Utilization of unlocked nucleic acid (UNA) to enhance siRNA performance *in vitro* and *in vivo*

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Small interfering RNAs (siRNAs) are now established as a favourite tool to reduce gene expression by RNA interference (RNAi) in mammalian cell culture. However, limitations in potency, duration, delivery and specificity of the gene knockdown (KD) are still major obstacles that need further addressing. Recent studies have successfully improved siRNA performance by the introduction of several types of chemical modifications. Here we explore the effect of incorporating unlocked nucleic acid (UNA) into siRNA designs. The acyclic UNA monomers lack the C2'–C3'-bond of the RNA ribose ring and additively decrease nucleic acid duplex thermostability. We show that UNA-modifications of siRNAs are compatible with efficient RNAi and can improve siRNA performance both *in vitro* and *in vivo*. In particular, we find that the destabilizing properties of UNA are well suited to enhance the potency of siRNAs which are heavily modified by other chemical modifications such as locked nucleic acid (LNA), C4'-hydroxymethyl-DNA (HM), 2'-O-methyl-RNA (OMe), DNA and 2'-Fluoro-DNA (F). Interestingly, we find that naked, but UNA-modified siRNAs have dramatically increased biostability in mice and can induce potent KD in a xenograft model of human pancreas cancer. Hereby UNA constitutes an important type of chemical modification for future siRNA designs.

Introduction

The use of siRNA to knock down gene function has truly revolutionized mammalian cell cultures studies and holds great promise in therapeutics.^{1–3} Several siRNA designs have been suggested,^{4,5} yet most studies utilizes a standard siRNA design comprising a 21 base pair (bp) RNA duplex containing a 19mer base pairing stem and 2 nucleotide (nt) 3' overhangs.⁶ Upon introduction into the cell cytoplasm siRNAs are taken up into the RNA-induced silencing complex (RISC), the non-guiding sense strand (SS) is degraded and the guiding antisense strand (AS) will subsequently direct the sequence-specific cleavage of perfectly complementary RNAs.^{7–13} In spite of the great potency and applicability of siRNAs, a number of obstacles still hamper their wider use. Most prominent is the

current lack of efficient means of siRNA delivery under the harsh condition *in vivo* where naked siRNA are quickly degraded by extra- and intracellular ribonucleases.^{14,15} Other concerns are reports of poor KD specificity as siRNAs can both induce innate immune responses¹⁶ and exert broad off-target effects on non-intended targets sharing only partial sequence complementarity with the siRNA.^{17,18} To address these problem we and others have successfully modified siRNAs and identified highly active, more nuclease resistant and target-specific siRNAs.^{5,14,15,19–24} Recently, a large scale comparison of 21 types of chemical modifications in siRNA design found unlocked nucleic acid (UNA) to have several interesting properties.²³ UNA monomers are acyclic derivatives of RNA lacking the C2'–C3'-bond of the RNA ribose ring, yet still structurally mimicking unmodified RNA upon incorporation into RNA duplexes. Incorporation of UNA monomers into siRNAs induces additive instability by 5–8 °C per UNA monomer thereby providing a unique possibility to locally destabilize the siRNA duplex.²⁵ Here we extend our analysis of incorporating UNA into siRNA designs and show how UNA can be utilized to improve siRNA performance. We find that low levels of UNA modifications in siRNAs are compatible with efficient RNAi whereas more extensive UNA modification lowers siRNA efficiency and serum stability. Yet, even low levels of UNA modification can be highly beneficial; especially the strong destabilizing property of UNA can be strategically used both in the SS and AS to significantly improve the potency of extensively

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modified siRNAs that are otherwise too stable or rigid to support RNAi. Interestingly, lightly UNA-modified siRNA has prolonged biostability *in vivo*, even compared to extensively LNA modified siRNA, and produces efficient gene KD in a mouse model bearing human pancreas tumour xenografts. Hereby, UNA constitutes an important type of siRNA modification that has several interesting properties for siRNA function both *in vitro* and *in vivo*.

