Competition for RISC binding predicts \textit{in vitro} potency of siRNA

Erich Koller*, Stephanie Propp, Heather Murray, Walter Lima, Balkrishen Bhat, Thaza P. Prakash, Charles R. Allerson, Eric E. Swayze, Eric G. Marcusson and Nicholas M. Dean

ISIS Pharmaceuticals, Carlsbad Research Center, 1896 Rutherford Road, Carlsbad, CA 92008, USA

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ABSTRACT

Short interfering RNAs (siRNA) guide degradation of target RNA by the RNA-induced silencing complex (RISC). The use of siRNA in animals is limited partially due to the short half-life of siRNAs in tissues. Chemically modified siRNAs are necessary that maintain mRNA degradation activity, but are more stable to nucleases. In this study, we utilized alternating 2′-O-methyl and 2′-deoxy-2′-fluoro (OMe/F) chemically modified siRNA targeting PTEN and Eg5. OMe/F-modified siRNA consistently reduced mRNA and protein levels with equal or greater potency and efficacy than unmodified siRNA. We showed that modified siRNAs use the RISC mechanism and lead to cleavage of target mRNA at the same position as unmodified siRNA. We further demonstrated that siRNAs can compete with each other, where highly potent siRNAs can compete with less potent siRNAs, thus limiting the ability of siRNAs with lower potency to mediate mRNA degradation. In contrast, a siRNA with low potency cannot compete with a highly efficient siRNA. We established a correlation between siRNA potency and ability to compete with other siRNAs. Thus, siRNAs that are more potent inhibitors for mRNA destruction have the potential to out-compete less potent siRNAs indicating that the amount of a cellular component, perhaps RISC, limits siRNA activity.

INTRODUCTION

Short interfering RNAs (siRNA) are 21–23 nt long double stranded RNA molecules that lead to degradation of specific mRNAs through a multi-protein complex termed the RNA induced silencing complex (RISC) (1). Once introduced into cells, siRNAs are bound to the RISC complex and the double stranded siRNA is unwound by the action of a helicase. The resulting RISC-bound strand is then able to duplex to a complementary mRNA, which leads to enzymatic cleavage of the target mRNA, resulting in post-transcriptional gene silencing. This cleavage is performed by Ago2 (eIF2C2), a RISC associated RNase (2). siRNA has become a useful and widely used tool for gene function studies \textit{in vitro} (3). However, the use of siRNA for both gene function analysis \textit{in vivo} and therapeutic development is limited due to the poor pharmacodynamic and pharmacokinetic properties, such as low stability due to serum nucleases and poor delivery to tissues.

To overcome these limitations chemically modified siRNAs with improved pharmacodynamic and pharmacokinetic properties and equal or greater potency have been identified (4–7). These chemically modified siRNAs are more potent than unmodified siRNAs and are much more stable to serum nucleases. For example, 2′-O-methyl (OMe) modifications have been introduced into the antisense strand of tissue factor siRNA (8–10). siRNA activity containing full OMe substitutions in the sense strand combined with phosphorothioate linkages has been shown to be mediated by RISC and strand-specific loading or binding to hAgo2 may be modulated through selective incorporation of these modifications (11). Recently, a double strand siRNA construct which has been fully modified on both strands with alternating OMe and 2′-fluoro (F) nucleotides has been shown to have greatly increased potency for silencing activity compared to an unmodified construct with the same sequence (6). Since this modified construct contained no RNA nucleotides, it was not clear whether it was using a RISC-mediated siRNA method of action.

In these preliminary studies, we demonstrate that OMe/F-modified siRNA designed to hybridize to the gene Eg5 are 10-fold more potent than unmodified siRNA. We have confirmed that the modified siRNA activity is mediated by the RISC. Ago2 leads to cleavage of the mRNA target at the same position with both unmodified siRNA and OMe/F-modified siRNA. Previously, it has been shown with unmodified siRNAs that inactive siRNAs can compete with active siRNAs, but the mechanism of this competitive
inhibition is unknown (9,12,13). Here, we show that in much the same manner, the OMe/F-modified siRNAs can compete with unmodified siRNA for RISC-mediated cleavage of target RNA. Thus, these fully modified siRNAs appear to utilize the same mechanism as unmodified siRNAs and serve as a potent tool to inhibit target genes in vitro.

