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RNAi and small interfering RNAs in human disease therapeutic applications

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Abstract

Small interfering RNAs (siRNAs) have shown to effectively down-regulate gene expression in human cells, giving them potential to eradicate disease. Prospects for clinical applications are discussed in this review, along with an overview of recent history and our current understanding of siRNAs used for therapeutic application in human diseases, such as cancer and viral infections. Over recent years, progress has been made in lipids, ligands, nanoparticles, polymers and viral vectors as delivery agents and for gene-based expression of siRNA to enhance the efficacy and specificity of these methods while at the same time reducing toxicity. It has become apparent that given the recent advances in chemistry and delivery, RNAi will soon prove to be an important and widely used therapeutic modality.

Introduction

Since the first small interfering RNA (siRNA)-related clinical trial in 2004, in which Acuity Pharmaceuticals (now Opko) introduced siRNA by intravitreal injection in patients with age-related macular degeneration (AMD) to target the vascular endothelial growth factor (VEGF) messenger RNA (mRNA) (Table 1a), it has become clear that every gene associated with a disease is a potential target for siRNA. Given that the majority of diseases affecting the human population involve some form of aberrant gene regulation, the potential use of siRNA and RNA interference (RNAi), as well as nucleic-acid-based inhibitors (Figure 1b) as therapeutic agents is extremely attractive, with a broad range of disease applications, including cancers, viral infections and genetic and metabolic disorders (Table 1). Even with our current extensive information about the human transcriptome, development of siRNA-based therapeutics represents an enormous challenge, and only a few candidate compounds have made it to clinical trials (Box 1). Nevertheless, increasing knowledge about the RNAi mechanism and machinery should help the scientific community to advance RNAi as a successful therapeutic modality.

Box 1

Clinical trials in RNAi therapeutics: past, present and future

The eye has been selected by biotech firms because siRNA can be delivered directly to the diseased tissue. Opko's siRNA-drug compound Bevasiranib, an siRNA that targets VEGF for the treatment of AMD, was the first siRNA in the industry to enter a Phase III clinical

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trial (Table 1). In March 2009, Opko decided to interrupt the trial of Bevasiranib because it was determined that Bevasiranib was unlikely to achieve its primary endpoint of reducing vision loss.

Bevasiranib was not the only compound to be stopped in its progression towards a drug. Sirna-034, a drug developed for inhibition of HCV, was supposed to reach Phase I clinical trials by 2006. The acquisition of Sirna therapeutics by Merck in 2006 led to modified therapeutic objectives, thereby relegating Sirna-034 as an orphan compound. Other promising projects concern small RNAs have been tossed out due to company reorganization, such as the development of ribozymes by Ribozyme Pharmaceuticals, which became Sirna therapeutics in 2003 (now Merck).

Companies merge with others to combine expertise in RNAi therapeutics and drug delivery, such as MDRNA, previously Nantech, which acquired Cequent recently (Table 1). MDRNA now owns two validated RNAi drug discovery platforms – MDRNA's UsiRNA (unlocked nucleic acid-siRNA [74])/DiLA2 platform and Cequent's TransKingdom RNAi platform [75] – which utilize two distinct, proprietary delivery technologies for systemic, local and oral delivery. Alnylam, who shares partnership with other companies as Tekmira and Medtronic, is advancing ALN-TTR—an RNAi therapeutic targeting transthyretin (TTR) for the treatment of TTR amyloidosis—and ALN-HTT, for the treatment of Huntington's disease. Despite its setback with Bevasiranib, Opko has developed siRNA against other molecular targets involved in the pathogenesis of AMD and cancers, such as the hypoxia inducible factor 1 alpha (HIF-1 α), in which Santaris Pharma also shows interest (Table 1).

Other biotech firms will stake out their places in RNAi-based gene therapy. Regulus is advancing miRNA therapeutics in several areas, including hepatitis C infection (miR-122), fibrosis (miR-21) and cancer (miR-21 and miR-34). By using its own version of RNAi compounds, Rxi Pharma should provide an alternative to conventional siRNAs; one of them, *sd-rxRNA*, has the unique ability to be 'self delivering', without the need for any additional delivery vehicle.

Small interfering RNA

siRNAs are either found in a variety of organisms or produced synthetically to target a specific mRNA in mammalian cells (Figure 1b-xi) [1]. They are usually ~19–23 base pairs (bp) with 2-nucleotide (nt) 3' overhangs that mimic the natural products of the RNase III enzyme Dicer. It is generally accepted that siRNA duplexes less than 30 bp do not induce the antiviral interferon (IFN) response [2]. Recently, the same has been shown for 34–40 bp Dicer-substrate siRNAs [3]. siRNAs are known to guide sequence-specific gene silencing of target mRNAs to which they are perfectly complementary by directing the RNA-induced silencing complex (RISC) to mediate site-specific cleavage, and, hence, destruction of the targeted mRNA (Figure 1) [4]. Exogenous siRNAs can also direct transcriptional gene silencing by inducing heterochromatin formation, leading to histone methylation and/or deacetylation, and ultimately DNA methylation [5].

