Nanoparticles and siRNA – Partners on the Pathway to New Cancer Therapies

Seven short years ago, in an October 1999 paper published in the journal *Science*, the research community learned of a radically new mechanism that cells can use to control protein production. Discovered by plant geneticists David Baulcombe, Ph.D., and Andrew Hamilton, Ph.D., this new process relied on small pieces of double-stranded RNA molecules that could bind to and neutralize specific messenger RNA molecules, which then prevented the cell from translating that particular message into a protein. Then, in early 2002, a paper in *Nature* from Thomas Tuschl, Ph.D., and his collaborators at the Max-Planck Institute for Biophysical Chemistry in Gottingen, Germany, showed that small interfering RNA (siRNA) molecules could block protein production in mammalian cells.

Today, RNA silencing – the process triggered by siRNA molecules – is poised to have a major impact on the treatment of human ailments, particularly cancer. Using siRNA molecules, researchers believe they can turn off the ability of cancer cells to produce the key proteins that make them different from normal cells, and by doing so, stop malignancy in its tracks. Early proof-of-principle experiments in various tumor cells showed quickly that RNA silencing had great potential as a means for treating cancer.

But almost as soon as those early results were in, a major obstacle surfaced: The human body is well-equipped to destroy double-stranded RNA circulating in blood and prevent it from entering cells. As a result, getting these potential therapeutic agents to tumors and getting them into cancer cells was not going to be easy.

Enter nanotechnology. Nanoparticles, it turns out, are proving to be ideal carriers for siRNA molecules. "With nanoparticles, we have the ability to load large numbers of these molecules into a protected environment, target them to cancer cells, and then have them taken up efficiently by those cells and release the RNA molecules into the interior of those cells," says Mark Davis, Ph.D., at the California Institute of Technology (Caltech).

In fact, the marriage between nanoparticles and siRNA is proving to be such a good one that several companies are already pushing nanoparticle-delivered anticancer siRNA agents toward human clinical trials. Intradigm, in Rockville, MD, expects to file an Investigational New Drug application with the U.S. Food and Drug Administration next year and begin clinical trials with its nanoparticulate formulation of an siRNA agent designed to stop tumor-associated angiogenesis. And in February of this year, Calando Pharmaceuticals, in Pasadena, CA, and the National Cancer Institute (NCI) entered into a collaborative development program for a nanoparticle-based siRNA therapeutic aimed at treating neuroblastoma, the most common extracranial solid tumor in children younger than five years old.

Targeting Is a Key

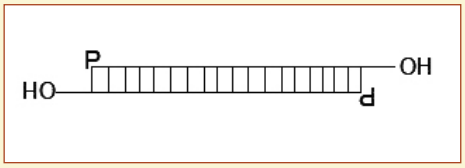
One characteristic of nanoparticles that has made them a favorite among cancer researchers in general, and not just those using siRNA, is the ability to decorate the surfaces of these drug vehicles with tumor-targeting agents. But with siRNA, this property is particularly attractive because there is some concern that siRNA delivered to the wrong cells may have unintended consequences. "The siRNA field itself is so young that we just aren't sure yet how important it is to target just one particular type of cell," says Vincent Rotello, Ph.D., of the University of Massachusetts in Amherst. "But the versatility of nanoparticles gives us the ability to better target cancer cells and to even develop nanoparticles that only deliver their RNA contents once they've entered the target cell. We hope that by taking advantage of these opportunities, that we can head off potential problems."

At Caltech, for example, the Davis group has developed nanoparticles that provide effective release of their contents under the low pH conditions characteristic of intracellular environments experienced when nanoparticles enter cells via targeted cancer cell surface receptors. The resulting nanoparticles can carry significant amounts of chemically unmodified siRNA and effectively deliver them to targeted cells as his group showed in a paper published in *Cancer Research* last year. Davis, who is a founder of Calando and has licensed his lab's work to the company, says he hopes to have an anticancer siRNA agent in clinical trials next year.

