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RNAi therapeutics: An update on delivery

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RNA interference (RNAi) has rapidly advanced from a laboratory observation into a major area of research within biology and medicine. RNAi is triggered by short interfering RNAs (siRNAs) of between 19 and 21 nucleotides in length, which induces the targeted cleavage of mRNA with sequences of homology to the siRNA. Because of its high degree of specificity and efficacy, the potential for RNAi-based therapeutics was recognized at an early stage. However, development of RNAi-based agents has been hindered because siRNAs are unstable in serum and delivery across the cell membrane is highly inefficient. Numerous methods have been developed to facilitate delivery of RNAi in animals and patients, each with their own set of advantages and disadvantages. This review discusses publications between 2005 to 2007 in the area of RNAi delivery, with a particular focus on in vivo application and clinical trials.

Keywords Gene therapy, liposome, RNAi, RNAi delivery, RNAi therapeutic, shRNA, siRNA, viral vector

Introduction

The phenomenon of RNA interference (RNAi) began with the seminal publication in 1998 by Andrew Fire, Craig Mello and coworkers, who were the first to elucidate requirements for the structure and delivery of short interfering RNAs (siRNAs) to induce gene knockdown in the nematode *Caenorhabditis elegans* [1••]. In 2001, Thomas Tuschl *et al* described the structural requirements for the siRNAs that trigger RNAi in mammalian cells, which consist of between 19 and 21 nucleotides (19 to 21mer) with 2 nucleotide overhangs on each 3' end [2••,3].

RNAi can be initiated by direct delivery of siRNAs into the target cell [2••] or by short hairpin RNAs (shRNAs), which are transcribed from DNA-based plasmids and are processed into siRNAs in the cytoplasm by Dicer RNase III (Dicer), a protein required for miRNA function [4,5••]. Although direct delivery of siRNAs is simple and usually produces potent gene silencing the effect is transient, whereas DNA-based strategies offer the potential of long-term silencing [4,6••].

Since its discovery, RNAi has quickly evolved into a highly useful tool for molecular biology research. It has also become the focal point of a race for a new class of biotechnological agents, RNAi-based therapeutics. After Tuschl and coworkers had described the basic structure required for an active siRNA in mammalian cells, early research on RNAi focused on predicting active sequences, as not every 21mer is equally active within a particular gene [7•]. This was determined by computing algorithms that utilized data from large screening trials [8•]. Such algorithms are now freely available to predict siRNAs with high activity [9,10]. However, it has now been recognized that the major challenges for the development of RNAi-based therapeutics are administration and delivery.

Delivery of siRNA refers to three different, although interdependent phenomena: (i) stability of siRNA – in serum, siRNAs exhibit low stability because of the presence of nucleases, which minimize the half-life in serum to less than 15 min [11••]; (ii) cell penetration – because of the polar nature of siRNA molecules, they cannot be easily taken up through the lipophilic cell membrane; and (iii) tissue targeting, which would allow systemic administration of the therapeutic with preferential distribution to the tissue or organ of interest.

While these three areas are interconnected and many studies address more than one of these problems, no clear 'front runner' has emerged so far from the various proposed delivery solutions; a number of excellent reviews have been published on the topic of RNAi delivery [12,13]. In this review, RNAi delivery methods that have been proposed or validated between 2005 to 2007 are summarized, with a focus on those with potential as clinically useful carriers. This review highlights the most important developments in the area of RNAi delivery *in vivo*, and aims to assist in the selection of appropriate delivery methods for specific disease indications or target organs.

Delivery methods for RNAi

RNAi delivery *in vivo* is an exciting and fast-moving field of research that is expected to be beneficial in therapeutic applications in the near-term. RNAi treatments are already

entering various stages of clinical testing. However, a variety of methods may be utilized for treating different disease indications and target tissues and it is highly likely that different solutions will be developed for different diseases and organs [14]. The majority of the ongoing clinical trials are utilizing unmodified or minimally chemically modified siRNAs, which are injected or inhaled directly into target organs (see Table 1).

However, targeted delivery of siRNA into parenchymal organs or solid tumors after systemic application is more challenging than local injection, mainly because of the rapid elimination of siRNAs from the blood stream by degradation in the serum and renal filtration, and an efficient delivery method to achieve this has yet to be developed [15]. Success in safety and efficacy in such an application would be considered a major milestone for the field of RNAi therapeutics. Besides delivery to target tissues localized in the gastrointestinal (GI) tract, systemic delivery of siRNA through oral application remains a challenge [16•].

