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Review

Designing Chemically Modified Oligonucleotides for Targeted Gene Silencing

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Oligonucleotides (ONs), and their chemically modified mimics, are now routinely used in the laboratory as a means to control the expression of fundamentally interesting or therapeutically relevant genes. ONs are also under active investigation in the clinic, with many expressing cautious optimism that at least some ON-based therapies will succeed in the coming years. In this review, we will discuss several classes of ONs used for controlling gene expression, with an emphasis on antisense ONs (AONs), small interfering RNAs (siRNAs), and microRNA-targeting ONs (anti-miRNAs). This review provides a current and detailed account of ON chemical modification strategies for the optimization of biological activity and therapeutic application, while clarifying the biological pathways, chemical properties, benefits, and limitations of oligonucleotide analogs used in nucleic acids research.

The concept of using synthetic oligonucleotides (ONs) to control the expression of specific genes dates back to the late 1970s, when Zamecnik and Stephenson first demonstrated targeted gene silencing using a short synthetic oligonucleotide (Zamecnik and Stephenson, 1978; Stephenson and Zamecnik, 1978). From these seminal studies emerged the ''antisense'' approach for targeted gene silencing. Here, an exogenous synthetic ON (termed an antisense ON, or AON) complementary to a target mRNA is introduced into cells with the intent to block gene expression through either translational inhibition or enzymatic cleavage of the mRNA target. AON-based therapeutics have been under clinical investigation for more than 30 years, achieving one approved drug (Fomivirsen/Vitravene). The gradual progress of AON therapeutic development has led to some hesitation regarding the viability of the platform. Subsequent to Zamecnik's discoveries, elucidation of the RNA interference (RNAi) pathway for modulation of gene expression, and the role of small interfering RNAs (siRNAs) in the process, changed our understanding of posttranscriptional gene expression control, and renewed excitement in the nucleic acid therapeutics community (Fire et al., 1998; Elbashir et al., 2001).

It is well established that properly designed AONs or siRNAs can cause cleavage of specific messenger RNA (mRNA) strands, taking advantage of endogenous cellular pathways to potently silence the expression of specific genes. These synthetic ON-directed approaches target mRNAs directly, before translation, eliminating the need for protein/enzyme inhibition using small molecules. Although siRNAs and AONs are perhaps the most commonly discussed ON-based agents under development, they are certainly not the only promising short synthetic ONs. Other relevant ON agents include microRNA-targeting ONs (anti-miRNAs) and antagomirs (discussed below), aptamers (Keefe et al., 2010), DNA/RNAzymes (Chan and Khachigian, 2009; Mulhbacher et al., 2010), exon-skipping and splice-switching compounds (Kole et al., 2012; Saleh et al., 2012), and immunostimulatory nucleic acids (Barchet et al., 2008).

The most significant of obstacles impeding the path to ON therapeutics include (1) their poor extracellular and intracellular stability, (2) low efficiency of intracellular delivery to targets cells or tissues, and (3) the potential for ''off-target'' gene silencing, immunostimulation, and other side effects. Fortunately, in attempts to overcome the therapeutically limiting features of DNA and RNA, a vast array of ON chemical modifications has been developed. These nucleic acid analogs are often rationally designed, allowing specific alterations to many of the inherent properties of ONs affecting their biological application and potency (e.g., target binding affinity, nucleoside/nucleotide/duplex conformation, hydrophobicity, enzyme interaction, nuclease resistance, and immunostimulatory properties).

Keeping with the aim of this review, biological pathways relevant to AON, siRNA, and anti-miRNA ON agents are briefly described, providing a foundation for subsequent discussions of therapeutic ON analogs. The underlying chemistries driving innovative strategies to optimize ON therapeutics are the focus of this review, and are best considered in relation to the biological pathways in which these ONs function. Concepts essential to the design of AONs, siRNAs, and anti-miRNAs are presented, along with perspectives on the current and future state of the art. Combining advances in biological understanding with chemical know-how is key to advancing this promising field.

Biological Pathways

Although specific details involved in some of the biological pathways described remain to be elucidated, the fundamental events of these processes are now understood to the point that chemical modifications can be utilized in an effort to enhance the potency and therapeutic potential of ONs. Here, AON-mediated gene silencing, miRNA- and siRNA-induced gene knockdown through RNAi, and gene expression modulation by anti-miRNAs are introduced. Focus is given to mechanism and nucleic acid-enzyme interactions where possible, such that subsequent discussions of oligonucleotide modifications and pathway compatibility can be made.

AON-Mediated Gene Silencing

AONs are perhaps the oldest and most studied class of gene-targeting ONs. Indeed, AONs have been reported on and studied

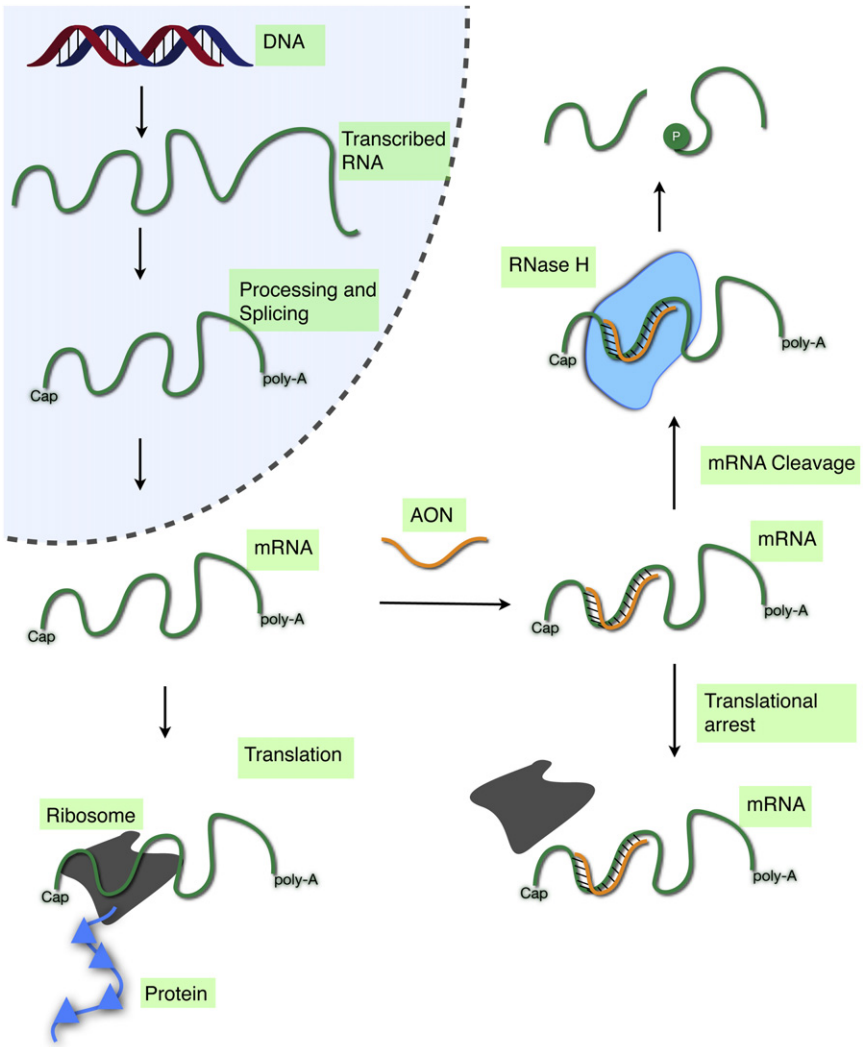

Figure 1. Fundamentals of mRNA-Targeting by AONs
Exogenously introduced AONs can recognize and bind to target mRNA sequences. This can result in either mRNA cleavage via recruitment of RNase H1 (in humans), or translational arrest. In both situations, the result is downregulation of gene expression. Although shown in the cytoplasm, RNase H may also function in the nucleus.

for nearly two decades before the initial reports on the RNAi pathway appeared. AON-based therapeutics have seen periods of both optimism and pessimism, perhaps partly owing to the longer than anticipated development time (Watts and Corey, 2012). Nonetheless, significant progress has been made in recent years, with one approved drug on the market (Fomiversen) and more than 20 candidates in early to late-stage clinical trials (Sanghvi, 2011; Bennett and Swayze, 2010; Watts and Corey, 2012).

A wide variety of RNA-targeting ONs can be considered as AONs, although they may act through different mechanisms. Specific and strong AON recognition and binding to the mRNA target is accomplished through Watson-Crick base pairing, which may or may not be augmented by chemical modifications to the AON internucleotide phosphate linkages, backbone sugars, or nucleobases. The majority of AONs can be divided into one of two groups: those that direct cleavage of the target mRNA, and those that alter mRNA translation without causing mRNA cleavage. The basic concepts of these two modes of action are outlined in Figure 1. In both cases, the cellular target of the AON is a region of the mRNA from the gene of interest.

Initially, the concept of gene silencing AONs that do not induce mRNA cleavage as part of their biological function appears to be quite straightforward: AONs are chemically modified to have strong binding affinity to their mRNA targets, and function by binding RNA tightly, preventing ribosomal assembly (Bennett and Swayze, 2010). Translational inhibition by these AONs is difficult to detect and measure (Bennett and Swayze, 2010), but can indeed be detected and assayed (e.g., via polysome profiling experiments) (Baker et al., 1997). However, the situation can become considerably more complex. In some cases, AONs can be designed to bind mRNA regions that prevent ribosomal assembly at the 5′ cap (Bennett and Swayze, 2010), prevent polyadenylation during mRNA maturation (Bennett and Swayze, 2010), or even affect splicing events (Watts and Corey, 2012; Kole et al., 2012; Saleh et al., 2012).

AONs that achieve gene silencing by directing mRNA cleavage events are widely studied and used in both research applications and in therapeutic development, with several candidates explored in the clinic. In almost all cases, AONs stimulate mRNA cleavage through the recruitment of an endogenous endonuclease known as RNase H. RNase H enzymes, which are involved in essential steps of the DNA replication process, are known to cleave the RNA strand of a DNA-RNA duplex (Stein and Hausen, 1969; Lima et al., 2007; Cerritelli and Crouch, 2009; Bennett and Swayze, 2010). In humans, the specific enzyme recruited by AON-mRNA duplexes is RNase H1 (Wu et al., 2004).

The cleavage mechanism through which RNase H mediates hydrolysis of an internucleotide phosphate linkage in the RNA strand of a DNA/RNA hybrid duplex is under active investigation, and relies on divalent metal ion(s) (Mg^{2+}) for catalysis (Ho et al., 2010; Hollis and Shaban, 2011). A widely accepted mechanism for RNase H phosphodiester hydrolysis involves a model in which two highly coordinated metal cations (often described as "metal A" oriented 3′ to the scissile phosphate, and "metal B" oriented to the 5′) are present in the active site, coordinated by acidic amino acid residues, nonbriding oxygen in the ON

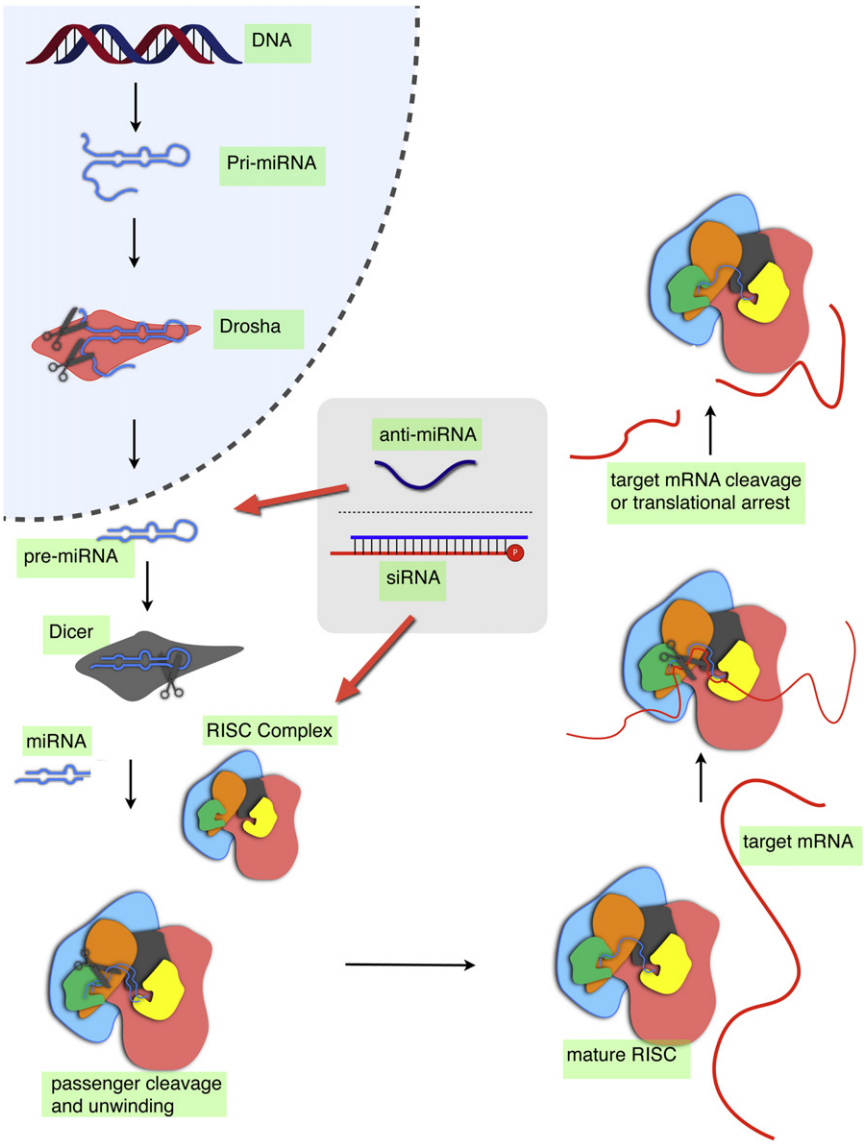

Figure 2. mRNA-Targeting by miRNA and siRNA

Naturally produced miRNAs, loaded into RISC, are capable of recognizing and binding partially complementary mRNAs, especially those with sequence complementarity to the miRNA "seed region." The result is a decrease in gene expression, adding an additional layer of post-transcriptional control over gene expression. Anti-miRNAs can target and inhibit these miRNAs. As well, synthetic siRNAs, mimicking natural miRNAs, can be introduced into cells to utilize the cellular RISC machinery for targeted mRNA gene silencing.

backbone, and water (Ho et al., 2010; Hollis and Shaban, 2011). Metal A may facilitate activation of water for in-line S_N2-like nucleophilic attack on the internucleotide linkage, with metal B in position to stabilize the transition state leading to 3′-OH and 5′-phosphate RNA cleavage products without cleaving the DNA strand. There is also evidence that a third Mg^{2+} cation may be involved in facilitating enzymatic activity, which can help explain the sensitivity of RNase H activity toward Mg^{2+} concentration changes (Ho et al., 2010).

Duplex conformation and flexibility in the AON/RNA hybrid is critical for the stimulation of RNase H activity, and could provide an explanation for the specificity of RNase H for DNA/RNA hybrid duplexes, but not dsDNA or dsRNA (Noy et al., 2004, 2005, 2008). Structurally, dsDNA duplexes are B-form, and do not closely resemble DNA/RNA heteroduplexes. A-form dsRNA is more similar in conformation to DNA/RNA hybrid duplexes, but the rigidity of the two strands in dsRNA duplexes may compromise RNase-H function (Mangos et al., 2003 and references therein).

RNAi-, miRNA-, and siRNA-Mediated Gene Silencing

The RNAi pathway is an important cellular process triggered by endogenous nucleic acids, as a means through which cells can achieve posttranscriptional gene expression control through the use of miRNA expression (Figure 2). RNAi was discovered following the observation that dsRNA sharing sequence with a cellular mRNA (Onc-22) silenced expression of that gene in *Caenorhabditis elegans* (Fire et al., 1998). It was subsequently found that RNAi could be triggered in mammalian cells using 21 nt-long dsRNA duplexes (Elbashir et al., 2001). The RNAi pathway in the mammalian system is the focus in this overview.

