



Chemical modification of siRNA plays an essential role in moving siRNA toward the clinic.

Chemically modified siRNA: tools and applications

Jonathan K. Watts, Glen F. Deleavey and Masad J. Damha

Department of Chemistry, McGill University, 801 Sherbrooke Street West, Montreal, QC H3A 2K6, Canada

Chemical modification provides solutions to many of the challenges facing siRNA therapeutics. This review examines the various siRNA modifications available, including every aspect of the RNA structure and siRNA duplex architecture. The applications of chemically modified siRNA are then examined, with a focus on specificity (elimination of immune effects and hybridization-dependent off-target effects) and delivery. We also discuss improvement of nuclease stability and potency.

Introduction

It has been ten years since the publication of the seminal paper demonstrating the high potency of long double-stranded RNA in gene knockdown [1]. Shortly thereafter, it was discovered that the same effect could be produced in mammalian cells using synthetic short RNA duplexes [2]. The relatively few years since then have seen an explosion of research into therapeutic applications of RNAi. Several companies have been formed to pursue the technology, and transactions involving these companies have recently been measured in the billions of dollars.

The reason for the excitement is that RNAi allows potent knockdown of virtually any gene. This in turn allows rapid progression from target selection to preclinical trials. siRNA has become the most common tool in functional genomics, and therefore can often also help at the target identification stage. Furthermore, some targets that are not druggable by traditional methods can be targeted by gene knockdown.

In spite of the immense attractiveness of gene knockdown as a therapeutic strategy, siRNA duplexes are not optimal drug-like molecules. RNA is highly vulnerable to serum exo- and endonucleases, leading to a short half-life in serum. siRNA duplexes are composed of two strands that can drift apart in a dilute environment-like serum. Because oligonucleotides are polyanions they do not easily cross cell membranes and, because this charge density leads to extensive hydration, they do not easily interact with albumin and other serum proteins, leading to rapid elimination. Unmodified oligonucleotides have limited tissue distribution. And finally, oligonucleotides can have off-target effects, either by stimulating the immune system or by entering other endogenous gene regulation pathways.

A wide variety of chemical modifications have been proposed to address these issues. In this review, we examine the principles of chemical modification of siRNA duplexes. We will briefly look into the toolbox; that is, summarize the possible ways that siRNA duplexes can be modified.

JONATHAN K. WATTS

Jonathan K. Watts received his BSc in chemistry from Dalhousie University in Halifax, Canada. He has just finished his PhD in the group of Masad Damha at McGill University. He has been awarded a Natural Sciences and Engineering Research Council of Canada (NSERC) postgraduate fellowship, the McGill Tomlinson Fellowship and a postdoctoral fellowship from FQRNT Quebec. His primary research interest is the interface of chemistry and biology in the field of small RNAs.

GLEN F. DELEAVEY

Glen F. Deleavey received his BSc in biology-chemistry from the University of New Brunswick in Fredericton, Canada. He is currently a PhD candidate in the group of Masad Damha at McGill University. He has been awarded a postgraduate fellowship from NSERC and a 2007 SCI Merit Award (Canadian section). His research interests lie in the field of chemical biology, with a focus on chemically modified siRNAs.

MASAD J. DAMHA

Masad J. Damha received his BSc and PhD degrees from McGill University, the latter in the group of Prof. Kelvin Ogilvie, on synthesis and conformational analysis of RNA and its analogues. After beginning his academic career at the University of Toronto, he returned to McGill in 1992, where he is currently James McGill Professor of Chemistry. His research interests include synthesis of RNA (including novel RNA structures) and the application of oligonucleotide derivatives as therapeutics. He was awarded the 2007 Bernard Belleau Award from the Canadian Society for Chemistry, honoring significant contributions to the field of medicinal chemistry, for the development of 2'-F-ANA.

GLOSSARY

RNA interference (RNAi) An evolutionarily conserved cellular mechanism for gene knockdown found in fungi, plants, and animals, in which double-stranded RNA (dsRNA) triggers the specific cleavage of complementary mRNA molecules via endogenous cellular machinery.

Short interfering RNAs (siRNAs) These triggers of RNAi are dsRNAs that typically contain 19–21 bp and 2-nt 3'-overhangs. siRNAs are naturally produced by Dicer-mediated cleavage of larger dsRNAs, but may also be introduced into cells exogenously.

microRNAs (miRNAs) Endogenous small non-coding RNAs that play an important role in the regulation of many genes. Precursor miRNAs (pri- and pre-miRNAs) contain hairpin structures, often with bulged regions, which are processed into duplexes resembling siRNAs.

Short hairpin RNAs (shRNAs) ssRNA molecules that fold into hairpin-shaped structures containing dsRNA stems and a ssRNA loop. While shRNAs resemble naturally occurring pre-miRNAs, shRNA typically refers to exogenous RNA molecules introduced into cells, or produced within cells by introducing exogenous DNA.

Antisense (guide) strand The strand of an siRNA duplex that is loaded into the RISC complex and which guides RISC to complementary mRNA.

Sense (passenger) strand The strand of an siRNA duplex that is not loaded into the RISC complex. It should have the same sequence as the target mRNA.

Seed region A 6–7-nt region at the 5' end of the antisense RNA strand (from nucleotides 2–7 or 2–8). The seed region is especially important for mRNA target recognition, and complementarity to the seed region is often sufficient to reduce gene expression through a miRNA-type mechanism.

Innate Immune Response A nonspecific cellular response to foreign material. The innate immune response is often associated with the production of cytokines.

Cytokines A class of signaling proteins and glycoproteins including interferons (INF), interleukins (IL) and tumor necrosis factors (TNF). They are stimulated as part of an innate immune response, and activate further immune system responses.

Toll-like receptors (TLRs) A class of pattern-recognition receptors that recognize molecular structures generally associated with pathogens. Human TLR3 can recognize dsRNA, and human TLR7 and TLR8, primarily considered ssRNA receptors, can also be stimulated by siRNA. Stimulation of TLRs leads to activation of innate immune responses.

Photocaging Temporarily blocking the activity of a drug by appending a photolabile group to it. The group can later be cleaved by treatment with light, activating the drug.

Following this, we will review the ways these tools have been applied to move siRNA toward the clinic, including the use of chemical modifications to improve potency, serum stability, specificity and delivery. We will point out the most useful and universal modifications as well as some of the most creative modifications and applications, which stretch our paradigms and open new avenues of research into RNAi-based drugs.

Much excellent work has led to significant growth in understanding the mechanism of RNAi [3–5] (Fig. 1, also see Glossary of specialist terms). When an exogenous 19–21 bp siRNA is introduced into a mammalian cell the 5'-end is phosphorylated. The

duplex is then assembled into the RNA-induced silencing complex (RISC), a multiprotein complex including Argonaute2 (AGO2), Dicer, TRBP (HIV-1 TAR RNA-binding protein) and PACT (a dsRNA-binding protein), as well as other proteins, some of which are yet unknown [3]. The strand with the lower binding affinity at its 5'-end becomes the antisense (guide) strand [6,7], and the other strand (known as the sense or passenger strand) is cleaved and unwound, to leave a single-stranded RNA associated with Argonaute2 (AGO2), an endonuclease at the heart of RISC that promotes location of complementary mRNA, hybridization and cleavage of the mRNA target. When modifying an siRNA duplex it is important to remember that different modification approaches are required for the sense and antisense strands, because of these very different roles [8,9].

In most cases it is simply assumed that the RNAi mechanism is unaffected by chemical modification of siRNA duplexes. A few studies using modified siRNA have confirmed this by showing that the cleavage of complementary mRNA occurs between bases 10 and 11, counting from the 5'-end of the guide strand, as is the case for unmodified duplexes [10–12]. However, in principle, this should be verified for each new pattern of modification.

Toolbox

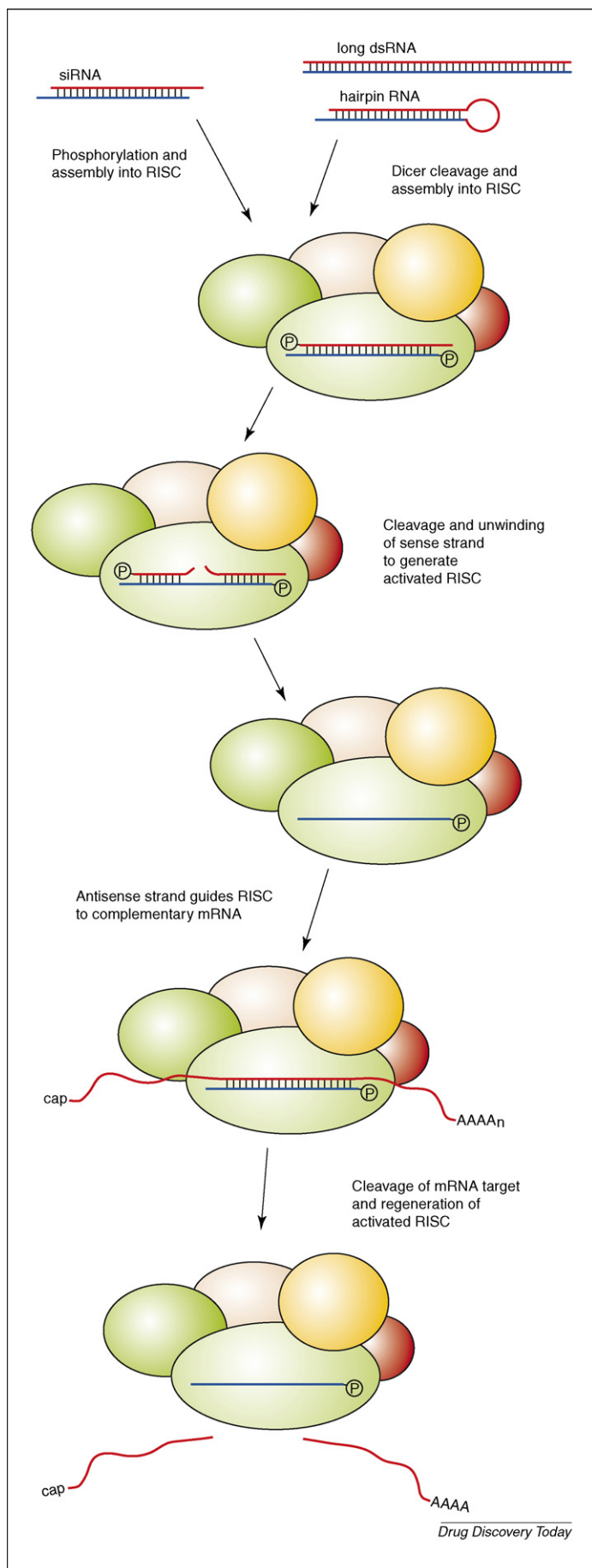
siRNA duplexes have been chemically modified in a wide variety of ways. Some of the results in the literature, however, seem to contradict one another, or to work on one system but not another. This field is still very young, and it will take time for the more robust and universal modifications to be recognized as such. In the meantime, it is useful to have many options so that at least one of the chemistries can be used to modify an siRNA without compromising its potency.

In this section, we will briefly review the most significant siRNA modifications in the literature, drawing attention to those that have proven most useful and robust up to now. Our goal in this section is not to explore the advantages of each modification in detail but simply to present all the known possibilities in a straightforward way. In the second half of this review we will explore how these modifications can help move siRNA toward the clinic. We hope that by examining the 'toolbox' of chemical modifications separately from the 'task list' of required properties we will inspire creative new combinations and applications.