In contrast to exogenously supplied siRNAs, the endogenous miRNA-guided silencing pathway (Figure 1a) uses post-transcriptional repression to down-regulate genes (reviewed in Refs. [6, 7]). mRNA regulation by endogenous miRNAs is often deregulated in human diseases or viral infections. Antisense targeting or ectopic expression of miRNA represents another way to use RNAi therapeutically. For example, Santaris Pharma is conducting a clinical trial using an antisense approach against miR-122 (Table 1) because it has been shown that miR-122 aids hepatitis C virus (HCV) replication in hepatocytes [8]. Based on what we are learning from the RNAi interference and miRNA-guided silencing pathway, future therapeutic applications

will include not only gene targeting with siRNAs, but also targeting or ectopic applications of miRNAs.

Targeting relevant genes or genome regions: HIV case report

RNAi has a relatively short history with respect to mammalian cell applications, but targeted knockdown of gene expression via RNAi is one of the most widely used experimental tools. Human immunodeficiency virus (HIV) was one of the first targets for therapeutic application of RNAi. The virus has been targeted by either synthetic siRNA or promoter-expressed short hairpin RNA (shRNA), which are processed by Dicer into siRNAs [9]. However, for HIV and other viruses [10], the use of a single siRNA often results in the emergence of RNAi-escape mutants [11]. To avoid this problem, cellular transcripts involved in the HIV life cycle, such as NF- κ B, the CD4 receptor and the chemokine (C-C motif) receptors 5 (CCR5), have been targeted [12] [13].

One approach to counteract escape mutants is to anticipate those that would abrogate the RNAi mechanism. It has been shown that viral escape routes are highly conserved when some HIV genes are targeted with shRNA [14]; as such, second-generation siRNAs, forming a complete match with the viral escape sequences, have been designed [15]. Although major escape routes are blocked, minor routes will eventually be chosen by the virus; this approach helps to understand virus evolution under RNAi pressure, and could be useful to force the production of attenuated or defective viruses.

Another approach for blocking HIV replication involves transcriptional gene silencing of the integrated long terminal region (LTR) viral promoter [16]. This approach has been used to silence a lentiviral-encoded luciferase reporter transgene with RNA [17]. However, despite high levels of suppression and sequence specificity to the target, off-target gene silencing can induce the suppression or the activation of other genes [5,18].

Delivery approaches

siRNA has the potential to be a powerful therapeutic compound. Non-encapsulated small RNAs have been delivered directly to cells (Figure 1b-i; ophthalmology and inflammation applications in Table 11) by gymnosis in Namalwa B cells, which are notoriously resistant to lipofection [19]. Unfortunately, this method of delivery is not generally feasible for most therapeutic applications. Due to their instability, vulnerability to degradation, and tendency to trigger an immune response, a robust and systemic method of delivery is usually required to ensure that siRNAs reach their target cells and tissues, and remain functional long enough to effectively knock down the expression of targeted mRNAs.

Chemically modified nucleic acids

Chemical modifications of RNA (Figure 2) lead to more suitable molecules for therapeutic applications [20]. These modifications, whether they are in the base, sugar or backbone, lead to an increase in resistance to nuclease activity, improve RNAi silencing, increase the melting temperature of siRNA duplexes, and lead to siRNAs with more favorable pharmacokinetic properties, including higher target specificity [20]. Selective incorporation of 2'-O-methyl (2' OMe)-modified (Figure 2b) uridine or guanosine nucleoside into one strand of the siRNA duplex has created a non-inflammatory siRNA. This approach has been shown to be effective for targeting apolipoprotein B (ApoB) without cytokine induction, toxicity or off-target effects associated with the use of unmodified siRNAs [21]. However, overuse of any modification can lead to toxicity or decreased efficiency of RNAi. Synergistic effects are observed when these modifications are used in combination. Modifications to siRNA are position-sensitive and are overall better-tolerated in the passenger strand (reviewed by Ref. [20]).