A summary of the RNAi pathway is shown in Figure 2. The miRNA triggers of RNAi are typically produced from transcription of intergenic or intronic DNA regions (Duroux-Richard et al., 2011), to form primary miRNA (pri-miRNA) hairpins that are further processed into dsRNA duplexes featuring a hairpin loop with imperfect sequence complementarity, termed precursor miRNA (pre-miRNA). Pre-miRNAs are exported out of the nucleus and processed by an endoribonuclease called Dicer (usually with an associated dsRNA-binding protein, TRBP) to form mature miRNA (for reviews, see Filipowicz et al., 2008; Kawamata and Tomari, 2010; Lennox and Behlke, 2011).

miRNAs, which are 21 nt-long dsRNAs with 2 nt-long 3′ overhangs and imperfect complementarity, are recognized and loaded into a complex of enzymes and proteins known as the RNA-induced silencing complex (RISC). One of the two miRNA strands, called the "guide strand" or miRNA strand, is selected and used in the RISC complex. The other, called the "passenger strand" or miRNA* strand, is discarded. The guide strand is "antisense," or complementary, to the sequence of the targeted mRNA, whereas the passenger strand is "sense" to the mRNA sequence. The RISC complex subsequently finds cellular mRNAs partially complementary to the loaded guide

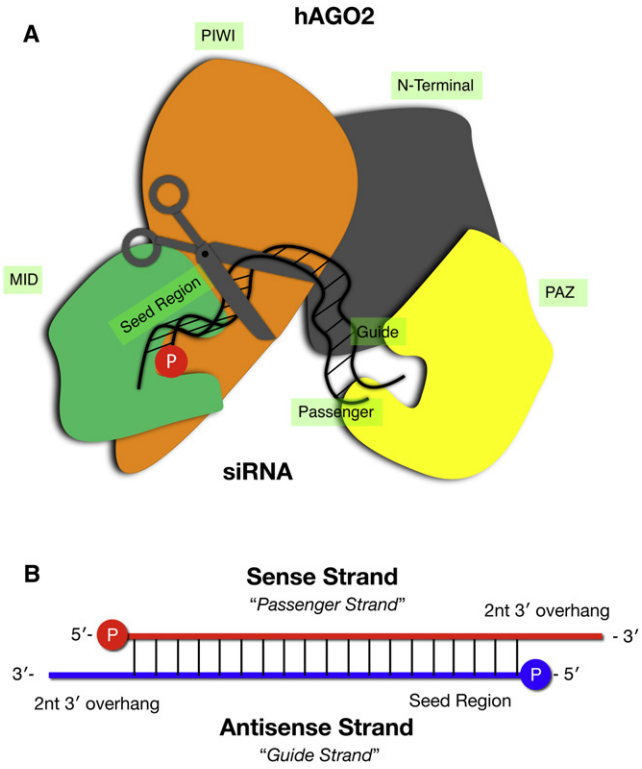

Figure 3. hAGO2 with Loaded siRNA

(A) A simplified diagram of AGO2 domains, and their interaction with siRNA. The MID sub-domain binds the 5′P and nucleotide of the guide strand. The PIWI sub-domain contains the RNase H-like fold with endonucleolytic cleavage activity, which cuts the siRNA passenger strand during loading, and the mRNA during gene silencing. The PAZ domain binds the guide strand 3′OH.

(B) Classical siRNA structure; 21 nt RNA duplex with 2 nt 3′ overhangs. The antisense strand ("guide" strand) is complementary to target mRNA. The sense strand ("passenger" strand) is complementary to the guide strand. The guide strand contains the seed region at the 5′.

strand sequence and prevents translation, either via translational arrest or mRNA cleavage. The extent of sequence complementarity between the miRNA guide strand and the mRNA target is thought to determine whether translation arrest (partial complementarity) or mRNA cleavage (near full complementarity) results from mRNA recognition by RISC (Humphreys et al., 2005). Most miRNAs are only partially complementary to their mRNA targets, thus translational arrest is more common. For the gene silencing event to occur at all, a short region composed of nucleotides 2–8 counting from the 5′ end of the guide strand (the "seed region") must recognize a complementary sequence in the mRNA, usually within the 3′ UTR (Humphreys et al., 2005).

From a chemical perspective, miRNAs are essentially short dsRNA molecules, mimics of which can be readily synthesized. Small interfering RNAs, or siRNAs (Figure 3), are exogenously produced double-stranded RNAs, typically 21–24 nt in length with 2 nt 3′ overhangs, designed to mimic miRNAs. siRNAs function very similarly to miRNAs in gene silencing, and naming convention with siRNAs is the same, the loaded strand is the guide and the discarded strand is the passenger. Unlike miRNAs, siRNAs are designed to share full sequence complementarity with a single target mRNA.

During RISC loading with siRNA, one of the two siRNA strands is loaded into RISC, while the other is cleaved and unwound from the guide strand to be discarded (Matranga et al., 2005; Leuschner et al., 2006), although cleavage is not obligatory (Matranga et al., 2005). In flies, Dicer-2 and R2D2 (an associated dsRNA binding protein) interact with siRNA, and facilitate this loading into RISC (Kawamata and Tomari, 2010; Betancur and Tomari, 2012). In mammals, RISC loading is somewhat less understood, and at least in some cases, Dicer is not required for siRNA loading (Betancur and Tomari, 2012). The strand selection process in which RISC designates guide and passenger strands from duplex siRNAs and miRNAs is based on several factors, including duplex thermodynamics (where the strand with the least tightly bound 5′ end is usually designated the guide strand; Khvorova et al., 2003; Schwarz et al., 2003); the presence of a 5′ phosphate (that is required for binding of the guide strand within RISC; Schwarz et al., 2002); and the sequence at the 5′ of the strands (U and A 5′ nucleotides may be preferred over G and C for AGO2; Frank et al., 2010). After loading, siRNA directs gene silencing through mRNA cleavage and catalytic turnover mediated by a component of RISC, an endoribonuclease called Argonaute-2 (AGO2) (Figures 2 and 3).

A particularly elegant model for siRNA guide strand selection and RISC loading has recently been proposed, in which dsRNA is processed in a catalytic region of Dicer to form an siRNA or miRNA product, which is subsequently released and repositioned to make contacts with the Dicer helicase domain. Dicer interacts with the dsRNA on the less stable end of the duplex, and TRBP (or PACT) on the more stable end, spatially orienting the duplex for a subsequent AGO2 loading step in which TRBP (or PACT) may hand off the stable end of the duplex to the PAZ domain (the guide strand 3′-binding domain) of AGO2 (Noland et al., 2011). This proposed model suggests a mechanism through which guide strand selection can occur based on siRNA duplex thermodynamic asymmetry, corresponding to observations that the siRNA strand with the least tightly bound 5′ end frequently becomes the RISC guide (Khvorova et al., 2003; Schwarz et al., 2003).

The fate of siRNA passenger strand during RISC loading has yet to be fully elucidated. Currently, there are three proposed mechanisms for dealing with the siRNA passenger strand during loading: (1) the siRNA passenger strand is cleaved by the hAGO2 PIWI subdomain upon loading of the guide strand and released (Gaynor et al., 2010); (2) a bypass mechanism independent of passenger strand cleavage exists, in which an ATP-dependent helicase activity is invoked for passenger strand unwinding (Gaynor et al., 2010); and (3) the passenger strand is "nicked" by hAGO2, triggering C3PO (component 3 promoter of RISC) to degrade the remaining passenger strand fragments (Ye et al., 2011). Interfering with passenger strand cleavage through introduction of 2′ modifications at the scissile nucleotide position can significantly impair gene silencing, although not in all cases (Martinez and Tuschl, 2004; Matranga et al., 2005; Muhonen et al., 2007). RISC-mediated ON cleavage produces a 3′-OH fragment and a 5′-phosphate fragment (Martinez and Tuschl, 2004), suggesting a free 2′ hydroxyl group available to form the 2′–3′ cyclic phosphate observed for some other nucleases is not necessary. These observations should be carefully considered

when designing chemically modified siRNAs, especially those modified near the hAGO2 cut site.

Clearly, the RISC complex plays a central role in the RNAi pathway, performing essential functions in RNAi-mediated gene silencing. Optimizing interactions with RISC may be the key to improving the potency of siRNA therapeutics. At the core of the RISC complex lies an endoribonuclease belonging to the Argonaute (AGO) family, which both anchors the loaded guide strand from miRNA/siRNA, and performs the key endonuclease function of the RISC complex via an RNase H-like fold (Liu et al., 2004). In humans, the AGO clade of the Argonaute family has four members: hAGO1, hAGO2, hAGO3, and hAGO4 (Gaynor et al., 2010; Gagnon and Corey, 2012). Of these, only hAGO2 has Slicer-type activity (Meister et al., 2004), and thus hAGO2 is of particular interest as the core catalytic component of RNAi gene silencing.

Human AGO2, hAGO2, has four principal domains: N-terminal, PAZ, MID, and PIWI (see Figure 3) (Jinek and Doudna, 2009; Gaynor et al., 2010; Gagnon and Corey, 2012). When an siRNA is loaded into RISC, the guide strand becomes highly associated with hAGO2. The 3′ end of the guide strand is recognized by the PAZ domain (Ma et al., 2004), and the 5′ nucleotide (with an essential 5′ phosphate) is anchored in the MID domain 5′ binding pocket (Ma et al., 2005; Frank et al., 2010). The 5′ phosphate can be added synthetically or by an endogenous kinase (i.e., Clp1) (Weitzer and Martinez, 2007). The anchoring of the 5′ nucleotide to the 5′ binding pocket bends it out and away from the rest of the guide strand, making it unavailable for the target mRNA base-pairing interactions seen in the neighboring seed region nucleotides (Frank et al., 2010; Gaynor et al., 2010). The PIWI subdomain of hAGO2 contains an RNase H-like activity dependent on two Magnesium ions (as described above), and catalyzes the mRNA target cleavage (Liu et al., 2004; Gaynor et al., 2010). The cleavage of the target RNA, either the siRNA passenger strand, or the mRNA, occurs between the nucleotides paired to nucleotides 10 and 11 of the hAGO2 guide strand (measured from the 5′ end) (Soutschek et al., 2004; Kraynack and Baker, 2006; Ui-Tei et al., 2008; Judge et al., 2009).

Anti-miRNAs and Gene Regulation

As our understanding of the complex roles played by miRNAs continues to grow, so does the view that a wide range of disease might be treatable through miRNA targeting (for a good commentary, see Ambros, 2008). We now recognize that miRNAs play strikingly important functions. Hundreds of miRNAs have been identified in the human genome, which may be capable of regulating up to 60% of protein-coding genes (Filipowicz et al., 2008; Friedman et al., 2009). Because seed-region complementarity is all that is necessary to produce mRNA downregulation, and many different mRNAs can all contain the same short sequences in their UTRs, a single miRNA sequence is capable of exerting control over multiple genes. Indeed, miRNAs are involved in regulating numerous biological processes, ranging from cell differentiation and development to apoptosis, which has motivated significant efforts to develop means for exerting control over miRNA activity both in vitro and in vivo. Malfunctions in the miRNA regulation system have been implicated in developmental changes, metabolism, rheumatoid arthritis (Duroux-Richard et al., 2011), viral infection, and cancer (Sonenberg and Hinnebusch, 2009; Lennox and Behlke, 2011; Bhayani et al., 2012).

The importance of miRNA gene regulation has attracted a substantial amount of research into the development of agents and techniques for exerting control over miRNA function. Fortunately, successes in RNAi and AON research can be readily applied to miRNA study. Elucidation of the RNAi pathway has revealed the importance of miRNAs, their structure, and their production in the cell, and advances in AON design have facilitated the development of miRNA-targeting ONs. Termed anti-miRNA ONs (sometimes called AMOs) (Lennox and Behlke, 2011), the majority of this class of ONs function via a steric block mechanism, in which miRNA function is inhibited by strong hybridization with exogenously introduced anti-miRNAs in order to block RISC loading (Figure 2). Anti-miRNAs may be designed to trigger miRNA cleavage by RNase H as well, but this has been less common thus far (Lennox and Behlke, 2011). A typical anti-miRNA is a perfect complement to the miRNA target (Lennox and Behlke, 2011), and typically features chemical modifications to enhance nuclease stability and target binding affinity.

Challenges in the Field

The development of ON therapeutics based on AON, siRNA, and anti-miRNA platforms have all proven highly potent and effective in in vitro cell assays. However, as previously mentioned, translation from the bench to the clinic has been hampered by significant challenges arising from: (1) the poor in vivo stability of nucleic acids, (2) ineffective uptake of nucleic acids to target cells, and (3) the potential for off target effects (OTEs) and immunostimulation. Chemical modifications to native DNA and RNA structures have gone a long way in abrogating many of these obstacles, and will be discussed in the subsequent section, following a brief description of the specific challenges facing ON therapeutics.

Nuclease Stability

Nucleic acids, RNAs in particular, are rapidly degraded in cells. AONs, siRNAs, and anti-miRNAs all suffer from these cellular degradation mechanisms, leading to shortened duration of activity and systemic delivery challenges. Duplex RNAs, such as siRNAs, are more nuclease resistant than RNA single strands, however unmodified siRNAs are also degraded quickly in serum (Turner et al., 2007). A human ortholog of *ERI-1* may be a major contributor in siRNA degradation (Kennedy et al., 2004). In the case of siRNAs, it appears that cleavage frequently happens after pyrimidines (Turner et al., 2007), which could be useful to consider when designing chemically modified siRNAs. As discussed below, chemical modification strategies have been developed to improve the nuclease resistance of ONs, without altering the nucleic acid sequence.

Effective and Targeted Delivery

Primary approaches being explored for achieving efficient cellular delivery of therapeutic ONs include both the development of ON conjugates (therapeutic ONs are covalently attached to moieties facilitating cellular uptake), and the development of delivery vehicles designed to encapsulate and shield ONs, as well as facilitate and target their cellular uptake. Strategies may be designed for either local or systemic administration, and systemic delivery approaches are now being investigated in clinical trials with some success (Yuan et al., 2011). However, the

delivery challenge remains formidable, and new, improved delivery technologies will be welcome additions to the field.

Nucleic acids can benefit tremendously from conjugation with biologically relevant moieties. There are examples in which nucleic acid conjugates have demonstrated increased circulation time, improved cellular uptake and endosomal release, and targeted tissue uptake and cellular localization. For thorough reviews of ON conjugates, their biological activity, and the synthetic strategies employed in oligonucleotide conjugate chemistry, please refer to these excellent reviews: Manoharan (2002), Lönnberg (2009), and Juliano et al. (2012).

The use of delivery vehicles to transport siRNA has proven a very promising mode of ON delivery, protecting siRNA from degradation and increasing cellular uptake. In fact, the first example of targeted systemic delivery of siRNAs in humans was recently reported, in which cyclodextrin-based nanoparticles were used to deliver siRNAs to patient tumors, effecting reduction of target gene mRNA and protein (Davis et al., 2010). An RNAi mechanism was evidenced by 5′-RACE PCR, which demonstrated that mRNA cleavage occurred precisely at the predicted AGO2 cleavage site in the mRNA (Davis et al., 2010). However, delivery vehicles often enter cells via endocytosis, which can lead to entrapment of siRNAs in endosomes and lysosomes rather than achieving their efficient release into the cytoplasm (Li and Huang, 2006). Additionally, delivery vehicles often expose siRNAs to endosomal immune receptors, potentially leading to undesirable immunostimulatory responses.(Whitehead et al., 2011) Again, chemical modification of the siRNAs themselves may be the only option for abrogating immune responses. Further discussion of current ON delivery strategies (including in vivo strategies) can be found in recent reviews (Yuan et al., 2011; Juliano et al., 2012; Rettig and Behlke, 2012).

OTEs and Immunostimulation

The knockdown of unintended genes (mRNAs) following ON treatment is a well-known side effect of both siRNA and AON-mediated gene silencing, although these effects are more frequently associated with siRNAs. These hybridization-dependent OTEs are generally observed as a result of partial complementarity between the ON agent and mRNAs other than the intended target. In the case of siRNAs, OTEs can happen when the siRNA passenger strand is selected as the RISC guide, or when the loaded guide strand functions in miRNA fashion, recognizing targets mainly on the basis of seed region complementarity, which is a short sequence more likely to be found in multiple mRNAs (Jackson et al., 2006b). In the case of siRNAs, two sets of OTEs are possible. Careful sequence selection when designing siRNAs and AONs, as well as chemical modifications to reduce the likelihood of siRNA passenger strands acting as guide strands, can be beneficial for minimizing OTEs (Deleavey et al., 2009). In addition, chemical modifications of the siRNA seed region can be used to reduce OTEs (Jackson et al., 2006a). In the case of anti-miRNAs, sequence selection is limited due to the inherent small size of the miRNA target, which could be problematic when targeting one miRNA from a family with significant sequence homology. Microarray technology can be used to monitor changes in cellular gene expression following oligonucleotide treatment (Jackson et al., 2003).