Meanwhile, Rotello and his colleagues are creating nanoparticles studded with molecules of folic acid, a well-studied nanoparticle targeting agent that binds to a receptor found in abundance on many types of can-

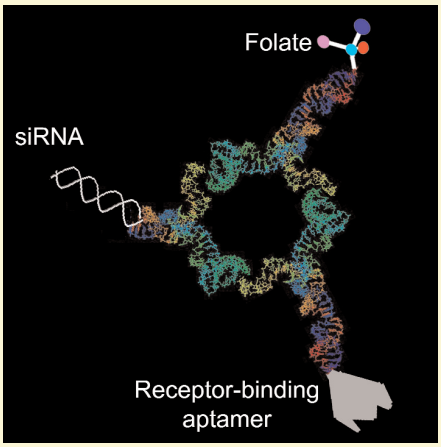
What is an siRNA?

siRNAs have a well-defined structure: a short (usually 21-nucleotide-long) double-strand of RNA (dsRNA) with 2-nucleotide 3' overhangs on either end:



Each strand has a 5' phosphate group and a 3' hydroxyl (-OH) group. This structure is the result of processing by an enzyme that converts either long dsRNAs or hairpin RNAs into siRNAs. siRNAs can also be introduced into cells using nanoparticles and other methods to bring about the specific knockdown of a gene of interest. Essentially any gene of which the sequence is known can thus be targeted based on sequence complementarity with an appropriately tailored siRNA. This method has made siRNAs an important tool for gene function and drug target validation studies in the post-genomic era.

Source: Wikipedia http://en.wikipedia.org/wiki/SiRNA



A triangular nanoparticle made of RNA carriers uses both folic acid and a receptor-binding aptamer as tumor-targeting agents to deliver anticancer siRNA molecules.

Courtesy: Peixuan Guo, Purdue University

cer. But in addition, the surface of these nanoparticles is covered with a sulfur-containing chemical group known as a thiol, with the sulfur bonding to the surface of the gold nanoparticle. Cells contain large amounts of the molecule glutathione, which is also a thiol. The abundance of glutathione inside a cell results in the displacement of the thiols on the particle, triggering the release of the RNA payloads.

Peixuan Guo, Ph.D., at Purdue University's Cancer Research Center, is taking advantage of another natural process to achieve targeted delivery and release of siRNA inside cancer cells. Nearly 20 years ago, Guo discovered that a particular bacteria-infecting virus called phi29 uses a novel type of RNA to package its DNA into its protein shell. Guo named his discovery pRNA, for packaging RNA, and today he and his colleagues are using it as a component of an all-RNA nanoparticle that resembles a triangle. Tests in both cultured human tumor cells and in mouse models of cancer have shown these RNA nanoparticles have potential as anticancer agents.

Guo's team created their nanoparticles by linking together two different kinds of RNA: pRNA, which acts as the delivery vehicle, and siRNA, which acts as the therapeutic agent. The team then added folic acid molecules as the targeting agent. "Using techniques we learned from our earlier work," explains Guo, "we were able to combine [these molecules] into triangles that are between 25 and 40 nanometers wide. This is the Goldilocks size for any nanoparticle that is to be used in the body – not too big, not too small."

Particles larger than about 100 nanometers are generally too large to pass through cell membranes into the cell's interior, Guo says, and the body has a hard time retaining particles smaller than 10 nanometers. But the tiny triangles fit, and they worked well enough to interrupt the growth of human breast cancer cells and leukemia model lymphocytes in laboratory experiments. The use of such protein-free particles could avoid the induction of immune response and avoid antibody production and cell immunity. Therefore, it facilitates the long-term treatment in chronic diseases such as cancer, hepatitis B, or AIDS.

