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EMERGING AREA

Unlocked nucleic acid – an RNA modification with broad potential

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The first unlocked nucleic acid (UNA) monomer was described more than a decade ago, but only recent reports have revealed the true potential applications of this acyclic RNA mimic. UNA monomers enable the modulation of the thermodynamic stability of various nucleic acid structures such as RNA and DNA duplexes, quadruplexes or i-motifs. Moreover, UNA monomers were found to be compatible with RNase H activity, a property which is important for single stranded antisense constructs. Notably, UNA monomers can be applied in the design of superior siRNAs, combining potent gene silencing and dramatically reduced off-target effects.

Introduction

Naturally occurring nucleic acids adopt various structures and functions. The chemical modification of nucleic acids or oligonucleotides leads to novel molecules with unique properties. Chemically modified nucleic acids constitute a driving force in the development of molecular diagnostics and gene- or RNA-targeting therapeutics.[1–4] The ability to design well-defined, nanometer-scale secondary structures composed of nucleic acids has further contributed to the progress in nanotechnology, *e.g.* the self-assembly of nanostructures or the construction of functional nanomachines.[5–8] Despite the fact that many chemical modifications of nucleic acids have already been characterized and established there is still a requirement for the development of modified nucleotides with unique or improved properties.

Chemistry of UNA monomers

An unlocked nucleic acid (UNA, 2′,3′-*seco*-RNA) monomer is an acyclic RNA mimic (Fig. 1). A characteristic feature of UNA is the lack of a bond between the C2′ and C3′ atoms of the ribose ring. Such a simple modification, without the introduction of additional functional groups, makes UNA closely related to natural RNA monomers while simultaneously increasing the flexibility.

Incorporation of the UNA thymidine monomer into DNA oligonucleotides was first reported in 1995.[9] The synthesis of the thymine UNA phosphoramidite building block started from 2′-hydroxythymidine and was accomplished using only three simple synthetic steps.[9–13] Recently, we have reported the chemical synthesis of the UNA phosphoramidite building blocks of adenine, cytosine, guanine and uracil (Fig. 2) starting from the 5′-DMT-protected ribonucleosides and consisting of three steps, oxidative cleavage/reduction, selective O2′-benzoylation, and O3′-phosphitylation.[14] The chemical synthesis of UNA phosphoramidite building blocks[14,15] for oligonucleotide synthesis is thus simple and efficient and compares advantageously with the multistep syntheses of the building blocks of many other modified nucleotides needed for synthesis of, *e.g.*, locked nucleic acids (LNA).[16] Moreover, UNA monomers can be easily incorporated into DNA or RNA oligonucleotides using automated oligonucleotide synthesisers and standard phosphoramidite chemistry with a step-wise coupling efficiency exceeding 99%.[9,14,†]

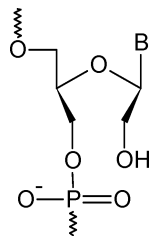

Fig. 1 Structure of a UNA monomer.

Hybridization properties of UNA oligonucleotides

Thermal stability studies have so far been described for UNA-modified DNA:DNA, RNA:DNA and RNA:RNA duplexes.[9,14,17,18] Acyclic nucleosides in general decrease the stability of the duplexes [9,19–21] and in this respect UNA monomers show similar behavior. The incorporation of a single UNA monomer into DNA:DNA duplexes (17-mers) results in a decrease in the thermal stability by about 10 °C.[9] A slightly weaker destabilization (5–8 °C) is observed for UNA monomers within RNA:RNA or RNA:DNA duplexes (21-mers).[14,17] Despite the destabilization of the duplexes upon incorporation of the UNA monomers, the Watson–Crick base-pairing rules are still obeyed.[14] Importantly, the incorporation of one or a few UNA monomers into the

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† UNA phosphoramidite monomers and UNA-modified oligonucleotides are commercially available from RiboTask ApS (www.ribotask.dk).

