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**REVIEW**

# Chemical Modification of siRNAs for *In Vivo* Use

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Well over a hundred reports have been published describing use of synthetic small-interfering RNAs (siRNAs) in animals. The majority of these reports employed unmodified RNA duplexes. While unmodified RNA is the natural effector molecule of RNA interference, certain problems arise with experimental or therapeutic use of RNA duplexes *in vivo*, some of which can be improved or solved through use of chemical modifications. Judicious use of chemical modifications can improve the nuclease stability of an RNA duplex, decrease the likelihood of triggering an innate immune response, lower the incidence of off-target effects (OTEs), and improve pharmacodynamics. This review will examine studies that document the utility of various chemical modifications for use in siRNAs, both *in vitro* and *in vivo*, with close attention given to reports demonstrating actual performance in animal model systems.

## Introduction

The year 2008 marks the 30th anniversary of the first experiments that employed synthetic oligonucleotides to specifically alter expression of targeted genes. In this early pioneering work, antisense DNA oligonucleotides were used to prevent translation of Rous Sarcoma virus RNA (Stephenson and Zamecnik, 1978; Zamecnik and Stephenson, 1978). Soon thereafter, antisense RNA was also shown to work as an experimental tool to inhibit gene expression (Melton, 1985; Graessmann et al., 1991). The efficacy of these early attempts to interfere with gene expression were limited by the short half-life of unmodified DNA or RNA molecules, which are quickly degraded by the nucleases present in serum or living cells. Synthetic nucleic acids can be chemically modified in ways that decrease their susceptibility to nuclease degradation and also changes their properties in ways that can be useful for *in vivo* applications, such as reducing their ability to trigger an innate immune response and improving their general pharmacodynamic properties. Hundreds of different modifications have been studied for utility in antisense applications (Matteucci, 1997; Manoharan, 2002; Jason et al., 2004). A variety of chemically modified antisense compounds have been successfully used for *in vivo* experimental biological studies and over a dozen candidate compounds are in various stages of clinical testing for use as therapeutic agents for a variety of disease indications (Crooke, 2004). So far, only a single antisense agent has been approved for clinical use by the US Food and Drug Administration (FDA) [Vitravene, a 21-mer phosphorothioate (PS) DNA oligonucleotide which was approved in 1998 to treat CMV retinitis by direct intravitreal injection (Grillone and Lanz, 2001; Jabs and Griffiths, 2002)].

While "antisense" technology was the first method that employed synthetic nucleic acids to alter gene expression, a number of other methodologies have since emerged that employ oligonucleotides as tools to regulate gene expression levels that work through very different mechanisms of action. These methods include ribozymes, aptamers, and most recently, RNA interference (RNAi). RNAi is a natural gene regulatory network wherein short double-stranded RNAs (dsRNAs) interfere with the expression of complementary genes (Hannon and Rossi, 2004; Meister and Tuschl, 2004; Mello and Conte, 2004). It is an ancient pathway that is present in plants, mammals, and even some fungi. In primitive systems, RNAi can be effectively triggered by the presence of long dsRNA species. In higher organisms, long dsRNAs also trigger innate immune responses and their use can be toxic. Long dsRNAs are processed into shorter species which are the actual effector molecules that trigger an RNAi response. These shorter species, called "small-interfering RNAs" (siRNAs), are functional in mammalian cells and synthetic siRNAs can be safely used to experimentally activate RNAi (Elbashir et al., 2001).

RNAi operates at multiple different sites within a cell and can suppress gene expression at the DNA level by inhibiting transcription (in the nucleus), by degrading messenger RNA (mRNA) (in the cytoplasm), or by directly suppressing translation (in the cytoplasm). The biochemistry of RNAi is

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complex and the interaction of many different proteins is required to achieve gene suppression. Functioning in the cytoplasm, a heterodimer complex comprising TRBP and the endoribonuclease Dicer processes dsRNAs (23-base length or longer) into 21–22-mer siRNAs which have 2-base 3′-overhangs and 5′-phosphates. Argonaute 2 (Ago2) joins Dicer/TRBP and together with the siRNA forms the simplest functional RISC (RNA-Induced Silencing Complex). In RISC, the siRNA is passed from Dicer/TRBP to Ago2 and one strand (the "passenger" strand) of the siRNA is degraded (or unwound and discarded) and the remaining single-stranded RNA (ssRNA) (the "guide" strand) remains associated with Ago2 and directs the sequence specificity of silencing by RISC. Ago2 contains an RNase H like domain which functions to cleave one strand of an RNA:RNA duplex and is the catalytic component of RISC that degrades a target RNA. Typically, perfect or near perfect base pairing between the siRNA guide strand and the target mRNA is required for Ago2 cleavage to occur.

Of the four Ago proteins in mammalian cells, only Ago2 has nuclease activity. The other three Ago proteins can nevertheless still be active components of RISC and it appears that some of these (at least Ago1) are involved in mediating the translational suppression arm of RNAi. Although any RNA loaded into RISC can theoretically trigger the translational suppression pathway, endogenous microRNAs (miRNAs) are natural regulatory molecules that employ this mechanism to regulate gene expression. Endogenous miRNAs are encoded in genomic DNA and are expressed as long hairpin transcripts in the nucleus. These long hairpins are processed by the endoribonuclease Drosha in the nucleus and are exported to the cytoplasm where processing is completed by Dicer/TRBP prior to loading into RISC. Unlike siRNAs, imperfect base pairing between the target RNA and a miRNA triggers the translational repression activity of RISC. Small-interfering RNAs and miRNAs can function in either pathway so that imperfect base pairing between a siRNA and a target mRNA can result in unintended translational suppression of that target and can cause undesired side effects (so called "Off Target Effects", or OTEs) (Doench et al., 2003; Saxena et al., 2003; Yekta et al., 2004; Bagga et al., 2005). More details about the biochemistry of RNAi can be found in recent reviews (Hannon and Rossi, 2004; Meister and Tuschl, 2004; Mello and Conte, 2004; Filipowicz et al., 2005; Sontheimer, 2005; Grewal and Elgin, 2007; Rana, 2007; Filipowicz et al., 2008; Hutvagner and Simard, 2008). This review will primarily focus on the chemistry of the siRNA effector molecules that activate the degradative RNAi pathway.

RNAi has rapidly progressed beyond use as an *in vitro* research tool and hundreds of reports already have been published describing use in animals. A variety of pharmaceutical and biotechnology companies are actively working toward development of RNAi-based therapeutics and human patients have already received siRNA drugs in clinical trials (Behlke, 2006; de Fougerolles et al., 2007; Kim and Rossi, 2007). While many of the early *in vivo* studies were done using unmodified RNAs, much of the current work relating to RNAi therapeutics involves use of chemically modified compounds.

