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RNAi: A novel antisense technology and its therapeutic potential

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Summary

Antisense oligonucleotide agents induce the inhibition of target gene expression in a sequence-specific manner by exploiting the ability of oligonucleotides to bind to target RNAs via Watson-Crick hybridization. Once bound, the antisense agent either disables or induces the degradation of the target RNA. This technology may be used for therapeutic purposes, functional genomics, and target validation. There are three major categories of gene-silencing molecules: (1) antisense oligonucleotide derivatives that, depending on their type, recruit RNase H to cleave the target mRNA or inhibit translation by steric hindrance; (2) ribozymes and deoxyribozymes – catalytically active oligonucleotides that cause RNA cleavage; (3) small interfering double-stranded RNA molecules that induce RNA degradation through a natural gene-silencing pathway called RNA interference (RNAi). RNAi is the latest addition to the family of antisense technologies and has rapidly become the most widely used approach for gene knockdown because of its potency. In this mini-review, we introduce the RNAi effect, briefly compare it with existing antisense technologies, and discuss its therapeutic potential, focusing on recent animal studies and ongoing clinical trials. RNAi may provide new therapeutics for treating viral infections, neurodegenerative diseases, septic shock, macular degeneration, cancer, and other illnesses, although *in vivo* delivery of small interfering RNAs remains a significant obstacle.

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RNA interference • siRNA • antisense technology • animal studies

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BACKGROUND

Because a number of diseases involve over-expression of a particular gene, much effort has gone towards finding drugs that can downregulate gene expression on the DNA, RNA or protein level. Similar approaches are being tested to fight viruses that, upon infection, turn host cells into factories that produce multiple copies of their viral genomes. One approach to alter levels of gene expression occurs on the post-transcriptional level through the use of antisense (AS)-based technologies. The antisense approach involves the delivery of oligonucleotides that are complementary to the mRNA or viral RNA of interest into cells, which are then able to seek out and bind to the RNA target (Figure 1). This leads to the suppression of expression of the protein either through degradation of mRNA or by sterically blocking critical steps of the translation process (depending on the type of AS used). The specificity of this approach is based on the assumption that any sequence longer than a minimal number of nucleotides (~20 nt) occurs only once within the human genome. In addition to therapeutic applications, other common applications for this technology include characterization of the roles of specific genes, discovery and validation of new targets for therapeutics, and the production of knock-down mice.

The focus of this mini-review will be the recently discovered and most powerful AS technology, which uses short interfering RNA (siRNA) molecules to induce gene silencing by RNA interference (RNAi), a naturally occurring gene regulatory pathway. The aim of this article is to introduce the RNAi effect, briefly compare it with previously developed antisense technologies, and discuss potential clinical applications, the most exciting of which is the development of a new generation of drugs. We will not focus on the detailed molecular mechanism of RNAi, cell studies, or biological applications, but rather on animal studies and ongoing clinical trials. We would like to apologize to the authors whose work was not cited in this mini-review due to size limitations.

ANTISENSE TECHNOLOGIES: FROM ANTISENSE OLIGONUCLEOTIDES TO siRNA

The original antisense technology that was developed in 1978 used antisense oligodeoxynucleotides complementary to sequences within their target mRNAs to inhibit gene expression [1]. Since then, many varieties of modified and unmodified DNA and RNA oligonucleotides of typical length 18–25 nucleotides have been used in antisense studies. All of these oligonucleotides share the first step in the mechanism of gene knockdown in common: they find and hybridize to their target RNAs in the cell. Once hybridized to their targets, negatively charged oligonucleotides, such as phosphodiester and phosphorothioates, are recognized by the cellular enzyme RNase H, which specifically cleaves the RNA strand of the complex and thereby degrades the target mRNA [2–4] (Figure 2A). Another class of antisense molecules does not activate RNase H because of the nature of the duplex formed with the RNA target, and instead inhibits translation by steric hindrance [5] or interferes with splicing of pre-mRNA [6] (Figure 2B). This class includes such modified nucleic acid derivatives as morpholinos, 2'-O-methyls, 2'-O-allyls, locked nucleic acids and peptide nucleic acids [7,8].

