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A screen of chemical modifications identifies position-specific modification by UNA to most potently reduce siRNA off-target effects

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ABSTRACT

Small interfering RNAs (siRNAs) are now established as the preferred tool to inhibit gene function in mammalian cells yet trigger unintended gene silencing due to their inherent miRNA-like behavior. Such off-target effects are primarily mediated by the sequence-specific interaction between the siRNA seed regions (position 2–8 of either siRNA strand counting from the 5'-end) and complementary sequences in the 3' UTR of (off-) targets. It was previously shown that chemical modification of siRNAs can reduce off-targeting but only very few modifications have been tested leaving more to be identified. Here we developed a luciferase reporter-based assay suitable to monitor siRNA off-targeting in a high throughput manner using stable cell lines. We investigated the impact of chemically modifying single nucleotide positions within the siRNA seed on siRNA function and off-targeting using 10 different types of chemical modifications, three different target sequences and three siRNA concentrations. We found several differently modified siRNAs to exercise reduced off-targeting yet incorporation of the strongly destabilizing unlocked nucleic acid (UNA) modification into position 7 of the siRNA most potently reduced off-targeting for all tested sequences. Notably, such position-specific destabilization of siRNA–target interactions did not significantly reduce siRNA potency and is therefore well suited for future siRNA designs especially for applications in vivo where siRNA concentrations, expectedly, will be low.

INTRODUCTION

Small interfering RNAs (siRNAs) are widely used as triggers of RNA interference (RNAi) to knock down (KD) gene expression in several experimental settings (1). The standard siRNA design is comprised of a 21-nt long antisense (AS) and sense strand (SS) annealed to form a siRNA duplex with a 19-bp double-stranded (ds) RNA stem and 2-nt 3’ overhangs (2). Upon introduction into the target cell cytoplasm, the siRNA is recognized by a RISC-loading complex (RLC), the passenger SS is cleaved and released and the guiding AS is incorporated into the so-called RNA-induced silencing complex (RISC) containing the effector endonuclease Argonaute 2 (Ago2) (3). RISC subsequently binds and cleaves RNAs having (near-) perfect sequence complementary to the AS thereby resulting in sequence-specific KD of the intended gene (4).

The application of siRNAs in therapeutics holds enormous potential, yet recent studies have identified unspecific side effects such as induction of host immune responses (5,6) and unspecific gene deregulation related to the method of siRNA delivery (7). In particular, microarray analysis have shown that siRNAs cause a reduction

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in the levels of hundreds of non-targeted messages (8–11). Such off-target effects presumably reflects the inherent miRNA-like behaviors of all investigated siRNA constructs (9) and is primarily mediated by the interaction between the seed region of the RISC-associated guiding structs (9) and is primarily mediated by the interaction between the seed region of the RISC-associated guiding strand (nucleotide position 2–8 counting from the 5’-end) and complementary sites in the 3’-UTR of the target mRNA (12,13). We and other groups have incorporated chemical modifications into siRNAs and successfully reduced the off-target effects of the SS (14–16) and the AS (16,17); especially 2’-O-methyl (OMe) modification of the second position of the SS and AS has been reported to significantly reduce off-targeting (16). However, off-targeting has never been completely avoided and seed modification does in many cases reduce siRNA potency thereby requiring higher siRNA concentrations for efficient silencing which complicates applications in vivo.

Here, we have developed reporter cell lines to compare the influence of chemical modification on siRNA potency and off-target effect. This screen evaluates 10 types of chemical modifications inserted throughout the AS seed region at several siRNA concentrations. We identify several potent siRNAs with reduced off-target effects however, insertion of the destabilized unlocked nucleic acid (UNA) proved most efficient in reduced off-targeting using several siRNA sequences.

MATERIALS AND METHODS

Synthesis of oligonucleotides and siRNAs

The phosphoramidites and oligonucleotides were synthesized as previously described (18) using standard solid-phase DNA/RNA synthesis on automated nucleic acid synthesizers using a standard RNA synthesis cycle (1–5μmol scale) and commercially available 2’-O-TBDMs protected RNA phosphoramidites, DNA phosphoramidites, LNA phosphoramidites, UNA phosphoramidites, chemically modified phosphoramidites (18) and common reagents. The stepwise coupling yield of all monomers was >99%. Following standard deprotection, purification and work-up, the composition and purity (>80%) of the resulting oligonucleotides was confirmed by MALDI–MS and MALDI–MS analysis and ion exchange HPLC. The oligomers are named according to the nomenclature of previously published work (18) to allow easy comparison of results between individual studies. For siRNA annealing SS and AS were mixed in 1× Dharmaco® annealing buffer (Dharmacon) and incubated for 1 min at 95°C followed by 1 h at 37°C and siRNA concentrations was quantified using the Quant-iT RiboGreen RNA Assay Kit from Molecular Probes (Invitrogen) on a FLUOstar luminometer (BMG labtech).

