Supporting Information (ESI)

Anti-HIV activity of new higher-order G-quadruplex aptamers obtained from tetra-end-linked oligonucleotides

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Reagents and equipment. Chemicals and solvents were purchased from Fluka-Sigma-Aldrich and used as such. Reagents, phosphoramidites, linkers and the CPG support used for DNA syntheses were purchased from Glen Research. HPLC analyses and purifications were performed with a Jasco PU2089 pump system equipped with the Jasco 2075 Plus UV detector, using an anion-exchange Macherey-Nagel column. The ODNs were assembled by a PerSeptive Biosystems Expedite DNA synthesizer using standard phosphoramidite chemistry. UV spectra were registered on a Jasco V 530 spectrophotometer. CD spectra and thermal denaturation experiments were obtained with a Jasco 715 circular dichroism spectropolarimeter equipped with a Jasco 505T temperature controller unit. NMR spectra were recorded either on a Varian Unity Inova 500 MHz spectrometer equipped with a broadband inverse probe with z-field gradient or on a Varian Unity INOVA 700 MHz spectrometer equipped with a triple resonance cryoprobe and processed using the Varian VNMR and iNMR (http://www.inmr.net) software packages.

Synthesis and purification of TEL-ODNs 1–3. The syntheses of TEL-ODNs **1–3** were performed using a solid-phase automated DNA synthesizer according to a previously described procedure.[1] To obtain the final products **2** and **3**, a DMT-on protocol, not including the final DMT deblocking step, was adopted. In the case of TEL-ODN **3**, the DMT-protected glucose phosphoramidite derivative, obtained through a previously reported protocol,[2,3] was used at a concentration of 50 mg/mL in anhydrous acetonitrile solution in the last coupling step. After the automated synthesis, the DMT-ODNs were detached from the support and deprotected by treatment with concentrated aqueous ammonia at 55 °C for 17 h. The combined filtrates and washings were dried, redissolved in water, analyzed and purified by HPLC on an anion exchange column (Macherey-Nagel, 1000-8/46, 4.4×50 mm, 5 μm) using a linear gradient from 0 to 100% B in 30 min, flow rate = 0.3 mL/min and detection at 260 nm (buffer A: 20 mM NaH_2PO_4 aq. solution pH 7.0, containing 20% (v/v) CH_3CN; buffer B: 20 mM NaH_2PO_4 aq. solution pH 7.0, containing 1 M NaCl and 20% (v/v) CH_3CN). After HPLC purification, the ODN samples were desalted on a Biogel column (BIORAD) eluted with H_2O/CH_3CH_2OH (9:1, v/v). After drying, the desalted **2** and the DMT-protected precursor of **3** (DMT-**3**) were further purified by HPLC on a RP-18 column (Purosphere STAR, 5 μm, 250 x 10 mm, Merck) eluted with a linear gradient from 0 to 100% B 30 min, flow rate 0.7 mL min^{-1} and detection at 260 nm (eluent A: triethylammonium bicarbonate buffer (TEAB) 0.1 μM; eluent B: CH_3CN). The retention time of **2** (23.1 min) and DMT-**3** (23.4 min) corresponded to that of the most retained peak in each HPLC profile due to the presence of the terminal DMT lipophilic groups. The collected peaks were dried under reduced pressure and lyophilised two times after solubilization in water. The purified **2** and DMT-**3** were quantified by UV spectroscopic analyses in pure water by measuring the absorbance at 260 nm at 90 °C using the molar extinction coefficient of 5'-CGGAGG-3' as calculated by the nearest-neighbor model[4] multiplied by 4 (the number of strands in each TEL-ODN complex). The calculated ε = 246,000 cm^{-1} M^{-1} was augmented by 1% due to the contribution of DMT groups.[5] The DMT deprotected **1** and **3** were obtained treating the corresponding precursors with a mixture of acetic acid and water (8:2, v/v, 3 mL) for 40 min at room temperature. After the addition of water (5 mL) the solutions were extracted with diethyl ether (4 x 10 mL) in a separating funnel. The acqueous layer was dried under reduced pressure (max 40°C) and co-evaporated with water three times to remove the residual acetic acid. Finally, the fully deprotected TEL-ODN **1** and **3** were dissolved in water, lyophilised and quantified by UV.

Characterization of TEL-ODNs 1–3 by quantitation of DMT cations released from 2 and DMT-3. 0.1 μmol of **2** or DMT-**3** was treated with a solution of 70% $HClO_4$/Ethanol (3:2, v/v) and the red solution was analysed by spectroscopic measurements at 498 nm (ε = 71,700 cm^{-1} M^{-1}). 0.1 μmol of **2** and DMT-**3** furnished 0.39 and 0.42 μmol of DMT, respectively, thus supporting the assigned

structures and the purity for both products.