## The Rationale for Chemical Modification

Most siRNAs used in research today are made by chemical synthesis using phosphoramidite building blocks as single-stranded oligonucleotides and are annealed into double-stranded form. This approach permits incorporation of a wide variety of natural and artificial modifications into the siRNA that can help solve some of the problems associated with administration of synthetic nucleic acids into cells or animals. Some problems that can be addressed using chemical modification include:

1. Susceptibility to nuclease degradation
2. Activation of the innate immune system (OTE)
3. Unwanted participation in miRNA pathways (OTE)
4. Cell uptake and pharmacokinetics.

The precise choice of chemical modifications to employ can vary with the design of the siRNA used, specific sequence, intended application, and method of delivery.

Most siRNAs used today for *in vivo* research applications are synthetic 21-mer RNA duplexes that mimic the design of natural siRNAs. Similarly, 21-mer siRNAs are currently the lead compounds for a number of clinical and preclinical RNAi drug development programs. Alternative designs are also in use for both research and drug development, including blunt 19-mers (Czauderna et al., 2003; Allerson et al., 2005; Prakash et al., 2005; Kraynack and Baker, 2006), blunt 25-mers (Chen et al., 2005), blunt 27-mers (Kim et al., 2005), and asymmetric 25/27-mers or 27/29-mers (Rose et al., 2005; Dore-Savard et al., 2008; Nishina et al., 2008). Some of these compounds directly load into RISC whereas others are substrates for Dicer and are processed into shorter species before RISC loading. The precise design of the siRNA employed can influence the choice or pattern of chemical modifications suitable for use.

Site selection is critical to the performance of siRNA compounds and a large amount of work from many different groups has led to the development of excellent site selection and design criteria as well as computer assisted algorithms that facilitate this process (Pei and Tuschl, 2006; Peek and Behlke, 2007). These design rules and algorithms were all developed using data from studies performed using unmodified siRNAs. It is important to note that the use of chemical modifications can alter the potency of a siRNA and frequently a chemically modified siRNA will show lower potency than the unmodified RNA version of that same sequence, especially when extensively modified. Specifically, certain modification patterns have been demonstrated to impair the ability of an RNA sequence to trigger an RNAi effect whereas other patterns have less of an impact on potency. These effects vary with sequence, such that a given modification pattern can be effective at one site yet reduce potency of a siRNA at a second site. Thus empiric testing is usually necessary to ensure that a modified siRNA is effective when a known potent unmodified siRNA is converted to a modified form. Alternatively, initial site screening can be done using only modified duplexes, bypassing use of unmodified RNA entirely. Design rules and site selection criteria specific for modified siRNAs have not been reported.

## Susceptibility to Nuclease Degradation

Single-stranded nucleic acids are rapidly degraded in serum or inside cells. Double-stranded nucleic acids, including siRNAs, are more stable than their single-stranded counterparts, but are still degraded and must be protected from nuclease attack if use includes exposure to serum. Protection can be provided externally through use of a suitable delivery tool (such as complexation with a nanoparticle or encapsulation within a liposome) or intrinsically through use of nuclease resistant chemical modification of the nucleic acid itself. The primary nuclease activity in serum is a processive 3′-exonuclease which may be ERI-1 (THEX1) (Eder et al., 1991; Kennedy et al., 2004), making 3′ end stabilization particularly important. The single-stranded 3′-overhangs present in traditional 21-mer siRNA designs are particularly susceptible to degradation. In serum, nuclease attack probably initiates at the 3′-overhangs and proceeds in a 3′ → 5′ direction (Zou et al., 2008). Placement of an inverted-dT base or other non-nucleotide groups at this position can protect against exonuclease attack. Interestingly, an RNase A like activity has also been implicated in the degradation of siRNAs in serum (Haupenthal et al., 2007; Turner et al., 2007) and inhibiting RNase A activity can improve stability of siRNAs (Haupenthal et al., 2006). RNase A cleaves at pyrimidine bases in ssRNA so it is not entirely clear how degradation of double-stranded siRNAs proceeds; it is possible that transient transitions to single-stranded form ("breathing") in these short duplexes might provide a suitable substrate for attack by this class of nuclease. Longer RNAs, such as 27-mer Dicer-substrate duplexes, appear to have slightly greater inherent stability to serum nucleases (Kubo et al., 2007, 2008).

When introducing chemical modifications into a siRNA, it is important to recall that the RNA must interact with a number of different cellular proteins, many or all of which may be sensitive to changes in RNA structure caused by the modifying group. The simplest approach to increase nuclease stability is to directly modify the internucleotide phosphate linkage. Replacement of a non-bridging oxygen with sulfur (PS), boron (boranophosphate), nitrogen (phosphoramidate), or methyl (methylphosphonate) groups will provide nuclease resistance and have all been used to help stabilize single-stranded antisense oligonucleotides. Figure 1 shows various modifications that improve nuclease stability and can be employed in siRNAs. Phosphoramidate and methylphosphonate derivatives were extensively explored for use in antisense applications and were found to significantly alter interactions between the nucleic acid and cellular enzymes, such as RNase H. Their use has not been systematically studied for use in RNAi. Boranophosphate modified DNA or RNA is resistant to nuclease degradation and the boron modification appears to be compatible with siRNA function; however, boranophosphates are not easily made using chemical synthesis (Hall et al., 2004; Hall et al., 2006). The PS modification is easily made and has been extensively used to improve nuclease stability of both antisense oligonucleotides and siRNAs. Phosphorothioate modified nucleic acids are sulfated polyanions that are "sticky" and can nonspecifically bind to a variety of cellular proteins, potentially causing unwanted side effects (Krieg and Stein, 1995). Nevertheless, this modification can be safely used to improve stability of a siRNA (Amarzguioui et al., 2003; Braasch et al., 2003; Chiu and Rana, 2003; Harborth et al., 2003; Li et al., 2005; Choung et al., 2006). Restricting placement of PS-modified bonds to the ends of the oligonucleotides will provide resistance to exonucleases while minimizing the overall PS content of the oligo, thereby limiting unwanted side effects. Given the long history of use of PS-modified antisense oligonucleotides, the potential toxicity of this modification is well understood and PS-modified compounds can be safely administered (Levin, 1999).

Modification of the 2′-position of the ribose can indirectly improve nuclease resistance of the internucleotide phosphate bond and at the same time can increase duplex stability ($T_m$) and may also provide protection from immune activation. 2′-*O*-methyl RNA (2′OMe) is a naturally occurring RNA variant found in mammalian ribosomal RNAs and transfer RNAs. It is nontoxic and can be placed within either the S or AS strands of a siRNA (Amarzguioui et al., 2003; Chiu and Rana, 2003; Czauderna et al., 2003; Harborth et al., 2003; Choung et al., 2006). Heavy modification with 2′OMe RNA can reduce potency or completely inactivate a siRNA; alternating 2′OMe with RNA bases generally retains siRNA function while conferring significant nuclease stabilization (Czauderna et al., 2003; Choung et al., 2006; Santel et al., 2006a; Santel et al., 2006b). 2′OMe RNA can be combined with other 2′-modifications which are not naturally occurring bases with good results.

