ChemComm

Cite this: *Chem. Commun.*, 2012, **48**, 9516–9518

www.rsc.org/chemcomm

COMMUNICATION

New *anti*-HIV aptamers based on tetra-end-linked DNA G-quadruplexes: effect of the base sequence on *anti*-HIV activity†

Valentina D'Atri,[a] Giorgia Oliviero,[a] Jussara Amato,[a] Nicola Borbone,*[a] Stefano D'Errico,[a] Luciano Mayol,[a] Vincenzo Piccialli,[b] Shozeb Haider,[c] Bart Hoorelbeke,[d] Jan Balzarini[d] and Gennaro Piccialli[a]

Received 19th June 2012, Accepted 9th August 2012
DOI: 10.1039/c2cc34399a

This communication reports on the synthesis and biophysical, biological and SAR studies of a small library of new *anti*-HIV aptamers based on the tetra-end-linked G-quadruplex structure. The new aptamers showed EC_{50} values against HIV-1 in the range of 0.04–0.15 μM as well as affinities for the HIV-1 gp120 envelope in the same order of magnitude.

The first stage of the HIV infection requires the entry of human immunodeficiency virus (HIV) into host cells. This stage involves the sequential interaction of the virion surface glycoprotein gp120 with the CD4 glycoprotein and a chemokine receptor, either CCR5 or CXCR4, on the host cell surface.[1] The CD4 glycoprotein is expressed on the surface of T-lymphocytes, monocytes, dendritic cells and brain microglia, and its expression makes these cells a target for HIV *in vivo*. Furthermore, an interesting function of CD4 binding is to induce conformational changes in gp120 that allow binding to the co-receptor, which is essential for viral entry.[2] The third variable region of gp120 (from now on designated V3 loop) is a pivotal component of the co-receptor binding site, typically consisting of a 35 amino acid-loop (range 31 to 39), closed by two cysteines that form a disulfide bridge. The crystal structure of gp120 complexed with the CD4 receptor and a neutralizing antibody (PDB ID 2B4C) revealed that the V3 loop is extended away from the gp120 protein and it is involved in co-receptor binding and selection, acting as a "molecular hook" that organizes associations within the viral spike.[3] The importance of gp120 and the role of the V3 loop in HIV-1 entry and pathogenesis have led to the recent pursuit of drugs targeted against it. One of the most studied alternatives is the use of aptamer technology.[4] Aptamers are single stranded DNA or RNA molecules selected among large pools of nucleic acid sequences for their ability to bind selectively and with high affinity to a biomedically relevant target. For their properties, aptamers can be considered as the nucleic acid analogues of antibodies. Like the antibodies, several aptamers proved to be valuable diagnostic tools[5] and promising therapeutics.[6,7]

In 1994 H. Hotoda (SA-1042)[8] and J. R. Wyatt (ISIS 5320)[9] independently reported the first *anti*-HIV aptamers targeted against the V3 loop. In both cases the ODN sequences were chemically modified to improve their resistance against nucleases by capping the 5′-ends with DMT groups (SA-1042) or by using the phosphorothioate backbone (ISIS 5320). Subsequently, other analogues of SA-1042 were obtained and tested,[10,11] and the 6-mer TGGGAG ODN (known as "Hotoda's sequence") bearing 3,4-dibenzyloxybenzyl groups at the 5′-ends and 2-hydroxyethylphosphates at the 3′-ends (R-95288) showed the highest *anti*-HIV activity.[11] CD investigations on ISIS 5320, R95288 and their analogues suggested their structuration into parallel tetramolecular quadruplexes. Following biophysical studies established that the presence of aromatic[12,13] moieties at 5′- and/or 3′-ends of TGGGAG dramatically enhances the rate of formation of quadruplex complexes. Moreover, the overall stability of quadruplex complexes was found to correlate with the reported *anti*-HIV activity.[12]

