FEBS Letters 581 (2007) 771–774

Systematic screening of LNA/2′-*O*-methyl chimeric derivatives of a TAR RNA aptamer

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Received 11 January 2007; accepted 22 January 2007

Available online 30 January 2007

Edited by Hans-Dieter Klenk

Abstract **We synthesized and evaluated by surface plasmon resonance 64 LNA/2′-*O*-methyl sequences corresponding to all possible combinations of such residues in a kissing aptamer loop complementary to the 6-nt loop of the TAR element of HIV-1. Three combinations of LNA/2′-*O*-methyl nucleoside analogues where one or two LNA units are located on the 3′ side of the aptamer loop display an affinity for TAR below 1 nM, i.e. one order of magnitude higher than the parent RNA aptamer. One of these combinations inhibits the TAR-dependent luciferase expression in a cell assay.**

Keywords: Aptamer; Locked nucleic acid; HIV-1; Surface plasmon resonance; 2′-*O*-methyl; TAR; Tat

1. Introduction

Locked nucleic acid (LNA) nucleotides are ribonucleotide analogues that contain a methylene bridge between the 2′ oxygen and the 4′ carbon of the ribose moiety (Fig. 1A). Structural studies on LNA:DNA and LNA:RNA duplexes [1–3] have shown that this modification locks the furanose ring in the C′3-*endo* conformation, similar to that of A-type RNA and 2′-*O*-methyl nucleotides, and perturbs neighbouring sugars towards the A-type conformation even in a quadruplex context [4]. This results in a very high affinity of LNA oligomers for DNA and RNA complementary sequences [2]. In addition an oligonucleotide including such a modification is more resistant to nuclease digestion [5–7].

In the past few years the use of LNA nucleotides has significantly increased. Studies were devoted to the understanding of the thermodynamics of heteroduplexes [8,9]. New structural insights were obtained with LNA modified quadruplexes [4] and dsDNA:LNA triplexes [10]. LNA probes were shown to improve mismatch discrimination [11], to mediate improvements in siRNA stability and functionality [7] and potentially to rival siRNA for gene silencing [12].

In a recent work [5], we used LNA to generate a modified version of an RNA kissing aptamer, R06, previously identified against the TAR RNA element of HIV-1 [13]. On the basis of structural studies on heteroduplexes containing LNA nucleotides, we reasoned that derivatives in which DNA and LNA monomers would be interspersed might generate interesting analogues of the parent RNA aptamer. Indeed the LNA–DNA derivative, resistant to nuclease digestion, containing LNA units at positions 2, 4, 6 and 7 Fig. 1B and DNA at positions 3 and 5 of the aptamer loop was shown to bind to TAR with a dissociation constant in the low nanomolar range, similar to that of the parent RNA aptamer. In this previous study, 16 sequences were synthesized and assayed for binding [5].

In an attempt to identify new anti-TAR aptamers with improved properties, we systematically explored the 2^6 combinations, that is 64 sequences, involving LNA residues in the loop, with the exception of the G, A residues that close the aptamer loop, which were shown to be crucial for stable complexes [5]. In order to get fully nuclease resistant oligomers, these chimeras were synthesized in a 2′-*O*-methyl ribonucleotide background. Indeed, we previously showed that a fully modified 2′-*O*-methyl R06 analogue displayed affinity for TAR close to that of the parent RNA aptamer and inhibited HIV-1 protein Tat-mediated transcription in an in vitro assay [14].

We report here that three combinations out of 64 displayed an affinity for TAR below 1 nM, i.e. one order and two orders of magnitude higher than the parent RNA aptamer and the LNA–DNA derivative previously identified, respectively [5,13]. In addition, one combination inhibits the TAR-dependent luciferase expression in a cell assay.

2. Materials and methods

LNA/2′-*O*-methyl derivatives were synthesized on an ABI 394 automated DNA/RNA synthesiser and purified by HPLC. The biotinylated miniTAR (Fig. 1B), corresponding to the top part of the retroviral TAR element, was synthesized on an Expedite 8908 synthesizer (Applied Biosystems) and purified as described previously [15].