Sugar modifications

The most widely used siRNA modifications are on the sugar moiety (Fig. 2). One of the earliest studies on chemically modified siRNA showed that, while A-form duplex structure is important, the 2'-OH is not required for active siRNA [13]. Therefore, the 2' position has been extensively modified.

2'-O-Methylation of RNA increases binding affinity and nuclease stability, and the resulting 2'-O-Me-RNA can be well-tolerated throughout the duplex, making it one of the most popular and versatile siRNA modifications. Many groups have found that large numbers of 2'-O-Me modifications (in either strand) decrease siRNA activity [13–16], but others have found that fully modified 2'-O-Me sense strands are functional [11,17]. Kravynack and Baker attribute these differences to their finding that 2'-O-Me modifications work best in blunt-ended duplexes [11], but at least one



group found that, even in this context, activity is greatly reduced by heavy 2'-O-Me modification [16].

In general, bulkier 2'-modifications are not well-tolerated in siRNA duplexes, except small numbers of modifications on the termini. Davis *et al.* demonstrated that bulky 2'-substituents correlate with poor activity as siRNA sense strands and, for the same reasons, excellent activity as 'anti-miRNA oligonucleotides' (to block miRNAs) [18].

Thus, the 2'-O-methoxyethyl (2'-O-MOE) modification has been used in the 3'-overhangs of an siRNA targeting the pain-related cation-channel P2X3, and resulted in successful gene targeting *in vivo* [19]. Another group found that 2'-O-MOE modifications could be included in the sense strand, especially at the termini, but not in the antisense strand [20]. Similarly, 2'-O-allyl-modifications caused a reduction in activity at most positions in the duplex, but can be used in the 3'-overhangs [21].

siRNAs in which 70% of the 2'-OH groups in both strands are converted at random into 2,4-dinitrophenyl ethers (2'-O-DNP) show a variety of improved properties, including higher binding affinity, nuclease resistance and potency [22].

Instead of a hydroxyl, alkoxy or aryloxy substituent, functional siRNAs can contain fluorine at the 2'-position. 2'-F-RNA is one of the best-known siRNA modifications, and partial 2'-F-RNA modification is tolerated throughout the sense and antisense strands [13,14,23], and some fully modified 2'-F-RNA siRNAs are also active [24]. 2'-F-RNA-modified siRNA duplexes have significantly increased serum stability [25]. 2'-F-RNA also increases the binding affinity of the duplex.

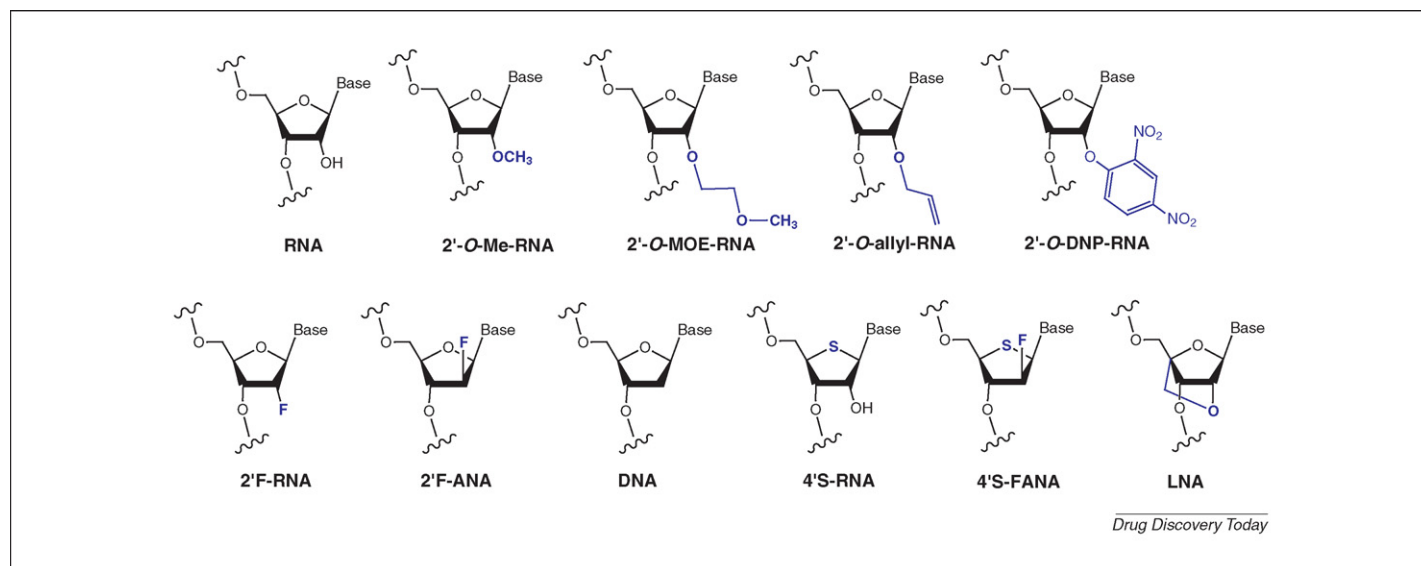
Changing the stereochemistry of the fluorine of 2'-F-RNA gives 2'-F-ANA, which was originally developed as a DNA mimic [26,27]. Considering this fact, it is somewhat surprising that 2'-F-ANA is also well-tolerated in siRNA duplexes, including fully modified sense strands and partial modification of the antisense strand [28,29]. Like its epimer 2'-F-RNA, it binds with high affinity and increases nuclease stability.

DNA itself, a 'modification' containing no electronegative substituent at 2', can also be accepted within siRNA duplexes. For example, use of DNA in the 3'-overhangs has been well-known since the earliest days of synthetic siRNA research [2] and DNA can also be tolerated, in limited numbers, within the base-paired region of an siRNA duplex [9,12,15]. An antisense strand made entirely of DNA purines and 2'-F-RNA pyrimidines is functional [13], since 2'-F-RNA strongly favors a northern sugar pucker and A-form helical structure, and presumably directs the conformation of the more flexible 2'-deoxynucleotides. Substitution with dsDNA in the 8-bp region at the 5'-end of the guide strand gives active duplexes with reduced off-target effects [12].

The ring oxygen has also been modified: 4'-S-RNA is a high-affinity modification that gives a significant advantage in nuclease stability. 4'-S-RNA is very well tolerated near the termini of siRNA duplexes [30–32]. In the antisense strand some loss of potency was observed, but not as much as with 2'-O-Me-RNA at the same positions [30]. The 5'-end of the antisense strand could be modified with a few 4'-S-RNA inserts without significant loss of potency

FIGURE 1

The mechanism of RNAi in human cells. The largest of the ellipses signifies AGO2, the catalytic engine of RISC.

**FIGURE 2**

Sugar units that have been successfully used to modify siRNA duplexes. Top row: 2'-O-alkyl (2'-O-Me, 2'-O-MOE, 2'-O-allyl) and 2'-O-aryl (2'-O-DNP) modifications. Bottom row, from left to right: other 2'-modifications (2'-F-RNA, 2'-F-ANA, DNA), 4'-modifications (4'-S-RNA, 4'-S-FANA) and a conformationally constrained modification (LNA).

[31]. The center of the antisense strand cannot be modified with 4'-S-RNA without significant loss of potency [30,31]. A strand architecture consisting of four 4'-thioribonucleotides on each end of the sense strand and four at the 3'-end of the antisense strand worked consistently well against two target genes in three cell lines [32]. Combinations of 4'-S-RNA with 2'-O-Me and 2'-O-MOE modifications at the termini of both strands showed excellent potency and serum stability [31].

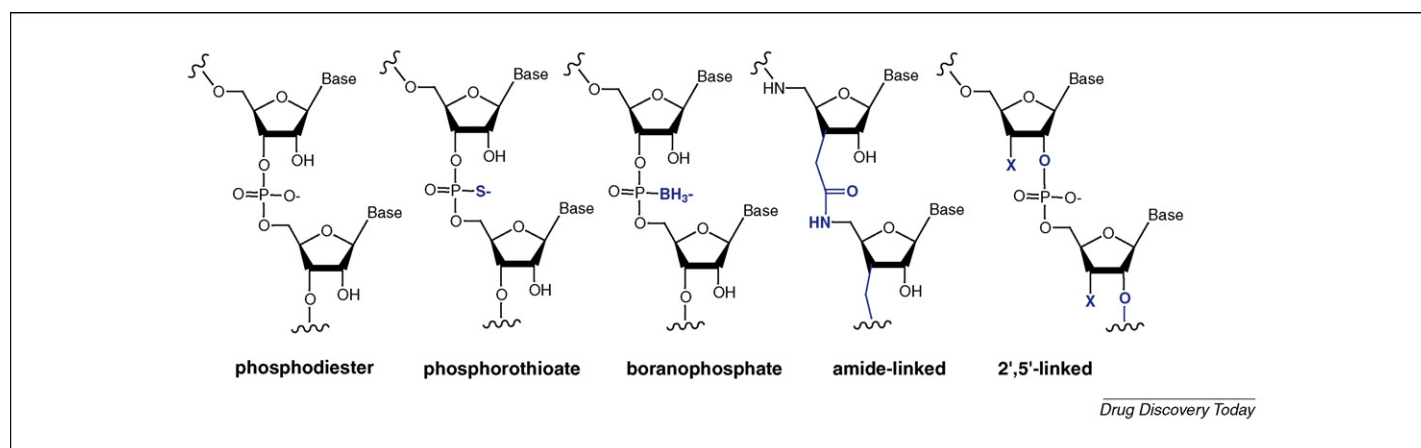
4'-S-FANA, with modifications at the 2' and 4' positions, has a northern, RNA-like conformation [29]. It has also shown siRNA activity at various positions in both strands. When 4'-S-2'-F-ANA is in the antisense strand it shows synergy with 2'-F-ANA sense strand modifications [29]. However, its low-binding affinity makes it suitable only for a small number of modifications in a duplex.

The conformationally constrained nucleotide LNA [33] has also been included in siRNA [14,34–37]. Its conformational rigidity

leads to significant increases in binding affinity. Careful placement of LNA in siRNA duplexes has led to functional duplexes of various types. The most common sites of modification are the termini of the sense strand [37] and the 3'-overhangs of the antisense strand [34,36]. Minimal modification of most internal positions of the antisense strand is also tolerated [14,34], but heavier modification of the antisense strand is tolerated only in combination with a segmented sense strand (described in more detail below) [35].

Phosphate linkage modifications

Several variations on the phosphodiester linkage are also accepted by the RNAi machinery (Fig. 3). Phosphorothioate (PS) linkages can be used, with comparable [21,23] or lower potency [13,38] to that of native siRNA. Some groups have found that PS linkages are not accepted at the center of the duplex, especially at the scissile phosphate [39]. However, the ability to accept fully modified PS

**FIGURE 3**

Internucleotide linkages used in siRNA. The phosphodiester linkage can be modified as a phosphorothioate or boranophosphate, which retain the negative charge of a phosphate, or a neutral amide-linked RNA can be used at select positions. Either 2',5'-linked DNA (X=H) or RNA (X=OH) can be used in the sense strand.

strands may depend on strand architecture [11]. Some cytotoxicity has been observed with extensive PS modification [21]. PS modifications do not appear to have a major effect on biodistribution of siRNA [40].

siRNAs with boranophosphate linkages are functional and show increased potency relative to PS-modified siRNAs and often to native siRNAs as well but, to maximize potency, the center of the antisense strand should be unmodified [38]. Boranophosphate siRNAs provide a significant increase in nuclease stability over native RNA [38].

