

Novel monomolecular derivatives of the anti-HIV-1 G-quadruplex-forming Hotoda's aptamer containing inversion of polarity sites

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ABSTRACT

Here we report on the design, preparation and investigation of four analogues of the anti-HIV G-quadruplex-forming Hotoda's aptamer, based on an unprecedented linear topology. In these derivatives, four TGGGAGT tracts have been joined together by exploiting 3'-3' and 5'-5' inversion of polarity sites formed by canonical phosphodiester bonds or a glycerol-based linker. Circular dichroism data suggest that all oligodeoxynucleotides fold in monomolecular G-quadruplex structures characterized by a parallel strand orientation and three side loops connecting 3'- or 5'-ends. The derivative bearing two lipophilic groups, namely HT353LGly, inhibited virus entry into the host cell, with anti-HIV-1 activity in the low nanomolar range; the other derivatives, albeit sharing the same base sequence and similar topology, were inactive. These results highlight that monomolecular Hotoda's aptamers with inversion of polarity sites represent a successful alternative strategy that merges the easiness of synthesis with the maintenance of remarkable activity. They also indicate that two lipophilic groups are necessary and sufficient for biological activity. Our data will inspire the design of further simplified derivatives with improved biophysical and antiviral properties.

KEYWORDS

Aptamer, G-quadruplex, HIV-1, Hotoda.

1. INTRODUCTION

DNA and RNA aptamers are relatively short synthetic nucleic acid fragments able to adopt distinctive three-dimensional structures and bind, with high affinity and specificity, a wide variety of molecular targets ranging from small molecules to large biomolecules, as proteins and other nucleic acids [1]. Thanks to their outstanding ligand properties, aptamers can find effective applications in many fields, including diagnostics and therapeutics [2]. In particular, there is an increasing interest in the application of aptamers in virus detection and antiviral therapy [3]. The development of suitable aptamers as potential therapeutic agents is especially important in the case of AIDS and anti-HIV therapy due to the increasing emergence of drug resistance that urges the development of innovative unconventional drugs [4,5]. Several aptamers have been successfully designed to target HIV at different stages of its life cycle [6,7]. Among them, G-rich oligomers comprise a large group of aptamers with the ability to fold into stable G-quadruplexes (G4s) under physiological conditions. G4s are highly polymorphic non-canonical nucleic acid secondary structures stabilized by the stacking interactions

of G-quartets that are planar arrangements of four guanines held together by Hoogsteen hydrogen bonding [8]. In this regard, the peculiar G4 structure of the aptamer has been shown to enhance target recognition [9]. G4-aptamers display several other advantages compared to unstructured sequences, including thermodynamic and chemical stability, no immunogenicity and resistance to numerous serum nucleases [10]. Anti-HIV G4-aptamers have aroused broad interest not only because of their promising efficiency but also because G4s are among the best-characterized DNA secondary structures and are present in regulatory regions of the genome of several viruses, thus indicating involvement of these structures in the control of key viral processes [11]. A wide range of anti-HIV G4-aptamers has been developed against crucial proteins that participate to the HIV life cycle. For example, a family of G- and T-containing aptamers (including T30923 [12], T30175 [13,14], their partially phosphorothioate versions, T30695 [12,15,16] and T30177 [13–16], and a modified version of T30175 [17]) bind and/or inhibit integrase (IN), the enzyme that catalyzes the integration of the newly synthesized double-stranded proviral DNA genome into the host genome. Further G4-aptamers, in particular oligodeoxyribonucleotides (ODNs) 93 and 112, target reverse transcriptase (RT) inhibiting its RNaseH activity [18]. Interestingly, shorter DNA G4-aptamers derived from ODNs 93 and 112, called 93del and 112del respectively, were able to inhibit HIV-1 integrase in the nanomolar range [19].

Taking into account the typically poor oligonucleotide cellular uptake that leads to high extracellular aptamer concentration, an additional attractive target protein for anti-HIV G4-aptamers is the surface glycoprotein (gp120), which is crucial for virus entry into the host cells since it mediates fusion of the viral envelope with the target cell membrane by binding to CD4 and a chemokine coreceptor. ISIS 5320 was the first G4-aptamer reported to bind gp120 and inhibit virus adsorption and cell fusion [20]. Afterwards, another group of G-rich ODNs was synthesized: the 6-mer TGGGAG, known as Hotoda's sequence, was identified and proven to bind gp120 [21]. This ODN was active against HIV-1 only if bearing bulky aromatic moieties at the 5'-position: these were necessary to establish hydrophobic interactions between the aptamer and gp120 [22]. The Hotoda's sequence and its derivatives adopt a tetramolecular parallel G4, stabilized by an A-tetrad and four G-tetrads in which all guanines adopt *anti* glycosidic conformations [23]. This G4-aptamer has been extensively investigated and has been used as lead sequence to carry out a number of modifications aimed at finding molecules with improved anti-HIV activity [24]. Most of the investigations have focused on the conjugation of the 3'- and 5'-ends with several types of bulky lipophilic moieties [25–27] or mono- and disaccharides [28], affording in some cases a general improvement of the aptamer properties. Enhancement of the biological activity has been also obtained by modifying the residues of the parent sequence [29–31]. However, the really significant and innovative modifications have been reached by modifying the Hotoda's aptamer molecularity from tetramolecular to bi- and monomolecular, to circumvent the poor aggregation rate of four strands to form the tetramolecular G4 structure. Bimolecular Hotoda's aptamer derivatives have been synthesized by joining the 3'- or 5'-ends of two TGGGAG 6-mers through a hexaethylenglycol loop [32], while the preparation of a family of monomolecular Hotoda's aptamer derivatives has been performed by chemically connecting the 3'- or 5'-ends of four TGGGAG strands by suitable spacers (tetra-end-linked G4s) [33]. In both cases, the original parallel strand orientation was preserved and a substantial improvement of the biological and physical-chemical properties was obtained [32,33].

