Antisense Has Growing Pains

Efforts to develop antisense compounds as therapies for cancer, AIDS, and other diseases have encountered some unexpected questions about how the drugs really work

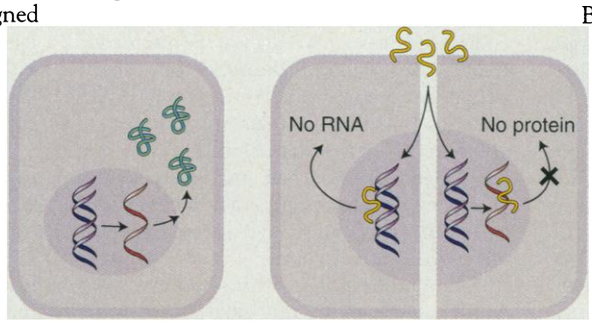
When *Science* named the gene-blocking technique known as antisense technology runner-up for its 1992 "Molecule of the Year," the accolade seemed well deserved. At the time, the technology appeared to offer a promising way to turn specific genes on or off at will. And that had made it potentially a powerful tool for uses ranging from fundamental molecular biology to the development of pharmaceuticals. Indeed, firms, both new and established, were rushing to exploit the technology to produce novel, rationally designed drugs for treating conditions ranging from genetic diseases to viral infections, including AIDS, and even cancer.

But during the past few years, the technique has run into unforeseen problems, and some of that early gloss has begun to wear off. Although several clinical trials have already begun, and there have been some promising results, researchers have encountered difficulties in getting antisense drugs—usually short pieces of DNA (called oligonucleotides) that have been designed to recognize and bind to specific genes—into target tissues. And potentially toxic side effects, including decreased blood clotting and cardiovascular problems such as increased blood pressure and decreased heart rate, have shown up in animal studies that have served as the basis for early human trials. But the biggest concern is that antisense compounds simply don't work the way researchers once thought they did.

"The assumption is that we are designing oligonucleotides that don't interact with anything besides [their targets]," says Cy Stein, an assistant professor of medicine and pharmacology at Columbia University's College of Physicians and Surgeons in New York City. "Many people are worried that a lot of the positive effects reported are not just antisense but other nonantisense mechanisms as well."

This uncertainty about what antisense drugs are doing inside the body has caused some experts in the field to argue that clinical trials have begun far too soon. "It is too early to take these things to human beings ... when we don't even know how they are working in a test tube," contends Ramaswamy Narayanan, who studies antisense drugs at Hoffmann-La Roche Inc. in New Jersey, but is not involved in any of the trials.

Others argue that even if the basic researchers haven't yet worked out the drugs' mechanisms of action, clinical trials are justified as long as the compounds show signs of efficacy. "As a clinician, what matters to me is if the drug works," says Jeffrey Holt, a pathologist at Vanderbilt University in Nashville, Tennessee, who is currently trying to use antisense DNA to fight advanced-stage breast cancer. "In medicine, people give drugs that we don't know the mechanism for." As one example, he cites aspirin, whose mode of action was not understood until relatively recently, even though it's been widely used for a century.



Holding on. In an untreated cell *(left)*, a gene's double-stranded DNA is transcribed into RNA, which then makes the protein *(green)*. Antisense drugs *(yellow)* are supposed to block this, by binding to the gene *(near right)* or the RNA *(far right)*. But do they?

Early promise

One reason antisense technology looked like the answer to drug designers' prayers is that it seemed to be simple and straightforward. During the first step of protein synthesis, in which genes are copied into RNA, only one strand of the double-helical DNA is so transcribed. The original idea, developed in the late 1970s and first published by Harvard Medical School researcher Paul Zamecnik, was to create a second RNA or DNA with a particular gene's complementary sequence—the so-called antisense molecule—that could recognize and bind to the RNA. This was supposed to prevent the RNA from manufacturing its protein, either directly or by causing it to be broken down by RNA-cutting enzymes. In the years since then, the technology has undergone several modifications, however.

To try and produce new drugs, researchers chemically string together a sequence of about 20 DNA bases—the oligonucleotide—that mirrors a short stretch of the gene scientists want to block. These may act either by binding to the RNAs, as the longer molecules do, or by binding directly to a target gene, thereby preventing it from being transcribed into RNA in the first place. (This latter approach is sometimes called "triplex" technology because a third DNA strand is being added to the two already in the DNA double helix.)

But however they work, such short oligonucleotides are much easier to synthesize than long antisense RNAs or DNAs. Researchers also made them more resistant to the many enzymes that break down nucleic acids by replacing a critical oxygen atom in each nucleotide building block with a sulfur atom. That's an important plus for a drug that has to be administered to a live human being, as it helps ensure that the drug will last long enough to do its job.