A 2',5'-linkage (either 2',5'-DNA or 2',5'-RNA) can substitute for the native 3',5' linkage, but only in the sense strand of the duplex and with some reduction in potency [41]. A nonionic amide linkage has been used in the 3'-overhangs of siRNA duplexes [42].

Base modifications

Use of modified bases in siRNA has been somewhat more limited, but there have been several examples (Fig. 4). Modified bases that stabilize A-U base pairs (5-Br-Ura and 5-I-Ura instead of uracil, and diaminopurine instead of adenine) were tolerated in siRNA duplexes, although their activity was somewhat reduced [13]. 4-Thiouracil has also been used [9]. 2-Thiouracil [43,44] and the C-linked base pseudouracil [43,44] increase binding affinity and can be used to increase potency and specificity if placed appropriately within the duplex (see the sections: *Increasing potency* and *Modulating immunostimulatory activity*). 5-Methylation of pyrimidines (i.e. use of T and 5-Me-C instead of U and C) is common in

conjunction with sugar modifications such as DNA, 2'-F-ANA and LNA.

Surprisingly, some atypical base structures have been used in siRNA. A difluorotoluy base, which has the same shape as thymine but cannot form hydrogen bonds, can replace uracil at single positions throughout an siRNA duplex, without significantly decreasing the potency or changing the mechanism of cleavage [45]. A nonaromatic base, dihydrouracil, can also be used, but because it cannot contribute to base stacking it lowers the binding affinity of the duplex and is best placed at the 5'-end of the duplex, as defined by the antisense strand [43]. Bulky or cationic base modifications have not been well-tolerated [9].

Modifications to the overhangs and termini

Early studies showed that the ideal siRNA consisted of 21-nt in both strands, including 2-nt 3'-overhangs [15]. These overhangs can be modified in various ways: from the beginning deoxy units have often been used in the 3' overhangs to reduce costs and possibly increase resistance to 3'-exonucleases [2]. However, RNA units also work well [46], most of the chemistries reviewed above work well in the overhangs, and blunt-ended siRNAs have also been used [16]. Blunt-ended duplexes are more resistant to 3'-exonucleases, and one study reported a greater tolerance to chemical modifications in combination with blunt-ended duplexes [11]. However, they are more immunogenic than duplexes with 3'-overhangs [47].

The termini of the strands can also be modified. Chemical phosphorylation of the 5'-end of the antisense strand helps ensure high potency, and is often necessary when the strand is modified. By contrast, 5'-phosphorylation of the sense strand can be blocked without loss of activity [16,48], and can in fact be beneficial [49].

Furthermore, various groups can be conjugated to the ends of siRNA duplexes, especially the termini of the sense strand. These groups can include an inverted abasic end cap [50,51], which helps with exonuclease stability. Conjugation of fluorescent dyes [23] or biotin [52] has allowed important biophysical/biochemical studies. Finally, conjugation of membrane-penetrating peptides [53] and lipophilic groups including steroids and lipids [10,54] has helped with delivery (see the section *Delivering siRNA*). An intriguing recent study showed that including 5–8 dA and dT units on the 3'-ends of the strands can lead to reversible concatemerization through these sticky ends, which in turn leads to higher efficiency delivery in complex with PEI [55].

The general consensus is that the 5'-end of the antisense strand is most sensitive to modifications [16,52,56] and does not tolerate most of the above-mentioned modifications. However, one group observed that fluorescein could be conjugated to any of the termini except the 3'-end of the antisense strand [23]. As long as an antisense 5'-phosphate is present, attaching a group to it does not necessarily eliminate RNAi activity [57].

Modifications to the duplex architecture

The duplex architecture itself can be modified through chemical synthesis (Fig. 5). While most siRNA duplexes are made up of two strands, it has been shown that an siRNA made of three strands (an intact antisense strand with two 9–13 nt sense strands) can reduce off-target effects and increase potency; the resulting duplex is termed small internally segmented interfering RNA (sisiRNA)

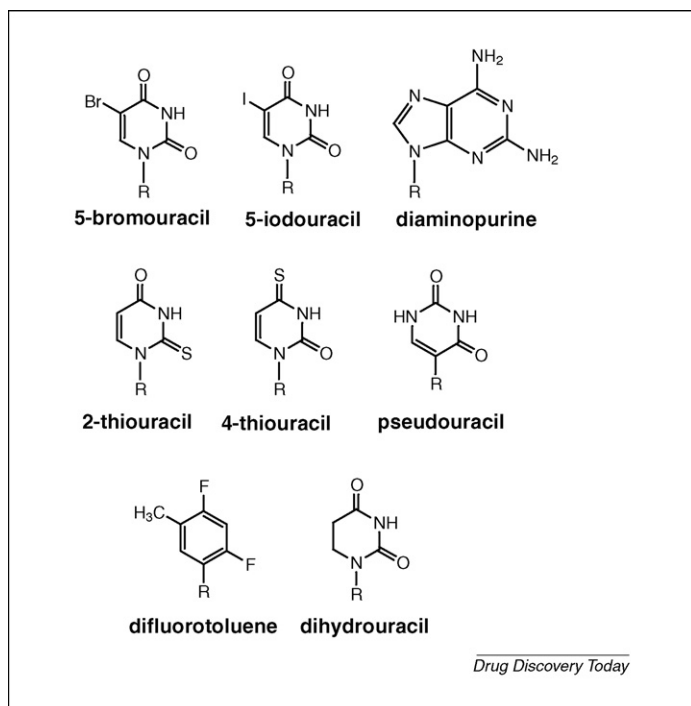
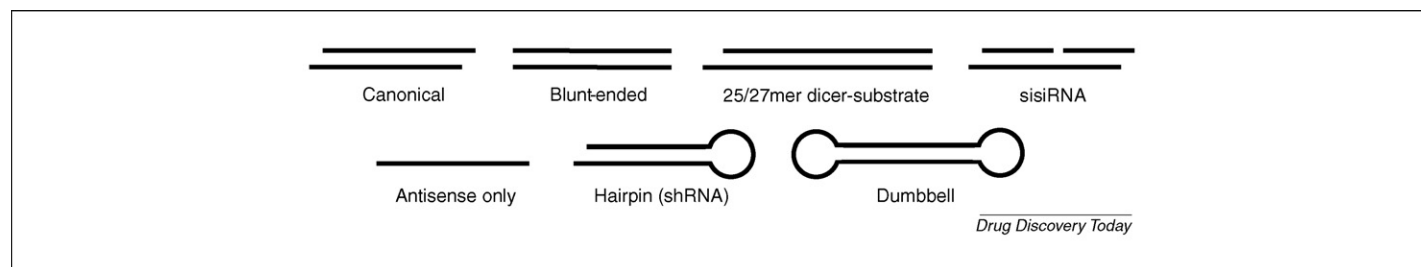


FIGURE 4

Nucleobase modifications used in siRNA. R represents the ribose phosphate backbone. Top two rows: 5-halouracils, 2-thiouracil, pseudouracil and diaminopurine increase the strength of A-U base pairs. Bottom row: highly atypical modified bases including a base that cannot form hydrogen bonds (difluorotoluene) and a non-aromatic base (dihydrouracil).

**FIGURE 5**

Functional siRNA architectures. The sense strand is always shown on top, in the 5' to 3' direction, and the antisense strand is on the bottom, in the 3' to 5' direction. Note that three of the structures (25/27mer, hairpin and dumbbell) require the activity of Dicer before incorporation into RISC.

[35]. Functional siRNA can also be made from just one strand, in one of the various ways. Hairpin-type duplexes, made from a single strand, can be introduced exogenously [58] or expressed within a cell [59,60]. Closing the other end of the hairpin results in a dumbbell or nanocircle which retains RNAi activity while providing complete protection from exonucleases [61]. And finally, a single-stranded antisense RNA (which does not fold into a duplex at all) has been shown to enter the RNAi pathway, with potency approaching that of the duplex siRNA in some cases [21,56,62].

The length of an siRNA duplex can also be changed. Most synthetic duplexes are 19–21 bp in length, mimicking the natural products of the Dicer enzyme. However, increasing the length of an siRNA duplex makes it a substrate for Dicer and has been found to increase its potency [63]. It is important to keep the length below 30 nt, to avoid triggering the interferon response [64].

Applications

Improving serum stability

Unprotected RNA is very quickly degraded in cells. The fact that siRNA is double-stranded provides it with some degree of protection, but not enough for *in vivo* use. A nuclease called *eri-1* has been found to play a key part in the degradation of siRNA [65], and expression levels of *eri-1* inversely correlate with duration of siRNA activity [66]. This and other data suggest that increasing the nuclease resistance of siRNAs can prolong their activity. Chemical modification is the principal strategy used to improve the nuclease resistance of siRNAs.

Essentially all of the modifications in the toolbox can be used to increase the serum half-life of siRNAs. Within the therapeutic siRNA community, however, two schools of thought have emerged regarding the best paradigm for protecting siRNAs against nucleases. The first strategy favors extensive or entire chemical modification. This paradigm is exemplified by the research of Sirna Therapeutics (<http://www.sirna.com/>), who have published work on heavily modified siRNA duplexes. For example, a fully modified siRNA with significantly increased potency in a hepatitis B virus (HBV) mouse model consisted of a sense strand made of 2'-F-RNA pyrimidines, DNA purines, and 5' and 3' inverted abasic end caps. The antisense strand was made of 2'-F-RNA pyrimidines, 2'-O-Me purines and a single phosphorothioate linkage at the 3'-terminus [51]. This fully modified duplex had a half-life in serum of two to three days, as compared with 3–5 min for the unmodified duplex [51]. This improved stability got translated into higher efficacy *in vivo*. Higher potency was later obtained by including one to three RNA inserts at the 5'-end of the antisense strand, and this heavily

modified siRNA still had a serum half-life nearly 30 times longer than that of unmodified siRNA [50].

A few other examples of fully modified duplexes have been reported. An siRNA made entirely of alternating 2'-F-RNA and 2'-O-Me units was found, unsurprisingly, to have greatly increased stability in serum [67]. This architecture also maintains or improves potency, as discussed below. A functional duplex made with DNA overhangs, a 2'-O-Me-RNA sense strand and a PS-RNA antisense strand were nearly all intact after 48 h in serum [17], and a functional fully modified 2'-F-RNA siRNA has also shown excellent nuclease resistance [24].

Such a large degree of modification, however, may not always be necessary. The second paradigm for creating stabilized siRNAs involves minimal, selective modification. It is exemplified in the research of, among others, Alnylam Pharmaceuticals (<http://www.alnylam.com/>). Because endonuclease degradation is a major mechanism of degradation of siRNAs [16], the endonuclease cleavage pattern of a given siRNA duplex is first characterized (this is often dominated by cleavage after a pyrimidine nucleotide, and can be readily characterized by mass spectrometry [68]). The vulnerable positions are then selectively modified, usually with 2'-O-Me or 2'-F-RNA nucleotides, which considerably increases the stability of the siRNA with minimal modification [69,70].

Besides these empirically determined internal positions, key positions for modification include the termini of the strands, especially the 3'-termini, protecting the duplex from 3'-exonuclease degradation [17].

