THE EFFECT OF L-THYMIDINE, ACYCLIC THYMINE AND 8-BROMOGUANINE ON THE STABILITY OF MODEL G-QUADRUPLEX STRUCTURES

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Abstract

Background: Guanine-rich oligonucleotides are capable of forming tetrahelical structures known as G-quadruplexes with interesting biological properties. We have investigated the effects of site-specific substitution in the loops and in the tetrads model G-quadruplexes using thymine glycol nucleic acid (GNA) units, L-thymidine and 8-Br-2'-deoxyguanosine.

Methods: Modified oligonucleotides were chemically synthesized and spectroscopic techniques were used to determine the relative stability of the modified G-quadruplex. The double 8-BrdU-modified quadruplexes were further characterized by Nuclear Magnetic Resonance. Binding to thrombin of selected quadruplex was analyzed by gel electrophoresis retention assay.

Results: The most interesting results were found with a 8-bromoG substitution that had the larger stabilization of the quadruplex. NMR studies indicate a tight relationship between the loops and the tetrads to accommodate 8-bromoG modifications within the TBA.

Conclusions: The substitutions of loop positions with GNA T affect the TBA stability except for single modification in T7 position. Single L-thymidine substitutions produced destabilization of TBA. Larger changes on quadruplex stability are observed with the use of 8-bromoG finding a

single substitution with the highest thermal stabilization found in thrombin binding aptamers modified at the guanine residues and having good affinity for thrombin. Double 8-BrdG modification in anti positions of different tetrads produce a conformational flip from *syn* to *anti* conformation of 8-Br-dG to favor loop-tetrad interaction and preserve the overall TBA stability.

General Significance: Modified guanine-rich oligonucleotides are valuable tools for the search for G-quadruplex structures with higher thermal stability and may provide compounds with interesting protein-nucleic acid binding properties.

Keywords

Thrombin Binding Aptamer, Thymine Glycol Nucleic Acid, L-Thymidine, 8-Bromo-2'-deoxyguanine, G-quadruplex, NMR

1. Introduction

Oligonucleotides are currently used in several areas such as diagnostics, therapeutics or in nanotechnology. Most of these applications rely on the fact that nucleic acids are versatile molecules that can adopt several specific structures. Among them, G-quadruplex has been highlighted in recent years. This structure is formed in G-rich sequences by the association of several units of G-tetrads that are stabilized by cations [1]. This structure is particularly interesting as it has been found in relevant regions such as in the promoter or telomeric regions, as well as in oncogenes [2]. It is possible to modulate the diverse biological functions of nucleic acids by the incorporation of site-specific chemical modifications. In this regard, thrombin binding aptamer (TBA) has attracted considerable attention and several modifications have been described to improve their thermal stability, nuclease resistance and affinity for the target thrombin [3, 4]. TBA is a 15-base sequence, d(GGTTGGTGTGGTTGG) with anticoagulant properties. It folds into antiparallel G-quadruplex structure consisting in two G-tetrads connected by two TT loops and a central TGT loop (Fig. 1a). Particularly, the two TT loops interact with the thrombin anion exosite I. The central loop TGT is in close proximity to the heparin binding site of a different thrombin molecule resulting in TBA interacting with two thrombin molecules but only inactivating one of them [5, 7-8]

Engineering TBA can provide a significant amount of relevant information for biomedical and structural studies. The structural factors controlling the structure of TBA are not only restricted to the G-tetrads; loops play an important role in the folding structure and in the binding to the target. We recently demonstrated that a higher-order structure of TBA can be stabilized by simple specific loop modifications [9]. Nucleotides with special structural or physicochemical properties have been specifically substituted by natural nucleotides of TBA [3]. For example, locked nucleic acids (LNA) [10,11], unlocked nucleic acids (UNA) [12] or 2'-deoxy-2'-fluoro- β -D-arabinonucleic acid (2'-F-ANA) [13] are derivatives with interesting backbone features that

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4 have been introduced in different positions of TBA. In addition, modification of nucleobases
5 containing alkylating moieties or bases with different electronic density has been studied in
6 order to understand the folding topology and stability of TBA [14].

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8 TBA is attracting considerable attention since it shows remarkable *in vitro* anticoagulant
9 properties and some mutants based on their structure are in advanced clinical trials [15].
10 Moreover, some G-quadruplex structures, including TBA, have shown antiproliferative activity
11 in cancer cells [16]. Although there is no clear relationship between the structure and the
12 biophysical properties in the antiproliferative behavior [16] recent research has taken this
13 direction [17-19].

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15 The studies based on the folding topology of TBA may be also exploited in diagnostics or in
16 biosensing applications. Recently, TBA was used as a DNA aptasensor for metal determination
17 [20].

