Drugs of the Future 2011, 36(3): 229-239

CCC: 0377-8282/2011
DOI: 10.1358/dof.2011.36.3.1583187

REVIEW ARTICLE

PROGRESS IN THE DELIVERY OF siRNA THERAPEUTICS: POTENTIAL IN UVEAL MELANOMA

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SUMMARY

Since 2006, when Fire and Mello were awarded the Nobel Prize in Medicine for their work on RNA interference (RNAi), there has been significant progress towards transforming what began as an important laboratory tool for understanding gene function into a drug development platform capable of revolutionizing medicine. Major advances have been made in both local and systemic delivery of small interfering RNAs (siRNAs) in animal models of disease. In particular, novel lipid nanoparticles and targeted polymer- and cyclodextrin-based particles are providing new ways of delivering siRNAs to liver and to tumors, and these have led to the initiation of a number of clinical trials across various disease areas that are providing useful information regarding safety, drug delivery and pharmacodynamic activity. Oncology is a key testing ground for RNAi therapeutics, as the emergence of new targets that are not druggable by traditional means provides an opportunity for using RNAi-based drug platforms to expand the reach of targeted therapy. The recent discovery of activating mutations in GNAQ and GNA11 in the majority of uveal melanoma patients raises the possibility that targeting these genes with systemically delivered siRNA may provide a treatment breakthrough for this devastating disease following spread to the liver.

INTRODUCTION

Our increasing knowledge of the cancer genome and the dysregulated growth and survival pathways central to the malignant phenotype of specific tumor types is providing more opportunities for the targeted therapy of cancer (1-3). Drugs targeting dysregulated vascular endothelial growth factor (VEGF) and mammalian target of rapamycin (mTOR) signaling have provided improvements in the treatment of renal cell cancer, hepatocellular carcinoma, colorectal/lung/breast cancers, glioblastoma and pancreatic neuroendocrine tumors (4-10), whereas drugs targeting epidermal growth factor receptor (EGFR) have impacted breast and K-Ras wild-type colorectal cancers (11, 12). The most dramatic effects have been observed with the successful targeting of so-called oncogenic "driver" mutations, including c-Kit (gastrointestinal stromal tumor), BCR/ABL (chronic myelogenous leukemia), EGFR and EML4-ALK (lung adenocarcinoma), and V600E B-Raf (cutaneous melanoma) (13-17).

Historically, in order to be druggable, targets of interest needed to be accessible to inhibition by small molecules or protein therapeutics. However, it has been estimated that only 3,000 of the approximately 25,000 proteins encoded in the human genome have a suitable hydrophobic pocket required for small-molecule inhibition (18). For protein therapeutics such as monoclonal antibodies, the target is typically extracellular, and < 10% of proteins are located on the cell surface or are secreted. This means that approximately 75-80% of all possible protein targets are beyond the reach of small molecules and traditional protein therapeutics. It is therefore clear that novel approaches beyond small molecules and antibodies will be required to expand the number of druggable oncology targets. Novel drug development approaches that harness the RNA interference pathway provide a unique opportunity to expand the reach of targeted therapy in oncology.

BIOLOGY OF RNA INTERFERENCE

RNA interference (RNAi) is mediated by small duplex RNA molecules 19-23 base pairs in length that are typically staggered and contain 2-nucleotide overhangs at the 3' ends (19-21). When synthesized or derived from long double-stranded RNA molecules following cleavage by the cytoplasmic enzyme Dicer, these duplex RNAs are termed small interfering RNA (siRNA). In the cytoplasm, siRNA binds to a protein complex termed RISC (RNA-induced silencing complex), following which the sense, or passenger, strand is removed and the antisense, or guide, strand is retained in the complex. When the siRNA antisense strand bound to RISC engages its complementary mRNA, it is cleaved by an endonuclease (argonaute-2 [hAgo2]) within RISC, leading to degradation of the mRNA and downregulation of protein expression (Fig. 1). This sequence-

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specific mRNA degradation can be confirmed through detection of the expected cleavage product using the 5′ rapid amplification of cDNA ends (RACE) analysis (22). A single siRNA antisense strand loaded onto RISC can mediate the cleavage of multiple copies of its cognate mRNA, thereby accounting for the substantial potency of siRNAs (23). In addition to sequence-specific downregulation of mRNA expression, siRNAs can also have nonspecific effects that may include immune stimulation mediated through Toll-like receptors or through cytoplasmic proteins such as RIG-I (retinoic acid-inducible gene I protein) or PKR (dsRNA-dependent protein kinase) (24-27). The immunostimulatory potential of siRNAs can be greatly diminished through various modifications to the RNA backbone, including the addition of 2′-*O*-methyl groups (28).

The term microRNA (miRNA) refers to duplex RNAs derived from endogenous hairpin loop transcripts that are first processed by the nuclear enzymes Drosha and DGCR8/Pasha before transport to the cytoplasm and further cleavage by the Dicer/TARBP2 complex (29). The mechanism by which miRNA downregulates protein expression is still being elucidated, but appears to differ from siRNA in that the binding of an miRNA to the 3′-untranslated region of its cognate mRNA leads to translational repression.

From an oncology therapy standpoint, siRNAs, which are the subject of this review, could be used to target the expression of proteins with gain-of-function mutations or proteins that are components of signaling pathways contributing to the malignant phenotype of a particular cancer. MicroRNAs can act as either tumor suppressors if they are directed at oncogenes or as oncogenes if they are directed at tumor suppressors (30). Therefore, miRNA mimetics could be used to restore the lost expression of miRNAs acting as tumor suppressors, whereas so-called "antagomirs" (single-strand antisense DNA molecules) could be used to downregulate the overexpression of miRNAs acting as oncogenes.

