siRNA-optimized Modifications for Enhanced *In Vivo* Activity

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Current modifications used in small interfering RNAs (siRNAs), such as 2′-methoxy (2′-OMe) and 2′-fluoro (2′-F), improve stability, specificity or immunogenic properties but do not improve potency. These modifications were previously designed for use in antisense and not siRNA. We show, for the first time, that the siRNA-optimized novel 2′-O modifications, 2′-O-benzyl, and 2′-O-methyl-4-pyridine (2′-O-CH2Py(4)), are tolerated at multiple positions on the guide strand of siRNA sequences *in vivo*. 2′-O-benzyl and 2′-O-CH2Py(4) modifications were tested at each position individually along the guide strand in five sequences to determine positions that tolerated the modifications. The positions were combined together and found to increase potency and duration of siRNAs *in vivo* compared to their unmodified counterparts when delivered using lipid nanoparticles. For 2′-O-benzyl, four incorporations were tolerated with similar activity to the unmodified siRNA *in vivo*, while for 2′-O-CH2Py(4) six incorporations were tolerated. Increased *in vivo* activity was observed when the modifications were combined at positions 8 and 15 on the guide strand. Understanding the optimal placement of siRNA-optimized modifications needed for maximal *in vivo* activity is necessary for development of RNA-based therapeutics.

Introduction

Double-stranded RNAs, such as a small interfering RNAs (siRNAs), induce degradation of sequence-specific homologous mRNA via RNA interference (RNAi), a mechanism of post-translational gene silencing. In order for an siRNA to be effective upon entering the cell, the siRNA must become phosphorylated on the 5′-end by Clp1 kinase and be incorporated into the endogenous RNA-induced silencing complex, which consists of Ago2, Dicer, and TRBP.[1–5] Nucleotides 2 through 8 of the siRNA guide strand are preassembled in an A-form helix and the guide strand makes contact with the surface of Ago2 through its sugar and phosphodiester backbone.[6,7] The guide strand can then associate with the complementary mRNA strand, causing consequent cleavage of the mRNA, and thereby regulates gene expression.

RNAi-based therapy presents an attractive opportunity to engage targets not accessible through conventional small molecules.[8] While the activity of unmodified, all ribonucleotide, siRNAs are suitable for *in vitro* experiments, siRNA use *in vivo* requires higher standards for siRNA potency, specificity, and safety that can be achieved through modifications to the individual nucleosides on the siRNA.[9–11] Once the best siRNA sequence has been chosen for a target, the siRNA can be optimized through chemical and structural modifications.[12–15] Modified siRNA duplexes are expected to reduce ribonuclease degradation in plasma,[16] immunogenicity, the off-target effects from genes having sequence complementarity to either siRNA strand, and poor pharmacokinetic properties.[17–19] The chemical modifications that can potentially optimize the performance of an siRNA include: the ribose ring to alter the sugar pucker and helical properties of the siRNA;[20] the bases to reshape hydrogen-binding properties that target mRNA;[21] or the phosphodiester backbone to adjust charge interactions.[22]

The most widely used and commercially available modifications in siRNAs have been limited to ones discovered over 10 years ago in the antisense field and developed at the 2′ position of the ribose ring including 2′-methoxy (2′-OMe), 2′-fluoro (2′-F), and 2′-O-methoxyethyl (2′-MOE).[23] 2′-OMe and 2′-F modifications are well tolerated at multiple positions in the siRNA guide strand due to their small size that is comparable to the natural RNA 2′-OH. They provide increased stability, increased specificity and reduced immunogenicity.[24] The structures of other, larger, 2′-O-modifications such as 2′-O-MOE and 2′-O-allyl modifications caused attenuated silencing activity. These modifications were tolerated in only a very position-specific manner within the guide strand.[25–28] They appeared to cause steric clashes with Ago2 residues thereby preventing guide strand loading into RISC.[7,28] These studies demonstrate that siRNA-optimized modifications are needed to improve siRNA activity. Even though discovery of chemical modifications that are beneficial and universally favorable to siRNAs *in vivo* have been elusive, identification, and design of siRNAs to achieve maximal activity will be necessary for therapeutic development.[9]

Recently, we identified a new siRNA 2′-O-modification, 2′-O-benzyl, which was tolerated at multiple positions

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throughout the guide strand, in contrast to what has been reported previously with 2′-MOE.[29] Due to this surprising finding, we decided to further evaluate this new modification for its potential to be used in RNAi therapeutics. In this study we evaluated, *in vivo*, 2′-O-benzyl, and its pyridine derivative 2′-O-methyl-4-pyridine (2′-O-CH2Py(4)). In contrast to the benzyl functional group, pyridine contains a set of lone pair electrons from the nitrogen atom which contributes to its shorter C-N bond length, planarity of the six-membered ring, and ability to hydrogen bond. We identified positions that tolerated these modifications and then combined these positions to optimize multiple siRNA sequences to demonstrate the applicability of these modifications to any sequence of choice. Combinations of four 2′-O-benzyls or six 2′-O-CH2Py(4) were tolerated in siRNA guide strands and had similar activity to unmodified siRNAs *in vivo*, demonstrating modifications can be tolerated in the guide strand and convey beneficial properties *in vivo*. The optimal combination that increased *in vivo* activity over unmodified siRNAs contained only two modifications, at positions 8 and 15. This study shows the importance of using optimized modifications specific for siRNAs and that placement of these new modifications is critical for obtaining maximal *in vivo* silencing activity.

