

A large-scale chemical modification screen identifies design rules to generate siRNAs with high activity, high stability and low toxicity

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ABSTRACT

The use of chemically synthesized short interfering RNAs (siRNAs) is currently the method of choice to manipulate gene expression in mammalian cell culture, yet improvements of siRNA design is expectably required for successful application *in vivo*. Several studies have aimed at improving siRNA performance through the introduction of chemical modifications but a direct comparison of these results is difficult. We have directly compared the effect of 21 types of chemical modifications on siRNA activity and toxicity in a total of 2160 siRNA duplexes. We demonstrate that siRNA activity is primarily enhanced by favouring the incorporation of the intended antisense strand during RNA-induced silencing complex (RISC) loading by modulation of siRNA thermodynamic asymmetry and engineering of siRNA 3'-overhangs. Collectively, our results provide unique insights into the tolerance for chemical modifications and provide a simple guide to successful chemical modification

of siRNAs with improved activity, stability and low toxicity.

INTRODUCTION

The use of RNA interference (RNAi)-based strategies has recently become the technique of choice to silence gene expression in mammalian cell culture. In typical strategies, 21-bp dsRNA molecules, termed short interfering RNAs (siRNAs), with perfect complementarity to the target RNA are used as experimental triggers of RNAi (1). Upon delivery into the cell, siRNAs are incorporated into the RNA-induced silencing complex (RISC) containing the signature component of the RNAi machinery, Argonaute 2 (Ago2) (2–4). The siRNA strand containing the thermodynamically less stable 5'-end is preferentially incorporated as the guiding strand of RISC (5,6), while the non-guiding sense strand (SS) of the siRNA duplex is cleaved by Ago2 and liberated (7–9). This generates the activated RISC* containing the guiding antisense strand (AS), which binds the complementary target RNAs and leads to their cleavage by Ago2 (10). Whereas unmodified siRNAs are highly efficient in cell culture, chemical

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modification is generally considered a prerequisite for fulfilling the potential of siRNAs *in vivo* and indeed successful experiments in animal models have relied on injection of chemically modified siRNAs (11–13). In particular, the half-life of unmodified siRNAs *in vivo* is in the range of minutes, but this can be significantly improved by chemical modifications, albeit often at the price of reduced siRNA activity (14–18). In addition, chemical modification of siRNAs may be required for delivery strategies, e.g. by conjugation to lipids (11,19), membrane-penetrating peptides (20) or RNA aptamer sequences (21). Furthermore, modified siRNAs with superior potency will reduce the dose required for gene silencing (14,22,23), and specific chemical modifications can minimize siRNA side-effects, such as the induction of recipient immune responses and inherent off-targeting effects (24–26). Therefore, a primary objective in the optimization of siRNA performance through chemical modification is to identify the type and position of specific alterations that can be introduced without compromising siRNA activity. A direct comparison of the various chemistries used for siRNA optimization is currently hampered by the fact that existing studies evaluate isolated chemistries in a variety of assays and sequence contexts. In addition, multiple chemistries are rarely included in the same siRNA duplex rendering identification of positive or negative synergy impossible.

In this study, we have made a direct comparison of the impact of 21 different types of chemical modifications on siRNA efficiency and cell viability using a total of 2160 siRNAs. The results from this extensive screen provide important insights into where and how siRNA strands can be modified by particular chemistries and how chemistries can be combined; moreover it allows us to formulate design rules to create superior siRNAs in terms of activity, stability and toxicity.

MATERIALS AND METHODS

Cell culture

HeLa cells stably expressing eGFP (HeLa-eGFP) were obtained by transfection with pEGFP-C1 (Clontech Laboratories, USA). A clone of the transfected pool was derived by G418 selection and cultivated in D-MEM (GIBCO-Invitrogen Corporation, USA) containing 10% FBS (foetal bovine serum) and 1% penicillin/streptomycin. The human lung cancer cell line H1299 was grown in RPMI-1640 (GIBCO) containing 10% FBS and 1% penicillin/streptomycin.

Synthesis of oligonucleotides

The phosphoramidites were incorporated into the siRNA sequence through solid-phase DNA/RNA synthesis (27) on an automated DNA/RNA synthesizer (for structures of chemically modified nucleotide monomers, see Figure 1). For a standard RNA synthesis cycle (1–5 μ mol scale), O^{2'}-TBDMS protected RNA phosphoramidites and common reagents were used and the stepwise coupling yield of all monomers was >99%. For incorporation of modified nucleotides, a coupling time of 10 min was used.

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. UNA (unlocked nucleic acid), LNA (locked nucleic acid) and ALN (α -L-LNA) modified oligonucleotides were obtained by using commercially available UNA (www.ribotask.com), LNA (www.exiqon.com) and α -L-LNA (www.exiqon.com). The UNA phosphoramidites were synthesized as O^{2'}-TBDMS derivatives by an optimized version of the published procedure for synthesis of the thymine monomer (28). The chemical synthesis of the remaining modified phosphoramidites is previously described; HM (4'-C-hydroxymethyl-DNA) (29), ADA (2'-N-adamantylmethylcarbonyl-2'-amino-LNA) (Babu, B.W. *et al.*, Manuscript in preparation), PYR (2'-N-pyren-1-ylmethyl-2'-amino-LNA) (30), EA (2'-aminoethyl), GE (2'-guanidinoethyl), CE (2'-cyanoethyl) (31,32), AP (2'-aminopropyl) (33), OX (oxetane-LNA) [(34) and references therein], CLNA (2',4'-carbocyclic-LNA-locked nucleic acid), CENA (2',4'-carbocyclic-ENA-locked nucleic acid) (35), AENA (2'-deoxy-2'-N,4'-C-ethylene-LNA) (36), OME (2'-O-methyl), F (2'-fluoro) (22), ANA (altritol nucleic acid) (37), HNA (hexitol nucleic acid) (38), AEM (2'-aminoethoxymethyl) and APM (2'-aminopropoxy-methyl) (39).

Evaluation of siRNA effect on eGFP expression and cell viability

A library of 2160 siRNAs was derived by annealing 48 ASs and 45 SSs in Tris–Borate–EDTA buffer (Ambion Applied Biosystem Inc., USA) by heating to 90°C for 2 min and slow cooling to 25°C for 45 min using a Gene Amp-R PCR System 9700 thermocycler (Applied Biosystems, USA). HeLa-eGFP were plated in a 384-well format in 50 μ l complete medium 24 h prior to transfection with 10 nM siRNAs (final concentration) complexed with 0.17 μ l INTERFERin/50 μ l medium (Polyplus-transfection SA, France) using an automated robotic platform (EVO-Freedom, TECAN Ltd, Switzerland). Cells were fixed 72 h post-transfection with 3.7% paraformaldehyde (PFA) and stained with a mix of 4,6-diamidino-2-phenylindole (DAPI) and SytoBlue 42 for nuclear and cytosolic counter-stain and imaged using an automated confocal microscope (Opera, PerkinElmer Cellular Technologies Germany GmbH) equipped with a 20 \times water immersion objective. Images were analysed in a blind manner using automated image analysis software acquiring 10 fields per well corresponding to \sim 1000 cells (Acapella, PerkinElmer Cellular Technologies Germany GmbH). Cell number, nuclei size and eGFP intensity data were analysed using the R-Project statistical package (40). The eGFP intensity threshold was calculated by taking the mean of the values detected in the positive controls in each plate plus three times the standard deviation; eGFP values below this threshold point were considered as eGFP down-regulation. The results represent average values from triplicate assays repeated twice. eGFP expression was normalized to cells transfected with siEGFP mismatch control (Dharmacon-, Dharmacon,

Inc.) and cell viability was normalized to cells treated with transfection reagent alone.

siRNA stability assay

Annealed siRNAs were incubated at 37°C in 80% FBS in D-MEM (Gibco) for the indicated time. Aliquots of 5 µl (each containing 20 pmol of siRNA) were diluted in 25 µl 1.2× TBE loading buffer (1.2× TBE, 10% glycerol, bromophenol blue) and snap frozen on dry ice. Samples were run on a 6% agarose gel and stained using SYBR Gold® (Invitrogen).

Dual-luciferase assay

The reporter constructs pISO_{AS-target} and pISO_{SS-target} are described previously (41). H1299 cells were plated in 48-well plates in RPMI supplemented with 10% FBS and grown to 40–60% confluency overnight. pISO_{AS-target} and pISO_{SS-target} (25 ng) were co-transfected with 0.2 ng pRluc-N2 (Perkin–Elmer) and siRNA duplexes (5–50 nM final concentration) by simultaneous use of 0.5 µl TransIT-LT1 (Mirus) and 0.5 µl TransIT-TKO (Mirus) according to manufacturer's protocol. Luciferase activity was measured 48 h post-transfection using the 'Dual-luciferase reporter assay system' (Promega) according to the manufacturer's protocol on a FLUOstar luminometer (BMG labtech); firefly luciferase signals (sample) were normalized to the renilla luciferase signals (transfection control).

RESULTS

We have evaluated the impact of 21 different types of chemical modifications on the activity and toxicity of a standard 21–22 bp siRNA targeting eGFP using all combinations of 48 ASs and 45 SSs, thereby generating a total of 2160 chemically different siRNA duplexes (see Table 1 for AS and SS sequences). The modifications can be broadly categorized into 2'-substituted RNAs (OMe, F, DNA, AEM, APM, EA, AP, CE, GE), 4'-modified RNAs (HM), Locked RNAs (LNA, ALN, ADA, PYR, OX, AENA, CENA, CLNA) and RNAs with radical modifications of the ribose sugar ring (UNA, ANA and HNA; Figure 1; see 'Material and Methods' section for full names). In brief, HeLa cells stably expressing eGFP were transfected with siRNA duplexes (10 nM final concentration), and eGFP levels and cell viability were evaluated 72 h post-transfection.

