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Research Article

Unusual Chair-Like G-Quadruplex Structures: Heterochiral TBA Analogues Containing Inversion of Polarity Sites

Antonella Virgilio, Luciano Mayol, Michela Varra, Aldo Galeone, and Veronica Esposito

Dipartimento di Farmacia, Università degli Studi di Napoli Federico II, Via D. Montesano, 49-80131 Naples, Italy

Correspondence should be addressed to Antonella Virgilio; antonella.virgilio@unina.it and Veronica Esposito; verespos@unina.it

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Heterochiral oligodeoxynucleotides based on the thrombin binding aptamer sequence, namely, 5′gg3′-3′TT5′-5′ggtgtgg3′-3′TT5′-5′gg3′ (**H1**), 5′gg3′-3′TT5′-5′gg3′-3′TGT5′-5′gg3′-3′TT5′-5′gg3′ (**H2**), and 5′gGTTGgtgtgGTTGg3′ (**H3**), where lower case letters indicate L-residues, have been investigated in their ability to fold in G-quadruplex structures through a combination of gel electrophoresis, circular dichroism, and UV spectroscopy techniques. In **H1** and **H2** inversions of polarity sites have been introduced to control the strand direction in the loop regions. Collected data suggest that all modified sequences are able to fold in chair-like G-quadruplexes mimicking the original **TBA** structure.

1. Introduction

The oligodeoxynucleotide (ODN) sequence 5′GGTTGGTGTGGTTGG3′ folds in a "chair-like" G-quadruplex structure formed by two G-tetrads and three lateral loops (Figure 1) [1–3]. This sequence represents one of the first aptamers isolated to bind the thrombin (**TBA**: thrombin binding aptamer) through a process known as SELEX (Systematic Evolution of Ligands by Exponential Enrichment) [4]. Although the early applications of this aptamer were connected with its anticoagulant properties, the strong dependence of the structural folding on the potassium ions presence in solution and the ability of the adopted G-quadruplex structure to bind other types of cations have expanded its utilization also as sensor for thrombin [5], K^+ ions [6], and the detection of heavy metal ions as Pb^{2+} and Hg^{2+} [7]. With the aim to improve its properties, **TBA** sequence has been subjected to a plethora of structural modifications concerning both the aromatic bases and the sugar-phosphate backbone [8]. Particularly, the applications of **TBA** analogues in biological environments, both as anticoagulant agent and sensor, require a suitable resistance to ubiquitous nucleases. Because of our particular interest in this frame, we have recently reported investigations concerning the properties of heterochiral **TBA** analogues, in which the sequence 5′ggTTggtgtggTTgg3′ (Table 1, **D13**, where lower case letters indicate L-residues) was proven to fold in a structure very similar to the **TBA** "chair-like" G-quadruplex and be suitably resistant to serum nucleases [9]. This sequence was expressly designed to show the 3′- and 5′-ends formed by L-residues and the lateral TT loops composed by natural residues, being these moieties mainly responsible for the interaction with the target protein, according to several authors [10–12]. However, a close comparison of the structures of **TBA** and **D13** reveals that, for the latter, the strand orientations of the TT loops relative to the adjacent G-tetrad are inverted with respect to the original chair-like structure.

In this paper we describe the preparation and investigation by circular dichroism (CD), UV, and electrophoretic techniques of two further heterochiral **TBA** analogues, namely, **H1** and **H2** (Table 1), in which the strand orientation of the TT loops has been controlled through the introduction of inversion of polarity sites along the heterochiral sequence, with the aim to obtain an analogue better mimicking the original **TBA**. The sequence of **H1** has been suitably designed in such a way to (1) preserve the structural stability and the nuclease resistance by maintaining the residues of the TGT loop and the adjacent G-tetrad with the same chirality

