What's new, or not, in siRNA

By Tim Fulmer, Staff Writer

A *Nature* paper suggests that locally delivered naked short interfering RNAs exert their effects mainly by stimulating the innate immune response and not by directly silencing target genes.¹ But biotechs polled by *SciBX* maintain that the potential effects of siRNAs on the innate immune response via toll-like receptors are not new news.^{2,3} And siRNA therapeutic developers do not plan on changing their preclinical or clinical development strategies.

Indeed, many research programs already focus on the design of siRNA molecules and delivery systems to avoid immunostimulatory off-target effects while effectively silencing genes within the cell (see **Table 1, "Selected short interfering RNA compounds in or about to enter the clinic"** and **Figure 1, "Staking out the RNAi landscape"**). Such strategies include chemically modifying siRNA molecules to prevent their recognition by the immune system.⁴

In the *Nature* paper, Jayakrishna Ambati, professor of physiology, ophthalmology and visual sciences at the **University of Kentucky**, and colleagues report that a panel of nontargeted and random-sequence double-stranded siRNAs at least 21 nucleotides in length, directly

delivered intravitreally as naked nucleic acid, significantly reduced angiogenesis in a mouse model of age-related macular degeneration (AMD) compared with buffer or no injection ($p < 0.05$).

In experiments with toll-like receptor 3 (TLR3) knockout mice of the same AMD model, the antiangiogenic effects of the siRNAs disappeared ($p < 0.05$). According to the authors, these results suggest that antiangiogenic effects of the siRNA molecules are not a result of targeted effects but rather a result of agonism of cell-surface TLR3.

The panel of siRNAs included bevasiranib (Cand5) from **Opko Health Inc.** and Sirna-027 (AGN211745) from **Merck & Co. Inc.**, two clinical-stage siRNAs targeted against the mRNAs of VEGF and VEGF receptor 1 (FLT1).

"We believe our results unequivocally show that siRNAs, including the two that we tested that are in clinical trials for macular degeneration, activated TLR3 and function solely through TLR3," Ambati told *SciBX*. "Given the wealth of data showing that, in animal models and in human cells, TLR3 activation can induce adverse side effects, it is my considered opinion that prudence dictates that siRNAs in ongoing and planned trials be rigorously evaluated for their ability to activate TLR3."

By contrast, Sam Reich, EVP of Opko's ophthalmologics division, told *SciBX* that "based on the data Ambati has presented in his paper, it is not possible to determine at what level, if any, the TLR3 mechanism may contribute to the efficacy or safety profile of our drug."

The *Nature* paper "will have no effect" on the design of the company's Phase III trial of bevasiranib to treat AMD, he said.

"Our previously conducted trials in more than 200 patients have demonstrated the safety of bevasiranib, and our ongoing Phase III trial is designed to definitively prove the efficacy of this drug," Reich said.

Table 1. Selected short interfering RNA compounds in or about to enter the clinic.

Company	Product	Target	Formulation	Delivery	Indication	Status
Opko Health Inc.	Bevasiranib	VEGF	Naked	Local	Wet age-related macular degeneration (AMD)	Phase III
Alnylam Pharmaceuticals Inc. (NASDAQ:ALNY)	ALN-RSV01	Respiratory syncytial virus (RSV) nucleocapsid (n)	Naked	Local	RSV	Phase II
Merck & Co. Inc. (NYSE:MRK)/ Allergan Inc. (NYSE:AGN)	Sirna-027	VEGF receptor 1 (FLT1)	Naked	Local	Wet AMD	Phase II
Quark Pharmaceuticals Inc./ Pfizer Inc. (NYSE:PFE)/ Silence Therapeutics plc (LSE:SLN)	RTP801i-14	RTP801, a gene discovered by Quark	Naked	Local	AMD	Phase I/II
Quark/Silence	AKli-5	p53	Naked	Systemic	Acute kidney injury	Phase I
Calando Pharmaceuticals Inc.	CALAA-01	Ribonucleotide reductase	Nanoparticle carrier	Systemic	Cancer	IND
Silence	Atu027	Protein kinase N3 (PKN3)	Lipid carrier	Systemic	Gastrointestinal cancers	Start Phase I in 2008
Protiva Biotherapeutics Inc., merging with Tekmira Pharmaceuticals Corp. (TSX:TKM)	ApoB SNALP	Apolipoprotein B (ApoB)	Lipid carrier	Systemic	Hypercholesterolemia	Start Phase I in 2008/early 2009

