VIEWPOINT

SCIENTIFIC DISCOVERY AND THE FUTURE OF MEDICINE

Harnessing RNA Interference for Therapy
The Silent Treatment

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RNA interference (RNAi) is a ubiquitous pathway that regulates gene expression. It uses small, imperfectly paired, double-stranded RNAs approximately 21 nucleotides long, called microRNAs, that are processed from longer stem-loop transcripts.[1] MicroRNAs are taken up by the cytoplasmic RNA-induced silencing complex (RISC), which removes 1 strand, leaving an unpaired strand that binds to messenger RNAs (mRNAs) with a partially complementary sequence. RISC suppresses the expression of bound mRNAs by accelerating their degradation and suppressing their translation into protein. MicroRNAs and the RNAi gene-silencing pathway were first discovered in the 1990s in plants, worms, and flies. In those organisms, microRNAs play an important role in regulating changes in gene expression that occur in development and in protection from viruses.

In 2001, Elbashir et al[2] discovered that the RNA interference (RNAi) pathway could be directly accessed in mammals. They showed that transfection of small double-stranded RNAs (called small interfering RNAs [siRNAs]), which are exactly complementary to sequences of a cellular mRNA, cause the targeted mRNA to be degraded—selectively knocking down or silencing the expression of the gene. These siRNAs enter the microRNA pathway by binding to the RISC, which directs cleavage of the target mRNA. This discovery led to the development of therapies designed to knock down disease-causing genes. Researchers quickly demonstrated that knocking down HIV genes or HIV receptors could inhibit HIV replication in vitro, and injection of siRNAs targeting the Fas cell surface death receptor (*FAS* [NCBI Entrez Gene 14 102]) could protect mice from lethal hepatitis.[3]

In principle, RNAi could be harnessed to knock down any mRNA, expanding the universe of drug targets beyond the enzymes and receptors targeted by conventional small molecules. Although gene knockdown was not as specific as initially thought, because of incremental silencing of genes possessing partially complementary sequences and stimulation of innate immune viral RNA receptors that induce interferons (and indeed the promising clinical results of early siRNA trials were probably due to off-target interferons), soon chemical modifications of siRNAs were identified. These chemical modifications virtually eliminated these off-target effects without sacrificing on-target silencing of the intended target gene. Taken together, these results spurred some to hail siRNAs as the next new class of drugs. Very quickly hundreds of biotech companies were started and major pharmaceutical companies prepared to develop RNAi-based drugs.

However, enthusiasm waned when delivering small RNAs into the cytoplasm of cells to the RISC proved difficult. There are 2 bottlenecks to delivering negatively charged RNAs into cells—crossing the plasma membrane and, once in the cell, getting out of endosomes. These barriers also plague development of other small oligonucleotide drugs that use other mechanisms, such as antisense inhibition or inhibition of splicing, to modulate gene expression. However, siRNAs have advantages over other antisense oligonucleotide strategies. Once taken up into the RISC, the active strand of the siRNA is protected from digestion by cellular RNA-degrading enzymes (RNases) and is typically stable and active in the cell for weeks. More importantly, the same siRNA complex catalytically cleaves multiple mRNAs. Because of the catalytic nature of RNAi, it is estimated that less than a few hundred siRNAs per cell are needed to silence a target gene.

Because the liver is the main blood-filtering organ that separates useful metabolites from harmful toxins and particulates, it is easier to deliver RNAi-based therapeutics to liver cells than to other internal organs. Accordingly, development of siRNA drugs has mostly focused on targets in the liver. Since the liver is also the body's major producer of many metabolites and secreted proteins, gene knockdown in the liver can potentially be used to treat many diseases. These include rare genetic diseases caused by mutations of genes encoding proteins produced in the liver, such as enzymes needed for heme biosynthesis, cholesterol metabolism, clotting proteins and complement, common metabolic syndromes in which proteins synthesized in the liver play a critical role, and diseases primarily involving the liver such as infectious hepatitis and nonalcoholic fatty liver.

Lipid nanoparticles (LNPs), incorporating cationic lipids that bind negatively charged RNAs, lodge in the liver where they are taken up by hepatocytes to knock down gene expression. The earliest clinical trials targeting the liver used first-generation LNPs that had a low therapeutic index. By modifying their lipid composition and the chemistry of the RNAs they carry, current-generation LNPs are able to knock down expression of a target gene transthyretin (mutation of this gene causes amyloidosis) in the liver by 80% to 95% for 1 month—without any serious adverse events.[4] Currently transthyretin-targeting LNPs (patisiran, 0.3 mg/kg given every 3 weeks) are in phase 3 studies for familial amyloidotic polyneuropathy, a progressive fatal disease. An open-label extension study of patisiran showed an encouraging slight improvement in neurological score in patients with this disorder when compared with the decline expected (based on historical control patients).