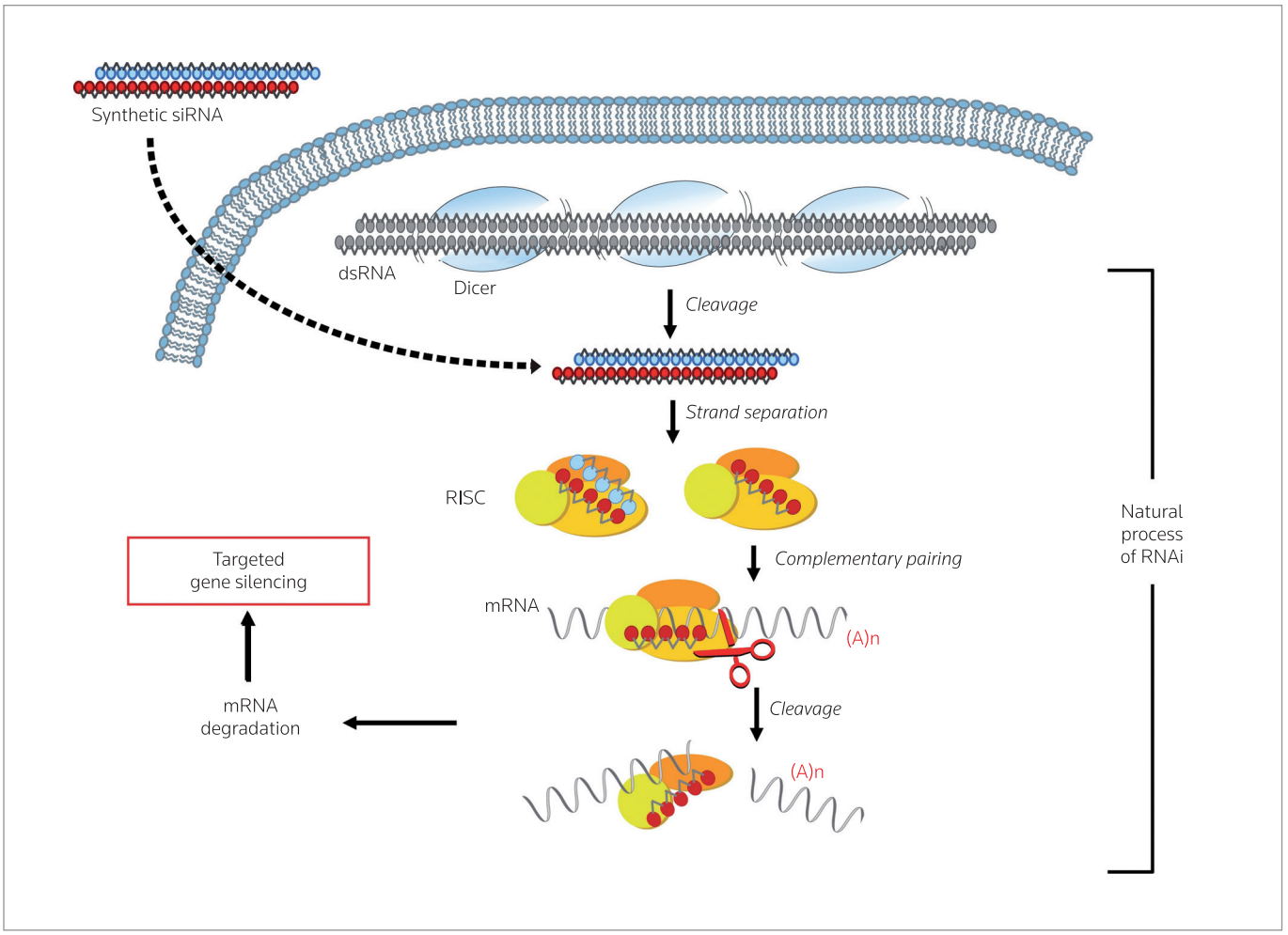

Figure 1. Schematic representation of RNA interference (RNAi) mechanism. The endogenous RNAi pathway within a cell is shown, as is the point of entry for a synthetic small interfering RNA (siRNA) once it crosses the cell membrane and is released into the cytoplasm. The guide (antisense) RNA strand is shown in red, the passenger strand in blue. Once bound to RISC (RNA-induced silencing complex), the two strands separate and the guide strand is then able to bind to its complementary mRNA and mediate cleavage via the argonaute-2 endonuclease present within RISC. This leads to further mRNA degradation and ultimately to downregulation of expression of the corresponding protein. A, adenosine.