Although a comparison of the biological and physical-chemical properties among Hotoda's aptamer derivatives belonging to different families is not always straightforward mainly due to their structural differences and the variety of biological assays used, the emerging general framework suggests that several factors affect the

properties of these aptamers [24]: 1) presence, position, number and nature of the lipophilic moieties; 2) rate of formation and thermal stability of the parallel G4 structure (in turn, affected by its structural topology); 3) resistance in the biological environment.

In this framework, we have prepared and investigated a new category of G4-aptamers based on the Hotoda's sequence, in which four TGGGAGT fragments have been linearly connected through 3'-3' and 5'-5' inversion of polarity sites involving canonical phosphodiester bonds or a glycerol moiety. Our data indicate that most of the derivatives adopt a monomolecular G4 structure preserving the parallel strand orientation. In particular, the aptamer bearing lipophilic groups at the 5'-ends, showed high formation rates and noteworthy anti-HIV activity.

2. RESULTS AND DISCUSSION

2.1. Design and synthesis of the new monomolecular Hotoda's aptamers

The design and synthesis of the proposed ODNs were formulated with the aim both to overcome the flaws of the Hotoda's aptamer (i.e. its tetramolecularity) and, at the same time, to take into account its key features and those of its more promising and recently reported derivatives. First, the parallel strand orientation and the presence of lipophilic groups at the 5'-ends, required to establish hydrophobic interactions with the target protein, are crucial characteristics for the anti-HIV activity. Second, the occurrence of a monomolecular ODN allows the increase of the formation rate and, third, the absence of free 3'-ends improves its resistance in the biological environment. Further important factors are a straightforward synthetic approach and the use of low-cost reagents.

We used two different synthetic strategies for derivatives HT535/HT353 and HT353Gly/HT353LGly, respectively (Table 1). Preparation of HT535 and HT353 was simply carried out on a DNA synthesizer using commercially available 3'-phosphoramidite and 5'-phosphoramidite synthons, that allowed the introduction of 3'-3' and 5'-5' inversion of polarity sites required to afford the parallel strand orientation. The synthetic approach for HT353Gly and HT353LGly [34] exploited the same synthons and in addition a glycerol-based linker that allowed the simultaneous synthesis of the two identical halves of the sequence, thus reducing costs and affording ODNs prone to adopt a parallel monomolecular G4 structures devoid of free 3'-ends and, in the case of HT353LGly, endowed with two thymidines at the 5'-ends bearing lipophilic groups (Fig. 1). As reference ODN, we employed the Hotoda's sequence with four lipophilic groups (**L-Hotoda**) (Fig.1).

2.2 CD analysis and structural insights

G4 folding of the newly synthesized Hotoda's aptamers was assessed by circular dichroism (CD), a key tool to ascertain the presence and type of G4 folding in solution, as G4s show distinctive CD spectra [35]. CD profiles of the investigated ODNs are shown in Fig. 2. **HT353Gly** and **HT353LGly** displayed CD spectra very similar to that of the control **L-Hotoda**, characterized by a negative band around 240 nm and a positive band around 265 nm, typical CD signature of parallel structures in which all 2'-deoxyguanosines adopt *anti* glycosidic conformations. On the other hand, the CD profiles of **HT535** and **HT353** were characterized by two positive bands (at 255 and 295 nm for **HT535**, and at 264 and 295 nm for **HT353**) that were not straightforwardly ascribable to a specific G4 topology [35]. However, considering that the CD signal depends on the type of G-tetrad stacking [36], we noted that this type of CD profiles were similar to those of tetramolecular G4s formed by two modified Hotoda's sequences containing an 8-methyl-2'-deoxyguanosine, the NMR of which indicated

the presence of an all-*syn* G-tetrad at the 5'-end [23]. In addition, monomolecular G4s (namely, mTG₃T and mTG₄T) that share with **HT535** both the sequence and type of inversion of polarity sites in the loops, present all-*syn* G-tetrads at their 5'-end [37]. This evidence suggests that **HT535** also presents this feature. On the other hand, the CD spectrum of **HT353** showed an evident band at 264 nm, that is consistent with the presence of a parallel species with all G-residues in *anti* glycosidic conformation, coexisting with the other one characterized by an all-*syn* G-tetrad at the 5'-end, similarly to **HT535** (Fig. 1).

