

Engineering miRNA features into siRNAs: Guide-strand bulges are compatible with gene repression

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Synthetic siRNA guide strands are typically designed with perfect complementarity to the passenger strand and the target mRNA. We examined whether siRNAs with intentional guide-strand bulges are functional *in vitro* and *in vivo*. Importantly, this was done by systematic shortening of the passenger strand, evaluating identical 19-mer guide-strand sequences but forcing them into conformations with 1- to 4-nt bulges after annealing. We demonstrate that guide-strand bulges can be well tolerated at several positions of unmodified and modified siRNAs. Beyond that, we show that GalNAc-conjugated siRNAs with bulges at certain positions of the guide strand repress transthyretin in murine primary hepatocytes and *in vivo* in mice. *In vivo*, a GalNAc-conjugated siRNA with a 1-nt bulge at position 14 of the guide strand was as active as the perfectly complementary siRNA. Finally, in a luciferase reporter system, mRNA target sequences were systematically shortened so that RNA-induced silencing complex activity could only occur with a guide-strand bulge. Here, luciferase reporters were repressed when 1- and 2-nt deletions of the reporter were applied to the edges of the sequence. We conclude that some guide-strand bulges versus target transcript can result in target repression and therefore should be evaluated as off-target risks.

INTRODUCTION

Mammalian microRNAs (miRNAs) and short interfering RNAs (siRNAs) mediate gene repression via Argonaute (Ago) proteins. The two small RNA species differ in their intrinsic properties and modes of action. Typically, siRNAs consist of two perfectly complementary strands, termed the “guide” and “passenger” strands.¹ When the siRNA duplex is loaded to Ago proteins, the passenger strand is removed while the guide strand remains associated with the Ago protein via its phosphate-sugar backbone; ultimately, it is the guide strand that binds to its mRNA targets with perfect or near-perfect base complementarity.² Among the four human Argonaute proteins, Ago2 is the only one with endonucleolytic activity.^{3,4} Ago2 exerts this activity as one of the protein components of the RNA-induced silencing complex (RISC). Extensive complementarity with the siRNA guide strand is essential for mRNA targets to be sliced in RISC.^{3–6} It is noteworthy that slicing activity is not restricted to mRNA targets but has also been observed for passenger strands, thereby contributing to RISC maturation.^{7–9}

In contrast to traditional siRNAs, mature miRNA duplexes are characterized not by complete sequence homology but by inclusion of various mismatches and bulges.¹⁰ miRNAs are functionally loaded to all human Argonaute proteins (Ago1–4).^{11,12} In non-slicing Ago proteins, removal of passenger strands and the inactive miRNA (designated miRNA*) strands cannot rely on endonucleolytic cleavage. It has been hypothesized that mismatched and bulged structures represent an alternative strategy to achieve thermodynamically destabilized structures and allow for successful miRNA strand separation in these proteins.^{7,10,13,14} Park and Shin demonstrated the existence of such a slicer-independent mechanism of duplex unwinding in mammals.¹⁵ Even siRNAs were shown to be processed this way, but the contribution of slicer-dependent and -independent unwinding at physiological temperatures is not clear. It has also been demonstrated that slicer-independent unwinding is less efficient for 2'-O-methyl (2'-OMe) ribose-modified siRNAs than for unmodified siRNA.¹⁵

The chemical structures of therapeutic siRNAs are often heavily modified. This is also the case for the hepatocyte-specific *N*-acetylgalactosamine (GalNAc) conjugated siRNAs, which are applied subcutaneously and need both internal and terminal stabilization to resist exo- and endonucleases during their route to the target site.¹⁶ However, the modifications applied for nucleic acid stabilization need to be compatible with the overall function of the siRNA. 2'-OMe and 2'-fluoro (2'-F) sugar modifications are commonly used in oligonucleotide therapeutics; both modifications, however, increase the hybridization temperature of double-stranded siRNAs.¹⁷ If slicer-independent unwinding is a major contributing factor during formation of mature RISC, the higher hybridization temperatures of chemically modified siRNAs could represent an unfavorable starting point for siRNA maturation relative to equivalent unmodified siRNAs (Figure 1). Additionally, if slicer-dependent unwinding is impaired—e.g., by a certain chemical modification at the passenger-strand cleavage site—requirements for functional slicer-independent unwinding would need to be fulfilled to

Received 7 October 2021; accepted 6 February 2022;
<https://doi.org/10.1016/j.omtn.2022.02.004>.

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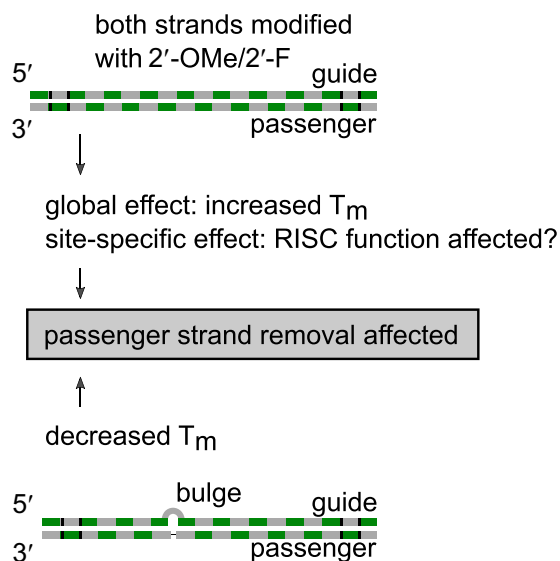


Figure 1. A possible model for bulges in modified siRNAs

Commonly used modifications increase the melting temperature, and site-specific modifications may interfere with RISC function. Bulges in the guide:passenger duplex may result in a decreased melting temperature and subsequently affect passenger-strand removal.

achieve efficient mRNA cleavage (Figure 1). Based on these observations, we hypothesized that if slicer-independent passenger strand removal contributes significantly to RISC maturation, the introduction of bulges into double-stranded siRNAs could contribute to increased overall siRNA knockdown activity.