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19 *Oxytricha nova* telomere repeat oligonucleotide d(G₄T₄G₄) consists of 1.5 units of the repeat in
20 telomers of *Oxytricha nova*. It is a symmetric dimeric G-quadruplex structure with four G-
21 tetrads and T4 loops which span the diagonal of the end G-tetrads (Fig. 1a) [21].

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23 In this study, we prepared a group of TBA variants with several types of modifications
24 including glycol nucleic acid thymine (GNA T) and L-thymidine (Fig. 1b), which were
25 introduced in different loop residues. These two different types of nucleic acids backbones were
26 studied in the loop positions of TBA. First, we studied the effect of substituting TBA's
27 thymidines for GNA T units. This thymine derivative is a propylene glycol nucleotide building
28 block with three carbon atoms linked by phosphodiester bonds [22]. GNAs form antiparallel
29 duplex structures according to the Watson-Crick rules [22]. These derivatives have never been
30 tested in the loops of quadruplex.

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32 Secondly, we have modified single positions in the loop regions of TBA with L-thymidine in an
33 effort to understand the effect of nucleotide chirality in specific loop residues. Oligonucleotides
34 containing L-nucleotides are interesting derivatives due to their good nuclease resistance
35 properties and have found different applications in aptamers or in the nanotechnological field.
36 L-thymidine has been introduced in a tetramolecular G-quadruplex [TG₄T]₄ [23] or in a
37 monomolecular G-quadruplex such as anti-HIV aptamer [24] or TBA [25]. In addition, TBA
38 has been modified with enantiomeric nucleosides in different positions. Stability and bioactivity
39 of TBAs modified with D/L-isothymidine showed a position-dependent effect [26].

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41 Then, we prepared several TBA variants with modified tetrads. We selected 8-Br-dG (Fig. 1b)
42 that is a nucleotide that stabilizes a *syn* conformation of the glycosidic bond by steric hindrance
43 between the Br and the ribose moiety. This modification has been shown to stabilize quadruplex
44 structures when is substituting guanines that are in *syn* position [27-29]. Specifically, it has
45 been demonstrated that 8-Br-dG in appropriate positions increases the affinity of TBA due to
46 the stabilizing enhancement of the G-quadruplex structure. Nevertheless, the effect of this
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derivative in *anti* positions or the additions of more than one modification was not yet studied. In addition, 8-Br-2'-deoxyguanosine was used to replace G in specific positions of G-tetrads of the *Oxytricha nova* telomere repeat oligonucleotide (Fig. 1).

Here we report the effect of the substitution of natural nucleosides by modified nucleotides with special structural features either in the loops or in the G-tetrads of model G-quadruplexes focusing on the folding topology and structural stability of the resulting variants.

Fig.1. (a) Scheme of the TBA and *Oxytricha nova* telomere repeat oligonucleotide, in red dG in *syn* conformation and in green dG in *anti*. (b) Chemical structure of L-thymidine and (S)-T-GNA, 8-Br-2'-deoxyguanosine residues used for the synthesis of modified G-quadruplex.

2. Materials and methods

2.1 Oligonucleotide synthesis

The oligonucleotides listed in Table 1 were synthesized on a DNA/RNA Applied Biosystems 394 synthesizer by solid-phase 2-cyanoethylphosphoramidite chemistry at 1 μ mol scale. Sequences containing 8-BrdG were prepared using the commercially available 5'-O-(4,4'-dimethoxytrityl)-8-Br-2'-deoxyguanosine-3'-O-(2-cyanoethyl-N,N-diisopropyl)phosphoramidite (Link Technologies).

The 2'-Deoxy-L-thymidine (Carbosynth) was protected with the dimethoxytrityl (DMT) group and further converted to the corresponding β -cyanoethylphosphoramidite according to standard procedures [24].

The GNA Thymine monomer (2-(S)-1-(4,4'-dimethoxytrytloxy)-4-(thyminy)butyl (2-cyanoethyl) (N,N'-diisopropyl) phosphoramidite) was obtained from (R)-(+)-glycidol as described [22].

The oligonucleotides were synthesized in the DMT-ON mode. After the standard ammonia treatment (conc. aq. NH_3 , 55°C, overnight), the oligonucleotides were purified using Glen-

PackTM DNA purification cartridges (Glen Research) except for those containing GNA-T. In this case, the solid support was previously treated 0.1 M DBU/ACN for five minutes and then washed with 1% Et₃N/ CAN and finally treated with concentrated ammonia.