CD thermal unfolding analysis was next employed to evaluate the thermal stability of the modified ODNs (Fig. S1). In the case of **L-Hotoda**, **HT353Gly** and **HT353LGly**, well-defined sigmoidal CD melting profiles allowed determination of melting temperatures (T_m) (Table 1). **HT353LGly**, bearing two *tert*-butyldiphenylsilyl groups, is 10°C more stable than **HT353Gly**, which lacks the lipophilic groups; **L-Hotoda**, which possesses four *tert*-butyldiphenylsilyl groups, has the highest T_m (82.5 ± 0.6 °C). These data indicate that the lipophilic groups drive the structural stability of this series of G4-aptamers. The non-sigmoidal heating profile of **HT353** prevented the obtainment of a reliable T_m , thus reinforcing the hypothesis of the presence of more than one conformation, as already suggested by the CD profile. In the case of **HT535**, the G4 signature in the CD spectrum was still evident up to 90°C, indicating a partial melting of the G4, and thus inferring high thermal stability. Although **HT535** is devoid of lipophilic groups, it is the only Hotoda's derivative bearing two 5'-5' inversion of polarity sites that, with the two long spacers connecting the G-runs, likely facilitate strand association and thus endow the molecule with the observed noteworthy stability. These results are in agreement with data reported for G4 structures sharing this type of topology [37], characterized by a parallel strand orientation, and two and one side loops with 5'-5' and 3'-3' inversions of polarity, respectively.

Next, the kinetics of formation of the investigated G4 structures were evaluated by monitoring CD ellipticity at the maximum Cotton effect wavelengths as a function of time after denaturing the sample by heating it at 95 °C for 5 min and rapidly cooling it down. The ellipticity vs time profile at 15°C of **HT353LGly** is shown in Fig. 3. Similar profiles were obtained for the other Hotoda's derivatives (Fig. S2). For all the synthesized G4s, far higher rates of formation were observed compared to **L-Hotoda** [38], in agreement with the reported folding of monomolecular and tetramolecular Hotoda's aptamer derivatives [39]. Taking into account the structural results obtained for similar ODNs [23,37], the whole data are consistent with the G4 structures proposed in Fig. 1.

2.3 Anti-HIV-1 activity

The Hotoda's aptamer had been shown to display strong anti-HIV-1 activity due to its ability in preventing virus entry into the host cell by blocking the binding of the viral glycoprotein gp120 to the cellular receptor CD4 [24]. To evaluate whether the modified ODNs improved or maintained such anti-HIV-1 effect, TZM-bl cells were infected with HIV-1 pNL4.3 strain and treated with increasing amounts of ODNs. The antiviral activity was measured 48 h post infection. **HT353LGly** displayed a potent antiviral effect with IC_{50} value in the low nanomolar range, similar to that of **L-Hotoda**. To note that the antiviral activity of **HT353LGly** was higher than that of **L-Hotoda** when considering the ODN concentration of the latter, while it was about half when considering the concentration of the G4 formed by the four ODN strands in **L-Hotoda**. Both **HT353LGly** and **L-Hotoda** displayed low cytotoxicity and hence very good selectivity indexes (Table 2 and Fig. 4). In contrast, **HT535**, **HT353**, **HT353Gly** did not show antiviral activity (Table 2).

Interestingly, **L-Hotoda** and **HT353LGly** are the only ODNs among the series characterized by the presence of lipophilic groups (Fig.1). Since this is the only difference between the active **HT353LGly** and inactive **HT353Gly**, these data clearly indicate the fundamental role of the lipophilic groups in the anti-HIV-1 activity of this series of ODNs, and show that two lipophilic groups are sufficient for the biological activity.

To assess the main and temporally last viral step targeted by **HT353LGly**, the time of addition assay (TOA) was performed. This time-based approach determines how long the addition of a drug can be postponed before losing antiviral activity in cell culture. In such an assay, the target of the antiviral compound can be identified by comparing its activity in the time scale to that of reference drugs. After infection of TZM-bl cells with HIV-1 pNL4.3, **HT353LGly** and **L-Hotoda** were added at different time points and virus production was assessed 31 h post infection. Dextrane sulfate (DS) was used as entry inhibitor reference compound, while azidothymidine (AZT) and nevirapine (NEV) were used as markers of the reverse transcriptase step. As reported in Fig. 5, **HT353LGly** displayed antiviral activity only when added at the time of infection (0 h post infection), while no effect was observed when added at later times post infection. This behavior was shared with that of the attachment inhibitor DS and **L-Hotoda**. In contrast, the reverse transcriptase inhibitors AZT e NEV maintained antiviral activity when added up to 3 and 4 h after infection, respectively. The remarkable overlapping TOA profiles of **HT353LGly** and **L-Hotoda** are consistent with inhibition of virus entry into the host cell by both aptamers. Since the activity of **L-Hotoda** is mediated by its binding to the HIV-1 gp120 envelope glycoprotein [24], we investigated gp120 interaction with **HT353LGly** measuring steady state binding affinity by surface plasmon resonance (SPR) analysis. Indeed both aptamers were able to interact with gp120, albeit **HT353LGly** ($K_D = 8.2 \pm 1.2 \mu\text{M}$) with lower affinity compared to **L-Hotoda** ($K_D = 1.0 \pm 0.1 \mu\text{M}$) (Fig. S3).