Results

Novel 2′-O- modifications are tolerated at multiple positions in the guide strand. In order to determine which positions in the siRNA guide strand to place 2′-O-benzyl and 2′-O-CH2Py(4) for *in vivo* studies we first evaluated them *in vitro*. Five different double-stranded 21-mer siRNA sequences were evaluated *in vitro*. The gene names and start target sites are: ApoB(9514), ApoB(10162), PHD2(196), PHD2(384), and PCSK9(1965). Positions 1–19 of both strands were ribonucleotides, and the overhangs at positions 20 and 21 contained 2′-OMe nucleotides. The passenger strands contained inverted abasics at the 5′ and 3′ ends to block loading into Ago2.[10] siRNAs containing 2′-O-CH2Py(4) at position 1 also had a 5′-P but 2′-O-benzyl modifications at position 1 did not, which contributed to the attenuated activity for 2′-O-benzyl at that position. These unmodified siRNAs were the template for systematic evaluation of siRNAs containing a single 2′-O-benzyl or 2′-O-CH2Py(4) modification at every position along the guide strand corresponding to the base that naturally occurs at that position (**Figure 1a**).

All siRNAs demonstrated target-specific mRNA degradation in cell-based assays as detected by quantitative PCR (qPCR). Hepa 1-6 cells were transfected with either an unmodified siRNA that had a natural 2′-OH or with an siRNA-containing 2′-O-benzyl (**Figure 1b**) or 2′-O-CH2Py(4) (**Figure 1c**) on the guide strand to test for maximal mRNA degradation. The data is normalized using the unmodified version of each sequence and taking the log2 ratio of the modified to the unmodified siRNA for each position, with a bar indicating the median. Unmodified siRNA corresponds to zero on the graph. If a modification has similar activity to unmodified siRNA the ratio value is zero (log2 of 1 is zero). If a modification attenuates activity it has a negative ratio value in relationship to its unmodified siRNA control; if the modification increases
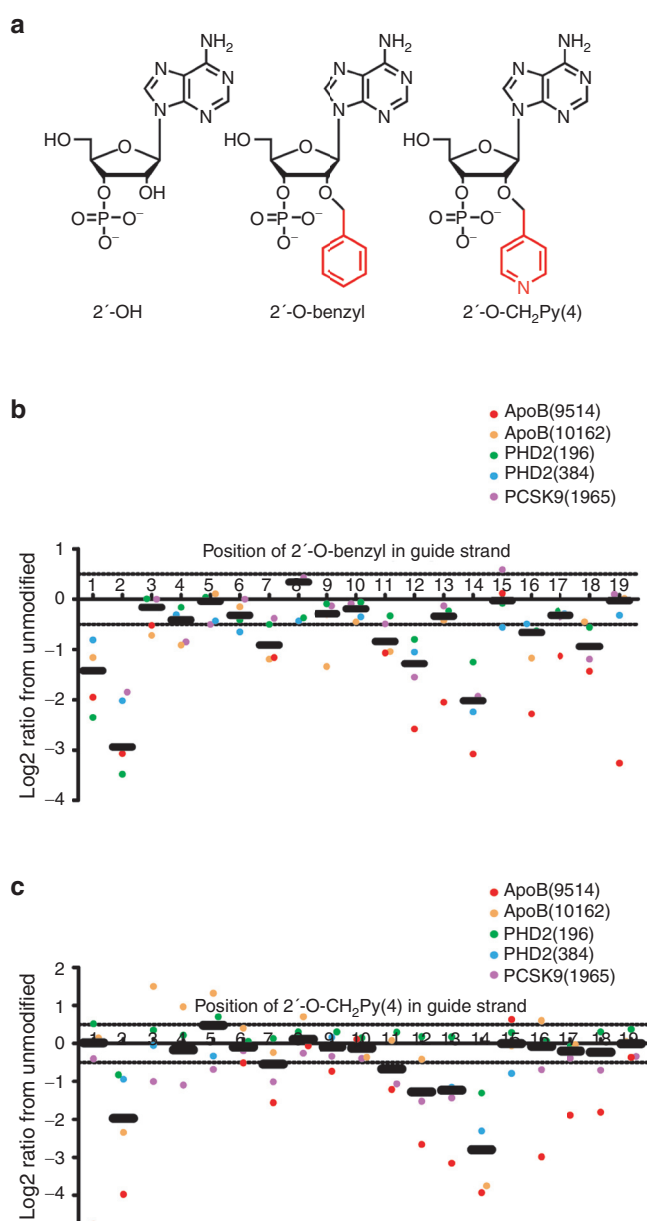

Figure 1   *In vitro* activity of 2′-o-benzyl and 2′-o-CH2Py (4). **(a)** Structure of the nucleotides used for oligonucleotide synthesis with 2′-OH or 2′-O-benzyl (in red). Adenosine is representative of the modifications to the other bases, guanosine, cytosine and uracil. (**b**, **c**) mRNA degradation of target as determined by quantitative PCR (qPCR) and compared to unmodified. Values are shown as log 2 deviations from unmodified. A log ratio of 1 represents a twofold change. Each of the five small interfering RNA (siRNA) sequences, represented by a different colored dot, contain complementary all ribose guide and passenger strands and were transfected into Hep1-6 cells at 10 nmol/l. The median value at each position is represented by a black bar. The dotted lines represent the experimental variation generated from the qPCR. Individual (**b**) 2′-O-benzyl or (**c**) 2′-O-CH2Py(4) were substituted at the indicated position on the guide strand of the duplex. Activity of the chemical unmodified (all ribose) siRNA is at the zero line. Increased mRNA degradation activity compared to an unmodified siRNA would result in a ratio higher than zero and would be advantageous to the siRNA and all points below the zero line have decreased activity.

