Tech News

RNAi Therapeutics: The Teenage Years

By any measure, the ascendance of RNA interference has been meteoric. Discovered in 1998, and garnering a Nobel Prize in Medicine just eight years later, the topic of RNAi had by that point racked up nearly 8,900 citations on PubMed, and at least one therapeutic in the clinic.

In the intervening years, the pace of RNAi therapeutic research and development has dramatically accelerated. A 2011 review in *Nature Reviews Genetics* counted 25 RNAi-based clinical trials (1), and more are coming. Just one Boston-area pharmaceuticals developer, Alnylam, has four products in various stages of clinical development.

The remarkable speed, and interest, stem in part from the fact that RNAi potentially represents a unique class of therapeutics, one that targets biological processes not with some random hit from a chemical library, but via an endogenous, programmable gene silencing mechanism. As a result any protein, not just enzymes and surface receptors, can theoretically be targeted – a distinct advantage over small molecule and antibody therapeutics.

But as is often the case, the euphoria of the technology's early years has given way to a reality check in adolescence, and for RNAi therapeutics that means delivery. RNAs must be packaged such that they aren't filtered out of the blood by the kidneys. They must be chemically tweaked to resist nuclease digestion and minimize off-target effects. Ideally, they should be targeted so they direct their payloads only to desired cells. And simply finding the target isn't enough, either: Once the therapeutic finds the desired tissue or tumor, it has to bind and enter the cell, escape the endosome, and find the cytoplasmic RISC (RNA-induced silencing complex) to silence the targeted, complementary mRNA.

"To this day, delivery remains an issue," says Beverly Davidson, the Roy J. Carver Biomedical Research Chair of Internal Medicine at the University of Iowa College of Medicine, who co-authored the 2011 review.

Phillip Zamore, the Gretchen Stone Cook Professor of Biomedical Sciences at the University of Massachusetts Medical School, agrees. "The way most of us who are financially conflicted would say it, I think, is that many of us underestimated the delivery hurdle," says Zamore, who co-founded Alnylam and sits on its advisory board ("You don't get more financially conflicted than that," he quips).

The recognition that RNAi would not quickly yield up blockbuster drugs, led the previously bullish speculation in RNAi therapeutics to turn decidedly bearish. Companies like Novartis and Roche, which just a few years earlier had invested hundreds of millions in the technology, were by 2010 and 2011 shifting their priorities. Investors, too, backed away. From a high of $36.55 in October 2007, for instance, Alnylam was trading at $6 per share four years later, and when Novartis declined to expand their collaboration in September 2010, the company had to lay off nearly 50 of its 225 employees.

"When you have a new technology like RNAi it takes a while to turn it into a drug," says Alan Carr, a senior analyst with Needham & Co., who tracks the RNAi therapeutics market. "I think people began to realize there were some challenges there and it was going to take a while to solve them."

Today enthusiasm is building again. A string of exciting, albeit preliminary preclinical and clinical results have to some extent reinvigorated investor interest. "There's still a way to go," says Carr, "this is Phase 1 data. But I think it was a very important proof-of-concept, something we didn't have before."