Ribozymes, RNA enzymes that catalyze chemical reactions without any protein co-factors, are another important category of sequence-specific gene-silencing molecules. Ribozymes used for gene-knockdown applications have a catalytic domain that is flanked by sequences complementary to the target RNA. The mechanism of gene silencing involves binding of the ribozyme to RNA via Watson-Crick base pairing and cleavage of the phosphodiester backbone of the RNA target by transesterification (Figure 2C) [2,9–12]. Once the target RNA is destroyed, ribozymes dissociate and subsequently can repeat cleavage on additional substrates. The hammerhead ribozyme is the mostly widely used ribozyme in molecular biology and biotechnology. It was first isolated from viroid RNAs that undergo site-specific self-cleavage as part of their replication process. By separating the catalytic and substrate strands of the ribozyme, it can be transformed from a *cis*-cleaving RNA enzyme to a target-specific *trans*-cleaving molecule [13,14]. Like antisense oligonucleotides, hammerhead ribozymes have been modified to generate molecules with advantageous properties such as increased nuclease resistance [15], enhanced activity under physiological concentrations of Mg^{2+} [16], and improved accessibility to target sequences [17].

Yet another category of nucleic acid-based agents for gene inhibition that has received considerable attention in the past several years are catalytic DNAs (deoxyribozymes) [18,19]. Deoxyribozymes bind to their RNA substrates via Watson-Crick base pairing and site specifically cleave the target RNA, as do ribozymes (Figure 2C). These molecules were produced by *in vitro* evolution since no natural examples of DNA enzymes are known. Two different catalytic motifs (8–17, 10–23), with different cleavage site specificities, were originally found via this route [20]. Recently, more deoxyribozymes were produced with different cleavage specificities, allowing researchers to target all possible dinucleotide sequences [21].

In the past few years RNA interference (RNAi) has become the most widely used technology for gene knockdown. RNAi is a natural powerful mechanism that is thought to have arisen for protection from viruses and transposons. It was originally discovered as a naturally occurring pathway in plants and invertebrates [22,23]. When long double-stranded RNA molecules are introduced into these organisms, they are processed by the endonuclease Dicer into 21- to 23-nt small interfering RNAs (siRNAs). siRNAs are then incorporated into the multicomponent RNA-induced silencing complex (RISC), which unwinds the duplex and uses the AS strand as a guide to seek and degrade homologous mRNAs (Figure 2D) [24–27].

However, in mammalian systems, the introduction of long double-stranded RNA (>30 bp) results in systemic, nonspecific inhibition of translation due to activation of the interferon response. A breakthrough occurred when it was found that this formidable obstacle could be overcome by the use of synthetic short siRNAs (20–25 bp) that can be either delivered exogenously [28] or expressed endogenously from RNA polymerase II or III promoters (in the form of siRNAs or short hairpin (sh)RNAs that are processed by Dicer into functional siRNAs), resulting in a powerful tool for achieving specific down-regulation of target mRNAs [29–31]. RNAi is the most potent AS technology discov-

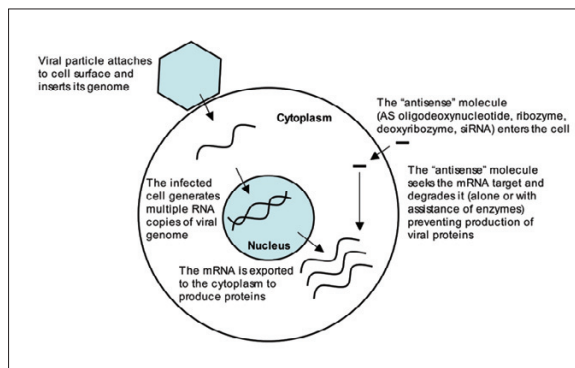


Figure 1. General scheme of inhibition of gene expression with "antisense" technology. The example given is for viral infection. However, any intracellular RNA can be down-regulated by this pathway.

ered thus far. It is estimated that the half-maximal inhibition levels (IC₅₀) of the siRNAs are some 100- to 1,000-fold lower than an optimal phosphorothioate oligodeoxynucleotide directed against the same target [32–34]. Announced by Science journal as the "Breakthrough of the Year" for 2002 [35], siRNA attracts ever-growing attention from academic researchers, the medical community, and the pharmaceutical industry.