Modifications are well tolerated in the SS

We initially evaluated the impact of chemical modification on siRNA activity for all ASs and SSs in combination with an unmodified RNA strand (SS and AS termed W207 and W053, respectively). Modifications in the SS were well tolerated, and even heavily modified SSs supported knock down (KD) of eGFP levels to below 20% [e.g. HNA (GS2536), AEM (GS2543), APM (GS2548), HM (W044), OMe/F (JC5) and LNA (W037)] (Figure 2a and Table 1). Only in a few cases did introduction of multiple modifications result in reduced silencing potency, as seen for ANA (GS2366), HNA (GS2370), an

additional UNA residue (W130) and full substitution using DNA (W007) and DNA/LNA (W008, W009). Bulky modifications were relatively well tolerated in the SS [e.g. ADA (W069), AEM (GS2542, GS2543) and APM (GS2547, GS2548)] as opposed to in the AS (see below). Interestingly, thermodynamic stabilization of the siRNA duplex by LNA could be achieved via SS modification, whereas a similar level of LNA modification in the AS abolished siRNA activity (compare SS W037 and AS W010; Figure 2). This illustrates that the SS can be functionalized by extensive modifications as long as the RNA duplex structure is not grossly distorted.

Position-dependent tolerance for AS modification

Modification of the AS had a strong impact on siRNA silencing activity with the most efficient ASs being only modestly modified (Figure 2b and Table 1). Accordingly, extensive modification reduced activity [e.g. HM (JW1187), LNA (W010), ANA (GS2373) and HNA (GS2540)] with the notable exception of the fully OMe/F-substituted AS (JC10). We generally found bulky modifications to be poorly tolerated in both the AS seed region [e.g. ADA (W068), PYR (W096), Figure 2b; AEM and APM in position 3, data not shown] and the 3'-end [e.g. ADA (W095), PYR (W097), AEM (GS2549), APM (GS2544), OMe/EA (DO1002)], presumably due to helix distortion or altered strand selection.

The AS seed region (positions 2–8) guides the initial target recognition by RISC* (42), and modifications in this region should therefore influence silencing activity. We found that single modifications in the seed region were generally well tolerated for non-bulky groups that do not strongly influence the thermodynamic stability [e.g. HNA (GS2538, GS2539), ANA (GS2378), OMe-(DHARM1), CENA (JC-F1, JC-F2, JC-F3) and HM (JW1186), Figure 2b and Supplementary Figure 1]. In contrast, modifications that stabilize the seed region, such as LNA (Supplementary Figure 1) and AENA (JC-A1, JC-A2 and JC-A3; Figure 2b) had a negative effect on silencing activity, presumably by altering strand selection or seed–target interactions. The strongly destabilizing UNA was better tolerated in the seed, but reduced silencing activity when introduced near the 5'-end where it could destabilize AS–target interactions (Figure 2b, compare W124 and W123, and Supplementary Figure 1). We also found some sensitivity to modification in the central positions of the AS, which must be capable of perfect base-pairing to the target to allow cleavage by Ago2 (7); UNA (W126) and alternating OMe/F (JC10) were well tolerated, while HNA and ANA impaired silencing (GS2373 vs. GS2378 and GS2540, Figure 2b). As expected, most modifications were well tolerated in the AS 3'-region [e.g. UNA (W125), HNA (GS2537), HM (W042), LNA (W075) F/OMe (JC10)] and 3'-overhang [e.g. ANA (GS2372), HNA (GS2537), ALN (W047), EA (JE1001), single HM (W059) and LNA (W006)], although UNA- (W127), double HM- (W054) and LNA modifications (W180) decreased silencing activity slightly (Figure 2b).

