

# RNA interference and chemically modified small interfering RNAs

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RNA interference (RNAi) is a powerful biological process for specific silencing of gene expression in diversified eukaryotic cells and has tremendous potential for functional genomics, drug discovery through *in vivo* target validation, and development of novel gene-specific medicine. The future success of this technology relies on identifying appropriate chemical modifications to improve stability, potency and *in vivo* cellular delivery. The present review summarizes the role of the chemist's toolbox in this emerging technology.

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## Abbreviations

<b>ENA</b>	ethylene-bridge nucleic acids
<b>LNA</b>	locked nucleic acid
<b>MOE</b>	2'-O-methoxyethyl
<b>RISC</b>	RNA-induced silencing complex
<b>RNAi</b>	RNA interference
<b>siRNA</b>	small interfering RNA
<b>T<sub>m</sub></b>	melting temperature

## Introduction

RNA interference (RNAi) is an evolutionarily conserved process for the control of gene expression. During gene silencing by RNAi, double-stranded RNA (dsRNA) guides the degradation of mRNAs that are homologous in sequence to the dsRNA. Gene silencing by long 299 base-paired dsRNAs was first observed in *Caenorhabditis elegans* by Fire and Mello [1] and silencing by small RNAs in plants was observed by Hamilton and Baulcombe [2]. The pioneering work on the structure and origin of the small interfering RNAs (siRNAs) came from biochemical analysis in *Drosophila melanogaster* carried out by Zamore *et al.* and Elbashir *et al.* [3,4]. These studies clearly established that dsRNA is processed to 21 or 22 nucleotide siRNAs that in turn mediate gene silencing.

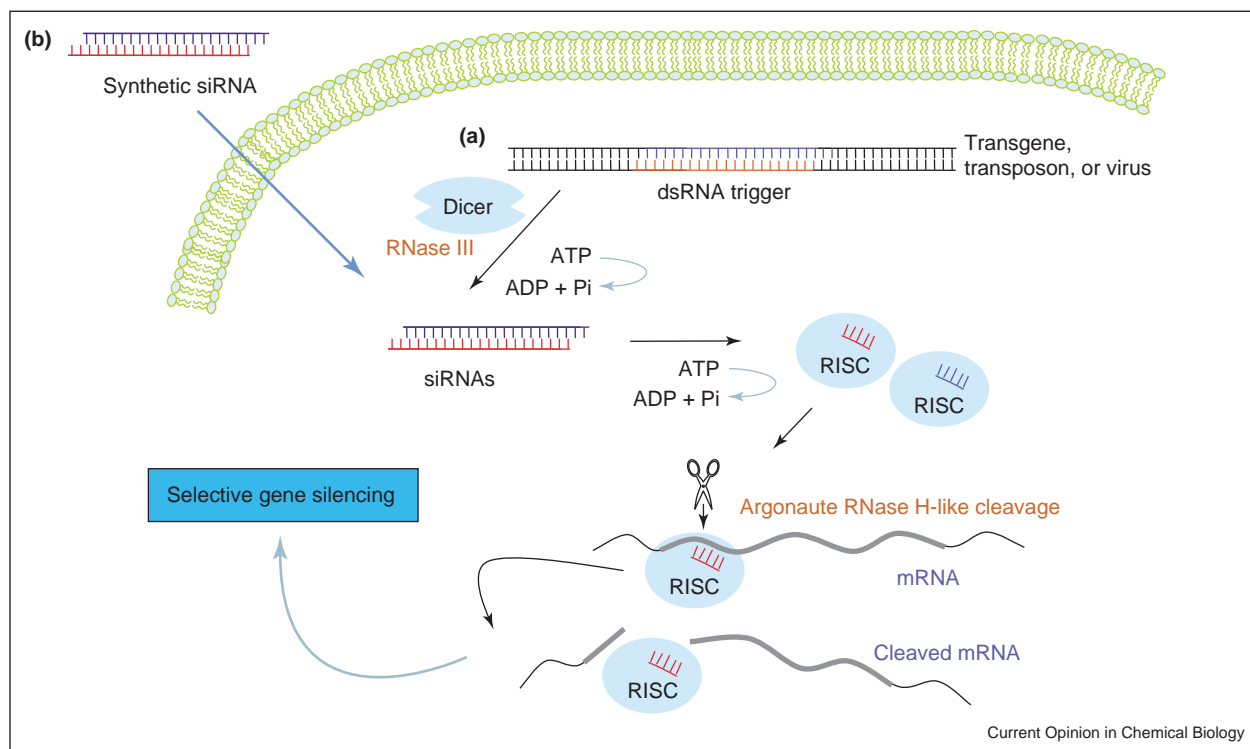
Subsequently, synthetic RNAi has been shown to silence genes *in vitro* in cultured human cells [5]. After the RNAi assembly into a protein–RNA complex (described below in detail), one of the strands of the siRNA duplex, the antisense strand or guide strand, hybridizes to the mRNA through formation of the specific Watson–Crick base pairs. The RNAi pathway activated by the hybridization of the guide strand results in degradation of the targeted RNA. Thus, a disease-causing protein, for example, is not produced [6–9]. This review attempts to summarize the mechanism of RNAi and describes some of the biological challenges associated with using synthetic siRNAs to effect RNAi, different chemical modifications being used to overcome these challenges, and future prospects.