DEVELOPMENT OF siRNA THERAPEUTICS: FROM DESIGN TO DELIVERY

Once a target gene is chosen for down-regulation, several hurdles must be overcome and decisions made to identify candidate therapeutics for clinical trials. First, an effective target site on the mRNA or viral RNA must be chosen that also does not cause off-target effects on gene expression. Also, one must decide whether to use siRNA or shRNA, and whether or not it should be chemically synthesized or expressed *in vivo* from a range of vectors. These decisions will, in turn, affect the method of delivery of the therapeutic. There are advantages and disadvantages to each, which we will discuss briefly in this section.

One of the biggest challenges for all AS-based approaches for gene knockdown is to identify not just an effective sequence, but the sequence that provides the most potent knockdown at the lowest possible concentration (dose) of the agent [36]. For traditional antisense approaches, finding an effective target site within an mRNA is not trivial. Factors affecting whether a given site is a good candidate include its primary sequence, the accessibility of target site because of local, internal secondary structure or long range tertiary structure, and steric occlusion as many sites may be blocked *in vivo* by proteins and polycations. Most of these factors are either not known or predictable a priori. Part of what makes the RNAi approach so attractive is that many sequences show a measurable knockdown of gene expression, in contrast to other AS technologies. Statistically, one in five sequences has been reported to be effective. Currently, there are several algorithms that are used to aid in selection of the most potent siRNAs for a given target such that one in two sequences tested will be capable of inhibition of gene expression on average. These algorithms take into account many factors compiling similar traits among experimen-

