Aptamer RA36 Inhibits of Human, Rabbit, and Rat Plasma Coagulation Activated with Thrombin or Snake Venom Coagulases

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 156, No. 7, pp. 54-58, July, 2013
Original article submitted June 29, 2012

RA36 DNA aptamer is a direct anticoagulant prolonging clotting time of human, rabbit, and rat plasma in the thrombin time test. Anticoagulant activity of RA36 is lower than that of recombinant hirudin. During inhibition of human plasma clotting activated with echitox (coagulase from *Echis multisquamatus* venom), the aptamer presumably binds to meisothrombin exosite I. The sensitivity of human plasma to the aptamer 5-fold surpasses that of rat plasma. Analysis of RA36 binding to coagulase of *Agkistrodon halys* venom (ancistron) is required for proving the effect of aptamer on polymerization of human fibrinogen.

Key Words: *aptamer; plasma coagulation; thrombin; snake venom coagulases*

Aptamers are synthetic DNA or RNA oligonucleotides specifically interacting with the target molecule [8]. The DNA aptamer binds to and inhibits activity of thrombin (blood clotting system serine protease), involved in catalysis of soluble fibrinogen transformation into one of the clot bases - insoluble fibrin [3]. In addition to the active center, thrombin has a positively charged substrate-binding exosite I. Heparin-binding exosite II is located on the thrombin molecule side contralateral to exosite I; heparin fixation facilitates thrombin interactions with antithrombin (blood clotting system serine protease plasma inhibitor).

Laboratory tests for evaluation of human plasma clotting are carried out with coagulation activators thrombin, thromboplastin, $CaCl_2$ solution, *etc.* [1]. Central Asian snake venoms contain enzymes with amidolytic and proacogulant activities (coagulases) [13]. Ecarin clotting time is used in clinical practice for monitoring preventive courses and therapy with native or recombinant hirudins (RH) [5]. *Echis carinatus* venom coagulase ecarin used in this test catalyzes prothrombin transformation into meisothrombin (mIIa), autocatalytically transformed into mIIa (desF1). Both meisothrombins are involved in the clot formation.

Prolongation of human plasma clotting time in the reptilase (batroxobine) time (with *Bothrops atrox* snake venom coagulase for clotting activation) can indicate inhibition of fibrinogen transformation into fibrin. The clot emerges despite the fact that reptilase added to the plasma cleaves only fibrinopeptide A from fibrinogen [11]. Fibrin degradation products, inhibiting T fibrinogenolytic effect and polymerization of fibrin-monomers, prolong both the thrombin and reptilase time.

We compared inhibitory activity of DNA aptamer RA35, RH, and nonfractionated heparin (NFH) *in vitro* in human, rabbit, and rat plasma after clotting activation with thrombin and coagulases isolated from *Echis multisquamatus* and *Agkistrodon halys* snake venoms.

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MATERIALS AND METHODS

The following reagents were used: porcine intestinal mucosal NFH (Bio-pharm Co Ltd.), mean molecular weight 15,000 Da, antithrombin (aIIa) activity 160 U/mg, activity against activated factor X (aXa activity) 148 U/mg; RH (Namos GmbH), specific activity 75.3 U/mg; NIBSC reference thrombin calibrated thrombin (Renam); *Echis multisquamatus* and *Agkistrodon halys* venom coagulases (Technologiya-Standard). The compounds were dissolved in 0.05 M Tris-HCl buffer with 0.0075 M Na_2-EDTA, 0.175 M NaCl, 0.1% polyethylene glycol 6000 (pH 7.4). A pool of platelet-poor human plasma from 10 donors was prepared as follows. The specimens were collected at Blood Transfusion Center of Hematological Research Center. Blood from the ulnar vein was collected into an S-Monovette 5 ml 9NC plastic syringe (Sardstedt) with 0.11 M sodium citrate. Stabilized blood was centrifuged at 1200-1400*g* (15-20 min, 4°C) on a Sigma 2K15 centrifuge. Rabbit platelet-poor plasma was pooled from specimens of citrate blood, collected from an incision in the marginal ear vein in 10 Soviet Chinchilla rabbits of both genders (3.5-5.2 kg). A pool of platelet-poor plasma from male Wistar rats (170-240 g) was prepared from citrate blood of 10 animals; the blood was collected from the jugular vein in rats narcotized intraperitoneally with Nembutal (60 mg/kg).

The rabbits and rats were obtained from the Stolbovaya Breeding Center of the Russian Academy of Medical Sciences. The animals were kept in boxes on standard rations. The studies were carried out in accordance with all appropriate regulations and international recommendations of the European Convention for Protection of Vertebrates Used in Experimental Studies.

