Pharm Res (2010) 27:1788–1799
DOI 10.1007/s11095-010-0156-0

RESEARCH PAPER

A Direct Comparison of Anti-microRNA Oligonucleotide Potency

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Received: 22 January 2010 / Accepted: 8 April 2010 / Published online: 28 April 2010

ABSTRACT

Purpose Cataloguing endogenous miRNA targets by inhibiting miRNA function is fundamental to understanding the biological importance of each miRNA in gene regulatory pathways. Methods to down-regulate miRNA activity may help treat diseases where over-expression of miRNAs relates to the underlying pathophysiology. This study objectively evaluates the *in vitro* potency of different anti-miRNA oligonucleotides (AMOs) using various design and modification strategies described in the literature as well as some novel modification strategies.

Methods MiR21 and miR16 AMOs, containing chemical modifications such as 2′-*O*-methyl RNA, locked nucleic acid and 2′-Fluoro bases with or without phosphorothioate linkages, were directly compared by transfection into HeLa cells using a dual-luciferase reporter assay to quantify miRNA inhibition.

Results Potency for the various AMOs ranged from inactive at high dose (50 nM) to strongly inhibitory at both high and low dose (1 nM). Including phosphorothioate linkages improved nuclease stability and generally increased functional potency.

Conclusions Incorporating high binding affinity modifications, such as LNA and 2′F bases, increases AMO potency while maintaining specificity; nevertheless, use of low dose is preferred when using high potency reagents to minimize the potential for cross reactivity. 2′OMe/LNA chimeras with PS modifications were the most potent constructs tested for miRNA inhibition *in vitro*.

KEY WORDS antagonist · antisense · LNA · miRNA · potency

ABBREVIATIONS

2′F	2′-F RNA
2′OMe	2′-O-methyl RNA
AMO	anti-miRNA oligonucleotide
ASO	antisense oligonucleotide
LNA	locked nucleic acids
miRNA	microRNA
RISC	RNA induced silencing complex
RNAi	RNA interference
Tm	melting temperature
UTR	untranslated region

INTRODUCTION

MicroRNAs (miRNAs) are a class of small, highly conserved endogenous RNAs that are triggers for sequence-specific post-transcriptional gene regulation. These regulatory RNAs are initially transcribed as long primary transcripts, or pri-miRNAs, which are processed into ~70nt stem loop intermediate pre-miRNAs by the RNase III enzyme Drosha in the nucleus and then are exported to the cytoplasm where they are further processed into short 21–24 nucleotide mature miRNAs by a second double-stranded RNA endoribonuclease, Dicer. In animals, mature miRNAs typically bind imperfectly in the 3′UTR of target genes and are responsible for impeding translation by one of several proposed mechanisms, including blocking the initiation step, interrupting elongation, deadenylation and degradation of the target mRNA or proteolysis of the nascent polypeptide

Electronic Supplementary Material The online version of this article (doi:10.1007/s11095-010-0156-0) contains supplementary material, which is available to authorized users.

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(1). MiRNAs in plants often bind with perfect or near perfect complementarity in the coding region of genes and can also down-regulate gene expression by cleaving the transcript via the degradative RNAi pathway (2).

In recent years, the key roles that miRNAs play in diverse cellular processes have been appreciated and their widespread importance is increasingly acknowledged. There are currently >700 known human miRNAs responsible for regulating up to 30% of all human genes (3,4) which function to control processes such as development, cell cycle regulation, cell differentiation and proliferation, neuronal asymmetry, metabolic pathways and immune responses (5–8). Dysregulation of miRNAs can lead to various disorders, including neurological diseases, coronary artery disease, and immune function disorders, and has also been implicated in many types of cancer (9–15).

The biological importance of miRNAs makes it desirable to catalogue their targets and decipher their regulatory networks. Because the miRNA binding sites in the 3′UTR are typically imperfectly matched, it is very difficult to computationally predict miRNA targets with certainty. MiRNA targets can alternatively be confirmed empirically by down-regulating miRNAs using antisense oligonucleotide (ASO) antagonists. In addition, interest in the potential for therapeutic use of miRNA antagonists continues to grow with increasing efforts to treat diseases resulting from over-expression of miRNAs.

Various steps during the biogenesis of mature miRNAs may be targeted by ASOs for down-regulation. Degradation of pri-miRNA transcripts using RNase H active ASOs is possible and may be the preferred approach if the goal is to simultaneously inhibit the activity of several miRNAs linked in a polycistronic transcript. However, mature miRNA turnover rate in the cytoplasm is slow, allowing the existing mature miRNA population to continually remain active long after pri-miRNA cleavage, complicating short-term *in vitro* studies (16–18). Additionally, a small proportion of pri-miRNAs are located in or overlap with the 3′UTR of protein-coding genes (19), and down-regulating the pre-miRNA may result in the inadvertent inhibition of these genes. Alternatively, ASOs may be designed to target the intermediate pre-miRNA stem-loop structure which theoretically could impede Dicer processing into the mature miRNA. This approach has design limitations, as it is thermodynamically challenging to invade hairpin structures with ASOs, and detection of inhibition is again negatively impacted by the stability of existing mature miRNAs in the cytoplasm. To date, the most promising approach is to design miRNA antagonists perfectly complementary to the mature miRNA intended for down-regulation. These anti-miRNA oligonucleotides (AMOs or "anti-miRs") sterically block the mature miRNA from binding to its target and may sequester the miRNA in P-bodies for storage or later degradation (20,21).

Each of the chemical modifications evaluated in this study has distinct thermodynamics, specificity, nuclease sensitivity and toxicity profile. Phosphorothioate (PS) linkages exchange a non-bridging oxygen of a phosphodiester bond with sulfur which confers nuclease resistance at this site; however, each linkage decreases the binding affinity (Tm) of the AMO by ~0.4°C/linkage (22). Depending upon the extent of modification, PS linkages can bind non-specifically to proteins, which may cause toxicity with AMOs that are fully PS modified (23). Alternatively, this characteristic can also improve cellular uptake upon direct injection *in vivo* by binding serum albumin and reducing plasma clearance (24), making full-PS-modified AMOs desirable for some *in vivo* applications. Incorporation of 2′-*O*-methyl RNA (2′OMe) bases can stabilize an AMO against nucleases and also increase binding affinity to the target RNA by ~0.7°C/modification (22). Because 2′OMe RNA bases naturally occur in mammalian ribosomal RNAs and transfer RNAs, toxicity from this modification is minimal. The 2′-Fluoro (2′F) modification also offers some protection for single-stranded oligonucleotides from nuclease degradation and increases the binding affinity of an AMO by an average of ~1.2°C/modification depending on the sequence (25), leading to potent inhibition of miRNAs (21). Locked nucleic acids (LNAs) connect the 2′-*O* with the 4′-C of the ribose with a methylene bridge, locking the sugar in the 3′-*endo* conformation (26). This base configuration is extremely stable against nucleases (27–29) and significantly increases the Tm of the oligonucleotide against an RNA target by ~2.4°C/modification (22,30) making it the highest affinity modification tested in this study.

