Optimized DNA-targeting using triplex forming C5-alkynyl functionalized LNA†

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Triplex forming oligonucleotides (TFOs) modified with C5-alkynyl functionalized LNA (locked nucleic acid) monomers display extraordinary thermal affinity toward double stranded DNA targets, excellent discrimination of Hoogsteen-mismatched targets, and high stability against 3′-exonucleases.

Substantial efforts have been invested to develop probes for use in the antigene technology, *i.e.*, sequence-specific targeting of double stranded DNA (dsDNA).[1] The efforts are motivated by the prospect of developing fundamental research tools and new types of therapeutic and diagnostic agents.[2,3] (TC)-motif triplex forming oligonucleotides (TFOs), which bind to polypurine regions in dsDNA targets *via* Hoogsteen base pairs in the major groove, are the most widely studied DNA-targeting agents,[1] although promising alternatives are emerging.[4] A drawback of the TFO-approach is the instability of triplexes at physiological pH due to electrostatic repulsion between the three strands and insufficient N3-protonation of cytosines resulting in weak Hoogsteen base pairing. Chemical modification of TFOs is therefore essential to increase triplex stability as well as to protect TFOs from enzymatic degradation.[1] Three classes of nucleotide building blocks have found particular use as TFO-modifications: (a) ribonucleotides and O2′-alkylated analogs hereof as they favorably preorganize the TFO strand for hybridization to dsDNA targets,[5] (b) conformationally restricted nucleotides with RNA-like furanose puckers such as locked nucleic acid (LNA, monomer **V**, Fig. 1)[6] or analogs thereof[7] in what is an extension of the TFO preorganization concept, and (c) C5-alkynyl functionalized pyrimidine DNA monomers, as they promote enhanced base stacking interactions in triplexes.[8] Interestingly, building blocks that combine C5-alkynyl functionalization and RNA-character induce greater triplex stabilization than the respective C5-alkynyl DNA and RNA TFO monomers by themselves.[9] In light of this, we hypothesized that C5-alkynyl functionalized LNA monomers

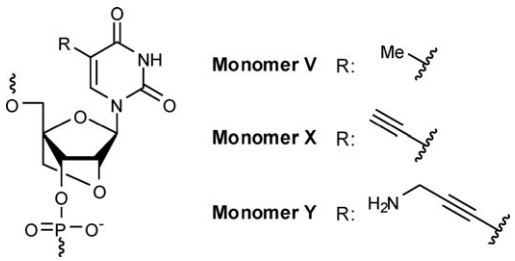

Fig. 1 Structures of LNA and novel C5-alkynyl functionalized LNA monomers.

X and **Y** (Fig. 1) would synergistically integrate beneficial properties from two compound classes, *i.e.*, (1) high thermal affinity toward dsDNA and partial resistance toward nucleases (LNA-component), and (2) resistance toward nucleases due to the presence of steric shields along with favorable contributions to binding affinity (C5-substituent).

The corresponding phosphoramidites[10] of the C5-ethynyl and C5-propargylamine functionalized LNA monomers **X** and **Y** and the commercially available LNA thymine phosphoramidite were incorporated one, three or six times into a 15-mer (TC)-motif DNA TFO sequence (Table 1) that has previously been used to evaluate hybridization properties of LNA,[7d] ENA[7d] and 2′,4′-BNA^{NC}[7e] TFOs. TFOs were synthesized on automated DNA synthesizers (0.2 μmol scale) using standard conditions except for extended coupling (15 min, using 4,5-dicyanoimidazole as activator) and oxidation times (45 s).† This resulted in stepwise coupling yields of >98% for the LNA monomers. 5-Methyl-deoxycytidine rather than deoxycytidine monomers were used to ameliorate the normal pH dependence of (TC)-motif TFOs.[11]‡ Following workup and purification, the composition and purity (>90%) of all synthesized TFOs were verified by MALDI-TOF MS (Table S2)† and HPLC, respectively.†

