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ORIGINAL ARTICLE

Improvement of spatial fibrin formation by the anti-TFPI aptamer BAX499: changing clot size by targeting extrinsic pathway initiation

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Summary. *Background:* Tissue factor pathway inhibitor (TFPI) is a major regulator of clotting initiation and a promising target for pro- and anticoagulation therapy. The aptamer BAX499 (formerly ARC19499) is a high-affinity specific TFPI antagonist designed to improve hemostasis. However, it is not clear how stimulation of coagulation onset by inactivating TFPI will affect spatial and temporal clot propagation. *Objective:* To examine the BAX499 effect on clotting in a spatial, reaction-diffusion experimental system in comparison with that of recombinant activated factor VII (rVIIa). *Methods:* Clotting in plasma activated by immobilized tissue factor (TF) was monitored by videomicroscopy. *Results:* BAX499 dose-dependently improved coagulation in normal and hemophilia A plasma activated with TF at 2 pmole m^{-2} by shortening lag time and increasing clot size by up to ~2-fold. The effect was TFPI specific as confirmed by experiments in TFPI-depleted plasma with or without TFPI supplementation. Clotting improvement was half-maximal at 0.7 nM of BAX499 and reached a plateau at 10 nM, remaining there at concentrations up to 1000 nM. The BAX499 effect decreased with TF surface density increase. RVIIa improved clotting in hemophilia A plasma activated with TF at 2 or 20 pmole m^{-2}, both by shortening lag time and increasing spatial velocity of clot propagation; its effects were strongly concentration dependent. *Conclusions:* BAX499 significantly improves spatial coagulation by inhibiting TFPI in a spatially localized manner that is different to that observed with rVIIa.

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Introduction

Pro- and anticoagulant reactions of blood coagulation are normally balanced. Insufficient concentration or functionality of factor VIII (FVIII), a predecessor of cofactor FVIIIa in the intrinsic factor X(FX)-activating (tenase) complex, leads to a bleeding disorder called hemophilia A, which is usually treated by supplementation with FVIII concentrates. However, this treatment can lead to development of anti-FVIII antibodies, which can be resolved by bypassing agents [1].

Bleeding in hemophilia is due to insufficient FX production by the intrinsic tenase complex; this is overcome *in vitro* by potent extrinsic pathway activation, such as that used in a prothrombin time assay. Accordingly, it had been proposed early on to treat patients by increasing their extrinsic FX activation, which led to the generation of prothrombin complex concentrates and recombinant activated FVII [1]. An alternative strategy for stimulating the extrinsic pathway can be inactivation of tissue factor pathway inhibitor (TFPI), a vitally important regulator of the extrinsic FX-activating complex [2]. Indeed, inhibition of TFPI shortened bleeding time in rabbits with antibody-induced hemophilia A [3]. This line of research was recently pursued by generation of a new TFPI antagonist, a highly specific inhibitor aptamer BAX499 (formerly ARC19499) that improved thrombin generation in hemophilia plasma and thrombelastography parameters in anti-FVIII-antibody-treated whole blood, and reduced bleeding time in a monkey model of hemophilia A [4].

Coagulation *in vivo* is a spatially heterogeneous process triggered by tissue factor (TF) at the site of damage and followed by propagation in space by means of FIXa diffusion and FX activation on the activated platelets' surface [5–7]. Because of this transmission of the procoagulant signal from

the TF-bearing cells to platelets, FXa generated by the extrinsic tenase is not always equivalent to that produced by the intrinsic tenase [5,8]. Thus, the importance of TFPI in a spatially heterogeneous system may significantly differ from that in conventional homogeneous experiments.

In order to understand to what extent and in what manner TFPI inactivation with BAX499 regulates spatial clot formation, we carried out experiments in normal, hemophilia A and TFPI-depleted plasma using a spatially heterogeneous reaction-diffusion system [1,6,8–10], where coagulation is activated by a surface with immobilized TF and then propagates into the bulk of plasma. Previous studies of this experimental model showed that spatial propagation of the fibrin clot is disrupted in hemophilia plasma [6,10,11]. This experimental system is also naturally suited to studies of the procoagulant signal transmission from the activating surface [8], which also supports its choice for the study of the action of BAX499.

Materials and methods

Reagents

The following materials were obtained from the sources shown in parentheses: PEG-6000 (Serva, Heidelberg, Germany), glycine (Panreac Quimica S.A.U., Barcelona, Spain), polyethylene imine (ICN Biomedicals, Irvine, CA, USA), factor rVIIa (Novoseven®; Novo Nordisk, Bagsværd, Denmark), thromboplastin (Renam, Moscow, Russia), FXa-specific chromogenic substrate S-2765 (Chromogenix, Lexington, MA, USA), glutaraldehyde 25% (TED Pella, Redding, CA, USA), TFPI-immunodepleted plasma (American Diagnostica, Stamford, CT, USA), rTFPI (R&D Systems, Minneapolis, MN, USA), FX (Enzyme Research Laboratories, South Bend, IL, USA). Other reagents were from Sigma-Aldrich (St Louis, MO, USA). Specific FXIIa inhibitor CTI prepared from corn seeds essentially as described [12] was obtained from the Institute of Protein Research of the Russian Academy of Sciences (Pushchino, Russia). The TFPI antagonist aptamer BAX499 was synthesized as described [4].