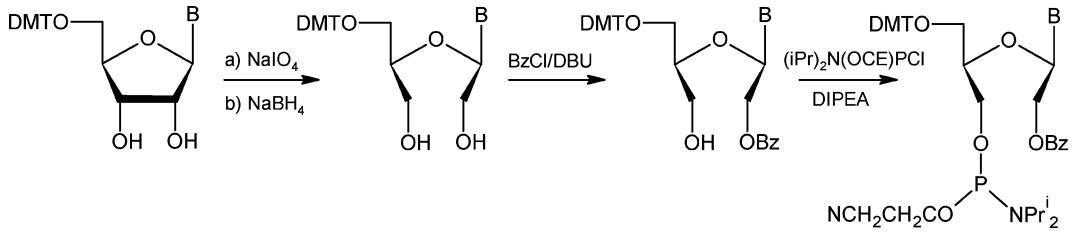

Fig. 2 The chemical synthesis of UNA phosphoramidite building blocks for automated oligonucleotide synthesis, B = nucleobases; DMT = 4,4′-dimethoxytrityl; OCE = cyanoethoxy.[14]

RNA:RNA duplexes do not alter the overall structure relative to that of the unmodified duplex.[18]

UNA monomers induce attractive properties when incorporated at two definite positions within an oligonucleotide. Thermal stability studies of RNA:RNA and RNA:DNA duplexes revealed that the presence of two UNA monomers directly flanking a mismatched RNA residue strongly decrease mismatch discrimination (Table 1). On the contrary, two UNA monomers interspaced by nine RNA residues significantly increase the base-pairing specificity of the RNA monomer placed centrally between those two modified monomers (Table 1).[14]

A detailed thermodynamic analysis of UNA-modified RNA duplexes indicates that UNA monomers destabilise more at internal than terminal positions, that UNA purine monomers decrease thermodynamic stability less than UNA pyrimidine monomers, that the stacking interactions of UNA residues are weaker than those of RNA residues, and that single substitution effects are additive, suggesting the possibility of establishing a reliable stability prediction tool for UNA-modified RNA duplexes.[18]

UNA monomers are potent modulators of the thermodynamic stability of highly-ordered structures like i-motifs or G-quadruplexes. It was found that a single UNA monomer may increase the stability of C-rich human telomere DNA (HT-DNA; i-motif structure; Fig. 3a) by about two-fold, or strongly destabilise the i-motif structure depending on its position.[22] Similarly, the introduction of UNA monomers into a quadruplex forming thrombin binding aptamer (TBA, Fig. 3b) results in a significant increase or decrease of the thermodynamic stability depending on its position within the structure.[23] UNA monomers have a stabilising effect when introduced at specific positions of the loops of the i-motif or TBA structures, while UNA modification of the core elements leads to a substantial destabilization.

UNA in aptamers

Aptamers are single stranded oligonucleotides which fold by intramolecular base pairing into structures which are able to bind to small molecules, proteins or whole cells.[24–27] Based on the thermodynamic results discussed in the previous paragraph, UNA monomers appear applicable for the design of aptamer drugs based on a quadruplex scaffold. The influence of UNA on the kinetic and biological properties of TBA was recently reported.[23] Unmodified TBA is marked by strong anticoagulant properties with a rapid onset of action and a short *in vivo* lifetime which facilitates the reversal of its activity. This eliminates dose-adjusting complications observed for other, commonly used anticoagulants and indicates possible applications in coronary surgery, the prevention and treatment of cardiovascular diseases or cancer therapy.[28–31] Kinetic and blood-clotting studies of UNA-modified TBA revealed that UNA monomers are allowed in many positions without a significant change in thrombin affinity or anticoagulant properties. Notably, one of the UNA-modified TBA variants (UNA in position 7, Fig. 3b), characterised by the highest thermodynamic stability of all variants, was found to have improved properties relative to unmodified TBA, *i.e.* superior binding affinity and more potent inhibition of fibrin-clot formation.

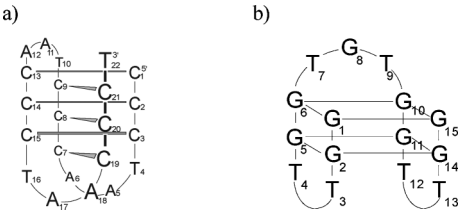

Fig. 3 Structures of HT-DNA (a) and TBA (b).