Immunostimulation following ON treatment is another potential side effect that is of concern in the development of ON therapeutics (Marques and Williams, 2005; Watts et al., 2008a), and can confuse experiments designed to measure gene silencing potencies of AONs and siRNAs (Marques and Williams, 2005; Kleinman et al., 2008). For example, the nonspecific innate immune responses triggered by siRNAs and AONs can cause changes in cellular gene expression levels, affecting gene silencing data and antiviral activity measurements, and can lead to phenotypic changes such as reduced tumor angiogenesis (Kleinman et al., 2008). Immune system responses to short nucleic acids such as siRNAs is a complex topic, and has been reviewed in detail elsewhere (Whitehead et al., 2011).

For the purposes of this review, it should be noted that different cellular immune receptors, positioned in different cellular locations, detect AONs and siRNAs, potentially leading to cytokine release and changes in gene expression. siRNA receptors include TLR3 (dsRNA, cell surface, and endosomal), TLR7 (ssRNA, endosomal), TLR8 (ssRNA, endosomal), MDA5 (cytoplasm), RIG-I (cytoplasm), and PKR (cytoplasm).(Takeda and Akira, 2005; Judge and MacLachlan, 2008; Kleinman et al., 2008; Watts et al., 2008a; Zamanian-Daryoush et al., 2008; Deleavey et al., 2009; Whitehead et al., 2011) AONs can be immunostimulatory as well. For example, when DNA sequences such as AONs contain unmodified 5′-CpG motif(s), they can be immunostimulatory upon recognition by TLR9 (endosomal) (Hemmi et al., 2000; Krieg, 2012). Immunostimulatory sequence motifs have also been identified for siRNAs, such as the 5′-UGU GUU (Judge et al., 2005) and 5′-GUCCUUCAA motifs (Hornung et al., 2005). In addition, it should be noted that the type of delivery vehicle used will influence the mode of cellular uptake, which can determine the number and type of immune receptors to which the oligonucleotides can be exposed (Whitehead et al., 2011). Many of the chemical modifications described below can dramatically reduce the immunostimulatory properties of nucleic acids, providing an effective means for avoiding these potential side effects. It should be noted that abrogating immunostimulation is not always beneficial, as in the cases of isRNAs (Schlee et al., 2006) and oligonucleotide adjuvants (Klinman, 2004), but in siRNAs, AONs, and anti-miRNAs, minimizing immunostimulation is often desired. Discussion of immunostimulatory oligonucleotides can be found elsewhere (Barchet et al., 2008; Whitehead et al., 2011).

Chemical Modifications of ONs

The continued development of chemically modified nucleoside analogs has provided nucleic acid chemists with the tools necessary to exert a remarkable amount of control over many important nucleic acid properties, including: binding affinity for RNA targets, structural preferences, nuclease stability, and immunostimulatory properties. An exhaustive list of all nucleic acid chemical modifications is beyond the scope of this review; however, this section will discuss many of the frequently utilized chemical modifications, as well as some interesting recent additions, keeping in mind the biological pathways and nucleic acid characteristics already described.

Generally speaking, chemical modifications of nucleic acids can be classified into three distinct categories: (1) internucleotide linkage modifications, (2) sugar modifications, and (3) nucleobase modifications. Below we elaborate on each of these categories.

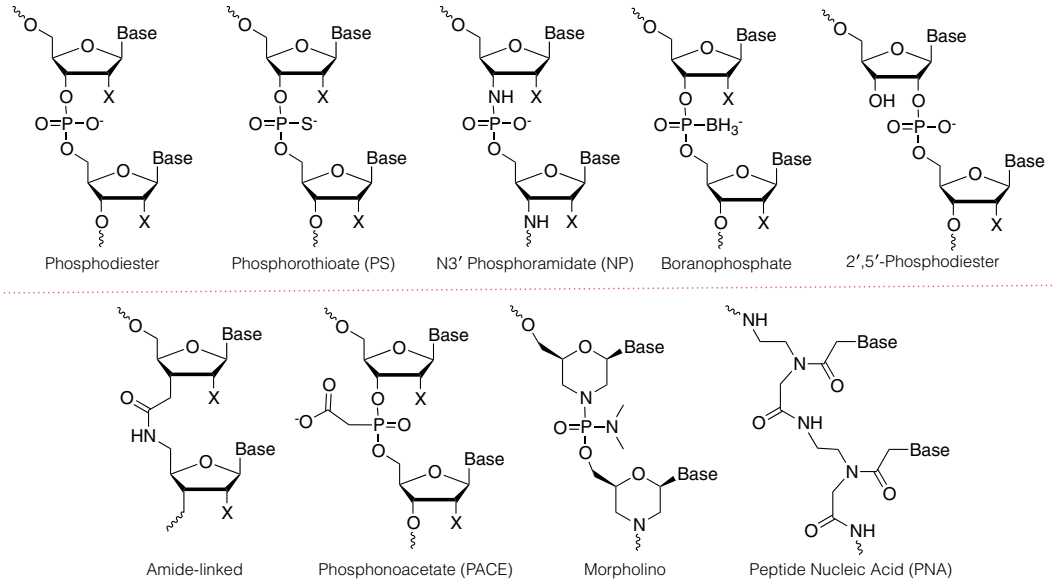

Figure 4. Selected Chemical Modifications of Internucleotide Linkages

Phosphodiester linkages form the backbone of natural DNA and RNA. Minor chemical modification of this chemical functionality can impart significant changes with respect to nuclease resistance, biological activity, duplex thermal stability, and cellular uptake. In some cases, significant departure from natural structure can be advantageous as well (e.g., Morpholino, peptide nucleic acid). X = H (DNA) or OH (RNA). Note: Some modifications have only been reported for only deoxyribose or ribose sugars, and not both.

Internucleotide Linkage Modifications

The internucleotide phosphodiester linkages of DNA and RNA are negatively charged at physiological pH (pK_a ~2), and can be readily cleaved by endo- and exonucleases found in serum and within mammalian cells. Chemical modifications of these bonds have been extensively studied, and several chemical strategies have proven very successful for improving nuclease resistance, some of which are still compatible with an RNase-H-mediated mRNA cleavage mechanism, and a few impart additional beneficial characteristics to ONs. Internucleotide linkage modifications are often combined with sugar modifications, allowing for additional control over ON properties. Figure 4 presents some of the frequently used internucleotide linkage modifications, and this section will briefly describe some of the features imparted by these design strategies.

The phosphorothioate (PS) linkage, where sulfur substitutes for one nonbridging phosphate oxygen (Eckstein, 1966, 1967, 2000), imparts significant resistance to nuclease degradation, and is widely used in AON applications. PS linkages are cost-effective modifications, readily incorporated using standard solid-phase ON synthesis protocols (Sanghvi, 2011). PS modification is a major component of first generation antisense therapeutic candidates (Sanghvi, 2011; Bennett and Swayze, 2010), and although some toxicity has been observed, PS modifications are found in a FDA-approved ON drug, Formivirsen (Sanghvi, 2011). Although the PS modification can reduce the binding affinity for a complementary strand (Kibler-Herzog et al., 1991; Milligan et al., 1993), this modification is still very compatible with RNase H-mediated mRNA cleavage (Agrawal and Kandimalla, 2000). The PS linkage introduces chirality to internucleotide linkages, and one stereochemistry (S_p linkage) is highly resistant to nuclease-mediated cleavage (Eckstein, 2002). Strategies for diastereoselective synthesis of PS linkages have been described (Guga and Stec, 2003). It is common to synthesize PS-modified AONs as a diastereomeric mixture, because each AON will contain multiple resistant linkages, and the overall stability of the population is enhanced. PS linkages in nucleic acids can also enhance their affinity for binding with serum albumin (Bennett and Swayze, 2010), which can improve pharmacokinetics and circulation time. PS-modified AONs can also be taken up by cells without the use of transfection or electroporation (Stein et al., 2010). PS linkages have also been incorporated into siRNAs without significant loss of potency, but can reduce activity in some cases (Deleavey et al., 2009), including modifications at the AGO2 cleavage site (Leuschner et al., 2006).

N3′ phosphoramidate (NP) linkages replace 3′-OH groups for 3′-amine functionality (Chen et al., 1995). NP modifications adopt a North sugar conformation, making them suitable mimics for RNA substitution (Gryaznov, 2010). As a result, NP containing ONs lack the ability to activate RNase-H-mediated cleavage (Heidenreich et al., 1997), but feature good binding affinity for target sequences and high nuclease resistance. Indeed, NP oligonucleotides can serve as potent RNase H-independent antisense oligonucleotides. Target-binding affinity can be further enhanced by combining 2′-OH or 2′-F functionality with NP-modified sugars (Gryaznov, 2010). These linkages can substitute a sulfur for a nonbridging oxygen in the internucleotide linkage as well, mimicking the PS linkage modification in some ways (for example, internucleotide linkages become chiral upon substitution with sulfur), while retaining enhanced binding affinity (Gryaznov, 2010).

Boranophosphate internucleotide linkages also impart chirality, and can be incorporated in siRNAs, with the resulting constructs being capable of triggering potent gene silencing (Hall et al., 2004). These modifications, not unlike PSs, impart improved nuclease stability as well. Boranophosphate-modified

AONs can be potent triggers of RNase H for a target RNA cleavage mechanism of action (Rait and Shaw, 1999).

Phosphonoacetate (PACE) linkages substitute an acetic acid group for a nonbridging oxygen in the internucleotide linkage, again introducing chirality to the linkage. PACE modifications retain the negative charge character of unmodified DNA (PACE pK_a ~3.8), demonstrate enhanced nuclease resistance (Dellinger et al., 2003; Sheehan et al., 2003), and adopt A-form duplexes when bound to RNA targets (Sheehan et al., 2003). PACE ONs are able to activate RNase H activity (Dellinger et al., 2003; Sheehan et al., 2003). PACE modification slightly reduces binding affinity for RNA complement strands (~1.3°C per insert for an RNA target in high salt conditions) (Sheehan et al., 2003). Interestingly, neutral esters of PACE ONs can be taken up by cells unassisted, perhaps allowing cellular esterases to subsequently release anionic acetate ONs inside cells, in a ''prodrug'' approach to ON delivery (Sheehan et al., 2003; Yamada et al., 2007). Although de-esterification does not produce natural phosphodiester linkages, these modified ONs retain the ability to trigger RNase H.

The nonbridging oxygen can also be replaced by sulfur to produce thio-PACE, which shares many of the qualities observed for PACE modifications (Sheehan et al., 2003). 2′-*O*-Me versions of PACE and thio-PACE ONs have recently been synthesized and tested in siRNA and anti-miRNA constructs as well (Threlfall et al., 2012). Modification of RNA with 2′-*O*-Me PACE was stabilizing relative to an RNA-RNA duplex in most cases, and 2′-*O*-Me thio-PACE was only slightly destabilizing. Both modifications were tolerated in siRNA constructs, with the 2′-*O*-Me thio-PACE demonstrating a slight advantage over 2′-*O*-Me PACE modifications. With lipid transfection, both modifications performed slightly worse than unmodified siRNA, but were able to effect some gene silencing in the absence of transfecting agent, indicating some unassisted uptake of siRNA duplexes with modified passenger strands. Single stranded 2′-*O*-Me thio-PACE ONs were effective at unassisted cell uptake, and notably improved the potency of a 2′-*O*-Me-based anti-miRNA targeting miRNA-122 in the absence of lipid transfection (Threlfall et al., 2012).

Morpholino phosphoramidates are uncharged substitutes of the internucleotide phosphodiester linkages and the furanose sugars of nucleic acids. These modified ONs can have similar or even improved binding affinity for target strands (Summerton, 1999; Corey and Abrams, 2001; Du and Gatti, 2011), and can be used as translational inhibitors (Bennett and Swayze, 2010) and anti-miRNAs (Bennett and Swayze, 2010). Although morpholino phosphoramidates lack the ability to trigger RNase H (Summerton and Weller, 1997), they have proven resistant to nuclease degradation (Summerton and Weller, 1997). Morpholino ONs have proven particularly successful as RNase H-independent AONs and anti-miRNAs in Zebrafish systems for conducting functional genomics studies (Lennox and Behlke, 2011), and as pre-mRNA splicing modulators (Du and Gatti, 2011; Kole et al., 2012).

Peptide nucleic acid (PNA) has a neutral charge backbone composed of *N*-(2-aminoethyl)glycine, exhibits significant nuclease and protease resistance (Demidov et al., 1994), binds ONs with higher affinity than DNA or RNA (Shakeel et al., 2006), obeys base pairing rules (Egholm et al., 1993), and features a number of interesting properties. PNAs do not activate RNase H, and can act via a translational inhibition mechanism (Bennett and Swayze, 2010), in siRNA overhangs (Potenza et al., 2008), and in numerous other biological pathways including splice-switching activities (Nielsen, 2010). PNA is also particularly useful in anti-miRNA applications. PNA has an interesting dsDNA invasion property (Peffer et al., 1993; Nielsen, 2010), in which it can invade dsDNA to form a variety of complex PNA/DNA structures (Nielsen, 2010). The structure of PNA is a major departure from typical nucleic acid structure, substituting both the internucleotide linkages and sugars for a peptide-based backbone. The nucleobases, however, can remain unchanged, and target recognition occurs via base pairing (mainly base stacking) (Nielsen, 2010) in either antiparallel or parallel orientations (Peffer et al., 1993; Nielsen, 2010). Since the discovery of PNA (Nielsen et al., 1991), a wide variety of PNA analogs have been developed, and are reviewed elsewhere (Pensato et al., 2007).

Sugar Modifications

Chemically modified sugars provide a remarkable level of control over nucleotide sugar puckering preferences, which is a physical property intimately related to ON binding affinity toward complementary strands, duplex conformation, and, by extension, quality of enzyme interactions. Sugar conformation can be altered through manipulation of gauche and anomeric effects, or via steric restraints. The conformation of sugar rings is readily described using phase angle values, calculated from the dihedral angles of the furanose rings. In turn, these phase angle values can be represented on a so-called ''pseudorotational wheel'' that provides an intuitive means for describing nucleotide sugar ''puckering'' or conformation (Altona and Sundaralingam, 1972; Blackburn et al., 2006) Most nucleotide sugars and analogs adopt conformations characterized on the pseudorotational wheel as either ''North'' (C3′-*endo*, C2′-*exo*), or ''South'' (C2′-*endo*, C3′-*exo*), passing through the ''East'' (O4′-*endo*) when moving between these conformational minima (Figure 5). In B-form, dsDNA sugars adopt the ''South'' puckering, whereas in A-form dsRNA sugars prefer the ''North'' conformation. These structural considerations can be particularly important when considering chemical modification strategies for gene silencing ONs, and will be discussed for the sugar modifications presented in this section. The cellular enzymes involved in both siRNA- and AON-mediated gene silencing are tolerant toward several chemically modified sugars, some of which are highlighted in Figure 6 and described below.