MiRNA antagonists with assorted chemical modification composition and placement have been studied by many groups to find the most potent AMOs *in vitro* (21,31–35) and *in vivo* (20,36–41). An ideal reagent will have high potency, which typically involves use of high affinity modifications for steric blocking AMOs, will have substantial resistance to serum and intracellular nucleases and will retain sufficient specificity to not unduly cross-react with related yet distinct sequences and cause unintended off-target effects (OTEs). The present study directly compares function of a variety of the different AMOs using a luciferase reporter system in HeLa cells, looking at potency, specificity, and stability against nucleases.

MATERIALS AND METHODS

Oligonucleotide Synthesis

All anti-miRNA oligonucleotides described in this study were synthesized using standard phosphoramidite chemistry and purified by reversed phase high performance liquid

chromatography (RP-HPLC) (Integrated DNA Technologies, Coralville, IA). Oligonucleotides were characterized by electrospray-ionization mass spectrometry (ESI-MS) and were within ±0.02% predicted mass, and capillary electrophoresis confirmed >90% purity. All oligonucleotides were used as the Na+ salt form and were quantified by ultraviolet spectroscopy using modification-specific extinction coefficients prior to transfection. All AMO sequences used in this study are listed in Table S1, available in the online Supplemental Materials.

Nuclease Stability Assays

A male mouse was sacrificed using cervical dislocation. One gram of liver tissue was immediately dissected out and placed into 10 ml of T-PER tissue protein extraction reagent (Pierce, Rockford, IL), and a cocktail of protease inhibitors containing 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), pepstatin-A, E-64, bestatin, leupeptin, and aprotinin (Sigma-Aldritch, St. Louis, MO) was added at 1:100 of the volume of the T-PER reagent. The liver-extraction reagent mixture was immediately homogenized for 1 min at 35,000 RPMs using an Omni TH homogenizer with a 10 mm stainless steel probe (Omni International, Kennesaw, GA). The extract was centrifuged at 10,000× *g* for 5 min, and the supernatant was stored at −80°C.

In a 25 µl reaction volume, 8 µM AMOs were diluted in PBS and incubated in 20% mouse liver protein extract or 10% non-heat-inactivated fetal bovine serum (FBS) at 37°C for 0, 2, 6 or 24 h (hrs). Reactions were stopped by adding an equal volume of 2X formamide gel-loading buffer, flash frozen on dry ice and stored at −80°C. Ten µl (40 pmoles) of each of the reactions were separated with denaturing polyacrylamide gel electrophoresis (PAGE) using 8M urea and 20% formamide denaturing 14% polyacrylamide gels. Gels were stained for 20 min with 1X GelStar (Lonza, Rockland, ME) and visualized by UV excitation.

Plasmid Construction and Preparation

Both strands containing one perfect match miR21 binding site (5′-pTCGAGCGAGCCGGTCTCAACATCAGTCTGATAAGCTACCGGATCGCGGGCTGC-3′, 5′-pGGCCGCAGCCCGCGATCCGGTAGCTTATCAGACTGATGTTGAGACCGGCTCGC-3′), one perfect match miR16 binding site (5′-pTCGAGCGAGCCGGTCCGCCAATATTTACGTGCTGCTACCGGATCGCGGGCTGC-3′, 5′-pGGCCGCAGCCCGCGATCCGGTAGCAGCACGTAAATATTGGCGGACCGGCTCGC) or an arbitrary scrambled control 5′-pTCGAGCGAGCCGGTCAAGCCAGACTTTGTTGGATTTGAAATTCCGGATCGCGGGCTGC, 5′pGGCCGCAGCCCGCGATCCGGAATTTCAAATCCAACAAAGTCTGGCTTGACCGGCTCGC were synthesized (Integrated DNA Technologies), annealed and ligated into the psiCHECK™-2 vector (Promega, Madison, WI) using the Xho1/Not1 sites in the 3′UTR of *Renilla* luciferase. DNA sequence was confirmed by sequencing both strands. Endotoxins were removed from the engineered psiCHECK™-2 plasmids using the MiraCLEAN® Endotoxin Removal Kit (Mirus Bio LLC, Madison, WI), and the plasmids were filtered through a 0.2 micron filter and quantified by ultraviolet spectroscopy prior to transfection. The underlined portion of the sequences above represents the miRNA binding domain.

Cell Culture and Transfections

HeLa cells were cultured in Dulbecco's Modified Essential Medium (DMEM) with 10% FBS (Invitrogen, Carlsbad, CA) in a 100 mm dish. At 90% confluency, 5 µg of the psiCHECK™-2-miRNA plasmid was transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Six hours post-transfection, cells were washed with PBS and replated in 48-well plates to achieve ~70% confluency the following morning. At 24 h after the plasmid transfection, the AMOs were transfected using TriFECTin™ (Integrated DNA Technologies) in serum-free media according to recommended guidelines. All transfections were performed in triplicate. After 6 h, the transfection media was replaced with DMEM+10% FBS for the remainder of the experiment. Luciferase assays were performed 24 h after AMO transfection.

Dual Luciferase Assay

Firefly and *Renilla* luciferase activities were measured using the Dual Luciferase Reporter Assay System (Promega) with a GloMax 96 Microplate Luminometer (Promega). Results are reported as a fold increase in *Renilla* luciferase relative light units (RLUs) compared to the lipid reagent control. All wells are normalized to the internal firefly luciferase control.

RESULTS

Nuclease Stability of AMOs

A series of miR21 AMOs were synthesized representing designs based upon previously published work as well as some novel modification patterns. The synthetic oligonucleotides were comprised of different combinations of modifications, including DNA, 2′OMe RNA, LNA and 2′

F bases. Further, subsets of these AMOs were made with PS-modified linkages throughout the entire sequence, three PS linkages on both ends or no PS linkages (Supplementary Table S1).

