Filling the gap in LNA antisense oligo gapmers: the effects of unlocked nucleic acid (UNA) and 4′-*C*-hydroxymethyl-DNA modifications on RNase H recruitment and efficacy of an LNA gapmer

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Stability against nucleases, affinity for the targeted mRNA and the ability to recruit RNase H are prerequisites for antisense oligonucleotide (AON) applications where gene expression knockdown is required. Typically chimeric gapmer AON designs are used with a central continuous stretch of RNase H recruiting nucleotides (*e.g.* phosphorothioate DNA), flanked by affinity and stability-enhancing modified nucleotides. However, many types of nucleotide modifications in the central DNA gap can disturb RNase H function. Here we present studies into two different types of nucleotide modifications, a flexible acyclic RNA analog named unlocked nucleic acid (UNA) and 4′-*C*-hydroxymethyl-DNA in the gap of an LNA (locked nucleic acid) flanked gapmer. We compared the efficacy of mRNA degradation by the gap modified LNA antisense gapmers in cell-free assays and cultured cells. This study shows that both UNA and 4′-*C*-hydroxymethyl-DNA gap insertions are compatible with RNase H activity when used sparingly. However, multiple 4′-*C*-hydroxymethyl-DNA modifications are better tolerated by RNase H than multiple UNA modifications in the gap. Furthermore, this report shows that LNA gapmer AONs with multiple 4′-*C*-hydroxymethyl-DNA moieties in the gap can mediate target knockdown *in vivo*.

Introduction

Nucleotide analogues with constrained furanose ring conformations such as locked nucleic acids (LNA) are very useful in antisense oligonucleotides (AONs), providing both strong binding towards an RNA target and stability against nucleases. An LNA monomer contains an O2′–C4′ linkage (Fig. 1) that locks the furanose ring in an N-type conformation. This results in high binding affinity towards complementary RNA for AONs composed of a mixture of LNA and RNA or LNA and DNA nucleotides. Incorporation of LNA nucleotides into AONs induce the formation of almost canonical A-form helix structures when duplexed to complementary RNA. Thus LNA can be characterized as a structural mimic of RNA. Importantly, LNA nucleotides induce high binding affinities with increases in thermal denaturation temperatures (Tm values) of 2–8 °C per modification.[1,2] These properties make LNA a prime option to modify AONs for use *in vitro* and *in vivo*.

Classic DNA antisense oligonucleotides (AONs) derive much of their efficacy in mRNA knockdown through recruitment of the ubiquitous RNase H. However, RNase H is incompatible with nucleotide modifications which increase the stability and affinity of AONs. Substrate duplexes with N-type nucleotides like LNA or O2′-alkylated-RNA nucleotides are notoriously unable to recruit RNase H.[3,4] This problem can be solved by the use of the so-called gapmers, which are chimeric oligonucleotides with a central continuous stretch of RNase H recruiting nucleotides (typically phosphorothioate DNA) flanked by affinity or stability enhancing modified nucleotides (*e.g.* LNA, α-L-LNA or O2′-alkylated-RNA nucleotides). Systematic studies concluded that a gap of seven or eight DNA nucleotides is necessary for activation of RNase H.[5–7] The high affinity of the LNA flanks in the gapmer results in a significantly improved access to otherwise difficult to access mRNA target sites (increased hit-rate) and allows the use of shorter length ODNs (*e.g.* 16 mers instead of the typically used 20 mer phosphorothioates) with an increased efficacy of target knockdown at nanomolar concentrations.[6–8]

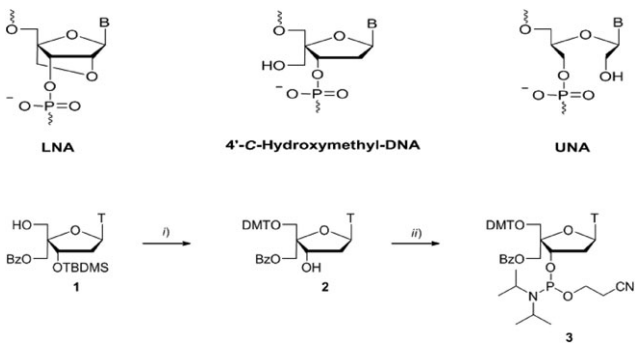

Fig. 1 Top, the chemical structures of LNA, 4′-*C*-hydroxymethyl-DNA and UNA monomers, and below the synthesis scheme used to prepare the novel 4′-*C*-hydroxymethyl-T phosphoramidite **3** as described in the experimental section.