MATERIALS AND METHODS

Cells and reagents

Tissue culture medium, trypsin and Lipofectamine2000 were purchased from Invitrogen (Carlsbad, CA). HeLa, and U87-MG cells were obtained from the American Type Tissue Collection (Manassas, VA) and cultured in DMEM supplemented with 10% fetal calf serum, streptomycin (0.1 µg/ml), and penicillin (100 U/ml). siRNA treatment was performed using Opti-MEM (Invitrogen) containing 5 µg/ml Lipofectamine2000 and the indicated amount of siRNA for 4 h at 37°C, as described previously (14,15).

Preparation of synthetic siRNA

Synthetic unmodified siRNAs were purchased from Dharmacon Research, Inc (Boulder, CO). Sequences of the oligoribonucleotides and their respective modifications are shown below. siRNA duplexes were formed according to the manufacturer’s instructions. In brief, 1.6 µl of a 250 µM antisense stock was combined with 1.6 µl of a 250 µM sense stock, 4 µl of 5X universal buffer (500 mM potassium acetate, 150 mM HEPES–KOH, pH 7.4, 10 mM magnesium acetate) and 12.8 µl of ultra-pure water followed by heating at 90°C for 1 min. The reaction was then allowed to cool to ambient temperature. The final concentration of the duplex was 20 µM in 1X universal buffer (100 mM potassium acetate, 30 mM HEPES–KOH, pH 7.4, 2 mM magnesium acetate). The sequence of the PTEN siRNA (341401_341391) is AAGTAAGGACCAGAGGACAA (sense) and TTGTCTCTCTCTGTCTAT (antisense). The sequence of the EG5 siRNA (371864_371854) is CACACAGGATGAGTGTC for the probe. The sequence for the PTEN siRNA (341401_341391) is AAGTAAGGACCAGAGGACAA (sense) and TTGTCTCTCTCTGTCTAT (antisense). The sequence of the PTEN siRNA (341401_341391) is AAGTAAGGACCAGAGGACAA (sense) and TTGTCTCTCTCTGTCTAT (antisense). The sequence of the PTEN siRNA (341401_341391) is AAGTAAGGACCAGAGGACAA (sense) and TTGTCTCTCTCTGTCTAT (antisense).

Cell-cycle analysis

Cells were grown on collagen coated 24-well plates and were treated with the siRNAs (four replicates per group). Cells were harvested 48 and 72 h after oligonucleotide treatment, fixed with 80% ethanol and stained with propidium iodide (PI; Sigma, St Louis) containing RNAse cocktail, (Ambion, Austin, TX). The PI-stained cells were analyzed on a FACScan (BD Biosciences, San Jose). Cell-cycle profile was calculated with ModFit LT (Verity Software House, Topsham, ME).

Tagman RT–PCR

Total RNA was harvested at 16–24 h post-transfection using an RNeasy 3000 BioRobot (Qiagen, Valencia, CA). Reduction of target mRNA expression was determined by real time RT–PCR using an ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA). The sequence for the human EG5 primer/probe set used in the RT–PCR reaction is GTGGTGAGATGCGAGACATTATAAT for the forward primer, CTTTCTACAGATCACATTCCTATTG for the reverse primer and TGCCAGAGCAGAAGCTAGCGC for the probe. The sequence for the human PTEN primer/probe set is AATGGCTAAAGTGAGTAGACAAT for the forward primer, TGCCATATCTA TTACAGCATCCGT for the reverse primer and AGATGC GGCTTTGATGGCTCCAGC for the probe.

Western blotting

siRNA treated cells were lysed in RIPA lysis buffer containing 1% Triton X-100, 0.1% SDS, 0.25% Sodium deoxycholate, 150 mM NaCl, Tris pH 7.5 and complete protease inhibitor mix with EDTA (Roche, Indianapolis, IN). Equal amounts of protein were resolved on a SDS–PAGE gel and transferred to PVDF membranes. The membranes were blocked for 1 h in PBS containing 0.1% Tween-20 and 5% non-fat milk powder. Proteins were detected using mouse anti-human EG5 (BD Transduction Laboratories). After incubation with peroxidase-coupled secondary antibodies, blots were developed using ECL-plus reagent (NEN) and exposed to X-ray films (Kodak).