When incorporated into an oligonucleotide, the different chemical modifications studied here confer varying levels of resistance to endo- and exonucleases. It is well established that nuclease stability plays an important role in the function of antisense oligonucleotides of all types (42), so stability studies were performed on compounds representing each class of chemistry/design in the collection. Representative subsets of the miR21 AMOs (Fig. 1A, Fig. S1A) were incubated in 10% FBS (Fig. 1B) or 20% mouse liver protein extract (Fig. S1B) for 0, 2, 6 or 24 h at 37°C and examined by gel electrophoresis to establish the effectiveness of the various chemical modification patterns to prevent degradation. The "2′OMe" AMO comprising 2′ OMe RNA bases alone was quickly degraded in serum; however, protecting the 5′ and 3′ ends of the "2′OMe" AMO with either PS linkages ("2′OMe 3PSends") or flanking hairpin secondary structure ("HP+RC+HP 2′ OMe") stabilized the compounds. The "2′OMe 3PSends" and the "HP+RC+HP 2′OMe" AMOs, which have single-stranded 2′OMe phosphodiester domains in the central region of the oligonucleotides, remained intact after incubation in both serum and liver protein extract. This suggests that the 2′OMe RNA bases alone may be sufficient to protect against any endonucleases which may be present in these environments. The "DNA/LNA" AMO, a DNA/LNA mixmer containing all phosphodiester linkages, was rapidly degraded, and little full length oligonucleotide remained intact after 2 h in 10% FBS or 20% liver protein extract; after 24 h incubation, it was severely truncated in both 10% FBS and 20% liver protein extract. The addition of PS linkages to the DNA/LNA mixmer improved stability, and no degradation of this compound was seen at any time point in either 10% serum or 20% liver protein extract. The phosphorothioate-modified versions of the 2′ OMe/LNA mixmers behaved similarly to the phosphorothioate-modified DNA/LNA mixmers when incubated in serum or liver protein extract; however, substitution of 2′ OMe RNA bases for DNA did improve nuclease resistance for the all-phosphodiester versions ("DNA/LNA" *vs.* "2′ OMe/LNA"). The "2′F LNAends" AMO has 18 2′F bases positioned centrally with two LNA bases on each end and was stable in 10% FBS. The primary nuclease activity in serum is derived from 3′-exonucleases; therefore, protecting the 3′ ends of the AMO is most important for stability in serum. Additional protection of the central domain may be needed in the intracellular environment where more endonucleolytic activity is present (43,44). This is evident with complete degradation of the "2′F LNAends" AMO in 20% liver protein extract, while the "2′F LNAends PS" AMO, which was protected with PS linkages, was stable under all conditions.

The AMOs that are end-protected with PS linkages usually showed a small amount of truncated product consistent with 1–2 bases of exonuclease "nibbling"; this is expected as synthesis of PS-modified oligonucleotides leads to generation of two stereoisomers at each linkage, and the Sp isomer is significantly more nuclease resistant than the Rp isomer to exonucleolytic attack (45,46).

Direct Comparison of AMO Potencies

To directly compare functional potency of the different AMO modification patterns, a psiCHECK™-2 luciferase expression plasmid was constructed containing a single perfect match binding site for miR21 in the 3′UTR of the *Renilla* luciferase gene. Having perfect complementarity to miR21 permits cleavage of the *Renilla* transcript directed by endogenous miR21 via the Ago2 degradative RNAi pathway, increasing the sensitivity and the dynamic range of the assay to detect when functional miR21 levels are reduced by transfection of an AMO (35). The psiCHECK™-2-miR21 plasmid was tested in HeLa cells for miRNA-mediated down-regulation of *Renilla* luciferase when compared to firefly luciferase, also expressed from the same plasmid and used as an internal normalizing control. From an average of 100 transfections, the psiCHECK™-2-miR21 plasmid shows >70-fold decrease in the *Renilla* luciferase/firefly luciferase ratio when compared to the psiCHECK™-2-Scr control vector. These results confirm that the psiCHECK™-2-miR21 plasmid is functional, that miR21 is highly expressed in HeLa cells and that a large dynamic range for detecting miRNA inhibition is available.

The luciferase reporter plasmids were transfected into HeLa cells, and 24 h later the AMOs (Fig. 2A) were transfected (in biological triplicates) with 5 doses ranging from 50 nM to 1 nM. Cells were harvested 24 h post-transfection and assayed for *Renilla* and firefly luciferase activity (Fig. 2B). A wide range of AMO activity was seen, ranging from no change in *Renilla* luciferase levels to approaching a 120-fold boost in activity, indicating very effective antagonism of functional miR21 levels. The "DNA-PS" AMO was inactive, presumably due to the low binding affinity (Tm) of a short DNA oligonucleotide that is fully PS modified. The "2′OMe" AMO showed very low levels of miR21 inhibition, as has been previously reported for this chemistry (21,35); the poor performance of this design might result from nuclease sensitivity coupled with some ability to function as a passenger strand when duplexed with the cognate miRNA (21). When three PS linkages were placed on each end ("2′OMe 3PSends"), stability against exonuclease degradation was improved and

