Part 1

Introduction and Basics of RNAi

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Mechanisms and Barriers to RNAi Delivery

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1.1 Introduction

RNA interference (RNAi) is an evolutionarily conserved, endogenous process for post-transcriptional regulation of gene expression. The main classes of small regulatory RNAs that silence target RNAs in a sequence-specific manner include small interfering RNAs (siRNAs) and microRNAs (miRNAs). The first class, siRNAs, are derived from longer double-stranded RNA (dsRNA) molecules (Figure 1.1). The endonuclease Dicer cleaves the dsRNA into 21–25 nt small RNAs, which are incorporated into the RNA-induced silencing complex) (RISC) and then downregulates target mRNA through site-specific cleavage. The second class, miRNAs encoded in the genome, are transcribed from endogenous miRNA genes as primary transcripts (pri-miRNAs), containing ∼65–70 nt stem-loop structures. The mechanism of miRNA-mediated silencing is repression of target mRNA translation accompanied by deadenylation and subsequent degradation of the mRNA targets.

Several years after the revolutionary discovery of RNAi in *Caenorhabditis elegans* by Fire and Mello [1], Elbashir *et al.* successfully demonstrated that a synthetic 21-mer siRNA duplex could induce sequence-specific gene silencing in mammalian cells without triggering interferon responses [2]. Since then, RNAi has captured the attention of scientists and has rapidly become a powerful tool for gene function research as well as an

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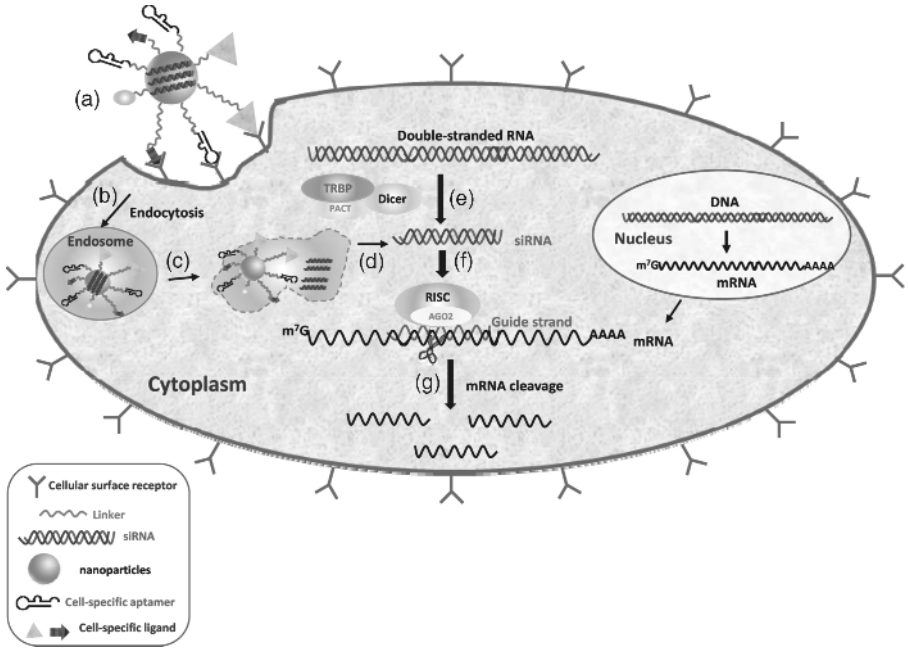

Figure 1.1 *Proposed mechanism for combinatorial targeting for targeted RNAi delivery. (a) Upon binding of the cell-specific aptamer portion or ligands of the nanocarrier-siRNA system to the target receptor on the cell surface, (b) the complex is internalized into cells, probably through an EPR effect (passive targeting) and receptor-mediated endocytosis pathway (active targeting). It is presumed that the complex shuttles into the endosome; (c) subsequently the nanocarrier-siRNA complex escapes from the endosome and then (d) siRNAs are dissociated from the complex and released into the cytoplasm. Or (e) Cytoplasmic double-stranded RNAs (dsRNAs) are processed by a complex consisting of Dicer, TAR RNA-binding protein (TRBP) and protein activator of protein kinase PKR (PACT) into small interfering RNAs (siRNAs). (f) siRNAs are loaded into Argonaute 2 (AGO2) and the RISC. The siRNA guide strand recognizes target sites to direct mRNA cleavage, which is carried out by the catalytic domain of AGO2. (g) siRNAs complementary to promoter regions direct transcriptional gene silencing in the nucleus through chromatin changes involving histone methylation. (Abbreviation: m7G, 7-methylguanylate.) (See color figure in color plate section).*