Binding to a central target region in a 21-mer dsDNA was first characterized by non-denaturing PAGE using equimolar quantities of TFOs and dsDNA target in a pH 7.2 HEPES buffer containing Mg^{2+}. Conventional LNA and C5-ethynyl functionalized LNA TFOs form stable triplexes with dsDNA targets under these conditions (**V**- and **X**-series, Fig. S1)† as a single band of lower mobility than the corresponding dsDNA target was observed in all instances. Triplex formation between C5-propargylamine functionalized LNA TFOs and dsDNA target is incomplete as a band (~20–40% intensity) of identical mobility as the corresponding dsDNA target is observed in addition to the triplex band (**Y**-series, Fig. S2).† Triplex formation was, however, driven toward completion

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† Electronic supplementary information (ESI) available: Synthetic outline of phosphoramidites, protocols for synthesis/purification of TFOs, determination of T_m- and k_{on}-values, and exonuclease studies; MALDI-TOF-MS data of TFOs; thermal denaturation profiles; PAGE experiments; absorption decay profiles; discussion regarding parallel duplex formation. See DOI: 10.1039/b917312a

Table 1 T_m-values and association rate constants (k_{on}) for triplexes formed by C5-alkynyl functionalized LNA, conventional LNA or DNA TFOs with dsDNA target[a]

			T_m [ΔT_m/mod]/°C			k_{on}/M^{-1} s^{-1}		
ON	Seq. 5′ → 3′	**B** =	**V**	**X**	**Y**	**V**	**X**	**Y**
B1	TTT TT^{m}C T**B**T ^{m}CT^{m}C T^{m}CT		40.0 [+11.0]	42.5 [+13.5]	42.5 [+13.5]	3800	4400	ND
B2	TTT T**B**^{m}C TT**B**^{m}CT^{m}C **B**^{m}CT		48.5 [+6.5]	54.0 [+8.3]	57.0 [+9.3]	3600	4000	3700
B3	TTT T**B**^{m}C T**B**T ^{m}C**B**^{m}C T^{m}CT		53.5 [+8.2]	59.5 [+10.2]	60.0 [+10.3]	3900	4100	3900
B4	TT**B** T**B**^{m}C **B**T**B**^{m}C**B**^{m}C **B**^{m}CT		61.0 [+5.3]	67.5 [+6.4]	74.5 [+7.6]	4300	4400	5500

[a] T_m's were determined as the first derivative of differential thermal denaturation curves (A_{260}*vs.* T)[6c]† recorded in a pH 7.0 phosphate buffer solution containing 140 mM KCl using 1.0 μM of each strand. T_m-values are averages of at least two independent measurements within 1 °C. k_{on}'s were measured in pH 7.2 HEPES buffer.† k_{on}'s are averages of at least three independent measurements. Data for DNA reference TFO **T1** (**B** = **T**): T_m = 29.0 °C, k_{on} = 4400 M^{-1} s^{-1}. Structures of monomers are shown in Fig. 1. ^{m}C denotes 5-methyldeoxycytidine monomers. dsDNA target sequence (TFO binding region underlined): 5′-GCT AAA AAG AAA GAG AGA TCG-3′, 3′-CGA TTT TTC TTT CTC TCT ACG-5′. ND = not determined.

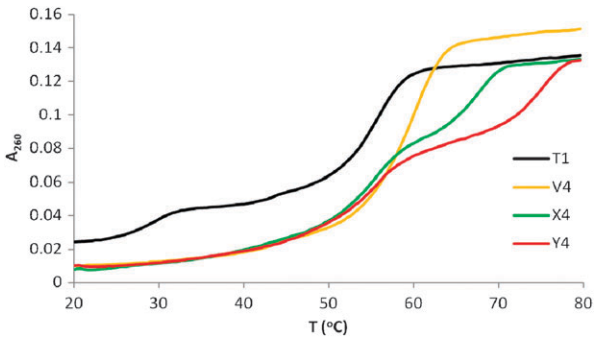

Fig. 2 Representative thermal denaturation profiles of triplexes between reference DNA **T1** or TFOs from the **B4**-series, and complementary dsDNA target. For sequences and conditions see Table 1.

