BIOPHYSICS AND BIOCHEMISTRY

Anticoagulant Effects of Thioanalogs of Thrombin-Binding DNA-Aptamer and Their Stability in the Plasma

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In order to create effective therapeutically significant oligonucleotide structures, a series of analogs of thrombin-binding aptamer d(GGTTGGTGTGGTTGG) containing thiophosphoryl substitutions were synthesized. The anticoagulation effects of the resultant thrombin-binding aptamer analogs were evaluated and the effects of local thiomodifications on their structure and function were studied, including the effects on stability in blood plasma and resistance to DNA nucleases. A promising modified oligonucleotide (LL11) was found with the structure modified only in TT loops. It retains antithrombin properties of thrombin-binding aptamer, but is more resistant to biodegradation.

Key Words: *DNA aptamers; oligonucleotide thiophosphoryl analogs; anticoagulation effects; MALDI mass spectrometry*

One of the problems in development of effective oligonucleotide drugs with targeted action is the problem of rapid biodegradation of these drugs. A possible solution is creation of structural analogs of oligonucleotides [1,7], including those containing the sucrose-phosphate base modifications [8,10].

Thiophosphoryl analogs of oligonucleotides, their application and metabolism were studied best of all [2,8,10]. These derivatives are sufficiently stable in biological fluids, membranotropic, and retain hybridization characteristics of natural DNA. On the other hand, completely thiophosphorylated oligonucleotides are characterized by high systemic toxicity [2,8,10] and therefore, the search for candidates resistant to biodegradation is carried out only among partially modified and hence, less toxic oligonucleotide sequences. It was shown, for example, that replacement of just two 3'-terminal internucleotide bonds with thiophosphoryl ones prolongs the lifespan of antisense oligonucleotides *in vivo* [2].

The use of antisense oligomers resistant to enzyme hydrolysis proved to be in general effective [2, 8]. However, in cases when their functional characteristics are due to primarily the tertiary structure of the oligonucleotide chain (for example, DNA aptamers), thiophosphoryl substitution led to failures [12,13]. The main cause of this result was the appearance of conformation distortions leading to loss of the targeted characteristics [5,6,11].

The task of this work was analysis of the stability of modified thioanalogs of thrombin-binding aptamer (TBA) under conditions of RQ1 DNA nuclease treatment, their degradation in blood plasma, and anticoagulation characteristics.

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A well-known TBA d(GGTTGGTGTGGTTGG) [9,13] was selected as the model. In solution it exists as a intramolecular G-quadruplex (DNA strand is folded 4 times, the structure consists of 2 guanine quartets stabilized with hydrogen bonds; Fig. 1, *a*). It is a highly selective thrombin inhibitor. However, short lifespan of TBA in the blood prevents its practical use. In order to detect the stabilizing modifications not reducing biological activity of G-quadruplex oligonucleotides, we obtained a set of TBA thioanalogs. Some of them were selectively thiophosphorylated in the loop regions, others in the TBA steric structure planes (Fig. 1, *a*). Sequences and main characteristics of TBA analogs are presented in Table 1.

MATERIALS AND METHODS

Oligonucleotides were synthesized by solid phase amidophosphite method on an ASM 800 DNA synthesizer (Biosset) using modified reaction cycle programs developed previously [3]. Standard commercial reagents were used: nucleoside, nucleoside polymer, and 3H-1,2-benzodithiol-3-ONE-1,1-dioxide amidophosphite derivatives (Glen Research) and solvents (Lekbiofarm, Panreac). The resultant oligomers were characterized by UV spectrophotometry and matrix-assisted laser desorption ionization (MALDI) mass spectrometry as described previously [3], analyzed by high pressure liquid chromatography and PAAG electrophoresis.

Oligonucleotide melting temperature was evaluated as described previously [5] using Jasco 715 spectrometer with temperature-controlled cuvette holder with a Peletier element.

Oligonucleotide solution (10-30 nmol/ml) in buffer (40 mM Tris-HCl, 10 mM $MgSO_4$, 1 mM $CaCl_2$, pH 8.0) was incubated at 95°C for 3 min and rapidly cooled on ice. After addition of RQ1 RNAse-Free DNAse (Promega), the mixture was incubated at 37°C. The samples were desalted and analyzed by MALDI mass spectrometry as described below.