Chemically modified RNAs can also be used to make anti-miRNA oligos. Using this strategy to inhibit the function of endogenous miRNA is conceptually quite different than using siRNA to selectively downregulate gene expression. Every miRNA can potentially regulate the expression of several hundred different mRNAs; thus, knocking down the levels of a single miRNA species can have a major impact on multiple endogenous pathways. Sugar-modified 2'OMe, 2'-fluoro or 2'-O-methoxyethyl (2'OMOE), and locked nucleic acid (LNA) (Figure 1b-iv; Figure 2b) anti-miRNAs, as well as combinations of these backbone modifications, have been tested for anti-miRNA function. Of particular interest is the LNA sugar modification (Figure 2b) – a bi-cyclic nucleic acid in which a ribonucleoside is linked with a methylene unit between the 2'-oxygen and the 4'-carbon. This modification has shown remarkable serum stability and the capacity to inhibit miRNA function [22], and is being tested for HCV as a trial drug called SPC3649 (Table 1).

Ligand-based targeting molecules

Aptamers are nucleic acid sequences with unique secondary and tertiary structures that have been selected for their high affinity to a chosen target and can be used for cell type-specific delivery of siRNA. An aptamer-siRNA chimera can be generated as a single, covalently joined polymer; as such, they are relatively easy to produce by *in vitro* transcription. Aptamer-siRNA chimeras (Figure 1b-ii) serve dual roles as receptor targeting agents and as siRNA delivery reagents. The first demonstration of an aptamer-delivered siRNA was for targeted delivery to the prostate-specific membrane antigen (PSMA) receptor expressed on the surface of prostate cancer cells. The aptamer-siRNA chimera was injected intratumorally and then internalized to trigger silencing of the siRNA target [23]. The chimera circulation and bio-availability *in vivo* was substantially enhanced with a 20-kDa PEG moiety added to the 5'-terminus of one of the siRNA strands [24]. This led to prolonged silencing effects on the target and inhibition of tumor growth following systemic injection in a murine human xenograft model.

RNA aptamers that bind to the HIV-1 Ba-L gp120 protein have also been shown to selectively deliver anti-HIV siRNAs into HIV-infected cells. Two different versions of the anti-gp120 aptamer have been developed. One is an aptamer-siRNA chimera; the other has a GC-rich bridge sequence that facilitates the interchange of different siRNAs with the same aptamer. The aptamer alone has inhibited HIV function, but the aptamer-siRNA chimeras have provided more potent inhibition via RNAi-mediated knockdown of the HIV target transcripts [25].

Like aptamers, antibodies and peptides have been shown to bind specific receptors on targeted cells and ensure proper delivery of siRNA. An antibody, scFv, has been used to target the T cell surface protein CD7 [26]. The antibody was modified to include a cysteine residue at its C-terminus for conjugation to a nona-d-arginine peptide, allowing for charge interaction with siRNA [26]. A fusion protein, protamine, which binds nucleic acids, has also been used as a bridge between the C-terminus of the heavy chain Fab fragment of an HIV-1 envelope antibody and the siRNA (Figure 1b-iii) [27] in order to selectively silence gene expression in HIV-infected cells.

Antibodies have also been used on the surface of liposomal carriers of siRNAs to direct these particles to specific cell types. Leukocytes were specifically targeted using integrin-targeted, stabilized nanoparticles containing siRNA and showed highly efficient gene silencing *in vivo* [28]. These nanoparticles look like liposomes (Table 2), with hyaluronan covalently linked to the surface, stabilizing the particles. A monoclonal antibody was then covalently attached to hyaluronan, allowing for cell-specific targeting.

Cholesterol has also been useful in a ligand-based approach to carry siRNAs, allowing increased stability, cellular uptake in certain tissues such as the liver, and membrane fusion through lipid-raft-mediated endocytosis (Table 2) [29]. Given the waxy nature of cholesterol,

it easily incorporates into cell membranes and lipid-based carriers; however, the role of cholesterol in targeting requires further investigation.

Lipid-based delivery

Lipid-based delivery facilitates protection from nuclease degradation and allows for systemic delivery of nucleic acids to target cells across cell membranes (Figure 1b-iv). DOTMA (*N*-[1-(2,3-dioleoyloxy) propyl]-*N,N,N*-trimethylammonium chloride) was the first synthetic lipid used to transfer plasmid DNA into mammalian cells [30]. Other cationic lipids commonly used as building blocks for delivery systems include DOTAP (1,2-dioleoyl-3-trimethylammoniumpropane), Transfectam and 98N12-5 (Table 2). Well-designed lipid carriers allow escape from the endosome and release of the siRNAs for incorporation into the RNAi machinery. The endosomal pathway represents the major challenge for drug delivery into the cytoplasm because endosome internalization often leads to degradation of the siRNA. If the siRNAs are in association with cationic lipid complexes, then association of these cationic lipids with cellular anionic lipids would allow for neutralization and release of the siRNA

Modifications of the liposomes can help delivery and uptake. Coating with a specialized polyethylene glycol (PEG)-lipid conjugate, as with the Tekmira and Alnylam siRNA delivery systems, termed SNALP (stable nucleic acid-lipid particles) (oncology and metabolic disease applications in Table 1), enables cellular uptake and endosomal release [31]. SNALP liposomal particles have been successfully used in nonhuman primate models [32]. PEG residues as long as C-18 have been used to increase plasma concentrations of siRNA embedded in liposomes when compared to short-chain PEG, and a 3-fold higher level of accumulation has been shown for PEG-liposomes in tumors as compared to non-PEG-containing liposomes [33].