Figure 1. Staking out the RNAi landscape. RNAi is a means of lowering the expression of disease-associated proteins that exploits a set of naturally occurring RNA processing enzymes. Several steps along the process serve as entry points for therapeutic approaches being developed by companies in this space. RNAi begins with a transgenic construct that expresses a short, double-stranded transcript, which resembles naturally occurring microRNA [a]. The transcript is trimmed down by the nuclear RNase Drosha complex [b] and exported into the cytoplasm [c]. Here, the transcript is further processed by the Dicer complex, first into a 21-nucleotide duplex, termed short interfering RNA, and then into two single strands, called the ‘guide’ and ‘passenger’ [d]. The guide gets incorporated into the RNA-induced silencing complex (RISC) [e], which binds to mRNA that is complementary to the guide sequence [f]. This process results in inactivation of the target mRNA through a variety of mechanisms, including cleavage and degradation, that prevent protein synthesis from happening [g].

“We are continuing to closely monitor the safety of bevasiranib in the latter trial as well.”

Merck partner **Allergan Inc.** is running a Phase II AMD trial of Sirna-027. Allergan spokesperson Heather Katt said the *Nature* paper “has not impacted the way Allergan is monitoring the safety and efficacy” of the trial.

She also noted the compound in the study “has some chemical modifications to the published sequence that make it different from the compound Ambati used. And in any case, we actually want an anti-angiogenic effect since we are treating the neovascular form of AMD.”

In a Phase I trial, Sirna-027 was well tolerated and produced no signs of inflammation.

Ambati told *SciBX* that one key next step for his research is “carrying out experiments that measure the differential contribution of the two mechanisms—targeted gene silencing and nontargeted TLR3 activation—to the overall effect of siRNA treatment.”

“The bottom line is that one has to be conscious of the TLRs since these are going to be activated by double-stranded RNA, single-stranded RNA and DNAs that lack the proper backbone modifications

that block TLR recognition,” said John Rossi, associate director for laboratory research in the Comprehensive Cancer Center and professor of molecular biology at the **City of Hope National Medical Center**.

Rossi is cofounder of **Dicerna Pharmaceuticals Inc.**, a biotech founded in November of last year and focused on the design of double-stranded RNAs for *in situ* processing by the Dicer complex into the corresponding siRNAs. The company is developing therapeutics in the field of ophthalmology and against some undisclosed viral targets.

No surprises

Most siRNA companies polled by *SciBX* were not surprised by the findings in *Nature*. Reports of TLR activation by double-stranded RNA go back as far as 2001.⁵ Also, many companies are focused on systemically delivering carriers of siRNA and are not concerned with locally delivering naked siRNA to diseased tissue^{6,7} as Ambati did in his paper.

For companies with naked siRNA therapeutics, the *Nature* findings mostly reinforce the designs of preclinical or clinical studies that are

already in place.

John Maraganore, CEO of **Alnylam Pharmaceuticals Inc.**, said the article “is a solid piece of work, though in many ways not especially surprising. It points out the importance of a preclinical siRNA drug development strategy that has been pursued by Alnylam from the beginning.”

Maraganore said Alnylam first designs some 200 siRNAs per target and screens them for *in vitro* potency and TLR activation. “A subset of promising candidates will then be chemically modified to increase stability, and these modified forms are also tested for immunostimulatory activity,” he said. “At that point, we’ve usually winnowed down the group to 10 or fewer siRNAs that are optimally potent without toxic immunostimulatory effects.”

These are then characterized in mouse models for their ability to silence target mRNA without causing a toxic immune response. “The latter can be measured by looking at cytokine levels downstream of TLRs in tissues and/or in circulation,” Maraganore said. “Our *in vivo* studies also include appropriate siRNA controls and molecular proof of RNAi using a nucleic acid amplification technique called 5'-RACE.”

Alnylam's lead therapeutic is ALN-RSV01, an aerosolized naked siRNA in a Phase II trial to treat respiratory syncytial virus (RSV) infection.

Maraganore said the company has found that ALN-RSV01's effects in the RSV study are independent of fluctuations in the patient's cytokine levels. Thus, he said, “activation of the innate immune response is almost certainly not occurring in our patients. At least for Alnylam, I don't believe Ambati's paper will have any impact on the way we are running our RSV trial or designing future studies on naked, modified or formulated siRNAs.”

