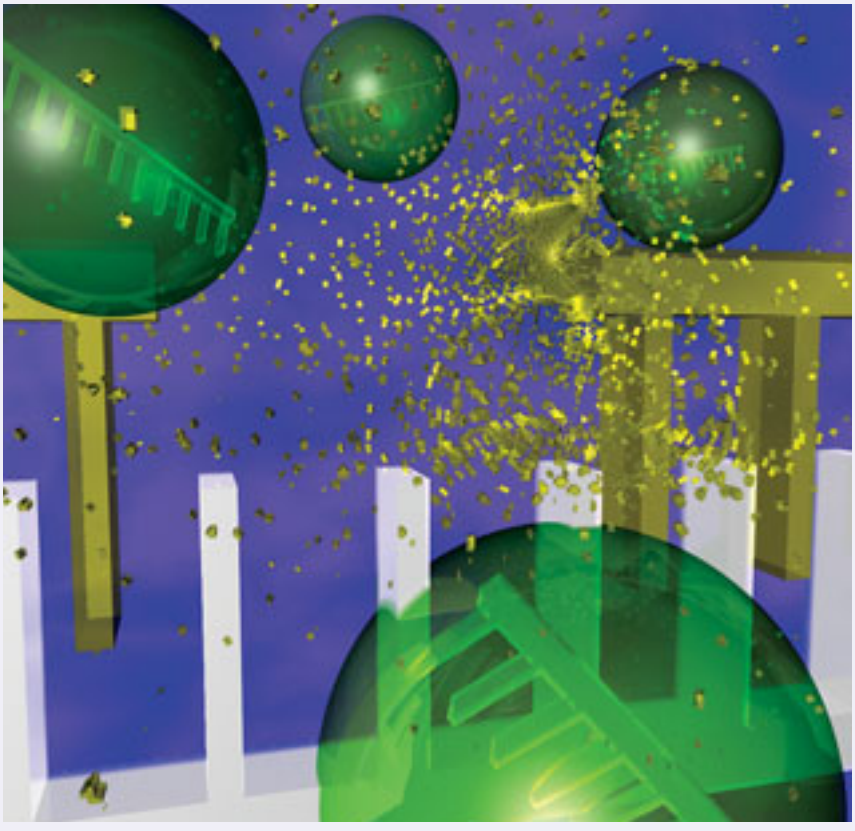
Therapeutic Interference

Small RNA molecules act as blockers of disease metabolism.

BY CHARLES W. SCHMIDT

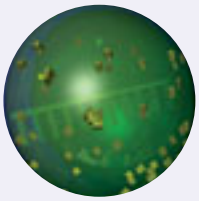
To say that biomedical researchers are enthusiastic about the new technology called RNA interference (RNAi) would be a blatant understatement. It would be more correct to say that the excitement surrounding RNAi—whereby gene expression is selectively turned off using short strands of RNA—is cranked up full tilt.

Last December, the editors of *Science* described RNAi as the "breakthrough of the year." Nobel laureate Phillip Sharp, a biologist at the Massachusetts Institute of Technology (*www.mit.edu*) agreed, calling RNAi the "most important and exciting breakthrough of the last decade, perhaps multiple decades." Several companies are exploring RNAi opportunities for drug therapy and drug target validation (Table 1), and one of them—Alnylam Pharmaceuticals (*www.alnylam.com*), co-founded by Sharp—was able to secure $17 million in venture capital funding last July even though it was barely off the ground. Alnylam CEO John Maraganore predicts that in the future, RNAi-based therapies will be used for many illnesses, including cardiovascular disease, rheumatoid arthritis, diabetes, and cancer. Similarly, Ribopharma (*www.ribopharma.de*) in Kulmbach, Germany, claims to be on the verge of clinical trials with RNA compounds targeted at malignant melanoma and pancreatic cancer.



Those are high expectations, but don't expect them to be met soon: RNAi has much to overcome before it delivers revolutionary

new drugs to the market. According to Luc Van Parijs, an immunologist at MIT, the cost of producing RNAi-based therapeutics is high, side effects are potentially severe, and physical delivery of the drugs to diseased cells is extremely challenging. Nevertheless, RNAi protagonists are bullish about the technology's future. "The more we learn about RNAi, the more useful it becomes," says Alnylam co-founder Phillip Zamore, who is also an associate professor at the University of Massachusetts Medical School (*www.umassmed.edu*). "We've only scratched the surface of its potential."



"RNAi will be used to dissect signaling pathways, to discover genes important to embryonic development, and to elucidate the function of novel genes in other fundamental biological processes," Ruprecht says.

