Published in final edited form as:
Biotechnol J. 2011 September ; 6(9): 1130–1146. doi:10.1002/biot.201100054.

Current Progress of siRNA/shRNA Therapeutics in Clinical Trials

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Abstract

Through a mechanism known as RNA interference (RNAi), small interfering RNA (siRNA) molecules can target complementary mRNA strands for degradation, thus specifically inhibiting gene expression. The ability of siRNAs to inhibit gene expression offers a mechanism that can be exploited for novel therapeutics. Indeed, over the past decade, at least 21 siRNA therapeutics have been developed for more than a dozen diseases, including various cancers, viruses, and genetic disorders. Like other biological drugs, RNAi-based therapeutics often require a delivery vehicle to transport them to the targeted cells. Thus, the clinical advancement of numerous siRNA drugs has relied on the development of siRNA carriers including biodegradable nanoparticles, lipids, bacteria, and attenuated viruses. Most therapies permit systemic delivery of the siRNA drug, while others use *ex vivo* delivery by autologous cell therapy. For some of the drugs, advancements in bioengineering and nanotechnology have led to improved control of delivery and release of the siRNA. Likewise, progress in molecular biology has allowed for improved design of the siRNA molecules. Here, we provide an overview of siRNA therapeutics in clinical trials, including their clinical progress, the challenges they have encountered, and the future they hold in the treatment of human diseases.

Keywords

RNAi; siRNA; shRNA; clinical trial; delivery

1 Introduction

Over the past two decades, advancements in biological drugs have generated a variety of therapeutics, including the clinical applications of recombinant proteins, monoclonal antibodies, nucleic acid-based aptamers, ribozymes, and many others. Like these biological drugs, RNA interference (RNAi)-based therapeutics have emerged for the treatment of cancer, infectious diseases, and other diseases associated with specific gene disorders. Since the discovery of RNAi, there have been more than 30 clinical trials involving 21 different biological drugs known as small-interfering RNAs (siRNAs) or the precursors to siRNA known as short-hairpin RNAs (shRNAs). In this review we will discuss siRNA/shRNA therapeutics under clinical investigation, including the diseases they target, the method of delivery, and the progress and challenges surrounding these therapeutics.

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Conflict of Interest
JJR is a co-founder of Dicerna Pharmaceuticals, an RNAi company.

To date, 14 diseases have been targeted in these clinical trials, with numerous more siRNA therapeutics in preclinical development. The advancement delivery methods for siRNA drugs continues, as researchers aim to maximize the specificity of the siRNA delivery while minimizing toxicity and degradation effects that compromise drug efficacy [1]. Strategies exist using synthetic materials or natural carriers (viruses and bacteria), and the preferred method typically depends on the application, the required duration of the therapeutic, and the targeted tissue. Synthetic carriers deliver synthesized siRNAs to targeted cells, while bacterial and viral carriers are typically engineered to express shRNAs that undergo intracellular processing and become functional siRNAs (Figure 1). Bacterial and viral carriers of shRNA are often delivered to target cells *ex vivo*, and these modified cells are then re-infused back into the patient. Systemic delivery methods that often require injection of the siRNA into the bloodstream are being tested for siRNA delivery to the kidney, liver, and some solid tumors. Similarly, the RNAi drug can be injected directly into the desired tissue or tumor to release the drug with the degradation of a biodegradable carrier. Tissues such as the eye, lung, skin are suitable for topical delivery siRNA [2].

2 *Ex vivo* delivery

Three clinical trials have used *ex vivo* delivery of the siRNA/shRNA therapeutic, in which cells are removed from patients and modified with siRNAs/shRNAs and reimplanted/ reinfused into the patient. This procedure, known as autologous cell therapy, has been used to deliver lentiviral-delivered shRNA for the treatment of HIV/AIDS, an anti-tumor bifunctional siRNA (bi-shRNA), and siRNA for the treatment of metastatic melanoma.

2.1 Lentivirus transduced autologous CD34+ cells for HIV/AIDS

In collaboration with Benitec (Melbourne, Australia), the City of Hope (Duarte, California, USA), conducted a human pilot feasibility study that employs RNA-based gene therapy to inhibit HIV infection and replication. This trial was designed for patients undergoing transplantation for AIDS-related non-Hodgkin's lymphoma (NHL), as all patients in the trial received autologous CD34+ hematopoietic progenitor cells (HPC) [3]. Four of these patients also received autologous HPCs transformed to express three RNA-based anti-HIV components (pHIV7-shI-TAR-CCR5RZ): a shRNA targeting the *tat/rev* viral mRNA, a decoy of the TAR mRNA hairpin, and a ribozyme that targets chemokine receptor 5 (CCR5).

CCR5 is an endogenous chemokine receptor on CD4+ T cells that serves as a co-receptor for HIV-1 infection. This receptor is an ideal target for anti-HIV therapeutics, as it is not essential for normal T-cell function and offers an endogenous target that, unlike viral targets, is not prone to mutational escape [4]. Tat and Rev are two early viral proteins from spliced mRNA transcripts that enhance viral replication. Tat protein recruits cellular factors, including P-TEFb, to the transactivating region (TAR) hairpin at the 5' end of viral mRNA transcripts to enhance the processivity of RNA Polymerase II (RNAPII). Rev protein mediates nuclear export of unspliced viral mRNA transcripts. Thus, siRNA-knockdown of *tat* and *rev* mRNAs are designed to inhibit viral replication at two distinct early steps. Similarly, the TAR decoy is a RNA hairpin designed to bind Tat protein within the cellular nucleolus, thus sequestering Tat away from the TAR hairpin on nascent viral transcripts. The processed *tat/rev* siRNA recognize viral mRNA transcripts within the first exons of both *tat* and *rev* reading frames. However, since the overlapping *tat* and *rev* reading frames are frame-shifted, possible mutations of the viral sequence would alter two viral factors and escape could not occur simply by silent mutations at the codon wobble base.

A replication incompetent lentivirus, which genetically encodes all RNA components, was transduced into the autologous HPCs *ex vivo*. Within the modified lentiviral genome, the

shRNA and TAR decoy are each transcribed from separate human U6 RNA polymerase III (U6 pol III) promoters, while expression of the CCR5 ribozyme is driven by the adenoviral VA1 pol III promoter.

Patients underwent chemotherapy-based conditioning prior to transplantation, which enhances the engraftment of the transduced HPCs [5]. Since patients in this trial required treatment of NHL in addition to HIV, autologous stem cell therapy offered a potential therapy for both diseases. In other studies, the same research team demonstrated that HIV-positive and HIV-negative patients with NHL have similar levels of nonrelapse mortality, two-year disease-free survival, and overall survival after autologous stem cell transplant with unmodified cells [6, 7]. Thus, HIV-positive NHL patients are strong candidates for anti-HIV, RNA-based autologous stem cell therapy [3].

In the pilot feasibility study, five NHL patients mobilized sufficient HPCs by apheresis (2.5×10^6 CD34+ cells per kilogram of weight). This particular study required two separate HPC collections for reinfusion, including one as an unmanipulated therapeutic fraction (HPC-A-Rx) and the other as anti-HIV experimental fraction (HPC-A-Exp) [3]. One of the five patients was able to mobilize sufficient APCs for only one of the two apheresis collections, and was thus assigned as a HPC-A-Rx only control. The other four patients received transplantations of both untransduced and transduced HPC fractions.