Results and discussion

Tolerance for UNA-modification in the SS

To investigate the impact of UNA-modification on siRNA activity we generated a H1299 cell line stably expressing a dual luciferase reporter harbouring a single perfect eGFP target site within the renilla luciferase 3'UTR. This cell line was transfected with chemically modified siRNAs at either 1 or 10 nM concentration (final concentration in the medium) using the Lipofectamine 2000 transfection reagent and the relative KD of renilla luciferase activity was evaluated 48 h after transfection (see Table 1 for an overview of the chemically modified ASs and SSs). We initially tested the tolerance for UNA modification at various positions within the SS or AS in combination with an unmodified opposing strand (Fig. 1A and 1B, respectively and see Fig. 1E for a structural representation of UNA). As reported for many other chemistries⁵ we find that single UNA modifications are well tolerated in the SS 3' overhang (W131) and additionally also in the SS 5'end (W132), central region (W308), 3'end (ID1276; Fig. 1A). Also triple UNA-modifications alone (W282) or in combination with 5'end LNA modification (W321) only reduced siRNA potency slightly as compared to the unmodified siRNA against eGFP, denoted siEGFP (Fig. 1A).

Tolerance for UNA-modification in the AS

We found that UNA-modification of the central part of the siRNA AS (W320, W126), 3'end (W125) and overhang (W127) are well tolerated, whereas UNA-modification of the AS seed region (position 2–8 of counting from the 5'end) can lead to slightly reduced potency (W315, W317, W318; Fig. 1B). Also double and triple modifications within the base-pairing stem of the siRNA duplex (W329, W187, W281 and W186) resulted in reduced silencing efficiency (Fig. 1B). Since these ASs efficiently form siRNA duplexes upon annealing to the unmodified SS, W207 (Fig. 4A) we speculate that several UNA-modifications may excessively destabilize AS interactions with the target site.

Tolerance for UNA-modification of siRNA duplexes

We next combined the functional UNA-modified SSs and ASs to test the tolerance for simultaneous UNA-modification in both siRNA strands (Fig. 1C). As expected, siRNA efficiency was largely dependent on the activity of the UNA-modified AS; all single-modified ASs retained their high silencing activity in combination with all tested SSs irrespectively of the positioning of the UNA modifications within the AS and SS (see all combinations of ASs W317, W126, W125 with

SSs W132, W308, ID1278, W282; Fig. 1C). The activity of the double and triple UNA-modified ASs (W329, W187 and W186, respectively) were more sensitive to SS modification; in particular the silencing activity of these ASs declined in combination with the double UNA-modified SS, W282 (Fig. 1C). This reduction in silencing potency for extensively UNA-modified siRNAs may well be due to excessive thermodynamic destabilization of the siRNA duplex and indeed we were largely unable to anneal several of the highly modified siRNAs (such as W281–W308, W330–W282 and W281–W282; Fig. 4A). We therefore recommend not to incorporate more than two UNA modifications in total within the base-pairing siRNA stem unless the siRNA is stabilized by other types of chemical modifications (see below).

Combining UNA and LNA allow higher levels of siRNA modification

As incorporation of more than one UNA modification into the AS resulted in reduced silencing (W329, W187, W186, W281; Fig. 1B) and reduced serum stability (Fig. 4A), we speculated that their potency may be enhanced in combination with a stabilizing, LNA-modified SS, W037. LNA monomers additively increase thermal stability by 2–10 °C per modification²⁶ and thereby counteract siRNA destabilization by UNA. We have previously reported that the activity of 36 out of 48 chemically modified ASs was reduced in combination with the LNA modified SS, W037, as compared to an unmodified SS, W207, and we never found any improvement in AS potency by W037.²³ Similarly, the potency of the unmodified AS (W053) is significantly reduced in combination with the LNA modified SS, W037 as compared to the unmodified SS, W207 (Fig. 3A). Here we found that the activity of both UNA modified AS, W329 and W187, was even enhanced in combination with W037 (Fig. 1D) and that the stability was greatly increased (stability is shown for W329 in Fig. 4B, data not shown for W187). This shows that balancing the use of the strongly stabilizing LNA and the destabilizing UNA can allow higher levels of siRNA modification and thereby enhance siRNA stability without compromising silencing efficiency.