Establishment of stable reporter cell lines

The human lung cancer cell line H1299 was grown in RPMI-1640 containing 10% FBS, 1% penicillin/streptomycin. Stable luciferase siRNA and miRNA seed/full reporters were generated by transfection and subsequent pyromycin selection (1 μg/ml medium) of H1299 cells with a psiCHECK2 vector (Promega) modified to contain a pyromycin selection cassette and the following DNA sequences in the multiple cloning site located in the 3’UTR of the Renilla luciferase gene:

- siSensor1: 5’-ACGACGTAAACGGCCACAAGTTGCAAATTC-3
- miRNA seed sensor1: 5’-AACCAACAGATACTACTGAACAA-3
- miRNA seed sensor2: 5’-GTAATTGACCCCAACGATACTTGCTGAATGAA-3
- miRNA seed sensor3: 5’-GGCAAGTCCCTGAAGCTCCG-3
- miRNA seed sensor4: 5’-GTCGAATAATATTACATAAATAAAACCTGAAG-3

siRNA transfections, quantitative PCR (qPCR) and dual luciferase assays

The experiment shown in Figure 1B was performed by transfection of stable siRNA or miRNA full sensor1 cell lines with 5 nM siRNA using lipofectamine 2000 (Invitrogen) in a six-well format using 450 000 cells per well. After 48 h both total RNA was collected using Trizol reagent (Invitrogen) and cell lysate for Dual luciferase assay, as described below. Relative Renilla luciferase mRNA levels were determined by quantitative PCR (qPCR) using the Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen) and the random hexamer-primed cDNA as template. The following primer sequences were used: rluc-F: 5’-TGCTGAATGTAACGGCACTGCTGAAGTTGGCGGCC-3’; siSensor1: 5’-AAATAAAATATTTTCAAAAAATCTACATAATCTTCAATCTTCTCTAATTCTACATAATCTCTTCTCTAACAACTAC-3’; miRNA full sensor1: 5’-CAAGCAGTCCCTGAAGTATAGGGAAGCACGACGTAAACGGCCACAAGTGCGGTGAATGTAACGGCACTGCTGAAGTTGGCGGCC-3’; miRNA seed sensor3: 5’-GTCGAATAATATTACATAAATAAAACCTGAAG-3’; GAPDH-R: 5’-GAGGTCGTGAGGCA-3’.

The experiment shown in Figures 2–5 siRNA transfections were performed in triplicates using the Lipofectamine 2000 reagent (Invitrogen) in a 96-well format in the presence of 0.25 μl lipofectamine 2000 and 15 000 cells per well in a total volume of 125 μl. The experiments performed in Figure 2B was performed in triplicates twice using 1 nM siRNAs in the presence of 10% FCS during transfection and the results represented here represents average values. All other transfections were performed in the absence of serum during siRNA transfection and 10% FCS was instead added 4 h after transfection.
Figure 1. Development of siRNA and miRNA sensor cell lines to monitor siRNA off-target effects. (A) Schematic view of the luciferase based sensors used to monitor siRNA and off-target effects in stable cell lines. The siRNA sensor harbors a single target site in the Renilla 3' UTR perfectly complementary to the siRNA AS thereby leading to Renilla luciferase mRNA cleavage by Ago2. The miRNA seed sensor instead contain four copies of a target sequence matching only position 1–8 of the siRNA (seed match) whereas the miRNA full sensor additionally matches the bases at position 13–19. Both miRNA sensors monitor Ago non-cleavage mediated KD of Renilla luciferase levels indicative of siRNA off-targeting. Firefly luciferase is used as endogenous non-regulated control to normalize for differences in cell numbers. Drawings are not to scale, siRNAs are shown in gray. P, Promoters; renilla luc., Renilla luciferase; firefly luc, Firefly luciferase; pA, polyA site. (B) Validation of siRNA sensor and miRNA sensor behaviors. Unmodified siRNA, siEGFP1, was transfected into the siRNA sensor1 and miRNA full sensor1 and the relative Renilla luciferase protein and mRNA levels were determined 48 h after transfection by dual luciferase assays and qPCR, respectively. Relative Renilla luciferase levels were normalized to cells transfected with the unrelated siRNA, siBCR-ABL.

Figure 2. Chemical modification of AS seed regions can reduce off-targeting. (A) Schematic overview of the chemical modifications investigated in this study. (B) Initial screen of siRNA potency (using the siRNA sensor) and off-targeting (miRNA seed sensor) of all chemically modified versions of the unmodified AS, W053 (see Table 1 for overview). Results from all ASs are shown in gray squares whereas selected oligos are shown in color-coding as indicated. ASs exhibiting reduced off-targeting relative to their siRNA potency are framed by the green triangle, whereas the majority of ASs exhibit a linear correlation between siRNA and off-target potency (marked by red square).
The presence/absence of 10% FCS during transfection slightly affects transfection efficiencies (and thereby silencing efficiencies) but not the behaviors of the tested siRNAs. Transient reporter–siRNA co-transfection experiments (Supplementary Figure S2) were performed by co-transfecting 0.025\(\mu\)g reporter plasmid (see above) and the siRNA duplexes by simultaneous use of 0.25\(\mu\)l TransIT-LT1 (Mirus) and 0.25\(\mu\)l TransIT-TKO (Mirus) according to the manufactures protocol. Cell lysates were harvested after 48 h and dual-luciferase assays were performed using the 'Dual-luciferase reporter assay system' (Promega) according to the manufacturer’s protocol on a FLUOstar luminometer (BMG labtech); Renilla luciferase signals (sample) were normalized to the firefly luciferase signals (cell number/transfection control). All results were normalized to the control siRNA, siBCR-ABL (19), which has minimal sequence similarity to the used siRNA and miRNA sensors.