The mammalian small RNA machinery cannot distinguish between siRNAs and miRNAs. As endogenous miRNAs may contain bulges, RISC must be able to tolerate these structures to a certain extent.¹⁸ This may occur when the duplex is loaded and unwound. We hypothesize that bulge-containing siRNAs, especially ribose-modified siRNAs, could benefit from thermal destabilization during RISC maturation, ultimately accelerating passenger-strand removal. RISC may also tolerate bulges in the “ternary complex”—consisting of Ago protein, Ago-associated small RNA, and bound target mRNA—with functional consequences for target binding and repression.

The activity of siRNAs with bulges on the guide strand has been previously investigated.¹⁹ Dua et al. designed siRNAs with bulges of the guide strand by the insertion of additional nucleotides into this strand.¹⁹ These siRNAs were described to reduce off-target effects.¹⁹ Importantly, insertion of additional nucleotides into the guide-strand sequence will not only affect the siRNA secondary structure, but is also likely to reduce on-target activity, as it will disrupt the alignment between guide strand and target mRNA at and after the initial bulge location once the passenger strand has been cleaved and removed. To address this and ensure that the only functional effect of such guide-strand bulges is the putative change in unwinding before engaging the target mRNA, we designed siRNAs where the guide-strand bulge is

formed by deletion of one or more nucleotides from the passenger strand rather than the insertion of nucleotides into the guide strand itself. This approach does not change the guide-strand sequence, which is essential when the activities of different bulge-containing variants are compared. We found that certain siRNAs with guide-strand bulges are functional and mediate gene repression both *in vitro* and *in vivo*. Tolerance for bulges depends on several factors, including (1) the length of the bulge, (2) the position of the bulge within the guide strand, and (3) the sequence-specific context. Despite this observed tolerance for guide-strand bulges, none of the tested variants showed improved activity compared with the same guide strand lacking bulges versus the passenger strand.

In ternary RISC, bulges of the target RNA have been previously identified, and in fact constitute a substantial proportion of miRNA targets in certain tissues.^{20,21} Beyond that, Becker et al. described guide:target duplexes where bulges were positioned in both target and guide strand, either by addition of nucleotides to the target or by deletion of nucleotides from the target sequence.²² They characterized different variants in a high-throughput binding and cleavage approach and found that most guide-strand bulges are compatible with target binding. However, only a subset is compatible with target cleavage.²²

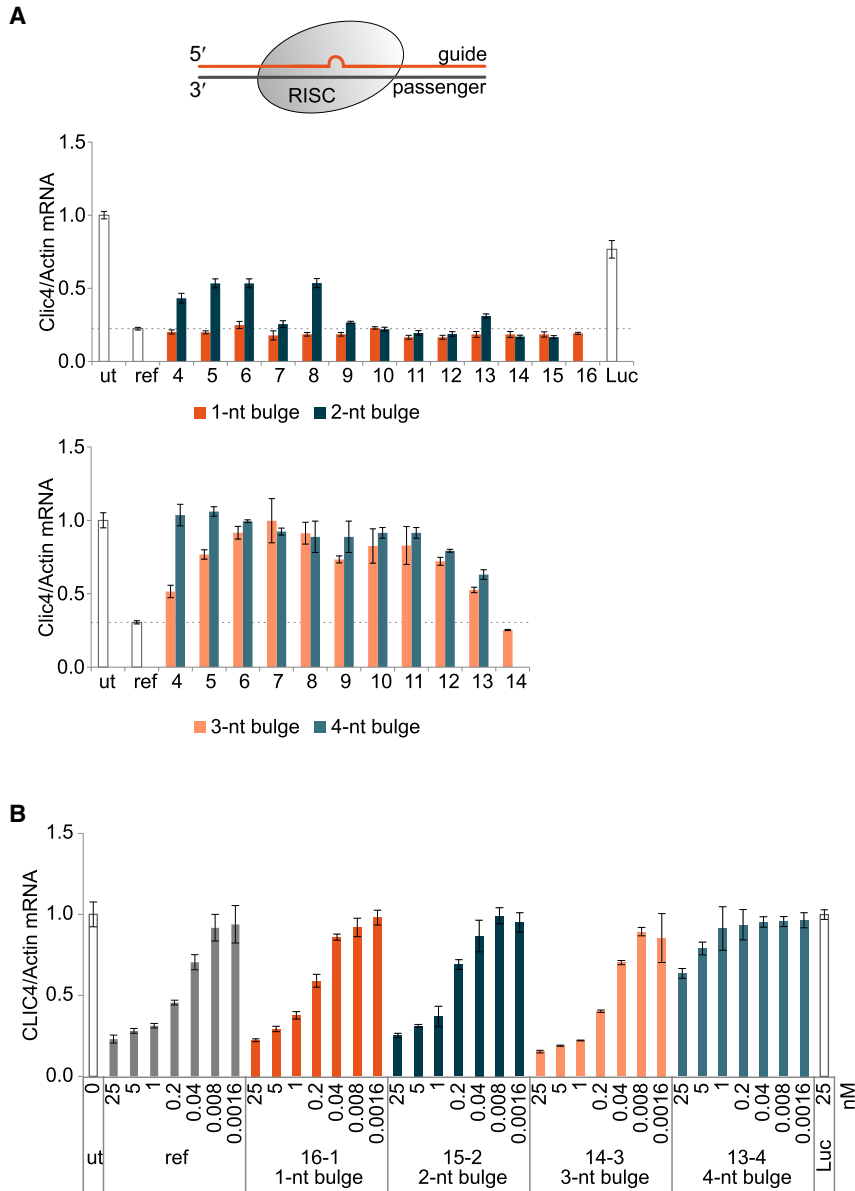
Here, we use ribose-modified siRNAs and a reporter system with degenerate target sequences that allow us to systematically screen bulge tolerance at different positions in ternary RISC. We show that a subset of bulges, specifically at the 5' and 3' termini of the guide strand, can indeed mediate off-target repression *in vitro*. We suggest that these observations should be considered during *in silico* siRNA design to evaluate and reduce the risk of off-target interactions; if such bulges are tolerated for on-target siRNA activity, they may also represent a risk for unintended off-target effects.