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Moreover, the use of LNA in these gapmers increased stability against nucleases. LNA modified gapmer ODNs have also been shown to be very efficacious in several *in vivo* model systems.[8–10]

In contrast to LNA, UNA (unlocked nucleic acid) is an acyclic analogue of RNA in which the bond between the C2′ and C3′ atoms has been cleaved.[11] However, whereas the additional methylene group linking the O2′ and C4′ atoms of LNA locks its furanose ring into a C3′-*endo* conformation, the cleaved ribose ring of UNA makes this molecule very flexible. Unlike LNA, UNA was shown to induce decreased binding affinity towards a complementary DNA strand.[11] Oligonucleotides containing acyclic inter-residue units such as UNA are reported to support RNase H-promoted cleavage of complementary RNA when used in gapmers.[12] Manipulation of the backbone geometries using conformationally labile monomers may provide benefits in the enzymatic recognition of the nucleic acid hybrids, eliciting high RNase H activity. Following on from this we wondered whether UNA modifications could be used to fill in the DNA gap in an LNA flanked gapmer and to what extent we could push the amount of gap filling without losing RNase H recruitment and efficacy of target mRNA knockdown.

In addition, recent studies revealed that 4′-*C*-hydroxymethyl-DNA was tolerated by RNase H in *in vitro* assays.[13] This prompted us to see whether 4′-*C*-hydroxymethyl-DNA modifications can be used in the gap of a 16 mer LNA flanked gapmer oligonucleotide while retaining its efficacy in target mRNA knockdown in cells. In this study we compared the effects of introducing 4′-*C*-hydroxymethyl-DNA modifications in the gap of an LNA gapmer with introduction of acyclic unlocked nucleotides (unlocked nucleic acid) UNA in the gap. For this study we used a well characterized LNA gapmer against H-Ras, and we tested the efficacy of target mRNA knockdown in cell free systems and a cancer cell line growing in culture and as xenograft in a mouse. We show that for both 4′-*C*-hydroxymethyl-DNA and UNA single modifications are compatible with RNase H activity at different insertion sites in the gap. However, multiple 4′-*C*-hydroxymethyl-DNA insertions are far better tolerated by RNase H than multiple UNA modifications in the gap.

Results and discussion

Efficacy *in vitro*

For this study we tested two new chemical modifications: 4′-*C*-hydroxymethyl-DNA and UNA using a well-characterized LNA gapmer against H-Ras. We tested the efficacy of the different gap modifications in cultured cancer cell lines. Synthesis of UNA phosphoramidites has been described previously.[14] The synthesis route used to prepare the novel 4′-*C*-hydroxymethyl-T phosphoramidite is depicted in Fig. 1. The AON gapmer selected as the study model was designed as previously described.[8] The gapmer contains 3 LNA moieties per flank (ends) and a stretch of 9 DNA moieties in the center (gap). 4′-*C*-Hydroxymethyl-DNA (T) or UNA (U) nucleotides were inserted in the gap sequence as indicated in Table 1. This means that the gap modifications are located (counting from the 5′ position) at either the second, fifth or ninth positions of the gap, or at all three positions at the same time.

Table 1 Gapmer antisense oligonucleotide sequences used in this study with coding numbers displayed to the right[a]

AON	4′-*C*-Hydroxymethyl-DNA	UNA
T_**C**_**C**gtcatcgct_**C**_**C**_**T**_c (2091)	—	—
T_**C**_**C**g**X**catcgct_**C**_**C**_**T**_c	2092	2301
T_**C**_**C**gtca**X**cgct_**C**_**C**_**T**_c	2093	2302
T_**C**_**C**gtcatcgc**X**_**C**_**C**_**T**_c	2094	2303
T_**C**_**C**g**X**ca**X**cgc**X**_**C**_**C**_**T**_c	2095	2304

[a] LNA nucleotides are shown in bold capitals, DNA in lowercase, positions of the UNA (uracil-1-yl derivative) or 4′-*C*-hydroxymethyl-DNA (thymin-1-yl derivative) monomers are indicated in the sequences by the symbol "**X**".