Evaluation of oligonucleotide annealing

To evaluate annealing of modified ASs to a 13-mer RNA sequence (5’-GCCACAAGUUCU-3; bold nucleotides indicate positions base paring to position 1–9 of the siRNA AS) 20 pmol of the relevant AS and 13-mer were mixed in a physiological salt solution (0.9% NaCl), heated to 95\(^\circ\)C for 1 min followed by 37\(^\circ\)C for 1 h. Annealing was evaluated on a 4% native agarose gel stained with SYBR Gold® (Invitrogen) (Figure 4A).

Evaluation of siRNA on-target and off-target activity by qPCR

Wild-type H1299 cells were transfected at 70% confluence in a six-well format with OMe, UNA or unmodified siRNAs at 1, 10 and 100 nM concentration (final concentration in the medium) using Lipofectamine 2000 (Invitrogen) (Figure 6). Total RNA was harvested after 24 h using Trizol (Invitrogen) and reverse transcription was performed using the SuperScript\textsuperscript{TM} III Reverse Transcriptase (Invitrogen) and random hexamer primers. qPCR quantification of mRNA levels was performed using the Taqman Gene Expression Master mix (Applied Biosystems) on a Stratagene Mx3005 qPCR thermocycler (Stratagene, La Jolla CA, USA) using the following Taqman Gene Expression Assays (Applied

![Figure 3. UNA-modification of the AS seed most dramatically reduces siRNA off-targeting. Evaluation of siRNA potency and off-targeting potential of selected ASs at 0.1, 1 and 10 nM concentrations using the siRNA sensor1 (light gray lines) and miRNA seed/full sensor1’s (dark gray lines), respectively.](nar.oxfordjournals.org)
RESULTS

Establishment of siRNA and off-target sensor cell lines

To generate an assay to monitor siRNA activity and off-target effects in a high throughput setting we established three stable H1299 cell lines expressing the dual luciferase reporters denoted siRNA sensor, miRNA seed sensor and miRNA full sensor (Figure 1A). The siRNA sensor harbors a single 21-nt target site for eGFP (denoted eGFP-1) placed in the 3' UTR of the Renilla luciferase mRNA thereby leading to Ago2-mediated cleavage upon introduction of an siRNA targeting eGFP-1, siEGFP1. Both the miRNA seed and full sensor instead contain four copies of similar, but truncated eGFP-1 target sequences bearing central mismatches around the Ago2 cleavage site (position 9–12 counting from the 5'-end of the siRNA). The miRNA seed sensor contains four target sites for the seed region only (position 2–8 of the siRNA) which is sufficient for off-target effects (8), yet we also designed the miRNA full sensor having additional base pairs in the 3'-end (position 13–19 of the siRNA) in order to mimic very extensively base pairing off-targets (Figure 1A). The central mismatches in the miRNA sensors will expectably prevent Ago2-mediated cleavage of the Renilla luciferase mRNAs and will thereby lead to only slightly reduced mRNA levels, typically <2-fold, through various mechanisms of mRNA decay (21,22) although the degree of mRNA decay differs between target sites (23–25). The miRNA-like behavior of siEGFP1 resulting in translation repression (and some mRNA destabilization) should still, however, be reflected as strongly reduced Renilla luciferase protein levels. In accordance, we observe significantly higher Renilla luciferase mRNA levels in the miRNA full sensor as compared to the siRNA sensor cell line although the protein activities are reduced to similar levels (Figure 1B).

Evaluating the potency and off-target effects of chemically modified siRNAs

Using the reporter cell lines we next investigated the impact of modifying single positions of the siEGFP1 AS seed region by 10 different types of chemical modifications (see Figure 2A for a structural overview of the chemical modification types and Table 1 for oligo sequences); hexitol nucleic acid (HNA) (26), altitol nucleic acid (ANA) (27), LNA (28), UNA (29), 2′-O-aminoethyl
We decided to preferentially modify the AS seed as this region guides initial miRNA–target interactions (34), is responsible for off-target effects (12) and is more tolerant to single mismatches (and thereby expectably chemical modification) towards target mRNAs as compared to the central and 3′-end region of the AS (35). The unmodified AS (W053) and all modified AS were annealed to an unmodified SS (W207) and transfected into the siRNA sensor and miRNA seed sensor cell line at 1 nM concentration in the medium (see MM for details) to compare their siRNA potency and off-targeting. We find a positive correlation between siRNA potency and off-target effects for most investigated siRNAs (dashed red box; Figure 2B) in agreement with previous studies (9,36). In particular, the unmodified AS (W053) trigger a very potent KD of the siRNA sensor, yet also reduces the seed miRNA sensor by 50% indicative of a high off-target potential. Interestingly, we identify a subset of siRNAs that silences the siRNA sensor yet have less impact on the seed miRNA target sensor (dashed green triangle; Figure 1B). Among these we find the AS Dharm1 which contains a OMe-modification at position 2 previously found to reduce siRNA off-targeting (16) hereby validating our miRNA-sensor based approach. Furthermore, we found ASs having AENA, CLNA, CENA, HNA and EA modifications at position 3 of the AS hereby suggesting this position to have special relevance to reducing off-targeting by certain chemistries [JC-A1 (AENA position 3,18), JC-seed3 (CLNA position 3), JC-S1 (CLNA position 3,18), JC-F1 (CENA position 3,18), GS2662, (HNA position 3), DO1119 (EA position 3); Figure 2B and Table 1].