The effects of oligonucleotides on blood clotting rate were evaluated by thrombin time (TT) according to the standard procedure and Thrombin-Test (Renam) kit protocol on a Minilab-701 coagulometer (Unimed). In order to obtain the plasma, donor blood was mixed with 3.8% sodium citrate (9:1 volume proportion) and centrifuged (10 min, 1600*g*). Oligonucleotide solution was added to plasma specimen simultaneously with thrombin solution, the resultant oligomer concentration in the mixture was 0.1-1.5 nmol/ml. The relationship between the recorded TT values and thrombin activity was evaluated using a calibration curve plotted for thrombin concentrations of 0.1 to 6.0 U.

Oligonucleotide stability in the plasma was then evaluated. Oligonucleotide mixture was added to 0.2 ml citrate-stabilized plasma (the concentration 2 nmol/ml for each oligomer) and the samples were incubated at 37°C. After incubation the oligonucleotide antidote solution ATCA (5'-CCAACCACACCAACC) in phosphate buffer (pH 7.4) was added to the plasma to the final concentration of 4-6 nmol/ml. After 30 sec, 1.5 ml acetone (Merck) was added to the

TABLE 1. Sequences and Main Characteristics of TBA Thioanalogs

Name	Modified oligonucleotide sequence	Number of thiophosphoryl substitutions	Molecular weight, Da	Melting temperature, °C
TBA	GGTTGGTGTGGTTGG	0	4726	52
P4	gGTTgGTGTgGTTgG*	4	4790	40
SATR	ggttggtgtggttgG	14	4950	45
P511	GGTTgGTGTgGTTGG	2	4758	46
P111	gGTTGGTGTgGTTGG	2	4758	47
P1011	GGTTGGTGTgGTTGG	1	4742	49
L3	GGttgGTGTGGTTGG	3	4774	52
LL11	GgttGGTGTGgttGG	6	4822	54
L1	GGTTGGTGTGGtTGG	1	4742	55
L11	GGtTGGTGTGGtTGG	2	4758	55
LL	GgttGgtgtGgttGG	10	4886	55
L121	GGtTGGtgTGGtTGG	4	4790	56

Note. *Capital letters show 3'-thiophosphoryl nucleoside components. **L shows modifications in the TBA loop region, P in G-quartet plane regions (Fig. 1, *a*).

mixture, shaken, and incubated for 2 h at -20°C. The precipitate was separated by centrifugation (20 min, 14,500 rpm) and resuspended in 0.2 ml water. The supernatant was concentrated in vacuum to the volume of 0.4-0.5 ml and analyzed by mass spectrometry.

Mass spectrometric analysis was carried out as follows. The measurements were carried out on a Microflex time-jet MALDI mass spectrometer (Bruker Daltonics, Billerica) in the linear mode for positive ions, at accelerating voltage of 20 kV, accumulating voltage 18.65 kV, and 400 nsec delay. Each spectrum was obtained by accumulation from 500 laser pulses from different positions in specimen's spot. The specimens were prepared as follows. Oligonucleotides were separated from residual admixture of plasma proteins and peptides or from endonuclease reaction mixture in the SAX Zip Tip disposable cation exchange microcolumns (Millipore, Billerica) with subsequent concentration/desalination in C-18 Zip Tip reverse phase microcolumns (Millipore, Billerica). At the last stage, the eluate containing the analyzed products was mixed with the matrix (0.25 M water solution of 3-hydroxy-

