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# The "Janus face" of the thrombin binding aptamer: Investigating the anticoagulant and antiproliferative properties through straightforward chemical modifications

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ABSTRACT

*Background:* The thrombin binding aptamer (TBA) is endowed with both anticoagulant and antiproliferative activities. Its chemico-physical and/or biological properties can be tuned by the site-specific replacement of selected residues.
*Methods:* Four oligodeoxynucleotides (ODNs) based on the TBA sequence (5′-GGTTGGTGTGGTTGG-3′) and containing 2′-deoxyuridine (**U**) or 5-bromo-2′-deoxyuridine (**B**) residues at positions 4 or 13 have been investigated by NMR and CD techniques. Furthermore, their anticoagulant (PT assay) and antiproliferative properties (MTT assay) have been tested and compared with two further ODNs containing 5-hydroxymethyl-2′-deoxyuridine (**H**) residues in the same positions, previously investigated.
*Results:* The CD and NMR data suggest that all the investigated ODNs are able to form G-quadruplexes strictly resembling that of TBA. The introduction of **B** residues in positions 4 or 13 increases the melting temperature of the modified aptamers by 7 °C. The replacement of thymidines with **U** in the same positions results in an enhanced anticoagulant activity compared to TBA, also at low ODN concentration. Although all ODNs show antiproliferative properties, only TBA derivatives containing **H** in the positions 4 and 13 lose the anticoagulant activity and remarkably preserve the antiproliferative one.
*Conclusions:* All ODNs have shown antiproliferative activities against two cancer cell lines but only those with **U** and **B** are endowed with anticoagulant activities similar or improved compared to TBA.
General significance:
The appropriate site-specific replacement of the residues in the TT loops of TBA with commercially available thymine analogues is a useful strategy either to improve the anticoagulant activity or to preserve the antiproliferative properties by quenching the anticoagulant ones.


## 1. Introduction

Thrombin binding aptamer (TBA) is a 15-base long oligodeoxynucleotide (5′-GGTTGGTGTGGTTGG-3′) endowed with interesting anticoagulant properties. According to both X-ray and NMR spectroscopy investigations, TBA adopts a monomolecular antiparallel G-quadruplex structure, characterized by two stacked G-tetrads and three edge loops [1,2] (two TT loops and one TGT loop, Fig. 1). Several studies have shown that G-tetrads are mostly responsible for the thermal stability of the aptamer, while loops are involved in the interaction with its target protein namely thrombin, which is a serine protease playing a key role in the blood coagulation pathway. In particular, these studies indicated that the minor loops TT interact with the thrombin anion exosite I, while the larger TGT loop is in close proximity to the heparin binding site of a further thrombin molecule [2–4].

After its discovery, TBA has been subjected to a plethora of chemical modifications aimed at improving thermal stability, enhancing nuclease resistance and increasing anticoagulant activity [5]. Both modifications concerning the sugar-phosphate backbone and investigations regarding the substitution of single bases in the loops have allowed the obtainment of useful data about the role of each residue in the structural stability and/or anticoagulant activity. Limiting our discussion to the TT loops being mainly involved in the interaction with the target, the general overview, which has arisen from literature data, suggests that improvements

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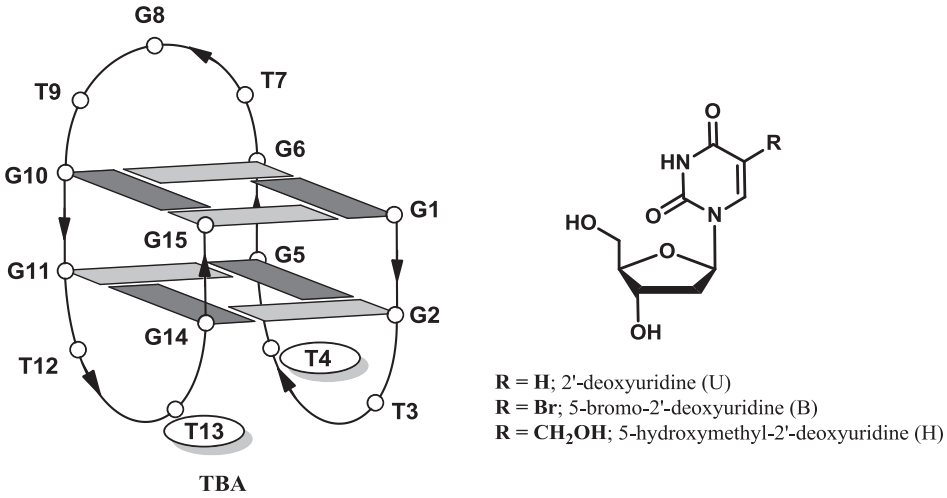


**Fig. 1.** Schematic representation of the G-quadruplex structure adopted by TBA and chemical structures of the nucleosides introduced in positions 4 and 13. Guanosines in *syn* and *anti* glycosidic conformations are in dark and light grey, respectively.

of anticoagulant activity are more probable if the modifications concern residues T3 and T12 [6–11]. On the other hand, residues T4 and T13 have proven to be very crucial for the anticoagulant activity, thus making almost all modifications concerning these residues detrimental to the biological properties [7,8,12]. However, contrary to the above general rules, in a recent paper, we have shown that the site-specific replacement of the thymine methyl group of residues 4 and 13 by fluorine significantly improves the TBA structural stability and anticoagulant activity [13]. This result raises the possibility of modulating the TBA properties by using simple tiny modifications concerning specific positions.

