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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 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 destabilizing 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 (1–5  $\mu$ mol 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 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 $\times$  Dharmacon 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

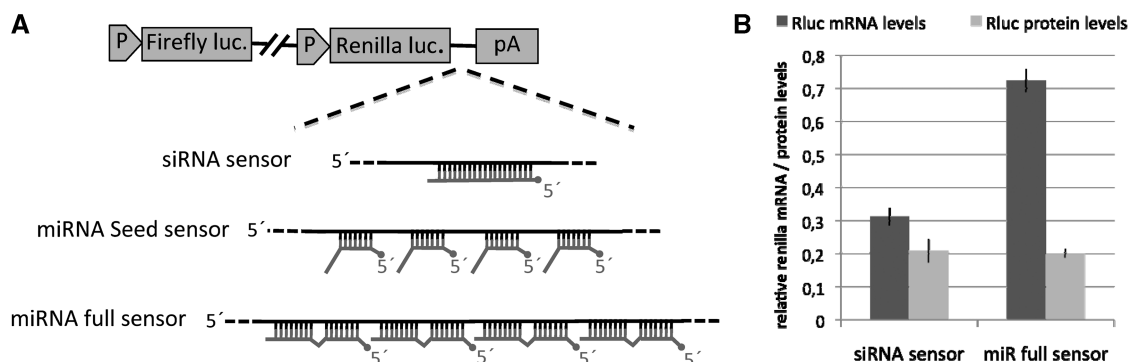
The human lung cancer cell line H1299 was grown in RPMI-1640 containing 10% FBS, 1% penicillin/streptomycin. Stable luciferase siRNA and miRNA seed/full reporters were generated by transfection and subsequent pyromycin selection (1  $\mu$ g/ml medium) of H1299 cells

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;

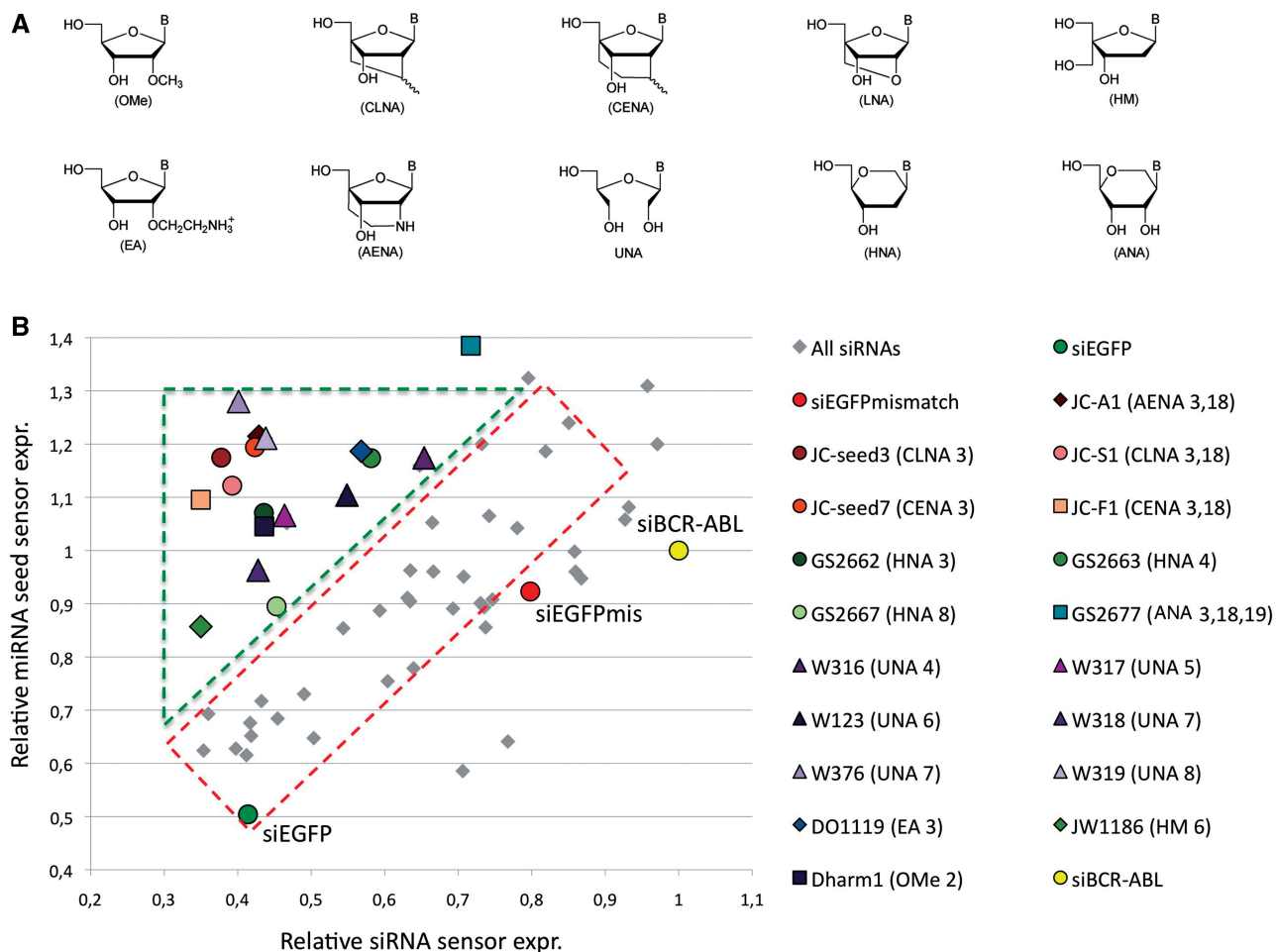
siSensor1: 5'-ACGACGTAAACGGCCACAAGTTTCG AATTC-3'; miRNA seed sensor1: 5'-AACCACAAGATA CTACTGAACCACAAGATACTACTGAACCACAAG ATACTACTGAACCACAAGA-3'; miRNA full sensor1: 5'-GTAATGAACCACAAGATACTACCGTAATGAA CCACAAGATACTAACGTAATGAACCACAAGATA CTACCGTAATGAACCACAAGA-3'; siSensor2: 5'-GG CAAGCTGACCCTGAAGTACCGGT-3'; miRNA seed sensor2: 5'-CCTGAAGTAATATTACATAAATAAAAA CCTGAAGTAATATTACATAAATAAACCTGAAG TAATATTACATAAATAAACCTGAAGT-3'; miRNA full sensor2: 5'-CAAGCACTGCCTGAAGTAT AGGCAAGCACTGCCTGAAGTATAGGCAAGCAC TGCCTGAAGTATAGGCAAGCACTGCCTGAAGT GCGGCC-3'; siSensor3: 5'-GCAGCAGCACTTCTTCA AGACCGGT-3'; miRNA seed sensor3: 5'-CTTTACAT AAATAAATAATCTTCAAAATCTACATAAATCTTCA AAATCTACATAATCTTCAAAATCTACATAATCTT CAACTAC-3'; miRNA full sensor3: 5'-CTACAGCAC ATAATCTTCAAACCTACAGCACATAATCTTCAA CTACAGCACATAATCTTCAAACCTACAGCACATA ATCTTCAAACCTAC-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<sup>®</sup> SYBR<sup>®</sup> Green qPCR SuperMix-UDG (Invitrogen) and the random hexamer-primed cDNA as template. The following primer sequences were used: rluc-F: 5'-TGCAGAAAGTT GGTCTGTGAGGCA, rLuc-R: 5'-TCTAGCCTTAAGA GCTGTAATTGAACTGG, GAPDH-F: 5'-GAAGGTG AAGGTCGGAGT, GAPDH-R: 5'-GAAGATGGTGA TGGGATTTC. Renilla luciferase mRNA levels were normalized to GAPDH mRNA levels and subsequently to cells transfected with an unrelated siRNA, siBCR-ABL. Relative quantification of mRNAs levels were done using the  $\Delta\Delta$ CT-method. In the experiments 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  $\mu$ l lipofectamine 2000 and 15 000 cells per well in a total volume of 125  $\mu$ l. The experiments performed in Figure 2B were 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.