## Mechanism of RNA interference

The underlying mechanism of RNAi is probably evolved from a natural defense mechanism against foreign DNA that is transcribed in cells into long dsRNA; dsRNA is also a key intermediate in the life cycle of many viruses. Unlike the cells of simpler organisms, such as plants and insects, mammalian cells react to the presence of long strands of dsRNA by triggering cellular suicide. Long dsRNA (independent of its sequence) is known to induce the interferon response that can lead to cell suicide. Concomitant with the interferon response, it is sometimes possible to detect a sequence-specific RNAi response. Researchers have shown that the RNAi pathway is independent of the interferon pathway and is activated in mammalian cells by short synthetic siRNA duplexes of 21–25 base pairs [5]. Analysis of the siRNAs isolated from *Drosophila melanogaster* showed that approximately 50% of the siRNAs were exactly 21 nucleotides in length. The siRNAs had 5' monophosphates and 3' hydroxyl groups and no sequence bias was observed for the 5' and 3' nucleotides at the cleavage sites. When short duplexes were used, gene silencing was more efficient when the fragments had two nucleotide 3' overhangs at each end than when the duplex was blunt ended [4].

The mechanism of RNAi is shown in Figure 1. Within cells, the RNAi process comprises at least four sequential assembly steps. First is the ATP-dependent processing of double-stranded RNA by the action of 'Dicer', an RNase III-family enzyme, into siRNAs. In the natural process (Figure 1a), long dsRNAs are processed to siRNAs. In the second step, these naturally derived siRNAs, or synthetic siRNAs introduced into the cell (Figure 1b), are incorporated into an inactive ribonucleoprotein complex. In the third step, ATP-dependent unwinding of the siRNA

Figure 1



The mechanism of RNAi. RNAi has been observed in plants, fungi, mammals, worms, and flies and offers significant therapeutic potential.

(a) RNAi as a natural process. (b) RNAi using synthetic siRNAs.

duplex generates an active RNA-induced silencing complex (RISC). ATP is used to maintain 5' phosphates on siRNAs. Finally, in an ATP-independent reaction, the RNA target is cleaved. RISC is a sequence-specific endonuclease complex that contains Argonaute proteins and the single-stranded guide siRNA strand. RISC can cleave target RNAs with multiple turnovers without additional ATP [10,14,15] (and reviewed in [11–13]). The high specificity of the process results from the Watson–Crick base pairing of the siRNA antisense oligonucleotide with the target mRNA. The mRNA cleavage products are presumably degraded due to the lack of either a poly (A) tail or 5'-cap [16]. Very recently it was demonstrated that the endonuclease, or 'Slicer', activity responsible for mRNA cleavage is exclusively associated with a particular member of the Argonaute family, Ago2 [17,18]. Recent crystal structures indicate that the Argonaute PIWI domain responsible for the 'Slicer' activity is very similar to known RNase H structures [19].

In a 21-mer siRNA duplex with mRNA, the cleavage position on the mRNA is located between nucleotides 10 and 11 when counting from the 5'-end of the antisense strand upstream from the target nucleotide paired to the 5' terminal of the antisense strand [16]. Recently the Tuschl and Zamore groups [20,21] independently

established the mechanism of mRNA cleavage by RISC. The Tuschl group used affinity-purified minimal RISC from human cells and showed that cleavage proceeds via hydrolysis and the release of a 3'-hydroxyl and a 5'-phosphate. Zamore's group confirmed the cleavage products and found that the RISC-mediated cleavage requires  $Mg^{2+}$  and that  $Ca^{2+}$  will not substitute [21]. Moreover, a single phosphorothioate in place of the scissile phosphate blocked cleavage; the phosphorothioate effect was rescued by the thiophilic cation  $Mn^{2+}$ , but not by  $Ca^{2+}$  or  $Mg^{2+}$ . These results suggest that during catalysis,  $Mg^{2+}$  is bound to the RNA substrate through a non-bridging oxygen of the scissile phosphate. In this regard, the mechanism is similar to that of RNase H and other RNase III superfamily enzymes.