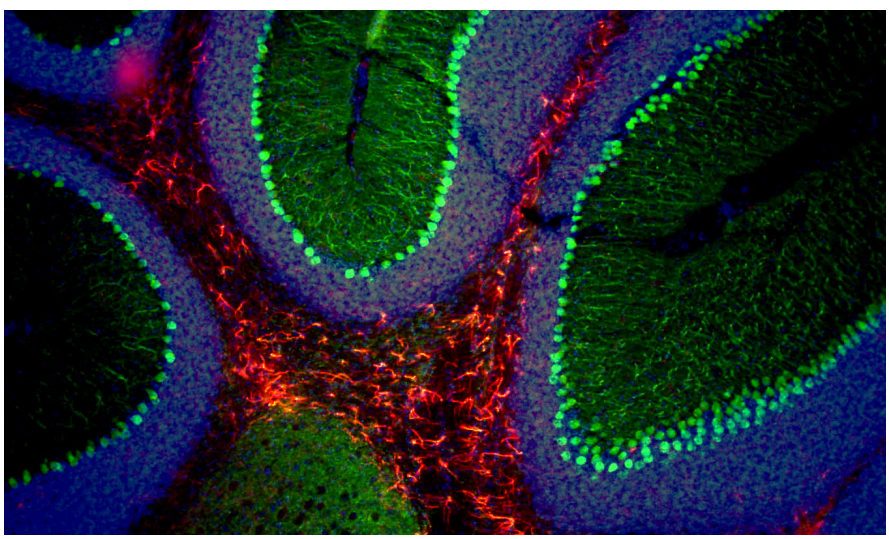
Of lipids and nanoparticles

After a decade of work on RNAi therapeutics, the one organ most readily and commonly targeted remains the liver. Other targets are coming along, albeit slowly. Yet according to MIT Chemical Engineering professor Daniel Anderson, even if siRNA therapy were limited to that organ, it would still have important utility. "Look, if you could knock out any gene in the liver that you wanted, I think that could be a tremendously powerful technology for a collection of important diseases," he says.

Alnylam specifically targets diseases that can be impacted via the liver. In April, the company announced data from its Phase 1 trial of ALN-PCS, an siRNA targeting PCSK9 in severe hypercholesterolemia. PCSK9 is a protein that marks the low-density lipoprotein (bad cholesterol) receptor for degradation. The idea is that by blocking the expression of PCSK9 in the liver, LDL receptor abundance should increase, causing LDL levels to fall.

The study was a Phase 1, "randomized, single-blind, placebo-controlled, single-dose escalation study" in 32 healthy volunteers with elevated LDL cholesterol, administering anywhere from 0.015 — 0.400 mg/kg of ALN-PCS intravenously. As a Phase 1 study, the nominal goal was to assess safety. Yet after just a single dose, the researchers observed a decrease in plasma PCSK9 levels of up to 84% in the highest-dose group, with concomitant reduction in LDL cholesterol of up to 50%. The effect, the company says, lasted at least a month.

In May, the company announced the final data from its Phase 1 trial of another



Beverly Davidson and colleagues use viruses to target RNAi to the mouse cerebellum in spinocerebellar ataxias. Shown here are Purkinje cells (green), astrocytes (red), and nuclei (blue). Courtesy: Megan Keiser and Beverly Davidson

therapeutic, ALN-TTR01, which hits the transthyreitin gene in TTR-mediated amyloidosis. "ALN-TTR01 demonstrated a dose-dependent reduction in serum TTR levels with a statistically significant mean reduction of 38% at approximately day 7 to 10 in the 1.0 mg/kg group," the company announced. One patient exhibited 63% knockdown at 48 hours, and 50% knockdown at 30 days.

"I was impressed," says Carr. The effect from both studies was "profound and durable. In both cases it was a single infusion and the effect lasted up to a month with dramatic reductions. So, it's a very good start."

Neither therapeutic is delivered as a naked siRNA; they are chemically modified, for instance with 2′-*O*-methyl groups, to reduce nuclease sensitivity and immunostimulatory side-effects, and packaged inside lipid nanoparticles (LNPs). The specific formulation, says Barry Greene, Alnylam's President and Chief Operating Officer, "is a set of lipids that encapsulate the siRNA that targets it to the liver and then helps with escaping to the cytoplasm, which is where the RNAi machinery works."

In ALN-PCS, that formulation is called MC3, a "second-generation" lipid that "confers benefit at much lower doses" than first-generation formulations, says Greene. Now the company is using that same lipid to package the siRNA from ALN-TTR01, producing a new therapeutic called ALN-TTR02.

The ALN-PCS data, says Greene, "gives us tremendous enthusiasm that this second-generation LNP should work very well." Preclinical data in nonhuman primates with ALN-TTR02 indicate that 0.3 mg/kg yields a 70% gene knockdown at approximately 30 days post-injection, compared to essentially no effect with ALN-TTR01 at 1mg/ml at that point.