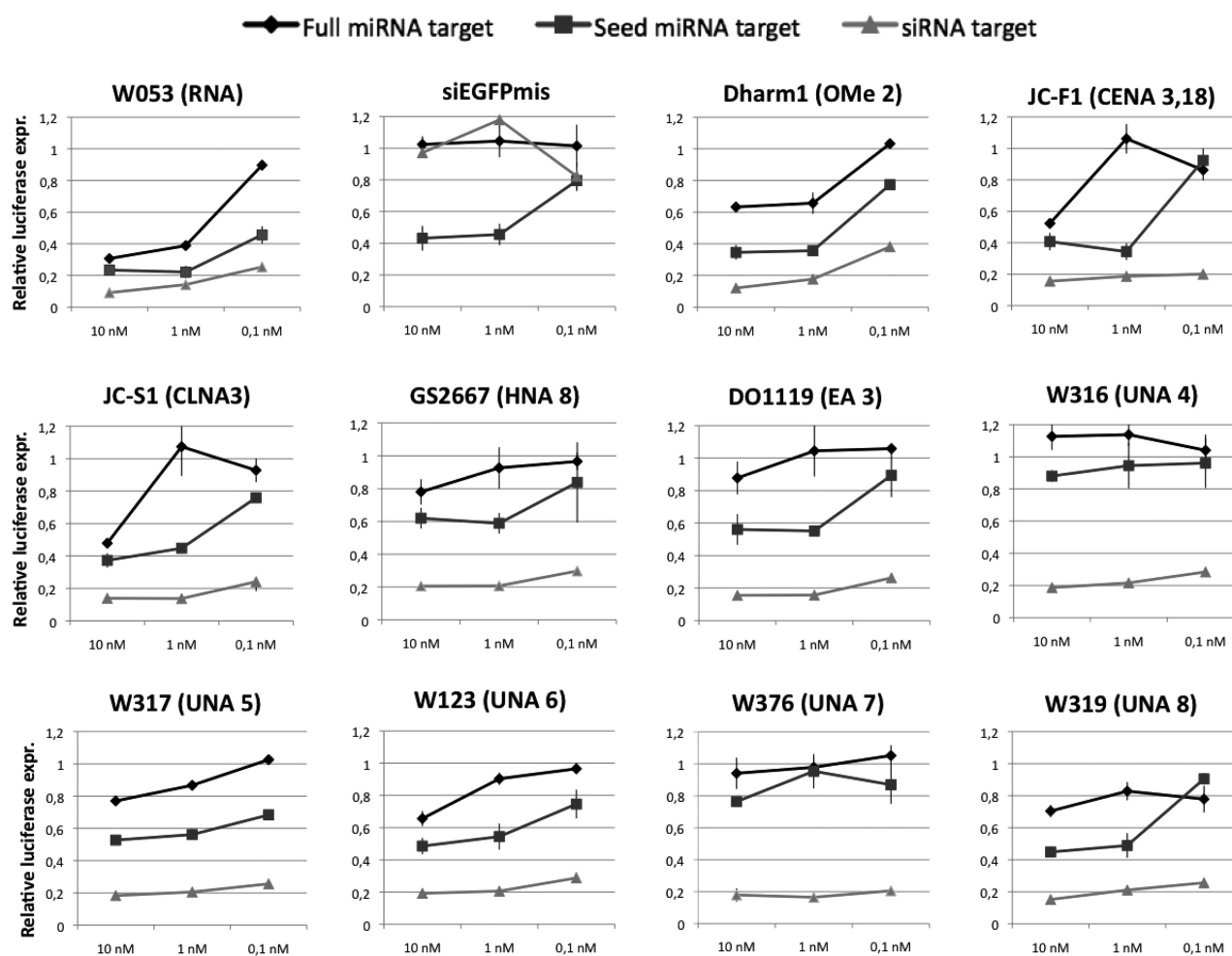


**Figure 1.** Development of siRNA and miRNA sensor cell lines to monitor siRNA off-target effects. (A) Schematic view of the luciferase based sensors used to monitor siRNA and off-target effects in stable cell lines. The siRNA sensor harbors a single target site in the Renilla 3' UTR perfectly complementary 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).





**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.

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 pairing 