Table 1. Overview of chemically modified ASs and SSs

Name	AS/SS	Modification	eGFP levels ^a	Viability ^a	Sequence (5'-3')
DHARM1	AS	OMe	0.24 ± 0.02	0.82 ± 0.11	AC _{OMe} UUGUGGCCGUUUACGUCGC
DO1001	AS	OMe/EA	0.20 ± 0.04	0.88 ± 0.11	A _{OMe} CUUGUGGCCGUUUACGUCGC _{EA} U
DO1002	AS	OMe/CE	0.49 ± 0.06	1.01 ± 0.24	A _{OMe} CUUGUGGCCGUUUACGU _{CE} CGCU
GS2371	AS	ANA	0.41 ± 0.03	0.58 ± 0.15	A _{ANA} C _{ANA} UUGUGGCCGUUUACGUCGC
GS2372	AS	ANA	0.11 ± 0.01	0.59 ± 0.08	ACUUGUGGCCGUUUACGUCG _{ANA} C _{ANA} U
GS2373	AS	ANA	1.12 ± 0.08	0.73 ± 0.19	ACUUG _{ANA} UGG _{ANA} CCG _{ANA} UUU _{ANA} ACG _{ANA} UCG _{ANA} CU
GS2378	AS	ANA	0.25 ± 0.03	0.51 ± 0.07	ACU _{ANA} UGU _{ANA} GGCCG _{ANA} UUU _{ANA} ACGU _{ANA} CG _{ANA} C _{ANA} U
GS2537	AS	HNA/DNA	0.19 ± 0.02	0.57 ± 0.04	ACUUGUGGCCGUUUACGU _{HNA} C _{HNA} G _{HNA} T _{DNA}
GS2538	AS	HNA/DNA	0.21 ± 0.04	0.48 ± 0.07	AC _{HNA} UUGUGGCCGUUUACGU _{HNA} C _{HNA} G _{HNA} T _{DNA}
GS2539	AS	HNA/DNA	0.20 ± 0.02	0.80 ± 0.12	ACU _{HNA} UGUGGCCGUUUACGU _{HNA} C _{HNA} G _{HNA} T _{DNA}
GS2540	AS	HNA/DNA	0.63 ± 0.08	0.71 ± 0.12	AC _{HNA} UUGUGGC _{HNA} CGUUUACGU _{HNA} C _{HNA} G _{HNA} T _{DNA}
GS2544	AS	AEM/DNA	0.59 ± 0.06	0.94 ± 0.12	ACUUGUGGCCGUUUACGU _{AEM} CAEMGT _{DNA}
GS2549	AS	APM/DNA	0.87 ± 0.11	0.90 ± 0.19	ACUUGUGGCCGUUUACGU _{APM} C _{APM} GT _{DNA}
JC10	AS	F/OMe	0.32 ± 0.03	0.70 ± 0.28	A _F C _{OMe} U _F U _{OMe} G _F U _{OMe} G _F G _{OMe} C _F C _{OMe} G _F U _{OMe} U _F U _{OMe} A _F C _{OMe} G _F U _{OMe} C _F G _{OMe} C _F
JC-A1	AS	AENA	0.26 ± 0.02	0.88 ± 0.18	ACU _{AENA} UGUGGCCGUUUACGU _{AENA} CGC
JC-A2	AS	AENA	0.63 ± 0.05	0.55 ± 0.15	ACUU _{AENA} GUGGCCGUUUACGU _{AENA} CGC
JC-A3	AS	AENA	0.69 ± 0.11	1.05 ± 0.18	ACU _{AENA} U _{AENA} GUGGCCGUUUACGU _{AENA} CGC
JC-F1	AS	CENA	0.10 ± 0.02	0.87 ± 0.17	ACU _{CENA} UGUGGCCGUUUACGU _{CENA} CGC
JC-F2	AS	CENA	0.18 ± 0.05	0.80 ± 0.19	ACUU _{CENA} GUGGCCGUUUACGU _{CENA} CGC
JC-F3	AS	CENA	0.19 ± 0.04	1.08 ± 0.12	ACU _{CENA} U _{CENA} GUGGCCGUUUACGU _{CENA} CGC
JC-S1	AS	CLNA	0.12 ± 0.01	0.80 ± 0.12	ACU _{CLNA} UGUGGCCGUUUACGU _{CLNA} CGC
JC-S2	AS	CLNA	0.28 ± 0.01	0.65 ± 0.16	ACUU _{CLNA} GUGGCCGUUUACGU _{CLNA} CGC
JC-S3	AS	CLNA	0.31 ± 0.04	0.86 ± 0.08	ACU _{CLNA} U _{CLNA} GUGGCCGUUUACGU _{CLNA} CGC
JE1001	AS	EA	0.13 ± 0.01	0.62 ± 0.17	ACUUGUGGCCGUUUACGUCG _{EA} C _{EA} U
JH1001	AS	AP	0.45 ± 0.09	0.78 ± 0.17	A _{AP} CUUGUGGCCGUUUACGUCGCU
JW1186	AS	HM/LNA	0.10 ± 0.04	0.95 ± 0.22	ACUUGT _{HM} GGCCGUUUACGUCG _{LNA} C _{LNA} U
JW1187	AS	HM/LNA	0.76 ± 0.13	1.01 ± 0.25	ACT _{HM} UGT _{HM} GGCCGUUT _{HM} ACGT _{HM} CG _{LNA} C _{LNA} U
W006	AS	LNA	0.09 ± 0.01	0.49 ± 0.11	ACUUGUGGCCGUUUACGUCG _{LNA} C _{LNA} U
W010	AS	LNA	0.54 ± 0.08	0.67 ± 0.07	ACT _{LNA} UGT _{LNA} GGCCGUUT _{LNA} ACGT _{LNA} CG _{LNA} C _{LNA} U
W042	AS	HM	0.18 ± 0.03	0.44 ± 0.09	ACUUGUGGCCGUUUACGUC _{HM} G _{HM} U
W047	AS	ALN	0.14 ± 0.03	0.46 ± 0.04	ACUUGUGGCCGUUUACGUCG _{ALN} U
W053	AS		0.14 ± 0.02	0.43 ± 0.09	ACUUGUGGCCGUUUACGUCGC
W054	AS	HM	0.33 ± 0.06	0.69 ± 0.12	ACUUGUGGCCGUUUACGUCT _{HM} T _{HM} U
W059	AS	HM	0.18 ± 0.02	0.72 ± 0.14	ACUUGUGGCCGUUUACGUCGT _{HM} U
W068	AS	ADA/LNA	0.54 ± 0.11	0.64 ± 0.12	ACUUGT _{ADA} GGCCGUUUACGUCG _{LNA} C _{LNA} U
W075	AS	LNA	0.14 ± 0.02	0.86 ± 0.15	ACUUGUGGCCGUUUACGUC _{LNA} G _{LNA} C
W095	AS	ADA/LNA	0.36 ± 0.04	0.78 ± 0.15	ACUUGUGGCCGUUUACGT _{ADA} CG _{LNA} C _{LNA} U
W096	AS	PYR/LNA	0.38 ± 0.06	0.52 ± 0.15	ACUUGT _{PYR} GGCCGUUUACGUCG _{LNA} C _{LNA} U
W097	AS	PYR/LNA	0.46 ± 0.05	0.73 ± 0.19	ACUUGUGGCCGUUUACGT _{PYR} CG _{LNA} C _{LNA} U
W106	AS	OMe	1.11 ± 0.12	0.73 ± 0.31	A _{OMe} C _{OMe} U _{OMe} U _{OMe} G _{OMe} U _{OMe} G _{OMe} G _{OMe} C _{OMe} C _{OMe} G _{OMe} U _{OMe} Me _{OMe} U _{OMe} A _{OMe} C _{OMe} G _{OMe} C _{OMe} C _{OMe} C _{OMe} U _{OMe}
W123	AS	UNA/LNA	0.11 ± 0.02	1.08 ± 0.22	ACUUGU _{UNA} GGCCGUUUACGUCG _{LNA} C _{LNA} U
W124	AS	UNA/LNA	0.16 ± 0.05	0.89 ± 0.22	ACU _{UNA} UGUGGCCGUUUACGUCG _{LNA} C _{LNA} U
W125	AS	UNA/LNA	0.11 ± 0.03	0.89 ± 0.13	ACUUGUGGCCGUUUACGU _{UNA} CG _{LNA} C _{LNA} U
W126	AS	UNA/LNA	0.13 ± 0.03	0.72 ± 0.15	ACUUGUGGCCG _{UNA} UUACGUCG _{LNA} C _{LNA} U
W127	AS	UNA	0.26 ± 0.05	0.66 ± 0.12	ACUUGUGGCCGUUUACGUCG _{UNA} U
W128	AS	UNA	0.56 ± 0.11	0.80 ± 0.23	ACU _{UNA} UGUGGCCGUUUACGUCG _{CUNA} U
W180	AS	LNA	0.27 ± 0.04	0.78 ± 0.22	ACUUGUGGCCGUUUACGUCG _{LNA} C _{LNA}
W209	AS	LNA	1.18 ± 0.23	0.88 ± 0.22	ACT _{LNA} UGT _{LNA} GGCCGUUT _{LNA} ACGT _{LNA} CG _{LNA} C _{LNA}
DO003	SS	EA	0.10 ± 0.01	0.67 ± 0.10	GACGUAACGGCCACA _{EA} GUUC
DO004	SS	GE	0.12 ± 0.01	0.62 ± 0.05	GACGUAACGGCCACA _{GE} GUUC
GS2332	SS	HNA	0.15 ± 0.01	0.55 ± 0.12	GA _{HNA} CGUAAA _{HNA} CGGCCA _{HNA} CAA _{HNA} GUUC
GS2366	SS	HNA	0.40 ± 0.06	0.65 ± 0.60	GACGU _{HNA} AAA _{HNA} CGGCCA _{HNA} CAA _{HNA} GUUCT _{DNA}
GS2368	SS	ANA/DNA	0.11 ± 0.02	0.71 ± 0.11	G _{ANA} A _{ANA} CGUAAACGGCCACAAGUUCT _{DNA}
GS2369	SS	ANA/DNA	0.10 ± 0.01	0.64 ± 0.18	GACGUAACGGCCACAAG _{ANA} U _{ANA} UUCT _{DNA}
GS2370	SS	ANA/DNA	0.31 ± 0.03	0.73 ± 0.22	GACGU _{ANA} AAA _{ANA} CGG _{ANA} CCA _{ANA} CAA _{ANA} GUUCT _{DNA}
GS2374	SS	ANA	0.16 ± 0.03	0.60 ± 0.14	GACGUAACGGCCACAAGU _{ANA} C _{ANA} U
GS2383	SS	HNA	0.11 ± 0.01	0.78 ± 0.16	GACGUAACGGCCACAAG _{HNA} U _{HNA} C
GS2534	SS	HNA/DNA	0.11 ± 0.01	0.58 ± 0.15	G _{HNA} A _{HNA} C _{HNA} GUAACGGCCACAAG _{HNA} U _{HNA} U _{HNA} T _{DNA}
GS2535	SS	HNA/DNA	0.19 ± 0.03	0.64 ± 0.09	GACGU _{HNA} AAA _{HNA} CGG _{HNA} CCA _{HNA} CAAGUUT _{DNA}
GS2536	SS	HNA/DNA	0.13 ± 0.02	0.74 ± 0.12	GACGU _{HNA} AAA _{HNA} CGG _{HNA} CCA _{HNA} CAAGU _{HNA} U _{HNA} T _{DNA}
GS2542	SS	AEM/DNA	0.11 ± 0.01	0.66 ± 0.15	GACGUAACGGCCACAAG _{AEM} U _{AEM} T _{DNA}
GS2543	SS	AEM/DNA	0.11 ± 0.02	0.62 ± 0.20	GAC _{AEM} GU _{AEM} AAAC _{AEM} GGCCACAAG _{AEM} U _{AEM} T _{DNA}
GS2547	SS	APM/DNA	0.12 ± 0.01	0.64 ± 0.18	GACGUAACGGCCACAAG _{APM} U _{APM} T _{DNA}
GS2548	SS	APM/DNA	0.17 ± 0.02	0.61 ± 0.08	GAC _{APM} GU _{APM} AAAC _{APM} GGCCACAAG _{APM} U _{APM} T _{DNA}
JC1	SS	OX	0.11 ± 0.01	0.63 ± 0.20	GACGUAACGGCCACA _{OX} AG _{OX} UUC
JC2	SS	OX	0.13 ± 0.02	0.81 ± 0.19	GA _{OX} CG _{OX} UAAACGGCCACAAGUUC

(continued)

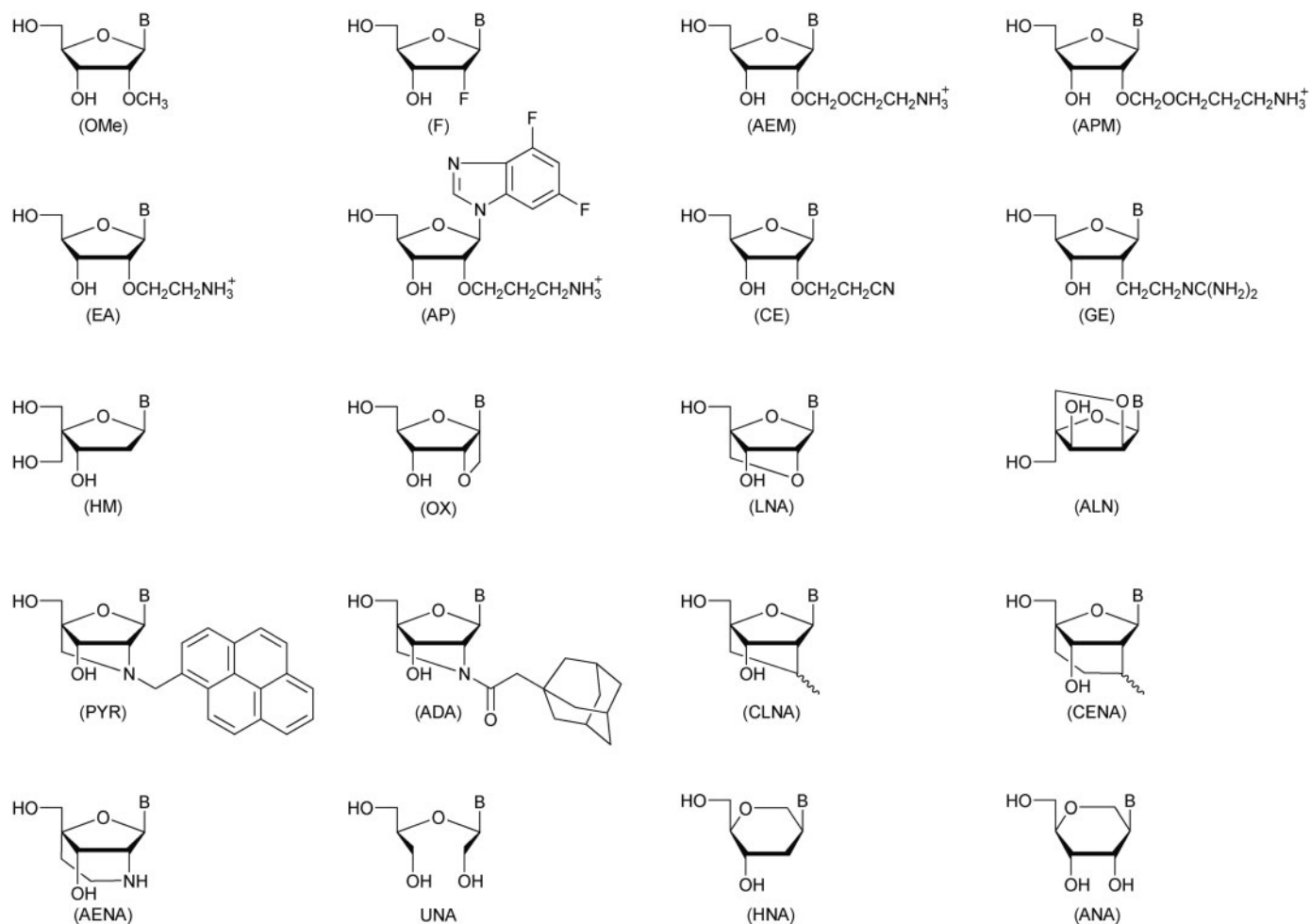


Figure 1. Structural overview of chemical modifications investigated.