ADVANCES IN siRNA DELIVERY

Local delivery

In order for an siRNA to mediate RNAi, it must enter the cytoplasm and become loaded onto RISC. The large size (~14 kD for a typical 21-bp duplex) and polyanionic charge of an siRNA pose a potential challenge for delivery of such a hydrophilic macromolecule across the cell membrane. Mammalian cells cannot take up "naked" siRNA in vitro; instead, it must be complexed first with a cationic lipid or transfected by some other means (31, 32). Yet, despite this requirement for transfection agents in vitro, there is evidence in various animal systems that naked siRNA formulated in saline can mediate RNAi in vivo. When administered via local delivery, this effect has been most notably demonstrated in neurons and oligodendrocytes in mice, rats and nonhuman primates (33, 34), and in the lungs in murine models of respiratory syncytial virus and parainfluenza virus infection (35, 36). While intravitreal injections of siRNA targeting VEGF or VEGFR have been shown to suppress choroidal neovascularization in mice (37), it was subsequently demonstrated that this effect was due to siRNA-mediated immunostimulation rather than to RNAi (38). The conjugation of cholesterol to siRNA to increase its hydrophobicity and facilitate cellular uptake has also been used successfully for local delivery applications in the central nervous system (CNS; transgenic mouse model of Huntington's disease) (39) and genitourinary tract (murine model of vaginal herpes simplex virus type 2 [human herpesvirus 2, HHV-2] infection) (40). However, in a murine model of bladder cancer, local delivery into the bladder of an siRNA targeting the mitotic kinase polo-like kinase 1 (PLK-1) was successful only when administered as a lipoplex (41).Over the past 5 years, several drug development programs have moved forward using local delivery of siRNA (Table I). ALN-RSV01, an siRNA targeting the nucleocapsid *N* gene involved in the replication of respiratory syncytial virus (RSV), is farthest along in development. Compelling data in a murine model of RSV infection showing that the antiviral activity of ALN-RSV01 was due to RNAi and not to any nonspecific immunostimulation (36) led to testing in a randomized, placebo-controlled phase II trial of intranasal ALN-RSV01 in 88 healthy adults experimentally infected with RSV (42). That study showed a statistically significant reduction in the rate of acquired RSV infection in subjects who received ALN-RSV01. A subsequent small, randomized, placebo-controlled trial in adult lung transplant recipients infected with RSV showed a significant reduction in both clinical symptoms and the incidence of bronchiolitis obliterans syndrome at day 90 postinfection in patients treated with inhaled ALN-RSV01 (43). These encouraging results have led to a larger, ongoing, randomized phase IIb trial in RSV-infected lung transplant recipients.

Other drugs in development using local delivery approaches (Table I) include ZaBeCor's Excellair™ (inhaled siRNA targeting tyrosine-protein kinase SYK) for asthma, Pfizer/Quark's PF-04523655 (intravitreal siRNA targeting RTP801) for acute macular degeneration and diabetic macular edema, Sylentis' SYL-040012 (topical siRNA targeting the β_2-adrenoceptor) for glaucoma, and Transderm's TD-101 (intralesional siRNA targeting mutant keratin-6A) for pachyonychia congenita (44). While intravitreal siRNAs targeting vascular endothelial growth factor (VEGF) (bevasiranib; Opko Health) or VEGFR-1 (AGN-211745; Allergan) were previously in clinical development for wet age-related macular degeneration (AMD), both of these programs have been terminated after phase III (bevasiranib) (45) or phase II (AGN-211745) (46) due to insufficient activity. No serious drug-related adverse events have been reported to date with these drugs or with ALN-RSV01, suggesting that the local delivery of siRNA is well tolerated.

Systemic delivery: liver targeting

The kinds of simple siRNA formulations being used for local delivery applications are inadequate for systemic delivery, since the duplex RNA in these formulations is rapidly degraded by nucleases in the blood and/or rapidly cleared by the kidneys. For systemic delivery, siRNAs need to be endowed with drug-like properties to increase circulation time, promote uptake by target tissues and facilitate cellular uptake and delivery into the cytoplasm.

Table I. *Local delivery siRNA programs.*

Sponsor	Program (stage of development)	Status	Target	Mode of delivery	Indication
Alnylam/Cubist/ Kyowa Hakko Kirin	ALN-RSV (phase IIb)	Ongoing	Respiratory syncytial virus (RSV) nucleocapsid *N* gene	Inhalation	Adult RSV infection
Zabecor	Excellair (phase II)	Ongoing	SYK	Inhalation	Asthma
Pfizer/Quark	PF-04523655 (phase II)	Ongoing	RTP801	Intravitreal	(1) Age-related macular degeneration (AMD) (2) Diabetic macular edema
Transderm	TD-101 (phase I) completed) (44)	Completed	Mutant keratin-6A	Intralesional injection	Pachyonychia congenita
Opko	Bevasiranib (phase III) (45)	Terminated	VEGF-A	Intravitreal	AMD
Allergan/SIRNA	AGN-211745 (phase II) (46)	Terminated	VEGFR-1	Intravitreal	AMD
Sylentis	SYL-040012 (phase I)	Ongoing	β_2-Adrenoceptor	Topical eyedrops	Glaucoma

One of the first approaches to demonstrate systemic delivery involved the use of a cholesterol conjugate. Using an siRNA targeting apolipoprotein B (Apo B), the cholesterol conjugate was significantly more effective in mice than unconjugated siRNA in suppressing Apo B mRNA in hepatocytes, which led to substantial reductions in plasma Apo B and low-density lipoprotein (LDL) cholesterol (47). Studies showed that the pharmacokinetics of the cholesterol conjugate (elimination half-life and distribution to multiple different tissues, including liver, small intestine, heart, kidney and lung) were improved through binding of the conjugate to circulating LDL and high-density lipoprotein (HDL) particles, and distribution to the liver in particular was mediated through binding to both LDL and scavenger (SR-BI) receptors on hepatocytes (48). In addition, there was evidence that uptake by hepatocytes was mediated by the mammalian homolog of the *Caenorhabditis elegans* transmembrane protein systemic RNA interference defective protein 1.

A major advance in the delivery of siRNA to the liver has been the development of a lipid nanoparticle (LNP) termed SNALP (stable nucleic acid lipid particle). This particle, which has a neutral charge at physiological pH, measures approximately 80 nm in diameter and consists of siRNA encapsulated within a particle comprised of four different lipid components: an ionizable lipid that is cationic at low pH, a neutral helper lipid, cholesterol and a pegylated lipid (Fig. 2). In addition to facilitating the condensation of the lipid with the anionic siRNA during particle formation, the ionizable lipid, upon becoming positively charged under acidic pH within endosomes involved in cellular uptake of SNALP, mediates fusion of SNALP with the endosomal membrane and release of the siRNA into the cytoplasm. Biodistribution studies in rodents and non-human primates have shown that SNALP localizes predominantly to the liver and spleen after i.v. injection, due in part to the fenestrated endothelium in those organs that permits the egress of 80-nm particles. In addition, liver uptake of SNALP appears to require opsonization of the particle by endogenous apolipoprotein E (Apo-E) and subsequent binding to LDL receptors on hepatocytes (49) (Fig. 3). For LNPs that contain a cationic, nonionizable lipid and are therefore positively charged at physiological pH, liver uptake appears to be Apo-E-independent.