In 2010, with the aim to overcome the unfavourable entropic factor and to stabilize the A-tetrad in Hotoda's analogues, we synthesized a series of new monomolecular *anti*-HIV aptamers by using the Tetra-End-Linker (TEL) strategy proposed by some of us in 2004.[14,15] Several TEL-$(TGGGAG)_4$ aptamers were prepared and analyzed in order to probe the influence of lipophilic groups and TEL size and position on the structural and *anti*-HIV properties of the resulting TEL-quadruplexes.[16] The results showed that (i) the presence of the TEL at either 5′- or 3′-ends was required for the *anti*-HIV activity, and (ii) lipophilic *tert*-butyldiphenylsilyl (TBDPS) groups at the 5′-ends strongly enhanced both the stability of TEL-quadruplexes and their *anti*-HIV activity. The EC_{50} of the best aptamer (**I**, Fig. 1), bearing TBDPS groups at the 5′-ends and the longer TEL at the 3′-ends was 0.082 ± 0.04 μM.

With the aim to further improve the *anti*-HIV activity of TEL-ODN aptamers, we used **I** as a molecular synthon for the synthesis of a series of new TEL-ODN aptamers embodying

[a] *Dipartimento di Chimica delle Sostanze Naturali, Università degli Studi di Napoli Federico II, Via D. Montesano 49, I-80131 Napoli, Italy. E-mail: Nicola.borbone@unina.it*
[b] *Dipartimento di Chimica, Università degli Studi di Napoli Federico II, Via Cynthia 4, I-80126 Napoli, Italy*
[c] *Centre for Cancer Research and Cell Biology, Queen's University of Belfast, 97 Lisburn Road, BT9 7BL, Belfast, UK*
[d] *Rega Institute for Medical Research, KU Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium*

† Electronic supplementary information (ESI) available: Synthesis and characterization of TEL-ODNs, as well as CD, SPR and docking studies and biological evaluation assays. See DOI: 10.1039/c2cc34399a

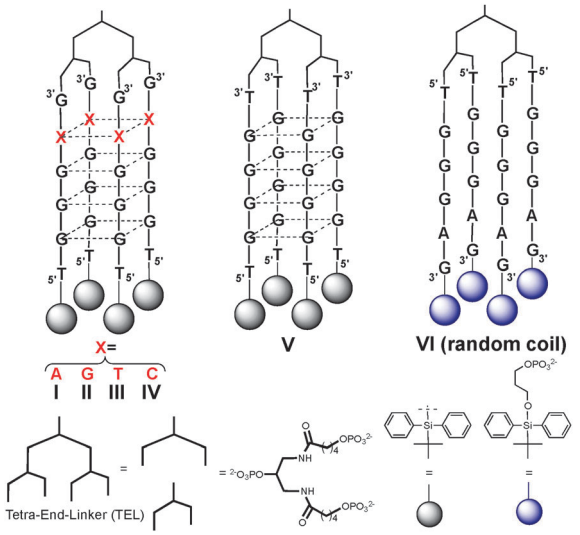

Fig. 1 Schematic representation of investigated TEL-ODNs.

the TGGGXG sequence (**II–IV**, Fig. 1). TEL-ODNs **V** and **VI** (a TEL-$(TGGGAG)_4$ analogue unable to form a TEL-quadruplex) were also synthesized and studied in order to investigate the role played by the G-tetrad at the 3′-terminus of TEL-quadruplexes **I–IV** and to further corroborate the requirement of quadruplex formation for the inhibition of HIV cytopathicity. In this communication we report the results of biophysical, biological and SAR studies performed on **I–VI**.

