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Conformation and thermostability of oligonucleotide d(GGTTGGTGTGGTTGG) containing thiophosphoryl internucleotide bonds at different positions

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ABSTRACT

The thrombin-binding aptamer d(GGTTGGTGTGGTTGG) (TBA) is an efficient tool for the inhibition of thrombin function. We have studied conformations and thermodynamic stability of a number of modified TBA oligonucleotides containing thiophosphoryl substitution at different internucleotide sites. Using circular dichroism such modifications were found not to disrupt the antiparallel intramolecular quadruplex specific for TBA. Nevertheless, the presence of a single thiophosphoryl bond between two G-quartet planes led to a significant decrease in the quadruplex thermostability. On the contrary, modifications in each of the loop regions either stabilized an aptamer structure or did not reduce its stability. According to the thrombin time test, the aptamer with thio-modifications in both TT loops (LL11) exhibits the same antithrombin efficiency as the original TBA. This aptamer shows better stability against DNA nuclease compared to that of TBA. We conclude that such thio-modification patterns are very promising for the design of anticoagulation agents.

1. Introduction

Inhibitory influence on thrombin activity was found for several short, single-stranded DNA sequences. It has been reported that all the sequences have a highly conserved 15-base region d(GGTTGGTGTGGTTGG) [1], called thrombin-binding aptamer (TBA). This sequence is able to fold in monomolecular antiparallel quadruplex (Fig. 1), as was shown by NMR, X-ray and circular dichroism spectrometry [2–5]. It was discovered that such structural motif is playing a major role in an aptamer's antithrombin activity [5]. However, practical applications of TBA are hampered by it's extremely short in vivo half-life, estimated to be ~100 s [6]. One way to overcome this problem is to use thiophosphoryl modification of TBA, which is a common approach to increase in vivo stability of synthetic oligonucleotides, since a sugar–phosphate backbone modification improves oligomers' resistance to various nucleases [7,8]. Nevertheless, these modifications can alter the spatial structure and target affinity of the oligonucleotides as well as potentially lead to system toxicity.

The completely thio-modified oligonucleotide (SATR) had been shown to maintain the G-quadruplex conformation [9]. However, SATR exhibits a high system toxicity, which could be decreased if a lesser number of phosphorothioate modifications would be introduced in the backbone. Our goal was to design TBA-derived oligonucleotides with a few numbers of phosphorothioates in such positions, that G-quadruplex structure and stability would be practically unaffected. With this in mind, we studied the influence of a number and localization of the backbone phosphorothioate modifications and its stability. Besides that, all synthesized oligonucleotides were preliminarily evaluated for their stability in blood serum and more importantly for their antithrombin properties.

2. Materials and methods

2.1. Phosphodiester and phosphorothioate oligonucleotides

In this study phosphodiester oligonucleotide TBA with sequence d(GGTTGGTGTGGTTGG) and 13 other oligonucleotides with different local phosphorothioate modifications were used (Fig. 1). The modifications were localized between guanines of G-quartets in P1011, P4, P1415, P111 and P511 oligonucleotides, or in the loops connecting G-quartets in L1, L11, L121, LL11 and LL (Fig. 1). Phosphorothioate modifications in oligonucleotides L343, L3 and SATR were localized as within G-quartets, as in the loops (Fig. 1). All phosphodiester and thiophosphoryl oligonucleotides were synthesized as in [10], purified by reverse-phase HPLC, and

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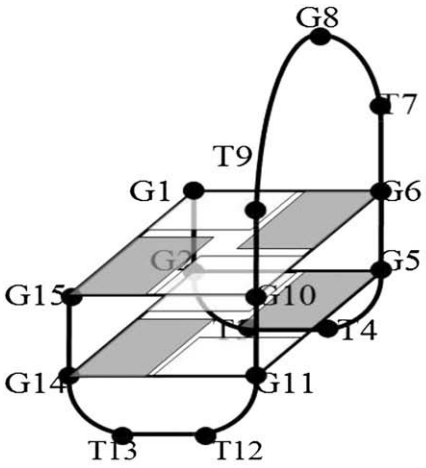

TBA	d(GGTTGGTGTGGTTGG)
Modification in plane only:	
P1011	d(GGTTGGTGTGsGTTGG)
P4	d(GsGTTGsGTGTGsGTTGsG)
P1415	d(GGTTGGTGTGGTTGsG)
P111	d(GsGTTGGTGTGsGTTGG)
P511	d(GGTTGsGTGTGsGTTGG)
Modification in loop only:	
L1	d(GGTTGGTGTGGTsTGG)
L11	d(GGTsTGGTGTGGTsTGG)
L121	d(GGTsTGGTsGTGGTsTGG)
LL11	d(GGsTsTGGTGTGGsTsTGG)
LL	d(GGsTsTGGTsGTsGTGGsTsTGG)
Mixed modification:	
L343	d(GGTsTsGGTsGTsGGTsTsGG)
L3	d(GGTsTsGGTGTGGTTGG)
SATR	d(GsGsTsTGGTsGTsGGTsTsGG)