RESULTS

Short guide-strand bulges are well tolerated in unmodified siRNAs

First, we addressed whether bulges of the guide strand are tolerated in unmodified siRNAs. We used an siRNA targeting the mouse chloride intracellular channel protein 4 (Clic4) transcript to generate various bulge-containing variants. To this end, we used the full-length guide strand (19 nt in length) and annealed it to shortened passenger strands. Bulges of 1–4 nt in length were tested at different positions of the guide strand. All positions named hereafter refer to the guide strand in the 5' → 3' orientation. All tested siRNAs were blunt-ended 19-mers. To improve the chances of the formation of a bulge rather than a “floppy end,” all constructs included three hybridizing base pairs at the siRNA ends. Therefore, 1-nt bulges were introduced at guide-strand positions 4–16, while 4-nt bulges were introduced at guide-strand positions 4–13.

Using the perfectly complementary siRNA as a control, these siRNAs with different guide-strand bulges were transfected at 5 nM concentration, and target mRNA levels were quantified 2 days after transfection. Bulges of 1 nt were well tolerated at most of the positions tested (Figure 2A). More distinct position-dependent effects on siRNA activity



were observed for siRNAs with 2-nt bulges at different positions; more specifically, the activity of siRNAs with bulges around positions 4–8 was reduced while siRNAs with bulges at positions 9–15 remained active. siRNA activity was clearly reduced when longer bulges were tested. A 3-nt bulge at position 14 is the only 3-nt bulge variant where siRNA activity was comparable with that of a perfectly complementary siRNA duplex. Finally, bulges 4 nt in length induced a complete loss of activity at most positions, with some residual activity remaining for bulges placed at positions 12 and 13 (Figure 2A). Variants with 1- to 4-nt bulges at the 3' end of the guide strand were further characterized by transfection across an extended range of siRNA concentration (0.0016–25 nM). In all variants, the three terminal nucleotides at the 3' end could theoretically pair to complementary bases in the passenger

Figure 2. Length-dependent limitations for bulges of the guide strand

(A) Bulges of different lengths were introduced at different positions (numbered 4–16) of an siRNA targeting mouse *Clc4*. To avoid “floppy ends,” the terminal positions 1–3 and 17–19 were not modified. One to four nucleotides were deleted from the passenger strand, thus introducing a bulge into the guide strand. 5 nM siRNA was liposomally transfected into MS1 cells and RNA was extracted 2 days after transfection. Target mRNA levels were analyzed by TaqMan qRT-PCR. One-nucleotide bulges were well tolerated at most positions; 2-nt bulges were tolerated only at selected positions. siRNAs with bulges 3 and 4 nt in length had clearly reduced activity. (B) 1-nt (16-1), 2-nt (15-2), 3-nt (14-3), and 4-nt (13-4) bulges at the 3' end of the guide strand were further characterized in a broader siRNA concentration range of 0.0016–25 nM to determine dose responses. Normalized means and standard deviations of three technical replicates are shown. ut, untreated sample; Luc, non-targeting control siRNA; ref, perfectly complementary siRNA. Experiments were repeated twice.

strand, avoiding the formation of “floppy ends.” Variants with 1-, 2-, and 3-nt bulges were as active as the perfectly complementary duplex (“ref”), while the variant with a 4-nt bulge showed clearly reduced activity (Figure 2B), indicating that the effects observed in the single-dose screen were representative. Taken together, guide-strand bulges of 1- and 2-nt length were shown to be tolerated at certain positions in unmodified siRNAs and to facilitate target gene repression. The majority of guide-strand bulges of 3- and 4-nt length is not tolerated and does not facilitate target gene repression.

The functional tolerance of guide-strand bulges depends on position and siRNA sequence

After we had demonstrated that short bulges at several positions of the guide strand remain compatible with siRNA activity, we asked how the introduction of bulges might affect the activity of chemically modified siRNAs. Therefore, we tested the activity of two additional sets of siRNAs based on previously characterized positive controls targeting either human *ALDH2* (aldehyde dehydrogenase) or mouse *Ttr* (transthyretin). These positive control siRNAs were fully ribose modified with alternating 2'-OME and 2'-F on both strands with two phosphorothioate internucleotide linkages at the 5' and 3' termini, respectively (Figure 3A).²³ We introduced 1- and 2-nt bulges at different positions of these chemically modified siRNAs. It should be noted that after deletion of passenger-strand nucleotides, we did not adapt the modification pattern of the remaining nucleotides. Hence, the resulting passenger-strand patterns are not fully alternating for 1-nt bulge variants

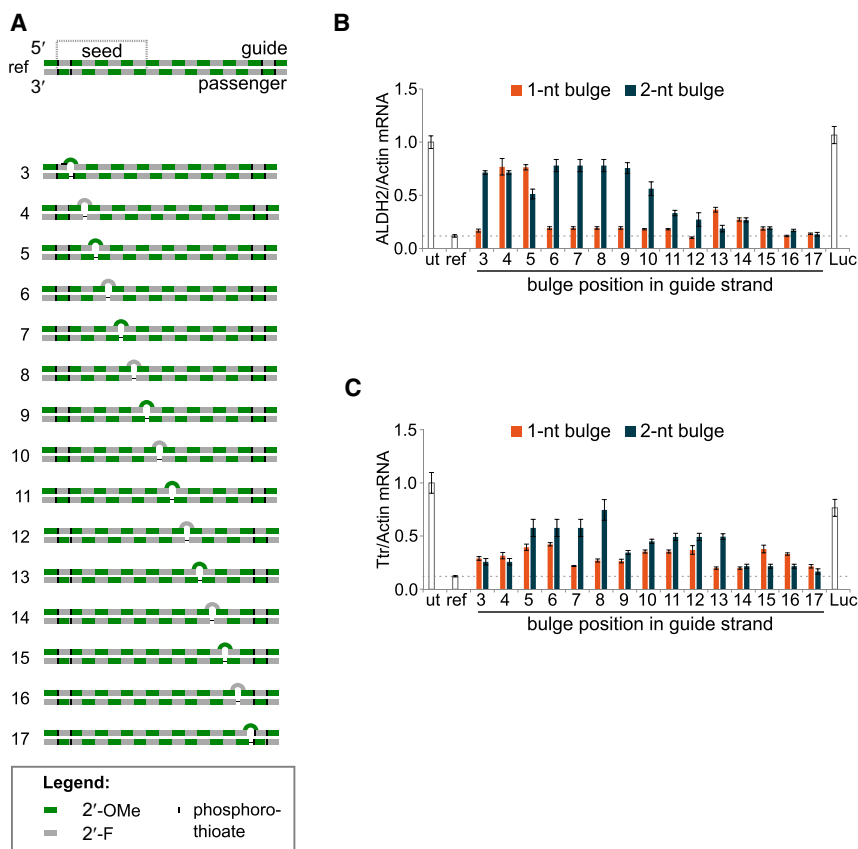


Figure 3. Guide-strand bulges are tolerated at certain positions in modified siRNAs

