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New findings on the d(TGGGAG) sequence: surprising anti-HIV-1 activity.

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Highlights

- Anti-HIV screening of d(TGGGAG) sequences with different G-quadruplex concentrations.
- Contrary to what reported, natural sequence (IC_{50}=14 nM) showed the highest activity.
- Investigation of the site/target of intervention by d(TGGGAG) ODNs.

Abstract

The biological relevance of tetramolecular G-quadruplexes especially as anti-HIV agents has been extensively reported in the literature over the last years. In the light of our recent results regarding the slow G-quadruplex folding kinetics of ODNs based on d(TGGGAG) sequence, here, we report a systematic anti-HIV screening to investigate the impact of the G-quadruplex folding on their anti-HIV activity. In particular, varying the single stranded concentrations of ODNs, it has been tested a pool of ODN sample solutions with different G-quadruplex concentrations. The anti-HIV assays have been designed favouring the limited kinetics involved in the tetramolecular G4-association based on the d(TGGGAG) sequence. Aiming to determine the stoichiometry of G-quadruplex structures in the same experimental conditions of the anti-HIV assays, a native gel electrophoresis was performed. The gel confirmed the G-quadruplex formation for almost all sample solutions while showing the formation of high order G4 structures for the more concentrated ODNs solutions. The most significant result is the discovery of a potent anti-HIV activity of the G-quadruplex formed by the natural d(TGGGAG)

sequence (IC_{50} = 14 nM) that, until now, has been reported to be completely inactive against HIV infection.

Graphical abstract

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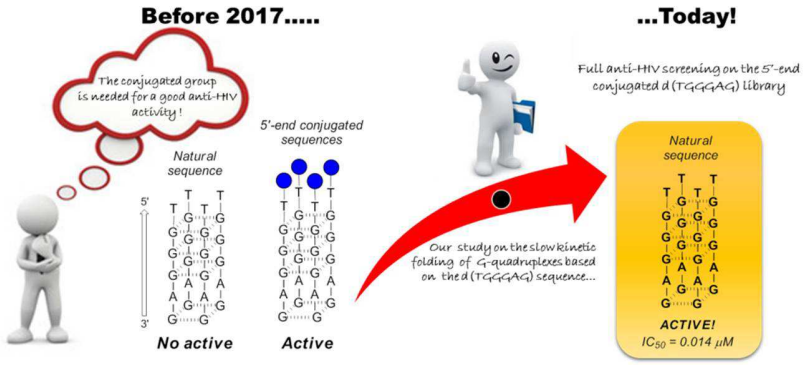


Keywords: anti-HIV-1, tetramolecular G- quadruplexes, modified oligonucleotides, aptamers.

1. Introduction

The discovery, in the '90s, of G-rich DNA sequences forming G-quadruplex structures irreversibly changed the way of thinking with respect to DNA encoded genetic information. Since then, scientific research has increasingly focused its attention at the therapeutic potential of oligonucleotides (ODNs) able to fold in G-quadruplex structures (G4s) [1]. G-quadruplexes are one of the most attractive "unusual" DNA classes formed by G-rich DNA sequences in the presence of different cations (K^+, Na^+; $NH4^+$). This class of DNA is characterized by an extensive conformational polymorphism that amplifies its biological potentialities [2-4]. The peculiar arrangements of G-quadruplexes have been reported to be crucial for aptamer recognition. In fact, thanks to the elevated specificity and selectivity of G4 structures, many G-quadruplex aptamers have been developed in the last few years. In this regard, a number of synthetic oligonucleotides forming G-quadruplexes have been developed as antiviral aptamers [5]. G-quadruplex-based aptamers have been successfully de-signed to target HIV at different stages of its life cycle [6]. The potential targets of these G-quadruplexes are the surface glycoprotein gp120 [7], the HIV-1 reverse transcrip-tase [8], the RNase H [9] and integrase (IN) [10]. The first G-quadruplex IN inhibitor aptamer tested in clinical trials (Zintevir™ developed by Aronex Pharmaceuticals in 1996) was T30177 (Zintevir). This aptamer is formed by a dimeric structure in which two subunits of propeller-type parallel-stranded G-quadruplexes are involved [11]. The inhibitory effect of Zintevir was initially attributed to its IN inhibition based on enzymatic assays but was later found to be due to the inhibition of HIV entry, targeting the viral glycoprotein gp120 [12]. Analogously to Zintevir, other G-quadruplex aptamers as 93del and T30923, showed multimodal inhibition of HIV [13,14]. In particular, the 93del presented a similar G-quadruplex arrangement to that of Zintevir, consisting of two G4 subunits strongly interlocked in a dimer parallel structure [15], which is stable even at temperatures over 90 °C.