All the oligonucleotides were analyzed by HPLC. Semipreparative column: X-bridgeTM OST C₁₈ (10x50 mm, 2.5 μ m); 10 min linear gradient from 0 % to 30%, flow rate 2 mL/min; solution A was 5% ACN in 0.1 M aqueous TEAA and B 70% ACN in 0.1 M aqueous TEAA and confirmed by Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) spectra (Table 1).

2.2 UV and CD spectroscopy of TBA variants

Oligonucleotides were resuspended in 10 mM cacodilate buffer and 100 mM KCl pH 7.0 to form solutions with a concentration between 2-6 μ M. These solutions were heated at 85 °C and slowly cooled down to room temperature for annealing. The resulting solutions were stored at 4°C for one night. The thermal (denaturing and annealing) curves were obtained following the change of the absorption at 295 nm from 20°C to 80°C with a linear temperature ramp of 0.5 °C/min on a JASCO V-650 spectrophotometer equipped with a Peltier temperature control. Melting temperatures (T_m) were determined as described elsewhere [30] using home-made routines written in Matlab code (Table 1).

Thermal difference spectra (TDS) were obtained by subtraction of the spectra obtained at 20°C from spectra obtained at 80°C using the same buffer [31]. The scan speed was 100nm/min with data collection of 1 point/nm. Results are shown in the supplementary content.

The CD spectra were recorded on a JASCO spectropolarimeter J-815. Spectra were registered at 15°C over a range of 220-320 nm with a scanning speed of 100 nm/min, a response time of 4 s, 0.5 nm data pitch and 1nm bandwidth. The samples were dissolved in the above buffer annealed, then slowly cooled down to room temperature and left at 4°C at least one night.

Table 1. Sequence, melting temperature (T_m) and mass spectra of TBA and G₄T₄G₄ derivatives used in this study. T^L: L-thymidine, T^N: (S)-GNA-T, G^{Br}: 8-Br-2'-deoxyguanosine.

Name	Sequence	T_m^a (°C)	ΔT_m	M _{calc}	M _{found}
TBA	GGTTGGTGTGGTTGG	49.6	-	4726.0	4726.6
TBA4GNAT	GGTT ^N GGTGTGGTTGG	36.9	-12.7	4684.4	4682.8
TBA7GNAT	GGTTGGT ^N GTGGTTGG	54.8	+5.2	4684.4	4686.4
TBA9GNAT	GGTTGGTGT ^N GGTTGG	40.1	-9.5	4684.4	4683.8
TBA7,9GNAT	GGTTGGT ^N GT ^N GGTTGG	38.6	-11	4642.2	4643.7
TBA3,12GNAT	GGT ^N TGGTGTGGT ^N TGG	47.1	-2.5	4642.2	4644.7

TBA4,13GNAT	GGTT ^N GGTGTGGTT ^N GG	37.5	-12.1	4642.2	4644.6
TBA7LT	GGTTGGT ^L GTGGTTGG	48.9	-0.7	4726.0	4726.1
TBA4LT	GGTT ^L GGTGTGGTTGG	46.7	-2.9	4726.0	4729.3
TBA3LT	GGT ^L T ^L GGTGTGGTTGG	45.5	-4.1	4726.0	4729.3
TBA2BrG	GG ^{Br} TTGGTGTGGTTGG	47.1	-2.5	4804.9	4802.5
TBA5BrG	GGTTG ^{Br} GTGTGGTTGG	56.9	+7.3	4804.9	4803.6
TBA2,11BrG	GG ^{Br} TTGGGTGTGG ^{Br} TTGG	53.2	+3.6	4883.8	4480.8
TBA2,6BrG	GG ^{Br} TTGG ^{Br} TGTGGTTGG	40.8	-8.8	4883.8	4885.1
G ₄ T ₄ G ₄	GGGGTTTTGGGG	59	-	3786.4	3785.2
G ₄ T ₄ G ₄ 2BrG	GG ^{Br} GGTTTTGGGG	-	-	3865.3	3864.4
G ₄ T ₄ G ₄ 3BrG	GGG ^{Br} GTTTTGGGG	71.3	+12.3	3865.3	3864.4

^a 10 mM sodium cacodylate, 100 mM KCl pH 7. Uncertainty 0.5°C

2.3. NMR spectroscopy of TBA variants

The oligonucleotides were dissolved in H₂O/D₂O (90:10 v/v) at a 0.2 mM concentration, in presence of 5 mM KCl, 10 mM K₂HPO₄/KH₂PO₄ phosphate buffer, pH 6.9. Samples were heated at 85°C for 1 minute and then cooled down to room temperature and left at 4°C overnight.