(A) Schematic representation of the tested variants. Gray, 2'-F; green, 2'-OMe; both termini were stabilized with two phosphorothioate internucleotide linkages each, indicated by black bars. (B and C) Bulges 1-nt and 2-nt in length were systematically tested at different positions (numbered 3–17) of the guide strand. Modified siRNAs targeting human ALDH2 (B) and mouse Ttr (C) were tested. In these sequences, bulges were tolerated well toward the 3' end of the guide strand and tended to reduce activity when present in the seed region. In addition, sequence-specific effects were observed. Experiments were conducted in Hep3B (B) and AML12 (C) cells. The cells were liposomally transfected with 0.1 nM (B) or 1 nM (C) siRNA. Target gene repression was analyzed by TaqMan qRT-PCR 2 days after transfection. Normalized means and standard deviations of three technical replicates are shown. ref, perfectly complementary siRNA; ut, untreated sample; Luc, non-targeting control siRNA.

(Figure 3A). The modified siRNAs were transfected into Hep3B and AML12 cells, and target mRNA levels were quantified 2 days after treatment.

In the siRNAs targeting ALDH2, 1-nt bulges were well tolerated at several positions, e.g., positions 3, 6–11, and the 3'-terminal positions. Activity was slightly reduced when 1-nt bulges were present at guide-strand positions 13 and 14. Interestingly, 1-nt bulges at guide-strand positions 4 and 5 clearly reduced activity (Figure 3B). With the same guide-strand sequence targeting ALDH2, bulges 2 nt in length were largely tolerated, with no or minimal loss of activity at guide-strand positions 11–17 (Figure 3B). A strong reduction of siRNA activity was observed when the bulges were present at positions 3–10 (Figure 3B).

We introduced 1- and 2-nt bulges into a third siRNA targeting mouse Ttr, again with alternating 2'-OMe and 2'-F modifications on both strands. Here, most siRNAs with a 1-nt bulge in the guide strand had reduced activity compared with that of the perfectly complementary siRNA duplex. Some activity was retained when guide-strand bulges were present at positions 7, 13, and 14. siRNA activity was considerably reduced when bulges were present at guide-strand positions 5, 6, 10–12, and 15 (Figure 3C). Introduction of 2-nt bulges reduced siRNA activity even further, with the strongest effects seen at positions 5–8 (Figure 3C). However, siRNAs with 2-nt guide-

strand bulges at their 5' and 3' termini still reduced target gene levels at the single concentration tested here.

Taken together, guide-strand bulges are tolerated in chemically modified siRNAs, with siRNA activity retained or only slightly reduced in several variants, especially when the bulge is only a single nucleotide in size. However, we did not observe improved siRNA activity of the introduced bulges relative to the perfectly complementary siRNA controls.

In summary, we have tested guide-strand bulges of 1- and 2-nt length in three different siRNA sequences, one of them unmodified (Figure 2A) and two fully modified with alternating 2'-OMe and 2'-F and terminal phosphorothioates (Figure 3). It should be noted that for the three tested sequences, bulges in the seed region resulted in less activity than bulges introduced toward the 3' end of the guide strand. The comparison of the three tested siRNA sequences shows that there are not only positional but also sequence-specific effects (Figures 2 and 3).

GalNAc-siRNA conjugates with bulges of the guide strand are active *in vitro*

Next, we addressed the activity of GalNAc-siRNA conjugates with guide-strand bulges to determine whether such bulges are compatible with siRNA activity when the molecule enters the cell via a receptor-mediated pathway. Based on the positional screening conducted in mouse Ttr (Figure 3C), we chose siRNAs with 1- and 2-nt guide-strand bulges at positions 7 and 14 for further characterization. siRNAs with 1-nt bulges at these positions had been as active as the perfectly complementary siRNA. The respective variants were modified with 2'-OMe, 2'-F, and terminal phosphorothioates, as described

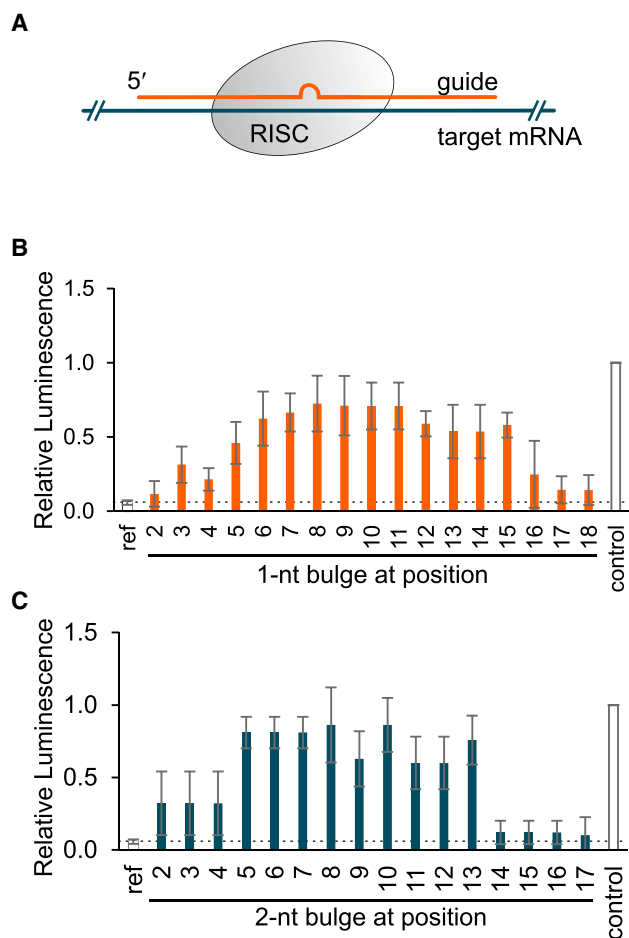


Figure 5. Guide-strand bulge formation and repression activity upon target mRNA binding