All products were synthesized and characterized following the previously described approach.[14,16] In order to verify that the presence of one (**II–IV**) or two (**V**) different bases did not affect the parallel quadruplex arrangement observed for the parent TEL-ODN **I**, CD measurements were performed (Fig. S2 in ESI†). The overall CD profiles, recorded in 100 mM K^+ buffer, were in agreement with the formation of G-quadruplex structures, showing two positive maxima at 220 and 260 nm and a negative minimum close to 240 nm, which are characteristic of head-to-tail arrangement of guanines, as typically found in parallel G-quadruplexes.[17–19] Unexpectedly, CD spectra of **VI** were similar to those of a random coil, thus evidencing that the insertion of a propylphosphate moiety between the OH at 3′-ends and TBDPS groups is detrimental for quadruplex stability. CD melting analyses on **II–IV** confirmed the expected great thermal stability of the new TEL-quadruplexes (Fig. S3 in ESI†). In fact, as for **I**, the apparent melting temperature of **II–IV** could not be determined since no derivatizable melting curve was obtained.

The formation of thermally stable TEL-quadruplexes for **II–IV** was also confirmed by ^{1}H NMR evidence (Fig. S4 and S5 in ESI†). The G-quadruplex diagnostic exchange-protected imino proton signals were observed in all recorded spectra up to 90 °C (100 mM K^+ buffer; H_2O/D_2O 9 : 1). As expected, the intensity of imino proton signals reflected the stability of the X-tetrad within the G-quadruplex structure. Stronger imino signals were observed for **II** (five stacked G-tetrads), whereas weaker imino signals were observed for **I**, **III** and **IV** due to the presence of the less stable A-, T- or C-tetrad, respectively, that accounts for the faster exchange of imino protons with the solvent.

The antiviral activity of **II–IV** against HIV-1 and HIV-2 was determined as previously reported for **I**[16] and is shown in Table 1. The binding properties of active aptamers **II–IV** to HIV-1(IIIB) gp120 were also determined through Surface Plasmon Resonance (SPR) technology (Table 1; Fig. S6 in ESI†).

All new TEL-$(TGGGXG)_4$ aptamers reported in this communication (**II–IV**) retained potent *anti*-HIV-1 activity (nanomolar EC_{50} values). They displayed comparable on (k_a) and off (k_d) rates for HIV-1 gp120 binding resulting in very similar gp120 binding affinities (K_D) to **I**, thus confirming that the residues of the gp120 V3 loop interact mainly with the grooves and the sugar–phosphate backbone of the aptamers. However, considering that the EC_{50}'s of **II** and **IV** were significantly lower than those of **I** and **III** (0.039–0.041 *versus* 0.11–0.15 μM), and that **V** proved to be markedly less active notwithstanding its structuration in a stable TEL-quadruplex structure, we hypothesized that some specific interactions between the X_5G_6 bases of **I–IV** and the V3 loop must occur. It is well-described that binding of agents (*i.e.* monoclonal antibodies) to the viral envelope does not necessarily result in efficient virus neutralization. Their effect on viral infectivity depends on the molecular epitope that is recognized on gp120.[20] Thus, it seems that **I** and **III** bind nearly as efficiently to gp120 as **II** and **IV** but neutralize ~3-fold less efficiently HIV-1 possibly due to subtile differences in epitope recognition. Alternatively, it cannot be excluded that the different TEL-quadruplexes have slightly different cellular uptake efficiencies or intracellular stability.

To obtain insight into the nature of the atomistic interactions between the TEL-aptamers **I–IV** and the V3 loop, we carried out molecular modelling studies by docking **I–IV** to the V3 loop of gp120 (PDB ID 2B4C). Our results revealed that (i) the V3 loop interacts with all aptamers with a similar binding mode involving the 3′-end tetrad and the TEL (Fig. S7 in ESI†), (ii) the type of nucleobases at the aptamer-V3 loop interface determines the chemical groups available for the interaction, and (iii) the number and type of interactions between the aptamer and the protein are responsible for the subtle differences in the binding energies (Table S1 in ESI†).