This review discusses studies in which siRNA was administered by direct injection or inhalation into the target tissues, mainly in instances where there is a direct path for clinical development. Such applications were initially utilized by researchers in the field, including companies such as Allergan Inc and Alnylam Pharmaceuticals Inc (see Table 1), but it is doubtful that RNAi-based therapeutics will ultimately be limited to these 'direct delivery' approaches, which are unlikely to develop into broad applications for clinical treatment. For this reason, a large number of studies that reported direction of injection of siRNAs into the target tissues (for example, tumors and brain) are not discussed in this review. The current methods for RNAi delivery are summarized in the following sections.

Viral delivery

Fewer studies on the use of viral vectors for RNAi delivery have been published between 2005 to 2007, compared with 2002 to 2005 [14]. Among these is a study that utilized virosomes derived from influenza as a means of delivering siRNA *in vivo* [17]. Virosomes, which are empty influenza virus envelopes, were complexed with cationic lipids for protection from nuclease activity and, following intraperitoneal injection, were demonstrated to successfully encapsulate siRNA duplexes and deliver them to cells in the peritoneal cavity of mice [17]. Virosomes may also serve as an effective means to target cells of the respiratory epithelium and cells that possess Fc receptors, such as dendritic-cells, macrophages and natural-killer cells [17].

Viral vectors were also utilized for *ex vivo* treatment of blood progenitor cells [18]. This method was used to suppress the expression of the HIV human co-receptor chemokine (C-C motif) receptor 5 (*huCCR5*) to prevent HIV penetration into lymphocytes [18]. CD34+ cells from rhesus monkeys (harvested after cytokine mobilization) were treated with lentiviral vectors expressing siRNAs directed at rhesus-CCR5, which were re-infused after the donor monkeys had undergone myeloablative treatment [18]. Following this treatment, lymphocytes expressing siRNAs against rhesus-CCR5 persisted for 14 months after transplantation and downregulation of CCR5 was observed [18]. Similar

Table 1. RNAi therapeutics: Delivery methods in clinical trials.

Compound	Developing company	Clinical phase	Study population	Indication	Route of administration and target tissue	Reference
Bevasiranib sodium (Cand5)	Opko Health Inc	III	Patients with AMD	AMD	Intravitreal injection (eye)	[94-96]
AGN-211745 (Sirna-027)	Allergan Inc	II	Patients with AMD	AMD and CNV infection	Intravitreal injection (eye)	[97]
ALN-RSV01	Alnylam Pharmaceuticals Inc	II	Adult volunteers	RSV infection	Intranasal (respiratory tract)	[98]
Bevasiranib sodium (Cand5)	Opko Health Inc	II	Patients with DME	DME	Intravitreal injection (eye)	[99]
AKIi-5 (I-5NP)	Quark Pharmaceuticals Inc	I	Patients with AKI after cardiac bypass surgery	AKI/ARF	Intravenous injection (systemic)	[100]
NUCB-1000	Nucleonics Inc	I	Patients with HBV	Chronic HBV infection	Intravenous injection (systemic)	[84]
RTP-801i-14 (REDD-14-NP)	Pfizer Inc	I	Patients with CNV secondary to AMD	AMD	Intravitreal injection (eye)	[101]
BLT-HIV (rHIV7-shl-TAR-CCR5RZ)	Benitec Ltd	I	Patients with AIDS-related lymphoma	HIV infection	Lentiviral (*ex vivo*)	[87]
TD-101	TransDerm Inc	I	Patients with PC	PC (orphan status)	Intradermal injection (skin)	[89]

AKI acute kidney injury, **AMD** age-related macular degeneration, **ARF** acute renal failure, **CNV** choroidal neovascularization, **DME** diabetic macular edema, **PC** pachyonychia congenital, **RSV** respiratory syncytial virus.

technology (BLT-HIV/rHIV7-shI-TAR-CCR5RZ; Benitec Ltd) is undergoing tests in a clinical trial (see Table 1).

