymmetric siRNAs. A symmetric siRNA contains two equally stable ends and thus, both the strands of the siRNA are assembled into the RISC with equivalent efficiency. An asymmetric siRNA contains one end with less stability than the other. siRNA can be unwind easily from the less stable end and one strand of the siRNA can be process referred to as the asymmetric assembly of RISCs [158]. Gene silencing by siRNA includes it’s binding to corresponding mRNA and degradation of target mRNA. In mammalian cells, synthetic siRNA duplexes can activate RNAi which knock down target mRNA sequence and hence, corresponding protein production. A specific endogenous siRNA is originated either from a long double-stranded RNA (dsRNA ~ 200 nucleotides) coded by a certain gene, or from an exogenous source such as non-viral and viral vectors. This long dsRNA is then fragmented into the 19 – 23 base pair siRNAs by RNase-III like enzyme called Dicer and then this siRNA forms complex known as the RNA-inducing silencing complex (RISC). The sense strand of siRNA guides the RISC to the appropriate target mRNA molecule, where it cleaves and destroys the complementary mRNA. The broken mRNA is rapidly degraded and protein expression is reduced or abolished [106, 159]. Principle of antisense oligonucleotide therapy helps in the development of RNA interference RNAi by using siRNA. Antisense oligonucleotides contain the single strands of DNA or RNA that are complementary to a specific sequence of mRNA. It inhibits translation of a complementary mRNA molecule by binding to it and physically obstructing the translation machinery. However, antisense RNA often lacks effective design, biological activity, and an efficient route of administration and because of that it has been replaced by the new technology of RNAi. Specific siRNA sequences for many target mRNAs can be predicted by using current bioinformatics
technologies. These artificial siRNAs are capable of silencing their complementary mRNAs by mechanisms similar to those of endogenous siRNA. These artificial siRNAs can either be synthesized chemically as oligos (siRNAs) or cloned into a plasmid or virus vector like adenovirus, retrovirus or lentivirus as short hairpin RNAs (shRNAs). To block gene expression by using siRNA have many advantages over other methods, like chemical inhibitors and dominant negative mutants. Knockdown the expression of any class of genes including both protein-encoding genes and non-coding RNAs can be targeted by siRNA. On the other hand, there are only a limited number of chemical inhibitors available against certain proteins or pathways, and many of them are not specific. siRNA have number of advantages like is highly specific, can be easily synthesized or cloned into expression vectors, siRNA-mediated silencing is more specific and less toxic compared with both chemical inhibitors and dominant negative mutants. Therefore, siRNA silencing is overall an excellent tool in various diseases to knock down the overexpressed genes involved in it, where conventional treatment often fails.

2.5.6. RNAi as Therapeutics

RNAi technology is also currently being evaluated as a potentially useful method to develop highly specific RNA-based gene-silencing therapeutics. As a new therapeutic approach, RNAi might be specific enough to allow the use of multiple RNAi targets at the same time, without the toxic effects often observed during chemotherapy and the sequence-independent toxic effects of antisense therapy. Gene expression is silenced by fundamental cellular mechanism of RNAi. Overexpression of pathological proteins is suppressed through RNAi and is applicable to all classes of molecular targets, including those which are difficult to modulate selectively with traditional pharmaceutical approaches. The target mRNA is enzymatically cleaved by RNAi which leads to suppression of the overexpressed protein. RNAi therapeutics as a drug class has the potential to exert a transformational effect on modern medicine [160]. RNAi is used in analysis of the biological function of individual genes or genes known to be associated with diseases [161]. RNAi is an emerging field for basic and biomedical research that may lead to a number of clinical applications. Various studies have been published demonstrating efficacious silencing of disease genes by local and systemic administration of RNAi in animal models of human disease. Both exogenous and endogenous genes have been silenced, and promising in
vivo results have been obtained across multiple organs and tissues. Efficacy has been demonstrated for viral infection (respiratory and vaginal), ocular disease, disorders of the nervous system, cancer and inflammatory bowel disease.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Route of Administration</th>
<th>Potential organ target</th>
<th>Disease Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Local/Direct</td>
<td>Eye</td>
<td>Macular degeneration, Diabetic macular oedema</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Skin</td>
<td>Atopic dermatitis</td>
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<tr>
<td></td>
<td></td>
<td>Vagina</td>
<td>Herpes simplex virus</td>
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<tr>
<td></td>
<td></td>
<td>Rectum</td>
<td>Inflammatory Bowel disease</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lung</td>
<td>SARS, RSV, Flu</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Brain</td>
<td>Huntington’s disease, Depression, Alzheimer’s disease, Spinoencephalitis, Ataxia, ALS, Encephalitis, Neuropathic pain</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Spinal cord</td>
<td>Chronic pain</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vagina</td>
<td>HSV</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Isolated tumour</td>
<td>Glioblastoma multiforme, Prostate, Adenocarcinoma, Human papillomavirus</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Digestive system</td>
<td>Irritable Bowel disease</td>
</tr>
<tr>
<td>2</td>
<td>Systemic</td>
<td>Liver</td>
<td>Hypercholesterolemia, HBV</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Heart</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kidney</td>
<td>Acute kidney injury</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Metastasized tumours</td>
<td>Ewing’s sarcoma</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Joints</td>
<td>Rheumatoid arthritis</td>
</tr>
</tbody>
</table>
Now a day, several researchers have explored the use of RNAi to limit infection by viruses in cultured cells. Jacque et al. directed siRNAs against the HIV-1 genome, including the viral long terminal repeat (LTR), vif and nef [162]. Gitlin et al. attenuated poliovirus infection after transfection with siRNAs that targeted either a capsid-protein mRNA or the viral polymerase mRNA.[163] Similarly, RNAi has been used to attenuate infection by Rous sarcoma virus in chick embryos, and sequences within the hepatitis C virus have been successfully targeted in living mice when present as a fusion with a reporter construct [92]. HIV-resistant progeny T cells and macrophages were produced by transplanting hematopoietic stem cells transduced with a lentivirus expressing an anti-HIV shRNA [164]. Intravenous injection of shRNA-encoding DNA vectors as well as intratracheal administration of shRNA vectors, have provided possible approaches to treat respiratory viruses such as influenza or respiratory syncytial virus [165]. shRNAs directed against the structural protein 1D (Ad5-NT21) or polymerase 3D (Ad5-POL) of foot and mouth disease virus (FMDV), delivered by adenovirus are capable of inhibiting virus replication in both cultured porcine cells and in guinea pigs [166]. The LNA-antimiR against miR-122 decreases total plasma cholesterol level without hepatotoxicity in African green monkeys [109]. Anti-miR-126 antagonizes to miR-126 and suppresses the asthmatic phenotype in mice model of allergic asthma [167]. SPC3649 (LNA-antimiRTM-122) is being developed as a new potential approach in treatment of Hepatitis C infection [168].