Interestingly, we also find single UNA-modifications at position 4–8 to dramatically reduce off-targeting hereby suggesting that this highly destabilizing modification is particularly interesting in the 3′-end of the AS seed [W316 (UNA position 4), W317 (UNA position 5).
W123 (UNA position 6), W318 (UNA position 7), W376 (UNA position 7) and W319 (UNA position 8); Figure 2B and Table 1].

**siRNA titrations reveal reduced off-targeting by siRNA UNA modification**

We next investigated the most promising siRNAs at three concentrations (0.1, 1 and 10 nM) in order to closely evaluate the concentration-dependency of siRNA potency and off-targeting (Figure 3 and Supplementary Figure S1). The unmodified siEGFP produced a potent knockdown of siRNA and miRNA seed sensor expression at all three concentrations whereas the full miRNA sensor was less affected (Figure 3). This likely reflects that the target sites in the full miRNA sensor are in too close proximity to allow the simultaneous binding of four RISCs (Figures 1A and 3). Again we found that the OMe modified AS Dharm1 exhibited reduced off-targeting at all concentrations however, the siRNA potency was significantly reduced at the 0.1 nM concentration (Figure 3). This underscores the necessity of testing siRNAs at a gradient of concentrations to truly distinguish subtle differences in potency, especially as lower concentrations relevant to applications *in vivo*. The ASs modified with AENA, CLNA, CENA and HNA modifications at position 3 exhibited a very similar profile with off-targeting being more pronounced a 10 nM concentrations.

**Figure 6.** UNA-modification of siRNA AS and SS reduces off-targeting of well-characterized endogenous off-targets while preserving on-target activity. (A) Evaluation of on-target (GRK4 mRNA) and off-target activity (hif-1α mRNA) for a siRNA directed against GRK4. (B) Evaluation of on-target (ICAM1 mRNA) and off-target activity (TNFR1 mRNA) for a siRNA directed against ICAM1. H1299 were transfected with the siRNAs at the indicated concentrations and mRNA levels were evaluated after 24 h by qPCR. The presented values are normalized to values from cells transfected with the unrelated siEGFP1.
Table 1. Overview of chemically modified ASs and SSs

