THERAPEUTIC siRNA: DELIVERY CHALLENGES

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ABSTRACT

Within few years of rnai discovery in 1998, enormous studies have been done in the development of rnai based therapeutic products, bringing them significantly closer to the market. Many big pharmaceutical companies are capitalizing billions on this technology through licensing delivery platforms and strategic alliances with specialized leading players in the market. Favourable results from ongoing clinical trials for the treatment of diseases like age-related macular degeneration, respiratory syncytial virus, cancer as well as rare diseases like pachyonychia congenita reflects the hope for breakthrough in pharmaceutical world. Today the key challenge for rnai application in disease prevention and treatment is the development of clinically suitable, safe and effective delivery carriers. Broadly, sirna delivery has been achieved using both viral and non-viral carriers. However, this review highlights only non-viral delivery strategies and updates its clinical use.

Keywords: *siRNA delivery, age-related macular degeneration, non-viral carrier*

Introduction

Right from the observation of down regulation of pigmentation in petunia flowers in 1990 by Napoli and Jorgensen and then explanation of double stranded RNA (dsRNA) triggers for RNAi in 1998 by Fire and Mello and further efforts for chemical synthesis of dsRNA by Elbashir and colleagues in 2001, the global awareness for RNAi applications in therapeutics has spread widely (1). Since then, an exhaustive research has been done on animals for applying RNAi in disease treatments and now progressing to push short interfering RNA (siRNA) mediated gene silencing into clinical applications for diseases like cancer, viral infections, age related macular degeneration and rare diseases like pachyonychia congenita. Bevasiranib is the first therapy based on the Nobel Prize-winning RNA interference (RNAi) technology to advance to Phase III clinical trials.

A seminal paper published by Fire and Mello provided an explanation for the hypothesis that the trigger for RNAi, a post transcriptional gene silencing, was not single-stranded RNA (ssRNA) but double-stranded RNA (dsRNA). The double stranded RNA precursors get hydrolyzed into short sequences of RNA (short interfering RNA; siRNA) by a cellular RNaseIII endonuclease enzyme called the "Dicer." Small interfering RNA (siRNA) thus formed or exogenously chemically synthesized siRNA can enter the RNA-induced silencing complex (RISC), which is activated upon guide (antisense) strand selection (2). The selection is based on the relative thermodynamic stabilities of the two duplex ends, and it is least stable 5′ end of the duplex that is recognized and asymmetrically unwound by the Piwi-Argonaute-Zwille (PAZ) domain of argonaute 2, a multifunctional protein within the RISC. The incorporated strand acts as a guide for the activated RISC complex to selectively degrade the complementary mRNA almost from the middle of the bound area with siRNA. Other strand of the duplex called sense or passenger strand gets degraded by the enzymes in cytoplasm. The activated RISC complex can then move on to destroy additional mRNA targets, which leads to persistent gene silencing effect for days or weeks depending on the specific mRNA target, the cell type and the organism (3).

RNAi has emerged as an idea of commercial interest in recent years to cash in a novel, quick, and cost-effective way of controlling diseases by knocking down the expression of almost any specific genes. It is a valuable way particularly to accelerate the functional characterization of disease-relevant genes for drug discovery and basic research. Presently the main challenge to harnessing RNAi is its delivery to get enough in the right cells at the right time through an appropriate siRNA design and by tagging it with suitable delivery system. The RNAi response is triggered by the presence of double-stranded RNA

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(dsRNA; over 100 nt) in cells. Due to large molecular weight (~13 kDa) and polyanionic nature (~40 negative phosphate charges) of naked siRNA, it can not easily cross the cell membrane. Thus appropriate delivery carriers are required to navigate siRNA inside the cellular sites of action. Several viral and non-viral strategies have been proposed to improve the delivery of siRNAs expressing vectors and synthetic siRNAs. *In vivo* delivery of siRNA, with the exception of mucosal tissues where siRNA uptake is extremely efficient, remains a major issue for the development of siRNA as small-molecule drugs (4, 5).

Modes of siRNA administration

The size and the charge of siRNA pose major hurdles in its targeting to the desired tissues and transfection (entry into the cells) to elicit gene specific silencing. Local or topical delivery of siRNA through various techniques like hydrodynamic i.v. injection and electroporation possess the advantage of targeting siRNA to the desired tissue with less toxic effects that are faced during systemic delivery (6, 7). Local siRNA delivery has been studied through various routes for many diseases like age related macular degeneration, delivery through nasal and pulmonary route for various respiratory diseases including cystic fibrosis, asthma, influenza, common cold and respiratory syncytial virus, vaginal route to deliver siRNA-microbicide for sexually transmitted diseases, delivery of PEI conjugated siRNA for tumor inhibition via intra-tumoral route and direct subcutaneous injection for cervical cancer etc. Upon systemic delivery, siRNA is passively targeted to the liver, spleen and reticuloendothelial system (8). While targeting, many other tissues require suitably designed delivery system that can overcome the barriers on its way to the destination.