Devastating problems may be arising due to many neurological diseases which are progressive and untreatable. RNAi-based gene silencing which is having high-order of specificity is more beneficial than other therapeutic approaches in the treatment of neurological disorders. Diseases like neurodegenerative disorders (Huntington’s disease, spinobulbar muscular atrophy, frontotemporal dementia with parkinsonism, dystonia, and slow channel congenital myasthenic syndrome), CNS tumors, chronic pain, prion diseases, trinucleotide repeat diseases, infectious diseases, are likely candidates to benefit from RNAi.[92] Silencing of mutant SOD1 expression possibly treats Familial Amyotrophic Lateral Sclerosis (FALS) and allele-specific silencing of mutant SOD1 using siRNA has been demonstrated by Ding et al. [169]. Potential therapeutic targets for siRNA-mediated gene silencing in Alzheimer’s disease (AD) are the β- and γ-secretases. These enzymes helps in the cleavage of APP to β - amyloid and thus provide logical targets for AD therapy by either direct inhibition or down regulation of expression using siRNA.
Various researchers have shown that siRNAs can be used against viral targets [170]. Equine Infectious Anemia Virus (EIAV) mediated silencing of mutant SOD1 expression in vulnerable motor neuron populations using shRNA causes reversal of a dominantly inherited form of Amyotrophic lateral sclerosis (ALS) in a transgenic mouse model [171]. Long-term in vivo expression of two different rAAV5-shRNA vectors led to significant reduction in striatal mHtt mRNA and protein levels which can ameliorate the Huntington’s disease (HD) phenotype of R6/1 mice [172]. There are some important problems which have to be solved before clinical use of RNAi. For successful therapeutic application of RNAi in humans, refinement of delivery methods seems to be the major barrier. Efficient and suitable delivery system should be used for successful therapeutic application of RNAi.

Cancers are often caused by deregulated expression of genes that lead to uninhibited cell growth. Bcl-2 and p53 are the particular interest of genes which involved in apoptotic pathways. A study showed that siRNAs directed against BCR/ABL transcripts induced apoptosis [173]. siRNAs have been used to target K-RASV12 which constituatively activates KAS leading to pancreatic and colon cancer. Knockdown of K-RASV12 resulted in specific degradation of K-RASV12 and inhibition of colony growth in soft agar [174]. Many diseases like cancer and angiogenesis-related diseases are characterized by the uncontrolled growth of new blood vessels because of the overexpression of multiple endogenous and exogenous pathogenic genes. Combination of multiple drugs is used when disease progression and the development of drug resistance stop the effect of single-drug treatments. Combination of multiple-siRNA to target multiple disease-causing genes provide a unique advantage for combination therapy. Improved anti-angiogenesis potency has been observed in combination of siRNAs targeting VEGF-A, VEGFR1 and VEGFR2 when compared with siRNAs targeting only one factor [175, 176]. Combination of multiple siRNAs targeting to angiogenic factors in each category may enable the identification of potent anti-angiogenic agents for potential therapeutic applications. Several attractive siRNA targets are available to fight against cancer and angiogenesis. Intratumoral injection of an adenoviral vector encoding a shRNA to target S phase kinase-associated protein 2, effectively inhibited a small cell lung carcinoma in mice [177]. Plasmid vectors of shRNA specific against STAT6 gene induced apoptosis in colon cancer cells [178, 179]. Inhibition of p16 expression in squamous cell carcinoma using shRNA and integrated these shRNA into adenoviral and
retroviral vectors for transient and integrated expression in human cells [178]. Antisense inhibitor to miR-27a, miR-96 and miR-182 leads to a significant increase in endogenous FOXO1 expression in breast cancer cells [180]. Fluiter et al., demonstrated that in vitro H-Ras knockdown and in vivo tumor growth inhibition in prostate tumor xenografts by anti-H-Ras ODN containing alpha-L-LNA,[181] p53 gene upregulate the mammalian miR-34 in response to radiation. miR-34 is responsible for a normal cellular response to DNA damage in vivo and it points to a potential therapeutic use for anti-miR-34 as a radio-sensitizing agent in p53-mutant breast cancer [182]. Delivery of vessel-targeted nanoparticle containing anti–miR-132 restored p120RasGAP expression in the tumor endothelium which results into suppression of angiogenesis and decreased tumor burden in human breast carcinoma [183]. Transfection of antimiR-146a OND into balloon-injured rat carotid arteries markedly decreased neointimal hyperplasia [184]. Inhibition of the formation of capillary-like structures stimulated by hypoxia and decreased cell migration in response to VEGF (vascular endothelial growth factor) was achieved through miR-210 blockade via anti-miRNA transfection [185].

