First published online as a Review in Advance on April 17, 2006

Running Interference: Prospects and Obstacles to Using Small Interfering RNAs as Small Molecule Drugs

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Annu. Rev. Biomed. Eng. 2006. 8:15.1–15.26

The *Annual Review of Biomedical Engineering* is online at bioeng.annualreviews.org

doi: 10.1146/annurev.bioeng.8.061505.095848

1523-9829/06/0815-0001$20.00

Key Words

RNA interference, drug development, in vivo delivery, therapy

Abstract

RNA interference (RNAi) is a well-conserved, ubiquitous, endogenous mechanism that uses small noncoding RNAs to silence gene expression. The endogenous small RNAs, called microRNAs, are processed from hairpin precursors and regulate important genes involved in cell death, differentiation, and development. RNAi also protects the genome from invading genetic elements, encoded by transposons and viruses. When small double-stranded RNAs, called small interfering (si)RNAs, are introduced into cells, they bind to the endogenous RNAi machinery to disrupt the expression of mRNAs containing homologous sequences with high specificity. Any disease-causing gene and any cell type or tissue can potentially be targeted. This technique has been rapidly utilized for gene-function analysis and drug-target discovery and validation. Harnessing RNAi also holds great promise for therapy, although introducing siRNAs into cells in vivo remains an important obstacle. Pilot siRNA clinical studies began just three years after the discovery that RNAi works in mammalian cells. This review discusses recent progress and obstacles to using siRNAs as small molecule drugs.

INTRODUCTION

Two approaches can be used to harness the RNA interference (RNAi) machinery to induce specific suppression of gene expression in cells. The first approach involves transducing cells with small double-stranded (ds)RNAs that are either small interfering (si)RNAs or siRNA precursors, which are rapidly turned into siRNAs within cells (1). Because siRNAs are not readily taken up by most cells, this approach requires strategies for in vivo siRNA delivery into the cytoplasm of target cells. The second approach involves using plasmids or viral vectors to express short hairpin (sh)RNAs (resembling endogenous microRNA precursors) that are processed by the endogenous microRNA machinery into siRNAs. This gene therapy approach has the potential for long-term silencing of a disease-causing gene and may be especially suitable for correcting primary genetic defects or for treating chronic conditions (2, 3). This approach requires efficient transduction and long-term expression of the shRNA in the targeted cell and is associated with potential dangers from vector toxicity or insertional mutagenesis (4). Because the immediate hurdles of developing a small molecule drug at present are less formidable than those associated with gene therapy, this review focuses on the opportunities and obstacles for developing siRNA-based small molecule drugs.

In the past year, solutions to some of the anticipated difficulties of developing siRNA therapy have begun to emerge. The first phase I studies of intravitreal siRNA injection targeting vascular endothelial growth factor (VEGF) or its receptors to treat age-related macular degeneration were completed without any untoward toxicity (5). Consequently, because therapeutic benefit is increasingly being shown in a variety of in vivo disease models, there is considerable optimism that siRNAs may constitute the next new class of drugs, providing potential approaches for diseases that have thus far proven intractable. We do not review here all the in vivo disease studies that demonstrate the promise of siRNA small drug therapy, as these have recently been reviewed (6).

This review begins by describing our current understanding of the mechanisms of RNAi, which is still a work in progress. We then discuss the relative merits of siRNA therapies compared with other approaches involving antisense oligonucleotides (ASOs) or ribozymes. siRNA drug development generally requires chemical modifications to improve their pharmacokinetic properties without crippling their biological activity because unmodified siRNAs are otherwise rapidly eliminated by renal excretion and degradation by endogenous nucleases. In some tissues, particularly the mucosal surfaces such as the lung and vagina, siRNAs—either mixed with a transfection lipid or on their own—are efficiently taken up and silence gene expression. For indications that only require local delivery, drug delivery is not much of a problem, and clinical benefit has been shown in a variety of animal disease models. However, for systemic delivery, other strategies for siRNA delivery into cells are required. This review discusses some of the recent approaches to systemic siRNA delivery. Although intracerebral siRNA injection can introduce siRNAs into neurons, the blood-brain barrier remains a significant obstacle for the practical use of siRNAs as small molecule drugs in the central nervous system.

RNAi MECHANISM

Because siRNAs are taken up and processed by the endogenous RNAi machinery, intelligent drug development requires an understanding of the RNAi mechanism. The regulation of gene expression by RNAi operates primarily through two post-transcriptional methods—targeted mRNA degradation and the inhibition of translation (1). The mRNA degradation pathway is more efficient at silencing gene expression and is the mechanism being harnessed for siRNA-based therapeutics (7). RNAi is also used to inhibit transcription by forming and maintaining regions of silenced chromatin, but this mechanism is not as well understood (8). These different approaches are all unified in that the specificity of silencing is determined by small RNA species, typically ~19–23 nucleotides (nt) long, with complementarity to the target. Many of the same proteins, including the highly conserved Argonaute (Ago) and RNase III family proteins, are involved in each of the RNAi pathways (1). The mechanism of gene silencing depends on the degree of complementarity between the guide small RNA and the target RNA, with sequences having almost complete base pairing targeting mRNA cleavage and degradation and sequences with less complementarity blocking translation.

Fire and colleagues (9) stumbled upon RNAi when they found that dsRNA introduced into *Caenorhabditis elegans* silenced expression of a homologous target gene approximately 10–100-fold more efficiently than the corresponding antisense RNA. The RNAi response was recapitulated in vitro when long dsRNA was added to a *Drosophila* embryo extract, silencing expression of a homologous reporter gene by directing degradation of its mRNA (10). Following the fate of the long dsRNA introduced into *Drosophila* embryo extracts, Zamore and colleagues (11) found that the long dsRNA was rapidly cleaved into shorter dsRNA segments of approximately 21–23 nt, termed siRNAs. Similarly, small RNAs were found in vivo in *Drosophila* cells transfected with long dsRNA and in fly embryos and *C. elegans* injected with long dsRNA (12–14). Biochemical analysis of the siRNAs showed that these molecules had 2–3-nt 3′ overhangs and a monophosphate group on the 5′-terminal nucleotide, indicative of the cleavage products of an RNase III–type endonuclease (15) (**Figure 1**). This led to the rapid identification of Dicer as the enzyme required for cleaving dsRNAs into siRNAs (16). Chemically synthesized siRNAs could also direct target mRNA cleavage with the same efficiency as long dsRNA, confirming that siRNAs were the RNAi effector molecules (17). siRNAs, generated by Dicer or introduced exogenously, are taken up by a multiprotein complex, the RNA-induced silencing complex (RISC), and direct the complex to the homologous site on the target mRNA (1). Only one of the two strands of the siRNA can direct RISC-mediated cleavage. The strand of the siRNA with the lower thermodynamic stability for unwinding at its 5′′ end predominates in the RISC (18, 19).