UNA in antisense oligonucleotides

The antisense strategy is aimed at suppressing gene expression by mRNA targeting, thus preventing the biosynthesis of pathogenic proteins.[32] The main advantage of this strategy is the ability to target, in principle, any genetic-based disease with high specificity.[32–34]

Table 1 Thermal stability of RNA:DNA and RNA:RNA duplexes containing one or two UNA monomers, and the effects of mismatches[14]

	T_m value (°C)	
RNA strand	DNA complement	RNA complement
5′-UGC ACU GUA UGU CUG UAC CAU	58 [–11, –9, –5]	71 [–10, –8, –6]
5′-UGC ACU GUA **U**G**U** CUG UAC CAU	42 [–1, ±0, –1]	57 [+1, ±0, –1]
5′-UGC AC**U** GUA **U**GU CUG **U**AC CAU	41 [–17, –13, –7]	47 [–12, –10, –9]

ΔT_m values for mismatches opposite position 11 (central G monomer) of the RNA strands are shown in brackets; the ΔT_m values in brackets are shown in the following order: [G:A mismatch, G:G mismatch and G:T/U mismatch] and are calculated relative to the fully matched duplexes (G:C). A = adenin-9-yl monomer, C = cytosin-1-yl monomer, G = guanine-9-yl monomer, U = uracil-1-yl monomer; UNA monomers are bold underlined letters.

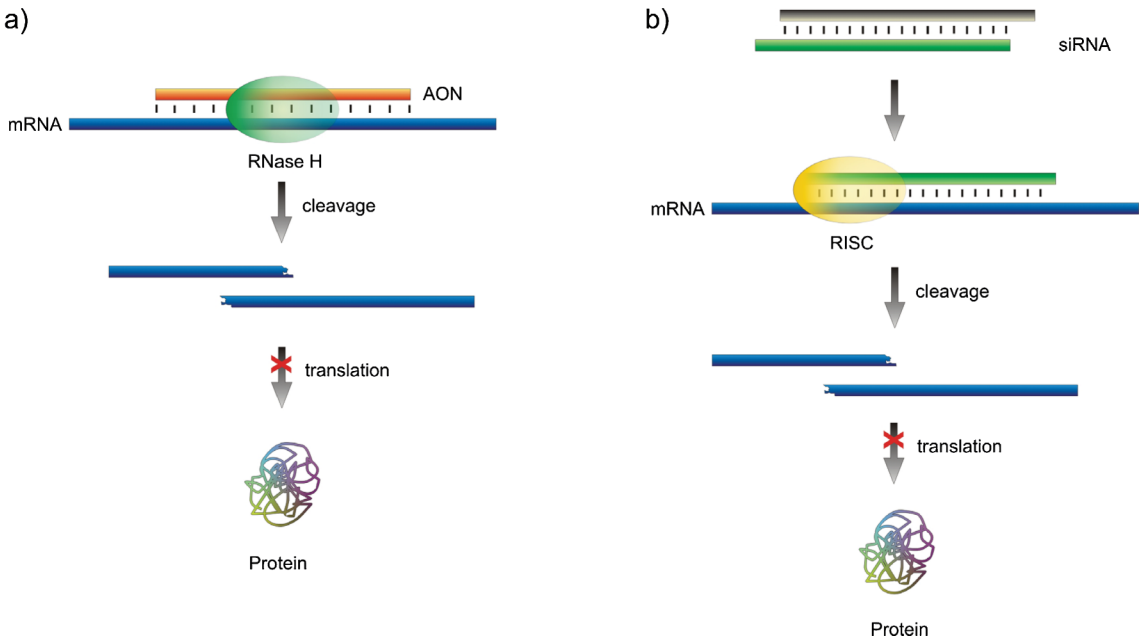

Fig. 4 Gene silencing approaches assisted by RNase H (a) and RISC (b).