using a two-fold excess of C5-propargylamine functionalized LNA TFOs (Fig. S2).†

After qualitatively establishing triplex formation, the thermal affinity of C5-functionalized LNA TFOs toward the dsDNA target was determined by UV thermal denaturation experiments at pH 7.0 in the absence of Mg^{2+}-ions and compared relative to reference DNA and LNA TFOs (Table 1). The thermal denaturation curves of the studied triplexes either exhibit (a) biphasic profiles when thermal denaturation temperatures (T_m's) of triplexes differed substantially from the T_m of the dsDNA target (T_m = 56.0 °C), or (b) monophasic profiles when denaturation curves for triplexes and dsDNA targets overlapped substantially (Fig. 2, Fig. S3 and S4).† Similar observations in identical sequence contexts have been made with other LNA analogs.[7b,d] To facilitate determination of triplex T_m's, differential thermal denaturation curves[6c] were obtained by 'subtracting' the denaturation curve of the dsDNA target from the 'raw' triplex denaturation curve (Fig. S3).†

Singly modified C5-alkynyl functionalized LNA TFOs exhibit dramatically increased thermal affinity toward dsDNA targets relative to reference DNA TFO **T1** ($\Delta T_m \approx +13.5$ °C, **B1**-series, Table 1). Additional modification of TFOs with **X**/**Y** monomers results in progressively more stabilized triplexes although less pronounced ΔT_m/mod-values are observed (*e.g.*, compare data of **B1**-, **B2**- and **B4**-series, Table 1). C5-Propargylamine functionalized LNAs (**Y**-series) stabilize triplexes slightly more efficiently than the corresponding C5-ethynyl functionalized LNAs (**X**-series), which likely results from reduced electrostatic repulsion between the triplex strands due to partial protonation of the propargylamino substituent, or from improved π-stacking with neighboring nucleobases.[8b] Consequentially, C5-propargylamine functionalized LNA TFO **Y4** containing six **Y** monomers as next-nearest neighbours forms exceedingly stable triplexes with dsDNA targets (T_m = 74.5 °C, Table 1).

Importantly, the observed stabilization of triplexes by C5-alkynyl LNA TFOs compares favourably with the corresponding parent LNA TFOs (**V**-series) as *additional increases* in T_m-values of between +1.1 °C and +2.8 °C *per modification* are observed (compare T_m-data for **X**-, **Y**- and **V**-series, Table 1). In fact, direct comparison with previously reported hybridization data for ENA[7d] and 2′,4′-BNA^{NC}[7e] TFOs in the same sequence, strongly suggests that C5-alkynyl LNA TFOs stabilize triplexes more efficiently than these 'gold standard' TFO building blocks. Thus, incorporation of C5-alkynyl functionalized LNA monomers into DNA TFOs is a simple,† yet powerful approach to improve thermal affinity of TFOs toward dsDNA targets.

To evaluate the influence of C5-alkynyl functionalized LNA monomers on association kinetics of triplex formation, the association rate constant k_{on} was determined for TFOs by fitting a second order rate equation to the A_{260} decay curve[13] obtained by mixing pre-annealed dsDNA target with equimolar quantities of TFOs (Fig. S6).† Generally little variation in the k_{on}-values between DNA, LNA or C5-functionalized LNA TFOs was observed (~4000 M^{-1} s^{-1}, Table 1), which verifies that LNA-type TFOs primarily influence triplex dissociation kinetics.[6a] As an exception hereto, densely modified C5-propargylamine functionalized LNA **Y4** exhibits faster triplex association kinetics which most likely is linked to the presence of partially positively charged 'patches' of the C5-propargylamine substituent.[9b]

Increased affinity of TFOs toward dsDNA targets should preferably be accompanied by concomitant improvements in mismatch discrimination to avoid off-targets effects.[5b] Accordingly, the ability of singly modified C5-alkynyl LNA TFOs (**B1**-series) to discriminate dsDNA targets with a single Hoogsteen base pair mismatch opposite of the modification was evaluated in a thermal denaturation buffer containing 10 mM Mg^{2+} (see footnote Table 2), which is known to accelerate and stabilize triplex formation.[14]§†