| Name         | AS/SS | Modification | Sequence (5'-3') |
|--------------|-------|--------------|-----------------|
| W053         | AS1   | RNA          | ACUGUGGCCGUGGUAAGCUGC |
| W207         | SS1   | RNA          | GACGUAACCAACCCAGGAA |
| siEGFPmis    | SS1   | RNA          | GACUUGGACCAACCAAGGG |
| siEGFPmis    | AS1   | RNA          | ACUGUGGCCGUGGUAAGCUGC |
| JC-A1        | AS1   | AENA 3,18    | ACUGUGGCCGUGGUAAGCUGC |
| JC-A2        | AS1   | AENA 4,18    | ACUGUGGCCGUGGUAAGCUGC |
| JC-Seed4     | AS1   | CLNA 2       | ACUGUGGCCGUGGUAAGCUGC |
| JC-Seed3     | AS1   | CLNA 3       | ACUGUGGCCGUGGUAAGCUGC |
| JC-S1        | AS1   | CLNA 4,18    | ACUGUGGCCGUGGUAAGCUGC |
| JC-Seed2     | AS1   | CLNA 4       | ACUGUGGCCGUGGUAAGCUGC |
| JC-Seed1     | AS1   | CLNA 6       | ACUGUGGCCGUGGUAAGCUGC |
| JC-Seed8     | AS1   | CENA 2       | ACUGUGGCCGUGGUAAGCUGC |
| JC-Seed7     | AS1   | CENA 3       | ACUGUGGCCGUGGUAAGCUGC |
| JC-F1        | AS1   | CENA 3,18    | ACUGUGGCCGUGGUAAGCUGC |
| JC-Seed6     | AS1   | CENA 4       | ACUGUGGCCGUGGUAAGCUGC |
| JC-Seed5     | AS1   | CENA 6       | ACUGUGGCCGUGGUAAGCUGC |
| GS2660       | AS1   | HNA 1        | ACUGUGGCCGUGGUAAGCUGC |
| GS2661       | AS1   | HNA 2        | ACUGUGGCCGUGGUAAGCUGC |
| GS2662       | AS1   | HNA 3        | ACUGUGGCCGUGGUAAGCUGC |
| GS2663       | AS1   | HNA 4        | ACUGUGGCCGUGGUAAGCUGC |
| GS2664       | AS1   | HNA 5        | ACUGUGGCCGUGGUAAGCUGC |
| GS2665       | AS1   | HNA 6        | ACUGUGGCCGUGGUAAGCUGC |
| GS2666       | AS1   | HNA 7,8     | ACUGUGGCCGUGGUAAGCUGC |
| GS2667       | AS1   | HNA 8        | ACUGUGGCCGUGGUAAGCUGC |
| GS2670       | AS1   | ANA 2        | ACUGUGGCCGUGGUAAGCUGC |
| GS2672       | AS1   | ANA 4        | ACUGUGGCCGUGGUAAGCUGC |
| GS2673       | AS1   | ANA 5        | ACUGUGGCCGUGGUAAGCUGC |
| GS2674       | AS1   | ANA 6        | ACUGUGGCCGUGGUAAGCUGC |
| GS2675       | AS1   | ANA 7        | ACUGUGGCCGUGGUAAGCUGC |
| GS2676       | AS1   | ANA 8        | ACUGUGGCCGUGGUAAGCUGC |
| GS2677       | AS1   | ANA 3,18,19 | ACUGUGGCCGUGGUAAGCUGC |
| SWC2         | AS1   | EA 2         | ACUGUGGCCGUGGUAAGCUGC |
| DO1119       | AS1   | EA 3         | ACUGUGGCCGUGGUAAGCUGC |
| DO1118       | AS1   | EA 4         | ACUGUGGCCGUGGUAAGCUGC |
| SWG5         | AS1   | EA 5         | ACUGUGGCCGUGGUAAGCUGC |
| DO1116       | AS1   | EA 6         | ACUGUGGCCGUGGUAAGCUGC |
| SWG7         | AS1   | EA 7         | ACUGUGGCCGUGGUAAGCUGC |
| SWG8         | AS1   | EA 8         | ACUGUGGCCGUGGUAAGCUGC |
| SWC9         | AS1   | EA 9         | ACUGUGGCCGUGGUAAGCUGC |
| SWC10        | AS1   | EA 10        | ACUGUGGCCGUGGUAAGCUGC |
| DO1110       | AS1   | EA 12        | ACUGUGGCCGUGGUAAGCUGC |
| DO1109       | AS1   | EA 13        | ACUGUGGCCGUGGUAAGCUGC |
| W259         | AS1   | LNA 1        | ACUGUGGCCGUGGUAAGCUGC |
| W260         | AS1   | LNA 2        | ACUGUGGCCGUGGUAAGCUGC |
| W261         | AS1   | LNA 3        | ACUGUGGCCGUGGUAAGCUGC |
| W262         | AS1   | LNA 4        | ACUGUGGCCGUGGUAAGCUGC |
| W263         | AS1   | LNA 5        | ACUGUGGCCGUGGUAAGCUGC |
| W264         | AS1   | LNA 6        | ACUGUGGCCGUGGUAAGCUGC |
| W265         | AS1   | LNA 7        | ACUGUGGCCGUGGUAAGCUGC |
| W266         | AS1   | LNA 8        | ACUGUGGCCGUGGUAAGCUGC |
| W313         | AS1   | UNA 1        | ACUGUGGCCGUGGUAAGCUGC |
| W314         | AS1   | UNA 2        | ACUGUGGCCGUGGUAAGCUGC |
| W315         | AS1   | UNA 3        | ACUGUGGCCGUGGUAAGCUGC |
| W316         | AS1   | UNA 4        | ACUGUGGCCGUGGUAAGCUGC |
| W317         | AS1   | UNA 5        | ACUGUGGCCGUGGUAAGCUGC |
| W123         | AS1   | UNA 6        | ACUGUGGCCGUGGUAAGCUGC |
| W318         | AS1   | UNA 7        | ACUGUGGCCGUGGUAAGCUGC |
| W319         | AS1   | UNA 8        | ACUGUGGCCGUGGUAAGCUGC |
| W376         | AS1   | UNA 7        | ACUGUGGCCGUGGUAAGCUGC |
| W380         | AS1   | UNA 8        | ACUGUGGCCGUGGUAAGCUGC |
| W381         | AS1   | dSpacer 7    | ACUGUGGCCGUGGUAAGCUGC |
| Dharm1       | AS1   | OmC 2        | ACUGUGGCCGUGGUAAGCUGC |
| W340         | SS3   | RNA          | GCACACAGCUCCUUUCAAGTACNN |
| W341         | AS3   | RNA          | CUCUUGGAAAGAAGCUAGGCC |
| W342         | AS3   | OmC 2        | CUCUUGGAAAGAAGCUAGGCC |
| W343         | AS3   | UNA 6        | CUCUUGGAAAGAAGCUAGGCC |
| W344         | AS3   | UNA 5        | CUCUUGGAAAGAAGCUAGGCC |
| W345         | AS3   | UNA 4        | CUCUUGGAAAGAAGCUAGGCC |
| W346         | AS3   | UNA 7        | CUCUUGGAAAGAAGCUAGGCC |
| W360         | AS2   | RNA          | ACUUCAGGGUGCAAGCUAGCT |

(continued)
The name, target sequence number, type and position of chemical modification and sequence of the investigated ASs and SSs are given. Only modifications found within the base pairing siRNA stem is listed in ‘modification’, as the modified overhangs do not contribute to siRNA off-target potentials. The oligos are named according to the nomenclature of previously published work (18) to allow easy comparison of results between individual studies. Base positions with mismatches to the target are underlined.