Table 2.4 Therapeutic intervention using siRNA

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Disease</th>
<th>Type</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Viral</td>
<td>HIV-1</td>
<td>LTR, vif, nef, Tat, Rev, Gag, CD4, CCR5, p24, Pol Capsid, viral polymerase</td>
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<tr>
<td></td>
<td></td>
<td>Poliovirus</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Hepatitis B</td>
<td>Core region (3.5 kb RNA), Pregenomic RNA</td>
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<tr>
<td></td>
<td></td>
<td>Rous sarcoma virus</td>
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<tr>
<td></td>
<td></td>
<td>Hepatitis C</td>
<td>EMCV-IRE5, NS3, NS5B, NA, Core, NS4B, 5’ UTR, NS5A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Respiratory Syncytial Virus</td>
<td>Phosphoprotein (P), Fusion protein (F)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Influenza A</td>
<td>NP, PA, PB1, PB2, M, NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rotavirus</td>
<td>VP4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adenovirus (group B)</td>
<td>CD46 (cellular coreceptor)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>γ herpes virus</td>
<td>Rta, ORF45</td>
</tr>
<tr>
<td></td>
<td>Cancer</td>
<td>Leukemia</td>
<td>c-raf, bcl-2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cervical carcinoma</td>
<td>E6, E7 (HPV)</td>
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<tr>
<td></td>
<td></td>
<td>Pancreatic carcinoma</td>
<td>K-RASV12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Melanoma</td>
<td>ATF2, BRAFV599R</td>
</tr>
<tr>
<td>3.</td>
<td>Angiogenesis</td>
<td>Ovarian carcinoma</td>
<td>H-Ras, mVEGF, COX-2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Prostate cancer</td>
<td>P110a, p110B of PI 3 kinase</td>
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<tr>
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<td></td>
<td>Wilms' tumor</td>
<td>Wt1, Pax2, Wnt4</td>
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<tr>
<td></td>
<td>Tumor angiogenesis</td>
<td></td>
<td>VEGF</td>
</tr>
<tr>
<td></td>
<td>Ocular neovascularization</td>
<td></td>
<td>VEGF, VEGFR1 and VEGFR2</td>
</tr>
<tr>
<td></td>
<td>Rheumatoid arthritis</td>
<td></td>
<td>Akt, GG2-1, ASC</td>
</tr>
</tbody>
</table>

| 4. | Neurological Disorders | Alzheimer's disease | β-, γ-Secretase, Protein kinases (GSK-3, Cdk-5) |
|     |             | Parkinson's disease | α-Synuclein, LRRK2 |
|     |             | Huntington's disease | Huntington |
|     | Familial amyotrophic lateral sclerosis |             | SOD-1 |
|     | Spinocerebral ataxia |             | SCA-1, SCA-2 |
|     | DYT1 dystonia |             | TOR 1A |

2.5.7. *In Vivo* Delivery Vectors

Most challenging task in the RNAi delivery is efficient intracellular accumulation of RNA macromolecule. Variety of approaches, including viral and nonviral delivery vectors, administration through local and systemic route, has been utilized to down regulate target protein. Different formulations ranging from saline solution of naked siRNA to lipid, protein or cholesterol conjugates, aptamers etc have been used to elicit the RNAi response *in vivo*.

Each of these has distinct merits and demerits to use them in clinical application. RNAi molecules require a delivery vector for many reasons. These include high negative charge, molecular weight, and degradation by nucleases [102]. Viral vectors are more beneficial when transfection efficiency is in a question. However, non-viral vectors own substantial advantages i.e. less in-vivo toxicity, immunogenicity and insertional mutagenesis [186]. An ideal RNAi delivery vector should be equipped with a cationic group for effective transfection, an endosomolytic group for endosomal escape, a surface modifier to decrease steric hindrance that ultimately enhances circulation time in blood, and a targeting moiety to direct a delivery system at target cells or tissue [112]. As far as systemic delivery is concerned, size of delivery vectors plays an enormous role in biological system. To avoid glomerular filtration, size of the delivery vector should not be less than 5 nm. At the same time, delivery system should be big enough to
avoid leakage to interstitial spaces of hepatic sinusoid and entrapment by hepatic Kuffer cells, which requires particle size greater than 100 nm [112]. Further, to avoid macrophage uptake in to systemic circulation, size should not be more than 200nm. Hence, ideal size of deliver vectors for systemic delivery should lie between 100-200 nm.

Viral vectors usually elicit long term inhibition of target protein in a single administration, but these vectors suffer from a major risk of immune response in host, which has been highlighted recently [187]. Earlier with the use of AAV mediated shRNA delivery in to mice, diversion of RNAi mechanism was observed that ultimately manifested in marked toxicity.

While working with viral vectors, it is extremely difficult to forcast RNAi exposure with respect to its amount and duration. Furthermore, it may be possible that viral vector encoded with high level of shRNA interfere with endogenous miRNA pathway. Non-viral delivery vectors have been extensively utilized for the delivery of nucleic acids, locally and systemically. These mainly include lipids, polymers, and peptides. Various lipid complexes, liposomes, polymers, proteins and antibodies have been used to deliver RNAi to target site. Cationic lipids and polymers have shown some cytotoxic effects that might limit the use of these carriers in RNAi delivery for particular disease indications and dosing paradigms [159]. Ultimately, one should go ahead with a carrier system, which is having least in-vivo toxicity with enhanced transfection efficacy [188, 189].