The 2′-fluoro (2′-F) modification is compatible with siRNA function and also helps stabilize the duplex against nuclease degradation. Incorporation of 2′-F at pyrimidine positions maintains siRNA activity *in vitro* (Allerson et al., 2005; Prakash et al., 2005; Kraynack and Baker, 2006) and *in vivo* (Layzer et al., 2004). The 2′-F modification is even tolerated at the site of Ago2 cleavage (Muhonen et al., 2007). The combined use of 2′-F pyrimidines with 2′OMe purines can results in RNA duplexes with extreme stability in serum and improved *in vivo* performance (Morrissey et al., 2005b). The 2′-*O*-(2-methoxyethyl) RNA (MOE) modification has been extensively used in antisense oligonucleotides and confers significant nuclease stability to an oligonucleotide as well as increases $T_m$. 2′-MOE residues can be incorporated into siRNAs much like 2′OMe or 2′-F (Prakash et al., 2005), however this modification is not generally available for use. The 2′-fluoro-$\beta$-D-arabinonucleotide (FANA) modification has also shown promise in antisense oligonucleotide applications and can also be placed in siRNAs. Substitution of FANA for RNA in the entire S-strand confers significant stabilization to nucleases while maintaining functional potency of the duplex; however, the AS-strand is less tolerant of the FANA modification (Dowler et al., 2006).

Locked nucleic acids (LNAs) contain a methylene bridge which connects the 2′-*O* with the 4′-*C* of the ribose. The methylene bridge "locks" the sugar in the 3′-*endo* conformation, providing both a significant increase in $T_m$ as well as nuclease resistance. Extensive modification of a siRNA with LNA bases generally results in decreased activity (even more so than 2′OMe); however, siRNAs with limited incorporation retain functionality and offer significant nuclease stabilization (Braasch et al., 2003; Grunweller et al., 2003; Elmen et al., 2005; Mook et al., 2007).

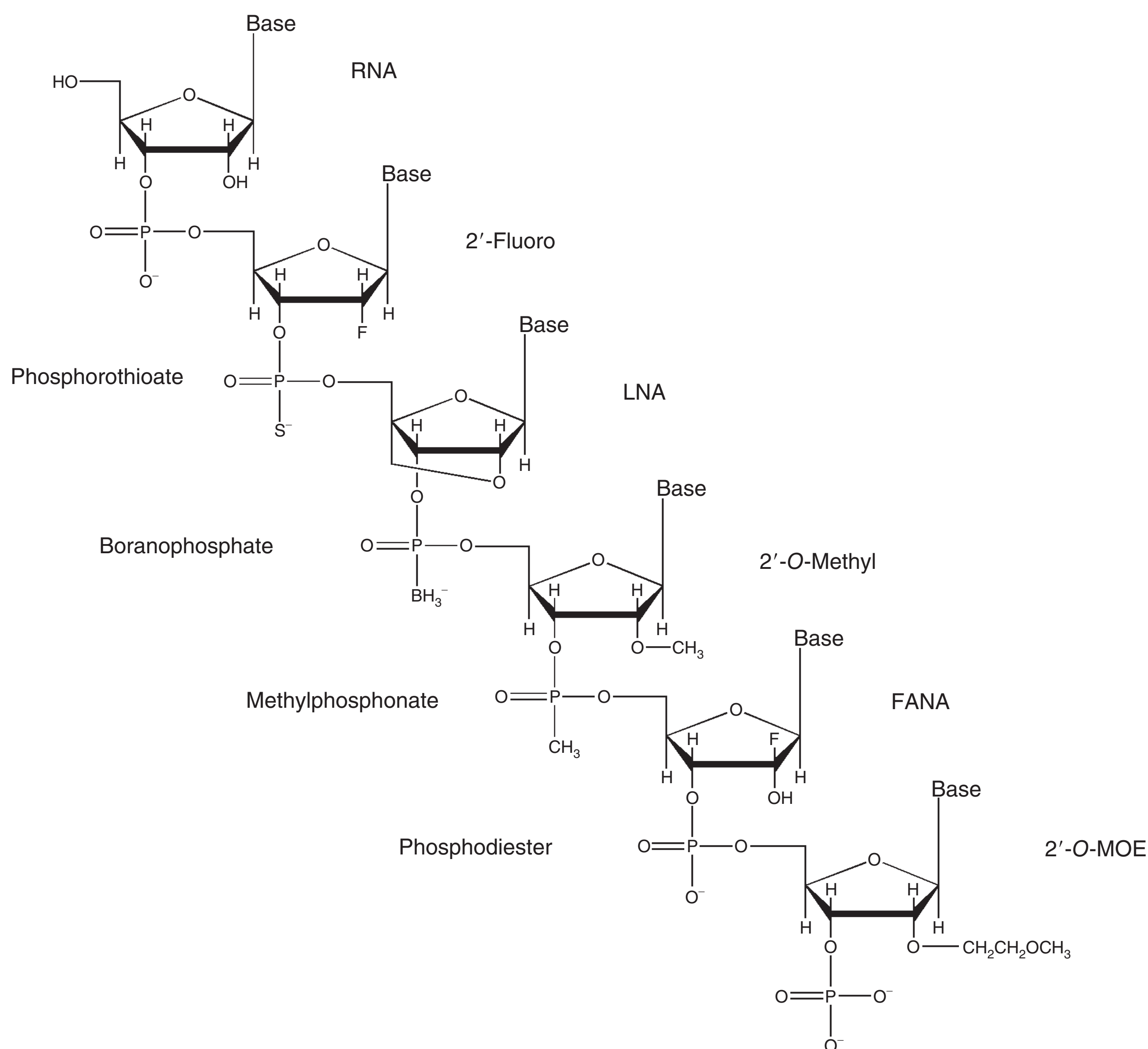


**FIG. 1.** Select chemical modifications that can be incorporated into siRNAs. Modifications to the phosphodiester backbone are shown, including phosphorothioate, boranophosphate, and methylphosphonate. Modification to the sugar backbone are shown, including 2′-fluoro, LNA, 2′OMe, FANA, and 2′MOE.

The 2′OMe modification is a naturally occurring RNA variant and its use in synthetic siRNAs is not anticipated to present significant toxicity. Other 2′-modifications discussed here are not naturally occurring and their potential for toxic side effects needs to be considered. The 2′-F modification has been studied for safety as a component of synthetic oligonucleotides. While substantially stabilized to nuclease attack, even heavily modified duplexes are eventually degraded in serum or in cells and processive 3′-exonuclease attack appears to be the primary route of degradation (Zou et al., 2008); thus it is expected that free 2′-modified nucleosides will be released into cells. When administered to rodents, 2′-F residues can later be found incorporated into both endogenous DNA and RNA in a variety of tissues (Richardson et al., 1999; Richardson et al., 2002). However, no obvious toxicity was observed to result from exposure to 2′-F modified nucleic acids. In contrast, some dose-dependent hepatic toxicity has been reported when LNA-containing oligonucleotides were administered to mice, ranging from mild elevation of hepatic serum transaminases (Fluiter et al., 2003) to frank hepatic necrosis (Swayze et al., 2007). In another study, no significant toxicity was observed in intravenous (i.v.) dosing of LNA-modified oligonucleotides to African green monkeys (Elmen et al., 2008). More studies need to be done to fully assess potential toxicity of the LNA modification, perhaps considering the possibility of species-specific variation in such responses.