Yet development on LNP formulations continues. In a 2010 study partially funded by Alnylam, Anderson's team described a "lipidoid" called C12-200 that promotes hepatocyte uptake in both non-human primates and mice at doses two orders of magnitude lower than an earlier formulation. Packaging a TTR-targeting siRNA with C12-200 reduced mRNA levels in cynomolgus monkeys by nearly 70% at just 0.03 mg/kg (2). To date, no Alnylam clinical trial has been based on Anderson's formulations, but that could change: Anderson's work, says Zamore, "is just stellar."

Moving away from the liver

One approach to move beyond the liver is to guide siRNAs to the desired cells using a specific targeting molecule, such as an antibody. "You get the drug largely to the cells that you want to target, so there's less toxicity to cells that aren't the intended targets, as well as, you need less of the drug to do the job," says Judy Lieberman, a Senior Investigator at Children's Hospital Boston.

MIT researcher Daniel Anderson, whose work on RNAi delivery Zamore calls "stellar." Courtesy: Chemical Engineering Department, MIT

Lieberman, along with Erwei Song at Sun Yat-sen University in China, recently highlighted the targeted approach in *Science Translational Medicine* (3). Using a fusion protein containing a single-chain antibody to the Her2 receptor on one end, and a highly basic peptide called protamine on the other, Lieberman and Song were able to knock-down Polo-like kinase 1 (PLK1) expression by about 75% in a mouse model of breast cancer. More significantly, treatment blocked growth of Her2-positive tumors at 7 weeks post-transplantion, as well as metastasis.

"This paper shows really impressive results in treating both primary Her2-positive breast cancer as well as a metastatic model of Her2 breast cancer," says Lieberman, who suggests the key to the approach's efficacy could be the protamine peptide, which helps it escape the endosomal compartment. "People who work on drug delivery have a theory that you need to have a basic charge in the endosomal vesicle for the endosome to release what's inside, and protamine is one of the most basic chemicals in the body." Protamine also condenses the nucleic acids into a more compact form — a result of the protein's normal function condensing DNA in sperm. "That may be partly why it works so well systemically, because it's so small that it can get into all the tissues of the body."

At the University of Iowa Carver College of Medicine, Paloma Giangrande takes a different approach — rather than targeting siRNAs using antibodies, Giangrande takes advantage of RNA aptamers.

"The advantage of the small RNA approach is you are dealing with a one-component system," Giangrande explains. That's in contrast with multicomponent systems like Lieberman's in which each individual component must be separately approved for human use. RNA offers other advantages, too: It is relatively stable in solution, and non-immunogenic.

In a 2006 proof-of-concept study, Giangrande's team described a 71-nucleotide aptamer targeting prostate-specific membrane antigen linked to the 21-nucleotide "passenger" (ie, non-silencing) strand of an siRNA targeting PLK1. This 92-mer was then annealed to the silencing strand (ie, the strand loaded into RISC) of the siRNA to produce the final formulation. In cells, the complex is processed to release a functional siRNA able to silence its target both in vitro and when injected directly into tumors in vivo.

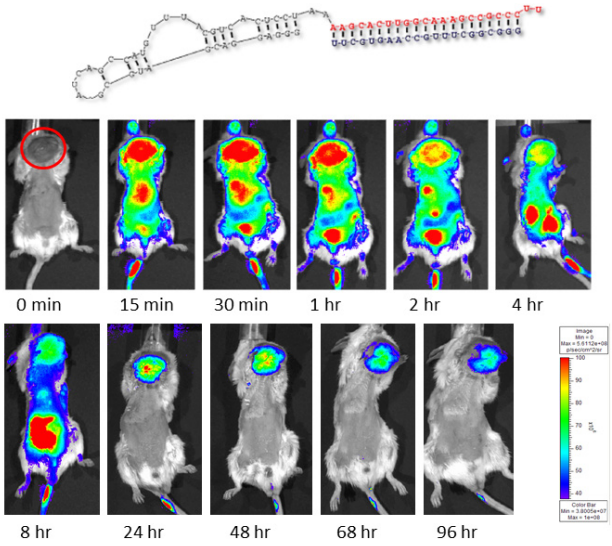
Three years later, Giangrande has optimized the aptamer's design, shrinking it from 71 bases to 41 and reversing the siRNA orientation so the silencing strand is coupled to the aptamer instead of the passenger strand. Those changes, says Giangrande, make the design amenable to large-scale chemical synthesis (a struggle with longer RNAs) and more potent. "That enabled us to see efficacy upon systemic administration of the RNAs," she says.