Some of the marketed lipid based non-viral RNAi vectors for transfection are Oligofectamin [190], Lipofectamine, TransIT-TKO and DharmFECT [191], all of which have been employed delivering RNAi macromolecules in-vivo. Usually, positively charged lipids are employed for complexation and delivery of RNAi; however, few of the neutral and anionic lipids have also been tried [192]. According to few reports, in vivo, these Cationic lipids possess poor in vivo stability and reproducibility with cytotoxicity [193]. For efficient delivery of RNAi, with minimum side effects, optimization of a charge ration between vector and nucleic acid is must, because it is the negative charge of RNAi molecule which complexes with cationic group of the vector. Cationic lipids or liposomes made up from these lipids are normally more toxic than their neutral counterparts. In contrast to lipid based vectors, polymer vectors possess relatively less
immune response, though not much safer in unmodified form [194]. Polymers give flexibility for use in terms of its physical and chemical properties that might be the major cause for extensive investigation on polymeric delivery of RNAi therapeutics. Charge density, molecular weight, and pH markedly affect the complex formation between polycations and RNAi, also known as polyplex. Polycations interacts weakly with RNAi as contrast to DNA molecule and hence, finally leads to the formation of loose polyplex. However, increment in charge ratio can overcome this drawback but, ultimately increased charge density results into decreased margin of safety with respect to cytotoxicity [195]. Cationic polymer Polyethylenimine (PEI) is one of the most extensively investigated non-viral delivery vector to transfer RNAi intracellularly for systemic and local applications [159]. PEI has also been utilized as a reference standard for many in-vitro and in-vivo studies. A proton sponge effect of this cationic polymer results into endosomal release of RNAi into cytoplasm and assures high efficiency of transported RNAi [149]. However, in-vivo toxicity of PEI has forced researchers to develop newer modified polymers and polycations for safe and effective RNAi delivery. Apart from this, nanoparticles made up of hydrophobic polymeric matrix encapsulating RNAi macromolecule is one of the alternate means for delivering RNAi. This system offers appreciable protection of RNAi against nucleases but negotiates with loading capacity of genomic materials [196]. Peptide vectors have also been utilized to transfect RNAi in-vivo. Various cell penetrating peptides and its modifications which are studied for in-vivo intracellular delivery of nucleic acids are TAT, transportan, penetratin, CADY, MPG and VP22 [159].

Many times small molecules, proteins and antibodies are used as conjugates with RNAi for efficient targeted delivery, which should also be focused with regard to the biological activity. Alteration may occur in normal physiology if a specific receptor or other endogenous molecule is used, which has a potential role in normal body functions. This finally causes undesirable side effects. Conclusively, non-RNAi part in to delivery system increases intricacy during manufacturing, especially at commercial scale. However, non-RNAi part is much essential to balance a well-recognized transfection to toxicity poser. Development of novel biodegradable polymers and less cytotoxic lipids may come out with more efficient and less immune active delivery vectors [160, 197].
2.5.8. In Vivo Delivery of RNAi

Delivery of RNA macromolecules in vivo can be achieved by two ways, systemic and local. Larger amount of nucleic acids is required when administered systematically into biological system to achieve down regulation of target gene. In contrary, local delivery of these macromolecules at desired sites is more preferable, as therapeutic effect can be governed at low dose with reduced systemic side effects [170, 198, 199]. Vast numbers of studies have been conducted for delivering RNAi therapeutics in-vivo. These includes direct injection of macromolecules, pulmonary administration via inhalers and nebulizers, intravenous injection using naked or vector mediated delivery approaches etc [200-202]. Many researchers have utilized vector mediated delivery for RNA macromolecules which includes delivery by using lipids, peptid, and polymers. These further can be surface modified with suitable ligand molecules [203, 204]. Recently, aptamer approach has also been employed to deliver siRNA intracellularly [205, 206].

A key concept behind considering a selection between local and systemic RNAi administration is the frequency and amount of doses required to accomplish adequate nucleic acid concentration in the target site and the probable unwanted effects due to exposure of non-targeted tissues to these RNAi molecules. Current scenario in silencing technology suggests that so far efficacy has only been shown by local RNAi application when local and systemic exposure come to the same platform. However, systemic administration has a defiant advantage when tissue like a liver is considered, where majority of systemically administered drug molecules get localized [160].

2.5.8.1. Systemic Delivery of RNAi Therapeutics

After numerous successes in mammalian cell culture system, RNAi therapeutics were successfully tried in animal models to elicit desired down regulation of target protein. Previously, in-vivo delivery of RNAi was attained by giving hydrodynamic injection into the tail vein of the mice, which resulted in significant suppression of a Luciferase gene [207]. In continuation of this, studies were also conducted in using high-pressure intravenous tail injection of siRNA and shRNA in adult as well as postnatal mice. This caused marked reduction of gene expression, up to 90%, in the liver and also in other organs such as lung, kidney, spleen and pancreas [208, 209]. Accumulation of large amount of the siRNA in to the liver prompted
researcher to think about application of RNAi therapeutics in the treatment of liver diseases. One such study involved RNAi administration in to acute liver failure induced mice model. This involved endogenous genes expressing FAS cell death receptor and caspase 8, both are involved in apoptosis during hepatic injury that is initiated by viruses or transplant rejection [174, 210]. It was observed that significant liver protection was obtained following pretreatment with FAS and caspase-8 targeting siRNAs, where liver failure was induced using different chemicals. Further, expression of FAS was found to be inhibited up to 10 days, which suggests in-vivo stability of siRNA in mice.

Liver is readily targeted via systemic RNAi delivery as compared to other organs. Recently, systemic administration of adenoviral vector expressing siRNA against HBV demonstrated reduction in viral load and almost restricted the replication of HBV for 26 days. Even though liver is assumed to be port of systemically delivered molecules, findings from this study put forward the application of RNAi in liver diseases [211]. Efforts have also been made to deliver RNAi across the BBB but it remained the most challenging task because, RNAi macromolecules cannot cross BBB. However, several researchers tried different strategies to deliver RNAi into the CNS including “Trojan Horse” technique using liposomes [212]. In this technique, RNAi macromolecules are encapsulated within the liposomes, which may be surface modified using polyethylene glycol (PEG). The PEG surface modification serves to protect liposomes, and so as RNAi, against macrophage uptake to impart long systemic circulation. This PEG can also help to graft cell specific monoclonal antibodies which can target a definite tissue or cells. Moreover, more than one targeting ligands can also be grafted onto liposomal surface for multiple targeting. For example, two distinct monoclonal antibodies or aptamers attached onto liposomal surface helps to target specific site into the CNS, since one targeting moiety can be distinct to BBB and thus allow transport across it and other one can be distinct to a cell type receptor inside the CNS [213]. In recent times, Alvarez-Erviti et al. have showed the application of exosomes to deliver RNAi across the BBB. Exosomes are biological nanovesicles, which help in transportation of RNAs and proteins [214]. Exosomes were obtained from dendritic cells and purified to reduce immunogenicity. To attain higher concentration inside the brain, dendritic cells were bioengineered to express an exosomal membrane protein known as Lamp2b fused to CNS rabies viral glycoprotein (RVG). RVG selectively binds to acetylcholine receptors in the CNS [215].
Finally, intravenous injection of RVG attached exosomes containing siRNA caused marked accumulation of siRNA in to the neurons and oligodendrocytes without having any immune response.[216] Efforts have also been made to target HIV using RNAi stratagies and this has been promoted by decreased viral load following RNAi application against HIV infection [217]. However, pathogenesis of HIV limits the use of RNAi macromolecule in the treatment of this disease. HIV can mutate to run away from RNAi trap. HIV may escape RNAi through recently recognized function of its Tat protein, which interferes with dicer activity [218]. Thus, betterment in the formulation, encoding RNAi molecule, may overcome these situations in near future.