The patients tolerated the therapy well and observed toxicities were strictly procedure-related events typical of HCT. No short-term toxicity was associated with the genetically modified HPCs. To evaluate long-term toxicity, the researchers analyzed the persistence of genetically modified cells over time. Two patients exhibited persistent expression of the *tat/rev* shRNA up to 18 and 24 months, respectively, suggesting that the expression of the shRNA had no detrimental effect on the survival of peripheral blood cells. Likewise, genetic marking of gene modified peripheral blood cells revealed normal development of T cells, B cells, and granulocytes, indicating that the lentiviral vector did not perturb differentiation of HPCs [3]. Interestingly, the levels of gene marking increased in two patients after increases of viremia. A logical explanation of these observations is that increased viral loads deplete the population of unprotected cells, while protected cells undergo selective expansion. Based upon the results of the first trial, a second 5 patient trial is planned 2011 for this same AIDS/lymphoma patient population.

2.2 Autologous tumor cells for cancer vaccine

Gradalis Inc. (Carrollton, Texas, USA) developed a therapeutic known as FANG™ Vaccine as a potential treatment for advanced solid cancers, including stage IIIc ovarian cancer. FANG™ Vaccine, currently in the recruiting stages for Phase I and Phase II trials, expresses both recombinant human granulocyte-macrophage colony stimulating factor (GM-CSF) and Furin bifunctional shRNA (bi-shRNA^{furin}) [8]. The bi-shRNA^{furin} consists of two stem loop structures with miR-30a backbone. The first stem loop has complete complementary guide and passenger strands while the second has mismatches at position 9, 10, and 11 of the passenger strand. Both stem loops target the same sequence. These bi-shRNAs are capable of triggering both cleavage-dependent and cleavage–independent RISC-mediated inhibition of target mRNA and hence show increased target gene knockdown [9].

Furin is a proprotein converstase which activates TGFβ1 and TGFβ2, thus the bi-shRNA is designed to indirectly reduce levels of these TGFβ isoforms. Many tumors including colon, pancreatic, breast, bladder, and lung produce high levels of TGFβ isoforms [10–13], while tumor progression and poor prognosis have also been correlated to high levels of TGFβ [14, 15]. TGFβ inhibits GM-CSF-induced maturation of bone-marrow-derived dendritic cells (DCs) as well as expression of the major histocompatibility complex II (MHC II) and co-

stimulatory molecules [16]. This leads to T-cell unresponsiveness by antigen presentation of immature DCs [17]. Furthermore, TGFβ inhibits the activation of macrophages and their antigen presenting function [18–20]. Thus, the down-regulation of TGFβ1 and β2 plus the expression of GM-CSF is designed to enhance the immune reaction towards the tumors and help the body to kill tumor cells evading the immune system. Patient tumor samples treated with the FANG™ Vaccine by *in vitro* electroporation result in a 5-fold decrease of TGFβ1 coupled with reduced Furin and increased GM-CSF [8].

In both Phase I and II clinical trials, patient tumor cells are collected and electroporated with the FANG™ Vaccine (http://www.gradalisinc.com/). The cells are then incubated for 24h to ensure the expression of the GM-CSF and the bi-shRNA^{Furin}. One day after transfection the cells are harvested, enumerated, and irradiated at 10,000 cGy and frozen until used. The irradiation arrests the cell proliferation to ensure no new tumor growth when injected back into the patient. In the Phase I clinical trial 19 patients have received intradermal injection of the vaccine. According to Gradalis Inc. co-founder Dr. John Nemunaitis, preliminary data indicate no significant toxicity and a subset of patients demonstrated partial remission (PR) or stable disease (SD) after 4 months (personal communication, October 1, 2010). As of February 2011, enrollment began for Phase II trial that will evaluate the efficacy and safety of the FANG™ Vaccine for patients with stage IIIC ovarian cancer (http://clinicaltrials.gov/ct2/show/NCT01309230).

2.3 Autologous dendritic cells for metastatic melanoma

Duke University (Durham, North Carolina, USA) is conducting a Phase I study with a siRNA drug to treat metastatic melanoma, a form of cancer that originates in melanocytes. The World Health Organization (WHO) reports that this disease accounts for about 48,000 deaths annually. In this study patients are treated with autologous dendritic cells transfected with siRNA and an mRNA that encodes for a tumor antigen. The goal of this siRNA therapy is to induce an anti-melanoma immune response by alteration of the proteasome-mediated antigen processing [21, 22]. Monocytes of patients are harvested and transfected *ex vivo* with siRNAs that target inducible immunoproteasome subunits LMP2, LMP7 and MECL1. These cells then undergo differentiation into DCs *in vitro*. After maturation the DCs are transfected with mRNAs encoding melanoma antigens MART, MAGE-3, gp100, and tyrosinase. The siRNA-induced knockdown of the proteasome is thought to enhance the melanoma antigen presentation by the DCs with the aim to provoke a strong immune response against the melanoma cells in these patients. Hence, cancerous cells will be killed by the patient's own immune system.

According to investigator Dr. Scott Pruitt from Duke University, 10 patients have been enrolled with none to date experiencing any toxicity from tumor antigen RNA-transfected DCs derived from siRNA-transfected monocytes (personal communication, October 19, 2010). Moreover, he reports that antigen specific immune responses have been detected in all subjects by IFN-gamma Elispot assays of primary blood lypmphocytes. The status of this clinical trial is still recruiting and no preliminary results have been published.

3. Intravenous (IV) injection of siRNA therapeutics

To date nine clinically tested siRNA therapeutics require intravenous (IV) injection for drug administration. Eight of these use a synthetic carrier for the siRNA payload. Among these are five that use a cationic liposome technology, including four that use the stable nucleic-acid lipid particle (SNALP) carrier developed by Tekmira and one that uses a siRNA-lipoplex known as AtuPLEX™. In contrast to cationic liposomal carriers, one siRNA from the University of Duisburg-Essen is incorporated into an anionic liposome. Two siRNA drugs use polymeric carriers, including one that uses a cyclodextrin nanoparticle from

Calando and another than uses a biodegradable polymeric matrix known as LODER. Finally, Quark's I5NP is administered as a naked siRNA, as it passes through the bloodstream to the liver without a carrier.

3.1 Stable nucleic acid-lipid particle (SNALP) carrier of siRNA

Tekmira Pharmaceuticals Corporation (Burnaby, British Columbia, Canada) developed a siRNA therapeutic for the treatment of hypercholesterolemia known as TKM-ApoB (PRO-040201). TKM-ApoB is a systemically delivered RNAi therapeutic incorporated into a first generation lipid nanoparticle, known as stable nucleic acid-lipid particle (SNALP). The siRNA drug is designed to specifically knockdown expression of ApoB, which is the main apolipoprotein of low density lipoproteins (LDL) and its precursor, very low density lipoprotein (VLDL). As LDL transports cholesterol and triglycerides through the blood stream, ApoB facilitates the uptake of LDL into various cell types and tissues [1]. Thus, inhibition of ApoB by siRNA knockdown is designed to reduce LDL cholesterol levels in hypercholesterolemia patients. A Phase I, placebo-controlled, single-blind, single-ascending dose clinical study was designed to assess the safety, tolerability, pharmacokinetics, and pharmacodynamics of TKM-ApoB in hypercholesterolemia patients (http://clinicaltrials.gov/ct2/show/NCT00927459). Of the 23 subjects, 17 received treatment with a single dose IV infusion of the TKM-ApoB at seven different dosing levels. The remaining six subjects received the placebo control. The drug was well tolerated with no evidence of liver toxicity. One of the two subjects at the highest dosage level reported flu-like symptoms that were consistent with immune system stimulation by the siRNA payload (http://www.tekmirapharm.com/Programs/Products.asp). However, for the two patients receiving the highest dosage of TKM-ApoB, ApoB protein and LDL cholesterol was transiently reduced by an average of 21.1% and 16.3%, respectively. Therefore, though the Phase I trial was terminated, the company continues to develop improved nanoparticle carriers and siRNA chemistry to recommence a clinical trial for the ApoB siRNA therapy [23].

