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SiRNA Delivery: challenges and role of carrier systems

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Gene silencing by RNA interference is a rapidly growing therapeutic area for managing diseases. Despite research advances in this direction, the poor cellular uptake of synthetic small interfering RNAs is a major impediment to their clinical applications. Polymer and lipid-based systems are attractive carrier systems for delivery of siRNA and provide options for desirable engineering of carrier particles. In this review, there is a detailed discussion of RNAi delivery systems and recent advances in the field.

1. Introduction

RNAi was first observed by plant biologists in the late 1980's (Bernstein et al. 2001). The first report of post-transcriptional gene silencing was in petunia flowers appeared in 1990. In cells, RNAi, is induced via short double stranded RNA molecules (siRNA, 21–23nt) generated by a cellular enzyme called Dicer (Dykxhoorn et al. 2003), that, in the presence of endogenous RNA-induced silencing complex (RISC), unwinds and binds to specific sequences of messenger RNA (mRNA) (Doi et al. 2003; Lu et al. 2003). This binding subsequently helps to mediate the destruction of the target mRNA by an endogenous cellular mechanism and elicit highly sequence-specific gene silencing. In this way, RNAi has the potential to inhibit selectively the expression of disease-associated genes in humans. The mechanism of gene suppression was established by Fire et al. (1998) using the 23mer to 25mer nucleotide sequence of the RNA molecule. This discovery was described as the "breakthrough of the year 2002" by Science magazine and also resulted in the award of the Nobel Prize for Medicine or Physiology to Andrew Fire and Craig Mello, in 2006.

More than one-third of the human genome is predicted to be targeted by miRNAs (Lewis et al. 2005), suggesting that numerous genetic networks may also be affected by miRNAs. Small RNAs (siRNA) play an entirely different role in the transcription and expression process for short-term non-viral nucleic acid delivery (Zou et al. 2010). SiRNA are potent and specific inhibitors of gene expression and are being used as a new technology for drug target validation, studying functional genomics, transgenic design and as promising therapeutic agents for diseases with a genetic etiology (Akhtar and Benter 2007b). The field encompassing therapeutic applications of siRNA is versatile and includes siRNAs in therapies for the central nervous system, inflammation or the cardiovascular system, as antiviral and anticancer agents, and in pain research as well as many other therapeutic applications.

Major drawbacks of siRNA include poor pharmacokinetic properties and biological permeability restrictions, off-target effects and interferon response (Dorsett and Tuschl 2004). In particular, siRNAs longer than 30 nucleotides, in specialized highly sensitive cell lines and at high concentrations, lead to activation of the immune system (Sledz et al. 2003). They also have a low transfection efficiency, and poor tissue penetration because of their polyanionic nature (Akhtar and Benter 2007a). SiRNA stability is itself also a major issue as, like most RNA molecules, they are readily degraded by RNAs, which are ubiquitous both in the extracellular and the intracellular space.

The lack of an efficient delivery system to target and deliver the siRNA to the desired cells is a particular limitation to the full therapeutic potential of this approach. When formulated as a delivery system, the stability of the delivery system is another issue, as is avoidance of nonspecific uptake by the reticuloendothelial system (RES), especially Kupffer cells and macrophages. There is a need for suitable carriers that will provide a stable complex and give protection to ensure targeting and delivery of siRNA as well as enabling them to cross the gap between cell culture and animal models to allow efficient siRNA delivery. Most of the current gene therapy approaches makes use of viral vectors due to the high transduction efficiency. However, viral delivery systems require development of technique which could potentially solve the problems of toxicity and the relatively strong host responses resulting from the activation of the human immune system. Multiple non-virally-based delivery methods have been used *in vivo* for delivering siRNA, including hydrodynamic injection, cationic liposome encapsulation, formation of cationic polymer complexes, and antibody-specific targeted delivery systems. Various approaches like lipid nanoparticles, polymeric nanoparticles, antibody conjugates, cyclodextrin nanoparticles (Hu-Lieskovan et al. 2005) and aptamer-siRNA conjugates (McNamara et al. 2006) can be used to knockdown genes.