The 2′-*O*-Me nucleoside analog is one of the most widely used modifications. Compared with a DNA strand, incorporation of 2′-*O*-Me units within ONs increases binding affinity for RNA complements (Majlessi et al., 1998), and increases nuclease stability (Rettig and Behlke, 2012). 2′-*O*-Me sugars prefer a North sugar conformation (Kawai et al., 1992; Nishizaki et al., 1997), resembling RNA, and form A-form duplexes (Nishizaki et al., 1997). This means that these units are particularly well suited for siRNA modification. Indeed, 2′-*O*-Me units are well tolerated in siRNAs, although in at least some situations extensive modification should be avoided (Czauderna et al., 2003; Deleavey et al., 2009). 2′-*O*-Me modification has been shown to reduce immunostimulatory properties of siRNAs (Whitehead et al., 2011), which is an attractive feature because innate immune response side effects have become increasingly concerning in recent

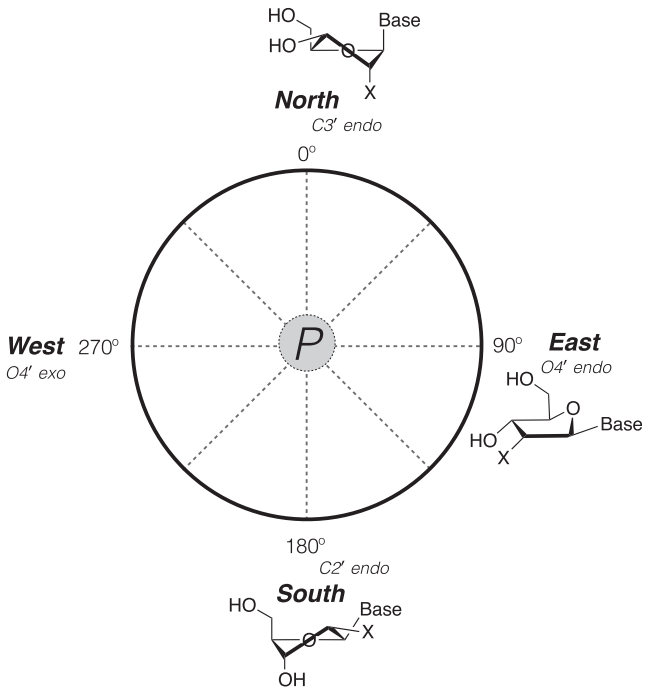

Figure 5. The Pseudorotational Wheel and Corresponding Sugar Pucker Notations

Based on the calculated phase angle (*P*), nucleoside conformations can be readily classified. For example, A-form RNA sugars are "North" or C3′-*endo*, and B-form DNA sugars are "South" or C2′-*endo*. X = H (DNA), X = OH (RNA).

years (Whitehead et al., 2011). The 2′-*O*-Me modification has been widely applied in AON research, especially when incorporated in so-called "gapmer" AON constructs (described below). 2′-*O*-Me modified ONs are in clinical trials (Sanghvi, 2011), and can be found in the first FDA-approved aptamer, Macugen (Ng et al., 2006).

The 2′F-RNA modification is another popular chemical modification in gene silencing/anti-miRNA research. 2′F-RNA, like 2′-*O*-Me, is an RNA mimic, preferentially adopting a North sugar pucker (Ikeda et al., 1998 and contained references), at least partly due to the strong gauche effect imparted by the 2′-fluorine. Much like 2′-*O*-Me, 2′F-RNA increases binding affinity for target RNA sequences (~2°C–3°C per insert versus DNA) (Kawasaki et al., 1993; Viazovkina et al., 2002; Bennett and Swayze, 2010), which may be partly due to conformational preorganization of the sugar for formation of A-form duplexes, but perhaps more importantly, due to enthalpy benefits from enhanced base-pairing and stacking interactions arising from the electronegative fluorine (Pallan et al., 2011). 2′F-RNA modification is very well tolerated in siRNA, in both the guide and passenger strands (Watts et al., 2008a; Deleavey et al., 2009). Phosphodiester 2′F-RNA ONs do not demonstrate significant resistance to exonucleases (Manoharan, 1999), although 2′F-RNA imparts resistance to pyrimidine-rich sequences toward endonuclease degradation. 2′F-RNA can also modulate splicing upon pre-mRNA binding through recruitment of the interleukin enhancer-binding factor 2 and 3 complex (ILF2/3), something not observed with 2′-*O*-MOE or 2′F-ANA (Rigo et al., 2012). The 2′F-RNA modification very closely mimics RNA, making it a versatile and well-tolerated chemical modification for many applications. Like 2′-*O*-Me, 2′F-RNA modifications can also be found in the FDA-approved aptamer, Macugen (Ng et al., 2006).

The 2′-deoxy-2′-fluoro-β-D-arabino nucleic acid (2′F-ANA) modification is an epimer of 2′F-RNA, structurally identical to 2′F-RNA in all respects with the single exception of the fluorine atom substitution at the 2′ position, which corresponds to the furanose form of arabinose rather than ribose. As a result, 2′F-ANA is a structural mimic of DNA rather than RNA, preferentially adopting a South/East sugar pucker (Trempe et al., 2001, Berger et al., 1998; Ikeda et al., 1998). 2′F-ANA enhances binding to RNA complements (~1.2°C) (Damha et al., 1998; Wilds and Damha, 2000), enhances nuclease stability in both AON (Kalota et al., 2006; Watts et al., 2009) and siRNA constructs (Dowler et al., 2006; Deleavey et al., 2010), and is fully compatible with RNase H activation for target mRNA cleavage (Damha et al., 1998). 2′F-ANA modification of AONs in gapmer or "altimer" constructs (see below) have beneficial effects on target binding stability, nuclease resistance, cellular uptake, level of RNA target degradation, duration of activity, and potency (Min et al., 2002; Kalota et al., 2006; Watts and Damha, 2008). Perhaps somewhat surprisingly, 2′F-ANA (a DNA-like modification) is also compatible with siRNA chemical modification, especially in the passenger strand (Dowler et al., 2006; Deleavey et al., 2010). In addition, combining 2′F-ANA with RNA-like chemical modifications (LNA, 2′F-RNA) can produce fully modified siRNAs with enhanced gene silencing potency, increased nuclease stability, and significantly reduced immunostimulatory properties (Deleavey et al., 2010). Chimeric siRNAs fully modified with 2′F-ANA and 2′F-RNA can maintain or improve siRNA potency, and can be designed to introduce thermodynamic asymmetry in siRNA duplexes (Deleavey et al., 2010). Perhaps because 2′F-ANA is an excellent siRNA passenger strand modification, these chemical modifications likely have poor potency in anti-miRNA applications. 2′F-ANA-modified PS AONs are compatible with gymnotic delivery techniques for achieving gene silencing without transfection, which has already been observed with PS LNA-DNA AONs (Stein et al., 2010). The improved thermal stability of 2′F-ANA-RNA duplexes are partly due to pseudohydrogen bonding between the 2′-fluorine and purine H8 (Watts et al., 2010; Anzahaee et al., 2011), an observation that can be rationally applied to AON design (Anzahaee et al., 2011). A related nucleoside analog, the RNA-like 4′S-FANA, is also compatible with siRNA-mediated gene silencing (Watts et al., 2007).

Locked nucleic acid (LNA) is a chemically modified RNA analog featuring a methylene bridge joining the 2′-OH to the C4′, essentially forming a conformationally restricted bicyclic nucleoside (Koshkin et al., 1998; Obika et al., 1998). These nucleoside analogs are essentially locked in a North sugar conformation that closely mimics A-form RNA, preorganizing LNAs for RNA-binding (Koshkin et al., 1998; Obika et al., 1998; Braasch and Corey, 2001). LNA units impart the most impressive duplex stability effects of the available chemical modifications, with stabilizations of ~5.6°C per insert (Koshkin et al., 1998). As a result, LNAs have proven very useful in anti-miRNA (Lennox and Behlke, 2011), AON (Braasch and Corey, 2001), and siRNA (Braasch et al., 2003; Elmén et al., 2005; Hornung et al., 2005; Bramsen et al., 2007; Mook et al., 2007; Deleavey et al., 2009) applications. The strong binding properties of LNA make them

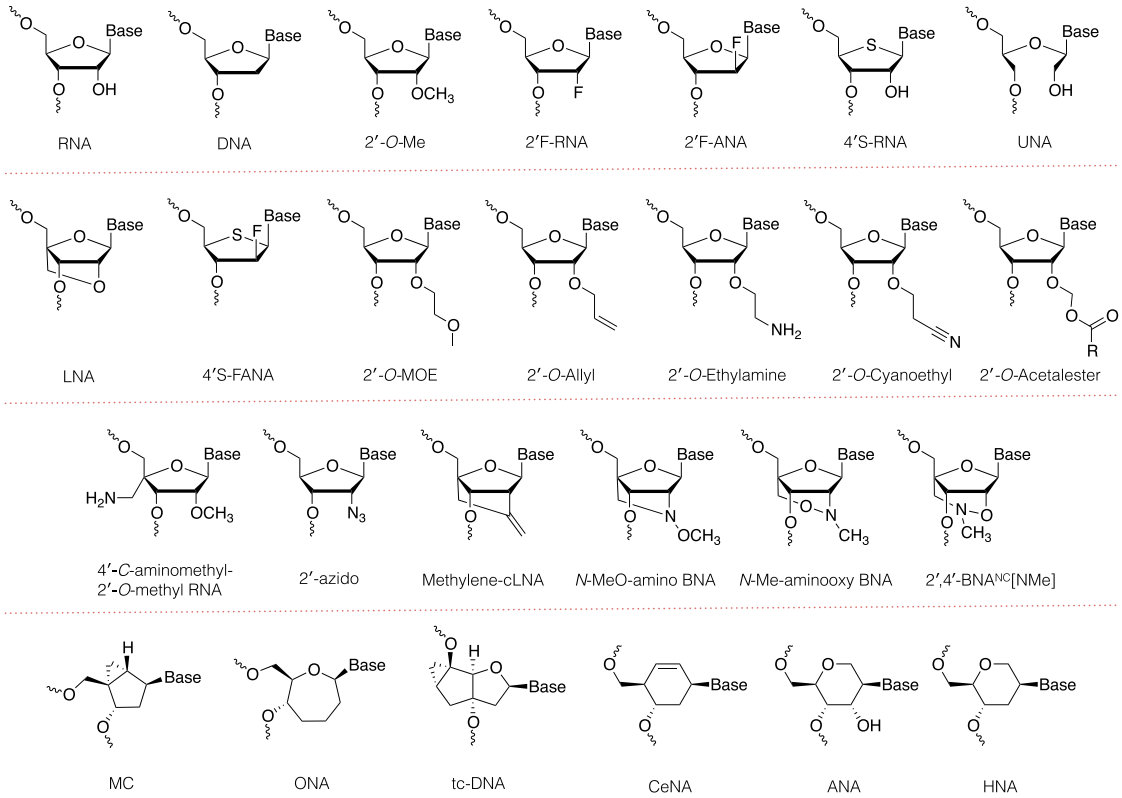

Figure 6. Selected Chemical Modifications of Oligonucleotide Sugars

A wide variety of chemical modifications have been developed and applied in the design of AONs, siRNAs, and anti-miRNAs. The majority of chemical modifications involve modification of the 2′ position of RNA. Other modifications include replacement of the ring oxygen with sulfur (4′S-RNA, 4′S-FANA); bridges between the C2′ and C4′ positions; modified C4′ nucleotides, and replacement of furanose with bicyclic, tricyclic, or expanded ring systems.

particularly useful in anti-miRNA applications, where short sequences can be necessary for miRNA specificity, and LNAs are excellent AON modifications when used in gapmer constructs (Wahlestedt et al., 2000). LNA modifications are also compatible with siRNA chemical modification (Braasch et al., 2003; Elmén et al., 2005; Hornung et al., 2005; Bramsen et al., 2007; Mook et al., 2007). LNAs have contributed to the development of sisiRNAs, which are siRNAs with nicked sense strands to reduce sense strand-dependent OTEs (Bramsen et al., 2007). In addition, LNA modification improves nuclease resistance (Wahlestedt et al., 2000), and can reduce siRNA immunostimulation (Whitehead et al., 2011).

Contrasting the rigid nature of the LNA modification, the highly flexible unlocked nucleic acid (UNA) (or ''seconucleoside'') modification is also being developed for application in ON therapeutics. UNA, missing the covalent C2′-C3′ bond of a ribose sugar, is not conformationally restrained, and can be used to influence ON flexibility (Mangos et al., 2003). UNA inserts can reduce duplex T_m by 5°C–10°C per insert in some cases (Mangos et al., 2003; Campbell and Wengel, 2011), can facilitate antisense strand selection as the RISC guide, and UNA modifications to the seed region of an siRNA guide strand can significantly reduce OTEs (Vaish et al., 2011). UNA has also been used in 2′F-ANA modified AONs, in efforts to increase duplex flexibility to accommodate RNase H (Mangos et al., 2003). In fact, 2′F-ANA modified AONs with acyclic residues (UNA and butyl linkers) amplify RNase H-catalyzed target RNA degradation, suggesting the added flexibility imparted to the substrate structure can enhance the protein/nucleic acid interaction. Biophysical studies revealed that the enhanced flexibility associated with a particular UNA modification remain globally undetectable, indicating the acyclic residues induce only local structural deformations to the hybrid architecture (Mangos et al., 2003).

The LNA modification is a member of a larger family of nucleoside analogs referred to as bicyclic nucleic acids (BNAs). Extensive research has been done on the development of several BNA nucleosides (Prakash, 2011), only a few of which are shown in Figure 6. Some of the examples of BNAs that have been applied in AON research include methylene carbocyclic LNA (methylene-cLNA) (Seth et al., 2010), *N*-MeO-amino BNA, *N*-Me-aminooxy BNA, and 2′,4′-BNA^{NC}[NMe] (Prakash et al., 2010). Bicyclo [3.1.0]hexane-based nucleoside analogs (2′-deoxy-methanocarba nucleosides [MC]) adopt a North sugar conformation, enhance thermal stability of duplexes, and enhance siRNA serum stability (Marquez et al., 1996; Terrazas et al., 2011). A conformationally constrained nucleoside analog, tricyclo-DNA (tc-DNA), has also been developed and tested (Renneberg et al., 2002; Ittig et al., 2010), based on a tricyclic (rather than bicyclic) structure. tc-DNA has enhanced binding affinity for RNA, does not activate RNase H, and is stable to nucleases (Renneberg et al., 2002; Ittig et al., 2010).

The 2′-*O*-MOE (2′-*O*-(2-methoxyethyl)) modification has been used in several therapeutic candidates undergoing clinical trials (Bennett and Swayze, 2010). These chemical modifications feature a methoxyethyl modification at the RNA 2′-OH, which increases target binding affinity (~2°C per insert) (Manoharan, 1999; Viazovkina et al., 2002), and improves nuclease stability (comparable with phosphorothioate modification) (Manoharan, 1999). 2′-*O*-MOE modifications adopt a North sugar conformation preorganized for RNA binding, which is also stabilized by hydration effects (Manoharan, 1999; Bennett and Swayze, 2010). 2′-*O*-MOE groups have been successfully applied to AON constructs (RNase H compatible when used in gapmer designs, Manoharan, 1999), and in siRNA, especially in sense strands (Prakash et al., 2005). One highly promising clinical candidate successfully advancing in clinical trials, Mipomersen, is a second generation AON containing PS linkages and 2′-*O*-MOE sugars (Kole et al., 2012; Kastelein et al., 2006). A number of similar 2′ modifications have been developed for use as gene silencing agents, including the 2′-*O*-Allyl (Amarzguioui et al., 2003; Odadzic et al., 2008), 2′-*O*-Ethylamine (Odadzic et al., 2008; Bramsen et al., 2009), 2′-*O*-Cyanoethyl modifications (Saneyoshi et al., 2005; Bramsen et al., 2009), and 2′-*O*-acetalester (Martin et al., 2009). Another North-type sugar modification, 2′-azido modified RNA, has also been used for siRNA modification (Aigner et al., 2011; Fauster et al., 2012).

Although the majority of sugar analog alterations are localized to the 2′ position, many other sites are amenable to modification, including the 4′ position, as evidenced by modified nucleosides such as 4′S-RNA and 4′-*C*-aminomethyl-2′-*O*-Me RNA. 4′S-RNA enhances nuclease stability (Leydier et al., 1995; Dande et al., 2006), has been applied to siRNA chemical modification (Dande et al., 2006), and is compatible with RISC-mediated gene silencing. Selenium modification of the 4′ position (4′Se-RNA) provides significant stabilization to RNA/RNA and RNA/DNA hybrid duplexes (Watts et al., 2008b). Other 4′ modifications have been developed, including 4′-*C*-aminomethyl-2′-*O*-Me RNA, which features modified 4′ functionality external to the 5-membered ring (Gore et al., 2012). 4′-*C*-aminomethyl-2′-*O*-Me RNA is slightly destabilizing versus RNA, stabilizes siRNA toward nuclease degradation, and does not significantly alter gene silencing potency (Gore et al., 2012).