The NMR spectra were recorded on a Bruker Avance 600 spectrometer operating at a frequency of 600.10 MHz for ¹H with a TXI probe (inverse triple-resonance). The spectra were recorded at variable temperature ranging from 5 °C to 55°C with TOPSPIN (v.1.3). Chemical shifts (δ) were measured in ppm and referenced to external DSS (2,2-dimethyl-2-silapentane-5-sulfonate sodium salt) set at 0.00 ppm.

Phase sensitive NOESY spectra were acquired at 25 °C and 5°C in TPPI mode, with 2 K x 512 complex FIDs. Mixing times of 100 and 300 ms. TOCSY spectra were acquired with the use of a MLEV-17 [32] spin-lock pulse (field strength 10.000 Hz, 60 ms total duration). The program Sparky (UCSF) [33] was used to assign the NOESY cross-peaks following the well-established strategy.

2.4. Thrombin-binding assay

The TBAs variants (0.3 μM) were mixed with increasing concentrations of thrombin. The mixtures were left to react for 1 hr at room temperature. Complexes formation were analyzed on a 10% polyacrylamide gel at 80V, at 4 °C for 4 h in 100 mM Tris pH 7.4, 50 mM AcOH, 5 mM EDTA, 12.5 mM Mg(OAc)₂. Images were taken using Fujifilm las1000 Intelligent Dark Box using SyBR-Green staining and analyze with ImageJ

3. Results and discussion

3.1. Rigid versus flexible nucleoside analogues in the loop regions

Extensive work has been done introducing conformationally restricted nucleosides into TBA. For example, we introduced guanine pseudosugars containing bicyclo[3.1.0]hexane instead of the ribose unit with restricted sugar puckering (*North* or *South*) and glycosidic angle (*anti* or *syn*) [35]. The results showed that when dG's in *syn* positions were substituted by *syn* methanocarba-dG nucleotides, the resulting TBA variants have similar stability than native TBA. However, the modification was differently tolerated in the two G-tetrads of the TBA. In addition, the glycosyl conformation is more restrictive for TBA stability than the sugar puckering [35]. Similarly, 2'F-ANA residues that favor *anti* conformation were introduced in the TBA. In this case, when the *syn* dGs of the tetrads were substituted, the resulted variant showed alternative G-quadruplex conformation [13]. LNA derivatives, which are characterized by a restricted C3'-endo sugar pucker and *syn* conformation were also introduced in several positions of TBA. The results confirmed that LNA substitution in defined positions produced, *syn-anti* switch of glycosidic angle and, therefore, a mixture of alternative G-quadruplex structures were produced [10,11].

Flexible nucleic acids derivatives have been also introduced in the TBA. Unlocked Nucleic Acids (UNA) derivatives have been introduced in TBA loop positions inducing a site-dependent stabilization of TBA [12]. For this reason, here we have studied the effect of the unlocked GNA T modification in the loop regions of TBA. This compound contains a flexible and short linker between the nucleobase and phosphate that leads to a decrease in duplex stability respect to a natural thymidine when incorporated into DNA and to an increase in T_m 's on all GNA duplexes with respect to DNA duplexes [22]. Specifically we have prepared TBA variants having thymidines 4, 7 and 9 replaced by GNA-T and double-modified TBA variants having modified thymidines in the upper TGT loop (positions 7 and 9), and thymidines in the TT lower loops (positions 3 and 12 and positions 4 and 13).

In order to analyze the effect of these chemical modifications in the stability of the TBA structure we have recorded the corresponding melting curves. Unmodified TBA has a T_m of 49.5 °C and shows a CD spectrum with positive peaks at 295 and 245 nm as well as a negative peak at 265 nm (Fig 2). These features agree with those reported previously for antiparallel G-quadruplex [1,7]. The thermal difference spectra (TDS) of TBA also exhibited a signature characteristic of an antiparallel G-quadruplex structure with two negative peaks (265nm, 295 nm) and two positive peaks (245nm, 275 nm) [31] (Fig S1).

Fig. 2. CD spectra of TBA variant containing a) 8-BrdG, b) GNAT and c) LT in 10mM Sodium cacodilate 100mM KCl pH7.