One of the first demonstrations of liver targeting using SNALP was in a mouse model of hepatitis B virus (HBV) infection (50). In that system, an siRNA targeting HBV RNA substantially inhibited viral replication at a dose of 3 mg/kg. In a subsequent study in non-human primates using an SNALP-formulated siRNA targeting Apo B (51), the elimination half-life of SNALP-formulated Apo B siRNA

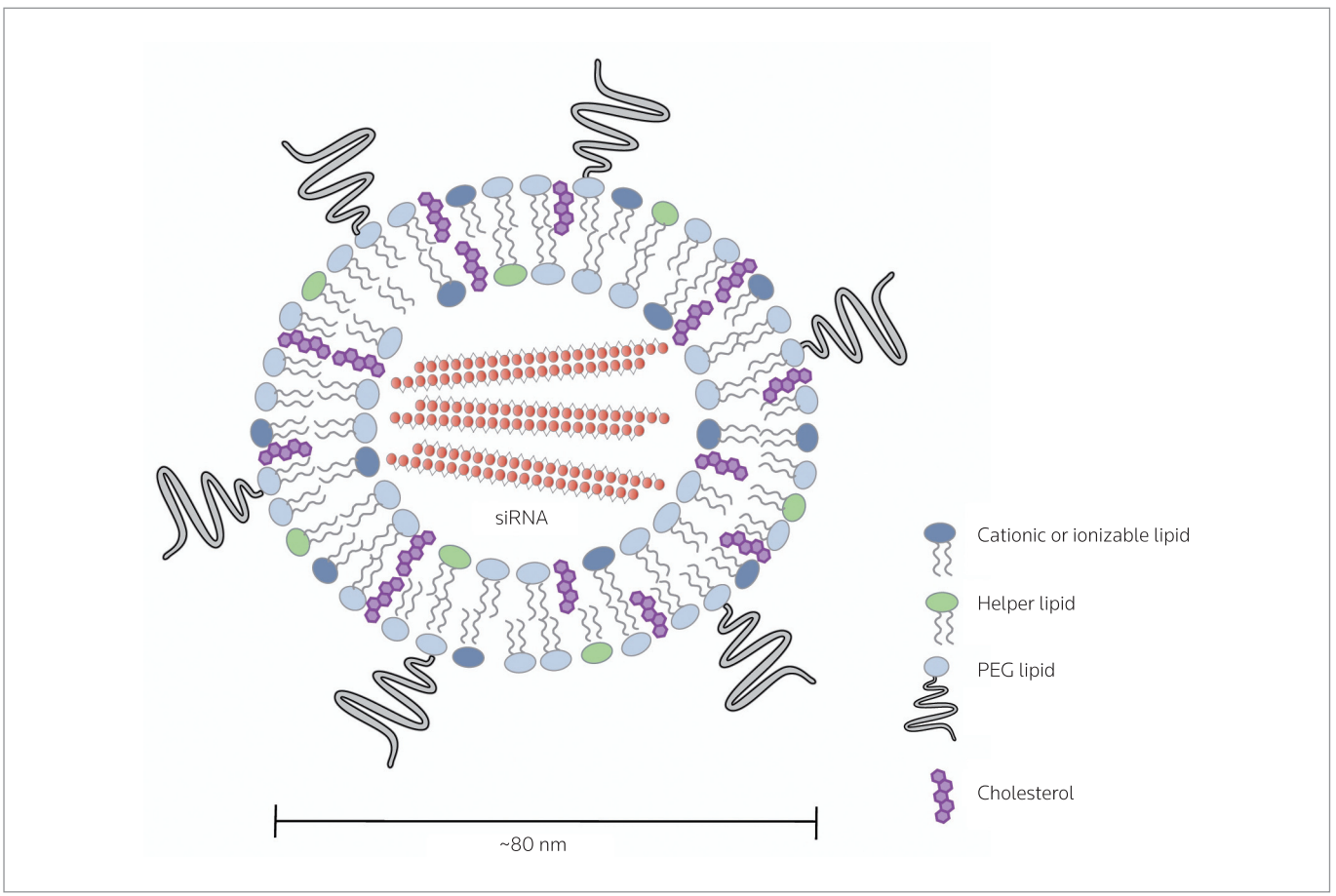

Figure 2. Components of SNALP (stable nucleic acid lipid particle). Shown are the four lipid components which encapsulate the siRNA payload.