## Design of siRNA

### Sequence considerations for design of siRNA

Generally, each strand of the siRNA duplex is 21 nucleotides in length, designed with two nucleotide 3' overhangs on each strand (the double overhang structure). It is the natural structure derived from the RNAi mechanism described above. Sequence constraints mostly derive from synthetic considerations, either to simplify chemical synthesis or to make use of pol III expression vectors. As with primer/probe design, a G/C content of around 50% is

usually selected and oligonucleotides with three or more of any purine nucleotide in a row are avoided. A BLAST search is carried out to ensure that the chosen sequence does not target unwanted genes.

The sites of active duplexes are not predictable, but several groups have systematically studied numerous siRNAs targeting particular messages in attempts to correlate duplex features with function. In the first comprehensive siRNA 'gene walk' study published, Harborth *et al.* evaluated 25 duplexes targeting the open reading frame and 3'-untranslated region of lamin A/C [22]. The G/C content of the oligonucleotides varied from 32% to 74% and had 3' dTdT overhangs. All siRNAs substantially reduced message levels, with 23 of the 25 reducing expression by >75%. There was no bias based on position within the message. It has been observed that siRNA efficiency varies with small positional shifts [22,23]. The positional effects could be due to many factors. Possibilities include competition for binding with protein factors, thermodynamic stability, or sequence-dependence of the RISC complex. Naturally, the structure and accessibility of the target message will also play an important role [24].

Recently, prediction algorithms have begun to offer some guidance for siRNA sequence selection [25]. Khvorova *et al.* [26] and Schwarz *et al.* [27] have shown that the most effective siRNAs tend to have weaker base pairing at the 5' antisense side of the duplex relative to the 3' antisense side of the duplex. Reynolds *et al.* [28] have proposed several characteristics that correlate with efficacy from the dataset of Khvorova *et al.* [26]. Reynolds *et al.* [28] used their criteria, including base composition, strength of base pairing at particular positions in the duplex, overall thermodynamic stability, and base identities, to select siRNA duplexes against five genes. The duplexes selected using their criteria had a mean percent silencing of 76% while those selected randomly gave only 39% mean silencing. Whereas introduction of mismatches in the central region of the antisense strand abolished silencing activity [16], the sense strand can tolerate limited mismatches [29]. Schwarz *et al.* have intentionally incorporated mismatches in the terminal four base wing region between the 5' end of the antisense strand and the 3' end of the sense strand to improve activity [27]. These so called 'forked' siRNAs gave a mean silencing of 87% whereas non-mismatched duplexes had a mean silencing of 75%.

In *Drosophila*, silencing was more efficient when the dsRNA had two nucleotide 3' overhangs at each end than when the duplex was blunt ended [3]. Most researchers still construct siRNA duplexes with 3'-TT overhangs (the 'Tuschl Design') on both strands. Alternative designs are possible: for example, siRNAs without 3' overhangs were active in silencing in mammalian cells [30], and single 3'-overhang structures in the antisense strand were also active [31]. Several groups have also shown that dsRNA

joined by a hairpin loop can effect target RNA cleavage through the RNAi pathway [22,32–34]. As single-stranded RNA is much more susceptible to nuclease degradation than double-stranded, and the recruitment by the RISC seems to require siRNA duplexes, most antisense-only single-stranded siRNAs have significantly less activity than the corresponding duplexes [35].