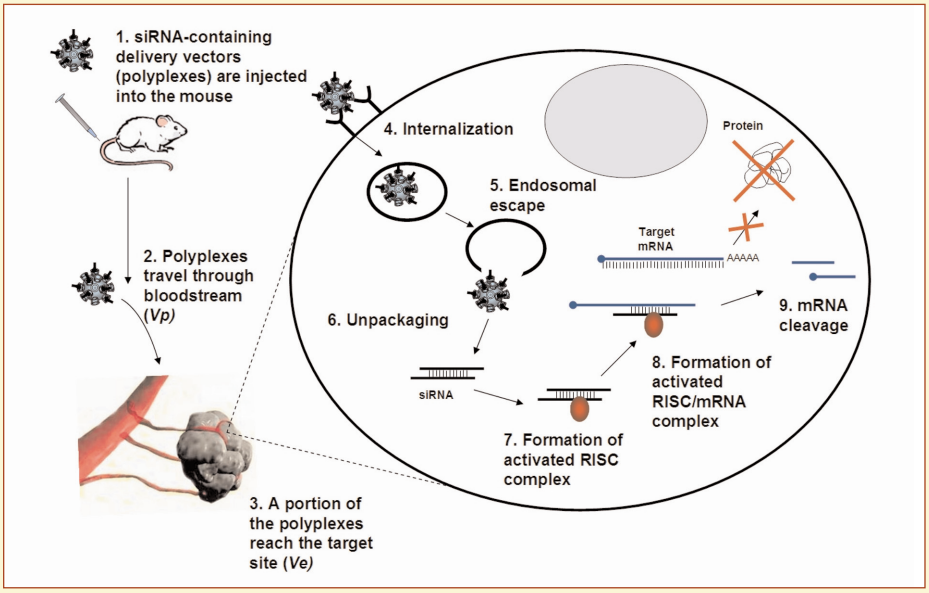
"One characteristic of cancer cells is that they do not stop growing, which is one reason tumors develop," Guo says. "Once inside, the siRNA essentially instructs the cells to 'stop not stopping.' The nanoparticles had done their work on the breast cancer cell cultures within a few days."

Additionally, the team found that the nanoparticles completely block cancer development in living mice. A group of mice that was in the process of developing cancer was tested with the nanoparticles, and they did not develop the disease. A second group, that was tested with mutated inactive RNA, all developed tumors.

"The results are very promising, but we still have several hurdles to jump before we can test this therapy on people," Guo says. "First and foremost, we must ensure that it is as safe as we think it is. Some RNA can be toxic to non-cancerous cells as well, and though our nanoparticles appear to go straight to the cancer cells, where we want them to go, we have to be sure they do not go anywhere else before we can inject them into a living person."

Stability of the RNA also is a factor the team must consider. Although his group had previously published data indicating that phi29 RNA nanoparticles are more stable than other RNA, Guo said the team has also introduced chemical modifications into the RNA backbone that further stabilizes it against degradation.

Intradigm's approach to successfully deliver and target its lead anticancer siRNA agent is to use a multicomponent polymer system that self-assembles into a rugged nanoparticle when mixed with siRNA molecules. Martin Woodle, Ph.D., chief scientific officer at the company, leads the company's efforts, which are aimed at developing an siRNA agent that silences the production of a receptor for vascular endothelial growth factor (VEGF) on the newly developing blood vessels surrounding tumors. The idea behind this approach is that if these blood vessels cannot respond to VEGF, angiogenesis will stop and tumors



Simplified schematic of the key steps required for siRNA delivery to and function within mammalian cells.

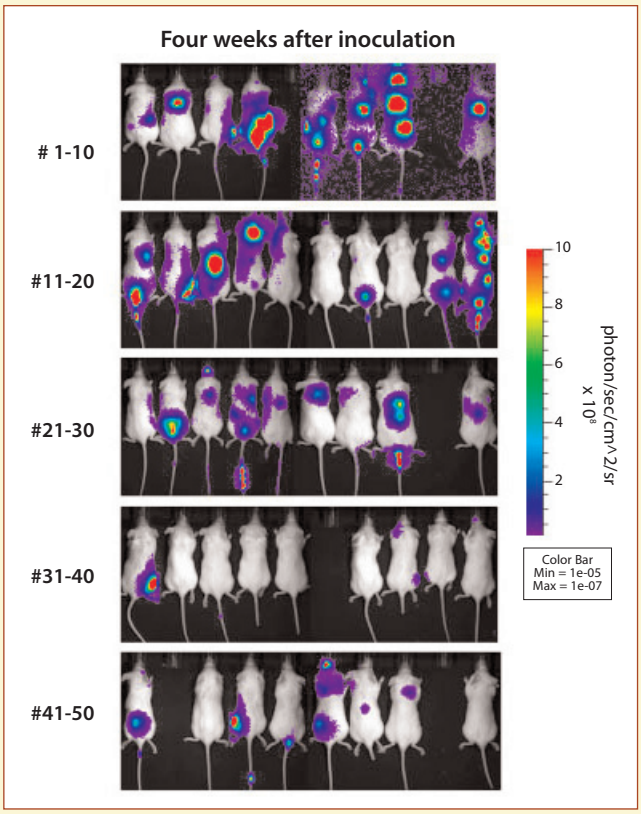
Courtesy: Derek Bartlett and Mark Davis, Nucleic Acids Research and Oxford University Press. Nucleic Acids Res. 2006; 34(1): 322–333. Published online 2006 January 12. doi: 10.1093/nar/gkj439

will not be able to procure the nourishment they need to thrive and metastasize.