3. CONCLUSIONS

The G4-forming Hotoda's aptamer is the lead compound of a large class of derivatives designed and prepared over the last years with the aim to improve its physical-chemical and biological properties. In general, the antiviral activity of these derivatives depends on three main factors [24]: (i) the intrinsic ability of the G4-structure to interact with the target gp120 protein; (ii) the structural thermal stability and kinetics of formation and (iii) the resistance to nucleases. The implemented modifications of the Hotoda's aptamer have mainly concerned three structural features [24]: the end-linked lipophilic groups, the ODN sequence and, more recently, the G4 topology. Although the biological and physical-chemical properties are affected by a complex interplay among the different structural features, the general overview emerging from the available data is that the presence of lipophilic groups at the 5'-ends and the parallel strand orientation are two crucial characteristics for the antiviral activity. In the original Hotoda's aptamer, the parallel orientation of the strands is achieved through a tetramolecular G4-complex [21] (Fig. 1), which is characterized by a relatively slow formation rate. In recent Hotoda's aptamer derivatives, the parallel strand orientation has been obtained either through formation of bimolecular G4 complexes formed by hairpin ODNs or by joining the 3'-ends of four ODNs by means of a suitable linker [32]. In this paper, we investigated the properties of a new class of Hotoda's aptamer derivatives in which four ODNs are linearly joined through their 3'- or 5'-ends. CD data indicated formation of parallel G4 structures characterized by three loops (Fig. 1). All derivatives displayed outstanding formation rates, typical of the monomolecular G4-structures. One derivative, namely **HT353LGly**, inhibited virus entry into the host cell, similarly to the parent Hotoda's aptamer (**L-Hotoda**), confirming that the lipophilic groups are fundamental for the antiviral activity, but also indicating that two of these groups are sufficient to obtain activity

comparable to that of the **L-Hotoda**. The Hotoda's derivatives we have described can be prepared through a synthetic approach based on standard methods that affords to obtain parallel monomolecular G4-structures characterized by high formation rates and, in the case of **HT353LGly**, endowed with two lipophilic groups at the 5'-ends but devoid of free 3'-ends. Our synthetic approach can be adapted to prepare Hotoda's aptamer derivatives with additional lipophilic groups.

Although the HIV envelope glycoprotein gp120 is the proven target of the Hotoda's aptamer and its derivatives, detailed information on the aptamer/target-protein interaction is still rather limited. In particular, the role played by the lipophilic groups is still far to be clarified. A molecular modeling study on the interaction between a tetra-end-linked (TEL) version of the Hotoda's aptamer and gp120 highlights the importance of electrostatic interactions between the conserved residue R190 in the protein hypervariable V3 loop and one of the G4 grooves [33]. In our Hotoda's aptamer derivatives, the presence of side-loops at the 3'- and 5'-ends does not prevent G4 groove/V3 loop interaction, consistently with the data available so far. However, recent investigations suggest higher complexity in G4-protein interactions [40,41], the full disclosure of which will help future aptamer design.

In conclusions, we have presented synthetically simplified monomolecular Hotoda's derivatives that maintained the original parallel conformation and half of the original lipophilic groups and preserved a remarkable antiviral activity. Our data will help the design of aptamers with even further improvements.

4. EXPERIMENTAL SECTION

4.1 Oligodeoxyribonucleotides synthesis and preparation

The oligodeoxyribonucleotides in Table 1 were synthesized on an ABI 394 DNA synthesizer using solid phase β -cyanoethyl phosphoramidite chemistry at 10 μ mol scale, adopting the DMT-on protocol, for oligomers **HT353LGly** and **L-Hotoda**, and the DMT-off protocol, for all other oligomers. The 5'-modified thymidine phosphoramidite, namely 5'-O-tert-butylidiphenylsilylthymidine-3'-O-(2-cyanoethyl)-N,N-diisopropylphosphoramidite, was synthesized following the reactions described in D'Onofrio et al. [38]. The synthesis of the 3'-5' tracts were performed by using normal 3'-phosphoramidites, whereas the 5'-3' tracts were synthesized by using 5'-phosphoramidites. For all ODNs an universal support was used. For the synthesis of modified oligonucleotides containing the glycerol moiety, a non-nucleosidic phosphoramidite, synthesized as previously reported by other authors [34,42], was coupled to the universal solid support before using 5'-phosphoramidites. The oligomers were detached from the support and deprotected by treatment with concentrated aqueous ammonia at 80 °C overnight. The combined filtrates and washings were concentrated under reduced pressure, redissolved in H₂O, analyzed and purified by high-performance liquid chromatography on a Nucleogel SAX column (Macherey–Nagel, 1000-8/46, Düren-Germany), using buffer A: 20 mM NaH₂PO₄/Na₂HPO₄ aqueous solution (pH 7.0) containing 20% (v/v) CH₃CN and buffer B: 1 M NaCl, 20 mM NaH₂PO₄/Na₂HPO₄ aqueous solution (pH 7.0) containing 20% (v/v) CH₃CN; a linear gradient from 0 to 100% B for 45 min and flow rate 1 mL/min were used. The fractions of the oligomers were collected and successively desalted by Sep-pak cartridges (C-18). The isolated oligomers proved to be > 98% pure by HPLC. ODNs were resuspended in TE buffer (Tris 10 mM, EDTA 1 mM) to final stock concentration (1 mM) and stored at -20 °C.

