CHEMICAL MODIFICATIONS TO IMPROVE RNA INTERFERENCE

Reported by Seth M. Parmley February 25, 2008

INTRODUCTION

Discovery of RNA Interference

In 1998, Fire and Mello reported that double-stranded RNA had potent and specific interference in the expression of mRNA in *C. elegans*, whereas either single strand produced only modest interference.[1] Only a few molecules of double-stranded RNA per affected cell were able to cause interference, suggesting a catalytic mechanism of post-transcriptional gene silencing, which is called RNA interference (RNAi). This differs from post-transcriptional gene silencing by antisense oligonucleotides, which occurs stochiometrically by direct hybridization with the targeted mRNA to sterically block translation[2] and/or catalytically by RNAse H recognition of the DNA:RNA double helix and subsequent cleavage of mRNA.[3] RNAi occurs when double-stranded RNA is associated with a set of proteins called the RNA-induced silencing complex (RISC).[4] The guide strand is then unwound by helicase and is used by RISC to recognize complementary mRNA, which is cleaved with active endonucleases. Both exogenous and endogenous RNAs activate RISC. Exogenous, long double-stranded RNA (dsRNA) is cleaved to form 20-25 nt short interfering RNA (siRNA) that binds to RISC and targets viral mRNA for cleavage. Endogenous, single-stranded, non-coding transcripts form a hairpin loop that is processed to form a double-stranded micro RNA (miRNA) that activates RISC. RISC activated by miRNA can repress translation with or without inducing cleavage.[5]

Current Therapies

In 2001, the first report of RNAi in mammalian cells was published,[6] ushering an explosive increase in research toward the goal of developing RNAi therapeutics, with no fewer than six siRNA's advancing to clinical trials.[7] Therapeutic introduction of siRNA to silence mRNA by using a natural, catalytic mechanism provides a novel pharmacological approach to treating disease.

The development of siRNA for clinical use has been greatly informed by previous efforts to develop antisense oligonucleotides (ASO) for the same purpose. The first (and only) antisense drug, fomivirsen (Vitravene), is delivered locally by intraocular injection for treatment of cytomegalovirus (CMV) retinitis. Systemic (intravenous) delivery is currently in phase III trials for an antisense drug, oblimersen sodium (Genasense), for the treatment of advanced melanoma and chronic lymphocytic

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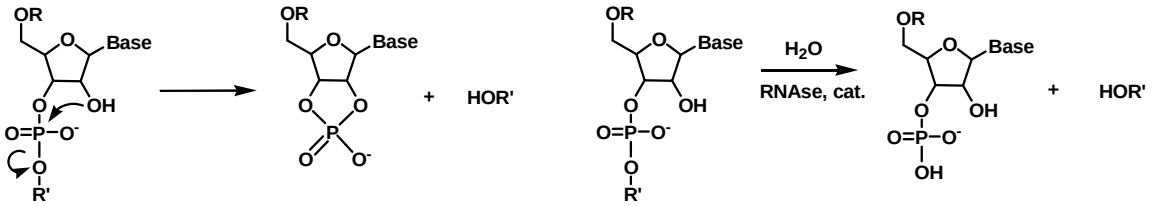
leukemia. The challenge for developing either ASO or siRNA drugs is to optimize in vitro properties of nuclease stability, potency and specificity, and then develop in vivo delivery methods.

DEVELOPING RNAI THERAPEUTICS: THE ROLE OF CHEMICAL MODIFICATIONS

Oligonucleotide drugs must have optimized nuclease stability, potency and specificity. Endo- and exonucleases rapidly degrade RNA (see Scheme 1), which has a half-life of only minutes in human plasma. In vitro transfection agents such as cationic liposomes provide protection against degradation in

Scheme 1. Degradation of RNA



addition to their cellular delivery function. However, chemical modifications of the phosphate backbone and ribose sugars of siRNA are needed to increase stability to intracellular nucleases and thus increase silencing persistance. These modifications must not impinge upon interactions with RISC or the target.