Barriers to systemic siRNA delivery *in vivo*

Upon systemic injection, siRNA needs to cross many steps like escape from the plasma active nucleases, glomerular filtration and uptake by the components of RES to reach the target tissue, then crossing the endothelial barrier of desired cells and entering the cytoplasm and finally endosomal release in the cytoplasm to be available for binding its complementary mRNA for its translational arrest. Unmodified, naked siRNAs are relatively unstable in blood and serum, and have short half-lives in vivo. Suitable chemical modifications to the backbone, base, or sugar of the RNA can be introduced into the RNA duplex structure so as to enhance biological stability without adversely affecting gene-silencing activity. While 'naked', chemically modified siRNA has shown efficacy in certain physiological settings such as the brain and the lung, there are many tissues in the body that require an additional delivery system to facilitate transfection. These can be achieved either by chemical modification of siRNA or by formulating it with a delivery system that not only enhances cell uptake but also affords biological stability for gene-silencing activity sustained for several days (8, 9). First, we will have an overview of siRNA delivery in chemically modified and conjugated form.

Chemical modification and conjugation of siRNA for improved delivery

Many chemical modifications have been shown to improve the stability of siRNA towards nucleases so as to allow the delivery through systemic injection. 2′-*O*-methyl modifications have also been shown to confer resistance to endonuclease activity and to abrogate off-target effects when incorporated into the seed region, which corresponds to nucleotides 2–8 on the antisense strand (9). Further, the introduction of phosphorothioate backbone linkages at the 3′-end of the RNA strands has been shown to reduce susceptibility to exonucleases. Modifications in sugar backbones, for example, 2′ sugar modifications like a fluorine substitution) increased resistance to endonucleases. Besides this, siRNA can also be directly attached to a targeting ligand to increase target-gene knockdown *in vitro*, including membrane-permeant peptides, polyethylene glycol (PEG) and cholesterol-modified siRNAs (9-12). Conjugate siRNA directly to the poly (L-lysine) (PLL) backbone and other end of PLL is conjugated with an endosomolytic peptide which promotes the endosomal release of siRNA. Conjugates of siRNA with monoclonal antibody against the human insulin receptor via a stable avidin-biotin linkage have been reported (13). Several lipid conjugates including lipofectamine, cardiolipin analogues have been successfully used for the delivery of siRNA. Lipid-mediated siRNA transfection is

shown to be at least a thousand fold more efficient than that of naked siRNA due to the fusogenic mechanism of intracellular entry. Moreover, cholesterol conjugated siRNA demonstrated increased binding to serum albumin, resulting in improved bio-distribution to certain targets including liver, heart, kidney and lung tissues. Cholesterol-modified siRNA were capable of silencing apolipoprotein B (ApoB) targets in mouse liver and jejunum, and of ultimately reducing total cholesterol levels (14). Another study by DiFiglia and co-workers details the ability of a cholesterol-modified siRNA to knockdown a gene associated with Huntington's disease. A promising silencing in hepatocytes via the HDl receptor has been observed with bile salt derivatives and longer chain fatty-acid conjugates. Chemical modification of siRNA does not always resolve the delivery issues for majority of the applications. Thus, in many cases it seems necessary to carry siRNA in suitable delivery carriers to shield siRNA degradation and to protect nonspecific delivery.