Table 1. Continued

| Name     | AS/SS | Modification | Sequence (5‘-3’) |
|----------|-------|--------------|-----------------|
| W361     | AS2   | UNA 7        | ACUUCAUGNAAGUCUUGCUUGCTTLNAU |
| W362     | AS2   | OMe 2        | AGCMUCAGGUUGCUUGCTTLNAU |
| W363     | AS2   | DNA 1-8      | AGDNAUUGDNAADNAUGDNAAGCUCUUUGCTTLNAU |
| W364     | SS2   | RNA          | TGCCAAGCUGACCUGAGAG |
| W367     | AS2   | UNA 2        | ACUUNAAGCGGAUUGCUUGCTTLNAU |
| W368     | AS2   | UNA 3        | ACGUUNAAGCGGAUUGCUUGCTTLNAU |
| W369     | AS2   | UNA 4        | AGCUUNAAGCGGAUUGCUUGCTTLNAU |
| W370     | AS2   | UNA 5        | AGCUUNAAGCGGAUUGCUUGCTTLNAU |
| W371     | AS2   | UNA 6        | AGCUUNAAGCGGAUUGCUUGCTTLNAU |
| W372     | AS2   | UNA 8        | AGCUUNAAGCGGAUUGCUUGCTTLNAU |
| GRK4     | AS2   | UNA 9        | AGCUUNAAGCGGAUUGCUUGCTTLNAU |
| ICAM1    | AS    | RNA          | AUCUGCCUGAAGAGAGCAGCUU |
| ICAM1    | AS    | OMe 2        | AGCUUNAAGCGGAUUGCUUGCTTLNAU |
| ICAM1    | SS    | RNA          | AUCUGCCUGAAGAGAGCAGCUU |
| ICAM1    | UNA   | RNA          | AUCUGCCUGAAGAGAGCAGCUU |
| ICAM1    | OMe   | RNA          | AUCUGCCUGAAGAGAGCAGCUU |

[Shown for CLNA (JC-S1) and CENA (JC-F1) in Figure 3 and see Supplementary Figure S1 for JC-A1 (AENA position 3), ICAM1-3 (CLNA position 3), ICAM1-7 (CENA position 3) and GS2662, (HNA position 3). More promising is HNA-modification at position 8 (GS2667) and EA-modifications at position 3 (DO1119) which further reduced off-targeting at 10 nM concentration while preserving high siRNA potency. Importantly, the most target specific siRNAs were found among the UNA-modified siRNAs; ASs modified at position 4 (W316) and position 7 (W367) exhibited reduced off-targeting at 10 nM concentration while maintaining a very potent KD of the siRNA sensors at 10 nM concentration whereas the top-performing AS modified with UNA at position 7 (W318) exhibited a clearly reduced off-targeting profile even at 100 nM concentration (Supplementary Figure S2).

Off-targeting is reduced in siRNA design by UNA modification at position 7

To investigate the ability of UNA to reduce off-targeting for other siRNA sequences we designed a second set of modified siEGFPs targeting a different sequence in eGFP, eGFP-2 and corresponding sensor cell lines. We performed a full ‘UNA seed walk’ by incorporation of single UNA-modified nucleotide positions at position 2–9 of the siRNA AS (Table 1). Furthermore, we included the unmodified AS (W360), an AS modified with OMe at position 2 (W362) and an AS containing a DNA seed region (W363), which has previously been reported to reduce off-target effects (17). As for siEGFP1 we found the unmodified siEGFP2 (W360–W364) to trigger a potent KD of both the seed and full miRNA sensor at 1–10 nM concentrations indicative of potent off-targeting (Figure 4). The ASs W362 (OMe at position 2) and W363 (DNA position 1–8) did indeed exhibit slightly reduced off-targeting at 10 nM, yet the corresponding siRNA potency was dramatically reduced, especially at 1 and 0.1 nM concentrations. UNA-modification of positions 2, 3, 4, 5, 9 similarly resulted in significantly reduced siRNA potency, especially at 0.1 nM. Interestingly, UNA modification at 3’end of the AS seed (position 6, 7 or 8) was fairly well tolerated; again the AS modified with UNA at position 7 (W361) had similar siRNA activity as unmodified siRNAs (siEGFP2) with very less effect on any of the two miRNA sensors (Figure 4). Hereby UNA modification of position 7 dramatically reduced siRNA off-targeting with minor loss of

<sup>1</sup>Shown for CLNA (JC-S1) and CENA (JC-F1) in Figure 3 and see Supplementary Figure S1 for JC-A1 (AENA position 3), ICAM1-3 (CLNA position 3), ICAM1-7 (CENA position 3) and GS2662, (HNA position 3). More promising is HNA-modification at position 8 (GS2667) and EA-modifications at position 3 (DO1119) which further reduced off-targeting at 10 nM concentration while preserving high siRNA potency. Importantly, the most target specific siRNAs were found among the UNA-modified siRNAs; ASs modified at position 4 (W316) and position 7 (W367) exhibited reduced off-targeting at 10 nM concentration while maintaining a very potent KD of the siRNA sensors at 10 nM concentration whereas the top-performing AS modified with UNA at position 7 (W318) exhibited a clearly reduced off-targeting profile even at 100 nM concentration (Supplementary Figure S2).

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siRNA potency as compared to all other modifications tested in this study.