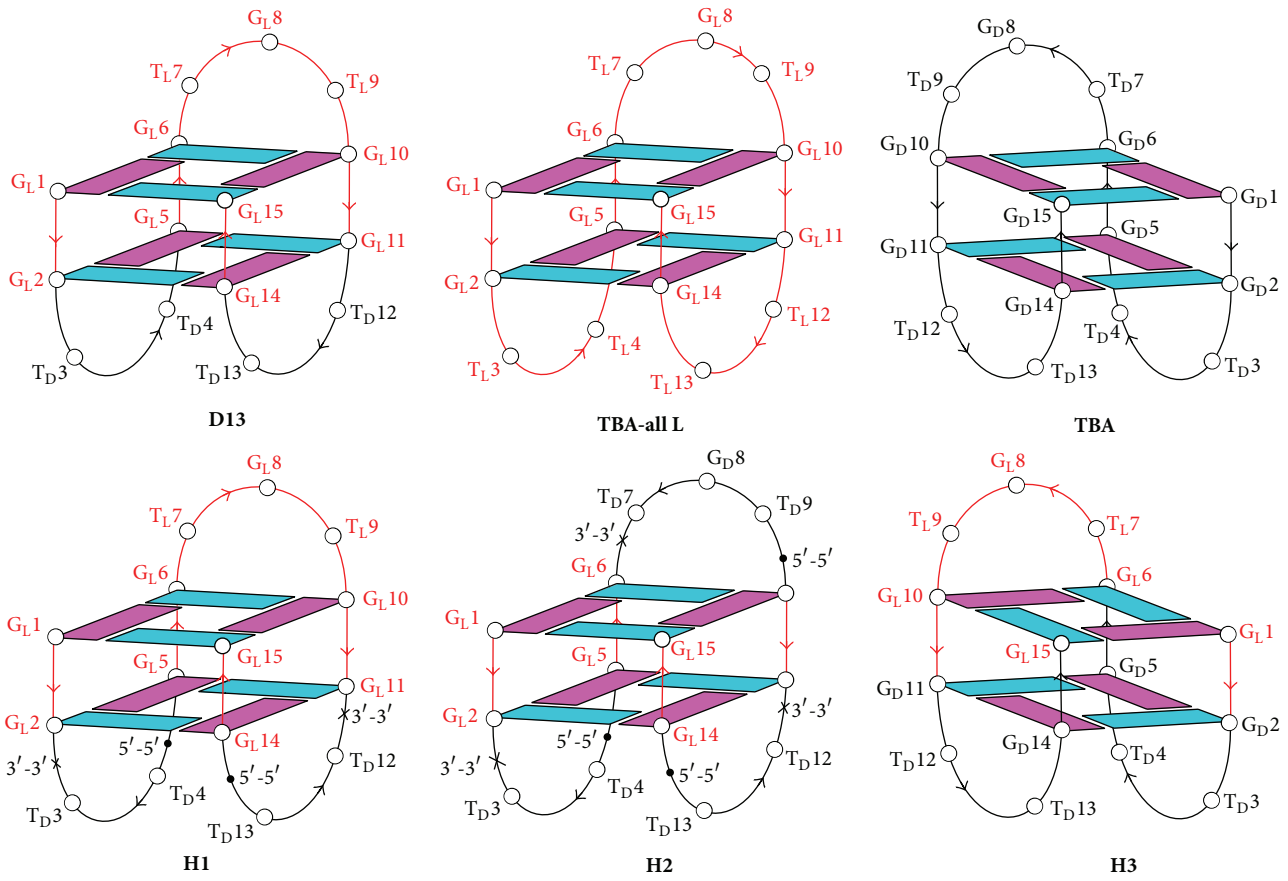

Figure 1: Structures proposed for the heterochiral sequences investigated and their parents G-quadruplexes. *syn*- and *anti*-Guanosines are in purple and light blue, respectively.

Table 1: Heterochiral sequences investigated, their parent ODNs, and melting temperatures (T_m).

Name	Sequence[a]	T_m (°C)
TBA	5′GGTTGGTGTGGTTGG3′	50
TBA-all L	5′ggttggtgtggttgg3′	50[b]
D13	5′ggTTggtgtggTTgg3′	52[b]
H1	5′gg3′-3′TT5′-5′ggtgtgg3′-3′TT5′-5′gg3′	50
H2	5′gg3′-3′TT5′-5′gg3′-3′TGT5′-5′gg3′-3′TT5′-5′gg3′	41
H3	5′gGTTGgtgtgGTTGg3′	42

[a]L-residues are in lower case letters; [b]see [9].

