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A straightforward modification in the thrombin binding aptamer improving stability, affinity to thrombin and nuclease resistance

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Abstract

Degradation of nucleic acids in biological environments is a major drawback of the therapeutic use of aptamers. Among approaches used to circumvent this negative aspect, the introduction of 3'-3' inversion of polarity sites at the sequence 3'-end has successfully been proposed. However the introduction of inversion of polarity at the ends of the sequence has never been exploited for G-quadruplex forming aptamers. In this communication we describe CD, UV, electrophoretic and biochemical investigations concerning thrombin binding aptamer analogues containing one or two inversion of polarity sites at the oligonucleotide ends. Data indicate that, in some cases, this straightforward chemical modification is able to improve, at the same time, thermal stability, affinity to thrombin and nuclease resistance in biological environments, thus suggesting its general application as a post-SELEX modification also for other therapeutically promising aptamers adopting G-quadruplex structures.

Among the aptamers based on G-quadruplex structures, the thrombin binding aptamer (TBA) has been the first one to be discovered and it is still the subject of several investigations.[1] It folds in a distinctive antiparallel G-quadruplex structure characterized by two overlapping G-tetrads and three lateral loops ("chair-like" G-quadruplex). Although several DNA/RNA aptamers are endowed with interesting and promising pharmacological properties, rarely they could be used unchanged as therapeutic agents without suitable structural modifications aimed at improving thermal stability and target affinity. Moreover, development of several aptamers as drugs has been often delayed by pharmacokinetic drawbacks mostly due to their inadequate resistance in biological environments. In the case of TBA, while most of the chemical modifications investigated have addressed the requirement to improve its thermodynamic properties and biological activity or to shed light on the details of interaction with the thrombin, less efforts have been made to improve also the resistance to nucleases. For example, modified TBA containing 2'-deoxy-2'-fluoroarabinonucleotide residues,[2] L-residues,[3] thiophosphoryl[4] or triazole internucleotide bonds[5] have proven to be more resistant to nucleases than the natural TBA, although no noteworthy improvement of the biological activity compared to TBA has been reported in these cases. Surprisingly, the approach used for pegaptanib (the first aptamer approved in therapy by the FDA)[6] to increase the stability in biological environments, namely the introduction of an inversion of polarity site (IPS, Fig. S1 in ESI) at the oligonucleotide 3'-end, has never been investigated for aptamers forming G-quadruplex structures.

In this communication we report investigations concerning TBA analogues containing one or two inversion of polarity sites at the ends of the sequence (Tab. 1), suitably designed with the aim to increase resistance to 3'-exonucleases or both to 5'- and 3'-exonucleases.

Since TBA G-quadruplex structure shows a characteristic CD profile with two positive bands at 247 and 295 nm and a negative band at 266 nm, we have firstly used this technique in order to assess the presence of G-quadruplexes and the effects of the IPSs on the structure adopted by the modified sequences.

In Fig. 1 the CD spectra of the structures formed by the TBA analogues are shown in comparison with that of the TBA. All modified aptamers except G-TBA-C, show profiles very similar to that of the parent TBA, with only slight different signal intensities, thus strongly suggesting that these modified sequences are able to fold in G-quadruplex structures resembling the "chair-like" G-

quadruplex adopted by the parent TBA. On the other hand, the modified aptamer G-TBA-C shows a CD profile quite different from that of the original TBA, being characterized by a distinct shift towards higher wavelengths of the positive band at 244 nm and by more marked differences in the band intensities. This datum would suggest for G-TBA-C the presence of additional conformations different from the original "chair-like" G-quadruplex structure.

The occurring of G-quadruplex structures for the modified sequences has been further corroborated by thermal difference spectrum (TDS) measurements[7] (Fig. 2). A thermal difference spectrum of an oligonucleotide is the difference between the UV absorbance spectra of the unfolded and folded states, then, at temperatures above and below the melting temperature of the formed structure (T_m). Since the TDS shows a specific shape that is unique for each type of nucleic acid structure this technique has been used to confirm the presence of G-quadruplex structures for the TBA analogues. According to Mergny et. al.[7] the TDS of the G-quadruplex structures investigated, share some common characteristics, namely a positive band at about 275 nm and a negative one at about 295 nm, while the region 220-270 nm shows the main differences among the various types of G-quadruplexes. Analogously, the TDS of the modified TBAs show a strict resemblance with that of the parent TBA in the region 275-320 nm, thus confirming the presence of G-quadruplex structures.