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°C for 1 min followed by 37°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™ 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

Biosystems): GRK4 (HS00178384\_m1); HIF1A (hs00153153\_m1); ICAM1 (hs99999152\_m1); TNFR1 (hs00533560\_m1); GAPDH (Hs99999905\_m1). Relative quantification of mRNAs levels were done by using the  $\Delta\Delta CT$ -method and all values were normalized to GAPDH mRNA levels.

## RESULTS

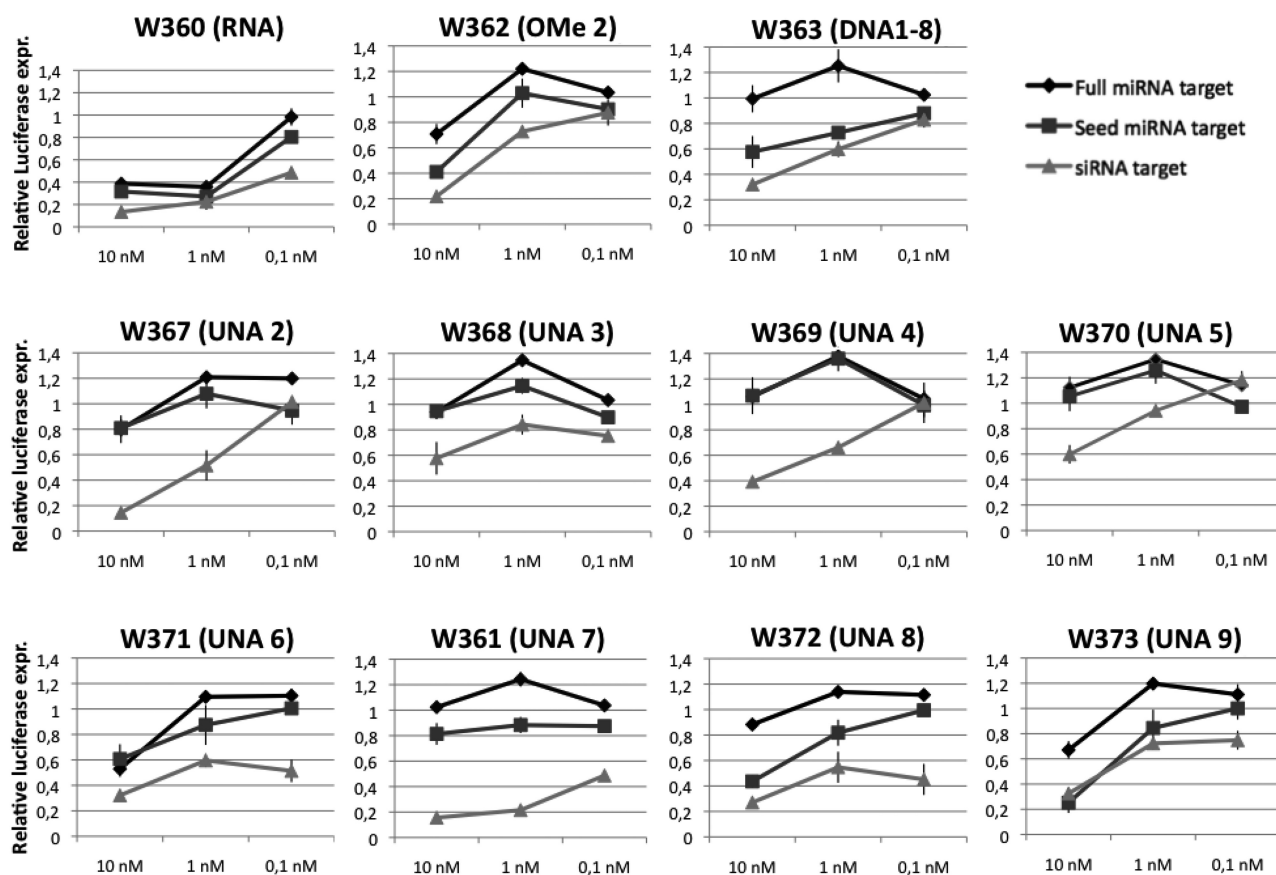
### Establishment of siRNA and off-target sensor cell lines