emerging therapeutic strategy to suppress disease genes, including wet agerelated macular degeneration (AMD), asthma, pancreatic cancer, liver cancer, advanced solid tumor, respiratory syncytial virus (RSV), hepatitis B virus (HBV), HIV-1 etc [3,4]. Over the past few years, a number of preclinical and phase I or II clinical trials on RNAi therapy fueled the excitement for the potential clinical development of RNAi in the treatment of various diseases [5], such as cancer, autoimmune diseases, renal disease and viral infections.

Although conceptually elegant, RNAi as a therapeutic has met some frustrating challenges [6]. The most common method used to harness the RNAi pathway for targeted gene silencing is to transfect synthetic triggers (such as siRNAs or Dicer substrate siRNAs – DsiRNAs) into cells, so here we focus on the mechanism and major barriers to successful use of the siRNA-mediated RNAi pathway. The main barriers have been

(i) inefficient delivery to the correct cells or tissues; (ii) suboptimal gene-silencing activity owing to instability and poor bioavailability of RNAi agents; (iii) toxicity due to off-target effects or immune activation. Amongst these, the most formidable impediment to clinical translation of RNAi is effective delivery of siRNAs to specific cells or tissues at therapeutic doses [7,8]. Proper delivery formulations must therefore be developed to overcome this key hurdle before RNAi-based therapy can be realized eventually as a general approach to treat human diseases [3,9]. In this regard, the advent of versatile nanotechnology platforms is triggering the development of multifunctional delivery formulations for targeted RNAi therapeutics [10,11]. In parallel, extensive efforts have been made to explore the molecular mechanisms of RNAi delivery [12], which might facilitate the clinical development of RNAi-based therapeutics. In this chapter, the mechanisms for, and major barriers to, the achievement of efficient RNAi delivery, and the current status of clinical trials of siRNA therapeutics are introduced. Recent advances in overcoming these challenges by using aptamer-functionalized delivery formulations are discussed.

1.2 Barriers to Systemic RNAi Delivery

The average diameter of a single siRNA duplex is below 10 nm, so nonformulated siRNAs administered into the blood stream, even using stabilizing backbone modifications, result in rapid excretion through renal filtration [7]. Although siRNAs can be administered directly to a target, for many diseases systemic administration is required, which generally requires greater therapeutic doses, leading to higher costs and potentially harmful side effects such as off-targeting and innate immune activation. Off-target effects refers to the potential for siRNAs to silence not only the intended target but also other transcripts. From a therapeutic standpoint, off-target effects can result in toxic effects that limit the clinical development of siRNAs. Various local alignment algorithms (such as BLAST and Smith-Waterman) should already be used to minimize off-targeting potential when designing an siRNA. High-level dosing of siRNAs may also result in competition for the RNAi machinery, which would negatively affect endogenous microRNA function. In addition, before reaching the targeted cells, it is hard for a nonformulated siRNA to pass through the blood-vessel endothelial wall and multiple tissue barriers including liver, kidney and lymphoid organs [12,13]. The blood supply to tissues and organs depends on endothelial cells that form the linings of the blood vessels and regulate exchanges between the bloodstream and the surrounding tissues. The endothelial cells line the entire circulatory system from the heart to the smallest capillaries and have very distinct and unique functions, including fluid filtration and barrier functions. By serving as a semiselective barrier, the endothelium controls the passage and the transit of materials. The kidney is also the body's natural filtration system, which removes waste products like urea and toxins, along with excess fluids, from the bloodstream in the form of urine. As a part of the circulatory system, the lymphatic system is responsible for the removal of interstitial fluid from tissue. These natural barriers and filters prevent a nonformulated, negatively charged nucleic acids from penetrating cellular membranes. Even if entering the cells *via* endoscytosis, siRNAs have to escape from endosomes and subsequently be incorporated into the RISC to trigger the RNAi mechanism [8] (Figure 1.1). In order to achieve efficient RNAi potency, several requirements are crucial, such as (i) optimal

gene-silencing activity without nonspecific immune stimulation or off-target effects; (ii) prolonged circulation time in the body and effective accumulation in the desired target tissues/organs; (iii) selective binding and uptake into target cells; (iv) rapid release into the cytoplasm and effective incorporation to RISC.

