Double Strand and Deliver

Before RNAi-based drugs get into the clinic, gene silencing molecules need to get into humans. Luckily, the delivery dilemma has attracted an army of investigators whose efforts are already widening the bottleneck.

By Jennifer Crebs

When Tom Tuschl and colleagues broke the story about RNA interference in mammalian cells, it didn't take long for researchers to start thinking about the possibility of harnessing this gene silencing technology for human disease. At this point, most in the know agree that the last major hurdle is one of delivery. A bit of chemistry, a bit of biology, and a whole lot of experimentation have resulted in several promising avenues — and even some RNAi-based drug clinical trials.

Harnessing the power of RNAi for therapeutics could spell the end of certain diseases that aren't served well by current drugs. It's a very potent technology, says Anastasia Khvorova of Dharmacon, because "the job is being done by protein complexes that have evolved to do it for millions of years."

Stimulating the RNAi pathway can be accomplished by either smuggling siRNAs or promoters encoding short hairpin RNAs into cells. In the latter case, the shRNA is processed along the lines of microRNA precursors inside the cellular cytoplasm.

Researchers agree that the main challenge for RNAi-based drug development is finding delivery methods to get small RNAs into selected cells or tissues with high specificity, low toxicity, and at a reasonable concentration. Depending on the disease target, siRNAs and shRNAs can be delivered either locally or systemically. To keep siRNAs stable, chemical modifications and formulations abound. It is a complex area of research and "there will probably be many solutions to the problem of delivery," says Judy Lieberman, whose Harvard lab succeeded in using small interfering RNAs to achieve disease protection in a mouse model.

DELIVER LOCALLY

When Sailen Barik of the University of South Alabama first learned of Tuschl's results, he thought about the potential of RNAi to prevent and treat respiratory virus infection, his own bailiwick.

"Viral messages are pretty much cytoplasmic, they are translated with the same machinery, so we thought, 'Why wouldn't they be subject to the same pathway as RNAi?'" First with cell culture, then via intranasal delivery in mouse models, he found that siRNAs could in fact prevent infections caused by respiratory syncytial virus and parainfluenza virus. Intranasal administration is ideal for these indications, Barik says, as siRNAs remain restricted to the lung and are thus unlikely to cause systemic side effects. Moreover, siRNAs delivered for RS virus don't seem to require specialized packaging to enter cells, Barik says, perhaps because those virus-infected cells have more compromised, more damaged, and more permeable membranes that allow uptake of siRNAs. "In a way, that's a blessing because it makes the siRNA more specific to infected cells," Barik says. "And if they don't go into uninfected cells, that's fine."

These benefits have not escaped the notice of pharmaceutical companies, and some early clinical trials for drugs targeting respiratory conditions and age-related macular degeneration are yielding promising results. Some companies with drug candidates in the works include Acuity Pharmaceuticals, Alnylam Pharmaceuticals, and Sirna Therapeutics. Why these indications? "The mucosal surfaces of the body seem to be amenable to RNAi delivery," Lieberman says, so the first RNAi-based drugs to hit the clinic will likely target tissues that are relatively easily accessible. For some indications, however, the story may be more complicated.

SILENCE GLOBALLY

Many groups are contending with how to protect siRNAs from degradation on their trip to the cell. Liposomal encapsulation, transfection reagents, and chemical modifications are a few strategies to get siRNAs where they need to go. It's rough terrain, and RNAi researchers want to avoid off-target effects and activation of toll-like receptor pathways, particularly those activating interferon response or NF-kappa B.

"I think the ideal method would be one that gives systemic delivery, but [is] modifiable so that ... you can selectively go after certain types of tissue," says John Rossi of City of Hope Comprehensive Cancer Center. It would also have staying power at reasonable concentra-

tions, he says, and "would not be rapidly cleared through the kidney or liver."

Dharmacon's Khvorova echoes these concerns, warning that "whenever you hijack a natural pathway, it has to be done very carefully to ensure that there are no long-term consequences." To thwart off-target effects, her team has applied bioinformatics, chemical modifications of both siRNA strands, and a pooling strategy to mimic the natural combinatorial effects of mixtures of siRNAs working in concert.