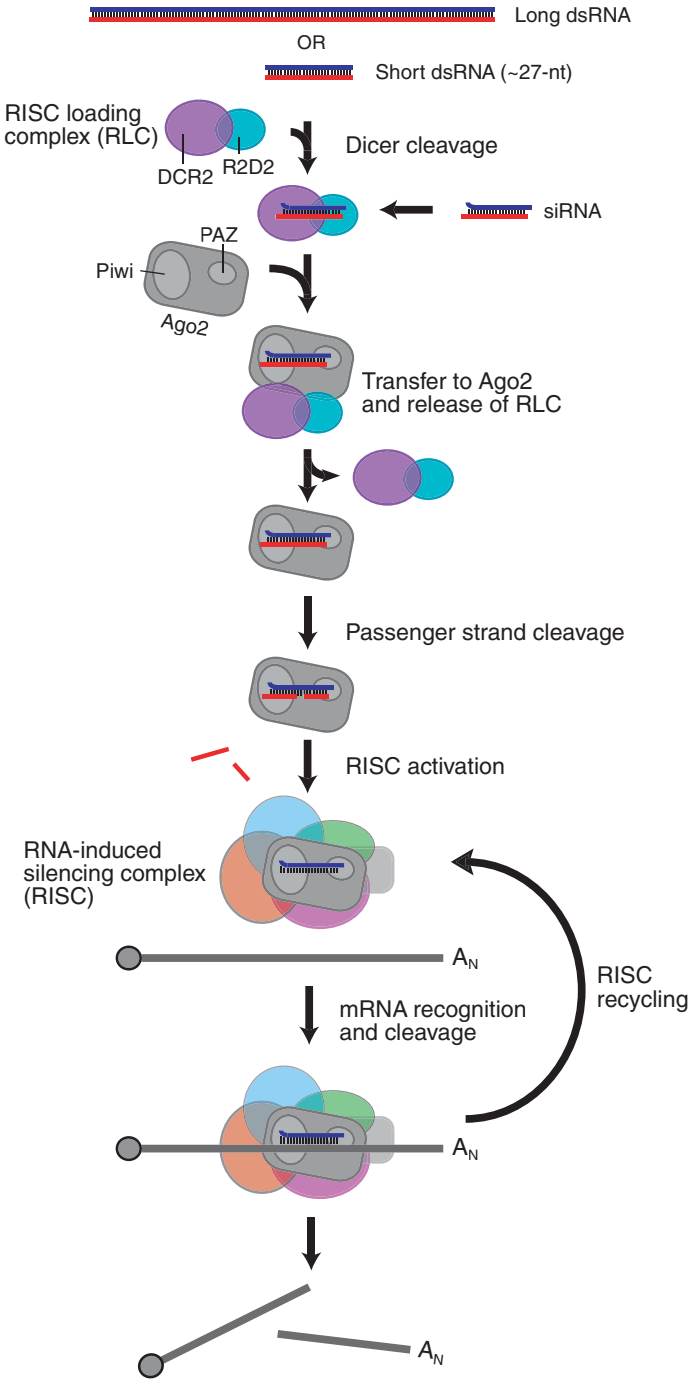
In *D. melanogaster*, the species in which the RNAi machinery has been best characterized, the loading of the siRNA into the RISC requires the RISC loading complex (RLC) that contains the double-stranded siRNA, DCR2 (one of two Dicer molecules in *Drosophila*) and a dsRNA-binding domain-containing protein, R2D2 (20). R2D2 helps to determine the asymmetry of the siRNA by binding to the more

thermodynamically stable end of the siRNA, orienting DCR2 to bind to the less thermodynamically stable end (21). How does the double-stranded siRNA found in the RLC transition to a single-stranded siRNA in the RISC? Ago2, a core component of the RISC and the endonuclease that cleaves the target mRNA, binds to the siRNA, displacing it from the RLC components, DCR2 and R2D2 (22–24). Transfer of the siRNA is facilitated by Armitage, a DEAD-box helicase (25). Ago2 cleaves the passenger strand of the siRNA, preparing the way for the guide strand to pair with a complementary mRNA sequence (26, 27). The phosphorylated 5′-terminal nucleotide of the siRNA guide strand burrows into a positively charged pocket of Ago2 and consequently does not participate in recognition and binding to the target mRNA (28). Nucleotides 2–8 of the siRNA guide strand are exposed on the surface of the RISC, forming a seed sequence that directs target recognition (28, 29). The paired siRNA-mRNA stretch is thought to form an A-type helix that aligns the cleavage site [10 nt from the 5′-end of the guide siRNA (15)] on the target mRNA with the Ago2 PIWI endonuclease domain (30). Ago2 cleaves the phosphodiester bond on the mRNA in the middle of the siRNA:mRNA recognition site. mRNA cleavage requires Mg^{2+} and produces 5′-monophosphate and 3′ hydroxyl-terminal groups (31, 32). The mutation of key residues that disrupt siRNA:mRNA pairing within this central region disrupts cleavage but has no effect on the binding of the siRNA guide strand (33). Once the target mRNA is cleaved, the activated RISC containing the siRNA guide strand is released to direct subsequent rounds of target mRNA cleavage (34). The catalytic character of the siRNA is likely an important determinant of its bioefficiency at gene silencing.

The endogenously generated microRNAs differ from siRNAs in their biogenesis but have overlapping functions (35). siRNA and microRNAs can both direct cleavage of homologous targets or repress the translation of partially complementary targets (36). The biogenesis of microRNAs involves the stepwise action of several RNase III–type endonucleases (37). microRNAs are expressed as highly structured hairpin

Figure 1

The RNA interference (RNAi) pathway in *Drosophila melanogaster*. Small interfering (si)RNAs are produced by the cleavage of longer double-stranded (ds)RNA substrates by Dicer (DCR2), a member of the RNase III family of enzymes (11, 16). Although DCR2 can cleave dsRNA without involving additional factors, DCR2's association with the dsRNA binding protein R2D2 to form the RISC loading complex (RLC) facilitates uptake of the siRNA into the RNA-induced silencing complex (RISC) (20). R2D2 binds to the most thermodynamically stable 5′ terminus of the duplexed siRNA, leaving DCR2 to interact with the less stable 5′ terminus (21). In this manner, RLC binding defines the siRNA strand that will enter the RISC to guide mRNA cleavage. Chemically synthesized siRNAs introduced into cells can enter the RNAi pathway either by associating with the RLC or binding directly to the RISC. The siRNA is transferred from the RLC to Argonaute (Ago)2, the RISC endonuclease (120). RISC activation requires the release of the passenger or sense strand of the siRNA by Ago2 cleavage, leaving the single-stranded guide (antisense) strand to direct the recognition of the target mRNA and position it for Ago2 cleavage (23, 24, 26, 27). After cleaving the mRNA, the activated RISC is released and is competent for multiple rounds of mRNA recognition and cleavage (34).