RA36 aptamer was synthesized on CPG columns (Bio Automation, 5'-DMT-dG(dmf) MerMade columns, MM1-1200F-1, 1 μM, Lot No. 025295-10). Oligonucleotides were synthesized in dry nitrogen atmosphere on a MerMade 48 automated synthesizer (Bio-Automation) using a standard synthetic cycle. The molecular weight of RA36 was measured by mass-spectrometry.

The effects of NFH, RA36, and RH on human, rabbit, and rat plasma clotting time after thrombin activation was studied in the thrombin time (TT) test [1].

The effects of the studied compounds on human, rabbit, and rat plasma clotting after activation with echitox, *Echis carinatus multisquamatus* venom coagulase, were evaluated as described previously [1] by the echitox test (ET). Plasma (0.2 ml) with NFH, RA36, or RH (0.3-104 μg/ml) was incubated (1 min, 37°C), 0.2 ml working solution of *Echis multisquamatus* venom (0.002-0.020 mg/ml distilled water) was added, and the time of clotting was recorded on a Minilab 701M coagulometer.

The effects of NFH, RA36, and RH on human, rabbit, and rat plasma clotting after activation with ancistron (*Agkistrodon halys* venom coagulase) were evaluated as described previously by the ancistron test (AT) [1]. Plasma (0.2 ml) with the studied anticoagulants (0.05-815 μg/ml) was incubated (1 min, 37°C), 0.2 ml ancistron solution with activity equivalent to 3 U thrombin/ml distilled water was added, and the time of the clot emergence was recorded on a Minilab 701M coagulometer.

In order to evaluate the anticoagulant potentials of the compounds, their effective concentrations 2TT, 2ET, and 2AT were determined. These concentrations served as the abscissa points on the concentration-effect (clotting time) curves; the ordinates were [2×(clotting time without anticoagulants).

The results were presented as the arithmetic means with the standard errors in the arithmetic means. The significance of differences in the values ($p<0.05$) was evaluated by nonparametric Kolmogorov—Smirnov test. The data were statistically processed using Statgraphics Plus software.

RESULTS

The molecular weight of synthesized RA36 was 10,000 Da. In a previous study, 31-meric RA36 aptamer inhibited thrombin activity by binding to the enzyme [14]. In our study human, rabbit, and rat plasma clotting time in the TT test increased with increase of RA36, RH, and HFH concentrations (Fig. 1). Human plasma clotting time in TT test was 2-fold prolonged *vs.* control (14.4±0.5 sec) by an 18-fold higher amount of RA36 than of RH ($p=0.023$). The concentrations of $2TT_{RA36}$ and $2TT_{NFH}$ were 2.53±0.20 and 2.36±0.45 μg/ml ($p=0.699$ for $2TT_{RA36}$ *vs.* $2TT_{NFH}$), respectively (Fig. 1, *a*). The 2TT concentrations in rabbit plasma for RA36, RH, and NFH were 5.25±0.88, 1.00±0.21, and 1.61±0.23 μg/ml, respectively (14.1±1.9 sec in control); $p=0.037$ for $2TT_{RA36}$ *vs.* $2TT_{RH}$ and $2TT_{RA36}$ *vs.* $2TT_{NFH}$ (Fig. 1, *b*). Rat plasma clotting time in TT test was 2-fold prolonged *vs.* control (15.60±0.42 sec) by RA36 quantity 9-10-fold higher than that of RH and NFH ($p=0.037$); the 2TT concentrations of RA36, RH, and NFH were 8.13±0.06, 0.83±0.03, and 0.93±0.05 μg/ml, respectively (Fig. 1, *c*). Effective RA36 concentrations in human, rabbit, and rat plasma, 5-18-fold higher than RH concentrations, indicated its lesser anticoagulant activity. Addition of any direct anticoagulant to human or mammalian plasma led to prolongation of the clotting time, while addition of NFH activated the antithrombin inhibition of thrombin and aXa activities [10].

Thrombin appeared in the plasma as a result of prothrombin activation with prothrombinase complex. Meisothrombin (mIIa) appeared as a result of Arg323 bond cleavage, the subsequent cleavage of Arg274 bond leading to thrombin generation [6].

