Characteristics of a New DNA Aptamer, Direct Inhibitor of Thrombin

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Characteristics of a new antithrombin DNA-aptamer RE31 were studied. This aptamer inhibited thrombin formation in human plasma catalyzed by exogenous (lengthening of thrombin time) and endogenous thrombin (lengthening of partial prothrombin time and activated partial thromboplastin time). In addition, the aptamer completely suppressed thrombin-induced aggregation of human platelets. On the other hand, RE31 did not reduce amidolytic activity of thrombin towards the short peptide substrate, in other words, did not modify the state of enzyme active center. By the capacity to inhibit clotting reactions, RE31 was superior to the previously described highly effective 31-component antithrombin aptamer 31TBA (thrombin binding aptamer, TBA). The effect of RE31 was species-specific: it inhibited human thrombin activity more effectively than activities of rat and rabbit thrombins.

Key Words: *thrombin; aptamers; fibrinogen; blood clotting; platelets*

Aptamers are DNA or RNA oligonucleotides (up to several tens bases long) forming complex 3D structures and due to this specifically interacting with numerous target molecules, from small ligands to large proteins. Some aptamers not only recognize their targets, but also inhibit their activities. Aptamers are first selected from random sequences library by binding to the target molecule and then purposeful design and/or modification of the selected aptamer is carried out, if necessary. By their affinity for protein targets, the aptamers are comparable to antibodies. However, in contrast to antibodies (polypeptides), the oligonucleotide aptamers are nonimmunogenic compounds. By the present time, aptamers are regarded as potential pharmacological substances, which can be used for drug creation [2,6,11].

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A possible pharmacological applications of aptamers is creation on their basis of an antithrombotic drug (direct inhibitor of thrombin) [3,9]. The most widely used antithrombin drugs, heparin and its low-molecular derivatives, inhibit thrombin functions by stimulating antithrombin III. Direct thrombin inhibitors modulate the enzyme proper. Bivalent inhibitors, such as hirudin and its derivatives (bivalirudin), inhibit thrombin activity by interacting with active center and substrate binding site (exosite 1), while monovalent synthetic inhibitors (argathrobane, dabigatrane) inhibit only its active center. In addition, in contrast to heparin, direct inhibitors do not interact with other plasma and cellular proteins and cause no such untoward effect as thrombocytopenia [5]. The first antithrombin DNA aptamer was obtained [4]. This thrombin-binding aptamer (TBA) consists of 15 nucleotides (15TBA, 15-mer, or HD1 aptamer) and includes eight conservative guanine (G) residues, forming the so-called G-quartet structure. In thrombin molecule, 15TBA binds to exosite 1 responsible for its reactions with sub-

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strates, fibrinogen and cellular PAR (protease activated receptors). Due to this it inhibits thrombin-catalyzed transformation of fibrinogen into fibrin and thrombin-induced platelet aggregation without directly modulating the active center of the enzyme [4,10]. Numerous analogs were created on the basis of 15TBA sequence. Some of them bind thrombin with high affinity and more effectively inhibit its activity [1,8].

We studied the capacity of RE31, a new antithrombin DNA aptamer, to inhibit thrombin-stimulated clotting reactions and platelet aggregation. The characteristics of RE31 were studied by comparing the new aptamer to previously described 31-component aptamer (31TBA). 31TBA includes 15TBA sequence and is one of the most effective antithrombin aptamers known by the present time [1,8].

MATERIALS AND METHODS

Aptamers 31TBA (CACTGGTAGGTTGGTGTGGTTGGGGCCAGTG) and RE31 were synthesized on Applied BioSystems 380b automated synthesizer. Human, rat (Haematologic Technologies Inc.), and rabbit (Sigma) thrombins with specific activities of 4400, 2500, and 2900 U/mg protein, respectively, were used.