As reported by Honig and co-workers,[21] charged residues are important in protein–DNA interactions. In this case, an important role is played by the side chain of residue R181 in the V3 loop that binds into the groove created by the phosphodiester

Table 1 Anti-HIV and SPR studies on **I–V**

	EC_{50}[a] (μM)		SPR *vs.* HIV-1(III_B) gp120[b]		
ODN	HIV-1	HIV-2	K_D (nM)	k_a (1/Ms)	k_d (1/s)
I	0.11 ± 0.05	1.4 ± 0.9	256 ± 63	(1.72 ± 0.28) × 10^4	(4.31 ± 0.37) × 10^{-3}
II	0.041 ± 0.007	≥ 2	183 ± 40	(2.40 ± 0.20) × 10^4	(4.35 ± 0.61) × 10^{-3}
III	0.15 ± 0.0015	1.4 ± 0.87	248 ± 28	(2.55 ± 0.35) × 10^4	(6.29 ± 0.14) × 10^{-3}
IV	0.039 ± 0.005	0.73 ± 0.16	196 ± 69	(2.15 ± 0.40) × 10^4	(4.07 ± 0.71) × 10^{-3}
V	≥ 2	2	—	—	

[a] EC_{50} represents the 50% effective concentration required to inhibit virus-induced cytopathicity in CEM cell cultures by 50%. [b] HIV-1(III_B) gp120 was obtained from recombinantly-expressed gp120 in CHO cell cultures.

Table 2 Interaction energies of top docks (Kcal mol^{-1}) and number of H bonds between **I–V** and the V3 loop of gp120

Aptamer	Interactions energy	Number of H bonds
I	−24.11	8
II	−28.45	15
III	−13.74	9
IV	−28.09	11
V	−13.74	8

backbone atoms (see Table S1 in ESI†). In particular, in **II** and **IV**, R181 makes multiple interactions with both phosphates and purine bases (G5 and G6 in **II**, G6 in **IV**). Furthermore, the side chains of R190, T195 and E197 and the nitrogen backbone atom of T194 of the V3 loop interact with the oxygen atoms of the TEL, giving additional stability to the complexes. In the **II**–V3 loop complex, the side chain of Y193 and the nitrogen backbone atom of Y193, T195 and I198 established additional interactions with the TEL, thereby resulting in lower interaction energy of this complex with respect to the other ones (Table 2). It should also be noted that when the side chain of Y193 is involved in the interaction (**I**, **II** and **IV**), the resulting complexes are found to have a better biological activity. Thus, the G5 nucleobase in **II** presents additional points for hydrogen bonding with the V3 loop. This is not seen in other bases. Therefore we infer that the differences in activity can arise from the thermal stability of the structures. Furthermore, this is also rationalised by the SPR experiments.

In order to better understand the structural features critical for the biological activity, we also carried out a molecular modelling study between the V3 loop and **V**, a quadruplex structure lacking marked *anti*-HIV activity. Our results revealed that the V3 loop interacts with **V** by using a different binding mode (Fig. S7 in ESI†). Differently from what was observed in the above-described complexes, **V** interacts with the V3 loop primarily *via* the TEL atoms and no atom of nucleobases is involved. Furthermore, except for R181, different residues of the V3 loop, such as K182, S183, I184, and I186, are involved in the interactions (Table S1 in ESI†). A plausible reason for the different binding mode of **V** is due to the presence of the T-tetrad at the 3′-terminus. As shown in Fig. S7 in ESI†, when there is a G-tetrad at the 3′-position, the oxygen atoms of guanines are involved in the interaction with the V3 loop through the side chains of R181 (and Y193 in the case of **IV**). In the **V**–V3 loop complex the methyl groups of the thymines are positioned in the groove formed by the phosphodiester backbone atoms, not allowing the formation of H bonds with R181 and Y193 of the V3 loop. All together, the structural evidence suggests that the T-tetrad at the 3′-position markedly affects the biological activity. In accordance with this finding and with the experimental data, the aptamer **II** showed the best docking score and the highest number of hydrogen bonds with the protein (Table 2).

