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## ABSTRACT

The ability to manipulate the RNA interference (RNAi) machinery to specifically silence the expression of target genes could be a powerful therapeutic strategy. Since the discovery that RNAi can be triggered in mammalian cells by short double-stranded RNAs (small interfering RNA, siRNA), there has been a tremendous push by researchers, from academia to big pharma, to move siRNAs into clinical application. The challenges facing siRNA therapeutics are significant. The inherent properties of siRNAs (polyanionic, vulnerable to nuclease cleavage) make clinical application difficult due to poor cellular uptake and rapid clearance. Side effects of siRNAs have also proven to be a further complication. Fortunately, numerous chemical modification strategies have been identified that allow many of these obstacles to be overcome. This unit will present an overview of (1) the chemical modifications available to the nucleic acid chemist for modifying siRNAs, (2) the application of chemical modifications to address specific therapeutic obstacles, and (3) the factors that must be considered when assessing the activity of modified siRNAs. *Curr. Protoc. Nucleic Acid Chem.* 39:16.3.1-16.3.22. © 2009 by John Wiley & Sons, Inc.

Keywords: chemical modification • siRNA • oligonucleotide • immunostimulatory effects • off-target effects

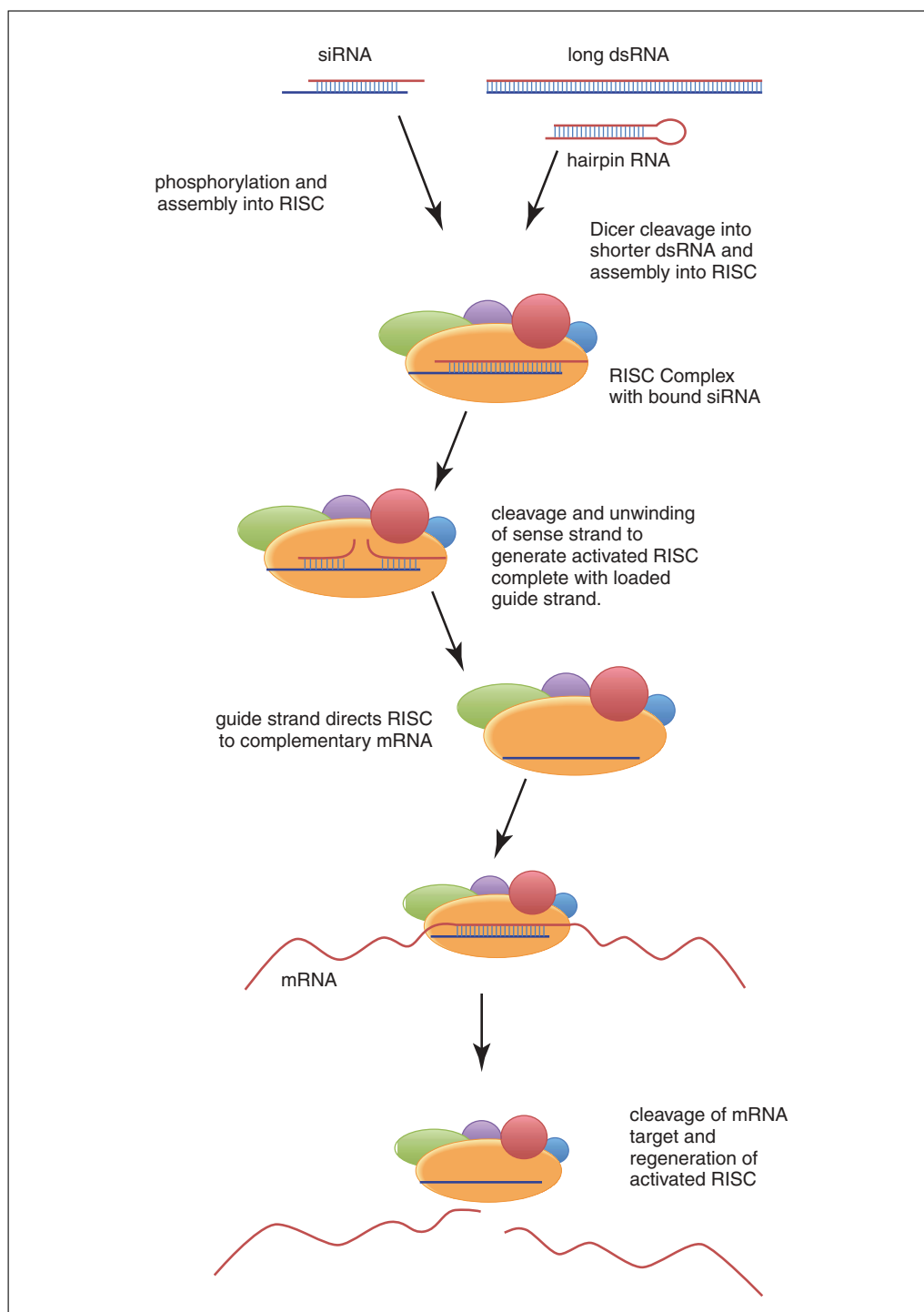
## INTRODUCTION

The first report that long double-stranded RNA could elicit specific gene silencing was published in 1998 (Fire et al., 1998). This discovery prompted a tremendous research effort to understand and manipulate the gene-silencing pathway now referred to as RNA interference (RNAi). Three years later a synthetic trigger of the RNAi pathway in mammalian cells was identified (Elbashir et al., 2001a). These short RNAi triggers are 21-nt double-stranded RNAs aligned such that each strand has a 2-nt 3'-overhang, and are referred to as siRNAs. In recent years, siRNAs have generated tremendous interest in the therapeutic field, and rightly so. siRNAs can be used to silence the expression of virtually any gene. The potential applications of siRNAs in drug development are immediately obvious: siRNAs can be used in cell culture to inhibit specific genes as a means of target validation, and/or siRNAs can themselves be drugs used to silence therapeutic targets.

The transition of siRNAs from the laboratory to the clinic has met with some significant obstacles. The polyanionic phosphodiester backbone of siRNA hinders cellular uptake, and numerous cellular enzymes are capable of rapidly degrading RNA strands, making for poor pharmacokinetics. Further-

more, it is becoming increasingly clear that siRNAs can elicit undesirable side effects. siRNAs can act like microRNAs (miRNAs) and affect the expression of unintended genes through partial complementarity. More recently, it has been discovered that siRNAs can be potent stimulants of the innate immune system (Robbins et al., 2009). Mammalian cells have receptors that recognize dsRNA (often a hallmark of viral infection), which can be triggered by siRNAs to produce inflammatory cytokines. Recent findings suggest that these immune responses to siRNAs may confuse gene-silencing outcomes, and may produce unwanted and potentially dangerous side effects. All of these side effects are collectively termed “off-target” effects.

Fortunately, a variety of chemical modifications have been proposed to address these issues. This overview describes a wide range of chemical modifications that have been applied to siRNAs, and provides examples of how these modifications can improve potency and serum stability, and reduce off-target and immunostimulatory side effects. The goal of this review is to provide the nucleic acid researcher with an overview of the state of the art, and a comprehensive guide for the design of chemically modified siRNAs.



**Figure 16.3.1** The mechanism of RNAi in human cells triggered by exogenously introduced dsRNAs. The largest of the ellipses represents AGO2, the catalytic engine of RISC. Adapted from Watts et al. (2008), with permission from Elsevier.