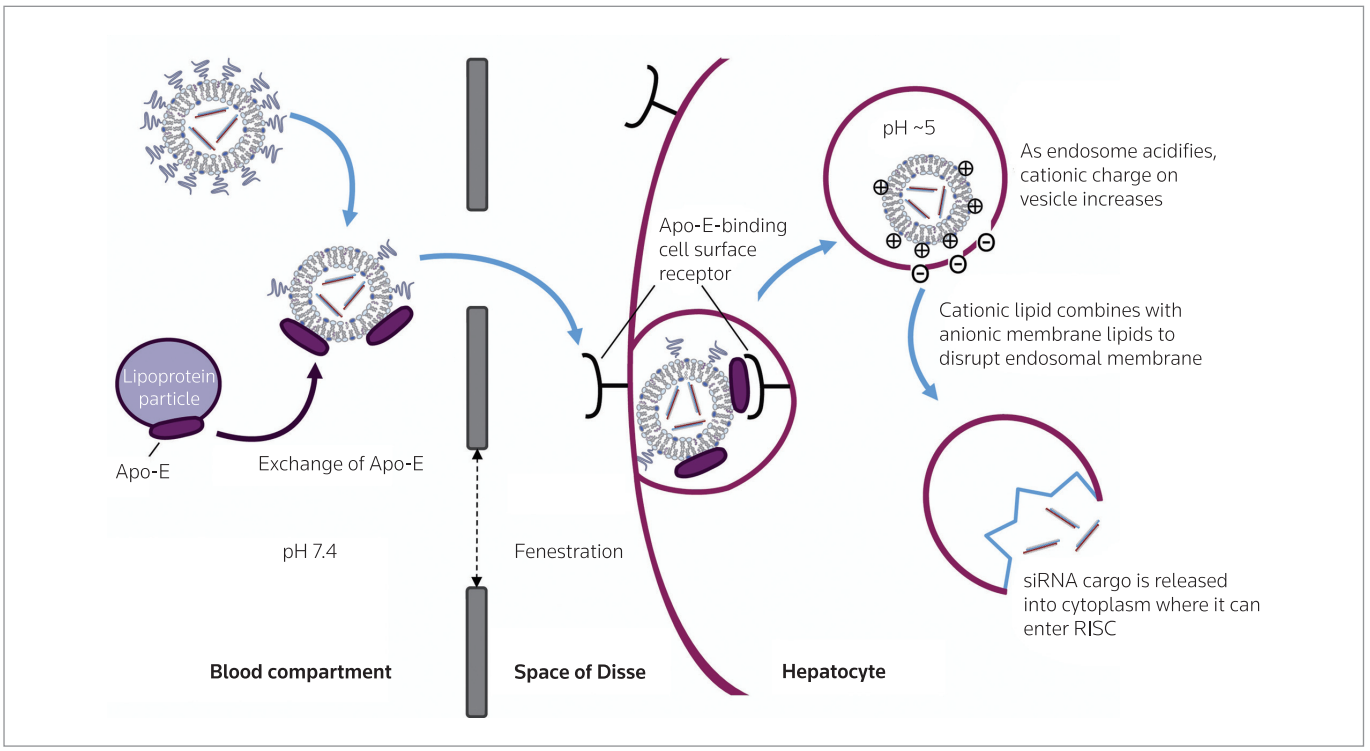

Figure 3. Apolipoprotein E (Apo-E)-dependent uptake of SNALP (stable nucleic acid lipid particle) by hepatocytes. At physiological pH in the circulation, SNALP is essentially neutral and is bound to Apo-E. It gains access to the liver by passing through sinusoidal fenestrations, where it then binds to low-density lipoprotein (LDL) receptors on hepatocytes and is internalized via endosomes. When the pH within the endosome drops, the ionizable lipid within SNALP becomes cationic, thereby facilitating fusion with the endosomal membrane and leading to release of the siRNA into the cytoplasm, where it can bind to RISC.

was 72 minutes following i.v. injection, and 68% and 90% suppression, respectively, of Apo B mRNA in the liver was observed at 48 hours after a single dose of 1.0 or 2.5 mg/kg. This effect on Apo B was accompanied by a significant drop in total and LDL cholesterol. At the dose of 2.5 mg/kg, continuous target suppression was seen for up to 11 days after a single dose. Use of the 5′ RACE analysis identified the predicted Apo B mRNA cleavage site, thereby confirming that mRNA silencing was mediated by RNAi. Multiple other hepatocyte targets, including proprotein convertase subtilisin/kexin type 9 (PC9, *PSCK9*) (52) and factor VII, have been silenced using SNALP-formulated siRNAs in multiple different species, including rats, mice, non-human primates, hamsters and guinea pigs. Across these different studies, there is a consistency of observations with respect to onset of target suppression, which generally occurs within 24 hours, with peak effects at 48-72 hours; duration, which lasts several weeks depending on the potency (and perhaps stability) of the siRNA concerned; strict dose-dependency; and return of target mRNA levels to baseline. Overall, the potency, predictable and reproducible pharmacology across different targets and species (as anticipated for an endogenous mechanism), and the selectivity and clear mechanism of action suggest a robust translational potential for LNP-based hepatic delivery of RNAi therapeutics.

To date, two clinical programs targeting hepatocytes have been initiated using SNALP-formulated siRNAs (Table II). One of these, a phase I single-dose study of SNALP Apo B in adult volunteers with elevated LDL cholesterol, was recently completed (53). In that placebo-controlled study, 17 subjects received a single dose of SNALP Apo B across 7 dose levels. One patient at the highest dose experienced flu-like symptoms consistent with immune stimulation, and a decision was made to conclude the trial. Transient reductions of plasma Apo B and LDL cholesterol of 21.1% and 16.3%, respectively, were seen in two subjects treated at the highest dose.

The second program is ALN-TTR, which targets hepatocyte production of mutant and wild-type transthyretin (TTR) to treat TTR amyloidosis (ATTR). Three ATTR syndromes have been described: familial amyloidotic polyneuropathy (FAP), familial amyloidotic cardiomyopathy (FAC) and senile systemic amyloidosis (SSA) (54). The first two are caused by autosomal dominant mutations in TTR, while the last is caused by wild-type TTR. The vast majority of circulating TTR is produced by hepatocytes, and in mice transgenic for the Val30Met mutation common to FAP, a single dose of ALN-TTR was able to potently suppress circulating TTR (mutant and wild-type) by > 90% and prevent the deposition of TTR in nerves, gut and heart (55). A placebo-controlled, dose-escalation, single-dose phase I trial of ALN-TTR in patients with ATTR was initiated in July 2010. In addition to evaluating safety, this study will examine the impact of ALN-TTR on circulating mutant and wild-type TTR levels. Another LNP-based program in the preclinical phase is ALN-PCS, which is targeting hepatocyte PC9 expression to treat hypercholesterolemia.