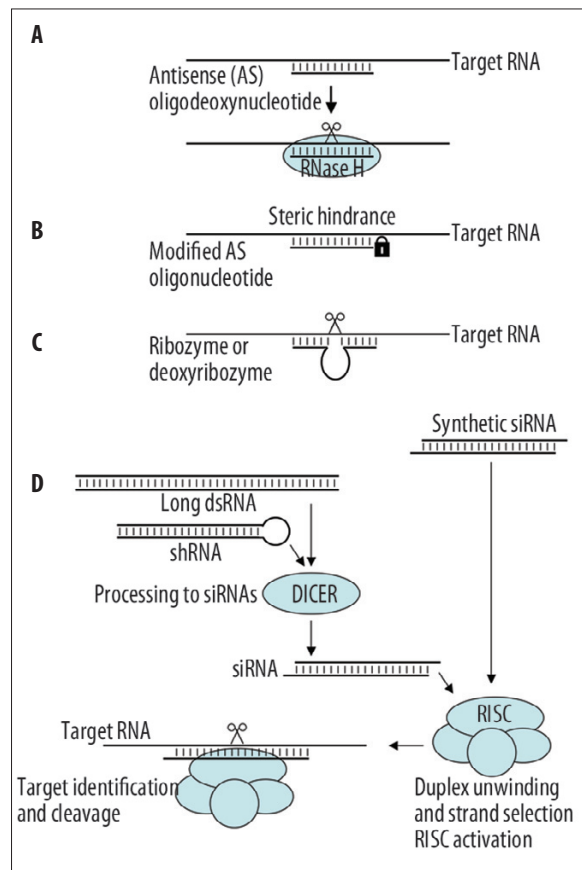


Figure 2. Mechanisms of the inhibition of gene expression through various "antisense" technologies. (A) An antisense oligodeoxynucleotide hybridizes to the target mRNA, which is then degraded by the intracellular enzyme RNase H that cleaves the RNA strand of the RNA/DNA duplex; (B) Certain chemically modified antisense molecules upon hybridization with target mRNA are not recognized by RNase H and they inhibit translation by steric hindrance or interfere with splicing of pre-mRNA; (C) Ribozymes and deoxyribozymes cleave the target RNA directly due to their intrinsic catalytic activities; (D) siRNA uses multicomponent enzyme complex to degrade target mRNA. Synthetic siRNAs (21–23 bp duplexes) are directly incorporated in the RNA-Induced Silencing Complex (RISC). If siRNA precursors are delivered (short hairpin (sh)RNAs or long double-stranded RNAs), they have to be processed by the enzyme Dicer to yield siRNAs. The RISC complex, using AS strand of the siRNA as a guide, searches for the mRNA target and degrades it.

tally tested effective sequences (e.g., overall G-C sequence content and identity of a certain nucleotide at a given position). By analyzing target gene sequences, a shortlist of siRNAs with the greatest probability of success is selected. Free, useful web sites for the design of siRNAs include: jura.wi.mit.edu/siRNAext (Whitehead Institute); www.ambion.com/techlib/misc/siRNA_finder.html (Ambion); www.rockefeller.edu/labheads/tuschl/sirna.html (Tuschl lab). After identifying sequences with good potential, the field can be further narrowed by experimentally testing each sequence in tissue culture. However, since the algorithms are not perfect, ideally, all possible target-specific siRNA sequences should be tested in cells to assure finding the best inhibitor(s) for a

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given mRNA. This could be done by individual screening of all possible sequences or, preferentially, by cell-based selection using libraries comprised of all oligonucleotide sequences represented within the target gene [37,38]. The potential importance of this was underscored in a recent study where it was found that some effective sequences for gene knockdown would not have been suggested by any of the publicly available algorithms [37].

For development of therapeutics, it is also important to demonstrate that each inhibitor affects expression of only the intended gene and not other unrelated genes. Although the original studies of siRNA silencing suggested high specificity, off-target and other toxic effects have been reported recently in cell culture experiments [39]. This potential toxicity may result from mRNA cleavage or translational repression of genes with partial homology to either strand of the duplex siRNA. Initially it was thought that effective RNAi requires almost perfect complementarity throughout the length of the sequence; it now appears that as few as 7 contiguous complementary base pairs can direct RNAi-mediated silencing, particularly by repressing translation as in the endogenous microRNA pathway [39]. Also, in some cases the sense strand may be selected preferentially by the RISC complex rather than the antisense strand, which may result in inhibition of unintended genes. Induction of an interferon response that could potentially result in global suppression of protein translation and other off-target effects has also been reported with both synthetic and vector-expressed siRNA in highly sensitive reporter cell lines at high concentrations of siRNAs [40]. The non-specific effects of siRNAs on gene expression depend on siRNA concentration and specific sequence. Thus, it is very important to identify the most potent sequences and to ensure that the sequence is specific to the target gene by performing a BLAST search (<http://www.ncbi.nlm.nih.gov/BLAST>) and by monitoring genome-wide expression profiles with microarray screens [41].

As with other forms of nucleic acid-based therapies, a major bottleneck in the development of siRNA therapies is the delivery of these molecules to the desired cell type, tissue or organ. RNAs do not readily cross the cell membrane on their own because of their large molecular mass and their high negative charge. There are two delivery strategies: use of chemically synthesized RNAs that have been modified for improved pharmacokinetic properties or use of viral or non-viral vectors to express RNA within cells. siRNAs are easy to synthesize chemically since each strand is of short length (~20 nt). Once antisense and sense strands are annealed, the duplex can be used in studies; it will bypass Dicer-processing and directly enter into the RISC complex (Figure 2D). However, if expressed from a vector with opposing promoters, the two strands hybridize inefficiently, presumably due to low local concentration, which leads to poor silencing activity. An alternative is to use shRNA, an siRNA precursor that must be processed by Dicer before it enters the RISC complex (Figure 2D). In contrast to siRNA, shRNA is more difficult to synthesize chemically since it is >50 nt in length, but it has an advantage over siRNA in the case of viral delivery since it is expressed as a single molecule whose duplex should be perfectly folded, which results in a high level of activity. Thus, for delivery of chemically synthesized molecules, siRNAs are the preferred format, while for viral vector delivery, shRNA is advantageous.