These modifications seemed to put drug designers on the right track: In initial tests with cultured cells, the sulfur-modified oligonucleotides, called phosphorothioates, appeared to work. For example, a team at Hybridon Inc., a biotech firm in Worcester, Massachusetts, found that one of their phosphorothioates, which they called GEM91, blocks replication of the AIDS virus, HIV-1, by targeting a viral life cycle gene called *gag*. "The antisense compound can suppress viral activity in vitro by up to 100%, depending on the concentration we use," says Sudhir Agrawal, vice president of drug discovery and chief scientific officer of the company. Other researchers also had early success in blocking reproduction of HIV-1 and other viruses with the sulfur-modified antisense constructs.

The successes quickly spurred the start-up of several biotech enterprises, such as Gilead Sciences Inc., an 8-year-old biotechnology company based in Foster City, California. "When we began, we said, 'Obviously from the literature, the technology works,' " recalls Richard Wagner, a molecular biologist at the company. "We thought that all we needed to do was bring in a few chemists and we were going to be rich."

But shortly after setting up shop, Gilead researchers realized it wouldn't be that simple. They quickly found that antisense

SOME CURRENT U.S. ANTISENSE CLINICAL TRIALS			
Company	**Disease**	**Rationale**	**Number of Patients**
Isis Pharmaceuticals	CMV retinitis in AIDS patients	Block CMV reproduction	200+
Isis Pharmaceuticals	Genital warts	Block human papilloma-virus reproduction	70+
Isis Pharmaceuticals	Kidney transplant rejection	Block immune cell activities	20 to 40
Isis Pharmaceuticals	Rheumatoid arthritis and other autoimmune diseases	Block immune cell activities	20 to 40 per disease
Lynx Therapeutics	Chronic myelogenous leukemia	Block cancer gene activities	50+
Hybridon	AIDS	Block HIV reproduction	125

compounds applied to a strain of human blood cells did not even get into the nucleus, the site of their RNA or DNA targets, Wagner explains. To get around that problem, they were forced to inject the compounds directly into the cells, a technique that works well in laboratories but cannot be applied to patients.

They did get some encouraging results, though: When they performed the injections, Gilead workers found that compounds directed at the *rev* or *gag* genes located in HIV-1 inhibited viral replication in the cells. In other experiments, antisense oligonucleotides targeted to the *c-myc* gene of blood cells from leukemic patients shut down cancer cell proliferation. But in both sets of experiments, yet another glitch cropped up.

To their surprise, researchers found that oligonucleotides they were using as controls, which couldn't recognize the *rev*, *gag*, or *c-myc* genes, either shut down virus replication or blocked cell proliferation almost as effectively as the ones they were testing as drugs. "While we could repeat many of the biological effects caused in cell culture, in every case our controls would show the same response," Wagner notes. "When we went back to the original papers, we found that often these controls were missing."

At first, Gilead researchers kept their concerns quiet. "There were a significant number of people claiming that these things worked," Wagner explains. "We really didn't want to go public with our negative results until we were sure that we weren't doing something wrong in our system." By the early 1990s, however, other researchers were echoing Wagner's concerns.

One example comes from Arthur Krieg of the University of Iowa, Iowa City, and his colleagues, who were attempting to develop antisense compounds that could be used to treat autoimmune diseases, such as rheumatoid arthritis, in which the immune system begins attacking the body's own tissues. "The B cells in autoimmune disease are hyperactive," Krieg explains. "We were trying to identify the genes responsible and shut them down."

When the researchers tried to inhibit B cells in culture with antisense DNA, however, the molecules turned B cell function up instead of down. That result was a mixed blessing, because it suggested that while the compounds tested would not be useful for treating autoimmune diseases, they might help buttress immune cell function in AIDS patients. But the Iowa team encountered an anomaly in their system similar to the one the Gilead workers had previously found. "Later, we got concerned as a number of controls also turned out to be B cell activators as well," Krieg recalls.

The immunologist, who says he worked "full time" to figure out what was causing this, came up with a solution earlier this year. In a paper published in the 6 April issue of *Nature*, Krieg and his colleagues reported evidence suggesting that antisense oligonucleotides mimic bacterial DNA in triggering a potent response by mammalian immune cells. They based this conclusion on experiments in which they showed that DNA fragments containing the two-base sequence CpG (where C stands for the nucleotide base cytosine, the G for guanine, and p for phosphate) activate mammalian B cells and natural killer cells in culture.