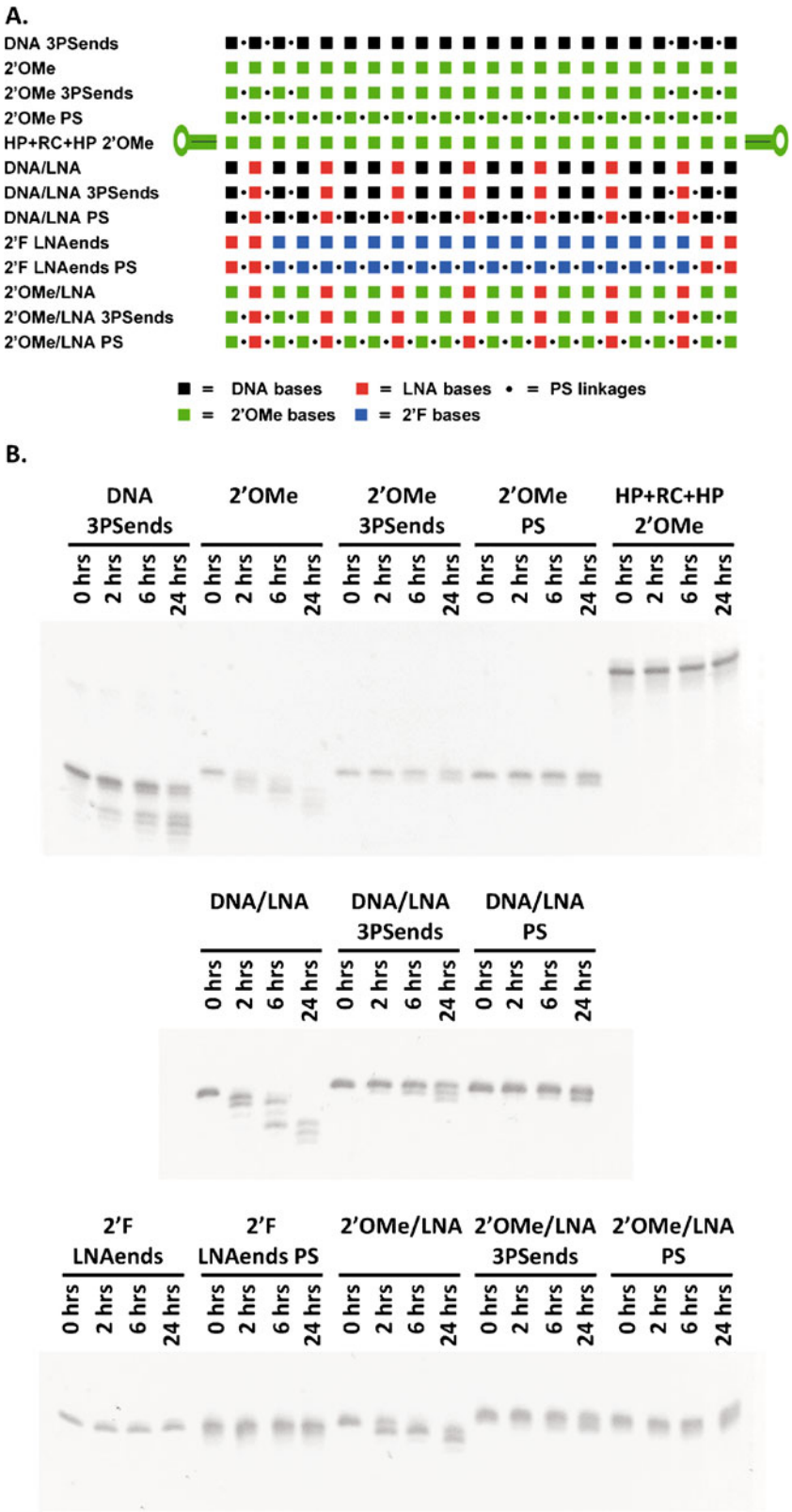

Fig. 1 Effect of chemical modifications on the stability of oligonucleotides in 10% FBS. **A** AMOs were designed using DNA, 2′-*O*-methyl RNA (2′OMe), locked nucleic acid (LNA) and 2′-Fluoro (2′F) bases with varying degrees of phosphorothioate (PS) linkages. **B** 8 μM of each AMO was incubated in 10% non heat inactivated FBS for 0, 2, 6 or 24 h, with the reactions stopped by adding equal volumes to 2X formamide gel loading buffer, flash freezing on dry ice and storage at −80°C. 40 pmoles of each AMO was separated on 14% polyacrylamide gels supplemented with 8M urea and 20% formamide, stained with 1X GelStar, and visualized by UV excitation.

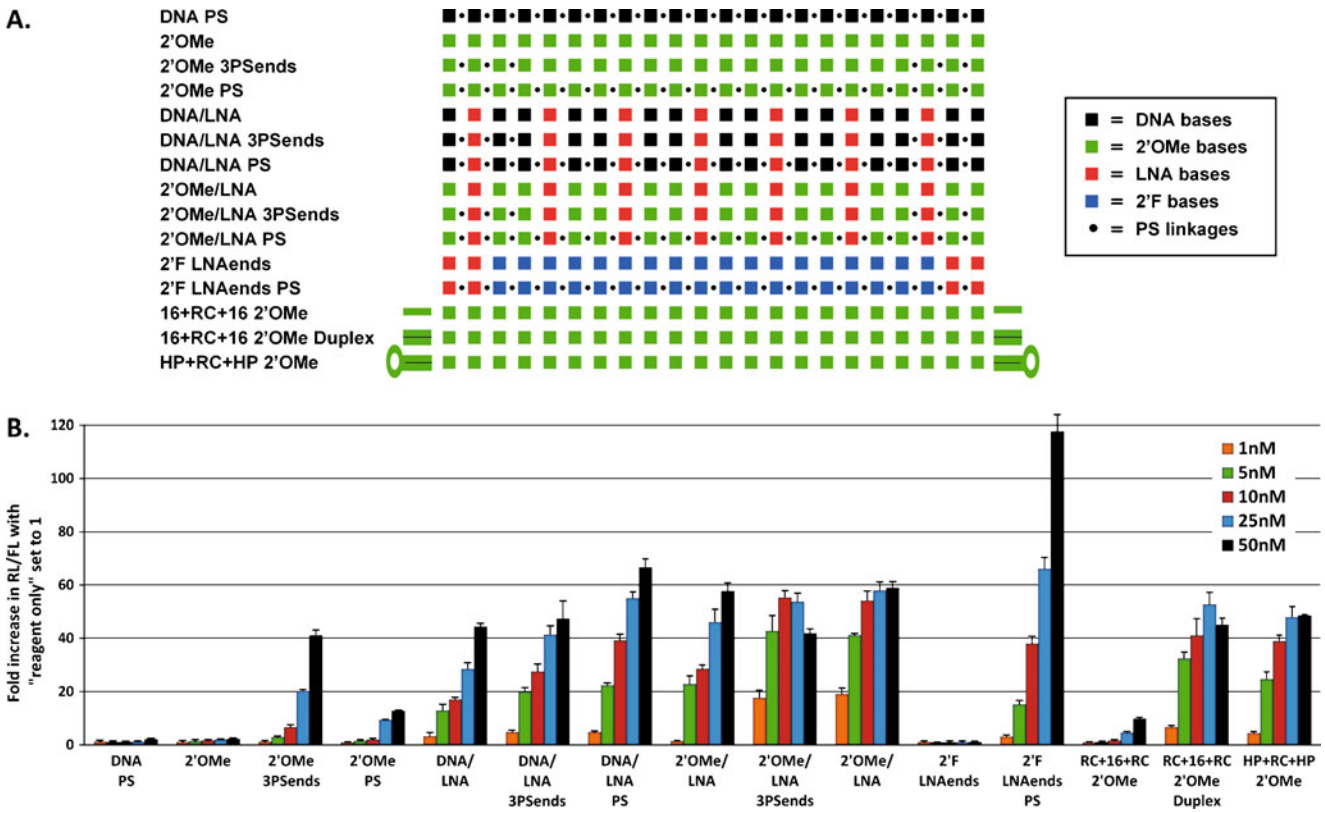

Fig. 2 A direct comparison of the potency of several miR21 AMOs. **A** AMOs were designed using DNA, 2′-*O*-methyl RNA (2′OMe), locked nucleic acid (LNA) and 2′-Fluoro (2′F) bases with varying degrees of phosphorothioate (PS) linkages. **B** HeLa cells expressing the psiCHECK™-2-miR21 plasmid were transfected at various concentrations in triplicate with AMOs targeting miR21. Cells were lysed and analyzed for luciferase expression 24 h post-transfection. All values are reported as a fold increase in *Renilla* luciferase when compared to the lipid reagent control and normalized with the internal firefly luciferase control.