UNA-modification of SS 3'end enhance siRNA performance

As strand selection during RISC loading is dependent on the thermodynamic profile of the siRNA duplex^{27,28} we speculated that UNA destabilization of the SS 3'end may enhance AS selection and thereby siRNA performance. To test this we generated a set of six ASs having single LNA-modifications at position 1–6 from the 5'end. As LNA-modifications are strongly stabilizing this generates a series of siRNA with a highly asymmetric thermodynamic profile unfavourable for AS incorporation into RISC. We found that UNA-modification at the 3' double stranded end of the SS (ID1276) did indeed improve the performance of siRNAs having LNA-modifications at positions 1–3 of the AS whereas no effect was seen for ASs modified at pos. 4–6 (Fig. 2A). This is in good agreement with previous studies showing that strand selection is primarily determined by the thermodynamic profile of the outermost positions of the stem.^{27,28} We next asked if UNA-modification of the SS 3'end may also improve

Fig. 2 UNA-modification of the SS 3' can improve siRNA performance by altering siRNA strand selection. (A) Silencing activity of ASs modified by LNA in position 1 to 6 counting from the AS 5' end in combination with UNA-modified SSs either with or without UNA in the 3' end (ID1276 and W131 respectively) (20 nM final concentration). (B) Silencing activity of extensively LNA- (W209) or HM-modified (JW1187) ASs in combinations with the unmodified SS W207 or SS modified by UNA in their 3' end (ID1276, ID1277) (10 nM final siRNA concentration). (C) Silencing activity of AS (on AS-sensor) and SS (on SS-sensor) of LNA- (pos. 1, pos. 2, W010) and HM-modified (JW1187) ASs in combination with UNA-modified SSs either with or without UNA in the 3' end (ID1276 and W131, respectively) (20 nM final siRNA concentration). The position of the UNA modifications or LNA modifications are shown schematically right to the figures as black crosses (UNA) or grey lines (LNA), respectively. siEGFP is an unmodified siRNA duplex containing AS W053 and SS W207. siEGFPmis is a mismatch control.

W131 for all tested ASs whereas AS performance is improved (Fig. 2C). This strongly suggests that SS 3' end UNA-modification does indeed disfavour SS and thereby favour AS incorporation during RISC loading.

UNA-modification of the SS enhance the activity of heavily modified siRNA duplexes

We have previously reported that the so-called sisiRNA design characterized by a segmented LNA-modified SS enhances the potency of heavily modified siRNA likely by destabilizing or introducing flexibility into the siRNA duplex.⁵ Therefore, we speculated that thermodynamic destabilization imposed by the UNA modification would similarly introduce flexibility into extensive modified siRNAs and thereby enhance their potency. To test this we introduced two UNA-modifications into a heavily LNA-modified SS (compare W037 and NAC3168) and tested its impact on silencing in combinations with AS modified by OMe (Dharm1), alternating OMe/F (JC10), HM (JW1187) and LNA (W010) (see Table 1). Indeed we found that both the UNA-modified SS (NAC3168) and sisiRNA design (W004 + W179) enhanced the potency of most ASs (Dharm1, JC10, HM1187 and unmodified AS (W053) to levels comparable to the unmodified SS (W207; Fig. 3). Only the heavily LNA-modified AS (W010) were significantly more efficient in combination with the sisiRNA design

(W004 + W179) as compared to the UNA/LNA-modified SS (NAC3168), yet the silencing efficiency was still significantly improved by NAC3168 to levels indistinguishable from the unmodified SS, W207 (Fig. 3A). This shows that UNA-modification of the SS may be utilized in extensively modified siRNA designs to enhance silencing activities.