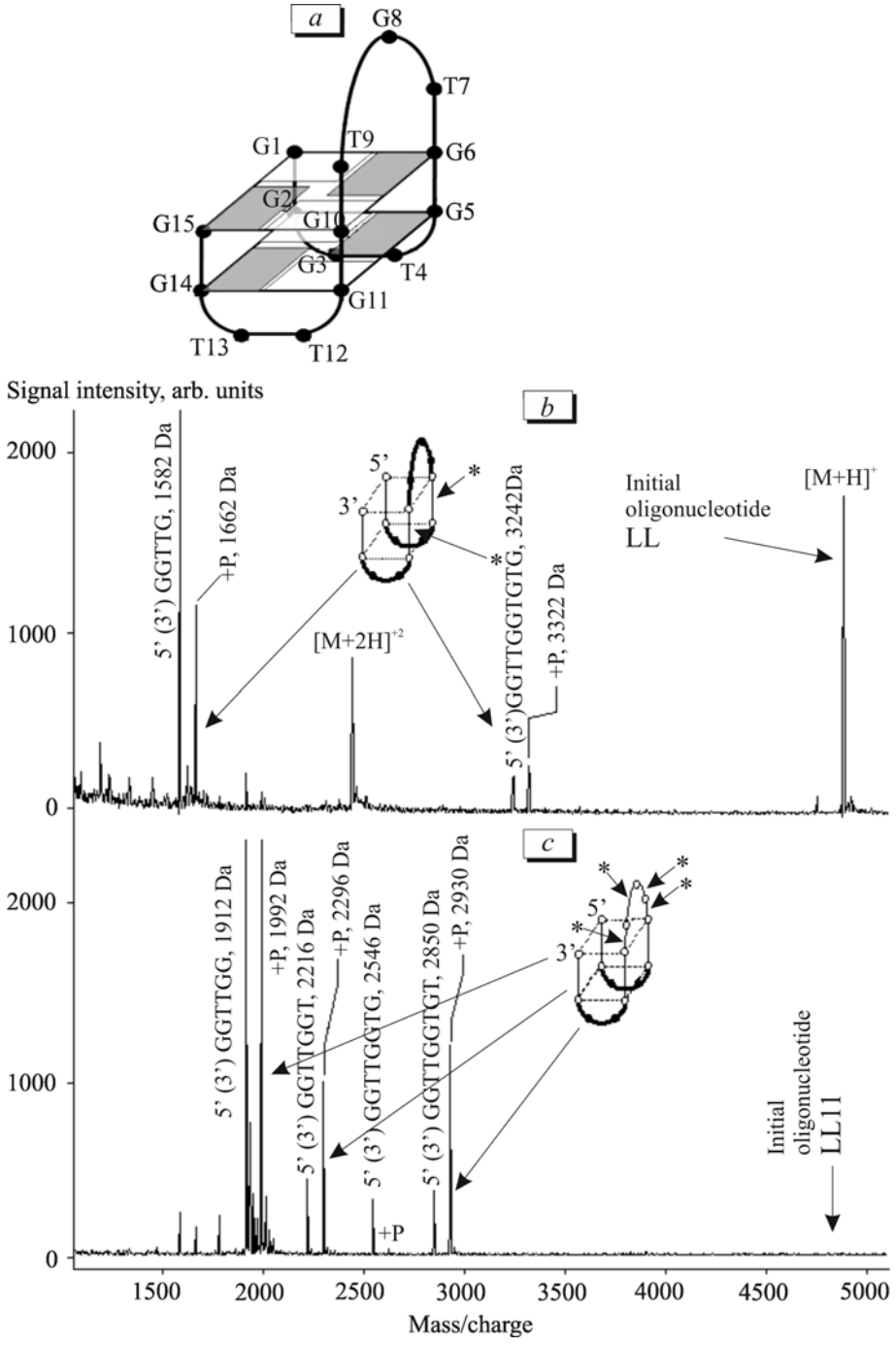

Fig. 1. TBA d(GGTTGGTGTGGTTGG) intramolecular G-quadriduplex structure (*a*) and TBA thioanalog hydrolysis product mass spectra under the effect of RQ1 endonuclease treatment (12 h, 37°C): LL (*b*) and LL11 (*c*). *Positions of predominant cleavage of oligonucleotides.

picolinic acid) and dried in air flow on the MALDI target.

RESULTS

In order to select TBA analogs most resistant to biodegradation and retaining the inhibitory characteristics, the melting temperatures of synthesized oligonucleotides were compared. The most stable synthesized oligomers with the maximum melting temperatures of intramolecular structures were selected for further studies: L3, LL11, L1, L11, LL, and L121 (Table 1). Interestingly, all of them had modifications only in the G-quadruplex loops.