It is becoming increasingly clear that the pool of available modified nucleosides for therapeutic ON analog development is not limited to 5-membered ring mimics of ribose. In fact, a number of expanded ring systems have been developed and applied in gene silencing applications. We have already discussed morpholino chemistry, which could be considered an expanded ring ON analog. In addition, cyclohexene nucleic acid (CeNA) (Verbeure et al., 2001; Nauwelaerts et al., 2007), altritol nucleic acid (ANA) (Fisher et al., 2007, 2009), oxepane NAs (ONA) (Sabatino and Damha, 2007), and hexitol NA (HNA) (Fisher et al., 2009) modifications have all been used in gene silencing experiments. CeNA binds RNA more tightly than a DNA strand, increases serum stability, and can activate RNase H (Wang et al., 2000b; Verbeure et al., 2001). ANA favors A-form duplex formation due to conformational preorganization, making it a stable RNA-binding modification. Oxepane nucleic acids (ONA) are also expanded ring modifications, which are nuclease resistant and can trigger RNase H (Sabatino and Damha, 2007).

Nucleobase Modifications

Chemically modified nucleobases have also found application in ON analog development. Although perhaps less common than sugar and backbone modifications, modified nucleobases are numerous, and can be used to affect thermal stability, reduce immunostimulation, and affect siRNA OTEs. A comprehensive review of nucleobase modifications can be found elsewhere (Herdewijn, 2000), in which nucleobase modifications and effects on RNA-binding are discussed. As well, the reader is directed to a thorough review of nucleobase modifications in siRNA (Peacock et al., 2011). Nucleobase modifications in PNA have also been reviewed (Wojciechowski and Hudson, 2007; Nielsen, 2010), as well as fluorescent nucleobase analogs (Dodd and Hudson, 2009). Here we only provide some brief highlights.

A popular goal in modified nucleobase design is to achieve increased duplex stability while maintaining and improving native base pairing recognition and hydrogen bonding. The 5-bromo-Ura and 5-iodo-Ura substitutes of Ura, as well as 2,6-diaminopurine in place of Ade, are excellent examples of this strategy, and can be used to stabilize A-U base pairs in ON duplexes (Chiu and Rana, 2003; Watts et al., 2008a; Deleavey et al., 2009). C-5 propynyl pyrimidine base modifications also increase duplex thermal stability, and are compatible with RNase H-mediated gene silencing, but have shown some associated toxicity (Herdewijn, 2000; Shen et al., 2003; Bennett and Swayze, 2010). In contrast to these modifications, other examples of modified nucleobases (i.e., difluorotoluene, difluorobenzene, dichlorobenzene) lacking oxygen, nitrogen, or classical hydrogen bond donating groups, have been reported to be beneficial in siRNA modification (Xia et al., 2006; Somoza et al., 2008; Addepalli et al., 2010). Naturally occurring modified nucleobases have also been used to chemically modify siRNA (2-thiouridine, pseudouridine, and dihydrouridine) (Sipa et al., 2007). Generally speaking, nucleobase modifications can provide a means for probing and altering duplex thermal stability, sugar conformation, and nucleobase hydrogen-bonding and steric interactions (Peacock et al., 2011).

In addition, intrinsically fluorescent modified nucleobases can be exceptionally useful in studying siRNAs, AONs, and anti-miRNAs due to their ability to impart fluorescent properties to nucleic acids. Fluorescent nucleobases can be "base discriminating," and have been used in a variety of nucleic acid studies (Dodd and Hudson, 2009). 6-phenylpyrrolocytidine (phpC) RNA (Wahba et al., 2010, 2011) is an interesting nucleobase modification, which has allowed development of enzymatic assays for RNase H-mediated cleavage (Wahba et al., 2010) and detection of siRNA cellular uptake (Wahba et al., 2011). Finally, a modified nucleobase (N2-alkyl 8-oxoguanosine) can be used as a "switch" to control steric effects in duplex major and minor grooves, modulating siRNA interactions with off-target proteins, while retaining gene silencing potency (Kannan et al., 2011).

A variety of modified nucleobases with minor-groove projections can be used to abrogate immunostimulatory responses to siRNAs and miRNA mimics, perhaps by preventing interactions with TLR and PKR receptors (Peacock et al., 2011). Additionally, AON immunostimulation responses to CpG motifs can be abrogated by substitution with 5-methyl cytosine (Krieg, 2012).

Therapeutic ON Analogues

AON Design Strategies

In order to improve activity and therapeutic potential of AON sequences, many chemical modifications discussed above have been designed and applied to the AON gene silencing approach. For example, PS-modified AONs can be considered as 1st generation AON technology, which has produced at least one successful therapeutic (Fomivirsen) (Sanghvi, 2011). The second and third generations of AON strategies, building upon the first generation, utilize additional chemical modifications (mainly sugar modifications) to further improve therapeutic potential. Second and third generation AONs typically contain 2′-MOE, LNA, 2′-*O*-Me, 2′F-ANA, or 2′F-RNA sugar modifications, and are designed to improve potency, mRNA target binding, nuclease stability, and to reduce immunostimulation.

In order to maintain potency in cases where RNase H-mediated mRNA cleavage is desired, it is generally considered a requirement that modified AONs retain a polyanionic character (Sheehan et al., 2003). As well, because RNase H recognizes DNA/RNA hybrids that tend to adopt conformations intermediate between A- and B-form, AON-based therapeutics aiming to elicit target mRNA degradation should retain a continuous region of DNA or DNA-like modification in order to ensure RNase H recruitment and cleavage. The second and third generation sugar modifications are often implemented in a ''gapmer'' design, in which sugar modified nucleotides are on the ON termini, flanking a central DNA core. This strategy works very well with 2′-MOE, LNA, 2′-*O*-Me, 2′F-ANA, or 2′F-RNA sugar modifications, and allows high-affinity RNA-like modifications to be used without interfering with RNase H activity. In the case of 2′F-ANA, a DNA-like chemical modification, an ''altimer'' design strategy can also be employed to activate RNase H, in which 2′F-ANA and DNA regions are alternated every 3 nucleotides (Min et al., 2002; Kalota et al., 2006).

Anti-miRNA Design Strategies

In general, anti-miRNAs are designed to act as steric blocks, strongly binding with a miRNA target so as to prevent RISC loading and mRNA targeting. Anti-miRNAs designed to trigger RNase H upon miRNA binding have also been tested, but steric block anti-miRNAs have been somewhat more common and successful thus far (Lennox and Behlke, 2011). A typical anti-miRNA is a perfect complement to the miRNA target (Lennox and Behlke, 2011), and can be designed to tightly bind the entire miRNA, especially at the seed region of the miRNA, which is the region critical for miRNA-mediated gene silencing. In fact, binding affinity to the 5′ end of a miRNA target at the seed region has been shown to influence anti-miRNA specificity and activity (Davis et al., 2006; Threlfall et al., 2012).

Overall, anti-miRNAs have many similarities to AONs, and have been developed using many of the same chemical modifications utilized for AON modification. Some of the most widely used anti-miRNA modifications are locked nucleic acid (LNA), 2′F-RNA, 2′*O*-Me, peptide nucleic acid (PNA), and morpholinos (Lennox and Behlke, 2011), all of which are described above. Antagomirs, which are 2′*O*-Me ONs containing phosphorothioate linkages and a conjugated cholesterol moiety (Krützfeldt et al., 2005, 2007) have also become a successful strategy for targeting miRNAs (Krützfeldt et al., 2005), and can be considered an addition to the anti-miRNA family.

In the case of anti-miRNAs, the cellular RNA target is a comparatively short miRNA sequence, significantly limiting the target binding region compared to mRNA targeting AONs and siRNAs. Short anti-miRNA constructs are desirable, particularly those with very high binding affinity for the miRNA target. Thus, LNA has proven a very attractive chemical modification in the design of anti-miRNAs, owing to its highly stabilizing effect in RNA hybridization. A correlation between the thermal stability of binding with the target and overall potency is generally observed, although not predictive in all cases (Lennox and Behlke, 2011), and so target-binding affinity should not be the only consideration in the design of anti-miRNAs. It has been hypothesized that there is a threshold stability that must be reached for good potency, after which further affinity increases have less impact on potency (Lennox and Behlke, 2011).

When an anti-miRNA binds a miRNA target, the result is an unfolded miRNA annealed to an anti-miRNA ON, which in some ways could resemble the structure of an siRNA duplex. Of course, siRNAs are capable of interaction with the RISC complex and performing gene knockdown, and so care should be taken when designing anti-miRNAs. More specifically, it has been shown that chemically modified ONs that are functional as siRNA passenger strands are low potency anti-miRNAs, whereas ON chemistries detrimental in siRNA passenger strands can be highly active anti-miRNAs (Davis et al., 2006).

siRNA Design Strategies

The double-stranded nature of this class of gene silencing ONs makes siRNA design more complex in some respects compared with AONs and anti-miRNAs. Because the two strands serve very different functions, chemical modification of siRNAs must carefully consider which strands are modified and in what fashion. Additionally, siRNAs can be generated from longer RNAs by upstream cellular enzymes, in much the same way that miRNAs are generated. Therefore, RNAi-mediated gene silencing can be triggered using a variety of longer RNA molecules, including hairpins (termed shRNAs, often produced from expression vectors, and reviewed elsewhere; Rao et al., 2009), longer dsRNAs (termed dicer-substrate siRNAs, which are processed by Dicer prior to RISC loading) (Kim et al., 2005), and circular RNAs (including dumbbell siRNAs) (Abe et al., 2007). Many of these RNA architectures are potent triggers of RNAi, processed by upstream enzymes to produce siRNAs that can be introduced into the RISC complex in similar fashion to miRNAs. Dicer substrate siRNAs demonstrate excellent potency, and highly effective design rules have been elucidated (Amarzguioui et al., 2006; Amarzguioui and Rossi, 2008). sisiRNAs have also been described, in which the siRNA passenger strand consists of two shorter fragments, thermally stabilized by LNA inserts, in order to create precleaved constructs that are potent gene silencing agents with reduced OTEs (Bramsen et al., 2007). Antisense siRNAs, in which single stranded antisense strands are introduced to trigger the RISC complex, have also been described, although in some cases reduced potency was observed versus standard duplex siRNAs (Martinez et al., 2002; Haringsma et al., 2012).

In general, the first step of siRNA design (as with AON design) is the identification of an ON sequence capable of potent knock down of the mRNA target. The siRNA sequence selection process can often be assisted by computational algorithms

designed to scan an input mRNA target to identify unique sequences that siRNAs may target with reduced chance of creating OTEs from partial complementarity to other unintended cellular mRNAs (Muhonen and Holthofer, 2010). Additionally, it is wise to avoid the described immunostimulatory sequence motifs to reduce immunostimulation.

Chemical modifications may then be selected and applied to the siRNA sequence (although, if a screen is performed to check and compare potencies of many siRNA sequences targeting an mRNA, it is often most effective to directly use chemically modified siRNAs as part of the screen). Careful consideration of which chemical modifications to employ, and at which positions within the duplexes they should be located, can be crucial for maintaining high potency.

RNAi is triggered by short A-form dsRNAs, and RNA-like nucleotide analogs preferring a North sugar conformation are frequently the most beneficial modifications for siRNA design (e.g., 2′-*O*-Me, 2′F-RNA, LNA, 2′-*O*-MOE).

Modifications at the cleavage site of the passenger strand can impair siRNA potency in some (but not all) cases because, upon RISC loading, the siRNA passenger strand is typically cleaved and removed from the guide strand (Martinez and Tuschl, 2004; Matranga et al., 2005; Muhonen et al., 2007).

The guide strand 5′ phosphate binds to the MID domain of AGO2, and caution should be taken when using 5′ modifications. Cellular kinases (i.e., CLP-1) (Weitzer and Martinez, 2007) can add this phosphate to siRNAs, but this function can be impaired by some modifications. Chemically adding a 5′ phosphate to guide strands during synthesis can be beneficial. Modifications preventing phosphorylation of the siRNA sense strand 5′ terminus should reduce OTEs arising from improper RISC loading.

siRNA guide strand selection relies on duplex thermodynamics (the strand with the least tightly-bound 5′ terminus is typically selected). Chemical modification influencing siRNA thermodynamics to favor antisense strand loading can help improve siRNA potency and reduce OTEs.

The siRNA guide strand is sensitive to chemical modifications, especially in the seed region, and so modification strategies typically minimize guide strand modifications, or emphasize RNA-like analogs for the guide strand. Structural insights revealing specific interactions between AGO2 and siRNAs (Wang et al., 2008a, 2008b, 2009; Schirle and MacRae, 2012) can be very useful when predicting chemical modifications that will be well tolerated in siRNAs (Shukla et al., 2010).

Finally, it is important to consider the nuclease resistance, immunostimulatory potential, and OTEs during siRNA design. As previously discussed, nuclease resistance can be effectively enhanced by a wide variety of chemical modifications, and many of the chemical modifications described in this review are able to reduce the immunostimulatory characteristics of siRNAs (for additional information, refer to Watts et al., 2008a; Deleavey et al., 2009; and Whitehead et al., 2011). OTEs can also be reduced through chemical modification. Modifications to the sense strand 5′ terminus to prevent phosphorylation and the use of thermally stabilizing (or destabilizing) nucleoside analogs to bias duplex thermodynamics can prevent OTEs arising from sense strand loading in RISC. In addition, OTEs resulting when siRNAs act through seed region complementarity in a miRNA-like fashion can be reduced through seed region modification (i.e., UNA insertion in the seed region, as described above).

Box 1. The following Relevant Topics Were Not Discussed but Are Well-Described in the following Suggested Reviews

(1) Aptamers
Keefe et al., 2010
(2) Modulating Splicing Events
Kole et al., 2012
Saleh et al., 2012
(3) Immunostimulatory oligonucleotides
Barchet et al., 2008
(4) Clinical Trials of Olignucleotide Therapeutics
Burnett and Rossi, 2012
Sanghvi, 2011
Watts and Corey, 2012
(5) Delivery technology and strategies
Juliano et al., 2012
Rettig and Behlke, 2012
Yuan et al., 2011

Conclusions

ONs, and chemically modified ON analogs, constitute an active and truly promising field of therapeutic research. Impressive advances have been made in the understanding of the biological pathways involved in ON-mediated gene silencing, and synthetic accessibility to an extensive array of ON chemical modifications has provided valuable tools for combating the hurdles facing ON therapeutics. Despite these advancements, clinical success has thus far been restricted to only two ON drugs (Vitravene and Macugen), which has garnered some disappointment in the field. Nevertheless, our understanding of the biological applications of synthetic nucleic acids is continuing to advance, and the number of ON therapeutic candidates in clinical trials has continued to grow. Drug discovery programs can benefit from more chemical modification tools and mechanistic understanding than ever before. ON chemical modifications will likely play an integral role in the development of many classes of ON therapeutics (for additional related topics not discussed here, refer to Box 1), and we hope this review has provided insight into the types of available modifications, the biological pathways through which they act, and the benefits that can be gained through their use. To date, several ON-based drugs have entered mid and late stage clinical trials (Sanghvi, 2011; Watts and Corey, 2012; Burnett and Rossi, 2012), the outcomes of which will hopefully foster confidence that ON therapeutics can make substantial contributions in the treatment of a variety of human diseases.

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REFERENCES

Abe, N., Abe, H., and Ito, Y. (2007). Dumbbell-shaped nanocircular RNAs for RNA interference. J. Am. Chem. Soc. *129*, 15108–15109.

Addepalli, H., Meena, Peng, C.G., Wang, G., Fan, Y., Charisse, K., Jayaprakash, K.N., Rajeev, K.G., Pandey, R.K., Lavine, G., et al. (2010). Modulation of thermal stability can enhance the potency of siRNA. Nucleic Acids Res. *38*, 7320–7331.

Agrawal, S., and Kandimalla, E.R. (2000). Antisense therapeutics: is it as simple as complementary base recognition? Mol. Med. Today *6*, 72–81.

Aigner, M., Hartl, M., Fauster, K., Steger, J., Bister, K., and Micura, R. (2011). Chemical synthesis of site-specifically 2′-azido-modified RNA and potential applications for bioconjugation and RNA interference. ChemBioChem *12*, 47–51.