UNA-modification of the AS can enhance the potency of heavily modified siRNA duplexes

We next asked if UNA modifications could similarly be incorporated into ASs to enhance siRNA performance, especially of the heavily LNA-modified duplexes. To test this we inserted a central UNA modification into a heavily LNA-modified AS (W188) and combined these with the heavily LNA-modified SSs, W037 and W013. We found that the introduction of UNA-modification in the central part of AS did indeed enhance the efficiency of the siRNA duplexes for both LNA-modified SSs, W037 and W013 as compared to the LNA-modified AS without UNA (W010) (Fig. 3B). Furthermore, we generated two SSs fully substituted with mixed DNA and LNA modifications (W008, W009) and tested these in combination with an AS containing a central UNA modification, W126 (Fig. 3C). Again we found a higher performance of W126 as compared to the unmodified AS, W053. Collectively, this clearly demonstrates that a

Table 1 Overview of chemically modified ASs and SSs. The name, type of chemical modification and sequence of the investigated ASs and SSs are given. The following modification abbreviations are used: 2'-O-methyl (OMe), 2'-fluoro (F), 2'-deoxy (DNA), 4'-C-hydroxymethyl-DNA (HM), locked nucleic acid (LNA), unlocked nucleic acid (UNA). The position of the modification is shown in bold in the oligo sequence. The oligoes are named according to the nomenclature of previously published work to allow easy comparison of results between individual studies

