On the in vitro and in vivo Properties of Four Locked Nucleic Acid Nucleotides Incorporated into an Anti-H-Ras Antisense Oligonucleotide

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Locked nucleic acid (β-D-LNA) monomers are conformationally restricted nucleotides bearing a methylene 2′-O, 4′-C linkage that have an unprecedented high affinity for matching DNA or RNA. In this study, we compared the in vitro and in vivo properties of four different LNAs, β-D-amino LNA (amino-LNA), β-D-thio LNA (thio-LNA), β-D-LNA (LNA), and its stereoisomer α-L-LNA in an antisense oligonucleotide (ODN). A well-known antisense ODN design against H-Ras was modified at the 5′- and 3′-ends with the different LNA analogues (LNA-DNA-LNA gapmer design). The resulting gapmers were tested in cancer-cell cultures and in a nude-mouse model bearing prostate tumor xenografts. The efficacy in target knockdown, the biodistribution, and the ability to inhibit tumor growth were measured. All anti H-Ras ODNs were very efficient in H-Ras mRNA knockdown in vitro, reaching maximum effect at concentrations below 5 nM. Moreover, the anti-H-Ras ODN containing α-L-LNA had clearly the highest efficacy in H-Ras knockdown. All LNA types displayed a great stability in serum. ODNs containing amino-LNA showed an increased uptake by heart, liver, and lungs as compared to the other LNA types. Both α-L-LNA and LNA gapmer ODNs had a high efficacy of tumor-growth inhibition and were nontoxic at the tested dosages. Remarkably, in vivo tumor-growth inhibition could be observed at dosages as low as 0.5 mg kg^{-1} per day. These results indicate that α-L-LNA is a very promising member of the family of LNA analogues in antisense applications.

Introduction

Several strategies are available to mediate specific gene knockdown. The oldest and best-understood method for inhibiting gene expression is the use of antisense oligonucleotides (ODNs). The most efficient mechanism of inhibition of gene expression by ODNs is mediated through RNase H-based cleavage of mRNA in the DNA–RNA heteroduplex following Watson–Crick base pairing.[1] Unmodified DNA or RNA ODNs are not of much use in vivo because they are easy prey for all sorts of nucleases present in biological systems. To circumvent these biological defense systems, three generations of chemical modifications to the sugar, base, and backbone have been used in ODNs. First, thiolation of the phosphodiester backbone of an ODN is the best-known modification, but this has also been associated with nonspecific effects caused by interactions with intracellular and cell-surface proteins.[2] The second generation of ODNs contain modifications of the 2′-*O* position of the ribose moiety.[3] Recently, interest has been focused on the third-generation antisense ODNs, which contain conformationally restricted derivatives. β-D-Locked nucleic acid (LNA), contains a methylene 2′-*O*, 4′-*C* linkage (Scheme 1).[4] This bridge reduces conformational flexibility and confers an RNA-like C3′-*endo* conformation on the sugar moiety of the nucleotide.[5] This greatly enhances affinity towards DNA and RNA targets (ΔT_m values from 4.0 to 9.3 °C per introduced LNA monomer compared to unmodified duplexes).[6] Very much like all 2′-*O*-modified ODNs, β-D-LNA is not a good substrate for RNase H. Systematic studies concluded that a DNA gap of 7 or 8 nt is necessary for activation of RNase H.[7] The high affinity of LNA results in significantly improved access to a RNA target, which allows the use of shorter-length ODNs (16-mers instead of the typically used 20-mer phosphorothioates), and in an increased efficacy of target knockdown at low concentrations.[7] Moreover, the use of LNA in these gapmers increased stability against nucleases more than tenfold. It was shown that LNA-modified ODNs can be very efficacious in several in vivo model systems.[8]

The clear benefits of LNA have prompted research into other derivatives.[9] These LNA analogues form a whole family of related conformationally restricted molecules with a methylene 2′-heteroatom, 4′-*C* linkage. In this study, we compared the properties of four of these LNA family members when incorporated into antisense ODNs both in vitro and in vivo. The classic β-D-LNA (LNA) chemistry was compared with its stereoisomer α-L-LNA together with more recent LNA family members such as amino-β-D-LNA (amino-LNA) and thio-β-D-LNA (thio-LNA; Scheme 1).[9c–h] Here we report the first comparative

