

Knocking down Disease with siRNAs

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Knocking down expression of disease-related genes using small interfering RNAs (siRNAs) has potential for treating a variety of illnesses. This Essay will examine the opportunities for harnessing RNA interference (RNAi) for therapy, as well as the obstacles and possible ways to circumvent them.

Five years ago, Tuschl and colleagues showed that RNA interference (RNAi) works in mammalian cells. They found that the endogenous RNAi pathway can be harnessed by introducing small double-stranded RNAs comprising 19–21 nucleotide complementary sequences (called small interfering RNAs, siRNAs) into mammalian cells to silence gene expression with exquisite specificity and without activating an interferon response (Elbashir et al., 2001). Translational researchers were quick to realize the potential for using RNAi to identify new drug targets and for designing small molecule drugs. siRNA-based drug development has proceeded at a dizzying pace. The first demonstration of disease protection using siRNAs in a mouse model was published three years ago (Song et al., 2003b), and human safety clinical studies began a little over a year later, well before much of the basic phenomena of RNAi were really understood. Some believe, and we agree, that siRNAs have the potential to become the next new class of drugs. Three phase I clinical studies investigating siRNAs for treating age-related macular degeneration and respiratory syncytial virus (RSV) infection have been completed with no reports of untoward toxicity. The ability to design siRNAs to any

host gene or pathogen once genetic sequences are known and test them rapidly highlights a key advantage of siRNA drug development compared to more conventional small molecule drugs. For example, the human phase I study of siRNAs targeting RSV by

Alnylam began just 15 months after the company starting working on this project (A. de Fougerolles, personal communication).

In the original RNAi studies, gene expression was silenced by transducing cultured mammalian cell lines with siRNAs or with viral vectors encoding short hairpin RNAs (shRNAs) processed within cells like endogenous microRNAs. These two approaches—siRNA small molecule drugs or shRNA gene therapy—are the two pathways available for harnessing RNAi in both research and the development of therapeutics. The gene therapy application of RNAi is likely to remain mostly an academic exercise for the immediate future. Early clinical studies have focused on using siRNAs as small molecule drugs. However, as we discuss in this Essay, there are many hurdles to be overcome before siRNA drugs become a reality.

Delivery

To be useful as drugs, siRNAs need to be delivered in vivo into the cytoplasm of cells. Here, the RNA-induced silencing complexes (RISC) take up the double-stranded siRNAs and cleave the sense passenger strand, leaving the activated RISC containing the antisense strand to search for mRNAs containing complementary sequences, which

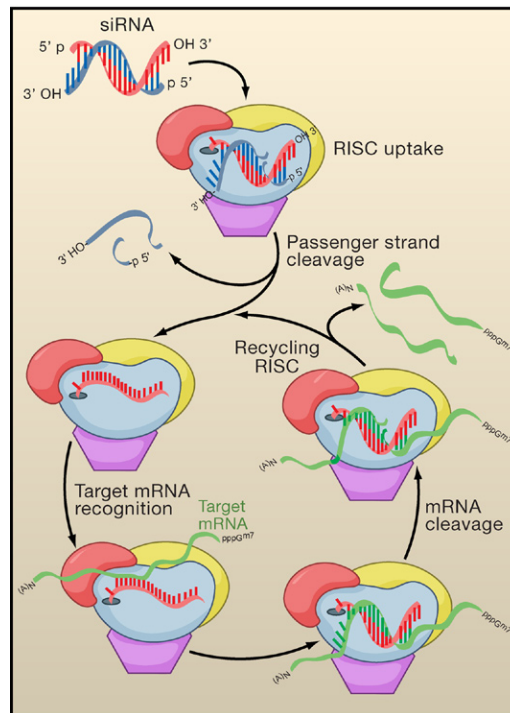


Figure 1. The Cellular Pathway for siRNA Drug Action Small interfering RNAs introduced into the cytoplasm are taken up by the RISC. The strand whose 5'-end is less tightly bound is incorporated as the active guide strand, whereas the other strand (the passenger strand) is cleaved. This activates the RISC, which then recognizes a target mRNA bearing a complementary sequence and cleaves it. Once the target mRNA is cleaved the RISC can be recycled to seek and destroy another mRNA. The potency of siRNA drugs is linked to their incorporation and stabilization in the endogenous RISC complex present in all cells, and the catalytic nature of the cleavage reaction.