Altona, C., and Sundaralingam, M. (1972). Conformational analysis of the sugar ring in nucleosides and nucleotides. A new description using the concept of pseudorotation. J. Am. Chem. Soc. *94*, 8205–8212.

Amarzguioui, M., and Rossi, J.J. (2008). Principles of Dicer substrate (D-siRNA) design and function. In Methods in Molecular Biology: RNAi: Design and Application, S. Barik, ed. (Totowa, NJ: The Humana Press), pp. 3–10.

Amarzguioui, M., Holen, T., Babaie, E., and Prydz, H. (2003). Tolerance for mutations and chemical modifications in a siRNA. Nucleic Acids Res. *31*, 589–595.

Amarzguioui, M., Lundberg, P., Cantin, E., Hagstrom, J., Behlke, M.A., and Rossi, J.J. (2006). Rational design and in vitro and in vivo delivery of Dicer substrate siRNA. Nat. Protoc. *1*, 508–517.

Ambros, V. (2008). The evolution of our thinking about microRNAs. Nat. Med. *14*, 1036–1040.

Anzahaee, M.Y., Watts, J.K., Alla, N.R., Nicholson, A.W., and Damha, M.J. (2011). Energetically important C-H···F-C pseudohydrogen bonding in water: evidence and application to rational design of oligonucleotides with high binding affinity. J. Am. Chem. Soc. *133*, 728–731.

Baker, B.F., Lot, S.S., Condon, T.P., Cheng-Flournoy, S., Lesnik, E.A., Sasmor, H.M., and Bennett, C.F. (1997). 2′-O-(2-Methoxy)ethyl-modified anti-intercellular adhesion molecule 1 (ICAM-1) oligonucleotides selectively increase the ICAM-1 mRNA level and inhibit formation of the ICAM-1 translation initiation complex in human umbilical vein endothelial cells. J. Biol. Chem. *272*, 11994–12000.

Barchet, W., Wimmenauer, V., Schlee, M., and Hartmann, G. (2008). Accessing the therapeutic potential of immunostimulatory nucleic acids. Curr. Opin. Immunol. *20*, 389–395.

Bennett, C.F., and Swayze, E.E. (2010). RNA targeting therapeutics: molecular mechanisms of antisense oligonucleotides as a therapeutic platform. Annu. Rev. Pharmacol. Toxicol. *50*, 259–293.

Berger, I., Tereshko, V., Ikeda, H., Marquez, V.E., and Egli, M. (1998). Crystal structures of B-DNA with incorporated 2′-deoxy-2′-fluoro-arabino-furanosyl thymines: implications of conformational preorganization for duplex stability. Nucleic Acids Res. *26*, 2473–2480.

Betancur, J.G., and Tomari, Y. (2012). Dicer is dispensable for asymmetric RISC loading in mammals. RNA *18*, 24–30.

Bhayani, M.K., Calin, G.A., and Lai, S.Y. (2012). Functional relevance of miRNA sequences in human disease. Mutat. Res. *731*, 14–19.

Blackburn G.M., Gait M.J., and Loakes D., eds. (2006). DNA and RNA structure. In Nucleic Acids in Chemistry and Biology (Cambridge, UK: RSC Publishing), pp. 13–76.

Braasch, D.A., and Corey, D.R. (2001). Locked nucleic acid (LNA): fine-tuning the recognition of DNA and RNA. Chem. Biol. *8*, 1–7.

Braasch, D.A., Jensen, S., Liu, Y., Kaur, K., Arar, K., White, M.A., and Corey, D.R. (2003). RNA interference in mammalian cells by chemically-modified RNA. Biochemistry *42*, 7967–7975.

Bramsen, J.B., Laursen, M.B., Damgaard, C.K., Lena, S.W., Babu, B.R., Wengel, J., and Kjems, J. (2007). Improved silencing properties using small internally segmented interfering RNAs. Nucleic Acids Res. *35*, 5886–5897.

Bramsen, J.B., Laursen, M.B., Nielsen, A.F., Hansen, T.B., Bus, C., Langkjaer, N., Babu, B.R., Højland, T., Abramov, M., Van Aerschot, A., et al. (2009). A large-scale chemical modification screen identifies design rules to generate siRNAs with high activity, high stability and low toxicity. Nucleic Acids Res. *37*, 2867–2881.

Burnett, J.C., and Rossi, J.J. (2012). RNA-based therapeutics: current progress and future prospects. Chem. Biol. *19*, 60–71.

Campbell, M.A., and Wengel, J. (2011). Locked vs. unlocked nucleic acids (LNA vs. UNA): contrasting structures work towards common therapeutic goals. Chem. Soc. Rev. *40*, 5680–5689.

Cerritelli, S.M., and Crouch, R.J. (2009). Ribonuclease H: the enzymes in eukaryotes. FEBS J. *276*, 1494–1505.

Chan, C.W.S., and Khachigian, L.M. (2009). DNAzymes and their therapeutic possibilities. Intern. Med. J. *39*, 249–251.

Chen, J.K., Schultz, R.G., Lloyd, D.H., and Gryaznov, S.M. (1995). Synthesis of oligodeoxyribonucleotide N3′—>P5′ phosphoramidates. Nucleic Acids Res. *23*, 2661–2668.

Chiu, Y.-L., and Rana, T.M. (2003). siRNA function in RNAi: a chemical modification analysis. RNA *9*, 1034–1048.

Corey, D.R., and Abrams, J.M. (2001). Morpholino antisense oligonucleotides: tools for investigating vertebrate development. Genome Biol. *2*, S1015.

Czauderna, F., Fechtner, M., Dames, S., Aygün, H., Klippel, A., Pronk, G.J., Giese, K., and Kaufmann, J. (2003). Structural variations and stabilising modifications of synthetic siRNAs in mammalian cells. Nucleic Acids Res. *31*, 2705–2716.

Damha, M., Wilds, C., Noronha, A., Brukner, I., Borkow, G., Arion, D., and Parniak, M. (1998). Hybrids of RNA and arabino nucleic acids (ANA and 2′F-ANA) are substrates of ribonuclease H. J. Am. Chem. Soc. *120*, 12976–12977.

Dande, P., Prakash, T.P., Sioufi, N., Gaus, H., Jarres, R., Berdeja, A., Swayze, E.E., Griffey, R.H., and Bhat, B. (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.

Davis, M.E., Zuckerman, J.E., Choi, C.H.J., Seligson, D., Tolcher, A., Alabi, C.A., Yen, Y., Heidel, J.D., and Ribas, A. (2010). Evidence of RNAi in humans from systemically administered siRNA via targeted nanoparticles. Nature *464*, 1067–1070.

Davis, S., Lollo, B., Freier, S., and Esau, C. (2006). Improved targeting of miRNA with antisense oligonucleotides. Nucleic Acids Res. *34*, 2294–2304.

Deleavey, G.F., Watts, J.K., and Damha, M.J. (2009). Chemical modification of siRNA. In Current Protocols in Nucleic Acids Chemistry, S.L. Beaucage, D.E. Bergstrom, P. Herdewijn, and A. Matsuda, eds. (Hoboken, NJ: John Wiley & Sons), pp. 16.3.1–16.3.22.

Deleavey, G.F., Watts, J.K., Alain, T., Robert, F., Kalota, A., Aishwarya, V., Pelletier, J., Gewirtz, A.M., Sonenberg, N., and Damha, M.J. (2010). Synergistic effects between analogs of DNA and RNA improve the potency of siRNA-mediated gene silencing. Nucleic Acids Res. *38*, 4547–4557.

Dellinger, D.J., Sheehan, D.M., Christensen, N.K., Lindberg, J.G., and Caruthers, M.H. (2003). Solid-phase chemical synthesis of phosphonoacetate and thiophosphonoacetate oligodeoxynucleotides. J. Am. Chem. Soc. *125*, 940–950.

Demidov, V.V., Potaman, V.N., Frank-Kamenetskii, M.D., Egholm, M., Buchard, O., Sönnichsen, S.H., and Nielsen, P.E. (1994). Stability of peptide nucleic acids in human serum and cellular extracts. Biochem. Pharmacol. *48*, 1310–1313.

Dodd, D.W., and Hudson, R.H.E. (2009). Intrinsically fluorescent base-discriminating nucleoside analogs. Mini Rev. Org. Chem. *6*, 378–391.

Dowler, T., Bergeron, D., Tedeschi, A.-L., Paquet, L., Ferrari, N., and Damha, M.J. (2006). Improvements in siRNA properties mediated by 2′-deoxy-2′-fluoro-β-D-arabinonucleic acid (FANA). Nucleic Acids Res. *34*, 1669–1675.

Du, L., and Gatti, R.A. (2011). Potential therapeutic applications of antisense morpholino oligonucleotides in modulation of splicing in primary immunodeficiency diseases. J. Immunol. Methods *365*, 1–7.

Duroux-Richard, I., Presumey, J., Courties, G., Gay, S., Gordeladze, J., Jorgensen, C., Kyburz, D., and Apparailly, F. (2011). MicroRNAs as new player in rheumatoid arthritis. Joint Bone Spine *78*, 17–22.

Eckstein, F. (1966). Nucleoside phosphorothioates. J. Am. Chem. Soc. *88*, 4292–4294.

Eckstein, F. (1967). A dinucleoside phosphorothioate. Tetrahedron Lett. *8*, 1157–1160.

Eckstein, F. (2000). Phosphorothioate oligodeoxynucleotides: what is their origin and what is unique about them? Antisense Nucleic Acid Drug Dev. *10*, 117–121.

Eckstein, F. (2002). Developments in RNA chemistry, a personal view. Biochimie *84*, 841–848.

Egholm, M., Buchardt, O., Christensen, L., Behrens, C., Freier, S.M., Driver, D.A., Berg, R.H., Kim, S.K., Norden, B., and Nielsen, P.E. (1993). PNA hybridizes to complementary oligonucleotides obeying the Watson-Crick hydrogen-bonding rules. Nature *365*, 566–568.

Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2001). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature *411*, 494–498.

Elmén, J., Thonberg, H., Ljungberg, K., Frieden, M., Westergaard, M., Xu, Y., Wahren, B., Liang, Z., Ørum, H., Koch, T., and Wahlestedt, C. (2005). Locked nucleic acid (LNA) mediated improvements in siRNA stability and functionality. Nucleic Acids Res. *33*, 439–447.

Fauster, K., Hartl, M., Santner, T., Aigner, M., Kreutz, C., Bister, K., Ennifar, E., and Micura, R. (2012). 2′-Azido RNA, a versatile tool for chemical biology: synthesis, X-ray structure, siRNA applications, click labeling. ACS Chem. Biol. *7*, 581–589.

Filipowicz, W., Bhattacharyya, S.N., and Sonenberg, N. (2008). Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat. Rev. Genet. *9*, 102–114.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., and Mello, C.C. (1998). Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature *391*, 806–811.

Fisher, M., Abramov, M., Van Aerschot, A., Xu, D., Juliano, R.L., and Herdewijn, P. (2007). Inhibition of MDR1 expression with altritol-modified siRNAs. Nucleic Acids Res. *35*, 1064–1074.

Fisher, M., Abramov, M., Van Aerschot, A., Rozenski, J., Dixit, V., Juliano, R.L., and Herdewijn, P. (2009). Biological effects of hexitol and altritol-modified siRNAs targeting B-Raf. Eur. J. Pharmacol. *606*, 38–44.

Frank, F., Sonenberg, N., and Nagar, B. (2010). Structural basis for 5′-nucleotide base-specific recognition of guide RNA by human AGO2. Nature *465*, 818–822.

Friedman, R.C., Farh, K.K.-H., Burge, C.B., and Bartel, D.P. (2009). Most mammalian mRNAs are conserved targets of microRNAs. Genome Res. *19*, 92–105.

Gagnon, K.T., and Corey, D.R. (2012). Argonaute and the nuclear RNAs: new pathways for RNA-mediated control of gene expression. Nucleic Acid Ther. *22*, 3–16.

Gaynor, J.W., Campbell, B.J., and Cosstick, R. (2010). RNA interference: a chemist's perspective. Chem. Soc. Rev. *39*, 4169–4184.

Gore, K.R., Nawale, G.N., Harikrishna, S., Chittoor, V.G., Pandey, S.K., Höbartner, C., Patankar, S., and Pradeepkumar, P.I. (2012). Synthesis, gene silencing, and molecular modeling studies of 4′-C-aminomethyl-2′-O-methyl modified small interfering RNAs. J. Org. Chem. *77*, 3233–3245.

Gryaznov, S.M. (2010). Oligonucleotide n3′—>p5′ phosphoramidates and thio-phoshoramidates as potential therapeutic agents. Chem. Biodivers. *7*, 477–493.

Guga, P., and Stec, W.J. (2003). Synthesis of phosphorothioate oligonucleotides with stereodefined phosphorothioate linkages. Curr. Protoc. Nucleic Acid Chem *Chapter 4*, 4.17.1–4.17.27.

Hall, A.H., Wan, J., Shaughnessy, E.E., Ramsay Shaw, B., and Alexander, K.A. (2004). RNA interference using boranophosphate siRNAs: structure-activity relationships. Nucleic Acids Res. *32*, 5991–6000.

Haringsma, H.J., Li, J.J., Soriano, F., Kenski, D.M., Flanagan, W.M., and Willingham, A.T. (2012). mRNA knockdown by single strand RNA is improved by chemical modifications. Nucleic Acids Res. *40*, 4125–4136.

Heidenreich, O., Gryaznov, S., and Nerenberg, M. (1997). RNase H-independent antisense activity of oligonucleotide N3′ —> P5′ phosphoramidates. Nucleic Acids Res. *25*, 776–780.

Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K., and Akira, S. (2000). A Toll-like receptor recognizes bacterial DNA. Nature *408*, 740–745.

Herdewijn, P. (2000). Heterocyclic modifications of oligonucleotides and antisense technology. Antisense Nucleic Acid Drug Dev. *10*, 297–310.

Ho, M.-H., De Vivo, M., Dal Peraro, M., and Klein, M.L. (2010). Understanding the effect of magnesium ion concentration on the catalytic activity of ribonuclease H through computation: does a third metal binding site modulate endonuclease catalysis? J. Am. Chem. Soc. *132*, 13702–13712.

Hollis, T., and Shaban, N.M. (2011). Structure and function of RNase H enzymes. In Ribonucleases, *Volume 26, Chapter 12*, N. Acids, M. Biology, and A.W Nicholson, eds. (Berlin, Germany: Springer Berlin Heidelberg), pp. 299–317.

Hornung, V., Guenthner-Biller, M., Bourquin, C., Ablasser, A., Schlee, M., Uematsu, S., Noronha, A., Manoharan, M., Akira, S., de Fougerolles, A., 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.

Humphreys, D.T., Westman, B.J., Martin, D.I.K., and Preiss, T. (2005). MicroRNAs control translation initiation by inhibiting eukaryotic initiation factor 4E/cap and poly(A) tail function. Proc. Natl. Acad. Sci. USA *102*, 16961–16966.

Ikeda, H., Fernandez, R., Wilk, A., Barchi, J.J., Jr., Huang, X., and Marquez, V.E. (1998). The effect of two antipodal fluorine-induced sugar puckers on the conformation and stability of the Dickerson-Drew dodecamer duplex $[d(CGCGAATTCGCG)]_2$. Nucleic Acids Res. *26*, 2237–2244.

Ittig, D., Luisier, S., Weiler, J., Schümperli, D., and Leumann, C.J. (2010). Improving gene silencing of siRNAs via tricyclo-DNA modification. Artif. DNA PNA XNA *1*, 9–16.

Jackson, A.L., Bartz, S.R., Schelter, J., Kobayashi, S.V., Burchard, J., Mao, M., Li, B., Cavet, G., and Linsley, P.S. (2003). Expression profiling reveals off-target gene regulation by RNAi. Nat. Biotechnol. *21*, 635–637.

Jackson, A.L., Burchard, J., Leake, D., Reynolds, A., Schelter, J., Guo, J., Johnson, J.M., Lim, L., Karpilow, J., Nichols, K., et al. (2006a). Position-specific chemical modification of siRNAs reduces "off-target" transcript silencing. RNA *12*, 1197–1205.