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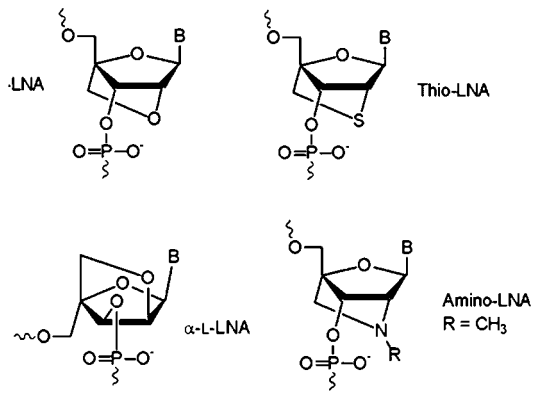

		T_m
Amino-LNA	Full match $T^N_s{}^{m}C^N_s{}^{m}C^N_s g_s t_s{}^{m}c_s a_s t_s{}^{m}c_s g_s{}^{m}c_s t_s{}^{m}C^N_s{}^{m}C^N_s T^N_s{}^{m}c_s$ Missmatch $T^N_s{}^{m}C^N_s T^N_s g_s t_s a_s a_s t_s a_s g_s{}^{m}c_s{}^{m}c_s{}^{m}C^N_s{}^{m}C^N_s{}^{m}c_s$	66.6 °C
Thio-LNA	Full match $T^S_s{}^{m}C^S_s{}^{m}C^S_s g_s t_s{}^{m}c_s a_s t_s{}^{m}c_s g_s{}^{m}c_s t_s{}^{m}C^S_s{}^{m}C^S_s T^S_s{}^{m}c_s$ Missmatch $T^S_s{}^{m}C^S_s T^S_s g_s t_s a_s a_s t_s a_s g_s{}^{m}c_s{}^{m}c_s{}^{m}C^S_s{}^{m}C^S_s{}^{m}c_s$	71.1 °C
α-L-LNA	Full match $T^\alpha_s{}^{m}C^\alpha_s{}^{m}C^\alpha_s g_s t_s{}^{m}c_s a_s t_s{}^{m}c_s g_s{}^{m}c_s t_s{}^{m}C^\alpha_s{}^{m}C^\alpha_s T^\alpha_s{}^{m}c_s$ Missmatch $T^\alpha_s{}^{m}C^\alpha_s T^\alpha_s g_s t_s a_s a_s t_s a_s g_s{}^{m}c_s{}^{m}c_s{}^{m}C^\alpha_s{}^{m}C^\alpha_s{}^{m}c_s$	69.5 °C
LNA	Full match $T_s{}^{m}C_s{}^{m}C_s g_s t_s{}^{m}c_s a_s t_s{}^{m}c_s g_s{}^{m}c_s t_s{}^{m}C_s{}^{m}C_s T_s{}^{m}c_s$ Missmatch $T_s{}^{m}C_s T_s g_s t_s a_s a_s t_s a_s g_s{}^{m}c_s{}^{m}c_s{}^{m}C_s{}^{m}C_s{}^{m}c_s$	69.4 °C

Scheme 1. Chemical structures of the four LNAs tested and the sequences of the ODNs used in this study. Abbreviations used; Capital letter: LNA, small letter: DNA. C^N: amino-LNA, C^S: thio-LNA, C^O: LNA, C^α: α-L-LNA, C_s: phosphorothiolated, ^mC: methyl-C. The T_m for each full match ODN as measured against full matched DNA oligonucleotides is also indicated.

study of the properties of the three LNA analogues and LNA when incorporated into a classic antisense ODN design and demonstrate that α-L-LNA is the most promising new member of the LNA family, pairing good efficacy with specificity.

Results and Discussion

Oligonucleotide design

In this study, we have used a 16-mer version of the classic 20-mer anti H-Ras ODN design ISIS 2503 as a model ODN. The incorporation of LNA moieties allows the shortening of a 20-mer to a 16-mer with improved efficacy in target knockdown.[10] This ODN design was chosen because we have used the ISIS 2503 design in previous studies and have shown that H-Ras is a valid and proven target in our model systems.[11] Secondly, both the properties of the first and second generations of antisense ODNs have been tested using the ISIS 2503 design in previous studies.[12]

A gapmer design was chosen with three LNA moieties per flank (ends) and a stretch of nine DNA bases in the center (gap) and a DNA base at the 3′-end. The ODNs were fully thiolated. For each type of LNA chemistry, a version of the ODN design and a five-mismatch control were synthesized. The T_m was approximately 70 °C and very similar for LNA, α-L-LNA, and thio LNA. Only amino-LNA showed a somewhat lower T_m of 66 °C (Scheme 1).