Polymers and other delivery strategies

Liposomes may be replaced by peptides. It has been shown that histidine-lysine (HK) polymers are more effective than liposomes in delivering nucleic acids to several cell lines [34]. Lysine residues bind to the negatively charged phosphate backbone of nucleic acids, and histidine enhances endosomal lysis by serving as a proton sponge, thereby enabling siRNAs to reach the cytosol.

Polymer delivery systems have provided a vessel to protect siRNAs from serum nucleases and they can be conjugated to ligands for delivery to specific cell types. The downside is that the transfection efficiency of these artificial nanocarriers is generally low. A PEG-modified polyetheleneimine (PEI)-siRNA targeting CD44v6 for delivery to gastric carcinoma cells has been used successfully *in vitro* and *in vivo* [43]. As PEI may induce cytotoxicity, PEG-modified PEI constructs displayed reduced cytotoxicity and maintained high transfection efficiency. The PEG modification has increased polymer solubility, stabilized the nanoparticles *in vivo*, and lessened non-specific interactions with serum proteins [35].

Another polymer that has been used for siRNA delivery is atelocollagen, a biocompatible biomaterial. Using siRNA against the androgen receptor, atelocollagen complexes have been employed to efficiently inhibit human prostate cancer cell growth *in vivo* [36]. Moreover, a bio-degradable arginine-conjugated cationic polymer has been introduced as a siRNA delivery vehicle for gene silencing in a cancer model [46]. This polymer allowed for efficient delivery of siRNA because the reductive environment of the cytoplasm cleaves the disulfide linkages in the polymeric backbone, resulting in de-complexing of the siRNA/polymer polyplex and release of siRNAs into the cytoplasm [37]. The results of the study demonstrated an increase in membrane permeability with the arginine modification and a similar level of cellular uptake as obtained for siRNA/PEI polyplexes.

The first in-human phase I clinical trial involving systemic siRNA administration to patients with solid tumors used a targeted nanoparticle delivery system (Figure 1b-iv) [38] (CALAA-01 for metastatic lymphoma in Table 1). The nanoparticles consist of a linear cyclodextrin-based polymer, a human transferrin protein ligand to target the transferrin receptor, a hydrophilic polymer (PEG) to promote nanoparticle stability, and anti-ribonucleotide reductase (RRM2) siRNA. Results from this study showed reductions in the RRM2 mRNA and protein levels. Moreover, the identification of the siRNA-directed cleavage products by RACE (rapid amplification of cDNA ends) PCR provided a direct demonstration that the target reduction was via RNAi. Tumor biopsies from the melanoma patients showed the presence of intracellular localized nanoparticles in amounts that correlated with the dosing levels of the nanoparticles [38].

An alternative to lipid or polymer-based carriers are bacteria-derived minicells. These carriers can be used to transport siRNA as well as cytotoxic drugs to tumors and cells even in the gastrointestinal tract. This interesting approach is further explored in Box 2.

Box 2

Bacteria as small RNA carriers for targeting tumors

Bacterial colonization in tumors has been recognized to have a beneficial effect in cancer cells [76] because those microorganisms naturally accumulate and replicate in a variety of solid tumors, leading to reduced tumor size. Bacteria, bacteriophages and bacteria-like particles, such as minicells, have been investigated as gene delivery agents.

Minicells that selectively target cancer cells via specific antibodies have been reported to cause substantial tumor stabilization and regression in a variety of tumor xenograft models [77]. Minicells were packaged with siRNAs or plasmid-encoding shRNAs that were designed to suppress expression of critical cell-cycle-associated proteins implicated in tumor cell proliferation. Dual sequential treatments that first targeted a known drug resistance mechanism by RNAi was followed by a second wave of minicells packaged with a cytotoxic drug. This two-step treatment rendered once-drug-resistant tumors drug-sensitive, thereby providing complete survival of mice bearing aggressive human tumor xenografts [79].

Salmonella typhimurium has also been demonstrated to be capable of delivering shRNA-expression vectors to mammalian cells and to induce RNAi *in vitro* and *in vivo* [78]. Upon oral administration, *S. typhimurium*, carrying shRNA-expressing vectors targeting Bcl-2, induced gene silencing in murine melanoma cells, leading to delayed tumor growth and prolonged survival in mice.