A detailed understanding of the mechanism of RNAi and the enzymes involved is invaluable when designing chemically modified siRNAs, an overview of which can be found in *UNIT 16.1*. For the purposes of this overview, a brief description of the RNAi process is included in Figure 16.3.1. Briefly, when an exogenous 19- to 21-bp siRNA is

introduced into a mammalian cell, the 5'-end is phosphorylated by Clp-1, a cellular kinase (Weitzer and Martinez, 2007). The siRNA is then loaded into the RNA-induced silencing complex (RISC). This multiprotein complex includes Argonaute2 (AGO2), Dicer, TRBP (HIV-1 TAR RNA-binding protein), and PACT (a dsRNA binding protein), as

well as other proteins, some of which remain unknown (Rana, 2007). The structural and mechanistic aspects of both Dicer and Argonaute2 have been recently reviewed (Jinek and Doudna, 2009).

siRNAs are double-stranded molecules, and the two strands play very different roles in RNAi. One strand, referred to as the guide or antisense strand, is loaded into the RISC complex and will function to direct RISC to complementary mRNAs for post-transcriptional gene silencing (PTGS). The other strand, referred to as the passenger or sense strand, is simply cleaved and/or unwound from the guide strand upon RISC loading. It appears that RISC differentiates between the guide and passenger strands based on the thermodynamics of the siRNA duplex. It has been reported that the strand with lower binding affinity at its 5'-end preferentially becomes the guide strand (Khvorova et al., 2003; Schwarz et al., 2003). The other strand is designated as the passenger strand. It is cleaved and unwound, generating a single RNA guide strand associated with AGO2, which is an endonuclease at the heart of RISC and promotes hybridization and cleavage of mRNAs complementary to the guide strand (Jinek and Doudna, 2009). When modifying an siRNA duplex, it is important to recognize that different modification approaches are required for the sense and antisense strands, because of their very different roles (Parrish et al., 2000; Hamada et al., 2002).

It is typically assumed that the mechanism of siRNA-mediated gene silencing is unchanged in chemically modified siRNAs. 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, which is the same result as for unmodified duplexes (Soutschek et al., 2004; Kraynack and Baker, 2006; Ui-Tei et al., 2008; Judge et al., 2009). In principle, this should be verified for each new pattern of modification.

## METHODS FOR DESIGNING MODIFIED siRNA

### Choosing the Right Sequence

Many factors must be considered in the design of an effective and specific siRNA. Firstly, the chosen siRNA sequence must be unique to the intended mRNA target. Even incomplete complementarity to unintended mRNAs can result in significant unwanted off-target effects (Jackson et al., 2006a). For exam-

ple, the silencing ability of siRNAs doubly mismatched to mRNA targets has been thoroughly analyzed. Results indicate that in some cases doubly mismatched siRNAs could still effect >50% gene silencing (Dahlgren et al., 2008). Because siRNAs can sometimes enter microRNA-type pathways, which silence gene expression based on complementarity to the miRNA "seed region" (the 6-7 nucleotide region at the 5' end of the guide strand), siRNAs should be designed to reduce seed-region complementarity to other genes as much as possible.

Additional considerations include the availability of the targeted region of the mRNA for RISC binding. Secondary structure can interfere with RISC-mediated mRNA cleavage, and so prediction of mRNA secondary structure can be useful (Brown et al., 2005; Gredell et al., 2008; Rudnick et al., 2008). Experiments can also be used to identify accessible mRNA regions (Sokol et al., 1998; Lee et al., 2002).

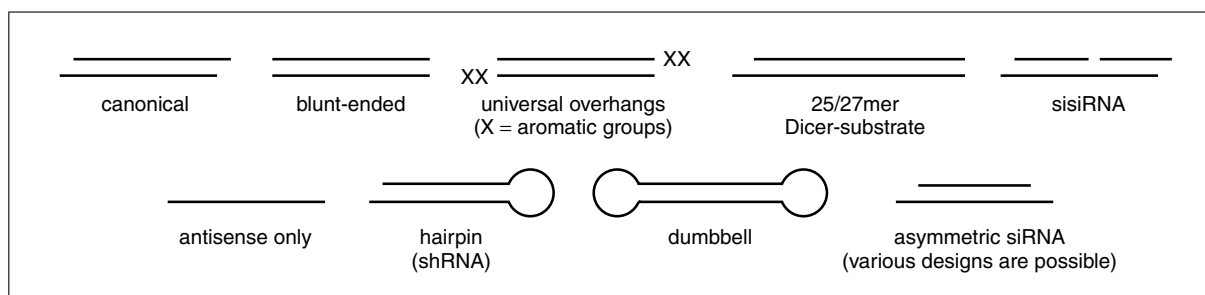
These factors can complicate siRNA design. Fortunately, computer programs have been developed that take these and other factors into account. Many of them are extremely user friendly, and can be invaluable when designing siRNAs. Some noteworthy programs include:

*BIOPREDsi* (<http://www.biopredsi.org>): This site was developed by the Novartis Institutes for BioMedical Research. It requires and provides only essential information. Input is a gene Accession number or gene sequence, and output is a user-defined number of optimized siRNA sequences (Huesken et al., 2005).

*Whitehead siRNA Selection Web Server* (<http://jura.wi.mit.edu/bioc/siRNAext>): This Web site was developed and is hosted by the Whitehead Institute. It is somewhat more complex, giving a large number of possible duplexes along with their properties, e.g., thermodynamic properties. An off-target search can be done for each duplex, within the site, but in a separate step (Yuan et al., 2004).

For an excellent guide to siRNA sequence selection, see Pei and Tuschl (2006). Other siRNA design algorithms are reviewed by Boese et al. (2005).

It is useful to test several duplexes targeting a gene, even after using a selection Web site. The most thorough way to identify highly active siRNA sequences is to test as many as 100 to 200 sequences against a given mRNA (Choung et al., 2006; Manoharan and Rajeev, 2007), looking for the duplexes with the best gene silencing activity and minimum off-target effects. Furthermore, individual sequences



**Figure 16.3.2** 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. Adapted from Watts et al. (2008), with permission from Elsevier.

respond differently to chemical modifications, and modifications beneficial for one siRNA sequence may be detrimental to another. Therefore, a variety of chemical modifications should be included in the sequence screen from fairly early in the process.

### Choosing an siRNA Architecture

The canonical siRNA architecture is a 21-nt antiparallel, double-stranded RNA with 2-nt 3' overhangs (Elbashir et al., 2001a). The canonical siRNA design mimics the product of Dicer processing of long dsRNAs (Zamore et al., 2000; Bernstein et al., 2001). In the naturally occurring cellular pathway, these Dicer cleavage products are the effector molecules loaded into RISC to effect gene silencing. However, researchers have discovered that a much wider variety of duplexes can be incorporated into the RNAi pathway.

Indeed, we now understand that siRNA overhangs are particularly amenable to modifications. The use of 2'-deoxynucleotide units in the 3'-overhangs has been common since the earliest siRNA experiments; it reduces the cost of oligonucleotide synthesis and may increase resistance to 3'-exonucleases (Elbashir et al., 2001a). Of course, RNA units also work well (Holen et al., 2002). Perhaps more interestingly, most of the chemistries reviewed below are well tolerated in the overhangs as well (i.e., accepted by the RNAi machinery without loss of potency).

Blunt-ended siRNAs have also been used (Czauderna et al., 2003) because blunt-ended duplexes are more resistant to 3'-exonucleases. In addition, one study reported a greater tolerance to chemical modifications in combination with blunt-ended duplexes (Kraynack and Baker, 2006). However, blunt-ended siRNAs can be more immunogenic than duplexes with 3'-overhangs (Marques et al., 2006).

It has been shown that the 3' overhang is bound within a hydrophobic pocket of the

PAZ domain (Lingel et al., 2003; Song et al., 2003; Yan et al., 2003; Ma et al., 2004). So, the 3' nucleotide overhangs can be replaced by aromatic groups (described as "universal overhangs" due to the lack of base pairing), improving nuclease stability without harming activity (Ueno et al., 2009).

Chemical phosphorylation of the 5'-end of the antisense strand can help ensure high potency, and may be necessary when the 5'-end of the strand is modified (Fisher et al., 2007; Watts et al., 2007; Zhang et al., 2009). Conversely, 5'-phosphorylation of the sense strand can be blocked without loss of activity (Nykanen et al., 2001; Czauderna et al., 2003). In fact, blocking sense strand phosphorylation can be beneficial, ensuring proper guide strand selection by RISC (Chen et al., 2008).

The siRNA architecture itself can be modified via chemical synthesis (Fig. 16.3.2). While most siRNA duplexes are made up of two strands, an siRNA made of three strands (an intact antisense strand and two 9- to 13-nt sense strands) can reduce off-target effects and increase potency; the resulting nicked duplex is termed small internally segmented interfering RNA (sisiRNA; Bramsen et al., 2007).