transcripts, containing numerous bulges and mismatches. The primary microRNA transcript (pri-miRNA) is recognized by a protein complex containing the RNase III–type endoribonuclease, Drosha, and a dsRNA binding protein called Pasha in *Drosophila*, Pash-1 in *C. elegans*, and DGCR8 in mammals (38–41). Drosha cleaves the pri-miRNA into a short stem loop pre-miRNA that is transported by Exportin 5 from the nucleus into the cytoplasm (42–45). In the cytoplasm, the pre-miRNA is recognized and cleaved by Dicer, in conjunction with another dsRNA binding protein (Loquacious in *Drosophila* and TRBP, *trans*-activator RNA–binding protein, in humans) into the mature microRNA (46–51). Subsequent steps in RNAi using microRNAs employ the same machinery as is used for silencing with exogenously generated siRNAs. Unlike plant microRNAs, which mostly function by cleaving target mRNAs (52–54), metazoan microRNAs are more likely to inhibit translation (55). The mechanism by which microRNAs inhibit translation remains poorly understood. mRNAs undergoing microRNA-induced translational inhibition and possibly siRNA-mediated cleavage, appear to be sequestered in distinct perinuclear cytoplasmic foci, referred to as processing (P) bodies, that contain factors associated with mRNA degradation, such as the decapping enzymes (DCP1 and DCP2), as well as the core components of the RISC, the Ago proteins (56–61).

A COMPARISON OF NUCLEIC ACID–BASED GENE-SILENCING APPROACHES

A variety of oligonucleotide approaches have been developed for silencing gene expression for therapeutics. Most notable are ASOs, ribozymes, and RNAi (62, 63). All take advantage of the recognition of a specific mRNA target site by a complementary oligomer, but they each silence gene expression by different mechanisms. Similar to RNAi, ASOs silence gene expression by either inhibiting translation or directing mRNA cleavage (62). However, unlike RNAi, where the degree of target site homology determines the mode of action, the charged characteristics of the ASO backbone largely determine the silencing mechanism (64, 65). ASOs with charged backbones (e.g., phosphodiester and phosphorothioate oligonucleotides) direct RNase H-mediated mRNA cleavage, whereas molecules with uncharged backbones (e.g., morpholinos, 2′-O-methyl and 2′-O-allyl substituted oligonucleotides, and locked nucleic acids) largely inhibit translation by steric hindrance (62). Ribozymes are highly structured, catalytic RNAs that guide the cleavage of complementary RNA sequences without the participation of proteins (62). Ribozymes, similar to siRNAs, can be engineered to silence alleles that differ by as little as 1 nt. ASOs and ribozymes hybridize to their mRNA targets on their own, which may be less efficient than RISC-facilitated binding of an siRNA to its mRNA target site. Therefore, relatively high concentrations of ASOs and ribozymes are required for efficient silencing, which increases the likelihood of nonspecific effects (63, 66, 67). In addition, the inhibition of translation mediated by steric hindrance of uncharged ASOs is relatively inefficient. This appears to be the case particularly for ASOs that target within the coding region of a gene. This inefficient targeting of the coding region by ASOs may be because the elongating ribosome can unwind regions of duplexed RNA to read through the steric

block (65). More success has been had with ASOs that target the 5′ or 3′ untranslated regions. ASOs can also interfere with other post-transcriptional events, such as splicing or nuclear export. siRNAs are incapable of interfering with these nuclear processes because the RISC RNAi effector complex is located in the cytoplasm (34, 62, 65). Because of the low stringency of RNase H, ASOs can direct the cleavage of mRNAs that have as little as 6–7 consecutive nt of complementarity, thereby reducing specificity (68). Off-target effects can be reduced by incorporating chemical modifications on the backbone, to improve hybridization or reduce susceptibility to nucleases, but often come at the cost of decreased activity.

Although studies that compare the different silencing approaches are limited, they generally have found that siRNAs silence gene expression more effectively than ASOs or ribozymes. Head-to-head comparison of an optimized phosphorothioate-modified ASO with an siRNA directed against the same target mRNA site found that the siRNA was approximately 100–1000-fold more efficient. siRNAs also produce more sustained silencing (69). This could be because the siRNA is protected from intracellular degradation by its incorporation into the RISC. Although virtually any gene can be specifically and efficiently silenced by RNAi, ASO approaches have only been found to work effectively in a limited number of cases. In fact, some ASOs that showed early promise as effective therapeutic agents were found to accomplish their antiviral or anticancer effects by stimulating an innate immune response owing to their high guanine-cytosine (GC) content, rather than by specifically silencing target gene expression (63, 70). Because these approaches use different mechanisms to silence gene expression, an effective strategy for therapeutic gene silencing might combine various antisense approaches. Such an approach has been applied to silence human immunodeficiency virus (HIV)-1 infection using a lentiviral vector encoding an shRNA, a ribozyme against CCR5, and a hairpin RNA decoy that mimics HIV-1 TAR (71).

CONVERTING siRNAs INTO THERAPEUTIC DRUGS

The application of siRNAs for therapeutic silencing of gene expression requires the introduction of drug-like properties, including increased in vivo stability and resistance to serum RNases, effective delivery to the tissue(s) of interest, and decreased nonspecific and immunostimulatory effects.

siRNA Sequence

The optimization of siRNAs for maximum potency will increase effectiveness and decrease potential nonspecific side effects because nonspecific effects are concentration dependent. By studying the functionality of large numbers of siRNAs, Reynolds et al. (72) were able to define characteristics associated with highly active siRNAs. These traits include a lack of secondary structure within the siRNA, low internal stability, moderate-to-low GC content, and low stability of binding interactions at the 5′ terminus of the guide siRNA strand (**Table 1**). The instability of the 5′ end of the guide strand imposes a functional asymmetry upon the siRNA

Table 1 Design considerations for maximally active siRNAs that have a low potential for off-target, unintended gene targeting

Design criterion	Rationale	References
Guanine-cytosine (GC) content between 30%–52%	Small interfering (si)RNAs with a GC content greater than 52% may have difficulty unwinding; siRNAs with a GC content lower than 30% may interact less well with the mRNA recognition site.	72, 73
Lack of internal secondary structure within the siRNA	Secondary structure could potentially interfere with the formation of a stable RNA duplex, which commonly adopts an A-type helix structure, in the siRNA and the siRNA:mRNA recognition site, or interfere with the interaction of the single-stranded siRNA with the RISC.	72, 73
Lower thermodynamic stability at the 5′-terminus of the guide siRNA strand	There is a bias toward an A residue at position 19 of the passenger strand and conversely, a bias against G and C nucleotides at this position. These biases favor looser binding at the 5′ end of the guide strand to promote its uptake into the RISC.	18, 19, 72, 73
A uridine residue at position 10 of the sense strand	Although Argonaute (Ago)2 will direct cleavage after any nucleotide, there is a bias toward cutting with a uridine base at position 10 of the sense strand.	72, 73
Specific sequence biases	The analysis of silencing by large numbers of siRNAs has shown there is a bias toward an A at position 3 and against a G at position 13 of the sense strand. These biases may be important for efficient mRNA cleavage, which might involve binding to the target mRNA, cleavage itself, or recycling of the activated RISC.	72, 73
Lack of immunostimulatory sequences within the siRNA	Recently, several sequence motifs (5′-UGUGU-3′ or 5′-GUCCUUCAA-3′) have been identified that activate Toll-like receptors.	108, 110, 114
Avoidance of sequences that have homology with unintended targets	Bioinformatics searches should be used to keep potential off-target effects at a minimum. This could involve performing a BLASTn search of the potential siRNAs or the Smith-Waterman dynamic programming sequence alignment algorithm. In particular, sequences that have a completely complementary seed sequence (nucleotides 2–8 of the guide strand) to important genes should be minimized.	29, 73, 116
Lack of secondary structure of the target site	It is not entirely clear what effect secondary structure in the complementary region of the target mRNA has on its binding to the siRNA-loaded RISC (the "activated" RISC). For ribozymes and ASOs that rely on the binding of naked oligonucleotides, the structure of the mRNA target site is an important consideration. However, because siRNAs are delivered as part of a ribonucloprotein complex that contains putative helicase activity, secondary structure may not be as important a determinant of activity.	73, 118, 119