mRNA RISC cleavage assay

HeLa cells were seeded at an initial density of 1 000 000 cells/100 mm plate (Becton Dickinson, Franklin Lakes, NJ) on the day prior to the initial transfection and incubated at 37°C, 5% CO2. Three 100 mm plates were used per siRNA sample to be tested. Plasmid pE2-N_HA (expressing Ago2) was delivered to cells (typically at 70–75% confluency) by using Effectene transfection reagent (Qiagen, Valencia, CA) according to manufacture’s instructions and incubated for 24 h at 37°C, 5% CO2. Synthetic siRNA (75 nM) was then delivered to cells (typically at 80–85% confluency) by using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to manufactures instructions. After 4 h at 37°C, 5% CO2, the media were aspirated from the cells and replaced with DMEM containing 10% FBS and antibiotics and returned to 37°C, 5% CO2 until cells were harvested 18 h later. Briefly, cells were trypsinized, three 100 mm plates per sample combined and cells pelleted by centrifugation. Cells were washed with 1 ml of cold PBS, and centrifuged at 1000 g. The cell pellet was resuspended in 500 µl lysis buffer (150 mM NaCl, 0.5% NP-40, 2 mM MgCl2, 2 mM CaCl2, 20 mM Tris, pH 7.5) containing a Complete Mini Protease Inhibitor Tablet (Roche, Indianapolis, IN) and 1 mM DTT, then passed through a 1 cm3 U-100 Insulin Syringe (Becton Dickinson, Franklin Lakes, NJ) 10 times, followed by a 10 min 10 000 g clearing spin. The supernatant (450 µl) was transferred to a new tube containing 15 µl of immobilized HA.11 beads (Covance, Richmond, CA) and 535 µl lysis buffer. Beads were rotated end-over-end for 2 h, then washed 3x in wash buffer (500 mM NaCl, 2 mM MgCl2, 2 mM CaCl2, 20 mM Tris, pH 7.5) followed by one wash with cleavage buffer (100 mM KCl, 2 mM MgCl2, 10 mM Tris, pH 7.5). Following the final wash, the
immunoprecipitated RISC complexes were incubated for 2 h at 30°C in a heated mixer. Final reaction volume was 30 μl containing 5 U/ml RNase, 1 mM ATP, 0.2 mM GTP, and labeled target substrate in cleavage buffer. The reaction was stopped by the addition of TRIZOL, followed by RNA extraction according to the manufacture’s protocol and PAGE analysis.

**In vitro cleavage assay**

1 x 10⁶ HeLa cells were plated in a 100 mm dish. The following day cells were transfected with 5 nM siRNA using 5 μg/ml Lipofectamine 2000 (Invitrogen, Carlsbad, CA). 18 h later total RNA was isolated with RNeasy spin columns (Qiagen, Valencia, CA). One microgram of total RNA was ligated to GeneRacer RNA oligo (Invitrogen, Carlsbad, CA). Omitting CIP and TAP treatment restricts ligating GeneRacer oligo to cleaved RNA. The ligated products were reverse transcribed using Superscript III Reverse transcriptase (Invitrogen, Carlsbad, CA) and a reverse Primer (5’-CTGGATTTGAC-GGCTCCTCTACTGT-3’ for PTEN and 5’-TGGATTTGAC-GTGAAAGCCATCAG-3’ for Eg5). A PCR step was performed with a nested gene specific reverse primer (5’-CGGCTAGGGAACCTCAGGATGA-3’ for PTEN and 5’-TCAGGTCTCTACTGTT-3’ for Eg5) and a forward primer to the GeneRacer oligo. The resulting PCR fragments were cloned into TOPO-TA cloning vector (Invitrogen, Carlsbad, CA) and sequenced using T3 and T7 universal primers.