2. Carrier systems employed to deliver siRNA

Optimal systemic delivery systems for siRNA should be biocompatible, biodegradable, and nonimmunogenic. The systems should provide efficient delivery of siRNA into target cells or tissues with protection of the active double-stranded siRNA products from attack by serum nucleases. Next, the delivery

systems must provide target tissue-specific distribution after systemic administration, avoiding rapid hepatic or renal clearance. Finally, after delivery into target cells via endocytosis, the systems should promote the endosomal release of siRNA into the cytoplasm, allowing the interaction of siRNA with the endogenous RISC (Juliano et al. 2008). To increase stability in the extracellular and intracellular environments, siRNA can be chemically modified by changing the oligo backbone, replacing individual nucleotides with nucleotide analogues or by addition of conjugates to the oligo. Various types of non-viral delivery strategies have been used such as hydrodynamic injection (Liu et al. 1999; Suda et al. 2008), particle bombardment (Belyantseva 2009) and electroporation (Liu and Huang 2002a, b), microinjection to calcium co-precipitation and chemical methods. Cationic polymers, proteins and peptides, and antibody or ligand-targeted conjugate mediated delivery of siRNA has been studied (Brown et al. 2002; Popielarski et al. 2005; Nguyen et al. 2009; Baker 2010; Sioud 2010; Tao et al. 2010).

The siRNA complexes with the delivery (or transfection) reagent are often specifically referred to as lipoplexes, dendriplexes or polyplexes depending on whether the vectors used are cationic lipids, dendrimers (branched polymer-like structures), polymeric micelles, cationic cell penetrating peptides or polymers, respectively.

2.1. Nanoemulsions

Water-in-oil nanoemulsions have been reported to improve absorption of water-soluble peptides but very few reports are available (Constantinides et al. 1996). Wu et al. (2001) describe the preparation of water-in-oil nanoemulsions (<40 nm) containing expression plasmid DNA for topical application. They observed much higher levels of transgene expression for nanoemulsions containing DNA than for those using aqueous DNA at both 24 and 48 h, and they showed no signs of dermal toxicity even after application of four daily doses of the nanoemulsion. Kaneda et al. studied negatively charged perfluorocarbon nanoparticle emulsions ($\sim$296 nm) containing a mixture of lipids They showed nanoparticle drug delivery by association of lipid raft components on the cell membrane allowing internalization and delivery to the cytosol without trafficking to the endoplasmic reticulum, suggesting a categorizing ability of the prepared nanoparticles (Kaneda et al. 2010). Unger et al. (Unger et al. 2001a, b) condensed plasmids into a solid phase which was further suspended within the perfluorocarbon interior phase of nanoemulsions. According to them, perfluorohexane present will be transformed to a gas bubble which will subsequently undergo cavitational activity and thereby enhance the transport of the plasmid into the cells.

2.2. Liposomes

Cationic liposomes comprise one of the most attractive vehicles, owing to their simplicity and reproducibility, liposomes forming complexes with the negatively charged siRNA. These liposomes are widely accepted due to their high transfection efficiency, enhanced pharmacokinetic properties, and relatively low toxicity and immunogenicity. Moreover, cationic liposomes can protect siRNA from enzymatic degradation, and provide reduced renal clearance (Lv et al. 2006). Kabanov et al. (1999) showed that following i.v. administration of plasmid DNA cationic liposomes in mice, 60% of the dose accumulated in the liver and the transgene expression per microgram level of DNA was 1000-fold lower in the liver than in the lung due to the rapid degradation of DNA following phagocytosis of the lipoplex by the Kupffer cells. Encoding a plasmid DNA, (shRNA) into a

Multifunctional Envelope type Nano Device (MEND) showed 96% inhibition of marker luciferase gene expression in the target cells by avoiding RES uptake (Khalil et al. 2007; Nakamura et al. 2007). This effect was due to the detergent-like activity of the PEG-phospholipid conjugate which lyses liposomes at high concentrations. Ion-pair formation (fusogenic property of liposomes and inverted hexagonal phase) as the mechanism by which the cationic lipoplex triggers endosome release has been proposed by Xu and Szoka (1996). The new liposome protamine-HA (haluronic acid) (LPH) nanoparticles significantly reduced serum cytokine levels and showed similar characteristics and siRNA delivery capabilities when compared to the previously developed liposome protamine-DNA (LPD) formulation (Chono et al. 2008). Cationic siRNA lipid delivery via the i.p route showed targeting (knockdown of >75%) to endogenous tissue necrosis factor (TNF-α) (Sorensen et al. 2003). Santel et al. (2006) designed a vasculature endothelium-targeted delivery system for siRNA, using cationic liposomes. Reduction of target protein levels was observed in vascular endothelium of the heart, liver, and lung.