In the '90s, two aptamers forming tetramolecular G quadruplex structures, ISIS-5320 [16] and the Hotoda's sequence d(TGGGAG) [17,18], were reported to target gp120. Recently, several short G-quadruplexes have been evaluated as potential anti-HIV drug candidates due to their binding with proteins related to HIV-1 infection. The first active modified d(TGGGAG) sequence was reported by Hotoda and contained a 4,4′-dimethoxytrityl (DMT) group at the 5′-end of the 6-mer. Through an extensive screening of modified d(TGGGAG) sequences, the R-95288 sequence was discovered to be the most potent inhibitor of HIV infection [19,20]. A SAR study conducted on this family of oligonucleotides has revealed that the anti-HIV-1 activity of 5'-end modified sequences relies on both, the conjugation with an aromatic group at the 5'-end and the folding in a G-quadruplex structure. As such, the biological activity of 5'-end modified d(TGGGAG) is related to the interaction of the G-

quadruplex structure with the positively charged V3 loop of the gp120 [17]. Since the structural stability of G-quadruplex-forming aptamers is closely related to their biological activities, a complete biophysical and structural investigation of Hotoda's modified sequences was provided by us in 2007 [21]. The overall biophysical and biological data have effectively suggested a correlation between the high thermal stability of 5'-modified G-quadruplexes and their biological role within the cell.

Inspired by these findings and aiming to expand the repertoire of potential end-modified d(TGGGAG) aptamers, we recently synthesized and biophysically characterized a variety of d(TGGGAG) ODN 5'-end conjugated with hydrophobic moieties via a phosphodiester bond [22-24]. All 5'-end modified sequences formed parallel tetramolecular G-quadruplexes with strongly increased thermal stability as confirmed by CD analysis. Particularly, it was reported that the major stability of the investigated G4 complexes is not directly correlated with their pronounced anti-HIV activity (nM range).

In the light of the acquired knowledge about the characteristics of these G4 structures, we have recently investigated the effect of the nature of modifications at 5′-end of the d(TGGGAG) sequence on kinetics of G-quadruplex folding. The kinetic study on the G4 folding was carried out on the most active 5'-end modified d(TGGGAG) by ESI-MS technique [25]. Very slow kinetics were observed for all G-quadruplexes along with a strong effect of ODN concentrations on the G4 folding kinetics. ESI-MS data suggested that the conjugation of d(TGGGAG) at the 5'-end does not necessarily increase the folding rate of the G-quadruplex structure. Unexpectedly, higher order G4 structures were observed by ESI-MS and unambiguously identified as octameric complexes. The formation of supramolecular complexes for the d(TGGGAG) sequence had been previously hypothesized by Virgilio [26] and V. Filichev and co-workers [27]. In the latter, the conjugation of the 5'-O-DMT-group in 5'-end modified sequences was reported to increase the thermal stability and facilitate the aggregation (formation of multimeric complexes) of G4s. Interestingly, they concluded that the anti-HIV activity seems to be correlated with the formation of higher order G-quadruplex structures.

To question whether G4 structures are implicated in the anti-HIV activity, in the present work, we have performed an extensive anti-HIV screening on some 5'-end modified d(TGGGAG) sequences in comparison with the unmodified sequence. For this reason, all experiments have been designed at different G4 concentrations and favouring the limited kinetic factor involved in the tetramolecular G4-association [28].

