Tetra-end-linked oligonucleotides forming DNA G-quadruplexes: a new class of aptamers showing anti-HIV activity†

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The biophysical and biological properties of unprecedented anti-HIV aptamers are presented. The most active aptamer (1L) shows a significant affinity to the HIV protein gp120.

Combinatorial chemistry techniques have proven to be particularly valuable in medicinal chemistry because they exploit the molecular diversity to design, prepare, and test new chemical structures in view of the discovery of unprecedented pharmacophores. In this frame, the SELEX (Systematic Evolution of Ligands by EXponential enrichment) techniques,¹ relying on DNA or RNA libraries, come out particularly advantageous since nucleic acid sequences can be automatically synthesized in high yields and in a large variety. Most importantly, the species selected for affinity to the target can be easily amplified and further selected thus obtaining few species (aptamers) endowed with high affinity and selectivity for the target. Aptamers can adopt a number of conformational arrangements. Among these, the four-stranded DNA or RNA structures known as G-quadruplexes have emerged as nucleic acid architectures of remarkable significance² due to their dramatic thermal stability. An important class of potentially therapeutic aptamers are those endowed with antiviral activity. They can adopt monomolecular (anti-HIV integrase aptamer, T30695),³ bimolecular (anti-HIV integrase aptamer, 93del)⁴ and tetramolecular parallel (anti-HIV glycoprotein gp120 aptamer, ODN phosphorothioate ISIS5320)⁵ G-quadruplex structures. Among these, the tetramolecular parallel ones, thanks to their shorter sequences and simpler strand arrangements, could be potentially more prone to be modified in order to improve their pharmacokinetic and pharmacodynamic properties in view of their use in therapy. In 1994 Hotoda *et al.* reported the anti-HIV-1 activity of a 6-mer ODN having the sequence TGGGAG and bearing a dimethoxytrityl group linked to its 5' hydroxyl function.⁶ Subsequently, other modified ODNs were prepared and tested and the 6-mer bearing a 3,4-dibenzyloxybenzyl group at the 5'-end and a 2-hydroxyethylphosphate at the 3'-end (R-95288) showed the most potent activity and the least cytotoxicity.^{7,8} CD investigations

on R-95288 and similar ODNs suggested that they form parallel tetramolecular quadruplexes.⁸ In a further study, the biophysical properties of the most interesting 5'-modified ODNs were investigated and it was established that the aromatic groups at the 5'-position of TGGGAG dramatically enhance the equilibrium and the rate of formation of the quadruplex complexes.^{9,10} Furthermore, the overall stability of the investigated complexes was found to correlate with the reported anti-HIV activity, thus strongly suggesting that the G-quadruplex structures are the species responsible for the biological activity.¹¹ In 2004, we described the synthesis and characterization of a new quadruplex structure (tetra-end-linked oligonucleotides: TEL-ODNs) formed by a cluster of four d(TG₄T) hexanucleotides linked together by their 3'-ends through a tetra-branched linker.¹² Subsequently, the new structure has been proven to possess a remarkable thermal stability compared to its natural counterpart.¹³ In following studies, the effects on structural properties of different orientations of the sequences and linker size have been investigated.¹⁴ In this communication we propose some TEL-ODNs containing the sequence TGGGAG as new modified aptamers and provide data on their properties and evidences for their noteworthy anti-HIV activity. Fig. 1 shows the ODNs involved in this study. TEL-ODNs were prepared according to the approach previously reported.¹¹

Fig. 1 Schematic structures of the investigated ODNs.

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TEL-ODNs **1L** and **1S** represent the tetra-end-linked versions of the Hotoda's natural quadruplex in which a lipophilic group (TBDPS) was attached to the 5'-end of each base sequence (**TH**). Subsequently, TEL-ODNs **2L**, **2S**, **3L** and **3S** (all devoid of the TBDPS groups and with the linker bound to the 3'- or 5'-ends for **2** or **3** analogues, respectively) were prepared in order to probe the influence of the lipophilic moieties and the TEL position on the structural and biological properties.