Recent advancement in systemic RNAi delivery is the targeting to specific cells or tissue by cell surface receptor. This strategy helps to provide maximum therapeutic benefit at least adverse events. Many targeting moieties including aptamers, monoclonal antibodies, and peptides in conjugation with RNAi, have been investigated to target specific cell surface receptor and thus to the desired site into the body [124, 219, 220]. Systemic administration of targeted RNAi was first time utilized in human during phase 1 clinical trial, which involved targeted RRM2 (Ribonucleotide reductase subunit M2) siRNA nanoparticles via intravenous route to the patients bearing solid tumor). Results demonstrated significant inhibition of RRM2 gene at both mRNA and protein stages [221].

Monoclonal antibody grafted liposomes, immunoliposomes, have been utilized to overcome barriers of BBB and transfer RNAi molecule into the brain.[222] RNAi expressed plasmid has been entrapped into the immunoliposomes, having particle size less than 100 nm. Further, surface of these liposomes was sterically stabilized to impart longer circulation into the blood. Results demonstrated that intravenous administration of RNAi immunoliposomes silenced significant gene expression in the brain. More specifically, intracranial brain cancer induced rats were administered plasmid DNA encoded with shRNA targeting Luciferase gene, which were expressed in brain tumor, by intravenous route. Results showed that 90% of Luciferase gene silencing was attained for not less than 5 days. In addition to this, mice bearing human brain cancer were also regressed and life span of the mice was increased by 90% after administering shRNA encoded plasmid containing immunoliposomes [223]. Despite of these tremendous efforts, RNAi requires repeated administration to achieve long term effect because, in-vivo
distribution of RNAi throughout the tissue and into the targeted cells is much heterogeneous. Hence, future approach to deliver sustained RNAi therapeutics may help to solve this issue and application of this novel technology in to clinical practice.

2.5.8.2. Clinical Trials and RNAi

The discovery of RNAi has been widely acknowledged as a major breakthrough in biology. This exciting technology has the potential to make a broad and significant impact in therapeutics. Much important scientific and clinical advancement are being made at a very rapid pace. Major companies demonstrating significant impact in clinical development of RNAi platform worldwide include Silence therapeutics, Alnylam therapeutics, Quark, Calando pharmaceuticals, Sirna, Allergan, Grada inc., Santaris Pharma A/S and Pfizer and Acuity as in collaborative research [145-150]. Alnylam is supporting the development of Direct RNAi™ therapeutics. These products are designed to be administered directly to sites of diseases in various parts of the body, such as the eye, the brain or the lungs. Calando is a clinical stage nano-biotechnology company at the forefront of RNAi therapeutics and develops nanoparticle therapeutics that use sugar (cyclodextrin)-based polymer technologies as a drug delivery system for siRNA. CALAA-01, the company’s leading drug candidate for treating cancer, is in phase II trial. Silence Therapeutics has several RNAi drugs in phase I and II trials for treating diabetic macular edema, age-related macular degeneration, acute kidney injury, and cancers. Quark pharmaceuticals has focused on diseases of the eye (e.g., wet diabetic macular edema, diabetic retinopathy, NAION, glaucoma etc.), lung (e.g., acute lung injury, primary graft dysfunction in lung transplantation), kidney (acute kidney injury, delayed graft function), inner ear (e.g. acute hearing loss, ototoxicity and Méniere’s disease), and spinal cord (spinal cord injury). These companies are concentrating majorly on siRNA therapeutics whereas Gradalis, inc. has made an effort to move shRNA base therapeutics into clinics including anti-cancer phi-shRNA™ STMN1 I.P to phase I and FANG™ autologous tumor cell vaccine to phase II. Miravirsen is the first microRNA-targeted drug to receive Investigational New Drug (IND) acceptance from FDA, paving the way to conduct Phase 2 trials for treatment of hepatitis C in the United States [6]. Santaris Pharma A/S advances Miravirsen, the first miRNA-Targeted Drug to enter clinical trials, into Phase 2 to treat patients infected with hepatitis C virus. Further research is in progress to understand the roles of miRNAs
in cancer and the potential for manipulating miRNAs for cancer therapy as these molecules make their way towards clinical trials [224].