This only takes place, however, when the CpG motif lacks methyl groups. Because such sequences are common in bacterial DNA, but not in mammalian DNA, where most nucleotides have an attached methyl group, the immune response may be a way of defending against bacterial infections, Krieg suggests. The finding applies to antisense technology because antisense manufacturers don't usually add methyl groups to their synthetic oligonucleotides. Thus, mammalian immune systems that encounter such compounds with the CpG motif may be tricked into thinking they have been invaded by bacterial aliens and consequently spring into action.

Krieg suggests that this response could be useful clinically, but he says researchers need to be aware that the drugs are working directly on the immune system, rather than, say, targeting the AIDS virus itself. "I am firmly convinced that synthetic oligonucleotides, like the ones in clinical trials now, will make useful drugs for human beings," Krieg says. "But I don't think they are working through true antisense mechanisms."

Side effects in animals

Besides not always working by "true antisense mechanisms," the synthetic oligonucleotides have also caused side effects in experimental animals. When administered by one-time injection in high doses to monkeys, for example, several phosphorothioate drugs were lethal to some of the animals, for reasons that are not yet understood. In others, the oligonucleotides caused a transient decrease in the total number of two kinds of white blood cells as well as changes in blood pressure and heart rate, according to Hybridon's Agrawal. In addition, phosphorothioates have been found by Hybridon and Isis researchers to accumulate in the liver, kidneys, and bone marrow of animals, although the long-term effects of this deposition are not clear.

Some of these effects may be explained by the drugs' propensity to bind to proteins, says Columbia's Stein. At a recent meeting on the "Art of Antisense,"* molecular pharmacologist Stein presented some of his teams' findings on why the compounds often don't make it to the nucleus. They've found that they end up instead in the endosomes, small membrane-bound vesicles in the cytoplasm. This apparently occurs because the oligonucleotides tend to bind to proteins, which are themselves incorporated in the endosomes. "Many cell types protect themselves by sequestering oligos in intracellular compartments," Stein says, but this could also contribute to the deposition of the drugs in liver and kidney.

In addition to getting entangled by proteins inside cells, the Columbia researcher found that many synthetic oligonucleotides, because of their highly negative charge, get hung up on proteins outside cells as well. Among these are growth factors and cell anchoring proteins such as fibronectin and laminin. The result is that antisense compounds block cell migration and adhesion to underlying tissue in vitro—an effect that may interfere with wound healing and arterial wall repair in living animals, Stein says.

Hybridon's Agrawal maintains, however, that the cardiovascular and other effects seen in animals can be minimized in patients by using low doses of the compounds and administering them gradually by continuous intravenous injection. That seems to be borne out by the early results of Hybridon's

* The meeting, which was sponsored by *Nature Medicine*, was held in New Orleans on 21 and 22 September.

clinical trial of GEM91 in AIDS patients, he told participants in the antisense meeting. Agrawal also reported that patients getting the higher doses are showing signs of clinical improvement in that their viral counts drop a few days after the treatments, although it is far too soon to tell whether this translates into improved survival. To Agrawal, it doesn't matter how the drugs work, if they end up helping AIDS patients. "Despite all the other properties [in addition to actual gene targeting], we feel that if we find an antisense effect ... then we have a new drug," Agrawal says.

Looking to the future

Agrawal is not the only one who hasn't lost faith in the technology. Biotechnology representatives argue that the problems turning up with antisense oligonucleotides are common in drug development, especially when untested, new technologies are being explored. "Every new technology starts at the bottom, in essence, getting your foot in the door," says Gerald Zon, vice president of medicinal chemistry at Lynx Therapeutics Inc., a biotech company in Hayward, California. He notes that every new drug has negative effects that must be weighed against clinical benefits. The answer, he says, is to design better second- and third-generation drugs in order to boost drug efficacy while, at the same time, minimizing unwanted side effects.

Indeed, researchers at companies such as Hybridon, Isis, and Gilead say they are applying the lessons they are learning from the animal studies and early clinical trials to try to come up with better and less toxic compounds. The options they are exploring include modifying the structures of oligonucleotides so that they bind less readily to proteins or more readily to their target genes. All three companies are also generating fat-soluble delivery molecules called cationic liposomes. The researchers hope these lipid-loving shuttles will help antisense compounds break through cellular barriers that prevent entrance into the nucleus.

These new compounds and delivery systems carry no guarantees that they will be any better than the phosphorothioates used in the current clinical trials. But even some of the critics, such as Stein, agree the field still holds great promise, as long as the researchers recognize that antisense drugs don't always work the way they are supposed to. "My guess is that we will find that the current generation of phosphorothioates are extremely active biological molecules," Stein concludes, "and that they work by many mechanisms, of which antisense is one. The truth is that we'll have to wait and see. None of us really knows what is going to come out of it."