Tekmira initiated a Phase I clinical trial for the siRNA drug TKM-PLK1 in September 2010. TKM-PLK-1 is being developed as a treatment for patients with advanced solid tumors whose current therapies are ineffective. Like TKM-ApoB, TKM-PLK1 consists of a siRNA targeting PLK1 encapsulated in the SNALP lipid nanoparticle. The siRNA targets polo-like kinase 1 (PLK1), which is involved in cell cycle progression, functions as an early trigger for G2/M transition [24]. PLK-1 is involved in two main steps of mitosis: the equal segregation of chromosomes with the help of the mitotic spindle and the occurrence of cell division without mistakes [25]. The deregulation of PLK1 has been shown in mammalian cells to cause formation of abnormal centrosomes, increased chromosomal instability, and tumor development [26]. Various cancers have elevated levels of PLK1 [27–31], but siRNA-knockdown of PLK1 induces growth inhibition and apoptosis of tumor cells *in vitro* [32, 33].

According to Tekmira pharmaceuticals TKM-PLK1 has the potential to provide both direct tumor cell killing and sensitization of tumor cells to the effects of chemotherapy (http://www.tekmirapharm.com/Programs/Products.asp). In preclinical studies TKM-PLK1 displayed potent and specific anti-tumor effects in a variety of tumor models in animals, thus providing significant survival benefits even when measured in aggressive liver cancer models. This clinical Phase I study will be a dose escalation study and is currently recruiting patients.

Alnylam Pharmaceuticals (Cambridge, Massachusetts, USA) has developed a siRNA therapeutic (ALN-VSP02) for the treatment of liver cancer and metastatic disease of the liver. In a licensing partnership with Tekmira, the Alnylam siRNA drug is packaged into a

first generation SNALP carrier. ALN-VSP02 is comprised of two separate siRNAs that each target genes critical for the growth and survival of cancer cells. One siRNA specifically targets the mRNA of vascular endothelial growth factor (VEGF), while the other inhibits kinesin spindle protein (KSP) mRNA.

A Phase I multi-center, open label, dose escalation trial for ALN-VSP02 was initiated in April 2009. This study, with locations in the USA and Spain, was designed to enroll approximately 55 patients with advanced solid tumors with liver involvement who have progressed or not responded to standard treatment. By November of 2010, reports indicate that the intravenous drug is well tolerated in most of the 28 initial patients. Preliminary pharmacodynamic data suggest an anti-VEGF effect in a majority of treated patients. A continued dose escalation Phase I trial will further evaluate the safety, tolerability, pharmacokinetics, and pharmacodynamics of ALN-VSP02.

Alnylam Pharmaceuticals has also initiated a Phase I study to evaluate safety and tolerability of a siRNA drug for the treatment of transthyretin-mediated amyloidosis (ATTR). ATTR is caused by mutations in the transthyretin (TTR) gene, which is expressed predominantly in the liver. Mutated TTR proteins are prone to misfolding, which can result in the accumulation of deposits of mutant and wild-type TTR protein as amyloid fibrils. Such deposits can further accumulate in the peripheral nervous system, heart, and gastrointestinal tract. Liver transplantation is the only treatment option for ATTR, but this invasive procedure is not available for most ATTR patients. The Alnylam siRNA (ALN-TTR01) is designed to specifically inhibit *TTR* mRNA, thereby reducing the accumulation of TTR protein [34]. In a partnership with Tekmira, Alnylam formulated the siRNA drug with a first generation SNALP carrier. These lipid nanoparticles are designed to be taken up by hepatocytes. The Phase I trial commenced in June of 2010, and includes treatment locations in Portugal, Sweden, and the UK. This randomized, single-blind, dose escalation trial is designed to enroll approximately 28 ATTR patients and will test the safety and tolerability of a single dose of ALN-TTR01. ALN-TTR01 will be administered by intravenous (IV) infusion, with dose levels between 0.01 and 0.4 mg/kg (http://alnylam.com/Programs-and-Pipeline/Programs/TTR-Amyloidosis.php).

3.2 Anionic liposome carrier of siRNA for Chronic Myeolid Leukemia (CML)

Approximately 24,500 people in the United States are living with CML with thousands more diagnosed each year. This cancer originates from transformed hematopoietic stem cells containing the hallmark of CML called the Philadelphia chromosome, a reciprocal translocation between chromosomes 22 and 9 (t(9;22)(q34;q11)) resulting in a fusion oncogene *bcr-abl* [35]. This fusion gene constitutively expresses a tyrosine kinase and leads to increased cell cycle progression. Furthermore, it also inhibits the DNA repair system, making the patients more susceptible to further genomic abnormalities. Apart from transplantation, current therapies include the selective inhibitor of *abl* known as Imatinib and the chimeric fusion protein BCR-ABL.

A clinical trial Phase I safety study, sponsored from the University of Duisburg-Essen (Germany), was employing three doses of a *bcr-abl* siRNA [36]. The siRNA in complex with anionic liposomes was injected intravenously and also subcutaneously into CML nodes of one female imatinib-resistant CML patient [37]. No severe side effects could be detected. Despite a reduction of BCR-ABL at nine days post-injection, knockdown was not maintained as BCR-ABL reached original levels 22 days post injection. Additional injections of siRNA complexes only reduced the levels slightly indicating a growing resistance to the siRNA therapeutic. One additional patient received the *bcr-abl* siRNA therapeutic but required combinatorial chemotherapeutics due to poor health unrelated to the siRNA.

3.3 siRNA-lipoplex for solid tumors

Silence Therapeutics (London, UK) is currently conducting a Phase I clinical test of a siRNA-lipoplex (Atu027) directed against protein kinase N3 (PKN3) for the treatment of advanced solid cancers. PKN3 is a downstream effector of phosphoinositide 3-kinase PI3K signaling pathway [38], which regulates diverse cellular responses including development, growth and survival [39]. PI3K is transiently activated by growth factors and is turned off through tumor suppressor PTEN. PTEN, one of the most frequently inactivated tumor suppressors in human cancers [40], regulates the PI3K signaling by dephosphorylation to ensure a transient and controlled activation of the pathway [41]. Loss of PTEN leads to permanent activation of PI3K, which correlates with increased metastatic behavior [42]. PKN3 was identified as downstream target mediating invasive cancer cell growth *in vitro* and *in vivo* [38]. Hence targeting PKN3 with siRNAs may result in inhibition of PI3K-dependent tumoral hemangiogenesis and/or lymphangiogenesis.

Atu027 is formulated into the previously tested liposomal AtuPLEX™ [43]. The trial is an open-label, single center, dose finding study (http://clinicaltrials.gov/ct2/show/NCT00938574). Patients are treated by a single dose followed by repeated treatment (8 treatments within 4 weeks) intravenously to determine toxicity and maximal tolerated dose. Completion of the study is expected by the second half of 2011.