### Chemically modified siRNAs: need for chemical modifications

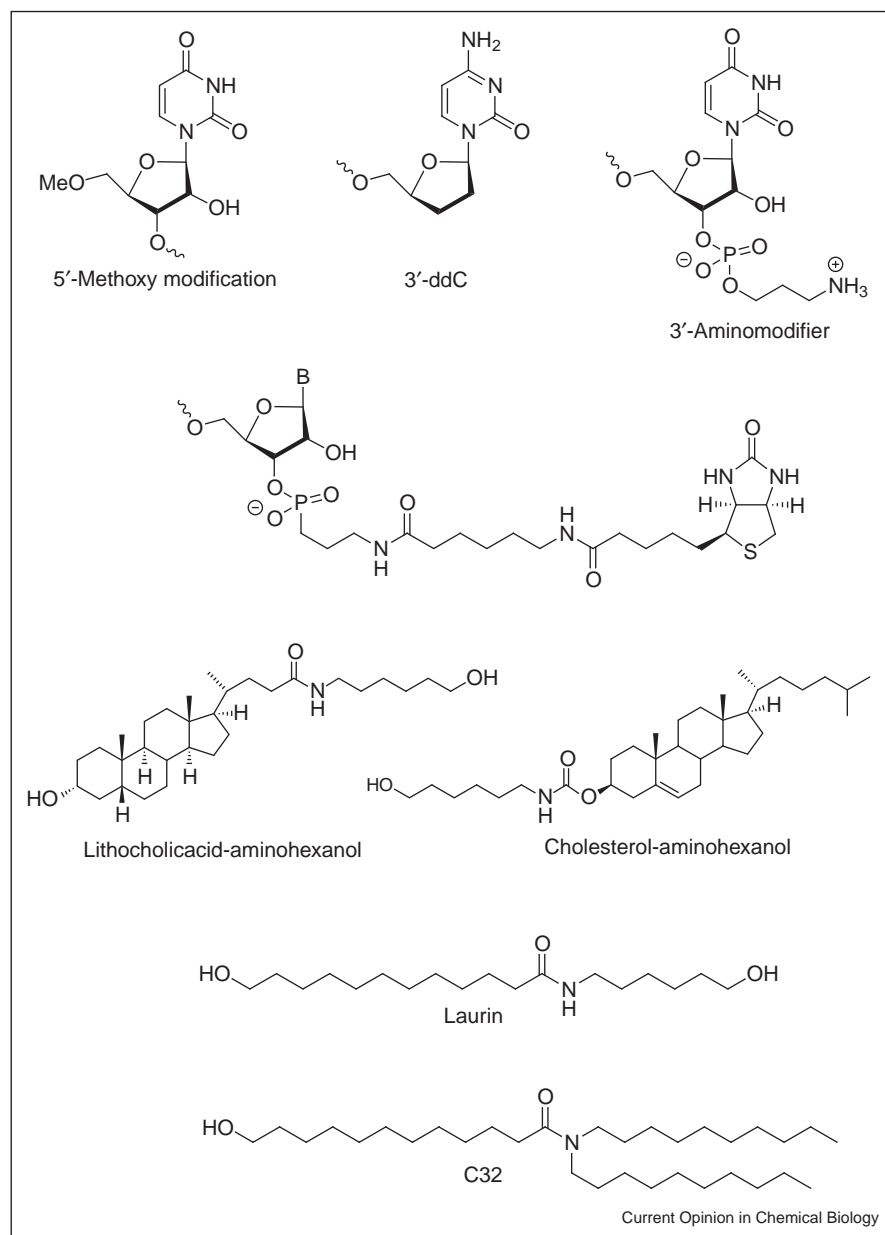
Although the short double-stranded RNA molecules used for silencing have a relatively high stability and activity in cell culture, there are important reasons why chemical modification of one or both of the strands may be desired [36–38]. First, further enhancement of thermodynamic and nuclease stability are important for therapeutic applications. Second, modifications will be required to increase the half-life of the siRNA duplexes in circulation *in vivo*. Third, chemical modifications are needed to improve the biodistribution and pharmacokinetic properties of siRNAs and to target siRNA to certain cell types. Fourth, one can envision improving the potency of siRNA with appropriate chemical modifications in terms of target binding affinity, favored conformational features of the modification such as RNA-like sugar pucker of the nucleoside (C3'-endo) and A-helix geometry, altering on-rates and off-rates of hybridization, and enhancing the product release. However, in designing chemical modifications, the safety issues of the chemical modifications and their cost must also be addressed.

### Chemical modification of the termini and conjugate groups

A 5'-phosphate group on the antisense strand, but not on the sense strand, is required for cleavage of target duplex [10,15]. Synthetic RNA bearing a 5'-hydroxyl group on the antisense strand can mediate RNAi function, as the RNA is phosphorylated by an endogenous kinase. When the 5'-hydroxyl termini of the antisense strands were blocked as 5'-OMe or other functionalities (Figure 2), silencing was abolished [10,15,35]; 5' modification of the sense strand had no effect [30,39]. As the antisense siRNA with a fluorescent label conjugated to the 5' phosphate group via a 6-carbon linker had the same activity as the unmodified oligonucleotide [22] and an siRNA with the antisense strand modified at the 5' end by a 6-amino-hexyl phosphodiester was effective [15], it appears that modifications that retain the 5' phosphodiester linkage are tolerated.

Structural studies [19,40] indicate that the 2-nucleotide 3' overhangs used for most siRNA duplexes appear to be specifically recognized by the PAZ protein domain, found in Dicer RNase III as well as in Argonaute proteins. Interactions with this domain must contribute to the formation and possibly also the stability or binding of the guide (antisense) siRNA strand in RISC. Synthetic

Figure 2



Illustrative terminal modifications evaluated.

siRNAs usually have two deoxy-thymidines at the 3' termini of both strands. However, the available data regarding modification of the 3' terminus of the antisense strand of the siRNA duplex is not conclusive. In mammalian cells, unpaired nucleotides at the 3' terminus were not required [30]. When the 3' end of the antisense strand and both termini of the sense strand were modified with either an inverted deoxy abasic residue or an amino group attached through a 6-carbon linker no reduction in activity was observed [30]. 3'-Puromycin and 3'-biotin modifications of the antisense strand had little or no effect on

activity [39]. When the antisense strand was modified at the 3'-end with ddC and an aminopropyl group was attached at the 3' end via the phosphate group, the siRNA was active [15]. By contrast, siRNA duplexes lost all activity when the 3'-end of the antisense strand was modified with either 2-hydroxyethylphosphate or 2'-O,4'-C-ethylene thymidine [29]. 2-hydroxyethylphosphate was tolerated in the sense strand [29].