In vitro properties

To test the biological properties in vitro we used the prostate-cancer cell line 15PC3, as described in previous studies.[8b,11] First, the efficiency of transfection was compared for each LNA analogue. By using radiolabeled and FAM-labeled ODNs, the amount of ODN uptake was determined after 5 h of transfection with lipofectamine 2000. For all LNA analogues, there was more than 95% transfection efficiency into the cells. Both LNA and its stereoisomer α-L-LNA showed similar levels of uptake and similar intracellular-distribution patterns, with clear nuclear, perinuclear, and diffuse cytosolic staining (Figure 1). Thio-LNA was taken up almost twice as much as LNA and α-L-LNA, but ended up for the most part in cytosolic vesicles. Amino-LNA was taken up in relatively low levels, but showed a similar intracellular distribution pattern to LNA and α-L-LNA. Amino-LNA, thio-LNA, and LNA showed very similar efficacies in mRNA knockdown as assayed by Northern blotting ($IC_{50} = 1.55$ nM; Figure 2). Remarkably, α-

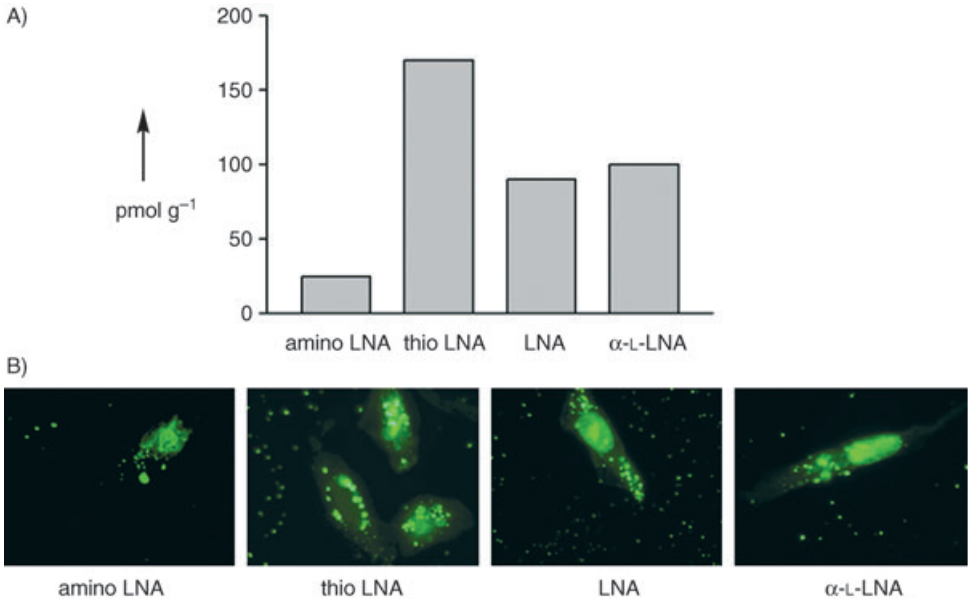

Figure 1. Cellular uptake and intracellular distribution of LNA-modified anti-H-Ras ODNs. 15PC3 Cells were transfected with tritiated or FAM-labeled LNA ODN by using lipofectamine 2000. After 5 h A) the total uptake of radiolabeled ODN per mg cell protein and B) the intracellular distribution were studied.

L-LNA had a significantly lower IC_{50} of 0.35 nM. In the 1–5 nM concentration range, none of the mismatch controls of the LNAs inhibited H-Ras mRNA expression significantly; this indicates sequence specificity.