4.2 Circular dichroism spectroscopy

For CD analysis, ODNs (Fig. 1) were diluted from stock to final concentration (8 μM) in potassium phosphate buffer (20 mM, pH 7.4) and KCl 80 mM. All samples were annealed by heating at 95 °C for 5 min and were left to cool down to room temperature overnight. CD spectra were recorded on a Chirascan-Plus spectropolarimeter (Applied Photophysics, Leatherhead, United Kingdom) equipped with a Peltier temperature controller, using a quartz cell of 5 mm optical path length, over a wavelength range of 230-320 nm. The reported spectrum of each sample represents the average of 2 scans at 20 °C and it is baseline corrected for signal contributions due to the buffer. Observed ellipticities were converted to mean residue ellipticity (θ) = $\text{deg}\times\text{cm}^2\times\text{dmol}^{-1}$ (molar ellipticity), where dmol^{-1} refers to the moles of G4 (in the case of **L-Hotoda** four molecules of ODN correspond to 1 molecule of G4). For the determination of T_m , spectra were recorded over a temperature range of 20-90 °C, with temperature increase steps of 5 °C. T_m values were calculated according to the van't Hoff equation, applied for a two-state transition from folded to unfolded state, assuming that the heat capacity of the folded and unfolded states are equal [43].

4.3 Isothermal Association Kinetics

The association of unfolded single strand into a well-defined G4 structure was monitored for each sequence in a buffer containing potassium phosphate 10 mM and KCl 70 mM, at pH 7.0. The experiments were carried out at 20 μM strand concentration. The samples were incubated at 95°C for 5 min to allow for dissociation. The structural transition from the unfolded strand to the monomolecular G4 was monitored at constant temperature (15 °C) by recording the change of the CD signal for all G4s at their maximum Cotton effect wavelengths as a function of time.

4.4 Surface Plasmon Resonance analysis

SPR experiments were performed on a Biacore™ T100 platform (GE Healthcare Europe, Milan, Italy). The HIV-1IIIB recombinant gp120 (ImmunoDiagnostics Inc., Woburn, MA) was immobilised on a Series S Sensor Chip CM5 (GE Healthcare Europe) by amine coupling in HEPES-NaCl running buffer (HEPES pH 7.4 10 mM, NaCl 150 mM, EDTA 3 mM). Protein gp120 was diluted in sodium acetate buffer at 20 ng/ μL and injected to reach about 6000 resonance units (RU). Binding analysis was performed at 20 $\mu\text{L}/\text{min}$ with contact and dissociation times of 120 s in HEPES-KCl buffer (HEPES pH 7.4 10 mM, KCl 200 mM, EDTA 3 mM). Aptamers HT353LGly and L-Hotoda were diluted to 40 μM in HEPES-KCl buffer, denatured at 95 °C for 5 min and slowly cooled at room temperature. Sensorgrams for HT353LGly and L-Hotoda were obtained in the concentration range of 250–16000 nM and 62.5–4000 nM, respectively. The chip surface was regenerated with 50 mM NaOH solution. All sensorgrams were corrected by subtraction of blank flow cell response and buffer injection response. The steady state affinity was evaluated by measuring RU response 5 sec before end of the association phase using BIAevaluation software (GE Healthcare).

4.5 Cell culture

TZM-bl cell line was provided by NIH AIDS Research Program. TZM-bl is a HeLa cell line stably expressing large amounts of CD4 and CCR5 and containing integrated copies of the reporter genes for firefly luciferase and E. coli β -galactosidase under control of the HIV-1 promoter. TZM-bl were grown in Dulbecco's modified Eagle Medium (DMEM) (Gibco, Life Technologies, Monza, Italy) supplemented with 10% heat-inactivated fetal

bovine serum (FBS) (Gibco, Life Technologies, Monza, Italy) and maintained as a monolayer in the logarithmic growth phase at 37 °C in a 5% CO₂-controlled humidified atmosphere.

4.6 Virus stock production

The HIV-1 strain NL4-3 viral stock was prepared by transfection of HEK 293T cells with wild-type X4 proviral genome (NIH AIDS Research and Reference Reagent Program), using CalPhos mammalian transfection kit (Clontech Laboratories, Santa Clara, CA, USA) according to the manufacturer's protocol. Viral particles in supernatants were collected and titrated by Reed and Muench method [44].

4.7 Antiviral assay in HIV-1 infected TZM-bl cells

HIV-1 infectivity was measured using the TZM-bl cells, in which the HIV infection drives transcription of the HIV-1 LTR-Luciferase reporter gene construct and thus it is possible to evaluate the antiviral effect of test compounds following the luciferase expression. For antiviral assay, ODNs were diluted from stock to final concentration (100 μM) in potassium phosphate buffer (20 mM, pH 7.4) and KCl 80 mM. Samples are annealed by heating at 95 °C for 5 min and gradually cooled to room temperature overnight. Cells were seeded in 96-well plates (10.000 cells/well) and grown overnight to permit adherence prior to treatment and viral infection. Cells were next infected with HIV-1 NL4.3 strain (MOI 0.5), treated with serial dilutions of ODNs (2-0.0625 μM) and incubated at 37 °C. After 48 h HIV-1 production was assessed following the LTR-luciferase signal using the briteliteTM plus Reporter Gene Assay System (Perkin Elmer, Waltman, Massachusetts, USA) according to the manufacturer's protocol. Cytotoxicity of tested ODNs was investigated in parallel to antiviral assays by MTT (Sigma-Aldrich, Milan, Italy). Briefly, 10 μL of freshly dissolved solution of MTT (5 mg/mL in phosphate buffered saline (PBS)) were added to each well, and after 4 h of incubation, MTT crystals were solubilized in solubilization solution (10% sodium dodecyl sulphate (SDS) and 0.01 M HCl). After overnight incubation at 37 °C, absorbance was read at 540 nm. Absorbance data of formazan are proportional to the number of viable cells. The percentage of cell survival was calculated as follows: $\text{cell survival} = (\text{A}_{\text{well}} - \text{A}_{\text{blank}}) / (\text{A}_{\text{control}} - \text{A}_{\text{blank}}) \times 100$, where blank denotes the medium without cells. Each concentration of ODN was tested in triplicated in at least two independent experiments.