A NEW TRICK FOR AN OLD IDEA

RNAi's therapeutic premise—that RNAs involved in disease can be selectively blocked with drugs—isn't new. For more than 10 years, scientists have tried to use "antisense" techniques to silence genes that contribute to disease. Antisense drugs are single-stranded chemically modified DNA molecules that are designed to bind directly to disease-related mRNA molecules and disable them (Figure 1). However, antisense has long been a problematic technology, and only one antisense drug—Vitravene, produced by Isis Pharmaceuticals (*www.isip.com*) to treat eye infections in AIDS patients—has won U.S. FDA approval. In most cases, antisense compounds degrade rapidly, so their effects are transitory and their potency is low. Furthermore, no one really knows how the process works. Antisense has perpetually frustrated researchers: The efficacy of new compounds can seem arbitrary, and gene-silencing effects are typically obtained only with considerable trial and error.

Like antisense, RNAi works by interfering with mRNA, but there are important differences. To begin with, whereas antisense compounds are wholly synthetic, RNAi relies on double-stranded short interference RNA molecules (siRNAs) that exist naturally in the cells of many species. "Therefore, their functioning is somehow connected to normal endogenous pathways for development," explains Christian Petersen, a researcher in Sharp's laboratory. "It's a natural way to silence genes."

In RNAi experiments, specific siRNAs are designed to bind with their mRNA counterpart. Then, once introduced to cells, the synthetic siRNAs are recognized by the intracellular machinery and guided toward specific proteins (Figure 1). The siRNAs bind with these proteins, and the resultant complexes migrate throughout the cell, disabling one mRNA after another in ways that are poorly understood. Exactly how many mRNAs a single siRNA–protein complex can disable remains unclear. Extrapolating from in vitro experiments, Zamore suggests that thousands might be disabled. Clearly, therefore, RNAi may have a major advantage over antisense in terms of drug potency.

Apart from drug development, Ruth Ruprecht, an immunologist at the Dana Farber Cancer Institutes and professor at the Harvard Medical School (*www.med.harvard.edu*), suggests that RNAi will also have an immediate impact on functional genomics. Silencing genes quickly and easily could give scientists new windows into how genes participate in cellular processes. Scientists could simply turn the genes off and then see what happens. This is an important advance for biology because even

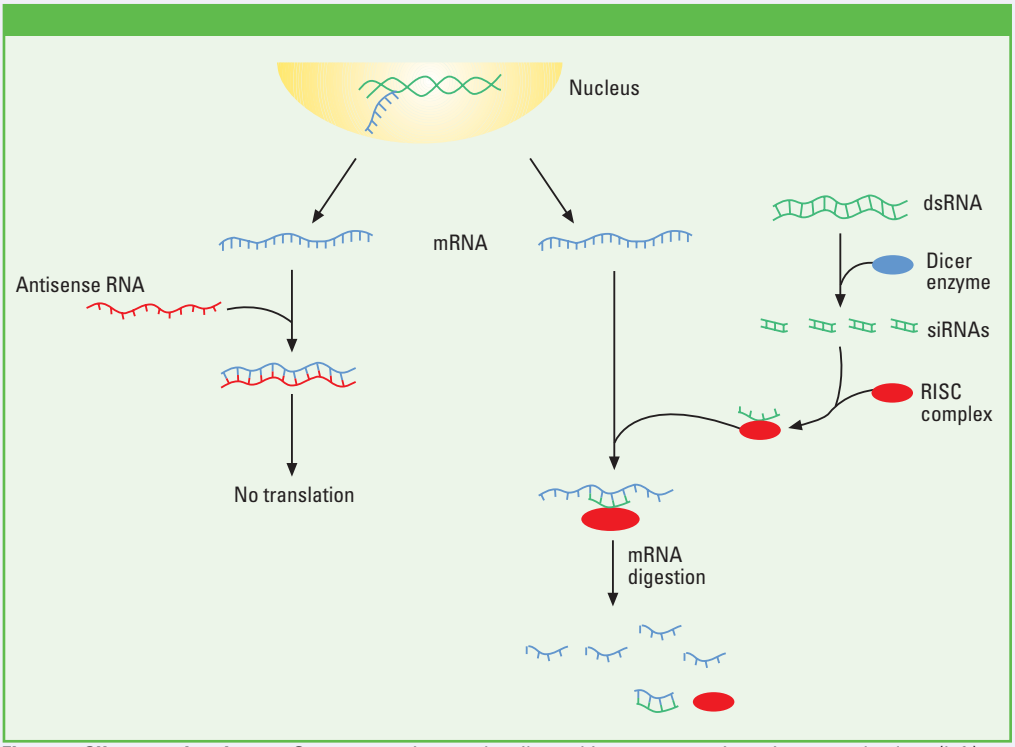
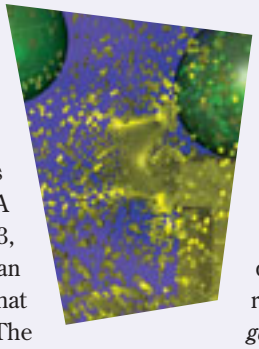