The approaches for selecting potent and specific siRNA's have been recently reviewed.[8] In silico algorithms aid this process, but ultimately in vitro screening of selected sequences is necessary to find the optimal complement for knockdown. Thus, careful sequence selection precedes efforts to maximize potency and specificity by chemical modification. The ideal specificity of RNAi is not entirely observed in practice. After specificity was demonstrated in early microarray experiments, researchers were surprised and disappointed to observe that sequence-specific off-target effects occur when partial complementarity allows silencing of untargeted genes.[9] Chemical modifications have been used to reduce these effects (see below).[10] It should also be noted that another type of off-target effect, immune response to double stranded RNA, is largely avoided by administering siRNA rather than long dsRNA, which triggers interferon production. However, some siRNA sequences do cause an immune response and must be identified and eliminated from the pool through screening. Also, 2' O-methyl modifications have been used to prevent TLR7 binding.[11,12]

Delivery is the most significant challenge to therapeutic RNAi. Cationic liposomes are used for in vitro silencing to facilitate cellular entry, but these formulations are not broadly applicable for in vivo application due to predominant liver accumulation. Great efforts have been made to develop platforms for delivery to specific cell types. These include aptamer-siRNA chimeras, nanoparticle encapsulated siRNA, and antibody-protamine bound siRNA, as discussed in a recent review of RNAi therapeutics.[13]

DNA-based viral vectors are also being explored.[14] Chemical modification has been used to improve biodistribution and cellular uptake (see below).[15]

CHEMICAL MODIFICATIONS TO IMPROVE NUCLEASE STABILITY

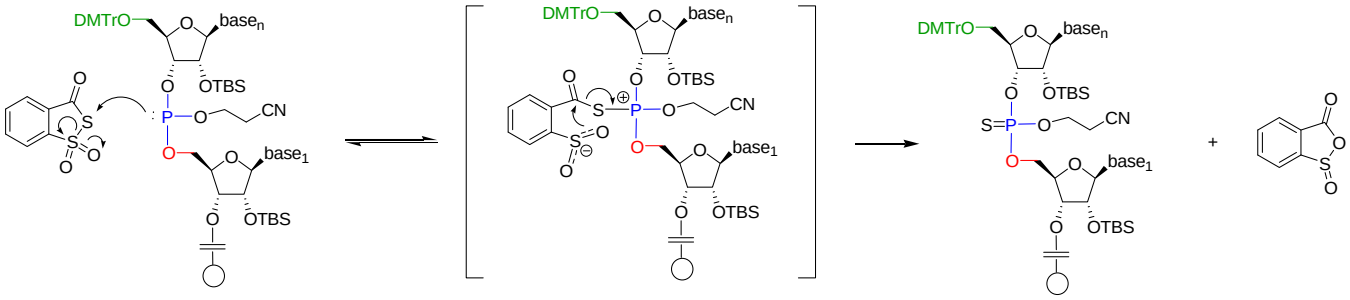
Modification of the Phosphate Backbone

First generation oligonucleotide modifications are those of the phosphate backbone. The phosphorothioate (PS) backbone, with a non-bridging sulfur atom in place of an oxygen atom, is a modification found in many antisense and siRNA drug candidates and in FDA-approved fomivirsen, which has complete PS substitution in the 21-nt single-stranded DNA. The earliest relevant observation, which was unexpected, was that nucleoside 5' phosphorothioates are more stable to alkaline phosphatase than the 5' phosphate. Furthermore, it was also observed that internal PS bonds were more stable to nuclease degradation than phosphodiester bonds; with PS modification, half-life in plasma can be extended from minutes to days. The magnitude of this difference is hard to account for by chemical explanation alone. Sulfur and oxygen are isoelectronic and van der Waals radii that are not drastically different (1.85 Å and 1.44 Å respectively). The P-S bond length is only slightly longer than P-O. Charge localization is predominantly on the sulfur atom according to some reports, while others suggest that charge is more evenly distributed. Localization is probably dependant upon the counter ion and the environment, for example, in the active site of an enzyme.

It is revealing that the two diastereomers of phosphorothioates (S_p and R_p) are degraded differently by enzymes. Some enzymes (such as ribonuclease T1 and snake venom phosphodiesterase) cleave the R_p diastereomer more readily and others (such as nuclease P1) cleave the S_p diastereomer.[16] Can these or other diastereomer differences provide mechanistic explanations of enhanced nuclease stability? *Escherichia coli* DNA polymerase I Klenow fragment has metal-dependant 3'-5' exonuclease activity against the R_p diastereomer, but the S_p diastereomer is inert. This stereospecificity is independent of the metal ion (Mg^{2+}, Mn^{2+} or Zn^{2+}) used. A crystal structure has shown that the sulfur of the S_p diastereomer excludes the two requisite metal ions.[17] Enzymes that do not require metal ions may react stereospecifically due to displacement of functional groups by the slightly larger sulfur atom. PS stability in the context of siRNA has been extensively shown by in vitro assays in cell extract.[18] These in vitro assays address general stability of PS siRNA, but nuclease activity is expected to be different in vivo. In vivo assays have shown that the in vitro stability of PS siRNA translates to greater persistence of mRNA knockdown.[19]