**RESULTS**

**Activity of unmodified siRNA versus chemically modified siRNA**

Unmodified siRNA are unstable in vivo, and it is generally agreed that chemical modification or formulation will be necessary to display potent systemic activity in vivo. For this reason, we performed a structure-activity relationship study to identify potent chemically-modified siRNA. We performed a screen with 19mer siRNAs to find chemistries that are more nuclease resistant. Previously, we have shown that PTEN siRNA, consisting entirely of alternating 2'-O-methyl and 2'-fluoro (OMe/F) nucleotides, display similar or increased in vitro potency compared to unmodified siRNA (Figure 1A), depending on the mRNA target site, increased Tm and enhanced plasma stability (6). The potencies of the PTEN siRNA and OMe/F used throughout the studies are shown in Figure 1A for both U87-MG human glioblastoma cells (left panel) and HeLa cells (right panel). To investigate whether the incorporation of the OMe/F modification into other siRNA sequences will increase potency against other molecular targets, we tested siRNAs with alternating OMe and 2'-fluoro targeting the cell-cycle regulating gene Eg5. Modified and unmodified siRNA duplexes were transfected into U87-MG cells (Figure 1B, left panel) and HeLa cells (Figure 1B, right panel) and in vitro potency was determined by measuring mRNA reduction by quantitative RT–PCR. The potency of the OMe/F-modified Eg5 siRNA compared to unmodified siRNA was increased 40-fold in U87-MG and 7-fold in HeLa cells (Figure 1B), respectively. Consistent with a decrease in Eg5 mRNA we also observed a dose-dependent decrease in Eg5 protein (Figure 1C).

Previously, we have demonstrated that inhibiting Eg5 with an RNase H antisense oligonucleotide (ASO) leads to a G2/M-arrest, followed by an increase in apoptosis (16). We also wanted to determine if inhibition of Eg5 with the modified siRNA resulted in the same phenotype. Both the unmodified siRNA and the OMe/F-modified siRNA led to an increase in G2/M and an increase in subG1 cells (hypodiploid cells) in U87-MG cells (Figure 2A) and HeLa cells (Figure 2B). In agreement with the Eg5 mRNA data, the OMe/F-modified siRNA was more potent and effective compared to the unmodified siRNA in arresting cells in G2/M and inducing apoptosis. The PTEN siRNAs did not affect any of the cell cycle parameters.

**Ago2 cleavage of mRNA with unmodified and chemically modified siRNA**

To investigate the mechanism by which the chemically modified siRNA reduced Eg5 mRNA, we next utilized an Ago2 based in vitro RNA cleavage assay to confirm that initial cleavage of the targeted mRNA by the modified OMe/F duplexes is a direct consequence of Ago2/RISC activity (17–20). In brief, HeLa cells were first transfected with a vector expressing HA-tagged Ago2, and then 24 h later transfected with the siRNAs. RISC complexes were allowed to assemble endogenously for an additional 24 h, after which cells were lysed and the HA-Ago2 RISC complexes were immunoprecipitated with the anti-HA antibody. Following isolation of the Ago2 complex, an RNA cleavage assay was performed using a 40 nM 5'-labeled RNA substrate corresponding to the region containing the target site for the transfected siRNA. Both the unmodified and the OMe/F-modified siRNA result in the same cleavage product, i.e. 10 nt from the 5’ end of the antisense-strand (Figure 3A). To confirm that the siRNA leads to the same cleavage of endogenous mRNA in cells, we also performed an in vivo cleavage assay. The cleaved mRNA products for both Eg5 (Figure 3B) and PTEN (Figure 3C) were cloned and sequenced. Both the unmodified and the OMe/F-modified siRNA result in the same cleavage product, i.e. 10 nt from the 5’ end of the guide-strand consistent with the in vitro results in Figure 3A. Both PTEN and Eg5 OMe/F-modified siRNAs led to cleavage at the same position as the unmodified siRNA. These results demonstrate that the OMe/F-modified siRNA complexes to Ago2 and cleaves targeted mRNA at the same site as the unmodified siRNA.