Miyawaki-Shimizu et al. (2006) showed that siRNA encapsulated cationic liposomes (i.v.) can target lung endothelium. In another study (Landen et al. 2005), intraperitoneal injection of neutral liposomes (DOPC) was used to treat ovarian cancer in mice. Sato et al. (2007) studied a galactosylated cationic liposome/siRNA complex (75 nm) for gene silencing of endogenous hepatic gene expression. No liver toxicity was observed for the galactosylated liposome/siRNA complex. Liposome-delivery of siRNAs is still evolving and it could be a promising tool for the therapeutic application of siRNAs (Li et al. 2006). Another report by Miyawaki-Shimizu et al. (2006) showed concentration-dependent down-regulation of caveoline-1 expression in regulating lung vascular permeability in male CD1 mice after injection of cationic liposome–siRNA complexes. Palliser et al. (2006) reported topical vaginal application of siRNA-encapsulated cationic liposomes directed toward herpes simplex virus–specific genes. The result showed sustained gene silencing and protection from lethal herpes simplex virus infection.

2.3. Polymeric nanoparticles

Cationic polymers are generally classified into synthetic and natural polymers, natural polymers being used because of their nontoxic nature, biocompatibility, and biodegradability. Synthetic polymers include branched or linear polyethylenimine (PEI), poly-L-lysine (PLL), and cyclodextrin-based polycations. Natural cationic polymers are exemplified by chitosan, atelocollagen, and cationic polypeptides. The PEIs, a class of extensively used cationic polymers with high transfection efficiency, have a wide range of molecular weights and many amino groups able to be protonated, leading to a high cationic charge density at physiological pH, the problem being their appreciable cytotoxicity (Grayson et al. 2006). PEIs have a buffering capability in the low pH of the endosome, releasing nucleic acids into the cytoplasm. PEI has been shown to induce cell death in a variety of cell lines through cellular mechanisms such as necrosis and apoptosis (Boeckle et al. 2004; Hunter 2006). ICS-283 of Intradigm is a cationic polymer with three major building blocks, including the cationic polymer PEI for interaction with SiRNA, the sterically stabilizing polymer PEG, and the RGD peptide for targeted delivery of siRNA to activated endothelial cells during angiogenesis (Schiffelers and Storm 2006). siRNA was complexed to the PEG-PEI-RGD polymer and administered intravenously (40 µg) to herpes simplex virus I infected mice, and showed a significant inhibitory effect against virus-induced angiogenesis

as well as against the development of the lesions of herpetic stromal keratitis (Kim et al. 2004).

Cyclodextrin-containing polycation nanoparticles self-assemble with siRNA to form colloidal particles (50 nm) (Davis et al. 2004). Moreover, cyclodextrin-containing polycation nanoparticles showed promising safety profiles in a non-human primate study (Heidel et al. 2007). Chitosan nanoparticles have been shown to be effective for *in vivo* delivery of siRNA. Chitosan((1/4) 2-amino-2-deoxy-b-D-glucan) is a non-toxic and biodegradable polymer and can be used for the development of polymer-based siRNA therapeutics (Gary et al. 2007). Chitosan (114 kDa) was complexed with siRNA and administered intranasally each day over 5 consecutive days to endogenously enhanced green fluorescent protein-transgenic mice (Howard et al. 2006). Chitosan/siRNA nanoparticle-mediated knockdown of endogenously enhanced green fluorescent protein was observed in bronchiole epithelial cells. Chitosan-coated polyisohexyl -cyanoacrylate (PIHCA) nanoparticles were used for intratumoral (de Martimprey et al. 2008) and intravenous delivery of RhoA-specific siRNA in mice. PIHCA nanoparticles were found to cause no toxic effects. Compared to controls, mice treated with the nanoparticles showed similar body weight gain, biochemical markers of hepatic, renal, and pancreatic function, and macroscopic appearance of organs (Pille et al. 2006). Other studies have reported the use of atelocollagen for *in vivo* siRNA delivery. Atelocollagen, a purified pepsin-treated type I collagen from calf dermis, is known to cause low immunogenicity owing to the lack of telopeptides. It exists as a liquid at 4 °C, but turns into a gel at 37 °C and has shown to increase cellular uptake, nuclease resistance, and prolonged release of genes and oligonucleotides (Hanai et al. 2006). siRNAs complexed with atelocollagen were administered to mice via intratumoral (Yamato et al. 2008) and intravenous routes (Takeshita et al. 2005). Given the numerous examples of *in vivo* siRNA delivery, the future of polymeric delivery systems seems to be as promising as that of lipid-based delivery systems. However, the major issue in the context of siRNA loaded polymeric nanoparticles is the aggregation that typically occurs due to the surface charge of siRNA loaded nanoparticles (typically net positive). Thus, the most common approach to prevent aggregation is to reduce the surface charge of the nanoparticles by introducing poly(ethylene glycol) (PEG) or sugar molecules (e.g., cyclodextrin), and hyaluronic acid (HA). Charge neutralization has the added benefits of increasing nanoparticle circulation time and allowing for nanoparticle targeting through limiting non-specific interactions between the positively charged nanoparticles and the negatively charged cell membrane (Bartlett et al. 2007; Heidel et al. 2007).