Preparation of G-quadruplexes (annealing). The G4s were formed by dissolving the TEL-ODNs **1–3** in 1 M K^+-containing aqueous buffer (900 mM KCl, 100 mM KH_2PO_4, pH 7) and annealed by heating at 90 °C for 5 min followed by quick cooling at 4 °C (kinetic annealing procedure). Stock solutions of 6 mM strand concentration for **1** and **2** and 3 mM for **3** were prepared. The samples were stored at 4 °C for 24 h before the measurements.

Native gel electrophoreses. Native gel electrophoresis experiments were run on 20% non-denaturing polyacrylamide gels in 1 × TBE buffer, pH 7.0, supplemented with 30 mM NaCl. 600 μM single strand ODN samples, obtained by diluting the annealed stock solutions just before the experiment, were loaded on the gel and 10% glycerol was added to each sample just before loading. The gels were run at room temperature at constant voltage (120 V) for 1 h. The bands were visualized by UV shadowing.

CD experiments. CD spectra of the annealed **1–3** were registered in a 0.1 cm path length cuvette at 5 °C at 10 μM G4 concentration. The spectra were recorded in the 360–200 nm wavelength range as an average of 3 scans (100 nm/min, 1 s response time, 1 nm bandwidth) and normalized by subtraction of the background scan containing only the buffer. CD melting experiments were performed monitoring the CD value (mdeg) at 264 nm in the temperature range 5–90 °C with 0.5 °C/min heating rate.

HPLC-SEC experiments. HPLC-SEC analyses were performed on a Yarra 200 SEC Column (Phenomenex, 300 x 7.8 mm, 3 um) equipped with a Phenomenex SecurityGuard pre-column eluted with 90 mM KCl and 10 mM KH_2PO_4/CH_3CN (80/20, v/v), flow rate 0.6 mL min^{-1}, detector at 260 nm. The analyses were performed at room temperature.

NMR experiments. NMR samples were prepared at 1.6 mM concentration by dissolving **1–3** in 250 μL of 1 M K^+-containing annealing buffer (900 mM KCl, 100 mM KH_2PO_4, pH = 7 in H_2O/D_2O 9:1 v/v). One-dimensional NMR spectra were acquired as 16,384 data points with a recycle delay of 1.0 s at 25 °C. Data sets were zero-filled to 32,768 points prior to Fourier transformation and apodized with a shifted sine bell squared window function. Water suppression was achieved by including a double pulsed-field gradient spin-echo (DPFGSE) module[6,7] in the pulse sequence prior to acquisition.

Anti-HIV activity assay. The anti-HIV activity and cytotoxicity of the ODNs were evaluated against wild-type (WT) HIV-1 strain III_B in MT-4 cell cultures using the 3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium bromide (MTT) method.[8] Briefly, stock solutions (10 × final concentration) of the tested compounds were added in 25 µL volumes to two series of triplicate wells so as to allow simultaneous evaluation of their effects on mock-and HIV-infected cells at the beginning of each experiment. Serial 5-fold dilutions of test compounds were made directly in flat-bottomed 96-well microtiter trays using a Biomek 3000 robot (Beckman instruments, Fullerton, CA). Untreated control HIV-and mock-infected cell samples were included for each sample. Virus stock (50 µL) at 100-300 $CCID_{50}$ (50% cell culture infectious dose) or culture medium was added to either the virus-infected or mock-infected wells of the microtiter tray. Mock-infected cells were used to evaluate the effect of test compounds on uninfected cells in order to assess the cytotoxicity of the tested compounds. Exponentially growing MT-4 cells were centrifuged for 5 min at 220 g and the supernatant was discarded. The MT-4 cells were resuspended at 6 x 10^5 cells/mL and 50-µL volumes were transferred to the microtiter tray wells. Five days after the infection, the viability of mock- and HIV-infected cells was examined spectrophotometrically by the MTT assay.

The MTT assay is based on the reduction of yellow colored 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Acros Organics) by mitochondrial dehydrogenase activity in metabolically active cells to a blue-purple formazan that can be spectrophotometrically measured. The absorbances were read in an eight-channel computer-controlled photometer (Infinite M1000, Tecan), at two wavelengths (540 and 690 nm). All data were calculated using the median absorbance value of three wells. The 50% cytotoxic concentration (CC_{50}) was defined as the concentration of the tested compound that reduced the absorbance (OD_{540}) of the mock-infected control sample by 50%. The concentration achieving 50% protection against the cytopathic effect of the virus in infected cells was defined as the 50% inhibitory concentration (IC_{50}).