We compared the efficacy of mRNA (H-Ras) knockdown by transfection of 15PC3 prostate tumor cells with the AONs and we used qPCR to measure H-Ras mRNA levels in the cells 24 hours post transfection. The well-described gapmer without gap modification showed near maximal efficacy (>70%) target knockdown at 0.5 nM concentration (Fig. 2). The single 4′-*C*-hydroxymethyl-DNA modifications did not interfere with the efficacy of the AON. The oligo 2093 with the modification in the center of the gap was similar efficient in target knockdown as the reference oligo 2091. However, our data in Fig. 2 suggests that AONs 2092 and 2094 where the single 4′-*C*-hydroxymethyl-DNA modification is located at either the 5′ or 3′ end of the gap have a slightly better efficacy in target knockdown at the 5 nM and 10 nM concentrations. When compared with the single UNA modifications in the gap there is only a slight reduction in target knockdown at the low 0.5 and 1 nM concentration, above 2 nM there is no discernable difference in efficacy when compared to the non-modified gap AON 2091. For UNA, three modifications is clearly detrimental leading to an essentially non-functional AON. However, the AON with three 4′-*C*-hydroxymethyl-DNA insertions can still knockdown H-Ras expression, albeit less efficiently than the reference gapmer at the lower concentrations. The 4′-*C*-hydroxymethyl-DNA modification disturbs the RNA–DNA duplex structure only to a limited extend which is reflected in duplex thermal stabilities which remain unchanged with either single or triple insertions (data not shown).

RNase H recruitment

In prokaryotes and eukaryotes two different enzymes with specific RNase H activity have been characterized as RNase H1 and RNase H2[15,16] The mammalian homologue of RNase H1 is the minor enzyme in mammalian cells, accounting for only 15% of total RNase H activity.[17] Nevertheless, *in vitro* liquid assays using cellular extracts containing either over-expressed RNase H1 or RNase H2 revealed that RNase H1 is the main mediator of AON mediated RNA degradation.[18] However, RNase H1 is easily hampered by substrate duplexes with N-type nucleotides like LNA or O2′-alkylated-RNA nucleotides.[4,5] Lima *et al.*[19] performed extensive studies of the effects of 2′-*O*-methoxyethyl (MOE) modifications on RNase H1 activity. They concluded that a conformational

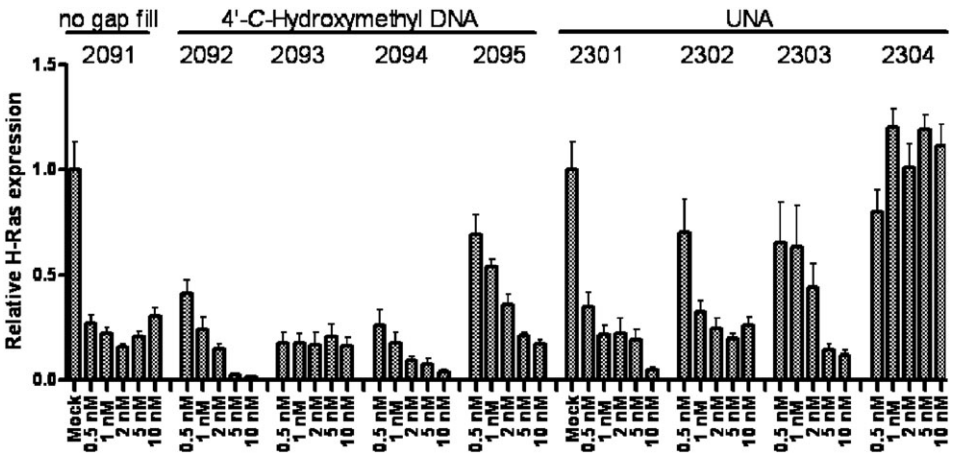

Fig. 2 Quantification of the knockdown of H-Ras mRNA levels in 15PC3 cells transfected with the indicated amounts of gapmer LNA AONs as measured using the qPCR-universal probe method. mRNA was isolated 24 hours post start of transfection. H-Ras levels were corrected using hATPase 6 as a housekeeping gene/loading control and normalized against mock transfected cells. Data depicted from three representative transfections.