CD melting measurements have been also used to evaluate the thermal stability of the modified aptamers (Tab. 1 and Fig. S2 in ESI). Data show that for sequences in which only a 3'-3' IPS has been introduced (namely, TBA-A, TBA-G, TBA-C and TBA-T) an increase of the melting temperatures of TBA analogues occurs, in comparison with the parent TBA (increases ranging from +4.5 to +12°C), regardless of the type of residue used. The enhancement of the thermal stability observed for TBA-A (+12°C) is noteworthy, particularly taking into account that it has been obtained through a simple and straightforward chemical modification. The comparison between ODNs containing only a 3'-3' IPS and their versions containing also a 5'-5' inverted residue complementary to the 3'-3' inverted one (pairs TBA-A and T-TBA-A, TBA-T and A-TBA-T, TBA-G and C-TBA-G, TBA-C and G-TBA-C) indicates that, in most of the cases, the simultaneous presence of a 5'-5' inverted residue contrasts the stabilizing effect given by a 3'-3' IPS, with the exception of ODN G-TBA-C for which a melting temperature increase has been observed compared with TBA-C. These data suggest intricate and contrasting effects of the type of IPS and the residue used on the thermal stability, whose full explanation is beyond the scope of this paper.

In order to evaluate their ability to compete with fibrinogen for thrombin anion binding exosite 1 (ABE I) the TBA analogues were subjected to purified fibrinogen clotting assay. The thrombin-induced clotting of fibrinogen was measured spectrophotometrically, following the increase in absorbance at 380 nm as a function of time. Among the series containing one 3'-3' IPS, two TBA derivatives, namely TBA-A and TBA-G, have shown increased affinities toward thrombin compared to that of TBA, while TBA-C and TBA-T have shown the same and a minor affinity than TBA, respectively (Fig. 3).

These data indicate a clear improvement of affinity to thrombin when a purine nucleotide is introduced at the 3'-end. On the other hand, only one modified aptamer (T-TBA-A) belonging to the series with two IPSs has shown a higher affinity to thrombin than TBA, while the other ones are characterized by no improvement (A-TBA-T) or a clear decreasing of the affinity (C-TBA-G and G-TBA-C). Although the three modified aptamers that have displayed better affinities to thrombin than TBA have also shown improved thermal stabilities, it has been not possible to establish a direct correlation between the activity and the melting temperature, as already observed in the case of other TBA derivatives.[8]

Finally, the resistance of all modified aptamers in biological environments has been tested through a degradation assay in fetal bovine serum (Fig. 4 and Fig. S3 in ESI) by using gel electrophoresis. Results clearly indicate that, in these conditions, all modified aptamers are resistant to nucleases up to 24 hours, while their natural counterpart is mostly degraded in 1 hour.[2] According to the results, the introduction of a 5'-5' IPS has not proven to further improve the resistance in biological environments compared to the derivatives only containing a 3'-3' IPS.

Conclusions

In G-quadruplex structures the IPSs have been proven to affect the strand arrangement,[9] glycosidic bond preference[10] and thermal stability.[10] Furthermore their behaviour as cation occupancy sites has been verified in an NMR study based on ^{15}N- labeled ammonium ion[11] and, recently, investigations on heterochiral TGGGGT analogue containing a 5'-5' IPS have highlighted unprecedented dynamic properties of these structures.[12] In addition, TBA analogues containing IPSs inside the sequence have been reported.[9,13] However this modification strongly influenced the strand arrangement and the only analogue investigated (3'-GGT-5'-5'-TGGTGTGGTTGG-3') didn't show improvements in the biological activity compared to the parent TBA. Furthermore, it can't be endowed with a particular resistance in biological environments being characterized by two 3'-ends that are straightforwardly cleaved by ubiquitous 3'-exonucleases. Conversely, in this communication we show that the introduction of suitable IPSs at the sequence ends of a G-quadruplex forming aptamer is able to simultaneously increase the thermal stability in most of the cases, enhance the affinity to the target and endow the aptamer with resistance to biological environments. Taking into account the general improvement of the properties obtained in the case of TBA, the introduction of suitable IPSs at the sequence ends can be regarded as an attractive post-SELEX modification that would be interesting to extend to other G-quadruplex forming aptamers,[14] particularly considering the approachability and simplicity in introducing this chemical modification in a DNA sequence.

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Figures and Tables captions

Table 1. Sequences investigated and their melting temperatures.

Fig. 1. CD spectra at 20°C of TBA (black), TBA-G (red), TBA-A (dark blue), TBA-C (light blue), TBA-T (green) (panel A) and G-TBA-C (red), T-TBA-A (green), C-TBA-G (light blue), TBA (black), A-TBA-T (dark blue) (panel B).

Fig. 2. Normalized TDS profiles of the TBA modified aptamers and their natural counterpart TBA. Panel A: TBA (black), TBA-A (dark blue), TBA-T (green), A-TBA-T (red), T-TBA-A (light blue). Panel B: TBA (black), TBA-G (dark blue), TBA-C (green), G-TBA-C (red), C-TBA-G (light blue).

Fig. 3. Prolonged fibrinogen clotting times measured in PBS buffer in presence of fibrinogen (2 mg/mL), thrombin (1.0 NIH) and TBA or each modified ODN at a concentration 20 nM.