the compound was much more active. The fully PS-modified "2′OMe PS" AMO showed slightly lower potency compared with the end-modified version, which may relate to the lower binding affinity associated with the additional PS linkages. This behavior pattern for the 2′OMe chemistry parallels earlier observations by other groups (47). The DNA/LNA mixmers were all potent compounds and, even without any PS linkages, were more effective than any of the unstructured 2′OMe AMOs. As before, incorporating PS linkages into the DNA/LNA mixmers further increased AMO potency, especially when fully modified, which probably relates to improved stability to intracellular endonucleases (Fig. S1). Similarly, all of the 2′OMe/LNA mixmers were very effective in inhibiting miR21 function. Once again, addition of PS modifications increased both the stability against nucleases and functional potency of these compounds, making the "2′OMe/LNA 3PSends" and the "2′OMe/LNA PS" constructs the most potent AMOs at low dose (1 nM) of all the compounds tested. The "2′F LNAends PS" construct was modeled after previously described AMOs that included 2′F bases with two 2′-*O*-2-methoxylethyl (MOE) bases on each end (41); the MOE modification is not commercially available, so LNA bases were substituted as the commercial chemistry with the most similar properties. The "2′F LNAends" construct was non-functional as a miRNA inhibitor. Although stable in serum, where a 3′-exonuclease activity is of primary concern and the LNA end caps provide protection, this compound is susceptible to intracellular endonucleases (Fig. S1), which limits its functional activity when transfected into HeLa cells. In support of this observation, the addition of PS linkages dramatically improved potency, making the "2′F LNAends PS" AMO the most active AMO tested at high dose (50 nM).

All of the AMOs studied so far were of similar length (22mers), matching the target miRNA. The last series of AMOs tested were 2′OMe oligonucleotides that were over twice the length of the target miRNA. These designs have a single-stranded central domain that is complementary to the target miRNA and extend on both the 3′ and 5′ ends with sequences complementary to the pri-miR21 sequence in either single-stranded or double-stranded form, or with hairpin structures that are unrelated to the miRNA target. These designs were previously reported to increase potency well above the short 2′OMe AMO parent compound (35).

In the anti-miR21 experiments conducted here, the version with single-stranded extensions, "16+RC+16 2′OMe," contained 16 additional bases on both the 5′ and 3′ ends of the 2′OMe AMO. Like the shorter 2′OMe single-stranded AMO, this compound proved to be a poor inhibitor of miR21 function. However, when the flanking sequence on both ends was duplexed with short complementary 2′OMe oligonucleotides or the flanking sequences formed hairpin structures, potency was markedly increased. There is precedent from previous antisense studies demonstrating that 3′-end hairpin structures can improve nuclease stability and significantly increase functional potency for knockdown of RNA targets (48,49). It was also suggested that the presence of flanking duplex domains improved the ability of the AMO to invade RISC and served a functional purpose beyond nuclease stabilization (35).

To establish whether these trends in AMO potency were translatable to other miRNA targets, six of the most potent designs were synthesized complementary to miR16 (Table S1 and Fig. 3A) and were tested against a miR16-target vector. Like the original miR21 vector, a new psiCHECK™-2-miR16 plasmid was made that contained a single perfectly matched binding site to miR16 in the 3′UTR of the *Renilla* luciferase gene. The new anti-miR16 AMOs were tested in HeLa cells transfected with the miR16 reporter plasmid for functional changes in miR16 levels. The psiCHECK™-2-miR16 plasmid showed an average of a 9-fold decrease in *Renilla* luciferase/firefly luciferase expression when compared to a psiCHECK™-2-Scr control, suggesting that miR16 is present in HeLa cells but that its expression (or at least functional activity) is significantly lower than miR21, which showed >70-fold change in the same assay. This reduces the useful dynamic range of the assay system; however, even with these reduced miR16 expression levels, miRNA inhibition was detectable, and the miR16 AMOs could be compared. Transfection of the miR16 AMOs into HeLa cells expressing the psiCHECK™-2-miR16 plasmid gave similar results as with miR21, with the exception that the "DNA/LNA 3PSends" design was inactive in this system (Fig. 3B). Given the compressed dynamic range of the miR16 assay system, the other five AMOs showed relatively similar performance, with the "2′OMe/LNA 3PSends" and "HP+RC+HP 2′OMe" compounds showing a slight advantage at the lower doses tested (1 nM and 0.5 nM).