1.3 Rational Design to Improve RNAi Efficacy

RNAi is a sequence-specific post-transcriptional gene-silencing process triggered by 21–25 nucleotide siRNAs, which can be chemically synthesized or produced by ribonuclease III Dicer cleavage from longer dsRNAs [1,14] (Figure 1.1e). The cleaved small RNA products possess a 5′-phosphate and two-base 3′-overhangs which facilitate their incorporation into the multi-protein RISC, where one of the two strands is selected as a "guide" for the sequence-specific degradation of the complementary messenger RNA, while the other strand – the "passenger" – is either rejected and/or degraded [15–17]. The selection of the guide strand is determined by the thermodynamic end properties of the siRNA duplex. The thermodynamic stability of the siRNA duplex ends is a major determinant for RISC incorporation efficacy with the 5′ end of the strand having the lowest stability being preferentially loaded [18–20]. The PAZ domain, a single-stranded RNA-binding domain of Dicer and the Argonaute proteins, also specifically recognizes the 3′ two-base overhang, suggesting it can function as a module for anchoring the 3′ end of the guide strand within the RISC [21–23]. For the Dicer substrate duplexes, the 3′ overhang affects the polarity of Dicer entry and hence cleavage as well as subsequent strand selectivity (binding to the RSIC catalytic component Ago2), ultimately influencing overall RNAi efficiency [24,25]. When using chemically synthesized siRNAs, the design of these RNAi triggers must take these factors into account. Moreover, the accessibility of the primary sequence and secondary structure of the targeted mRNA should be considered carefully as well. In order to avoid/minimize unwanted off-target effects, designed siRNA sequences should be screened against a transcriptome database as a safety measure. Sequences can be rationally designed by combining computer algorithms and experimental validation, optimized siRNA [26,27]. There have been several reviews on siRNA design, with in-depth discussions of some optimal design features of siRNA, including length/composition of 3′-terminus, GC content, inverted repeats, and so on [25,28,29].

The main strategy used to harness the RNAi mechanism for targeted gene silencing is to transfect chemically synthesized 21-nt traditional siRNAs or longer, 25–27 nt RNA duplexes that can be processed by Dicer into siRNAs – Dicer substrate siRNA (DsiRNA) – into cells. Previous studies have reported that Dicer substrate duplexes of 25–30 nt can be up to a hundred-fold more potent than conventional 21 nt duplexes for the same target [30,31]. The increased potency might be attributed to the fact that Dicer-generated 21–23 nt siRNAs are more efficiently incorporated into RISC through physical association of Dicer with the Argonaute proteins [32,33]. Since the overall RNAi efficacy of Dicer substrates critically depends on the composition and potency of the Dicer processing products, a random design of Dicer substrates may generate siRNAs with poor RNAi activity. In this regard, dicing patterns should be carefully considered in rational designs of Dicer substrate siRNAs.

Combinatorial siRNA approaches may be desirable for downregulating multiple targets associated with different human diseases. This is especially relevant for antiviral

applications where the viral targets have a high rate of mutation [34]. Contransfection of a mixture of siRNAs is typically used when simultaneous targeting of more than one mRNA is required. However, competition between siRNAs for RISC can occur, resulting in reduced knockdown efficiencies of some of the siRNAs within the mixture. A recent study showed that Dicer-substrate siRNAs (DsiRNAs) have reduced competitive potentials, presumably due to more efficient RISC loading [35]. The Dicer-substrate siRNAs may therefore offer a more efficient way of multiplexing siRNAs for multiple mRNA targets. Dual-targeting Dicer substrate siRNAs allow the use of a single duplex wherein each strand is selected separately into RISC and at least two different targets can be downregulated [36].