Certain terminal conjugates have been reported to improve cellular uptake (Figure 2). For example, siRNAs

conjugated at the 5'-end of the sense strand with cholesterol, lithocholic acid, lauric acid or long alkyl branched chains ('C32') improved *in vitro* cell permeation in liver cells [31]. Certain fluorescent chromophores did not perturb gene silencing when conjugated to the 5'-end or 3'-end of the sense siRNA strand or the 5'-end of the antisense siRNA strand, but conjugation to the 3'-end of the antisense siRNA abolished gene silencing [22].

siRNA conjugated to TAT peptide or TAT-derived oligocarbamate at the 3'-end of antisense strand had efficient RNAi activity and perinuclear localization in HeLa cells similar to activity and localization of siRNA duplexes delivered by a transfection agent, but distinctly different from free TAT peptide nucleolar localization. The silencing activity was observed without the need for transfection agent, although a higher concentration was needed compared with normal transfection [41].

## Chemical modifications within the oligonucleotides

### Backbone modifications

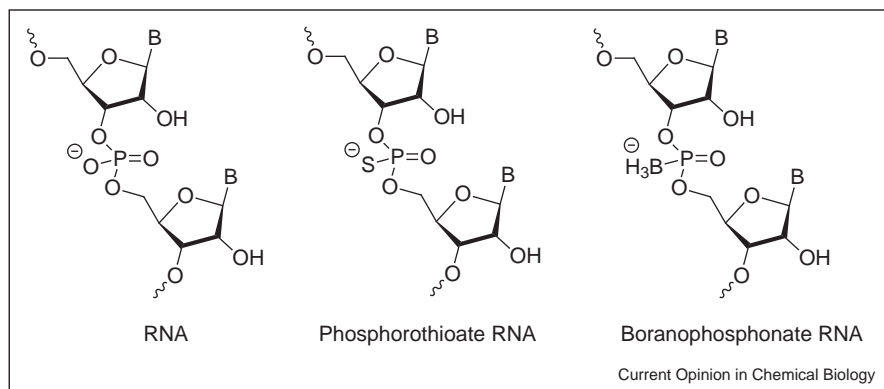
In general, backbone modifications cause a small loss in binding affinity, but offer nuclease resistance and may alter pharmacokinetic behavior of oligonucleotides. Harborth *et al.* [22] targeted endogenous lamin A/C mRNA in human HeLa or mouse SW3T3 cells, with siRNA duplexes that contained phosphorothioate (P=S) backbone modifications (Figure 3). This modification did not significantly affect silencing efficiency [22,42], although cytotoxic effects were observed when every second phosphate (P=O) of an siRNA duplex was replaced by phosphorothioate. Complete replacement of P=O by P=S also caused cytotoxicity [42]. Braasch *et al.* showed that phosphorothioate linkages did not significantly enhance siRNA nuclease stability and reduced the melting temperatures of the duplexes as compared with unmodified RNA [43]. In another study, P=S modification was shown to reduce siRNA activity [44].

RNAs with a boranophosphonate backbone (P=B, Figure 3) have been synthesized using T7 RNA polymerase from nucleotide triphosphates with specific Rp linkages [45]. The resultant compounds have Sp linkages and are chirally pure. They show improved nuclease resistance relative to racemic phosphorothioates and obviously have more nuclease resistance than unmodified RNAs. The chimeric 5'-modified compounds were resistant to digestion with the 5'-exonuclease bovine spleen phosphodiesterase. The oligonucleotides are lipophilic and maintain negative charge, which may contribute to important pharmacokinetic and pharmacodynamic properties. The P=B linkages were better tolerated in the sense strand than in the antisense strand of the siRNA duplex and exhibited good inhibition of green fluorescence protein activity [45]. While modifications in the central core region were not as well tolerated, siRNAs with many P=B modifications in the terminal regions of the oligonucleotides (equivalent to the 'wings' of antisense 'gappers') retained activity and silencing was observed for a longer time than with unmodified siRNAs.