The stability of the selected TBA analogs under conditions of nuclease treatment was evaluated by enzymatic hydrolysis with RQ1 DNAse. MALDI mass spectrometry proved to be the most informative method for analysis of the hydrolytic mixture composition. This method is characterized by higher sensitivity than electrophoresis or chromatography and identifies the oligomer fragments, that is, provides additional structural information on the nuclease effects on the TBA thioanalogs. According to mass spectrometry of hydrolysis products (Fig. 1, *b*, *c*), the loop region was most liable to cleavage. The most intense signals in the LL hydrolysis products spectrum (Fig. 1, *b*) corresponded to fragments 5'(3')-GGTTG (1582 Da) and 5'(3')-GGTTGGTGTG (3242 Da) and their phosphorylated derivatives (1662 and 3322 Da, respectively). The presence of these derivatives is explained by symmetrical structure of the quadruplex and the fact that nuclease cleavage starts from 3'- and 5'-terminals. As a result, a pair of fragments of the same composition forms, one of them containing the terminal phosphate group. Similar pairs of fragments, differing by 80 Da (phosphate group), are observed for LL11 analog (Fig. 1, *c*), also symmetrical. The LL oligomer, in which all loops are protected by thiogroups, is cleaved only by internucleotide bonds in the quartet planes. By the end of hydrolysis (12 h), an appreciable part of the initial oligonucleotide (4887 Da, $[M+H]^+$) remained intact (Fig. 1, *b*). The LL11 analog with intact large loop (TGT; Fig. 1, *c*) was cleaved completely during the same period (the initial signal disappeared). Characteristic large loop hydrolysis products (1912, 2216, 2545, and 2850 Da) and their phosphorylated pairs were mainly observed. These data emphasize good prospects of modifications of the G-quartet loop regions in construction of bioactive analogs.

Comparison of antithrombin characteristics of modified TBA aptamers is the most important step in screening for their optimal structures. The mechanism of anticoagulant activity of TBA is based on its capacity to form a strong complex with thrombin,

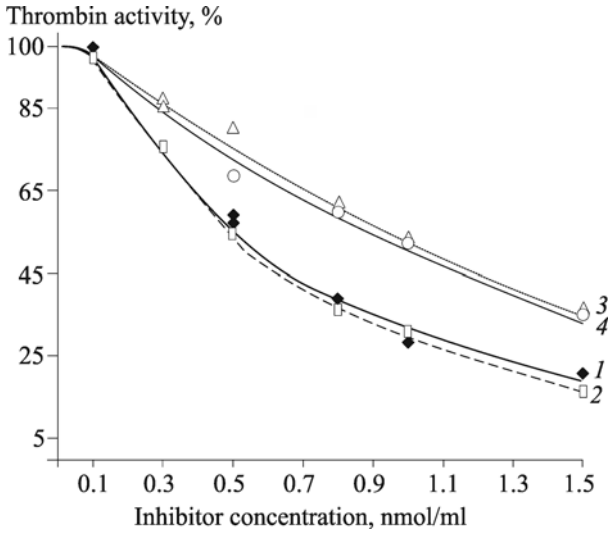

Fig. 2. Inhibition of activity of thrombin (6.0 arb. units/ml) added to blood plasma with oligonucleotides TBA (*1*), LL11 (*2*), LL (*3*), and P4 (*4*).

which leads to blockade of the entire clotting cascade [9]. The effects of addition of TBA and synthetic oligomers to blood plasma on clotting were studied by evaluating TT (a standard medical parameter). Oligomers were added to plasma in concentrations of 0.1-0.8 nmol/ml. Thrombin activity in this oligonucleotide concentration was evaluated by interpolation from the TT–thrombin concentration calibration curve. As we expected, completely modified TBA analog (SATR) did not inhibit the formation of the clot, but accelerated it, in comparison with the sample without oligonucleotide. This can be due to well-known hydrophilic characteristics of thio-oligomers and their capacity to nonspecifically bind various proteins [2,4,8,10]. Only some of the studied oligomers were close to the initial TBA aptamer by TT values. The rest analogs exhibited a rather low anticoagulant activity, for example, LL oligomer containing 10 thiophosphoryl groups and P4 oligomer with unstable conformation. The studied oligonucleotides can be ranked by their antithrombin activity as follows: SATR<<P4<L11<LL11=TBA. Typical concentration-dependent effects of L11, P4, LL11, and TBA inhibitors on activity of thrombin added to plasma are presented in Fig. 2. It is obvious that oligomer LL11 modified by only the quartet TT loops is the most close to the initial TBA.