Toxicity due to the induction of interferon response can be managed by controlling the structure, as well as the chemical composition of siRNAs, Khvorova says. More importantly, she says, "formulation itself might be reason or a trigger for substantial toxic effects." Many studies have focused on lipid nanoparticles, polynanions, or polymers used to formulate siRNAs, but, as Barik puts it, "there's no golden rule" for success in this area.

Troy Moore, CTO of Open Biosystems, a company that specializes in the delivery of shRNAs, points to the specificity and stability of shRNAs, which can also be hooked up with a molecular marker to monitor subtle changes in the cell. "Certainly we need to keep picking apart the mechanism" that underlies shRNA specificity, Moore says, "as we are getting to the point where we need to know what else is happening for eventual clinical use."

Rossi has had success in delivering shRNAs using viral vectors, both *in vivo* and *ex vivo*. Adenovirus and adeno-associated virus have the advantage of transducing cells efficiently *in vivo*, he says, while lentiviral vectors can slip into nondividing cells like those in the brain. Rossi has used lentiviral vector-borne shRNAs to transduce hematopoietic cells taken out of a patient, which were then reinfused to inhibit HIV-1 infection. Recombinant effects cropping up are not a huge concern with this method, he says, as "new vectors are so carefully designed that [risk] is minimal."

The consensus in the field is that there's still work to be done before your pharmacist will be doling out RNAi in a box. "There's a lot riding on RNAi," says Barik, pointing out that significant studies will be needed to bring this tool to the clinic. "We'll just have to see how it all pans out." GT

Many Roads to RNAi

This year has been marked by substantial activity in the RNAi-based drugs space. Relatively direct injection or aerosol methods have been used in early clinical trials to deliver naked siRNAs into easily accessible tissues, such as the eye, skin, or lungs. Here are details on a sampling of recently developed siRNA delivery technologies and techniques.

Antibody-mediated

Last year, Judy Lieberman's team published results on a non-toxic, antibody-based method of delivering siRNA into cells efficiently and with high specificity. The key to this technology is protamine, which compresses strings of DNA in sperm and can bind siRNAs. Lieberman's group engineered an antibody-protamine fusion protein capable of delivering siRNA cargo into HIV-infected or envelope transfected cells. The results show that antibody-protamine method is safe, flexible, and capable of delivering higher amounts of active siRNA. Alnylam licensed the technology, and may use it to develop therapeutics for cancer and certain viral diseases.

Aptamers

Bruce Sullenger and researchers at Duke University have combined aptamers — RNA ligands that bind to target proteins with high affinity and specificity — with siRNAs to create an agent capable of inhibiting tumor growth and regression. In a study published earlier this year in *Nature Biotechnology*, Sullenger's team cooked up a molecule in which the aptamer mediates binding with a cell-surface receptor that is overexpressed on the surface of prostate cancer cells. As the receptor is endocytosed, the siRNA package can be slipped into cells.

Cyclodextrin-based polymers

Calando Pharmaceuticals has developed a means of delivering its siRNA drug targeting solid tumors, such as neuroblastoma, via intravenous injection. Delivery of the drug is accomplished through a linear, cyclodextrin-containing polycation that piggybacks onto the siRNA backbone. The polymer and the siRNA join together to create nanoparticles, which are resistant to nuclease degradation in blood serum.

Nanoparticles

Copernicus Therapeutics is working on using nanoparticles to deliver siRNAs. The technology compresses single molecules of DNA to their smallest possible size in order to sneak them into cells. Once the molecules are compacted, they bind to nucleolin, a cell membrane protein present on the surface of ocular and neural cells, which ferries the resulting nanoparticles into the nucleus. Because the method doesn't involve endosomes or lysosomes, degradation isn't an issue.

SNALPs

Alnylam has an exclusive license from Inex Pharmaceuticals for the use of a liposomal-based drug delivery method. Inex spinout Protiva Therapeutics developed the strategy, called SNALPs for stable nucleic acid-lipid particles, which encapsulates oligos by cationic and fusogenic lipids, themselves surrounded by a polyethylene glycol coating to prevent clearance of the lipids from the bloodstream. Early this year, an Alnylam-led team of researchers showed that SNALPs can be used to systemically deliver siRNAs in non-human primates. —JC