(L) and (2) maintain the residues of the TT loops in the natural chirality (D) with the same orientation as the **TBA** structure. Furthermore, the properties of **H2** have been investigated, in which also the TGT loop is composed of natural residues, considering that a probable role of this moiety in the interaction with other plasma components has been proposed [13, 14]. CD, UV, and electrophoretic data have been obtained for a further heterochiral sequence, **H3** (Table 1), potentially able to adopt a chair-like G-quadruplex characterized by natural D-residues in the TT loops and the adjacent G-tetrad and by L-residues for the remaining part of the structure.

2. Materials and Methods

2.1. Oligonucleotides Synthesis and Purification. The oligonucleotides in Table 1 were synthesized on an Expedite 8909 DNA synthesizer using solid phase β-cyanoethyl phosphoramidite chemistry at 15 μmol scale. The synthesis of the 3′-5′ tracts was performed by using normal 3′-phosphoramidites, commercially available, and 5′-dimethoxytrityl-β-L-deoxyguanosine (iBu)-3′-phosphoramidite and 5′-dimethoxytrityl-β-L-deoxythymidine-3′-phosphoramidite prepared according to Urata et al. procedure [15]. The 5′-3′ tracts were synthesized by using 5′-phosphoramidites. For all ODNs an universal support was used.

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 H_2O, analyzed, and purified by high-performance liquid chromatography on a Nucleogel SAX column (Macherey-Nagel, 1000-8/46), using buffer A: 20 mM KH_2PO_4/K_2HPO_4 aqueous solution (pH 7.0) containing 20% (v/v) CH_3CN and buffer B: 1 M KCl, 20 mM KH_2PO_4/K_2HPO_4 aqueous

solution (pH 7.0) containing 20% (v/v) CH_3CN; 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 NMR.

2.2. Gel Electrophoresis. All oligonucleotides were analyzed by nondenaturing PAGE. Samples in the potassium buffer (10 mM KH_2PO_4, 70 mM KCl, pH = 7) were loaded on a 20% polyacrylamide gel containing Tris–Borate-EDTA (TBE) 2.5x and KCl 50 mM. The run buffer was TBE 1x containing 100 mM KCl. For all samples, a solution of glycerol/TBE 1x-100 mM KCl 2 : 1 was added just before loading. Electrophoresis was performed at 9.2 V/cm at a temperature close to 10°C. Bands were visualized by UV shadowing.

2.3. CD Spectroscopy and Melting. CD samples of modified oligonucleotides and their natural counterpart were prepared at a ODN concentration of 100 μM by using a potassium buffer 10 mM KH_2PO_4/K_2HPO_4 and 70 mM KCl (pH 7.0) and submitted to the annealing procedure (heating at 90°C and slowly cooling at room temperature). CD spectra of all quadruplexes and CD melting curves were registered on a Jasco 715 CD spectrophotometer. For the CD spectra, the wavelength was varied from 220 to 320 nm at 100 nm min^{-1} scan rate, and the spectra were recorded with a response of 16 s, at 2.0 nm bandwidth, and normalized by subtraction of the background scan with buffer. The temperature was kept constant at 20°C with a thermoelectrically controlled cell holder (Jasco PTC-348). CD melting curves were registered as a function of temperature (range: 20°C–90°C) for all quadruplexes at their maximum Cotton effect wavelengths. The CD data were recorded in a 0.1 cm path length cuvette with a scan rate of 0.5°C/min.

2.4. UV Thermal Difference Spectra (TDS). UV samples of investigated oligonucleotides were prepared using a buffer solution: 10 mM KH_2PO_4/K_2HPO_4, 70 mM KCl (pH 7.0). For each oligonucleotide sample, a UV spectrum was recorded above and below its melting temperature (T_m). All experiments were performed on a Jasco V 530 UV/Vis spectrophotometer using quartz cuvettes with an optical path of 1 cm and at 50 μM strand concentration. Absorbance spectra were recorded in the 220–320 nm range, with a scan speed of 100 nm min^{-1} and with a data interval of 1 nm. The difference between the UV spectra at high (90°C) and low (20°C) temperatures was defined as the TDS; this represents the spectral difference between the unfolded and folded forms [16]. The temperature (20 or 90°C) was kept constant with a thermoelectrically controlled cell holder (Jasco PTC-348). The thermal difference spectra were normalized (+1 for the highest positive peak).