activity of the siRNA relative to the unmodified sequence the ratio value is positive. siRNAs with 2′-O-benzyl had median values the same or better than unmodified siRNAs at positions 5, 8, 15, and 19 on the guide strand. Positions 5, 6, 8, 10, 15, and 19 were equivalent or better than unmodified siRNAs for 2′-O-CH2Py(4). These data demonstrate these new modifications are tolerated at the same positions for multiple sequences. Our findings indicate that there is potentially more optimization to be done of the siRNA/Ago2 complex than previously realized.

2′-O-benzyl modifications enhance *in vivo* activity and duration. We next assessed 2′-O-benzyl modifications *in vivo* using the most tolerated positions from the *in vitro* data above for 2′-O-benzyl. siRNAs containing 2′-O-benzyls at positions 5, 8, 15, or 19 were formulated into lipid nanoparticles and delivered by intravenous tail injection into mice for ApoB(10162) (**Figure 2**). Lipid nanoparticles localize to the liver, where apolipoprotein B (ApoB) is highly expressed. The livers were harvested and ApoB mRNA levels were evaluated by qPCR 3 and 14 days after injection. Mice treated with unmodified ApoB siRNAs showed ApoB mRNA degradation of 90%. siRNAs containing 2′-O-benzyls at positions 8 or 15 had statistically significantly higher mRNA degradation than the unmodified siRNA, each with 96% mRNA degradation at day 3 (**Figure 2a**). siRNAs with 2′-O-benzyls at positions 5 or 19 had similar activity to the unmodified siRNA at day 3.

Duration of activity was evaluated until day 14 for the same ApoB(10162) unmodified and 2′-O-benzyl modified siRNAs (**Figure 2b**). siRNAs containing 2′-O-benzyls at positions 5, 8, or 15 had statistically significantly better target mRNA degradation than unmodified siRNAs with 78, 88, and 88% mRNA degradation, respectively compared to unmodified siRNA with 64%. ApoB siRNAs with position 19 modified with a 2′-O-benzyl had similar activity to unmodified siRNA.

2′-O-benzyls combined at positions 8 and 15 in the guide strand increase activity *in vivo*. Based on the results above, 2′-O-benzyls were combined into the guide strand at positions 8 and 15 and tested *in vivo* (**Figure 3**). Three different siRNA sequences were tested: ApoB(9514), ApoB(10162), and Luc(80). 2′-O-benzyls at positions 8 and 15 in ApoB(10162) had statistically significantly better mRNA degradation than unmodified siRNA at day 3 and 14 (**Figure 3a**). While these differences may seem small, the clinical significance has yet to be determined for how much target silencing is needed to maintain a beneficial therapeutic effect. Another ApoB siRNA, ApoB(9514), was also tested *in vivo* with and without 2′-O-benzyls at positions 8 and 15 (**Figure 3b**). At day 3, the unmodified and 2′-O-benzyl containing ApoB(9514) siRNAs had similar activity *in vivo*. However, unmodified ApoB(9514) siRNAs have a shorter duration of activity *in vivo* compared to ApoB(10162), with the unmodified ApoB(9514) siRNAs having no activity by day 14. This duration increased when the siRNAs had 2′-O-benzyls at positions 8 and 15, with a statistically significant increase of 47% mRNA degradation compared to 3% from unmodified siRNA. A third siRNA, Luc(80) was also tested with 2′-O-benzyls (**Figure 3c**). Unmodified and 2′-O-benzyl containing
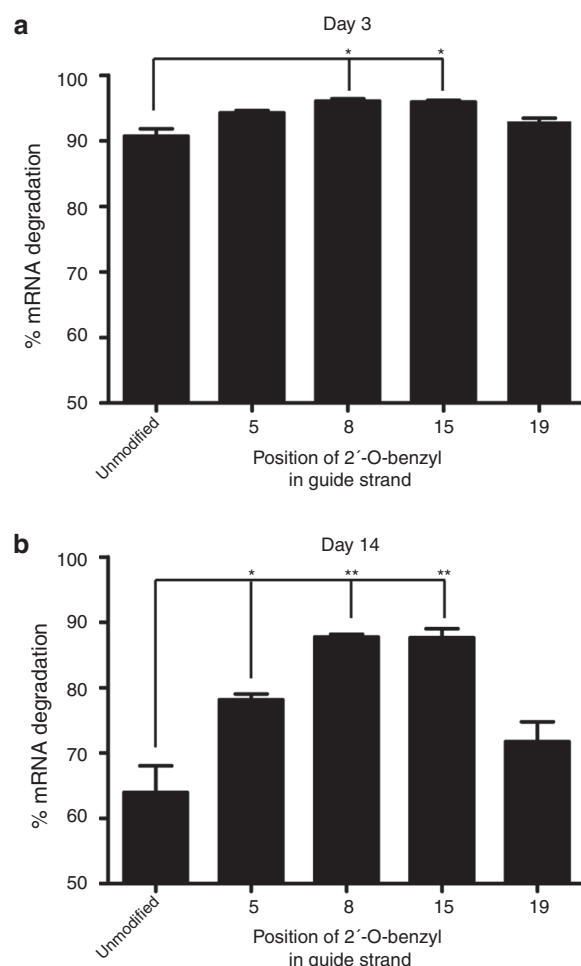
Figure 2 2′-O-benzyls demonstrate enhanced *in vivo* activity after intravenous (i.v.) injection. (**a**) Unmodified ApoB(10162) small interfering RNA (siRNA), siRNAs with an 2′-O-benzyl at either position 5, 8, 15, or 19 were formulated into lipid nanoparticles and injected in mice at 3 mg/kg. The livers were harvested and *ApoB* mRNA levels were evaluated by quantitative PCR (qPCR) 72 hours after injection (* = student's t-test, *P* < 0.05). Each dot represents an individual mouse and bars represent the mean and standard deviation of all mice per group. (**b**) Unmodified ApoB(10162) siRNA, siRNAs with an 2′-O-benzyl at either position 5, 8, 15, or 19 were formulated into lipid nanoparticles and injected in mice at 3 mg/kg. The livers were harvested and apolipoprotein B (*ApoB*) mRNA levels were evaluated by qPCR at 3, 7, and 14 days after injection. Data are presented as the mean and s.e.m. *P* < 0.05 versus unmodified; **P* < 0.005 versus unmodified.