Increasing potency

The RNAi pathway is very efficient and unmodified siRNA is a very potent gene silencing agent, although potency does depend on cell type, target and siRNA sequence. In general, increasing potency is not considered the primary objective of chemical modification: it is sufficient to maintain the potency of unmodified siRNA while increasing its serum half-life and its specificity. However, as the requirements for effective RNAi are increasingly well understood, we can foresee an increase in the use of chemical modifications to optimize potency as well, through features such as target-binding affinity (enhancing hybridization on-rates and off-rates), conformational preorganization (A-form helical structure) and flexibility.

It is very rare to find patterns of chemical modification that universally increase potency. By contrast, there are several known modifications that increase potency, sometimes very significantly, for particular sequences or systems. One of the most dramatic

increases was observed for a fully modified siRNA made of a combination of 2'-O-Me and 2'-F-RNA modified nucleotides, which was 500 times more potent than unmodified RNA [67]. Whereas such a high degree of improvement was not observed for other sequences, this architecture was consistently of equal or greater potency and efficacy than unmodified RNA [71].

Part of the increase in potency observed in some studies may be due to the increased nuclease stability of the chemically modified siRNA. Beyond this, however, in most cases where increased potency is observed, it is not yet clear why. Similarly, it is not yet clear why so many patterns of chemical modification work for one system/sequence and not for another. This reflects paucity in our understanding of the requirements of the RNAi pathway.

Rational chemical modification can, however, be used to improve potency by improving the selective loading of the antisense strand. In the normal RNAi pathway strand incorporation into RISC is determined by the thermodynamics of the siRNA duplex [6,7]. The strand with lowest binding at its 5'-end is preferentially incorporated into RISC. The sequence of an siRNA should therefore be chosen to be AU-rich at the 5'-end of the antisense strand. However, chemical modifications can be used to increase the selectivity of antisense strand incorporation, by placing high-affinity modifications like LNA at the 5'-end of the sense strand [34]. The activity of a moderately active siRNA duplex was significantly improved using the high-affinity 4'-S-RNA modification in both strands [32]. By contrast, a duplex that had optimal strand thermodynamics was not further improved by 4'-S-RNA modification [32].

Base modifications can be used to the same effect. Of a series of modified siRNA duplexes, the most potent siRNAs contained a high-affinity 2-thiouracil base at the 3'-end of the antisense strand and a low-affinity dihydrouracil base at the 3'-end of the sense strand [43].

Modulating immunostimulatory activity

RNAi is an evolutionarily conserved cellular mechanism believed to function as an early defense against pathogens. The presence of dsRNA in cells is often characteristic of viral infection as many viruses have a dsRNA stage in their life cycles. The cell responds to this perceived invader in several ways including specific gene knockdown through RNAi and other effects caused by innate immune responses [72]. Side-effects from an siRNA therapeutic may result from this immune response, unless the immunogenic potential of dsRNA can be addressed.

It has long been known that dsRNAs longer than 30 bp are potent activators of immune responses [64]. This fact prevented the application of RNAi to mammalian cells until it was discovered that 21mer siRNA duplexes were capable of effecting potent and specific gene knockdown in mammalian cells, without the immunostimulation characteristic of longer dsRNAs [2]. However, the immunogenic activity of short RNA duplexes is more significant than it was originally thought. A growing number of recent studies report off-target effects resulting from immunostimulation caused by dsRNAs that are shorter than 30 bp [37,44,50,72–76].

Immune response activation by short RNAs is a complex process, and has been reviewed elsewhere [72,73]. Briefly, RNAs are recognized by three main types of immunoreceptors: Toll-like receptors (specifically TLR3, TLR7 and TLR8; see Glossary), protein

kinase R (PKR) and helicases such as RIG-I and MDA5. Recognition by these receptors can lead to a number of cellular responses including release of cytokines and changes in gene expression [72]. The types of immunoreceptors involved, and the level of immune activation, depend on a number of factors including the length, sequence and cellular delivery method of the RNA, as well as the type of immune cell involved. These receptors exist on cell surfaces (TLR3) [77], in endosomes (TLR3/7/8) [78–80] and in the cytoplasm (RIG-I, MDA5, PKR) [79].

The sequence dependence of immune responses is not fully understood. Judge *et al.* have identified immunostimulatory motifs in mice and *in vitro* in human blood, most notably 5'-UGUGU-3' regions [74]. Hornung *et al.* demonstrated that 5'-GUCCUCAA-3' is also a potent immunostimulatory motif [37]. In fact, any U-rich RNA sequence may be sufficient for recognition by TLR7 [81]. The presence of a 5'-triphosphate or blunt end can lead to RIG-I-mediated immunostimulation [44,47,75]. Finally, duplexes between 23 and 30 bp may still induce the interferon response in a length-dependent manner, depending on cell type [76].

Chemical modifications can be used to reduce the immunostimulatory properties of siRNAs [79]. For example, siRNA duplexes >90% modified with 2'-F-RNA, 2'-O-Me and DNA residues were shown to cause no detectable effect on interferon levels or cytokines, while unmodified siRNA duplexes caused significant activation [50]. Because of immunostimulation, mice treated with unmodified siRNA showed increased levels of serum transaminases and signs of systemic toxicity such as decreased body weight, transient lymphopenia and thrombocytopenia, and piloerection, but these adverse effects were not evident in those mice treated with the modified siRNA [50]. More recently, it has been shown that a far smaller degree of 2'-O-Me modification is sufficient to eliminate immunostimulatory activity [82]. Almost any degree of modification was sufficient, with the exception that 2'-O-methylation of cytidine residues was ineffective in reducing immunostimulatory activity [82]. 2'-F-RNA and/or 2'-O-Me modification of uridine resulted in the elimination of immune off-target effects [44], including TLR-dependent and TLR-independent immune effects [83]. Strikingly, even the presence of 2'-O-Me-modified RNAs on separate, noncomplementary strands abrogates the TLR7-dependent immunostimulatory activity of unmodified siRNAs, indicating that 2'-O-Me-RNA itself is a potent antagonist of TLR7 [84].

2'-O-Me and 2'-F modifications are not alone in reducing immunostimulation. LNA has been used to modify the ends of an siRNA sense strand containing an immunostimulatory motif, abrogating the IFN- α immunostimulatory activity of the duplex without affecting the silencing activity [37]. Base modifications, too, can help reduce immune activation: modification with pseudouracil or 2-thiouracil prevented the RIG-I-mediated immunostimulation due to a 5'-triphosphate [44], and various base modifications abrogated immune effects mediated by TLR3, TLR7 and TLR8 [85].

Indeed, different types of chemical modifications can reduce the immune effects from different receptors. For example, of the base modifications useful for siRNA, one study found that only 2-thiouridine was able to reduce TLR3-mediated immunostimulation, but several others were able to reduce TLR7- or TLR8-mediated effects [85]. In another recent study, the cytokine

induction profile was different upon treatment with unmodified, 2'-O-Me-modified and 2'-F-modified siRNAs [80].

Immune stimulation can have a real effect on the results of siRNA experiments. For example, use of a control siRNA that is less immunostimulatory than the experimental siRNA can lead to false positives or overestimated efficacies [79].

Not all researchers have observed immunogenic side effects from siRNA administration. However, it is clear that when immune activation is a problem, there are many chemical modifications with immunomodulatory effects available to a nucleic acids researcher. Surely many more modifications will be tested and found successful in the years to come. Thus, much work lies ahead before the full immunomodulatory potential of chemical modifications can be both realized and understood.

In closing this section, we note that the immunostimulatory activity of siRNA may actually prove useful in some cases. A recent paper showed that the therapeutic anti-angiogenesis activity of an unmodified siRNA currently in clinical trials may actually be due to TLR3-mediated immune effects [77]. Furthermore, immunostimulatory siRNAs may find use as drugs capable of facing cancer and chronic viral infections on two fronts: eliciting gene knock-down and activation of immune responses [72]. Currently, however, immune activation is generally considered an unwanted and potentially dangerous off-target effect [79]. Thus, chemical modification provides yet another important tool for moving siRNA toward the clinic.

Reducing hybridization-dependent off-target effects

Another class of off-target effects of siRNA involves partial hybridization with the wrong mRNA [86]. This primarily occurs when siRNAs act as miRNAs, involving 6–8 nt complementarity at the 5'-end of the antisense strand [87,88], but can also occur with a region (~15 nt) of significant complementarity in the center of the antisense strand [86]. If some of the sense strand is mistakenly incorporated into RISC instead of the antisense strand it can cause its own set of off-target effects [86].

Thus two major strategies are used to reduce off-target effects: (i) increasing the selective uptake of the antisense strand (disfavoring incorporation of the sense strand) and (ii) disfavoring the miRNA-type pathway itself.

The first of these strategies, for obvious reasons, can have the benefit of increasing potency as well as reducing off-target effects. Typically, this is done by increasing the binding affinity of the 3'-end of the antisense strand and/or the 5'-end of the sense strand, as discussed in the section *Increasing potency*. However, to ensure that the sense strand makes absolutely no contribution to off-target effects, it can be further modified. The most thorough example of this is *sisiRNA*, in which the sense strand is nicked to prevent it from being incorporated into RISC (see Fig. 5) [35]. To allow such a strand architecture, the high affinity of LNA modifications is required [35], since 10–12 bp duplexes of unmodified RNA are not stable enough to be useful for gene silencing. Another method of reducing incorporation of the sense strand is to block phosphorylation of its 5'-terminus by methylation [49]. Finally, incorporation of eight DNA nucleotides at the 3'-end of the sense strand significantly reduces sense-strand-mediated off-target effects [12].

Even if the antisense strand is incorporated into RISC with perfect selectivity, the problem remains that siRNAs can function

as miRNAs [89,90] and downregulate many genes through partial complementarity [87,91], especially in the seed region. Furthermore, these off-target effects can cause toxicity [92]. Once again, however, chemical modification can be of significant help. Use of two 2'-O-Me nucleotides at the 5'-end of the antisense strand reduces miRNA-type off-target effects significantly, with little to no effect on siRNA gene silencing [93]. If the seed region is made of DNA instead of RNA miRNA-type off-target effects are also significantly reduced [12].

A third strategy for reducing off-target effects is adopted by Dharmacon RNA Technologies (<http://www.dharmacon.com/>) who have championed the idea of delivering four siRNA duplexes at once, against the same gene. In this way, the desired cleavage level is maintained but the off-target effects are diluted, because they are different for each of the siRNA duplexes. Thus, the overall off-target signature of a pool of siRNAs is usually less than that of any of the individual duplexes that make it up. This strategy is reflective of the natural siRNA pathway in which long dsRNA is diced into a large number of siRNA duplexes, each targeting the same gene but with a different off-target signature. This strategy can be combined with the chemical modification strategies described above.

To reduce or avoid immune-mediated and hybridization-dependent off-target effects, careful sequence selection is an essential first step (see Box 1). Chemical modifications can then provide further protection.

Delivering siRNA

Getting siRNA into cells is one of the biggest challenges of any application of RNAi. In this section, we will examine the most relevant methods used for local and systemic delivery of siRNAs *in vivo*.

Delivery of siRNA into cells in culture has become standard practice. Most commonly, cationic lipids are used as transfection agents but, for challenging cell types, microinjection, electroporation and calcium phosphate precipitation have also been used. All of these techniques have been reviewed elsewhere [94].