It typically involves the hybridization of short, typically 14–20 mer long, antisense oligonucleotides leading to the formation of a heteroduplex with mRNA which acts as a substrate for RNase H, which in turn leads to mRNA degradation (Fig. 4a). To make antisense oligonucleoties functional, chemical modifications need to be introduced in order to improve the hybridization specificity and affinity, resistance towards exo- and endonucleases, and the activation of RNase H. Other desirable properties include low toxicity and an efficient cellular uptake.[32,33]

UNA monomers were shown to inhibit degradation by the 3′-exonuclease snake venom phosphodiesterase (SVPDE).[9] More recent reports have revealed that UNA-modified oligonucleotides also fulfil a second essential criteria of functional antisense oligonucleotides, namely compatibility with RNase H activity.[15,36] Biological studies showed that the introduction of UNA monomers instead of DNA monomers in the center of 2′-F-ANA[35] 18-mer antisense oligonucleotides efficiently improves the RNase H-promoted degradation of target RNA. This study was the first to illustrate the relevance of UNA modification in gene silencing strategies.[15] Moreover, *in vitro* studies of mRNA knockdown efficacy in cultured prostate tumor cell lines confirmed the compatibility of UNA-modified oligonucleotides with RNase H-assisted cleavage of mRNA.[36] It was shown that the substitution of a DNA monomer by a UNA monomer at different positions in the central DNA gap of an LNA-DNA-LNA gapmer antisense oligonucleotide results in efficient target RNA knockdown, and that improved mRNA knockdown was even observed for the highest (10 nM) concentration of one of the UNA modified gapmers.

UNA in siRNA constructs

During recent years another antisense technology approach, based on the RNA interference (RNAi) mechanism, has been successfully developed for efficient and specific post-transcriptional gene silencing.[32,37–40] This naturally occurring phenomenon involves 19–23 nt, asymmetric and double stranded structures named small interfering RNAs (siRNAs) which act in conjunction with the so-called RNA-induced silencing complex (RISC) (Fig. 4b). The introduction of various chemical modifications into siRNAs have led to the improvement of their efficiency, stability and specificity, facilitating the utilization of siRNA for *in vivo* studies.[40–45]

Several reports concerning promising applications of UNA monomers in siRNA constructs have been published very recently.[46–51] Five UNA-modified siRNAs which additionally contained 4′-C-hydroxymethyl-DNA, LNA or cyclohexene nucleic acid (CeNA) monomers were evaluated among the ten best of 134 differently modified siRNA variants for the suppression of an eGFP gene.[46] It was furthermore shown that the thermodynamic destabilization of the siRNA duplex in the 3′-end of the sense strand by the introduction of a UNA monomer considerably improves the gene silencing activity. The same was observed when UNA monomers were placed in the 5′-region of the antisense strand, though not directly at the 5′-end. In contrast, the presence of a UNA monomer at the 5′-end of the sense strand or the 3′-end of the antisense strand reduces siRNA activity. The enhanced ability for gene suppression reported for some designs of UNA-modified siRNAs can be attributed to the duplex destabilizing properties of UNA monomers most likely affecting strand selection by RISC making antisense strand incorporation more favourable.[52–56] Moreover, UNA-modified siRNAs seem to induce high cell viability upon transfection which make them highly suitable for both high-throughput applications and *in vivo* gene silencing.[46]

Another comprehensive study on the degradation of a target *ApoB* gene involving siRNAs with singly UNA-modified antisense (guide) strands showed that the modification is allowed in many positions without any significant loss of potency (Fig. 5).[47] However, the modification of one of the first three positions of the 5′-region of the antisense strand as well as several central positions