to increase the rate of guide strand uptake (18, 19, 72, 73). In fact, an inefficient siRNA can be converted into a potent silencer by altering the thermodynamic properties of the 5′ ends of the guide and passenger strands (73a). Biochemical and bioinformatics studies have lead to algorithms, many of which are available on the web (**http://www.dharmacon.com**, **http://www1.qiagen.com/siRNA**, **http://www.ambion.com/techlib/misc/siRNA_finder.html**, **http://molecula.com/new/siRNA_inquiry.html**), that can aid in the choice of siRNAs to silence any gene, but these algorithms are imperfect and do not predict the most efficient

sequences. Ultimately only experimental testing can determine the most effective siRNA for a given target gene. For clinical use, comprehensive testing of a large array of sequences (or potentially all possible sequences) may be required not only to optimize silencing, but also to avoid sequence-specific off-target silencing of partially homologous genes or the stimulation of inflammatory responses by the activation of Toll-like receptors (TLRs) (see below).

Silencing may be improved by designing siRNA precursors that are slightly longer than the siRNA that is incorporated into the RISC but still smaller than the 30-nt threshold for triggering an interferon response (17). Kim et al. (74) suggests these dsRNAs will enter the endogenous microRNA pathway earlier than the shorter siRNAs and be taken up by Dicer and more efficiently passed on to the RISC. Whether these findings are generally true requires further experimental validation.

Stability and Nuclease Resistance

Knowledge about chemical modifications that improve the pharmacological properties of ASOs and ribozymes has been the starting point for increasing the in vivo stability of siRNAs. Ideally, modifications should increase siRNA stability while maintaining the potency of silencing. A variety of modifications can be incorporated at various positions on either strand. Generally, modifications of the passenger strand, which plays no direct role in silencing, have little adverse effect on silencing but contribute to enhancing the stability of the duplex siRNA. Because the 5′-terminal phosphate on the guide strand is required for binding to Ago2, chemical modifications that block phosphorylation of the 5′ end of the guide strand (e.g., 5′-O-Me) impair siRNA-mediated target silencing; however, this same modification on the passenger strand is well tolerated. In fact, because either strand can potentially direct silencing, alterations that reduce passenger strand uptake into the RISC are desirable to reduce potential off-target effects. In addition to removing the 5′ phosphate on the passenger strand, disrupting base-pairing of the siRNA at the 5′ end of the guide strand favors unwinding from that end and thereby enhances guide strand incorporation into the RISC (18, 19, 72). Incorporation of a 3′,5′-inverted deoxy abasic residue at the 5′- and 3′-terminus of the passenger strand and the 3′-terminus of the guide strand increases resistance to serum exonucleases without impairing activity (75–77). Similarly substituting phosphorothioate linkages in the phosphodiester backbone at the ends of the strands protects against exonuclease digestion without adversely affecting silencing. In addition to increasing the resistance to exonucleases, these modifications may also inhibit uptake of the passenger strand into the RISC. Although these modifications were found on siRNA that had greatly improved stability, the siRNAs tested had additional internal modifications that made it difficult to assess the effect of these modifications by themselves. Not all modifications on the 3′-terminus of the passenger strand are well tolerated—adding either a 2′-O,4′-C-ethylene thymidine or 2-hydroxyethylphosphate abrogates siRNA function.

In addition to the modification of terminal residues, internal modifications are used to increase resistance to endonuclease degradation. These include substituting chemical groups for the 2′-OH residue of the ribose, as well as changing the

phosphodiester backbone. Generally 2′-fluoro (2′-F) substitutions have been well tolerated. In fact, several groups have found that 2′-F modifications on all pyrimidines from both strands had no affect on silencing (75, 76, 78–80). These substitutions greatly enhanced stability and maintained effective silencing both in tissue-culture experiments (75, 78, 80) and in mice (75, 76, 80). However, this is not a universal finding because 2′-F substitutions for all the uridine residues in another study significantly decreased silencing (14). The full substitution of 2′-O-Me or 2′-deoxy residues in either strand leads to significantly reduced silencing (79, 81), whereas the modification of selected residues maintains silencing while conferring resistance to nucleases (77). Other attempts at increasing siRNA stability incorporate thioate linkages (P-S) in place of the phosphodiester backbone. The full substitution of the siRNA with thioate linkages decreased silencing by greater than 50% (79), whereas partial substitution retained activity (82, 83). However, the P-S substituted siRNAs were somewhat cytotoxic (83).

The most promising results have used a combination of chemical modifications to ensure stability and efficient gene silencing. An siRNA with the following modifications was significantly resistant to serum nucleases: a passenger strand containing 2′-F modifications on all the pyrimidines, deoxyribose for all the purines, and a 3′-,5′-inverted deoxy abasic residue at the 5′- and 3′-termini and a guide strand containing 2′-F on all the pyrmidines, 2′-O-Me-modified purines, and a single 3′-terminal thioate linkage. The guide strand of the modified duplexed siRNA had a half-life in 90% human serum of 3 days compared with 5 min for the unmodified siRNA (75). Despite the extensive modifications, this siRNA directed against hepatitis B virus (HBV) inhibited viral replication in tissue culture and upon hydrodynamic injection with an HBV replicon in mice. (Hydrodynamic injection involves rapid intravenous injection of siRNAs in a large-volume bolus that causes right-sided heart failure and elevated venous pressures that transiently disrupt the plasma membrane of cells in highly vascularized organs, such as the liver and lung, allowing transient siRNA uptake.) Similarly, modifying all the pyrmidines in both the target and guide strands of an siRNA increased resistance to serum nucleases. These modifications increased the plasma half-life to approximately 1 day, compared with a half-life of less than 1 min for unmodified siRNAs (80). The 2′-F-modified siRNAs and unmodified siRNAs showed roughly equivalent levels of silencing in cell-culture experiments and upon hydrodynamic injection with a luciferase expression construct in mice. Both 2′-F and unmodified siRNAs showed equivalent silencing in mouse livers when introduced by hydrodynamic injection, despite the increased resistance of the modified siRNA to serum nucleases. This suggests cellular uptake occurs rapidly after hydrodynamic injection, and once inside cells, unmodified siRNAs are as resistant to degradation as modified siRNAs (80). This could be because the RISC complex protects the siRNA guide strand from cellular nucleases. This conclusion is supported by earlier experiments that showed sustained silencing for 10 days or more when unmodified siRNAs were introduced into nondividing cells both in vitro and in vivo (84, 85).