Functional siRNA can also be made from just one strand, in any of the following ways: Hairpin-type duplexes, made from a single strand, can be introduced exogenously (Siolas et al., 2005) or expressed within a cell (Brummelkamp et al., 2002; reviewed in Banan and Puri, 2004). Dumbbells or nanocircles, made by closing the open end of the hairpin, retain RNAi activity while providing complete protection from exonucleases (Abe et al., 2007). Finally, a single-stranded antisense RNA (not folded into a duplex at all) has been shown to enter the RNAi pathway, with potency occasionally approaching that of duplex siRNA (Martinez et al., 2002; Amarzguoui et al., 2003; Holen et al., 2003).

Recent findings have also demonstrated that asymmetrically designed siRNA duplexes can be potent gene silencing agents, comparable to canonical siRNAs. Indeed, siRNA duplexes can vary significantly in the lengths of either the sense or antisense strands. Asymmetrical siRNAs can contain ssRNA regions and comparatively short dsRNA regions without impairing activity in many cases. Active asymmetrical designs include 15 (sense, S) + 21 (antisense, AS) (Sun et al., 2008), 16 (S) + 21 (AS), 18 (S) + 18 (AS) (Chu and Rana, 2008), and 16 (S) + 19 (AS) (Chang et al., 2009). Asymmetrical siRNA designs are intriguing because several of these designs significantly decrease off-targeting, especially the sense strand mediated off-targeting that occurs when the wrong strand is loaded into RISC.

Asymmetry in siRNA overhangs can also be beneficial in directing the proper guide strand into RISC. Asymmetric siRNAs designed such that the guide strand has a 2-nt 3'-overhang and the passenger strand lacks a 3'-overhang (blunt ended) can improve siRNA potency (Sano et al., 2008). This effect is useful for siRNAs with unfavorable thermodynamics of loading.

The length of an siRNA duplex can be altered. Most synthetic duplexes are 19 to 21 bp in length, designed to mimic the natural products of the Dicer enzyme, which cleaves long dsRNAs into substrates for RISC. On the other hand, using a longer siRNA duplex can make it a *substrate* for Dicer, and has been found to increase potency of gene silencing and stability to degradation (Kim et al., 2005; Amarzguioui et al., 2006; Kubo et al., 2007). Enhanced potency may result from a physical transfer of newly dicer-produced siRNA to RISC. It is important to maintain a length of <30 nt, to avoid triggering the interferon response (Minks et al., 1979).

## INCORPORATING MODIFIED NUCLEOSIDES AND BACKBONE LINKAGES

While careful sequence selection is essential and varying the architecture of an siRNA duplex can provide certain advantages, this review will focus on changing the chemical nature of the oligos themselves. The rationale for chemical modification is clear, and the right chemical modifications can address nearly all the shortcomings of siRNA therapeutics:

1. Nuclease stability can be significantly improved.

2. Cellular uptake can be increased.
3. Immunostimulation can be reduced (or increased, if desired).
4. Off-targeting can be reduced.

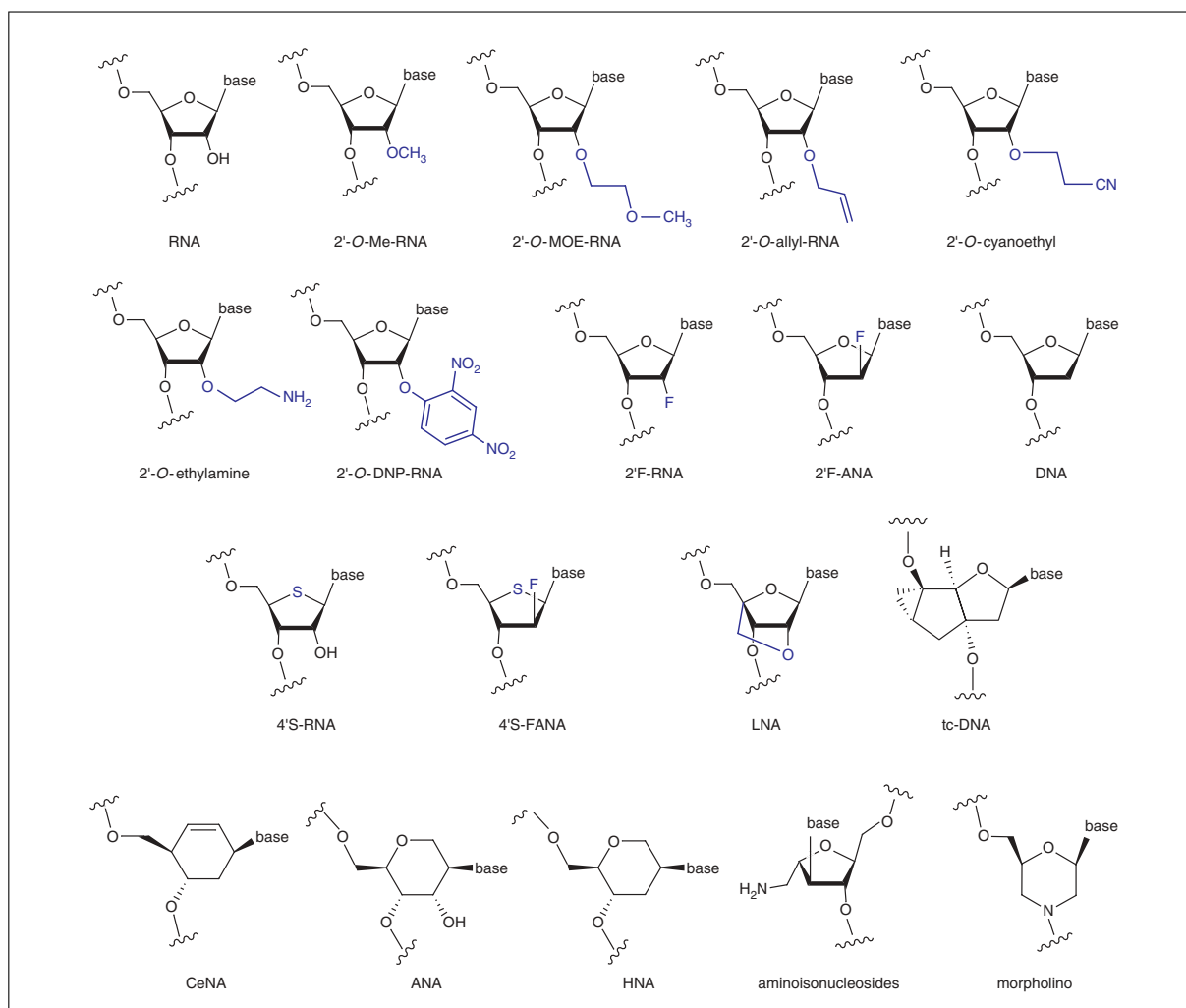
This section will describe a wide variety of chemical modifications compatible with siRNA.

## Sugar Modifications

The ribose sugar moiety is perhaps the most attractive component for chemical modification. Changes in the sugar chemistry can strongly influence sugar conformation, and thus overall siRNA duplex structure. Sugar modifications can improve endonuclease resistance. siRNAs containing RNA-like nucleoside analogues can pass under the immune receptor radar screen without impairing gene silencing activity. A wide variety of sugar modifications can be used to modify siRNAs, although often with limitations on position and quantity. Various sugar modifications compatible with RNAi are described below (see Fig. 16.3.3), with reference to features and properties that are useful when designing modified siRNAs.

A number of successful siRNA modifications have focused on modification of the 2' position. 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 (Chiu and Rana, 2003). DNA is perhaps the simplest modification accepted within siRNA duplexes. DNA has been used in the 3'-overhangs of synthetic siRNAs since the beginning (Elbashir et al., 2001a), and can be tolerated in limited numbers within the duplex region as well (Parrish et al., 2000; Elbashir et al., 2001b; Ui-Tei et al., 2008). An antisense strand made entirely of DNA purines and 2'-F-RNA pyrimidines is functional (Chiu and Rana, 2003), since 2'-F-RNA strongly favors a northern (C3'-*endo*) sugar pucker and A-form helical structure, presumably directing the conformation of the more flexible 2'-deoxynucleotides towards northern, RNA-like conformations. dsDNA can be used in the 8-bp region at the 5'-end of the guide strand to produce active siRNAs with reduced off-target effects (Ui-Tei et al., 2008).