Name	AS/SS	Modification	Sequence (5'–3')
Dharm1	AS	OMe	AC _{OMe} UUGUGCCGUUUACGUCGC
JC10	AS	F/OMe	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
JW1187	AS	HM/LNA	ACT _{HM} UGT _{HM} GGCCGUUT _{HM} ACGT _{HM} CG _{LNA} C _{LNA} U
W010	AS	LNA	ACT _{LNA} UGT _{LNA} GGCCGUUT _{LNA} ACGT _{LNA} CG _{LNA} C _{LNA} U
W053	AS		ACUUGUGCCGUUUACGUCGC
W095	AS	ADA/LNA	ACUUGUGCCGUUUACGT _{ADA} CG _{LNA} C _{LNA} U
W123	AS	UNA/LNA	ACUUGU _{UNA} GGCCGUUUACGUCG _{LNA} C _{LNA} U
W125	AS	UNA/LNA	ACUUGUGCCGUUUACGU _{UNA} CG _{LNA} C _{LNA} U
W126	AS	UNA/LNA	ACUUGUGCCGU _{UNA} UUACGUCG _{LNA} C _{LNA} U
W127	AS	UNA	ACUUGUGCCGUUUACGUCG _{CUNA} U
W128	AS	UNA	ACU _{UNA} UGUGCCGUUUACGUCG _{CUNA} U
W180	AS	LNA	ACUUGUGCCGUUUACGUCG _{LNA} C _{LNA}
W186	AS	UNA/LNA	ACUUGU _{UNA} GGCCGU _{UNA} UUACGU _{UNA} CG _{LNA} C _{LNA} U
W187	AS	UNA/LNA	ACUUGU _{UNA} GGCCGU _{UNA} UUACGUCG _{LNA} C _{LNA} U
W188	AS	UNA/LNA	ACT _{LNA} UGT _{LNA} GGCCGU _{UNA} UT _{LNA} ACGT _{LNA} CG _{LNA} C _{LNA} U
W209	AS	LNA	ACT _{LNA} UGT _{LNA} GGCCGUUT _{LNA} ACGT _{LNA} CG _{LNA} C _{LNA}
W281	AS	UNA	ACUUGUG _{UNA} GCCGUUU _{UNA} ACGUCG _{CUNA} U
W315	AS	UNA/LNA	ACU _{UNA} UGUGCCGUUUACGUCG _{LNA} C _{LNA} U
W317	AS	UNA/LNA	ACUUG _{UNA} UGGCCGUUUACGUCG _{LNA} C _{LNA} U
W318	AS	UNA/LNA	ACUUGUG _{UNA} GCCGUUUACGUCG _{LNA} C _{LNA} U
W320	AS	UNA/LNA	ACUUGUGG _{CUNA} CGUUUACGUCG _{LNA} C _{LNA} U
W329	AS	UNA/LNA	ACUUGUGCCGU _{UNA} UUACGU _{UNA} CG _{LNA} C _{LNA} U
W330	AS	UNA	ACUUGUGCCGU _{UNA} UUACGUCG _{CUNA} U
ID1276	SS	UNA	GACGUAACCGGCCACAA _{UNA} GUAC _{UNA} U
ID1277	SS	UNA/LNA	GACGUAACCGGCCACAA _{GUNA} UT _{LNA} C _{LNA} U
ID1278	SS	UNA	GACGUAACCGGCCACAA _{GUNA} UAC _{UNA} U
NAC3168	SS	UNA/LNA	GAC _{LNA} GUA _{UNA} AAC _{LNA} GGCC _{LNA} AC _{LNA} AA _{UNA} GUT _{LNA} C _{LNA} U
W004 + W179	SS	LNA	GAC _{LNA} GUAAC _{LNA} G + GCC _{LNA} AC _{LNA} AAGUT _{LNA} C _{LNA}
W008	SS	DNA/LNA	G _{DNA} A _{DNA} C _{LNA} G _{DNA} T _{DNA} A _{LNA} A _{DNA} A _{DNA} C _{LNA} G _{DNA} G _{DNA} C _{LNA} C _{DNA} A _{DNA} C _{LNA} A _{DNA}
W009	SS	DNA/LNA	A _{DNA} G _{LNA} T _{DNA} T _{LNA} C _{LNA} T _{DNA} G _{DNA} A _{LNA} C _{DNA} G _{LNA} T _{DNA} A _{LNA} A _{DNA} A _{DNA} C _{DNA} G _{LNA} G _{DNA} C _{LNA} C _{DNA} A _{LNA} C _{DNA} A _{LNA} A _{DNA} G _{LNA} T _{DNA} T _{LNA} C _{LNA} T _{DNA} GAC _{LNA} GT _{LNA} AA _{LNA} AC _{LNA} GGCCACAAGUT _{LNA} C _{LNA} U GAC _{LNA} GUAAC _{LNA} GGCC _{LNA} AC _{LNA} AAGUT _{LNA} C _{LNA} U GACGU _{UNA} AAACCGGCCACAAGUT _{LNA} C _{LNA} U GACGUAAC _{CUNA} GGCCACAAGUT _{LNA} C _{LNA} U GACGU _{UNA} AAACCGGCCACAAGUUU _{UNA} U GACGU _{UNA} AAACCGGCCACAAGUUU _{UNA} U GAC _{LNA} GUAAC _{LNA} GGCC _{LNA} AC _{LNA} AAGUT _{LNA} C _{LNA} U GAC _{UNA} GUAACCGGCCACAA _{UNA} GUUU _{UNA} U GACGUAAC _{GUNA} GCCACAAGUUU _{UNA} U GAC _{LNA} GUAAC _{LNA} G _{UNA} GCC _{UNA} AC _{UNA} AAGUT _{LNA} C _{LNA} U GACGUAACCGGCCACAAGUUC
W013	SS	LNA	GAC _{LNA} GT _{LNA} AA _{LNA} AC _{LNA} GGCCACAAGUT _{LNA} C _{LNA} U
W037	SS	LNA	GAC _{LNA} GUAAC _{LNA} GGCC _{LNA} AC _{LNA} AAGUT _{LNA} C _{LNA} U
W129	SS	UNA/LNA	GACGU _{UNA} AAACCGGCCACAAGUT _{LNA} C _{LNA} U
W130	SS	UNA/LNA	GACGUAAC _{CUNA} GGCCACAAGUT _{LNA} C _{LNA} U
W131	SS	UNA	GACGUAACCGGCCACAAGUUU _{UNA} U
W132	SS	UNA	GACGU _{UNA} AAACCGGCCACAAGUUU _{UNA} U
W181	SS	LNA	GAC _{LNA} GUAAC _{LNA} GGCC _{LNA} AC _{LNA} AAGUT _{LNA} C _{LNA} U
W282	SS	UNA	GAC _{UNA} GUAACCGGCCACAA _{UNA} GUUU _{UNA} U
W308	SS	UNA	GACGUAAC _{GUNA} GCCACAAGUUU _{UNA} U
W321	SS	UNA/LNA	GAC _{LNA} GUAAC _{LNA} G _{UNA} GCC _{UNA} AC _{UNA} AAGUT _{LNA} C _{LNA} U
W207	SS		GACGUAACCGGCCACAAGUUC

UNA-modification of both the SS and ASs can significantly help increasing the number of total modifications that are tolerated in siRNA designs.