In order to verify that in modified TEL-ODNs the presence of the TEL doesn't affect the original parallel arrangement of the parent Hotoda's ODN 5'-TGGGAG-3' (**H**), we performed CD measurements (Fig. S1 in ESI†). All spectra, including that of **H**, show very similar profiles characterized by two maximum Cotton effects at 220 and 260 nm and a minimum Cotton effect at 241 nm, which are typical of parallel stranded quadruplex structures with all G-residues in an *anti*-glycosidic conformation.¹⁵ Since in previous investigations concerning modified Hotoda's ODNs,¹¹ the anti-HIV activity has been related to the thermal stability of the formed quadruplex structures, we have evaluated the apparent melting temperatures ($T_{1/2}$) in physiological PBS solution (potassium ions concentration = 2.7 mM) for all quadruplexes formed by TEL-ODNs in comparison with the complexes formed by the parent ODN (**H**) and its modified version bearing a TBDPS group at the 5'-end (**TH**). Results are listed in Table 1.

In these conditions, the stability of quadruplex **H** results negligible since no suitable melting profile could be obtained. However, as already reported,¹¹ the presence of the TBDPS group at the 5'-end (**TH**) clearly results in a $T_{1/2}$ enhancement. On the other hand, complexes formed by ODNs **1L** and **1S** (in which both the 5'-lipophilic groups and the 3'-TEL are present) show a dramatic improvement of the thermal stability compared to the parent quadruplexes **H** and **TH**. The ability of the TEL to enhance the thermal stability was further confirmed in complex **2L** (containing only the long type 3'-TEL) showing higher $T_{1/2}$ than the parent quadruplex **H**. The effect of the TEL at the 5'-ends has also been verified in quadruplexes **3L** and **3S**. However, for these complexes and for **2S**, as well, the non-sigmoidal melting profiles (see Fig. S2 in ESI†) prevented us to obtain a reliable estimation of their $T_{1/2}$. In fact, their profiles point to a two-step melting process,

thus indicating that the quadruplexes begin to disaggregate at quite low temperatures (around 20 °C). The annealing profiles in PBS solution provide further noteworthy data. **HT** is characterized by a remarkable hysteresis between melting and annealing curves, as expected for a tetramolecular quadruplex.¹⁶ In contrast, sigmoidal melting and annealing curves of **1L**, **1S** and **2L** show negligible hysteresis, suggesting that the folding process is independent from the ODN concentration.

Results regarding the anti-HIV activities of quadruplex complexes are listed in Table 1. Concerning the anti-HIV-1 activity, all complexes formed by TEL-ODNs proved more active than their precursor complex **TH**. Particularly, quadruplexes **1L** and **1S** show an EC_{50} of 0.082 and 0.29 μ M, respectively, against HIV-1. These values are noteworthy lower than the other complexes being devoid of the lipophilic groups. It should be noticed that the anti-HIV activities of the quadruplexes are markedly higher for HIV-1 than for HIV-2. Also, the L-type derivatives are consistently more active than the S-type derivatives. None of the compounds showed pronounced cytostatic activity ($IC_{50} > 30 \mu$ M) pointing to a significant degree of antiviral selectivity. When examined for their inhibitory activity against HIV-1 reverse transcriptase (using two different homopolymeric (polypurine and polypyrimidine) templates), rather poor inhibitory effects were observed (Table 1). The anti-HIV-1 activity, in particular for **1L**, **1S** and **3L**, was markedly higher than their anti-HIV-1 RT activity. These findings virtually rule-out HIV RT as the principal target for the anti-HIV activity of the quadruplexes. Given their negatively charged nature, inhibition of viral adsorption to and/or viral entry into their target cells may be a more likely mechanism of action for the quadruplexes.

Compound **1L** was evaluated for its binding properties to HIV-1(III_B) gp120 through SPR technology. Interestingly, **1L** showed a clear kinetic and dose-dependent interaction (binding) to gp120 (Fig. 2), while we could not detect a significant binding of **2L** to gp120 at 4 μ M concentrations.

The K_D affinity constant was estimated to be 348 nM, a compound concentration that belongs to the same order of magnitude as its anti-HIV-1(III_B) activity in cell culture (EC_{50} : 82 nM). The on- and off-rates of the compound against gp120 were $4.23 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ and $1.28 \times 10^{-3} \text{ s}^{-1}$, respectively.