Despite initial promise as a vehicle for the delivery of RNAi into live animals, viral vectors no longer appear to be the tool of choice. It is unclear why this is the tendency, although it may be because of adverse effects observed in viral vector gene therapy trials [19••], and RNAi-induced hepatotoxicity and deaths in mice after treatment with virally-delivered shRNA [20••]. Interestingly, the reason for the observed hepatotoxicity, which included death through liver failure, was presumed to be competition of the highly expressed shRNA with the endogenous miRNAs for access to the RNAi machinery (see section on liver targeting).

Liposomal delivery

A major focus in the development of RNAi therapeutics are liposomal formulations for siRNA delivery to improve efficacy and safety. Numerous commercial and academic institutions are in the process of improving liposomal delivery. Silence Therapeutics plc (formerly Atugen AG) are developing a novel class of lipid delivery vehicles using 'AtuPLEX', a mixture of cationic and fusogenic lipids complexed with negatively charged siRNAs, to promote uptake of siRNAs into endothelial cells of blood vessels in the liver and tumors after systemic administration [21,22]. Intravenous injections of siRNA-AtuPLEX that targeted CD31/platelet endothelial cell adhesion molecule 1, a gene whose expression is localized to endothelial tissue, inhibited tumor growth and suppressed metastases in a PC-3 prostate xenograft model [21,22]. Repeated systemic administration of anti-CD31 siRNA-AtuPLEXes did not induce toxicity and could potentially be an effective gene target for RNAi-based antiangiogenic therapeutic interventions [21,22]. Silence Therapeutics emphasizes that this formulation would allow lyophilization for storage and shipping and that the siRNA can be resuspended without loss of activity [23]

Protiva Biotherapeutics Inc and Alnylam have utilized stable nucleic acid lipid particle (SNALP)-formulated siRNAs [24••]. SNALPs are specialized lipid nanoparticles that encapsulate siRNAs. They are coated with a diffusible PEG-lipid conjugate which stabilizes the particle during formulation by providing a neutral hydrophilic exterior and prevents rapid systemic clearance *in vivo* by shielding the cationic bilayer; therefore, SNALPs facilitate the cellular uptake and endosomal release of the particle's siRNA payload [25]. These studies showed for the first time that a systemically delivered RNAi therapeutic can potently silence an endogenous disease-causing gene in a clinically relevant manner in non-human primates [24••]. In addition, Alnylam is exploring a library of 'lipidoids' or lipid-like molecules developed by Robert Langer and Daniel Anderson, to develop siRNA therapeutics for two different disease targets in the liver: hypercholesterolemia and liver cancer [26].

Nanoparticle delivery

In order to overcome the limitations of difficult-to-transfect cells such as lymphocytes, numerous nanoparticle designs have been proposed and developed. Targeted nanoparticles were used successfully to deliver therapeutic siRNAs and some of these methods offer the advantage of tissue-targeting by utilizing surface markers such as antibodies or antibody fragments [27••]. Since primary lymphocytes are highly resistant to transfection using conventional transfection reagents (eg, cationic lipids and polymers), Peer *et al* have utilized antibody-protamine fusion bodies [28] and antibody-targeted liposomes [29], which target human integrin lymphocyte function-associated antigen-1 (LFA-1) expressed on all leukocytes. This fusion protein was constructed from an antibody capable of specifically recognizing activation-dependent conformational changes in LFA-1 and successfully targeted siRNA delivery to activated leukocytes in a SCID mouse model [28].

Calando Pharmaceuticals Inc has developed a cationic cyclodextrin-containing polymer that binds siRNAs for systemic delivery [30]. The delivery system consists of two parts: (i) the first component is a linear, cyclodextrin-containing polycation that, when mixed with siRNA, binds to the anionic backbone of the siRNA; (ii) the polymer and the siRNA then self-assemble into nanoparticles of approximately 50 nm in diameter [30]. The siRNA inside the particle is protected against nuclease degradation in serum and, additionally, the surface of the particles contain stabilizing agents and can also be modified by targeting ligands [30].

Direct administration into target organs

In ongoing clinical trials of RNAi therapeutics, direct application of modified or unmodified siRNAs into the organ of interest, for example, injection into the eye (Allergen Inc and Opko Health Inc), into the skin (TransDerm Inc) or by intranasal delivery (Alnylam) are the leading methods of administration (see Table 1). However, these delivery methods may be limited to a few indications in a small number of accessible organs such as the lung, eye and skin.