Although not generally considered to be a nuclease-stabilizing modification, substitution of DNA bases into a siRNA can alter its stability to ribonucleases and may offer certain

advantages. A surprisingly large amount of DNA can be incorporated into a dsRNA and retain potency to trigger RNAi; the entire S (passenger) strand can be DNA (Hogrefe et al., 2006; Pirollo et al., 2007). The guide strand is less tolerant to DNA modification than the passenger strand. Nevertheless, the 5′ end of the guide strand (AS-strand) can contain DNA residues and still retain most functionality (Ui-Tei et al., 2008). The ability to insert DNA bases in this area may have added benefit to alter OTEs (see below).

The modification strategies discussed above are intended to impart nuclease resistance to 21-mer siRNAs while retaining the ability of the duplexes to enter RISC and maintain guide-strand function with Ago2. The situation is somewhat more complex when introducing chemical modifications into longer Dicer-substrate siRNAs where some degree of nuclease sensitivity is desired so that Dicer cleavage can still occur. Thus slightly different modification rules apply for this class of siRNAs. So long as the "Dicing domain" remains unmodified, very similar patterns of modification can be employed with good results (Collingwood et al., 2008; Nishina et al., 2008), and it is possible to make duplexes having improved stability in serum while retaining the ability to be cleaved by Dicer.

The relative impact that nuclease stabilization of a siRNA has on functional gene knockdown *in vivo* will vary with the method of administration. "Naked" siRNA exposed to serum will obviously benefit the greatest from chemical modification. In one study, nuclease-resistant siRNA administered by direct hydrodynamic injection gave 4-fold greater reduction in target levels than unmodified siRNAs (Bartlett and Davis, 2007), however the kinetics of recovery after administration were similar. An even greater difference in potency between modified and unmodified duplexes was seen in a murine hepatitis B virus (HBV) model system (Morrissey et al., 2005a). Once the guide strand is associated with Ago2 in RISC, the protein components may themselves protect the RNA from intracellular nucleases, and unmodified siRNA can show 2–4 weeks duration of action in slowly dividing or nondividing cells (Song et al., 2003; Omi et al., 2004; Bartlett and Davis, 2007). Thus the greatest benefit from nuclease stability may be realized during delivery, and chemical stabilization of the siRNA might be less important when employing a delivery tool that protects the siRNA cargo.

### Off-Target Effect: Triggering the Innate Immune System

The innate immune system provides the body's first line of defense in the recognition and response to foreign substances. A variety of different specialized receptors have evolved that recognize "pathogen associated molecular patterns" and trigger immune responses when their ligands are present (Armant and Fenton, 2002; Tosi, 2005). Some of these receptors are restricted to immune cell lineages while others are present in all cells. Synthetic nucleic acids can be recognized as "foreign" by the innate immune system. These responses constitute one of the major types of OTEs which must be addressed when using antisense oligonucleotides, ribozymes, aptamers, or siRNAs *in vivo*.

Different receptors exist in the cytoplasm and/or in endosomal compartments that specifically recognize foreign motifs in DNA or RNA molecules (Marques and Williams, 2005; Schlee et al., 2006). Toll-like receptor 9 (TLR9) recognizes unmethylated CpG dinucleotides in DNA. In mammals, the cytosine in CpG pairs is methylated at the C5 position; in bacteria, cytosine is not methylated. Synthetic DNA made with unmethylated CpG motifs is recognized as foreign by TLR9 and triggers a type I interferon (IFN) response (Krieg et al., 1999; Krieg et al., 2000). Similarly, synthetic RNA can trigger an IFN response. TLR3 recognizes motifs in dsRNAs (Alexopoulou et al., 2001) and TLRs 7 and 8 recognize motifs in ssRNAs (Diebold et al., 2004). In the cytoplasm, oligoadenylate synthetase (OAS), dsRNA responsive kinase (PKR), retinoic acid inducible gene 1 (RIG-I), and melanoma differentiation-associated gene 5 (Mda5) are all capable of recognizing dsRNA in some form and can trigger immune responses (Stark et al., 1998; Yoneyama et al., 2004).

TLRs 3, 7, and 8 are largely restricted to endosomal compartments and exposure to these receptors is influenced by the mode of entry by which a synthetic RNA enters the cell. Delivery of siRNA using cationic lipids or liposomes maximizes exposure to the endosomal compartment and therefore increases the risk of triggering a TLR based response (Sioud and Sorensen, 2003; Ma et al., 2005; Morrissey et al., 2005b; Sioud, 2005). A known immunogenic sequence that is synthesized within a cell is less immunostimulatory than that same sequence transfected into the cell using lipid-based delivery (Robbins et al., 2006). Similarly, mechanical delivery of an immunostimulatory RNA in mice using hydrodynamic injection fails to produce an IFN response although that same sequence will trigger a response when delivered using lipid vehicles (Heidel et al., 2004). Thus the route and means of *in vivo* delivery of a siRNA can play a large role in the need to protect that sequence from detection from the innate immune system.

Certain RNA sequences have been identified that are particularly immunostimulatory, such as "UGUGU" (Heil et al., 2004; Judge et al., 2005) and "GUCCUUCAA" (Hornung et al., 2005). Unfortunately, avoiding these sequence motifs does not prevent immune activation. The presence of specific chemical modifications allows mammalian tRNAs and rRNAs to escape triggering an autoimmune response; protective modifications include pseudouridine, N6-methyl-A, and 2′OMe modified ribose (Kariko et al., 2005). Use of these same chemical modifications can similarly allow synthetic siRNAs to evade immune detection. In fact, inclusion of only two or three 2′OMe modified residues in an RNA duplex can be sufficient to prevent immune activation, and modification of rU and rG residues is most effective (Judge et al., 2006). Mechanistically, 2′OMe groups act as a competitive inhibitor of TLR7 (Robbins et al., 2007). As a result, 2′-modified groups can protect in *trans*, so that modification of only a single strand of a siRNA is necessary to block the immunostimulatory potential of that duplex. Other 2′-modifications such as 2′-F can confer protection from immune activation; it has not been reported if the LNA modification offers similar benefits. Interestingly, 2′-deoxy (DNA) bases have recently been reported to also block immune detection, particularly dT or dU bases (Eberle et al., 2008). Triggering TLR7 may

involve recognition of specific structures formed by ssRNAs involving internal hairpins, especially containing rU bases (Sioud, 2006; Gantier et al., 2008). Given the minimal impact that substitution of a limited number of 2′OMe-U or dT residues for rU has on functional potency of an siRNA, it may be prudent to routinely include either or both of these modifications in sequences intended for *in vivo* use, especially if lipid-based delivery tools are employed. Unfortunately, these modification strategies do not effectively block TLR3 activation, and immune stimulation through this receptor can still occur even in the absence of lipid-based delivery. It was recently reported that both modified and unmodified naked siRNAs administered by direct intraocular injection can trigger cell surface–localized TLR3 and affect angiogenesis pathways in a sequence nonspecific fashion (Kleinman et al., 2008).

