Tolerance for bulges in mature miRNAs, siRNA duplexes and target-bound guide strands

Judith Hauptmann, Sibylle Dames, Marie Wikström Lindholm
Silence Therapeutics GmbH, Robert-Rössle-Str. 10, 13125 Berlin, Germany
contact: j.hauptmann@silence-therapeutics.com

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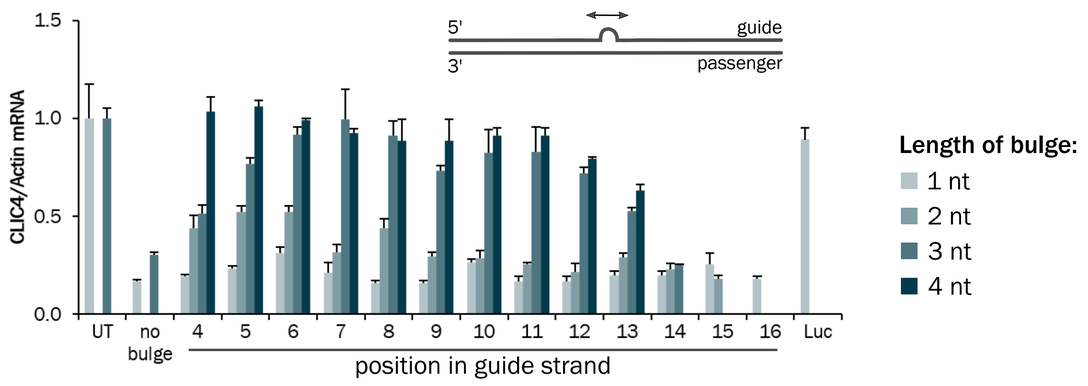
Abstract

Small non-coding RNAs are key players in gene regulation. Whereas miRNAs act as endogenous regulators of gene expression, siRNAs are well established as molecular biology tools and emerging as therapeutic agents. The siRNA guide strand is typically designed to form perfectly complementary duplexes both with the passenger strand and the target mRNA transcript. In contrast, nature designs miRNAs to contain elements that interrupt the perfect duplex, for example mismatches or bulges. Here, we tested if bulges of the guide strand are accepted in siRNA duplexes. Bulges of different lengths were introduced into siRNA duplexes by deleting one or more nucleotides from the passenger strand. Bulge tolerance at different positions was examined by systematically walking the bulge through the siRNA duplex. *In vitro* data were retrieved with unmodified and modified siRNAs of different nucleobase sequences. We also evaluated *in vivo* activity of selected, bulge-containing GalNAc-siRNA conjugates. Taken together, we find restrictions on bulge length and position in functional siRNAs and compare our insights to observations in the miRNA field. Finally, we asked if guide strand bulges may also form upon target RNA binding and evaluated this as a possible off-target risk.

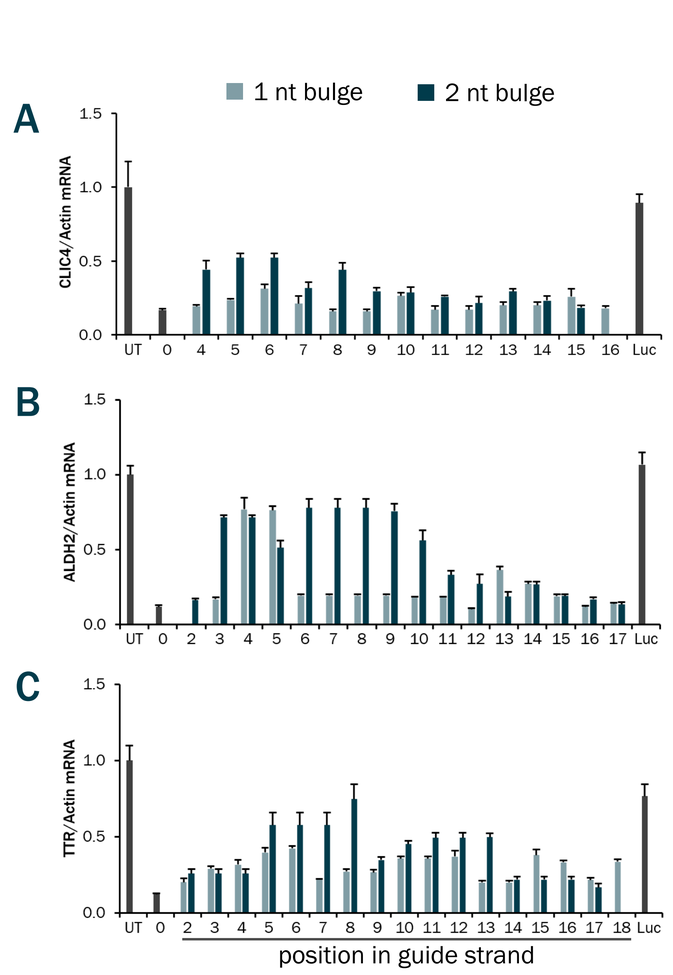
1 A tool siRNA tolerates guide strand bulges of 1-2 nt length