Other delivery methods

Several other delivery methods do not fall into any of the above named categories, but have shown promise for the therapeutic delivery of RNAi. These include extracorporeal, *ex vivo* treatments (for example, for autologous stem cells) [31], transkingdom RNAi (*tk*RNAi) which uses live bacteria to deliver shRNA [16•], gene-therapy based approaches that use eukaryotic transcription plasmids to produce shRNA inside of the target cells [4], and delivery of siRNA precursors (Dicer substrates) [32,33].

Injection of unformulated RNA

In contrast to the earlier years of RNAi research, a limited number of studies have reported efficacy of non-formulated ('naked') siRNAs after systemic injection between 2005 and 2007. This may be because efficacy is difficult to quantify as siRNAs are rapidly degraded in the blood stream by nucleases [11••,15].

Delivery methods for RNAi in individual organs

Respiratory tract

For indications in which endogenous genes (for example, growth factor genes, oncogenes and genes related to angiogenesis) are targeted, the level of gene suppression by RNAi might have to be titrated to avoid undesirable physiological effects caused by the complete suppression of a gene [34]. However, RNAi seems ideally suited to combat viral infections, since a complete suppression of viral genes is desired. Administration of unformulated siRNAs can also produce an IFN response through the recognition of dsRNA by TLRs located on endosomal membranes of the cell, in a manner that depends both on the target cell type and the sequence of the siRNA used [35,36]; for viral indications a certain level of IFN response may be acceptable as it might contribute to helping the immune system overcome the viral infection [37].

Progress has been made in the administration of unformulated and unmodified siRNAs to the respiratory epithelium by inhalation. Delivery of siRNAs to the lung was demonstrated using a variety of formulations from polymers to unformulated, 'naked' siRNAs, and were shown to be effective in combating viral respiratory infections caused by, for example, the parainfluenza virus (PIV) [38], respiratory syncytial virus (RSV) [38], and severe acute respiratory syndrome (SARS) [39] in mouse and monkey models.

Intranasal administration of siRNAs targeting RSV and PIV viral genes, unformulated or complexed with the cationic polymer transfection reagent, *Trans*IT-TKO, at a dose of 5 nmol or approximately 3.5 mg/kg, significantly reduced pulmonary RSV and PIV titers by more than 99% in mice with no signs of IFN induction [38]. RSV-induced disease symptoms such as respiratory distress, pulmonary inflammation and leukotriene induction were also lowered following local siRNA administration [38].

In a non-human primate model, siRNAs formulated in a 5% dextrose solution and targeting SARS coronavirus (SCV) were effective in suppressing SARS-like symptoms when delivered intranasally [39]. A reduction in SCV-induced SARS pathogenesis such as a reduction in fever, alveoli damage and a diminished presence of SCV RNA in the lung were observed following treatment and showed that intranasal siRNA delivery is a promising method for the treatment of respiratory viral diseases in humans [39]. It may also offer great potential as a prophylactic therapeutic because of its highly specific and efficacious viral inhibition at low doses. A clinical phase I trial is ongoing to test a nebulized non-modified antiviral siRNA targeting RSV (see Table 1).

Inhalation of RNAi-based agents would be particularly attractive as a prophylactic if they were formulated to protect against a range of the most relevant respiratory infections, for example, for patients using a mechanical respirator, in the setting of an intensive-care unit. The above mentioned study by Bitko *et al* also investigated if a combination of siRNAs could be useful against both RSV and PIV in a mixed infection; however, an excess of one siRNA moderated the inhibitory effect of the other, suggesting that both RNAis were competing for the RNAi machinery [38].

Eye

Direct, local delivery of RNAi to the target site is advantageous because a lower dose of siRNA is required to produce maximum efficacy and can mitigate any unwanted systemic side effects. Historically, ophthalmological application of "naked" siRNA has gained special consideration in the field of RNAi therapeutics, as this was the first disease area in which RNAi-based therapeutics were introduced into clinical trials for the treatment of age-related macular degeneration (AMD) [40]. For example, local delivery to the eye was successfully demonstrated in mouse and monkey models of ocular neovascularization [41-44]. Direct intravitreal injection of an siRNA against *VEGF* formulated in PBS reduced neovascularization in laser-induced models of AMD in mouse and non-human primates [41,42] and an siRNA directed against VEGF receptor (*VEGFR*)*1*, formulated in PBS and administered by intravitreal and periocular injections, was also effective in reducing neovascularization from 45 to 66% in a laser-induced choroidal neovascularization (CNV) murine model [43]. The siRNA was still present in retinal cells on day 5 following injection [43]. Furthermore, Cashman *et al* demonstrated therapeutic effects of shRNA-induced silencing of *VEGF* (84% reduction in neovascularization) in a mouse model of CNV [44].