will then be targeted for degradation (Figure 1). Although siRNAs are readily taken up by invertebrate cells, siRNAs on their own are not taken up by most mammalian cells in a way that preserves their activity. Even cells that are actively sampling their environment, such as dendritic cells and macrophages, do not take up siRNAs efficiently enough to activate gene silencing. Although lipid-based transfection can introduce siRNAs into a variety of cells in vitro, many important cells, such as primary lymphocytes or hematopoietic stem cells, are refractory to transfection (although nucleo-

Some tissues of the body that are easily accessible, including the respiratory and genital tracts and the eye, can take up siRNAs after topical application or direct injection of naked siRNAs alone or in complexes with cationic lipids used for in vitro transfection (Bitko et al., 2005; Palliser et al., 2006). Phase I clinical studies to treat age-related macular degeneration of the retina and RSV in the respiratory tract have demonstrated uptake in those target tissues after intravitreal injection and intranasal application of naked siRNAs, respectively. Cells

most pathogens, the use of topically applied siRNAs to prevent or treat intracellular infection is an attractive option, especially for viral infections for which few drugs exist. For example, local siRNA delivery to the lung was able to protect nonhuman primates from infection with the SARS coronavirus (Li et al., 2005). Other opportunities for local treatment include localized inflammatory diseases such as seasonal rhinitis or mucosal malignancies such as cancers of the head and neck, nasopharynx, or cervix (some of which are caused by transforming viruses, providing specific gene targets). Targeting inflammatory lung diseases, such as asthma, is also high on the agenda of at least one RNAi biotechnology company. This will require aerosolized delivery to introduce siRNAs into the appropriate epithelial and immune cells deep within the lung. However, it is still not clear which primary cell types in accessible tissues efficiently take up naked siRNAs or siRNA-lipid complexes and would be amenable to local siRNA therapeutic delivery. Moreover, the mechanism of uptake into mammalian cells is not understood. Answering these questions will be important for choosing the best disease targets for RNAi therapeutics.

The earliest studies showing siRNA effectiveness systemically in animal disease models relied on hydrodynamic injection. With this technique, siRNAs are injected rapidly in a large bolus that causes transient damage to cell membranes in highly vascularized organs enabling siRNAs to access the cytoplasm (McCaffrey et al., 2002; Song et al., 2003b). This method of injection is dangerous and not feasible for human use. However, by catheterizing the vein draining an organ, it is possible to raise vascular pressure locally to introduce siRNAs that are therapeutically beneficial (Hamar et al., 2004). These early studies suggested that naked siRNAs would not be internalized after conventional intravenous injection into most tissues. However, a sensitive detailed tissue survey has never

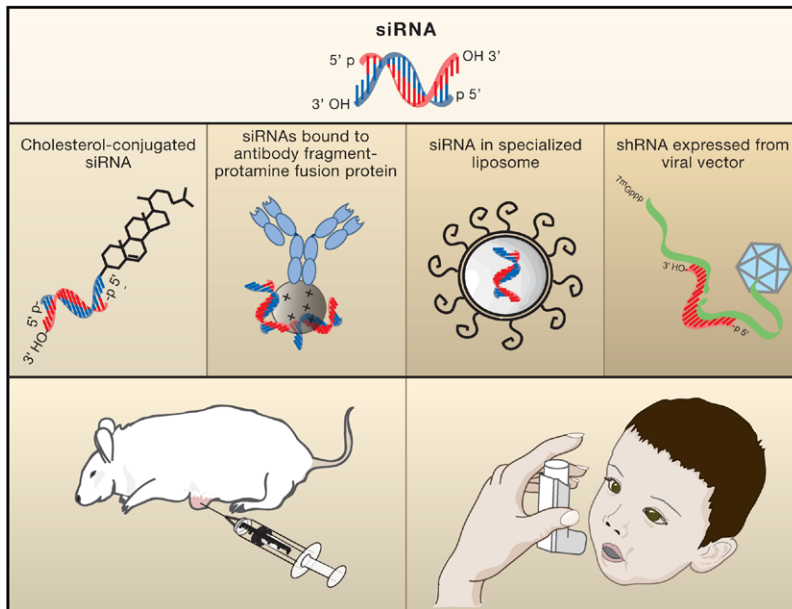


Figure 2. Delivery of siRNAs for Therapy

To be a useful drug, an siRNA (top) must be delivered into cells in vivo. Methods to accomplish this (middle) include chemical conjugation of the passenger strand to cholesterol; binding of the siRNA to an antibody fragment-protamine fusion protein for cell receptor-mediated uptake; incorporation of the siRNA into specialized liposomes or nanoparticles; or expression of an shRNA precursor from a viral vector. (Bottom) Delivered siRNAs can then be tested in small animals, nonhuman primates, and people. Application of siRNAs to mucosal tissues such as the lungs (using an inhaler, for example) does not require specialized delivery methods.