### 2'-sugar modifications

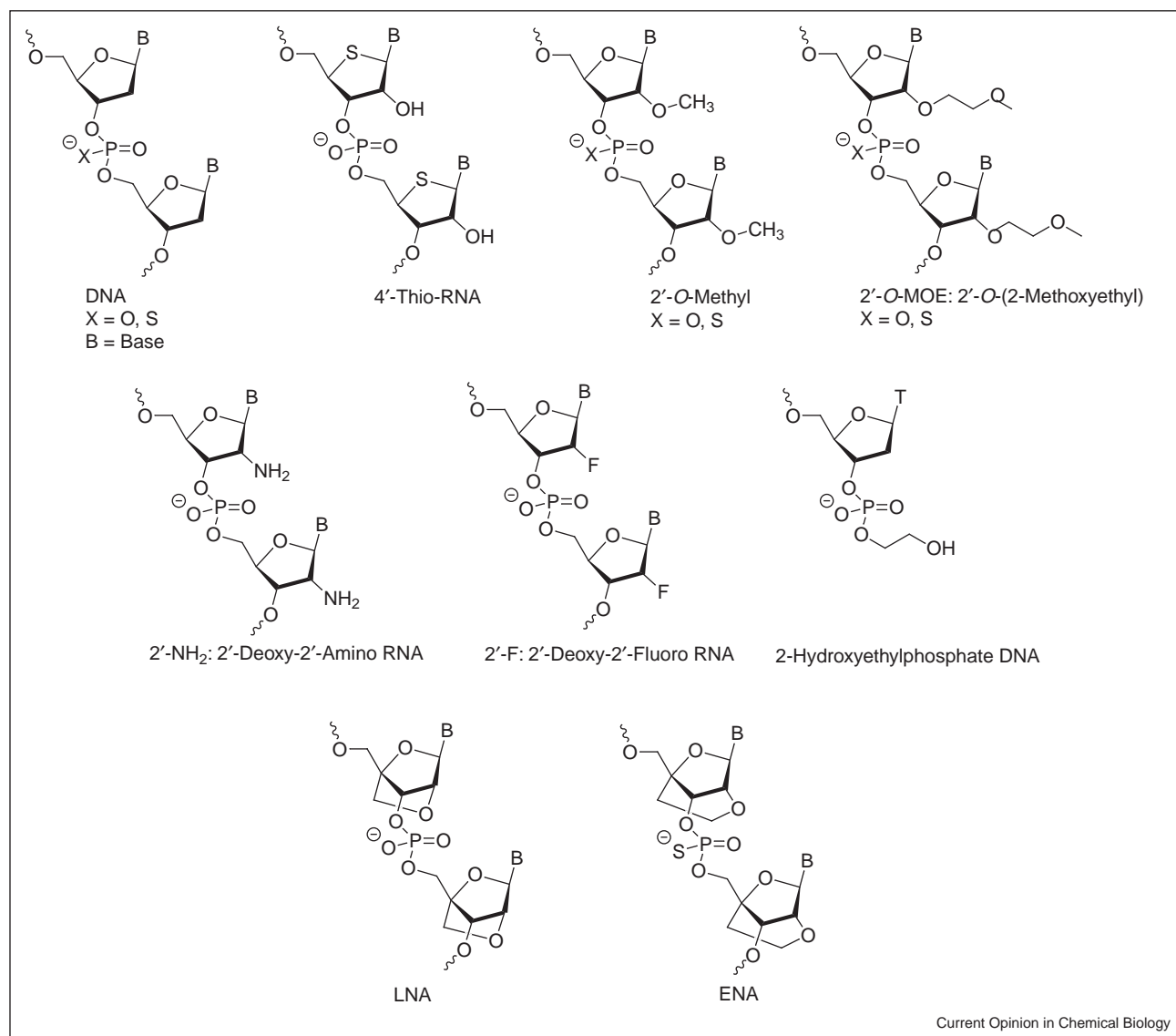
Silencing by siRNA duplexes is compatible with some types of 2'-sugar modifications within the strands and effects depend on whether the sense or antisense strand is modified. Some of the sugar modifications (2'-H, 2'-OMe, 2'-F, 2'-NH<sub>2</sub>, 2'-O-MOE, locked nucleic acid (LNA) and ethylene-bridge nucleic acids (ENA) evaluated are shown in Figure 4. Among these modifications, 2'-F, LNA and ENA provide significant increases in target binding affinities (with RNA hybridization melting temperature differences,  $\Delta T_m$ , of 2 to 4°C per modification), others did not have much effect, and 2'-H cause a decrease in  $T_m$  ( $\Delta T_m = -0.5^\circ\text{C}$  per modification). Furthermore, but not surprisingly, the duplex must retain conformationally RNA-like A-type helical characteristics for effective gene silencing. However, conformational features alone do not decide the activity: even in A-helix forming RNA-DNA

Figure 3



Backbone modifications evaluated.

Figure 4



Sugar modifications evaluated.

heteroduplexes, the activity was almost completely abolished when either of the strands was 2'-deoxy, but 2'-deoxy residues were tolerated in the overhangs and at the terminal ends [16,46]. Almost complete activity was retained if the 5' region of the antisense strand was free of modification [44]. On the other hand, several laboratories have shown that the 2'-fluoro modification with a high preference for C3'-endo sugar pucker, was well tolerated with or without a phosphorothioate backbone [22,43,44]. These results support the idea that the 2'-F modification is well accommodated during the RNAi pathway. Capodici *et al.* treated HIV-infected activated CD4(+) T cells with a 2'-F modified siRNA. The modified siRNA was resistant to RNase A, and the authors' observed activity without transfection agent. Under these

conditions, inhibition of HIV infection was observed while the unmodified siRNA was ineffective [47].