transmission of the MOE–RNA helical geometry extended 2–5 base pairs into the DNA–RNA portion of the heteroduplex depending on the 5′ or 3′ position of the modifications. Therefore few ''RNA-like'' chemical modifications are tolerated in a stretch of DNA in a gapmer oligonucleotide for it to be able to attract RNase H1. To assess whether differences in the ability to recruit RNase H by the different gap modifications caused the differences observed in the mRNA knockdown efficacy, we compared the RNase H recruitment capabilities of the different LNA gapmer AONs in a liquid *in vitro* assay, using eukaryotic RNase H. For this assay 15PC3 cancer cell extracts were used as the eukaryotic source of RNase H activity and run-off RNA as the substrate. The data in Fig. 3 evidently show RNase H mediated cleavage of the run-off substrate (45% cleaved product after 5 minutes of incubation) with similar levels for the unmodified gap and the single 4′-*C*-hydroxymethyl-DNA insertions. The UNA insertions hint at a slightly reduced cleavage of the target (25–30% cleaved product), but the difference is very pronounced when the triple insertions are being evaluated. In line with the transfection experiments the triple UNA modification did not elicit RNase H cleavage of the target, while the triple 4′-*C*-hydroxymethyl-DNA insertion was still able to produce a cleaved product (15% cleaved product after 5 minutes of incubation), albeit less than the single 4′-*C*-hydroxymethyl-DNA insertions.

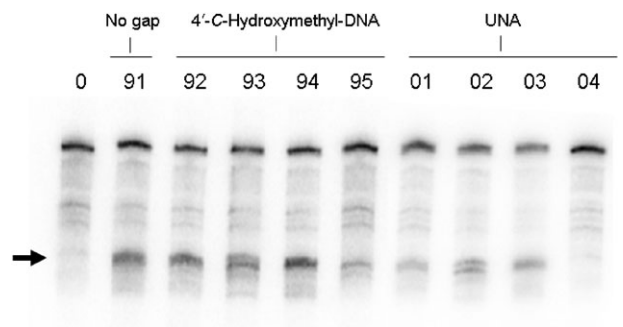

Fig. 3 *In vitro* RNase H assay. 15PC3 cancer cell extracts were used as the eukaryotic source of RNase H activity. The efficacy of H-Ras run-off mRNA cleavage as mediated by the AONs as indicated can be observed. The main cleavage product is indicated by an arrow.

In vivo knockdown of H-Ras

To study the *in vivo* efficacy of the gap filled AONs as compared to the classic LNA–DNA gapmer we used the in nude mouse model as described in previous studies with H-Ras targeting LNA gapmers.[8] We injected 15PC3 cells subcutaneously and after tumors had formed, AONs were injected intraperitoneal, once a day for two days, at a 2 mg kg^{-1} dose. Two days after the last dose, the tumors were removed and RNA was isolated to measure H-Ras levels by qPCR (Fig. 4). Compared with the control LNA gapmer that does not

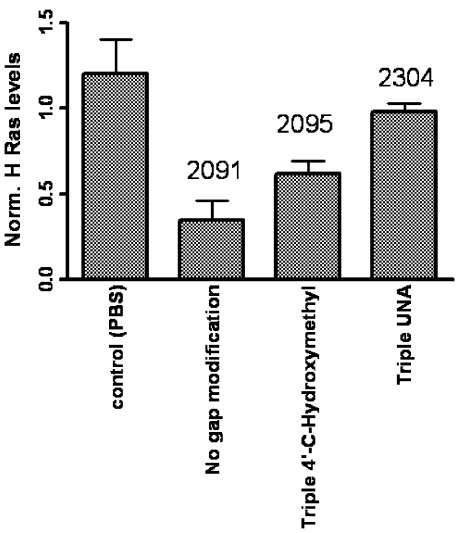

Fig. 4 Quantification of the knockdown of H-Ras mRNA levels in 15PC3 xenografts. Nude mice received two bolus injections (one injection per day at day one and two) of AONs as indicated with a dose of (2 mg kg^{-1} day^{-1}). The tumors were removed at day 4. RNA was isolated from the tumors and H-Ras levels were measured by qPCR.

contain any gap filling, the triple 4′-*C*-hydroxymethyl-DNA insertion reduced *in vivo* efficacy, but it could still be used to lower H-Ras levels in the tumors. In contrast, the AON modified with triple UNA insertions did not show any relevant knockdown of H-Ras levels *in vivo*.