The 50% cytotoxic concentration (CC₅₀) was defined as the concentration of the ODN that was able to reduce the absorbance of the mock-infected cells by 50%. The 50% inhibitory concentration (IC₅₀) was defined as the concentration of the ODN that inhibit 50% of LTR-Luciferase signal. The selectivity index (SI) is the relative effectiveness of the tested ODN in inhibiting viral replication compared to inducing cell death (CC₅₀ value/IC₅₀ value).

4.8 Time of addition assay

For TOA, dextran sulfate (DS), nevirapine (NVP) and Zidovudine (AZT) (Sigma-Aldrich, Milan, Italy) were used as reference drugs and handled according to manufacturer's protocols. ODNs were diluted from stock to final concentration (500 μM) in potassium phosphate buffer (20 mM, pH 7.4) and KCl 80 mM. Samples are annealed by heating at 95 °C for 5 min and gradually cooled to room temperature overnight. TZM-bl cells were seeded in 96-well plates (10.000 cells/well) and grown overnight to permit adherence. Cells were next infected with HIV-1 NL4.3 strain at MOI 0.5. After 1 h, cells were washed twice with phosphate buffered saline (PBS) (Gibco, Life Technologies, Monza, Italy), and incubated at 37 °C. ODNs and the reference compounds DS,

AZT and NVP were added at different time points p.i. ODNs were added at 10 μ M and the reference compounds were added at a concentration corresponding to 100-fold their 50% inhibitory concentration (IC_{50}). HIV-1 production was determined 31 h p.i. following the LTR-luciferase signal using the briteliteTM plus Reporter Gene Assay System (Perkin Elmer, Waltman, Massachusetts, USA) according to the manufacturer's protocol.

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Table 1. Sequence and melting temperature (T_m) of the investigated ODNs.

ODN	Sequence	T_m (°C) \pm s.d.
L-Hotoda	L-5'-TGGGAG-3'	82.5 \pm 0.6
HT535	3'-(TGAG ₃ T)-5'-5'-(TG ₃ AGT)-3'-3'-(TGAG ₃ T)-5'-5'-(TG ₃ AGT)-3'	> 90
HT353	5'-(TG ₃ AGT)-3'-3'-(TGAG ₃ T)-5'-5'-(TG ₃ AGT)-3'-3'-(TGAG ₃ T)-5'	N.D.
HT353Gly	5'-(TG ₃ AGT)-3'-3'-(TGAG ₃ T)-5'-Gly-5'-(TG ₃ AGT)-3'-3'-(TGAG ₃ T)-5'	66.0 \pm 0.7
HT353LGly	L-5'-(TG ₃ AGT)-3'-3'-(TGAG ₃ T)-5'-Gly-5'-(TG ₃ AGT)-3'-3'-(TGAG ₃ T)-5'-L	76.0 \pm 0.9

L = *tert*-butyldiphenylsilyl moiety; Gly = glycerol moiety; N.D. = not determined. See Fig. 1 and experimental section for details.

Table 2. Antiviral Effects of the ODNs against HIV-1 NL4.3 strain in TZM-bl cells.

ODN	CC₅₀ (μM)	IC₅₀ (μM)	SI
L-Hotoda	> 2.00	0.14 ± 0.03 / 0.04 ± 0.01	> 14.3 / > 50
HT535	> 2.00	> 2.00	/
HT353	> 2.00	1.07 ± 0.31	> 1.9
HT353Gly	> 2.00	> 2.00	/
HT353LGly	> 2.00	0.08 ± 0.02	> 25

IC₅₀ is the ODN concentration required to inhibit 50% of HIV-1 production; CC₅₀ is the ODN concentration at which 50% of cell toxicity is observed; SI is the selectivity index. For **L-Hotoda**, the IC₅₀ and SI values obtained considering the ODN or the G4 (four molecules of ODN) concentration are reported.