**F/OMe modified siRNAs compete with unmodified siRNA**

Previous work has suggested that RISC components may be rate limiting to siRNA potency, as combining two siRNA results in loss of activity (13). To determine whether chemically modified siRNA molecules can also compete with siRNA loading into RISC, and whether this correlates with increased potency, we tested PTEN and Eg5 siRNA for their ability to compete with each other in U87-MG cells. Cotransfecting either 10 or 50 nM unmodified PTEN siRNA shifted the dose response curve of Eg5 siRNA by 10- or 100-fold,
Figure 1. Target reduction with unmodified and OMe/F-modified siRNA. U87-MG and HeLa cells were transfected with increasing concentrations of PTEN (A) and Eg5 (B) targeting siRNA (closed diamond, OMe/F; closed square, unmodified siRNA) and 24 h later PTEN and Eg5 mRNA expression was determined, respectively. Data represent means ± SD of three replicates. (C) HeLa cells were transfected with increasing concentrations of Eg5 OMe/F and Eg5 protein expression was measured 48 h later by western blotting. Lane 1 and 7: untreated control, lane 2–6 control OMe/F-treated (0.05, 0.2, 1, 10 and 50 nM), lane 8–12 Eg5 OMe/F-treated (0.05, 0.25, 1, 10 and 50 nM).
Figure 2. Effect on inhibiting Eg5 expression on cell-cycle in U87-MG (A) and HeLa cells (B). Cells were transfected with increasing concentrations of Eg5 and PTEN targeting siRNA (closed diamond, Eg5 siRNA; closed square, Eg5 OMe/F; closed circle, PTEN siRNA; closed triangle, PTEN OMe/F), and 48 h later cell-cycle profile was determined. Hypodiploidy represents cells with fragmented DNA as a measure of apoptosis while 4N represents mitotic cells or cells with two nuclei.
respectively. We also tested if an increasing concentration of PTEN siRNA would compete with a constant concentration (5 nM) of Eg5 siRNA. No effect was observed at concentrations <5 nM. Concentrations >5 nM PTEN siRNA was required to compete with 5 nM Eg5 siRNA (Figure 4B). The PTEN siRNA with two dT-overhangs competes with Eg5 siRNA comparable to the blunt end PTEN siRNA (data not shown).

Next, we tested whether the OMe/F-modified siRNAs are capable of competing with unmodified siRNA. Activity of the unmodified Eg5 siRNA was inhibited to a similar degree with OMe/F-modified PTEN siRNA (Figure 4C) as it was with the unmodified PTEN siRNA in U87-MG cells (Figure 4A). In HeLa cells, unmodified PTEN siRNA competed with Eg5 siRNA (Figure 4D). Similarly, OMe/F-modified PTEN siRNA competed with unmodified Eg5 siRNA (Figure 4E). However, higher concentrations were required for competition than in U87-MG cells. To ensure the competition seen is specific for siRNA, we next evaluated the ability of a single stranded oligonucleotide that functions through an RNase H mechanism of action to compete with unmodified Eg5 siRNA. This compound had no effect on Eg5 siRNA potency (Figure 5A). As with unmodified Eg5 siRNA the RNAse H antisense ASO was unable to compete with the OMe/F-modified Eg5 siRNA using concentrations as high as 200 nM. In the same experiment, PTEN mRNA reduction with the PTEN ASO alone was the same as with the combination of the OMe/F-modified Eg5 siRNA and the PTEN ASO (data not shown).

**Correlation between siRNA potency and competition**

So far, we have tested if PTEN siRNA competes with Eg5 siRNA. We then evaluated whether the Eg5 unmodified
siRNA could compete with the unmodified PTEN siRNA. The unmodified Eg5 siRNA failed to compete with the unmodified PTEN siRNA (Figure 6A), while the OMe/F-modified Eg5 siRNA at 10 and 50 nM competed, resulting in a decrease in potency of the unmodified Eg5 siRNA (Figure 6B). The unmodified PTEN siRNA is about five times more potent than the unmodified Eg5 siRNA while the OMe/F-modified Eg5 siRNA is about four times as potent as unmodified PTEN siRNA suggesting that there is a correlation between potency and ability to compete. In addition, we used four PTEN siRNAs with different potencies to reduce PTEN mRNA (Figure 6C, upper panel). Three of the PTEN siRNAs competed with the Eg5 siRNA, while the PTEN siRNA with the lowest potency failed to compete with the Eg5 siRNA (Figure 6C, lower panel). This further indicates that very potent siRNAs also are good competitors.

DISCUSSION

In order to develop siRNA with improved pharmacodynamic and pharmacokinetic properties, we have synthesized a series of siRNAs with alternating 2'-O-methyl/2'-fluoro modifications in both the sense and antisense strands. These modified constructs have been shown to be more potent and more
nuclease resistant than chemically unmodified siRNA. Recently, an OMe/F-modified siRNA was found to have greatly increased potency compared to an unmodified siRNA (6). Here we found that OMe/F-modification of a siRNA targeting Eg5 results in a more potent construct in both U87-MG and HeLa cells. The higher potencies with OMe/F siRNA correlated directly with increased biological responses, including increasing 4N-DNA content and apoptosis. The same biologic effects have been observed when Eg5 expression was inhibited with RNase H-mediated ASOs (16) further demonstrating that the phenotype observed is characteristic for reduced Eg5 expression levels and not a reflection of unspecific toxicity. In addition, PTEN OMe/F-modified constructs displayed no obvious toxicities.