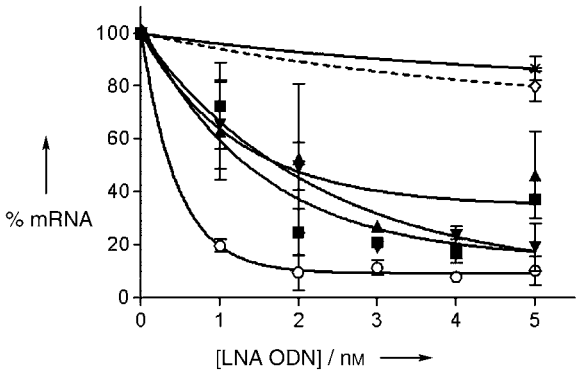

Figure 2. Efficacy of LNA-modified anti-H-Ras ODNs to knockdown H-Ras mRNA as measured by Northern blotting. 15PC3 cells were transfected with the indicated amounts of LNA ODN. ◇ mismatch LNA, * mismatch α-L-LNA, ▲ thio LNA, ▼ LNA, ■ amino LNA, ○ α-L-LNA. Twenty hours post transfection, the cells were harvested, and, after isolation, the RNA was blotted. The H-Ras mRNA levels were corrected for loading by using a 28S ribosomal RNA probe. The quantified data of three separate experiments are depicted ± SEM. As a control mismatched versions of LNA and α-L-LNA ODNs, as indicated in Scheme 1, were transfected at the highest 5 nM dosage only.

In vivo properties

To study the in vivo properties of the different LNA analogues, we used a model of nude mice with tumor xenografts.[11] The radiolabeled ODNs were administered by subcutaneously implanted osmotic minipumps. The biodistribution of each analogue was determined (Figure 3A). LNA and α-L-LNA showed very similar biodistributions, typical for LNA and thiolated DNA ODNs,[8b] except for in the kidneys, where the uptake of α-L-LNA was significantly higher. In contrast, amino- and thio-LNA showed an increased uptake by the liver. Compared with the other analogues, amino-LNA showed the most different biodistribution, with increased uptake in the heart, lung, muscle, and bone. In the 15PC3 tumor xenografts, the amounts of LNA and α-L-LNA taken up were more or less similar. In order to test whether the new LNA modifications confer nuclease resistance on ODN gapmers, we determined the stability of all four LNAs in serum at 37 °C. Like LNA, all the analogues showed a great stability in serum. The stability in serum of the four LNAs during 24 h of incubation was similar (Figure 3C).

Since the α-L-LNA was considerably more potent in vitro than the other LNA ODNs, we tested whether α-L-LNA was also more potent in vivo. The efficacy at tumor-growth inhibition of LNA ODN and α-L-LNA ODN was determined at four different dosages (0.25, 0.5, 1, and 2.5 mg kg^{-1} per day). Nude mice bearing 15PC3 xenografts were treated with anti H-Ras ODNs containing either LNA or α-L-LNA modifications by using osmotic minipumps. At a 1 mg kg^{-1} dosage, clear inhibition of tumor growth was seen with both LNA and α-L-LNA ODN (Figure 4). The mismatch control of α-L-LNA only slightly inhibited tumor growth at dosages up to 1 mg kg^{-1} per day. Figure 4B shows a summary of the tumor-growth rates in response to different dosages. Tumor growth was measured during the treatment period, and the slope of the growth curve was fitted to calculate the mean growth rate during the entire treatment period (Figure 4B). From these data, it is clear that α-L-LNA ODN is slightly more potent in inhibiting 15PC3 growth than LNA ODN. Both LNAs have superior efficiency in tumor-growth inhibition to the previously published 5 mg kg^{-1} per day dosages needed for 15PC3 xenograft-growth inhibition with the classic ISIS 2503 phosphorothioate ODN.[8b] However, increasing the dosage above 1 mg kg^{-1} per day resulted in a loss of specificity of the LNA ODNs, since the tumor growth was also inhibited by the mismatch control ODN at 2.5 mg kg^{-1} per day dosage. From our own experience and that of other groups,[13] it has become clear that significant LNA modifications can impose "mismatch tolerance" on oligonucleotides, and this needs to be considered when designing the compound. However, we note that, when dealing with rapidly mutating disease agents such as viruses and bacteria, a level of mutation tolerance can be desirable. To evaluate whether LNA and α-L-LNA caused any toxicity in the mouse liver (one of the principle uptake sites of ODNs in the body), aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), and alkaline phosphatase levels were measured after treatment. No significant adverse effects of either analogue were apparent when measuring the enzymatic activity levels in the serum after treatment (Figure 5). The body temperature of the mice was also monitored continuously during treatment, and no abnormal fluctuations were seen.(data not shown).