The impact of UNA modification on siRNA biostability *in vitro*

As UNA modification decreases siRNA thermodynamic stability,²⁵ we next investigated the impact of UNA modification on siRNA biostability *in vitro* (Fig. 4A&B) and upon injection of naked siRNAs in mice (Fig. 5A&B). Notably, we were unable to anneal the most heavily UNA-modified siRNA duplexes such as W330–W282, W281–W282 and W281–W308 (Fig. 4A) suggesting that care must be taken when incorporating more than two UNA-modifications into the stem of siRNAs. Furthermore all investigated siRNAs modified exclusively by UNA within the stem had significantly reduced stability and were largely degraded after 5 min of incubation in 80% FCS (Fig. 4A). We therefore recommend the use of

shielding delivery reagents when utilizing siRNA modified only by UNA within the base pairing stem. In contrast, insertion of UNA into the stem of very extensively modified siRNA such as OMe/F/UNA-modified and LNA-modified duplexes did not significantly reduce serum stability (compare JC10–W037 to JC10–NAC3168 and W209–ID1276 to W209–W207, respectively; Fig. 4B). Furthermore, the incorporation of UNA in either the SS or AS of extensively LNA-modified siRNA only marginally reduced siRNA serum stability (compare W010–NAC3168 and W188–W037 to W010–W037; Fig. 4B) although siRNA potency was significantly enhanced (Fig. 3).

The impact of UNA modification on siRNA biostability and knock down efficiency *in vivo*

We have previously observed that extensive LNA-stabilization can prolong the blood circulation time of naked siRNA in

Fig. 3 UNA-modification of ASs and SSs can enhance the potency of extensively modified siRNAs. **A.** Silencing activity of ASs modified by OMe (Dharm1), OMe/F (JC10), HM (JW1187), LNA (W010) in combination with either an extensively LNA modified SS (W037), segmented SS (W004 + W179), UNA/LNA-modified SS (NAC3168) or unmodified SS (W207) (1 nM final siRNA concentration). **B.** Silencing activity of extensively LNA-modified (W010) or LNA/UNA-modified (W188) ASs in combination with extensively LNA-modified SSs, W037 or W013 (10 nM final siRNA concentration). **C.** Silencing activity of unmodified AS (W053) and UNA modified (W126) AS in combination with fully DNA-LNA substituted SSs (W008, W009) (10 nM final siRNA concentration). The position of the UNA modifications or LNA modifications are shown schematically below or right to the figures as black crosses (UNA) or grey lines (LNA), respectively. siEGFP is an unmodified siRNA duplex containing the AS W053 and SS W207. siEGFPmis is a mismatch control.

mice upon intravenous injection²⁹ and mediate efficient KD of eGFP in the lungs.³⁰ Very surprisingly, siRNA modified with UNA only in the AS and SS overhangs, W127–W131, exhibited an even prolonged blood circulation time with siRNA species detectable 24 h after injection in blood and several organs such as kidney, spleen lung and hearth (Fig. 5A & B). This was unexpected as W127–W131 is only marginally more stable than unmodified siRNA upon incubation in 80% FCS (Fig. 4B). However, W127–W131 did exhibit surprisingly high stability upon incubation in freshly prepared mouse serum (Fig. 4B). This suggests that the composition of nuclease differs between FCS and mouse serum with the latter having relatively less ribonuclease activity. The result also underscores the necessity of testing siRNA stability in the biological fluids relevant to the particular siRNA application. Next we tested the potential of UNA modifications to enhance siRNA function *in vivo*. Notably, we observed a potent eGFP KD in a mice model bearing human eGFP-expressing pancreas tumor xenografts upon subcutaneous injection of the naked UNA-modified siRNA, W127–W131, whereas no effect was observed using non-modified siRNA. This demonstrates that the UNA-modified siRNA duplex was indeed biological functional and superior to unmodified siRNA (Fig. 5C) and hereby establishes UNA as an important type of chemical modification with great potential in siRNA therapeutics.