3. Results and Discussion

3.1. Gel Electrophoresis. Since some authors have shown that site-specific changes in the **TBA** sequence can result in the formation of bimolecular structures [17], before further investigations, ODNs **H1**, **H2**, and **H3** have been analyzed by PAGE, in which the **TBA** has been used as a reference (Figure 2). As it is evident from the electropherogram, all the three investigated heterochiral ODNs show a main band with electrophoretic motilities very similar to **TBA**, thus strongly suggesting the occurrence of major monomolecular G-quadruplex structures resembling the **TBA** chair-like G-quadruplex.

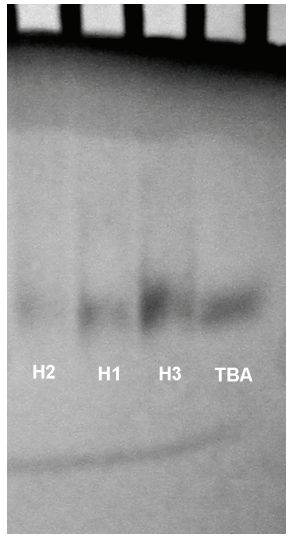

Figure 2: Nondenaturing PAGE of the heterochiral G-quadruplex structures investigated. **TBA** has been included as a reference.

3.2. CD Spectroscopy. Circular dichroism is generally accepted as a very useful technique to assess the presence of a G-quadruplex structure in solution, to obtain preliminary information concerning the quadruplex folding topology or to corroborate structural data afforded by different techniques, considering that several G-quadruplex types show distinctive CD spectra [18–20]. However, through a straightforward comparison of the CD profiles, this technique can be also more informative in obtaining topological data in the case of derivatives and analogues of specific G-quadruplex structures whose conformation has been ascertained and whose CD profile is known.

Figure 3(a) shows CD profiles of **H1**, **H2,** and **TBA-all L** (Table 1). Since the latter one is characterized by the same sequence as **TBA**, but it is formed by only L-residues, it adopts a G-quadruplex that is the mirror-image of the **TBA** structure [9]. Therefore, its CD spectrum is specular compared to natural **TBA**, showing two positive bands at ~230 and 268 nm and two negative bands at ~247 and 294 nm. Apart from slight differences concerning the band intensities and their wavelengths, CD spectra of **H1** and **H2** show profiles comparable to **TBA-all L**, thus suggesting very similar monomolecular G-quadruplex structures adopted by these heterochiral ODNs (Figure 1). On the other hand, the CD spectrum of **H3** is quite different from both the natural **TBA** and its mirror-image **TBA-all L**, since it shows a profile similar to that of a typical parallel G-quadruplex (a