A new approach introduced by Cequent Pharmaceuticals, termed TransKingdom RNAi (tkRNAi), uses non-pathogenic bacteria, engineered to invade target cells, to carry silencing shRNA. The silencing shRNAs are transcribed from a tkRNAi plasmid by T7 RNA polymerase, and are released into the cytoplasm once the bacteria are taken up by phagosomes and lysed. RNAi-mediated gene silencing is accomplished through the standard Dicer/RISC pathway. This approach appears to be clinically safe, proven by the use of non-pathogenic bacteria for decades without any safety concerns, as a treatment of gastrointestinal diseases [75].

Using bacteria for siRNA therapy provides yet another approach for safe delivery of siRNA and has been shown to effectively induce gene silencing without toxic side effects. Bacteria can be manipulated, or different strains used, in order to customize them for treatment of various diseases in a variety of tissues. With these characteristics, bacterial delivery of

siRNA is definitely an approach the community aiming to use siRNA therapeutically will be watching.

Viral vectors

Much has changed since the first small-scale clinical trial that cured an otherwise fatal immunodeficiency disorder in children using a γ -retrovirus several years ago [39]. The use of retrovirus- (e.g. murine leukemia virus, MLV), lentivirus- (e.g. HIV), adenovirus- (e.g. Adenovirus 8) and adeno-associated-virus- (e.g. AAV-8) based vectors for transgene expression (Figure 1b-v) is now better-documented. Vectors are also safer; third generation lentiviruses are self-inactivating, contain sequences to enhance the efficiency of packaging and transgene expression [40–42].

Both retroviruses and lentiviruses can integrate into the genome (Figure 1b-ix) and infect many cell types, including hematopoietic stem cells, but only lentiviruses can effectively infect non-dividing cells [44]. Recently, a novel chimeric vector carrying a mutant MLV promoter which was internal to a lentiviral vector backbone, and thereby contained useful properties of both types of vectors, was created to circumvent insertional mutagenesis of retroviruses while still providing the expression benefits of the MLV promoter element. In pluripotent stem cells, this allowed a temporal transgene expression in a non-pluripotent state before stem cell derivation [45].

shRNA sequences can be cloned into a lentiviral vector, which is packaged to produce a lentivirus. Following infection and integration into the host DNA, shRNA can be processed into siRNA, which can then target viral or endogenous transcripts (Figure 1b-iv–xi). A point of concern for anti-HIV shRNA vectors is that packaging and expression of the vector components may be compromised by the production of siRNAs targeting the HIV components of the packaging system, thereby reducing vector titers. A safety feature for shRNA expression specific for HIV-infected cells was developed wherein the Tat was used for activating expression of the anti-HIV shRNA, thus creating a negative feedback loop [43].

Off-target effects that could disrupt regulation of other genes are a cause for concern with this family of vectors. Although not yet observed, it is possible that integration could result in functional gene disruption or activation of adjacent oncogenes. A potential approach for avoiding integration of the lentiviral vectors into functional genes is to target them away from these sequences using an artificial tethering factor to develop safe and target-specific lentiviral vectors for gene therapy [46].

HIV-based expression vectors may be used to target the same host cells as wild-type HIV, but there is a major challenge with lentiviral vector-mediated shRNA gene delivery in cancer applications because of the lack of an efficient *in vivo* delivery mechanism. Even so, a lentivirus-based anti-VEGF-C shRNA-containing vector has been used to transduce breast cancer cells and reduce VEGF-C mRNA and protein expression, suppressing the metastatic activity of these cells in a murine model [47]. Other vectors, such as adenovirus or AAV, may be better-suited for anti-cancer siRNA gene delivery. Adenovirus and recombinant AAV (rAAV) do not integrate within the genome, but transient forms persist in the nucleus. Both vectors have been used for the expression of proteins or small inhibitor molecules in infected or cancerous cells (reviewed in Refs. [48,49]).

With respect to RNAi, adenovirus has recently been used to down-regulate the foot-and-mouth disease virus using multiple shRNAs driven by the U6 polymerase (Pol) III and CMV Pol II promoters [50]. A recombinant AAV-8, which is a robust vector for gene transfer to the liver [51], has been used to deliver shRNA against endogenous p53 in the livers of transgenic mice

harboring a conditional MYC expression construct, resulting in effective knockdown of p53 [52]. AAV uses a variety of cellular receptors/co-receptors to gain entry into cells, and a number of serotypes are now available; therefore, the cognate receptors/co-receptors for only a handful of these have thus far been identified [48]. AAV vectors have been used for expressing shRNAs or miRNA-mimic hairpins to downregulate genes involved in various cancer cells [48]. rAAV vectors are attractive as agents for shRNA mediated gene therapy because they are non-pathogenic for humans and cannot replicate by themselves,.