Other researchers are also pursuing all-RNA approaches. Lieberman has used it to block HIV transmission in mice by targeting CD4+ T cells in the female genital tract, while Eli Gilboa, the Dodson Professor of Microbiology and Immunology at the University of Miami Miller School of Medicine, applied the approach to essentially force tumor cells to become immunogenic. "We use aptamer-targeted siRNA to deliver siRNA to tumor cells in the body for the purpose of making them look more foreign, because we decorate them with new antigens," says Gilboa.

Giangrande is currently working on a third generation aptamer design that not only targets PSMA, but also inhibits its enzymatic activity, which has been linked to cancer. This makes the strategy potentially doubly powerful, hitting the cell at its receptor and with the target of the siRNA. Her team also recently published a new aptamer enrichment strategy to select for molecules that not only bind a given target in the context of the cell membrane, but get internalized as well. "Not all aptamers can do this," she notes.

In cell-internalization SELEX, a library of randomized aptamers is pre-cleared by incubation with cells not expressing the desired antigen, then incubated with cells that do. Internalized aptamers are collected by washing cells with salt, lysing, and amplifying the internalized molecules again for another cycle. Using this approach, Giangrande's team demonstrated the ability to deliver an siRNA targeting the anti-apoptotic gene Bcl-2 in Her2-expressing mouse mammary carcinoma cells, while sparing non-expressing controls.

"Rather than a trial-and-error approach to finding receptors that will serve as good targets for aptamer-siRNA cargos, this cell-based



Targeting with RNA: A fluorescently labeled RNA aptamer (top), injected through the tail vein, recognizes and targets a human prostate tumor (red circle) in an immunocompromised mouse. The chimera is cleared from the body within 24 hr but persists in the tumor up to 4 days. Source: Paloma Giangrande

selection strategy allows us to directly select for sequences that internalize into a particular cell type," the authors write (4).

RNAi on the brain

Giangrande's colleague at the University of Iowa, Beverly Davidson is pursuing yet another RNAi delivery approach. Davidson studies neurodegenerative diseases like Huntington's Disease and spinal cerebral ataxias, targeting them not with exogenous siRNAs but rather virally expressed small RNAs.

The viral approach, Davidson explains, offers delivery benefits not seen with exogenous siRNAs — resistance to nucleases and packaging, for instance, which can be especially beneficial for chronic diseases like Huntington's. "For chronic diseases ...where there may be a requirement for sustained activity or engagement of the RNAi machinery to silence the mutant allele, [the viral approach] may provide a one-stop shop where you introduce the material once and you get benefit for years on end," says Davidson.

To do that, her team infects the brains of lab animals with adeno-associated virus vectors expressing short RNAs that target the mutant huntingtin gene in the context of a microRNA called miR-30. Processing that miR-30 produces the active siRNA, which then silences the huntingtin gene.

Davidson's team first demonstrated this approach was safe and effective in rodents, and now, along with Jodi McBride at the Oregon Health and Science University, has shown it is also safe in non-human primates. When injected into a brain region called the putamen in normal (ie, non-Huntington) rhesus macaques, the virus directed suppression of the huntingtin gene by 45% at six weeks, without concomitant neurological or inflammatory side effects.

Although there have been some successes to date, RNAi therapeutics has yet to score a home run, says Davidson. Still, many believe the field has most definitely turned a corner; several years ago, researchers were grappling with both delivery and the technology of RNAi itself. The latter has largely been addressed, she says, while the former remains. The only way to close that gap, is to keep plugging along. It took monoclonal antibodies nearly two decades to achieve blockbuster status, and RNAi is just in its early teens. It's had its "irrational exuberance" stage, and it's had its inevitable doom-and-gloom letdown stage. Now, it seems, the clouds could be breaking, if only just. "We're still in phase 1, early phase 2 for RNAi," says Carr. "It's [still] a few years to market."

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Written by Jeffrey M. Perkel, Ph.D.

BioTechniques 52:355-357 (June 2012)
doi: 10.2144/000113871