1.4 Chemical Modifications to Enhance siRNA Stability and Reduce Immune Response

It is known that siRNAs without some protective backbone modifications are vulnerable to nuclease digestion in biological fluids such as serum. Systemic administration might ultimately result in poor pharmacokinetics (PK) and lack of target gene silencing. Unmodified siRNAs can also be potent triggers of the innate immune response, such as triggering interferon gene-mediated transcriptional upregulation, either through the double-stranded RNA-activated protein kinase (PKR) [37,38] or toll-like receptors (TLRs) [39,40]. In particular, it has been reported previously that siRNAs delivered by liposomes or polyplex reagents can nonspecifically activate inflammatory cytokine production (tumor necrosis factor-alpha, interleukin-6, and interleukin-12) as well as IFN-responsive genes; this, in turn, can trigger cellular toxicity [41,42]. In this regard, chemically modified siRNAs ameliorate these responses and are therefore more suitable for therapeutic application. Precisely placed chemical modifications in siRNAs therefore not only improve serum stability but also decrease TLR responses [43].

Various positions in the siRNA duplexes can be modified to alter the duplex melting temperature, confer resistance to nucleases and inhibit TLR activation without affecting the efficiency of RNAi [44–46]. Some examples of base modifications that can be used are 5-methyuridine, 3-methyuridine, and pesudourine, while examples of backbone modifications include phosphorothioates (PS), peptide nucleic acids (PNA), boranophosphates or the 2′ ribose modifications 2′-O-methyl, 2′-fluoro, and 2′-amino. Even the more conformationally strained locked nucleic acids (LNA) have been functionally incorporated into siRNAs. By selectively modifying 2′-O-methyl uridine or guanosine nucleosides in one strand of the siRNA duplex, the immune stimulation was completely abrogated [44]. However, it must be emphasized that modifications of bases or the ribose backbone are position sensitive and therefore empirical testing must be carried out for each individual siRNA. It has been demonstrated that hypermodification or improper positioning of the modification can compromise RNAi activity and even trigger cellular toxicity [46]. As an example, a siRNA with a boranophosphonate modification at the central position of the antisense strand resulted in improved stability but reduced the silencing potency [47].

1.5 Cellular Uptake and Intracellular Release of siRNA

Although it was recently reported that a minor pathway mediated by fusion between siRNA lipoplexes and the plasma membrane is responsible for functional siRNA delivery,

the major pathway of internalizing a nanocarrier-mediated siRNA delivery system involves two subtypes of endocytosis [12,48,49]: phagocytosis and macropinocytosis. Macropinocytosis usually occurs from highly ruffled regions of the plasma membrane. In this case, the cell membrane forms a pocket and subsequently pinches off the extracellular molecules to form a vesicle. The vesicle subsequently travels into the cytosol and fuses with other vesicles (endosomes and lysosomes). This process allows cells to internalize molecules from the external environment for metabolic purpose, recycling or degradation in lysosomes. Compared to phagocytosis, the macropinocytosis pathway is observed in almost every cell type and in uptake of small sized nanocarriers. In the phagocytosis process cells bind internalized particulates larger than around 0.75 μm in diameter. The composition, surface properties and other characteristics (rigidity and shape) of nanocarriers may affect the phagocytosis pathway of internalization [50].

Following cellular uptake, the endocytic vesicle generated by the nanocarrier-siRNA system travels along microtubules and subsequently fuses with early endosomes, which mature into late endosomes and finally enter into lysosomes, which are the last compartment of the endocytic pathway [48]. The lysosomal environment is acidic (approx. pH 4.8) with a high content of lysosomal membrane proteins and active lysosomal hydrolases, so lysosomes can break down cellular waste products, fats, carbohydrates, proteins and other macromolecules (such as nanocarriers, RNA, DNA) into simple compounds. The endosomal release of the internalized molecules has therefore been identified as a major impediment to achieving effective RNAi activity in siRNA delivery systems [51]. Different strategies have been conducted to improve endosomal escape of siRNA into the cytoplasm. For example, by taking advantage of the "proton-sponge" effect, cationic polymers (such as: poly(ethyleneimine) (PEI) or polycationic dendrimer-mediated delivery of siRNAs) show enhanced transfection efficiency in various cell lines. The "proton-sponge" effect [52,53] of the acidic lysosomal environment can result in a high protonation of amine groups in the PEI or dendrimer vehicles, thereby causing osmotic swelling and vacuole disruption accompanied by cytoplasmic release of the polymer and its siRNA cargo [54]. Moreover, some acid-sensitive components (e.g. endosome-disruptive fusogenic peptides [55,56] and acid-responsive disulfide bonds [57]) have also been incorporated into nanocarriers to accelerate endosomal escape of siRNA and overcome this intracellular hurdle.