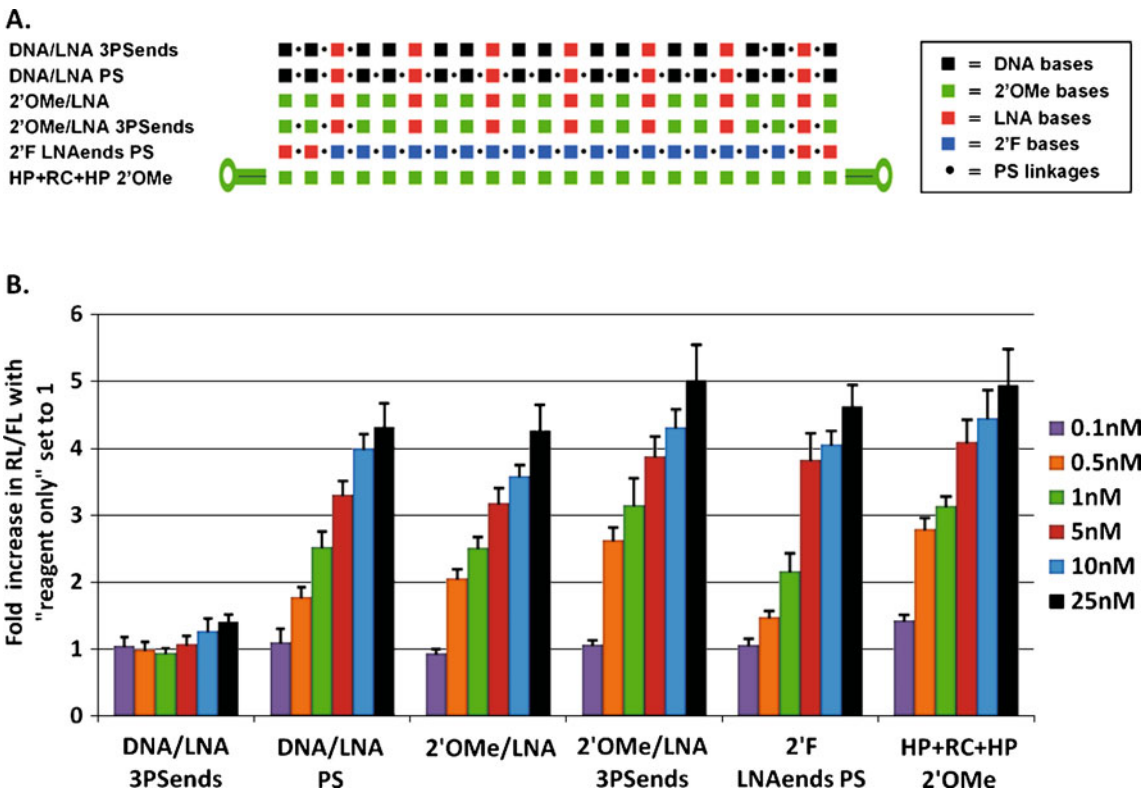

Fig. 3 A direct comparison of the potency of miR16 AMOs. **A** AMOs were designed using DNA, 2′-*O*-methyl RNA (2′OMe), locked nucleic acid (LNA) and 2′-Fluoro (2′F) bases with varying degrees of phosphorothioate (PS) linkages. **B** HeLa cells expressing the psiCHECK™-2-miR16 plasmid were transfected at various concentrations in triplicate with AMOs targeting miR16. Cells were lysed and analyzed for luciferase expression 24 h post-transfection. All values are reported as a fold increase in *Renilla* luciferase when compared to the lipid reagent control and normalized with the internal firefly luciferase control.

Comparing the Specificity of Potent AMOs

While incorporating high binding affinity modifications into AMOs can increase potency, it can also potentially cause unintended off-target effects by promoting cross-hybridization with closely related sequences. To test the specificity of some of the more potent inhibitors, new miR21 AMOs were synthesized with three mismatches placed throughout the sequence (Table S1 and Fig. 4A). These "3MUT" controls were directly compared with their perfect match counterparts in HeLa cells expressing the psiCHECK™-2-miR21 plasmid. Both the "DNA/LNA PS" and the "2′F LNAends PS" AMOs showed high specificity, and there was very little activity seen using the 3MUT variants of these designs even at the highest dose tested (50 nM) (Fig. 4B). Although the 2′OMe/LNA mixers were specific at the mid-range and lower doses, some off-target activity was observed using the 3MUT variant at the 50 nM dose. In general, it is prudent to administer synthetic oligonucleotides of any kind at the lowest dose that gives effective activity to minimize the risk of these kinds of unwanted cross-hybridization effects.

Effect of LNA Placement on AMOs Activity

The DNA/LNA and 2′OMe/LNA AMOs incorporated LNA residues once every three bases, resulting in seven LNA bases in a 22nt oligonucleotide sequence. This modification pattern in general seems to work well when designing AMOs. However, it is likely that blind application of this approach will result in hairpin structures being stabilized by the LNA residue placement, which will vary with the unique sequences of individual miRNAs and which may adversely affect function. It is likely that customizing the precise placement pattern for each miRNA could improve function; however, this would require empiric optimization for each sequence of interest. Careful optimization of this kind has been performed for a miR122 AMO as part of a drug development program and resulted in a compound that is a 15mer oligonucleotide comprising eight LNA bases and seven DNA bases and is fully PS modified. This compound has successfully been used via IV injection in non-human primates to reduce miR122 levels and shows promise in treating chronic Hepatitis C Virus infection (40,50).