Similarly, Elena Feinstein, CSO of **Quark Pharmaceuticals Inc.**, expects the *Nature* paper will not alter the development path for the company's RTP801i-14, a saline-formulated siRNA in Phase I/IIa testing to treat AMD. The 19-nucleotide siRNA inhibits expression of *RTP801*, a hypoxia-inducible gene.

Quark and **Silence Therapeutics plc** jointly discovered the RTP801i-14 (REDD1) siRNA. Quark then developed the compound for AMD under license to Silence's siRNA chemical structure and subsequently granted **Pfizer Inc.** exclusive rights in 2006.

In mouse and primate models, an intravitreal injection of the siRNA produced antiangiogenic activity.

“In preclinical studies, we saw that it mainly had an anti-inflammatory effect, whereas activation of TLRs is usually proinflammatory,” noted Feinstein. “Our compound's gene-specific antiangiogenic effects on the same AMD model that Ambati used were significantly more prominent compared to the TLR-mediated effects reported in the *Nature* paper. Finally, our siRNA is a 19-mer, whereas according to Ambati's paper, TLR3 activation occurs when siRNA is a 21-mer or longer.”

Quark investors were clearly not put off by the *Nature* paper—the company raised \$27 million in a series H round last week.

Special delivery

Although companies using naked siRNA believe they already have covered their bases via multiple assays for innate immune response activation, companies that use an siRNA delivery system say the

Nature findings are potentially less of an issue.

“Ambati's paper simply points out, among other things, that local injection of naked siRNA can trigger an immune response,” said Klaus Giese, CSO of Silence Therapeutics. “That effect is not new, and it likely has little direct relevance to an approach like ours, which packages the siRNA in a carrier designed to deliver siRNA therapeutic to the interior of the cell without invoking an immune response.”

Silence's AtuRNAi-lipoplex (AtuPLEX) technology—chemically modified siRNA designed for increased stability, activity and avoidance of immune response and formulated with three lipid components to allow for cellular uptake—has shown efficacy and safety in mouse models of cancer⁸ and other disease areas.

In 2007, Silence and **AstraZeneca plc** partnered to discover and develop siRNA molecules against up to five undisclosed pharma company targets for respiratory diseases using the biotech's siRNA technology. Earlier this year, the companies partnered to discover and develop undisclosed siRNA drug delivery technologies.

Like Silence, **Protiva Biotherapeutics Inc.** is using a lipid-based delivery vehicle. Protiva's ApoB SNALP, an siRNA against apolipoprotein B (ApoB) mRNA encapsulated in liposomal stable nucleic acid lipid particles (SNALP), is expected to begin a Phase I trial to treat hypercholesterolemia this year or early in 2009. The formulation demonstrated systemic safety and efficacy in nonhuman primates.⁹

In addition to the delivery vehicle, CSO Ian MacLachlan said Protiva “always monitors siRNA candidates for both immunostimulatory and RNAi activity. Moreover, we're careful to correlate reductions in target mRNA and protein with clinically relevant physiological endpoints.”

At the end of the day, MacLachlan expects Ambati's paper could be beneficial for the siRNA space. “It's hardly a setback for the field,” he said. “Over the long haul, the paper should contribute to improved strategies for identifying RNAi therapeutics with minimal immunostimulatory activity, where that's required.”

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COMPANIES AND INSTITUTIONS MENTIONED

Allergan Inc. (NYSE:AGN), Irvine, Calif.
Alnylam Pharmaceuticals Inc. (NASDAQ:ALNY), Cambridge, Mass.
AstraZeneca plc (LSE:AZN; NYSE:AZN), London, U.K.
City of Hope National Medical Center, Duarte, Calif.
Dicerna Pharmaceuticals Inc., Boston, Mass.
Merck & Co. Inc. (NYSE:MRK), Whitehouse Station, N.J.
Opko Health Inc. (AMEX:OPK), Miami, Fla.
Pfizer Inc. (NYSE:PFE), New York, N.Y.
Protiva Biotherapeutics Inc., Vancouver, British Columbia, Canada
Quark Pharmaceuticals Inc., Fremont, Calif.
Silence Therapeutics plc (LSE:SLN), London, U.K.
University of Kentucky, Lexington, Ky.