Local delivery of siRNA *in vivo* is somewhat more challenging, since most of the methods used in cell culture are impracticable *in vivo*: most of the cationic lipid-based transfection agents used in cell culture are too toxic, electroporation is usually too harsh and microinjection is not useful for most tissues. There are, however, various proven methods for local delivery of siRNA. Two oligonucleotide drugs are currently on the market, the antisense oligonucleotide Vitravene® and the aptamer Macugen®, both of which are chemically modified oligonucleotides injected directly into the eye. Other current sites of local siRNA delivery include intravaginal [95], intranasal/inhaled, intracerebral, intramuscular and intratumoral examples (reviewed in [96,97]). In all of these examples local delivery is attractive because it allows control over which tissues are exposed to the siRNA (reducing possible side effects), and because locally delivered siRNAs are not as vulnerable to serum degradation or to hepatic or renal elimination.

Systemic delivery of siRNA *in vivo* is therefore the greatest challenge. However, it is a challenge that cannot be avoided, since so many targets, including most cancers and viruses, are inaccessible via local delivery [96]. In order for systemically delivered

BOX 1

Resources for getting started with chemically modified siRNA

To select an siRNA sequence, one of the following websites can be used. Many other sequence prediction websites also exist, often associated with companies that sell siRNA. For more detailed background, including the biological basis for current sequence selection tools, see ref. [128].

- BIOPREDSi – <http://www.biopredsi.org> – Developed by the Novartis Institutes for BioMedical Research, this site features a very simple input and output, with only the essential information given (input is a gene Accession number or gene sequence, output is a user-defined number of optimized siRNA sequences). See Ref. [129] for more information.
- Whitehead siRNA Selection Web Server – <http://jura.wi.mit.edu/bioc/siRNAext> – Developed and hosted by the Whitehead Institute, this website is somewhat more complex, giving a large number of possible duplexes along with their thermodynamic properties, etc. An off-target search can be done for each duplex, within the site but in a separate step. See Ref. [130] for more information.

It is always worth testing several duplexes against the same gene, even after using one of these selection websites. In fact, if resources are available, the most thorough way to find highly active siRNA sequences is to test as many as 100–200 sequences against a given mRNA [17,131]. Look for the duplexes with the highest potency and minimum off-target effects. Take the best duplexes, and try applying one or more of the following classic patterns of chemical modification:

- Include two 2'-O-Me units at the 5'-end of the antisense strand, or both strands (reduces immunostimulatory and off-target effects) [82,93] and/or replace sense strand U and G nucleotides (other than those at position 9) with 2'-O-Me units (reduces immunostimulatory effects) [79]
- Include a few LNA units at the 5'-end of the sense strand (improves thermodynamic bias, reduces immunostimulatory and off-target effects) [34,37]
- Replace some or all of the pyrimidines with 2'-F-RNA units (improves endonuclease stability, may reduce immunostimulatory effects) [23]
- Include any of the sugar or phosphate modifications from the toolbox in the 3'-overhangs (improves 3'-exonuclease stability, may reduce immunostimulatory effects).

For more general background on siRNA and RNA interference, as well as relevant protocols, readers may wish to consult one of several useful books [132–135]. We also recommend the following reviews on chemically modified siRNA [136,137] and delivery methods [98,127,138], along with two full-scale reviews of RNAi that cover both topics [5,94].

siRNAs to reach their targets, many requirements must be satisfied: siRNAs must be stable to serum RNases, avoid urine excretion, reach their target tissues in sufficient quantities (which requires passing through blood vessel walls) and be capable of crossing the plasma membrane of target cells [96]. Naked siRNAs stand little chance of overcoming these obstacles; however, significant advances in siRNA delivery technologies have been made in a very short period of time, providing several effective delivery options for potential RNAi drugs.

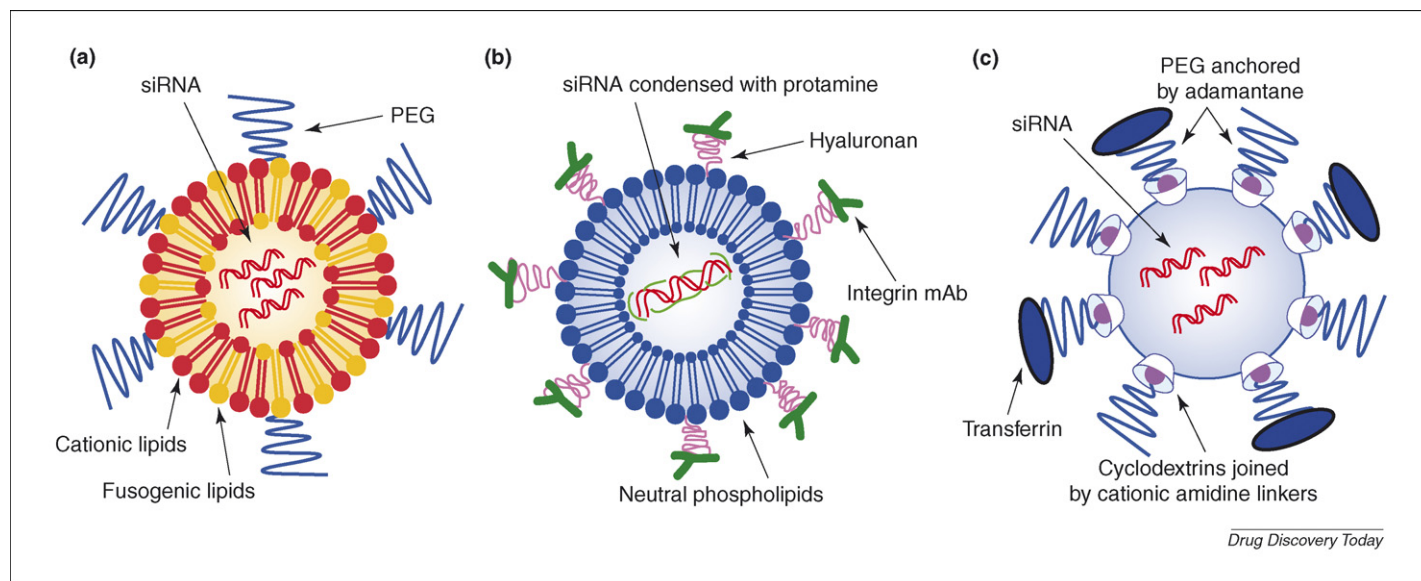
To improve the delivery of siRNA, it can be conjugated to various ligands [98,99]. Conjugation to steroids or lipids [54,100] makes the siRNA more hydrophobic, helps it cross cell membranes and increases its circulation time by allowing it to bind to circulating plasma proteins and lipoproteins. Membrane-penetrating peptide (MPP) conjugates allow siRNA to cross cell membranes very readily, and in one study penetratin or transportan was conjugated to the siRNA duplexes using a disulfide bond that is labile in the reducing environment of the cytoplasm, leading to effective gene silencing [53]. One drawback of cationic MPPs, however, is that they can interact electrostatically with the siRNA duplexes, causing them to precipitate. Furthermore, MPPs have been observed to cause nonspecific knockdown effects and to aggravate the immunogenic effects of siRNA [101].

Systemic application of siRNA must not be synonymous with delivery to all cell types. Various conjugation strategies can be used to ensure that siRNAs are delivered only (or mainly) to the relevant tissue. Because the liver is involved in processing steroids and lipids, cholesterol-conjugated siRNAs are selectively taken up by liver cells [54,100]. One cholesterol-conjugated siRNA has shown systemic activity against the liver target ApoB in mice [10]. In two other very interesting examples, an aptamer-siRNA conjugate was able to enter cells expressing prostate-specific membrane antigen selectively, whether the aptamer and siRNA were part of the same long RNA strand [102] or were conjugated using a modular streptavidin bridge [103].

In spite of the activity of naked and conjugated siRNA, a significant increase in potency is obtained through the formulation of siRNA into delivery vehicles (e.g. see Ref. [104]), and this has been the focus of most recent research. One advantage of encapsulation is that siRNA is protected from nuclease degradation until it is released into the cytoplasm. Many siRNA delivery vehicles are available, including some that have been adapted from technology originally designed for antisense drugs or gene therapy.

Most siRNA delivery vehicles are nanoparticles, made of cationic lipids or macromolecules capable of complexing with negatively charged siRNAs. Lipid-based delivery strategies have been reviewed elsewhere [105]. Combinatorial chemistry has recently been applied to develop new 'lipidoids' effective for systemic RNAi delivery in mice, rats and nonhuman primates [106]. Cationic macromolecules that have shown good results for siRNA administration include chitosan [107], poly(amidoamine) dendrimers [108], polyethyleneimine [109], lysine dendrimers [110], atelocollagen [111,112] and cyclodextrins [113,114]. The latter four systems have shown good results for systemic administration, and the cyclodextrin-based system is particularly well-adapted for targeted delivery (see below).

Liposome or vesicle-based siRNA delivery systems, lipid bilayers that enclose siRNA, are powerful delivery vehicles. One particularly promising example of this technology is the stable nucleic acid lipid particle (SNALP) delivery vehicle (Fig. 6a) developed by Protiva Biotherapeutics (<http://www.protivabio.com/>, recently acquired by Tekmira Pharmaceuticals). SNALPs are vesicles made of a mixture of lipids, including cationic lipids and so-called fusogenic lipids, which allow the SNALP to fuse to the endosomal membrane, releasing its siRNA payload into the cytoplasm [115]. Polyethylene glycol (PEG) groups coat the outside of the particle,

**FIGURE 6**

Schematics of three siRNA delivery vehicles: **(a)** a SNALP [50,104,115]; **(b)** a liposome-based $\beta 7$ integrin-targeted stabilized nanoparticle [120]; and **(c)** a transferrin-targeted cyclodextrin nanoparticle [113,114].

ensuring a long lifetime in serum. This technology has been used in key studies showing knockdown of medically relevant targets in the liver, including HBV replication in the mouse [50] and the lipoprotein ApoB in nonhuman primates [104]. In a comparative study between SNALP and PEI-based delivery, SNALP-encapsulated siRNA was more effective and was able to rescue guinea pigs from a lethal challenge of Ebola virus, even when the siRNA was delivered after exposure to the virus [116].

Targeting ligands can be included in siRNA delivery vehicles without the need for covalent linkage to the siRNA. In one example, fusion proteins were made consisting of an antibody and protamine, a cationic protein. Negatively charged siRNA was associated with the protamine moiety and could be delivered specifically into any of several classes of cells, including hard-to-transfect primary T cells and cancer cells [117]. Various other ligands can be used, including vitamin A [118], folate [119] and carbohydrates (reviewed in Ref. [98]).

Another class of targeted vesicle-type delivery agents consists of protamine-condensed siRNA surrounded by neutral phospholipids (Fig. 6b) [120]. The lipids are covalently linked to hyaluronan, which stabilizes the particle *in vivo*, and serves as a point of attachment for an anti-integrin monoclonal antibody. These particles were used to slow the growth of leukocytes involved in gut inflammation after systemic administration [120].

Cyclodextrin-based delivery systems can be targeted using the ability of cyclodextrins to form inclusion compounds with adamantane. Thus, adamantane-PEG-transferrin conjugates associate with siRNA-containing particles made of cationic cyclodextrins (Fig. 6c), and allow the particles to selectively enter tumor cells, which over-express transferrin receptors [114]. This delivery system is one of the first systems to be approved for clinical trials.