(A) Luciferase reporters were used to assess siRNA activity with degenerate target sequences. Therefore, reporter targets were designed so that they lacked one or two nucleotides at different positions. (B and C) Bulge reporters for 1-nt bulges (B) and 2-nt bulges (C) were co-transfected with 1 nM siRNA, and luciferase activity was assayed after 24 h and normalized to a control with non-complementary target sequence. Shown are means and standard deviations obtained from four biological replicates. Best repression of degenerate targets was achieved when the bulge was positioned in the 5'- or 3'-terminal regions of the guide strand.

present in the 1-nt bulge region (07-1 and 07-1 P1, Figure 4B). A 2-nt bulge at the same position reduced siRNA activity further (07-2, Figure 4B). This is in line with the activity of the respective non-conjugated siRNAs tested in transfection experiments (position 7, Figure 3C). Surprisingly, activity was reduced even more when phosphorothioates were present in the 2-nt bulge region (07-2 P2, Figure 4B), demonstrating that stabilizing the single-stranded bulge region with phosphorothioates was not contributing to siRNA activity, and further that endonuclease activity was not a major reason for the reduced activity for a guide-strand bulge in this construct. Perfectly complementary control conjugates with a comparable thiolation pattern showed activity loss only for the pattern that was applied for the 2-nt bulge (ref P1

and ref P2, Figure 4B). At the second position tested, position 14 of the guide strand, a 1-nt bulge reduced siRNA activity slightly (14-1, Figure 4C). Of note, an siRNA conjugate with a 2-nt bulge of the guide strand was as active as the perfectly complementary siRNA (14-2 and ref, Figure 4C). Activity was reduced when phosphorothioates were present in the 1-nt and 2-nt bulge regions (14-1 P3 and 14-2 P4, Figure 4C), and perfectly complementary control molecules with the same thiolation pattern showed slightly reduced activity (ref P3 and ref P4, Figure 4C). Additional stabilization of the bulge region by phosphorothioates did not seem to add benefits in the context of the *in vitro* experiments conducted here.

GalNAc-siRNA conjugates with guide-strand bulges can be active *in vivo*

As siRNAs with bulges at position 14 of the guide strand performed well *in vitro*, *in vivo* activity was assessed in C57BL/6 mice subcutaneously injected with equimolar amounts of different versions of a GalNAc-conjugated siRNA targeting Ttr. Target protein levels in serum were analyzed 10, 20, and 30 days after treatment. All tested conjugates showed clear time-dependent repression of the target gene. Specifically, a conjugate with a 1-nt bulge at position 14 was as active as the perfectly complementary siRNA (14-1 and ref, Figure 4D). Thiolation in the bulge region reduced activity in the bulge-containing and control siRNA (14-1 P3 and ref P3, Figure 4D). A 2-nt bulge at position 14 reduced siRNA activity compared with the perfectly complementary control molecule. Additional phosphorothioates in the bulge region did not further reduce activity, although the same phosphorylation pattern reduced activity of the perfectly complementary control compound (ref P4). Numerical values of this study, including means, standard deviations, and significance, are summarized in Table S2. Compared with the perfectly complementary control (“ref”), only the PBS-treated groups and the group treated with 14-1 P3 (day 10) are significantly different. Taken together, the activity of compound 14-1, a GalNAc-conjugated siRNA with a 1-nt bulge at position 14 of the guide strand, showed that potent target gene reduction could be achieved by bulge-containing siRNAs.

Can bulges of the guide strand occur during target binding?

We have shown that functional gene silencing takes place when bulges are present in double-stranded siRNA duplexes, i.e., between guide and passenger strand. Next, we asked whether bulges are tolerated in the duplex formed by the guide strand and the target mRNA. Formation of guide-strand bulges could be triggered when mature RISC, i.e., the Ago-loaded guide strand, hybridizes to target sites that lack one or more nucleotides at a certain position. Here, we call these sites “degenerate target sites.”

We aimed to systematically assess this possibility and at the same time compare it with our findings with bulges in double-stranded siRNAs. Therefore, we designed luciferase reporters with shortened Ttr target sites (Figure 5A). Target sites are identical to the Ttr passenger-strand sequences used before (Figures 3 and 4). We co-transfected different bulge reporters with the siRNA targeting mouse Ttr and compared them with a perfectly complementary reporter and with a control

reporter. Reporters were clearly repressed when 1-nt deletions were applied to the edges of the sequence (e.g., positions 4 and 17, Figure 5B). None of these reporter variants was as active as the reporter with the perfectly complementary sequence (“ref”). Reporter activity was reduced when nucleotides were deleted from the central region of the siRNA (e.g., position 8). A similar effect was observed when two consecutive nucleotides were deleted from different positions of the target sequence: reporter repression was maintained when deletions were applied in the 5'- and 3'-terminal regions of the siRNA, and repression was compromised when central nucleotides were deleted (Figure 5C). This is in contrast to the activity patterns observed in the guide:passenger duplexes, where at least 1-nt bulges were tolerated at several positions distributed throughout the whole length of the siRNA (Figure 3). Despite the strong overall loss of activity seen in many bulged guide:target duplexes, it must be noted that the target gene is repressed when guide-strand bulges are present at the 5'- and 3'-terminal regions of a guide:target duplex.