1.6 Combinatorial Targeting for Targeted RNAi Delivery

Typically, liposomes, nanoparticles and macromolecular drug molecules with diameters below 400 nm are able to extravasate and accumulate in the "leaky" vasculature of tumor tissue more effectively than in normal tissues, thereby providing a selective targeting of drugs for the tumors and enhancing the therapeutic index [58,59]. This passive leakage phenomenon has been characterized and termed the tumor-selective enhanced permeability and retention (EPR) effect [60,61]. Differing from macromolecular drugs, small molecules, which reach most normal tissues/organs as well as tumor tissues by diffusion-dependent equilibrium, do not discriminate between tumor tissue and normal tissue. Passive targeting relies on a size-flow-tissue filtration that is generally limited to tumors and lymph nodes, so the EPR concept is popularly applied in the design of new anticancer

agents [62], and is important for nanoparticle and liposome delivery to cancer tissue. Similarly, some studies have taken advantage of the EPR effect to achieve passive targeting of siRNA-nanocarriers within the size range optimal for EFP to the human tumor. Currently, various kinds of materials [54,63], including cationic lipids (e.g. liposome, micelle), natural materials (e.g. chitosan, cyclodextrin) or synthetic polymers (e.g. polyethylenimine PEI, dendrimer), cationic polypeptides (e.g. atelocollagen, poly(L-lysine) and inorganic nanoparticles (quantum dots) have been exploited to form nanocarriers for *in vitro* or *in vivo* delivery of siRNAs.

Although EPR-based passive targeting can result in a selective accumulation of drugs in tumor tissue, the low/moderate specificity may not be sufficient to direct the tissue distribution of siRNAs. Decoration of carriers with a ligand specific to the target tissue of interest can be used to promote carrier-specific binding to the cell membrane and facilitate cellular uptake *via* receptor-mediated endocytosis or cellular membrane permeation, thus increasing the local concentration of the drugs in the desired cells or tissues [9]. A wide variety of internalizing molecules such as antibodies, proteins, peptides, folate, carbohydrates, aptamers and other small molecule ligands have been adapted successfully for the targeted delivery [64–66]. For example, siRNAs have been covalently conjugated to a targeting ligand (e.g. cholesterol [45], alpha-tocopherol [67], lipophilic molecules [68,69], short peptides and antibodies [70,71], agonist molecules and nucleic acid-based aptamers [72,73]).

By combining two targeting strategies (passive targeting and active targeting) in one delivery module it may be possible to achieve therapeutic doses of siRNA drugs in the desired cell type, tumor or tissue/organ. Figure 1.1 shows a proposed mechanism for combinatorial targeted RNAi delivery. A precisely engineered nanocarrier system has an appropriate nano-scale size, thereby allowing preferential accumulation in the tumor/organ in the passive target mode. Meanwhile, such a nanocarrier system, functionalized with a cell type-specific internalizing ligand, will selectively bind to surface proteins on the targeted cells *via* the interaction of the ligand and cell-surface receptor, thereby facilitating selective internalization. Small interfering RNAs have been noncovalently assembled with active target recognition moieties and nanocarriers as multifunctional targeting delivery systems, such as a folate conjugated dendrimer [74], folate-conjugated phage RNA [11], a transferrin modified polymer/liposome [75–78], peptide-based nanovectors [79–82], cholesterol polymers [83], antibody-mediated delivery formulations [84–86], aptamer-coated cationic polymers or nanoparticles [87,88], and peptide-functionalized exosome nanovesicles [89].

1.7 Cell-Specific Aptamer-Functionalized Nanocarriers for RNAi Delivery

By functionalizing cell-specific aptamers with therapeutic agents or delivery vehicles, the cellular uptake of the therapeutic agent is enhanced and the local concentration of the therapeutics in the targeted cells or tissues is increased, thereby improving the therapeutic efficacy. Currently, a number of aptamer-functionalized therapeutics have been successfully utilized for targeted delivery [72]. In particular, with the technological maturation and increasing knowledge of RNAi, aptamers and their mechanism of action, it seems natural to partner these two types of therapeutic nucleic acids to expand the options for targeted RNAi delivery.