Finally, the concepts of conjugation and formulation can be combined. For example, lactose-PEG-siRNA conjugates can be combined with cationic poly-L-lysine (PLL) in a charge ratio of 1:1. This results in 'polyion complex micelles' containing a dense

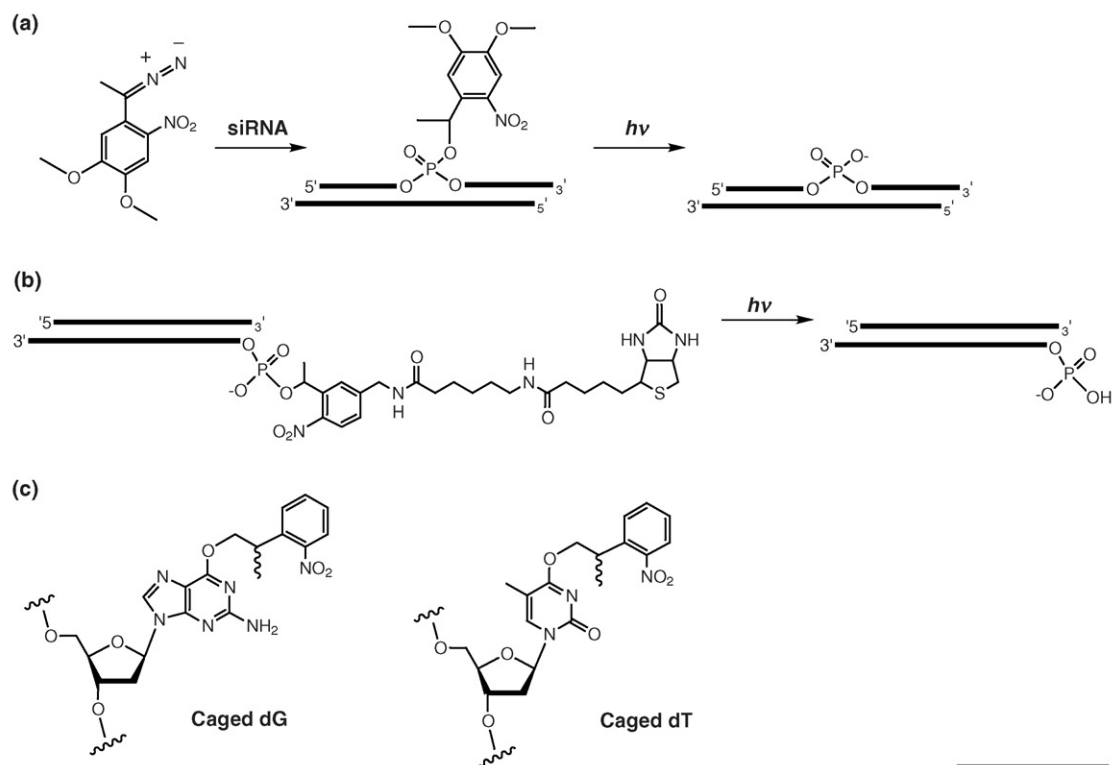
core of siRNA and PLL, surrounded by a flexible and hydrophilic PEG shell that increases biocompatibility and protects the particles from aggregation [121]. The lactose ligand triggers receptor-mediated endocytosis, and a pH-sensitive linker facilitates dissolution of the complex once inside the cell [121]. All of these properties led to very high siRNA potency in cultured human hepatoma cells [121].

Achieving effective delivery of siRNAs *in vivo* is a difficult task. The challenge becomes even greater when targeted delivery to specific cells is required. The effective delivery of siRNA has been a long-time barrier to the therapeutic application of RNAi. However, siRNA delivery technology is improving at an incredible rate, and now several approaches are under development, some of which have the potential to finally bring siRNA to the clinic.

Achieving temporal or spatial control of RNAi induction

Chemical modifications can be used to turn RNAi on or off at a given time or in a specific tissue. For example, siRNA activity can be brought under the control of light by caging (temporarily blocking) random phosphate groups with a photolabile group. A 4,5-dimethoxy-2-nitrophenylethyl (DMNPE) group can be linked to phosphate groups by using its diazo derivative (Fig. 7a) [122]. This reduces the activity of treated siRNAs until the photolabile groups are removed, at some point after transfection, by exposure to light [122].

Instead of photocaging random phosphate groups the antisense 5'-phosphate, which is required for activity, can be selectively caged (Fig. 7b) [123]. Use of a nitrophenylethyl photocaging group at the antisense 5'-phosphate was shown to reduce siRNA activity to 30–40% before irradiation, while allowing at least 80% activity afterward [123]. The 30–40% activity before irradiation was attributed to contaminating uncaged siRNA [123]. In many cases however, it has been shown that simply appending a group to the antisense 5'-phosphate is not sufficient to block siRNA activity completely [57,124].



Drug Discovery Today

FIGURE 7

siRNA photocaging strategies: (a) random phosphate caging [122]; (b) antisense 5'-phosphate caging [123]; and (c) nucleobase caging [125].

Instead of modifying the phosphates, it has been shown that caging of one or two antisense-strand nucleobases at positions around the cleavage site (bases 9–11) can effectively bring siRNA under the control of light (Fig. 7c) [125]. Using this strategy allows synthesis of samples containing very little uncaged siRNA, which helps ensure very low siRNA activity before UV irradiation. After irradiation, the activity was almost indistinguishable from that of unmodified siRNA.

siRNA drugs in clinical trials

Several siRNA drug candidates are now in clinical trials (Table 1), and several more are expected to start soon. At least three of the drugs in clinical trials are unmodified, and most are being delivered locally as naked siRNAs (to the eye, lung and skin). Nevertheless, a recent paper has called into question whether naked siRNAs delivered to the eye are really working via an RNAi mechanism [77]. This includes the siRNA closest to the market, OPKO's

TABLE 1

siRNA drug candidates in clinical trials (not including shRNA drug candidates [126])

Company	Drug name	Phase	Target gene and disease	Chemically modified?	Delivery
OPKO (formerly Acuity)	Bevasiranib (formerly Cand5)	III	All VEGF-A isoforms for wet AMD	No	Direct, intraocular
Sirna/Merck with Allergan	AGN-745 (formerly Sirna-027)	II	VEGF receptor (VEGF-R1) for wet AMD	Yes	Direct, intraocular
Alnylam	ALN-RSV01	II	RSV (viral nucleocapsid)	n.d. ^a	Direct, intranasal or inhaled
Quark/Silence with Pfizer	RTP801i-14	I/II	RTP801 for wet AMD	Yes	Direct, intraocular
Quark/Silence	AKI-i5	I	p53 for acute renal failure	Yes	Systemic, IV injection
TransDerm	TD101	Ib	Keratin 6a (N171K mutation) for Pachyonychia Congenita	No	Direct, intradermal
Calando	CALAA-01	Ia	Ribonucleotide reductase (RRM2) for cancer	No	Systemic, transferrin-targeted CD nanoparticles

The information in this table was obtained primarily from press releases on the individual company websites; see also Ref. [127].

^a Not disclosed.

Phase III candidate, bevasiranib. However, the same paper showed that cholesterol conjugation did allow siRNAs to enter cells and produce a genuine RNAi effect [77].

It is perhaps not surprising that most of the first siRNA drugs in the clinic are delivered locally, since the industry needs to start with the easiest targets. As for the importance of chemical modification, it will be interesting to see whether the local and systemic applications of unmodified siRNA currently in trials can overcome the challenges discussed in this review. One of the systemically delivered siRNAs in the clinic is formulated, and the other is chemically modified. These RNAi companies are offering us all a great service by testing such a wide variety of modifications, routes of delivery and tissues in clinical trials, the results of which will play a key part in defining the future possibilities for siRNA therapeutics.

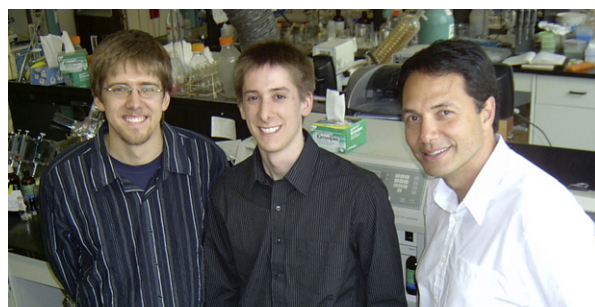
Conclusions and future perspectives

We believe that appropriate chemical modifications and delivery technologies will be essential for bringing RNAi to the clinic. Although investigation was initially focused on increasing serum stability, the need for chemical modification is much broader. The areas of specificity and delivery are among the greatest challenges to RNAi therapeutics, and chemistry will certainly be a big part of the solution to both problems.

Several frontiers remain in the field of chemically modified siRNAs. Most of the currently used strategies have significant sequence dependence; thus the remaining challenge is to find universal chemical modification strategies, or to predict reliably which modifications will be effective for a given sequence.

Many of the best-modified siRNAs consist of a combination of multiple chemical modifications [31,50,67,71]. Yet most of the research being done on new chemically modified siRNA involves only one type of modification. Therefore, there is an ongoing need to combine chemistries in creative ways, if the field is to move toward universal chemical modification architectures. These modified duplexes will in turn benefit from improved delivery vehicle designs.

Many obstacles remain on the road to therapeutic siRNA, but many of them are already being removed. Creative and wise use of chemistry will continue to make a key contribution to progress in almost all of these areas.



In their lab, from left to right: JKW, GFD, MJD.

Acknowledgements

We thank Prof. David R. Corey, Prof. Jean-Christophe Leroux and Dr. Muthiah Manoharan for their valuable comments during the preparation of this manuscript.