Combinatorial RNAi: strategies for gene-based expression of siRNA and shRNA

Promoters

Promoter-based expression systems enable a continuous production of shRNA or miRNA. Molecules expressed from RNA Pol III promoters such as U6 and H1, can be more effective than siRNAs for triggering RNAi-mediated gene silencing [53]. Transcription via Pol III promoters can give high levels of expression levels of shRNAs but over-expressed transcripts can also saturate endogenous miRNA-guided silencing components, eventually resulting in toxicity [54,55]. By comparison, Pol II transcripts can be expressed from a tissue or cell-specific promoter, their expression levels are lower and they induce less toxicity [54].

The choice between Pol II and Pol III promoters could be related to the targeted tissue or the level of expression desired. However, both can be incorporated in an inducible system. For gene therapy, an inducible vector dependent on the delivery of the antibiotic is preferred over one based on gene silencing, which necessitates constant administration of antibiotics unless transgene expression is required [57].

Dual-targeting and multiplexing

As mentioned for HIV, anti-viral applications of gene repression by siRNA or shRNA often results in resistance and escape mutants. The targeting of more than one gene, or the use of more than one small RNA to target the same gene, allows for the strongest effect on gene regulation; nevertheless, it can also induce off-target effects.

By taking advantage of the known pathways for siRNA-induced target cleavage and miRNA-directed translational inhibition, siRNAs have been designed to simultaneously direct cleavage of the mRNA encoding the HIV co-receptor CCR5 and suppress translation of HIV mRNAs by binding to sites in the 3'UTR [58]. This was accomplished by identifying siRNAs that were predicted to be highly effective against HIV-1 genes and had multiple seed site occurrences in the HIV 3'UTR. The effectiveness of siRNAs is based on an algorithm created from experimentally tested siRNA databases [59]. These bifunctional siRNAs were able to trigger inhibition of HIV infection and replication. The authors further emphasized that 90% of human genes have sites that make the design of bifunctional siRNAs possible.

Several different designs for multiplexing siRNAs to thwart viral resistance have been tested *in vitro*. Although using multiple siRNAs or shRNAs targeting different sequences can be effective, the use of long shRNA transcripts that can be processed into two or more siRNAs has also shown promise [60,61]. Long hairpins have the advantage of targeting multiple sites, and proper design of these hairpins is required to generate multiple, unique Dicer products. Inhibition of HIV-1 has been demonstrated using long hairpin RNAs (lhRNAs) of various lengths [62] [63].

With respect to endogenous miRNA structures, pre-miRNA mimics transcribed from RNA Pol II or Pol III promoters have been shown to produce functional siRNAs with reduced

cytotoxicity [56,64,65]. For other applications, such as in anti-cancer therapy, the choice of the appropriate mRNA targets for RNAi targeting in cancer applications is also highly relevant because the goal of cancer therapy is to trigger apoptosis or cell cycle arrest. As such, a thorough knowledge of the oncogene dependency and anti-apoptotic gene expression patterns for a given type of cancer need to be established prior to RNAi applications.

Combinatorial strategies

HIV co-receptor CXCR4 is essential for hematopoietic and immune systems. It has been shown that CCR5-tropic HIV can switch tropism to CXCR4, in which case CCR5 targeting should be combined with other anti-virals to provide a more potent combinatorial approach and to minimize viral escape. This has been successfully accomplished by co-expressing an anti-HIV tat/rev U6shRNA, a nucleolar-localizing TAR decoy, and an anti-CCR5 ribozyme in a single vector backbone [66]. This strategy, based on a lentiviral-expressing system, is currently in use in a small-scale clinical trial (Table 1, 6b) and has shown persistent vector expression in multiple cell lineages at low levels for up to 24 months without any toxic effect [67]. While this combinatorial approach involved expression of shRNA and other RNA molecules, co-expression of proteins, such as a humanized, transdominant negative mutant HIV Rev protein is also possible. A shRNA against Rev mRNA and transdominant protein has resulted in potent, long-term inhibition of HIV-1 gene expression and suppression of shRNA-resistant mutants [68]. It is clear that combinatorial approaches demonstrate great promise for anti-viral and anti-cancer therapeutic applications.

Immune response: good or bad?

Unmodified siRNA can be a potent trigger of the innate immune response, particularly when associated with delivery vehicles that facilitate intracellular uptake and activation of Toll-like receptors [69]. Intracellularly expressed shRNAs are less prone to IFN activation because they are not detected by the cell-surface receptors (reviewed by Ref. [70]). However, it is not well-understood how innate immune responses can help or antagonize the RNAi pathway.