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

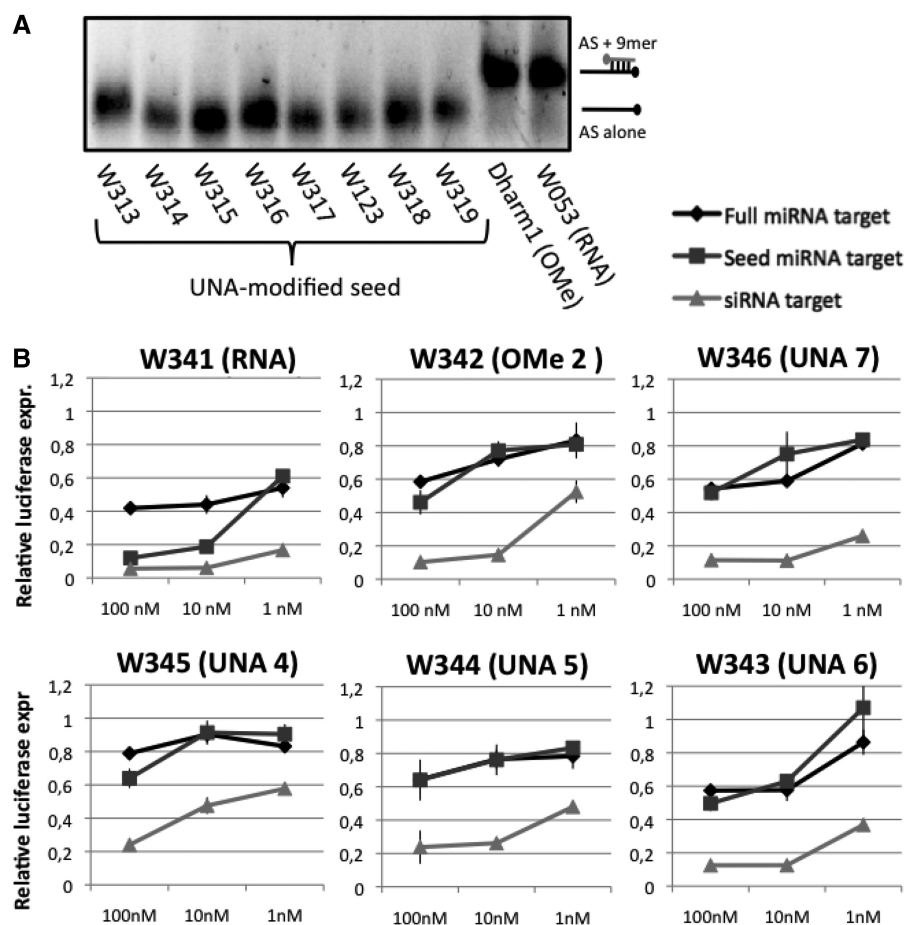
designed the miRNA full sensor having additional base pairs in the 3'-end (position 13–19 of the siRNA) in order to mimic very extensively base pairing off-targets (Figure 1A). The central mismatches in the miRNA sensors will expectably prevent Ago2-mediated cleavage of the Renilla luciferase mRNAs and will thereby lead to only slightly reduced mRNA levels, typically <2-fold (8,9,20), 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), altritol nucleic acid (ANA) (27), LNA (28), UNA (29), 2'-O-aminoethyl