Brain

The brain and other regions of the central nervous system have been targeted by local viral delivery of shRNA or intraventricular or intrathecal injections of siRNAs to the target site. For example, intrathecal injection for CNS delivery was shown to be useful for the management of pain [45]. In addition, local injection of an siRNA into the ventral tegmental and substantia nigra areas of the brain of adult wild-type or dopamine transporter knockout mice led to approximately 40% gene silencing of dopamine transporter (DAT) expression in the cell bodies of dopaminergic neurons [46]. Moreover, direct administration of unmodified siRNAs in buffered isotonic saline by intraventricular infusion was also shown to be effective in reducing the expression of both the enhanced GFP reporter gene and the endogenous *DAT* gene in mice [34]. However, the silencing effect was most pronounced in regions adjacent to the injection site and this approach required high amounts of siRNA [34].

Delivery across the blood-brain barrier is a challenge that has to be addressed if effective therapeutics to treat a multitude of central nervous system indications, such as Alzheimer's or Parkinson’s diseases, are to be developed. The use of targeted delivery vehicles, such as mAb-targeted liposomes, in which targeting antibodies are attached to the surface of pegylated liposomes, were developed to overcome this obstacle [47]. Using this approach, Zhang *et al* intravenously injected RNAi expression plasmids that were encapsulated in the interior of 85 nm pegylated immunoliposomes (PILs), and significant levels of gene silencing in the brain were observed [48]. Specifically, adult rats with luciferase-

expressing intracranial brain cancer were administered a single, intravenous injection of 10 μg of plasmid DNA per animal expressing anti-luciferase shRNA [48]. A 90% silencing of the luciferase gene expressed in the brain tumor was observed for at least 5 days. In a separate study by the same researchers, the lifespan of mice with intracranial human brain cancer was extended by approximately 90% following weekly intravenous injections of PILs carrying plasmid DNA that expressed shRNA against the human EGF receptor [49].

GI tract

The environment inside the GI tract is extremely unfavorable to protein- and oligonucleotide-based therapeutics. The GI tract can be considered as an exposed body surface, which interacts with an 'external' environment contained in the food. Digestive enzymes, denaturing pH values, a protective mucus layer, as well as the GI local flora all make this organ a difficult target for biological therapies.

However, in some cases direct application of siRNAs in combination with Lipofectamine transfection reagent were found to be effective in mouse models of disease [50]. For example, intrarectal administration of an siRNA complexed with Lipofectamine and directed against *TNFα*, was successful in alleviating inflammatory bowel disease symptoms by a reduction of TNFα and colonic inflammation [50].

Xiang *et al* proposed an alternative approach to deliver RNAi to the intestinal mucosa based on the use of live bacteria [16•]. Since bacteria (prokaryotes) express and deliver mediators of RNAi that target cells of mammalian organisms (eukaryotes), this concept was called *tk*RNAi. In a proof-of-concept study, noninvasive and non-pathogenic *Escherichia coli* was engineered to express shRNA [16•]. The *E coli* was also engineered to express two proteins: invasin and listeriolysin. Invasin is derived from the invasive enteropathogen, *Yersinia pseudotuberculosis*, and was expressed on their surface; it triggers uptake into the epithelial mucosa of the GI tract after contact with β-integrins present on the surface of epithelial cells [51]. After internalization, *tk*RNAi bacteria were degraded inside the host cell endosome and released the second component of the invasion mechanism, listeriolysin. This protein is derived from the invasive enteropathogen, *Listeria monocytogenes* and is a major virulence factor that allows *L monocytogenes* to escape from phagocytotic vesicles to the cytoplasm [52••]. *tk*RNAi is under development by Cequent Pharmaceuticals and the first clinical trials are anticipated to begin in late 2008 [53].