UNA destabilises siRNA–target interactions but still support the function of weak seeds

UNA is reported to destabilize RNA–RNA interactions (29) and can be strategically used in siRNA design to induce local thermodynamical destabilization (37–39). We therefore speculate that UNA-modification of the siRNA seed reduces off-target potential by exerting a relatively more profound impact on target interactions relying on partial sequence complementarity (i.e. off-targeting) rather than full complementarity (i.e. siRNA effect). Indeed, we found that single UNA-modification of position 2–8 of the AS seed (oligos W313–W319 and W123) prevented their annealing to a 13mer RNA oligo with sequence complementarity to position 1–9 of the AS thereby mimicking initial siRNA–target interactions (34) (Figure 5A). Yet as UNA-modification strongly destabilizes RNA–RNA interactions we worried that UNA-modification would be detrimental to siRNAs that rely on very week seed–target interactions. This would limit the general applicability of UNA-modifications to reduce off-targeting in siRNA design as seen for other modification types (16,17). The seed regions tested in siEGFP1 and siEGFP2 both form four GC base pairs with their (off-) targets and may be more permissive to UNA-modifications than seeds with a lower GC base pair content. We therefore designed a third siRNA targeting eGFP, denoted siEGFP3 (Table 1) which forms only two GC-pairs with (off-) targets at position 4 and 7. This would provide a very stringent test of introducing UNA at position 7 as on-target interactions will be particularly destabilized as compared to e.g. siEGFP3 and W342 (AS with OMe position 2; Table 1). We tested the modified ASs in stable reporter cell lines at higher concentrations (1–100 nM) as the weak binding siEGFP3 has a reduced siRNA potency (Figure 4B). Under these conditions the unmodified AS (W341) exhibited both strong siRNA potency and off-targeting at all concentrations tested. Off-targeting was significantly reduced by OMe modification at position 2 (W342) at all concentrations however, siRNA potency was significantly reduced at 1 nM giving only 50% KD. UNA-modification at position 4 and 5 similarly reduced siRNA potency at 1 nM confirming previous observations of central positions of the seed being less susceptible to UNA modification. Notably UNA modification of position 7 proved to have only little impact on siRNA potency at all concentrations while significantly reducing off-targeting (Figure 5B). This establishes that the highly destabilizing UNA modification can be used at position 7 in the AS to reduce off-targeting of even siRNAs with weak binding seeds thereby greatly enhancing its potential in siRNA design.

As UNA-modification at position 7 of the AS is well tolerated we speculated that base pairing at this position may not be required for siRNA action and that introduction of an abasic C-linker may even further reduce off-targeting potential. Indeed we find a dramatically reduced off-target effect on the seed miRNA target1 when position 7 was modified with aSpacerC3 or dSpacer linker. However, the siRNA potency was concurrently somewhat reduced (Supplementary Figure S3). Hereby UNA-modification has the least impact on siRNA potency of the tested modifications while dramatically reducing off-target effects, presumably by strongly destabilizing but not abrogating base pairing at position 7.

UNA modifications in the siRNA AS and SS reduce off-targeting of endogenous off-targets while preserving on-target activity

Examples of off-target regulation of endogenous genes by siRNAs has in some cases been very carefully dissected (13,40); e.g. Lin et al. (13) found that a siRNA directed towards the GRK4 mRNA triggered an equally potent downregulation of the hif-1α mRNA through a 7nt motif present within its 3’ UTR region and complementary to the siRNA AS seed region (13). To investigate if OMe modification at AS position 2 or UNA modification at AS position 7 of this GRK4-directed siRNA would alleviate hif-1α off-targeting we transfected H1299 cells with siRNAs at 1, 10 and 100 nM concentrations and evaluated GRK4 (on-target) and hif-1α (off-target) mRNA levels by qPCR (Table 1 and Figure 6A). We found that both OMe and UNA modifications dramatically reduced off-targeting of hif-1α at all three concentrations as compared to the unmodified siRNA (Figure 6A). However, the UNA-modified siRNA, but not the OMe-modified siRNA, was equally potent as the unmodified siRNA at 1 nM concentration hereby confirming our observations that UNA dramatically reduces off-targeting while preserving on-target activity. In another study Clark et al. (40) found that the SS of a siRNA directed towards ICAM1 produced a potent off-target regulation of the TNFR1 mRNA in HUVEC cells. Furthermore, OMe-modification of the SS position 2 fully alleviated off-targeting albeit somewhat reducing on-target activity (40). To investigate if UNA-modification can similarly reduce SS off-targeting we transfected H1299 cells with OMe-, UNA- and unmodified siRNA at 1, 10 and 100 nM concentration (Table 1 and Figure 6B). No significant KD of the on-target ICAM1 was seen at 1 nM for all siRNAs (data not shown). A very modest reduction of ICAM1 mRNA levels was seen for all siRNAs at 10 nM and the potencies of both modified siRNAs seemed slightly reduced (Figure 6B, dark gray bars) as previously observed (40). Instead all siRNAs very potently downregulated the off-target TNFR1, however, off-targeting by the OMe-modified and especially the UNA-modified siRNA were slightly less potent (Figure 6B, light gray bars). This trend was even more pronounced at 100 nM concentrations where the UNA-modified siRNA exhibited a much lower off-target regulation of TNFR1 while enhancing on-target activity as compared to both the unmodified and OMe-modified siRNA (Figure 6B).
DISCUSSION