Surface plasmon resonance experiments were performed on a BIAcore™ 3000 apparatus (Biacore AB, Sweden) as described previously [15]. Binding kinetics were performed at 23 °C in 20 mM HEPES buffer, pH 7.4, containing 20 mM sodium chloride, 140 mM potassium

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Abbreviations: LNA, locked nucleic acid

0014-5793/$32.00 © 2007 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/j.febslet.2007.01.047

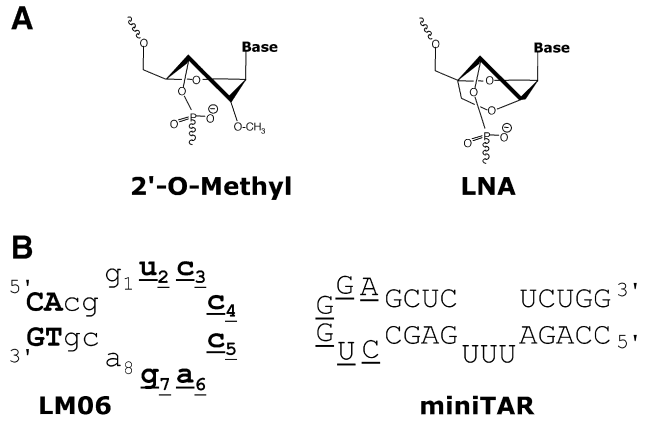

Fig. 1. LNA and 2′-*O*-methyl monomers and secondary structure of miniTAR and the LNA/2′-O-methyl aptamer. (A) Conformations of LNA and 2′-*O*-methyl monomers. (B) Sequence and secondary structure of miniTAR. The loop bases susceptible to base pairing are underlined. LM06 refers to derivatives containing LNA and 2′-*O*-methyl monomers in the loop (underlined nucleotides in bold). Capital letters in bold and lower case letters represent LNA and 2′-*O*-methyl bases, respectively.

chloride and 3 mM magnesium chloride. Samples (500 nM) were injected at 20 μl/min. Complexes were dissociated with one minute pulse of 20 mM EDTA. The kinetic parameters, k_{on} and k_{off}, were determined assuming a pseudo-first order model by direct curve fitting of the sensorgrams using the Bia-evaluation 4.1 software (Biacore AB). The dissociation equilibrium constant, K_d, was calculated as k_{off}/k_{on}.

2.1. Tet-off/Tat/luc-f/luc-R HeLa cell line

HeLa cells containing stably integrated HIV-1 Tat gene under Tet-off promoter control, firefly luciferase gene under HIV-1 long terminal repeat and *Renilla* luciferase gene under cytomegalovirus promoter control [16], were grown in DMEM medium supplemented with 10% Tet System Approved fetal bovine serum (FBS) (Clontech, Palo Alto, CA), penicillin and streptomycin at 37 °C under 5% CO_2/95% air.

2.2. Tat-dependent trans-activation in HeLa cells

Tat-dependent *trans*-activation experiments in the presence of LNA/2′-*O*-methyl derivatives were carried out as described previously [17]. Briefly, oligonucleotides were prepared at a concentration of 1 μM in serum-free Opti-MEM (Invitrogen, San Diego, CA). An equal volume of cationic lipid Lipofectamine 2000 in Opti-MEM (10 μl lipid per 1 ml medium) was added to each solution. Complexes were formed at room temperature for 20 min and subsequent dilutions were made from this oligonucleotide/lipid mixture. After 3 h transfection the medium was replaced with 100 μl DMEM/10% fetal bovine serum (FBS) and cells were incubated for 18 h at 37 °C. The luciferase and cell toxicity levels were then measured as described previously [17].

3. Results and discussion

The TAR RNA imperfect hairpin is specifically recognized by an RNA aptamer termed R06 that adopts a hairpin structure with a loop framed by crucial G, A residues [13,18]. The R06–TAR interaction is mediated by a loop–loop interaction, resulting in the formation of a 6-bp helix.

The LNA/2′-*O*-methyl oligonucleotides studied in the present work were synthesized on the basis of a 16-nt long LNA–DNA sequence identified previously [5]. The 4-bp stem ends with two LNA pairs that increase its thermodynamic stability and lock the aptamer into its hairpin conformation, a prerequisite for kissing complex formation (Fig. 1B). We analyzed the binding properties of the 64 LNA/2′-*O*-methyl combinations of the R06 loop sequence complementary to the TAR one (positions 2–7, Fig. 1B). The loop closing G, A residues were not subject to LNA modification, since our previous study showed that this was detrimental to the kissing interaction. The oligonucleotides were named according to their loop chemistry: LM06 stands for derivatives containing LNA (L) and 2′-*O*-methyl (M) monomers in the loop, LNA positions being indicated by a subscript.

The equilibrium dissociation constant of each aptamer derivative was determined by surface plasmon resonance. The results (Table 1) are discussed with respect to (i) the number of LNA residues and (ii) their locations (5′ versus 3′) in the loop sequence. These two parameters correlate reasonably well with the ranking from strong (K_d < 1 nM) to poor binders (K_d > 500 nM).