DISCUSSION

siRNAs with short guide-strand bulges can be active *in vitro* and *in vivo*

For certain prokaryotic Argonaute proteins, crystal structures with artificial bulges on either the guide strand or the target mRNA are available.^{24,25} However, structure determination has not always proved predictive for biochemical activity and emphasizes the importance of complementary functional assays.²⁴

Here we show that double-stranded siRNAs with bulges in the guide strand can repress target genes, in some cases with activities comparable with that of perfectly complementary siRNAs. In general, short guide-strand bulges 1–2 nt in length are more compatible with siRNA activity than bulges that are more than 2 nt in length. We have systematically tested all possible positions for guide-strand bulges in three different siRNA sequences and have found that bulges are less tolerated in the seed region than in the central and 3'-terminal regions. This finding is in line with previous reports showing that the seed region is sensitive to perturbations such as mismatches^{26–28} and bulky chemical modifications²⁹ across species. Beyond nucleobase sequence and nucleic acid modifications, structural features such as abasic bulges at different positions in the seed sequence are generally not well tolerated.²¹

In addition to unmodified siRNAs, we have also assessed bulge tolerance for chemically modified siRNAs. To our knowledge, this has not previously been assessed systematically. We find that ribose-modified siRNAs tolerate guide-strand bulges to a similar extent as unmodified siRNAs. Interestingly, the introduction of bulges did not result in improved activity for any of the tested variants. For the tested sequences and bulge variants, with or without ribose modifications of the strands, there were several examples of guide-strand bulge-containing siRNAs with on-target activity equal to that of perfectly complementary siRNAs with the same guide strand. However, we have not observed any examples of guide-strand bulge-containing siRNAs with increased activity compared with a perfectly comple-

mentary siRNA. It is still conceivable that bulges have a positive effect on strand separation and siRNA maturation, possibly at shorter time scales than the ones covered here.

Beyond this, we find that GalNAc-conjugated siRNAs with bulges of the guide strand can be active *in vivo*. We have even identified a variant that reduces target gene levels to a similar extent as the perfectly complementary siRNA with the same guide strand (14-2, Figure 4C).

It should be noted that terminally located bulges may form overhang structures or a mixture of bulge and overhang structures. This is likely to depend on the specific sequence composition and the chemical modifications used. However, we have provided evidence here that the conjugates we characterized are present as double-stranded siRNAs when injected *in vivo*.

Why are bulges tolerated at so many positions of the double-stranded siRNA? The eukaryotic silencing machinery does not distinguish between miRNAs and siRNAs. The different small RNA species rely on the same set of Argonaute proteins for loading and silencing. The tolerance we observe is likely a consequence of Argonaute molecular features needed for miRNA-mediated repression.

Assessing the off-target risk for target interactions with guide-strand bulges

We have shown here that in double-stranded siRNAs—unmodified, modified, and GalNAc-conjugated—guide-strand bulges at several positions may be tolerated, showing little to no loss of mRNA cleavage activity. We also find that such bulges can occur at the stage of guide:target pairing. Although we see that target-induced bulge formation is unlikely in central regions of the guide strand, we see reporter repression when short bulges are located at the 5' and 3' ends of the siRNA, more specifically at positions 2–4 and 16–18 of the guide strand. Apparently, RISC can skip nucleotides during target binding and accommodate them as either bulges or open structures.

The individual contributions of such degenerate targets compared with perfectly complementary targets still need to be addressed in competitive measurements. Overall, our observations reported here are in agreement with high-throughput binding and cleavage assays conducted with bulge-containing guide strands and a variety of degenerate target sequences.²² These analyses showed that target binding is impaired when bulges are positioned in the seed region and that target cleavage is impaired when bulges are positioned in central regions of the guide strand.²² Our luciferase reporter system is not restricted to siRNA-like mechanisms and may contain a contribution of miRNA-like repression mechanisms, especially by 3'-supplementary and 3'-compensatory target binding modes.³⁰ We cannot exclude the possibility that bulges do not form upon target binding but instead give way to a multitude of mismatches that eliminate activity. Finally, in the 5'- and 3'-terminal regions, overhang structures or a mixture of bulged and overhang structures may exist. Despite this, we show that target sequences with 5' and 3' degenerate sequences can be repressed.

As bulges occur in the guide strand, they can also occur in the target RNA. We had restricted ourselves to specifically characterize siRNAs with nucleotides deleted from the passenger strand. Notably, bulges of the bound target have been described before.^{20,21} Specifically, Chi et al. reported that in mouse brain miR-124 can bind to target sites that form so-called nucleation bulges, which are in that particular case sites with an additional G nucleotide positioned between positions 5 and 6 of the miRNA.²⁰ Lee et al. transferred that finding to the scope of seed-mediated off-target effects of siRNAs and suggest spacer modifications at the same position to circumvent binding to regular seed sites and bulged seed sites.²¹ In addition, there are several examples across species where bulge formation was observed in context of miRNA binding. Interestingly, human miRNAs miR-33 and miR-374 as well as *Caenorhabditis elegans* miRNAs let-7, miR-50, and miR-58 have in common that they bind to targets with a bulge opposite positions 4 and 5 of the small RNA.^{31,32}

In the context of *in silico* predictions, target sites are typically predicted by base complementarity (match versus mismatch).³³ Prediction parameters can be set to include degenerate targets when nucleotide omissions are allowed. Likewise, nucleotide additions can be considered and will represent bulge formation of the target mRNA. The general concept and the specific knowledge about guide and target positions that tolerate bulge structures during target repression can be applied to fine-tune off-target prediction of any siRNA.

Importantly, the positional tolerance we observed for guide:target bulges reflects a class of mechanism different from the positional tolerance of guide:passenger bulges. Bulges of the guide:passenger duplex provide information about steric acceptance during duplex loading and siRNA maturation. As soon as maturation is complete, no sign of the formerly bulge-containing siRNA will remain. On the other hand, bulges of the guide:target duplex are informative about steric acceptance during target binding and about enzymatic tolerance. The latter can here refer to siRNA- and miRNA-mediated silencing mechanisms; to this end, we have not tested the exact mechanism with which reporter repression is accomplished.

Summary

The study reported here demonstrates that guide-strand bulges can be well tolerated in unmodified and ribose-modified siRNAs. Thereby, siRNA activity depends on length and position of the bulge and is affected by the siRNA sequence. Certain modified siRNAs with bulges of the guide strand are compatible with GalNAc conjugation, receptor-mediated uptake, and target repression *in vivo*. We note that the bulged structures described herein do not show improved activity over perfectly complementary siRNA conjugates; i.e., if the structural flexibility impacted strand unwinding, it was not as apparent as improved knockdown activity compared with the perfectly complementary controls. We observed that the acceptance of bulges is lower in the seed region than in other regions of the siRNA. Importantly, we show here that the terminal positions of siRNA targets accept structural flexibility. Therefore, nucleotide omissions may need to be included for thorough off-target prediction during *in silico* siRNA design.