Similarly as ecarin, echitox coagulase from *Echis multisquamatus* venom was used for prothrombin activation to mIIa. The effects of RA36, NFH, and RH on human, rabbit, and rat plasma clotting time in ET were presented in Figure 2. Nonfractionated heparin in therapeutic concentrations of 0.3-0.6 μg/ml did not change the plasma clotting time in comparison with the control (31.1±1.1 sec), which was in line with published data [1]. With the aptamer, this effect could be attained with its quantity 3-fold greater ($p<0.037$) than that of RH, the effective concentrations being 6.53±0.28 and 1.99±0.07 μg/ml, respectively (Table 1, Fig. 2, *a*). The rabbit plasma clotting time did not increase *vs.* control (26.5±1.1 sec) at the aptamer concentrations of 2.5-100 μg/ml (Table 1; Fig. 2, *b*). Effective concentrations for NFH and RH were 7.68±0.21 and 0.51±0.01 μg/ml. Effective concentrations of the aptamer, NFH, and RH with rat plasma were 29.6±1.1, 0.51±0.013, and 7.68±0.21 μg/ml, respectively (Table 1; Fig. 2, *c*).

The time of fibrin clot formation initiated by echitox addition to human and rat plasma was prolonged with increasing RA36 concentration. The $2ET_{RA36}$ concentrations for human and rat plasma were higher than $2ET_{RH}$ and $2ET_{NFH}$ concentrations. This fact indicated a lesser effect of RA36 on plasma clotting caused by prothrombin activation to mIIa and mIIa (desF1). Activities of mIIa and mIIa (desF1) were inhibited with native hirudin and RH, but not with NFH, because of inavailability of exosite II [9] and incomplete availability of exosite I, the affinity for fibrinogen *vs.* thrombin being 1 and 10%, respectively [4]. Presumably, exosite I was essential for manifestation of RA36 anticoagulant activity (as the absence of aptamer binding to active thrombin center was demonstrated previously [2]), while RH was more effective than the aptamer, because it binds to exosite I and active site of thrombin [12]. Rat plasma $2ET_{RA36}$ concentration was 4.6 times higher than human plasma ET_{RA36} concentration (2980±110 and 650±30 nM, respectively). The $2ET_{RA36}$ concentration in the rabbit plasma had to be higher than 80 μg/ml (8000 nM), which seemed to indicate RA36 lesser affinity for the rabbit thrombin than for human thrombin. Aptamer HD1-22 (interacting with thrombin exosites I and II) in a concentration of 2000 nM prolonged the plasma clotting time in the ecarin time test 2-fold *vs.* control. This concentration of the aptamer was 1.5 times lower than RA36 concentration in ET test [9]. Single-stranded 29-nucleotide aptamer, binding only to human thrombin exosite II (dissociation constant 0.5 μM), had been synthesized previously [7].