Although modified siRNAs show no increase in silencing compared with their unmodified counterparts after hydrodynamic injection, hydrodynamic injection for systemic delivery is not suitable for human clinical use. For other systemic methods

of administration, in which cellular uptake of siRNAs may occur more slowly, improving the circulating half-life of the siRNA is likely to contribute significantly to therapeutic potency. However, for topical delivery to a variety of tissues, including the lung (86, 87), vagina (88) and eye (5), uptake may be rapid enough or the levels of nucleases low enough to allow for effective delivery and clinical benefit from unmodified siRNAs. In these situations, it is still unclear whether any benefit is gained by siRNA modification. Because endonucleases generally have sequence preferences and chemical modifications often reduce silencing, one sensible strategy is to customize and minimize the chemical modifications to the siRNA by identifying and modifying only the sites of degradation for each particular siRNA (89).

Because siRNAs are smaller than the size threshold for glomerular filtration, rapid renal excretion of unmodified siRNAs is the most important determinant of circulating half-life. Incorporating siRNAs into particles or developing methods for enhancing binding to serum proteins blocks rapid renal excretion and is essential to any effort to improve siRNA pharmacokinetics after systemic administration. One approach conjugated the 3′ end of the passenger strand to cholesterol, which enhanced cellular uptake via lipoprotein receptors but also enhanced serum half-life by binding to serum albumin (89) (**Figure 2**). The cholesterol-conjugated siRNA improved the elimination half-life of the siRNA from 6 min for an unconjugated siRNA to 95 min for the cholesterol conjugate after intravenous injection into rats. In another approach, an antibody fragment-protamine fusion protein was used to bind multiple siRNAs, creating a particle that bypassed kidney filtration and targeted the siRNAs only into cells bearing the cell surface receptor recognized by the antibody (103). In yet another approach, the encapsulation of an siRNA into a specialized liposome produced a stable-nucleic-acid-lipid particle that allowed for increased retention in the blood stream (the elimination half-life increased from ∼2 min to 6.5 h), effective siRNA delivery, and silencing of an HBV replicon in mouse liver cells after passive intravenous injection (76). Complexing siRNAs with low molecular weight polyethylenimine (PEI) protects the siRNAs from degradation and elimination and effectively delivers siRNAs to subcutaneous tumor cells after intravenous injection in mice (90). Although PEI may be too toxic for clinical use, combining siRNAs into other copolymers might be suitable.

Intracellular Delivery

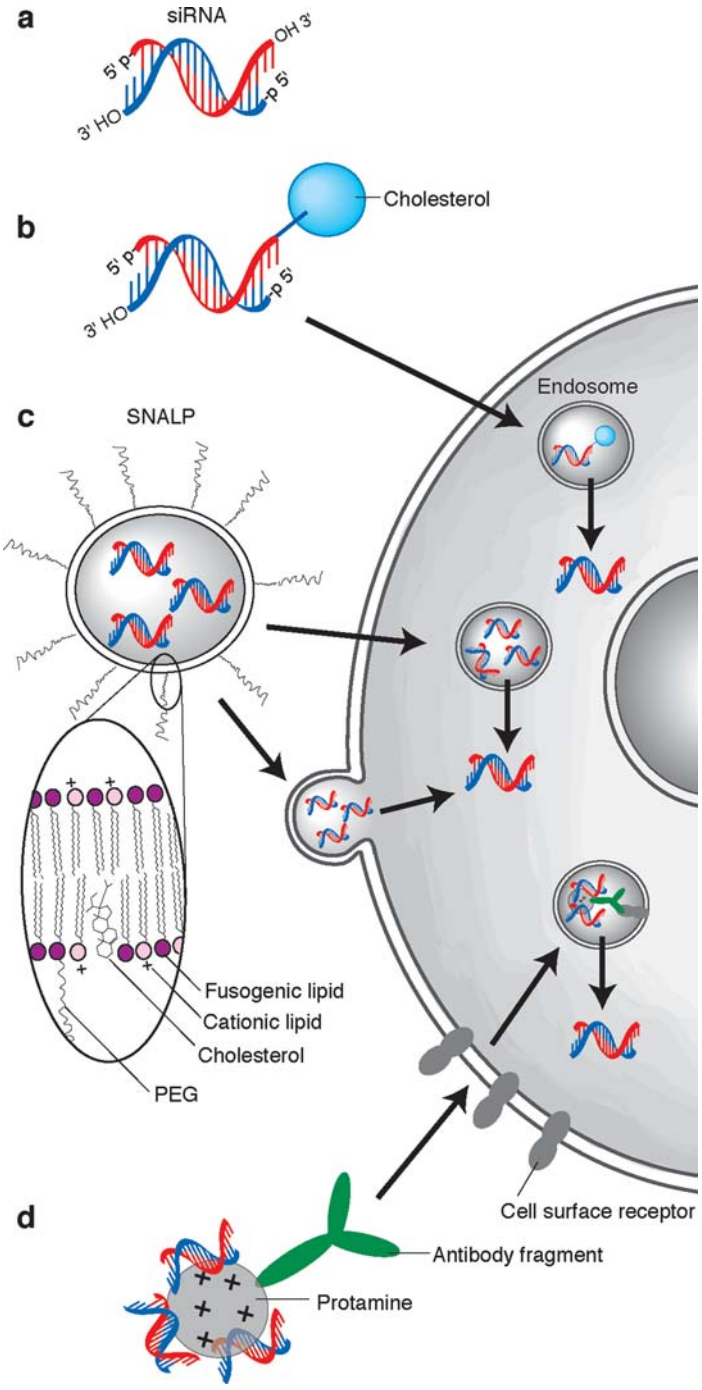
The major hurdle for the effective application of RNAi in vivo is the delivery of the siRNA to the target organ(s) in a manner that retains the siRNA's silencing activity. This requires siRNA uptake into the cytoplasm where it can be loaded onto the RISC. The accessibility of the target tissue influences the method used for the delivery of siRNAs. The most direct applications, and the ones most extensively explored, are topical or local delivery to easily accessible tissues. Animal studies using localized delivery to the eye, lung, muscle, subcutaneous tissues, and vagina have shown effective silencing and protection in disease models (**Figure 3**). Beyond the ease of delivery, localized administration of siRNAs also requires lower amounts of siRNAs because there is decreased uptake by unintended tissues and less elimination by renal

excretion. However, most tissues are not easily accessible and would require invasive methods for access. This makes the development of effective, clinically relevant methods for the systemic administration of siRNAs essential.