Experimental

Preparation of siRNA duplexes

The phosphoramidites were incorporated into the siRNA sequence through solid-phase DNA/RNA synthesis on an automated DNA/RNA synthesizer using O²'-TBDMS protected RNA phosphoramidites and common reagents as described in ref. 23 (for structures of chemically modified nucleotide monomers, see Fig. 1E). The oligos are named according to the nomenclature of previously published work to allow easy comparison of results between individual studies. For siRNA annealing SS and AS were mixed in 1x Dharmacon annealing buffer (Dharmacom) and incubated at 95 °C for 1 min and 1 h at 37 °C and siRNA concentration was quantified using the Quant-iT RiboGreen RNA Assay Kit from Molecular Probes (Invitrogen) on a FLUOstar luminometer (BMG labtech).

Cell culture, transfections and Dual-luciferase assay

The human lung cancer cell line H1299 was grown in RPMI-1640 containing 10% FBS, 1% penicillin/streptomycin. A stable luciferase reporter H1299 cell line was created by transfection and subsequent pyromycin selection (1 µg ml⁻¹ medium) of H1299 cells with a psiCHECK2 vector (Promega)

Fig. 4 Stability of UNA modified siRNAs in serum. **A.** Native PAGE analysis of siRNA integrity of UNA-modified AS and SS upon siRNA annealing (upper panel, no serum incubation) or after 5 min of incubation in 80% Foetal calf serum (FCS) (lower panel). The position of the UNA modifications or LNA modifications are shown schematically as black crosses (UNA) or grey lines (LNA), respectively. **B.** Native PAGE analysis of siRNA integrity of UNA/LNA modified siRNAs upon incubation in 80% FCS or 80% freshly prepared mouse serum (MS) for the indicated time-period.

modified to contain a perfect target site for eGFP in the multiple cloning site (MCS) and a pyromycin selection cassette. siRNA transfections were performed in triplicates using the Lipofectamine 2000 reagent (Invitrogen) in a 96 well format using 15,000 cells in 100 μ l serum-free RPMI. Growth medium was added 4 h after transfection and 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 (transfection control). Transient transfection experiments using the reporter constructs pISO-AS-target and pISO-SS-target to evaluate AS and SS efficiency (Fig. 2C) is described previously.⁵

Evaluation of siRNA serum stability

Annealed UNA/LNA-modified siRNAs were incubated at 37 °C in 80% fetal calf serum (FCS) or 80% freshly prepared

mouse serum. Aliquots of 5 μ l (each containing 20 pmol of siRNA) were diluted in 25 μ l 1.2 $\times$ TBE loading buffer (1.2 $\times$ TBE, 10% glycerol, bromophenol blue) and snap-frozen on dry ice immediately upon sample taking. Samples were run on a 15% native polyacrylamide gel and stained using SYBR Gold® (Invitrogen).

Biostability of siRNAs in mice

For investigation of siRNA biostability and biodistribution in mice 10 μ g unmodified, LNA- or UNA- modified siRNA duplex was administered i.v. in a total volume of 200 μ l. Total RNA was purified by Trizol reagent (Invitrogen, Carlsbad, CA) from frozen blood samples and weighed tissue in RNeasy lysis buffer (Qiagen). RNA from blood (2 μ g) and from tissues (4 μ g) was run on 15% denaturing polyacrylamide gels and transferred onto Hybond-N+ membrane (Amersham Biosciences, Uppsala, Sweden). After UV crosslinking, the membranes were analyzed on a phosphorimager to visualize the ³²P labelled AS of the siRNA. To perform northern blotting, the same