2. Results and discussion

Hence, in an attempt to characterize the site/target of intervention by ODNs (**A**-**F**), we tested the d(TGGGAG) sequences (**A**-**F**) against a variety of HIV-1 strains: the HIV-1 IIIB and NL4.3 WT-viruses and some resistant NL4.3 strains (AR177 and DS8000 resistant NL4.3 strains). The ODNs (**A**-**F**, Figure 1) stock solutions were prepared starting from different single stranded concentrations (ss, 0.3 mM, 0.6 mM, 1.2 mM) and tested 14 days after the addition of the G4 cation (100 mM KCl) in order to promote the G-quadruplex folding.

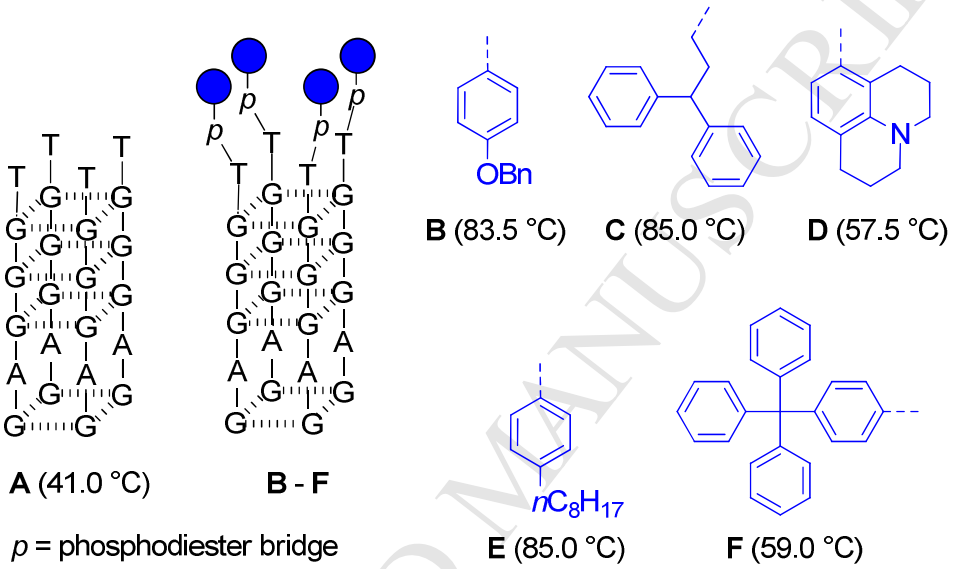


Figure 1. d(TGGGAG) ODNs carrying hydrophobic groups at the 5'-end; all 5'-end modified ODNs formed G-quadruplexes with higher thermal stability (Tm) that the unmodified sequence **A**.

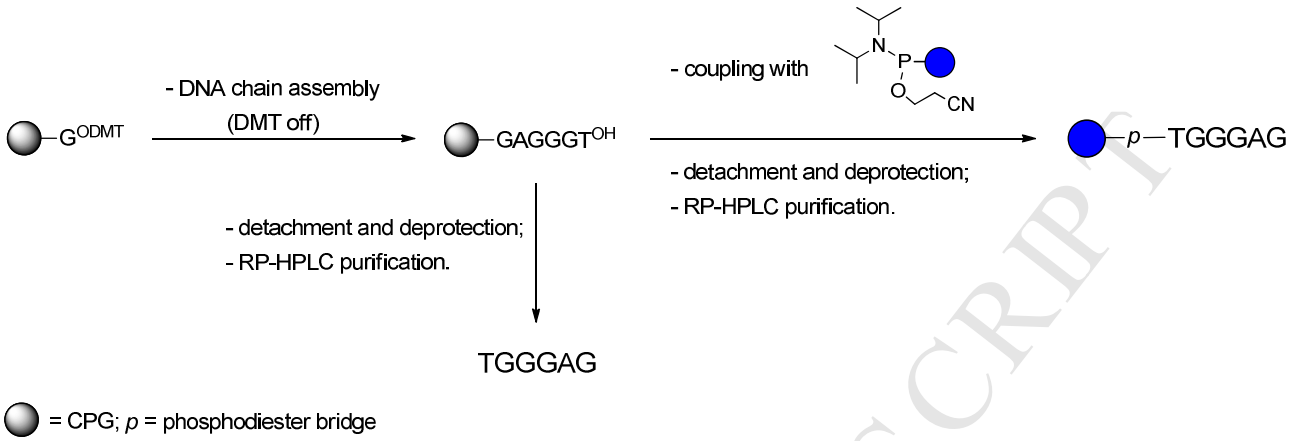
In addition to the anti-HIV assays, a native gel electrophoresis (PAGE) experiments under the same experimental conditions used for the HIV tests were conducted attempting to determine the stoichiometry of all structures formed.