Synthetic materials for siRNA delivery

Although viral vectors are highly efficient and most powerful tool for gene transfection, these show a limited loading capacity, are difficult to produce in large scale and, most importantly, pose severe safety risks due to their oncogenic potential and their inflammatory and immunogenic effects, which prevent them from repeated administration. Hence, several synthetic non-viral vectors like biodegradable cationic lipids and/or liposomes, cationic polymers, cationic dendrimers (branch-like polymer structures), and cationic cell-penetrating peptides (CPPs) biodegradable lipids and other high molecular weight cations have been developed to offer alternatives to viral vectors for nucleic acid delivery applications, and are carefully formulated to avoid cytotoxicity and stimulation of the immune system. An efficient delivery system can be formulated by designing it with important features like cationic group for ionic interaction with siRNA, Poly(ethylene glycol) (PEG) for steric hindrance, an endosomolytic group for endosomal disruption, and a targeting ligand (polysaccharide, antibody, antibody fragment and peptide) for site-specific delivery. Besides these surface characteristics, size of the final formulation is also an important factor in deciding its biodistribution. Most of these delivery agents and siRNA itself are passively moved towards liver, spleen, lungs, kidney and tumors through leaky vasculature in tumors and thus have been explored for diseases associated with these targets. Controlling the size of these formulations can prevent the glomerular filtration and inclusion of targeting ligand can navigate it to the desired site. Lipoplexes, dendriplexes, and polyplexes, are specifically referred to as siRNA delivery (or transfection) reagent complexes. Alternatively, these cationic lipids and polymers can entrap siRNA within its body to form nanoparticles. Complexes and nanoparticles generally have to be less than 100 nm to avoid renal excretion and be taken up by cells. Once inside cells, the inclusion of fusogenic lipids such as l- dioleoyl phosphatidyl ethanolamine (DOPE) and/or pH-sensitive peptides such as poly-histidine-lysine in siRNA delivery systems are thought to help destabilize endosomal membranes to aid their release into the cytosol. Further surface modification of complexes or nanoparticles with PEG can stabilize nanoparticles and prolong circulation times, and attachment of a targeting ligand or antibody for a cell-specific receptor or antigen can enable tissue or cell type targeting of siRNA.

1. Liposomes, Lipoplexes and denriplexes:

Liposomes consist of an aqueous compartment enclosed in lipid bilayer with hydrophilic drug typically entrapped in the center aqueous layer. The bilayer often contains a lipid component (regularly is a cationic and/or a fusogenic lipid), cholesterol and polyethylene glycol-lipid. The liposomes are particles with stable physicochemical characteristics. In contrast, lipoplexes are spontaneously formed via interaction of positively charged lipids and negatively charged nucleic acids. Lipoplexes should be prepared immediately before use because lipoplexes are unstable. In order to realize this potential the cationic lipids employed must satisfy at least four criteria. First, while a positive charge is required for formulation with nucleic acid-based drugs, the liposomal nanoparticles (LN) formed must have near neutral surface charge in order to avoid immediate uptake into the lymphoid tissue of the reticuloendothelial system. Second, cationic lipids are toxic molecules and must be biodegradable to limit this toxicity.

Third, cationic lipids appear to play an important role in destabilizing biological membranes by combining with anionic lipids to induce non-bilayer structure. Such propensities must be maximized. Finally, in order to be biologically active, the siRNA must dissociate from cationic lipid inside the cell, requiring that anionic lipids are able to compete for associated cationic lipid leaving the free oligonucleotide to exert its biological effect. Lipofectin, RNAifect, Oligofectamine, Lipofectamine and TransIT TKO are commercially available as potential enhancers of siRNA delivery in vitro. DOTAP (*N*-(1-(2,3-dioleoyloxy))-*N*,*N*,*N*trimethyl ammonium propane) and Oligofectamine were some of the first lipid formulations to be used for the in vivo delivery of siRNA and effective gene silencing of TNF-R and -catenin in mice. Although neutral liposomes have been successfully used to deliver siRNA in vivo, cationic liposomes are more commonly used for siRNA delivery. Cationic liposomes termed "solid nucleic acid lipid particles" (SNALPs) that have been stabilized by PEGylation for improved pharmacokinetics have also been successfully used to deliver siRNA in mice and nonhuman primates (15). In monkeys, ApoB were markedly suppressed at a dose of 2.5 mg/kg of SNALP-formulated siRNA. Furthermore, PEGylated liposomes are feasible option for delivering siRNA in humans as these are already approved clinically for doxorubicin. Polycationic dendrimers such as poly (amidoamine) (PAMAM) dendrimers offer many advantages including controllable molecular structure and size, high chemical and structural homogeneity, high ligand and functionality density etc. These characteristics can be explored to tailor a delivery system as per the need to deliver siRNA. These dendrimers bear primary amine groups on their surface, while having tertiary amine groups inside. The primary amine groups present in dendrimers participate in siRNA binding to form nanoscale particles and promote its cellular uptake and facilitate the release from endosomes in cytoplasm by proton sponge act of the buried tertiary amino groups act. Moreover, partially degraded PAMAM dendrimers were reported to have more flexible structures than intact dendrimers and therefore to interact more efficiently with RNA (16).