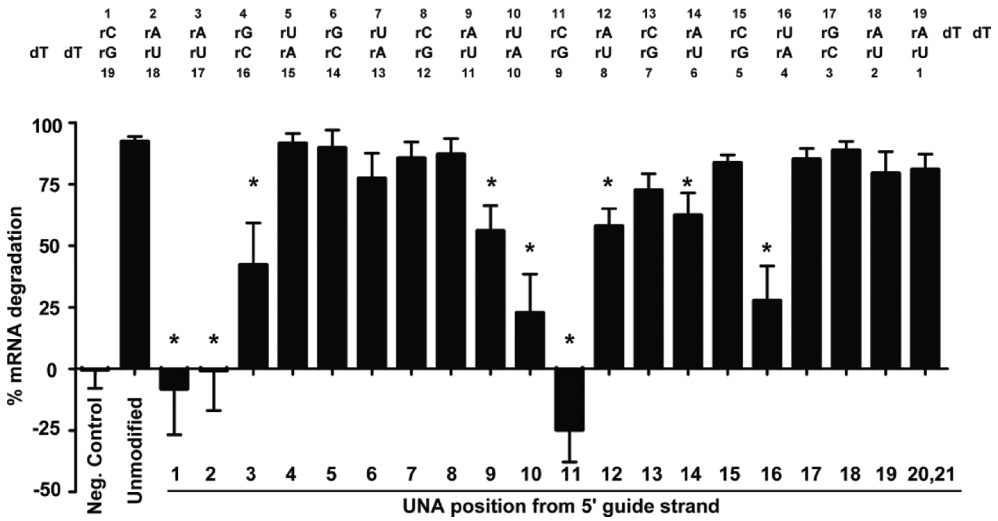

Fig. 5 Degradation of the target gene by UNA-modified siRNAs depend on the UNA position within the antisense strand. Kenski *et al.*, in an analysis of acyclic nucleoside modifications in siRNAs found that the sensitivity at position 1 was restored by 5′-terminal phosphorylation both *in vitro* and *in vivo*.[47] Reproduced by permission of Oxford University Press.