IFN induction can be used synergistically with siRNA function when activation of the immune system is used to destroy tumor cells. A siRNA that triggered Rig-1 activity and an IFN type I response from natural killer cells has been shown to mediate Bcl-2 silencing and enhance the apoptosis of tumor cells in lung metastases *in vivo* [71]. An immune-mediated rejection has also been induced in tumour cells targeted with a PSMA aptamer-siRNA conjugate which downregulates Smg1 or Upf2, both involved in nonsense-mediated messenger RNA decay [72]. This application shows great potential as a therapeutic modality for cancer.

Activation of the immune system can also be triggered by the introduction of a miRNA-like non-pairing uridine-bulge in the siRNA passenger strand. This design has been shown to increase the immuno-stimulatory activity in immune cells while conserving silencing efficiency [73]. To date, modulation of the immune system by siRNA/shRNA is not completely understood, and recent findings will hopefully unveil new combinatorial approaches of innate immune stimulation combined with siRNA/shRNA to treat human diseases.

Future perspectives for siRNA therapy

Before siRNA therapies can be realized as a general approach to treat human diseases, several issues need to be carefully addressed. The most critical is that therapeutic siRNAs must reach their intended targets; if not, they may cause damage to non-targeted cells. Protection from serum nuclease degradation, modulation of the innate immune system, and entry into cells are all hurdles that have been sufficiently addressed.

The promise of selective target knockdown of gene expression has prompted notable activity in developing RNAi-based drugs. Liposomal carriers provide protection and allow for easy uptake by cells, but offer little in targeting specific cells. Aptamers, antibodies and peptides are useful for cell-type-specific delivery of siRNAs, and these need to be further exploited. In our opinion, many promising results have been produced in the past several years, several of which are from clinical trials. Further testing and investigation of the mechanisms of siRNA-based therapies is clearly worth the time and effort because clinically useful RNAi-based products will contribute significantly to the future of medicine.

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Figure 1.

miRNA pathway and RNAi in cells targeted by small RNA-based therapies. (a) In healthy cells, (i) miRNA genes are expressed from genomic DNA, and (ii) the microprocessor complex processes the primary miRNA (pri-miRNA) into precursor miRNA (pre-miRNA), which is further exported to the cytoplasm. (iii) Dicer, in collaboration with TRBP (HIV-1 transactivating response RNA-binding protein), process the pre-miRNA into miRNA duplex, which is (iv) loaded into Argonaute (Ago) protein-containing complex. (v) One strand of the duplex, the 'guide strand', represses translation of mRNA by complementary binding affinity within the 3' UTR of the mRNA. (b) Small RNAs, such as siRNA and shRNAs, can be delivered into cells via different described methods; (i) naked siRNA can be delivered locally by injection in a tissue, while (ii) RNA-structured aptamer molecules and (iii) antibody-protamine conjugated siRNAs can be used for cell-specific delivery. (iv) Small RNAs, including locked nucleic acid (LNA) oligos, can be incorporated into liposomal preparations. (iv-v) shRNA and other RNA-based tools (e.g. ribozymes and decoys) may be cloned into plasmids and viral vectors for promoter-base long-term expression. (vi) Following entry into the cytoplasm, small RNAs (vii) and lentivirus (viii) are released from endosomal compartments. (ix) Lentiviruses can be further integrated within the host genome and can be transcribed by the host machinery. (x) siRNA/shRNA delivery into diseased cells are also loaded into the Ago-containing protein complex to allow gene regulation of misregulated genes by RNA interference (xi).

Figure 2. Common chemical modifications used with siRNA in therapeutic approaches. These modifications can be made on (a) the base, (b) the sugar or (c) other parts of the RNA backbone.

Table 1

Small RNA-based therapeutics in clinical trials

Diseases	Disease category	Drug name	Drug type	Target	Phase	Company/Affiliation	Notes ^ψ
AMD	Ophthalmology	Bevasinab	siRNA	VEGF	III	Opko Health	Phase III was interrupted by Opko in 2009 (Box 1)
		PF-4523655 (RTP801 ^{ti} -14)	siRNA	RTP801	II	Quark	RTP801 is a Quark proprietary target
		AGN211745	siRNA	VEGF-R1	I/II	Allergan	AGN211745 is known as Sirta-027
Macular edema	Ophthalmology	Bevasinab, Cand 5	siRNA	VEGF	II	Opko Health	
		PF-4523655 (RTP801 ^{ti} -14)	siRNA	RTP801	II	Quark	RTP801 is a Quark proprietary target
Chronic optic nerve atrophy	Ophthalmology	QPI-1007	siRNA	proNGF	I	Quark	
Pachyonychia congenita	Genetic disorder	TD1010	siRNA	Keratin K6a	I	Pachyonychia Congenita Project	
Chronic lymphocytic leukemia	Oncology	SPC2996	LNA oligo	Bcl-2	I/II	Santaris Pharma	
Metastatic lymphoma	Oncology	Proteasome siRNA	siRNA	Immuno-proteasome β-subunits LMP2, LMP7 and MECL1	I	Duke University	
		gPLK SNALP	siRNA	PLK1	Pre-clinical	Tekmira	siRNA embedded in SNALP
Solid tumors	Oncology	CALAA-01	siRNA	M2 subunit of ribonucleotide reductase	I	Pharmaceutical Inc	siRNA embedded in lipid nanoparticles
		Att027	siRNA	PKN3	I	Silence Therapeutics AG	
		EZN3042	LNA oligo	Survivin	I/II	Enzon	
		EZN2968	LNA oligo	HIF-1α	I/II	Santaris Pharma	
		FANG vaccine	shRNA	Furin	I	Gradalis	shRNA furin+ GMCSF expression
		ALN-VSP	siRNA	KSP and VEGF	I	Amylam	siRNA embedded in SNALP
		eIF-4E ASO	LNA oligo	eIF-4E	I	Lilly	
		Survivin ASO	LNA oligo	Survivin	II	Lilly	For prostate cancer