**Table 2.5 siRNA – Clinical Trials [145-150, 224] (http://clinicaltrials.gov/)**

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Product Details</th>
<th>Company and Strategic Alliance</th>
<th>Target tissue</th>
<th>Indication</th>
<th>Type and route of delivery</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>SiRNA TD101</td>
<td>TransDerm, Inc./Pachyonychia Congenital Project</td>
<td>Thick calluses, non-specific topical keratolytics, and oral retinoids</td>
<td>Pachyonychia Congenita</td>
<td>Injection into a callus</td>
<td>Phase Ib</td>
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<td>2.</td>
<td>SiRNA Sirna-027/AGN211745</td>
<td>Allergan/ Sirna Therapeutics Inc</td>
<td>Retina</td>
<td>Age-Related Macular Degeneration, Choroidal Neovascularization</td>
<td>Naked siRNA Intravitreal Injection</td>
<td>Phase I and II</td>
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<tr>
<td>3.</td>
<td>SiRNA AGN211745</td>
<td>Allergan</td>
<td>Retina</td>
<td>Choroid Neovascularization Age-Related Macular Degeneration</td>
<td>Injection</td>
<td>Phase II terminated</td>
</tr>
<tr>
<td>4.</td>
<td>SiRNA CALAA-01</td>
<td>Calando Pharmaceuticals</td>
<td>Cancer cells</td>
<td>Cancer/ Solid Tumor</td>
<td>Intravenous</td>
<td>Phase I</td>
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<tr>
<td>5.</td>
<td>Atu027</td>
<td>Silence Therapeutics AG</td>
<td>Targets PKN3 molecule in cancer cells</td>
<td>Advanced Solid Tumors</td>
<td>Intravenous infusion</td>
<td>Phase I</td>
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<td>6.</td>
<td>QPI-1007</td>
<td>Quark Pharmaceuticals</td>
<td>Eye</td>
<td>Optic Atrophy, Non-arteritic Anterior Ischemic Optic Neuropathy</td>
<td>Intravitreal Injection</td>
<td>Phase I</td>
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<tr>
<td>7.</td>
<td>SiRNA Cand5/bevasiranib</td>
<td>Acuity/later licensed by Opko</td>
<td>Eye</td>
<td>Age-Related Macular Degeneration</td>
<td>Naked siRNA Intravitreal</td>
<td>Terminated at Phase-III</td>
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<tr>
<td>8.</td>
<td>Akli-5</td>
<td>Silence Therapeutics, AtuRNAi technology sublicensed to Pfizer via Quark's license.</td>
<td>Kidney</td>
<td>Acute kidney injury in kidney transplantation</td>
<td>Chemically modified siRNA with AtuRNAi technology, IV</td>
<td>Phase I/II</td>
</tr>
<tr>
<td>9.</td>
<td>PF-655 (formerly REDD14NP)</td>
<td>Eye</td>
<td>Age-Related Macular Degeneration</td>
<td>Naked siRNA Intravitreal</td>
<td>Phase II</td>
<td></td>
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<tr>
<td>10.</td>
<td>DGFi</td>
<td>Kidney</td>
<td>Delayed graft function in kidney.</td>
<td>Chemically modified siRNA with AtuRNAi technology, IV</td>
<td>Phase I/II</td>
<td></td>
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<td>11.</td>
<td>TKM-080301</td>
<td>National Cancer Institute (NCI)/National Institutes of Health Clinical Center</td>
<td>Liver</td>
<td>Colorectal, Pancreas, Gastric, Breast and Ovarian cancer with Hepatic Metastase.</td>
<td>Intra-Arterial</td>
<td>Phase I</td>
</tr>
<tr>
<td>12.</td>
<td>SYL1001</td>
<td>Sylentis, S.A.</td>
<td>cornea and conjunctival sac</td>
<td>Ocular Pain, Dry Eye</td>
<td>Eye drops: Topical administration</td>
<td>Phase I</td>
</tr>
<tr>
<td>13.</td>
<td>QPI-1002 (I5NP)</td>
<td>Quark Pharmaceuticals</td>
<td>Temporarily inhibit expression of the pro-apoptotic protein, p53</td>
<td>Injury of Kidney, Acute renal failure.</td>
<td>IV injection</td>
<td>Phase I</td>
</tr>
<tr>
<td>14.</td>
<td>SYL040012</td>
<td>Sylentis, S.A.</td>
<td>Eye</td>
<td>Glaucoma, Ocular Hypertension</td>
<td>Ophthalmic drops</td>
<td>Phase I/II</td>
</tr>
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<td>15.</td>
<td>PF-04523655 (Stratum I) and PF-04523655 With/Without Ranibizumab</td>
<td>Quark Pharmaceuticals</td>
<td>Eye</td>
<td>Choroidal Neovascularization Diabetic Retinopathy and macular edema.</td>
<td>intravitreal (IVT)</td>
<td>Phase II</td>
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### Literature Review

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<tr>
<th></th>
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<th>Wild type and all mutant forms of TTR, Hepatocyte specific gene silencing</th>
<th>TTR amyloidosis</th>
<th>IV bolus</th>
<th>Phase I</th>
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<tr>
<td>16</td>
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<td>Alnylam pharmaceuticals</td>
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<td>kinesin spindle protein, or KSP and VEGF</td>
<td>Liver cancer</td>
<td>IV bolus</td>
<td>Phase II</td>
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<td>Hypercholesterolemia</td>
<td>Intravenous</td>
<td>Phase I</td>
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<td>nucleocapsid &quot;N&quot; gene of the RSV genome</td>
<td>Respiratory syncytial virus infection</td>
<td>Intravenous</td>
<td>Phase I</td>
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#### 2.5.9. Delivery of Therapeutic siRNA in cancer

The RNAi phenomenon and siRNA have provided newopportunities for the development of innovativemedicine to treat previously incurablediseases such as cancer. siRNA is of inherent potency because it exploits the endogenous RNAi pathway, allows specific reduction of diseaseassociated genes, and is applicable to any gene with a complementary sequence [225]. 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 throughwhich they act characterized [226]. 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 beeninvestigated 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 [227].

Nevertheless, most approaches to RNAi-mediated gene silencing for cancer therapy have been with cell cultures in the laboratory, and key impediments in the transition to the bedside due to delivery considerations still remain. Delivery systems that can improve siRNA stability and cancer cell-specificity need to be developed, involving the minimizing of off-target and nonspecific immune stimulatory effects. As the route of administration may differ depending on the nature of the cancer, the delivery systems must be optimized for specific cancers. The current status of siRNA delivery systems for various cancers is summarized in Table 2.6, and its particular application in lung cancer is discussed below.