Woodle and his colleagues start with the biocompatible polymer poly(ethyleneimine), or PEI, and link it to a second biocompatible polymer, poly(ethylene glycol), also known as PEG. As a targeting agent, the researchers chose a three-amino-acid peptide, arginine-glycine-aspartic acid (known by its amino acid code RCG) that binds to a class of proteins known as integrins. Found on the surface of new blood vessels, integrins are involved in a wide range of biological processes, including angiogenesis, tumor cell growth and metastasis, and inflammation. The researchers attached this integrin-targeting peptide to one end of the PEG polymer chain.

Mixing equal parts of the polymer-RGD construct and anti-VEGF receptor siRNA yields nanoparticles in which a core of PEI and siRNA is surrounded by a water-soluble shell of PEG, with the RGD-targeting peptide sticking out from the particle. Studies in cultured cells showed that this formulation entered only those cells containing integrins, suggesting that the RGD peptide was an effective targeting agent. In contrast, nanoparticles lacking RGD did not enter targeted cells. This study also showed that siRNA cargo was released within the cell and was able to silence VEGF receptor production.

Next, the Intradigm research team administered their nanoparticle to tumor-bearing mice; they also treated a second group of mice with a nanoparticle containing siRNA designed to silence a bacterial protein (to act as a control experiment) and left a third group of animals untreated. Within six days of intravenous injection, it was clear that the nanoparticle containing the anti-VEGF receptor siRNA was having the desired effect – tumor growth in the animals treated with that formulation was minimal, compared to significant growth seen in both of the two control groups. Intradigm hopes to begin clinical trials with this agent late next year.



Mice with Ewing's sarcoma were treated with a targeted nanoparticle containing anti-tumor siRNA (#31-40), as well as various control formulations. Only the targeted agent had a marked effect on tumor growth. Tumors show up as purple areas.

Courtesy: Calando Pharmaceuticals

Based on these results and others, the future of nanoparticle-based siRNA therapy for cancer looks promising. Certainly, questions remain concerning the safety of these agents, though clinical trials using siRNA to treat other illnesses have not yet come across any stop signs. Also, manufacturing RNA molecules is not trivial or inexpensive, though chemists have made marked improvements in reducing both the complexity and cost of RNA production.

"Obviously, all of these agents will have to prove themselves in clinical trials and until they do, they'll remain in the promising but unproven category," says Davis. "But combining siRNA with nanoparticle technology gives us a real opportunity to have an impact in the treatment of cancer."

—Joe Alper

Selected References

Devi RS. siRNA-based approaches in cancer therapy. *Cancer Gene Therapy* **13**: 819-29, 2006.

Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411** (6836): 494-8, 2002.

Guo S, Huang F, Guo P. Construction of a folate-conjugated pRNA of bacteriaphage phi29 DNA packaging motor for delivery of chimeric siRNA to nasopharyngeal carcinoma cells. *Gene Therapy* **13**: 814-20, 2006.

Hamilton AJ, Baulcombe DC. A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* **286** (5441): 950-2, 1999.

Hu-Lieskovan S, Heidel JD, Bartlett DW, Davis ME, Triche TJ. Sequence-specific knockdown of EWS-FLI1 by targeted, nonviral delivery of small interfering RNA inhibits tumor growth in a murine model of metastatic Ewing's sarcoma. *Cancer Res.* **65** (19): 8984-92, 2005.

Khaled A, Guo S, Li F, Fuo P. Controllable self-assembly of nanoparticles for specific delivery of multiple therapeutic molecules to cancer cells using RNA nanotechnology. *Nano Letters* **5** (9): 1797-1808, 2005.

Schiffelers RM, Ansari A, Xu J, Zhou Q, Tang Q, Storm G, Molema G, Lu PY, Scaria PV, Woodle MC. Cancer siRNA therapy by tumor selective delivery with ligand-targeted sterically stabilized nanoparticle. *Nucleic Acids Res.* **32** (19): e1492004, 2004.