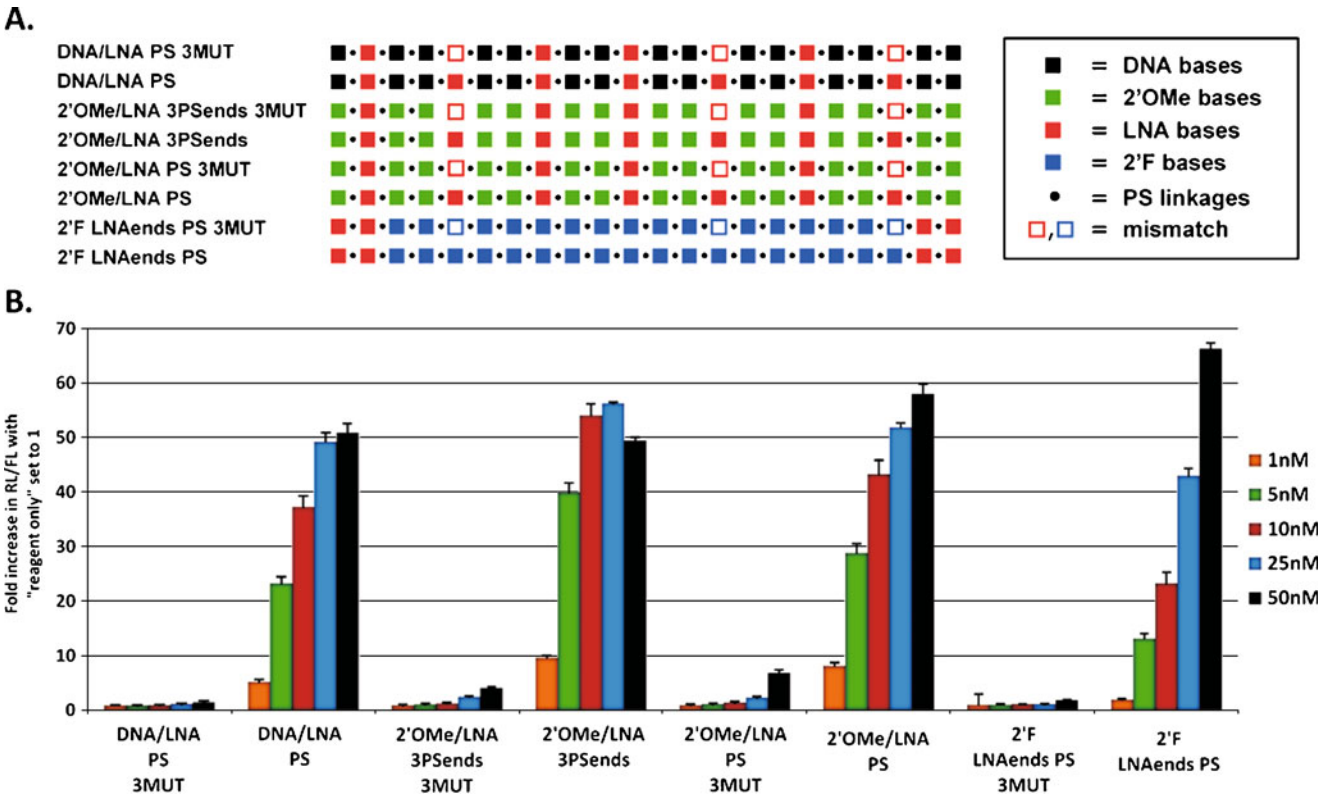

Fig. 4 Determining the specificity of miR21 AMOs containing high affinity modifications. **A** AMOs were designed using DNA, 2′-*O*-methyl RNA (2′OMe), locked nucleic acid (LNA) and 2′-Fluoro (2′F) bases with varying degrees of phosphorothioate (PS) linkages. To test for specificity, three mismatches were incorporated throughout the miR21 AMOs. **B** HeLa cells expressing the psiCHECK™-2-miR21 plasmid were transfected in triplicate with AMOs targeting miR21 at various concentrations. Cells were lysed and analyzed for luciferase expression 24 h post-transfection. All values are reported as a fold increase in *Renilla* luciferase when compared to the lipid reagent control and normalized with the internal firefly luciferase control.

As a first step in designing DNA/LNA mixmers or 2′OMe/LNA mixmers for use as AMOs, several basic design parameters must be considered. Minimizing the secondary structure (hairpin) within the AMO as well as the self-dimer hybridization potential may be beneficial in improving potency. Further, precise positioning of the LNAs with respect to the 5′end may influence AMO potency. With the miR21 DNA/LNA and 2′OMe/LNA mixmer AMOs studied here, every third base was LNA modified, starting with the second position from the 5′-end. This starting point was arbitrarily chosen, and it would have been also feasible to position, for example, the first LNA base at the third position from the 5′-end. Two new miR21 AMOs were synthesized using this alternate start point for LNA modification (which then proceeded with placement at every third base thereafter) (Table S1, called "DNA/LNA2 PS" and "2′OMe/LNA2 3PSends," and Fig. 5A). These new constructs were tested using the psiCHECK™-2-miR21 plasmid reporter system in HeLa cells and compared with the original versions of the compounds that started with the LNA base at position two. Interestingly, the "DNA/LNA2 PS" AMO, having the LNA modifications beginning at the third position of the sequence, lost potency compared with the original "DNA/LNA PS" AMO and was inactive at low dose (Fig. 5B). In contrast, the "2′OMe/LNA2 3PSends" AMO retained full activity compared with the original "2′OMe/LNA 3PSends" AMO. These findings were unexpected and are difficult to reconcile. Predictive algorithms indicate that the two modification patterns should result in oligonucleotides that vary in Tm by only 2°C and that hairpin or secondary structure is not obviously worse with one pattern over the other. It may relate in some way to the 2′OMe content of the second design which was more tolerant to changes in modification pattern. Additional sequences are being studied with this kind of comparison to better understand this observation.

DISCUSSION

This study directly compares the potency of 15 different AMO designs and found wide variation in degrees of stability, activity, and specificity among the different modification patterns. Even though transfections were performed in serum-free media, complexing the lipid reagent with the AMOs took place in reduced-serum media which may account for the correlation of the overall stability of the AMO in serum nucleases and AMO potency; those compounds that did not survive incubation in serum were ineffective at reducing functional miRNA levels. For example, the basic "2′OMe" AMO is rapidly degraded in serum, and although this design is reported to be capable of miRNA inhibition in some earlier work in other systems (32,38,51,52), it was inactive in the miR21-luciferase reporter system employed here. Other groups have observed similar problems with using phosphodiester 2′OMe oligonucleotides as AMOs (21). When comparing the higher binding affinity DNA/LNA mixmers with varying levels of PS modification, an increase in serum stability corresponded with an increase in potency. The

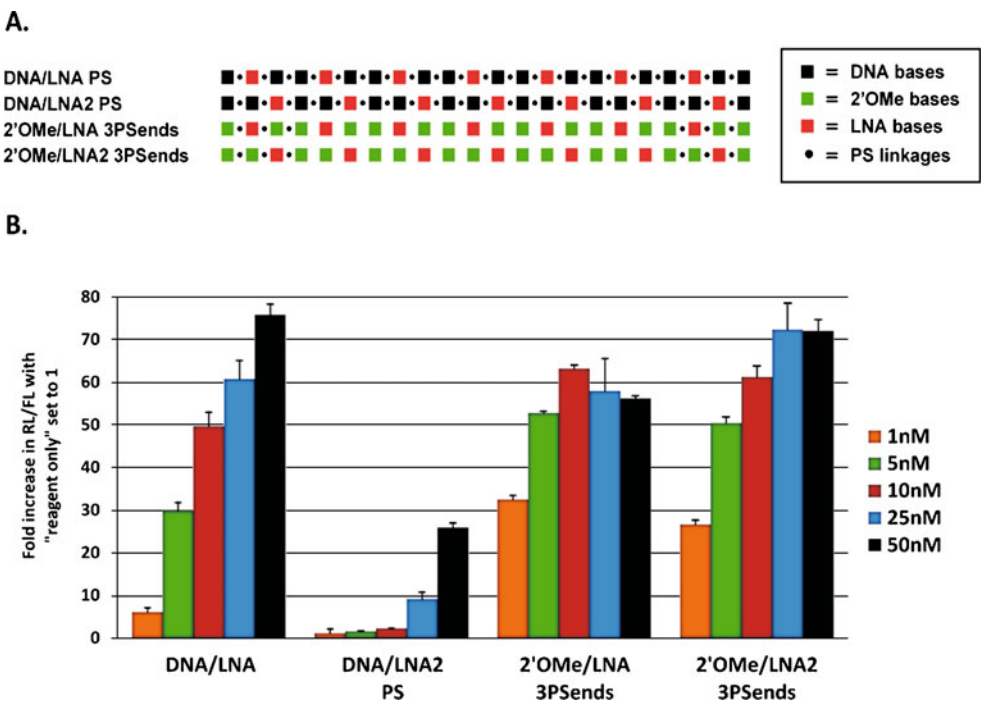

Fig. 5 Effects of LNA placement on DNA/LNA and 2′OMe/LNA mixmers. **A** AMOs were designed using DNA, 2′-*O*-methyl RNA (2′OMe) and locked nucleic acid (LNA) bases with varying degrees of phosphorothioate (PS) linkages. The LNA placement pattern was altered by positioning the LNA bases starting either two or three bases in from the 5′end of the AMO. **B** HeLa cells expressing the psiCHECK™-2-miR21 plasmid were transfected in triplicate with AMOs targeting miR21 at various concentrations. Cells were lysed and analyzed for luciferase expression 24 h post-transfection. All values are reported as a fold increase in *Renilla* luciferase when compared to the lipid reagent control and normalized with the internal firefly luciferase control.