3.4 Cyclodextrin nanoparticle carrier of siRNA for solid tumors

Calando Pharmaceuticals (Pasadena, California, USA) has developed a siRNA therapeutic (CALAA-01) that is also currently tested in a Phase I clinical trial for treatment of relapsed or refractory cancers. This siRNA is designed to inhibit tumor growth and to reduce tumor size by targeting the M2 subunit of ribonucleotide reductase (RRM2). The siRNA is encapsulated a cyclodextrin nanoparticle decorated with the human transferrin (TF) protein and polyethylene glycol (PEG) for stability [44]. The TF protein serves as a ligand for the transferrin receptor (TfR), which is highly expressed on cancer cell surfaces, to deliver the nanoparticle to malignant cells. This trial marks both the first demonstration of systemically delivered nanoparticles and of receptor-mediated delivery of siRNAs. Importantly, by confirming siRNA-induced mRNA cleavage with a modified 5'-RACE (rapid amplification of complementary DNA ends) PCR technique, this also provided the first mechanistic evidence of RNAi from siRNA in humans [44]. Therefore, this critical test indicates that the therapeutic effects result directly from the siRNA-mediated knockdown of RRM2 and not as a consequence of indirect effects, such as the nonspecific activation of Toll-like receptors (TLRs).

Ribonucleotide reductase (RNR), consisting of two subunits RRM1 and RRM2, catalyzes the formation of deoxyribonucleotides – the building blocks of DNA from – ribonucleotides. Hence RNR is required for DNA synthesis and repair [45]. The activity of RNR, and therefore DNA synthesis and cell proliferation, is controlled during the cell cycle by the synthesis and degradation of the RRM2 subunit [46]. A variety of tumor cells obtained from mouse and human tissues showed alteration in the RNR activity [47–49]. RRM1 and RRM2 play opposite roles in malignant progression of tumor cells. Overexpression of human *RRM1* or the murine homolog significantly inhibits *in vivo* tumor growth and decreases metastatic potential of tumors [50, 51]. On the contrary, overexpression of human RRM2 enhances the invasive and metastatic potential of various human cancer cells [52, 53]. Further studies demonstrated that the RRM2 protein level in human colon cancer was positively correlated to the metastasis of colon cancer [49, 54]. Patients with solid cancers are administered targeted nanoparticles on days 1, 3, 8 and 10 (21 day cycle) by IV infusion.

3.5 Biodegradable polymeric matrix for pancreatic ductal adenocarcinoma

As of November of 2010, Silenseed Ltd (Jerusalem, Israel) began enrolling for a Phase 0/I clinical trial to evaluate the safety and tolerability of a siRNA therapeutic for pancreatic ductal adenocarcinoma. Pancreatic ductal adenocarcinomas are usually caused by somatic mutations in the KRAS oncogene, including the most common KRASG12D mutation. Thus, by targeting KRASG12D with the siG12D LODER (Local Drug EluteR) therapeutic, the drug is designed to selectively knockdown KRASG12D expression and lead to apoptosis of the targeted cancer cells. The siG12D LODER is injected into the patient's tumor using an endoscopic ultrasound (EUS) biopsy needle. The siRNA is encapsulated in the LODER biodegradable polymeric matrix that is designed to release the drug locally within a pancreatic tumor and over an 8-week period. The Phase 0 study is designed to evaluate the safety and tolerability with treatment of one siG12D LODER in the tumor. The Phase I trial will utilize dose escalation of one, two, or four siG12D LODERs in the tumor. Secondary objectives for both trials include the analyses of local efficacy of the drug based on histopathology measurements (http://clinicaltrials.gov/ct2/show/NCT01188785).

3.6 IV injection of siRNA for acute kidney injury and delayed graft function

Quark Pharmaceuticals (Fremont, California, USA) has developed a kidney-targeted siRNA (I5NP or QPI-1002) that is designed to temporarily inhibit expression of the pro-apoptotic p53. The kidney is an attractive target for siRNA therapies, since, within a few hours after IV injection, unmodified siRNAs typically pass through the kidneys to the urine [23]. I5NP has entered two separate clinical trials to evaluate its safety, tolerability, and ability of reducing p53 levels in order to prevent acute kidney injury (AKI) or delayed graft function (DGF). The first trial was designed to temporarily reduce p53 levels to prevent AKI during cardiovascular surgery (http://clinicaltrials.gov/ct2/show/NCT00554359). This Phase I, randomized, double-blind, dose escalation study was designed to enroll 32 patients in the USA and Switzerland. Following cardiovascular surgery, patients received a single IV injection of I5NP or placebo. The study was completed in November of 2010, and though some details of the trial have not yet been publicly announced, favorable safety data were reported [23]. In the other Phase I/II trial, I5NP was administered to patients undergoing kidney transplantation. This controlled, double-blind, randomized, dose escalation study aims to evaluate the safety and tolerability of I5NP, as well as the prophylaxis of DGF in patients receiving kidney transplants (http://clinicaltrials.gov/ct2/show/NCT00802347). The dual-arm study will first require a 40 patient, 4 cohort dose escalation to determine the highest or maximum tolerated dose (MTD). The second arm will then utilize the MTD to further evaluate the safety and clinical efficacy of I5NP as a 326 patient (163 I5NP and 163 placebo), multicenter, Phase II study. As in the I5NP study for AKI, favorable safety data were reported for the Phase I trial for DGF, and an independent Data Safety Monitoring Board (DSMB) has recommended the continuation of clinical development of I5NP into Phase II trials.

4 Intravitreal (IVT) injection of siRNA therapeutics

Due to the self-containment of the eye, ocular tissue offers a suitable target for localized gene therapy including siRNA drugs. Intravitreal injection (injection directly into the eye) has been used to deliver three different siRNA therapeutics for age-related macular edema (AMD), diabetic macular edema (DME), or non-arteritic ischemic optic neuropathy (NAION). Due to the similar factors associated in AMD and DME, the same siRNA drug has been tested for both diseases.

4.1 IVT injection of siRNA for AMD and DME

AMD is the leading cause of legal blindness in over 55 year olds and affects more than 14 million people worldwide [55, 56]. Abnormal blood vessel growth (chorodial neovascularization) behind the retina and macula lead to vision loss. Bleeding and leaking of fluids from newly formed blood vessels result in bulging or lifting of the macula, which concludes in irreversible damage and loss of vision if left untreated. The cause of vision loss in DME patients is similar, though it afflicts patients with diabetes. The WHO estimates that 15 million people have diabetes in the US but only half are diagnosed and half of those receive proper eye care. If untreated there is an approximate 25% risk of developing DME with moderate visual losses. Microvascular changes such as thickening of the basement membrane and reduction in the number of pericytes lead to increased permeability and incompetence of retinal vasculature. The leakage of plasma constituents into the surrounding retina results in retinal edema.

VEGF is the predominant mediator of angiogenesis and is increased in neovascular membranes of AMD patients [57, 58] and in DME patients [59]. RNAi-mediated knockdown of VEGF or the VEGF Receptor (VEGF-R) in the retina may lead to regression of abnormal blood vessel growth and improvement of vision (Figure 2).

Opko Health Inc. (Morristown, New Jersey, USA) produced the first siRNA therapeutic to reach a Phase III clinical trial. Bevasiranib is a 21 nucleotide long siRNA with two deoxythymidine (dTs) on both 3’ ends and targets VEGF A mRNA [60]. The siRNA initially showed potential for treatment of AMD in Phase I and II clinical trials. Nevertheless, Opko terminated the Phase III study in March 2009, based on the recommendation of the Independent Data Monitoring Committee. The committee reasoned that siRNA drug was unlikely to achieve its primary endpoint of reducing vision loss in the AMD patients. In addition, Opko tested Bevasiranib for DME in another Phase II study, and despite demonstrating good safety and encouraging signs of biological activity, no Phase III trial has been announced.