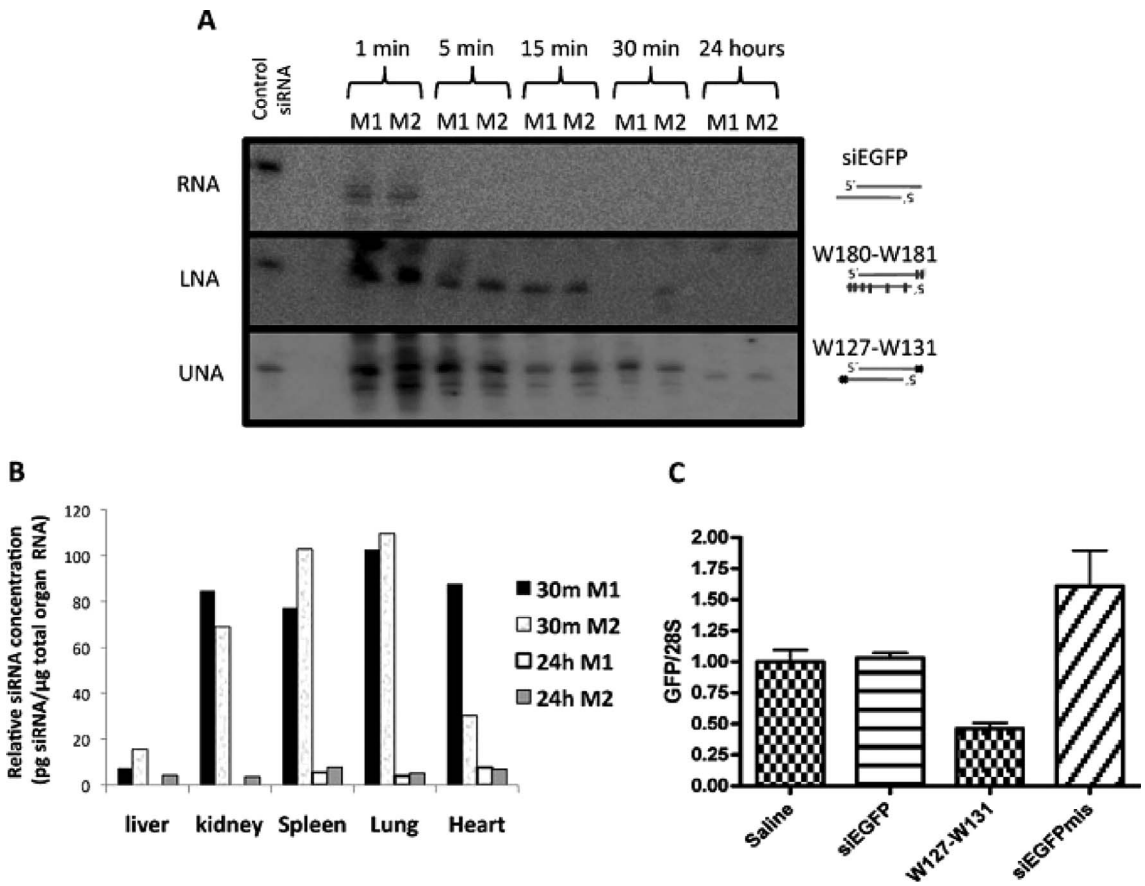

Fig. 6 Biostability of UNA-modified siRNA: stability in blood (**A**), biodistribution from various organs after 30 min and 24 h after intravenous injection (**B**), efficiency in eGFP KD in a human pancreas xenograft model in mice (**C**). siEGFP: unmodified siRNA, siEGFPmis: mismatch control, W180-W181: LNA-modified siRNA (AS: 5′ACUUGUGGCCGUUUACGUC**G**^{L}**C**^{L}, SS: 5′GA**C**^{L}GUAAA**C**^{L}GGC**C**^{L}A**C**^{L}AAGU**T**^{L}**C**^{L}U), W127-W131: UNA-modified siRNA (AS: 5′ACUUGUGGCCGUUUACGUCG**C**U, SS: 5′GACGUAAACGGCCACAAGUU**U**U). UNA monomers are marked by bold underlined letters, whereas LNA monomers by bold underlined letters with "L" superscript.[48] Reproduced by permission of The Royal Society of Chemistry.

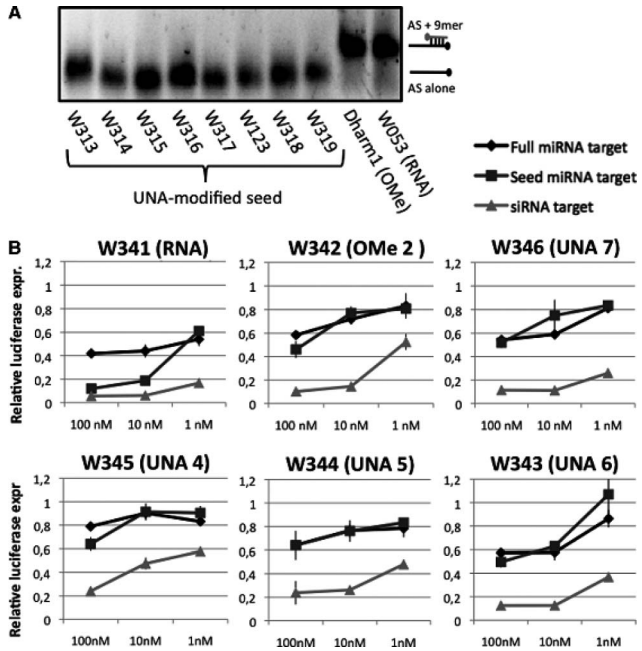

Fig. 7 Reduced off-targeting while retaining gene silencing activity by UNA-modified siRNA (W346(UNA7): UNA-modified seed of antisense strand at position 7). Destabilization of the initial (off-) target interaction between the UNA-modified seed region of the antisense strand and RNA (**A**). siRNA potency and off-targeting (**B**).[50] Reproduced by permission of Oxford University Press.