Synthetic siRNAs may be particularly useful in situations in which long-term silencing is not required, such as treating acute viral infections. Since siRNAs have a very short half-life in blood (less than minutes), they should be chemically modified to make them resistant to serum RNases, which can be accomplished without a significant decrease in biological activity [42]. Also, modification may improve the pharmacokinetic properties of siRNAs *in vivo* by mediating binding to blood components, thereby increasing the circulation time of the siRNAs. Finally, chemical modification can aid in broadly targeting siRNAs into cells and tissues, and certain conjugates can enhance uptake in specific cell types. Chemical modifications can be introduced at various positions within the siRNA duplex, including modifications at the termini, at the ribose, and within the backbone [43]. Encapsulation of siRNAs in lipid complexes, attachment to fusogenic peptides, antibodies or cell surface receptor ligands allows further improvement of potential drug candidates [43].

Viral vectors derived from adenovirus, adeno-associated virus, retrovirus, or lentivirus that are engineered to encode shRNAs can be used for more long-term gene knockdown, which would be useful for chronic infections such as hepatitis C and HIV. Although much progress has been made in developing gene-therapy vectors, there are still a number of obstacles to overcome [44]. These include the possibility of insertional mutagenesis and malignant transformation as well as the problem of the host developing an immune response to proteins expressed from viral vectors or intrinsic inflammatory and interferon responses to viral vectors. Furthermore, the effect of long-term expression of shRNA is not known. Despite these difficulties, one should keep in mind that viruses are naturally evolved machines for the delivery of nucleic acids into cells. It might take scientists many years to create artificial systems of similar efficacy, and thus the obvious solution is to try to use what is already available.

THE SPECTRUM OF POTENTIAL RNAI-BASED THERAPIES

There is growing enthusiasm regarding the potential for the development of a new class of powerful siRNA-based therapeutics against a broad range of diseases including viral infections, neurodegenerative diseases, septic shock, macular degeneration and cancer [26,45–47]. Inhibition of viral replication by RNA interference has been demonstrated *in vitro* for a variety of RNA viruses such as HIV, influenza virus, hepatitis C, hepatitis delta, rotavirus, respiratory syncytial virus, poliovirus, West Nile virus, foot and mouth disease and dengue virus, as well as DNA viruses such as human papillomavirus, hepatitis B and herpes simplex virus [48,49]. Currently, a growing number of studies are being performed in mouse models that clearly demonstrate the potential of RNAi for *in vivo* modulation of diverse diseases, using both chemically synthesized and vector-encoded si/shRNAs. Selected examples are shown in Table 1.

Many groups have focused on developing oligonucleotide-based therapeutics for eye-related disorders. Because localized delivery is achieved by direct injection, the amount of material required is much smaller (and thus cheaper) than would be required for systemic drug delivery. Also, there are inherent host defense and clearance mechanisms that may

Table 1. Studies of RNAi therapeutic efficacy in rodents.