GNA T substitution in position 7 of the central TGT loop produced a thermal stabilizing effect with ΔT_m 5.2 °C (Table 1). This position is very permissive to be modified [3]. However, thymidine located in position 9 although is present in the same TGT upper loop is very sensitive to the modification as the substitution of T by GNAT at this position decreases the thermal stability in 9.5 °C. Similarly, when T7 and T9 were substituted by GNAT the resulted variant was very unstable presenting a T_m of 38.6 °C. The lower TT loops of TBA were also very sensitive to modifications, especially changes in positions 4 and 13 that in the unmodified TBA forms a hydrogen bond [8]. In this case, TBA4GNAT is very unstable having a T_m of 36.9 °C. Similar results were obtained with TBA4,13GNAT that showed a very low thermal transition (T_m 37.5°C), whereas TBA3,12GNAT is more stable (T_m 47.1°C) indicating that the GNAT modification is more adapted in the overall TBA structure in positions 3 and 12. According to CD spectra, all the TBA GNAT variants showed antiparallel quadruplex structures. TBA7,9 GNAT was the most featureless, implying a less structured oligonucleotide (Fig. 2). TBA variants with GNAT modifications showed similar TDS than TBA. Based on these data we can conclude that, although GNAT contains a short backbone, it has a similar behavior as UNA derivatives [12,34]. Both derivatives increase TBA stability in position 7 [12]. In addition,

multiple UNA modifications in TBA were also studied [34]. Similar to our work with GNAT, a position-dependent effect was observed being position 7, 3 and 12 the most tolerant in the TBA stability.

3.2. Presence of L-thymidine in the loop regions

Next, we studied the introduction L-thymidine, the enantiomer of natural T, in single loop positions of TBA. According to thermal transition, the substitution of T by T^L produced a destabilization of the TBA. Position 7 was again the most permissive position with a T_m similar to that of TBA but modification in position 3 or 4 produced TBA variants less stable than TBA with a ΔT_m of -4.1 and -2.9 °C respectively. The CD spectrum of TBA3LT is almost flat but the rest of the modified TBA variants still maintained the CD antiparallel profile. TDS of TBA7LT is similar to TBA and TBA3LT is the most featureless. The modification with multiple L-derivatives in the tetrads and in the loops of TBA has been studied in the bibliography [25]. Heterochiral TBA analogues resulted in less stable structures than TBA. However, in the case that the central loop and tetrads are modified the resulted variant is more stable indicating their close relation. Tuning with L-nucleotides is a good strategy to improve nuclease activity without compromising thermal stability in certain positions of the G-quadruplex. However, from our work it is envisaged that small number of modifications seems to be detrimental for TBA quadruplex stability.

3.3 Unexpected stabilization effects from 8-bromo-guanine modifications

It has been described that modified TBA's carrying 8-BrdG substitutions for guanines that are in *syn* conformation improve the affinity for thrombin as well as the stability of the resulting TBA variant [27]. This is in agreement with the preference of 8-bromo-G to be in *syn* conformation. Additionally, this nucleoside was also used as a single chemical probe in a telomeric sequence [29].

We have prepared modified TBA carrying one 8-BrdG substituting a guanine in *syn* conformation in the native TBA (position 5) and a guanine in *anti* (position 2). As well as several double-modified TBA's. In the case of a single 8-BrdG substitution for a G in *anti*-conformation (position 2, TBA2BrG), the melting temperature decreases as expected but only by 2.5 °C. In contrast, in single 8-BrdG substitution for a G in *anti*-conformation (position 5, TBA5BrG), induce a high increase of the T_m (ΔT_m 7.3 °C). These results are expected as the addition of a derivative with preference for the *syn* conformation in a position that is occupied for a G residue in *anti* conformation produces a small penalty in term of thermal stability but the derivative is well adapted in an antiparallel-G-quadruplex structure. However, the introduction of 8-BrdG that prefer *syn* conformation in a *syn* position produces a modified TBA with much

more stability. In both cases, the CD spectra reveal an antiparallel G-quadruplex profile similar to that of the native TBA.

The double modification of TBA by 8-BrdG produced unexpected results. When we introduce in two 8-Br-dG modifications in position where guanines are in *anti* conformation in different G-tetrads of TBA the resulting variant (TBA2,6BrG) is less stable than TBA with a T_m of 40.8 °C. CD and TDS features also confirm the formation of a destabilized antiparallel TBA structure (Fig. 2 and S1). However, when the two guanines that are in *anti* conformation are replaced by 8-BrdG (that prefer *syn* conformation) in the same tetrad, the resulting variant (TBA2,11BrG) showed an unexpected increase of thermal stability compared with TBA (T_m 53.8°C, ΔT_m +3.6°C). Interestingly, the CD spectrum was similar to the CD spectrum of native TBA but the 245 nm positive band is slightly shifted, denoting small modifications in the structure. The TDS feature of TBA variants containing 8-BrdG are similar to that of TBA, except for TBA2,6BrG which even though the TDS signature is similar to that of TBA, the bands are more pronounced. TBA2,11BrG was expected to be less structured or having a different structure from native TBA but, on the contrary it shows to be more stable and very similar to native TBA.