Defending against the cytoplasmic RNA receptors OAS, PKR, RIG-I, and MDA5 may also be important. Oligoadenylate synthetase is not fully stimulated until it encounters dsRNAs in the 60–70 base size (Minks et al., 1979), so direct involvement of OAS in sensing siRNAs seems unlikely. Although PKR is generally thought to maximally respond to dsRNAs >30 bases long, it can sense the presence of species as short as 16 bases. Like TLR7, however, PKR recognition can be blocked by a variety of chemical modifications (Nallagatla and Bevilacqua, 2008). Interestingly, the 2′-F modification does not help evade PKR responses. The specific structural requirements for MDA5 to detect small synthetic RNAs have not been defined.

RIG-I is an important cytoplasmic receptor that can sense the presence of certain classes of synthetic RNAs and plays several different roles in immune surveillance (Yoneyama et al., 2004; Kato et al., 2006; Saito et al., 2008). It was reported that in certain cell lines synthetic RNA duplexes having 19-mer duplex domains with 2-base 3′-overhangs (Dicer-product 21-mer siRNAs) do not trigger any adverse response but that longer RNA duplexes can induce immune responses; TLR3 dependence was proposed (Reynolds et al., 2006). Although some element of TLR3 recognition of these sequences may occur, it appears that RIG-I is an important cytoplasmic receptor capable of recognizing these longer RNA species. Specifically, RIG-I can recognize RNA duplexes >21-mer length having blunt ends and that the presence of 3′-overhangs evades recognition even for duplexes of 27-mer length (Marques et al., 2006). Importantly, 2′OMe modification of the RNA duplex will prevent this response and, interestingly, it seems that the related 2′-F modification is less protective (Collingwood et al., 2008; Zamanian-Daryoush et al., 2008). RIG-I also recognizes the presence of a triphosphate at the 5′-end of nucleic acids (Kim et al., 2004; Hornung et al., 2006; Pichlmair et al., 2006), causing an immune response to transcripts made by viral polymerases (including *in vitro* transcripts).

### Off-Target Effect: Unwanted Participation in miRNA Pathways

Although it is possible to achieve single base discrimination with select siRNAs, single- or even double-base mismatches are often tolerated and can still reduce target levels by significant amounts (Du et al., 2005; Birmingham et al., 2006; Schwarz et al., 2006; Dahlgren et al., 2008). If the full sequence of the reference genome is known, a thorough homology screen of candidate siRNAs should permit exclusion of sites where unwanted homology exists with other genes and theoretically lead to high specificity. Unfortunately, this kind of traditional cross-hybridization analysis can be less effective than expected and even carefully screened siRNAs can cause significant changes in expression levels in unrelated genes (Jackson et al., 2003; Persengiev et al., 2004; Scacheri et al., 2004). It appears that many of these effects are mediated by the unintended participation of siRNAs in miRNA pathways.

The miRNA translational suppression pathway is directed by imperfect base pairing between target and guide strand (He and Hannon, 2004; Kim, 2005) and the specificity of this process is defined by a 6–7 base "seed region" at the 5′ end of the antisense strand of the miRNA (Doench and Sharp, 2004; Lin et al., 2005; Jackson et al., 2006b). Given the expected frequency of finding 6–7 base matches between a siRNA and nontargeted genes within the entire transcriptome, it is not surprising that OTEs mediated by this mechanism can simultaneously affect hundreds of genes (Lim et al., 2005; Birmingham et al., 2006). Target sites for miRNA binding seem to be enriched in the 3′-untranslated region (UTR) of genes and a careful focus on homology screening of the seed regions of candidate siRNA in the 3′-UTR of all genes may be prudent (Anderson et al., 2008). Unfortunately, 6-base matches are very common, and only a few of these matches actually prove to be real functional sites that can lead to target gene suppression. Some method of reducing miRNA-pathway derived OTEs is needed beyond seed region homology screening.

Although siRNAs have two strands, only functional participation of the guide strand is desired. Design of the siRNA can introduce strand bias into the siRNA so that one strand is preferentially incorporated into RISC; however, this bias is only relative and some variable amount of the passenger strand will be functionally loaded and can engage in undesired gene-knockdown events. For example, the S-strand of a specific anti-ICAM-1 (intercellular adhesion molecule-1) siRNA was found to directly mediate off-target suppression of the TNFa gene (tumor necrosis factor alpha)(Clark et al., 2008). Various strategies have been devised to reduce or totally eliminate participation of the S-strand of the siRNA in gene silencing. One approach is to cleave the S-strand so that the RNA duplex is comprised of an intact AS-strand which is annealed to two adjoining shorter S-strand RNA oligomers. This design has been called "small internally segmented siRNA" (Bramsen et al., 2007). To effectively hybridize and form a functional siRNA, the short S-strand fragments require inclusion of Tm increasing modifications, such as LNA bases. Another approach is use of modifications that block the 5′ end of the S-strand (passenger strand) of the siRNA. Both strands of endogenous siRNAs or miRNAs naturally have a 5′-phosphate (which results from Dicer cleavage), and synthetic siRNAs are usually made with a 5′-phosphate or a 5′-hydroxyl [in which case the siRNA is phosphorylated by the cellular RNA kinase hClp1(Weitzer and Martinez, 2007)]. Although synthetic siRNAs are tolerant of some

5′-modifications (Shah and Friedman, 2007), blocking the 5′ end of an RNA strand (by, e.g., for example, 5′-O-methylation) can reduce or eliminate participation of that strand in silencing (Chen et al., 2008).

The use of site-specific chemical modification may also permit reduction or elimination of OTEs derived from unwanted participation of the guide strand in miRNA pathways. Several groups have studied chemical modification patterns looking for selective modification strategies that retain potency of the siRNA guide strand to direct Ago2 cleavage of an mRNA target while reducing participation of that siRNA in miRNA-like seed-region directed events. Jackson and colleagues reported that placing a single 2′OMe residue at position +2 of the guide strand can substantially reduce seed region–related OTEs (Jackson et al., 2006a). Ui-Tei and colleagues reported that replacing the entire seed region with DNA residues maintained functional potency for knockdown of the intended target while reducing seed region–related OTEs (Ui-Tei et al., 2008). Selective placement of LNA residues may similarly improve specificity of a siRNA (S. Magdaleno, personal communication). Thus the use of chemical modifications may simultaneously reduce OTEs that arise from triggering the innate immune system as well as reduce the ability of a siRNA to participate in seed-region directed miRNA-pathway OTEs.