Table 1 $T_{1/2}$ and anti-HIV activities of the quadruplexes formed by the investigated ODNs (n.d. = not determined, n.a. = not applicable); EC_{50} represents the 50%-effective concentration required to inhibit virus-induced cytopathicity by 50%. IC_{50} represents the 50% inhibitory concentration required to inhibit the RT reaction by 50%

ODN	$T_{1/2}$ (PBS)	$EC_{50}/\mu\text{M}$		$IC_{50}/\mu\text{M}$	
		HIV-1	HIV-2	Pol.rC.dG	Pol.rA.dT
H	<5	Not active ^a	n.d.	n.d.	n.d.
TH	47	>30	>30	>30	>30
1L	56	0.082 ± 0.04	8.6 ± 2.1	>30	>30
1S	54	0.29 ± 0.057	7.3 ± 3.9	26 ± 2	>30
2L	41	5.3 ± 5.1	≥ 25	≥ 25	17 ± 13
2S	n.a.	15 ± 14	>25	18 ± 2	19 ± 11
3L	n.a.	0.89 ± 0.0	≥ 25	>30	>30
3S	n.a.	1.40	>25	≥ 30	>30

^a See ref. 9.

Fig. 2 Kinetic analysis of interactions of compound **1L** with HIV-1(III_B) gp120 isolated from CHO cell cultures using SPR technology (see ESI†). The experimental data (coloured curves) were fit using the 1 : 1 binding model (black lines) to determine the kinetic parameters. The data are a representative example of two independent experiments.

Fig. 3 Molecular models of interactions between the aptamer **1L** and the gp120 V3 loop (green). (A) Close-up view. The backbone of the aptamer is drawn as liquorice. Hydrogen bonding interactions between gp120 V3 loop and the aptamer are drawn as dashed lines. (B) The aptamer is represented as charged molecular surface and highlights how a conserved R190 slides in the groove generated by backbones in the aptamer.

We also carried out molecular modelling studies to gain insight into the nature of the interaction between G-quadruplex-forming TEL-ODNs and the glycoprotein (gp) 120 hypervariable V3 loop of HIV. It has been previously shown that the tetramolecular parallel anti-HIV gp120 G-quadruplex aptamers are responsible for anti-HIV activity.^{9,17} In the absence of any structural data, molecular models provide a rationalisation of potential interactions. The main purpose of this modelling study was to identify how the hypervariable V3 loop in gp120 interacts with these aptamers. Although speculative, our results suggest that the interactions between the V3 loop and the aptamer are primarily based on electrostatic interactions (Fig. 3 and ESI†).

R190 is the only conserved residue in the hypervariable V3 loop and it is a major contributor towards binding of the V3 loop to the aptamer. R190 slides in the grooves between the two ODN chains and makes multiple interactions with the adenine, guanine and phosphate-backbone. An earlier study by Honig and co-workers¹⁸ has shown that charged residues, mainly arginines and lysines, play an important role in protein–DNA interactions in a similar manner. Furthermore, the side chains of residues H185 and Y193 in the V3 loop interact with the charged phosphodiester backbone atoms of the aptamer. The carbonyl backbone atoms of I186 and P188 also make electrostatic interactions with the charged backbone in the aptamer. The V3 loop interacts with **1S** aptamer in a similar manner, while in the case of **2L** and **2S** a different orientation of the V3 loop was observed (see ESI†).

The biological activity of these parallel quadruplex complexes may mainly depend on two different factors: (i) the extent of interaction with the viral gp120 V3 loop and (ii) the amount of quadruplex able to interact with the target, in turn, depending on the quadruplex thermal stability, the formation kinetics and the resistance to nucleases. As far as the first factor is concerned, molecular modelling studies suggest that the V3 loop residues only interact with the grooves and the sugar-phosphate backbones of the aptamer.

For this reason, the higher biological activity of TEL-complexes could be ascribed to both their enhanced thermal stability and their monomolecular nature that probably improves the kinetic formation. Furthermore, it should be taken into account that complexes **1L** and **1S**, showing the highest anti-HIV activities, are both characterized by capped 3'- and 5'-ends that, definitively, improve their resistance to exonucleases. However, more in-depth investigations are required to reveal the exact mode of action of the quadruplex derivatives.