Jackson, A.L., Burchard, J., Schelter, J., Chau, B.N., Cleary, M., Lim, L., and Linsley, P.S. (2006b). Widespread siRNA "off-target" transcript silencing mediated by seed region sequence complementarity. RNA *12*, 1179–1187.

Jinek, M., and Doudna, J.A. (2009). A three-dimensional view of the molecular machinery of RNA interference. Nature *457*, 405–412.

Judge, A., and MacLachlan, I. (2008). Overcoming the innate immune response to small interfering RNA. Hum. Gene Ther. *19*, 111–124.

Judge, A.D., Sood, V., Shaw, J.R., Fang, D., McClintock, K., and MacLachlan, I. (2005). Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA. Nat. Biotechnol. *23*, 457–462.

Judge, A.D., Robbins, M., Tavakoli, I., Levi, J., Hu, L., Fronda, A., Ambegia, E., McClintock, K., and MacLachlan, I. (2009). Confirming the RNAi-mediated mechanism of action of siRNA-based cancer therapeutics in mice. J. Clin. Invest. *119*, 661–673.

Juliano, R.L., Ming, X., and Nakagawa, O. (2012). The chemistry and biology of oligonucleotide conjugates. Acc. Chem. Res. *45*, 1067–1076. http://dx.doi.org/10.1021/ar2002123.

Kalota, A., Karabon, L., Swider, C.R., Viazovkina, E., Elzagheid, M., Damha, M.J., and Gewirtz, A.M. (2006). 2′-deoxy-2′-fluoro-β-D-arabinonucleic acid (2′F-ANA) modified oligonucleotides (ON) effect highly efficient, and persistent, gene silencing. Nucleic Acids Res. *34*, 451–461.

Kannan, A., Fostvedt, E., Beal, P.A., and Burrows, C.J. (2011). 8-Oxoguanosine switches modulate the activity of alkylated siRNAs by controlling steric effects in the major versus minor grooves. J. Am. Chem. Soc. *133*, 6343–6351.

Kastelein, J.J.P., Wedel, M.K., Baker, B.F., Su, J., Bradley, J.D., Yu, R.Z., Chuang, E., Graham, M.J., and Crooke, R.M. (2006). Potent reduction of apolipoprotein B and low-density lipoprotein cholesterol by short-term administration of an antisense inhibitor of apolipoprotein B. Circulation *114*, 1729–1735.

Kawai, G., Yamamoto, Y., Kamimura, T., Masegi, T., Sekine, M., Hata, T., Iimori, T., Watanabe, T., Miyazawa, T., and Yokoyama, S. (1992).

Conformational rigidity of specific pyrimidine residues in tRNA arises from posttranscriptional modifications that enhance steric interaction between the base and the 2′-hydroxyl group. Biochemistry *31*, 1040–1046.

Kawamata, T., and Tomari, Y. (2010). Making RISC. Trends Biochem. Sci. *35*, 368–376.

Kawasaki, A.M., Casper, M.D., Freier, S.M., Lesnik, E.A., Zounes, M.C., Cummins, L.L., Gonzalez, C., and Cook, P.D. (1993). Uniformly modified 2′-deoxy-2′-fluoro phosphorothioate oligonucleotides as nuclease-resistant antisense compounds with high affinity and specificity for RNA targets. J. Med. Chem. *36*, 831–841.

Keefe, A.D., Pai, S., and Ellington, A. (2010). Aptamers as therapeutics. Nat. Rev. Drug Discov. *9*, 537–550.

Kennedy, S., Wang, D., and Ruvkun, G. (2004). A conserved siRNA-degrading RNase negatively regulates RNA interference in C. elegans. Nature *427*, 645–649.

Khvorova, A., Reynolds, A., and Jayasena, S.D. (2003). Functional siRNAs and miRNAs exhibit strand bias. Cell *115*, 209–216.

Kibler-Herzog, L., Zon, G., Uznanski, B., Whittier, G., and Wilson, W.D. (1991). Duplex stabilities of phosphorothioate, methylphosphonate, and RNA analogs of two DNA 14-mers. Nucleic Acids Res. *19*, 2979–2986.

Kim, D.-H., Behlke, M.A., Rose, S.D., Chang, M.-S., Choi, S., and Rossi, J.J. (2005). Synthetic dsRNA Dicer substrates enhance RNAi potency and efficacy. Nat. Biotechnol. *23*, 222–226.

Kleinman, M.E., Yamada, K., Takeda, A., Chandrasekaran, V., Nozaki, M., Baffi, J.Z., Albuquerque, R.J.C., Yamasaki, S., Itaya, M., Pan, Y., et al. (2008). Sequence- and target-independent angiogenesis suppression by siRNA via TLR3. Nature *452*, 591–597.

Klinman, D.M. (2004). Immunotherapeutic uses of CpG oligodeoxynucleotides. Nat. Rev. Immunol. *4*, 249–258.

Kole, R., Krainer, A.R., and Altman, S. (2012). RNA therapeutics: beyond RNA interference and antisense oligonucleotides. Nat. Rev. Drug Discov. *11*, 125–140.

Koshkin, A., Singh, S., Nielsen, P., Rajwanshi, V., Kumar, R., Meldgaard, M., Olsen, C.E., and Wengel, J. (1998). LNA (locked nucleic acids): synthesis of the adenine, cytosine, guanine, 5-methylcytosine, thymine and uracil bicyclonucleoside monomers, oligomerisation, and unprecedented nucleic acid recognition. Tetrahedron *54*, 3607–3630.

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.

Krieg, A.M. (2012). CpG still rocks! Update on an accidental drug. Nucleic Acid Ther. *22*, 77–89.

Krützfeldt, J., Rajewsky, N., Braich, R., Rajeev, K.G., Tuschl, T., Manoharan, M., and Stoffel, M. (2005). Silencing of microRNAs in vivo with 'antagomirs'. Nature *438*, 685–689.

Krützfeldt, J., Kuwajima, S., Braich, R., Rajeev, K.G., Pena, J., Tuschl, T., Manoharan, M., and Stoffel, M. (2007). Specificity, duplex degradation and subcellular localization of antagomirs. Nucleic Acids Res. *35*, 2885–2892.

Lennox, K.A., and Behlke, M.A. (2011). Chemical modification and design of anti-miRNA oligonucleotides. Gene Ther. *18*, 1111–1120.

Leuschner, P.J.F., Ameres, S.L., Kueng, S., and Martinez, J. (2006). Cleavage of the siRNA passenger strand during RISC assembly in human cells. EMBO Rep. *7*, 314–320.

Leydier, C., Bellon, L., Barascut, J.L., Morvan, F., Rayner, B., and Imbach, J.L. (1995). 4′-Thio-RNA: synthesis of mixed base 4′-thio-oligoribonucleotides, nuclease resistance, and base pairing properties with complementary single and double strand. Antisense Res. Dev. *5*, 167–174.

Li, S.-D., and Huang, L. (2006). Gene therapy progress and prospects: non-viral gene therapy by systemic delivery. Gene Ther. *13*, 1313–1319.

Lima, W.F., Rose, J.B., Nichols, J.G., Wu, H., Migawa, M.T., Wyrzykiewicz, T.K., Siwkowski, A.M., and Crooke, S.T. (2007). Human RNase H1 discriminates between subtle variations in the structure of the heteroduplex substrate. Mol. Pharmacol. *71*, 83–91.

Liu, J., Carmell, M.A., Rivas, F.V., Marsden, C.G., Thomson, J.M., Song, J.-J., Hammond, S.M., Joshua-Tor, L., and Hannon, G.J. (2004). Argonaute2 is the catalytic engine of mammalian RNAi. Science *305*, 1437–1441.

Lönnberg, H. (2009). Solid-phase synthesis of oligonucleotide conjugates useful for delivery and targeting of potential nucleic acid therapeutics. Bioconjug. Chem. *20*, 1065–1094.

Ma, J.-B., Ye, K., and Patel, D.J. (2004). Structural basis for overhang-specific small interfering RNA recognition by the PAZ domain. Nature *429*, 318–322.

Ma, J.-B., Yuan, Y.-R., Meister, G., Pei, Y., Tuschl, T., and Patel, D.J. (2005). Structural basis for 5′-end-specific recognition of guide RNA by the A. fulgidus Piwi protein. Nature *434*, 666–670.

Majlessi, M., Nelson, N.C., and Becker, M.M. (1998). Advantages of 2′-O-methyl oligoribonucleotide probes for detecting RNA targets. Nucleic Acids Res. *26*, 2224–2229.

Mangos, M.M., Min, K.L., Viazovkina, E., Galarneau, A., Elzagheid, M.I., Parniak, M.A., and Damha, M.J. (2003). Efficient RNase H-directed cleavage of RNA promoted by antisense DNA or 2'F-ANA constructs containing acyclic nucleotide inserts. J. Am. Chem. Soc. *125*, 654–661.

Manoharan, M. (1999). 2′-carbohydrate modifications in antisense oligonucleotide therapy: importance of conformation, configuration and conjugation. Biochim. Biophys. Acta *1489*, 117–130.

Manoharan, M. (2002). Oligonucleotide conjugates as potential antisense drugs with improved uptake, biodistribution, targeted delivery, and mechanism of action. Antisense Nucleic Acid Drug Dev. *12*, 103–128.

Marques, J.T., and Williams, B.R.G. (2005). Activation of the mammalian immune system by siRNAs. Nat. Biotechnol. *23*, 1399–1405.

Marquez, V.E., Siddiqui, M.A., Ezzitouni, A., Russ, P., Wang, J., Wagner, R.W., and Matteucci, M.D. (1996). Nucleosides with a twist. Can fixed forms of sugar ring pucker influence biological activity in nucleosides and oligonucleotides? J. Med. Chem. *39*, 3739–3747.

Martin, A.R., Lavergne, T., Vasseur, J.-J., and Debart, F. (2009). Assessment of new 2′-O-acetalester protecting groups for regular RNA synthesis and original 2′-modified proRNA. Bioorg. Med. Chem. Lett. *19*, 4046–4049.

Martinez, J., and Tuschl, T. (2004). RISC is a 5′ phosphomonoester-producing RNA endonuclease. Genes Dev. *18*, 975–980.

Martinez, J., Patkaniowska, A., Urlaub, H., Lührmann, R., and Tuschl, T. (2002). Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. Cell *110*, 563–574.

Matranga, C., Tomari, Y., Shin, C., Bartel, D.P., and Zamore, P.D. (2005). Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes. Cell *123*, 607–620.

Meister, G., Landthaler, M., Patkaniowska, A., Dorsett, Y., Teng, G., and Tuschl, T. (2004). Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. Mol. Cell *15*, 185–197.

Milligan, J.F., Matteucci, M.D., and Martin, J.C. (1993). Current concepts in antisense drug design. J. Med. Chem. *36*, 1923–1937.

Min, K.-L., Viazovkina, E., Galarneau, A., Parniak, M.A., and Damha, M.J. (2002). Oligonucleotides comprised of alternating 2′-deoxy-2′-fluoro-beta-D-arabinonucleosides and D-2′-deoxyribonucleosides (2'F-ANA/DNA 'altimers') induce efficient RNA cleavage mediated by RNase H. Bioorg. Med. Chem. Lett. *12*, 2651–2654.

Mook, O.R., Baas, F., de Wissel, M.B., and Fluiter, K. (2007). Evaluation of locked nucleic acid-modified small interfering RNA in vitro and in vivo. Mol. Cancer Ther. *6*, 833–843.

Muhonen, P., and Holthofer, H. (2010). Bioinformatic approaches to siRNA selection and optimization. In RNA Interference: From Biology to Clinical Applications, Methods in Molecular Biology, W.-P. Min and T. Ichim, eds. (Totowa, NJ: Humana Press), pp. 93–107.

Muhonen, P., Tennilä, T., Azhayeva, E., Parthasarathy, R.N., Janckila, A.J., Väänänen, H.K., Azhayev, A., and Laitala-Leinonen, T. (2007). RNA interference tolerates 2′-fluoro modifications at the Argonaute2 cleavage site. Chem. Biodivers. *4*, 858–873.

Mulhbacher, J., St-Pierre, P., and Lafontaine, D.A. (2010). Therapeutic applications of ribozymes and riboswitches. Curr. Opin. Pharmacol. *10*, 551–556.

Nauwelaerts, K., Fisher, M., Froeyen, M., Lescrinier, E., Aerschot, A.V., Xu, D., DeLong, R., Kang, H., Juliano, R.L., and Herdewijn, P. (2007). Structural characterization and biological evaluation of small interfering RNAs containing cyclohexenyl nucleosides. J. Am. Chem. Soc. *129*, 9340–9348.

Ng, E.W.M., Shima, D.T., Calias, P., Cunningham, E.T., Jr., Guyer, D.R., and Adamis, A.P. (2006). Pegaptanib, a targeted anti-VEGF aptamer for ocular vascular disease. Nat. Rev. Drug Discov. *5*, 123–132.

Nielsen, P.E. (2010). Peptide nucleic acids (PNA) in chemical biology and drug discovery. Chem. Biodivers. *7*, 786–804.

Nielsen, P.E., Egholm, M., Berg, R.H., and Buchardt, O. (1991). Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide. Science *254*, 1497–1500.

Nishizaki, T., Iwai, S., Ohtsuka, E., and Nakamura, H. (1997). Solution structure of an RNA.2′-O-methylated RNA hybrid duplex containing an RNA.DNA hybrid segment at the center. Biochemistry *36*, 2577–2585.

Noland, C.L., Ma, E., and Doudna, J.A. (2011). siRNA repositioning for guide strand selection by human Dicer complexes. Mol. Cell *43*, 110–121.

Noy, A., Pérez, A., Lankas, F., Javier Luque, F., and Orozco, M. (2004). Relative flexibility of DNA and RNA: a molecular dynamics study. J. Mol. Biol. *343*, 627–638.

Noy, A., Pérez, A., Márquez, M., Luque, F.J., and Orozco, M. (2005). Structure, recognition properties, and flexibility of the DNA.RNA hybrid. J. Am. Chem. Soc. *127*, 4910–4920.

Noy, A., Luque, F.J., and Orozco, M. (2008). Theoretical analysis of antisense duplexes: determinants of the RNase H susceptibility. J. Am. Chem. Soc. *130*, 3486–3496.

Obika, S., Nanbu, D., Hari, Y., Andoh, J., Morio, K., Doi, T., and Imanishi, T. (1998). Stability and structural features of the duplexes containing nucleoside analogues with a fixed N-type conformation, 2′-O,4′-C-methyleneribonucleosides. Tetrahedron Lett. *39*, 5401–5404.

Odadzic, D., Bramsen, J.B., Smicius, R., Bus, C., Kjems, J., and Engels, J.W. (2008). Synthesis of 2′-O-modified adenosine building blocks and application for RNA interference. Bioorg. Med. Chem. *16*, 518–529.

Pallan, P.S., Greene, E.M., Jicman, P.A., Pandey, R.K., Manoharan, M., Rozners, E., and Egli, M. (2011). Unexpected origins of the enhanced pairing affinity of 2′-fluoro-modified RNA. Nucleic Acids Res. *39*, 3482–3495.

Peacock, H., Kannan, A., Beal, P.A., and Burrows, C.J. (2011). Chemical modification of siRNA bases to probe and enhance RNA interference. J. Org. Chem. *76*, 7295–7300.

Peffer, N.J., Hanvey, J.C., Bisi, J.E., Thomson, S.A., Hassman, C.F., Noble, S.A., and Babiss, L.E. (1993). Strand-invasion of duplex DNA by peptide nucleic acid oligomers. Proc. Natl. Acad. Sci. USA *90*, 10648–10652.

Pensato, S., Saviano, M., and Romanelli, A. (2007). New peptide nucleic acid analogues: synthesis and applications. Expert Opin. Biol. Ther. *7*, 1219–1232.

Potenza, N., Moggio, L., Milano, G., Salvatore, V., Di Blasio, B., Russo, A., and Messere, A. (2008). RNA interference in mammalia cells by RNA-3′-PNA chimeras. Int. J. Mol. Sci. *9*, 299–315.

Prakash, T.P. (2011). An overview of sugar-modified oligonucleotides for antisense therapeutics. Chem. Biodivers. *8*, 1616–1641.