References

- 1 Fire, A. *et al.* (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391, 806–811
- 2 Elbashir, S.M. *et al.* (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 411, 494–498
- 3 Rana, T.M. (2007) Illuminating the silence: understanding the structure and function of small RNAs. *Nat. Rev. Mol. Cell Biol.* 8, 23–36
- 4 Tomari, Y. and Zamore, P.D. (2005) Perspective: machines for RNAi. *Genes Dev.* 19, 517–529
- 5 Grünweller, A. and Hartmann, R.K. (2005) RNA interference as a gene-specific approach for molecular medicine. *Curr. Med. Chem.* 12, 3143–3161
- 6 Schwarz, D.S. *et al.* (2003) Asymmetry in the assembly of the RNAi enzyme complex. *Cell* 115, 199–208
- 7 Khvorov, A. *et al.* (2003) Functional siRNAs and miRNAs exhibit strand bias. *Cell* 115, 209–216
- 8 Hamada, M. *et al.* (2002) Effects on RNA interference in gene expression (RNAi) in cultured mammalian cells of mismatches and the introduction of chemical modifications at the 3'-ends of siRNAs. *Antisense Nucl. Acid Drug Dev.* 12, 301–309
- 9 Parrish, S. *et al.* (2000) Functional anatomy of a dsRNA trigger: differential requirement for the two trigger strands in RNA interference. *Mol. Cell* 6, 1077–1087
- 10 Soutschek, J. *et al.* (2004) Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. *Nature* 432, 173–178
- 11 Kraynack, B.A. and Baker, B.F. (2006) Small interfering RNAs containing full 2'-O-methylribonucleotide-modified sense strands display Argonaute2/eIF2C2-dependent activity. *RNA* 12, 163–176
- 12 Ui-Tei, K. *et al.* (2008) Functional dissection of siRNA sequence by systematic DNA substitution: modified siRNA with a DNA seed arm is a powerful tool for mammalian gene silencing with significantly reduced off-target effect. *Nucl. Acids Res.* 36, 2136–2151
- 13 Chiu, Y.L. and Rana, T.M. (2003) siRNA function in RNAi: a chemical modification analysis. *RNA* 9, 1034–1048
- 14 Braasch, D.A. *et al.* (2003) RNA interference in mammalian cells by chemically modified RNA. *Biochemistry* 42, 7967–7975
- 15 Elbashir, S.M. *et al.* (2001) Functional anatomy of siRNAs for mediating efficient RNAi in *Drosophila melanogaster* embryo lysate. *EMBO J* 20, 6877–6888
- 16 Czauderna, F. *et al.* (2003) Structural variations and stabilising modifications of synthetic siRNAs in mammalian cells. *Nucl. Acids Res.* 31, 2705–2716
- 17 Choung, S. *et al.* (2006) Chemical modification of siRNAs to improve serum stability without loss of efficacy. *Biochem. Biophys. Res. Commun.* 342, 919–927
- 18 Davis, S. *et al.* (2006) Improved targeting of miRNA with antisense oligonucleotides. *Nucl. Acids Res.* 34, 2294–2304
- 19 Dorn, G. *et al.* (2004) siRNA relieves chronic neuropathic pain. *Nucl. Acids Res.* 32, e49
- 20 Prakash, T.P. *et al.* (2005) Positional effect of chemical modifications on short interference RNA activity in mammalian cells. *J. Med. Chem.* 48, 4247–4253
- 21 Amarzguoui, M. *et al.* (2003) Tolerance for mutations and chemical modifications in a siRNA. *Nucl. Acids Res.* 31, 589–595
- 22 Chen, X.L. *et al.* (2004) Poly-2'-DNP-RNAs with enhanced efficacy for inhibiting cancer cell growth. *Oligonucleotides* 14, 90–99
- 23 Harborth, J. *et al.* (2003) Sequence, chemical, and structural variation of small interfering RNAs and short hairpin RNAs and the effect on mammalian gene silencing. *Antisense Nucl. Acid Drug Dev.* 13, 83–105
- 24 Blidner, R.A. *et al.* (2007) Fully 2'-deoxy-2'-fluoro substituted nucleic acids induce RNA interference in mammalian cell culture. *Chem. Biol. Drug Des.* 70, 113–122
- 25 Layzer, J.M. *et al.* (2004) In vivo activity of nuclease-resistant siRNAs. *RNA* 10, 766–771
- 26 Damha, M.J. *et al.* (1998) Hybrids of RNA and arabinonucleic acids (ANA and 2'-F-ANA) are substrates of Ribonuclease H. *J. Am. Chem. Soc.* 120, 12976–12977
- 27 Wilds, C.J. and Damha, M.J. (2000) 2'-Deoxy-2'-fluoro-β-D-arabinonucleosides and oligonucleotides (2'-F-ANA): synthesis and physicochemical studies. *Nucl. Acids Res.* 28, 3625–3635

- 28 Dowler, T. *et al.* (2006) Improvements in siRNA properties mediated by 2'-deoxy-2'-fluoro- β -D-arabinonucleic acid (FANA). *Nucl. Acids Res.* 34, 1669–1675
- 29 Watts, J.K. *et al.* (2007) 2'-Fluoro-4'-thioarabino-modified oligonucleotides: conformational switches linked to siRNA activity. *Nucl. Acids Res.* 35, 1441–1451
- 30 Hoshika, S. *et al.* (2005) RNA interference induced by siRNAs modified with 4'-thioribonucleosides in cultured mammalian cells. *FEBS Lett.* 579, 3115–3118
- 31 Dande, P. *et al.* (2006) Improving RNA interference in mammalian cells by 4'-thio-modified small interfering RNA (siRNA): effect on siRNA activity and nuclease stability when used in combination with 2'-O-alkyl modifications. *J. Med. Chem.* 49, 1624–1634
- 32 Hoshika, S. *et al.* (2007) Study of modification pattern-RNAi activity relationships by using siRNAs modified with 4'-thioribonucleosides. *ChemBioChem* 8, 2133–2138
- 33 Petersen, M. and Wengel, J. (2003) LNA: a versatile tool for therapeutics and genomics. *Trends Biotechnol.* 21, 74–81
- 34 Elmen, J. *et al.* (2005) Locked nucleic acid (LNA) mediated improvements in siRNA stability and functionality. *Nucl. Acids Res.* 33, 439–447
- 35 Bramsen, J.B. *et al.* (2007) Improved silencing properties using small internally segmented interfering RNAs. *Nucl. Acids Res.* 35, 5886–5897
- 36 Mook, O.R. *et al.* (2007) Evaluation of locked nucleic acid-modified small interfering RNA in vitro and in vivo. *Mol. Cancer Therap.* 6, 833–843
- 37 Hornung, V. *et al.* (2005) Sequence-specific potent induction of IFN- α by short interfering RNA in plasmacytoid dendritic cells through TLR7. *Nat. Med.* 11, 263–270
- 38 Hall, A.H.S. *et al.* (2004) RNA interference using boranophosphate siRNAs: structure–activity relationships. *Nucl. Acids Res.* 32, 5991–6000
- 39 Schwarz, D.S. *et al.* (2004) The RNA-induced silencing complex is a Mg²⁺-dependent endonuclease. *Curr. Biol.* 14, 787–791
- 40 Braasch, D.A. *et al.* (2004) Biodistribution of phosphodiester and phosphorothioate siRNA. *Bioorg. Med. Chem. Lett.* 14, 1139–1143
- 41 Prakash, T.P. *et al.* (2006) RNA interference by 2',5'-linked nucleic acid duplexes in mammalian cells. *Bioorg. Med. Chem. Lett.* 16, 3238–3240
- 42 Iwase, R. *et al.* (2007) Solid-phase synthesis of modified RNAs containing amide-linked oligoribonucleosides at their 3'-end and their application to siRNA. *Nucleosides Nucleotides Nucl. Acids* 26, 1451–1454
- 43 Sipa, K. *et al.* (2007) Effect of base modifications on structure, thermodynamic stability, and gene silencing activity of short interfering RNA. *RNA* 13, 1301–1316
- 44 Hornung, V. *et al.* (2006) 5'-Triphosphate RNA is the ligand for RIG-I. *Science* 314, 994–997
- 45 Xia, J. *et al.* (2006) Gene silencing activity of siRNAs with a ribo-difluorotoluy nucleotide. *ACS Chem. Biol.* 1, 176–183
- 46 Holen, T. *et al.* (2002) Positional effects of short interfering RNAs targeting the human coagulation trigger tissue factor. *Nucl. Acids Res.* 30, 1757–1766
- 47 Marques, J.T. *et al.* (2006) A structural basis for discriminating between self and nonself double-stranded RNAs in mammalian cells. *Nat. Biotechnol.* 24, 559–565
- 48 Nykanen, A. *et al.* (2001) ATP requirements and small interfering RNA structure in the RNA interference pathway. *Cell* 107, 309–321
- 49 Chen, P.Y. *et al.* (2008) Strand-specific 5'-O-methylation of siRNA duplexes controls guide strand selection and targeting specificity. *RNA* 14, 263–274
- 50 Morrissey, D.V. *et al.* (2005) Potent and persistent in vivo anti-HBV activity of chemically modified siRNAs. *Nat. Biotechnol.* 23, 1002–1007
- 51 Morrissey, D.V. *et al.* (2005) Activity of stabilized short interfering RNA in a mouse model of hepatitis B virus replication. *Hepatology* 41, 1349–1356
- 52 Chiu, Y.-L. and Rana, T.M. (2002) RNAi in human cells: basic structural and functional features of small interfering RNA. *Mol. Cell* 10, 549–561
- 53 Muratovska, A. and Eccles, M.R. (2004) Conjugate for efficient delivery of short interfering RNA (siRNA) into mammalian cells. *FEBS Lett.* 558, 63–68
- 54 Lorenz, C. *et al.* (2004) Steroid and lipid conjugates of siRNAs to enhance cellular uptake and gene silencing in liver cells. *Bioorg. Med. Chem. Lett.* 14, 4975–4977
- 55 Bolcato-Bellemin, A.-L. *et al.* (2007) Sticky overhangs enhance siRNA-mediated gene silencing. *Proc. Natl. Acad. Sci. U. S. A.* 104, 16050–16055
- 56 Martinez, J. *et al.* (2002) Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. *Cell* 110, 563–574
- 57 Schwarz, D.S. *et al.* (2002) Evidence that siRNAs function as guides, not primers, in the Drosophila and human RNAi pathways. *Mol. Cell* 10, 537–548
- 58 Siolas, D. *et al.* (2005) Synthetic shRNAs as potent RNAi triggers. *Nat. Biotechnol.* 23, 227–231
- 59 Brummelkamp, T.R. *et al.* (2002) A system for stable expression of short interfering RNAs in mammalian cells. *Science* 296, 550–553
- 60 Banan, N. and Puri, M. (2004) The ins and outs of RNAi in mammalian cells. *Curr. Pharm. Biotechnol.* 5, 441–450
- 61 Abe, N. *et al.* (2007) Dumbbell-shaped nanocircular RNAs for RNA interference. *J. Am. Chem. Soc.* 129, 15108–15109
- 62 Holen, T. *et al.* (2003) Similar behaviour of single-strand and double-strand siRNAs suggests they act through a common RNAi pathway. *Nucl. Acids Res.* 31, 2401–2407
- 63 Kim, D.-H. *et al.* (2005) Synthetic dsRNA dicer substrates enhance RNAi potency and efficacy. *Nat. Biotechnol.* 23, 222–226
- 64 Minks, M.A. *et al.* (1979) Structural requirements of double-stranded RNA for the activation of 2',5'-Oligo(A) polymerase and protein kinase of interferon-treated HeLa-cells. *J. Biol. Chem.* 254, 180–183
- 65 Kennedy, S. *et al.* (2004) A conserved siRNA-degrading RNase negatively regulates RNA interference in *C. elegans*. *Nature* 427, 645–649
- 66 Takabatake, Y. *et al.* (2007) Chemically modified siRNA prolonged RNA interference in renal disease. *Biochem. Biophys. Res. Commun.* 363, 432–437
- 67 Allerson, C.R. *et al.* (2005) Fully 2'-modified oligonucleotide duplexes with improved in vitro potency and stability compared to unmodified small interfering RNA. *J. Med. Chem.* 48, 901–904
- 68 Turner, J.J. *et al.* (2007) MALDI-TOF mass spectral analysis of siRNA degradation in serum confirms an RNase A-like activity. *Mol. Biosyst.* 3, 43–50
- 69 de Fougerolles, A. *et al.* (2007) Interfering with disease: a progress report on siRNA-based therapeutics. *Nat. Rev. Drug Discov.* 6, 443–453
- 70 Manoharan, M. (2006) RNA interference and chemical modifications: conformational and stereochemical aspects. *Abstracts of Papers, 231st ACS National Meeting*, Atlanta, USA, March 26–30
- 71 Koller, E. *et al.* (2006) Competition for RISC binding predicts in vitro potency of siRNA. *Nucl. Acids Res.* 34, 4467–4476
- 72 Schlee, M. *et al.* (2006) siRNA and isRNA: two edges of one sword. *Mol. Ther.* 14, 463–470
- 73 Marques, J.T. and Williams, B.R.G. (2005) Activation of the mammalian immune system by siRNAs. *Nat. Biotechnol.* 23, 1399–1405
- 74 Judge, A.D. *et al.* (2005) Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA. *Nat. Biotechnol.* 23, 457–462
- 75 Kim, D.-H. *et al.* (2004) Interferon induction by siRNAs and ssRNAs synthesized by phage polymerase. *Nat. Biotechnol.* 22, 321–325
- 76 Reynolds, A. *et al.* (2006) Induction of the interferon response by siRNA is cell type- and duplex length-dependent. *RNA* 12, 988–993
- 77 Kleinman, M.E. *et al.* (2008) Sequence- and target-independent angiogenesis suppression by siRNA via TLR3. *Nature* 452, 591–597
- 78 Takeda, K. and Akira, S. (2005) Toll-like receptors in innate immunity. *Int. Immunol.* 17, 1–14
- 79 Judge, A. and MacLachlan, I. (2008) Overcoming the innate immune response to small interfering RNA. *Hum. Gene Ther.* 19, 111–124
- 80 Zamanian-Daryoush, M. *et al.* (2008) Determinants of cytokine induction by small interfering RNA in human peripheral blood mononuclear cells. *J. Interferon Cytokine Res.* 28, 221–233
- 81 Diebold, S.S. *et al.* (2006) Nucleic acid agonists for Toll-like receptor 7 are defined by the presence of uridine ribonucleotides. *Eur. J. Immunol.* 36, 3256–3267
- 82 Judge, A.D. *et al.* (2006) Design of noninflammatory synthetic siRNA mediating potent gene silencing in vivo. *Mol. Ther.* 13, 494–505
- 83 Cekaite, L. *et al.* (2007) Gene expression analysis in blood cells in response to unmodified and 2'-modified siRNAs reveals TLR-dependent and independent effects. *J. Mol. Biol.* 365, 90–108
- 84 Robbins, M. *et al.* (2007) 2'-O-Methyl-modified RNAs act as TLR7 antagonists. *Mol. Ther.* 15, 1663–1669
- 85 Karikó, K. *et al.* (2005) Suppression of RNA recognition by Toll-like receptors: the impact of nucleoside modification and the evolutionary origin of RNA. *Immunity* 23, 165–175
- 86 Jackson, A.L. *et al.* (2003) Expression profiling reveals off-target gene regulation by RNAi. *Nat. Biotechnol.* 21, 635–637
- 87 Jackson, A.L. *et al.* (2006) Widespread siRNA “off-target” transcript silencing mediated by seed region sequence complementarity. *RNA* 12, 1179–1187
- 88 Birmingham, A. *et al.* (2006) 3' UTR seed matches, but not overall identity, are associated with RNAi off-targets. *Nat. Methods* 3, 199–204
- 89 Zeng, Y. *et al.* (2003) MicroRNAs and small interfering RNAs can inhibit mRNA expression by similar mechanisms. *Proc. Natl. Acad. Sci. U. S. A.* 100, 9779–9784
- 90 Doench, J.G. *et al.* (2003) siRNAs can function as miRNAs. *Genes Dev.* 17, 438–442
- 91 Lim, L.P. *et al.* (2005) Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature* 433, 769–773
- 92 Fedorov, Y. *et al.* (2006) Off-target effects by siRNA can induce toxic phenotype. *RNA* 12, 1188–1196
- 93 Jackson, A.L. *et al.* (2006) Position-specific chemical modification of siRNAs reduces “off-target” transcript silencing. *RNA* 12, 1197–1205
- 94 Gilmore, I.R. *et al.* (2004) The design and exogenous delivery of siRNA for post-transcriptional gene silencing. *J. Drug Target.* 12, 315–340
- 95 Palliser, D. *et al.* (2006) An siRNA-based microbicide protects mice from lethal herpes simplex virus 2 infection. *Nature* 439, 89–94