Although *C. elegans* and *Drosophila* cells actively take up siRNAs (91, 92), mammalian cells, even those that actively sample their environment (e.g., dendritic cells and macrophages), do not effectively internalize these small molecules (84, 93). This difficulty is easily overcome in vitro for most cells by using cationic lipid transfection reagents to transduce the siRNAs into cells. Although many of these reagents have toxic side effects that limit their usefulness in vivo, several lipid-based transfection reagents have been successfully used for local in vivo application. For example, siRNAs complexed with Oligofectamine™ were taken up by epithelial and lamina propria cells throughout the vagina and ectocervix, leading to the effective silencing of green fluorescent protein expression in a transgenic mouse that ubiquitously expresses green fluorescent protein (88). This same delivery strategy was used to protect mice from a lethal intravaginal inoculation of herpes simplex virus (HSV)-2, even when the HSV-2 siRNAs were given 3 h after the viral challenge. Importantly, the siRNA-Oligofectamine-treated tissues showed no induction of interferon or interferon-responsive genes when analyzed by quantitative reverse transcription polymerase chain reaction, and no cytotoxic effects were seen upon histological examination for cell death or immune infiltration (88). The intranasal or intratracheal administration of siRNAs effectively silences gene expression in the lung. Although most of these studies used lipid-based transfection reagents, several studies have demonstrated effective delivery and silencing of gene expression in the lung in the absence of transfection reagents (94–96). In fact, the intranasal administration of siRNAs that were either naked or complexed with the transfection reagent Trans-IT TKO® effectively protected mice from respiratory syncytial virus and parainfluenza

Figure 2

Strategies for the systemic delivery of small interfering (si)RNAs. (*a*) Schematic representation of an siRNA molecule containing the characteristic 19-nt RNA duplex with 2–3-nt 3′ overhangs and phosphorylated 5′ termini (81). The in vivo delivery of siRNAs into cells in a therapeutically relevant manner remains one of the biggest challenges to using siRNAs as small molecule drugs. The small size of the siRNA leads to the rapid elimination of naked, unmodified siRNAs from the circulation by renal clearance (7). Therefore, successful siRNA delivery must increase the retention time of the siRNAs, facilitating their uptake into the tissue(s) of interest. (*b*) The conjugation of the 3′ terminus of the passenger strand of an siRNA to cholesterol greatly increases the retention of the siRNA within the circulation by binding to albumin (89). Cholesterol binds to cellular low-density-lipoprotein receptors, which direct the endocytosis of cholesterol-conjugated siRNAs into cells. (*c*) The encapsulation of siRNAs into modified liposomes, termed stable-nucleic-acid-lipid particles (SNALPs), increases the retention time of the siRNAs in the bloodstream of mice after intravenous injection and uptake into hepatocytes and other tissues (76). Depending on the liposome, this may involve direct membrane fusion or endocytosis. PEG, polyethylene glycol. (*d*) By combining the nucleic acid–binding properties of protamine and the specificity of an antibody, an antibody fragment-protamine fusion protein can noncovalently bind siRNAs and deliver siRNAs, probably by endocytosis, specifically to cells that express the surface receptor recognized by the antibody (103).

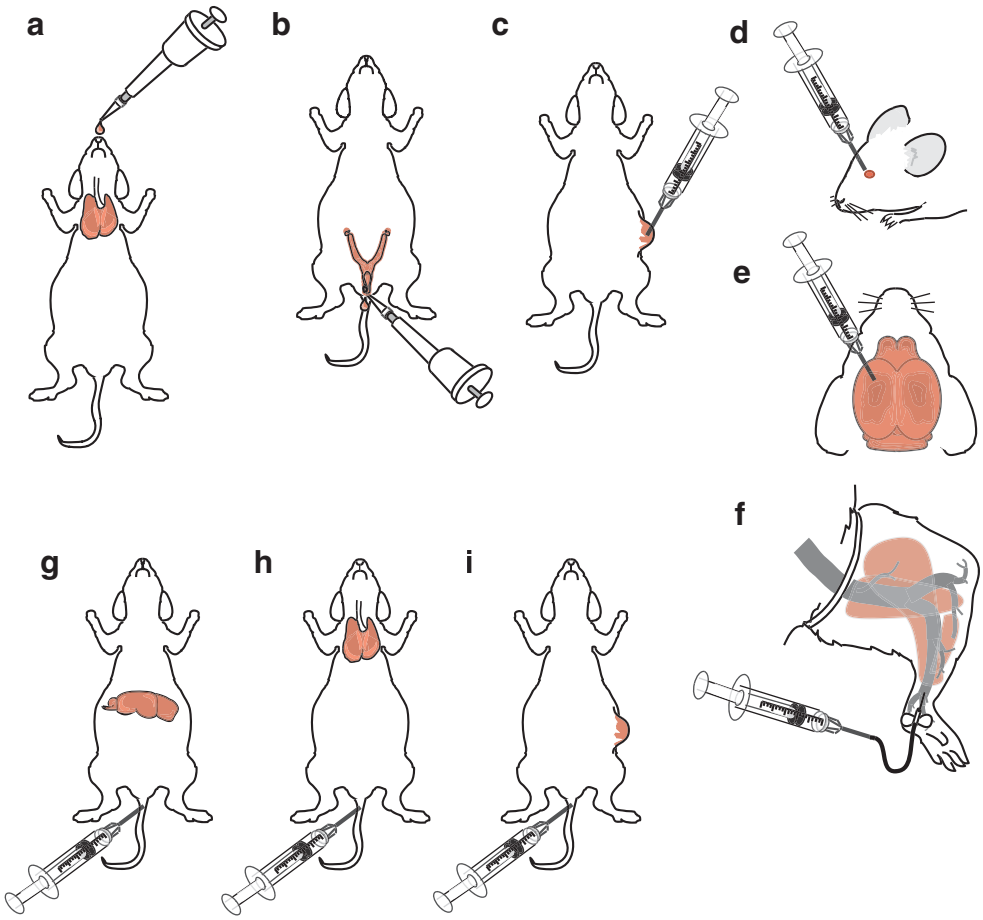


virus infections (94). Importantly, the intratracheal administration of siRNAs in a glucose solution efficiently delivered siRNAs to the lungs of Rhesus macaques (87). The prophylactic and therapeutic administration of siRNAs targeting the SARS (severe acute respiratory syndrome) coronavirus effectively inhibited SARS coronavirus replication and protected monkeys from developing SARS-like symptoms and lung pathology. This study was the first to demonstrate the effectiveness of siRNAs in a primate disease model.