Tissue	Disease	Target	RNAi formulation	Route of administration	Reference
Liver	Hepatitis B	HBsAg	siRNA	Hydrodynamic (intravenous)	[50]
		Viral genes	shRNA from plasmid DNA	Hydrodynamic (intravenous)	[51]
		Viral genes	siRNA stabilized	Hydrodynamic (intravenous)	[52]
		Viral genes	siRNA stabilized and complexed with lipid	Intravenous	[53]
	Hepatitis C	Viral genes	shRNA, T7 promoter transcribed	Hydrodynamic (intravenous)	[54]
		Viral genes	siRNA	Hydrodynamic (intravenous)	[55]
	Autoimmune hepatitis	Fas	siRNA	Hydrodynamic (intravenous)	[56]
Lung	Hypercholesterolemia	apo B	siRNA, modified and coupled to cholesterol	Intravenous	[57]
	Influenza	Viral genes	siRNA complexed to polyethyleneimine	Intravenous	[58]
		Viral genes	shRNA expressed from plasmid DNA	Intranasal + intravenous	[58]
		Viral genes	siRNA + siRNA-lipid complex	Hydrodynamic (intravenous) + intranasal	[59]
	Respiratory syncytial virus	Viral genes	siRNA with and without lipid	Intranasal	[60]
		NS1	shRNA expressed from plasmid DNA	Intranasal	[61]
CNS	Spinocerebellar ataxia-1	Ataxin-1	shRNA expressed from adeno-associated viral vector	Intracerebellar	[62]
	Neuropathic pain	Cation channel	siRNA	Intrathecal	[63]
Eye	Neovascularization	VEGF	siRNA	Intraocular	[64]
		VEGF	siRNA	Intravitreal	[65]
Kidney	Acute tubular necrosis	Fas	siRNA	Renal vein or hydrodynamic	[66]
Tumors	Germ-cell tumor	FGF-4	siRNA complexed to atelocollagen	Intratumoral	[67]
	Glioblastoma	MMP-9 + cathepsin B	shRNA expressed from plasmid DNA	Intratumoral	[68]
	Small-cell lung carcinoma	Skp-2	shRNA expressed from adenoviral vector	Intratumoral	[69]
	Pancreatic adenocarcinoma	CEACAM6	siRNA	Hydrodynamic (intravenous)	[70]

promote cellular uptake of siRNA in the eye [64]. It should be mentioned that the only current FDA-approved AS drug Fomiversen (Vitravene) from ISIS Pharmaceuticals is against CMV retinitis, targeting CMV IE2 [71], and is administered by intravitreal injection. Given these precedents and the general clinical validation of the vascular endothelial growth factor (VEGF) pathway in humans, a significant amount of work has been done with siRNA targeting the VEGF pathway in the eye. siRNAs targeting VEGF have been shown to be active in mouse and non-human primate models of choroidal neovascularization [64,65]. Although siRNA therapeutic development efforts were initiated only a few years ago, siRNA drug candidates have already entered Phase I clinical trials. Acuity Pharmaceuticals was first to enter Phase I

trials with the anti-VEGF Cand 5 siRNA drug candidate for Age-related Macular Degeneration (AMD), which has been completed with positive results [72]. They have now started recruiting patients for a Phase II trial. Sirna Therapeutics (formerly RPT) also began a Phase I clinical trial that is currently close to its successful completion using the chemically modified siRNA-027 that also targets VEGF [73].

The respiratory system is another example of a localized context in which the direct RNAi approach has already been shown to be very promising. Several groups have demonstrated that siRNA (both synthetic with or without transfection reagents and vector-produced) administered by simple intravenous injection or more importantly intranasally, effectively treat influ-

enza and respiratory syncytial virus [58–61]. This is a brilliant demonstration that low dosages of inhaled siRNA might offer a fast, potent and easily administratable antiviral regimen against respiratory viral diseases in humans. Alnylam has developed an siRNA drug candidate against respiratory syncytial virus and expects to enter Phase I trials in 2006 [74].

As most target tissues or organs cannot be accessed by local administration of potential siRNA therapeutics, a systemic route of delivery is the ultimate goal in developing siRNA drugs. Aside from the practical considerations of delivering therapeutics to internal organs, in some cases, inhibition of gene expression in multiple tissues is obligatory (e.g. treatment of highly metastatic tumors). One example of the successful systemic administration of siRNA is a study from Alnylam Pharmaceuticals [57,74]. In this work, the target is mRNA that encodes apolipoprotein B, a protein involved in the metabolism of cholesterol. The concentrations of this protein in human blood samples correlate with those of cholesterol, and higher levels of both compounds are associated with an increased risk of coronary heart disease. Chemically stabilized siRNAs joined to a cholesterol group in order to improve delivery were synthesized. Intravenous injections of the siRNA conjugates in mice resulted in significant uptake into several tissues and the siRNAs efficiently reduced the levels of apolipoprotein B mRNA by more than 50% in the liver and by 70% in the jejunum. This reduction resulted in a decrease in cholesterol in the blood comparable to levels observed in mice in which the apolipoprotein B gene had been deleted.