In another study, 2'-F modified siRNAs enhanced serum stability in cell culture and showed efficacy in silencing luciferase expressed from a co-injected plasmid in mice [48]. However, even though the modified siRNAs have greatly increased resistance to nuclease degradation in plasma, this increase in stability did not translate into enhanced or prolonged inhibition of target gene expression in mice following tail vein injection. It appears that 2'-F modified siRNAs are functional *in vivo*, but are not more potent than unmodified siRNAs in animals. The unfavorable, pharmacokinetic properties of the 2'-F-modified compound are likely to be the reasons, and



they compromised the pharmacodynamic features of this modification.

Even though it has a *C3'*-endo sugar pucker, the 2'-*O*-methyl modification ( $\Delta T_m$ , ca. 0°C to 1°C per modification) was not tolerated when oligonucleotides were fully modified [16,30,43,44]. Incorporation of two to four nucleotides in either strand was well tolerated. When every other nucleotide was modified with 2'-*O*-methyl, oligonucleotides were relatively resistant to serum-derived nucleases and significant enhancement in activity was observed [30]. In a different study, full replacement of 2'-OH by alternating 2'-OMe/2'-F improved the activity of an siRNA duplex significantly with an increase in nuclease resistance [49].

The 2'-*O*-allyl modification in the 3'-unpaired nucleotides had no effect on activity [42]. The 2'-*O*-methoxyethyl (MOE) terminal modification with P=S linkage has been used to improve nuclease resistance in certain local delivery applications [50]. In a study targeting the PTEN gene, the MOE modification was tolerated in the antisense strand of the siRNA duplex. It was shown that appropriate placement of the MOE modification, for example, an alternating MOE/OH format or several MOE at the termini (as in an antisense 'gapmer'), led to improved activity [51].

The 2'-amino modification has not been widely studied. One study reported some loss of activity when the mod-

ification was incorporated in the sense strand, and severe loss when the modification was in the antisense strand [46]. As the study involved long dsRNA in *C. elegans*, the loss of activity could have arisen from lack of efficient cleavage of the transcript by Dicer or at the amplification step. Additional studies with this modification are warranted.