Figure 1. Silence and antisense. Gene expression can be silenced in many ways. In antisense technology (left), an oligoribonucleotide binds with the mRNA molecule to prevent protein translation. With RNAi technology, however, the Dicer enzyme cleaves double-stranded (ds) RNA molecules into short interference (si) RNA molecules, which are shuttled by the RISC protein complex to the mRNA molecule. Once bound, the complex digests the mRNA molecule, preventing translation.

though most of the human genome has been decoded, little is known about what vast regions of the genome actually do. "RNAi will be used to dissect signaling pathways, to discover genes important to embryonic development, and to elucidate the function of novel genes in other fundamental biological processes," Ruprecht says.



RNAi techniques in these more functional studies are analogous to the gene knockout methods used to identify and validate drug targets (see "A knockouts tale", *Modern Drug Discovery*, June 2003, p 26). If, for example, silencing a particular gene can cure a diseased animal, then the protein encoded by that gene is an attractive target for drug development. The standard technique in these experiments involves breeding transgenic animal species such as the p53 knockout mouse, a mutated strain in which this tumor-suppressor gene has been inactivated. But the process of breeding transgenics is expensive and time-consuming. RNAi could dramatically accelerate this process, researchers say.

"Say you have 100 kinases and you want to know which one in that pathway could be a druggable molecule," Zamore explains. "Using RNAi, a mid-size academic laboratory could knock those 100 kinases down in a month. That's a whole lot faster than breeding 100 knockouts." RNAi might also allow researchers to "interrogate" several genes simultaneously, Zamore adds. This is crucial because disease typically involves complex networks of genes working in tandem through a range of interconnected pathways.

At the recent Applications of RNA Interference conference held in San Diego, researcher Thomas Kidd described some of the work using RNAi technology that was being done at Exelixis, Inc. (*www.exelixis.com*) (*1*). Using RNAi to knock down the expression of genes throughout the genomes of *Drosophila*, *Caenorhabditis elegans*, and zebrafish—standard model organisms—an Exelixis team disrupted the function of more than 2700 druggable gene targets in just 16 months. Among their discoveries were several genes involved in the ceramide pathway, which is critical to programmed cell death. The researchers then inhibited the functions of human versions of these genes in tumor cells and found that they could disrupt cell proliferation in at least two cell lines.

The road to RNAi discovery

The term RNAi was coined in 1998 by researchers Andrew Fire, then at the Carnegie Institution of Washington (*www.ciw.edu*), and Craig Mello from the University of Massachusetts Medical School upon their discovery that double-stranded RNA molecules were remarkably potent inhibitors of a targeted gene in *C. elegans* (*2*). The technique was later shown to work in flies and plants but not in mammalian cells. This is mainly because the RNAs were destroyed by mammalian immune systems. Later, Thomas Tuschl, a biochemist then at the Max Planck Institute for Biophysical Chemistry (*www.mpibpc.gwdg.de*) and now at Rockefeller University (*www.rockefeller.com*), overcame this problem by trimming the RNA molecules to 21–23 nucleotides. Tuschl's tiny siRNAs evaded the immune system to disable mRNAs in mammalian cells. Tuschl's discovery, announced at the RNA Society's annual meeting in Banff, Alberta, Canada, in May 2001, stunned researchers, who had essentially given up on RNA targets (*3*). Thus enabled, RNAi quickly leapt to the forefront of biomedical research.

The next key event took place in Sharp's laboratory at MIT. Collaborating with Judy Lieberman and Premlata Shankar of the Harvard University Center for Blood Research, Sharp demonstrated that siRNAs could stop HIV infections in cell cultures, thereby demonstrating the technology's clinical potential (*4*). Independently, Mario Stevenson at the University of Massachusetts Medical School confirmed these findings (*5*). Lieberman then showed that massive doses of siRNA injected directly into the bloodstreams of mice protected liver cells from the effects of chemically induced fulminating hepatitis (*6*). In this case, the siRNAs targeted *Fas*, a gene that induces

Table 1

Exploiting RNAi

Therapeutics

Company	Website	Target
AGY Therapeutics, Inc.	www.agyinc.com	CNS disorders
Alnylam Pharmaceuticals	www.alnylam.com	Diabetes, cancer, arthritis
Cenix BioScience	www.cenix-bioscience.com	Cancer
Devgen	www.devgen.com	CNS disorders
OSI Pharmaceuticals	www.osip.com	Cancer
Nucleonics Inc.	www.nucleonics inc.com	HIV, hepatitis
Ribopharma AG	www.ribopharma.de	Cancer
Ribozyme Pharmaceuticals	www.rpi.com	Hepatitis

Screening

Company	Website
Amgen Inc.	www.amgen.com
Benitec	www.benitec.com.au
Deltagen	www.deltagen.com
Eos Biotechnology	www.eosbiotech.com
Exelixis Pharmaceuticals	www.exelixis.com
Immusol Inc.	www.immusol.com
Millennium Pharmaceuticals	www.mlnm.com
Novartis Research Foundation	http://web.gnf.org
Regeneron Pharmaceuticals	www.regeneron.com

the apoptosis of liver cells when overexpressed. Human *Fas* is also involved in liver diseases.