(GS2373)]. The activity of certain ASs was improved by several optimal SSs (e.g. W097, GS2549, JC-A2 and GS2544) whereas other ASs were only rescued by one or few SSs (e.g. W209 by GS2542, JC-A3 by W194 and W106 by GS3543, Figure 3 and Supplementary Table 1). Conversely, certain ASs allowed the use of heavily modified SSs that failed to support RNAi when combined with an unmodified AS; the fully LNA/DNA substituted SS W009, produced a potent ~80% KD in combination with the UNA-modified AS W123 but gave only a 30% KD in combination with unmodified AS W053 (Supplementary Table 1). These observations imply that the reduced activity often observed upon extensive siRNA modification can be compensated by careful matching of specifically modified SSs and ASs. However, our data also identifies SSs that act as more 'general optimal SSs' to improve the performance of many ASs and are therefore broadly applicable in siRNA design. In particular, SSs modified in the 3'-overhang by UNA and HM (W131 and W043, respectively) or destabilized in the 3'-end by EA and OX (DO003 and JC1, respectively) produced a strong KD in combination with many ASs (Table 3 and Figure 3).

Strategies for enhancing siRNA activity

The HE siRNA duplexes were found to harbour modifications either in the 3'-overhang (e.g. W006, JE1001, GS2372, W043, W047, W060, W131), within the siRNA body (e.g. JC-F1, DO003, JC-S1, JC1, GS2369) or in both regions (e.g. GS2383, JW1189, GS2542, GS2534) (Tables 2 and 3), and we therefore speculated that two mechanisms were responsible for enhancing siRNA activity: (i) Modification of the siRNA body to introduce thermodynamic asymmetry to favour AS incorporation into RISC. (ii) Modification of SS and AS 3'-overhangs to enhance serum stability or affect strand selection by RISC.

Destabilization of SS 3'-ends enhances silencing efficiency

Given that strand selection during RISC loading, and thereby AS activity, is dependent on the thermodynamic profile of the siRNA duplex we investigated the impact of modifications on thermodynamic asymmetry. The chemical modifications used in this study have both stabilizing (OMe, F/OMe, HNA, ANA, ALN, LNA) and destabilizing (DNA, AEM, APM, OXE, EA, AP, CE, UNA) properties. We found 15 of the 20 most efficient siRNA

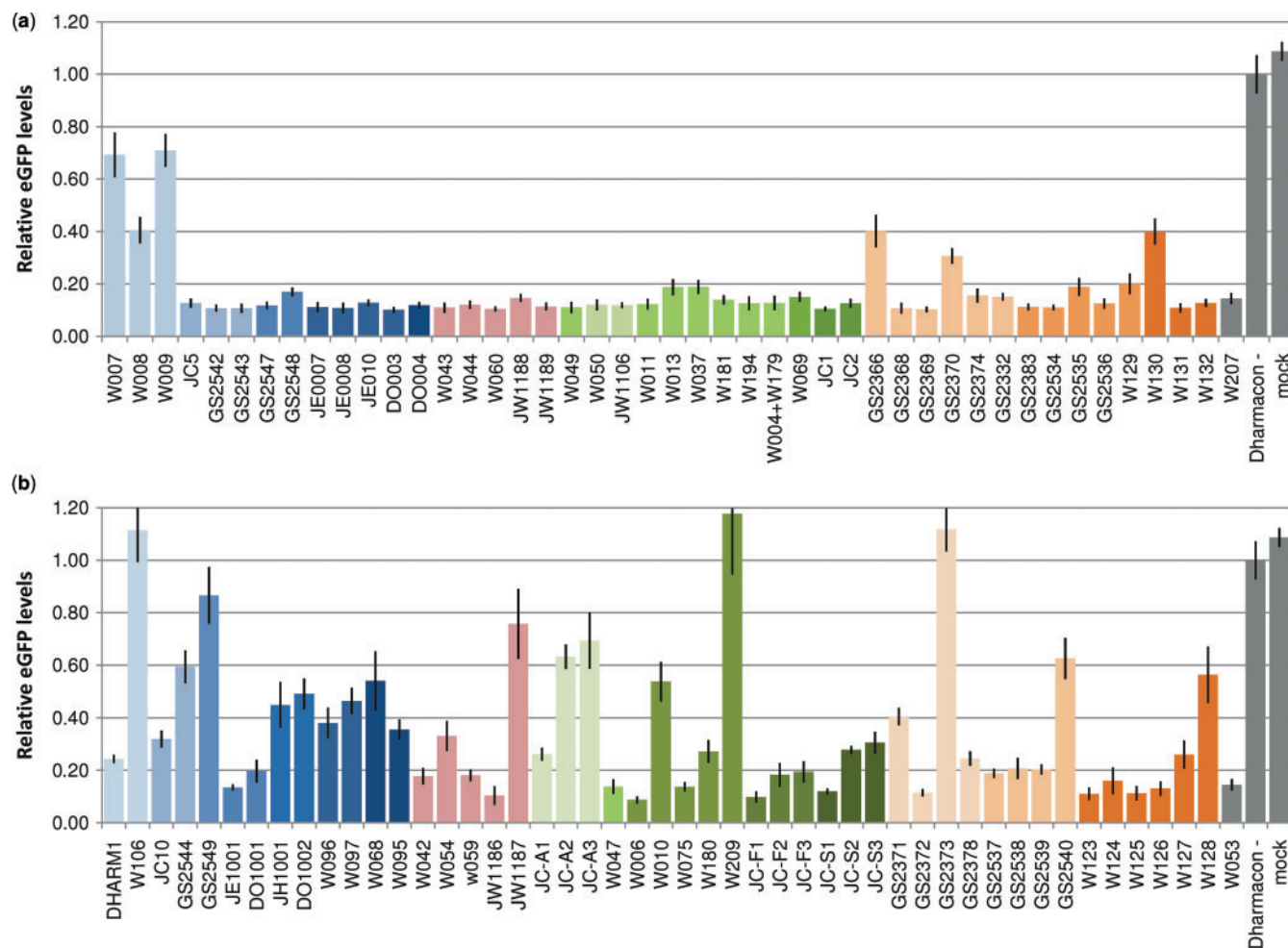


Figure 2. Silencing activity of chemically modified SSs and ASs. HeLa-eGFP were transfected with the indicated siRNAs (10 nM concentration) and eGFP levels were evaluated 72 h post-transfection. (a) Silencing activity of modified SSs in combination with the unmodified AS, W053. (b) Silencing activity of modified ASs in combination with the unmodified SS, W207. Colour-code: 2'-OH substituted oligos (blue), 4'-modified oligos (pink), 2'-locked oligos (green) and ribose ring modified oligos (orange).

duplexes to have chemical modifications favouring AS incorporation by altering the thermodynamic profile. W123 contained a 5'-end destabilizing UNA, whereas JC-F1 and JC-S1 contained CENA and CLNA resulting in stabilization of the AS 3'-end (see Tables 2 and 3 and Supplementary Table 1). Nearly a third of the HE siRNAs (38/134) contained these three ASs suggesting that altering the thermodynamic asymmetry through AS modification is a major determinant of siRNA activity.

A similar trend was found among the HE SSs; the 3'-end OX-destabilized JC-1 and the 3'-end EA destabilized DO003 improved the performance of most ASs (Figure 3, orange and green, respectively, and Figure 4a, columns 1, 2, 13,14) and resulted in an average 25% improvement in activity for 22 and 23 ASs, respectively (Table 3). Furthermore, JC1 and DO003 were highly represented among the 134 HE siRNAs and among the top three SSs for each AS (Table 3). Conversely, we found both SS 3'-end stabilization (e.g. GS2369 and GS2383; Figure 4a, columns 11, 23 and 12, 24) and SS 5'-end destabilization (e.g. JC2 and W129; Figure 4a,

columns 9, 21 and 10, 22) to negatively influence the activity of many ASs. The stabilization of the SS 5'-end should improve siRNA performance, and moderate 5'-end stabilization (e.g. GS2368; Figure 4a, columns 4, 16) did improve the performance of most ASs; however more stabilizing modifications (e.g. multiple HNAs (GS2534; Figure 4a, columns 5, 17), single and extensive LNA modifications [(W011 and W013; Figure 4a, columns 6, 18 and 7, 19)] impaired silencing. This suggests that, although favouring AS selection, extensive thermodynamic stabilization of an siRNA duplex is detrimental to silencing activity. In accordance, the activity of stabilized ASs (e.g. W106, W209 and GS2373) was enhanced both by the 3'-end destabilized SSs (DO003 and JC1) and by the 5'-end destabilized JC-2 although this SS should disfavour AS incorporation into RISC based on thermodynamic asymmetry (Figure 3 and Supplementary Table 1). This highlights that siRNA thermodynamic stability should fall within a range compatible with the components of the RNAi machinery, and that both siRNA thermodynamic asymmetry and stability are critical parameters to

consider during siRNA design. Based on our screen, relative destabilization of the SS 3'-end (using DO003 and JC1) represented the more reliable strategy to improve siRNA performance unless high stability of the duplex is specifically required.