Binding affinity and nuclease stability are increased by 2'-*O*-methylation of RNA, and 2'-*O*-Me-RNA can be well-tolerated throughout the siRNA duplex. This makes it a popular and versatile siRNA modification. Many groups have found that large numbers



**Figure 16.3.3** Sugar units that have been successfully used to modify siRNA duplexes. Top row, 2'-O-alkyl modifications (2'-O-Me, 2'-O-MOE, 2'-O-allyl, 2'-O-cyanoethyl); second row, other 2'-modifications (2'-O-ethylamine, 2'-O-DNP, 2'-F-RNA, 2'-F-ANA, and DNA); third row, 4'-modifications (4'-S-RNA and 4'-S-2'-F-ANA) and bicyclic/tricyclic modifications (LNA and tc-DNA, respectively); bottom row, six-membered ring modifications (CeNA, ANA, HNA) and dramatically redesigned sugar structures (aminoisounucleosides, morpholino). Adapted from Watts et al. (2008), with permission from Elsevier.

of 2'-O-Me modifications (in either strand) decrease siRNA activity (Elbashir et al., 2001b; Braasch et al., 2003; Chiu and Rana, 2003; Czauderna et al., 2003), but others report that fully modified 2'-O-Me sense strands are functional (Choung et al., 2006; Kraynack and Baker, 2006). The retention of functionality may be due to the 2'-O-Me modifications being made in blunt-ended duplexes (Kraynack and Baker, 2006), but at least one other group reports that even in this context, activity is greatly reduced by heavy 2'-O-Me modification (Czauderna et al., 2003). The 2'-O-methoxyethyl (2'-O-MOE) modification has been used in the 3'-overhangs of siRNA targeting the pain-related cation-channel P2X<sub>3</sub> and has resulted in successful gene targeting in vivo (Dorn et al., 2004). Another group found that

2'-O-MOE modifications could be used in the sense strand, especially at the termini, but not in the antisense strand (Prakash et al., 2005). Similarly, 2'-O-allyl-modifications reduce activity at most positions in the duplex, but can be used (Odadzic et al., 2008). These modifications do not reduce activity in the 3'-overhangs (Amarzguoui et al., 2003).

A cyanoethyl group at the 2' position increases binding affinity and nuclease resistance, and may find applications in siRNA (Saneyoshi et al., 2005; Bramsen et al., 2009). 2'-O-acetalester-protecting groups are being developed for production of "proRNA," which remains protected until demasked by cellular esterases (Martin et al., 2009). These molecules may find application as "pro-siRNA" (Martin et al., 2009). In a similar fashion, 2'-esterified units (levulinates) may

be used to create protected siRNA molecules (Lackey et al., 2007).

2'-*O*-alkylamines also appear to be active in siRNA duplexes (Odadzic et al., 2008; Bramsen et al., 2009). Minimal modification with 2'-*O*-ethylamine units at the 3' end of the antisense strand appears to be well-tolerated (Odadzic et al., 2008). Further characterization will improve our understanding of their gene silencing potential.

In most cases, bulkier 2'-modifications are not well tolerated in siRNA duplexes, except for limited modifications on the termini. Interestingly, siRNAs with 70% of the 2'-OH groups 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 (Chen et al., 2004). Recently Bramsen et al. (2009) have reported a large-scale screen of siRNA activity elicited by several nucleoside analogues, showing that a variety of 2' modifications can be tolerated, particularly in the sense strand. In some cases, bulky 2'-modifications were tolerated, but with slightly reduced activity.

Functional siRNAs can also contain fluorine at the 2'-position. One of the best-known and most versatile siRNA modifications is 2'-F-RNA. Partial 2'-F-RNA modification is tolerated throughout sense and antisense strands (Braasch et al., 2003; Chiu and Rana, 2003; Harborth et al., 2003), and there are reports of active siRNAs that are fully modified 2'-F-RNA (Blidner et al., 2007). 2'-F-RNA-modified siRNA duplexes have significantly increased serum stability (Layzer et al., 2004) and increased binding affinity of the duplex (Kawasaki et al., 1993; Monia et al., 2003). The 2'-F-RNA modification has also allowed the synthesis of siRNAs containing 5'-amino-2',5'-dideoxy-2'-fluororibonucleic acid units that confer improved hydrolytic stability (Ueno et al., 2008).

Changing the stereochemistry of the fluorine of 2'-F-RNA results in 2'-deoxy-2'-fluoroarabinonucleic acids (2'-F-ANA). Considering that 2'-F-ANA was originally developed as a DNA mimic (Rosenberg et al., 1993; Berger et al., 1998; Damha et al., 1998; Ikeda et al., 1998; Wilds and Damha, 2000), it is somewhat surprising that 2'-F-ANA is also well-tolerated in siRNA duplexes, including fully modified sense strands and partially modified antisense strands (Dowler et al., 2006; Watts et al., 2007). Like 2'-F-RNA, 2'-F-ANA binds with high affinity and increases nucle-

ase stability. Both 2'-fluorinated analogues are highly resistant to acidic conditions (Watts et al., 2009).

In addition to the oxygen at the 2'-position, the ring oxygen can be modified for siRNA applications. One example is 4'-S-RNA, a high-affinity modification that gives a significant advantage in nuclease stability. Incorporation of 4'-S-RNA units does not reduce silencing when placed near the termini of siRNA duplexes (Hoshika et al., 2005, 2007; Dande et al., 2006). An siRNA architecture consisting of four 4'-thioribonucleotides on each end of the sense strand and four at the 3'-end of the antisense strand has consistently worked well for silencing two target genes in three cell lines (Hoshika et al., 2007). When the antisense strand was modified with 4'-S-RNA, especially at the center, potency was reduced (Hoshika et al., 2005; Dande et al., 2006). Combinations of 4'-S-RNA with 2'-*O*-Me and 2'-*O*-MOE modifications at the termini of both strands have also shown excellent potency and serum stability (Dande et al., 2006).

4'-S-2'-F-ANA, featuring modifications at both the 2' and 4' positions, has a northern, RNA-like conformation (Watts et al., 2007), and is compatible with siRNA activity at various positions in both strands. 4'-S-2'-F-ANA in the antisense strand shows synergy with 2'-F-ANA modifications in the sense strand (Watts et al., 2007). However, the low-binding affinity of 4'-S-2'-F-ANA means that only a small number of modifications should be used.

Locked nucleic acid (LNA), a conformationally constrained nucleotide adopting a northern structure (Petersen and Wengel, 2003), has been extensively studied in siRNA (Braasch et al., 2003; Elmen et al., 2005; Hornung et al., 2005; Bramsen et al., 2007; Mook et al., 2007). Its conformational rigidity causes significant increases in binding affinity. Careful placement of LNA in siRNA duplexes can lead to functional duplexes of various types. Common terminal modification sites are the termini of the sense strand (Hornung et al., 2005) and the 3'-overhang of the antisense strand (Elmen et al., 2005; Mook et al., 2007). Minimal modification of most internal positions of the antisense strand is also tolerated (Braasch et al., 2003; Elmen et al., 2005), but heavier modification of the antisense strand is tolerated only in combination with a segmented sense strand (sisiRNA, described above; see Bramsen et al., 2007).

LNA is one example of a larger family of nucleoside analogues, the bicyclic nucleic

acids (BNAs). Another BNA, ethylene-bridge nucleic acid (ENA), has been tested in the siRNA overhang and been found to impair siRNA activity (Hamada et al., 2002). A rigid tricyclic modification (tc-DNA) has been successfully used at either end of the sense strand, and even improved activity when placed in the overhangs (Ittig et al., 2008). The development of a variety of BNAs has been reviewed by Obika (2004), although much remains to be understood regarding their compatibilities to elicit siRNA activity. Toward this end, Bramsen et al. (2009) have published a large-scale screen of siRNA activity elicited by several nucleoside analogues, including several members of the BNA family. For the siRNA sequence tested, several BNAs appear to be tolerated in siRNA, especially within the sense strand.