As anticipated, less stable triplexes are generally formed with mismatched dsDNA targets. Introduction of C5-functionalized LNA monomers into TFOs greatly improves discrimination of Hoogsteen TA- and CG-mismatches compared to reference

Table 2 T_m-values of matched and mismatched triplexes in the presence of Mg^{2+}-ions[a]

	$T_m[\Delta T_m]$/°C			
ON	A : T (match)	T : A	C : G	G : C
T1	37.0	10.0 [−27.0]	19.5 [−17.5]	13.5 [−23.5]
V1	49.0	10.0 [−39.0]	28.5 [−20.5]	24.0 [−25.0]
X1	51.5	10.0 [−41.5]	24.5 [−27.0]	27.5 [−24.0]
Y1	50.0	10.0 [−40.0]	26.5 [−23.5]	27.5 [−22.5]

[a] T_m-values (ΔT_m = change in T_m-value relative to matched triplex). Conditions and TFO-sequences (**B1**-series) as described in Table 1 except for addition of 10 mM $MgCl_2$. dsDNA targets = 5′-GCT AAA AAG A**M**A GAG AGA TCG-3′ : 3′-CGA TTT TTC T**M′**T CTC TCT ACG-5′, where **M** : **M′** = A : T, T : A, C : G and G : C, respectively.

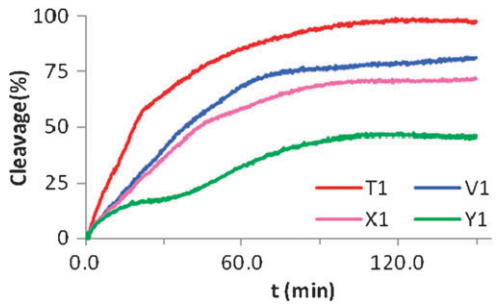

Fig. 3 Exonuclease (SVPDE) degradation of singly modified C5-functionalized LNA and reference TFOs. Nuclease degradation studies performed in Tris-buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, pH 9.0) at 37 °C using 2 μM of TFO strands and 0.43 μg of SVPDE (snake venom phosphordiesterase). For sequences of **T1–V1–X1–Y1**, see Table 1.

TFOs **T1** and **V1**, while discrimination of GC-mismatches is largely unaffected (Table 2).

Consequentially, the *most demanding mismatched dsDNA targets* are more efficiently discriminated by C5-functionalized LNA TFOs (ΔT_m = −24.0 °C and −22.5 °C for **X1** and **Y1**, respectively) than by reference TFOs (ΔT_m = −17.5 °C and −20.5 °C, for **T1** and **V1**, respectively). This data demonstrates that C5-alkynyl functionalized LNAs exhibit increased thermal affinity *and* specificity relative to parent LNAs.

Finally, the resistance of singly modified C5-alkynyl functionalized LNA TFOs (**B1**-series) against degradation by the 3′-exonuclease snake venom phosphodiesterase (SVPDE) was evaluated, by following the increase in absorbance (hyperchromicity) at 260 nm[15] of TFOs in the presence of SVPDE (Fig. 3). As expected, DNA TFO **T1** is rapidly degraded (>85% cleavage after 1 h) whereas singly modified LNA **V1** confers some protection against SVPDE-mediated degradation (~70% cleavage after 1 h). C5-Ethynyl and, in particular, C5-propargylamine functionalized LNA TFOs are considerably more resistant against nucleolytic degradation (**X1** ~60%, **Y1** ~35% after 1 h). This suggests that the C5-substituents act as a steric shield, to ensure greater biostability.

To conclude, C5-alkynyl functionalized LNAs exhibit unsurpassed TFO-hybridization properties, which are likely to enable efficient DNA-targeting under physiological conditions. Studies along these lines are ongoing.

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Notes and references

‡ O4-Triazolyl-dT-CE phosphoramidites[12] were incorporated into TFOs, and converted to 5-methyldeoxycytidine monomers during deprotection (32% aq. NH_3).

§ Triplex formation was complete within ~1 h in the presence of 10 mM Mg^{2+}, while requiring overnight incubation in absence of Mg^{2+}. Triplex stabilities increased by 8–10 °C (T_m-data Tables 1 and 2).

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