Bulges of different lengths were introduced at different positions of an siRNA targeting mouse CLIC4. Therefore, 1-4 nucleotides were deleted from the passenger strand, thus introducing a bulge into the guide strand. Importantly, the guide strand is a 19-mer of identical sequence in all variants.
1 nt bulges were well tolerated at most positions. 2 nt bulges were less tolerated in the seed region, but good activity was retained in variants with 2 nt bulges at positions towards the 3' end. siRNAs with bulges of 3 and 4 nucleotides length had clearly reduced activity.

Experimental conditions: MS1 cells, liposomal transfection of 5 nM siRNA, RNA extraction 2 days after transfection, analysis of target mRNA levels by Taqman qRT-PCR. Bars represent means and standard deviation from three technical replicates. UT, untreated sample; Luc, non-targeting control siRNA.

2 Sequence- and position-dependent limitations for 1-2 nt bulges of the guide strand



Bulges of 1 nt and 2 nt length were "walked" through all possible positions of the guide strand. Therefore, three different siRNA sequences targeting different genes were used: an unmodified siRNA targeting CLIC4 (A, also shown in Figure 1), a modified siRNA targeting ALDH2 (B) and a modified siRNA targeting TTR (C).
In all sequences, bulges were tolerated well towards the 3' end of the guide strand and tended to reduce activity when present in the seed region. In addition to that, sequence-specific activity patterns were observed.

Experiments were conducted in MS1, Hep3B and AML12 cells, respectively. The cells were transfected with 1 µg/ml liposomal transfection reagent and 5 nM (A), 0.1 nM (B) or 1 nM (C) siRNA. Target gene repression was analysed by Taqman qRT-PCR two days after transfection. Bars represent means and standard deviations from three technical replicates. 0, siRNA without bulge; UT, untreated sample; Luc, non-targeting control siRNA.

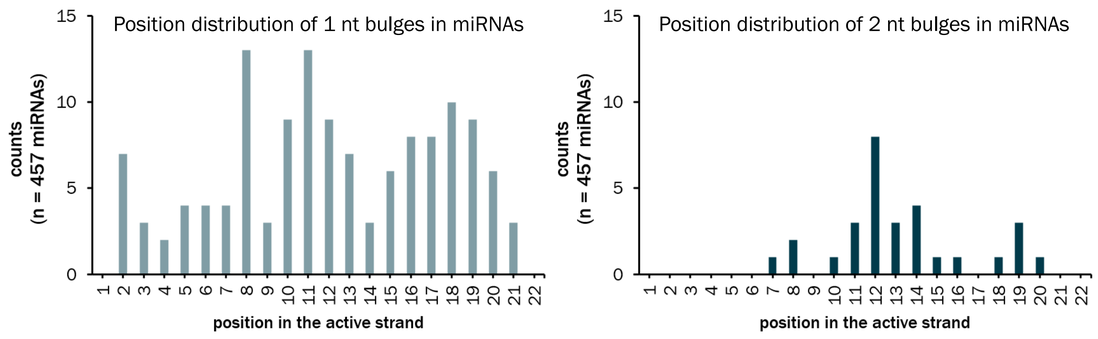
3 Descriptive analysis of bulges in human miRNAs

457 miRNAs analysed

56% contain bulges

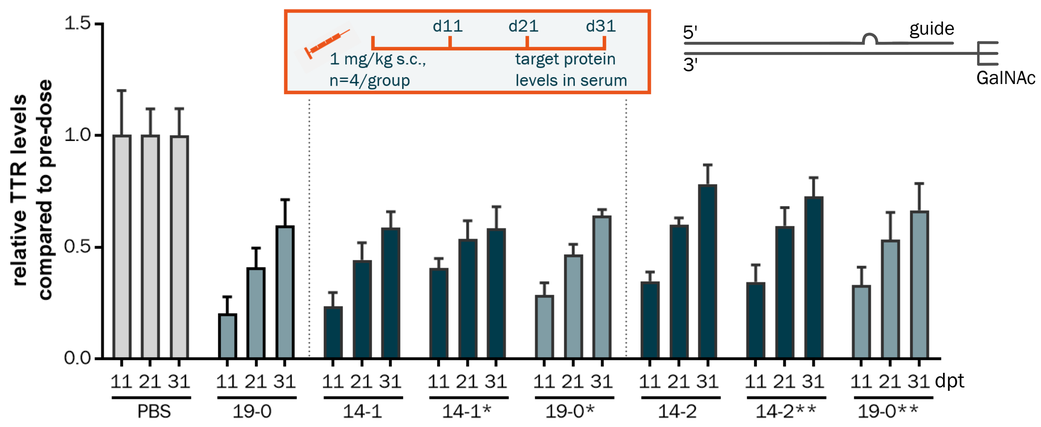
31% contain bulges of the predominantly loaded strand