The eye represents a prime target for the therapeutic administration of siRNAs owing to its relative isolation and the ease with which siRNAs can be delivered. In fact, the first phase I clinical studies using siRNAs were performed on patients with neovascular age-related macular degeneration. Neovascularization, the growth of new blood vessels, within the eye is a leading cause of vision loss among adults. Because of the central role of VEGF in stimulating the growth of new blood vessels, this molecule (as well as its receptors, VEGFR1 and VEGFR2) has been chosen for targeting. The subretinal injection of VEGF siRNAs suppressed choroidal neovascularization induced by laser photocoagulation in mice (97). Silencing of VEGF, VEGFR1, or VEGFR2 impaired corneal neovascularization in response to HSV-1 infection or treatment with proinflammatory CpG oligodeoxynucleotides (98). The suppression of new blood vessel growth in response to these insults was enhanced when the three siRNAs were combined. Both localized administration of uncomplexed siRNAs (subconjunctival injection) and systemic intravenous administration

Figure 3

Routes of small interfering (si)RNA in vivo administration. Effective in vivo delivery has been achieved using localized (*a–f*) and systemic (*g–i*) administration. (*a*) Intranasal and intratracheal instillation of siRNAs have been used to protect against respiratory viruses in mice and nonhuman primates (87, 94–96). This route of administration is particularly effective because siRNAs are readily taken up by lung tissue even in the absence of a transfection reagent (87, 94). (*b*) The use of siRNAs as a potential microbicide for a sexually transmitted disease was recently demonstrated by protecting mice from herpes simplex virus 2 infection (88). (*c*) Intratumoral injection of siRNAs complexed with various lipid formulations, atelocollagen, or an antibody-protamine fusion protein has been shown to inhibit tumor outgrowth (103, 121, 122). (*d*) Direct injection of siRNAs into the eye has been used for the first clinical studies testing siRNA therapy (97–99). (*e*) Direct injection into the brain or by continuous infusion into the ventricles protected mice from neuropathic pain and flavivirus infection (123). (*f*) Hydrodynamic delivery of siRNAs into an isolated tissue may be a viable therapeutic approach. This is accomplished here by the isolation of the limb using a tourniquet (124). Another approach is the injection by catheter into the vein training an internal organ, such as the kidney. A variety of internal tissues have been targeted by systemic intravenous administration, most notably the liver (*g*), lungs (*h*), and tumors (*i*). (*g*) Hydrodynamic (high-volume, high-pressure) injection of siRNAs was the first mechanism that successfully administered siRNAs systemically (85, 125–129), but this method, which causes right-sided heart failure, is unsuitable for human use. Passive injection of siRNAs either conjugated to cholesterol (89) or encapsulated in a lipid particle effectively delivers siRNAs to the liver (76) (see **Figure 2**). (*h*) Intravenous injection of siRNAs complexed with polyethyleneimine delivers siRNAs to the lungs and inhibits influenza infection (95). (*i*) Intravenous or intraperitoneal injection of siRNAs complexed with lipids or with antibody fragment-protamine fusion proteins inhibits tumor growth (90, 103).



of siRNAs incorporated into nanoparticles constructed with PEI conjugated to a modified polyethylene glycol containing an arginine–glycine–aspartic acid (RGD) peptide ligand inhibited corneal neovascularization. Similarly, the intravitreous or periocular injection of anti-VEGFR1 siRNAs significantly reduced neovascularization in mice with ischemic retinopathy (99). The effectiveness of siRNA delivery in the lung, vagina, and eye may indicate specialized mechanisms for siRNA uptake in surface tissues in mammals. These accessible sites will be the initial testing grounds for RNAi therapeutics.

For systemic administration, alternate delivery strategies are needed. One approach is to use peptide-based gene delivery. A novel recombinant protein that contains the fusion peptide domain of the HIV-1 gp41 protein and the nuclear localization sequence from the SV40 large T antigen effectively delivers DNA to the nucleus of cells. A variant of this protein that contains a point mutation in the nuclear localization sequence binds and delivers siRNAs to the cytoplasm of tissue culture cells (100, 101). Although this technology has not been applied to the delivery of siRNAs

in vivo, similar bifunctional proteins have been used for gene transfer experiments in mice (102). The conjugation of cholesterol to siRNAs improves the pharmacological properties of the siRNA (see above) and facilitates siRNA delivery because of uptake by ubiquitous cholesterol receptors to a wide variety of tissues (including the lung, heart, kidney, adipose, and liver) upon intravenous injection in mice and rats (89). The delivery of cholesterol-conjugated siRNAs targeting apolipoprotein B, a key protein in the metabolism of cholesterol and the formation of low-density lipoproteins, effectively silenced apolipoprotein B expression in the liver and jejunum, leading to a significant reduction in circulating total cholesterol and low-density-lipoprotein cholesterol.

An important consideration for the development of siRNA-based therapeutics is the dose necessary to achieve effective silencing. Hydrodynamic injection requires a large amount of siRNAs (approximately 50 μg per mouse per injection) because only a relatively small fraction of the siRNAs actually enters the tissues. Delivery agents that enhance the retention of the siRNA in the circulation, such as lipid nanoparticles and cholesterol conjugation, can decrease the required dosage (**Figure 2**). However, these approaches deliver the siRNAs to a variety of tissues as well as the intended target organ. This increases the amount of drug needed and increases the potential toxicity by targeting unintended tissues. Therefore, delivery strategies that can target specific cells or tissues would be of great therapeutic value. One approach takes advantage of the nucleic acid–binding properties of protamine to bind the siRNAs and the specificity of fragment antibodies (Fab) to deliver siRNAs to the cell type of interest (103). To achieve this, a bifunctional protein was produced that fused protamine to the carboxy terminus of the heavy chain Fab fragment that recognizes the HIV-1 envelope protein (gp120). This protein was able to bind siRNAs via a charge interaction with basic protamine and deliver them only to cells that expressed the HIV-1 envelope. To test the efficacy of delivery and silencing, T cells infected with HIV-1 that therefore expressed the HIV-1 envelope protein on their surface were treated with siRNAs targeting the HIV-1 capsid protein. This led to significant inhibition of HIV-1 replication. The efficient delivery of siRNAs into primary T lymphocytes was unexpected because these cells are refractory to lipid-based transfection. In vivo, the specificity of delivery was tested by subcutaneously implanting B16 mouse melanoma cells expressing the HIV-1 envelope protein and injecting, either intratumorally or intravenously, the fusion protein mixed with a fluorescently tagged siRNA. The fluorescent siRNA was targeted specifically to the HIV-1 envelope-expressing B16 cells and not the surrounding tissue or to B16 cells that lack envelope expression. This targeting was shown to have therapeutic benefit because the delivery of siRNAs targeting several oncogenes inhibited the growth of HIV-1 envelope-expressing tumors, but not envelope-negative tumors. Similar results were seen with a single chain antibody-protamine fusion protein targeting ErbB2+ breast cancer cells. Much lower amounts of siRNAs were used in this study (injections of 3 mg/kg) compared with the cholesterol-conjugated siRNA delivery study (injections of 50 mg/kg). However, conjugating the siRNA passenger strand to other cell surface receptor ligands besides cholesterol might also be used for specific targeting, particularly by choosing ligands to receptors expressed only on the subset of cells needing targeting.

If these methods use receptor-mediated endocytosis for siRNA uptake, as seems likely, the siRNAs must escape the endosome and enter the cytoplasm to direct gene silencing. The requirement for endosome escape is highlighted by a study that showed that a Tat-conjugated polyamidoamine dendrimer, a fusion protein with oligonucleotide binding and cell-penetrating characteristics, was able to deliver siRNAs to cells, but they were inactive (104). Microscopic analysis demonstrated fluorescent siRNAs localized to intracellular vesicles, presumably endosomes, but sequestered from the cytoplasm. How the effective delivery siRNA agents that use receptor-mediated endocytosis facilitate endosomal escape is not understood.