Conclusion

All the new LNA family members tested possess the characteristics of high affinity for matching mRNA and stability against nucleases. Like the parent LNA, they can be incorporated into ODNs without problems and they have a high efficacy in target knockdown in in vitro cell transfections. Our results suggest that α-L-LNA ODN can further improve upon the already great efficacy of the parent LNA in vitro and in vivo. α-L-LNA is better (ca. fivefold) than other LNA analogues at mediating target knockdown in vitro. It is well established that β-D-LNA is locked in a N-type conformation and thus gives rise to an A-form duplex with complementary DNA and an almost canonical A-type (the natural form of double-stranded RNA) with complementary RNA.[5] Duplexes between α-L-LNA and DNA adopt a B-form[14] (the natural form of double-stranded DNA), whereas duplexes of α-L-LNA with RNA generate an intermediate structure that is between the A and B forms[9g] and structurally closer to the natural substrate of RNase H.[7c,9h] However, it is still unclear whether this fact contributes to the in vivo efficacy of the antisense ODNs.

The data presented here underpin the exciting possibility of influencing pharmacokinetic parameters by choice of LNA chemistry. In vivo usage of amino- and thio-LNA results in a different biodistribution pattern from those of both stereo-

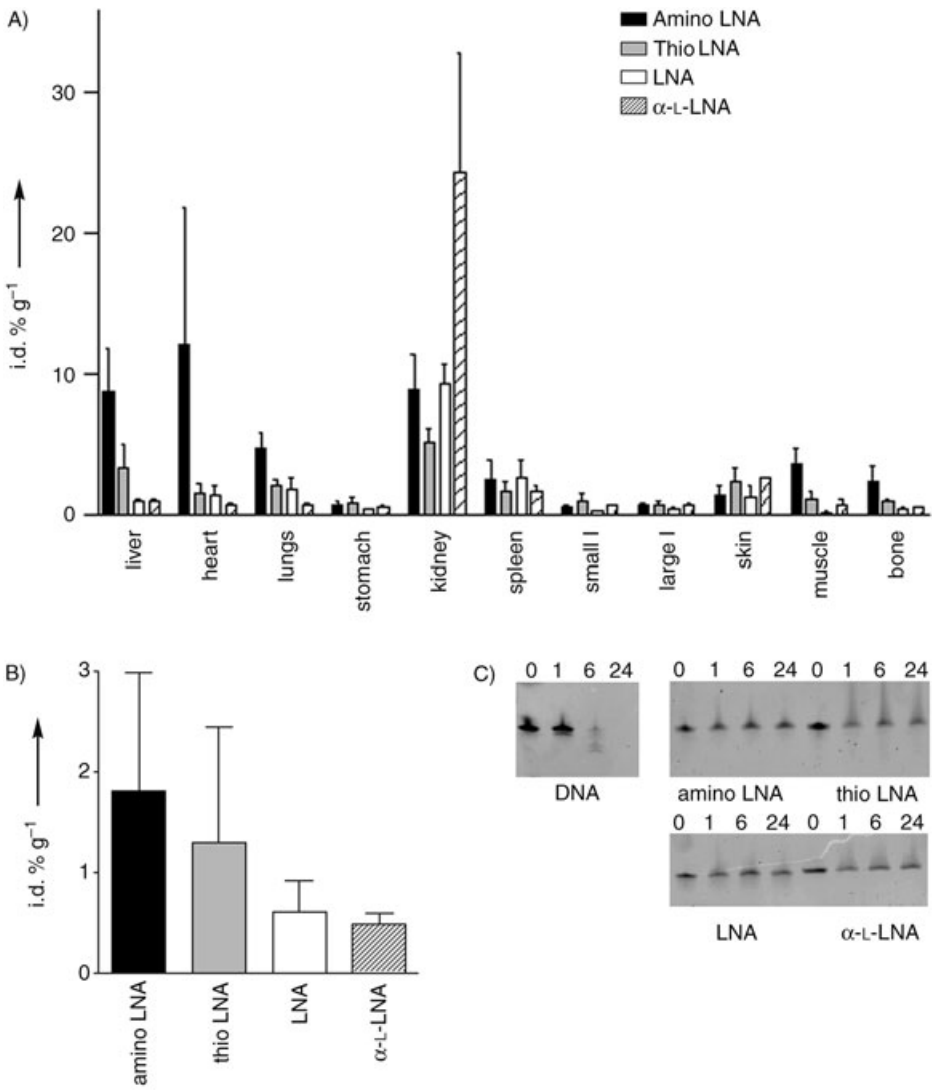

Figure 3. A) Biodistribution in female nude mice of radiolabeled ODNs modified with one of the four LNAs. The LNA ODNs were administered by using subcutaneously placed osmotic minipumps for 48 h. The mean specific uptake as a percentage of the given dosage (i.d.%) per gram of tissue in five mice per ODN is indicated. B) Uptake of the LNA ODNs, which were administered to 15PC3 tumor xenografts by using osmotic minipumps, given as a percentage of the given dosage per gram tissue (five mice per ODN). C) Stability of ODNs with the four LNAs in serum. The LNA ODNs were incubated in 50% fresh human serum at 37°C and, following the indicated incubation period, put on a 16% PAGE gel with 8 M urea. The LNA ODNs were stained with ethidium bromide. A 16 mer DNA ODN was used as control.