2. Cationic polymer and peptide delivery systems for siRNA:

Linear or branched cationic polymers including peptides readily bind and condense DNA and have thus been widely used as transfection reagents for genes, oligonucleotides, and now duplex siRNA. The high charge density of cationic polymers allows them to escape from endosomes and deliver their nucleic acid cargo into the cytosol through the so-called "proton-sponge" effect. The polycationic nature of the polymer is thought to buffer low endosomal pH through enhanced influx of protons and water, culminating in endosome rupture. Of the many cationic polymers, polyethyleneimine (PEI) has been widely studied for DNA, oligonucleotide, and siRNA delivery. PEI is available in either linear or branched form and in many molecular weights, ranging from 1 kDa to more than 1,000 kDa. Generally, low molecular- weight PEIs (<25 kDa) with a branched rather than linear architecture are thought to be superior transfection reagents, as higher molecular weights tend to be toxic (17). Now, a novel delivery strategy using PEGylated PEI with an RGD (Arg-Gly-Asp) peptide to deliver siRNA targeting VEGF has been demonstrated inhibit tumor growth and reduce angiogenesis after iv administration (18). The use of polyamidoamine polymers in vivo might be hindered owing to their nonspecific toxicity. Atelocollagen (300 kDa) has been used to administer siRNA systemically and locally in tumor models. In vivo, this polymer was also able to effectively deliver siRNA targeting VEGF to tumor vasculature in an orthotopic model of human testicular cancer, to bone metastases, and to a xenograft model of prostate cancer. A cyclodextrin polycation delivery system is well tolerated, and even repeat doses failed to elicit a significant delivery system-specific antibody response (19). More recently, there was a report that repeated administration of RVG (rabies virus glycoprotein)-9R-bound antiviral siRNA did not induce inflammatory cytokines or antipeptide antibodies (20). The study also reported for the first time that systemically delivered siRNA-peptide conjugates can cross the blood-brain barrier (BBB).

3. Nanoparticles with combination of lipids and peptides

The major components of the delivery systems are cationic lipids and functional peptides and/ or a cationic polypeptide like protamine, which can interact with negatively charged siRNA. Surface steric stabilization is introduced by PEGylation to prevent the aggregation of the resulting complex with serum components. Ligands are attached to the distal end of the PEG chain to increase cellular bioavailability. Cationic lipid is necessary for endosome lysis and intracellular release of siRNA. The mechanism of the endosome membrane destabilization is most likely due to the formation of ion pair complex between the cationic lipid in the nanoparticles and the negatively charged anionic lipids in the endosome membrane, as hypothesized by Xu and Szoka (21, 22). Also, a short amphipathic peptide, MPG, that is able to form stable nanoparticles with siRNA can enter the cell independently of the endosomal pathway and can efficiently deliver siRNA in a fully biologically active form into a variety of cell lines and *in vivo* (22). Another study in March 2009 demonstrated a novel lipid-polycation-DNA (LPD) nanoparticles containing DOTAP targeted with polyethylene glycol (PEG) tethered with anisamide (AA) to specifically deliver siRNA to H460 human lung carcinoma cells which express the sigma receptor. This specific nanoparticles formulation of siRNA prepared with new cationic lipid DSGLA, targets lung tumor cells and plays both roles of a delivery component and a therapeutic agent (23).

Many big pharma companies are licencing FDA approved delivery platforms to specialized leading players in the field of RNAi therapeutics. Recently, in Jan 2009, Abbott Laboratories has licensed Liquidia Technologies' PRINT nanoparticles technology for the delivery of siRNA-based therapeutics, which has the potential to improve the company's cancer portfolio. The two companies will collaborate on the development of the technology, which allows researchers to create nanoparticles of defined size, shape, surface chemistry and composition. Abbott will be using the technology to develop delivery methods for small interfering RNA, which has so far proved problematic for researchers. Another biotechnology company MDRNA is focused on the development and commercialization of therapeutic products based on RNA interference (RNAi). The DiLA2 Platform is MDRNA's proprietary platform for creating novel lipids from amino acids. The platform enables MDRNA to tailor the charge, linker and acyl chains in order to optimize the liposome for delivery to the target tissue of interest. In addition, the platform is designed to permit attachment of various peptides to improve a variety of delivery characteristics including nanoparticles formulation, cellular uptake, endosomal release and cell/tissue targeting. Such strategic alliances and plannings have accelerated the entry of siRNA based formulations into clinical trials.