To further examine the structural stability of double 8-BrdG modification, UV thermal melting analysis was conducted in dimeric quadruplex formed by the *Oxytricha nova* telomere repeat oligonucleotide (G₄T₄G₄) [21]. This oligonucleotide forms an antiparallel dimeric quadruplex with four G-quartets. When we introduce a single 8-BrdG derivative two modifications in two different G-tetrads are produced. The modification in position 3 (*syn*) produced as expected a more stable G-quadruplex structure with an increased thermal stability of more than 12°C. However, the modification in position 2 (*anti*) that altered the central G-tetrads produced a complete disruption of the G-quadruplex structure. CD analysis showed G₄T₄G₄ and G₄T₄G₄3BrG variants with an antiparallel feature, whereas G₄T₄G₄2BrG were almost featureless, thus implying an unstructured oligonucleotide. Modification in two different and consecutive tetrads produces partly (in the case of monomeric TBA) or completely (in the case of dimeric G₄T₄G₄) disruption of the quadruplex structures, while no *syn-anti* switch is assumed to produce a rearrangement of the quadruplex structure.

In order to understand the increased stability of the double modification in the same tetrad we performed NMR studies of double 8-BrdG modified TBA variants.

3.4. NMR structural characterization of TBA2,11BrG and TBA 2,6 BrG

The analogy with the native TBA together with the well established procedures [6, 36-38] were used to identify the imino, aromatic and ribose protons of two tetrads and loops. In Table S1 and S2 are selected chemical shifts for TBA2,11BrG and TBA2,6BrG, respectively, are reported. ¹H NMR spectra of the exchangeable resonances of TBA2,6BrG and TBA2,11BrG display sharp eight hydrogen-bonded G imino proton resonances (11.8-12.6 ppm) indicating the formation of

two G-tetrads as in the unmodified TBA (Fig S2). The spectra shown in Fig. S2 also display the exchangeable protons from the loop residue. All the exchangeable protons are characterized by small linewidths, confirming the presence of a folded antiparallel quadruplex structure. Downfield chemical shift variation of NH imino protons of the modified guanines in comparison with the unmodified TBA is in agreement with literature data [39] ($\Delta\delta=+0.32$ and $+0.37$ ppm for G2 and G11 in TBA2,11BrG and $\Delta\delta= +0.21$ and $+ 0.25$ ppm for G2 and G6 in TBA2,6BrG).

Inter and intraresidue NOEs were identified for both the modified aptamers as previously reported by different authors for the native TBA [6, 36-38]. In particular: *a*) the key NOEs between the methyl of one T with four different imino protons allowed to identify T9 and G1,G6,G10 and G15 in analogy with the unmodified TBA (Fig. 3). Moreover long-range connectivities between H8G8 and ribose protons H2' and H2'' of G6 and between H8 G15 with the anomeric proton of T9 were also detected (Fig. 4). These contacts are related to other NOEs between the anomeric protons of T9 and G8 with NH imino protons of the tetrad G1-G6-G10-G15.; *b*) the key NOEs between H2' and H2'' ribose protons of other two T with imino protons different to the previous ones allowed to identify T4 and T13 and G5 and G14 residues (Fig. 3). Complementary information are given by the NOEs between methyl T4 and H1', H2' and H2'' of G2 and from methyl of T13 with H1', H2' and H2'' of G11. All these findings allowed the G2-G5-G11-G14 tetrad to be assigned. Long-range NOE weak connectivities between methyl of T4 and methyl of T13 with G2 and G11 NH respectively were also detected for TBA2,11BrG whereas these connectivities were not observed for TBA2,6BrG. These long-range NOEs were also found in the Sr^{2+} -TBA complex by Mao et coworkers and this suggest that these two thymines for TBA2,11BrG were not-coplanar with the G tetrad and the methyl groups are oriented edgewise towards the quadruplex core [39].

The glycosidic angles for Gs in the tetrads, with the exception for the G residues missing of the aromatic H8 protons, were determined by the intensities of the NOEs interactions between H8 - H1' and H8-H2'/H2'' of the G residues. The alternation *syn-anti* in the tetrads is respected as in the native TBA for both modified derivatives (Fig. 4). These findings, together with the CD data, allowed us to conclude that both modified have a similar topology as the native TBA with a difference at the level of the TT loop for TBA2,11BrG.

Melting experiments performed in the above modified TBAs and in TBA5BrG confirm the UV and CD data (Fig. S3). After the melting experiments, the same spectra were obtained cooling the samples at the temperature of 25°C and 5°C.