Modification of 3'-overhangs influences siRNA activity

The observation that several highly efficient siRNAs (e.g. W006-W060, W006-W043 and W006-W131) are modified in the 3'-overhangs only, highlights the importance

Table 2. Highly efficient siRNA duplexes

AS	SS	eGFP levels	Viability	Rank
JW1186	W043	0.06 ± 0.01	0.62 ± 0.12	1
W123	W131	0.06 ± 0.02	1.05 ± 0.08	2
JW1186	W131	0.06 ± 0.01	0.66 ± 0.20	3
JC-S1	DO003	0.06 ± 0.01	0.65 ± 0.13	4
W123	W043	0.06 ± 0.01	0.89 ± 0.20	5
GS2372	GS2383	0.06 ± 0.01	0.60 ± 0.15	6
JC-F1	W131	0.07 ± 0.01	0.64 ± 0.17	7
W123	JW1189	0.07 ± 0.01	0.92 ± 0.25	8
GS2372	DO003	0.07 ± 0.01	0.47 ± 0.14	9
JC-S1	JC1	0.07 ± 0.01	0.62 ± 0.13	10
W053	W207	0.09 ± 0.01	0.73 ± 0.14	380
Dharmacon –	–	1.00 ± 0.07	0.78 ± 0.10	–
Mock	–	1.09 ± 0.04	1.00 ± 0.15	–
W006	W004 + W179	0.09 ± 0.01	0.57 ± 0.16	35
JW1186	W004 + W179	0.12 ± 0.02	0.90 ± 0.10	238
W123	W037	0.13 ± 0.02	1.01 ± 0.18	276
W006	W037	0.13 ± 0.02	0.65 ± 0.20	326
JC10	W004 + W179	0.32 ± 0.06	0.88 ± 0.11	983
W010	W004 + W179	0.34 ± 0.07	0.46 ± 0.27	1028

Relative silencing activity (eGFP levels), viability (# nuclei) and activity ranking of the most efficient siRNA duplexes. Upper panel: top 10 performing siRNA duplexes. Lower panel: selected siRNAs with high activity and LNA modifications in the SS for enhanced serum stability. The unmodified siRNA (W053-W207) is highlighted in bold.

Table 3. Highly efficient SSs and ASs

Strand name	AS/ SS	Sequence (5'–3')	Percentage in HE siRNAs/ top 3 siRNAs (%)	# impr. ASs/ avr. impr.	Remark
DO003	SS	GACGUA AACGGCCACAA _{EA} GUUC	7/15	23/27	Destabilized 3'-end favours AS selection
JC1	SS	GACGUA AACGGCCACA _{OxAGox} UUC	7/10	22/25	Destabilized 3'-end favours AS selection
W043	SS	GACGUA AACGGCCACAAGUT _{HM} C _{HM} U	6/13	25/17	Disfavoured 3'-overhang favours AS selection
W131	SS	GACGUA AACGGCCACAAGUUU _{UNA} U	7/11	21/13	Disfavoured 3'-overhang favours AS selection
GS2383	SS	GACGUA AACGGCCACAAGU _{HNA} U _{HNA} C	6/7	17/26	Disfavoured 3'-overhang favours AS selection
JW1189	SS	GACGT _{HM} AAACGGCCACAAGT _{HM} T _{HM} C	6/5	14/21	Disfavoured 3'-overhang favours AS selection
W004 + W179	SS	GAC _{LNA} GUA AAC _{LNA} G + GCC _{LNA} AC _{LNA} AAAGUT _{LNA} C _{LNA}	2/4	4/115	Stabilizing SS for highly modified ASs
W006	AS	ACUUGUGGCCGUUUACGUCG _{LNA} C _{LNA} U	24/–	–	HE ASs, favoured 5'-overhang
JE1001	AS	ACUUGUGGCCGUUUACGUCG _{EA} C _{EA} U	15/–	–	HE ASs, favoured 5'-overhang
W123	AS	ACUUGU _{UNA} GGCCGUUUACGUCG _{LNA} C _{LNA} U	13/–	–	HE ASs, favoured 5'-overhang
GS2372	AS	ACUUGUGGCCGUUUACGUCG _{ANA} C _{ANA} U	9/–	–	HE ASs, favoured 5'-overhang
JC-S1	AS	ACU _{CLNA} UGUGGCCGUUUACGUC _{CLNA} CGC	9/–	–	HE ASs, thermodynamically asymmetric
W047	AS	ACUUGUGGCCGUUUACGUCG _{ALN} U	9/–	–	HE ASs, favoured 5'-overhang
JW1186	AS	ACUUGT _{HM} GGCCGUUUACGUCG _{LNA} C _{LNA} U	8/–	–	HE ASs, favoured 5'-overhang
JC-F1	AS	ACU _{CENA} UGUGGCCGUUUACGU _{CENA} CGC	7/–	–	HE ASs, thermodynamically asymmetric

The name, sequence, statistical performance and remarks are given for the most efficient SSs and ASs. 'Percentage in HE siRNA' refers to the percentage of the 134 HE siRNAs containing the particular AS/SS. 'Percentage in top 3 siRNAs' refers to the summed representation of the particular SS among the three most efficient SSs for each AS (in %). '#impr. ASs/avr. improvement' refers to the number and average improvement (%) of ASs whose activity is significantly enhanced by the particular SS as compared with the unmodified SS (W207). Modifications are highlighted in bold in the sequences.

of overhangs for silencing activity (Figure 2, Tables 2 and 3). In fact, ASs modified exclusively in the 3'-overhang by LNA (W006), EA (JE1001), ALN (W047) and ANA (GS2372) were found in half (66/134) of the HE siRNAs. The impact of 3'-overhang modification was highly chemistry dependent; the UNA- (W127), LNA- (W180) and HM-modified (W054) ASs showed a significantly reduced silencing activity whereas ASs modified by ANA (GS2372), EA (JE1001) and ALN (W047) were equally efficient to unmodified RNA (W053) (Figure 2b). Interestingly, the LNA-modified overhang in W006 (5'-LNA-LNA-RNA-3') containing an additional 3'-RNA residue resulted in significantly improved silencing (Figure 2b; see the Discussion section below).

We speculated that the influence of 3'-overhang modifications could result from differences in siRNA serum stability; however, no clear correlation between stability and silencing activity was observed for overhang-modified siRNAs (Figures 2 and 5 and data not shown). Instead modification of the 3'-overhang was found to influence strand selection, as modifications disfavoured in the AS 3'-overhang [e.g. HM (W054), UNA (W127), LNA (W180); Figures 2b and 4b] were found to favour AS selection when incorporated into SS 3'-overhangs [HM (W043), LNA (W194) and UNA (W131); Figure 4b]. In detail, the silencing activity of the LNA-modified AS W180 (Figure 4b, column 9) was enhanced to a level indistinguishable from unmodified siRNA (Figure 4b, column 5) when paired to a SS with disfavoured overhangs, such as LNA (W194), UNA (W131) and HM (W043) (Figure 4b, columns 10–12). Similar SS 3'-overhang effects were seen for the UNA-modified AS W127 (Figure 4b, columns 13–16) and HM-modified AS W054 (Figure 4b, columns 17–20). The HM modification was particularly strongly disfavoured as the HM-modified W054 was a significantly poor AS (Figure 4b, column 17), while the