Surface Plasmon Resonance (SPR) experiments. The binding of gp120 on immobilized heparin was measured using Surface Plasmon Resonance (SPR). The experiments were performed at 25°C on a Biacore T200 (GE Healthcare, Uppsala, Sweden) in HBS-EP (10 mM HEPES, 150 mM NaCl, 3 mM EDTA and 0.05% (v/v) Tween20; pH 7.4). Heparin (Iduron) was minimally biotinylated at the reducing end using biotinamidohexanoic acid hydrazide (Sigma-Aldrich). Biotinylated-heparin was extensively dialyzed to remove unreacted biotin and 400 Resonance Units (RU) were captured on a Streptavidin Sensor Chip. Recombinant gp120 HIV-1(III_B) (ImmunoDiagnostics Inc., Woburn, MA) alone (2 µg/ml) or premixed with a concentration range of the G-quadruplex aptamers was injected for 2 minutes at a flow rate of 30 µl/min, followed by a dissociation phase of 2 minutes. The Sensor chip was regenerated by treatment with 1M NaCl in 10 mM NaOH. A reference flow was used as a

control for non-specific binding. Several buffer blanks were used for double referencing.

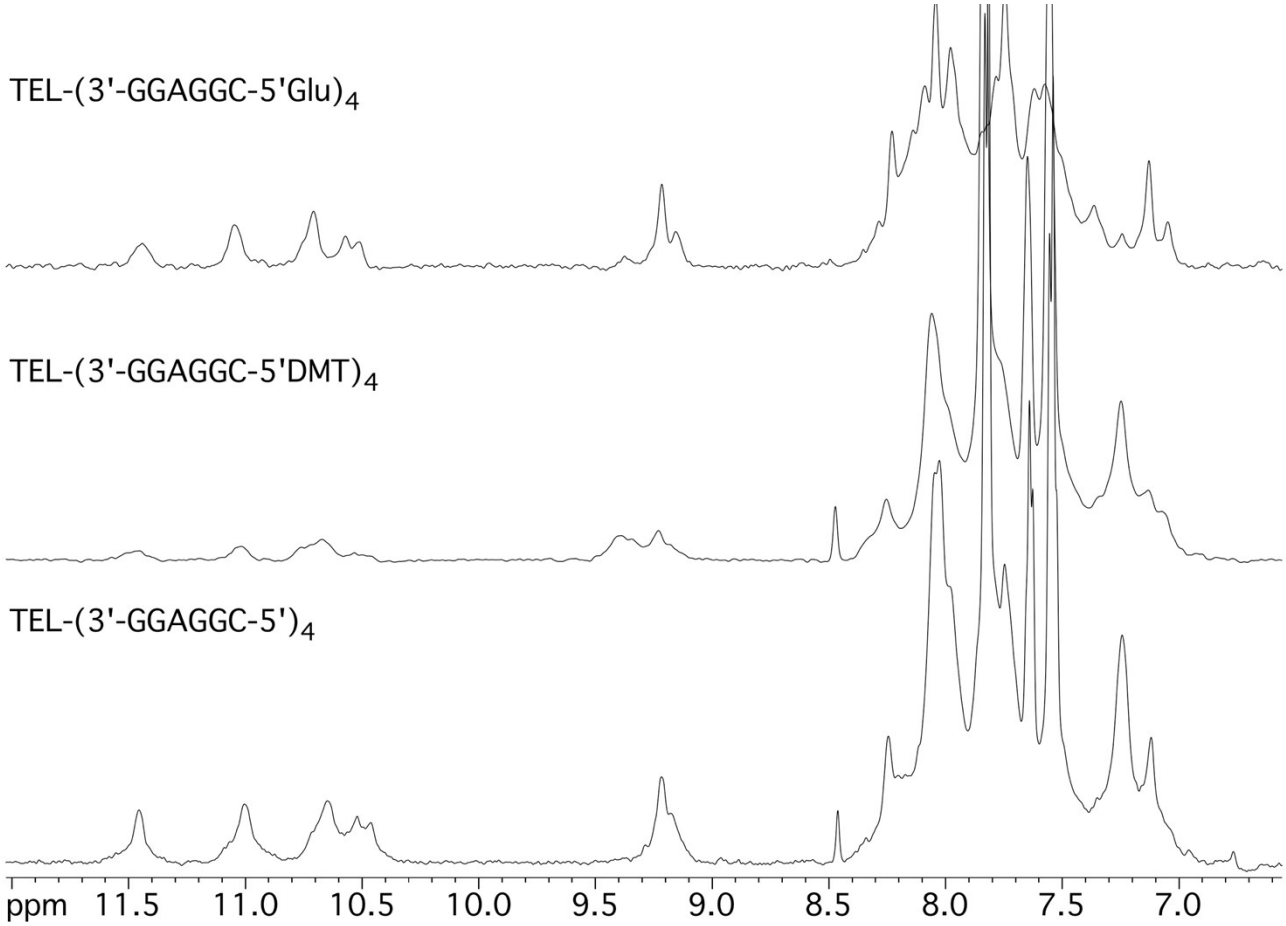

Fig. S1. Exchangeable and aromatic protons region of ^{1}H-NMR spectra of **1**–**3** registered at 25 °C in 1 M K^{+} buffer.

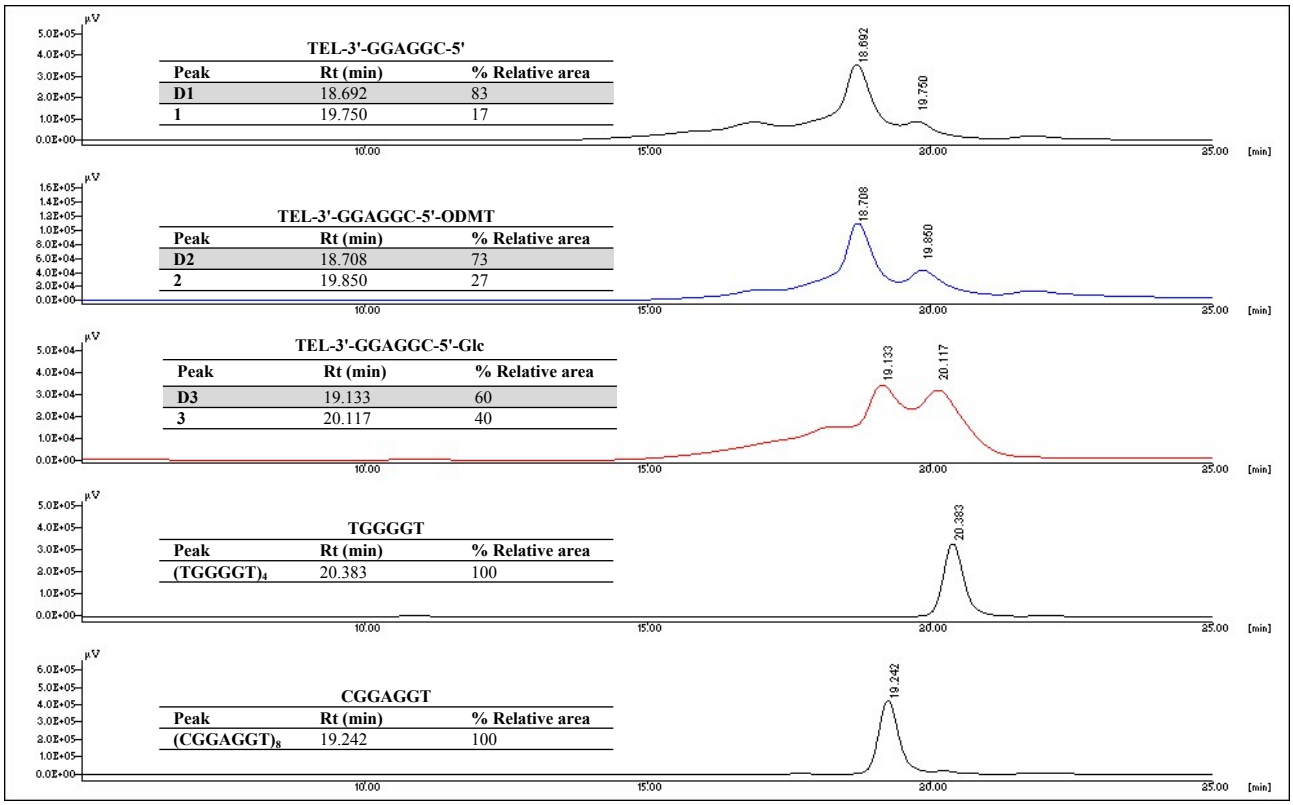

Fig. S2. HPLC-SEC profiles of TEL-ODNs **1–3** and monomeric (TGGGGT) and dimeric (CGGAGGT) G4 size markers annealed in 1.0 M K^+ buffer. The relative area of peaks belonging to monomeric and dimeric G4s formed by **1–3** is reported in the tables.

Notes and References

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