In conclusion, the 8BrGs need to flip from *syn* to *anti* in both modified TBA to assist the folding process. This process needs some energy requirement. This happens also for other aptamers previously studied [23]. This causes for TBA2,6 BrG a penalization in terms of stability due to the steric hindrance of the bromine in comparison with the native TBA. G2 and G6 units belong

to two different tetrads in the structure of the aptamer and experiment a different local structural environment in terms of loops. G2 and G6 are close to TT and TGT loops. After the sequential assignment we observed that, in the case of TBA2,6BrG, the imino and aromatic regions show weak signals due to minor conformations present in solution (Fig. 4). Strangely for the TBA2,11BrG, we observed a slight increasing of the stability in comparison with the native TBA. G2 and G11 belong to the same tetrad in the structure and experience an equal local structural environment in terms of loops. G2 and G11 are symmetrically positioned and close to TT loops with the methyl groups oriented in a different way compared with the native TBA. This results in a more favorable assistance in the folding arrangement.

Fig. 3. Selected region of NH imino protons in NOESY spectra showing cross peaks of methyl of T9 and H2' and H2'' resonances of T4 and T13. Assignments are reported in the figure. (a) TBA2,6BrG and (b) TBA2,11BrG, in 5 mM KCl, 10 mM K₂HPO₄/KH₂PO₄ phosphate buffer, pH 6.9, T=5°C.

Fig. 4. Expanded region of NOESY spectra showing aromatic protons and anomeric protons. Assignments are reported in the figure. (a) TBA2,6BrG and (b) TBA2,11BrG, in 5 mM KCl,

10 mM K_2HPO_4/KH_2PO_4 phosphate buffer, pH 6.9, $T=5^\circ C$. Minor conformation presents in solution for TBA2,6BrG are indicated with circles on the projection.

3.5. Thrombin binding affinity

We studied the complex formation between thrombin and the 8-Br-dG TBA variants by electrophoresis mobility shift assay (EMSA). Single substitutions facilitates the complex formation with thrombin at lower ratio however at 1:10 the complex formation is the similar to native TBA. On the contrary 8-Br-dG double substitutions in the TBA produced a decrease in the affinity of the TBA variants for thrombin. From these experiments we can conclude that one single modification of guanine by 8-BrdG is allowed for thrombin recognition but double modification is detrimental for thrombin binding and it confirms that thrombin binding is not dependent of the thermal stability of TBA variants as shown by other authors [17, 19].

Fig. 5. EMSA assay of complexes between thrombin and TBA variant in in 100 mM Tris pH 7.4, 50 mM AcOH, 5 mM EDTA, 12.5 mM $Mg(OAc)_2$.

4. Conclusions

In conclusion, we showed that chemical modifications could provide important information about TBA stability and protein-nucleic acids binding affinity. The substitutions of loop positions with GNA T affect the TBA stability except for single modification in T7 position. However, when the T9 or T4 positions are modified the resulting TBA are much less stable. Single L-thymidine substitutions produced also destabilization of TBA but compared with

GNAT a smaller destabilization in positions 3, 4 and 7 is observed, indicating that this modification is more permissive than GNA.

In addition, the presence of single 8-BrdG in the tetrads, that have preference to adopt a *syn* glycosidic conformation is the major responsible for the correct tetrad formation and subsequent TBA stability. Nevertheless, double 8-BrdG modification in *anti* positions of different tetrads produce a conformational flip from *syn* to *anti* conformation of 8-Br-dG to favor loop-tetrad interaction and preserve the overall TBA stability. EMSA experiments showed that double 8-BrdG produced TBA variants with less thrombin affinities than single modified.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at

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Supplementary Content

Fig. S1. Thermal difference spectra of a) TBAs modified with 8Br-dG b) TBAs modified with GNAT c) TBAs modified with LT and d) $G_4T_4G_4$ modified with 8Br-dG

Table S1 Proton chemical shift of TBA2,11BrG in 5 mM KCl, 10 mM K₂HPO₄/KH₂PO₄ phosphate buffer, pH 6.9, T=5°C.

Base	H8/H6	H1'	H2'	H2''	MeT	NH
G1	7.57	6.16	3.01	3.01	-	12.09
G2^{Br}	-	6.39	2.84	2.26	-	12.47
T3	7.83	6.39	2.69	2.28	1.93	11.48
T4	7.45	6.05	2.27	2.64	1.07	11.57
G5	7.40	6.01	3.44	2.82	-	12.17
G6	7.74	6.03	2.86	2.61	-	12.39
T7	7.94	6.49	2.53	2.60	1.97	11.48
G8	7.53	5.61	1.90	2.24	-	10.63
T9	7.16	5.69 ^a	2.03	2.48	1.89	10.28
G10	7.62	6.15	3.70	3.17	-	11.97
G11^{Br}	-	6.39	3.01	2.81	-	12.45
T12	7.85	6.37	2.26	2.69	1.96	11.48
T13	7.45	6.40	2.28	2.69	1.03	11.57
G14	7.42	6.03	3.60	2.88	-	12.04
G15	8.19	6.20	2.71	2.45	-	12.53

^a H1' T9 experiments a chemical exchange with another H1' T9 resonating at 5.80 ppm

Table S2 Proton chemical shift of TBA2,6BrG in 5 mM KCl, 10 mM K₂HPO₄/KH₂PO₄ phosphate buffer, pH 6.9, T=5°C.