These data show that LNA gapmer AONs with multiple 4′-*C*-hydroxymethyl-DNA insertions still retain enough efficacy for target mRNA knockdown in a tumor. Despite the fact that multiple 4′-*C*-hydroxymethyl-DNA insertions decrease the RNase H activity in the *in vitro* assay, *in vivo* loss of mRNA knockdown efficacy is limited. The multiple UNA modifications however do not elicit RNase H activity at all in the *in vitro* assay and consequently there is no significant knockdown *in vivo* of the target mRNA.

Experimental

LNA oligonucleotide synthesis

All ODNs were synthesized as all-phosphorothioate derivatives on an automated DNA synthesizer using commercial DNA and LNA phosphoramidites, 4′-*C*-hydroxymethyl-T phosphoramidite **3** and UNA-U phosphoramidite. The 4′-*C*-hydroxymethyl-T phosphoramidite used was prepared according to the procedure described below. The UNA-U phosphoramidite used [3′-*O*-(2-cyanoethoxy(diisopropylamino)-phosphino)-5′-*O*-(4,4′-dimethoxytrityl)-2′,3′-secouridine] was synthesized by an optimized version of the published procedure[11] for synthesis of the thymine monomer.[14] In all ODNs, 5-methyl-C was used. The DMT-ON ODNs were purified by reversed phase HPLC (RP-HPLC). After the removal of the DMT-group, the ODNs were characterized by AE-HPLC, and the expected molecular mass was confirmed by ESI-MS and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF) on a Biflex III MALDI (Bruker instruments, Leipzig, Germany). The sequences of the ODNs are depicted in Table 1.

Synthesis of 4′-*C*-hydroxymethyl-T phosphoramidite 3

4′-*C*-Benzoyloxymethyl-5′-*O*-(4,4′-dimethoxytrityl)thymidine (2). 4′-*C*-Benzoyloxymethyl-3′-*O*-(*tert*-butyldimethylsilyl)thymidine (1.01 g, 2.06 mmol)[20] was dissolved in anhydrous pyridine (75 ml) under nitrogen and 4,4′-dimethoxytrityl chloride (1.43 g, 4.22 mmol) was added at room temperature under stirring. After 4 h, the reaction mixture was concentrated to dryness under reduced pressure, and the residue was co-evaporated using MeCN. Methylene chloride (100 ml) was added and the organic phase was washed with sat. aq. $NaHCO_3$ (3 × 75 ml), dried over Na_2SO_4 and evaporated to dryness under reduced pressure. The residual yellowish oil was dissolved in anhydrous THF (50 ml) under nitrogen and 1.0 M tetrabutylammonium fluoride in THF (4.1 ml, 4.1 mmol) was added at room temperature under stirring. After 45 min, EtOAc (300 ml) was added and washing was performed using sat. aq. $NaHCO_3$ (3 × 150 ml). The organic phase was separated, dried over Na_2SO_4 and evaporated to dryness under reduced pressure. Nucleoside **2** was obtained as a white solid material after column chromatographic purification (0–80% EtOAc–petroleum ether, v/v) and evaporation under reduced pressure of the pooled fractions containing pure product. Yield 1.02 g (73%). ^{1}H NMR ($CDCl_3$) δ 1.54 (s, 3H, 5-CH_3), 2.37–2.58 (m, 2H, H-2′), 3.40 (d, 1H, J = 9.7 Hz, H-5′$_a$), 3.43 (br s, 1H, 3′-OH), 3.56 (d, 1H, J = 9.9 Hz, H-5′$_b$), 3.75 (s, 6H, 2 × OCH_3), 4.53 (d, 1H, J = 11.9 Hz, 4′-*C*-CH_{2a}), 4.62 (d, 1H, J = 11.8 Hz, 4′-*C*-CH_{2b}), 4.65–4.72 (m, H-3′), 6.49 (t, 1H, J = 6.9 Hz, H-1′), 6.79 (dd, 4H, J = 3.2 Hz, J = 9.0 Hz, H_{arom}), 7.20–7.56 (m, 13H, H_{arom}, H-6), 7.83 (d, 2H, J = 7.1 Hz, H_{arom}), 9.35 (br s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ 11.9, 40.7, 55.2, 64.4, 65.3, 73.6, 84.9, 87.2, 87.2, 111.3, 113.3, 113.4, 127.1, 127.8, 128.0, 128.0, 128.3, 128.6, 129.5, 129.6, 129.9, 130.1, 130.1, 133.1, 135.1, 135.2, 135.6, 144.2, 150.5, 158.6, 158.7, 163.8, 166.2. HRMS (ESI) m/z calculated for $C_{39}H_{38}N_2O_9Na^+$ (M + Na^+): 701.2470; found: 701.2453.