Model studies of enzymatic cleavage of aptamers showned the sites most sensitive to hydrolysis in the TBA quartet structure. Hydrolysis velocities of thioanalogs and TBA in blood plasma were then compared. Special methods for isolation and analysis of oligomers from blood plasma were developed, compatible with the MALDI mass spectrometry requirements. Fluorescent-labeled oligonucleotides of

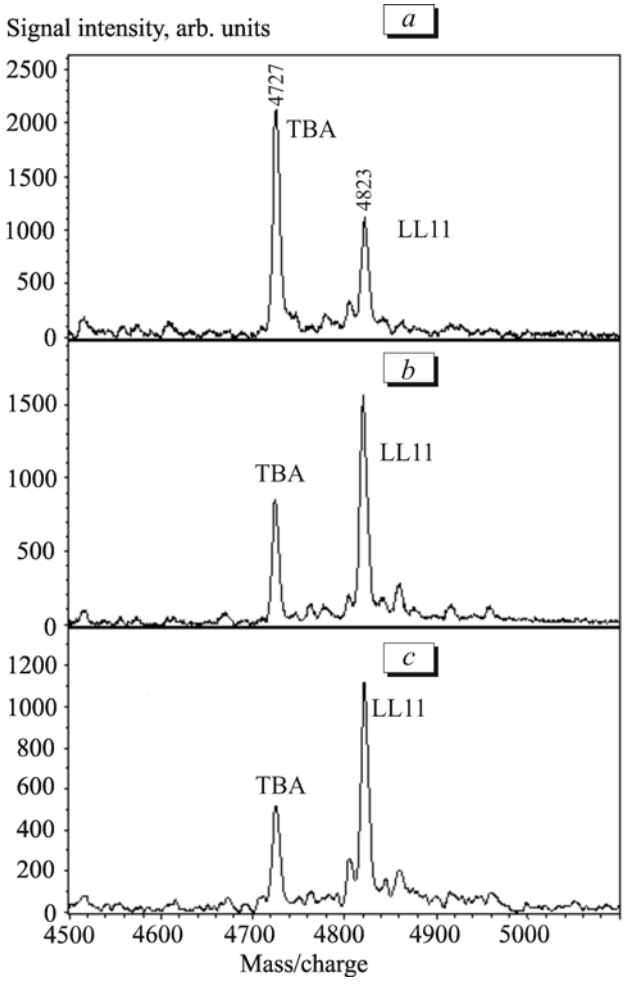

Fig. 3. Mass spectra of TBA oligonucleotide mixture and LL11 isolated from blood plasma directly after mixing (*a*) and after incubation at 37°C for 1 (*b*) and 5 min (*c*).

14-26 components were used for optimization of these methods, due to which all steps of isolation were visualized. An excess of oligomer antidote complementary to TBA was used for more complete destruction of the aptamer-thrombin complex. Oligomer mixtures isolated after incubation with blood plasma were desalinated and analyzed by MALDI mass spectrometry. It is noteworthy that the developed method for evaluation of oligonucleotide stability in blood plasma can become the base for future pharmacokinetic studies.

Mass spectra for TBA oligomer mixture (4627 Da, $[M+H]^+$) and LL11 (4823 Da, $[M+H]^+$) incubated with human plasma were obtained. Isolation was carried out directly (0 min, Fig. 3, *a*), after 1-min exposure (Fig. 3, *b*), and after 5-min exposure (Fig. 3, *c*) at 37°C. The initial proportion of TBA/LL11 signal values was ~1.5:1 (Fig. 3, *a*), while after just 5 min it decreased to ~0.4:1 (Fig. 3, *c*). These data indicate a significantly slower hydrolysis of LL11 modified oligomer in the plasma in comparison with TBA.

Hence, the data indicate that the LL11 oligonucleotide structure with modified loops is prospective for further studies. Importantly, this oligonucleotide is characterized by antithrombin properties at the TBA level and by better resistance to degradation under the effect of DNA nuclease. The regularities revealed in our study are important for targeted construction of other therapeutically important oligonucleotides, for example, of G-quadruplex conformation.

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