The *anti*-HIV activity against HIV-1 and HIV-2 and the binding properties with HIV gp120 of a small library of new TEL-aptamers (**II–IV**) have been reported. Results from TEL-ODNs **V** and **VI** confirmed that the formation of a quadruplex species by the aptamer is required, but not sufficient to exert the *anti*-HIV activity. The docking data suggest that the interaction of the V3 loop with both the backbone and the TEL of the aptamers is required. Furthermore, the direct involvement of nucleobases in the interaction with the V3 loop gives additional stability to the complexes and results in a better biological activity. Overall, the here reported results expand our knowledge about *anti*-HIV G-quadruplexes and provide the rational basis for the design of novel *anti*-HIV aptamers with improved biological activity.

The European Commission through the COST Action MP0802, the Italian MURST (PRIN 2009), KU Leuven (PF 10/18 and GOA 10/14) and the FWO are gratefully acknowledged for the financial support. Dr Luisa Cuorvo is also acknowledged for technical assistance.

Notes and references

1 P. D. Kwong, R. Wyatt, J. Robinson, R. W. Sweet, J. Sodroski and W. A. Hendrickson, *Nature*, 1998, **393**, 648–659.
2 R. Wyatt, *Science*, 1998, **280**, 1884–1888.
3 C. C. Huang, *Science*, 2005, **310**, 1025–1028.
4 S. Chou and K. Chin, *Trends Biochem. Sci.*, 2005, **30**, 231–234.
5 A. C. Perkins and S. Missailidis, *Q. J. Nucl. Med. Mol. Imaging*, 2007, **51**, 292–296.
6 A. D. Keefe, S. Pai and A. Ellington, *Nat. Rev. Drug Discovery*, 2010, **9**, 537–550.
7 J. Lee, G. Stovall and A. Ellington, *Curr. Opin. Chem. Biol.*, 2006, **10**, 282–289.
8 H. Hotoda, K. Momota, H. Furukawa, T. Nakamura, M. Kaneko, S. Kimura and K. Shimada, *Nucleosides Nucleotides*, 1994, **13**, 1375–1395.
9 J. R. Wyatt, T. A. Vickers, J. L. Roberson, R. W. Buckheit, 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.
10 H. Furukawa, K. Momota, T. Agatsuma, I. Yamamoto, S. Kimura and K. Shimada, *Antisense Nucleic Acid Drug Dev.*, 1997, **7**, 167–175.
11 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.
12 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.
13 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.
14 G. Oliviero, N. Borbone, A. Galeone, M. Varra, G. Piccialli and L. Mayol, *Tetrahedron Lett.*, 2004, **45**, 4869–4872.
15 G. Oliviero, J. Amato, N. Borbone, A. Galeone, M. Varra, G. Piccialli and L. Mayol, *Biopolymers*, 2006, **81**, 194–201.
16 G. Oliviero, J. Amato, N. Borbone, S. D'Errico, A. Galeone, L. Mayol, S. Haider, O. Olubiyi, B. Hoorelbeke, J. Balzarini and G. Piccialli, *Chem. Commun.*, 2010, **46**, 8971–8973.
17 S. Paramasivan, I. Rujan and P. H. Bolton, *Methods*, 2007, **43**, 324–331.
18 S. Masiero, R. Trotta, S. Pieraccini, S. De Titio, R. Perone, A. Randazzo and G. P. Spada, *Org. Biomol. Chem.*, 2010, **8**, 2653–2872.
19 J.-L. Mergny, A. De Cian, A. Ghelab, B. Saccà and L. Lacroix, *Nucleic Acids Res.*, 2005, **33**, 81–94.
20 L. M. Walker, M. Huber, K. J. Doores, E. Falkowska, R. Pejchal, J. P. Julien, S. K. Wang, A. Ramos, P. Y. Chan-Hui, M. Moyle, J. L. Mitcham, P. W. Hammond, O. A. Olsen, P. Phung, S. Fling, C. H. Wong, S. Phogat, T. Wrin, M. D. Simek, W. C. Koff, I. A. Wilson, D. R. Burton and P. Poignard, *Nature*, 2011, **477**, 466–470.
21 R. Rohs, S. M. West, A. Sosinsky, P. Liu, R. S. Mann and B. Honig, *Nature*, 2009, **461**, 1248–1253.