2.1 Chemistry

As a part of our continuing research effort on the synthesis of modified ODNs by exploiting phosphoramidite chemistry [30], here, we reported an efficient synthesis of 5'-end modified d(TGGGAG) sequences carrying at 5'-end different aromatic groups linked by phosphodiester bond.

ODN tracts were synthesised using standard solid-phase β-cyanoethyl phosphoramidite chemistry protocols on an automated DNA synthesizer, starting from a commercially available, DMT-protected, nucleoside-functionalised CPG supports. A CPG solid support that anchors 3′-O-(4,4′-dimethoxytrityl)-guanosine (Scheme 1) was used to synthesize the first DNA tract (synthesis by 5'→3' direction), leading to the d(TGGGAG) sequence **A**.

The insertion in the 5'-end of the aromatic groups was carried out using their phosphoramidites. Spectroscopic measurements monitored DMT cation formation, indicating chain assembly for each coupling cycle.



Scheme 1. Synthetic scheme of the ODNs (**A**-**F**) by automated DNA synthesis using the phosphoramidite chemistry.

Compound yields of at least 98% were obtained. Target oligomers **A** – **F** were detached from the solid support, deprotected and the crude materials were then analysed and purified using HPLC and gel filtration chromatography. The ODNs were characterized by MALDI-TOF MS, in all cases giving masses in accordance with their expected values [22].

2.2 Anti-HIV-1 screening on the d(TGGGAG) ODNs depending on the G4 folding process

The anti-HIV-1 screening was performed testing the d(TGGGAG) sequences (**A**-**F**) at three different G4 concentrations against the HIV-1 IIIB and NL4.3 WT-viruses and also against AR177 and DS8000 resistant NL4.3 strains. NL4.3 strains resistant to AR177 and DS8000 have been chosen because are both entry inhibitors. In particular, AR177 is a known entry inhibitor (target gp120) with structural (parallel G4) and biological similarities with our oligonucleotides and DS8000 presents the same polyanionic character. The mechanism of action of Zintevir-resistant NL4.3 strain was reported by Esté et al. in 1998 and revealed that the resistant phenotype was associated to the emergence of mutations in the gp120 [12]. Overall anti-HIV data are reported in Table 1. All ODN stock solutions were appropriately diluted before the assay. Analyzing the anti-HIV results, some compounds (**D** and **E**) prove inactive, others (**B**, **C** and **F**) are very potent against different HIV-1 strains. The ODN **B** shows a strong anti-HIV-1 activity at low stock solution concentrations (ss, 0.6 mM and 0.3 mM), in particular against the IIIB and NL4.3 WT strains. Similarly, the ODN **C** displays a pronounced anti-HIV activity at the same concentrations as **B** (0.6 mM and 0.3 mM) showing also a strong selectivity for WT viruses.

Regarding this oligonucleotide **C**, a loss of activity was observed when tested against NL4.3/AR177 strain.

Difflerently, the ODN **F** exhibits a good anti-HIV activity at 1.2 mM and 0.6 mM *ss* concentrations and mainly against WT viruses. Overall HIV data reported that the modified ODNs (**B**, **C** and **F**) are no longer able to inhibit entry or replication of the NL4.3/AR177 resistant strain. Therefore, it seems that the cross resistance observed with some 5'-end modified ODNs (**B**, **C** and **F**) points to a similar mode of action as reported for AR177 (Zintevir) [12].