Table II. *Systemic delivery siRNA programs.*

Sponsor	Program (stage of development)	Status	Target	Mode of delivery	Indication
Tekmira	ApoB SNALP (phase I) (53)	Completed	Apo B	SNALP	Hypercholesterolemia
Alnylam	ALN-TTR (phase I)	Ongoing	Transthyretin (TTR)	SNALP	TTR amyloidosis
Alnylam	ALN-PCS	Preclinical	PCSK9	LNP	Hypercholesterolemia
Alnylam	ALN-VSP (phase I) (62)	Ongoing	VEGF, KIF11	SNALP	1° and 2° liver cancer
Calando	CALAA-01 (phase I) (65)	Ongoing	RRM2	Targeted cyclodextrin-based nanoparticle	Cancer
Silence	ATU-027 (phase I)	Ongoing	PKN3	Lipoplex-siRNA	Cancer
Tekmira	SNALP PLK-1 (phase I)	IND submitted	PLK-1	SNALP	Cancer

In addition to LNPs, successful delivery of siRNA to hepatocytes has also been achieved using so-called Dynamic PolyConjugates. These 10-nm particles are comprised of an endosomolytic polymer backbone to which are attached polyethylene glycol (PEG) for shielding, *N*-acetylgalactosamine (NAG) for hepatocyte targeting via binding to the asialoglycoprotein receptor, and an siRNA payload. In mice, a single dose of 2.5 mg/kg of an Apo B-1 siRNA polyconjugate resulted in 84% inhibition of liver Apo B mRNA levels 2 days after injection, with an associated decrease in serum cholesterol (56).

Systemic delivery: tumor targeting

In addition to hepatocyte delivery, LNPs have also been successfully used to deliver siRNA to tumors. While LNPs and liposomes are not structurally identical, prior experience with liposomal drugs in oncology does nevertheless provide insight into the potential of this sort of delivery approach for siRNA therapeutics. Liposomal delivery of doxorubicin chemotherapy has been successful in reducing the risk of doxorubicin-mediated cardiotoxicity, while enhancing delivery to tumors (57). The size of this liposome (~100 nm) helps to limit its egress from the circulation (thereby reducing cardiac uptake), and surface pegylation helps avoid uptake by the reticuloendothelial system (thus the term "Stealth" liposome). These features greatly prolong the circulation time, which in turn provides a greater opportunity for uptake by tumors that have a porous microvasculature (so-called enhanced permeability and retention [EPR] effect) (58). Although Apo-E opsonization and binding to LDL receptors is essential to SNALP uptake by hepatocytes, it is not yet known whether this mechanism may also play a role in LNP uptake by tumor cells.

Using siRNAs against the mitotic proteins polo-like kinase 1 (PLK-1) and kinesin-like protein KIF11, Judge et al. showed that a single dose of SNALP-formulated siRNA administered i.v. to mice at 2 mg/kg suppressed target message by 50% in human hepatocellular carcinoma Hep 3B tumors implanted into the liver (59). This was accompanied by detection of the expected mRNA cleavage fragment by 5′ RACE for up to 7 days after a single dose of drug, as well as the demonstration of mitotic inhibition in tumor cells. The suppression of tumor growth in the liver was also shown in mice receiving multiple doses of SNALP PLK-1 or SNALP KIF11. While same SNALP formulation was also able to inhibit the growth of s.c. Hep 3B tumors in mice, it was even more effective when the elimination half-life was prolonged by using a PEG lipid with a larger alkyl chain that associates more strongly with the lipid particle and thereby reduces uptake by the reticuloendothelial system. An IND for SNALP-formulated PLK-1 siRNA was filed in August 2010 (60) and a phase I study in cancer patients was initiated in December 2010.

ALN-VSP is the first SNALP-formulated siRNA therapeutic to be tested in cancer patients (Table II). ALN-VSP is comprised of two different siRNAs targeting VEGF and KIF11 (1:1 molar ratio of the two siRNAs in each particle), and as such represents the first multitargeted siRNA therapeutic. Preclinical data with ALN-VSP administered to SCID/beige mice with orthotopic Hep 3B liver tumors essentially mirrored the results of Judge et al. with regard to demonstration of tumor cell delivery, pharmacodynamic effects of both VEGF and KIF11 inhibition, and suppression of tumor growth. In addition, studies with ALN-VSP in tumor-bearing mice have shown evidence of delivery to extrahepatic tumors and to other tumor histologies (e.g., colorectal) in addition to hepatocellular carcinoma (61). A phase I dose-escalation trial of ALN-VSP given as a 15-minute i.v. infusion every 2 weeks in patients with advanced solid tumors and liver involvement was initiated in March 2009. Interim safety and pharmacodynamic data on the first 19 patients enrolled were presented at the 2010 annual ASCO meeting in Chicago (62). These data showed that ALN-VSP was generally well tolerated at doses of up to 0.7 mg/kg, with no significant changes in liver function tests in the large majority of patients and no clinically apparent immune stimulation. Serial DCE-MRI performed on liver tumors showed a ≥ 40% drop in K^{trans} in 67% of patients with evaluable scans, suggestive of an anti-VEGF effect. The study has not yet reached the maximum tolerated dose and accrual continues to dose levels beyond 0.7 mg/kg. In addition to DCE-MRI, the study also includes serial plasma VEGF and sVEGFR-2 measurements, along with tumor biopsies performed before and after the first dose to assess drug levels, VEGF and KIF11 message levels, the presence of specific mRNA cleavage fragment by 5′ RACE and pharmacodynamic effects of VEGF and KIF11 inhibition.