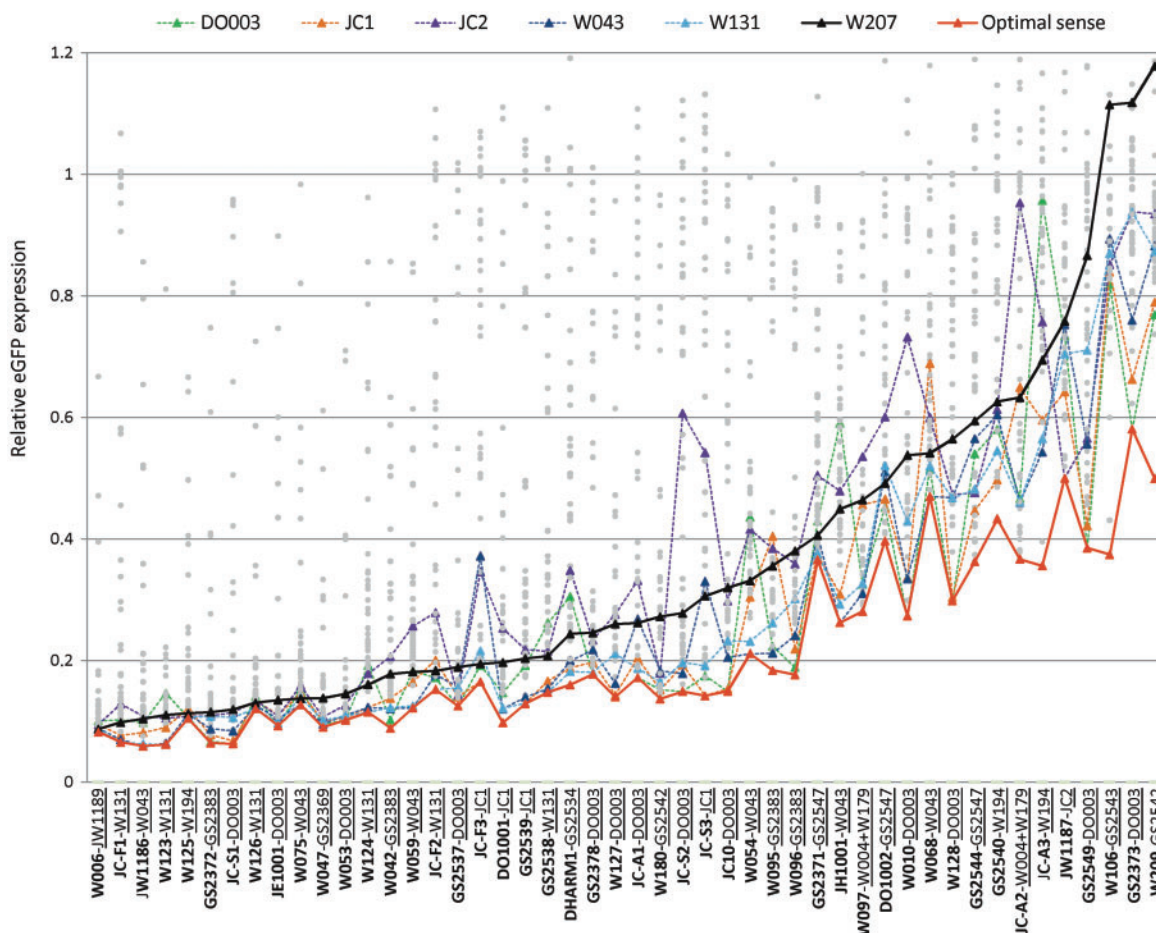


Figure 3. Optimal SSs enhance the activity of ASs. Relative eGFP expression of HeLa-eGFP cells transfected with all investigated ASs (X-axis, AS name given in bold) in combination with all 45 SSs (represented by grey dots) or with selected SSs (coloured triangles/lines). The activity of most ASs in combination with the unmodified SS, W207 (represented by black triangles/line) can be enhanced in combination with a specific, optimal SS for each AS (red triangles/line; name of the particular optimal SS for each AS is underlined). Furthermore the activity of many ASs is enhanced by the thermodynamically asymmetric SSs, DO003 (dark green triangles) and JC1 (orange triangles), as well as the 3'-overhang modified SSs, W043 (dark blue triangles) and W131 (light blue triangles). In contrast, the thermodynamically asymmetric SS, JC2, decreases the activity of many ASs (purple triangles).

HM-modified SS W043 had the most potent rescue effect on ASs with disfavoured overhangs (Figure 4b, columns 8, 12, 16, 29). This suggests that HM-modified overhangs may be more strongly disfavoured than UNA- (W131) and LNA-modified (W194) SS and can therefore be broadly used in SS design to favour AS-strand selection.

Modified overhangs alter strand selection by RISC

To further investigate the impact of 3'-overhang modifications on altered strand selection, we evaluated the activity of both strands in the siRNA duplex using reporters containing a perfect target site for either the SS or AS downstream of a luciferase open reading frame (Figure 4d). We expected disfavoured modifications in the AS 3'-overhang [LNA (W180), UNA (W127), HM (W054)] to increase incorporation of the unmodified SS (W207) into RISC*, thereby leading to higher SS silencing activity. Indeed, the modest silencing effect of the SS (W207) in an unmodified duplex (5 nM concentration) was significantly increased

when combined with an AS with a disfavoured overhang (Figure 4c, compare column 2 with 4, 6, 8). We therefore suggest that disfavoured modifications in 3'-overhangs can be used to favour the incorporation of opposing strands, irrespective of the thermodynamic asymmetry of the siRNA duplex.

Intriguingly, we found the AS W006 to be significantly more efficient than an unmodified AS (W053) when paired to W207 suggesting the 3-nt 5'-LNA-LNA-RNA-3' overhang motif to be favoured for RISC loading even over the natural RNA-RNA 3'-overhang (Figures 2b and 4b). In agreement, a SS (JW1104) with the 5'-LNA-LNA-RNA-3' overhang motif exhibited significantly enhanced activity compared with the unmodified SS (W207) (Figure 4c, compare columns 2 and 10) and a similar effect was obtained when combining JW1104 with the disfavoured ASs W180 (LNA), W127 (UNA) and W054 (HM) (Figure 4c, compare columns 4, 6, 8 and 12, 14, 16). We found other highly efficient ASs modified exclusively in the 3'-overhang by EA (JE1001), ANA (GS2372) and ALN (W047) (Table 3) to be favoured during RISC loading as

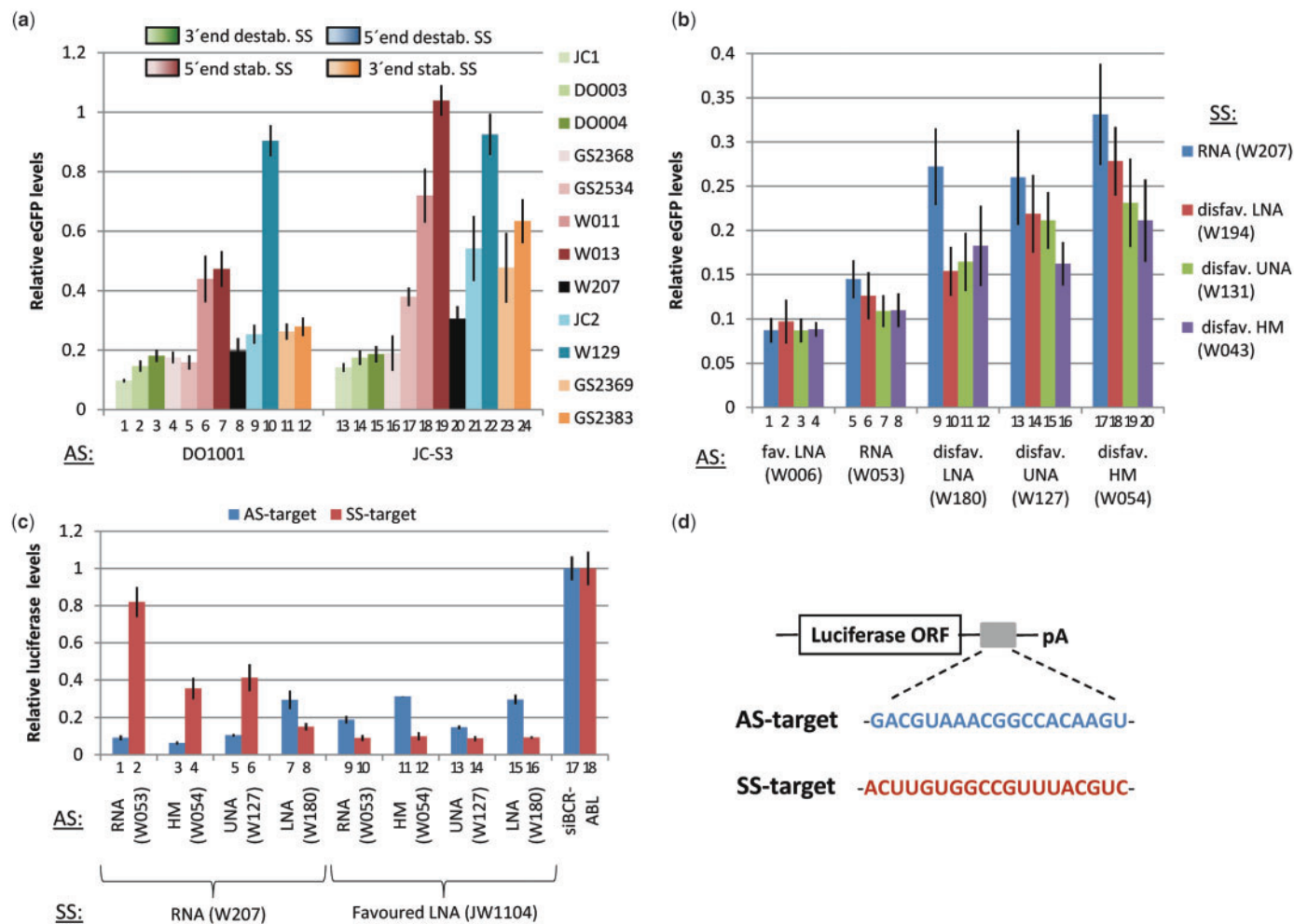


Figure 4. Improvement of siRNA performance by introduction of additional thermodynamic asymmetry and modification of 3'-overhangs. (a) The performance of ASs (exemplified by the representative ASs DO1001 and JC-S3) can be modified by altering the overall thermodynamic profile of the siRNA duplex by introduction of chemical modification in the SS. (b) The siRNA activity is influenced by the nature of the AS and SS 3'-overhangs. ASs modified in the 3'-overhang by LNA, UNA and HM have significantly lower activity than unmodified and LNA-modified AS in combination with the RNA SS (blue). This loss of activity can be partly rescued by using SSs with the disfavoured overhangs LNA, UNA or HM. (c) The LNA-LNA-RNA motif is a favoured 3'-overhang motif. The silencing activity of both the AS (on the AS-target, blue) and SS (on the S-target, red) is shown for the unmodified, HM-, UNA- and LNA-modified ASs in combination with unmodified and LNA-modified SSs. (d) Overview of the luciferase reporters used to evaluate the silencing activity of both the AS (denoted 'AS-target') and SS (denoted 'SS-target').

they lowered the activity of the opposing SS (W207) as compared with the unmodified AS W053 (Supplementary Figure 3). Consequently, favoured overhangs such as the 5'-LNA-LNA-RNA-3' motif and others can be broadly utilized to favour AS incorporation into RISC*, thereby enhancing siRNA potency.