Luc(80) siRNAs had similar maximal mRNA degradation at day 1 *in vivo*. However, by day 3 mRNA degradation from Luc(80) unmodified siRNA was 23% whereas siRNAs containing 2′-O-benzyls had a statistically significant threefold increase of 58% mRNA degradation. The duration of activity for Luc(80) siRNAs containing 2′-O-benzyl at positions 8 and 15 slowly decreased over 21 days to 0 while the unmodified siRNA activity was attenuated by day 11. Area under the curve was calculated to determine the difference in duration between Luc(80) siRNAs. The area under the curve was statistically significantly increased for the Luc(80)siRNAs containing 2′-O-benzyls at positions 8 and 15 compared to unmodified siRNA (**Figure 3d**). These data demonstrate two
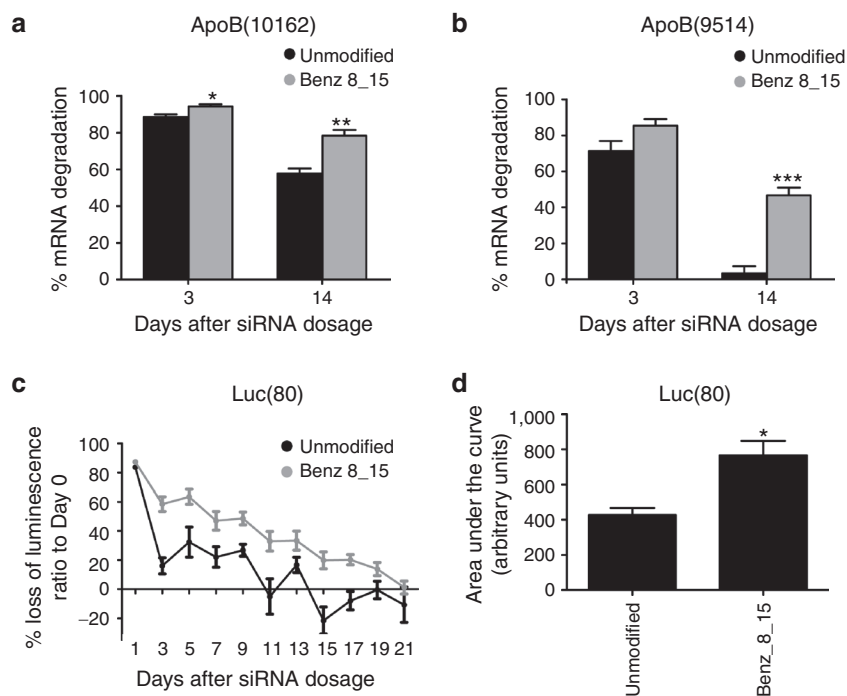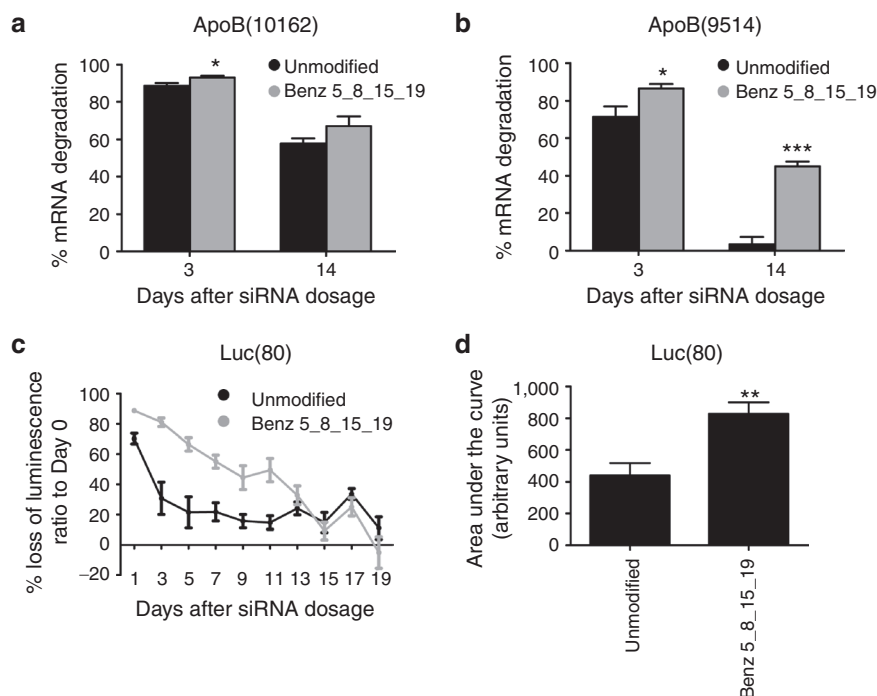
Figure 3   *In vivo* evaluation of 2′-O-benzyls at positions 8 and 15 on the small interfering RNA (siRNA) guide strand. (**a**) Unmodified ApoB(10162), (**b**) ApoB(9514), and (**c**) Luc(80) siRNAs and the same siRNAs with 2′-O-benzyls at positions 8 and 15 were formulated into lipid nanoparticles and injected in mice at 3 mg/kg. The livers were harvested for Apo(9514) and ApoB(10162) mice and mRNA levels were evaluated by quantitative PCR (qPCR) at 3 and 14 days after injection. Luciferase mice were imaged every other day after dosing. Area under the curve was calculated (**d**) for Luc(80) siRNAs as a measure of duration. Data are presented as the mean and s.e.m. *$P < 0.05$ versus unmodified; **$P < 0.005$ versus unmodified; ***$P < 0.0005$ versus unmodified.