A very surprising finding is the strong anti-HIV activity of the natural sequence **A** that was proved to be the most active ones. This result is in contrast to what has been extensively reported over the years regarding the inactivity of the natural d(TGGGAG) sequence. Interestingly, the unmodified d(TGGGAG) sequence **A** showed the highest anti-HIV-1 activity against all virus strains without any discrimination for WT and/or resistant strain viruses. This latter feature of the d(TGGGAG) sequence becomes interest concerning its mechanism of action as anti-HIV agent, implying a target for **A** different from that of the 5'-end conjugated ODNs. Unexpectedly the natural sequence showed a very significant anti-HIV-1 activity at lower G4 concentration (0.3 mM stock solution). Moreover the results about all sequences (**A**-**F**) tested suggest that does not exist a linear correlation between the available G4 amount and the anti-HIV activity of the **A**-**F** sequences.

Table 1 "Tetrazolium-based colorimetric assay" for the detection of HIV replication inhibitors based on the assessment of viability of cells and the protection against virus-induced cytopathogenic effect in MT-4 cells.[29]

Compound (mM)[a]	Strain	IC_{50}(μM)[b]	CC_{50}(μM)[§]	Compound (mM)[a]	Strain	IC_{50}(μM)[b]	CC_{50}(μM)[b]
A (1.2)	III_B	>5.00	>5.00	**C** (0.6)	III_B	0.061	>5.00
A (0.6)	III_B	0.068	>5.00		NL4.3/WT	1.54	>5.00
	NL4.3/WT	1.95	>5.00		NL4.3/AR177	≥4.19	>5.00
	NL4.3/AR177	1.96	>5.00		NL4.3/DS8000	2.15	>5.00
	NL4.3/DS8000	1.65	>5.00	**C** (0.3)	III_B	0.38	>5.00
A (0.3)	III_B	0.014	>5.00		NL4.3/WT	2.36	>5.00
	NL4.3/WT	0.21	>5.00		NL4.3/AR177	>5.00	>5.00
	NL4.3/AR177	0.078	>5.00		NL4.3/DS8000	>5.00	>5.00
	NL4.3/DS8000	0.21	>5.00	**D** (1.2;0.6;0.3)	III_B;NL4.3/WT/AR177/DS8000	>5.00	>5.00
B (1.2)	III_B	>5.00	>5.00	**E** (1.2;0.6;0.3)	III_B;NL4.3/WT/AR177/DS8000	>5.00	>5.00
B (0.6)	III_B	0.073	>5.00	**F** (1.2)	III_B	0.088	>5.00
	NL4.3/WT	1.83	>5.00		NL4.3/WT	2.21	>5.00
	NL4.3/AR177	≥2.09	>5.00		NL4.3/AR177	>5.00	>5.00
	NL4.3/DS8000	1.76	>5.00		NL4.3/DS8000	2.77	>5.00
B (0.3)	III_B	0.086	>5.00	**F** (0.6)	III_B	0.12	>5.00
	NL4.3/WT	1.35	>5.00		NL4.3/WT	0.91	>5.00
	NL4.3/AR177	0.89	>5.00		NL4.3/AR177	≥1.40	>5.00
	NL4.3/DS8000	3.35	>5.00		NL4.3/DS8000	0.79	>5.00
C (1.2)	III_B	2.05	>5.00	**F** (0.3)	III_B	2.29	>5.00
	NL4.3/WT	2.59	>5.00		NL4.3/WT	2.43	>5.00
	NL4.3/AR177	>5.00	>5.00		NL4.3/AR177	>5.00	>5.00
	NL4.3/DS8000	>5.00	>5.00		NL4.3/DS8000	2.37	>5.00
				AR177	NL4.3/WT	0.26	>50.00
					NL4.3/AR177	>50.00	>50.00
					NL4.3/DS8000	4.27	>50.00

[a] stock solution concentrations of ss ODNs; [b] The 50% cytotoxic concentration (CC_{50}) was defined as the concentration of the test 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}).

2.3 Native Gel Electrophoresis

In order to determine the stoichiometry of the structures that have been tested in the anti-HIV assays, we performed native gel electrophoresis (PAGE) experiments under the same experimental conditions used for the HIV tests. All ODN stock solutions (0.3 mM, 0.6 mM, 1.2 mM) were analyzed on the gel 14 days after the addition of potassium cation.