2.4. Lipid nanoparticles

Nanoparticle (NP) formulations allow the addition of targeting ligands to the surfaces of the nanoparticles. Furthermore, large-scale manufacturing of such particles is feasible (Shegokar et al. 2010), and in general, nanoparticles are less toxic than their liposome counterparts, and offer the advantage of being biodegradable (Müller et al. 2011). Engineered cationic lipids can encapsulate negatively charged nucleic acids. Various parameters such as temperature, concentration, charge ratio, and lipid composition may affect the transfection efficiency of these lipid complexes. Numerous cationic lipids generated by combinatorial synthesis have been screened for optimal siRNA delivery (Akinc et al. 2008). Kim et al. reported that the delivery of siRNA using solid lipid nanoparticles resulted in efficient target gene silencing and serum stability, with a minimal level of cytotoxicity (Kim et al. 2008).

Lipoplexes called stabilized nucleic acid particles (SNALP) of DSPC:cholesterol:PEG-C/DMA:DLINDMA have been prepared by an ethanol dialysis method to deliver siRNA [2.5 mg/kg] systemically for silencing the apoB gene in mice and cynomolgus monkeys (MacLachlan et al. 1999; Wheeler et al. 1999; Tam et al. 2000; Cullis 2002; Fenske et al. 2002). PEG-lipids are used not only to stabilize the particle during the formulation process but also to shield the cationic bi-layer, thereby preventing rapid systemic clearance (Leng Q. et al. 2007). Heyes et al. synthesized various cationic lipids, e.g., 1,2-distearyloxy-*N,N*-dimethyl-3-aminopropane (DSDMA), 1,2-dioleyloxy-N,Ndimethyl-3-aminopropane (DODMA), 1,2-dilinoleyloxy-*N,N*-dimethyl-3-aminopropane (DLinDMA) and 1,2-dilinolenyloxy-*N,N*-dimethyl-3-aminopropane (DLenDMA) (Heyes et al. 2005). Mevel et al. synthesized novel cationic lipids comprising cholesteryl-moieties linked to guanidinium functional groups, and cationic lipids comprising a dialkylglycylamide moiety conjugated with a polyamine or a guanidinium functional group. These cationic lipids were formulated into cationic liposomes with the neutral co-lipid dioleoyl-L-α-phosphatidylethanolamine (DOPE) (Ilies et al. 2002; Martin et al. 2005; Miller 2008; Bhattacharya and Bajaj 2009) or with a recently reported neutral lipophosphoramidate derivative of histamine (MM27). Immunolipoplexes with monoclonal antibodies for insulin and ligands for transferrin receptor were found to be efficient for crossing the blood-brain barrier and siRNA delivery to the brain (Boado 2007).

Yoshizawa et al. (2008) prepared folate-linked nanoparticles (NP-F), and evaluated their potential in human nasopharyngeal KB cells, which over expressed folate receptor (FR). NP-F showed a significantly higher intracellular siRNA level and stronger localization of siRNA in the cytoplasm than non-coated nanoparticles (NP-P) and significantly inhibited tumor growth. In *in vivo* tests, only an NP-F nanoplex of Her-2 siRNA significantly inhibited tumor growth of KB xenografts after intratumoral injection, compared with control siRNA (Elbashir et al. 2001). At Mirus Corporation has developed a lipopolyplex transfection reagent called *Trans*IT-TKO®, composed of a charge-dense polycation and a cationic lipid, to deliver siRNA. Charge-dense polymer forms complexes with siRNA that are resistant to disassembly in physiological solutions, including serum. This allows the complexes to be added directly to a cell culture media that contains serum. Li et al. (2009) developed a lipid coated calcium phosphate (LCP) nanoparticle formulation for efficient delivery of siRNA to a xenograft tumor model by intravenous administration. The LCP-NP was further surface modified by post-insertion of polyethylene glycol (PEG) with or without anisamide, a sigma-1 receptor ligand for systemic administration.