The derivative containing six LNA units in the loop, namely $LMO6_{234567}$, did not bind to miniTAR, in agreement with our previous work that showed that chimeric LNA–DNA hairpins with LNA loops were not good TAR ligands [5]. LNA monomers are locked because of the 2′-*O*-4′C methylene bridge. This brings stability to DNA/LNA or RNA/LNA linear heteroduplexes but clearly not to a loop–loop helix, demonstrating that it is probably not a canonical A-type helix, in agreement with results that showed that the minor groove of the loop–loop helix of a related system was narrower compared with a linear RNA helix [19]. This conclusion is supported by previous studies in which we used fully modified 2′-*O*-methyl [14] and N3′ → P5′ deoxyphosphoramidated [15] versions of the R06 aptamer. In contrast to the results obtained for linear duplexes, none of these modifications generated an aptamer analogue of increased affinity for the TAR RNA. Except for one sequence, $LMO6_{24567}$, that displayed a K_d between 25 and 100 nM (Table 1), all other combinations

Table 1
Dissociation equilibrium constants for LNA/2′-*O*-methyl derivative-miniTAR complexes

Number of LNA residues in the loop	K_d (nM)					
	K_d < 1	1 < K_d < 5	5 < K_d < 25	25 < K_d < 100	100 < K_d < 500	K_d > 500
1	5	6; 7	2; 3; 4			
2	56; 57	25; 26; 36; 46; 47; 67	24; 27; 35; 45; 37	23; 34		
3		236; 247; 356; 567	235; 246; 457; 357; 346; 456	245; 347	267	237; 256; 257; 467; 367; 234; 345
4		2367; 2467; 4567	3467; 2567	2347; 2456; 3567	2457; 2346; 3456	2356; 2357; 2345; 2347; 3457
5				24567	23467; 34567	23456; 23457; 23567
6						234567

Positions with LNA residues are indicated and numbered according to Fig. 1B.

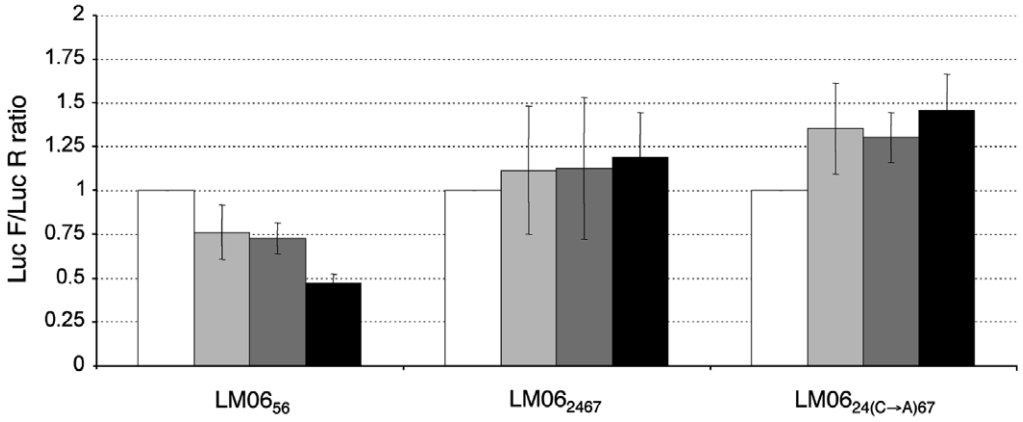

Fig. 2. Inhibitory effect of LNA/2′-*O*-methyl derivatives on TAR-dependent luciferase expression in HeLa cells. Inhibition is reported as the ratio of Firefly luciferase to *Renilla* luciferase expression, normalized to cell viability count, after cell incubation with LNA/2′-*O*-methyl derivatives. Left to right, oligonucleotide concentrations are 0, 62.5,125, and 250 nM.

with five LNA units were poor TAR ligands, suggesting that the loop conformation was too constrained for stable loop–loop interaction.