During chemical synthesis of nucleic acids, various functional groups can be readily incorporated into the aptamers to functionalize them as nanocarriers. For example, synthetic aptamers containing a 5′-NH_2 group can be conjugated with a terminal carboxylic acid on the surface of the nanoparticles using carbodiimide coupling chemistry. Recently, a 5′-NH_2-modified PSMA aptamer was chemically coupled with a branched polyethyleneimine-grafted-polyethylene glycol polymer (PEI-PEG) that serves as a vehicle for siRNA delivery [87].

Anaplastic large cell lymphoma (ALCL) cells, an aggressive T-cell lymphoma, exhibit an abnormal expression of the anaplastic lymphoma kinase (ALK) oncogene and unique surface expression of CD30 [90]. Similar to a CD30-specific antibody, a CD30 RNA aptamer is able to selectively bind to CD30-expressing lymphoma cells. Most recently, a nanocomplex was formulated by incorporating both an ALK siRNA and the CD30 RNA aptamer onto nanosized polyethyleneimine-citrate carriers *via* a noncovalent interaction [91]. Exposure of ALCL cells to this targeted nanocomplex, with a maximum hydrodynamic diameter of ∼140 nm, specifically silenced ALK gene expression and induced growth arrest and apoptosis.

Anti-CD4 RNA aptamers also have been fused into a multifunctional RNA-based nanoparticle for targeted siRNA delivery [92]. As an ideal RNA building block for bottom-up assembly, pRNAs (bacterial virus phi29 DNA packaging RNAs) were covalently fused with different therapeutics agents, targeting ligands or imaging agents (for example: siRNAs, a CD4 specific-aptamer, fluorescent molecules) and subsequently self-assembled into multifunctional nanoparticles, allowing aptamer-directed cell-specific gene silencing and drug tracking. In contrast to the average size of a normal single siRNA molecule that is well below 10 nm, aptamer-functionalized pRNA nanoparticles (dimer or trimer) have a size ranging from 20 to 40 nm, which have the potential to improve circulation time and biodistribution *in vivo*, as well as efficacy in therapeutic applications.

1.8 The Clinical Development and Challenges of siRNAs Therapeutics

Since Song *et al.* demonstrated the first *in vivo* evidence of RNAi-based therapeutic efficacy in a mouse disease model in 2003 [93], siRNAs have been proposed as an attractive therapeutic modality. The first in-human Phase I clinical trial using a targeted nanoparticle system to deliver siRNA to patients was recently conducted and showed direct evidence for siRNA-mediated gene silencing *via* RNAi pathway [94]. This targeted, nanoparticle formulation of an siRNA consists of a cyclodextrin-containing polymer (CDP), a polythethylene glycol (PEG) steric stabilization agent, and human tranferrin (Tf) as a targeting ligand for binding to transferrin receptors (TfR) that are typically upregulated on cancer cells. The four component formulation is self-assembled into nanoparticles in the pharmacy and administered intravenously (IV) to patients. The data demonstrate that siRNA administered systemically in humans can produce specific gene inhibition by an RNAi mechanism of action. So far, over 20 RNAi-based drugs have been evaluated in clinical trials for the treatment of various diseases [3].

Small interfering RNA drugs against the vascular endothelial growth factor (VEGF) gene and the VEGF receptor have been used in preclinical studies to suppress ocular neovascularization and vascular permeability in animal models. The first and most advanced