decreased or even abolished activity. Additionally, siRNAs having UNA monomers at positions 1, 2 and 3 of the antisense strand failed to be 5′-phosphorylated by mammalian RNA kinase (C1p1) which is a prerequisite for proper interactions between siRNA and RISC, and consequently target gene knockdown. Successful recovery of gene silencing was observed *in vitro* and *in vivo* after the chemical introduction of a 5′-phosphate group into the antisense strand modified with UNA at position 1, but not at positions 2 and 3. It was suggested that the increased flexibility introduced by UNA at position 2 or 3 hinders the proper interactions of siRNA with the Ago2 unit of RISC and the target mRNA. This data demonstrates the advantages of using siRNAs with a 5′-end UNA-modified sense strands to prevent its entry into RISC, thus eliminating off-target effects derived therefrom.[47]

An alternative approach to improving antisense strand incorporation into RISC by the introduction of a UNA monomer at the 3′-end of the sense strand has been reported.[48] It was also found that single UNA substitutions are well tolerated at many positions of the passenger strand. However, the introduction of more than one UNA monomer in the duplex-forming segment of the antisense strand usually has a detrimental effect on gene suppression due to the resulting substantial thermodynamic destabilization of the siRNA duplex or less favorable interactions with target mRNA. It was also shown that UNA monomers efficiently improve the activity of siRNAs which were heavily modified by other chemical modifications, *e.g.* strongly stabilizing LNA monomers. A similar effect has earlier been published for the so-called small internally segmented interfering RNA (sisiRNA) design involving a segmented sense strand.[57] *In vitro* biostability studies revealed that singly UNA-modified siRNAs are poorly resistant to degradation but also that the introduction of UNA monomers into siRNAs containing other modifications only slightly decreases the serum stability. Interestingly, an siRNA modified by a single UNA monomer in the 3′-end of each strand was found to be extremely stable *in vivo* (Fig. 6) and capable of efficiently knocking down human eGFP in a xenograft model of a pancreatic tumor, even without formulation.[48]

The potential for UNA-modification as a means to obtain superior siRNAs was confirmed in a study targeting coxsackievirus B3 (CVB-3) which is responsible for heart muscle infections.[49] The study revealed that siRNA constructs containing both UNA and LNA monomers at strategic positions are able to reduce virus propagation at least 10-fold *via* an RNAi mechanism. Moreover, LNA and UNA monomers were shown to be efficient tools to reverse the relative thermodynamic stability of the two siRNA ends, resulting in significant activity against the positively-oriented viral RNA, which is not silenced by unmodified siRNAs. Nevertheless, the activity of the modified siRNA was higher against the isolated target site than the full-length viral target.[49]

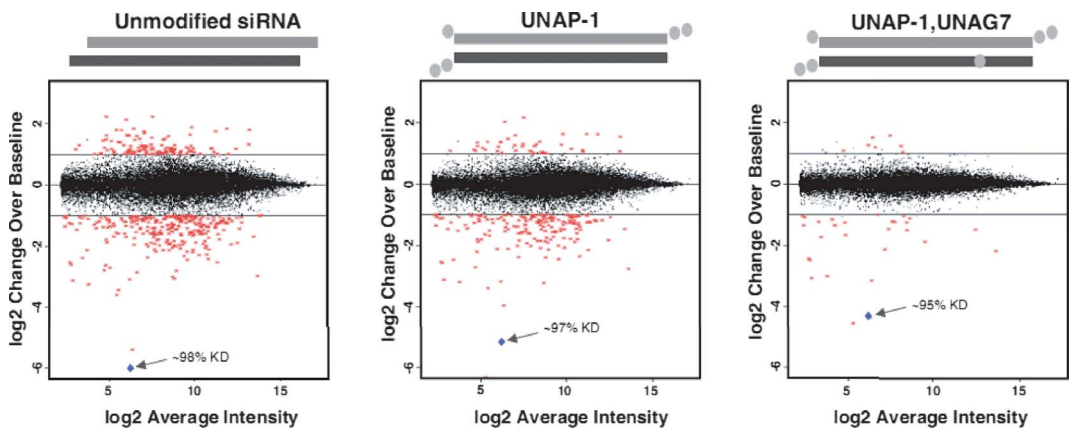

Fig. 8 The improvement in the siRNA silencing specificity is due to the introduction of UNA monomers. Plots of $\log_2$ change over the baseline *versus* the average intensity. Baseline: the average of no siRNA treatments. Unmodified siRNA treatment: 389 genes changed >2-fold ($P < 0.05$) from the no siRNA control baseline. UNAP-1 siRNA treatment: 215 genes changed >2-fold ($P < 0.05$). UNAP-1, UNAG7 siRNA treatment: 35 genes changed >2-fold ($P < 0.05$). Blue diamonds represent target gene.[51] Reproduced by permission of Oxford University Press.