Fig. 1. Scheme of G-quadruplex structure of TBA. The oligonucleotide sequences with thiophosphoryl modifications. Mark "s" denotes the thiophosphoryl internucleotide bond.

desalted. The number of thiophosphoryl bonds in oligomers was verified by MALDI TOF MS (Matrix assisted laser desorption ionization time-of-flight mass-spectrometry).

The concentration of each oligonucleotide in water solution was determined at 90 °C using the calculated extinction coefficient 158,100 $M^{-1}cm^{-1}$ at 260 nm [11]. For all further experiments 5 μM oligonucleotide solutions in PBS (10 mM K-phosphate, pH 7.4, 0.1 M KCl) were utilized. The solutions were heated at 95 °C for 15 min and quickly cooled on ice.

2.2. Absorption spectra and melting curves

Absorption spectra and UV melting curves were recorded using Jasco V-550 spectrophotometer with thermostated cuvette holder. Melting curves were registered at λ = 295 nm in the 15–90 °C temperature range. Thermodynamic parameters were defined by fitting procedure using the two-state model for monomolecular melting [12].

2.3. Fluorescence polarization and lifetime of EtBr bound to TBA oligonucleotides

The fluorescence polarization (P) of ethidium bromide (EtBr) bound to TBA oligonucleotides was measured with Cary Eclipse spectrofluorimeter at the temperature 3 °C. Excitation wavelength was 540 nm and the fluorescence intensity was registered at 610 nm (I_{610}). The fluorescence polarization P was calculated using the equation [13]:

$$P = (I_{||} - I_{\perp})/(I_{||} + I_{\perp}). \quad (1)$$

The vertical $I_{||}$ and horizontal $I_{\perp}$ components of bound EtBr fluorescence intensity were measured at excitation by the vertically polarized light. The free dye contribution was taken into account as described elsewhere [14]. Concentration of EtBr was 1 μM, concentration of TBA oligonucleotides — 5 μM.

The fluorescence lifetime (τ) of EtBr:TBA complexes was evaluated using Easy Life V. Fluorescence decay was registered through a RG610 long pass filter at excitation LED 525 nm.

2.4. Rotational relaxation times of EtBr:TBA complexes

Rotational relaxation time (ρ) for the EtBr:TBA complexes was estimated using the Perrin–Weber equation, valid for small particles having spherical or low-asymmetrical ellipsoidal shape [13]:

$$\rho = 3\tau(1/P_o - 1/3)/(1/P - 1/P_o). \quad (2)$$

P is the observed polarization, and $P_o = 41 \pm 1\%$ is its limiting value in the absence of rotational depolarization; τ is the fluorescence lifetime of adsorbed EtBr on TBA oligonucleotides, see also [14–16].

2.5. Circular dichroism

CD spectra of oligonucleotides were registered with a Jasco 715 spectropolarimeter using a thermostated cell. The CD values ($\Delta\varepsilon$) are given per moles of nucleotides. CD spectra were registered in 220–330 nm wavelengths and the 15–80 °C temperature range.

2.6. Impact of oligonucleotides on blood coagulation (thrombin time)

Thrombine time (TT) was measured according to the published procedure [17] and the protocols of an assay kit manufacturer ("Thrombin-TEST", Renam, Russia). Clotting times for the TT assay were initiated when 100 μl of citrate-stabilized serum was pipetted and incubated for 120 s. The last step – the addition of oligonucleotide to the final concentration of 0.1–1.5 pmol/μl – activated the final measurement with a coagulation analyzer (MiniLab-701, Unimed, Russia).

2.7. Oligonucleotide stability in blood serum

Citrate-stabilized serum specimens were obtained from healthy individuals, separated into 200 μl aliquots and stored at −20 °C until used. The hydrolysis was started by adding to the serum aliquot the mixture of the investigated oligonucleotide and TBA as an internal reference to the final concentration of 2 pmol/μl for each of them. Serum specimens were incubated at 37 °C for 120 s, followed by addition of the oligonucleotide–antidote A (5'dCCAACCACACCAACC) solution in PBS buffer (pH 7.4), which was added to the final concentration of 4–6 pmol/μl. Subsequently, after 30 s 1.5 ml of acetone