Allergan (Irvine, California, USA) sponsored a Phase II clinical trial targeting AMD. The drug AGN-745 (formerly known as Sirna-027 by Sirna Therapeutics, Inc.) also intravitreal administered is a chemically modified siRNA targeting the VEGF receptor (Figure 2). As the second RNAi drug reached clinical trials, early data were promising considering its efficacy and safety. However, the trial was terminated in May 2009, due to its lack of improvement of visual acuity.

Reports have indicated that 21-nt siRNAs suppress neovascularization independent of their sequence or target due to triggering the immune receptor Toll-like receptor 3 (TLR3) [61, 62]. These developments led to the discontinuation of AGN-745 and Bevasiranib as potential drug candidates, due to the uncertainty that the siRNA are functioning as designed as opposed to nonspecific, TLR3-mediated effects. With more than 700 patients enrolled in clinical trials for these two drugs, the drugs were largely well tolerated with no reported severe side effects. Hence from a safety perspective, these trials indicated that the siRNAs are well tolerated, but that the siRNA therapeutic must be improved to increase target-specific efficacy and to reduce off-target triggering of TLR3.

Pfizer (New York City, New York, USA) and collaborator Quark Pharmaceuticals have conducted three clinical trials to test a siRNA for the treatment of retinal diseases AMD and DME. The drug PF-655 (aka, PF-04523655, REDD14NP, and RTP801i) is a synthetic siRNA designed to target the pro-angiogenic factor RTP801, which is a direct transcriptional target of hypoxia inducible factor I (HIF-1) (Figure 2). HIF-1, alongside with apoptosis-inducing protein 53 (p53), constitutes an important part of the control of neovascularization

[63, 64]. HIF-1 levels, increased throughout the inner retina, play a role in up-regulating VEGF [65], while overexpression of RTP801 leads to the generation of reactive oxygen species (ROS) and apoptosis of neuron-like cells [66, 67].

PF-655 was well tolerated in a Phase I trial for patients with choroidal neovascularization secondary to AMD (http://clinicaltrials.gov/ct2/show/NCT00725686). A Phase II trial evaluated PF-655 versus laser treatment for patients with DME. However, the trial was terminated when the objectives of the study could no longer be achieved, but not for safety reasons (http://clinicaltrials.gov/ct2/show/NCT00701181). PF-655 is currently in a Phase II study for AMD and DME, and public information is limited while the trial continues (http://clinicaltrials.gov/ct2/show/NCT00713518).

4.2 IVT injection of chemically modified siRNA for NAOIN

Non-arteritic ischemic optic neuropathy (NAION) affects more than 10,000 people every year in the United States and is the most common cause of sudden optic nerve related visual loss. It primarily affects the elderly, though can occur at any age. Anterior Ischemic Optic Neuropathy (AION) is due to ischemia of the anterior part of the optic nerve (or optic nerve head ONH), which is supplied by the posterior ciliary artery [68] and can be divided into two subtypes. Arteritic AION (AAION) which is caused by giant cell arteritis and non-arteritic AION a condition in which insufficient blood supply damages the optic nerve resulting in the death of retinal ganglion cells and hence loss of vision.

Many factors play a role in blood flow to the ONH, hence NAOIN patients have a unique combination of systemic and local factors which taken together cause ONH ischemic damage [69]. Due to the multifaceted nature of this disease, no clinical treatment currently exists. Since the common feature among NAOIN patients is the death of retinal ganglion cells, any potential drug should protect these cells from apoptosis.

Apoptosis is a very complex cellular mechanism involving a cascade of caspases, a family of cysteine-dependent, aspartate-directed proteases [70]. Caspases exist as inactive proenzymes and undergo proteolytic processing into two subunits that dimerize to form the active enzyme. One of the earliest identified caspases, Caspase 2, induces apoptosis by releasing pro-apoptotic proteins from mitochondria [71, 72]. Quark Pharmaceuticals developed QPI-1007, a chemically modified siRNA targeting Caspase-2. According to Quark the siRNA utilizes a novel structure, which preserves efficacy but attenuates off-target and immunostimulatory effects. It has been developed as neuroprotectant for NAOIN but it might also be used for other similar diseases as glaucoma in the future. In animal models QPI-1007 displayed neuroprotective activity and efficacy for both diseases and is currently tested in a Phase I dose escalation study for treatment of NAOIN.

5 Intralesional delivery of siRNA for pachyonychia congenita

TransDerm (Santa Cruz, California, USA) and the International Pachyonychia Congenita Consortium (IPCC) have collaborated to develop a siRNA treatment for pachyonychia congenita (PC). PC is a rare autosomal dominant skin disorder characterized by thick and dystrophic nails, oral leukoplakia, and other skin defects [66]. PC results from dominant-negative mutations in genes that encode keratins. Such mutations, including single nucleotide mutation (N171K) of the *keratin 6a* (*KRT6A*) gene, are associated with keratin aggregate formation [73]. The research team has developed a mutant-specific siRNA (TD101) that can selectively target the N171K mutation *KRT6A* without affecting wild-type expression [73]. TD101 has been tested in a Phase Ib trial for a single patient, establishing the first-in-human usage of a mutation-targeted siRNA [74]. The safety and clinical efficacy TD101 were evaluated in this double-blind, split-body, vehicle-controlled, dose-escalation

study. TD101 was administered by intralesional injection into a plantar callus, with a dose escalation ranging from 0.10 mg to 17.0 mg over a 17-week schedule [74]. Unmodified siRNA, which would be quickly degraded if it entered the bloodstream, was used to limit any potential toxicity effects from TD101. Importantly, callus regression was observed in the TD101-treated (right) foot, while no effect was seen in the vehicle control (left) foot. No adverse effects occurred during the trial or 3-month washout period [74]. Although the regular injections were painful and difficult to tolerate, TransDerm is currently developing alternative delivery methods. These include a topical ointment with lipid-based carriers (GeneCreme) and a dissolvable microneedle array known as a Protrusion Array Device (PAD) [75].

6 Topical delivery of siRNA therapeutics

Topical delivery applications of siRNA therapeutics are attractive since they tend to be non-invasive, are often locally restricted, and can be administered by the patient. Three siRNA drugs that require topical delivery methods have been tested in clinical trials. These include siRNA eye drops for the treatment of ocular hypertension and glaucoma from Sylentis, an inhaled siRNA drug for asthma from ZaBeCor, and a siRNA drug for respiratory syncytial virus (RSV) from Alnylam that has been clinically delivered by nebulizer and nasal spray.

6.1 siRNA eye drops for ocular hypertension and glaucoma

Sylentis (Madrid, Spain) has developed a RNAi therapeutic for the treatment of ocular hypertension associated with glaucoma [23]. The siRNA drug (SYL040012) specifically targets the mRNA of beta-2 adrenergic receptor (ADRB2) [34]. Ocular hypertension can be relieved by blocking ADRB2 on the ciliary epithelium, which in turn may reduce the production of aqueous humour. SYL040012 is delivered to the eye by drops. The first Phase I clinical study testing SYL040012 was completed in June of 2010 (http://clinicaltrials.gov/ct2/show/NCT00990743). This trial assessed the safety and tolerance of the siRNA therapeutic over a two-part survey. They first examined one dose of SYL040012 over a three day period. In the second part, the drug was administered at one dose per day over a seven day period, with tolerance assessments until day 11. Both parts of this Phase I trial were safe and well tolerated. As of October 2010, the drug has entered a Phase I/II study, which will further evaluate the tolerance of SYL040012 and its effect on unmedicated patients with elevated intraocular pressure (IOP) (http://clinicaltrials.gov/ct2/show/NCT01227291). Like the initial trial, the latter will be conducted in Spain. Patients will receive a daily single dose of SYL040012 for seven days, and tolerance, adverse events, pharmacokinetics, and effect will be assessed after day 11.