### Table 2.6 Examples of siRNA delivery systems for treatment of cancers

<table>
<thead>
<tr>
<th>Delivery systems</th>
<th>Property</th>
<th>Targeted gene</th>
<th>Animal model</th>
<th>Route</th>
<th>Ref.</th>
</tr>
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<tr>
<td>Liposomes</td>
<td>SNALP</td>
<td>HBV</td>
<td>HBV vector-based mouse</td>
<td>i.v.</td>
<td>[228]</td>
</tr>
<tr>
<td></td>
<td>Cationic liposome</td>
<td>Bcl-2</td>
<td>Liver metastasis mouse model</td>
<td>i.v.</td>
<td>[229]</td>
</tr>
<tr>
<td></td>
<td>Cationic liposome</td>
<td>Integrin αν</td>
<td>Prostate cancer xenograft</td>
<td>i.t.</td>
<td>[230]</td>
</tr>
<tr>
<td></td>
<td>Cationic liposome</td>
<td>CD31</td>
<td>Prostate cancer xenograft</td>
<td>i.v.</td>
<td>[231]</td>
</tr>
<tr>
<td></td>
<td>Cationic liposome</td>
<td>Bcl-2</td>
<td>Prostate cancer xenograft</td>
<td>i.t.</td>
<td>[229]</td>
</tr>
<tr>
<td></td>
<td>Cationic cardiolipin liposome</td>
<td>Raf-1</td>
<td>Prostate cancer xenograft</td>
<td>i.v.</td>
<td>[232]</td>
</tr>
<tr>
<td></td>
<td>Cationic cardiolipin analogue-based liposomes</td>
<td>c-raf</td>
<td>Breast cancer xenograft</td>
<td>i.v.</td>
<td>[233]</td>
</tr>
<tr>
<td></td>
<td>Neutral liposomes (DOPC)</td>
<td>EphA2</td>
<td>Ovarian cancer xenograft</td>
<td>i.v./i.p.</td>
<td>[234, 235]</td>
</tr>
<tr>
<td></td>
<td>Neutral liposomes (DOPC)</td>
<td>FAK</td>
<td>Ovarian cancer xenograft</td>
<td>i.p.</td>
<td>[236]</td>
</tr>
<tr>
<td>Delivery systems</td>
<td>Property</td>
<td>Targeted gene</td>
<td>Animal model</td>
<td>Route</td>
<td>Ref.</td>
</tr>
<tr>
<td>--------------------------</td>
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<td>------</td>
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<tr>
<td>Neutral liposomes (DOPC)</td>
<td>ADRB2</td>
<td>Ovarian cancer xenograft</td>
<td>i.p.</td>
<td></td>
<td>[237]</td>
</tr>
<tr>
<td>Neutral liposomes (DOPC)</td>
<td>IL-8</td>
<td>Ovarian cancer xenograft</td>
<td>i.p.</td>
<td></td>
<td>[238]</td>
</tr>
<tr>
<td>Liposome-polycation-DNA</td>
<td>EGFR</td>
<td>Lung cancer xenograft</td>
<td>i.v.</td>
<td></td>
<td>[239]</td>
</tr>
<tr>
<td>Cationic immunoliposome</td>
<td></td>
<td>Lung metastasis</td>
<td>i.v.</td>
<td></td>
<td>[240]</td>
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<tr>
<td>Immunoliposome</td>
<td>Her-2</td>
<td>Breast cancer xenograft</td>
<td>i.v.</td>
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<td>CaCO$_3$ nanoparticle</td>
<td>VEGF</td>
<td>Gastric cancer xenograft</td>
<td>i.t.</td>
<td></td>
<td>[242]</td>
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<tr>
<td>Chitosan-coated nanoparticle</td>
<td>RhoA</td>
<td>Breast cancer xenograft</td>
<td>i.v.</td>
<td></td>
<td>[243]</td>
</tr>
<tr>
<td>Folated lipid nanoparticle</td>
<td>Her-2</td>
<td>Nasopharyngeal cancer xenograft</td>
<td>i.t.</td>
<td></td>
<td>[244]</td>
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</table>

**Nanoparticles**

<table>
<thead>
<tr>
<th>Polymers</th>
<th>Property</th>
<th>Targeted gene</th>
<th>Animal model</th>
<th>Route</th>
<th>Ref.</th>
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<tr>
<td>PEI</td>
<td>Her-2</td>
<td>Ovarian cancer xenograft</td>
<td>i.p.</td>
<td></td>
<td>[245]</td>
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<tr>
<td>PEI</td>
<td>PTN</td>
<td>Orthotopic glioblastoma</td>
<td>i.c.</td>
<td></td>
<td>[246]</td>
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<tr>
<td>Poly (ester amine)</td>
<td>Akt1</td>
<td>Urethane-induced lung cancer</td>
<td>Inhalation</td>
<td></td>
<td>[247]</td>
</tr>
<tr>
<td>Atelocollagen</td>
<td>HPV18 type E6 and E7</td>
<td>Cervical cancer xenograft</td>
<td>i.t.</td>
<td></td>
<td>[248]</td>
</tr>
<tr>
<td>Chemical modification</td>
<td>HBV</td>
<td>HBV vector-based mouse</td>
<td>i.v.</td>
<td></td>
<td>[249]</td>
</tr>
<tr>
<td>Carbon nanotube</td>
<td>TERT</td>
<td>Lewis lung tumor</td>
<td>i.t.</td>
<td></td>
<td>[196]</td>
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<tr>
<td>Cyclodextrin-containing polycation</td>
<td>EWS-FLI1</td>
<td>Metastatic Ewing's sarcoma</td>
<td>i.v.</td>
<td></td>
<td>[250]</td>
</tr>
<tr>
<td>Fusion protein (Protamine, HIV-1 envelop Ab)</td>
<td>c-myc, MDM2, VEGF</td>
<td>Subcutaneous B16 melanoma tumor</td>
<td>i.t./i.v.</td>
<td></td>
<td>[251]</td>
</tr>
<tr>
<td>Electroporation</td>
<td>EGFP</td>
<td>Subcutaneous B16F10</td>
<td>i.t.</td>
<td></td>
<td>[252]</td>
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</table>