Interestingly, siRNA potency can be retained, and in some cases improved, when the 5-membered sugar moiety is replaced with a 6-membered ring. Cyclohexenyl nucleic acids (CeNA), altritol nucleic acids (ANAs), and hexitol nucleic acids (HNAs) have all been successfully incorporated into siRNAs. Incorporation of one or two CeNA units can produce siRNAs with similar or enhanced activity compared to unmodified siRNA (Nauwelaerts et al., 2007). ANAs support A-form structures, and can improve potency and duration of activity when incorporated at certain positions (Fisher et al., 2007), but can also slightly decrease potency in some cases (Fisher et al., 2009). HNA can improve siRNA potency, and is tolerated in the sense strand opposite the position on the antisense strand at which mRNA cleavage occurs (Fisher et al., 2009).

siRNAs can be minimally modified with aminoisonucleosides (see Fig. 16.3.3) without reducing activity (Li et al., 2007). Aminoisonucleosides were better tolerated by the RNAi machinery in the sense strand than in the antisense strand, and a single insert could be used at either the 5' or the 3' region of the sense strand without impairing gene silencing. However, modifications were less tolerated internally in the sense strand, reduced activity when placed in the antisense strand, and may be detrimental to overall nuclease stability. Aminoisonucleoside modification of siRNA is awaiting optimization.

Morpholino nucleoside analogues can also be used in siRNA. Morpholino analogues are tolerated in the sense strand and on the overhangs, but reduce activity in the antisense strand (Zhang et al., 2009).

## Backbone Linkage Modifications

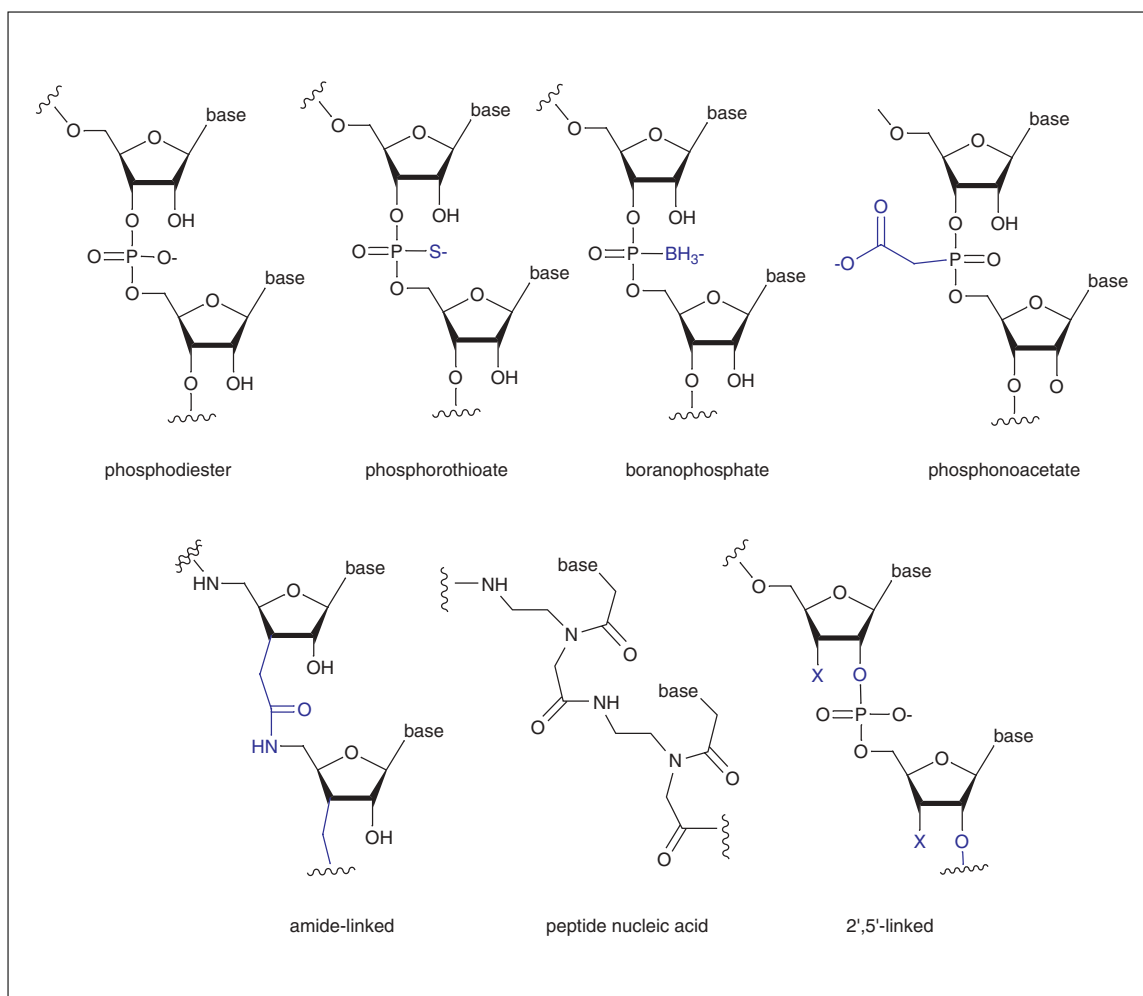
Chemical modifications to the oligonucleotide backbone are also tolerated by the RNAi machinery. Indeed, several variations on the phosphodiester linkage can be used for gene silencing (Fig. 16.3.4). Phosphorothioate (PS) linkages, a popular modification in antisense technology, can be used in siRNA, but they often reduce potency relative to native siRNA (Chiu and Rana, 2003; Hall et al., 2004). However, in some cases their potencies are comparable to native siRNAs (Amarzguioui et al., 2003; Harborth et al., 2003). Some observations indicate that PS linkages are not accepted at the center of the duplex, especially at the sense strand scissile phosphate (Leuschner et al., 2006). Some cytotoxicity has been observed with extensive PS modification (Amarzguioui et al., 2003). In marked contrast to PS-DNA antisense constructs (Geary et al., 2001), PS modifications do not appear to have a major effect on biodistribution of siRNA (Braasch et al., 2004).

siRNAs with boronophosphate linkages are functional. They show increased potency relative to PS-modified siRNAs, and often to native siRNAs as well. To maximize potency, the center of the antisense strand should not be modified (Hall et al., 2004). Boronophosphate linkages in siRNAs provide a significant increase in nuclease stability over native RNA (Hall et al., 2004).

Two nonionic amide linkages are tolerated in the 3' overhangs of siRNA: a simple amide linkage that maintains the ribose structure while increasing duplex thermodynamic stability and nuclease resistance (Iwase et al., 2007) and peptide nucleic acid, in which the entire sugar-phosphate backbone is replaced by an amide moiety (Potenza et al., 2008).

Phosphonoacetate linkages (Fig. 16.3.4) are recently described backbone modifications with exciting properties (Sheehan et al., 2003; Yamada et al., 2007). These linkages can be esterified, affording nucleic acids with neutral backbones, which can be taken up by cells without transfection reagent (Sheehan et al., 2003; Yamada et al., 2007). Cellular esterases are then believed to cleave the ester groups, producing phosphonoacetate backbones, which are negatively charged and thus retained inside cells (Yamada et al., 2007). The properties of phosphonoacetate backbones are beneficial for siRNA applications, and preliminary reports of siRNA activity elicited by these modifications are encouraging.





**Figure 16.3.4** Internucleotide linkages used in siRNA. The phosphodiester linkage can be replaced by phosphorothioate, boranophosphate, or phosphonoacetate linkages, which retain the negative charge of a phosphate. Neutral amide-linked RNA or peptide nucleic acid (PNA) can be used at select positions. Either 2',5'-linked DNA (X=H) or 2',5'-linked RNA (X=OH) can be used in the sense strand. Adapted from Watts et al. (2008), with permission from Elsevier.

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 (Prakash et al., 2006).