2′-O-benzyls can be incorporated into multiple siRNA guide strands at positions 8 and 15 with beneficial effects in duration of activity. It also indicates further optimization and SAR at positions 8 and 15 may be possible to bring about even larger increases in duration and activity *in vivo*.

2′-O-benzyls combined at positions 5, 8, 15, and 19 in the guide strand increase activity *in vivo*. We next wanted to test whether the number of 2′-O-benzyls in an siRNA would have a beneficial effect on *in vivo* activity. Since two 2′-O-benzyl modifications increased *in vivo* activity, if an siRNA contained all the positions where 2′-O-benzyl was tolerated, positions 5, 8, 15, and 19, there potentially could be an even greater increase *in vivo* activity. These four positions individually had activity comparable or better *in vitro* (**Figure 1b**) and *in vivo* (**Figure 2**) than the unmodified siRNA. Four incorporations of 2′-O-benyzls were tested *in vivo* on the guide strand at positions 5, 8, 15, and 19 (**Figure 4**). Three different siRNA sequences were again tested: ApoB(9514), ApoB(10162), and Luc(80). Four incorporations of 2′-O-benzyl in the guide strand statistically significantly increased maximum mRNA degradation for ApoB(10162) siRNA at day 3 *in vivo* (**Figure 4a**), from 89 to 93%, respectively. The duration of activity, as measured at day 14, for the 2′-O-benzyl modified ApoB(10162) siRNA was comparable to the unmodified siRNA, in contrast to the statistically significant increase observed when there were only two incorporations of 2′-O-benzyl (**Figures 3a** and **4a**). mRNA degradation for ApoB(9514) siRNAs containing

2′-O-benzyls at positions 5, 8, 15, and 19 at day 3 was statistically significantly increased compared to unmodified siRNA from 71 to 86% (**Figure 4b**). Duration was statistically significantly increased at day 14 for ApoB(9514) when the siRNAs contained 2′-O-benzyls at positions 5, 8, 15, and 19 with 45% mRNA degradation compared to 3% from unmodified siRNA. At day 3, unmodified Luc(80) siRNAs had 31% mRNA degradation compared to 81% from the siRNA with 2′-O-benzyls at positions 5, 8, 15, and 19 at day 3 (**Figure 4c**). The area under the curve was statistically significantly larger for Luc(80) siRNAs containing 2′-O-benzyl at positions 5, 8, 15, and 19 compared to unmodified siRNAs, indicating four 2′-O-benzyl modifications can favorably alter the duration of an siRNA *in vivo* (**Figure 4d**). For multiple siRNA sequences, four incorporations of 2′-O-benzyl produced favorable, statistically significant increases in maximal mRNA degradation and duration of siRNAs. However, four incorporations of 2′-O-benzyl in the guide strand did not have better activity over only two incorporations at positions 8 and 15. This demonstrates that while four incorporations can be tolerated in the guide strand, using the correct modification at the correct position for maximal *in vivo* benefits is crucial in the siRNA optimization process.