Derivatives that contained four LNA monomers showed more interesting properties: 5 of the 16 combinations displayed a K_d between 1 and 25 nM (Table 1). In a 2′-*O*-methyl context, the ligand with LNA substitutions at positions 2, 4, 6 and 7, LMO6$_{2467}$, displayed an affinity for TAR higher (K_d = 1.8 ± 1.0 nM) than its counterpart in a DNA context (K_d = 21.1 ± 2.1 nM, [5]). This may be related to a more favourable A-type geometry of the loop in LNA/2′-*O*-methyl derivatives compared to LNA/DNA sequences, where the N-type conformation adopted by the DNA sugars is induced by the neighbouring LNA monomers. When positions 6 and 7 were modified, low K_d's are generally observed. In contrast, LNA residues incorporated into positions 2 and 3, on the 5′ side of the loop, resulted in weak binding: five combinations yielded a K_d above 100 nM (Table 1). The only noticeable exception was compound 2367, thanks to the effect of the 2 LNA residues on the 3′ side of the loop. Thus, structural determinants for stable loop–loop interaction are different on the two sides of the aptamer loop, suggesting that in addition to loop–loop complementarity non-canonical interactions, such as base-stacking, are crucial for stability. This result agrees well with a previous study on a natural kissing complex from *E. coli*, RNAI-RNAII, which showed that inversion of the loop sequences of wild-type hairpins 5′–3′ resulted in a complex 350-fold more stable [20,21] than the original wild-type.

Further analysis of chimeric analogues with three, two or even one LNA substitution emphasizes this trend. Derivatives with three contiguous LNA units on the 3′ side of the loop (LMO6$_{456}$ and LMO6$_{567}$) are good TAR ligands (K_d between 1 and 5 nM), while those with LNA units of the 5′ side are poor ones (LMO6$_{234}$ and LMO6$_{345}$, K_d > 500 nM). In contrast to the observations made with four LNA substitutions, when positions 6 and 7 are LNAs, one additional LNA on the 5′ side of the loop is rather detrimental (see LMO6$_{267}$, LMO6$_{467}$ and LM06$_{367}$).

Analogues with two LNAs on the 3′ side of the aptamer loop are stronger ligands than those with two incorporations on the 5′ side. Two oligomers, LMO6$_{56}$ and LMO6$_{57}$, display a K_d below 1 nM, (0.27 nM ± 0.18 and 0.27 ± 0.12 nM, respectively), compared to the LM06$_{2467}$ derivative (K_d = 1.8 ± 1.0 nM) or the RNA aptamer (K_d = 1.31 ± 0.15 nM). Overall, the insertion of two LNA units is rather well tolerated, since most of the dissociation constants (13 out of 15) are below 25 nM. Chimeric analogues with a single LNA substitution behave similarly and confirm the trend. Substitutions at positions 2, 3 or 4 generate ligands displaying lower affinity for the RNA target compared with those modified at positions 5, 6 or 7. The best ligand, LM06$_5$, shows a K_d of 0.72 ± 0.27 nM.

The inhibitory effect of LNA/2′-*O*-methyl derivatives was tested in a TAR-dependent double-luciferase HeLa cell reporter system as described previously [16,17]. No effect was observed with LM06$_{2467}$ (Fig. 2) or with LM06$_{24(C\rightarrow A)67}$, where LNA monomers are at similar positions but with a C → A mismatch at position 4 in the loop. A clear dose-dependent reduction in the firefly luciferase/*Renilla* luciferase ratio was observed with LM006$_{56}$, one of the best ligands of mini-TAR, where the K_d (0.27 ± 0.18 nM) is almost 10-fold lower than for LM06$_{2467}$ (K_d = 1.8 ± 1.0 nM).

A recent study where LNA monomers were included in 2′-*O*-methyl RNA–RNA heteroduplexes [8] was aimed at rationalizing the influence of LNA on heteroduplex stability: (i) 3′ terminal U LNA and 5′ terminal LNAs were less stabilizing than interior and other 3′ terminal LNAs, (ii) most of the stability enhancement was observed when LNA nucleotides are separated by at least one 2′-*O*-methyl nucleotide, (iii) the effects of LNA modifications were more or less additive when LNA units were separated by at least one 2′-*O*-methyl monomer. These conclusions do not fit with our results and thus one cannot extrapolate trends observed for unconstrained linear duplexes to kissing complexes. The benefit to stability brought by LNA residues on the 3′ side of the aptamer loop might be related to the known stacking of the loop on the 3′ strand of the hairpin stem.

In conclusion, three LNA/2′-*O*-Me derivatives that displayed a higher binding affinity for the HIV-1 TAR element than the parent RNA aptamer were identified by a systematic screening of all combinations within the aptamer loop sequence. One of these winning chimeras inhibits the TAR-dependent transcription.

Acknowledgement: S. Reigadas has been supported by a grant from the Sidaction. We thank Isabelle Lebars and Eric Dausse for helpful discussions.

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