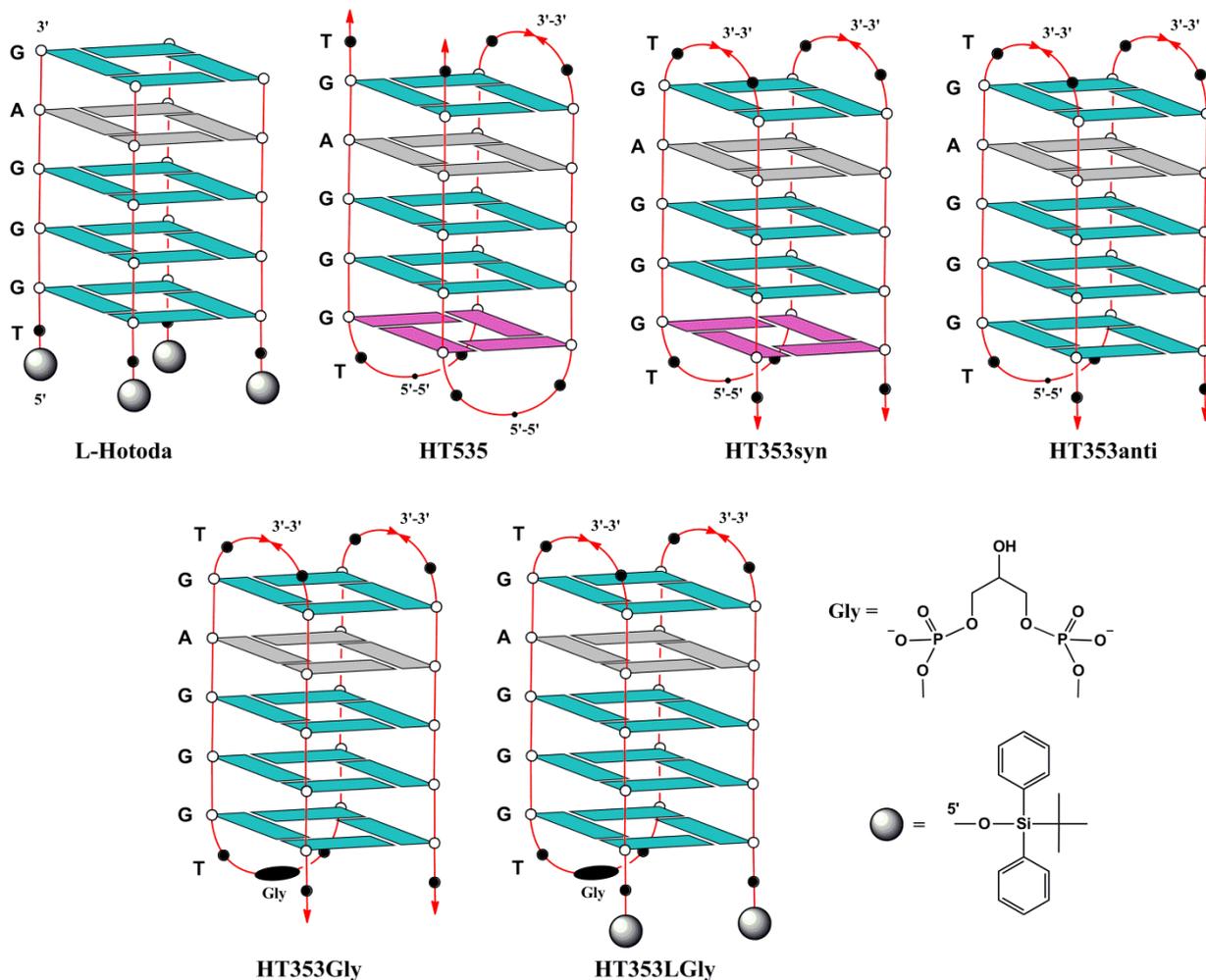


Fig. 1. Schematic representation of the G4 structures of the Hotoda's aptamer (**L-Hotoda**) and its monomolecular derivatives proposed in this study (Table 1). Guanosines in *syn* and *anti* glycosidic conformations are in pink and light blue, respectively, while adenosines are in grey. Thymidines are represented by black circles. See main text for details.

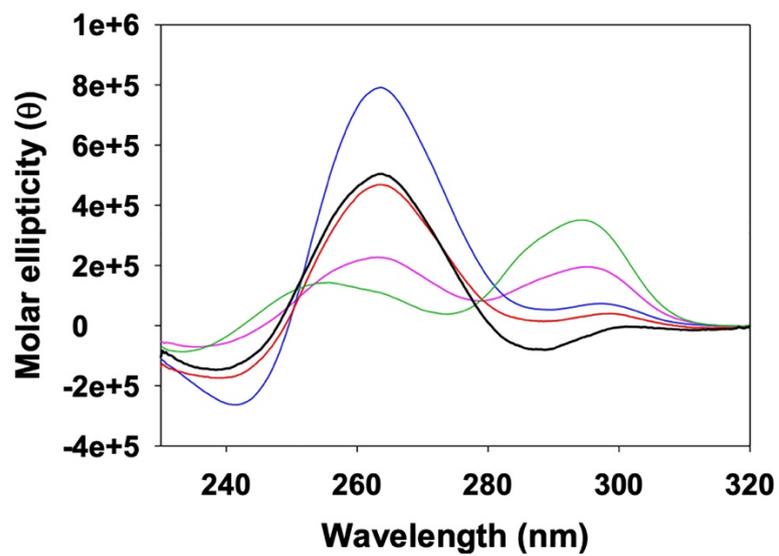


Fig. 2. Representative CD spectra of **L-Hotoda** (black), **HT535** (green), **HT353** (magenta), **HT353Gly** (blue) and **HT353LGly** (red) in potassium phosphate buffer (20 mM, pH 7.4) and KCl 80 mM.

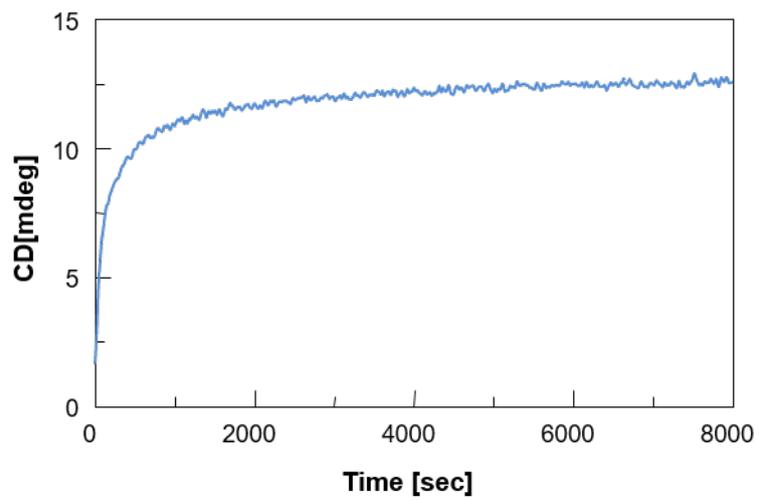


Fig. 3. Kinetics of G4 formation for **HT353LGly** at 15 °C in potassium phosphate buffer (10 mM pH 7.0) supplemented with 70 mM KCl (ODN concentration: 20 μ M). G4 formation leads to an increase of molar ellipticity at 263 nm.