6.2 Inhalation of siRNA for asthma

ZaBeCor Pharmaceuticals (Bala Cynwyd, Pennsylvania, USA) has developed a siRNA drug for the treatment of asthma. The siRNA therapeutic, known as Excellair™, targets the mRNA of spleen tyrosine kinase (Syk). Syk is involved in signaling from the B-cell receptor and is a key regulator of downstream signaling cascades that ultimately leads to the activation of several pro-inflammatory transcription factors. Therefore, the specific inhibition of Syk by Excellair™ is designed to alleviate inflammatory diseases such as asthma. In a Phase I study, patients with asthma received the inhaled siRNA therapeutic for 21 consecutive days [23]. The drug was well tolerated in all asthma patients, with no serious adverse effects. Moreover, 75% of treated patients reported improvement of breathing or reduced inhaler usage, while placebo patients reported no improvement. These results helped to drive Excellair™ into a Phase II clinical trial, which was announced in September of 2009 (http://www.zabecor.com/news/news092209.php).

6.3 Nasal spray or nebulizer delivery of siRNA for RSV

Alnylam Pharmaceuticals (Cambridge, Massachusetts, USA) has developed a siRNA therapeutic (ALN-RSV01) that targets the mRNA of a key viral protein of respiratory syncytial virus (RSV). RSV infects nearly 70% of infants during the first year of birth and is the leading cause of hospitalization of infants [76]. It can also produce severe respiratory diseases, such as pneumonia, in the elderly and in immunocompromised adolescents and adults. A RSV vaccine is not available and the only approved antiviral therapy for RSV is undesired in pediatric patients due to its potential teratogenicity and limited effectiveness. Thus, a safe and efficacious RSV therapy is long awaited for both pediatric and adult populations.

The ALN-RSV01 siRNA was the first antiviral RNAi therapy approved for clinical trials. RSV, like other lung diseases, replicates in the outermost layer of respiratory epithelium, including the lining of nasal passages, trachea, and bronchioles [77]. Thus, the location of the diseased tissue allows for topical delivery of siRNA to the affected cells by intranasal aerosol, where the epithelial cells take up the siRNAs [77]. ALN-RSV01 targets the mRNA of the RSV nucleocapsid (N) protein, which is a critical factor for viral replication. Moreover, the targeted region of the N mRNA is a well-conserved sequence across both major serotypes (A and B). The antiviral siRNA is a double-stranded RNA duplex with 19 base pairs of complementarity and 2-nt deoxythymidine (dT) overhangs at each 3’ end [78].

In an initial Phase I trial, two studies were conducted in a combined 101 healthy male volunteers (65 with various single and multiple doses of ALN-RSV01 and 36 with single and multiple doses of placebo), ranging from 18 to 45 years old. Both studies were randomized, placebo-controlled, observer-blind studies and both delivered the therapeutic siRNA by nasal spray [78]. One study in the United States employed single dose with dose-escalation, while the other in the European Union used single and multiple dose with dose-escalation. In the two-study Phase I trial, there were no serious adverse events (AEs) for any subject. Despite some mild and moderate AEs, the character, frequency, and severity of the AEs in the treated group were similar to those of the placebo group. Likewise, no trends were observed in severity or frequency of AEs with increasing dose or number of doses in any treatment group [78].

The initial safety trial was followed by a Phase II trial to test for efficacy. This 1:1 randomized, double-blind, parallel-group Phase II trial tested 85 healthy males ages 18–45 [76]. All subjects were screened for low neutralizing antibody concentrations, among other exclusion criteria [76]. This trial incorporated both prevention and treatment strategies in order to simultaneously maximize the likelihood of observing a statistically significant antiviral effect while also minimizing the risk of RSV infection to the subjects [76]. All subjects received RSV01 inoculation at day 0, and the ALN-RSV01 treated cohorts received the siRNA intranasal spray at days −1, 0, +1, +2, and +3. A statistically significant reduction in detected RSV by quantitative culture and real-time PCR was reported for patients receiving 150mg of study drug, the highest dosage tested. Averaged for all treated patients vs. placebo, an acquisition over time effect was observed by either PCR or quantitative culture. Indeed, the strongest effects of treatment with ALN-RSV01 were observed by its prophylactic efficacy. The drug provided an antiviral effect over an 11-day time course, which resulted in reduced infectionover time noticeable within 3–4 days after inoculation [76]. Multiple regression models – which incorporate variables for RSV inoculum, pretreatment RSV microneutralization titer, and various intranasal proinflammatory cytokine concentrations – demonstrated that the antiviral effect of ALN-RSV01 was statistically independent of other variables.

Another Phase II trial conducted by Alnylam assessed the safety and antiviral activity of ALN-RSV01 plus standard care for lung transplant patients infected with RSV (http://clinicaltrials.gov/ct2/show/NCT00658086). This randomized, double-blind, placebo-controlled, trial included 24 patients (ALN-RSV01 N=16; placebo N=8) with stratified randomization based on ribavirin use [79]. The therapy was well tolerated and no drug-related AEs were reported. In contrast to the intranasal delivery, this study utilized an electronic nebulizer for 8 to 10 minutes. The drug was administered once daily for three days, followed by daily symptom and virus scores until day 14. Over the first 6 days, the viral area under the curve was lower in the ALN-RSV01 than in the placebo group. However the individual mean daily load values were not statistically significant between data sets. There was, though, a significant reduction effect in the mean daily symptom scores of ALN-RSV01 treated vs. placebo patients [79]. Spirometry measurements collected at days 30 and 90 indicated a significant reduction in the incidence of new or progressive bronchiolitis obliterans syndrome (BOS) in ALN-RSV01 treated patients relative to placebo [79]. The encouraging results from this trial have driven ALN-RSV01 into a Phase IIb trial that will treat RSV infection across a broader group of lung transplant patients (http://clinicaltrials.gov/ct2/show/NCT01065935).

Although neither Phase II human study obtained direct evidence for the antiviral RNAi mechanism, previous reports from the same group demonstrate critical evidence in an RSV murine model [80]. These include no antiviral effect for mice treated with mismatched siRNAs and confirmed cleavage products of target RSV N-protein transcript by RACE PCR [80].

7 Oral delivery of siRNA for Familial Adenomatous Polyposis

Marina Biotech (Bothell, Washington, USA; formerly Cequent Pharmaceuticals) has initiated the first and only toxicology study of a siRNA drug that employs oral delivery. This 26 week long Phase I study uses a shRNA against β-catenin known as CEQ508 for the treatment of Familial Adenomatous Polyposis (FAP). FAP is a rare hereditary colon syndrome that when left untreated typically leads to colon cancer by the age of 40. It results from defects in the gene *adenomatous polyposis* (*APC*). Over-expression of APC leads to formation of polyps that can become malignant in the epithelium of the large intestine. More than 80% of sporadic and hereditary colon cancers are caused by aberrations in the β-catenin/Wnt signaling pathway [81–83]. The β-catenin stability is regulated by multiprotein complex that contains APC and by activation it accumulates in the nucleus where it can activate multiple target genes contributing to the development of cancer [84, 85]. In classical FAP β-catenin is dysregulated. Inhibition of β-catenin has been shown to slow cell growth of intestinal cells responsible for polyp growth.