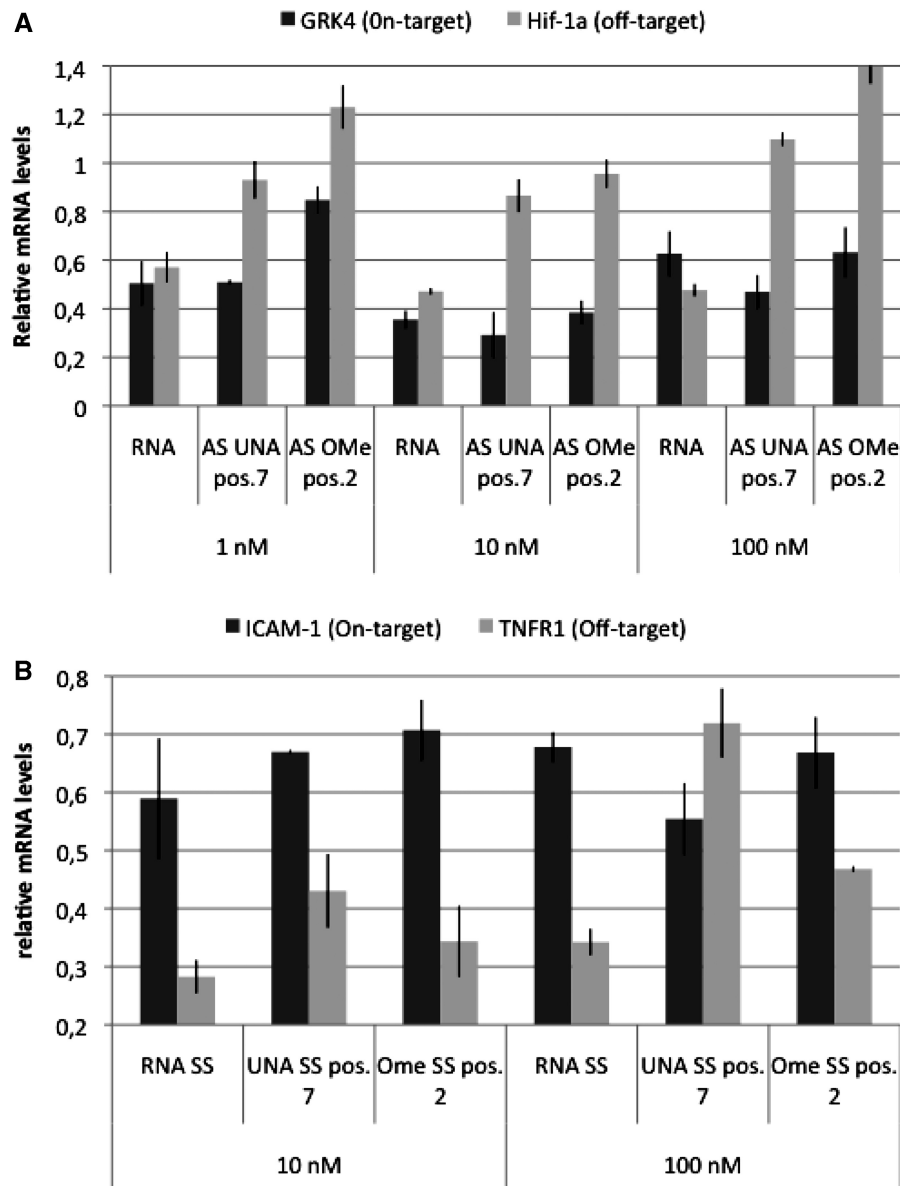
**Figure 4.** UNA-modification of AS position 7 dramatically reduces siRNA off-targeting while preserving siRNA potency. Evaluation of siRNA potency and off-targeting potential of selected ASs at 0.1, 1 and 10 nM concentrations using the siRNA sensor2 (light gray lines) and miRNA seed/full sensor2's (dark gray lines), respectively.



**Figure 5.** UNA-modification destabilizes (off-) target interactions but still supports the siRNA function of ASs with weak seed region. (A) UNA-modification of the AS seed region strongly destabilizes initial (off-) target interactions. UNA-modified ASs, OMe modified (Dharm1) and unmodified AS (W053) were annealed to a 13-mer RNA with sequence complementarity to position 1–9 of the ASs and analyzed by native gel electrophoresis. Sizes of annealed duplexes and single stranded ASs are indicated to the right. (B) Evaluation of siRNA potency and off-targeting potential of selected ASs at 0.1–10 nM concentrations using the siRNA sensor3 (light gray lines) and miRNA seed/full sensor3's (dark gray lines), respectively. The seed sequence of the siEGFP3 contains forms only two GC pairs with its target. ASs modified with UNA at position 7 further exhibits almost wild-type siRNA potency.

(EA) (30,31), 2',4'-carbocyclic-ENA-LNA (CENA), 2',4'-carbocyclic-LNA-locked nucleic acid (CLNA), 2'-deoxy-2'-N,4'-C-ethylene-locked nucleic acid (AENA) (32), 4'-C-hydroxymethyl-DNA (HM) (33) and 2'-O-methyl (OMe); (Table 1 and Figure 2B). 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),



**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-1 $\alpha$*  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 24h by qPCR. The presented values are normalized to values from cells transfected with the unrelated siEGFP1.

W123 (UNA position 6), W318 (UNA position 7), W376 (UNA position 7) and W319 (UNA pos 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 at 10 nM concentrations