2′-O-CH2Py(4) modifications enhance *in vivo* duration. We next tested whether small changes in SAR would have an effect on the siRNA guide strand. A 2′-O-CH2Py(4) modification, similar in size to 2′-O-benzyl, but with different structural and hydrogen-bonding properties, was used on the same set

Figure 4 *In vivo* evaluation of 2′-O-benzyls at positions 5, 8, 15, and 19 on the small interfering RNA (siRNA) guide strand. Unmodified (**a**) ApoB(10162), (**b**) ApoB(9514), and (**c**) Luc(80) siRNAs and the same siRNAs with 2′-O-benzyls at positions 8 and 15 were formulated into lipid nanoparticles and injected in mice at 3 mg/kg. The livers were harvested for Apo(9514) and ApoB(10162) mice and mRNA levels were evaluated by quantitative PCR (qPCR) at 3 and 14 days after injection. Luciferase mice were imaged every other day after dosing. Area under the curve was calculated (**d**) for Luc(80) siRNAs as a measure of duration. Data are presented as the mean and s.e.m. *$P < 0.05$ versus unmodified; **$P < 0.005$ versus unmodified; ***$P < 0.0005$ versus unmodified.

of siRNAs used throughout this study. *In vitro*, we observed positions 5, 6, 8, 10, 15, and 19 were equivalent or better than unmodified siRNAs for 2′-O-CH2Py(4) (**Figure 1c**). siRNAs were made with 2′-O-CH2Py(4) at positions 8 and 15, in order to directly compare to the 2′-O-benzyl results. siRNAs were also made with incorporations at positions 5, 6, 8, 10, 15, and 19 to understand the correlation between the number of modifications and *in vivo* activity as well as the maximal tolerance of the guide strand for modifications (**Figure 5**). ApoB(10162) siRNAs were created with and without 2′-O-CH2Py(4) and tested *in vivo* for mRNA degradation at days 3 and 14. When 2′-O-CH2Py(4) were placed at positions 8 and 15 there was a statistically significant increase in mRNA degradation at day 3 and 14 (**Figure 5a**). At day 3, the unmodified ApoB(10162) siRNAs had 90% mRNA degradation while the siRNAs with 2′-O-CH2Py(4) at positions 8 and 15 had 95%; and at day 14, the unmodified ApoB(10162) siRNAs had 68% mRNA degradation while the siRNAs with 2′-O-CH2Py(4) had 88%. ApoB(10162) siRNAs containing six incorporations of 2′-O-CH2Py(4) at positions 5, 6, 8, 10, 15, and 19 had comparable activity to unmodified siRNA at day 3 and 14 (**Figure 5b**). As with 2′-O-benzyl, this data shows the importance of proper placement of the modifications to have maximal activity gains. Interestingly, the statistically significant increases in silencing activity over unmodified siRNA came from siRNAs with two incorporations of 2′-O-CH2Py(4), not six. While six modifications were tolerated, they only had the same *in vivo* silencing activity as unmodified siRNAs. These data suggest that the 2′-O-CH2Py(4) is not the optimal

modification at all six positions and additional SAR at each position may be needed to confer optimal activity.

Discussion

Maximizing *in vivo* siRNA potency by chemical modification is therapeutically desirable in order to minimize the dose of delivered siRNA required for efficient RNAi, lower the cost of goods and increase the duration of the siRNA. We have identified and tested two novel 2′-O- modifications *in vivo* that were optimized specifically for use in siRNA. These modifications were tolerated in combination at multiple positions in the guide strand, with 2′-O-CH2Py(4) placed at six positions and 2′-O-benzyl at four positions. Increased *in vivo* duration was observed with these modifications over their unmodified counterparts.

The mechanism for the increased potency of these modifications remains to be elucidated. Since the lipid nanoparticle encapsulates the siRNAs and shields it from serum nucleases and provides high serum stability to the siRNA regardless of modification status, it is unlikely that improved serum stability is responsible for the improved *in vivo* activity. Also, if the modifications were to confer added serum stability resulting in increased potency we would predict that siRNAs with four incorporation of 2′-O-benzyls to have more activity than the siRNAs with only two incorporations. However, our data do not support this hypothesis. In fact, the opposite occurred. siRNAs with two chemical incorporations were superior to those siRNAs with four 2′-O-benzyls. Therefore, increased

a

ApoB(10162)

● Unmodified
● Py(4) 8_15

b

ApoB(10162)

● Unmodified
● Py(4) 5_6_8_10_15_19
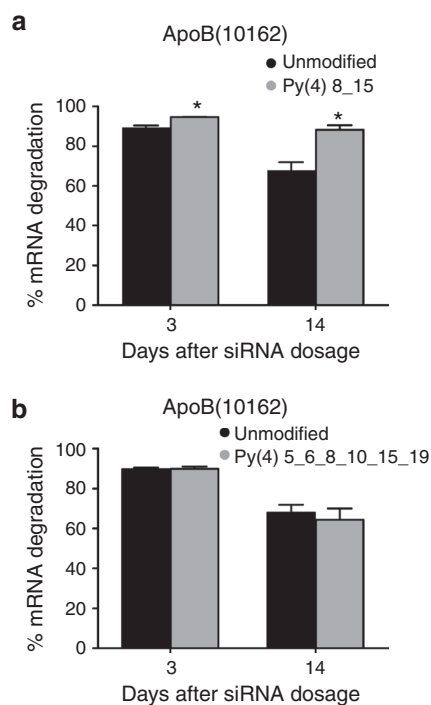
Figure 5 ***In vivo* duration of 2′-O-CH2Py(4) on the small interfering RNA (siRNA) guide strand**. Unmodified ApoB(10162) siRNA and the same siRNAs with 2′-O-CH2Py(4) at positions (**a**) 8 and 15 or (**b**) 5, 6, 8, 10, 15, and 19 on the guide strand were formulated into lipid nanoparticles and injected in mice at 3 mg/kg. The livers were harvested and mRNA levels were evaluated by quantitative PCR (qPCR) at 3 and 14 days after injection. Data are presented as the mean and s.e.m. *$P < 0.05$ versus unmodified.