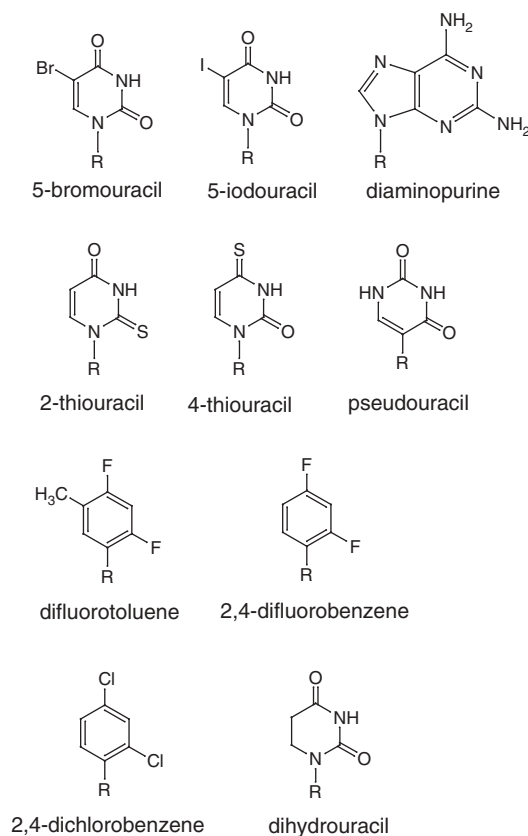
### Base Modifications

The use of modified bases in siRNA has been more limited than other modifications, but there have been several examples (Fig. 16.3.5). Modified bases that stabilize A-U base pairs (5-Br-Ura and 5-I-Ura instead of uracil, and diaminopurine instead of adenine) are tolerated in siRNA duplexes, although activity is somewhat reduced (Chiu and Rana, 2003).

4-Thiouracil has also been successfully used (Parrish et al., 2000). 2-Thiouracil (Hornung et al., 2006; Sipa et al., 2007) and the C-linked base pseudouracil (Hornung et al., 2006; Sipa et al., 2007) 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., 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. Indeed, small base modifications projecting into the RNA major groove (5-Me-U and 5-Me-C) can improve siRNA stability without impairing gene silencing, whereas bulkier groups (5-propynyl-U) are detrimental to activity (Terrazas and Kool, 2009). Bulky or cationic base modifications have not been well-tolerated (Parrish et al., 2000).

Some surprisingly atypical base structures have been used in siRNA. A difluorotoluoyl base, which has the same shape as thymine but cannot form classical hydrogen bonds, can replace uracil at single positions throughout



**Figure 16.3.5** Base-modified nucleotides used in siRNA. Top two rows: 5-halouracils, 2-thiouracil, pseudouracil, and diaminopurine increase the strength of A-U base pairs; bottom two rows: highly atypical modified bases including bases that cannot form classical hydrogen bonds (difluorotoluene, 2,4-difluorobenzene, 2,4-dichlorobenzene) and a non-aromatic base (dihydrouracil). R represents the ribose phosphate backbone. Adapted from Watts et al. (2008), with permission from Elsevier.

an siRNA duplex, without significantly decreasing the potency or changing the mechanism of cleavage (Xia et al., 2006). Non-polar nucleobases (2,4-difluorobenzene and 2,4-dichlorobenzene; see Fig. 16.3.5) are also tolerated in certain siRNA positions, and can in some cases improve siRNA specificity (Somoza et al., 2008). These studies provide evidence that non-hydrogen-bonding nucleobases can be tolerated at positions 1, 3, 4, 7, and 19 of the guide strand (Somoza et al., 2008). 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 (Sipa et al., 2007).

### Terminal Conjugates

Various groups can be conjugated to the termini of siRNA duplexes, especially the termini of the sense strand. An inverted abasic end cap

(David et al., 2005; Morrissey et al., 2005) helps with exonuclease stability. Conjugation of fluorescent dyes (Harborth et al., 2003) or biotin (Chiu and Rana, 2002; Schwartz et al., 2008) has allowed important biophysical/biochemical studies. Conjugation of lipophilic groups, including steroids and lipids (Lorenz et al., 2004; Soutschek et al., 2004), has helped with delivery. Cholesterol conjugation has even been shown to allow siRNA-mediated protection from HSV-2 following intravaginal administration of the siRNAs (Wu et al., 2009).

A combinatorial library of lipid-like conjugates, termed “lipidoids” has also been described as potential delivery agents (Akinc et al., 2008). siRNA delivery is a rapidly developing research field; a recent study showed that including 5 to 8 dA and dT units on the 3'-ends of the strands can lead to reversible concatemerization via sticky ends, which in turn leads to higher efficiency delivery in complex

with linear polyethylenimine (PEI) (Bolcato-Bellemin et al., 2007).

The general consensus is that the 5'-end of the antisense strand is the most sensitive to modifications (Chiu and Rana, 2002; Martinez et al., 2002; Czauderna et al., 2003). However, one group observed that fluorescein could be conjugated to any of the termini except the 3'-end of the antisense strand (Harborth et al., 2003). As long as an antisense 5'-phosphate is present, attaching a group to it does not necessarily eliminate RNAi activity (Schwarz et al., 2002).

### Combining Modifications

The various modifications described above can confer advantageous properties to siRNAs. However, it is also clear that the effectiveness of modifications has a strong sequence dependence. Many modifications are beneficial in some siRNA regions but detrimental in others, and enhance activity in some sequence contexts, but reduce potency in others. An interesting concept then becomes the combination of different, complementary modifications within the same siRNA. Most siRNA modification studies focus on individual modifications to minimize experimental variables. However, a few studies have demonstrated that combining modifications can produce siRNAs with impressive features. Indeed, many interesting modified siRNAs consist of a combination of multiple chemical modifications (Allerson et al., 2005; Morrissey et al., 2005; Dande et al., 2006; Koller et al., 2006; Bramsen et al., 2009).

Indeed, by combining various modifications, these researchers were able to design potent siRNAs completely free of the native ribose. We have been investigating the use of chimeric siRNAs composed entirely of 2'-F-ANA and 2'-F-RNA modifications, and have observed synergy between these two modifications when assessing gene silencing activity against a few different mRNA targets (G.F. Deleavey et al., unpub. observ.). Primarily, research on new chemically modified siRNA involves only one type of modification. However, if the field is to move toward widely applicable chemical modification strategies, there is an ongoing need to combine chemistries in creative ways.

### APPLYING MODIFICATIONS TO ADDRESS siRNA SHORTCOMINGS

The following sections were adapted from Watts et al. (2008), with permission from Elsevier.

### Serum Stability

RNA is rapidly degraded in cells. The double-stranded nature of siRNA improves stability relative to ssRNA, but these molecules are still not ideal for in vivo therapeutic applications. A nuclease called *eri-1* has been found to play a key role in degradation of siRNA (Kennedy et al., 2004), and expression levels of *eri-1* inversely correlate with duration of siRNA activity (Takabatake et al., 2007). 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 discussed can be used to increase the serum half-life of siRNAs. In the therapeutic siRNA community, two approaches are being studied for protecting siRNAs against nucleases. The first strategy favors extensive or full chemical modification of siRNA. Sirna Therapeutics (<http://www.sirna.com>) has published work on heavily modified siRNA duplexes, demonstrating the advantages of this strategy. A fully modified siRNA with a sense strand made of 2'-F-RNA pyrimidines, DNA purines, and 5' and 3' inverted abasic end caps, and an antisense strand made of 2'-F-RNA pyrimidines, 2'-O-Me purines, and a single phosphorothioate linkage at the 3'-terminus, had significantly increased potency in an HBV mouse model (David et al., 2005). This fully modified duplex had a half-life in serum of 2 to 3 days, as compared with 3 to 5 min for the unmodified duplex (David et al., 2005). This improved stability translated into higher efficacy in vivo. Still higher potency was later obtained by including 1 to 3 RNA inserts at the 5'-end of the antisense strand, and this heavily modified siRNA still had a serum half-life nearly 30× longer than that of unmodified siRNA (Morrissey et al., 2005).

Several other examples of fully modified duplexes have been reported. An siRNA made entirely of alternating 2'-F-RNA and 2'-O-Me units had greatly increased stability in serum (Allerson et al., 2005). This architecture maintains or improves potency, as discussed in the next section. An siRNA made with DNA overhangs, a 2'-O-Me-RNA sense strand, and a PS-RNA antisense strand was nearly all intact after 48 hr in serum (Choung et al., 2006), and a functional fully 2'-F-RNA-modified siRNA has also shown excellent nuclease resistance (Blidner et al., 2007).