Thrombin, prothrombin, and activated partial thromboplastin time (APTT) were measured on STA-compact analyzer (Diagnostica Stago) using human plasma pool as described previously [1]. Thrombin time was evaluated using human thrombin; in some experiments, rat and rabbit thrombins were used (thrombin solutions with initial activity of 6 unit/ml were used in all cases). STA Neoplastin Plus and STA APTT reagents (Diagnostica Stago) were used for measuring prothrombin time and APTT, respectively. Amidolytic activity of thrombin was analyzed on an FP-910 analyzer (Labsystems) by hydrolysis of the substrate Tos-Gly-Pro-Arg-pNA Chromozym TH (Boehringer Mannheim GmbH) [1]. Hydrolysis rate was evaluated by increase in optical density of the incubation mixture at 405 nm/min (ΔA_{405}/min). Thrombin-induced aggregation of human platelets was registered by changes in light transmission (T%) of the platelet suspension on a BIOLA aggregometer (BIOLA) [1]. The maximum aggregation (maximum T%) was evaluated by analysis of aggregation curves.

RESULTS

The characteristics of the new antithrombin DNA aptamer RE31 were compared to those of 31TBA aptamer. Both aptamers added to human plasma inhibited clotting activity of thrombin by lengthening thrombin time, prothrombin time, and APTT (Fig. 1). Measurements of thrombin time are used to evaluate the rate of