## Chemical Modifications Can Direct Cell Uptake and Alter Biodistribution

Various chemical groups can be covalently attached to synthetic RNA oligonucleotides that direct entry into cells and/or alter biodistribution. Along these lines, a substantial amount of work has already been done in the medicinal chemistry of antisense oligonucleotides that can be directly applied to siRNAs (Manoharan, 2002). Steroid and other hydrophobic lipid groups can be attached to siRNAs that alter protein binding in serum, extending circulation time, and can facilitate direct cellular uptake (Lorenz et al., 2004; Soutschek et al., 2004). For example, cholesterol has been successfully used as a ligand to direct delivery of siRNAs *in vivo* (Soutschek et al., 2004). Cholesterol directed uptake is more complex than originally believed and it appears that both the cell surface scavenger receptor SR-BI and Sid-1 (homolog of the *Caenorhabditis elegans* protein involved in propagation of systemic RNAi responses) are involved (Wolfrum et al., 2007). Direct conjugation of Vitamin-E ($\alpha$-tocopherol) to a siRNA has also been demonstrated to mediate delivery of siRNAs when administered i.v. in mice (Nishina et al., 2008). The potential of cell penetrating peptides to deliver siRNAs is just starting to be realized (Juliano, 2005; Juliano et al., 2008).

Intravenous administration of naked siRNAs leads to limited tissue uptake due to degradation of the siRNA and rapid renal clearance. Chemical stabilization of the siRNA alone simply results in renal clearance of intact siRNA without degradation. Biodistribution of synthetic siRNAs following intravenous administration is similar to that previously established for single-stranded DNA antisense oligonucleotides (Braasch et al., 2004; Viel et al., 2008), with the highest concentrations seen in the liver and kidneys. Modifications that increase protein binding, such as PS or hydrophobic ligands (cholesterol, etc.) will extend serum lifetime (Braasch et al., 2004; Soutschek et al., 2004); however, use of a nanoparticle or lipid-based delivery tools can have far greater effects (Morrissey et al., 2005b; Bartlett et al., 2007).

## Examples from Published Reports in Nonhuman Systems

There is no standard algorithm that helps guide where to position chemical modifications when designing siRNAs for *in vivo* use, and the approach used may need to vary with different applications and the intended route of delivery. Some insights can be gained by comparing modification patterns that have already been successfully employed *in vivo* in different nonhuman model systems. Representative examples of different siRNA designs that have been reported in published animal studies are shown in Table 1. These examples include siRNAs that range from minimal modification to near total replacement of native RNA residues.

Layzer and colleagues used an anti-Luciferase 21-mer siRNA with 3′-overhangs containing 2′-F modifications at all pyrimidine bases. This siRNA was administered to mice through tail vein hydrodynamic co-injection with a Luciferase expression plasmid (3 mg of reporter plasmid with 10 mg of siRNA, or 0.5 mg/kg siRNA). The compound functioned *in vivo* to suppress Luciferase expression but did not show any obvious benefit over unmodified RNA using this method of administration. In fact, it showed slightly less potent suppression at later time points (1 week after injection) (Layzer et al., 2004). Soutschek and colleagues employed standard low pressure i.v. administration in mice of an anti-ApoB siRNA that contained phosphorothioate 3′-end modification, two 2′OMe RNA residues, and a cholesterol targeting group (Soutschek et al., 2004). This siRNA employed an asymmetric design with a 21-mer S-strand and a 23-mer AS-strand, having a single 3′-overhang on the AS-strand. This "minimally modified" design was functional and protected the siRNA sufficiently to be administered without protection from another delivery aid, but did require a very high dose to show effective knockdown of ApoB (50 mg/kg).

Morrissey and colleagues compared activity of the same siRNA anti-HBV sequence in unmodified form with a highly modified version that included DNA bases, 2′OMe purines, 2′-F pyrimidines, terminal inverted ribose residues, and PS linkages at select positions, leaving only three unmodified RNA bases at the 5′ end of the AS-strand (Morrissey et al., 2005b). When "naked" siRNAs were administered intravenously, the unmodified compounds showed a plasma half-life of 2 minutes while the modified versions showed a half-life of ~45 minutes. When administered with a lipid-based nanoparticle, half-life was extended to 6.5 hours, highlighting the value of using a delivery tool even with heavily modified RNAs. Doses of 3 mg/kg showed good biological effects in reducing HBV replication. Importantly, when using the lipid-based delivery the unmodified siRNA triggered a strong innate immune response while the modified siRNA did not.

The only siRNA in Table 1 that is currently in clinical testing was originally described by Shen et al. (2006) in studies

Table 1. Examples of Chemically Modified siRNAs used *In Vivo*

| *Target* | *Sequence* | *S/AS* | *Source* |
|---|---|---|---|
| GL3 | 5′ cuuAcGcuGAGuAcuucGAtt 3′<br>3′ ttGAAuGcGAcucAuGAAGcu 5′ | 21/21 | Layzer et al. (2004) |
| APOB | 5′ GUCAUCACACUGAAUACCAAU*-Chol 3′<br>3′ C*A*CAGUAGUGUGACUUAUGGUUA 5′ | 21/23 | Soutschek et al. (2004) |
| HBV | 5′ B-ggacuucucucaauuuucutt-B 3′<br>3′ t*tccuGAAGAGAGuuAAAAGA 5′ | 21/21 | Morrissey et al. (2005b) |
| VEGFR-1 | 5′ B-CUGAGUUUAAAAGGCACCCtt-B 3′<br>3′ t*tGACUCAAAUUUUCCGUGGG 5′ | 21/21 | Shen et al. (2006) |
| PCSK9 | 5′ GCCUGGAGUUUAUUCGGAAt*t 3′<br>3′ t*tCGGACCUCAAAUAAGCCUU 5′ | 21/21 | Frank-Kamenetsky et al. (2008) |
| SOD1 | 5′ C*G*A*uGuGUCUAUUGAAG*A*u*u*C 3′<br>3′ C*G*G*c*u*AcACAGAUAACUuc u Ap 5′ | 21/21 | Wang et al. (2008) |
| PTEN | 5′ CCACCACAGCUAGAACUUA 3′<br>3′ GGUGGUGUCGAUCUUGAAU 5′ | 19/19 | Santel et al. (2006a, 2006b) |
| HER2 | 5′ tctctgcggtggttggcat 3′<br>3′ AGAGACGCCACCAACCGUA 5′ | 19/19 | Hogrefe et al. (2006)<br>Pirollo et al. (2007) |
| HER2 | 5′ titiitGCGGUGGUUgicit 3′<br>3′ AGAGACGCCACCAACCGUA 5′ | 19/19 | Hogrefe et al. (2006)<br>Pirollo et al. (2007) |
| APOB | 5′ GUCAUCACACUGAAUACCAAU GC U GG*A 3′<br>3′ C*A*CAGUAGUGUGACUUAUGGUUA*CG*A*CC U-Toc 5′ | 27/29 | Nishina et al. (2008) |