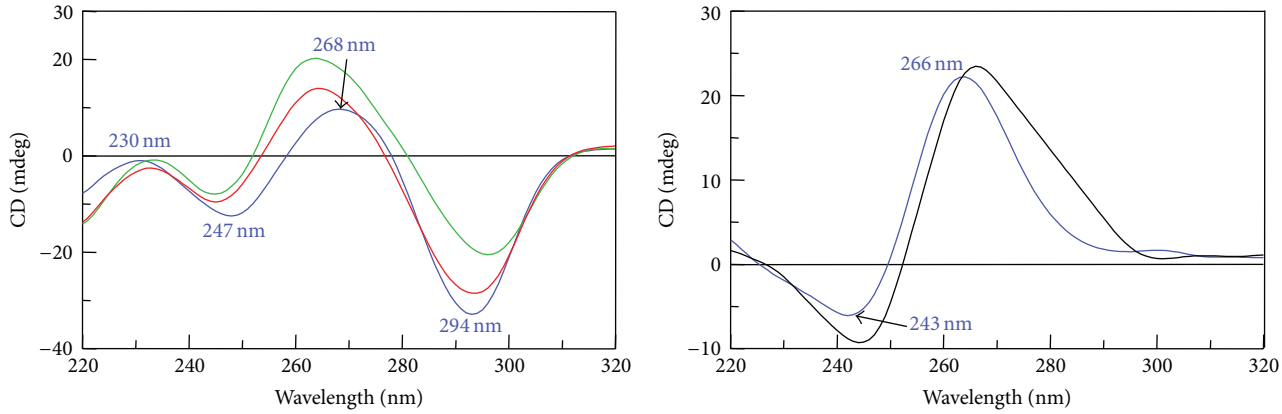

FIGURE 3: CD spectra at 20°C of **H2** (green), **H1** (red), and **TBA-all L** (blue) (a); **H3** (black) and TG_4T (blue) (b). Bands of the parent structures (blue curves) have been labelled.

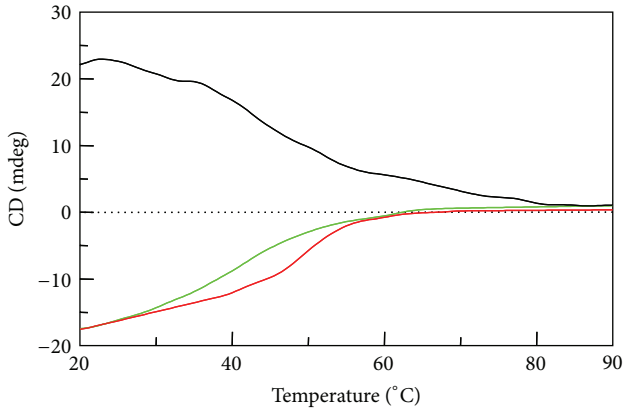

FIGURE 4: CD melting curves of **H3** (black), **H2** (green), and **H1** (red).

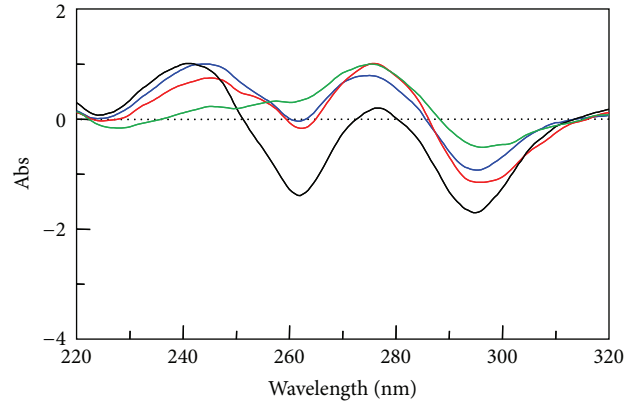

FIGURE 5: Normalized TDS profiles of **TBA-all L** (blue), **H1** (red), **H2** (green), and **H3** (black).

negative band at ~243 and a positive band at ~266 nm), as, for example, $[(TG_4T)]_4$, in which G-tetrads with the same H bonds orientation are stacked (Figure 3(b)) [21]. Taking into account its CD profile and the relationship between glycosidic bond of L-guanosines and strand orientation in heterochiral G-quadruplex structures previously investigated [22], in the case of **H3** we propose a right-handed G-quadruplex structure characterized by a G-tetrad formed by L-residues, adjacent to the all-L TGT loop, and a G-tetrad formed by natural D-residues, adjacent to the all-D TT loops (Figure 1).

