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Synthesis, characterization and *in vitro* activity of thrombin-binding DNA aptamers with triazole internucleotide linkages

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ABSTRACT

A series of DNA aptamers bearing triazole internucleotide linkages that bind to thrombin was synthesized. The novel aptamers are structurally analogous to the well-known thrombin-inhibiting G-quadruplexes TBA15 and TBA31. The secondary structure stability, binding affinity for thrombin and anticoagulant effects of the triazole-modified aptamers were measured. A modification in the central loop of the aptamer quadruplex resulted in increased nuclease resistance and an inhibition efficiency similar to that of TBA15. The likely aptamer-thrombin binding mode was determined by molecular dynamics simulations. Due to their relatively high activity and the increased resistance to nuclease digestion imparted by the triazole internucleotide linkages, the novel aptamers are a promising alternative to known DNA-based anticoagulant agents.

1. Introduction

DNA aptamers are increasingly recognized as drug candidates. Recently, DNA aptamers that bind to thrombin have emerged as potent inhibitors of thrombin function. These molecules are now extensively studied with respect to controlling blood clotting [1]. Currently available anticoagulants cause serious side effects, mainly due to low specificity or indirect action, and the development of new anticoagulants is of great importance due to the prevalence of cardiovascular disease. In the past two decades, a number of oligonucleotide-based thrombin inhibitors have been described [2–6], and two have entered clinical trials [7–9]. The two major problems limiting the application of the oligonucleotide aptamers to thrombin are rapid clearance from circulating blood and degradation by blood nucleases [10,11]. Rapid clearance may be advantageous in terms of tunability (reversibility) of the anticoagulation effect in a number of treatment options. However, it is important that the aptamers persist undigested for a period consistent with the desired dosing regimen. Chemical modifications (internucleotide, 2′-OMe, 2′-F and some other types) are commonly used to protect ONs from nucleases [12]. They are sometimes combined with bioconjugation (PEGylation in particular [12]) to improve pharmacokinetics and biodistribution of an aptamer, since nuclease resistance alone cannot dramatically increase its life span in blood.

The deoxyoligonucleotide TBA15 (thrombin binding aptamer), which forms an antiparallel two-tetrad G-quadruplex in solution, is the most efficient single-module unmodified DNA aptamer. Various modifications have been made to the TBA15 structure to improve its affinity to thrombin and its resistance to biodegradation [13–16]. Comparative analysis of known TBA15 analogs indicates that internucleotide modifications in the quadruplex loops are particularly promising [17–19]. Non-natural internucleotide linkages are likely to increase the biostability of aptamers [19,20].

Abbreviations: CD, circular dichroism; EDTA, ethylendiamine tetraacetic acid; EMSA, electrophoretic mobility shift assay; HPLC, high performance liquid chromatography; HR ES MS, high-resolution electrospray ionization mass spectrometry; MALDI TOF MS, matrix-assisted laser desorption/ionization-time of flight mass spectrometry; MD, molecular dynamics; MST, microscale thermophoresis; TBA, thrombin binding aptamer; Thr, thrombin; TLC, thin layer liquid chromatography; TT, thrombin time; XDA, X-ray diffraction analysis.

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Moreover, conformationally rigid linkages may restrict loop geometry in a manner that is favorable to specific aptamer activity. Triazole linkages are a type of rigid linkage and have been widely discussed due to their potential biocompatibility and pronounced effect on the hybridization properties of oligonucleotides [21–23].

In this study, we synthesized a series of DNA aptamers bearing triazole internucleotide linkages that bind to thrombin and assessed their stability, binding affinity for thrombin and anticoagulant effects.

2. Results and discussion

2.1. Synthesis and structure confirmation

We synthesized four analogs of TBA15 (A1-A4) in which triazole fragments were introduced into the central loop or in one or both TT loops and four analogs of 31TBA [24], a well-known double-module thrombin binding aptamer that contains a duplex fragment in addition to the quadruplex core (A5-A8). A5 and A7 carry triazole fragments in the duplex modules. A6 and A8 have modifications in TT loops (Fig. 1). Sequences of A5 and A6 are identical to that of 31TBA, while A7 and A8 has an 'inverted' duplex module and are generally similar to the recently reported 31TBA analog RE31 [24]. All of the modified aptamers were obtained via automated oligonucleotide synthesis using a modified dithymidine phosphoramidite block with a triazole internucleoside linkage. The modified phosphoramidite block was synthesized as described in Ref. [21].