The ease of synthesis of PS oligonucleotides comes from only a slight modification to the efficient and automated synthesis of unmodified oligonucleotides. The oxidation step of P(III) to P(V) is simply replaced by sulfurization, which is most often performed with the Beaucage reagent.[20]

Scheme 2. Sulfur Transfer By Beaucage Reagent



The nuclease stability conferred by a complete PS modification is substantial; phase III Genasense (an antisense drug) is administered intravenously without any protective encapsulation. However, adverse effects of toxicity and undesirable protein binding have been observed with PS oligonucleotides. Current design of siRNAs sometimes includes PS backbone, but 2' modifications are more commonly used.

2' Modifications For Stability

The chief goal of 2' modifications is to maximize nuclease stability without decreasing potency. One experiment measures potencies of modified siRNAs using a dual fluorescence system where a plasmid encoding green fluorescent protein (GFP) and red fluorescent protein (RFP) is transfected into HeLa cells. Silencing of the mRNA encoding GFP may then be determined by measuring fluorescence of GFP relative to RFP. [21] In this experiment the chosen 21-nt siRNA, which silences the exogenous gene encoding GFP, was modified to analyze structure-function relationship of 2'-deoxy (H), 2'-methoxy (OMe), and 2'-fluoro (F) substitutions. Complete 2'-H substitution in the guide strand removed all silencing effect. Replacement of about half of the 2'-OH throughout the guide strand with 2'-F did not preclude RNAi, with silencing comparable to that of wild type. Also, when the same 2'-F modified strand incorporated only three 2'-deoxynucleotides, silencing activity was still strong. The nucleotides complementary to the cleavage site of the targeted mRNA were 2'-F and 2'-deoxy modified, proving that 2'-OH groups are not required near the site of cleavage. In fact, it has been shown that no single 2'-OH in the siRNA double helix is indispensable for RNAi.[22]

After determining that 2'-F modifications were tolerable, the stabilities of various 2'-F modified oligonucleotides were explored. After 1 hour at 37°C, partially modified siRNAs were 80% (both strands partially modifed) and 68% (only antisense strand partially modified) intact. Only 7% of the unmodified siRNA remained intact. By comparison, a single strand (antisense) was almost completely degraded within 20 minutes. This in vitro assay was useful for analyzing the relative nuclease stabilities

of these siRNAs, but does not suggest anything about half-life of siRNA in vivo. The relevant meter for in vivo testing was to determine if the enhanced stability in cell extract corresponded enhanced silencing ability. The most stable fluoro siRNA and its unmodified analogue were used for kinetics study of GFP knockdown in HeLa cells. Maximal knockdown (approximately 85 - 95% 60 hours after transfection) is observed with both modified and unmodified siRNAs, but knockdown by modified siRNAs is more persistant. After 120 hours knockdown by modified siRNA is 80%, while knockdown by unmodified siRNA is almost 0%.

The reason that complete 2'-H substitution in the guide strand is intolerable is that RNAi requires a type-A double helix,[23,24] which chemical modifications should not disrupt. The type-A double helix is formed by RNA and is distinct from the type-B double helix formed by DNA (see Table 1). Helix characteristics arise from nucleotide conformations. The C3'-endo (north) and C2'-endo (south) conformations are most stable for RNA and DNA nucleotides, respectively. When the guide strand of siRNA is 2'-deoxy modified, the resulting hybrid of DNA:target mRNA has a helix that is somewhere between type A and type B. However, when 2'-OH is substituted for 2'-F the resulting double helix formed with mRNA has a type A twist.[25] These early studies gave insight into the structural requirements of siRNA, but 2'-F modifications are used lest often. The phosphoramidite is much more expensive than 2'-methoxy phosphoramidite, which also confers greater stability.

Methoxy groups are the most common 2' modification for improving nuclease stability. Alkoxy substitution has the added benefit of enhancing duplex stability, since the C2' endo conformation is greatly favored and leads to a type A helix. However, the extent and position of 2'-OMe substitutions can have an adverse effect on silencing. For example, complete substitution of 2'-OH for 2'-OMe in a siRNA results in great enhancement of nuclease stability, but essentially disallows RNAi. [26] Complete substitution of the passenger strand and no substitution of the guide strand had less negative effect on RNAi than the substitution of the guide strand and no substitution of the passenger strand. This is consistent with other results that suggest 2' modifications are less tolerable on the guide strand than the passenger strand. Effective silencing was achieved for siRNAs with only partial 2'-OMe substitution.