However, extensive chemical modification may not always be necessary. The second

paradigm for protecting siRNAs against nucleases involves minimal, selective modification. This strategy is favored by, among others, Alnylam Pharmaceuticals (<http://www.alnylam.com>). Endonucleolytic hydrolysis is a major mechanism of degradation of siRNAs (Czauderna et al., 2003), and so the endonuclease cleavage pattern of a given siRNA duplex is characterized. Degradation is often dominated by cleavage after a pyrimidine nucleotide, and can be readily characterized by mass spectrometry (Turner et al., 2007) or denaturing polyacrylamide gel electrophoresis (Xia et al., 2006). Vulnerable positions are selectively modified, usually with 2'-O-Me or 2'-F-RNA nucleotides, which considerably increase the stability of the siRNA with only minimal modification (Manoharan, 2006; de Fougères et al., 2007). Besides these experimentally determined vulnerable internal positions, key regions for modification include the strand termini (Choung et al., 2006; Hoerter and Walter, 2007).

### Increasing Potency

siRNA is a potent gene silencing agent, although potency depends on cell type, mRNA target, and siRNA sequence. Increasing potency is usually not considered the primary objective of chemical modification: it is sufficient to maintain the potency of unmodified siRNA. It is more important to increase serum half-life and specificity. However, as our understanding of the requirements for effective RNAi develops, we can foresee an increase in the use of chemical modifications to optimize potency as well, through features such as target-binding affinity (by enhancing hybridization on-rates and off-rates), preorganization into appropriate conformations (to reduce entropic penalties of binding), flexibility (to allow for enzyme dynamics), and optimized binding to RISC (in various other ways).

Unfortunately, patterns of chemical modification that universally increase potency are rare. However, 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, which was 500-fold more potent than unmodified siRNA (Allerson et al., 2005). Such impressive improvement was not observed for other sequences, but this architecture showed equal or greater potency and efficacy than unmodified siRNA for several other targets (Koller et al., 2006).

Part of the increase in potency observed in some studies may arise from 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. In addition, 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, and of the interplay between chemical modifications, nucleotide sequence, and duplex structure.

Rational chemical modification can, however, be used to improve potency through improving the selective loading of the antisense strand. Strand differentiation by RISC is determined by the thermodynamics of the siRNA duplex (Khvorova et al., 2003; Schwarz et al., 2003). The strand with lower binding at its 5'-end is preferentially selected as the guide strand by RISC. The sequence of an siRNA should therefore be chosen to be AU-rich at the 5'-end of the antisense strand. Chemical modifications can also be used to increase incorporation of the appropriate guide strand. By placing high-affinity modifications like LNA at the 5'-end of the sense strand (Elmen et al., 2005), duplex thermodynamics can be altered to affect strand loading. The activity of a moderately active siRNA duplex was significantly improved using the high-affinity 4'-S-RNA modification in both strands (Hoshika et al., 2007). However, a duplex that already had optimal strand thermodynamics was not further improved by 4'-S-RNA modification (Hoshika et al., 2007). Base modifications can be used for similar purposes. 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 (Sipa et al., 2007).

In addition to chemical modification, a recent report has demonstrated that a small molecule, enoxacin, can actually enhance siRNA-mediated mRNA degradation (Shan et al., 2008). Enoxacin is believed to enhance TRBP-binding to RNA (Shan et al., 2008), and enhanced interactions between TRBP and siRNA may facilitate RISC loading (Katoh and Suzuki, 2007). This interesting discovery suggests that RNAi potency can be improved via indirect methods as well as with direct chemical modification, and also suggests that it would be worth designing chemical modifications that optimize protein interactions directly.

## Immunostimulation

Long dsRNA can be a hallmark of viral infection. It has long been known that dsRNAs longer than 30 bp are potent activators of immune responses (Minks et al., 1979), preventing the application of RNAi to mammalian cells in the same fashion originally described in *C. elegans* (Fire et al., 1998). It was eventually discovered that 21-nt siRNA duplexes were capable of effecting potent and specific gene knockdown in mammalian cells. These molecules were below the 30-bp threshold, and were expected to avoid the immunostimulation characteristic of longer dsRNAs (Elbashir et al., 2001a).

However, the immunogenic activity of short RNA duplexes is more significant than originally perceived. Reports of off-target effects resulting from immunostimulation caused by dsRNAs shorter than 30 bp are widespread (Kim et al., 2004; Hornung et al., 2005, 2006; Judge et al., 2005; Marques and Williams, 2005; Morrissey et al., 2005; Reynolds et al., 2006; Schlee et al., 2006). Nonspecific immune responses to siRNA therapeutics may trigger unwanted side-effects unless the immunogenic properties of siRNA can be addressed.

Immune response activation by short RNAs is a complex process, and has been reviewed elsewhere (Marques and Williams, 2005; Schlee et al., 2006). Briefly, RNAs are recognized by three main types of immunoreceptors: Toll-like receptors (TLRs) (specifically TLR3, TLR7 and TLR8), protein kinase R (PKR), and helicases like RIG-I and MDA5. Receptor recognition can lead to a number of cellular responses including release of cytokines and changes in gene expression (Schlee et al., 2006). 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) (Kleinman et al., 2008), in endosomes (TLR3/7/8; Takeda and Akira, 2005; Judge and Maclachlan, 2008; Zamanian-Daryoush et al., 2008), and in the cytoplasm (RIG-I, MDA5, PKR; Judge and Maclachlan, 2008).

Immune responses are affected by RNA sequence; however, the sequence dependence is not fully understood. Judge et al. have identified immunostimulatory motifs in mice and in vitro human blood, most notably 5'-UGUGU-3' regions (Judge, et al., 2005). Hornung et al.

demonstrated that 5'-GUCCUCAA-3' is also a potent immunostimulatory motif (Hornung et al., 2005). In fact, any U-rich RNA sequence may be sufficient for recognition by TLR7 (Diebold et al., 2006). The presence of a 5'-triphosphate or blunt-end can lead to RIG-I-mediated immunostimulation (Kim et al., 2004; Hornung et al., 2006; Marques et al., 2006). Duplexes between 23 and 30 bp may still induce the interferon response in a length-dependent manner, depending on cell type (Reynolds et al., 2006).

Fortunately, chemical modifications can be used to reduce the immunostimulatory properties of siRNAs (Eberle et al., 2008; Judge and Maclachlan, 2008). 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 (Morrissey et al., 2005). 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 modified siRNA (Morrissey et al., 2005). Although minimal 2'-O-Me modification is sufficient to reduce immunostimulatory activity, 2'-O-methylation of cytidine residues was, however, found ineffective in fulfilling this function (Judge et al., 2006). 2'-F-RNA and/or 2'-O-Me modification of uridine resulted in elimination of immune off-target effects (Hornung et al., 2006), including both TLR-dependent and TLR-independent immune effects (Cekaite et al., 2007). Notably, even the presence of 2'-O-Me modified RNAs on separate, non-complementary strands abrogates the TLR7-dependent immunostimulatory activity of unmodified siRNAs, indicating that 2'-O-Me-RNA itself is a potent antagonist of TLR7 (Robbins et al., 2007).

In addition to 2'-O-Me and 2'-F-RNA modifications, LNA has been used to modify the ends of an siRNA sense strand containing an immunostimulatory motif, abrogating the IFN- $\alpha$  immunostimulatory activity of the duplex without affecting the silencing activity (Hornung et al., 2005). Base modifications can also reduce immune activation: modification with pseudouracil or 2-thiouracil prevented the RIG-I-mediated immunostimulation due to a 5'-triphosphate (Hornung et al., 2006), and various base modifications abrogated immune

effects mediated by TLR3, TLR7, and TLR8 (Karikó et al., 2005).

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 as well (Karikó et al., 2005). In another recent study, the cytokine induction profile was different upon treatment with unmodified, 2'-O-Me-modified, and 2'-F-RNA modified siRNAs (Zamanian-Daryoush et al., 2008).

Immunogenic side effects from siRNA administration have not always been observed. However, it is clear that when immune activation is a problem, many chemical modifications are available to minimize this predicament, and 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. Encouragingly, our understanding of dsRNA recognition by TLR-3 has been greatly improved by very recent reports of the structural basis of dsRNA recognition (Liu et al., 2008; Pirher et al., 2008). Studies such as these may allow the rational design of siRNAs that can avoid receptor recognition.