Another strategy for hepatocyte delivery uses siRNAs conjugated at one end with a polyvalent sugar

(GalNAc) specifically recognized by the liver cell asialoglycoprotein receptor.[5] GalNAc conjugates, given subcutaneously, knock down liver gene expression by approximately 95% for at least 1 month with little or no toxicity. GalNAc conjugates targeting transthyretin (revusiran) are now in phase 3 studies for treatment of the amyloidotic cardiomyopathy caused by mutations of the same gene. GalNAc conjugates are simpler to manufacture and, although with less experience, better tolerated than LNPs and have the advantage of subcutaneous vs intravenous administration. It is likely that in the future, these conjugates could become the method of choice for gene knockdown in the liver. Phase I studies using this platform have already begun to knock down proprotein convertase subtilisin/kexin type 9 ([*PCSK9*] NCBI Entrez Gene 255738]) for potential treatment of familial hypercholesterolemia and complement C5 for paroxysmal nocturnal hemoglobinuria, hemolytic uremic syndrome, and potentially other complement-mediated diseases.

An unconventional approach to treating hemophilia that uses GalNAc-conjugated siRNAs to knock down antithrombin 3 is also being assessed in phase 1 trials. Instead of replacing the missing clotting factors, this treatment attempts to stop bleeding by interfering with the inactivation of thrombin. This intervention may be especially valuable for patients with hemophilia who develop inhibitory antibodies to replacement clotting factors. Among the few patients with hemophilia who have received this treatment, thrombin generation and bleeding times improved and a patient with severe disease did not have clinically apparent bleeding episodes over a few months.

Another approach to harnessing RNAi therapeutically uses small oligonucleotides (synthetic versions with enhanced stability and binding) to mimic or antagonize endogenous microRNAs. Humans express more than 1000 different microRNAs and the expression pattern varies with cell type and state of differentiation and activation. Each microRNA suppresses hundreds of genes with partial complementarity, but the degree of suppression of each gene is usually much less than for siRNAs that have perfect complementarity to their target gene. Because they affect many genes, microRNA-based drugs may be more difficult to design and control than siRNAs. Intracellular delivery is again the main obstacle to miRNA-targeted therapeutics and the liver has been the initial focus of clinical studies. The most abundant microRNA in liver cells, miR-122, stabilizes the hepatitis C virus (HCV) genomic RNA and is needed for viral replication.

In a recent phase 2 study of HCV infection, administration of miR-122 antisense oligonucleotides (miravirsen) in 5 weekly doses caused a dose-dependent reduction in plasma viremia that was below the level of detection in 4 of 9 patients at the highest dose.[6] Of note, no drug resistance developed, presumably because all viral strains relied on miR-122. Interim results from a recent phase 1 study of GalNAc-conjugated miR-122 antagonist (RG-101) showed a 4-log reduction in viremia after a single dose without any serious toxicity. However, the recent approval and potency of viral inhibitor drugs that cure hepatitis C virus after a short course make it unlikely that antisense miRNAs will compete for this market. Some microRNAs are globally reduced in cancer cells, especially in the most malignant cancers such as mixed-lineage leukemia and triple-negative breast cancer, and replacement of microRNAs that act as tumor suppressors is an attractive strategy. MicroRNAs that enhance cardiac function have also been identified. The future of microRNA-based therapy for these indications, however, will require solving delivery outside the liver.

The potential applications of RNAi-based therapies could expand greatly if clinically suitable methods of delivering small RNAs to tissues beyond the liver are available. The mucosal surfaces of the body are accessible and relatively easy to target. Although replacing GalNAc with targeting moieties that bind to cell-type specific cell surface receptors would seem like an attractive approach to targeting internal tissues, some of the early attempts have not worked. Another attractive strategy for targeted RNA delivery is conjugation of 1 strand of the siRNA to an RNA aptamer, a structured piece of RNA selected for high-affinity binding to a cell surface receptor. Indeed aptamer-siRNAs have been used to knock down gene expression in cancers and lymphocytes to suppress tumor growth and HIV transmission and infection, respectively, in mice. Another popular approach is targeted nanoparticles, but efficient delivery into tissues, while avoiding trapping in the liver and other organs, remains a challenge.

In the past 2 years, clinical development of RNAi-based drugs has increased rapidly with impressive and durable gene knockdown in the liver in humans. Although there is always concern that potent knockdown of any gene could lead to toxicity, genetic deficiency of some of the genes being targeted is well-tolerated in adults. In addition to the indications discussed previously, within the next 2 years, clinical evaluation of siRNA treatment of porphyrias, iron overload, and hepatitis B infection should begin. In the near future, it is likely that a surge in preclinical and clinical studies will occur first to treat orphan diseases and then more common diseases in which knocking down a gene expressed in the liver could provide clinical improvement. The promising results of recent clinical studies and preclinical work in primates and smaller animals suggest that RNAi-based drugs are well-poised to become the next new class of drugs.

ARTICLE INFORMATION

Conflict of Interest Disclosures: Both authors have completed and submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Dr Lieberman reports being on the scientific advisory board and Dr Sharp reports being a founder, director, and chair of the scientific advisory board of Alnylam Pharmaceuticals. Dr Lieberman reports receipt of personal fees and nonfinancial support from Alnylam Pharmaceuticals outside the submitted work; having 3 patents issued, 1 patent issued and licensed (Alnylam; not actively being developed), 2 patents pending (patents allowed), and 1 provisional patent filed (awarded patents were for siRNA-based microbicides and treatments for HSV-2 [herpes simplex virus] and HIV, miRNA-based treatments for hematological cancer, and methods that use antibodies or aptamers for targeting small RNAs into specific cell types).

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