Base	H8/H6	H1'	H2'	H2''	MeT	NH
G1	7.58	6.16	3.13	3.13	-	11.96
G2^{Br}	-	6.41	2.92	2.74	-	12.35
T3	7.86	6.40	2.25	2.72	1.96	11.28
T4	7.37	6.00	2.25	2.59	1.09	11.40
G5	7.49	6.08	3.52	2.93	-	12.13
G6^{Br}	-	6.30	3.13	2.68	-	12.43
T7	7.74	6.40	2.51	2.57	1.95	11.28
G8	7.62	5.64	1.99	2.36	-	10.75
T9	7.19	5.83 ^a	1.97	2.34	1.58	10.30
G10	7.56	6.09	3.69	2.93	-	12.05
G11	8.23	6.07	3.00	2.34	-	12.21
T12	7.92	6.19	2.23	2.34	1.99	11.28
T13	7.31	6.15	2.12	2.73	0.96	11.40
G14	7.49	6.08	3.52	2.97	-	12.14
G15	8.15	6.17	2.72	2.43	-	12.40

^a H1' T9 experiments a chemical exchange with another H1' T9 resonating at 5.75 ppm

Fig. S2. Imino proton NMR spectra for (a) unmodified TBA, (b) TBA2,6BrG and (c) TBA2,11BrG in 5 mM KCl, 10 mM K_2HPO_4/KH_2PO_4 phosphate buffer, pH 6.9, $T=5^\circ C$.

Fig. S3. Imino proton region of a) TBA2,11BrG b) TBA2,6BrG and c) TBA5BrG

Table 1. Sequence, melting temperature (T_m) and mass spectra of TBA and $G_4T_4G_4$ derivatives used in this study. T^L : L-thymidine, T^N : (S)-GNA-T, G^{Br} : 8-Br-2'-deoxyguanosine.

Name	Sequence	$T_m^a(^{\circ}C)$	ΔT_m	M_{calc}	M_{found}
TBA	GGTTGGTGTGGTTGG	49.6	-	4726.0	4726.6
TBA4GNAT	GGTT ^N GGTGTGGTTGG	36.9	-12.7	4684.4	4682.8
TBA7GNAT	GGTTGGT ^N GTGGTTGG	54.8	+5.2	4684.4	4686.4
TBA9GNAT	GGTTGGTGT ^N GGTTGG	40.1	-9.5	4684.4	4683.8
TBA7,9GNAT	GGTTGGT ^N GT ^N GGTTGG	38.6	-11	4642.2	4643.7
TBA3,12GNAT	GGT ^N TGGTGTGGT ^N TGG	47.1	-2.5	4642.2	4644.7
TBA4,13GNAT	GGTT ^N GGTGTGGTT ^N GG	37.5	-12.1	4642.2	4644.6
TBA7LT	GGTTGGT ^L GTGGTTGG	48.9	-0.7	4726.0	4726.1
TBA4LT	GGTT ^L GGTGTGGTTGG	46.7	-2.9	4726.0	4729.3
TBA3LT	GGT ^L T ^L GGTGTGGTTGG	45.5	-4.1	4726.0	4729.3
TBA2BrG	GG ^{Br} TTGGTGTGGTTGG	47.1	-2.5	4804.9	4802.5
TBA5BrG	GGTTG ^{Br} GTGTGGTTGG	56.9	+7.3	4804.9	4803.6
TBA2,11BrG	GG ^{Br} TTGGGTGTGG ^{Br} TTGG	53.2	+3.6	4883.8	4480.8
TBA2,6BrG	GG ^{Br} TTGG ^{Br} TGTGGTTGG	40.8	-8.8	4883.8	4885.1
$G_4T_4G_4$	GGGGTTTTGGGG	59	-	3786.4	3785.2
$G_4T_4G_4$ 2BrG	GG ^{Br} GGTTTTGGGG	-	-	3865.3	3864.4
$G_4T_4G_4$ 3BrG	GGG ^{Br} GTTTTGGGG	71.3	+12.3	3865.3	3864.4

^a 10 mM sodium cacodilate, 100 mM KCl pH 7. Uncertainty 0.5°C

Figure 1

Figure 2

Figure 3

Figure 4

(a)

(b)

Figure 5