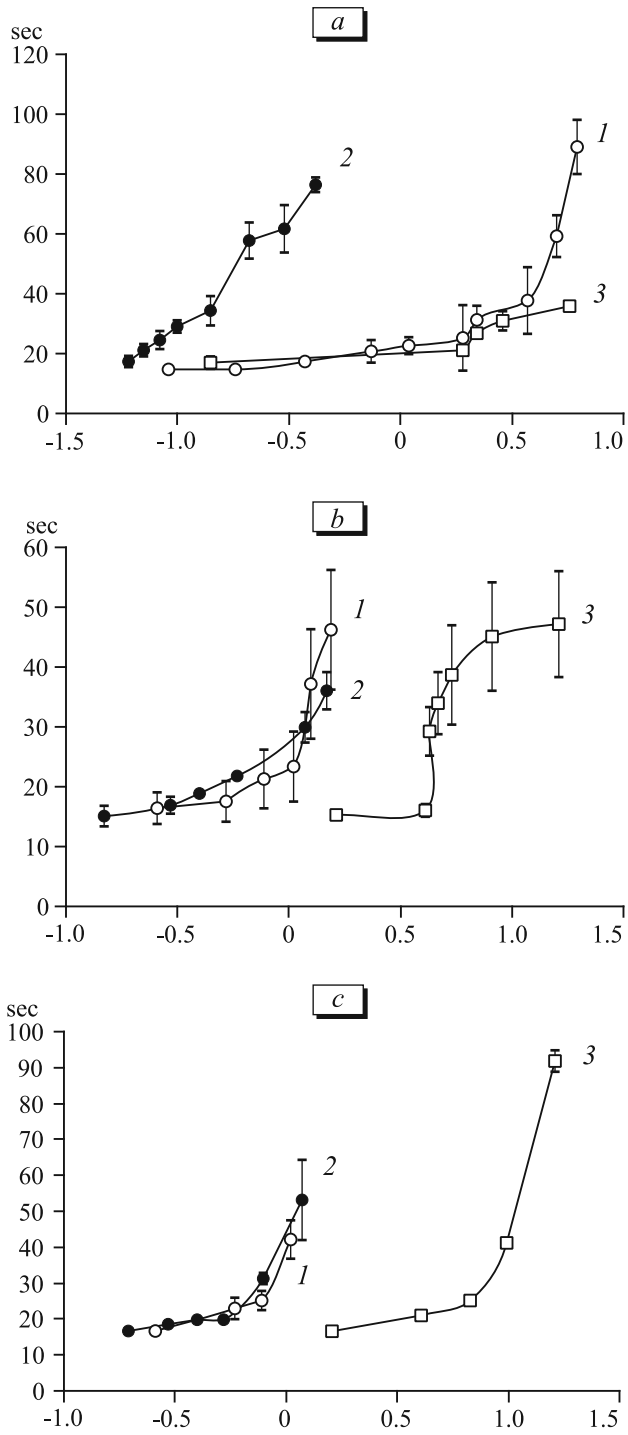

Fig. 1. Effects of NFH, RH, and RA36 on plasma clotting time in TT test. *a*) human plasma (control 14.4±0.5 sec); *b*) rabbit plasma (control 14.1±1.9 sec); *c*) rat plasma (control 15.60±0.42 sec); 4-5 independent experiments were carried out. Here and in Fig. 2: abscissa: NFH (*1*), RH (*2*), and RA36 (*3*) concentration decimal logarithms; ordinate: plasma clotting time.

Further analysis of the effect of intravenous aptamer on the plasma should be carried out on rats,

as the rabbit plasma was insensitive to the aptamer in ET test. The range of RA36 doses calculated with consideration for the TT and ET test sensitivities was estimated according to the formula: (effective RA36 concentration/effective RH concentration)×0.4 mg/kg), where 0.4 mg/kg was the RH dose for intravenous bolus injection [12]. The estimated dose range for humans and rats was 4-11 mg/kg.

Addition of ancistron, reptilase, or bathroxobine to the plasma for clotting activation triggered plasma clotting with fibrinogen transformation into fibrin (without involvement of other blood clotting factors) [1,2]. Ancistron activity differed from that of thrombin by cleaving only fibrinopeptide A from fibrinogen and not activating factor XIII. The effect of ancistron (and reptilase) could not be blocked by the heparin-antithrombin complex. In contrast to TT, ET could not be prolonged by heparin and hirudin. The ET test was used for measuring fibrinogen level in the plasma of patients treated with heparin, for evaluating the fibrinogen hypo- or dysfunction and the effects of fibrinogen derivatives (soluble fibrinogen, fibrin and fibrinogen lysis products), detection of blood clotting inhibitors modulating fibrinogen polymerization, and for more accurate evaluation of the mechanism of anticoagulant activities of the studied factors [1].

In contrast to NFH and RH, aptamer RA36 in plasma concentrations of 16.3-163 μg/ml prolonged human plasma clotting time in AT test (Fig. 2, *d*). The effective concentration of RA36, at which plasma clotting time was 2-fold prolonged *vs.* control (24.6±1.0 sec) was 62.7±2.1 μg/ml. Clotting prolongation in AT test could indicate its effect on ancistron activity or on fibrinogen polymerization. It was impossible to analyze the effects of the studied anticoagulants in the AT test with rabbit and rat plasma, as the clot did not form even after 5 min at higher ancistron concentrations.

Hence, the synthesized aptamer RA36 with molecular weight of 10,000 Da proved to be a direct anticoagulant, prolonging the human, rabbit, and rat plasma clotting in the TT test. Anticlotting activity of RA36 was lower than that of RH, as the aptamer effective concentrations in the TT test with human, rabbit, and rat plasma were higher by one order of magnitude. the ap-