CEQ508 is based on the company's TransKingdom RNA™ interference (*tk*RNAi) technology [86]. This technology is based on non-pathogenic *Escherichia coli* to produce and deliver shRNAs to target cells. The *tk*RNAi-mediating vector (TRIP) contains a T7 promoter to drive the expression of the shRNA along with a gene for the T7 polymerase. Furthermore, the vector contains the *Inv* locus from *Yersinia pseudotuberculosis* that encodes for Invasin protein, which permits the bacteria to enter epithelial cells that express the β-1 integrin surface receptor. The vector also encodes *HlyA from Listeria monocytogenes* for lysteriolysin O, a pore forming protein that enables the shRNA to escape their entry vehicles into the cytoplasm, where they can enter the RNAi pathway (Figure 3).

No viable pharmaceutical treatments for FAP are currently available, and the only choice of treatment is surgical. Hence, treatment of FAP with *tk*RNAi offers a promising new approach. *In vitro* and *in vivo* (APC^{min} mouse model) β-catenin is efficiently downregulated

on mRNA levels after treatment with CEQ508. Studies in non-human primates confirm that oral *tk*RNAi targeting β-catenin is safe, well tolerated, and is capable of reversibly attenuating β-catenin expression in the intestinal tract. The Phase I safety study is currently being initiated. The primary endpoint is safety and tolerability of daily oral administration of CEQ508 for 28 days as well as measuring β-catenin expression throughout biopsies taken at the beginning and endpoint of treatment.

8 Concluding remarks

The advancement of more than 20 therapeutic siRNAs into the clinic illustrates that RNAi-based medicine holds a pivotal place in the future treatment of human diseases. However, there remain several obstacles in the clinical development of RNAi-based therapeutics. One of the greatest challenges in RNAi therapy continues to be the delivery method of the therapeutic siRNA to the target cells.

The advancements of synthetic carriers of siRNA have broadened the usage of siRNA therapeutics for a variety of tissue-specific diseases and tumors. By providing a delivery vehicle for the siRNA payload to the target cell, nanoparticles can prevent non-specific delivery of the siRNA drug and may also protect the siRNA during transport. Moreover, the efficacy and safety of siRNA drugs heavily depends on its delivery to the intended target [87]. Lipid-based nanoparticles like SNALP from Tekmira can deliver siRNA to various tissues and tumors by IV injection. Other promising advancements in nanoparticle delivery include the cyclodextrin carrier from Calando and the LODER delivery system from Silence. Such biodegradable polymers can be engineered to release the siRNA over a localized area of tissue for an extended and controllable duration. Novel synthetic carriers, such as RNA-aptamers conjugated with siRNAs and next-generation lipid-based carriers will further drive siRNA drugs into clinical applications [1, 2, 88].

Naked siRNAs have been extensively tested, particularly for therapies that allow for administration through topical or IVT methods. Due to the fragility of RNA molecules and their susceptibility to degradation, prolonged expression of RNAi-based therapeutics has been notoriously difficult. Chemical modifications to siRNA molecules, including modifications to the base, the ribose sugar, and phosphodiester backbone may alleviate undesired inflammatory responses while also extending the half-life of the therapeutic drug for days or weeks [87].

In addition to the delivery methods based on synthetic materials, viruses, and bacteria offer an alternative for shRNA-based therapeutics encoded in DNA vectors. Bacterial or lentiviral shRNA-expressing vectors offer a long-term delivery method, in which the expression of the shRNA is designed to persist indefinitely. However, both delivery methods are currently limited to packaging of nucleic acids that encode shRNA precursors of siRNA, and *in vivo* delivery of RNAi viral vectors has not yet been clinically tested.

Other major challenges for RNAi-based therapeutics include controlling the specificity of the siRNA and minimizing potential off-target effects related to the sequence of both dsRNA strands. siRNAs are known to initiate off-target gene silencing by functioning like microRNAs (miRNAs) [89]. This effect may occur when the antisense strand of the siRNA is perfectly matched at positions 2–7 or 2–8 to the 3’ UTR sequence of a nonspecific mRNA [90]. Asymmetric 25-nt/27-nt dicer substrates can be designed to preferentially load the guide ssRNA strand of the dsRNA duplex, thus mitigating the potential off-target effects from the passenger ssRNA strand [91, 92]. Dual-targeting siRNAs are designed so that both strands target different sites within a single mRNA target or two separate target mRNAs [93]. Thus, dual-targeting siRNAs may reduce the potential for off-target gene silencing,

increase the opportunity to knockdown the desired target gene(s), and potentially provide additive or even synergistic effects by both strands.

As observed with the two *VEGF* siRNA drugs that were terminated during clinical testing, siRNAs may also have the tendency to activate TLRs to lead to inflammation and other off-target effects. Despite the promising advancement of the drugs to Phase II and III trials, these setbacks have drawn public concern and attention. However, it should be noted that despite the drawbacks, more than a thousand patients were treated with siRNAs and no major negative effects or adverse events were detected. Moreover, the pro-inflammatory effects of TLR activation may be beneficial for treatment of certain diseases, so these properties of siRNA drugs may eventually be therapeutically exploited.

Like most novel therapies, the progress of siRNA drugs in clinical trials has encountered challenges along the way. Encouraging signs have developed in a number of trials, most notably for the treatment of various cancers, acute kidney injury, RSV, and HIV. While the setbacks from the AMD and DME trials deserve thoughtful scrutiny, lessons have quickly been learned, including validating siRNA-directed mRNA cleavage using an advanced technology known as 5'-RACE PCR. Thus, despite mixed results for the 21 siRNA/shRNA drugs in clinical trials, the potential of RNAi therapeutics in the field remains strong. With the emergence of newer technologies, such as chemical modifications to siRNA and more advanced delivery systems, the discontinuation of a few drugs has not impeded the clinical progress or the pre-clinical developments of other RNAi-based therapeutics.

Acknowledgments

The authors would like to thank Dr. John Nemunaitis (Gradalis Inc.), Dr. Scott Pruitt (Duke University), Dr. Alison Silva (Cequent Pharmaceuticals) and Dr. James Thompson (Quark Therapeutics) for sharing unpublished information used in this manuscript.

Abbreviations

ACT	autologous cell therapy
AE	adverse event
AKI	acute kidney injury
AMD	age-related macular edema
ATTR	transthyretin-mediated amyloidosis
bi-shRNA	bifunctional-shRNA
CML	chronic myeolid leukemia
DC	dendritic cell
DGF	delayed graft function
DME	diabetic macular edema
dT	deoxythymidine
FAP	familial adenomatous dolyposis
HPC	hematopoietic progenitor cell
IOP	intraocular pressure
IV	intravenous

IVT	intravitreal
LDL	low density lipoprotein
MHC	major histocompatibility complex
miRNA	microRNA
MTD	maximum tolerated dose
NAION	non-arteritic ischemic optic neuropathy
NHL	non-Hodgkin's lymphoma
ONH	optic nerve head
PC	pachyonychia congenital
RACE	rapid amplification of complementary DNA ends
RNAi	RNA-interference
RSV	respiratory syncytial virus
shRNA	short-hairpin RNA
siRNA	small-interfering RNA
SNALP	stable nucleic-acid lipid particle
TLR	Toll-like receptors
TTR	transthyretin
VLDL	very low density lipoprotein