CALAA-01 is a targeted nanoparticle for siRNA delivery. These 70-nm particles consist of siRNA and a linear, cyclodextrin-based polymer decorated on the surface with adamantane–PEG and adamantane–PEG–transferrin, with the transferrin moiety used to target the particles to tumor cells expressing surface transferrin receptors. Studies in mice showed that while tumor localization of the nanoparticles could be achieved without targeting, silencing by the siRNA payload required targeting with transferrin, suggesting that such targeting was necessary for tumor cell uptake (63). In an orthotopic mouse model of metastatic Ewing's sarcoma, targeted nanoparticles containing an siRNA against the *EWS-FLI1* fusion gene product inhibited tumor growth (64). Based on these results, a phase I dose-escalation trial in cancer patients was initiated using an siRNA targeting ribonucleoside-diphosphate reductase subunit M2. In this study, patients received a 30-minute infusion twice weekly for the first 2 weeks of a 21-day cycle. CALAA-01 was generally well tolerated, with grade 1-2 fatigue, fever, chills and gastrointestinal symptoms seen in some patients across all dose levels (65). Encouraging results regarding drug delivery to tumor were seen in three patients with melanoma: nanoparticles were detected in cutaneous metastases, and in one of these patients treated at the highest dose level, the specific ribonucleoside-diphosphate reductase subunit M2 cleavage product was detected in tumor biopsies by 5′ RACE (66).

Another lipid-based siRNA therapeutic currently in phase I testing in oncology is ATU-027, which is a lipoplex-siRNA system that facilitates delivery to vascular endothelial cells. ATU-027 contains an siRNA targeting serine/threonine-protein kinase N3, and experiments in mice, rats and non-human primates have shown N3 downregulation in multiple tissues after dosing, most notably in lung and liver (67). In addition, ATU-027 treatment suppressed tumor growth in orthotopic mouse models of prostate and pancreatic cancer.

Taken together, these programs demonstrate that significant progress has been made in the development of delivery solutions for siRNA therapeutics in oncology. The translation of these delivery technologies into the clinic is beginning to provide valuable information in man regarding tolerability, tumor delivery and pharmacodynamic activity, which will facilitate the further expansion of these efforts across a variety of different tumor types and gene targets.

UVEAL MELANOMA: A UNIQUE OPPORTUNITY FOR RNAi THERAPEUTICS

Background

Uveal melanoma arises from melanocytes of the iris, ciliary body or choroid plexus; it is the most common intraocular malignancy in adults and represents 3% of all melanoma diagnoses. The annual U.S. incidence is 1,500-2,000, with a similar rate in Europe. Therapy of the primary tumor is radiation (plaque brachytherapy) plus laser therapy or enucleation for more advanced cases (68). Despite adequate local therapy, up to 60% of patients will develop metastatic disease within 2-5 years. The risk of metastatic disease is related to the size and location of the primary tumor and the presence or absence of abnormalities in chromosomes 3, 8q and 6. A gene expression profile has been developed that is now routinely used to discriminate between primary tumors with a low (class 1) or high (class 2) risk of subsequent metastatic spread and death (69). The liver is the initial and dominant site of metastasis in > 80% of patients who develop disseminated disease, and the median survival for patients with liver metastases is < 6 months. Uveal melanoma notably lacks mutations in B-Raf, N-Ras, PTEN and c-Kit found in cutaneous and mucosal melanomas.

There is no effective therapy for metastatic uveal melanoma. Systemic chemotherapy has produced response rates of only 5-9% and median survival of approximately 5 months, and there have been no randomized trials (70). Transarterial chemoembolization or infusion of chemotherapy into the hepatic artery can induce responses in the liver (71), but there can be substantial morbidity and mortality, and no conclusions can be drawn regarding the impact on survival in the absence of randomized trials. A recently reported randomized trial of percutaneous hepatic infusion with melphalan versus best supportive care showed a significant improvement in partial response rate and hepatic progression-free survival in the melphalan group, but no difference in overall survival (72). Treatment-related mortality was 7.5%, with deaths due to liver failure and infection.

Identification of *GNAQ* and *GNA11* as driver mutations in uveal melanoma

In 2009, van Raamsdonk et al. reported their discovery that *GNAQ* is mutated in 48% (23 of 48) of uveal melanoma samples analyzed (73). Heterotrimeric G proteins, consisting of Gα, Gβ and Gγ subunits, are ubiquitous mediators of signaling closely associated with G protein-coupled receptors (GPCRs) (74). The Gα subunit alternates between a guanosine diphosphate (GDP)-bound "off" state and a guanosine triphosphate (GTP)-bound "on" state; the intrinsic GTPase activity of Gα causes hydrolysis of GTP to GDP. The Gq class is one of four major Gα classes and consists of GNAQ, GNA11, GNA14 and GNA15. The structurally closely related proteins GNAQ and GNA11 make up the $G_{q/11}$ family. In uveal melanoma, the mutation in *GNAQ* occurs in codon 209 of exon 5, which encodes glutamine. This codon lies within a Ras-like domain essential for GTP hydrolysis, and mutations at this codon in Ras family members cause loss of GTPase activity, leading to constitutive activation of the protein. The oncogenic nature of this mutation in *GNAQ* was demonstrated by its ability to transform human and murine melanocytes, leading to anchorage-independent growth in vitro and the formation of tumors in nude mice. This cellular transformation by mutant *GNAQ* was also associated with activation of MAP kinase (ERK). Notably, in vitro inhibition of *GNAQ* expression using siRNA in a human uveal melanoma cell line containing mutant *GNAQ* led to a decrease in ERK activation and to growth inhibition and apoptosis. These results show that mutant *GNAQ* is a driver mutation in uveal melanoma and suggest that inhibition of *GNAQ* expression can reverse the malignant phenotype.