RNA, AGCU; DNA, agct; 2′OMe RNA, AGCU; 2′F RNA, acgu; Phos, p; PS bond, * Chol, cholesterol; Toc, α-tocopherol (Vitamin-E); B, inverted abasic deoxyribose; i, deoxyinosine; S/AS, length of the sense and antisense strands, respectively.

performed in mice. Sirna-027 is a 21-mer siRNA targeting the vascular endothelial growth factor receptor type 1 (VEGFR-1) and is being developed to treat the wet form of age-related macular degeneration (AMD). The duplex has DNA TT dinucleotide 3′-overhangs with a single phosphorothioate linkage positioned in the 3′-overhang on the AS-strand. The S-strand has inverted abasic ribose units positioned at each end to block exonuclease entry. A single intravitreal dose of 0.5 or 1.5 mg of Sirna-027 in saline resulted in significant reduction of intraocular VEGFR-1 mRNA and suppressed the choroidal neovascularization response that usually follows laser injury of the retina.

An intermediate level of modification was employed by Frank-Kamenetsky and colleagues who administered an anti-PCSK9 siRNA intravenously in "lipidoid" nanoparticles in mice, rats, and cynomolgus monkeys (Frank-Kamenetsky et al., 2008). The siRNAs employed were chemically modified, including: all 2′OMe pyrimidine residues on the S-strand, two 2′OMe residues on the AS-strand, and a single 3′ end PS bond on each strand. No evidence for immune stimulation was detected by either altered cytokine levels or elevated hepatic transaminases and evidence for biological action (knockdown of PCSK9 levels and lowering of plasma cholesterol) was detected for over 2 weeks following a single i.v. administration of 5 mg/kg in the lipidoid nanoparticle.

Wang and colleagues modified an anti-SOD1 siRNA with four 2′-F pyrimidine residues in the S-strand and six 2′-F pyrimidine residues in the AS-strand. A greater number of PS bonds were employed, including five bonds in the 3′-end of the AS-strand and 3–4 bonds at both ends of the S-strand (Wang et al., 2008). Naked siRNAs were delivered by slow continuous direct intrathecal infusion using an implanted osmotic pump into the central nervous system (CNS). Mouse spinal cord tissue was tested for levels of STAT1, OAS1, and IFIT following 28 days of continuous infusion. No immune stimulation was detected at a dose of 0.2 mg/kg/day but was seen at 0.8 mg/kg/day. The potential for immune stimulation within the CNS has not been well characterized for different modified forms of dsRNAs and it would be interesting to see what differences may exist between unmodified vs. 2′OMe modified vs. the 2′-F modified residues that were employed in this study. Intact siRNA was detected in CNS tissue after the infusion was complete, demonstrating nuclease stability of the siRNA, and evidence for biological activity was seen even at the lower dose.

Santel and colleagues administered several different blunt 19-mer siRNAs in mice by i.v. injection with a lipid nanoparticle system that selectively delivers cargo to vascular endothelium (Santel et al., 2006a; Santel et al., 2006b). The siRNAs were chemically modified with 2′OMe RNA in an alternating pattern on both strands which resulted in a nuclease stable compound that also appeared to avoid immune stimulation. Target knockdown was seen at doses below 2 mg/kg. Of note, Silence Therapeutics and Quark Pharmaceuticals are currently developing siRNA therapeutics using this precise modification pattern. Pirollo and colleagues described the use of different kinds of blunt 19-mer siRNA duplexes. In one version, the siRNAs had a DNA S-strand and an unmodified RNA AS-strand. In a second version, the S-strand had a central core of 2′OMe RNA residues flanked by DNA bases including 2′-deoxyinosine while the AS-strand was unmodified RNA (Hogrefe et al., 2006; Pirollo et al., 2007). Anti-HER-2 siRNAs were administered i.v. using lipid nanoparticles at doses up to 3 mg/kg. Specific target knockdown was demonstrated; no obvious toxicity was observed, however cytokine levels were not directly assayed.

Nishina and colleagues employed asymmetric Dicer-substrate siRNAs having a 27-mer S-strand and a 29-mer AS-strand targeting ApoB. A combination of 2′OMe residues and PS bonds were placed at select locations in both strands, leaving the predicted site of Dicer processing unmodified. Vitamin-E ($\alpha$-tocopherol) was covalently attached to the 5′ end of the AS-strand, a location predicted to be cleaved from the mature siRNA within the cell by Dicer. This modification pattern stabilized the naked siRNA in serum and allowed for direct i.v. administration using the Vitamin-E group to mediate cell uptake. Effective delivery was demonstrated in the liver with increasing target knockdown observed as the dose was increased from 2 to 32 mg/kg. No changes in IFN-$\alpha$ levels or hepatic transaminases were observed following treatment, demonstrating that chemical modifications can protect even longer RNAs of this type from both degradation and from triggering immune responses.

## Examples from Clinical Trials

Two synthetic oligonucleotide-based drugs have been approved by the FDA and are available for use in the clinic today, including Formivirsen (a phosphorothioate modified antisense DNA oligonucleotide to treat CMV retinitis) (Jabs and Griffiths, 2002) and Macugen (an aptamer to treat wet AMD) (Gragoudas et al., 2004). Both of these drugs treat ocular diseases and are administered by direct intravitreal injection. Seven synthetic siRNAs are currently in various stages of clinical trials for use in a wide range of therapeutic indications, which are summarized in Table 2 (de Fougerolles et al., 2007; Novobrantseva et al., 2008). Unfortunately, only a single report describing a clinical trial performed on siRNA drugs has been published thus far (DeVincenzo et al., 2008) and we are otherwise left with less definitive information from corporate press releases or presentations at conferences to follow progress in this field.

Due to the lower complexity of introducing new therapies that employ local administration compared with systemic i.v. administration, diseases of the eye have been the first siRNA drugs to enter clinical trials. Bevasiranib (also known as Cand5) is being developed by OPKO Health for treatment of wet AMD. It is an unmodified 21-mer siRNA targeting vascular endothelial growth factor (VEGF) which is administered by direct intravitreal injection in saline with no delivery assistance. Phase I and Phase II clinical trials have been completed with no significant adverse reactions encountered beyond those expected from the route of administration. Patients are currently being enrolled in a Phase III clinical trial.

AGN211745 (also known as Sirna-027) is being developed by Allergan and Merck, also for the treatment of wet AMD. It is a minimally modified 21-mer siRNA (see Table 1) targeting the vascular endothelial growth factor receptor (VEGR1, or FLT1) and is also administered by direct intravitreal injection in saline with no delivery assistance. Phase I and Phase II clinical trials have been completed with no significant adverse reactions encountered beyond those expected from the route of administration (Whelan, 2005). A Phase II clinical trial is ongoing comparing efficacy of AGN211745 with Ranibizumab (Lucentis), a monoclonal antibody targeting VEGF that is already FDA approved for this indication.