3.3. CD Melting Measurements. The signal at the maximum wavelength in the CD spectra has been used to evaluate the structural thermal stability through melting temperatures (T_m) and compare them with the **TBA** (Table 1). CD melting curves for **H1** and **H2** (Figure 4) show excellent sigmoid profiles that have allowed us to confidently determine T_m of 50 and 41°C, respectively. It should be noted that the T_m of **H1** is similar to those of **TBA** (or **TBA-all L**) (50°C) and the heterochiral G-quadruplex **D13** (52°C), thus corroborating the structure proposed for **H1**. In fact, all these three G-quadruplexes share a common structural feature: the stem moiety (that includes the two G-tetrads) and the TGT loop, being the parts mostly responsible for the thermal stability [23], are composed of residues with the same chirality. Conversely, **H2**, in which the stem (all-L) and the loops (all-D) do not share the same chirality, shows a lower T_m (41°C) than **TBA** (or its mirror version **TBA-all L**). As **H3** is concerned, although its melting profile is not perfectly sigmoid, an approximate T_m of ~41°C has been estimated. In this case, the decrease of the T_m compared to **TBA** is in agreement with the proposed structure (Figure 4) taking into account that the stabilizing effect of the homochiral TGT and the adjacent G-tetrad could be countered by the heterochiral stem moiety formed by an all-L G-tetrad and an all-D G-tetrad that, for this reason, could be not suitably stacked to each other as it occurs in a homochiral stem.

3.4. TDS. The profiles obtained by this technique reflect the subtleties of base stacking interactions and, for this

reason, it can be considered particularly useful to compare G-quadruplex structures. Figure 5 shows the normalized TDS profiles of the modified heterochiral aptamers under investigation and that of **TBA-all L** as a reference. All spectra show profiles typical of G-quadruplex structures. Furthermore, the strictly resemblance of profiles of **H1** and **TBA-all L** confirms the similarity between the structures adopted by them. On the other hand, profile of **H2** shows a lower intensity of the band around 245 nm while that of **H3** shows remarkable differences if compared to **TBA-all L**. Taking into account that, in the case of **H2**, the stacking of the TGT loop on the adjacent G-tetrad [10] could be less effective due their different chirality, these data are in agreement with the proposed structure (Figure 1). Similarly, the significant differences between the **H3** and **TBA-all L** profiles are in agreement with the structure proposed for **H3** according to CD data (Figure 1) since this is characterized by a quite different stacking type of the G-tetrads compared to **TBA-all L**.

4. Conclusions

The introduction of inversion of polarity sites in G-rich sequences has proven to affect several features of the G-quadruplex structures including the glycosidic bond preference of the adjacent residues [24–26], the strand arrangement [27], the thermal stability [18, 19], the cation occupancy [28], and the resistance in biological environments [29]. Furthermore the introduction of a 5′-5′ inversion in a heterochiral ODN 5′TGGGGT3′ analogue has allowed disclosing unprecedented dynamic properties of G-quadruplex structure [30]. In this paper inversions of polarity have been exploited to control the strand orientation of the TT loops in two heterochiral **TBA** analogues whose sequences have been suitably designed to form chair-like G-quadruplex structures, characterized by two main features: (1) 3′- and 5′-ends composed by L residues, which are not substrates for ubiquitous exonucleases, and (2) the TT loops composed by natural D residues and characterized by the same strand orientation as **TBA**, being these moieties involved in the interaction with the target [8]. PAGE analysis, CD, and UV data suggest that sequences **H1** and **H2** fold in chair-like G-quadruplex structures similar, apart from handedness, to **TBA**, although **H2** shows a thermal stability lower than its natural counterpart. A similar structure has been proposed also for the heterochiral ODN **H3**, although, in this case, the CD spectrum suggests a right-handed G-quadruplex in which the H bonds of the stacked G-tetrads are likewise oriented.

In order to test the ability of the modified aptamers to bind thrombin, purified fibrinogen clotting assays have been performed in which the **TBA** analogues compete with fibrinogen for thrombin anion binding exosite 1 (ABE I). All modified aptamers show rather decreased affinities to thrombin in comparison with the unmodified **TBA** (data not shown), thus suggesting that the presence of loop strand orientations similar to the original aptamer cannot be considered the only aspect affecting the aptamer-thrombin interaction. On the other hand it should be noted that, apart from interaction with thrombin, the anticoagulant activity of **TBA** and its analogues is a quite complicate process affected by a number of other factors. As a matter of fact, modified **TBA** aptamers are known in which an improved anticoagulant activity has been observed in spite of a reduced affinity to thrombin [14]. Experiments aimed at evaluating the aptitude of the described heterochiral **TBA** analogues to interact with other plasma components have been planned in our laboratory.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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