4′-*C*-Benzoyloxymethyl-3′-*O*-(2-cyanoethoxy(diisopropylamino)-phosphino)-5′-*O*-(4,4′-dimethoxytrityl)thymidine (3). Nucleoside **2** (560 mg, 0.825 mmol) was co-evaporated three times from anhydrous MeCN (3 × 10 ml) and the residue was dissolved in anhydrous methylene chloride (20 ml) under nitrogen at room temperature under stirring. *N*,*N*-Diisopropylethylamine (0.50 ml, 2.9 mmol) and then 2-cyanoethyl *N*,*N*-diisopropyl-phosphoramidochloridite (0.25 ml, 1.1 mmol) were added slowly. After 3 h, the reaction mixture was evaporated to dryness under reduced pressure to give a residue which was subjected to column chromatographic purification (50.0 : 49.5 : 0.5 EtOAc–petroleum ether–Et_3N, v/v/v) to give amidite **3** as a white solid material after evaporation under reduced pressure of the pooled fractions containing pure product. Yield 484 mg (67%). ^{31}P NMR ($CDCl_3$) δ 149.0, 150.1. HRMS (ESI) m/z calculated for $C_{48}H_{55}N_4O_{10}PNa^+$ (M + Na^+): 901.3548; found: 901.3523.

Cell culture and transfection of LNA gapmers

The prostate cancer cell line 15PC3 was maintained by serial passage in Dulbecco's modified Eagle's medium (DMEM). Cells were grown at 37 °C and 5% CO_2. Media were supplemented with fetal calf serum (10% v/v), L-glutamine (2 mM), penicillin (100 U ml^{-1}) and streptomycin (100 μg ml^{-1}). ODN transfections were performed in 6-well culture plates with Lipofectamine 2000 (Invitrogen) as liposomal transfection agent. Fluorescently (FAM) labeled LNA ODNs were used to determine the transfection efficiency.

For fluorescence microscopy, cells were plated on glass coverslips in a 6-well culture plate, and transfected with FAM-labeled LNA ODNs. For analysis, cells were fixed on the glass in PBS containing paraformaldehyde (4% m/v) and embedded in Vectashield Mounting Medium (Vector Laboratories Inc., Burlingame, CA, USA). Fluorescence microscopy was done with a Vanox microscope (Olympus) and appropriate filters.

In vivo experiments

For the *in vivo* experiments: eight to ten week old athymic nude NMRI nu/nu mice (Charles River, Maastricht, the Netherlands) were injected subcutaneously in the flank with 10^6 15PC3 cells in Matrigel (300 μl) (Collaborative Biomedical products, Bedford, MA, USA). When tumor take was positive (after 10 days), AONs (dissolved in PBS) were injected I.P.

(2 mg kg^{-1} day^{-1}) for two days, and tumors were removed at day 4. RNA was isolated from the tumors and H-Ras levels were measured by qPCR. All animal experiments were conducted in compliance with the law in the Netherlands and were sanctioned by the local animal ethics committee of the AMC.

qPCR

RNA was isolated using Trizol according to the instructions of the manufacturer (Invitrogen). cDNA was made using oligodT primer and SuperScriptII enzyme (Invitrogen). qPCR was done using Universal probe primers (Roche) and a Lightcycler 480 (Roche). Primer sets used (5′–3′): H-Ras U-Probe 32 and Forward cttttgaggacatccaccagt, Reverse acgtcatccgagtccttcac. All data was corrected using hATPase 6 as housekeeping gene/loading control Uprobe 23 Forward cataatgacccaccaatcaca, Reverse gagagggcccctgttagg. All reactions were done in quadruplicate and qPCR conditions were as standard recommended by the manufacturer (Roche).