2.5. Other approaches

2.5.1. Chemical modification

Chemical modifications of siRNAs that increase their stability include phosphorothioate addition to 39 ends and 29-O-methyl, 29-fluoro and locked nucleic acid–type substitutions to the ribose backbone. Substitution of the $2'$-OH group on the ribose ring with an O-methyl group ($2'$-O-Me), a fluoro ($2'$-F) group, or a 2-methoxyethyl ($2'$-O-MOE) group resulted in prolonged half-lives (due to enhanced binding to serum proteins such as albumin) and RNAi stability of RNAi without affecting efficiency (Braasch et al. 2003). Another modification includes phosphorothioate modification of the RNA backbone (Layzer et al. 2004). Strategies like an enzyme active linker, acid labile cross-linkers (Guo and Szoka 2003), pH-sensitive detergent
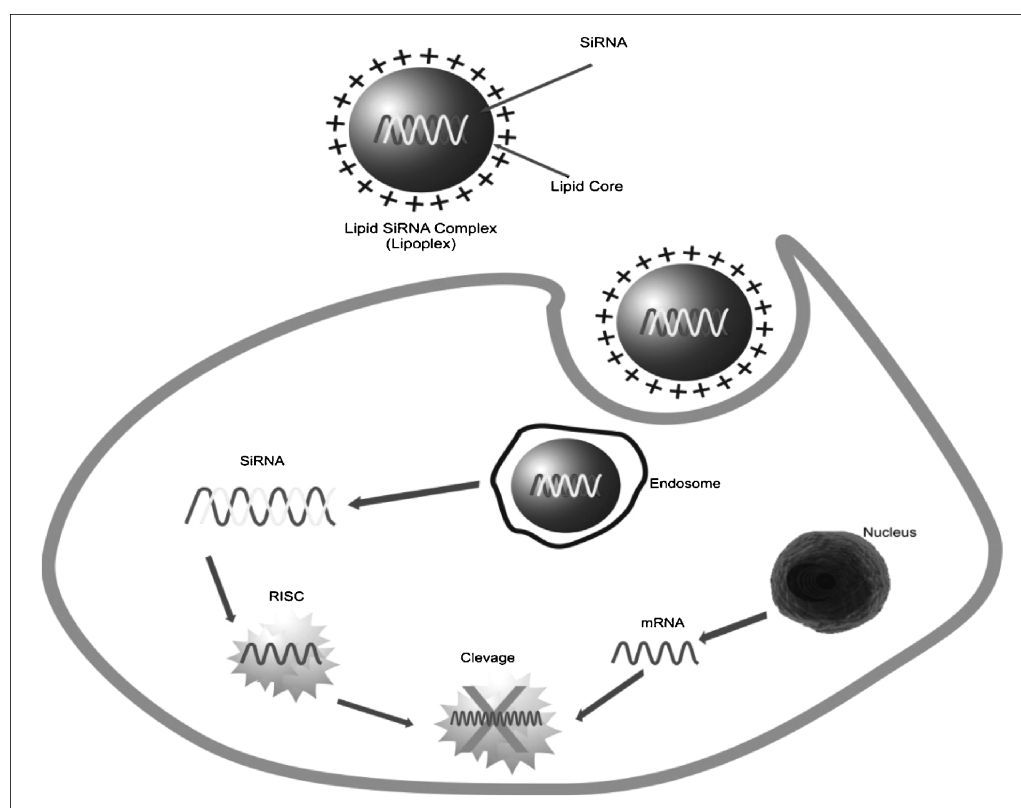
Fig. 1: Mechanism of siRNA uptake using lipid as surrogate vector

(Asokan and Cho 2002), thermal sensitive liposomes (Needham et al. 2000), or a reductive environment sensitive disulfide cross-linker (Austin et al. 2005) have been explored to improve efficient self de-assembly of the nanoparticles.