Genito-urinary tract

Multiple diseases and indications in the genitourinary tract could potentially benefit from RNAi-based treatments. In a proof-of concept study for RNAi-based agents as local antiviral microbicides, a lipid-formulated siRNA was shown to be effective in targeting the mucosal surfaces of the genitourinary tract [54••]. Intravaginal administration of the siRNA complexed with Oligofectamine transfection reagent and targeting HSV-2, protected mice from infection when administered before or after a lethal HSV-2 challenge [54••]. The lipid-formulated siRNA was taken up by the epithelial and lamina propria cells and reduced HSV-2 gene expression for 9 days [54••]. Intravaginal delivery of siRNA was advantageous since it did not induce inflammation or cause systemic silencing in distant organs [54••]. This observation may open the possibility of using local administration of siRNA-agents to protect against numerous sexually transmitted diseases, including HCV and HIV.

Specific tissue-targeting was achieved using the prostate-specific membrane antigen (PSMA), which is overexpressed on the cell surface of prostate cancer cells and tumor vascular endothelium [55]. McNamara *et al* linked an siRNA against polo-like kinase 1 gene with PSMA targeting aptamers to achieve targeted delivery into prostate cancer cells [55]. Repetitive treatment with targeted siRNA (intratumoral injection) produced significant growth reduction and tumor shrinkage in a prostate cancer xenograft model [55].

In addition, *Salmonella typhimurium* was previously shown to retard established tumor growth and has been investigated in clinical trials [56-58]. For example, bioengineered attenuated strains of *S typhimurium* were found to accumulate preferentially (> 1000-fold) in tumors compared with normal tissues after intravenous injection [56]. In a variation of the bacterial delivery method by Xiang *et al* [16•], Zhang *et al* investigated the potential of *S typhimurium* to deliver RNAi into solid tumors [59]. Following treatment with *S typhimurium* carrying the eukaryotic expression plasmid pSilencer combined with siRNA against signal transducer and activator of transcription 3 (*stat-3*), significant tumor growth inhibition was observed in a mouse model [59]. The researchers concluded that combination treatment with attenuated *S typhimurium* and a *stat-3*-specific siRNA can induce an enhanced antitumor effect [59].

Liver

Modulation of targets in the liver by siRNA treatment have provided important successes [60-63]. The liver seems an ideal target for an evolving technology such as RNAi therapeutics, as it has an active filter function and can therefore be targeted easily through intravenous injection [64,65]. In addition, the liver has numerous attractive treatment targets, including key genes involved in glucose and lipid metabolism. Other targets in the liver include infectious diseases, such as HCV and HBV. Furthermore, the liver is also an important location for metastasis. Chemically modified siRNAs [11••,66], as well as liposomal formulations, particularly SNALPs [24••,67], have played a major role in many of the successes of liver targeting. Cholesterol-bound siRNA was one of the earliest successes for systemically administered siRNA and targeted the endogenous gene apolipoprotein B (*apoB*) in the jejunum and liver [11••]. In an extension of this research, Wolfrum *et al* utilized stabilized siRNAs conjugated to various lipophilic conjugates, including several fatty and bile acids [68]. Importantly, LDL-bound siRNAs were mainly taken up by the liver and those bound to HDL were taken up by various tissues, including adrenal, ovary, kidney and liver tissues [68].

Kupffer cells are the primary source of TNFα (a proinflammatory cytokine) in the liver following exposure to LPS [69••]. Rats challenged with LPS and treated with liposomes encapsulating siRNA to target *TNFα*, showed an inhibition of TNFα secretion of over 60% in comparison to LPS challenge alone [70]. This method could be useful to treat numerous diseases, such as alcoholic liver disease, septic shock, and rheumatoid arthritis, in which a catastrophic phenotype is caused by the body's inflammatory response rather than the initial injury.

Merck & Co Inc (formerly Sirna) has successfully targeted HBV infection using a novel class of synthetic siRNAs; these are chemically modified to resist degradation by nucleases for prolonged stability by substituting 2'-OH residues for 2'-fluoro, 2'-*O*-methyl, or 2'-deoxy sugars [71]. This technology was combined with the above-mentioned delivery tool, SNALP [67] and was shown to be an effective method for administering RNAi-based therapeutics, since it conferred robust and persistent *in vivo* activity at a reduced dosing level and frequency [24••,67,69••].