**Others**
2.5.10. siRNA Application in Lung Cancer

Lung cancer is the most common cause of cancer-related death in men and the second most common cause of lethality in women. The main types of lung cancer are divided into small cell lung carcinoma and non-small cell lung carcinoma. The treatment modalities for these two types of cancer are different, so it is important to distinguish the two types. Recently, various new molecular targets for lung cancer therapies have been developed; for example, gefitinib (Iressa), which targets the tyrosine kinase domain of the epidermal growth factor receptor, another tyrosine kinase inhibitor known as erlotinib (Tarceva), and the angiogenesis inhibitor bevacizumab. For delivery into lung cancer cell lines, liposomes with arginine octamers on their surface were used for encapsulation of human double-minute gene 2-specific siRNA. The siRNA-loaded arginine octamer-liposomes showed a high stability in blood after 24 h of incubation and showed good transfection efficiencies into several lung cancer cell lines [253].

In another approach, LPD nanoparticles were developed for delivery of siRNA to lung cancer cells. siRNA targeting survivin in PEGylated LPD nanoparticles showed steric stabilization in the presence of serum and exerted antitumor effects by down-regulation of survivin expression, as measured by initiation of apoptosis, inhibition of tumor cell growth, and sensitization of tumor cells to anticancer drug treatment [254]. In an in vivo study, LPD nanoparticle formulations provided significant growth inhibition in a lung cancer xenograft mouse model.

LPD nanoparticle-mediated intravenous injection (1.2 mg/kg; 3 daily injections) of siRNAs specific for the epidermal growth factor receptor induced silencing of the target gene, and showed a synergistic effect on anti-lung cancer tumor activity when combined with cisplatin [239]. In a B16F10 lung metastatic mouse model, the LPD nanoparticles and siRNA complexes
afforded silencing of the target gene, and showed little immunotoxicity or development of organ defects after intravenous administration [255].

Cationic immunoliposomes have been used for systemic delivery of siRNA in an animal lung cancer metastasis model induced by the intravenous inoculation of MDA435/LCC6 cells. Cationic liposomes composed of DOTAP and DOPE were modified by conjugation with an anti-transferrin receptor single-chain antibody fragment. The complexes of cationic immunoliposomes with fluorescent siRNA were intravenously injected at a dose of 9 mg/kg into mice. A distribution of fluorescent siRNA was observed in the tumor-metastasized lung tissues, but not in the liver [240]. Polymer-based delivery systems have been studied in lung cancer models. Positively charged single-walled carbon nanotubes (–CONH– (CH2)6–NH3+) were used to carry siRNA targeting telomerase reverse transcriptase into cancer cells in vitro and in a lung tumor model. The siRNA coupled to single-walled cationic carbon nanotubes was shown to be internalized to tumor cells in vitro, and suppressed the expression of the target gene. The intratumoral injection of siRNA and the positively charged carbon nanotube complexes into subcutaneous Lewis lung tumors has been reported to reduce tumor growth in mice [256].

Recently, noninvasive aerosolized siRNA delivery systems were developed for lung cancer treatment. Poly(ester amine), a degradable PEI-alt-PEG copolymer, was synthesized by reaction of low-molecular-weight PEI with PEG diacrylate [196]. An aerosol of poly(ester amine)/ Akt1-targeting siRNA complex was delivered into mice with urethane-induced lung cancer through a nose-only inhalation system. Following aerosol delivery twice weekly for 4 weeks, Akt1 siRNA delivered in complexes with poly(ester amine) showed down-regulation of Akt related signals and inhibited the progression of tumors in the lung cancer model of K-rasLA1 mice [247].
2.6. RGD Peptide for Targeting

RGD-based strategies include RGD antagonists, RGD conjugates, and RGD nanoparticles. Because of the expression of integrins in various cell types and their role in tumor angiogenesis and progression, integrins have become important therapeutic targets. Integrin antagonists currently preclinically studied or in clinical trials include (i) monoclonal antibodies (such as etaracizumab, Abeclin), (ii) RGD-based antagonists (peptidic or peptidomimetic), (iii) non-RGD antagonists (such as ATN-161, a non-RGD-based peptide inhibitor of integrin α5β1), and (iv) integrin-targeted therapeutics. A cyclic RGD peptide antagonist of α5β3 and α6β5, cilengitide (EMD 121974) showed favorable safety profiles and no-dose-limiting toxicities in phase I clinical trials. Cilengitide is currently being tested in phase II trials in patients with lung and prostate cancer and glioblastomas. In addition, cilengitide has been shown to enhance radiotherapy efficiency in endothelial cell and non-small-cell lung cancer models. Nanocarriers like liposomes, nanoparticles, micelles, etc. can be grafted at their surface with a targeting ligand such as an RGD-based sequence. Several advantages are attributable to these nanocarriers: (i) the size of these nanocarriers (20 – 400 nm) leads to the “passive targeting” of tumors via the so-called enhanced permeability and retention (EPR) effect [257]; (ii) because of the size of these systems, renal filtration is avoided, leading to prolonged blood circulation times and longer accessibility of the ligand to target receptors within the tissue [258]; (iii) 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 [259]; (iv) 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 [260]. 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 αvβ3-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 as suggested by Judah Folkman. αvβ3 integrin is up-regulated in angiogenic endothelial cells but also in
several tumor cells, leading RGD-targeted nanocarriers to a potential double targeting. However, this double targeting is not yet exploited by systems delivering chemotherapeutics while it is described for integrin antagonists as etaracizumab or for RGD peptides.
2.7. References