Fig. 4. Stability of T-TBA-A in 10% foetal bovine serum (FBS), as monitored by denaturing PAGE (time points h, are shown in the figure).

Names	Sequence	T_m (°C)	ΔT_m
TBA	5'-$G_2T_2G_2TGTG_2T_2G_2$-3'	33.0	-
TBA-A	5'-$G_2T_2G_2TGTG_2T_2G_2$-3'-3'-A	45.0	+12
TBA-T	5'-$G_2T_2G_2TGTG_2T_2G_2$-3'-3'-T	41.0	+8
T-TBA-A	T-5'-5'- $G_2T_2G_2TGTG_2T_2G_2$-3'-3'-A	37.0	+4
A-TBA-T	A-5'-5'- $G_2T_2G_2TGTG_2T_2G_2$-3'-3'-T	32.0	-1
TBA-G	5'- $G_2T_2G_2TGTG_2T_2G_2$-3'-3'-G	38.5	+5.5
TBA-C	5'- $G_2T_2G_2TGTG_2T_2G_2$-3'-3'-C	37.5	+4.5
C-TBA-G	C-5'-5'- $G_2T_2G_2TGTG_2T_2G_2$-3'-3'-G	33.0	0
G-TBA-C	G-5'-5'- $G_2T_2G_2TGTG_2T_2G_2$-3'-3'-C	42.5	+9.5

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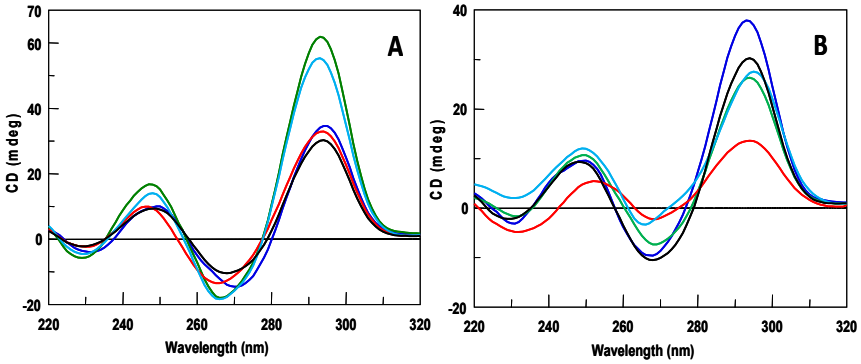

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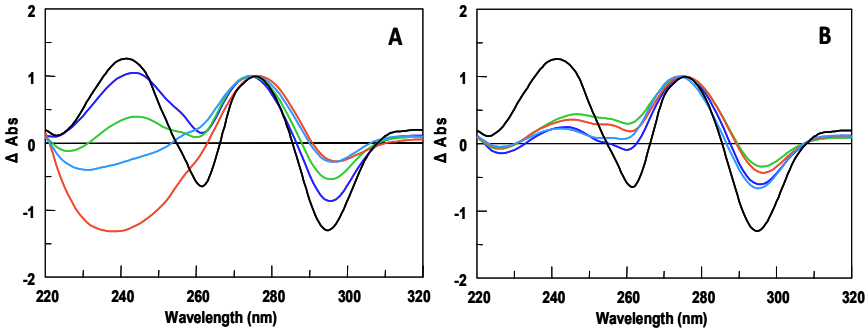

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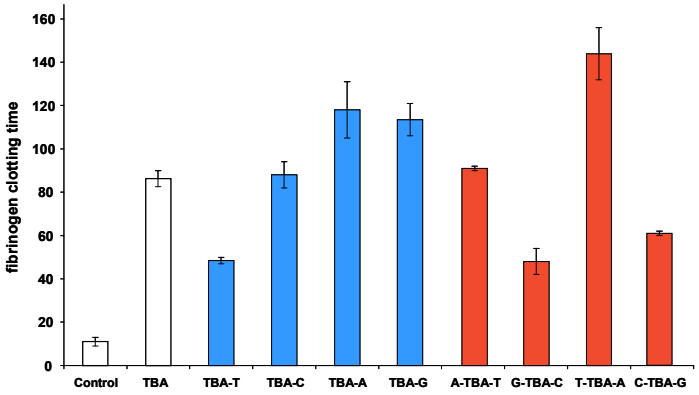

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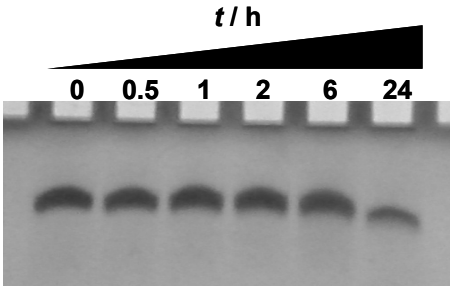

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