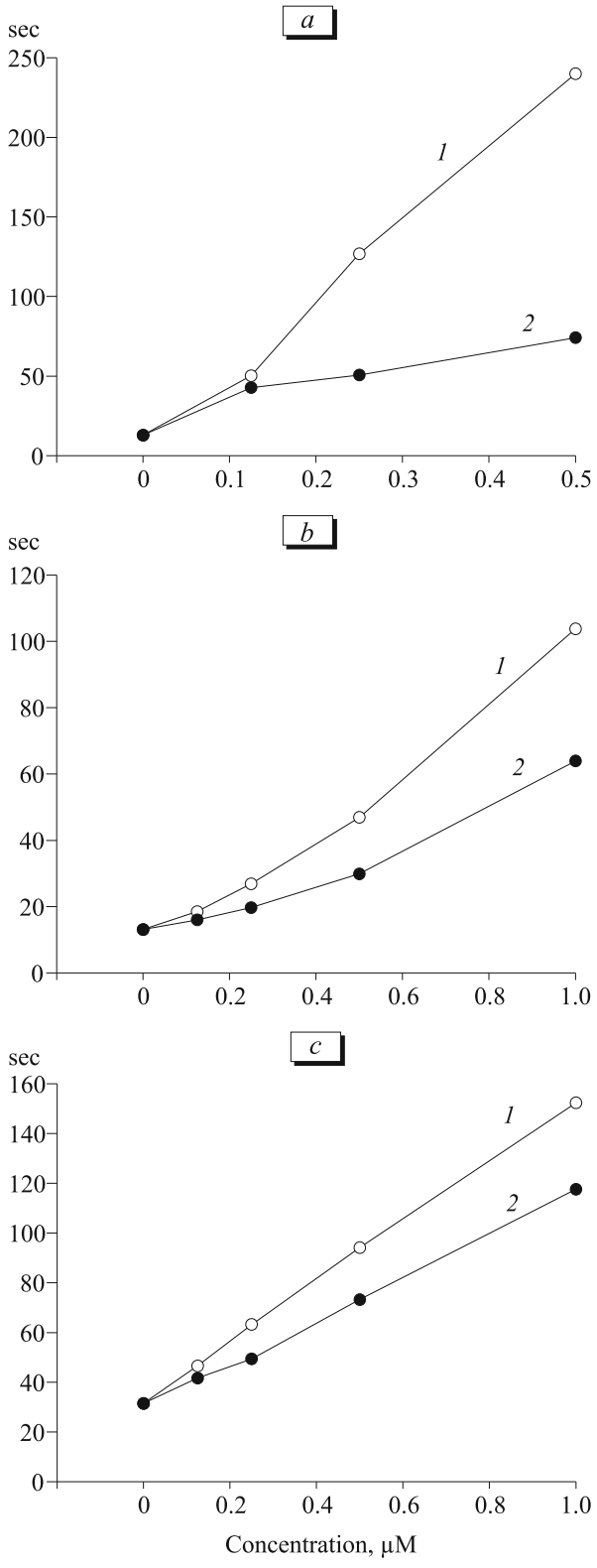

Fig. 1. Effects of RE31 and 31TBA aptamers on human plasma clotting. Aptamers RE31 (*1*) and 31TBA (*2*) were added in the specified concentrations to human plasma and thrombin time (*a*), prothrombin time (*b*), and APTT (*c*) were recorded in the presence of human thrombin. The time of fibrin clot formation was recorded in all tests. Results of 1 of 3-4 reproducible experiments are presented.

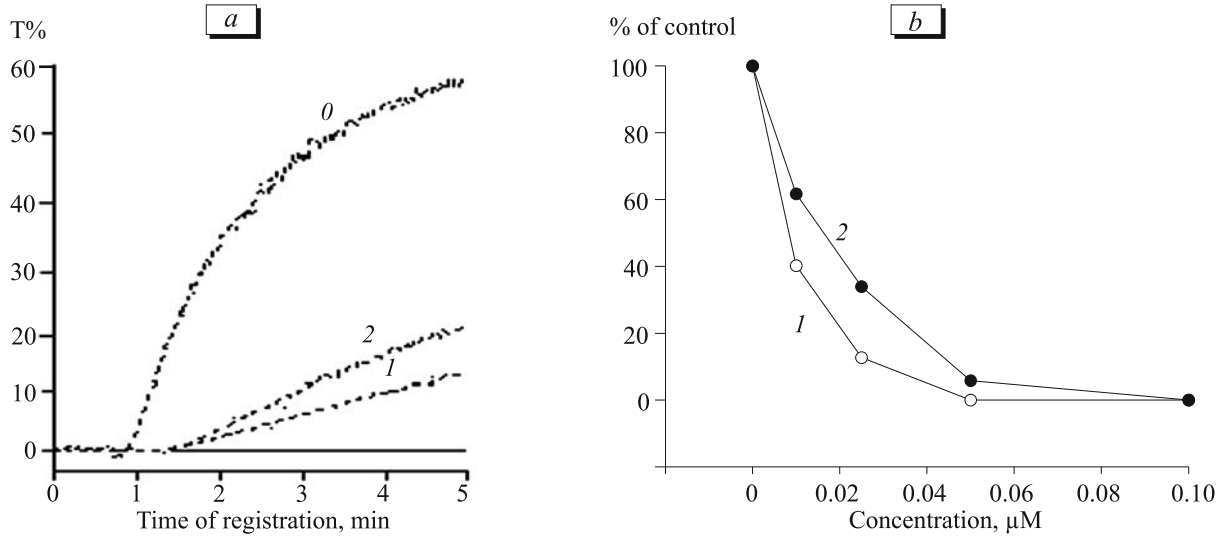

Fig. 2. Inhibition of thrombin-induced human platelet aggregation in the presence of RE31 and 31TBA aptamers. *a*) no aptamers added to washed platelets (*0*) or 0.025 μM RE31 (*1*) or 0.025 μM 31TBA (*3*) added. Platelet aggregation stimulated by addition of 0.25 U/ml human thrombin (added 30 sec after the beginning of light transmission recording). *b*) platelet aggregation stimulated by 0.25 U/ml thrombin without aptamers and in the presence of different concentrations of RE31 (*1*) and 31TBA (*2*). The maximum level of aggregation was evaluated in % of control (100% without aptamers). The results of 1 of 2 reproducible experiments are presented.