isomers of LNA. In conclusion, LNA-containing ODNs can be used for antisense gapmer ODNs with high efficacy, and α-L-LNA is the most promising new member of the LNA family, pairing good efficacy with specificity.

Experimental Section

LNA oligonucleotide synthesis: The amino-LNA, thio-LNA, and α-L-LNA monomers were prepared by following published procedures,[9c,d,15a,15b] with the final phosphitylation step being performed according to Pedersen et al.[15c] The β-D-LNA monomers were obtained from Exiqon A/S (Denmark). All ODNs were synthesized by Santaris Pharma AS (Hørsholm, Denmark), as described previously.[7c,8b] 5-Methyl-C was used in all the ODNs. All syntheses were carried out by using the phosphoamidite approach on an Expedite 8900 MOSS (Multiple Oligonucleotide Synthesis System) synthesizer at a 1 μmol scale. The ODNs were purified by reversed-phase HPLC (RP-HPLC). After the removal of the DMT-group, the ODNs were characterized by anion exchange (AE)-HPLC, and the molecular mass was further confirmed by ESI-MS and MALDI-TOF mass spectrometry on a Biflex III MALDI (Brucker instruments, Leipzig, Germany). The sequences of the ODNs are depicted in Scheme 1. Melting temperatures were measured as described[7c] with complementary DNA as the opposite strand. Tritium labeling of ODNs was performed by using the heat-exchange method described by Graham et al.[16]

Cell culture and in vitro experiments: 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

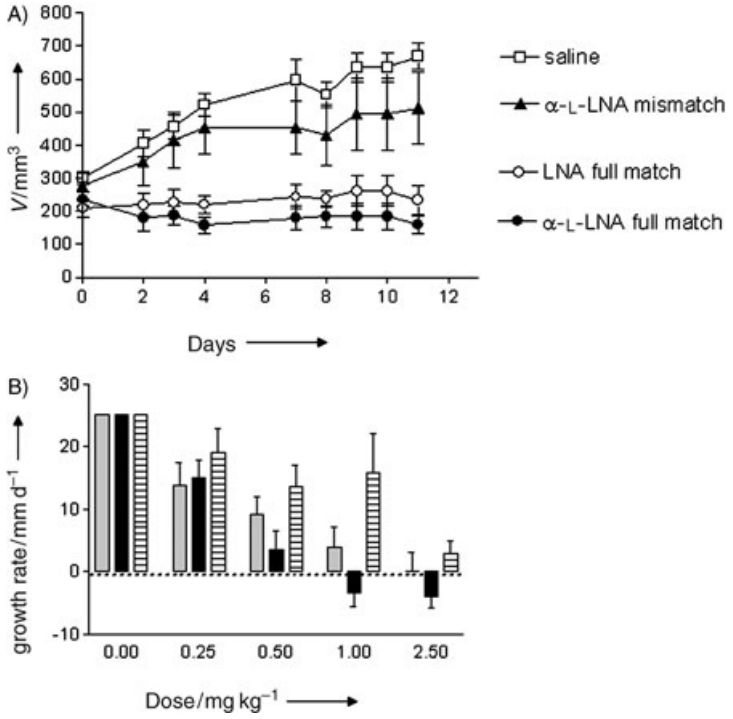

Figure 4. A) Comparison of the efficacy of tumor-growth inhibition by α-L-LNA and LNA. Example of tumor-growth inhibition of 15PC3 xenografts at a dosage of 1 mg kg^{-1} per day. The results are the means ± SEM of five mice per treatment. B) Comparison of tumor-growth rates (mm per day) during 11 days treatment with increasing dosages of full-match α-L-LNA (black bar), LNA (gray bar), and a mismatch control α-L-LNA (striped bar).