Fig. 5 Stability, distribution and activity of UNA-modified siRNAs *in vivo*. **A.** Stability of unmodified, UNA- or LNA-modified siRNA in blood upon intravenous injection in mice as evaluated by Northern blotting. Experiments were performed in duplicates and M1 and M2 denote the two mice used for the experiment. The position of the UNA modifications or LNA modifications are shown schematically as black crosses (UNA) or grey lines (LNA), respectively. **B.** Biodistribution of UNA-modified duplexes from various organs 30 min and 24 h after intravenous injection. Analysis of siRNA integrity was performed by Northern blotting (similar to Fig. 5A) and the siRNA levels were quantified and normalized to total RNA harvested from the specific organ. Experiments were performed in duplicates and M1 and M2 denoted the two mice used for the experiment. **C.** Efficiency in eGFP KD by UNA-modified siRNAs in a human pancreas cancer xenograft model in mice. siEGFP is an unmodified siRNA duplex containing the AS W053 and SS W207. siEGFPmis is a mismatch control.

filters were stored for four months (eight half-lives of 32P) and probed with [gamma-32P] adenosine triphosphate-labelled sense-strand LNA-modified siRNA according to standard procedures.

***In vivo* efficacy of siRNAs in human xenograft liver cancer model in mice**

siRNA was tested for its ability to repress EGFP expression *in vivo*. Subcutaneous tumours were induced in 8- to 10-week-old NMRI nu/nu mice (Charles River, Maastricht, the Netherlands) by injection of 10^6 MiaPaca II tumour cells in Matrigel (BD Biosciences). One week after tumour cell injection, when tumour take was positive, administration of the siRNA started. Administration was *via* osmotic minipumps (model 1007D; Alzet Corp., Palo Alto, CA) placed subcutaneously. Total RNA was isolated from the tumours and livers and EGFP mRNA levels were determined in these samples by Northern blotting.

Conclusion

Our current and previous work has identified UNA as an important modification in siRNA design. We have previously reported that UNA-modification of the SS 3' overhang can enhance siRNA potency and that UNA modification of ASs could enhance target cell viability.⁵ Recently we have also found that UNA-modification of the AS seed region can

reduce off-target effects (Bramsen *et al.*, in prep.). Here we have extended our analysis of UNA-modification in siRNA design and describe novel strategies to improve siRNA performance. We find that single UNA modifications are well tolerated at most tested positions in the AS and SS; However, additional UNA-modifications, especially in the AS, lead to reduced silencing efficiency (Fig. 1A–C) likely by destabilizing the siRNA duplex or interactions with the target mRNA. In accordance, UNA-modification of the siRNA stem often lead to reduction in serum stability and siRNA duplexes with several UNA-modifications are not efficiently formed under standard siRNA annealing conditions (Fig. 4A). Yet, we find that even few UNA-modifications can significantly improve siRNA performance if placed strategically within the siRNA duplex; introduction of UNA in the 3' end of the SS can enhance siRNA efficiency of thermodynamically asymmetric siRNA by favoring AS incorporation into RISC (Fig. 2). Furthermore, UNA modification seems well suited in siRNA designs in combination with stabilizing modifications such as *e.g.* LNA to enhance siRNA potency: Incorporation of UNA into an extensively LNA-modified SS enhanced the potency of ASs containing several types of chemical modifications (Fig. 3). Similarly, inserting UNA into both unmodified and extensively LNA-modified ASs enhanced siRNA potency in combination with several extensively modified SS (Fig. 3). Notably, introduction of UNA into the stem of extensively modified siRNA did not dramatically reduce serum stability in

contrast to siRNAs modified exclusively with UNA (Fig. 4B). Thus, UNA can be used to improve the KD efficiency of heavily modified siRNA without necessarily hampering the stability of the duplex. Interestingly, we find a prolonged survival of siRNA modified by UNA in their 3' overhangs upon intravenous injection in mice even when compared to extensively LNA-stabilized siRNAs (Fig. 5). Moreover, these UNA-modified siRNAs are biologically active leading to > 50% KD of eGFP expression in a pancreas tumor xenograft model in mice in contrast to unmodified siRNAs. Collectively, this makes the UNA modification a potent chemical modification for increasing efficiency, stability and specificity of siRNAs with great potential in therapeutics.

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Contributions

M.M.P., M.B.L., N.L., S.L.W., O.R.M. and J.B.B. performed research; J.B.B., J.K., J.W. designed research and J.B.B. wrote the article. Access publication charges for this article were provided by the EU-FP6 RIGHT project (no. LSHB-CT-2004-005276).

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