MATERIALS AND METHODS

siRNA sequences

Oligonucleotides for siRNAs were obtained as single strands from Kaneka Eurogentec (Seraing, Belgium).

rA, rU, rC, rG is unmodified RNA; mA, mU, mC, mG is 2'-OMe-modified RNA; fA, fU, fC, fG is 2'-F modified RNA; (ps) indicates phosphorothioate linkages.

The perfectly complementary 19-mer ("ref") sequences of the used siRNAs are as follows.

siRNA targeting mouse *Clic4*: antisense 5'-rArArGrArUrGrUrCrCrArUrUrCrCrArGrCrArG-3'; sense 5'-rCrUrGrCrUrGrGrArArUrGrGrArCrArUrCrUrU-3'.

siRNA targeting ALDH2 (aldehyde dehydrogenase): antisense 5'-mA (ps) fA (ps) mU fG mU fU mU fU mC fC mU fG mC fU mG fA mC (ps) fG (ps) mG-3'; sense 5'-fC (ps) mC (ps) fG mU fC mA fG mC fA mG fG mA fA mA fA mC fA (ps) mU (ps) fU-3'.

siRNA targeting mouse *Ttr* (transthyretin): antisense 5'-mU (ps) fU (ps) mA fU mA fG mA fG mC fA mA fG mA fA mC fA mC (ps) fU (ps) mG; sense 5'-fC (ps) mA (ps) fG mU fG mU fU mC fU mU fG mC fU mC fU mA fU (ps) mA (ps) fA-3'.

For each siRNA with a bulge, the 19-mer antisense (guide) strand and a shortened sense (passenger) strand were used. Therefore, 1–4 nucleotides were omitted at different positions of the sense strand. Bulged variants were named in the format xx-y, where "xx" indicates the bulge position according to 5'-3' numbering of the antisense strand and "y" indicates the length of the bulge. When modified RNA was used, the modification pattern of remaining nucleotides in the sense strand was not changed after omission of certain nucleotides. Both strands were hybridized in equimolar amounts.

Sequence and modification of GalNAc-conjugated siRNAs are listed in [Table S1](#).

Synthesis of GalNAc-conjugated siRNAs

Oligonucleotide GalNAc conjugates were synthesized using standard phosphoramidite chemistry according to an earlier published procedure.³⁴

For double-strand annealing, the respective single strands were reconstituted in water. To monitor the annealing process, a two-step annealing procedure was performed. First, the respective sense strand was provided in the calculated amount to afford the desired double-strand quantity. Native IP-RP-LCMS was used to afterward identify the single strand in the hybridization mixture. Next, the amount of the respective antisense strand needed to bind 75% of the provided sense strand was added and the mixture was heated to 80°C for 5 min. After cooling to room temperature, native IP-RP-LCMS was performed to identify the resulting double-stranded siRNA. A baseline separated peak shift to

higher retention time indicates the formation of the double-stranded product. The assigned single- and double-strand entity peak UV areas were used to calculate the needed amount of single strand for complete hybridization. After addition, heating, and cooling, the completion of the reaction (>90% UV area under assigned double-strand product peak) was demonstrated by native IP-RP-LC/MS. Chromatograms are collected in [Figure S3](#).

Native analytical IP-RP-LC/MS was performed using an XBridge BEH C18, 2.1 × 50 mm column, 2.5 μm particle size, 130 Å pore size (Waters, Eschborn, Germany), and applying a gradient from 5% B to 45% B over 15 min (A: 100 mM HFIP, 15 mM TEA in H₂O/MeOH [20:1, v/v]; B: 100 mM HFIP, 15 mM TEA, MeOH) at a flow rate of 0.3 mL/min at 25°C.

Oligonucleotides were quantified by their UV absorption at 260 nm in water using a NanoDrop Photometer (VWR, Darmstadt, Germany).

Melting curve analysis

Temperature-dependent absorption of GalNAc-conjugated siRNAs with and without guide-strand bulges was determined at a wavelength of λ = 260 nm (2 nm UV-visible bandwidth) on a Jasco (Pfungstadt, Germany) V-650 UV-visible spectrophotometer equipped with a Jasco PAC-743 temperature-controlled cell holder and a Julabo (Seelbach, Germany) F250 cooling system. A quartz cuvette (Hellma Analytics, Müllheim, Germany) with a path length of 1 cm was used. Samples were prepared in 1 mL of PBS buffer. Each sample was measured for three cycles of heating from 20°C to 95°C at a heating rate of 1 K/min and a sampling of 0.5 K. Melting curves were collected from the second and third cycles of each measurement. The measurement was repeated three times. All melting temperatures were determined from the first derivative of the absorbance temperature.

Cell culture

Cells were cultured in medium with 10% fetal bovine serum (FBS) and 2 mM glutamine at 37°C and 5% CO₂. Media and further additives were as follows. MS1: Dulbecco's modified Eagle's medium (DMEM), 20 mM HEPES; Hep3B: Eagle's minimal essential medium; AML12: DMEM/Ham's F12 (1:1), 1% insulin-transferrin-selenium-ethanolamine (Thermo Fisher Scientific, Waltham, MA), 40 ng/mL dexamethasone.

Primary murine hepatocytes (Life Technologies, Carlsbad, CA) were thawed and cryopreservation medium was exchanged for Williams' E medium supplemented with 5% FBS, 1 μM dexamethasone, 2 mM GlutaMAX, 1% penicillin-streptomycin, 4 μg/mL human recombinant insulin, and 15 mM HEPES (pH 7.4). For treatment with GalNAc-siRNA conjugates, 20,000–25,000 cells were seeded per well in collagen I-coated 96-well plates (Thermo Fisher Scientific) in a final volume of 100 μL.

Transfection and receptor-mediated uptake

Transfections were carried out in 6-well format using Atufect (Silence Therapeutics, Berlin, Germany) liposomal transfection reagent (1 μg/

mL) as described previously.³⁵ Samples were lysed 2 days after treatment, and total RNA was extracted with an InviTrap Spin Cell RNA Mini Kit (Invitex Molecular, Berlin, Germany) according to the manufacturer's protocol.