PF-04523655 (also known as REDD14NP) is being developed by Quark Pharmaceuticals and Pfizer for the treatment of wet AMD. The sequence of this compound has not been published; however, it is a blunt RNA duplex that employs an alternating 2′OMe RNA modification pattern similar to that shown in Table 1 (see the PTEN duplex, from Santel et al.). The siRNA reduces retinal neovascularization through a VEGF-independent pathway by suppressing levels of DDIT4 (also known as REDD1 or RTP801). A Phase I clinical trial is in progress. Like AGN211745, a Phase II clinical trial is also ongoing which will compare the efficacy of PF-04523655 with Ranibizumab (Lucentis).

Quark Pharmaceuticals is also involved in an another Phase I clinical trial of a siRNA drug called I5NP (also known as AKli-5) that is intended to treat the acute kidney injury

Table 2. siRNA Compounds in Active Clinical Trials

| *Drug* | *Source* | *Indication* | *Target* | *Administration* | *Form* | *Status* |
|---|---|---|---|---|---|---|
| Bevasiranib (Cand5) | OPKO Health | Wet AMD | VEGF | Intravitreal | Unmodified | Phase III |
| AGN211745 (Sirna-027) | Allergan/ Merck | Wet AMD | FLT1 (VEGFR1) | Intravitreal | Minimal modification | Phase II |
| PF-04523655 (REDD14NP) | Quark/Pfizer | Wet AMD | DDIT4 | Intravitreal | Alt. 2′OMe | Phase II |
| ALN-RSV01 | Alnylam | RSV | RSV-N | Inhaled | ? | Phase II |
| I5NP (AKli-5) | Quark | AKI | TP53 | Intravenous, saline | Alt. 2′OMe | Phase I |
| CALAA-01 | Calando | Solid tumor | RRM2 | Intravenous, polyplex | Unmodified | Phase I |
| TD101 | Pachyonychia Congenita Project | Pachyonychia Congenita | KRT6A | Subcutaneous, saline | Unmodified | Phase I |

AMD, age related macular degeneration; AKI, acute kidney injury; RSV, respiratory syncytial virus.

that follows the ischemic insult experienced during cardiac bypass surgery. The compound is a blunt RNA duplex targeting p53 that employs an alternating 2′OMe RNA modification pattern similar to that shown in Table 1 (see the PTEN duplex, from Santel et al.). The exact sequence of this siRNA has not been released. The compound will be administered by direct i.v. infusion in saline without a delivery aid. Using "naked" i.v. delivery, only the kidney proximal tubule shows sufficient uptake of the siRNA for biological activity, effectively making this delivery method tissue specific. Data from preclinical testing have not been published, but a representative of Quark Pharmaceuticals reported that toxicity was difficult to see in rats and that i.v. doses as high as 1 gm/kg were safety administered during toxicology evaluation (Jim Thompson, RNAi World Congress, Boston, May 2008).

ALN-RSV01 is being developed by Alnylam for treatment of respiratory syncytial virus (RSV). It is administered by inhalation in a nondisclosed formulation. The siRNA targets the N gene of RSV. The precise sequence and chemical composition of the siRNA have not been published. A Phase I clinical trial of the drug has been completed and these results have been published (DeVincenzo et al., 2008). Minor respiratory track irritation and nasal edema were seen throughout the dose escalation (from 1.5 to 150 mg, single dose); however, no significant adverse effects were reported. The compound is proceeding into Phase II clinical trials where healthy volunteers are infected with controlled doses of RSV and efficacy of treatment is experimentally evaluated.

Calando Pharmaceuticals is developing an unmodified 21-mer siRNA targeting the M2 subunit of ribonucleotide reductase (RRM2) for the treatment of solid tumors. CALAA-01 is a formulation of this siRNA with their proprietary polyplex delivery tool called Rondel™, which comprises a linear cyclodextrin-containing polycation linked to a transferrin-targeting ligand. Transferrin receptors are enriched on many malignant cells, making transferrin a good targeting ligand for oncology indications. Extensive preclinical characterization of this siRNA has been published from work done in rodents and nonhuman primates (Bartlett et al., 2007; Heidel et al., 2007a, 2007b; Bartlett and Davis, 2008). The formulated siRNA was tested for toxicity in cynomolgus monkeys at 3, 9, and 27 mg/kg using i.v. administration. No adverse effects were seen for the two lower doses. At the maximal dose tested (27 mg/kg), reversible elevation of blood urea nitrogen was seen (nephrotoxicity) as well as mild, reversible elevations of serum transaminases (hepatotoxicity). Further, transient elevation of the inflammatory cytokine interleukin-6 was seen in all animals receiving the highest dose. Note that the 27 mg/kg dose is likely to be 5–10-fold higher than the anticipated therapeutic dose in humans. A Phase I clinical trial is currently enrolling participants.

TransDerm and the Pachyonychia Congenita Project have developed a siRNA that can discriminate between one disease-causing mutant allele and the wild-type form of keratin (specifically KRT6A) that is responsible for some sporadic cases of pachyonychia congenita (Hickerson et al., 2008; Leachman et al., 2008). In a new Phase I clinical trial, one patient was treated with this compound (TD101, an unmodified 21-mer siRNA) by direct injection of 18 mg of the siRNA in saline into a lesion located on the plantar surface on one foot. Although transient improvement of the lesion was observed at the site of injection, pain at the site of injection was severe; alternative methods of administration are being considered for future studies, such as use of a topical cream (reported by Roger Kaspar at the fourth Oligonucleotide Therapeutics Society Meeting, Boston, October 2008).

Interestingly, the first systemic administration of a siRNA drug in humans may have been preformed in an experimental setting outside of a traditional clinical trial. A patient with Philadelphia chromosome positive, imatinib-resistant chronic myelogenous leukemia who relapsed after bone marrow transplantation was treated with intravenous siRNA against the *bcr-abl* oncogene (Koldehoff et al., 2007). The siRNA was an unmodified 21-mer which was delivered in a soy-lipid formulation (Lipovenos) that is typically used for i.v. nutrition; dose ranged from 10 to 30 mg/kg. The number of circulating *bcr-abl*$^+$ cells decreased significantly after the first dose, however, this was not observed after the second and third administrations. No adverse effects were observed that could be directly correlated to siRNA treatment in this very ill patient.

A number of additional siRNA drug candidates are currently in preclinical testing and IND applications for several new Phase I trials are anticipated over the next few years.

## Conclusions

Although it is possible to employ unmodified RNA duplexes to trigger RNAi responses *in vivo*, the use of chemical modifications can improve nuclease stability, reduce the risk of activating the innate immune system and decrease OTEs. Modifications can also be used to facilitate delivery, improve biodistribution and increase plasma circulation half-life. There is no single "best" modification pattern to recommend; rather, choice of the precise modification patterns employed can vary with the design of the siRNA, application, route of delivery, and optional use of delivery aids, such as liposomes or nanoparticles.

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