- 96 Xie, F.Y. *et al.* (2006) Harnessing in vivo siRNA delivery for drug discovery and therapeutic development. *Drug Discov. Today* 11, 67–73
- 97 Aigner, A. (2007) Applications of RNA interference: current state and prospects for siRNA-based strategies in vivo. *Appl. Microbiol. Biotechnol.* 76, 9–21
- 98 De Paula, D. *et al.* (2007) Hydrophobization and bioconjugation for enhanced siRNA delivery and targeting. *RNA* 13, 431–456
- 99 Ikeda, Y. and Taira, K. (2006) Ligand-targeted delivery of therapeutic siRNA. *Pharm. Res.* 23, 1631–1640
- 100 Wolfrum, C. *et al.* (2007) Mechanisms and optimization of in vivo delivery of lipophilic siRNAs. *Nat. Biotechnol.* 25, 1149–1157
- 101 Moschos, S.A. *et al.* (2007) Lung delivery studies using siRNA conjugated to TAT(48–60) and penetratin reveal peptide induced reduction in gene expression and induction of innate immunity. *Bioconjugate Chem.* 18, 1450–1459
- 102 McNamara, J.O. *et al.* (2006) Cell type-specific delivery of siRNAs with aptamer-siRNA chimeras. *Nat. Biotechnol.* 24, 1005–1015
- 103 Chu, T.C. *et al.* (2006) Aptamer mediated siRNA delivery. *Nucl. Acids Res.* 34, e73
- 104 Zimmermann, T.S. *et al.* (2006) RNAi-mediated gene silencing in non-human primates. *Nature* 441, 111–114
- 105 Li, W. and Szoka, F.C., Jr (2007) Lipid-based nanoparticles for nucleic acid delivery. *Pharm. Res.* 24, 438–449
- 106 Akinc, A. *et al.* (2008) A combinatorial library of lipid-like materials for delivery of RNAi therapeutics. *Nat. Biotechnol.* 26, 561–569
- 107 Howard, K.A. *et al.* (2006) RNA interference in vitro and in vivo using a chitosan/siRNA nanoparticle system. *Mol. Ther.* 14, 476–484
- 108 Zhou, J. *et al.* (2006) PAMAM dendrimers for efficient siRNA delivery and potent gene silencing. *Chem. Commun.* 2006, 2362–2364
- 109 Urban-Klein, B. *et al.* (2005) RNAi-mediated gene-targeting through systemic application of polyethylenimine (PEI)-complexed siRNA in vivo. *Gene Ther.* 12, 461–466
- 110 Baigude, H. *et al.* (2007) Design and creation of new nanomaterials for therapeutic RNAi. *ACS Chem. Biol.* 2, 237–241
- 111 Minakuchi, Y. *et al.* (2004) Atelocollagen-mediated synthetic small interfering RNA delivery for effective gene silencing in vitro and in vivo. *Nucl. Acids Res.* 32, e109
- 112 Takeshita, F. *et al.* (2005) Efficient delivery of small interfering RNA to bone-metastatic tumors by using atelocollagen in vivo. *Proc. Natl. Acad. Sci. U. S. A.* 102, 12177–12182
- 113 Heidel, J.D. *et al.* (2007) Administration in non-human primates of escalating intravenous doses of targeted nanoparticles containing ribonucleotide reductase subunit M2 siRNA. *Proc. Natl. Acad. Sci. U. S. A.* 104, 5715–5721
- 114 Hu-Lieskova, S. *et al.* (2005) Sequence-specific knockdown of EWS-FLI1 by targeted, nonviral delivery of small interfering RNA inhibits tumor growth in a murine model of metastatic Ewing's Sarcoma. *Cancer Res.* 65, 8984–8992
- 115 Heyes, J. *et al.* (2005) Cationic lipid saturation influences intracellular delivery of encapsulated nucleic acids. *J. Control. Rel.* 107, 276–287
- 116 Geisbert, T.W. *et al.* (2006) Postexposure protection of guinea pigs against a lethal Ebola virus challenge is conferred by RNA interference. *J. Infect. Dis.* 193, 1650–1657
- 117 Song, E. *et al.* (2005) Antibody mediated in vivo delivery of small interfering RNAs via cell-surface receptors. *Nat. Biotechnol.* 23, 709–717
- 118 Sato, Y. *et al.* (2008) Resolution of liver cirrhosis using vitamin A-coupled liposomes to deliver siRNA against a collagen-specific chaperone. *Nat. Biotechnol.* 26, 431–442
- 119 Zhang, K. *et al.* (2008) Receptor-mediated delivery of siRNAs by tethered nucleic acid base-paired interactions. *RNA* 14, 577–583
- 120 Peer, D. *et al.* (2008) Systemic leukocyte-directed siRNA delivery revealing cyclin D1 as an anti-inflammatory target. *Science* 319, 627–630
- 121 Oishi, M. *et al.* (2005) Lactosylated poly(ethylene glycol)-siRNA conjugate through acid-labile β -thiopropionate linkage to construct pH-sensitive polyion complex micelles achieving enhanced gene silencing in hepatoma cells. *J. Am. Chem. Soc.* 127, 1624–1625
- 122 Shah, S. *et al.* (2005) Light-activated RNA interference. *Angew. Chem. Int. Ed.* 44, 1328–1332
- 123 Nguyen, Q.N. *et al.* (2006) Light controllable siRNAs regulate gene suppression and phenotypes in cells. *BBA Biomembranes* 1758, 394–403
- 124 Shah, S. and Friedman, S.H. (2007) Tolerance of RNA interference toward modifications of the 5' antisense phosphate of small interfering RNA. *Oligonucleotides* 17, 35–43
- 125 Mikat, V. and Heckel, A. (2007) Light-dependent RNA interference with nucleobase-caged siRNAs. *RNA* 13, 2341–2347
- 126 Haussecker, D. (2008) The business of RNAi therapeutics. *Hum. Gene Ther.* 19, 451–462
- 127 de Fougères, A.R. (2008) Delivery vehicles for small interfering RNA in vivo. *Hum. Gene Ther.* 19, 125–132
- 128 Boese, Q. *et al.* (2005) Mechanistic insights aid computational short interfering RNA design. *Methods Enzymol.* 392, 73–96
- 129 Huesken, D. *et al.* (2005) Design of a genome-wide siRNA library using an artificial neural network. *Nat. Biotechnol.* 23, 995–1001
- 130 Yuan, B. *et al.* (2004) siRNA selection server: an automated siRNA oligonucleotide prediction server. *Nucl. Acids Res.* 32, W130–W134
- 131 Manoharan, M. and Rajeev, K.G. (2007) Utilizing chemistry to harness RNA interference pathways for therapeutics: chemically modified siRNAs and antagomirs. In *Antisense Drug Technology: Principles, Strategies, and Applications* (2nd edn) (Crooke, S.T., ed.), pp. 437–464, CRC Press
- 132 Appasani, K., ed. (2005) *RNA Interference Technology: From Basic Science to Drug Development*, Cambridge University Press
- 133 Schepers, U. (2005) *RNA Interference in Practice: Principles, Basics and Methods for Gene Silencing in C. elegans, Drosophila, and Mammals*. Wiley-VCH
- 134 Sohail, M., ed. (2005) *Gene Silencing by RNA Interference: Technology and Application*, CRC Press
- 135 Hannon, G.J., ed. (2003) *RNAi: A Guide to Gene Silencing*, Cold Spring Harbor Press
- 136 Manoharan, M. (2004) RNA interference and chemically modified small interfering RNAs. *Curr. Opin. Chem. Biol.* 8, 570–579
- 137 Corey, D.R. (2007) Chemical modification: the key to clinical application of RNA interference? *J. Clin. Invest.* 117, 3615–3622
- 138 Akhtar, S. and Benter, I.F. (2007) Nonviral delivery of synthetic siRNAs in vivo. *J. Clin. Invest.* 117, 3623–3632