Subsequently, Bastian's group reported finding the same exon 5, codon 209 mutation in *GNA11* (75), which is 90% homologous to *GNAQ* at the amino acid level. Approximately 35% of uveal melanoma specimens analyzed to date have a *GNA11* mutation. Additional mutations can be found in exon 4 at R183 in *GNAQ* and *GNA11* in a minority of uveal melanomas (approximately 5%). All mutations in both genes are mutually exclusive, with roughly 80% of uveal melanomas having an activating mutation in either *GNAQ* or *GNA11*. The role of Gα subunits in cancer has now expanded since the earlier observation that

GNAS mutations are associated with pituitary adenomas (76) (Table III), and a recent finding that *GNAS* mutations or amplifications, as well as *GNAO1* mutations, can be found in breast, ovarian, lung and prostate cancers (77) suggests that finding ways to target these undruggable proteins may open up new treatment opportunities across multiple different tumor types.

Opportunity to target GNAQ and GNA11 with siRNA to treat uveal melanoma

Uveal melanoma appears to be one of those rare solid tumors where a specific driver mutation is found in the large majority of patients. Other solid tumors where a driver mutation has been found in a majority of patients include gastrointestinal stromal tumors (GISTs), where activating mutations in c-Kit are found in 75% (13), pancreatic adenocarcinoma (*KRAS* mutations in 67%) (78) and cutaneous melanoma (*BRAF* mutations in over 50%) (79). The targeting of c-Kit with the small-molecule kinase inhibitor imatinib has resulted in one of the most dramatic success stories of targeted therapy in oncology, with a partial response rate of 84% in GIST patients with exon 11 *KIT* mutations (13). *KIT* mutations are also found in distinct clinical subsets of melanoma (80), where they have also been demonstrated to be responsive to c-Kit inhibitors such as imatinib (81). In patients with advanced cutaneous melanoma whose tumors have the V600E *BRAF* mutation, an oral inhibitor of mutated B-Raf induced partial responses in 75% of patients (17). These demonstrations of the clinical impact of targeting driver mutations in solid tumors provide a compelling rationale for targeting *GNAQ* and *GNA11* to treat uveal melanoma. However, at this time, there is no evidence that either *GNAQ* or *GNA11* are druggable using small molecules, and both are inaccessible to monoclonal antibodies. In contrast, both targets can be inhibited by siRNA, and given the substantial homology between *GNAQ* and *GNA11*, it is possible that a single siRNA could hit both.

An siRNA therapeutic directed at uveal melanoma could consist of a single siRNA targeting both *GNAQ* and *GNA11* or two siRNAs delivered together that target both genes. This way, one drug could be used for uveal melanoma patients with either the *GNAQ* or *GNA11* mutation. One of the key issues in the development of such a drug is whether any of the delivery solutions currently available for systemic administration of siRNA would be capable of delivering siRNAs to uveal melanoma tumors in the liver and outside the liver. Since the majority of patients with metastatic uveal melanoma have disease that is predominantly in the liver, and this is usually what has the greatest impact on survival, even delivery that favored silencing within liver tumors relative to extrahepatic tumors would likely still be quite impactful. In light of the promising results with SNALP-formulated siRNA in various orthotopic mouse liver tumor models and the encouraging, albeit early, results to date with ALN-VSP02 in cancer patients with liver involvement, SNALP, or a similar LNP, may be the best near-term delivery vehicle to be used with siRNAs targeting *GNAQ* and *GNA11*. Additional work showing that LNP-formulated siRNAs against *GNAQ* and *GNA11* have antitumor activity against uveal melanoma in mouse orthotopic liver tumor models would be important in this respect, as would data from the phase I ALN-VSP02 trial showing that siRNA can be delivered to uveal melanoma tumors in the liver.

In addition to the potential of siRNAs directed against *GNAQ* and *GNA11* to treat metastatic uveal melanoma through systemic delivery, there may also be local delivery opportunities for such siRNAs (82, 83) to treat the primary tumor in the eye. In particular, if it can be shown in animal models that intravitreal siRNA can silence targets within uveal melanoma tumors implanted in the choroid, it may be possible one day to use this method instead of plaque brachytherapy and laser treatment, or in combination with brachytherapy in order to obviate the need for laser therapy, and thereby better preserve vision in the treated eye. One might also envision treating systemically in the adjuvant setting following local therapy in patients with the class 2 gene expression signature who are at substantial risk of disease spread and death.

Table III. *Gα subunit mutations in cancer.*

Gene	Gα class	Mutated codon	Tumor type
GNAQ	Gq	Q209	Uveal melanoma (73)
GNA11	Gq	Q209	Uveal melanoma (75)
GNAS	Gs	R201	Pituitary adenomas, breast*, ovarian* (76, 77)
GNAO1	Go	R243	Breast, lung, ovarian, prostate (77)

**GNAS* also found to be amplified.

CONCLUSION

In summary, the progress that has been made over the past several years in both local and systemic delivery is starting to define the path forward for transforming RNAi from a laboratory research tool to an established drug platform. Potential advantages to an RNAi-based approach to cancer include an ability to hit otherwise undruggable targets, allele-specific targeting of mutated oncogenes, the capacity for high-specificity multitargeting and expanded opportunities for tailored pharmacotherapy ("personalized medicine"). The discovery of *GNAQ* and *GNA11* mutations in uveal melanoma provides a unique opportunity for demonstrating how siRNA therapeutics may extend the reach of targeted therapy in oncology. The clinic is the ultimate proving ground for this exciting new drug development technology, and future successes in development will depend upon matching well-validated target opportunities with appropriate delivery solutions.

DISCLOSURES

The authors (except B.C. Bastian) are employees of Alnylam Pharmaceuticals.

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