In vitro RNase H assay

The *in vitro* RNase H assay is a combination of two protocols described in the literature.[21,22] RNase H derived from eukaryotic cells was used in the assay. Whole cell extracts were prepared as follows: exponentially growing cells were harvested by scraping, were washed once in NaCl/Pi, and then resuspended in 100 μL hypotonic lysis buffer (7 mM Tris–HCl pH 7.5, 7 mM KCl, 1 mM $MgCl_2$, 1 mM 2-mercaptoethanol) per 106 cells. After 10 min incubation on ice, DNA was sheared by repeated passaging through a 27 Gauge needle. Then, 0.1 vol. of neutralization buffer (21 mm Tris–HCl pH 7.5, 116 mM KCl, 3.6 mM $MgCl_2$, 6 mM 2-mercaptoethanol) was added. Cell debris was removed by centrifugation for 10 min at 4 °C. The supernatant was transferred to a fresh tube on ice and glycerol was added to a final concentration of 45%. The RNase H activity in these extracts is relatively labile and susceptible to freezing or diluting of the extracts. The extracts used in one experiment were always isolated at the same time and treated in the same way. Therefore, within one experiment, the ratio of the extracts of different cell lines is comparable. Absolute levels differ between the experiments. Template RNA was prepared by *in vitro* transcription of linearised target (H-Ras) plasmid construct using T7 RNA polymerase (Promega) and the manufacturer's protocol. Run-off RNA and complementary ODN were denatured separately by boiling for 5 min in 100 mM KCl, 0.1 mM EDTA and slowly cooled to room temperature to allow folding of the template. Template RNA (50 ng) and 100 ng ODN were annealed at 37 °C for 15 min in 30 μL of 100 mM KCl, 0.1 mM EDTA. Then, RNase H mixture was added comprising of 8.4 μL 5 × buffer (250 mM Tris–HCl pH 7.5, 50 mM $MgCl_2$, 1 mM dithiothreitol, 2.5 mg mL^{-1} BSA), 1 μL RNasin (20 U μL^{-1}; Promega) and 5 μL cell extract, and incubated at 37 °C for 5 min. RNA was subsequently precipitated in the presence of glycogen, after removal of proteins by phenol extraction, and dissolved in gel loading buffer containing 95% formamide. Fragments were separated on a denaturing gel (6% acrylamide, 8 M urea), electroblotted (GeneSweep, Hoefer scientific instruments, San Francisco, CA) onto Hybond-N+ membrane (Amersham), and visualized by hybridization with a probe derived from the insert of the plasmid used for run-off RNA synthesis.

The run-off RNA used for H-Ras corresponds to position 145–280 in GenBank accession NM_176795. Hybridized probe was visualized and quantified on a phospho imager (Fuji BAS imager) using AIDA 2.46 software (Raytest Benelux, Tilburg, the Netherlands).

Conclusion

We showed that both 4′-*C*-hydroxymethyl-DNA and UNA single modifications are compatible with RNase H activity at different insertion sites in the gap. However it is clear that a 4′-*C*-hydroxymethyl-DNA modification is better tolerated by RNase H1 than an UNA modification, which is also clearly manifested for multiple insertions in the gap. This indicates that manipulation of the backbone geometries using conformationally labile monomers does not per se provide benefits in the enzymatic recognition of the nucleic acid hybrids by RNase H1 as described previously.[12] An AON with multiple 4′-*C*-hydroxymethyl-DNA insertions was still efficacious in target knockdown in a tumor xenograft indicating that these modifications could be useful for *in vivo* use.

In this study, we used LNA gapmers and it is likely that the LNA flanks extend a conformational transmission of an RNA-like helical geometry even when acyclic nucleotides are used. RNase H1 prefers a strict RNA–DNA heteroduplex, which apparently is less disturbed by 4′-*C*-hydroxymethyl-DNA modifications in the gap. In the fully phosphorothiolated LNA–DNA gapmer AONs there is little reason to modify the DNA gap for stability issues. With systemic delivery, phosphorothiolated LNA gapmers are very stable *in vivo*[8] and additional modifications inducing extra stabilization do not increase bio-availability per se because of the efficient elimination by renal excretion.[23] However, gap modifications might prove useful when non-phosphorothiolated AONs are to be used with a topical delivery strategy, since thiolated AONs, at least when used at higher doses, have been reported to increase the risk for non-specific side effects *in vivo*.[23,24] In addition, the ability to incorporate novel and effective gap modifications, which retain the ability to recruit RNase H1, might provide extra options for intellectual property rights considerations which might open up new avenues for the use of these compounds.

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