Recently, Francis Chisari and colleagues at The Scripps Research Institute (*www.scripps.edu*) tested the capability of RNAi to inhibit hepatitis C virus (HCV) RNA replication (*7*). The researchers developed a series of siRNAs against various host and viral genes and transfected the RNA molecules into cultured HCV-infected cells, looking for signs of viral RNA replication using reverse transcriptase real-time PCR and Northern blot analysis. The researchers found that several of the siRNAs inhibited viral replication within two days of transfection, suggesting that RNAi might provide a valuable therapeutic tool. Furthermore, they speculated that RNAi might play a role in viral clearance during natural HCV infections and that "therapeutic induction of RNAi, either alone or in combination with [interferon] treatment, might represent an alternative approach for the treatment of chronic HCV infection."

The road ahead

Although promising, these findings also illustrate how far RNAi has to go before it yields human drugs. The injections in this case, given in three bolus doses, were equivalent to nearly half the animal's total blood volume. When asked what major challenges lie ahead, Zamore said, "Delivery, delivery, delivery."

Presently, researchers are embedding siRNAs in cationic lipids that pass through cell membranes and deliver their products into the interior of the cell. These methods are fine for cultured cells, says Zamore, but he adds, "I wouldn't want anyone injecting cationic lipids into my bloodstream."

An alternative method currently being developed by Van Parijs involves attenuated lentiviruses that deliver genes encoding for siRNAs. Van Parijs says that this approach could be especially useful for designing gene-knockout species for functional studies or drug target validation. But he's skeptical that the technique, which is analogous to gene therapy, could be applied to making RNAi-based drugs. "Whenever you talk about introducing a genetic element using a retrovirus, you have to be concerned about screwing up the genome and producing cancer," he explains. "That's a valid concern and one that has to be addressed."

Another key difficulty, Van Parijs adds, is the expense of synthesizing the required RNA molecules. It costs thousands of dollars to produce enough RNA for experiments in tissue cultures, he says. "In humans, every injection might run you $1000." He adds that the costs could conceivably be reduced by economies of scale: Should the technology prove therapeutically successful, pharmaceutical plants could make RNA in sufficient volume to bring down costs.

As its protagonists point out (in what seems to be a growing turf battle between the two approaches), antisense is farther along in terms of drug development than is RNAi. Frank Bennett, vice president for antisense research at Isis, says the company now has a group of 12 new antisense drugs in various stages of clinical trials. "It's not relevant to say one approach is better than the other," Bennett argues. "The reality is that RNAi is a new and exciting area in biology, and it has demonstrated value in target validation as have other antisense mechanisms. Our conclusion from investigating these mechanisms is that there is no single mechanism that is vastly superior to the others. They all have potential utility."

On this point, however, Alnylam's Maraganore begs to disagree. "RNAi is a catalytic process that uses the cell's own machinery to disable mRNA," he says. "So the process is very different, and so is the potency, which is up to a thousandfold greater. This is an important aspect of how we think RNAi therapeutics are going to be developed and made successful."

Table 2

Some suppliers of RNAi tools and reagents

Company	Website
Amaxa Biosystems	www.amaxa.com
Ambion	www.ambion.com
Compugen	www.cgen.com
Dharmacon	www.dharmacon.com
Eurogentec	www.eurogentec.com
Galapagos Genomics	www.galapagos genomics.com
Gene Therapy Systems	www.genetherapy systems.com
GenScript Corp.	www.genscript.com
Imgenex Corp.	www.imgenex.com
Intradigm Corp.	www.intradigm.com
InvivoGen	www.invivogen.com
Invitrogen Corp.	www.invitrogen.com
Qiagen	www.qiagen.com/siRNA
Mirus Corp.	http://genetransfer.com
Molecula Research Labs	www.molecula.com
MWG Biotech Inc.	www.mwgbiotech.com
New England Biolabs, Inc.	www.neb.com
Novagen	www.novagen.com
Oligoengine	www.oligoengine.com
Proligo LLC	www.proligo.com
Promega Corp.	www.promega.com
Sequitur, Inc.	www.sequiturinc.com
Spring Bioscience	www.springbio.com
Stratagene	www.stratagene.com

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