fection, a proprietary electroporation method developed by Amaxa, does work for these cells). Moreover, these methods of intracellular delivery are not suitable for in vivo use. Therefore, siRNA intracellular delivery has been the most formidable obstacle to siRNA drug development. Practical solutions to the delivery problem are, however, becoming available.

in the epithelium and lamina propria of the mouse vagina and cervix very efficiently take up siRNA-lipid complexes. It may well be that most cells at the mucosal surfaces of the body efficiently internalize siRNAs, suggesting that topical siRNA therapies have the fewest hurdles to overcome. Given that mucosal surfaces are the portals of entry for

been reported, raising the possibility that some cell types or tissues might actually be amenable to systemic targeting.

To target most tissues deep within the body, clinically feasible noninvasive strategies need to be devised for directing siRNAs into cells. In the past year, several approaches have achieved impressive therapeutic benefit following intravenous injection into mice and primates using reasonable doses of siRNAs without apparent limiting toxicities (Figure 2). One approach involves covalently coupling the passenger strand of the siRNA to cholesterol to facilitate uptake through ubiquitously expressed cell-surface LDL receptors (Soutschek et al., 2004). This approach targeted siRNAs to the liver and jejunum. In fact, because of its rich vascular supply and role as the blood filtering and detoxifying organ, the liver is the internal organ that is probably easiest to target. Silencing the cholesterol transport protein ApoB with cholesterol-conjugated chemically stabilized ApoB siRNAs reduced serum cholesterol in mice by about 30%. However, this therapeutic effect required a high siRNA dose (~50 mg/kg) that is unlikely to be applicable to humans. A similarly impressive decrease in serum cholesterol could be achieved by designing a cholesterol-conjugated stabilized siRNA, with an active strand that is complementary to an endogenous microRNA (miR-122) expressed primarily in the liver. Such an siRNA is designed to interfere with the ability of the microRNA to silence its targets (Krutzfeldt et al., 2005). Drugs based on siRNAs that mimic or antagonize endogenous microRNA function may be of value in treating cancer because microRNAs are important for regulating cellular differentiation, which goes awry during tumor formation (Croce and Calin, 2005; Hammond, 2006). Conjugating siRNAs to other small molecules, either natural ligands for cell-surface receptors or small molecule drugs, would theoretically provide alternate ways to target specific subsets of cells bearing the specified

receptor. Although all cells bear cholesterol receptors, steering siRNAs into specific cell types by chemically conjugating them to ligands for cell-type-specific receptors would be likely to reduce the necessary drug dose as well as limit toxicity to unintended bystander cells.

Another strategy for delivering siRNAs to the liver involves encapsulating siRNAs into specialized liposomes formed using polyethylene glycol to reduce uptake by scavenger cells and enhance time spent in the circulation. These specialized nucleic acid particles (stable nucleic acid-lipid particles or SNALPs) delivered siRNAs effectively to the liver (and not to other organs), reducing the replication of hepatitis B replicons in mice (Morrissey et al., 2005) and ApoB expression in nonhuman primates (Zimmermann et al., 2006). Delivery by SNALPs, a strategy developed by Protiva, reduced the effective siRNA dose to ≤ 3 mg/kg/day, a practical therapeutic dose. Liposomes, or other lipoplex or nanoparticle siRNA formulations, can at least in principle be varied with respect to size, lipid composition, and incorporation of targeting molecules to direct siRNAs to other treatment sites.

A cell-specific targeting strategy, effective at ~1 mg/kg siRNA dose in a mouse tumor model, involves mixing siRNAs with a fusion protein composed of a targeting antibody fragment linked to protamine, the basic protein that nucleates DNA in sperm and binds siRNAs by charge (Song et al., 2005). These fusion proteins can deliver siRNAs with exquisite specificity to a wide variety of cells, including primary hematopoietic cells that are ordinarily difficult to transfect even *in vitro*. Such fusion proteins have delivered siRNAs to HIV-infected lymphocytes but not uninfected cells (Song et al., 2005) and to activated leukocytes but not resting leukocytes (D. Peer, P. Zhu, M. Shimaoka, and J.L., unpublished data). Because the siRNA is not covalently bound, the same delivery agent can be used to deliver different siRNAs or cocktails of siRNAs.