**Table 1.** Overview of chemically modified ASs and SSs

Name	AS/SS	Modification	Sequence (5'-3')
W053	AS1	RNA	ACUUGUGGCCGUUUACGUCGC
W207	SS1	RNA	GACGUAAACGGCCACAAGUUC
siEGFPmis	SS1	RNA	GACCUAAU <sup>U</sup> CGGGCACA <sup>U</sup> GUUC
siEGFPmis	AS1	RNA	ACA <sup>U</sup> UGUG <sup>U</sup> CCCG <sup>A</sup> UUUAGGUCGC
JC-A1	AS1	AENA 3,18	ACU <sup>AENA</sup> UGUGGCCG <sup>U</sup> UUUACGU <sup>AENA</sup> CGC
JC-A2	AS1	AENA 4,18	ACUU <sup>AENA</sup> GUGGCCG <sup>U</sup> UUUACGU <sup>AENA</sup> CGC
JC-Seed4	AS1	CLNA 2	AC <sup>CLNA</sup> UUUGUGGCCG <sup>U</sup> UUUACGUCGC
JC-Seed3	AS1	CLNA 3	ACU <sup>CLNA</sup> UGUGGCCG <sup>U</sup> UUUACGUCGC
JC-S1	AS1	CLNA 3,18	ACU <sup>CLNA</sup> UGUGGCCG <sup>U</sup> UUUACGU <sup>CLNA</sup> CGC
JC-Seed2	AS1	CLNA 4	ACUU <sup>CLNA</sup> GUGGCCG <sup>U</sup> UUUACGUCGC
JC-Seed1	AS1	CLNA 6	ACUUGU <sup>CLNA</sup> GGCCG <sup>U</sup> UUUACGUCGC
JC-Seed8	AS1	CENA 2	AC <sup>CENA</sup> UUUGUGGCCG <sup>U</sup> UUUACGUCGC
JC-Seed7	AS1	CENA 3	ACU <sup>CENA</sup> UGUGGCCG <sup>U</sup> UUUACGUCGC
JC-F1	AS1	CENA 3,18	ACU <sup>CENA</sup> UGUGGCCG <sup>U</sup> UUUACGU <sup>CENA</sup> CGC
JC-Seed6	AS1	CENA 4	ACUU <sup>CENA</sup> GUGGCCG <sup>U</sup> UUUACGUCGC
JC-Seed5	AS1	CENA 6	ACUUGU <sup>CENA</sup> GGCCG <sup>U</sup> UUUACGUCGC
GS2660	AS1	HNA 1	A <sup>HNA</sup> CUUGUGCCG <sup>U</sup> UUUACGUCGCT <sup>DNA</sup>
GS2661	AS1	HNA 2	AC <sup>HNA</sup> UUUGUGCCG <sup>U</sup> UUUACGUCGCT <sup>DNA</sup>
GS2662	AS1	HNA 3	ACU <sup>HNA</sup> UGUGCCG <sup>U</sup> UUUACGUCGCT <sup>DNA</sup>
GS2663	AS1	HNA 4	ACUU <sup>HNA</sup> GUGCCG <sup>U</sup> UUUACGUCGCT <sup>DNA</sup>
GS2664	AS1	HNA 5	ACUUG <sup>HNA</sup> UGGCCG <sup>U</sup> UUUACGUCGCT <sup>DNA</sup>
GS2665	AS1	HNA 6	ACUUGU <sup>HNA</sup> GGCCG <sup>U</sup> UUUACGUCGCT <sup>DNA</sup>
GS2666	AS1	HNA 7	ACUUGUG <sup>HNA</sup> GCCG <sup>U</sup> UUUACGUCGCT <sup>DNA</sup>
GS2667	AS1	HNA 8	ACUUGUGG <sup>HNA</sup> CCG <sup>U</sup> UUUACGUCGCT <sup>DNA</sup>
GS2670	AS1	ANA 2	AC <sup>ANA</sup> UUUGUGCCG <sup>U</sup> UUUACGUCGCT <sup>DNA</sup>
GS2672	AS1	ANA 4	ACUUGU <sup>ANA</sup> GGCCG <sup>U</sup> UUUACGUCGCT <sup>DNA</sup>
GS2673	AS1	ANA 5	ACUUG <sup>ANA</sup> UGGCCG <sup>U</sup> UUUACGUCGCT <sup>DNA</sup>
GS2674	AS1	ANA 6	ACUUGU <sup>ANA</sup> GGCCG <sup>U</sup> UUUACGUCGCT <sup>DNA</sup>
GS2675	AS1	ANA 7	ACUUGUG <sup>ANA</sup> GCCG <sup>U</sup> UUUACGUCGCT <sup>DNA</sup>
GS2676	AS1	ANA 8	ACUUGUGG <sup>ANA</sup> CCG <sup>U</sup> UUUACGUCGCT <sup>DNA</sup>
GS2677	AS1	ANA 3,18,19	ACU <sup>ANA</sup> UGUGCCG <sup>U</sup> UUUACGUC <sup>ANA</sup> G <sup>ANA</sup> CT <sup>DNA</sup>
SWC2	AS1	EA 2	AC <sup>EA</sup> UUUGUGGCCG <sup>U</sup> UUUACGUCGC
DO1119	AS1	EA 3	ACU <sup>EA</sup> UGUGGCCG <sup>U</sup> UUUACGUCGC
DO1118	AS1	EA 4	ACUU <sup>EA</sup> GUGGCCG <sup>U</sup> UUUACGUCGC
SWG5	AS1	EA 5	ACUUG <sup>EA</sup> UGGCCG <sup>U</sup> UUUACGUCGC
DO1116	AS1	EA 6	ACUUGU <sup>EA</sup> GGCCG <sup>U</sup> UUUACGUCGC