serum stability, delivery efficiency, or uptake would probably not be a cause for the increased potency observed. Increased Ago2 binding is an additional possibility for increased potency. Analysis of the Ago crystal structure showed that positions 2–6 of the guide strand form a stacked helical conformation and has extensive surface and charge complementarity with the PIWI domain.[30,31] The bases at the 5′-end, from positions 2–8 also contribute to target mRNA-binding energy. The 2′-O-benzyl and 2′-O-CH2Py(4) modifications at positions 5 or 8 may therefore play a role in the enhancing the siRNA conformation and interactions with Ago2 and its target. To examine this further, we measured siRNA binding to Ago2 via stem-loop PCR.[32] However, these large 2′-modifications could not be interpreted by and were not compatible with the polymerases used in stem-loop PCR. No meaningful data was obtained. In previous work, we have also measured the Tm's of the 2′-O-benzyl modifications in the siRNA walkthrough and found no differences at any position between the modified and unmodified siRNAs.[29]

The chemical modified ribose rings have the potential to be further optimized. We have also demonstrated that siRNAs can tolerate 2′-O-benzyl and 2′-O-CH2Py(4) modifications that are larger than conventional modifications such as 2′-OMe and 2′-F. However, even between 2′-O-benzyl and 2′-O-CH2Py(4) there were positional preferences where 2′-O-CH2Py(4) was tolerated at positions 6 and 10 and benzyl was not. The small change of a carbon to nitrogen

provided additional benefits at positions 6 and 10 in an siRNA that benzyl was unable to provide. Yet, 2′-O-benzyl and 2′-O-CH2Py(4) modifications are limited and more SAR can be done at the specific positions they are tolerated to understand and harness the interactions with Ago2, such as hydrogen bonding and lipophilicity, to achieve maximal siRNA activity. 2′-O-benzyl and 2′-O-CH2Py(4) can potentially have their ortho-, meta-, and para-carbons further modified to understand the constraints of every position in an siRNA when it interacts with Ago2. There is the potential to alter the length and width of the modification using different ring sizes. Once the chemical space is identified, each position can have its own unique modification tailor-fit for maximum potency and the combinations of such modifications will allow the generation of superior siRNAs that can form the basis for potent and safe siRNA therapeutics in the near future.

One of the most critical issues in developing modified siRNAs is the placement of the modification in the duplex. The wrong modification at the wrong position can obliterate activity. We have shown a simple walkthrough of a modification successfully predicts the positions to use in combinations. These combinations improved siRNA performance *in vivo*. In addition, more of a modification does not equal better activity. Four 2′-O-benzyl modifications on an siRNA had increased siRNA activity *in vivo* but were not better than only two 2′-O-benzyls at positions 8 and 15. A systematic study is required covering as much chemical space as possible to understand the role of modifications at each position of the siRNA guide strand and its interactions in the RISC complex. The strategic placement of modifications at each position will be critical for optimal siRNA design.

The universally applicability of a modification pattern has not emerged, mostly due in part to 2′-OMe and 2′-F maintaining activity at many positions on the guide strand which allows for many different combinations to be created and have activity. In this study, multiple sequences were used and the same positions in all of them, both individually and in combination, had similar activity *in vitro* and *in vivo*. The findings from this set of siRNA sequences used *in vitro* were also able to be applied to the Luc(80) sequence that was not included originally in the walkthrough data. The Luc(80) siRNA had similar results to the ApoB sequences also tested *in vivo*, indicating that using a small test set of sequences can potentially identify the specific positions a modification should be placed in any siRNA of interest. As more specific modifications are created, a pattern that is applicable to any siRNA for maximal potency, stability and specificity will emerge.

siRNA design will require modifications to balance between delivery, potency, stability, and pharmacokinetics properties. Clear mechanistic information is needed about how best to select and modify siRNAs for a given target and application. Expansion of novel modifications available for the guide strand will be useful for future therapeutics.

Materials and methods

2′-O-benzyl and 2′-O-CH2Py(4) synthesis. The 2′-O-benzyl and 2′-O-CH2Py(4) modified phosphoramidites were synthesized using procedures analogous to those described.[29] All

nonhydrolytic reactions, unless indicated otherwise were carried out in dry solvents purchased from Aldrich (St. Louis, MO). High-performance liquid chromatography analyses, except for the amidites, were performed at 60°C using an Agilent Zorbax Eclipse Plus C18, 2.1 × 50mm, 1.8 micron column, at 0.8ml/minute flow rate, eluted with a gradient (5–95%) of acetonitrile and water with formic acid (0.1%) as a modifier. The amidites were analyzed using a Supelco Ascentis C18, 100 × 4.6mm, 2.7 micron column and ammonium formate (3 mmol/l) as a modifier, under otherwise identical conditions. UV traces were recorded at 220 nm and mass spectra were obtained using an Agilent Technologies 6140 Quadrupole LC/MS mass spectrometer in both positive and negative ion mode. NMR spectra were recorded on a Varian Unity 600, 500, or 400 spectrometers.