(100 μg mL^{-1}). ODN transfections were performed in six-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 six-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 performed with a Vanox microscope (Olympus) and appropriate filters.

Northern blot analysis of RNA was as described.[8b] The hybridized probe was visualized and quantified on a phospho imager (Fuji BAS imager) by using AIDA 2.46 software (Raytest Benelux, Tilburg, the Netherlands). cDNA fragments to be used as probes were generated by RT-PCR and subsequent cloning into the pGEM-T Easy vector (Promega). Probes used were Ha-Ras (GenBank accession no. NM_176956, position 233–762) and 28S rRNA (GenBank accession M11167, position 1635–1973).

In vivo experiments: Eight to ten-week-old athymic nude NMRI nu/nu mice (Charles River, 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, an osmotic mini pump (Alzet model 1002, Durect Co., Cupertino, CA, USA) was implanted dorsally according to the manufacturer's instructions. The osmotic minipumps were filled with oligonucleotides (with the dosages indicated in the figure legends) or physiological saline. Five mice per group were used for each treatment. Tumor growth was monitored daily following the implantation of the osmotic mini pump. Tumor volume was measured as described previously.[11] Tissue-distribution studies of tritiated ODNs were performed according to Bijsterbosch et al.,[17] with the exception that the ODNs were administered by using Alzet model 1002 osmotic minipumps. The radioactivity in the different organs was corrected for serum present at the time of sampling as determined by the distribution of ^{125}I-BSA.

Serum incubations were performed with ODN (400 pmol) in fresh human serum (50% *v/v*) from healthy volunteers. After incubation at 37°C, the samples were mixed 1:1 with loading dye containing formamide (95% *v/v*) and loaded on denaturing PAGE (16%, 19:1) gels containing 8 M urea. After running of the gel, the ODNs were stained with ethidium bromide and visualized by using a Fuji LAS3000 darkbox (Raytest Benelux, Tilburg, the Netherlands).

Aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), and alkaline phosphatase levels in serum were determined by using standard diagnostic procedures with the H747 (Hitachi/Roche) with the appropriate kits (Roche Diagnostics). Body temperature was monitored daily for each mouse by using IPTT-200 transponder chips and a DAS 5002 chip reader (Biomedic Data Systems, Seaford, Dellaware, USA).

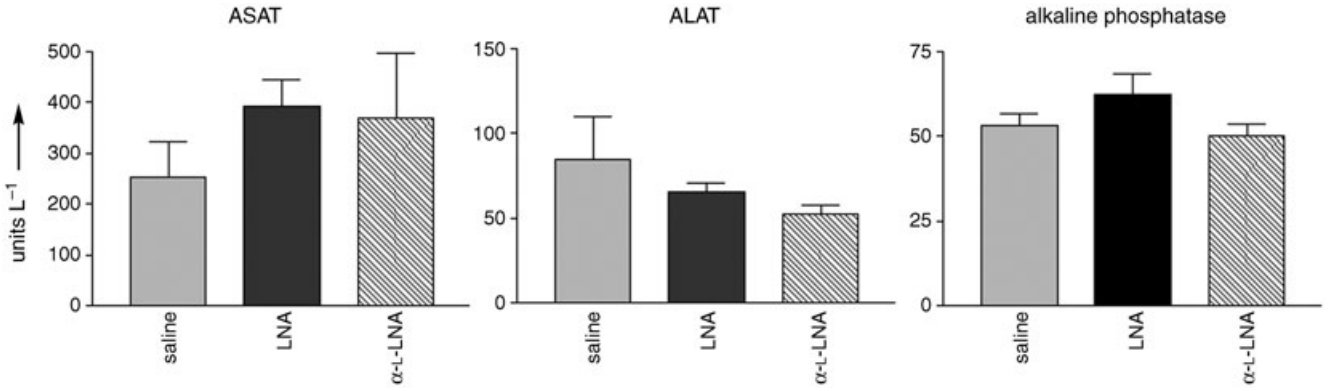

Figure 5. ASAT, ALAT, and alkaline phosphatase levels (units per liter at 37°C) in mouse serum after treatment with 0.9% saline, LNA or α-L-LNA at 1 mg kg^{-1} per day dosage. The results are the mean values ± SEM of five mice per treatment.

All animal experiments were conducted under the institutional guidelines and according to the law; they were sanctioned by the animal ethics committee.

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