Prakash, T.P., Allerson, C.R., Dande, P., Vickers, T.A., Sioufi, N., Jarres, R., Baker, B.F., Swayze, E.E., Griffey, R.H., and Bhat, B. (2005). Positional effect of chemical modifications on short interference RNA activity in mammalian cells. J. Med. Chem. *48*, 4247–4253.

Prakash, T.P., Siwkowski, A., Allerson, C.R., Migawa, M.T., Lee, S., Gaus, H.J., Black, C., Seth, P.P., Swayze, E.E., and Bhat, B. (2010). Antisense oligonucleotides containing conformationally constrained 2′,4′-(N-methoxy)aminomethylene and 2′,4′-aminooxymethylene and 2′-O,4′-C-aminomethylene bridged nucleoside analogues show improved potency in animal models. J. Med. Chem. *53*, 1636–1650.

Rait, V.K., and Shaw, B.R. (1999). Boranophosphates support the RNase H cleavage of polyribonucleotides. Antisense Nucleic Acid Drug Dev. *9*, 53–60.

Rao, D.D., Vorhies, J.S., Senzer, N., and Nemunaitis, J. (2009). siRNA vs. shRNA: similarities and differences. Adv. Drug Deliv. Rev. *61*, 746–759.

Renneberg, D., Bouliong, E., Reber, U., Schümperli, D., and Leumann, C.J. (2002). Antisense properties of tricyclo-DNA. Nucleic Acids Res. *30*, 2751–2757.

Rettig, G.R., and Behlke, M.A. (2012). Progress toward in vivo use of siRNAs-II. Mol. Ther. *20*, 483–512.

Rigo, F., Hua, Y., Chun, S.J., Prakash, T.P., Krainer, A.R., and Bennett, C.F. (2012). Synthetic oligonucleotides recruit ILF2/3 to RNA transcripts to modulate splicing. Nat. Chem. Biol. *8*, 555–561.

Sabatino, D., and Damha, M.J. (2007). Oxepane nucleic acids: synthesis, characterization, and properties of oligonucleotides bearing a seven-membered carbohydrate ring. J. Am. Chem. Soc. *129*, 8259–8270.

Saleh, A.F., Arzumanov, A.A., and Gait, M.J. (2012). Overview of alternative oligonucleotide chemistries for exon skipping. In Exon Skipping: Methods and Protocols, A. Aartsma-Rus, ed. (New York: Humana Press), pp. 365–378.

Saneyoshi, H., Seio, K., and Sekine, M. (2005). A general method for the synthesis of 2′-O-cyanoethylated oligoribonucleotides having promising hybridization affinity for DNA and RNA and enhanced nuclease resistance. J. Org. Chem. *70*, 10453–10460.

Sanghvi, Y.S. (2011). A status update of modified oligonucleotides for chemotherapeutics applications. In Current Protocols in Nucleic Acid Chemistry, S.L. Beaucage, D.E. Bergstrom, P. Herdewijn, and A. Matsuda, eds. (Hoboken, NJ: John Wiley & Sons), pp. 4.1.1–4.1.22.

Schirle, N.T., and MacRae, I.J. (2012). The crystal structure of human Argonaute2. Science *336*, 1037–1040.

Schlee, M., Hornung, V., and Hartmann, G. (2006). siRNA and isRNA: two edges of one sword. Mol. Ther. *14*, 463–470.

Schwarz, D.S., Hutvágner, G., Haley, B., and Zamore, P.D. (2002). Evidence that siRNAs function as guides, not primers, in the Drosophila and human RNAi pathways. Mol. Cell *10*, 537–548.

Schwarz, D.S., Hutvágner, G., Du, T., Xu, Z., Aronin, N., and Zamore, P.D. (2003). Asymmetry in the assembly of the RNAi enzyme complex. Cell *115*, 199–208.

Seth, P.P., Allerson, C.R., Berdeja, A., Siwkowski, A., Pallan, P.S., Gaus, H., Prakash, T.P., Watt, A.T., Egli, M., and Swayze, E.E. (2010). An exocyclic methylene group acts as a bioisostere of the 2′-oxygen atom in LNA. J. Am. Chem. Soc. *132*, 14942–14950.

Shakeel, S., Karim, S., and Ali, A. (2006). Peptide nucleic acid (PNA)—a review. J. Chem. Technol. Biotechnol. *81*, 892–899.

Sheehan, D., Lunstad, B., Yamada, C.M., Stell, B.G., Caruthers, M.H., and Dellinger, D.J. (2003). Biochemical properties of phosphonoacetate and thiophosphonoacetate oligodeoxyribonucleotides. Nucleic Acids Res. *31*, 4109–4118.

Shen, L., Siwkowski, A., Wancewicz, E.V., Lesnik, E., Butler, M., Witchell, D., Vasquez, G., Ross, B., Acevedo, O., Inamati, G., et al. (2003). Evaluation of C-5 propynyl pyrimidine-containing oligonucleotides in vitro and in vivo. Antisense Nucleic Acid Drug Dev. *13*, 129–142.

Shukla, S., Sumaria, C.S., and Pradeepkumar, P.I. (2010). Exploring chemical modifications for siRNA therapeutics: a structural and functional outlook. ChemMedChem *5*, 328–349.

Sipa, K., Sochacka, E., Kazmierczak-Baranska, J., Maszewska, M., Janicka, M., Nowak, G., and Nawrot, B. (2007). Effect of base modifications on structure, thermodynamic stability, and gene silencing activity of short interfering RNA. RNA *13*, 1301–1316.

Somoza, A., Silverman, A.P., Miller, R.M., Chelliserrykattil, J., and Kool, E.T. (2008). Steric effects in RNA interference: probing the influence of nucleobase size and shape. Chemistry *14*, 7978–7987.

Sonenberg, N., and Hinnebusch, A.G. (2009). Regulation of translation initiation in eukaryotes: mechanisms and biological targets. Cell *136*, 731–745.

Soutschek, J., Akinc, A., Bramlage, B., Charisse, K., Constien, R., Donoghue, M., Elbashir, S., Geick, A., Hadwiger, P., Harborth, J., et al. (2004). Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. Nature *432*, 173–178.

Stein, C.A., Hansen, J.B., Lai, J., Wu, S., Voskresenskiy, A., Høg, A., Worm, J., Hedtjärn, M., Souleimanian, N., Miller, P., et al. (2010). Efficient gene silencing by delivery of locked nucleic acid antisense oligonucleotides, unassisted by transfection reagents. Nucleic Acids Res. *38*, e3.

Stein, H., and Hausen, P. (1969). Enzyme from calf thymus degrading the RNA moiety of DNA-RNA Hybrids: effect on DNA-dependent RNA polymerase. Science *166*, 393–395.

Stephenson, M.L., and Zamecnik, P.C. (1978). Inhibition of Rous sarcoma viral RNA translation by a specific oligodeoxyribonucleotide. Proc. Natl. Acad. Sci. USA *75*, 285–288.

Summerton, J. (1999). Morpholino antisense oligomers: the case for an RNase H-independent structural type. Biochim. Biophys. Acta *1489*, 141–158.

Summerton, J., and Weller, D. (1997). Morpholino antisense oligomers: design, preparation, and properties. Antisense Nucleic Acid Drug Dev. *7*, 187–195.

Takeda, K., and Akira, S. (2005). Toll-like receptors in innate immunity. Int. Immunol. *17*, 1–14.

Terrazas, M., Ocampo, S.M., Perales, J.C., Marquez, V.E., and Eritja, R. (2011). Effect of north bicyclo[3.1.0]hexane 2′-deoxy-pseudosugars on RNA interference: a novel class of siRNA modification. ChemBioChem *12*, 1056–1065.

Threlfall, R.N., Torres, A.G., Krivenko, A., Gait, M.J., and Caruthers, M.H. (2012). Synthesis and biological activity of phosphonoacetate- and thiophosphonoacetate-modified 2′-O-methyl oligoribonucleotides. Org. Biomol. Chem. *10*, 746–754.

Trempe, J.F., Wilds, C.J., Denisov, A.Y., Pon, R.T., Damha, M.J., and Gehring, K. (2001). NMR solution structure of an oligonucleotide hairpin with a 2'F-ANA/RNA stem: implications for RNase H specificity toward DNA/RNA hybrid duplexes. J. Am. Chem. Soc. *123*, 4896–4903.

Turner, J.J., Jones, S.W., Moschos, S.A., Lindsay, M.A., and Gait, M.J. (2007). MALDI-TOF mass spectral analysis of siRNA degradation in serum confirms an RNAse A-like activity. Mol. Biosyst. *3*, 43–50.

Ui-Tei, K., Naito, Y., Zenno, S., Nishi, K., Yamato, K., Takahashi, F., Juni, A., and Saigo, K. (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. Nucleic Acids Res. *36*, 2136–2151.

Vaish, N., Chen, F., Seth, S., Fosnaugh, K., Liu, Y., Adami, R., Brown, T., Chen, Y., Harvie, P., Johns, R., et al. (2011). Improved specificity of gene silencing by siRNAs containing unlocked nucleobase analogs. Nucleic Acids Res. *39*, 1823–1832.

Verbeure, B., Lescrinier, E., Wang, J., and Herdewijn, P. (2001). RNase H mediated cleavage of RNA by cyclohexene nucleic acid (CeNA). Nucleic Acids Res. *29*, 4941–4947.

Viazovkina, E., Mangos, M., Elzagheid, M.I., and Damha, M.J. (2002). Solid-phase synthesis of 2′-deoxy-2′-fluoro-β-D-oligoarabinonucleotides (2′-F-ANA) and their phosphorothioate derivatives. In Current Protocols in Nucleic Acids Chemistry, S.L. Beaucage, D.E. Bergstrom, P. Herdewijn, and A. Matsude, eds. (Hoboken, NJ: John Wiley & Sons), pp. 4.15.1–4.15.22.

Wahba, A.S., Esmaeili, A., Damha, M.J., and Hudson, R.H.E. (2010). A single-label phenylpyrrolocytidine provides a molecular beacon-like response reporting HIV-1 RT RNase H activity. Nucleic Acids Res. *38*, 1048–1056.

Wahba, A.S., Azizi, F., Deleavey, G.F., Brown, C., Robert, F., Carrier, M., Kalota, A., Gewirtz, A.M., Pelletier, J., Hudson, R.H.E., and Damha, M.J. (2011). Phenylpyrrolocytosine as an unobtrusive base modification for monitoring activity and cellular trafficking of siRNA. ACS Chem. Biol. *6*, 912–919.

Wahlestedt, C., Salmi, P., Good, L., Kela, J., Johnsson, T., Hökfelt, T., Broberger, C., Porreca, F., Lai, J., Ren, K., et al. (2000). Potent and nontoxic antisense oligonucleotides containing locked nucleic acids. Proc. Natl. Acad. Sci. USA *97*, 5633–5638.

Wang, J., Verbeure, B., Luyten, I., Lescrinier, E., Froeyen, M., Hendrix, C., Rosemeyer, H., Seela, F., Van Aerschot, A., and Herdewijn, P. (2000). Cyclohexene nucleic acids (CeNA): serum stable oligonucleotides that activate RNase H and increase duplex stability with complementary RNA. J. Am. Chem. Soc. *122*, 8595–8602.

Wang, Y., Juranek, S., Li, H., Sheng, G., Tuschl, T., and Patel, D.J. (2008a). Structure of an argonaute silencing complex with a seed-containing guide DNA and target RNA duplex. Nature *456*, 921–926.

Wang, Y., Sheng, G., Juranek, S., Tuschl, T., and Patel, D.J. (2008b). Structure of the guide-strand-containing argonaute silencing complex. Nature *456*, 209–213.

Wang, Y., Juranek, S., Li, H., Sheng, G., Wardle, G.S., Tuschl, T., and Patel, D.J. (2009). Nucleation, propagation and cleavage of target RNAs in Ago silencing complexes. Nature *461*, 754–761.

Watts, J.K., and Damha, M.J. (2008). 2′F-Arabinonucleic acids (2′F-ANA)—history, properties, and new frontiers. Can. J. Chem. *86*, 641–656.

Watts, J.K., and Corey, D.R. (2012). Silencing disease genes in the laboratory and the clinic. J. Pathol. *226*, 365–379.

Watts, J.K., Choubdar, N., Sadalapure, K., Robert, F., Wahba, A.S., Pelletier, J., Pinto, B.M., and Damha, M.J. (2007). 2′-fluoro-4′-thioarabino-modified oligonucleotides: conformational switches linked to siRNA activity. Nucleic Acids Res. *35*, 1441–1451.

Watts, J.K., Deleavey, G.F., and Damha, M.J. (2008a). Chemically modified siRNA: tools and applications. Drug Discov. Today *13*, 842–855.

Watts, J.K., Johnston, B.D., Jayakanthan, K., Wahba, A.S., Pinto, B.M., and Damha, M.J. (2008b). Synthesis and biophysical characterization of oligonucleotides containing a 4′-selenonucleotide. J. Am. Chem. Soc. *130*, 8578–8579.

Watts, J.K., Katolik, A., Viladoms, J., and Damha, M.J. (2009). Studies on the hydrolytic stability of 2′-fluoroarabinonucleic acid (2'F-ANA). Org. Biomol. Chem. *7*, 1904–1910.

Watts, J.K., Martín-Pintado, N., Gómez-Pinto, I., Schwartzentruber, J., Portella, G., Orozco, M., González, C., and Damha, M.J. (2010). Differential stability of 2'F-ANA*RNA and ANA*RNA hybrid duplexes: roles of structure, pseudohydrogen bonding, hydration, ion uptake and flexibility. Nucleic Acids Res. *38*, 2498–2511.

Weitzer, S., and Martinez, J. (2007). The human RNA kinase hClp1 is active on 3′ transfer RNA exons and short interfering RNAs. Nature *447*, 222–226.

Whitehead, K.A., Dahlman, J.E., Langer, R.S., and Anderson, D.G. (2011). Silencing or stimulation? siRNA delivery and the immune system. Annu. Rev. Chem. Biomol. Eng. *2*, 77–96.

Wilds, C.J., and Damha, M.J. (2000). 2′-Deoxy-2′-fluoro-β-D-arabinonucleosides and oligonucleotides (2'F-ANA): synthesis and physicochemical studies. Nucleic Acids Res. *28*, 3625–3635.

Wojciechowski, F., and Hudson, R.H.E. (2007). Nucleobase modifications in peptide nucleic acids. Curr. Top. Med. Chem. *7*, 667–679.

Wu, H., Lima, W.F., Zhang, H., Fan, A., Sun, H., and Crooke, S.T. (2004). Determination of the role of the human RNase H1 in the pharmacology of DNA-like antisense drugs. J. Biol. Chem. *279*, 17181–17189.

Xia, J., Noronha, A., Toudjarska, I., Li, F., Akinc, A., Braich, R., Frank-Kamenetsky, M., Rajeev, K.G., Egli, M., and Manoharan, M. (2006). Gene silencing activity of siRNAs with a ribo-difluorotoluyl nucleotide. ACS Chem. Biol. *1*, 176–183.

Yamada, C.M., Dellinger, D.J., and Caruthers, M.H. (2007). Synthesis and biological activity of phosphonocarboxylate DNA. Nucleosides Nucleotides Nucleic Acids *26*, 539–546.

Ye, X., Huang, N., Liu, Y., Paroo, Z., Huerta, C., Li, P., Chen, S., Liu, Q., and Zhang, H. (2011). Structure of C3PO and mechanism of human RISC activation. Nat. Struct. Mol. Biol. *18*, 650–657.

Yuan, X., Naguib, S., and Wu, Z. (2011). Recent advances of siRNA delivery by nanoparticles. Expert Opin. Drug Deliv. *8*, 521–536.

Zamanian-Daryoush, M., Marques, J.T., Gantier, M.P., Behlke, M.A., John, M., Rayman, P., Finke, J., and Williams, B.R.G. (2008). Determinants of cytokine induction by small interfering RNA in human peripheral blood mononuclear cells. J. Interferon Cytokine Res. *28*, 221–233.

Zamecnik, P.C., and Stephenson, M.L. (1978). Inhibition of Rous sarcoma virus replication and cell transformation by a specific oligodeoxynucleotide. Proc. Natl. Acad. Sci. USA *75*, 280–284.