These examples of systemic siRNA delivery are only the beginning but suggest that the delivery hurdle is not insurmountable. Which approach works best will likely depend on the target cell and indication.

Pharmacokinetics

Unmodified and uncomplexed siRNAs have a very short half-life in the blood (~minutes) that limits their usefulness as drugs. They are rapidly eliminated by renal excretion, which is the rate-limiting factor, and also degraded somewhat more slowly ($T_{1/2}$ ~1 hr) by serum RNases. Information about the half-life of unmodified siRNAs in other bodily fluids, such as those bathing the mucosal portals of entry and the eye, is not available. Given their rapid elimination, the dramatic therapeutic effects of unmodified siRNAs in some animal disease models (Bitko et al., 2005; Palliser et al., 2006; Song et al., 2003b) are indeed impressive and bode well for the effectiveness of modified siRNA drugs with improved pharmacokinetic properties. Once inside a cell, the active strand of the siRNA incorporated into the RISC appears to be incredibly stable as silencing in some tissues persists for weeks (Palliser et al., 2006; Song et al., 2003b; Zimmermann et al., 2006). Two factors seem to control the persistence of silencing—the rate of cell division of the targeted cell, where siRNA dilution with each cell division reduces the siRNA concentration, and the presence of the target mRNA within the cell. Silencing is sustained in slowly dividing cells expressing the target mRNA (Song et al., 2003a). Infrequent dosing may be possible for some nondividing or slowly dividing tissues, such as the brain and the liver, but not for other targets, such as cancer cells. Whether and how the presence of the target mRNA enhances stability of the siRNA requires further investigation but needs to be considered when designing interventions. For example, an siRNA-based microbicide used to prevent viral infection might last longer if the siRNAs target host receptor genes rather than viral genes.

The plasma half-life of siRNAs can be extended substantially (from minutes to days) by the siRNAs forming complexes with other molecules or becoming incorporated into particles (to bypass the renal filtration cutoff of ~50 kDa) and by chemical modification to avoid exonuclease and endonuclease digestion. These modifications generally come at the price of reduced intracellular silencing efficiency, presumably because the RNAi machinery is optimized to handle unmodified endogenous RNAs and because the unmodified active strand is stable once inside the cell (at least if the target is present). Given that nuclease sensitivity is sequence dependent, one intelligent strategy is to minimize the amount of chemical modification and restrict it to the linkages or residues that are most susceptible to attack (Soutschek et al., 2004).

Off-Target Effects

All drugs, and siRNAs are no exception, have unintended off-target effects. Some genes containing sequences with imperfect complementarity may be unintentionally silenced by mRNA cleavage or by translational inhibition (Jackson et al., 2003). Off-target effects occur by siRNAs mimicking microRNA target recognition and gene silencing (Jackson et al., 2006b). An attractive method for minimizing off-target effects was recently described: chemical modification of the second residue in the active strand of the siRNA (a key residue in the seed region for endogenous microRNA activity) may suppress unintended off-target effects without interfering with silencing of the target gene (Jackson et al., 2006a). The rules for predicting off-target gene regulation are still too crude to be completely useful, although sequences with a high degree of similarity to the target by BLAST search need to be avoided. Unintended off-target changes in mRNA can be surveyed using mRNA microarrays, which generally show that few off-target mRNAs are reduced by more than 2-fold. However, for some genes changes of 2-

fold or less may be clinically significant. It is more difficult to evaluate the extent of unintended changes in protein expression. As translational inhibition is less effective at silencing gene expression than mRNA cleavage, the hope is that most of these off-target effects will be too weak to cause clinical problems. Indeed there has been an encouraging lack of toxicity so far in both animal and early clinical studies. It is unlikely that microarray analyses will be helpful to screen and modify siRNA sequences to predict and minimize off-target effects before their clinical testing. More likely they will provide useful tools to guide modifications if unexplained toxicity is observed.