Clinical update of siRNA

Lots of clinical trials are ongoing and are planned for taking siRNA into the clinic in the treatment of important diseases such as macular degeneration, cancer, hepatitis, inflammatory, respiratory diseases and skin diseases like pachyonychia congenita through various systemic and local routes like skin and intranasal. Table 1 updates the clinical involvement of various pharmaceutical, biotechnological companies and universities leading to direct different siRNA formulations towards FDA approvals.

Bevasiranib is a first-in-class small interfering RNA (siRNA) drug developed by Acuity pharma and later licenced by Opko healthcare, designed to silence the genes that produce vascular endothelial growth factor (VEGF), believed to be largely responsible for the vision loss of wet AMD. Although, Opko Health announced in March 2009 to terminate phase 3 trial of bevasiranib for treating wet age-related macular degeneration due to disappointing preliminary results, yet indications of activity are encouraging with no systemic or ocular safety issues. Thus company remains committed to the continued development of siRNA portfolio targeting vascular endothelial growth factor, including recently announced VEGFA165b sparing siRNA.

Future directions and conclusion

The pace of siRNAs revolution as robust and gene specific drug development tool is astounding. Tremendous efforts have been put in by research scientists in appropriate designing of siRNA by critical screening of gene libraries and improvements in its delivery constructs. Appreciating these inputs there are judicious hopes to see this wonderful new therapeutic tool as gene

Table 1: Current clinical status of siRNA therapeutics

S.No.	Product details	Company & strategic alliances	Drug target/ tissue	Indication	Delivery method	Status
1.	siRNA Cand5/ Bevasiranib	Acuity/ later licenced by Opko	VEGF/ Eye	Age related macular degeneration (AMD)	Naked siRNA, Intravitreal injections	Terminated at Phase-III
			VEGF/ Eye	Diabetic retinopathy	Naked siRNA, Intravitreal injections	
2.	Sirna-027/ Now AGN-745	Sirna Therapeutics/ Later acquired by Allergan	VEGF/ Eye	AMD	Naked siRNA, Intravitreal injections	Terminated at Phase II
3.	RTP-801i	Silence Therapeutics's (formerly Atugen) AtuRNAi technology sublicensed to Pfizer via Quark's license	VEGF/ Eye	AMD	Naked siRNA, Intravitreal injections	Phase II
4.	Atu027		Targets PKN3 molecule in cancer cells	Cancer	siRNA incorporated in AtuPLEX delivery platform, Intravenous	Phase I
5.	AKIi-5		P53 gene/ Kidney	acute kidney injury in kidney transplantation	Chemically modified siRNA with AtuRNAi technology, intravenous	Phase I/II
6.	DGFi			delayed graft function in kidney transplantation		Phase I/II
7	ALN-RSV01	Alnylam Pharmaceuticals	RSV nucleocapsid / Lungs	Respiratory Syncytial Virus (RSV) infection	Naked siRNA, intranasal	Phase III
8	ALN-VSP	Alnylam Pharmaceuticals	kinesin spindle protein(KSP) and VEGF/ Liver	Liver cancer	Two siRNA molecules formulated in lipid nanoparticles, intravenous	Phase I
9	NUC B1000	Nucleonics	4 HBV genes/ Liver	Hepatitis B antiviral agent	Plasmid DNA formulated in cationic lipid delivery system, Intravenous	Phase I
10	TD101	TransDerm (Santa Cruz, CA)	Targets the N171K mutant form of the gene/ Skin	Pachyonychia congenita	Two delivery methods: 1. Soluble Tip Microneedle Array 2. Topical gene cream, lipid based technology	Phase Ib
11	CALAA-01	Calando Pharmaceuticals	M2 subunit of ribonucleotide reductase/ solid tumors	Anticancer	RONDEL (RNAi/Oligonucleotide Nanoparticle Delivery/ Intravenous	PhaseI
12	MDR-03030	MDRNA Inc.	Targets conserved region of the influenza viral genome	Acts on influenza viral genome its ability to mutate around the compound	Combined UsiRNAs with DiLA2 delivery platform, Intranasal	Preclinical phase

specific drug therapy for impossible disease treatments like diabetes, cancer and HIV. Looking further, the entry of modified siRNA formulations and to some extent synthetic nano-particles composed of polymers, lipids, lipidoids or conjugates in clinics have attracted the interest of many pharmaceutical and biotechnological companies to extract billions of monetary benefits out of it. Focus of current research should be towards refining safe and effective development of siRNA delivery systems to ensure FDA approvals through key efficacy in clinical trials. I wish young pharmaceutical scientists to adopt the intricacies, bring India in forefront of siRNA delivery research, and provide cure/prevention of otherwise untreatable diseases or diseases of masses in India.

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