SWG7	AS1	EA 7	ACUUGUG <sup>EA</sup> GCCG <sup>U</sup> UUUACGUCGC
SWG8	AS1	EA 8	ACUUGUGG <sup>EA</sup> CCG <sup>U</sup> UUUACGUCGC
SWC9	AS1	EA 9	ACUUGUGGC <sup>EA</sup> CG <sup>U</sup> UUUACGUCGC
SWC10	AS1	EA 10	ACUUGUGGCC <sup>EA</sup> GU <sup>U</sup> UUUACGUCGC
DO1110	AS1	EA 12	ACUUGUGGCCG <sup>EA</sup> UUUACGUCGC
DO1109	AS1	EA 13	ACUUGUGGCCG <sup>EA</sup> U <sup>U</sup> UACGUCGC
W259	AS1	LNA 1	A <sup>LNA</sup> CUUGUGCCG <sup>U</sup> UUUACGUCG <sup>LNA</sup> C <sup>LNA</sup>
W260	AS1	LNA 2	AC <sup>LNA</sup> UUUGUGCCG <sup>U</sup> UUUACGUCG <sup>LNA</sup> C <sup>LNA</sup>
W261	AS1	LNA 3	ACU <sup>LNA</sup> UGUGCCG <sup>U</sup> UUUACGUCG <sup>LNA</sup> C <sup>LNA</sup>
W262	AS1	LNA 4	ACUU <sup>LNA</sup> GUGCCG <sup>U</sup> UUUACGUCG <sup>LNA</sup> C <sup>LNA</sup>
W263	AS1	LNA 5	ACUUG <sup>LNA</sup> UGGCCG <sup>U</sup> UUUACGUCG <sup>LNA</sup> C <sup>LNA</sup>
W264	AS1	LNA 6	ACUUGU <sup>LNA</sup> GGCCG <sup>U</sup> UUUACGUCG <sup>LNA</sup> C <sup>LNA</sup>
W265	AS1	LNA 7	ACUUGUG <sup>LNA</sup> GCCG <sup>U</sup> UUUACGUCG <sup>LNA</sup> C <sup>LNA</sup>
W266	AS1	LNA 8	ACUUGUGG <sup>LNA</sup> CCG <sup>U</sup> UUUACGUCG <sup>LNA</sup> C <sup>LNA</sup>
W313	AS1	UNA 1	A <sup>UNA</sup> CUUGUGCCG <sup>U</sup> UUUACGUCG <sup>LNA</sup> C <sup>LNA</sup> U
W314	AS1	UNA 2	AC <sup>UNA</sup> UUUGUGCCG <sup>U</sup> UUUACGUCG <sup>LNA</sup> C <sup>LNA</sup> U
W315	AS1	UNA 3	ACU <sup>UNA</sup> UGUGCCG <sup>U</sup> UUUACGUCG <sup>LNA</sup> C <sup>LNA</sup> U
W316	AS1	UNA 4	ACUU <sup>UNA</sup> GUGCCG <sup>U</sup> UUUACGUCG <sup>LNA</sup> C <sup>LNA</sup> U
W317	AS1	UNA 5	ACUUG <sup>UNA</sup> UGGCCG <sup>U</sup> UUUACGUCG <sup>LNA</sup> C <sup>LNA</sup> U
W123	AS1	UNA 6	ACUUGU <sup>UNA</sup> GGCCG <sup>U</sup> UUUACGUCG <sup>LNA</sup> C <sup>LNA</sup> U
W318	AS1	UNA 7	ACUUGUG <sup>UNA</sup> GCCG <sup>U</sup> UUUACGUCG <sup>LNA</sup> C <sup>LNA</sup> U
W319	AS1	UNA 8	ACUUGUGG <sup>UNA</sup> CCG <sup>U</sup> UUUACGUCG <sup>LNA</sup> C <sup>LNA</sup> U
W376	AS1	UNA 7	ACUUGUG <sup>UNA</sup> GCCG <sup>U</sup> UUUACGUCGC
W380	AS1	dSpacer 7	ACUUGU-DSpacer-GCCG <sup>U</sup> UUUACGUCG <sup>LNA</sup> C <sup>LNA</sup> U
W381	AS1	SpacerC3 7	ACUUGU-SpacerC3-GCCG <sup>U</sup> UUUACGUCG <sup>LNA</sup> C <sup>LNA</sup> U
Dharm1	AS1	OMe 2	CU <sup>OME</sup> UGAAGAAGUCGUGCUGCT <sup>LNA</sup> T <sup>LNA</sup>
W340	SS3	RNA	GCAGCAGGACUUCUUAAGT <sup>LNA</sup> C <sup>LNA</sup>
W341	AS3	RNA	CUUGAAGAAGUCGUGCUGCT <sup>LNA</sup> T <sup>LNA</sup>
W342	AS3	OMe 2	CU <sup>OME</sup> UGAAGAAGUCGUGCUGCT <sup>LNA</sup> T <sup>LNA</sup>
W343	AS3	UNA 6	CUUGAA <sup>UNA</sup> GAAGUCGUGCUGCT <sup>LNA</sup> T <sup>LNA</sup>
W344	AS3	UNA 5	CUUGA <sup>UNA</sup> AGAAGUCGUGCUGCT <sup>LNA</sup> T <sup>LNA</sup>
W345	AS3	UNA 4	CUUG <sup>UNA</sup> AAGAAGUCGUGCUGCT <sup>LNA</sup> T <sup>LNA</sup>
W346	AS3	UNA 7	CUUGAA <sup>UNA</sup> AAGUCGUGCUGCT <sup>LNA</sup> T <sup>LNA</sup>
W360	AS2	RNA	ACUUCAGGGUCAGCUUGCCT <sup>LNA</sup> T <sup>LNA</sup> U