Enhancing siRNA serum stability by chemical modification

The identification of highly nuclease-resistant siRNAs is a key concern for *in vivo* applications and great efforts have been applied to enhance stability by chemical modification (14,16,22,43). Like other studies, we find siRNA serum stability to be positively correlated with the level of RNA modification for most chemistries. While substitution of 3'-overhangs led to a modest increase in serum stability (e.g. UNA, ALN, HM, LNA, ANA; Figure 5a, left panel), partial or full modification of the siRNA body led to efficient stabilization (OMe/F, OMe, DNA/LNA

LNA; Figure 5a, right panel). Although a large number of substitutions within an siRNA does indeed enhance stability, highly modified siRNAs generally displayed poor silencing. Fully OMe/F-substituted siRNAs have previously been reported to be both highly stable and more potent than standard siRNAs (30); however, in our hands a similar siRNA (JC5-JC10) exhibited high stability, but produced a very modest 26% KD (Figure 5a and Supplementary Table 1). This highlights the importance of identifying other strategies for siRNA stabilization that support high silencing activity. Interestingly, we found that LNA-mediated stabilization of selected positions within the siRNA duplex led to similar or even enhanced stability as compared with fully substituted duplexes (Figure 5a, compare W010-W037, W010-W004-W179, JC10-W037 with JC5-JC10 and W106-W009). In fact, the partially LNA-modified W006-W037 and W010-W004-W179 exhibited higher

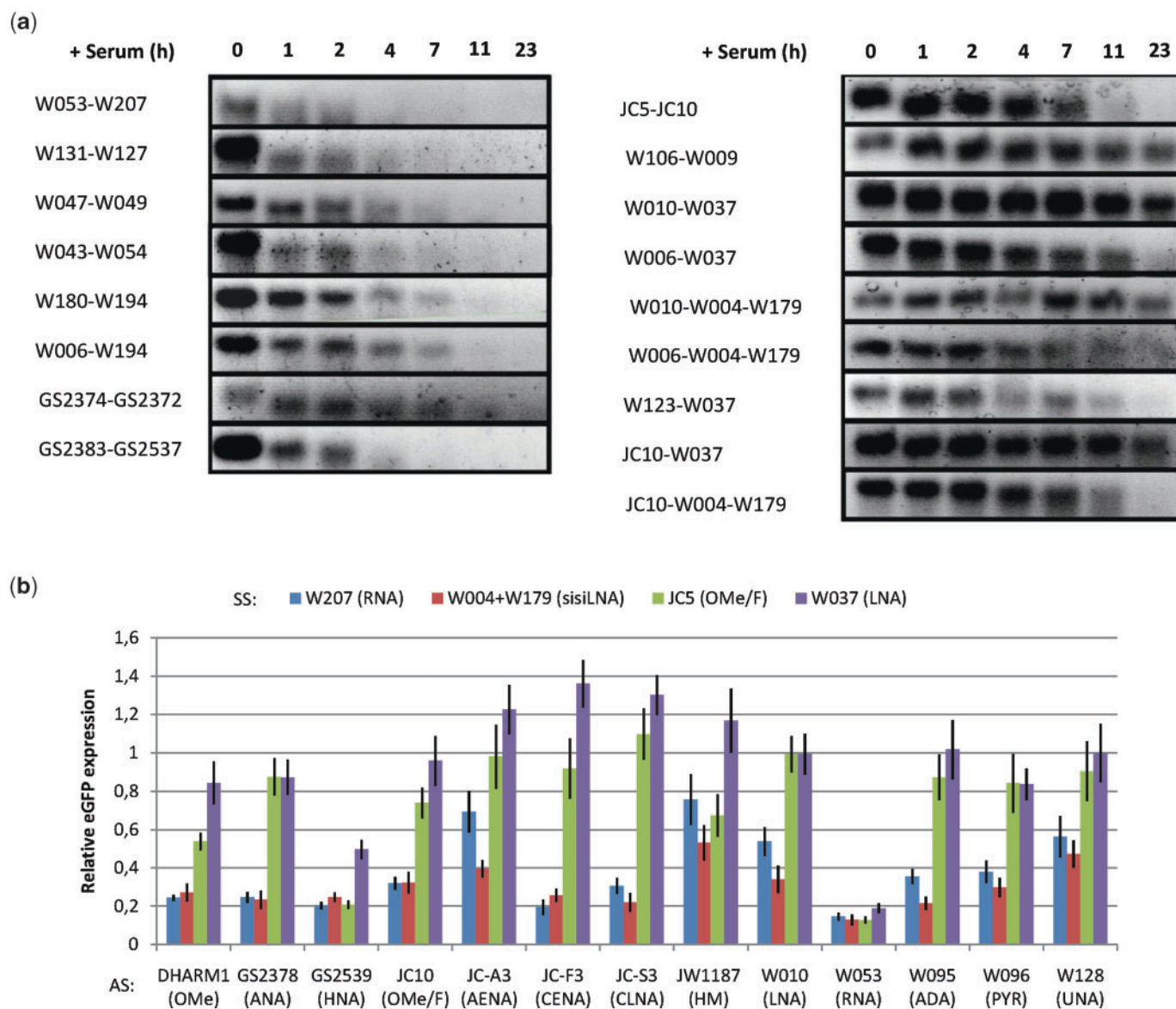


Figure 5. Enhancing serum stability of siRNAs with minor loss of activity. **(a)** The biostability of modified siRNAs was evaluated by incubation in 80% FBS. While a low level of chemical modification results in only modest increase in stability (left panel), more extensive and full substitutions result in dramatically improved siRNA stability (right panel). The incubation time is given in hours (h). **(b)** The eGFP levels of cells transfected with modified ASs paired with either unmodified SS (W207), LNA-modified segmented SS (W004 + W179), fully OMe/F substituted SS (JC5) and LNA-modified SS (W037) are given. The segmented LNA-modified SS (W004 + W179) generally prevented the loss in activity imposed by the LNA-modified unsegmented SS (W037).

stability than the fully OMe/F-substituted JC5-JC10, while retaining a potent 87% and 66% KD activity, respectively (Table 2 and Figure 5a). Moreover, we found that the sisiRNA design (41) that utilizes an LNA-stabilized segmented SS (W004 + W179) may be broadly applied to introduce LNA-stabilization with minor impact on activity. The sisiRNA constructs W010-W004-W179 and JC10-W004-W179 retained both high silencing activity and serum stability when modified extensively with LNAs, whereas an intact LNA-modified SS (W037) was detrimental to siRNA activity (W010-W037 and JC10-W037, Figure 5). In fact, the sisiRNA design allowed the LNA-stabilization of several

differentially modified ASs without major loss of silencing activity (Figure 5b). This implies that siRNA stabilization is preferentially achieved by introducing few LNA modifications, preferentially using the sisiRNA design, rather than generating fully substituted duplexes. Furthermore, selected modifications allow further functionalization of the siRNA duplex through modification of the remaining nucleotide positions.

Reducing siRNA toxicity through chemical modification

Chemical modifications have previously been shown to affect the toxicity of siRNAs (44) and we therefore

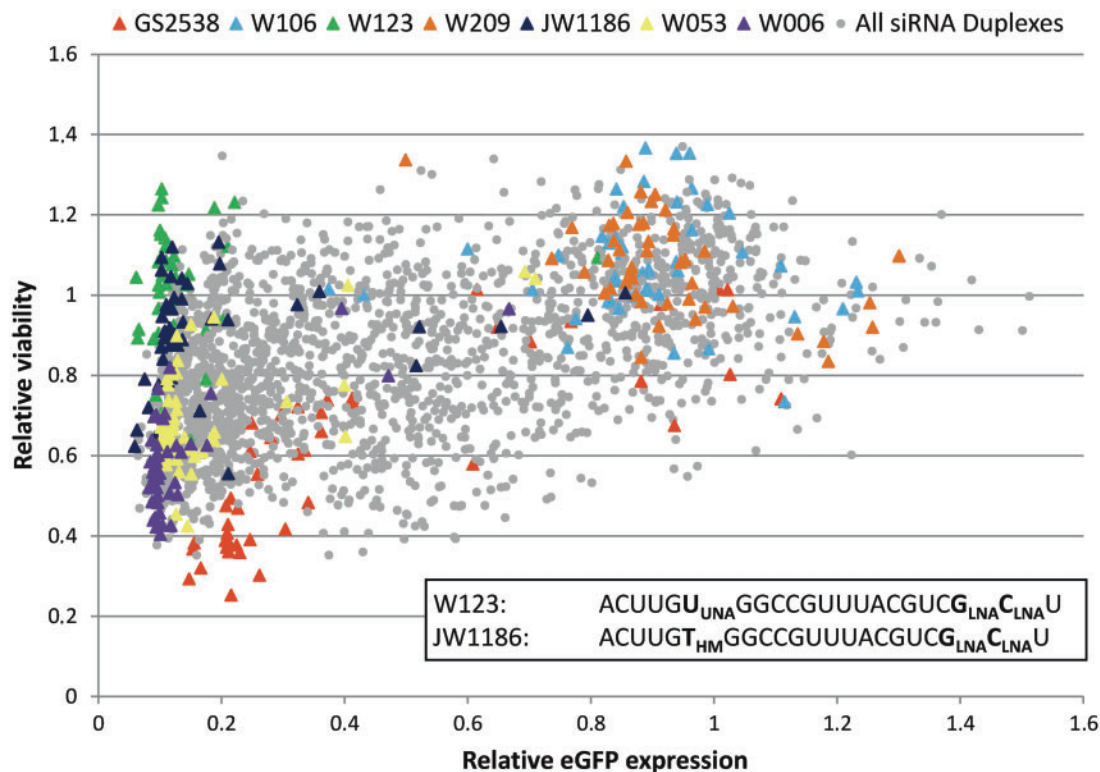


Figure 6. Identification of highly efficient siRNAs with low toxicity. Scatter plot showing target cell viability versus eGFP expression (siRNA activity) for all tested siRNAs (grey dots) and for selected ASs in combination with all 45 SS (coloured dots). Silencing activity correlates with toxicity for most siRNAs. The highly active, unmodified (W053, yellow triangles), HNA- (GS2538, red triangles) and LNA- (W006, purple triangles) modified ASs have high activity and poor viability, whereas heavily LNA- (W209, light brown triangles) and OME- (W106, light blue triangles) modified ASs have poor activity, but high viability. In contrast, the UNA- (W123, green triangles) and HM- (JW1186, dark blue triangles) modified ASs have both high activity and viability.