Besides the anticoagulant activity, just like other G-rich oligonucleotides, TBA has shown also antiproliferative properties [14]. In this frame, the simultaneous anticoagulant activity of TBA represents a drawback in exploiting this additional biological property. However, recent papers concerning both the replacement of specific residues with an expressly prepared dibenzyl linker [15] and substantial modifications of the sugar-phosphate backbone [16] have shown that it is possible to develop TBA analogues in which the anticoagulant activity has been removed or considerably reduced but still endowed with a remarkable antiproliferative activity. In an effort to continue and improve our previous research, we have prepared TBA analogues, in which residues T4 and T13 have been replaced with commercially available thymine derivatives (Table 1) with the aims of: i) increasing their structural and anticoagulant properties through straightforward tiny modifications or, on the contrary, ii) reducing or completely eliminating the anticoagulant activity in order to preserve only the antiproliferative one as the biological property. Specifically, TBA derivatives containing 2′-deoxyuridine (**U**) and 5-bromo-2′-deoxyuridine (**B**) have been investigated by CD and NMR techniques and their anticoagulant and antiproliferative properties evaluated and compared with those of their natural counterpart, TBA, and of analogous derivatives containing 5-hydroxymethyl-2′-deoxyuridine (**H**) previously investigated [17]. In order to estimate the contribution of the guanine-based products derived from biological degradation to the antiproliferative activity, we have also investigated a scrambled TBA derivative (**sTBA**) in which the base composition and length of the parent aptamer have been preserved.

**Table 1**
Name, sequence and melting temperature of the ODNs investigated. $\Delta T_m$ indicates the difference between the $T_m$ of the modified aptamer and that of TBA.

| Name | Sequence* | $T_m$ (°C) (±1) | $\Delta T_m$ (°C) |
|---|---|---|---|
| TBA | 5′-GGTTGGTGTGGTTGG-3′ | 50 | – |
| TBA-U4 | 5′-GGT**U**GGTGTGGTTGG-3′ | 51 | +1 |
| TBA-U13 | 5′-GGTTGGTGTGGT**U**GG-3′ | 50 | 0 |
| TBA-B4 | 5′-GGT**B**GGTGTGGTTGG-3′ | 57 | +7 |
| TBA-B13 | 5′-GGTTGGTGTGGT**B**GG-3′ | 57 | +7 |
| TBA-H4[a] | 5′-GGT**H**GGTGTGGTTGG-3′ | 50 | 0 |
| TBA-H13[a] | 5′-GGTTGGTGTGGT**H**GG-3′ | 50 | 0 |
| sTBA | 5′-GTGGTGTGTGTGTGG-3′ | – | – |

* U = 2′-deoxyuridine; B = 5-bromo-2′-deoxyuridine; H = 5-hydroxymethyl-2′-deoxyuridine.
[a] See reference [17]

## 2. Materials and methods

### 2.1. Oligonucleotides synthesis and purification

The oligonucleotides reported in Table 1 were synthesized on a Millipore Cyclone Plus DNA synthesizer using solid phase β-cyanoethyl phosphoramidite chemistry at 10 μmol scale. The modified monomers were introduced in the sequences using commercially available 5′-dimethoxytrityl-5-bromo-2′-deoxyuridine, 3′-[(2-cyanoethyl)-(*N*,*N*-diisopropyl)]-phosphoramidite, 5′-dimethoxytrityl-2′-deoxyuridine,3′-[(2-cyanoethyl)-(*N*,*N*-diisopropyl)]-phosphoramidite and 5′-dimethoxytrityl-5-acetoxymethyl-2′-deoxyuridine,3′-[(2-cyanoethyl)-(*N*,*N*-diisopropyl)]-phosphoramidite (Glen Research). The oligomers were detached from the support and deprotected by treatment with concentrated aqueous ammonia at room temperature for 24 h (**TBA-B4** and **TBA-B13**) or at 55 °C overnight (all the others). 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 $NaH_2PO_4/Na_2HPO_4$ aqueous solution (pH 7.0) containing 20% (v/v) $CH_3CN$ and buffer B: 1 M NaCl, 20 mM $NaH_2PO_4/Na_2HPO_4$ aqueous solution (pH 7.0) containing 20% (v/v) $CH_3CN$; a linear gradient from 0 to 100% B for 45 min and a 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. CD spectroscopy

CD samples of oligonucleotides reported in Table 1 were prepared at a ODN concentration of 100 μM using a potassium