For receptor-mediated uptake in mouse primary hepatocytes, siRNA conjugates were prediluted in 100 μL of medium, and 25 μL of this prediluted siRNA or medium only was added to the cells. Samples were lysed, and total RNA was extracted 24 h after treatment by an InviTrap RNA Cell HTS 96 Kit (Invitex Molecular).

Quantitative real-time PCR (TaqMan)

In the case of 96-well extractions, 10 μL of isolated RNA was used. In the case of RNA extraction from 6-well plates, RNA concentration was determined and 100 ng RNA was used per well. The indicated amount or volume of total RNA was mixed with 10 μL of PCR Takyon Low Rox Probe MasterMix dTTP (Eurogentec), containing 300 nM primers (in the case of actin 100 nM), 100 nM probe, 5 U of MMLV reverse transcriptase, and 5 U of RNase inhibitor. TaqMan analysis was performed in 96-well format. Reverse transcription was carried out for 10 min at 48°C. Thereafter, 3 min of initial denaturation at 95°C and 40 cycles of 95°C for 10 s and 60°C for 1 min were run. Depicted bars represent normalized means and standard deviations from three technical replicates. The following primers and probes were used:

mACTB (5'-CCT AAG GCC AAC CGT GAA AAG-3', 5'-AGG CAT ACA GGG ACA GCA CAG-3', probe 5'-YY-TGA GAC CTT CAA CAC CCC AGC CAT GTA C-BHQ1-3')

hACTB (5'-GCA TGG GTC AGA AGG ATT CCT AT-3', 5'-TGT AGA AGG TGT GGT GCC AGA TT-3', probe 5'-YY-TCG AGC ACG GCA TCG TCA CCA A-BHQ1-3')

mCLIC4 (5'-ACA GCG AAG TCA AGA CGG ATG-3', 5'-GAC TCT GGG TGC TTT GGT GAA-3', probe 5'-FAM-TCG AAG AAG TCT TGT GCC CAC CCA AGT A-BHQ1-3')

mTtr (5'-TGG ACA CCA AAT CGT ACT GGA A-3', 5'-CAG AGT CGT TGG CTG TGA AAA C-3', probe 5'-FAM-ACT TGG CAT TTC CCC GTT CCA TGA ATT-BHQ1-3')

hALDH2 (5'-GGC AAG CCC TAT GTC ATC TCC T-3', 5'-GGA TGG TTT TCC CGT GGT ACT T-3', probe 5'-FAM-TGG TCC TCA AAT GTC TCC GGT ATT ATG CC-BHQ1-3')

PTEN (5'-CAC CGC CAA ATT TAA CTG CAG A-3', 5'-AAG GGT TTG ATA AGT TCT AGC TGT-3', probe 5'-FAM-TGC ACA GTA TCC TTT TGA AGA CCA TAA CCC A-BHQ1-3')

FAM, 6-carboxyfluorescein; BHQ1, Black Hole Quencher 1; YY, Yakima Yellow.

Luciferase assay

Luciferase reporter assays were performed with the Ttr target sequence (5'-CAG TGT TCT TGC TCT ATA A-3'). The sequence was cloned into psiCHECK-2 (Promega, Madison, WI) via SgfI/

PmeI. The reverse target sequence (5'-AAT ATC TCG TTC TTG TGA C-3') was used to create a control reporter. Bulge reporter constructs were cloned via site-directed mutagenesis. Reporter constructs were obtained from GenScript (Piscataway, NJ). MCF-7 cells were seeded into 96-well plates 24 h before transfection at a density of 6,000–8,000 cells per well. Per well, 10 ng of reporter and 0.1–1 nM siRNA were co-transfected using 1 µg/mL Atufect liposomal transfection reagent.³⁵ Dual-Glo luciferase reagent (Promega) was added to the samples 24 h after transfection, and Firefly and Renilla luminescence were determined according to the manufacturer's protocol. Luminescence values were background-subtracted. For each well, Renilla luminescence was normalized to Firefly luminescence. Mean values and standard deviations were calculated from three technical replicates and normalized to samples that were treated with a luciferase reporter lacking the target site. The assay was carried out four times. The data shown in Figure 5 represent mean values and standard deviations from four biological replicates.

In vivo experiments

In vivo experiments were performed at Experimental Pharmacology & Oncology Berlin-Buch (Germany) in accordance with the German Animal Protection Law and approved by the local responsible authorities.

A single dose of vehicle or test items was subcutaneously administered in the scapular region of male C57BL/6 mice aged 8 weeks. The test items and the vehicle were administered at 10 mL/kg. One week before treatment and at all indicated points in time, blood samples were collected from the retro-orbital sinus of each animal under isoflurane anesthesia. Serum was separated from the cells after clot formation by centrifugation. The blood samples were centrifuged at 4,000×g for 10 min at room temperature. The separated serum was frozen immediately and stored at –20°C until analysis.

Serum Ttr levels in mice were analyzed with a mouse Prealbumin ELISA kit (Alpco 41-PALMS-E01) according the manufacturer's protocol (ALPCO, Salem, MA). For each animal, measured Ttr levels were normalized to predose values. Group means were normalized to the mean of the PBS-treated group at the same time point.

Statistical analysis

Statistical analysis was performed using Kruskal-Wallis with Dunn's multiple comparisons test (GraphPad Prism 9.3.0.463; GraphPad, San Diego, CA) comparing each group with the data for the perfectly complementary control molecule ("ref") at each time point.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.omtn.2022.02.004>.

ACKNOWLEDGMENTS

We thank Dmitry Samarsky for scientific discussions, Gabi Anlauf and Jens Endruschat for siRNA conjugate synthesis, Pablo Lores

Lareo for siRNA analytics, and Eliot Morrison for critically reading the manuscript.

AUTHOR CONTRIBUTIONS

J.H.: conceptualization, supervision, writing – original draft. V.H.: investigation, validation. M.B.: investigation, validation. L.B.: resources, supervision. M.W.L.: conceptualization, writing – review & editing.

DECLARATION OF INTERESTS

All authors are employees of and have stock options in Silence Therapeutics GmbH.

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