In addition to silencing unintended targets, siRNAs could potentially cause problems by triggering immune and inflammatory pathways. These may include the interferon response and toll-like receptor (TLR) pathways, which are designed to recognize double-stranded RNAs of invading viral pathogens (Sledz et al., 2003). Innate immune cells, which sound the alarm during viral invasion, need to tolerate endogenous microRNAs and can distinguish between foreign RNAs and host RNAs. The interferon pathway is not efficiently triggered by double-stranded RNAs less than 30 nucleotides in length, providing an adequate size window for siRNA drugs (the active strand of endogenous single-stranded RNAs binding to RISC is only about 21 nucleotides long). Longer siRNAs that need to be processed by Dicer but are still below the 30 nucleotide cutoff may be more effective at silencing than 21 nucleotide siRNAs, possibly because Dicer processing helps to incorporate siRNAs into the RISC (Kim et al., 2005). In a few animal studies showing siRNA therapeutic benefit, induction of interferon-responsive genes has not been detected, even with sensitive real-time RT-PCR assays. Activating TLRs turns out to be sequence specific, with the preferred trigger being GU-rich sequences (Judge et al., 2005). Candidate siRNAs for clinical use (and for some types of research) will need

to be screened to verify that they do not activate inflammatory pathways. Because the number of effective siRNAs for any gene is generally large, excluding siRNAs that bind to TLRs is not likely to preclude silencing any target gene. In addition to avoiding TLR-activating sequences, chemical modifications of the siRNAs that do not abrogate silencing can be used to block TLR activation. Several strategies are available to bypass the unintended immunostimulatory effects of siRNA drugs, and this potential toxicity is not likely to impede siRNA drug development.

Drug Resistance

For some important clinical applications, notably treating viral infection and cancer, siRNA drug resistance by selection of escape mutations of the target sequence during viral replication or cancer cell division is likely to develop. This may be more of a problem for siRNAs than for other types of small molecule drugs, as synonymous mutations can interfere with siRNA recognition while leaving the encoded protein untouched. One way around this potential problem is to design siRNAs that target sequences that are conserved at the nucleotide level (these exist even for highly variable viruses like HIV). Another strategy is to use both strands of an siRNA to target more than one gene or sequence, including drug-resistant variants. Alternatively, siRNA cocktails can be used to hit multiple genes and, in the case of viruses, to target both viral genes and host genes. By focusing on targets that are essential for viral replication or cell division, resistance mutations are more likely to interfere with the underlying fitness of the virus or cell. Whether drug resistance develops more readily for siRNAs than for other types of drugs is hard to predict without doing clinical studies. If resistance does prove to be a clinical problem, one important advantage of siRNA drugs is the relative ease, compared to other small molecule drugs, with which resistance can be overcome by simple changes in the siRNA sequence. The same strate-

gies for preventing anticipated drug resistance will also lead to the development of drugs suitable for treating the diversity of viral strains or cancer variants.

Interfering with Endogenous microRNAs

A potential cause of toxicity of siRNA drugs is that introducing exogenous siRNAs into a cell can interfere with the processing and function of endogenous microRNAs. Copiously expressing microRNA-like shRNAs, either using a virus (Lu and Cullen, 2004) or gene therapy vector (Grimm et al., 2006), interferes with endogenous microRNA nuclear export by exportin 5. Although siRNA drugs, introduced directly into the cytoplasm, will not compete with microRNAs at this stage, large intracellular siRNA concentrations might compete for limiting amounts of Dicer (for longer siRNA precursor drugs) or RISC. This consideration might set a limit on the number of different siRNAs that could be incorporated into a drug cocktail. The catalytic nature of mRNA cleavage by siRNAs, where the same RISC can be recycled over and over to cleave many mRNAs, works to the advantage of siRNA drugs. In fact, it may take only about 1000 siRNA molecules/cell to silence gene expression efficiently (a rough estimate derived from the frequencies of individual endogenous microRNAs in cells). Quantitative information about the relative numbers of Dicer and RISC molecules and endogenous microRNAs in different cells, together with information about the numbers of siRNAs required for efficient gene silencing, would be helpful for anticipating whether this toxicity might be an issue. There is a slim hint from the

SiRNA Therapeutics phase I study of intraocular injection of siRNAs targeting the VEGF pathway to treat age-related macular degeneration. The study results suggested that too high a concentration of siRNAs might be less effective than lower concentrations, but the number of subjects treated at each dose is too small to draw any conclusions.

Although many of the potential hurdles that stand in the way of harnessing RNAi for therapy seem surmountable, cautious optimism is in order. Although the anticipated problems seem to be solvable, it is often the unanticipated problems that sidetrack drug development.

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