SNALPs were also utilized by Alnylam to encapsulate siRNA targeting *apoB* in mice and non-human primates [24••]. ApoB is an essential protein required for the transport and metabolism of cholesterol, as well as for the assembly and secretion of very LDLs and LDLs. *apoB*, which is predominantly expressed in the liver and jejunum, was silenced by over 90% in the liver in cynomolgus monkeys following a single intravenous injection 48 h after administration [24••]. SNALP-siRNA targeting *apoB* significantly reduced ApoB protein, serum cholesterol and LDL levels that led to a specific, dose-dependent and long-lasting effect observed up to 11 days after treatment [24••].

In a study to evaluate the delivery efficacy of hepatic RNAi using adeno-associated vector type 8, Grimm *et al* noted hepatic toxicity and deaths in mice at high levels of siRNA [20••]. Toxicity was found to be correlated with reduced levels of natural miRNAs and is presumed to be caused by competition for the siRNA/miRNA pathway machinery [20••]. In the second part of this study, toxicity was avoided and therapeutic effects could be observed against HBV when shRNA expression levels were controlled below a certain threshold [20••].

This issue of liver toxicity through competitive inhibition of the miRNA pathway was also investigated in a study by Alnylam [66]. Hepatocyte-specific genes, including *apoB* and Factor VII (*FVII*) were targeted by siRNAs in the mouse and hamster [66]. siRNAs were formulated in liposomal nanoparticles and administered by a single intravenous bolus injection [66]. High doses of si-*apoB* and si*FVII* (5mg/kg) achieved high levels of gene silencing (78 ± 3% and 83 ± 2%, respectively, 2 days after administration), with no significant differences in miRNA levels [66]. This data was interpreted as reassuring and in support of the safety of RNAi therapeutics based on synthetic siRNAs.

Overview of current clinical trials with RNAi therapeutics

The use of RNAi as a tool for drug development has progressed significantly from initial *in vitro* studies. Data from previous and ongoing preclinical programs have suggested that RNAi therapeutics have huge potential. These preclinical studies have led to ongoing clinical programs at various stages; many with positive preliminary data (see Table 1).

Alnylam has focused on developing RNAi-based therapeutics targeting RSV infection in the lungs. In a phase I clinical trial, ALN-RSV01, a naked, unmodified siRNA against the mRNA encoding the nucleocapsid protein of RSV that is essential for viral replication, was administered intranasally to evaluate safety and pharmacology [72]. In a follow-up phase I clinical trial, this agent was administered via inhalation using a nebulizer [73]. Single and multiple doses of ALN-RSV01 were evaluated ranging from 0.1 to 3 mg/kg and 0.01 to 0.6 mg/kg, administered once daily for 3 days in the multidose arm; no severe or serious adverse events were observed [73]. Interestingly, the delivery efficiency of inhaled ALN-RSV01 was significantly greater in humans than observed in preclinical models [73]. In a phase II trial, healthy adults were infected with a wild-type strain of RSV [74]. ALN-RSV01 was administered intranasally for five consecutive days – two days prior and three days after viral inoculation; treatment showed statistically significant antiviral activity [74]. Alnylam has announced plans to conduct another phase II trial with naturally infected adult patients [74].

Intravitreal injection of siRNAs offers the advantage that this ocular compartment is low in endonuclease activity [12] and has previously proved successful for antisense oligonucleotide treatment [75]. Building on the experiences gained with oligonucleotides, such as Vitravene (Fomivirsen) [75] and the aptamer Macugen [76], companies including Opko (formerly Acuity Pharmaceuticals), Allergan Inc and Merck (formerly Sirna) targeted either *VEGF* or VEGF receptor (*VEGFR)* genes, which are believed to be mainly responsible for vision loss in patients with wet AMD [77]. Animal studies have demonstrated that VEGF was necessary and sufficient for the formation of new blood vessels [43]. The validation of the *VEGF* gene and its essential role in the development of ocular neovascularization has rapidly advanced competing RNAi-based therapies [77]. For example, in clinical trials either the unmodified siRNA, bevasiranib sodium (Cand5; Opko Health Inc) [78] or the chemically modified siRNA, AGN-211745 (Sirna-027; Allergan Inc) were injected directly into the eye [79]. Positive data have been obtained so far; AGN-211745 was well tolerated and patients have experienced visual acuity stabilization [79].