By PAGE gels (Figure 2), we observed a pool of structures, ranging from the single stranded form (Figure 2, *line 1*) to the G-quadruplex and higher order G4 structures (Figure 2, *lines 2* and *3* respectively). Notably, G4 forms is promoted by increasing ss concentrations but almost all ODNs (**A**-**E**) presented a more intense band for the G-quadruplex structure (Figure 2, *line 2*), confirming that, at these concentrations (*ss*, 0.3 mM, 0.6 mM, 1.2 mM), the G4 structure is the more abundant species.
Another striking result is the detection of different retarded migrations with respect to the G-quadruplex (Figure 2, *line 3*). These bands confirmed the formation of high order G4 structures as recently described by us, in the ESI-MS study [25]. In the specific case of sequence **F**, a unique smearing band was observed at all tested concentrations, suggesting a more complex aggregation phenomenon. Surprisingly, the multimeric behaviour of **F** involves also its potent anti-HIV activity especially against WT viruses. In conclusion, it seems that the aggregation makes all stock solutions of **F** almost equipotent.

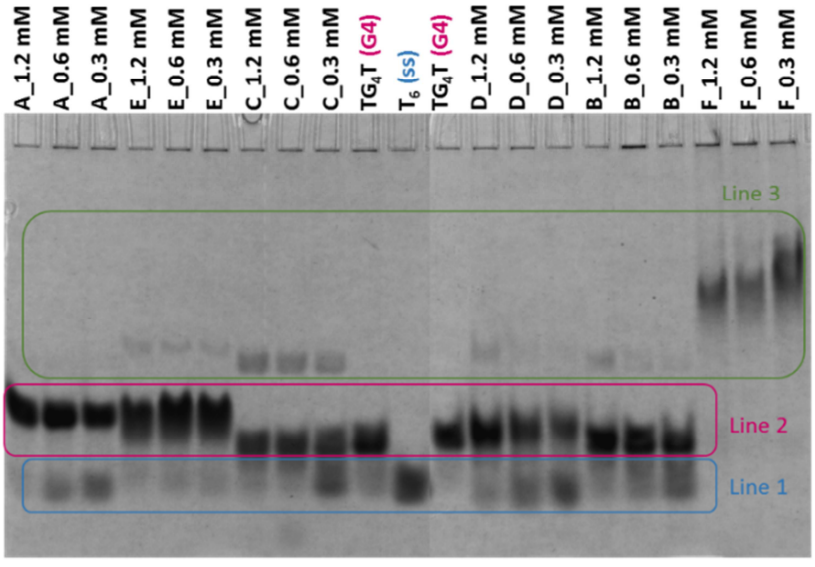


Figure 2. Non-denaturing polyacrylamide gel electrophoresis (PAGE) was carried out on 15 % polyacrylamide gel using running buffer (TBE 0.5 X) containing 10 mM KCl; 100 mM of the ODN samples were loaded on the gel.

3. Conclusion

In conclusion, we have reported a systematic anti-HIV screening on some 5'-end modified d(TGGGAG) sequences with the aim to clarify the role of the G4 structure in the anti-HIV activity. The anti-HIV screening was performed at different initial ss concentrations of ODNs (**A**-**F**) (0.3 mM, 0.6 mM, 1.2 mM), also considering the slow kinetics of tetramolecular G-quadruplex folding.

Native PAGE experiments have demonstrated that the ODNs (**A**-**F**) fold into G4 structures as well as into high order G4 structures and that at increasing of the initial ODNs concentrations turns into an increase of both complexes (G4 and multimers). Comparing these results with the anti-HIV ones, it seems that the **A**, **B** and **C** sequences show a higher activity for the more diluted stock solutions (ss, 0.6 mM and 0.3 mM) and therefore for a minor amount of G4. However, from the native gels it is also observed that for the **A**-**E** ODNs, G4 structure remains at all concentrations tested the most abundant species. In addition, the ODNs **B**, **C** and **F** showed a cross resistance against the NL4.3/AR177 resistant strain that suggests a similar mode of action as AR177 (Zintevir) [12]. The most significant result achieved by the current anti-HIV screening regards the strong anti-HIV activity observed for the natural sequence **A** (0.6 mM and 0.3 mM stock solutions), which has been reported to be absolutely inactive [17].