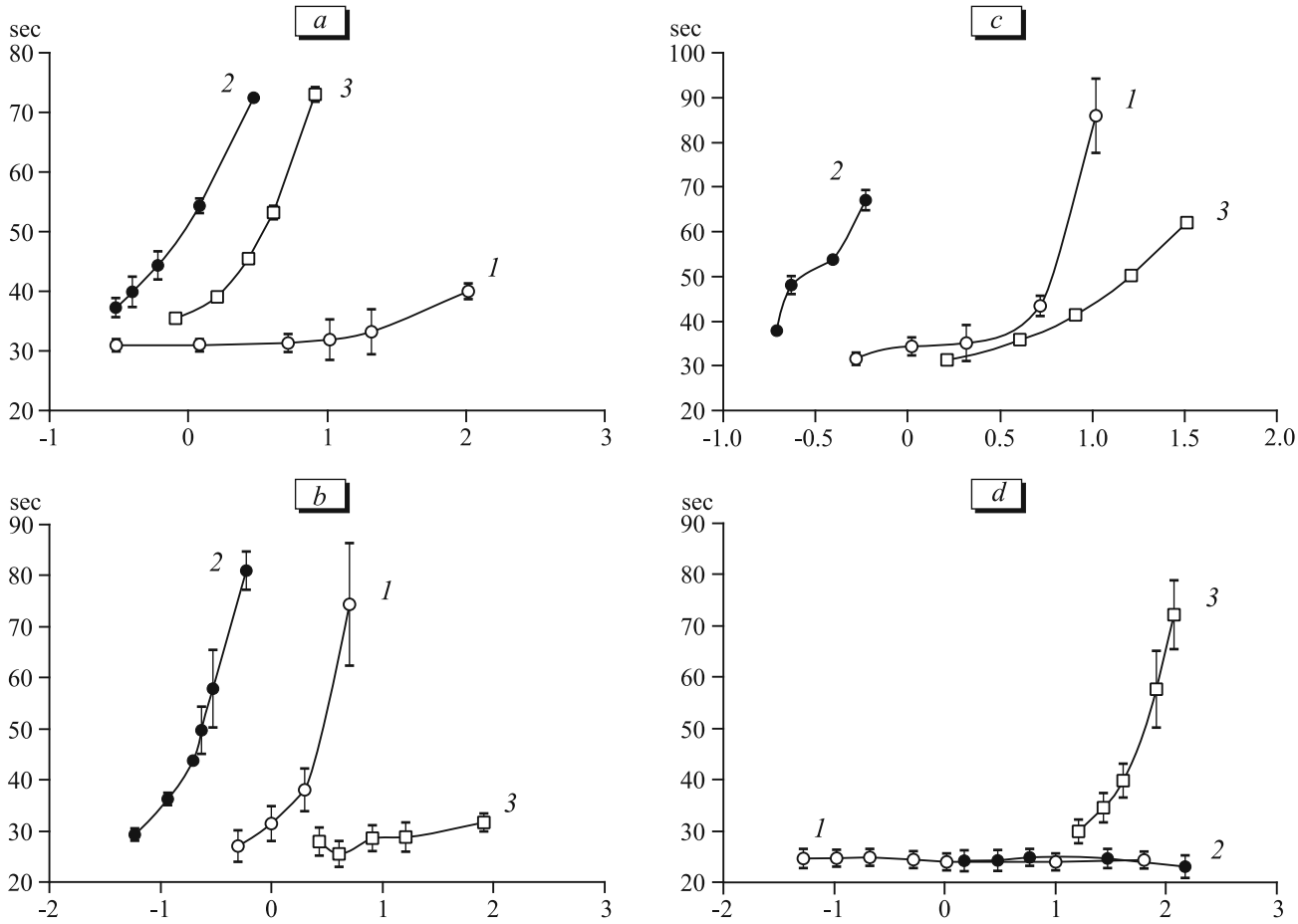

Fig. 2. Effects of NFH, RH, and RA36 on plasma clotting time in ET (*a-c*) and AT (*d*). a) human plasma (control 31.1±1.1 sec); *b*) rabbit plasma (control 26.5±1.1 sec); *c*) rat plasma (control 29.5±1.0 sec); *d*) human plasma in AT (control 24.6±1.0 sec); 4 independent experiments were carried out.

TABLE 1. Effective Concentrations of RA36, RH, and NFH in ET Test with Human, Rabbit, and Rat Plasma

Human plasma			Rabbit plasma			Rat plasma		
RA36	RH	NFH	RA36	RH	NFH	RA36	RH	NFH
6.53±0.28 p_1=0.0366	1.99±0.07	>100	>80	0.25±0.03	3.66±0.38 p_1=0.0366 p_2=0.0366	29.1±1.41	0.51±0.013	7.68±0.21

Note. At effective concentration (2ET, μg/ml) the plasma clotting time was 2-fold prolonged vs. control (buffer added instead of anticoagulants); p_1: significance of differences between the results for RA36 and RH; p_2: significance of difference between the results for RA36 and NFH; 4-7 independent experiments for each compound.

tamer Inhibiting human plasma clotting activated with *Echis multisquamatus* venom coagulase presumably fixed to thrombin exosite I (provided its interaction with coagulase were not demonstrated in later studies). The aptamer sensitivity of human plasma was 5-fold higher than that of rat plasma. In order to prove the aptamer effect on human fibrinogen polymerization, it would be essential to analyze RA36 binding to ancistron - *Agkistrodon halys* venom coagulase.

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