We demonstrated that the chemically modified siRNAs like unmodified siRNA result in cleavage of mRNA through Ago2 (Figure 3A). To further support the hypothesis that OMe/F-modified siRNA use the same mechanism as unmodified siRNA, we conducted competition studies. We found that both unmodified and OMe/F-modified PTEN siRNA competed with unmodified Eg5 siRNA. These two PTEN constructs were approximately equipotent at reducing PTEN mRNA and were equally good competitors of Eg5 siRNA. In contrast, only the OMe/F-modified Eg5 construct could effectively compete with the unmodified PTEN construct. The unmodified Eg5 construct had little or no effect on the ability of the PTEN siRNA to reduce PTEN mRNA. The OMe/F modified Eg5 is much more potent than the unmodified Eg5 construct which led us to believe that the ability to compete may be correlated to the potency of the constructs in reducing their targeted mRNA. To test this, we used chemically-modified PTEN siRNA constructs that had varying potencies for reducing PTEN mRNA. The ability of these constructs to compete with an unmodified Eg5 siRNA construct was correlated to their intrinsic potencies. However, the correlation was not perfect, so there may

Figure 5. siRNA can not compete with ASOs. (A) U87-MG cells were transfected with increasing concentrations of PTEN ASO alone (closed diamond) or in combination with 50 nM Eg5 siRNA (closed square). (B) U87-MG cells were transfected with increasing concentrations of Eg5 OMe/F Eg5 alone (closed square), PTEN ASO alone (closed diamond) or Eg5 F/OMe in combination with PTEN ASO (×). Increasing doses of PTEN ASO were unable to compete with 5 nM of Eg5 OMe/F.
be other factors that also play a role in determining ability of siRNAs to compete with each other.

There are also cell type differences in the competition of siRNA constructs. In general, higher concentrations of competitor siRNA were needed to see effects in HeLa cells compared to U87-MG cells. This is demonstrated by the fact that it required 50 nM PTEN siRNA to compete with Eg5 siRNA in HeLa cells, but only 10 nM in U87-MG cells. Also, the low potency unmodified Eg5 siRNA shows a limited ability to compete with PTEN siRNA in U87-MG cells, but no competition in HeLa cells. HeLa cells are probably the most frequently used cell type in published siRNA studies. They seem to have an excellent capacity for siRNA-mediated mRNA cleavage relative to other cell lines. This may be due to expression levels of proteins like Ago2, TRBP and PACT. However, novel RISC components are still being discovered and their roles in siRNA activity and cell type differences remains to be elucidated (21).

The ability of siRNAs to compete is not limited to these two targets as we also tested other siRNA constructs for their ability to compete. We found siRNAs to genes other than Eg5 and PTEN and also unspecific control siRNAs were able to compete (data not shown). Despite the ability of siRNAs to compete with each other, it is still possible to inhibit more than one target simultaneously using appropriate concentrations of siRNA. We have tested this with simultaneous treatment of PTEN and Eg5 siRNA and observed good target reduction for both PTEN and Eg5 (data not shown). However, the target reduction observed might be better when cells are treated with one siRNA at a time. Simultaneous silencing of two genes has also been shown by others (22). However, it is now clear that when attempting to silence...
two or more genes at once, one should be careful to use appropriate siRNA concentrations to inhibit each target.

While we were writing this manuscript, Grimm et al. (23) published the interesting finding that mice treated with high doses of viral vectors carrying genes encoding shRNAs can suffer from hepatotoxicity. This effect appeared to be caused by the shRNAs competing with endogenous microRNAs for mediated export from the nucleus. Exogenously added siRNAs were found to enter the nucleus and quickly get exported by exportin-5 (24). Although in addition to exportin-5 there might be other limiting factors contributing to saturation of endogenous small RNA pathways. The exact implications of this and our study for application of siRNA in drug discovery efforts needs to be further evaluated.

The wide spread in vivo use of siRNA still awaits improvements in pharmacokinetic properties of siRNA constructs and additional work will be needed to optimize efficacy while taking care to prevent interference with crucial endogenous siRNA/microRNA pathways that might result in toxicity. Clearly more work is needed to understand the molecular mechanism of how siRNAs can compete with each other and how exogenous siRNA affects endogenous siRNA/microRNA pathways.

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