Bevasiranib, is the first-ever siRNA agent to enter phase III clinical trials [78]. Bevasiranib, which was designed to silence the *VEGF* gene, will be compared with ranibizumab (Lucentis), a humanized anti-VEGF-A antibody fragment approved by the FDA [78]. The study will determine equivalent efficacy of bevasiranib administered every 8 to 12 weeks versus the administration of ranibizumab every

4 weeks. In another ophthalmological indication, Pfizer Inc (under license from Quark Pharmaceuticals Inc) is developing an siRNA therapeutic candidate (RTP-802i-14) to inhibit expression of the hypoxia-inducible gene *RTP801*, inhibition of which leads to a reduction of CNV [80].

In November 2007, Quark announced another breakthrough development in the field with the first systemic dosing of an RNAi therapeutic in humans [81]. Quark licensed the AtuRNAi structure from Silence Therapeutics [82] and has developed AKIi-5, which targets the p53 tumor suppressor gene. AKIi-5, a chemically modified siRNA, is intended for the treatment of acute kidney injury (AKI), also known as acute renal failure (ARF) [83]. The p53 gene plays an important role in ARF by inducing tubular cell death (apoptosis) in response to injury and the aim of treatment is to temporarily inhibit p53 in acute settings such as AKI, to delay apoptosis and thereby allow natural repair mechanisms to restore cellular integrity [83]. Preclinical studies were conducted in rats and non-human primates. Rats treated with a single bolus injection of AKIi-5 were significantly protected from ischemia/reperfusion-induced AKI [83]. The pharmacokinetic, distribution, and toxicity studies in rats and monkeys demonstrated that AKIi-5 had a favorable safety profile and a short residence time in the kidney [83]. In an ongoing phase I clinical trial, AKIi-5 was administered intravenously as a single dose to patients undergoing major cardiac surgery [83].

Nucleonics Inc has initiated a phase I clinical trial of the systemically administrated RNAi-based therapeutic NUCB-1000 for the potential treatment of HBV infection [84]. NUCB-1000 consists of a plasmid DNA construct designed to produce four different shRNAs, targeting different sequences of the HBV genome, under the control of an RNA polymerase III promoter [84]. The plasmid DNA was formulated with a proprietary cationic-lipid delivery system [85].

Another antiviral strategy that has received much attention is currently under development in a phase I clinical trial for the potential treatment of HIV [86,87]. In this approach, CD34+ cells were collected from patients after induction and treated *ex vivo*. A mixture of shRNA, ribozyme and RNA decoy targeting three different HIV-related genes: trans-activator of transcription/regulator of virion (*tat/rev*), *CCR5*, and transactivation-response genes, respectively, were delivered by a lentiviral vector [88]. The trial combined gene therapy with RNAi, and involved transfecting CD34+ hematopoietic progenitor cells *ex vivo* and then returning the cells to the patients; RNAi treatment thereby influences all the CD34+ hematopoietic progenitor cells' future progeny, including T-cells. This represents an important trial since it is the first in which a lentivirus was used as a vector in combination with DNA-directed shRNAs. Preclinical safety and efficacy parameters were encouraging and the pluripotent precursor cells were able to differentiate normally after lentiviral transduction [87].

TransDerm Inc became the latest RNAi therapeutic developer to transition siRNA therapeutics into the clinic. TD-101 is designed to inhibit target gene expression in the skin for the treatment of pachyonychia congenita (PC) [89]. PC is a rare dominant-negative epithelial fragility disorder (with 500 patients worldwide) caused by the mutation of a keratin gene [90]. Single-nucleotide specific siRNAs targeting the mutant keratin have been shown to reverse the aggregation phenotype *in vitro* and *in vivo* [91,92]. In a phase I clinical trial, unmodified siRNA was injected intradermally [89]. A topical delivery technology called gene creme for TD-101 is in development [93].

Conclusion

The field of RNAi has evolved rapidly and the technology has transitioned from *in vitro* cell culture verification to advanced clinical trials within just six years. RNAi holds great promise for the treatment of a range of human diseases such as macular degeneration, hepatitis infection, Huntington's disease, HIV, respiratory infections and cancer. Early delivery approaches are currently under development in clinical trials and the need for new and improved delivery vehicles still exists. It is our prediction that various different delivery technologies are expected to evolve, to co-exist depending on the specific challenges posed by each disease area and target organ. Many researchers are investigating innovative solutions for delivery of RNAi therapeutics and to maximize safety and efficacy.

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