fibrin clot formation catalyzed by exogenous thrombin while measurements of prothrombin time and APTT show the rate of this clot formation catalyzed by endogenous thrombin, forming as a result of stimulation of the blood clotting system. The clotting cascade of reactions in the prothrombin time test is triggered in the plasma by the exogenous pathway, while in the APTT test the endogenous pathway is used. Inhibitory activity of RE31 aptamer is higher than that of 31TBA: in all three tests similar effects (prolongation of plasma clotting time) were observed at lower concentrations of RE31. We previously showed that 31TBA, similarly as another shorter aptamer 15TBA with the G-quartet structure in its composition, does not modify thrombin amidolytic activity [1]. Aptamer RE31 is also based on the G-quartet and does not modify thrombin capacity to cleave short peptide substrates. The rate of chromogenous substrate Tos-Gly-Pro-Arg-pNA cleavage was the same without and with 1 μM RE31: 0.259 and 0.253 ΔA_{405}/min, respectively (mean values from 2 experiments). Similarly as 31TBA, RE31 suppressed thrombin-induced platelet aggregation. The inhibitory effect of RE31 in this case manifested at somewhat lower concentrations (Fig. 2). The effect of RE31 aptamer on thrombin was species-specific. Prolongation of thrombin time in the presence of RE31 after stimulation with human thrombin was significantly more pronounced than after stimulation with rat thrombin (at some concentrations of the aptamer, 3- to 4-fold differences were observed), and no inhibitory effect of the aptamer in the studied concentrations was observed in the presence of rabbit thrombin (Fig. 3). In control specimens (without aptamer),

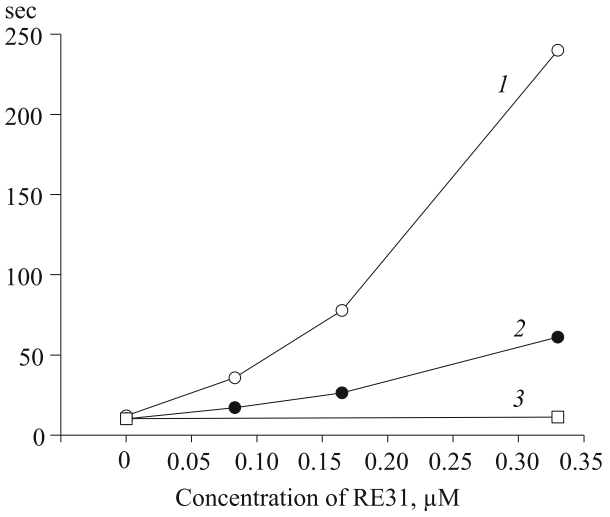

Fig. 3. Effect of RE31 aptamer on human plasma clotting, stimulated by human, rat, and rabbit thrombins. Aptamer RE31 was added to the plasma in the concentrations shown and thrombin time of the clot formation was recorded after addition of human (*1*), rat (*2*), and rabbit (*3*) thrombin.

the clot formation time was about the same with all thrombins (about 10 sec).

Hence, the new DNA aptamer RE31, similarly as other aptamers designed on the basis of the G-quartet structure, inhibits clotting activity of thrombin and thrombin-induced platelet aggregation. It seems that this aptamer also binds to exosite 1 of the thrombin molecule and prevents its interaction with fibrinogen and platelet PAR without modulating the active center and proteolytic effect of the enzyme towards short substrates. Aptamer RE31 is species-specific: it more

intensely inhibits activity of human thrombin that of rat and rabbit thrombin. These data confirm previously noted similarity of the aptamer and antibody characteristics [2,3,9], in this case, their high specificity towards the target. By the capacity to inhibit thrombin activity, RE31 is superior to 31TBA, one of the most effective previously described antithrombin aptamers [1,8]. The results suggest using RE31 as the basis for creation of a new drug, direct inhibitor of thrombin. However, it should be borne in mind that aptamers, specifically, antithrombin DNA aptamers, are very rapidly eliminated from circulation [7], and therefore, in order to create the drug, basic oligonucleotide aptamer has to be modified in order to prolong its life span in the circulation.

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