Oligo synthesis and sequences. Chemically modified siRNAs contained either ribo (r), deoxy (d), inverted abasics (iB) or O-methyl (ome) 2′ modifications as described previously.[33] The siRNA sequences and modification are as follows from 5′-3′:

Luciferase(80) unmodified siRNA:
Passenger: iB;rA;rU;rA;rA;rG;rG;rC;rU;rA;rU;rG;rA;rA;rG; rA;rG;rA;rU;rA;dT;dT;iB
Guide: rU;rA;rU;rC;rU;rC;rU;rU;rC;rA;rU;rA;rG;rC;rC;rU;rU; rA;rU;dT;dT

ApoB(9514) unmodified siRNA:
Passenger: iB;rC;rU;rU;rU;rA;rA;rC;rA;rA;rU;rU;rC;rC;rU; rG;rA;rA;rA;rU;omeU;omeU;iB
Guide: rA;rU;rU;rU;rC;rA;rG;rG;rA;rA;rU;rU;rG;rU;rU;rA;rA; rA;rG;omeU;omeU

ApoB(10162) unmodified siRNA:
Passenger: iB;rC;rA;rA;rG;rU;rG;rU;rC;rA;rU;rC;rA;rC;rA; rC;rU;rG;rA;rA;omeU;omeU;iB
Guide: rU;rU;rC;rA;rG;rU;rG;rU;rG;rA;rU;rG;rA;rC;rA;rC;rU; rU;rG;omeU;omeU

PHD2(196) unmodified siRNA:
Passenger: iB;rC;rA;rU;rU;rG;rA;rA;rC;rC;rC;rA;rA;rA;rU;rU; rU;rG;rA;rU;omeU;omeU;iB
Guide: rA;rU;rC;rA;rA;rA;rU;rU;rU;rG;rG;rG;rU;rU;rC;rA;rA; rU;rG;omeU;omeU

PHD2(384) unmodified siRNA:
Passenger: iB;rC;rA;rG;rU;rC;rA;rG;rC;rA;rA;rG;rA;rC; rG;rU;rC;rU;rA;omeU;omeU;iB
Guide: rU;rA;rG;rA;rC;rG;rU;rC;rU;rU;rU;rG;rC;rU;rG;rA;rC; rU;rG;omeU;omeU

Transfection and qPCR. Transfection and qPCR for *in vitro* and *in vivo* studies were done as described previously.[34] Hepa1-6 cells were cultured in Dubelco 's modified Eagle's medium (Mediatech Cellgro, Manassas, VA) containing 10% serum. Cells were plated in a 96-well plate (3,500 cells/well) and were transfected 24 hours after plating in Opti-MEM I Reduced Serum Media (Gibco, Grand Island, NY) and Lipofectamine RNAiMax reagent (Invitrogen, Grand Island, NY) for a final concentration of 10 nmol/l siRNA for the initial screening. For IC$_{50}$ curves the final concentration ranged

from 0.15 pmol/l to 160 nmol/l along a 12-point titration curve. Approximately 24 hours after transfection, cells were washed with phosphate-buffered saline and lysed in Cells-to-CT Lysis Buffer (Ambion, Grand Island, NY) with rDNase I (RNase-free) added. Stop solution (Ambion) was used to halt the reaction. Reverse transcription-PCR was performed using 7 μl of cell lysate in 2× RT buffer with 20× RT Enzyme Mix (Ambion) added. Conditions were as follows: 37°C for 60 minutes and 95°C for 5 minutes. mRNA levels were detected by qPCR with target-specific probes from Applied Biosystems. All complementary DNA samples were added to a 10-μl reaction volume with the following cycling conditions: 2 minutes at 50°C, 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. qPCR was assayed using an ABI Prism 7900 sequence detector using 2× Taqman Gene Expression Master Mix (Applied Biosystems). GAPDH mRNA levels were used for data normalization.

siRNA formulation. Lipid nanoparticles were made using the cationic lipid 2-{4-[(3b)-cholest-5-en-3-yloxy]-octyl}-*N*,*N*-dimethyl-3-[(9*Z*,12*Z*)-octadeca-9,12-dien-1-yloxy]propan-1-amine (Merck and Co., Whitehouse Station, NJ), cholesterol (Northern Lipids, Burnaby, British Columbia, Canada), and monomethoxypolyethyleneglycol-1,2-dimyristoylglycerol (NOF, Tokyo, Japan) at a 60:38:2 mol/l ratio, respectively.

In vivo. C57BL/6 male mice 20–23g were purchased from Taconic Farms. Mice were injected intravenously with 200μl containing 3mg/kg siRNA formulated in a lipid nanoparticle. Four mice per group were sacrificed at indicated time points following siRNA injection. Livers were harvested and processed to assess target mRNA levels by qPCR as described above. To determine statistical significance a Student's *t*-test was used to obtain *P* values. A *P* value was considered "statistically significant" if it was <0.05 and was denoted on the bar graphs: *P < 0.05; **P < 0.005; ***P < 0.0005. The use of the words "comparable" or "similar" in the text refers to the lack of statistical significance.

Luciferase mouse model. The luciferase mouse model and imaging measurements and analysis were done as described previously.[32]

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Supplementary Information accompanies this paper on the Molecular Therapy–Nucleic Acids website (http://www.nature.com/mtna)