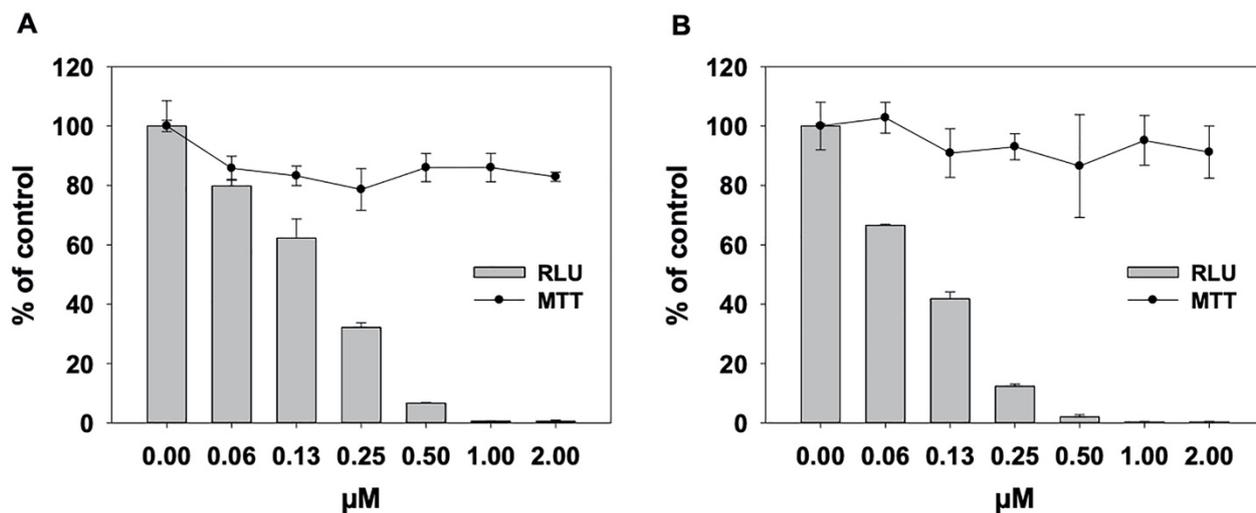


Fig. 4. TZM-bl cells were infected with HIV-1 NL4-3 strain and treated with increasing concentrations (0.06-2.00 μM) of **L-Hotoda (A)** or **HT353LGly (B)**. Uninfected cells were treated in the same conditions. Antiviral activity, expressed as relative luciferase units (RLU), was measured on infected cells, while cell viability was obtained in parallel by MTT assay on the uninfected cells.

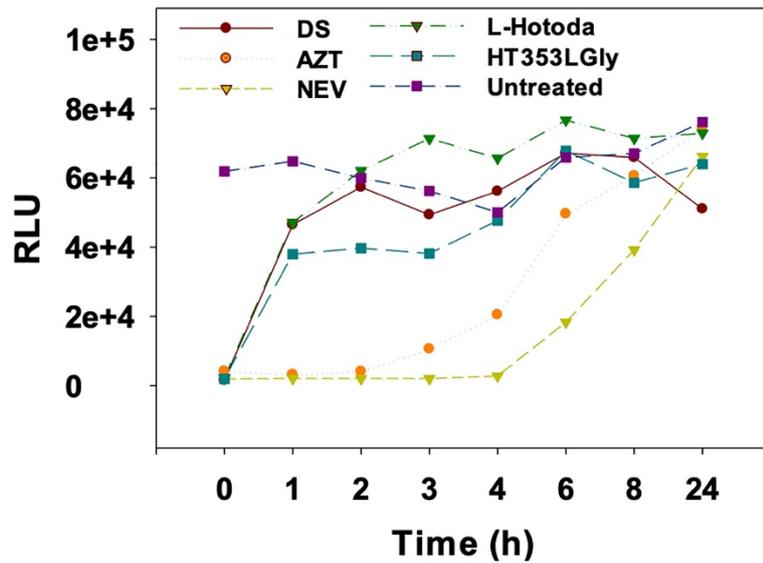


Fig. 5. Time of addition assay. TZM-bl cells were infected with HIV-1 NL4-3 strain and test ODNs or reference compounds were added at different time points after infection. HIV-1 production was determined 31 h p.i. HIV-1 following the LTR-luciferase signal. Uninfected cells were treated in the same conditions. The antiviral activity of test ODNs was compared with that of the negative control and with reference drugs.

Declaration of competing interest

The authors declare no conflict of interest.

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Abbreviations used

AZT, zidovudine; DS, dextran sulfate; G4, G-quadruplex; NVP, nevirapine; ODN, Oligodeoxyribonucleotide; TOA, time of addition.