clinical study was the phase III trial of Bevasiranib, an unmodified siRNA targeting vascular endothelial growth factor (VEGF) [95]. Opko Heath (previous Acuity Pharmaceuticals) announced the first siRNA-related clinical trial in 2004, in which Bevasiranib was given in patients with wet age-related macular degeneration (AMD). Because it was unlikely to achieve its primary endpoint of reducing vision loss, Opko terminated the Phase III clinical trial of bevasiranib on March of 2009. In this case, it was administered as an unmodified siRNA without a delivery formulation that was given by intravitreal injection. Since negatively charged nucleic acids do not readily traverse cellular membranes and are vulnerable to degradation without some protective covering and/or appropriate chemical modifications, such direct administration might ultimately result in poor pharmacokinetics and lack of gene silencing. In addition to Bevasiranib, there are two clinical trials focusing on the intravitreal treatment of AMD: VEGFA165b siRNA against VEGF A165 isoform and Sirna-027 [96] (also known as AGN 211745, a chemically modified siRNA, Allergan Inc.) against a conserved region VEGF receptor-1 mRNA. In the phase I study, a single intravitreal dose of Sirna-027 between 100 μg to 1600 μg was well tolerated in patients. The 84-day follow-up data indicated that 14.4% patients had an improvement in visual acuity. Although a randomized phase II trial was conducted, the study finally failed to meet the efficacy endpoints. Recently, the specificity and mechanism of the anti-VEGF siRNA drugs mentioned above for treating AMD was called into question [97]. This study showed that the siRNA-mediated inhibitory activity of neovascularization may be attributed to a nonspecific immune response associated with activation of the cell surface toll-like receptor 3 (TLR3), rather than to a target sequence-specific interaction. In this regard, careful attention must be to nonspecific activation of the innate immune response receptors such as the TLRs in the future development of siRNA drugs.

RNAi therapy for respiratory syncytial virus (RSV) has rapidly progressed from laboratory investigations to clinical trials. ALN-RSV01, an siRNA targeting a highly conserved region of the mRNA encoding the nucleocapsid (NC) protein of RSV, has been shown to exert a potent antiviral effect against RSV *in vitro* and in murine models by intranasal delivery [98–100]. In 2007 Alynylam Pharmaceuticals started phase II clinical studies for ALN-RSV01. The siRNA was shown to reduce the rate of RSV lung infection in experimentally infected adult patients. In 2011 the first randomized trials of ALN-RSV01 siRNA to treat a naturally occurring RSV infection in lung transplant (LTX) recipients were reported [101]. Aerosolized ALN-RSV01 (0.6 mg/kg) or placebo was administered daily for three days. The 90-day followup results demonstrated that ALN-RSV01 was safe, well tolerated and a significant improvement in symptoms accompanied by a decrease in the incidence of new or progressive bronchiolitis obliterans syndrome (BOS), suggesting it may have beneficial effects for allografts in LTX patients infected with RSV. Due to its high specificity for its target RSV mRNA, ALN-RSV01 did not have unintended off-target adverse effects. In this case, the genetic characteristics and tropism of RSV make it an excellent target for siRNA therapy. The tropism of RSV to the airway epithelial cells allows for direct delivery of the siRNA, so naked, aerosolized ALN-RSV01 can be delivered directly to the airway mucosa *via* a nebulizer device and still remain stable and active. However, siRNA was rapidly degraded upon absorption to the circulatory system. It was found that ALN-RSV01 levels were undetectable or transiently low in the plasma of all of the patients treated with the siRNA drug. Therefore, a proper

delivery formulation for the siRNA would be necessary to enhance the siRNA stability and achieve the maximal cellular uptake and duration of therapeutic efficacy.

1.9 Conclusion and Perspectives

RNAi technology is widely employed for biological applications but is also being harnessed to suppress pathogenic genes for therapeutic applications. Since the first siRNA clinical trials in 2004, siRNAs have been proposed as an attractive treatment for a wide variety of diseases. However, some trials have been put on the back burner or are still in development because of current technical challenges. Safe and efficient RNAi delivery remains a major challenge.

Although the early excitement in siRNA therapy might be tempered by the reality of impediments to clinical development, extensive efforts are being made to surmount the key hurdles to the widespread use of RNAi as a therapy, which most importantly includes development of intelligent delivery formulations for siRNAs. Rational design and precise chemical modifications can improve siRNA potency and stability but these features are not sufficient to solve the major hurdle of targeted systemic delivery. Precisely engineered, multifunctional nanocarriers with combined passive and active targeting capabilities may therefore be the best solution for therapeutic applications. Such carriers could protect siRNAs from serum nucleases, enhance the pharmacokinetics and biodistribution and allow targeted delivery only to the desired cells or tissues. Once inside the cellular environment, these must be engineered to release the siRNA drugs rapidly into the cytosol and achieve RNAi and hence therapeutic efficacy in the absence of nonspecific toxicities. With continued efforts directed towards understanding the cellular mechanisms of uptake and siRNA release into the RISC, RNAi-based therapy should eventually be realized as a general approach to treat human diseases.

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