According to CD spectroscopy (Fig. 2) and UV melting studies, all of the modified aptamers, with the exception of A7, fold into rather stable antiparallel G-quadruplexes. The UV melting curves can be found in Supplementary material, while the melting temperatures are presented in Table 1. The quadruplex module of A7 appeared to be unstable at room temperature. Interestingly, this dramatic decrease in the quadruplex melting temperature is caused by a modification in the duplex module. The quadruplex core is known to be essential for aptamer function; therefore, A7 was excluded from further investigations. The modification of the duplex module in aptamer A5 also caused destabilization, although this effect was less significant. The unfavorable effects of the introduction of triazole fragments into the duplex module are in agreement with recently published data on the hybridization properties of triazole-linked oligonucleotides. Modifications in one or both of the TT-loops (aptamers A1–A3, A6 and A8) had relatively insignificant effects on quadruplex thermostability, while modification of the central loop (A4) resulted in destabilization. It should be noted that we had to modify the sequence of the central loop in A4 to introduce the triazole fragment and the destabilization can be caused by the sequential change. All aptamers exhibited CD spectra that were generally similar to that of TBA15, with a positive band at 295 nm and a negative band at approximately 268 nm, which is characteristic of antiparallel quadruplexes. A hypochromic shift of the negative band and an increase in its amplitude was observed in the spectra of A4 and the double-module aptamers.

The monomolecular folding of the single-module aptamers was confirmed by comparing the UV melting curves obtained at different aptamer concentrations (see Supplementary material). No significant concentration dependence of the Tm values was observed. The monomolecular folding of A5–A8 was confirmed by rotational relaxation time analysis, in which the fluorescence polarization (P) and fluorescence lifetime (τ) of EtBr bound to the aptamers were measured, and the rotational relaxation time (ρ), which is proportional to the molecular hydrodynamic volume, was calculated. The obtained values for the rotational relaxation time are consistent with monomolecular folding (see Supplementary material).

2.2. Binding to thrombin

The binding affinity of the aptamers was analyzed by electrophoretic mobility shift assays (EMSA) (see Supplementary material). We labeled single-module aptamers with FAM for better visualization in these experiments. The affinities of A5 and A6 for thrombin were comparable to that of 31TBA. Other double-module aptamers demonstrated weak or no affinity for thrombin. EMSA data on the single-module aptamers were less clear. A4 was the only single-module aptamer which demonstrated significant binding. However, thrombin complexes with A4 and TBA15 are poorly seen on electropherograms, so for an accurate comparison we performed a microscale thermophoresis assay (MST) (Fig. 3). A K_D value of 97 nM ± 1 nM was determined for the TBA/Thr complex in the MST assay, in agreement with the value found in the literature [25]. The K_D value determined for A4 (127 nM ± 1.4 nM) was very close to that of TBA15. The addition of 10% blood plasma to the working buffer changed the K_D values significantly (K_D^{TBA} = 1690 nM ± 15 nM; K_D^{A4} = 3040 nM ± 65 nM), presumably due to unspecific interactions between the aptamers and plasma proteins, but the K_D^{TBA}/K_D^{A4} ratio remained below 2.

The thrombin-A4 binding mode was investigated by molecular dynamics (MD) simulations. The CD data indicate that the structure of A4 is generally close to that of TBA. Thus, we assumed that the two aptamers are likely to have similar patterns of interaction with thrombin, and our initial models of the A4/Thr complex were based on published data for the unmodified TBA/Thr complex (PDB: 1hao; PDB: 1hut). Two mutually inconsistent structures of the TBA/Thr complex have been reported: the NMR-resolved structure and the X-ray-resolved structure. In the NMR-based model (PDB: 1hao), TBA binds the Thr exosite-1 through the TT loops [26], while in the X-ray-based (XDA) model (PDB: 1hut), TBA binds through the TGT

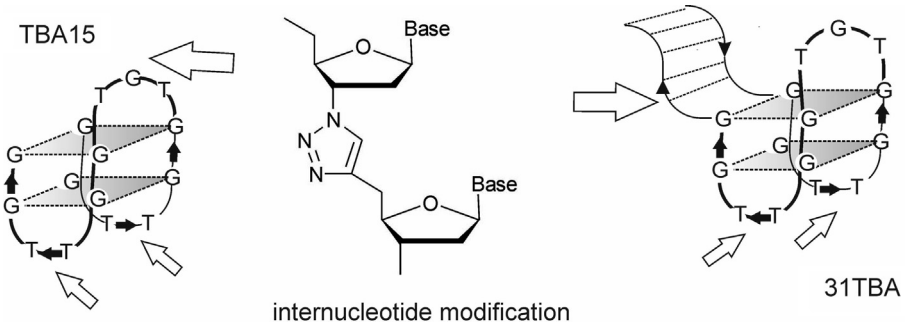

Fig. 1. Schematic representation of the thrombin binding aptamers TBA15 and 31TBA. The arrows indicate the modification positions. The modified dinucleoside fragment is shown in the center.