A major problem towards realizing the prospects of siRNA-mediated gene silencing *in vivo* is the off-target effects caused by seed region of siRNA antisense strands (nucleotides 2–8) acting in a partially complementary manner with the mRNA targets.[58–62] Such microRNA-type off-target effects significantly decrease the specificity of the gene suppression. Strategic positioning of UNA monomers may however resolve this problem. The first paper to report on this comprised of a comprehensive analysis of 75 chemically diverse antisense strands containing 10 types of nucleotide modifications.[50] It was revealed that only a UNA monomer, when placed at position 7 in the seed region of the antisense strand efficiently decreases such micro-RNA related off-target effects without compromising the silencing activity (Fig. 7). It was suggested that a UNA monomer at position 7 significantly destabilizes off-target RNA–RNA interactions by decreasing the number of potential off-target mRNAs within the cell. More importantly, the presence of a UNA monomer still retains the position of nucleobases within the seed region which is crucial for siRNA activity. Alternative approaches such as the introduction of RNA mismatches, DNA monomers, or 2′-O-Me-RNA monomers within the seed region were previously described as effective methods to reduce off-target effects.[53,54,56] However, the application of UNA-modified siRNAs not only efficiently decreases off-target silencing but also maintains a high silencing potency, thereby eliminating undesirable processes accompanied by other approaches such as shifting off-targeting to another pool of mRNAs (RNA mismatches[53]) or reducing siRNA on-target effectiveness (DNA[54] and 2′-O-Me-RNA[50,56]).

A more recent study confirms that the unique properties of UNA monomers lead to an improved specificity of siRNA-mediated gene suppression by reducing the off-target effects originating from both sense and antisense strands.[51] It was reported that the presence of a UNA monomer‡ at the 5′-end of the sense strand efficiently abolishes its interactions with RISC, enhances antisense strand efficacy and significantly diminishes the RNAi-mediated sense strand activity leading to improved potency of siRNAs. Additional UNA monomers placed at the 3′-ends of both siRNA strands reduce off-target effects by about 2-fold (Fig. 8). Notably, the introduction of UNA monomers at position 7 of the antisense strand, at the 5′-end of the sense strand, and at the 3′-ends of both siRNA strands decreases the number of off-target effects more than 10-fold without affecting on-target siRNA activity (compared to unmodified siRNA). Moreover, UNA substitution at strategic positions does not result in any new off-target effects.

Conclusions

Recent studies concerning UNA as an RNA mimicking nucleotide modifier in oligonucleotides have revealed interesting results. The chemical synthesis of UNA monomers and their incorporation into DNA and RNA oligonucleotides is simple and efficient. UNA monomers were found to significantly and cumulatively decrease the thermodynamic stability of DNA:DNA, RNA:RNA and DNA:RNA duplexes, while at the same time for oligonucleotides modified with two UNA monomers, enable increased or decreased base-pairing specificity depending on the exact positioning of the UNA monomers. Moreover, a UNA monomer can modulate the thermodynamic stability of i-motif and G-quadruplex structures and constitutes a new option in the design of quadruplex-based aptamer drugs. Most notably, the resistance towards 3′-exonucleolytic degradation, low toxicity, and the enabling characteristics of high silencing activity and dramatically reduced off-target effects make UNA monomers highly attractive for therapeutic applications as a constituent in superior siRNA constructs.

‡ The terms "unlocked nucleic acid monomer" as used herein, and "unlocked nucleobase analogs" as used by Vaish *et al.*[51] concern the same chemical structure.

Acknowledgements

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