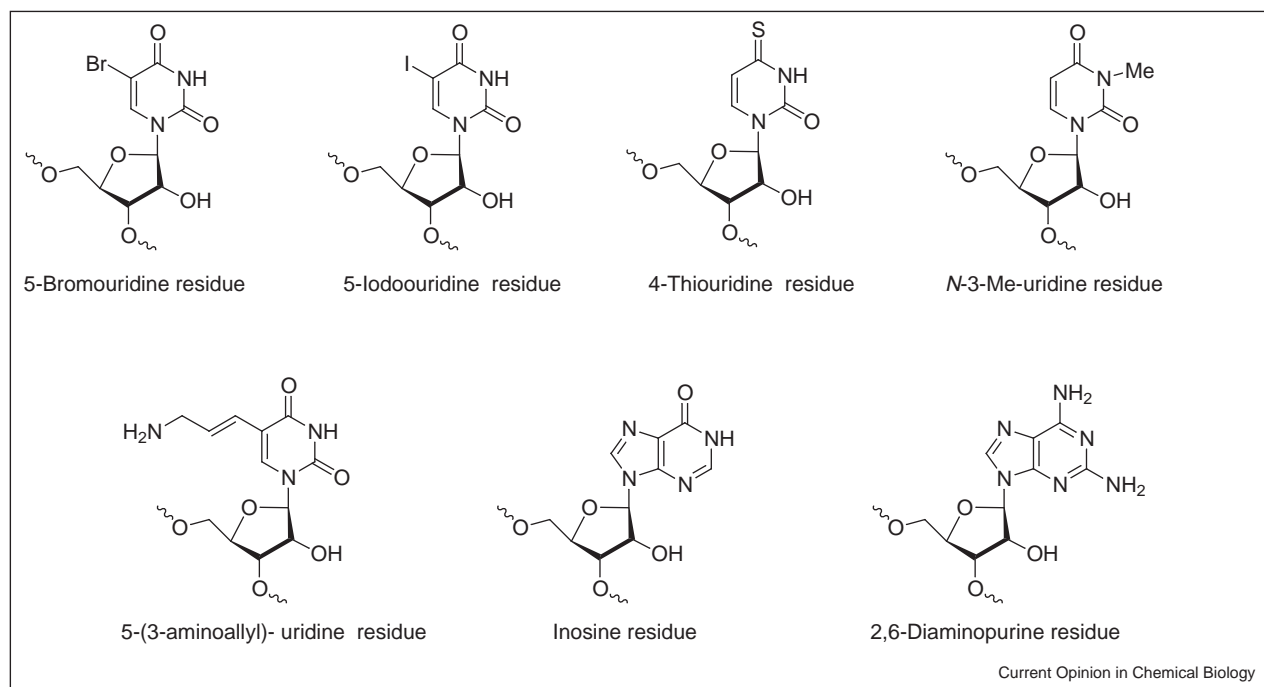
#### 4'-Thio-RNA

4'-Thio-RNA modifications showed nearly 1°C per modification increase in  $T_m$  in a hybridization assay. When both strands were modified, the increase was even higher. However, the increase in  $T_m$  per modification was less than that observed for 2'-OMe RNA for the same sequence. In a nuclease assay using isolated enzymes having 5'-exonuclease, 3'-exonuclease, endonuclease S1, or ribonuclease A activity, 4'-thio RNA showed very high nuclease resistance in each case. For gene silencing, this modification was better tolerated in the sense strand than in the antisense strand. In the sense strand, the modification was most effective when it was located at the termini of the strand rather than in the center [52,53]. In another study, the 4'-thio modification was tolerated at the terminal ends of antisense and showed enhanced nuclease resistance [54].

#### LNA modification

The very high binding affinity modification LNA (Figure 4) has been evaluated as a modification for siRNA. Duplexes modified with LNA at the 5'-termini, the 3'-termini, or

Figure 5



Nucleobase modifications, evaluated.

both in both strands, were able to block gene expression [43]. This is in contrast with data from Hamada *et al.* [29] who did similar experiments with 2'-O,4'-C-ethylene thymidine, a component of ethylene-bridge nucleic acids (ENA, Figure 4), at the termini of siRNA duplexes and observed complete loss of activity with ENA. An siRNA duplex with certain wing region LNA modifications was able to block protein expression with efficiency equal to the unmodified duplex [43]. As this duplex had a melting temperature of over 95°C, the experiment demonstrated that the  $T_m$  of duplexes can be extremely high without affecting the function of the RISC. This is consistent with the assumption that ATP hydrolysis drives the separation of the siRNA strands [10]. Some duplexes with LNA modifications in the central core were inactive. Both number and placement of LNA modifications affect activity, and the inactive duplexes apparently do not bind to RISC as they did not inhibit silencing of a control gene.

### Nucleobase modifications

The nucleobase modifications evaluated in siRNA duplexes are shown in Figure 5. In their work in *C. elegans*, Parrish *et al.* showed that while 4-thiouridine and 5-bromouridine residues were tolerated in both sense and antisense strands of siRNA duplexes [46], bulky 5-iodo or cationic 5-(3-aminoallyl) modifications were not well tolerated. Replacement of guanosine by inosine reduced the activity of siRNA duplexes [46]. As this analysis was carried out with *in vitro* transcribed long dsRNA, the lack of gene inhibition could be due to inhibition of Dicer processing rather than to the base modification. In a different study in HeLa cells, *N*-Me-uridine in place of uridine abolished the siRNA activity [44], and as a general conclusion from this study, all base modifications produced deleterious effects of various magnitudes compared with the wild-type RNA. Surprisingly, this was the case with even 2,6-diaminopurine in place of adenine [44].

### Perspectives

RNAi holds great promise as a tool for understanding biological functions of genes and as a therapeutic. Chemical modification of one or both strands of the siRNA duplex has allowed numerous groups to understand the mechanism of siRNA processing and target cleavage. The effects of chemical modifications are being evaluated and certain rules are emerging, although some mixed observations exist. This may be due to differences among the gene targets employed or to the different sequences employed. Repeating the experiments with well-characterized siRNA duplexes in multiple targets and with multiple sequences will yield valuable information for better design of chemical motifs for efficacious siRNAs. Crystal structures of enzymes in the RISC pathway are being solved and RNA docking has allowed further insight into the mechanistic path-

ways [18,19,40]. Chemical modifications can be designed for efficient RNAi using the results from these studies. Apart from such 'rational drug design' approaches, chemical modifications will also be needed to improve pharmacokinetic properties of siRNA in order for effective use in human therapeutic applications. The issues associated with tissue and cell-specific *in vivo* delivery of siRNAs have not been rigorously analyzed. The siRNA duplexes have at least 40 negative charges, high molecular weight and are very hydrophilic, and chemical modification will be required to allow broad biodistribution and effective cell permeation. Because of interest in antisense RNA and ribozymes over the past decade, the efficiency of RNA synthesis and analysis have improved considerably, but to be practical and competitive more efficient synthetic processes, especially with better protecting groups, improved purification techniques, and faster analytical methods need to be developed.

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