Diseases	Disease category	Drug name	Drug type	Target	Phase	Company/Affiliation	Notes ^y
Delayed Graft Function	Inflammation	ISNP	siRNA	p53	I/II	Quark	Also known as QPI-1002
Acute kidney injury	Inflammation	ISNP	siRNA	p53	I	Quark	Also known as QPI-1002
Familial adenomatous polyposis	Inflammation	CEQ508	shRNA	β-catenin	I	Cequent	Oral administration MDRNA acquired Cequent in 2010
Hypercholesterolemia	Metabolic disease	PRO-040201	siRNA	ApoB	I	Pharmaceuticals Corp.	siRNA embedded in SNALP
		ApoB SNALP	siRNA	ApoB	I	Tekmira	
		SPC4955	siRNA	ApoB	Pre-clinical	Santaris Pharma	
		ALN-PCS	siRNA	PCSK9	Pre-clinical	Alnylam	
HCV	Viral infection	SPC3649	LNA oligo	miR-122	I	Santaris Pharma	
HIV	Viral infection	Lentivirus expressing shRNAs/SIL-TAR decoy and anti CCR5 ribozyme	shRNA +TAR decoy +CCR5 ribozyme	HIV Tat and Rev proteins, HIV TAR RNA, and human CCR5	I	City of Hope Medical Center/ Benitec	Lentiviral expression in autologous CD4 ⁺
		pHIV7-sh1-TAR-CCR5RZ	shRNA +TAR decoy +CCR5 ribozyme	HIV Tat protein, HIV TAR RNA, and human CCR5	Pre-clinical	City of Hope Medical Center/ Benitec	
RSV	Viral infection	ALN-RSV01	siRNA	RSV nucleocapsids	II	Alnylam/Cubist	

Abbreviations: Bcl-2, B-cell CLL/lymphoma 2; eIF-4E, eukaryotic translation initiation factor 4E; GM-CSF, granulocyte macrophage colony stimulating factor; HIF-1α, hypoxia-inducible factor 1 alpha; KSP, kinesin spindle protein; LMP2, large multifunctional peptidase 2; LMP7, large multifunctional peptidase 7; MECL1, proteasome subunit beta type-10; PLK1, polo-like kinase 1; PNK3, protein kinase N3; PCSK9, proprotein convertase subtilisin/kexin; proNGF, proform of nerve growth factor; RSV, respiratory syncytial virus; RTP801, DNA-damage-inducible transcript 4; TAR, Transactivating region.

^y This column provides useful information about RNA-based drugs in clinical trials, but does not reflect all the information found in literature. Sources: Company web sites, press releases and www.clinicaltrials.gov

Table 2

Structure	Description	Illustration
Lipid-raft compartments ^a	Membrane microdomains that function as signaling platforms. Rich in caveolin, cholesterol, glycosphingolipids, glycosylphosphatidylinositol (GPL)-anchored proteins, and signaling molecules.
Liposomes ^b	Spherical vesicles in which a single or several continuous lipid bilayers separate the external aqueous medium from the intraliposomal aqueous core opposed to a hydrophobic core.
Lipidoids	Conjugation of alkyl-acrylates or alkyl-acrylamides to primary or secondary amines. Depending on the number of additions to amino monomer, lipidoids can form 1 to 7 tails.
Lipoplexes	Cationic lipids interact with negatively charged nucleic acids through electrostatic interactions, thus forming a complex of multilamellar structures with alternating positively charged lipid bilayers and negatively charged nucleic acids.

^a Image by Artur Jan Fijałkowski, which has been released into the public domain by its author.

^b Image has been released into the public domain by its author, Mariana Ruiz Villarreal from LadyofHats.