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 might actually be due to TLR3-mediated immune effects (Kleinman et al., 2008). Furthermore, immunostimulatory siRNAs may find use as drugs capable of facing cancer and chronic viral infections on two fronts: eliciting both gene knockdown and activation of immune responses (Schlee et al., 2006; Poeck et al., 2008). Currently, however, immune activation is generally considered an unwanted and potentially dangerous off-target effect (Judge and Maclachlan, 2008).

### Hybridization-Dependent Off-Target Effects

Off-target side effects of siRNA can arise from partial hybridization with unintended mRNAs (Jackson et al., 2003). This primarily occurs when siRNAs function as miRNAs via 6 to 8 nt complementarity at the 5'-end of the antisense strand (Birmingham et al., 2006; Jackson et al., 2006a), but can also occur with a

~15-nt region of significant complementarity in the center of the antisense strand (Jackson et al., 2003). If some of the sense strand is mistakenly incorporated into RISC instead of the antisense strand, it can cause even more off-target effects (Jackson et al., 2003).

Two major strategies are used to minimize off-target effects: (1) increasing the selective loading of the antisense strand (disfavoring incorporation of the sense strand) as the RISC guide, and (2) disfavoring miRNA-type activity.

The first of these strategies can have the benefit of increasing potency, as well as reducing off-target effects, and is typically accomplished 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*. To ensure that the sense strand makes absolutely no contribution to off-target effects, it can be further modified. An extreme example of this is small internally segmented interfering RNA (sisiRNA), in which the sense strand is nicked to prevent it from being incorporated into RISC (see Fig. 16.3.2, and *Choosing an siRNA Architecture*) (Bramsen et al., 2007). The high affinity of LNA modifications is essential for such strand architecture (Bramsen, et al., 2007), since 10- to 12-bp duplexes of unmodified RNA are too unstable to effect gene silencing.

Another method of reducing RISC loading of the sense strand is to block 5'-phosphorylation by methylation (Chen et al., 2008). Finally, incorporation of eight DNA nucleotides at the 3'-end of the sense strand significantly reduces sense-strand mediated off-target effects (Ui-Tei et al., 2008). However, even if the antisense strand is incorporated into RISC with perfect selectivity, the problem remains that siRNAs can function as miRNAs (Doench et al., 2003; Zeng et al., 2003) and downregulate many genes through partial complementarity (Lim et al., 2005; Jackson et al., 2006a), especially in the seed region. Furthermore, these off-target effects can cause toxicity (Fedorov et al., 2006). 2'-O-Me nucleotides at the 5'-end of the antisense strand can reduce miRNA-type off-target effects significantly, with little effect on siRNA gene silencing (Jackson et al., 2006b). If the seed region is made of DNA instead of RNA, miRNA-type off-target effects are also significantly reduced (Ui-Tei et al., 2008).

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

different siRNA duplexes at once against the target gene. In this way, the desired gene silencing is maintained, but hybridization-dependent off-target effects are diluted, since 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 mimics 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 both immune-mediated and hybridization-dependent off-target effects, careful sequence selection is an essential first step (see Choosing the Right Sequence above). Chemical modifications can then provide further protection.

### **CONSIDERATIONS WHEN ASSESSING THE ACTIVITY OF MODIFIED siRNAs**

The wide range of nonspecific and off-target effects that can be triggered by siRNAs can complicate experimental results. Controlling for off-target and immunostimulation effects is an essential aspect of siRNA experimentation. At a minimum, siRNA experiments should include mismatch, scrambled, and nontargeting (e.g., luciferase) controls. These measures control for nonspecific effects. Several positive controls (independent siRNA sequences targeting the same mRNA) should also be used. Unfortunately, these simple controls do not provide the full picture of nonspecific effects caused by siRNA. The following section will look in more detail at ways to rule out nonspecific effects when using siRNAs.

#### **Off-Target Effects**

siRNA off-target effects can significantly affect the phenotypic outcome of gene silencing experiments. Off-target effects can arise from partial complementarity of unintended mRNA targets to either the sense or antisense strand of an siRNA. Several methods have been developed to determine the off-target effects of siRNAs.

Perhaps the most thorough method to observe off-target effects is the use of microarray technology to monitor changes in cellular gene expression following siRNA treatment. (Jackson et al., 2003)

Another technique for observing sense-strand mediated versus guide-strand mediated mRNA targeting involves the use of two psiCheck plasmids containing sequence complementarity to either the sense strand or the guide strand, which allows determination of the extent of sense-strand mediated off-targeting (Ui-Tei et al., 2008).

In order to verify that the observed phenotype resulting from RNAi-mediated gene silencing is a result of gene knockdown and not off-target effects, it is often useful to attempt to obtain the phenotype using different siRNA sequences targeting the same gene. This technique should control for off-target effects since different siRNA sequences have different off-target effects, but in each case, the intended gene should be silenced (Echeverri and Perrimon, 2006). Conversely, rescue experiments can be performed, whereby the target mRNA can be modified to remove complementarity to an siRNA without changing the coded protein (Echeverri and Perrimon, 2006). In the rescue condition, the phenotype should disappear since the mRNA is no longer degraded. If the phenotype remains, it could be attributed to siRNA-mediated off-targeting (Kittler et al., 2005).

Finally, chemically modified siRNAs with reduced off-targeting properties can be used to further verify that effects are arising from specific gene silencing.

#### **Immunostimulation**

siRNA knockdown experiments can be further confused by immunostimulation triggered by siRNA. Indeed, when siRNA is being used to target cancer and viral mRNAs, experimental outcomes must be carefully interpreted due to the high sensitivity many of these diseases have towards innate immune responses (Robbins et al., 2008). When comparisons are made with scrambled controls (duplexes that have been rearranged to prevent base pairing to the mRNA), caution should also be observed due to the possibility that the scrambled controls may possess significantly different immunostimulatory properties in comparison to the targeting siRNAs under study (Robbins et al., 2008). Use of a control siRNA that is less immunostimulatory than the experimental siRNA can lead to false positives or overestimated efficacies, if a phenotype mediated by the innate immune response is attributed to the RNAi pathway (Judge and MacLachlan, 2008). Use of chemically modified analogues of siRNAs can also help to

rule out immune effects, as discussed above. Two recent studies that demonstrated the potential of siRNAs to induce immune effects also rigorously demonstrated that chemically modified siRNA analogues operated through the RNAi mechanism in vivo (Kleinman et al., 2008; Judge et al., 2009).

### Metabolites

Depending on endo and exonuclease cleavage patterns of siRNAs, several degradation products can be created when siRNAs are metabolized in vivo, all of which should be tested for safety. This is equally true for chemically modified siRNAs. Mass spectroscopy analysis following serum incubation is a method for determining metabolic products. Mass spectroscopy is a common tool for oligonucleotide characterization, and effective protocols have been developed for siRNA analysis (Shah and Friedman, 2008; Yan et al., 2008). The ultimate test of safety in humans, however, is clinical trials. Several siRNA clinical trials are underway and include both modified and unmodified duplexes.

### CONCLUSIONS

The chemical modification of siRNA is a rapidly developing field of study. New and exciting advancements are reported on a regular basis. The discovery of siRNA-mediated gene silencing by short double-stranded RNAs generated tremendous interest in the therapeutics field. Nevertheless, the progression of siRNAs from discovery to clinical application has been hampered by a number of obstacles, including stability issues, cellular uptake problems, and undesirable side effects arising from off-targeting and immunostimulation. Encouragingly, in the 8 years since the discovery of siRNA, a vast array of chemical modifications has become available to the nucleic acid chemist. Indeed, rapid development in this field, owing in no small part to the successes of research on previous and ongoing antisense technologies (Corey, 2007), has provided the understanding and tools with which siRNA therapeutics may begin to realize their full potential.

While rapid progress has been made in this field of study, several frontiers remain to be explored with chemically modified siRNAs. Most of the currently used strategies have significant sequence dependence; thus, a remaining challenge is to find universal chemical modification strategies, or to elucidate rules

to allow reliable prediction of which modifications and architectures will be effective for a given sequence. Significant advances in siRNA delivery technologies have also been achieved, and in combination with chemical modification strategies, the prospects for siRNA therapeutics are looking up.

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