number of miR bulges	1 nt	2 nt	3 nt	4 nt
165	131	29	5	0
100%	79%	18%	3%	0%



We analysed the occurrence of bulges in human miRNAs based on miRBase Release 22 and the precursor structures predicted therein. We identified 165 examples where a bulge is formed in the predominantly loaded strand. Most of these are 1 nt bulges. 2 nt and 3 nt bulges occur at lower frequencies. Positional analysis of 1 nt bulges showed low frequencies in the seed region and at positions 9 and 14 (left). Bulges of 2 nt length predominantly occurred at central positions (right).

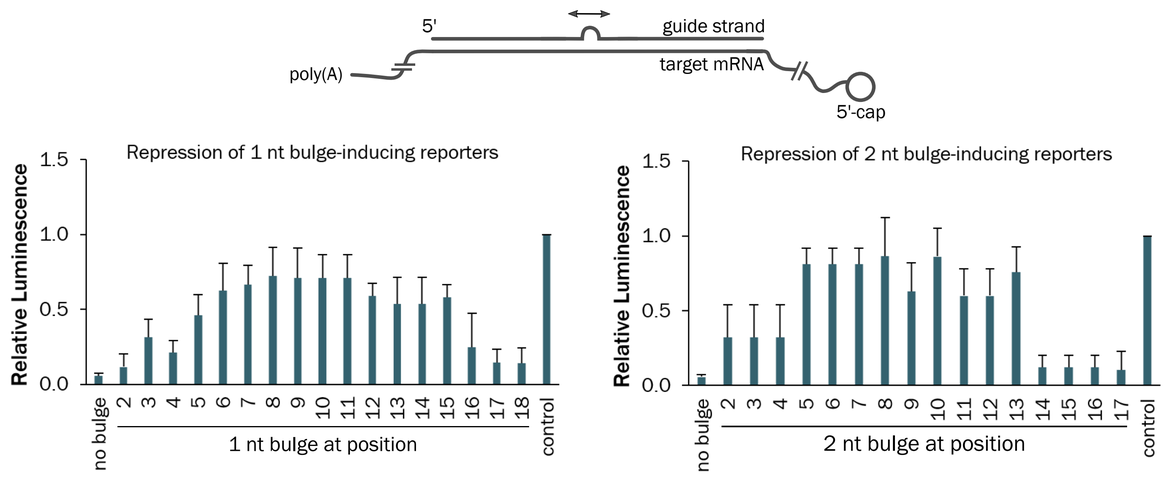
4 Bulged GalNAc-siRNA conjugates reduce target mRNA expression *in vivo*



Two variants targeting TTR with high *in vitro* activity were chosen and synthesised with a triantennary GalNAc moiety at the 5' end of the sense strand. The siRNA was modified with alternating 2'-OMe/2'-F and stabilised with each two phosphorothioates at all non-conjugated ends. Bulge forming regions were optionally stabilised with additional phosphorothioates (ps) and these two phosphorylation patterns were also applied to the duplex without bulges. *: ps between guide positions 12-15; **: ps between guide positions 13-17.

Experimental conditions: male C57BL/6 mice, target protein levels analysed from serum by ELISA, shown are means and standard deviations relative to PBS-treated control group for each point in time, sense sequence (5'-3'): CAGUGUUCUUGCUCUAUAA

5 Can target binding induce bulges of the guide strand?



Luciferase reporters were used to assess formation of guide strand bulges upon target mRNA binding. Therefore, target sequences were designed so that they lacked one or two nucleotides at different positions. Best repression of bulge-inducing targets was achieved when the bulge (or alternative structure) was positioned in the 5' or 3' terminal regions of the guide strand. Off-targeting may occur through this mechanism.

Experimental conditions: co-transfection of bulge-inducing reporters and 1 nM siRNA into MCF-7 cells. Luciferase activity was assayed after 24 h and normalised to a control with non-complementary target sequence. Shown are means and standard deviations obtained from four biological replicates.

Conclusion

- siRNAs tolerate bulges of 1 and 2 nucleotides length *in vitro* and *in vivo*. These bulges do not provide improved activity.
- We found position-dependent restrictions that can also be found in human miRNAs.
- Bulges can form during target binding. Positional acceptance differs from that observed with small RNA duplexes. Tolerance is highest at the siRNA 5' and 3' termini and should be considered in off-target predictions.