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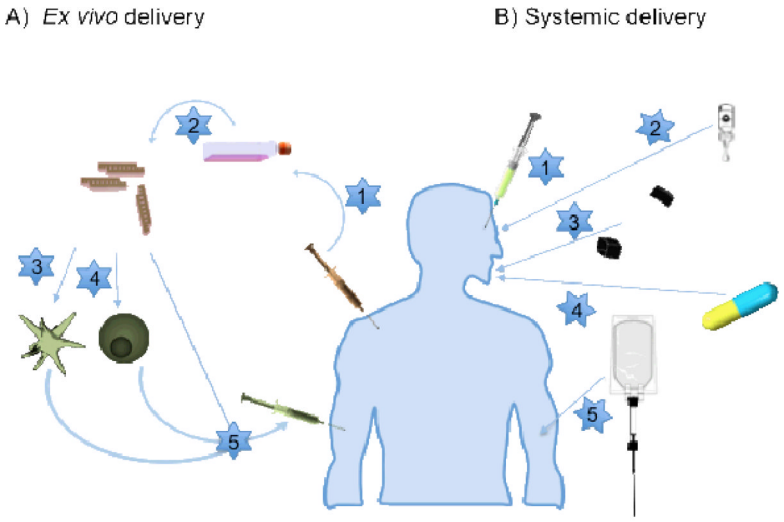

Figure 1. Delivery systems from current clinical trials

(A) *Ex vivo* delivery of RNAi therapeutics. Cells (monocytes, hematopoietic stem cells, and tumor cells) are extracted from patient, cultured (1) and transfected with siRNA via electroporation or lipofection or infected with shRNA expressing lentivirus (2). The monocytes are differentiated to dendritic cells (3) the modified tumor cells are irradiated (4) and injected back to the patient (5). (B) Systemic delivery of RNAi therapeutics in clinical trials. Therapeutic siRNAs can be administered by IVT injection (1), as eye drops (2), via inhalation (3), oral (4), or injected either IV or directly into the tumor (5).

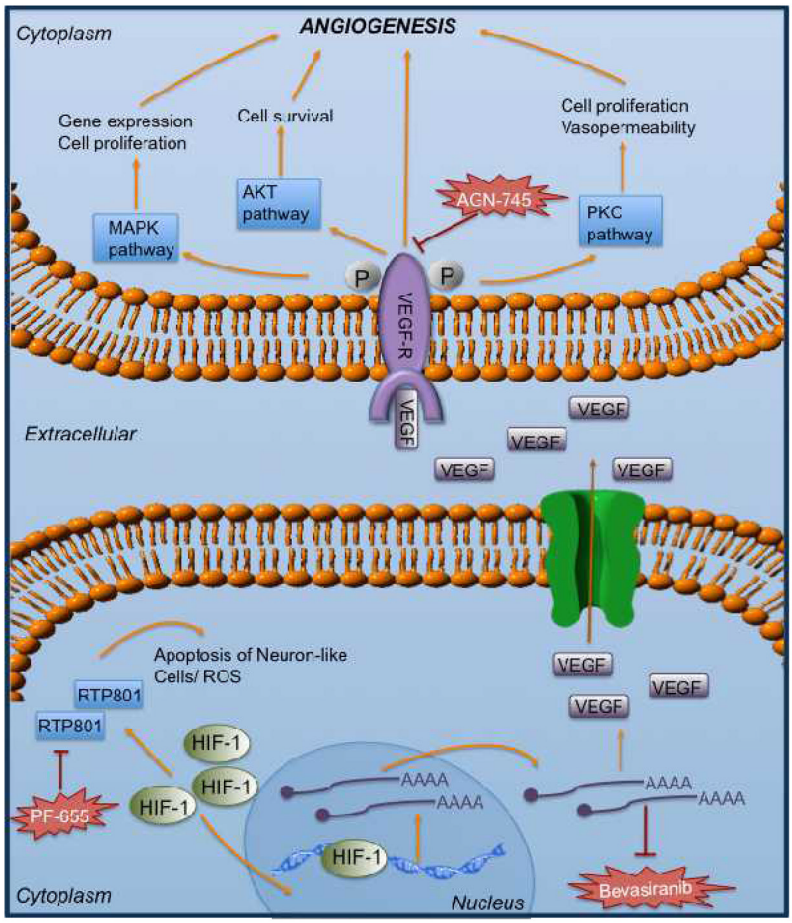

Figure 2. Inhibition of the VEGF, VEGF-R and RTP801 by novel potential RNAi drugs

HIF-1 is a transcription factor for VEGF and RTP801. RTP801 leads to apoptosis of neuron-like cells and reactive oxygen species (ROS), which in turn can cause retinopathy. PF-655 targets *RTP801* mRNA to attenuate retinopathy. HIF-1 binds to VEGF DNA in the nucleus to initiate transcription of *VEGF*. The *VEGF* mRNA is then transported into the cytoplasm where the translation of VEGF protein takes place. VEGF is transported out of the cells and can bind to VEGF receptor. The VEGF-receptor is part of many pathways that lead to angiogenesis. The siRNA Bevasiranib targets *VEGF* mRNA for degradation. AGN-745 targets the *VEGF-R* mRNA.

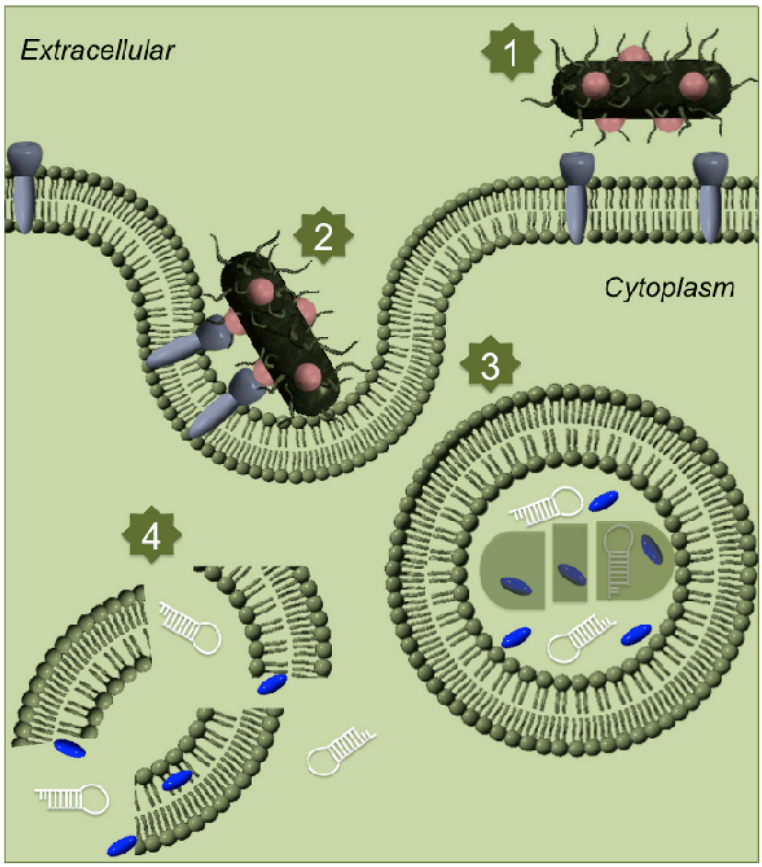

Figure 3. ***Escherichia coli*** **delivery of shRNA**

(1) The *E. coli* containing the shRNA have Invasin on their cell surface, which can bind to invasin receptors (β-1 integrin) on the cell surface in the intestinal tract. (2) Bacteria are then taken up via receptor-mediated endocytosis. (3) The bacteria are lysed in the endosome releasing the shRNAs and listeriolysin. (4) The listeriolysin breaks down the endosome membrane and releases the shRNAs into the cytoplasm where it can then be processed by the RNAi machinery.