Instead to the literature and similar to other natural ODNs, the natural sequence is the most active ones and its strong anti-HIV activity is independent from the conjugation at 5'-end with a lipophilic group. Moreover, this study suggests that the highest anti-HIV activity displayed at low concentration of the G4 structure in combination with the lack of cross-resistance against AR177 resistant strain, could be not combined to its peculiar G4 arrangement but related to a different mechanism of action of the d(TGGGAG) sequence. Future studies will aim to the understanding of the structure-activity relationships of the natural sequence.

4. Experimental procedures

*4.1 Synthesis of the ODNs (**A**-**F**)*

Starting with 100 mg of commercially available CPG-dG support with 0.10 meq/g initial loading, and after the assembly of the sequence d(TGGGAG), to obtain the sequences **B**-**F**, an additional coupling step was carried out with the corrisponding phosphoramidite building block. To obtain the sequence **A**, the corresponding support was treated directly with conc. aq. ammonia at 50 °C for 5 h. Indeed for the **B**–**F** sequences, the supports were first treated with Et_3N/pyridine (1:1, v/v) at 50 °C for 1 h and then with conc. aq. ammonia at 50 °C for 5 h (Scheme 1). The combined filtrates and washings were concentrated under reduced pressure, re-dissolved in H_2O, and analysed and purified by RP-HPLC. Purification of the crude conjugated oligonucleotides was carried out on Phenomenex RP18 column (Gemini, 10 um C18, 21.2 x 250 mm) using a linear gradient of CH_3CN and an aqueous solution of 0.1M TEAA (triethylammonium acetate) at pH 7.0 from 10% to 100% over 30 min at a flow rate of 7 mL/min with detection at 260 nm. In all cases 50–80 OD units of pure **A**–**F** could be on average recovered starting from 100 mg of functionalized solid support (average yields 16–25%).

4.2 Quadruplex Preparation

Stock ODNs solutions were prepared at 1.2 mM, 0.6 mM and 0.3 mM in 100 mM KCl, The stock concentrations were determined by UV absorbance at 260 nm measured on an Agilent Cary 100 UV spectrophotometer using the nearest-neighbour calculated molar extinction coefficient of 5'TGGGAG3' ε=62500 cm-1 M-1, not taking into consideration the contribution of the 5'-aromatic label. Annealing experiments were performed at 80 °C heating for 5 minutes and then a fast cooling to room temperature was carried out. The solutions were stored at 20 °C for 14 days before the starting of both gel electrophoresis experiments and anti-HIV assays.

4.3 Native gel electrophoresis

All stock solutions of (**A**-**F**) were loaded on the gels after dilution to 100 µM (ss concentration) and with the addition of 25% of glycerol. The oligonucleotide $d(T_6)$ was used as a single-stranded 6-mer marker and $d(TG_4T)$ as a tetramolecular G-quadruplex marker, $[d(TG_4T)]_4$. Native gel electrophoresis was performed on 15% polyacrylamide gel (acrylamide/bi-acrylamide 19:1) and the running buffer (TBE 0.5X) was supported by 10 mM KCl in order to preserve the native conditions. The gel was run at 26 °C at constant voltage (100 V) for 2.5 h. The bands were visualized by UV shadowing after ‘all stains’ coloration.

4.4 Anti-HIV assays

The anti-HIV activity and cytotoxicity of the oligodeoxynucleotides were evaluated against wild-type (WT) HIV-1 strains IIIB and NL4.3, T30177 (Zintevir) resistant strain derived from NL4.3 (NL4.3/AR177) and dextran sulfate 8000 (DS8000) resistant strain derived from NL4.3 (NL4.3/DS8000) in MT-4 cell cultures using the 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) method [29]. Briefly, stock solutions (10 x final concentration) of test 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 test 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 105 cells/mL and 50-µL volumes were transferred to the microtiter tray wells. Five days after 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 measured spectrophotometrically. 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 test 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}).

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