–Trisha Gura

Trisha Gura is a reporter on leave from the Chicago Tribune.

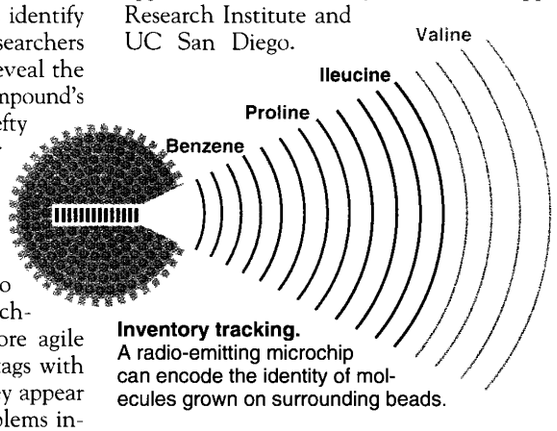
CHEMISTRY

Radio Tags Speed Compound Synthesis

Like aging computers, it doesn't take long for scientific techniques to seem slow and cumbersome. Take combinatorial chemistry. When it was introduced a few years ago, it was the supercomputer of chemical synthesis. The technique allows chemists to quickly paste together several different chemical building blocks into millions of combinations, in hopes that one will prove to be a new drug or a useful material. To identify each one of the new compounds, researchers typically affix chemical tags that reveal the unique arrangement of each compound's components. But these tags carry a hefty price: Their use doubles the number of chemical steps—and the time—involved in the assembly process, and their fragility prevents the synthesis of some compounds.

In the past 2 weeks, however, two separate groups of California researchers have unveiled a faster and more agile model. By replacing chemical ID tags with tiny radio-emitting microchips, they appear to have overcome both of the problems inherent in the old one. "The upshot is that it makes the whole process of drug discovery more efficient," says Rob Armstrong, a chemist at the University of California (UC), Los Angeles, who led one of the research groups, which includes scientists from Ontogen Corp. in Carlsbad, California. "This has the potential to be a significant advance in simplifying the encoding process," adds Michael Pavia, who heads combinatorial research at Sphinx Pharmaceuticals in Cambridge, Massachusetts. The technique not only saves time, says Pavia, "it gives you a wider range of chemical diversity to select from in building your new molecules."

Armstrong's group presented its findings at last week's meeting of the Western Biotech Conference in San Diego, as did the second team, led by Michael Nova at IRORI Quantum Microchemistry in La Jolla, California, and K. C. Nicolaou, who holds dual appointments at the La Jolla–based Scripps Research Institute and UC San Diego.



Inventory tracking. A radio-emitting microchip can encode the identity of molecules grown on surrounding beads.

The IRORI group was, however, the first in print, with a paper in the 15 October issue of *Angewandte Chemie*.

Both techniques add considerable power to combinatorial chemistry, which already made traditional synthetic chemistry look like an old IBM punch card. Traditionally, novel compounds are synthesized one at a time, but combinatorial chemists create huge numbers in a single process by assembling a few chemical building blocks—each of which has a corresponding ID tag, such as a short nucleotide sequence—in all possible combinations. Chemists need these tags to decipher the makeup of compounds that show promise in an assay, such as the ability to kill cancer cells (*Science*, 3 June 1994, p. 1399).

But because a tag has to be added with each building block, assembling a 10-component molecule actually involves at least 20 time-consuming chemical steps. And the technique runs into trouble when creating small organic molecules, which constitute most of today's drugs. Some of the synthetic reactions involve potent reagents, such as hydrofluoric acid, which can rip ID tags apart.

The new microchip tags appear to solve both these problems at once. A chip, which emits a binary code, is inserted into a mesh capsule loaded with polymer beads—the "seeds" to which combinatorial building blocks are added by dunking the capsule in a series of beakers. In the Ontogen approach, a nearby radio scanner registers both the identity of a capsule and the contents of each beaker it enters. These data are uploaded to a computer that keeps track of the order of building blocks in the growing molecule. In the IRORI approach, the information is stored on the microchip itself, using a transmitter that writes the information to the chip. The information is uploaded to the computer only when the assembly run is complete.

By eliminating the chemical tags, both approaches do away with half the synthetic steps involved, yet end up with an instantly available computer record of the precise structure of the compounds in each capsule. Moreover, says Nicolaou, "now you are free to use any chemistry you want to build your molecules." Speed and flexibility—for chemists, it's a winning combination.

–Robert F. Service