(continued)

Table 1. Continued

Name	AS/SS	Modification	Sequence (5'-3')
W361	AS2	UNA 7	ACUUCAG <sub>UNA</sub> GGUCAGCUUGCCT <sub>LNA</sub> T <sub>LNA</sub> U
W362	AS2	OMe 2	AC <sub>OMe</sub> UUCAGGGUCAGCUUGCCT <sub>LNA</sub> T <sub>LNA</sub> U
W363	AS2	DNA 1-8	A <sub>DNA</sub> C <sub>DNA</sub> U <sub>DNA</sub> U <sub>DNA</sub> C <sub>DNA</sub> A <sub>DNA</sub> G <sub>DNA</sub> G <sub>DNA</sub> GUCAGCUUGCCT <sub>LNA</sub> T <sub>LNA</sub> U
W364	SS2	RNA	GGCAAGCUGACCCUGAAGUUC
W367	AS2	UNA 2	AC <sub>UNA</sub> UUCAGGGUCAGCUUGCCT <sub>LNA</sub> T <sub>LNA</sub> U
W368	AS2	UNA 3	ACU <sub>UNA</sub> UCAGGGUCAGCUUGCCT <sub>LNA</sub> T <sub>LNA</sub> U
W369	AS2	UNA 4	ACU <sub>UNA</sub> CAGGGUCAGCUUGCCT <sub>LNA</sub> T <sub>LNA</sub> U
W370	AS2	UNA 5	ACUUC <sub>UNA</sub> AGGGUCAGCUUGCCT <sub>LNA</sub> T <sub>LNA</sub> U
W371	AS2	UNA 6	ACUUC <sub>UNA</sub> GGGUCAGCUUGCCT <sub>LNA</sub> T <sub>LNA</sub> U
W372	AS2	UNA 8	ACUUCAGG <sub>UNA</sub> GUCAGCUUGCCT <sub>LNA</sub> T <sub>LNA</sub> U
W373	AS2	UNA 9	ACUUCAGGG <sub>UNA</sub> UCAGCUUGCCT <sub>LNA</sub> T <sub>LNA</sub> U
GRK4 SS	SS	RNA	GACGUCUCUUCAGGCAGUUUU
GRK4 AS	AS	RNA	AACUGCCUGAAGAGACGUCUU
GRK4 UNA	AS	UNA 7	AACUGCC <sub>UNA</sub> UGAAGAGACGUCUU
GRK4 OMe	AS	OMe 2	AA <sub>UNA</sub> CUGCCUGAAGAGACGUCUU
ICAM1 AS	AS	RNA	GUGGCCUUCAGCAGGAGCUUU
ICAM1 SS	SS	RNA	AGCUCCUGCUGAAGGCCACUU
ICAM1 UNA	SS	UNA 7	AGCUCC <sub>UNA</sub> GCUGAAGGCCACUU
ICAM1 OMe	SS	OMe 2	AG <sub>OMe</sub> CUCCUGCUGAAGGCCACUU

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.

[Shown for CLNA (JC-S1) and CENA (JC-F1) in Figure 3 and see Supplementary Figure S1 for JC-A1 (AENA position 3,18), JC-seed3 (CLNA position 3), JC-seed7 (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 (W376 (shown in Figure 3)) and W318 (Supplementary Figure S1) exhibited only minimal KD of the miRNA sensors at 10 nM concentration while maintaining a very potent KD of the siRNA sensors even at 0.1 nM. UNA modification of position 5, 6 or 8 (W123, W317 and W319, respectively) also exhibited reduced off-targeting, yet to a lesser degree (Figure 3 and Supplementary Figure S1). Notably, the mismatch control siRNA (siEGFP<sub>mis</sub>) produced strong repression of the miRNA sensor at 1 and 10 nM although it contains two mismatches in the seed region (at position 3 and 8; Table 1) and did not reduce siRNA sensor expression. This shows that merely five matching bases in the seed region can produce potent off-targeting hereby substantiating the need for chemical modifications to circumvent extensive off-targeting.

We found that the strong off-target effect observed for siEGFP on the seed miRNA-sensor cell line was not due to the absence of an endogenous siRNA-target; upon co-transfecting an eGFP-expressing H1299 cell line with relevant siRNAs and the seed miRNA-reporter plasmid DNA we could evaluate siRNA effects and off-targeting in the same cell population by flow cytometry and dual luciferase assays, respectively. As expected, siEGFP exhibits strong off-target repression of the co-transfected

miRNA-sensor at all concentrations 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 the 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 little 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

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 weak 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 a SpacerC3 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-1 $\alpha$*  mRNA through a 7-nt 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-1 $\alpha$*  off-targeting we transfected H1299 cells with siRNAs at 1, 10 and 100 nM concentrations and evaluated GRK4 (on-target) and *hif-1 $\alpha$*  (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-1 $\alpha$*  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 an 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 with 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 thermodynamical stability of siRNA duplexes by 5–8°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 siRNA 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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