behavior was similarly observed with the 2′OMe/LNA series. The exception to this trend was the "2′F LNAends" AMO, which was stable in serum yet was inactive as a miRNA inhibitor. This seeming discordance is probably due to the dominance of 3′-exonuclease activity in serum, with the LNA bases positioned at the 3′ and 5′ ends protecting the "2′F LNAends" AMO in serum. When the "2′F LNAends" AMO was incubated in liver protein extract, it was rapidly degraded by the intracellular endonucleases. The addition of full PS modification to this design in the "2′F LNAends PS" version resulted in a compound that was stable in both serum and liver protein extract and showed high potency. Some of the AMOs showed evidence for some end "nibbling" when incubated in serum, such as the DNA/LNA PS-modified constructs, but were nevertheless active, potent AMOs. An examination of "DNA/LNA PS"-modified miR21 AMOs with lengths varying between 18 and 22nts did not show any significant differences in potency (data not shown), and modified compounds of this general design as short as 15nts have been used with success (40,50). Other groups have observed that longer AMOs are needed when using only the 2′OMe chemistry (47). Thus, it seems that shorter oligonucleotides may function well as AMOs, at least when employing high affinity modifications such as LNA bases so that small amounts of "end nibbling" has no functional impact when using the longer oligonucleotides tested here.

When using high affinity chemical modifications in antisense applications, there is often a trade-off between potency and specificity, with the highly modified, high Tm variants providing increased potency at the cost of having lower specificity. The specificity survey of AMOs presented here demonstrated that compounds containing high binding affinity modifications are specific to their intended target. However, specificity is dose dependent and can decrease when the compounds are used at high concentrations. For example, the "2′OMe/LNA 3PSends 3MUT" and "2′OMe/LNA PS 3MUT" AMOs showed some inhibition of miR21 at the highest doses studied, but this was around 10% or less of the activity seen with the perfect match compounds. The "2′F LNAends PS 3MUT" and "DNA/LNA PS 3MUT" AMOs did not show appreciable cross-reactivity with the miR21 reporter even at the highest dose tested. It is possible that these designs might begin to show cross-reactivity if only 1–2 mismatches were tested. Typically, shorter sequences show higher mismatch discrimination than longer sequences; the AMOs tested here were 22mers, and use of shorter sequences will likely display even better specificity (LNA-modified AMOs as short as 15mers can show high potency). These data serve as a reminder that it is always prudent to employ as low a dose of synthetic oligonucleotide in biological experiments that produces the desired effects to minimize the risk of cross-reactivity and off-target effects. For the higher potency compounds studied here *in vitro*, little benefit was realized by increasing dose above 10 nM. As an added benefit, using lower doses also lowers cost. Lower Tm AMOs made fully of the 2′OMe chemistry can show specificity for even a single base mismatch in certain sequence contexts (47).

The mismatches tested in this study were evenly spaced throughout the miRNA sequence and may not necessarily reflect the placement of mismatches between closely related miRNAs encountered in nature. Non-targeted miRNAs containing mismatches that are clustered on the 5′ or 3′ end might still be unintentionally inhibited by the AMO. As mentioned previously, LNA-modified AMOs can be optimized for a specific sequence context, and if cross-reactivity with related species is of concern, intelligent design rules for LNA placement to maximize mismatch discrimination are available from thermodynamic studies that should be applicable to this need (53). In some instances, closely related family members of miRNAs, such as the Let7 family members, can have as little as a single mismatch between species, which would be difficult to discriminate between no matter which chemical modification strategy was used. However, these miRNA family members are predicted to regulate the same genes (54), and it actually may be desirable to target the entire family using a single AMO that cross-reacts with all family members. It should be fairly straightforward to design compounds like this using sequences that span the full length of the miRNA and incorporate a larger number of high affinity modifications (such as LNAs), exploiting where possible mismatches which are favorable for base pairing, like G:U wobble bases. Obviously mismatched controls should be included in any study that tests specificity, and, if possible, inadvertent inhibition of any closely related miRNAs that may exist should be looked for.

In summary, several potent AMO design strategies exist. The design strategy of choice should be tailored to the experimental goal. Depending on the experimental system, no PS linkages, partial PS modified or fully PS modified may be desirable. The AMO design that seemed least sensitive to varying levels of PS modification are the 2′OMe/LNA mixmers, as similar efficacy was seen between the no-PS-modification, PS-end-modified, or fully PS-modified variants. This may be due in part to the intrinsic nuclease stability provided by the mixture of 2′OMe and LNA bases. We nevertheless note that the two PS-modified 2′OMe/LNA mixmers ("2′OMe/LNA 3PSends" and "2′OMe/LNA PS") were the most potent compounds seen at low dose in the more sensitive miR21 assay system. Also of note, the long 2′OMe hairpin design does not seem to need PS modification, at least in the cell culture application studied here. When working *in vivo*, it will likely be important to employ the more highly modified, nuclease-resistant versions of AMOs, as exposure to nucleases is

generally higher here than *in vitro*. Additional considerations arise for *in vivo* use of AMOs relating to toxicity and pharmacokinetics, but these issues are not addressed by the cell culture experiments presented here.

CONCLUSION

Our findings are generally concordant with previous studies. AMOs previously reported to be highly active, such as LNA-modified or 2′F-modified short linear oligonucleotides and longer 2′OMe-modified oligonucleotides containing flanking secondary structure, were very potent *in vitro* using miR21 and miR16 reporter assay systems. A new modification scheme, the 2′OMe/LNA mixmers, showed the highest efficacy at low dose of any of the compounds tested. Selecting the appropriate AMO for the experimental system is important, and these data should aid researchers in making informed decisions when selecting a modification scheme to suit their individual needs and designing AMOs with the appropriate controls.

ACKNOWLEDGEMENTS

We thank Dr. Scott Rose for making the mouse liver protein extracts used in the nuclease stability studies. This research was entirely supported by internal funding from Integrated DNA Technologies, Inc.

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