evaluated the viability of the siRNA transfected cells after 72 h (as number of nuclei per well relative to mock transfections). The majority of modified SSs resulted in a similar high degree of viability in combination with unmodified RNA AS whereas the viability varied more dramatically among the modified ASs in combination with the RNA SS (Table 1). We found cell viability to correlate with siRNA potency for the majority of ASs (Supplementary Figure 4) with only a few non-functional siRNAs resulting in low cell viability (Figure 6). This suggests that the observed siRNA toxicity arises from efficient ASs interfering with the endogenous RNAi pathway. Indeed, plotting the relative eGFP levels versus relative viability allowed us to identify distinct subgroups of ASs; the unmodified W053, and the LNA-modified W006 displayed high silencing potency and moderate cell viability (Figure 6, yellow and purple triangles, respectively), the OMe- (W106) and heavily LNA- (W209) modified ASs had poor activity and high viability (Figure 6, light blue and brown triangles, respectively), while the HNA-modified GS2538 had high activity and very low viability (Figure 6, red triangles). Interestingly, silencing activity and viability of particularly the UNA- (W123) but also the HM- (JW1186) modified ASs were both high in combination with most SSs (Figure 6, green and dark blue triangles, respectively). Hereby our data shows that

although most potent siRNAs cause reduced viability, it is possible to generate highly potent and non-toxic siRNAs by selecting particular chemical modifications.

DISCUSSION

Optimization of siRNA performance by chemical modification has been addressed in several studies (14–18,22,23,31,41); however, in most cases cross-study comparisons of chemistries are difficult. In the present study, we have directly compared the impact of 21 types of chemical modifications on siRNA activity and cell viability using 2160 chemically different siRNA duplexes, thus providing unique insight into the ways specific chemistries can be combined to generate siRNAs with superior properties. As a result we have identified 134 siRNA duplexes displaying higher silencing activity than standard siRNAs. The majority of these highly efficient siRNAs are only modestly modified, and especially the AS exhibited a limited and position-dependent tolerance for modification. This is expected as the AS must be bound at the 3'-end by Ago2 during RISC loading (42,45), guide initial target interactions in the seed region (positions 2–8) (42,46,47) and be perfectly base paired in the central region to support Ago2-mediated target cleavage (7,42). Modification of the SS was generally well tolerated, and this strand

would therefore be useful as an entry route for extensive modifications in the siRNA duplex.

Our data highlights that improvement of siRNA performance by chemical modifications should focus primarily on ensuring preferential loading of the intended AS into RISC*, as chemical modifications affecting the thermodynamic asymmetry of the siRNA duplex or the recognition of 3'-overhangs were the major contributors to improved silencing activity in our screen. Previous results have demonstrated the strand containing the least stable 5'-end to be preferentially utilized as the guide strand in RISC* (5,6). In agreement, we found chemical modifications leading to relative destabilization of the AS 5'-end to be highly represented among the most potent siRNA duplexes. In contrast, strong destabilization of the AS seed region reduces siRNA silencing activity presumably by weakening its interactions with the target mRNA. AS selection can also be favoured by incorporation of stabilizing modifications in the SS 5'-end or AS 3'-end, and accordingly previous studies have successfully incorporated LNA into SS 5'-ends to favour AS selection (17,23). Although our results support this observation, we found a large increase in thermostability to reduce siRNA activity, and we therefore suggest favouring AS selection by destabilization of the SS 3'-end, unless high stability of the siRNA duplex is specifically required.

Previous studies have given little attention to the choice of siRNA 3'-overhangs, and chemical modification of 3'-overhangs appears to be generally well tolerated [this study and (18,41,48)]. Interestingly, our study shows that chemical modification of siRNA 3'-overhangs can be utilized to favour AS selection during RISC loading irrespective of the thermodynamic asymmetry of the duplex. We have identified 3'-overhang motifs that are either favoured (5'-LNA-LNA-RNA-3' and others) or disfavoured (5'-RNA-HM-3' and 5'-RNA-UNA-RNA-3') by the RNAi machinery, and we therefore propose the incorporation of disfavoured overhangs in the SS and favoured overhang in the AS for optimal siRNA performance.

For many applications, particularly *in vivo*, extensive modification is required to enhance siRNA stability or support delivery strategies. Improved resistance towards nucleases has been a primary goal of siRNA modification and several highly stabilized siRNAs have already been developed, albeit often at the expense of activity [this study and (14,16,22,49)]. Interestingly, we found that the poor activity of many modified ASs can be partially rescued by combination with an 'optimal' SS (Figure 3), suggesting that heavily modified ASs are incompatible with recognition by the RNAi machinery rather than incompetent for target cleavage. Hereby our data provides unique insight into synergistic effects between chemistries that maximize the level of modification and minimize the loss of activity.

Chemical modification of siRNA overhangs could provide protection against exonucleases and was found to elicit a modest increase in serum stability, while resistance towards endonucleases was achieved by substituting multiple positions in the siRNA body (Figure 5). We evaluated the serum stability of the heavily modified, yet functional siRNAs in our screen and found that

partially LNA-substituted JC10-W004-W179 and W010-W004-W179 exhibited stabilities similar to highly and fully modified siRNAs. This suggests that enhancement of siRNA stability need not focus on substituting all positions of the siRNA duplex, but can be restricted to few selected positions. We speculate that resistance towards endonucleases could be influenced by the thermodynamic stability of the duplex; in this regard, the segmented siRNA design (Table 1: SS W004-W179) provides a convenient way of introducing highly stabilizing LNA modifications in a configuration well tolerated by the RNAi machinery (41). Importantly, this design allows the generation of more functionalized siRNA duplexes in which other positions could be modified to minimize siRNA off-target effects and toxicity or favour delivery strategies. It is noteworthy that extensive stabilization of siRNAs should only be applied when explicitly needed, e.g. upon injections of naked siRNA *in vivo*. When using siRNAs shielded by transfection reagents in cell culture or even *in vivo*, chemical modification should primarily aim at maximizing silencing activity as exemplified by the 134 HE siRNAs identified in this study.

It is interesting that the most highly active siRNA duplexes, including the unmodified siRNA (W053-W207), have a negative impact on cell viability compared with mock transfections. Only 26 out of 134 highly efficient siRNAs resulted in viabilities indistinguishable from mock controls, and among these, the UNA- (W123) and HM- (W1186) modified ASs were represented 15 and 6 times, respectively, indicating that very few ASs are both highly active and non-toxic. siRNA toxicity may have several sources ((50–52) but we suspect that highly active siRNA duplexes interfere with endogenous RNAi pathways as most non-functional siRNAs in our screen are non-toxic. This type of competition between exogenous RNAi substrates and endogenous miRNAs has been observed both in cell culture (53) and *in vivo* (54). Alternatively, the toxicity may arise from off-target effects due to a miRNA-like behaviour on mRNAs with 6–7 nt matches with the AS/SS seed region (25). Interestingly, the efficient, non-toxic ASs W123 and JW1186 both contain modifications within their seed region, and we speculate that this may lower the potential for off-target effects by destabilizing miRNA-like interactions. Indeed, recent work has demonstrated that siRNA off-targeting can be minimized by a slight destabilization of the siRNA–target interaction by the introduction of DNA in the AS seed (55).

In summary, our results have allowed us to formulate the following guidelines for enhancing siRNA activity through chemical modifications:

- (1) siRNA overhangs can be chemically modified to favour AS incorporation into RISC*; the SS should contain disfavoured overhangs such as UNA, HM and LNA-LNA, whereas the AS should contain favoured overhang motifs such as LNA-LNA-RNA.
- (2) In order to favour AS incorporation into RISC* the siRNA duplex should preferably be destabilized by modifications in the SS 3'-end rather than stabilized in the SS 5'-end. Additionally, the AS should not

contain strongly destabilizing/stabilizing/bulky modifications within the 5'-end of the seed region.

- (3) The use of single UNA and HM modifications in the AS seed region increases cell viability upon siRNA transfection.
- (4) Enhanced serum stability can be obtained by selective thermodynamic stabilization of a few positions rather than full substitution of the entire siRNA duplex. The siRNA design may provide a convenient way to introduce strong LNA stabilization for many types of AS.

As demonstrated here, using the above guidelines to combine various types of chemical modifications will lead to the generation of superior siRNAs with high activity, high stability and low toxicity.

SUPPLEMENTARY DATA

Supplementary Data is available at NAR Online.

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