Another example is a study by Sirna Therapeutics [52,53,73] where the efficacy of chemically modified siRNA targeted to hepatitis B virus was examined in an *in vivo* mouse model of HBV replication. siRNA (alone or incorporated into a liposome) administered by intravenous injection into mice efficiently reduced the level of serum HBV DNA $>1.0 \log(10)$. It should be emphasized that these studies were carried out with regular low pressure intravenous injections as opposed to earlier studies aimed at Hepatitis B and C [50,55] where the siRNAs were delivered by hydrodynamic injection, which is not practical for human patients.

However, a potential pitfall in the development of anti-viral drugs is that some viruses have been found to carry natural defense mechanisms against or have evolved resistance to either the siRNAs or components of the RNAi machinery. For example, siRNAs can inhibit the production of progeny virus for respiratory syncytial virus, hepatitis delta virus, and rotavirus, but their genomic RNAs are resistant to RNAi either because of tight shielding by proteins or localization in subcellular compartments inaccessible to siRNAs [75–77]. Some viruses such as influenza and vaccinia produce proteins that actively suppress silencing by RNAi [78]. In addition, it was recently reported that the tat protein in HIV encodes a suppressor of RNA silencing by functional abrogation of Dicer, a key enzyme in the RNAi pathway [79]. Adenovirus was recently shown to block the processing of shRNAs in mammalian cells by expressing a viral noncoding RNA at such high levels that it binds most of the available RNAi processing machinery [80]. To avoid the adverse effects of long double-stranded RNAs, viruses have evolved double-stranded RNA binding proteins that can inhibit the effects of the interferon response and antiviral RNA interference.

An additional problem is that most viruses, for example HCV and HIV, mutate rapidly because of the high rate of infidelity of their replicases and a lack of proofreading activity [81]. Although siRNA molecules are typically designed to target highly conserved sites, viral mutants resistant to therapy may arise rather fast. For this reason, cocktails of siRNAs that target multiple viral sequences may be the best option to prevent viral 'escape mutants'. For example, Benitec has an HCV drug candidate consisting of three siRNA sequences targeting the HCV RNA genome where each component was shown individually to be a potent inhibitor of hepatitis C virus derivatives in both tissue culture and rodent models [82]. Benitec expects to be in Phase I trials with this three-in-one drug by the end of 2006. However, combination therapy with current treatments for HCV infection (e.g. interferon and/or ribavirin [83,84]) might be necessary to completely clear infection. Also, Benitec expects to enter Phase I trials to treat HIV patients with lymphoma with a multi-RNA therapeutic that combines siRNA, ribozyme and RNA decoy molecules delivered with a lentiviral vector [82]. Summarizing, the steady improvements in the design of siRNA, methods for local and systemic delivery and the absence of apparent toxicity in the mouse models are positive signs that RNAi therapeutics are close to becoming a reality.

CONCLUSIONS

RNA interference is a unique approach for therapeutic applications by gene silencing since it uses an ancient natural, robust pathway. However, the mechanism is complex and not fully understood at the moment. Problems including identification of effective sites in the target RNAs, minimization of off-target effects, enhanced stability and efficient delivery for siRNA, as well as evolution of anti-RNAi defense systems by some viruses must be addressed. The good news is that previously used AS oligonucleotides and ribozymes have been studied in much more detail, and knowledge in solving problems common for these gene knockdown approaches may be directly applied to siRNA. Summarizing, the development of new siRNA-based drugs is feasible, but it will take at least several more years.

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