2.5.2. Surface modified or ligand targeted delivery

Ligand-targeted lipoplexes have been developed using different kinds of ligands such as antibodies, receptors, peptides, vitamins, oligonucleotides, or carbohydrates (Song et al. 2005; Watts et al. 2008; Rao 2010). Attachment of ligands to the nanoparticles was achieved either by directly coupling the

Fig. 2: Status of RNAi candidates in different clinical trials

ligand to the phospholipid or by attaching the ligand to the distal end of the PEG-lipid. Schiffelers et al. (2004) demonstrated efficient systemic delivery of the siRNA to tumor and ocular neovasculature tissue in a herpes simplexvirus (HSV) eye infection model through a Arg-Gly-Asp (RGD)-motif peptide ligand-targeted nanoparticle. siRNAs specific for human immunodeficiency virus (HIV)-1capsid protein gag were complexed to a fusion protein composed of cationic protamine and HIV-1 envelope antibody. Antiviral siRNAs complexed to the antibody conjugate were shown to be delivered to naïve T cells and suppress the replication of HIV in infected mice (Kumar et al. 2008). The surface charge can be masked by covering the vector with hydrophilic polymers (such as (poly)ethyleneglycol (PEG), (poly)hydroxylpropyl methacrylamide (pHPMA) or (poly)vinyl pyrrolidine (PVP)) (Ogris and Wagner 2002) which form a dense, hydrophilic network around the vector and limit hydrophobic or electrostatic interaction with the extracellular medium. The result is a longer circulation time by avoiding MPS-uptake (Gref et al. 1994) and a vector with stability and stealth properties. Other ligands for tumour cells are peptides with an arginine-glycine-aspartic acid (RGD) motif, which facilitate binding between the delivery system and the receptor, situated at the target cell surface, and which can help to internalise the vector with the siRNA.
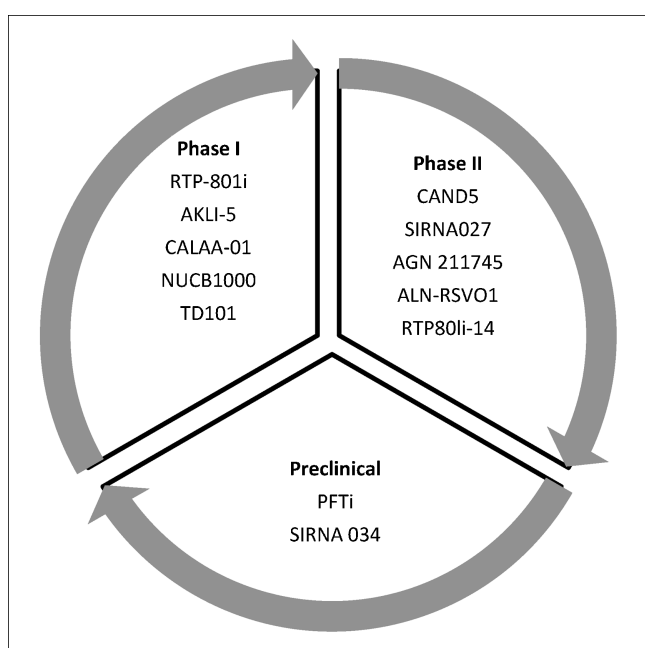
2.6. Present scenario

Most siRNAs in clinical trials are administered by local delivery. Vitravene, a local intravitreal injection for the treatment of cytomegalovirus retinitis, is the only licensed antisense oligonucleotide on the market. Currently, several clinical trials are underway that use siRNAs for treatment of a variety of diseases and disorders, and many more are in the pipeline to commence phase I clinical trials. Sirna Therapeutics is exploring the potential for local delivery of siRNAs to the lung for the treatment of respiratory diseases including asthma, chronic obstructive pulmonary disease (COPD), and viral

infections. In the near future, Alnylam Pharmaceutical's ALN-VSP01 (targeting VEGF and kinesin spindle protein) is scheduled to enter trials for the treatment of liver cancer or solid tumors. Calando Pharmaceuticals have launched a clinical trial for an investigational new drug (IND) CALAA-01 (a transferrin-tagged cyclodextrin polymeric nanoparticle) for targeting the M2 sub unit of ribonucleotide reductase for tumor treatment (U.S. Food and Drug Administration). This phase I trial would be the first clinical trial using a targeted and systemic delivery system of siRNA for cancer treatment.

3. Conclusion

RNAi therapeutics is an emerging field that has quickly established effective gene silencing. Despite promising results, their use is restricted by many drug delivery factors, and a suitable nanotechnology based carrier system needs to be developed. That is why only a very limited number of siRNA delivery systems are in clinical trials. The future prospects depend strongly upon advances in formulation of carrier systems, and the availability and validity of animal model and cell line experimentation.

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