Another study focused on positional effects of 2'-F, -OMe, and -O-Methoxyethyl (-O-MOE) substitutions (see Chart 1). [27] Using a blockmer approach, 3-nt stretches of modified nucleotides were placed throughout the guide strand of a 21-mer siRNA targeting PTEN mRNA in HeLa cells. It was clearly shown that 2'-OMe modifications of the guide strand near the 3'-end and center of the guide strand allowed better knockdown than modifications near the 5'-end. For example, quantitative RT-PCR showed that three tandem 2'OMe nucleotides at the 3'-end of the guide strand permitted virtually the same knockdown (~80%) at [siRNA] = 15 nM after 16 hours incubation, whereas knockdown by the 5'-

end modified siRNA was only ~52%. The bulky 2'-MOE siRNAs resulted in lower knockdown compared to the unmodified form, regardless of position, and 2'-F groups were well-tolerated regardless of position. As for the passenger strand, 2'-OMe and -OMOE groups were well-tolerated and showed no positional preference.

Locked Nucleic Acids

Locked nucleic acids (LNA), have a conformation that makes hybridization with complementary oligonucleotides more energetically favorable. The modified strand is pre-organized for forming a type A double helix, and therefore the entropy penalty paid upon binding to the targeted mRNA is reduced. LNAs increase melting temperatures of double helices and can increase serum stability in classic antisense oligonucleotides.[28] A current clinical trial for an ASO uses LNA and DNA nucleotides.[29] LNA in siRNA has also been explored. The permissible positions and extent of LNA modifications has been shown.[30] Furthermore, LNA near the 5' end of the sense strand has been shown to favor incorporation of the antisense strand into RISC, thereby reducing sequence-specific off-targeting effects that would result if the sense strand were to activate RISC.

CHEMICAL MODIFICATION TO IMPROVE SPECIFICITY

As mentioned previously, siRNAs can be chemically modified to reduce sequence-specific silencing of untargeted genes.[31] Silencing of partially-complementary mRNA occurs by a mechanism that is slightly different from silencing of perfect complements. (This mechanism is also observed in silencing by endogenous micro RNAs (miRNAs).[32]) Thus, statistical exclusion of sequences identical to the 21-nt target is not sufficient for perfect silencing specificity. The crucial pairing region that is often responsible for off-target silencing is the seed region, which is made of nucleotides 2 – 8 at the 5' end of the guide strand.[33,34] Chemical modifications to the ribose sugars of nucleotides in the seed region reduce off-target silencing and still allow RNAi of the targeted mRNA (see below).[35,36] In a recent experiment expression profiling that found 52 untargeted genes were silenced to varying degrees. Modification with 2'-OMe in the seed region was explored for reducing off-target effects.[37] It was found that placement of a single 2'-OMe on nucleotide 2 (from the 5' end) of the guide strand significantly reduced undesired silencing and still allowed silencing of the target mRNA. This phenomenon was shown to be position specific and sequence independent in 10 different siRNAs. On average, this modification reduced off-target silencing by 66%.

The differential control of this modification is not fully understood. The authors pointed out that the mechanistic differences between perfect complementary-mediated silencing and the miRNA-like pathway for silencing of partially complementary targets could be the source of this differential.

Alternatively, the selective reduction of off-target silencing could reflect simply quantitative differences in silencing by perfectly and partially matched guide strands. (More strongly hybridizing targets may simply be able to overcome the disruptive modification.) The guide strand modification is located on position 2, which is shown by crystal structures to have limited space available for accommodating the modification while bound to RISC proteins. Indeed, position 2 has the only 2'-O that is within 4 Å of any PIWI (domain of RISC) residue.[38]

CONCLUSION

Phase III and II clinical trials are in progress for treatment of macular degeneration (intraocular injection) and respiratory syncytial virus (RSV) (inhalation). The first siRNA clinical trial candidates have not required chemical modification since they are delivered locally. (For example, the eyeball, considered a "test tube" within the body, is ideal for local delivery due to its small, contained volume.) However, the requirements of siRNAs administered systemically are greater. This includes requirement of larger doses, which may result in greater off-target silencing.

In addition to chemical modifications, delivery platforms for tissue-specific targeting should reduce problems of off-target silencing and immune response by reducing the desired dosages. Currently drug companies are most intent on developing such delivery platforms. If significant achievements are made in this area, chemical modifications may become less important for effective therapy. However, chemical modifications have been extensively studied and have been shown to improve properties of siRNAs. Therefore, chemical modifications must be considered in the transition from first-in-class to best-in-class therapies.

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