Notes and references

- 1 K. A. Marshall and A. D. Ellington, *Methods Enzymol.*, 2000, **318**, 193–214.
- 2 H. J. Lipps and D. Rhodes, *Trends Cell Biol.*, 2009, **19**, 414–422.
- 3 N. Jing, C. Marchand, J. Liu, R. Mitra, M. E. Hogan and Y. Pommier, *J. Biol. Chem.*, 2000, **275**, 21460–21467.
- 4 V. R. de Soultrait, P.-Y. Lozach, R. Altmeyer, L. Tarrago-Litvak, S. Litvak and M. L. Andreola, *J. Mol. Biol.*, 2002, **324**, 195–203.
- 5 (a) J. R. Wyatt, T. A. Vickers, J. L. Roberson, R. W. Buckheit, Jr., T. Klimkait, E. DeBaets, P. W. Davis, B. Rayner, J. L. Imbach and D. J. Ecker, *Proc. Natl. Acad. Sci. U. S. A.*, 1994, **91**, 1356–1360; (b) D. J. Ecker, J. R. Wyatt, T. Vickers, R. Buckheit, J. Roberson and J.-L. Imbach, *Nucleosides, Nucleotides Nucleic Acids*, 1995, **14**, 1117–1127.
- 6 H. Hotoda, K. Momota, H. Furukawa, T. Nakamura, M. Kaneko, S. Kimura and K. Shimada, *Nucleosides, Nucleotides Nucleic Acids*, 1994, **13**, 1375–1395.
- 7 M. Koizumi, R. Koga, H. Hotoda, K. Momota, T. Ohmine, H. Furukawa, T. Nishigaki, K. Abe, T. Kosaka, M. Kaneko, S. Kimura and K. Shimada, *Nucleosides, Nucleotides Nucleic Acids*, 1997, **16**, 1205–1208.
- 8 M. Koizumi, R. Koga, H. Hotoda, K. Momota, T. Ohmine, H. Furukawa, T. Agatsuma, T. Nishigaki, K. Abe, T. Kosaka, S. Tsutsumi, J. Sone, M. Kaneko, S. Kimura and K. Shimada, *Bioorg. Med. Chem.*, 1997, **5**, 2235–2243.
- 9 H. Hotoda, M. Koizumi, R. Koga, M. Kaneko, K. Momota, T. Ohmine, H. Furukawa, T. Agatsuma, T. Nishigaki, J. Sone, S. Tsutsumi, T. Kosaka, K. Abe, S. Kimura and K. Shimada, *J. Med. Chem.*, 1998, **41**, 3655–3663.
- 10 M. Koizumi, R. Koga, H. Hotoda, T. Ohmine, H. Furukawa, T. Agatsuma, T. Nishigaki, K. Abe, T. Kosaka, S. Tsutsumi, J. Sone, M. Kaneko, S. Kimura and K. Shimada, *Bioorg. Med. Chem.*, 1998, **6**, 2469–2475.
- 11 J. D'Onofrio, L. Petraccone, E. Erra, L. Martino, G. Di Fabio, L. De Napoli, C. Giancola and D. Montesarchio, *Bioconjugate Chem.*, 2007, **18**, 1194–1204.
- 12 G. Oliviero, N. Borbone, A. Galeone, M. Varra, G. Piccialli and L. Mayol, *Tetrahedron Lett.*, 2004, **45**, 4869–4872.
- 13 G. Oliviero, J. Amato, N. Borbone, A. Galeone, L. Petraccone, M. Varra, G. Piccialli and L. Mayol, *Bioconjugate Chem.*, 2006, **17**, 889–898.
- 14 (a) L. Petraccone, L. Martino, I. Duro, G. Oliviero, N. Borbone, G. Piccialli and C. Giancola, *Int. J. Biol. Macromol.*, 2007, **40**, 242–247; (b) G. Oliviero, N. Borbone, J. Amato, S. D'Errico, A. Galeone, G. Piccialli, M. Varra and L. Mayol, *Biopolymers*, 2009, **91**, 466–477.
- 15 S. Paramasivan, I. Rujan and P. H. Bolton, *Methods Enzymol.*, 2007, **43**, 324–331.
- 16 J. L. Mergny, A. De Cian, A. Ghelab, B. Saccà and L. Lacroix, *Nucleic Acids Res.*, 2005, **33**, 81–94.
- 17 T. Agatsuma, I. Yamamoto, H. Furukawa and T. Nishigaki, *Antiviral Res.*, 1996, **31**, 137–148.
- 18 R. Rohs, S. M. West, A. Sosinsky, P. Liu, R. S. Mann and B. Honig, *Nature*, 2009, **461**, 1248–1253.