Gene off-targeting by siRNAs is a serious concern that can severely complicate the interpretation of RNAi experiments (7,13) and delay clinical applications. Some success in reducing siRNA off-target effects has been achieved by chemically modifying siRNAs (16,17) and by utilizing siRNA pools to minimize the contribution of the individual siRNAs to off-targeting while preserving on-target activity. In the present study we identified several chemical modifications that highly efficiently reduce gene off-targeting by siRNA when introduced into the AS seed region. We investigated a total of 75 chemically different ASs using 10 different modification types and three different target sequences and concentrations. We decided to develop luciferase-based off-target reporters in stable cell lines for several reasons. First, homology between the seed region of siRNA and the 3'-UTR but not coding sequence of mRNA is important for siRNA off-target effects (12) thereby making UTR reporters biologically highly relevant. Secondly, Ui-Tei et al. (17) found a very high correlation between results obtained using a similar luciferase reporter (upon transient co-transfection) and mRNA arrays/qPCR hereby validating the reporter-based approach. In accordance, we find modification types (such as in Dharm1, W342, W362 and W363) previously reported to exhibit less off-targeting as evaluated by mRNA array to behave similarly in our luciferase assay. Furthermore the UNA modification initially identified using stable cell line reporters similarly reduces the well characterized off-target effects of siRNAs targeting endogenous targets (Figure 6). Thirdly, stable cell line reporters are suitable for HTS of many siRNAs at multiple concentrations, which is required to truly uncover siRNA potency/off-target correlations under non-saturating conditions relevant for applications in vivo. As an example, the investigations performed in this study would require the use of ~150 mRNA arrays per biological replicate, which is technically and financially challenging.

We find that chemical modifications of position 3 (using AENA, CLNA, CENA, HNA and EA) and in the positions 6–8 (using e.g HNA, HM and UNA) of the AS significantly reduced off-targeting showing that these distal seed region are particular susceptible for modifications to reduce off-targeting. In contrast central bases of the seed seem important for siRNA function in good agreement with these being positioned for nucleation of the mRNA target as evaluated by the crystal structure of Thermus thermophilus Ago2 (34). We do not know the precise mechanism behind reduced off-targeting by modifying position 3. Jackson and coworkers previously found 2’OMe modification of position 2 of the AS to reduce off-targeting and proposed that the strict size constraints of Ago2 to accommodate a 2’-OMe group at this position would affect conformational adjustments in RISC (16). We agree with this idea and speculate that AENA, CLNA and CENA, which are all LNAs, would similarly impose conformational constraint into RISC to reduce off-targeting potentials. Notably, HNA and EA-modification of position 3 exhibited even further reduced off-targeting at higher concentrations (Figures 2B and 3); the larger size of these nucleotide analogs as compared to RNA (Figure 2A) may similarly conformationally change RISC, or EA may simply thermodynamically destabilize seed-off-target interactions as for UNA modifications (see below). Collectively, this study reveals that several modifications of position 3 of the AS have interesting potentials that should be investigated further.

Most importantly, we find that modification of several positions of the AS seed using the destabilizing UNA greatly reduces off-targeting; particularly UNA-modification at position 7 efficiently reduced off-targeting without significantly compromising siRNA activity for all tested sequences. This observation is in good agreement with previous studies showing that position 7 of the siRNA AS is more tolerant to target site mismatches than other positions of the seed (12,35). It is well described that siRNA off-target effects primarily correlate with the thermodynamic stability of the interaction between the seed region of the guiding strand within RISC and complementary sites in the 3’ UTR of the target mRNA (12,17). Ui-Tei et al. (17) previously showed that full substitution of the AS seed by DNA (pos 1–8) reduced siRNA off-targeting arguably by lowering the thermostability of seed–target interactions. Very similarly, UNA destabilizes off-target RNA–RNA interactions to reduce the pool of potential off-target mRNAs within the cell and indeed we were unable to anneal UNA-modified ASs to a 9mer RNA target mimic, even at room temperature (Figure 5A). Hence, UNA-modification will increase the requirement for additional base pairing between the AS and mRNA to allow efficient RNAi thereby preferentially eliminating off-targeting that relies on partial complementarity.

We propose that UNA modification is particular suitable for lowering off-target effect by destabilizing seed off-target interactions; UNA-modification additively decrease the thermostability of siRNA duplexes by ~5°C per UNA monomer (29) thereby allowing dramatic lowering of RNA duplex stability at defined positions within the siRNA seed. Hereby base positions crucial for siRNA function, such as the central seed positions, can be left unmodified whereas positions important for off-target interactions, yet dispensable for siRNA function, can be strongly destabilized. We find that abasic dSpacer and SpacerC3 linkers at AS position 7 reduces siRNA potency in addition to off-targeting (Supplementary Figure S3). We therefore believe that UNA modification of position 7 will more efficiently reduce off-target and preserve siRNA potency than non-site-specific seed destabilization by e.g. DNA which will affect positions important for siRNA potency [see W363 Figure 3 and (17)]. Importantly, local destabilization allows even thermodynamically very weak seed region to be UNA-modified without significant reduction of siRNA potency (Figure 4B) suggesting that UNA-7 modification can be widely used in siRNA designs. However, as UNA modifications internally in siRNA duplexes lower the thermodynamic stability and thereby the nuclease resistance of siRNA duplexes (37) UNA modified ASs in combination with the sisiRNA design harboring
LNA-stabilized segmented passenger strands is one appealing option for applications in vivo. This will not only greatly enhance serum stability, yet also completely alleviate the contribution of the passenger strand to off-targeting (14).

In conclusion, we find that UNA-modification at position 7 of the AS represent the most potent modification for reducing off-targeting while preserving siRNA potency which may prove especially important in clinical settings in vivo where siRNA concentrations are expected to be low.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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Conflict of interest statement
J.W. and S.L.W. are co-founders of RiboTask ApS which is marketing UNA amides and UNA oligonucleotides.

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