Specificity of Silencing

Initial reports suggested an siRNA would require nearly complete complementarity with its target mRNA over its entire 19 or more nt sequence for efficient silencing. In fact, a single nucleotide change within the siRNA was capable of completely abrogating the functioning of the siRNA. However, this high degree of specificity was not always the case, and siRNAs were quickly found that could effectively silence gene expression despite mismatches between the target site and the siRNA guide. Off-target silencing must be taken into consideration in developing RNAi-based therapies. Off-target effects can be divided into two types of responses: (*a*) the induction of nonsequence-specific silencing pathways and (*b*) the silencing of targets that have partial complementarity to the siRNA.

dsRNAs, produced as an intermediate in the life cycle of many viruses, trigger an antiviral interferon response that globally shuts down gene expression by interfering with translation. Because this response is usually efficiently triggered only by dsRNAs greater than 30 nt in length, shorter siRNAs do not efficiently trigger an interferon response. Although initial studies found no activation of the interferon pathway by siRNAs, subsequent studies performed using sensitive microarray analysis found that treatment of highly sensitive cells, particularly at high concentrations of siRNAs, upregulates the expression of subsets of interferon genes (105–107). This upregulation does not lead to increased cytotoxicity, suggesting only an attenuated interferon response is induced. Moreover, this response is sequence dependent. In addition, different subsets of interferon-responsive genes are induced under different treatment regimes and by different siRNAs. Vector-mediated expression of shRNAs by polymerase III promoters and siRNAs in vitro synthesized by T7 polymerase is particularly potent at inducing interferon genes (105). This may be the result of particular sequence preferences for aspects of transcription by these promoters (e.g., the need for a run of uridines for the termination of transcription). The nonspecific results demonstrated in these studies are not universal, and some studies using vector-mediated siRNA delivery found no evidence of interferon gene upregulation. By mimicking the structure of endogenous microRNAs, generally expressed from pol II promoters, it may be possible to express siRNA precursors that effectively silence gene expression without inducing nonspecific gene silencing.

Much of the interferon induction by siRNAs may not come from direct activation of the dsRNA-dependent kinase of the interferon pathway, but by indirect

triggering of TLR activation as part of the innate immune danger response that recognizes pathogen-related RNAs. Recent studies show that TLR receptors recognize specific immunostimulatory sequence motifs in the siRNAs, explaining why nonspecific effects have been seen with only some siRNAs. A number of recent studies have found a dose-dependent and sequence-dependent stimulation of inflammatory cytokine release after systemic administration to mice (108, 109). Plasmacytoid dendritic cells were highly sensitive to the stimulatory siRNAs, whereas monocytes produced very little IFNα when treated with the same siRNAs. The immunostimulatory activity correlated with specific GU-rich sequences, in particular 5′-UGUGU-3′ and 5′-GUCCUUCAA-3′, suggesting recognition occurs through TLR7 and TLR8 (108, 110, 111). Consistent with the role of TLR7 in this process, TLR7 knockout mice did not mount an inflammatory response to siRNAs (108, 110, 112). Nucleotide changes within this sequence decreased the immunostimulatory properties of the siRNA and the inclusion of an activating GU-rich sequence could convert a nonstimulatory siRNA into one with immunostimulatory properties (108). Immunostimulatory motifs that activate the innate immune response should be avoided when designing siRNAs. Because all the sequences that activate TLRs are not known, the potential for TLR activation by a given siRNA needs to be determined experimentally for candidate siRNAs. TLR activation was found after administering siRNAs containing immunostimulatory motifs in vitro or as lipid nanoparticles (but not naked siRNAs) in mice (108, 112). That these effects were seen only in the context of liposomes suggests the mechanism by which siRNAs are taken up by the cells will influence the potential induction of nonspecific silencing. Consistent with these findings, a separate study found that treatment with a lipid transfection reagent alone was capable of altering the pattern of off-target gene expression and that siRNAs introduced by electroporation had minimal nonspecific gene upregulation (113). Alternatively, liposome-mediated delivery may increase uptake, leading to higher intracellular siRNA concentrations and a greater stimulation of nonspecific responses. Chemical modification of the siRNAs may reduce TLR activation. When delivered in vivo within stable-nucleic-acid-lipid particles, unmodified siRNAs induced an innate immune response, whereas the same siRNA sequence when extensively modified with 2′-F, 2′-O-Me, and deoxyribose residues was nonstimulatory (75). Similarly, the incorporation of 2′-O-Me uridine and guanine nucleosides into an immunostimulatory siRNA sequence completely abrogated the immune response to the siRNA but did not reduce silencing (114). Therefore, by prudent sequence choice and appropriate chemical modifications, the nonspecific induction of TLR signaling and inflammation can be avoided.

Another potential source of toxicity is the silencing of mRNA targets that are only partially homologous to the siRNA sequence (115, 116). In some cases, an siRNA can inhibit gene expression when the target has only 15 complementary nt, with as few as 11 contiguous nt (116). In general, mRNA microarray studies have found that most off-target effects are small, usually resulting in less than a twofold decrease in mRNA levels, but a few genes may be more severely affected (115–117). However, these studies may underestimate off-target effects because they measure changes in mRNA and do not account for differences in protein expression resulting from

translational inhibition. The rules that govern potential translational inhibition are not well understood, making the identification of potential off-target effects difficult to predict. Of particular concern is the possibility that the inhibition of translation may require base-pairing of only the 7-nt seed sequence. Generally the effect of any single small RNA on blocking translation is small, and significant translational inhibition requires the concerted action of several targeting events acting on the same mRNA (55). Therefore, these sorts of translational off-target effects (although impossible to avoid because of the presence of any 7-nt sequence in multiple mRNAs) may not contribute substantially to altering protein expression of unintended targets (36). Because proteome screens are not as advanced or extensively available as mRNA microarray technology and target prediction is not reliable, identifying potential targets of translational inhibition at early stages of clinical development may prove challenging. It is too early to tell whether silencing genes with partial homology will prove a significant problem in practice. Once a significant off-target effect is identified, it may be possible to bypass the problem with minor alterations of the siRNA sequence.

SUMMARY

The clinical development of siRNA drugs has advanced rapidly. In just four years since RNAi was shown to work in mammalian cells (17), the endogenous molecular RNAi pathways and their importance in regulating gene expression in mammalian cells are rapidly being elucidated. These scientific advances are swiftly being translated into therapeutic approaches to delivering siRNAs into cells to tackle a variety of diseases. The major obstacles of drug delivery, stability, and potential inflammatory side effects seem to be solvable. As siRNA-based therapies begin to be evaluated in clinical studies, the next few years will test the promise of RNAi-based drugs. It should be an exciting time.

ACKNOWLEDGMENTS

We thank members of the Lieberman laboratory and our collaborators for useful discussions. This work was supported by NIH AI56900 and AI056695.

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