Blood collection and plasma preparation

Blood was collected from healthy donors and hemophilia A patients under approval of the Center for Theoretical Problems of Physicochemical Pharmacology and National Research Center for Hematology Ethical Committees. Blood was drawn into 3.8% sodium citrate (pH 5.5) at a 9:1 blood:anticoagulant volume ratio in the presence of CTI (final concentration 0.2 mg mL^{-1}). Blood was processed by centrifugation at 1500 $\times g$ for 15 min to obtain platelet-poor plasma, then at 10 000 $\times g$ for 5 min to obtain platelet-free plasma. Fresh pools of normal plasma were prepared from three healthy donors. Lyophilized TFPI-depleted plasma was dissolved in distilled water and thoroughly mixed in the vial. CTI was added to plasma at 0.2 mg mL^{-1}, mixed on a shaker for 30 min, and centrifuged at 10 000 $\times g$ to remove undissolved particles. Plasma pH was stabilized at 7.2–7.4 by lactic acid treatment [13].

Activator preparation

Thromboplastin was immobilized on a polystyrene surface by the chemical sorption method, essentially as described [9]. A detailed description is available in the Supporting Information.

Experimental design of the spatial model

Plasma clotting was activated by a surface covered with immobilized TF as shown in Fig. 1. The process of clotting was measured by light scattering from the fibrin gel formed [9,10]. The acquired series of images was processed by computer and parameters of spatial dynamics of blood clotting were calculated. A detailed description is available in the Supporting Information.

Mathematical modeling

Computer simulations of blood clotting were carried out using a mathematical model of clotting in a reaction-diffusion system [6] with minor modifications as detailed in the Supporting Information.

Results

Dynamics of spatial fibrin clot formation

Typical experiments on spatial fibrin clot formation are shown in Fig. 1(A,B) in order to illustrate the experimental design, as well as overall consequences of FVIII deficiency and its correction with bypassing agents. In normal plasma activated with low density TF, clotting began within 20 min and rapidly propagated from the activator. Spatial propagation was significantly impaired in hemophilia A plasma. Addition of BAX499 at 300 nM, which was previously found to be a saturating concentration [4], greatly accelerated clotting onset and initial propagation in hemophilia A plasma, such that the clot size at 60 min was similar to that observed in normal plasma. RVIIa shortened the lag time, accelerated the spatial propagation and stimulated TF-independent clotting. At high rVIIa concentrations, clotting occurs throughout the chamber and, as such, there was no spatial propagation in a strict sense of the word.

Dependence of spatial clot formation on the TF density

To study how the role of TFPI depends on the surface TF density, we performed experiments in normal plasma activated by different TF densities from 0 to 100 pmole m^{-2}, with the addition of no or saturating amounts (300 nM) of BAX499. The effect of TFPI inhibition on spatial clotting parameters was dependent on the TF density and was more pronounced at lower TF densities (Fig. 2). Specifically, the lag time (Fig. 2A)

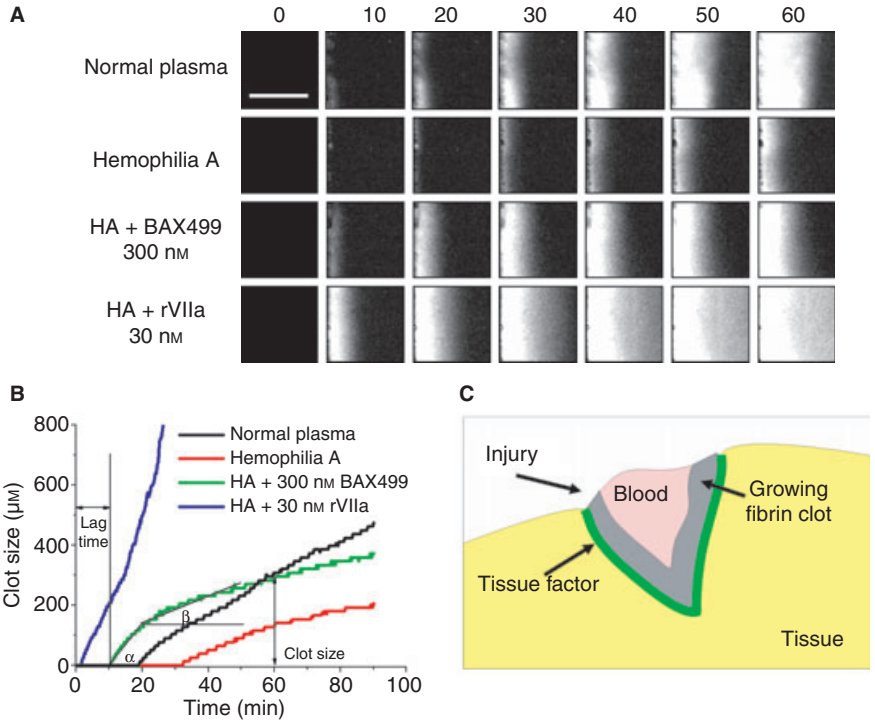

Fig. 1. Effects of BAX499 and rVIIa on spatial clotting in hemophilia A. (A) Typical light-scattering images of clot growth in plasma initiated by immobilized TF at a surface density of 2 pmole m^{-2}. TF-coated activator is seen as a vertical black strip on the left side of each image. The white bar shows the scale of 0.5 mm. (B) Clot size vs. time plots for the experiments shown in (A). The panel also illustrates parameters used for experiment analysis throughout the study: lag time (time of clot growth initiation); α, initial velocity (mean slope over the first 10 min); β, stationary velocity (mean slope over the following 30 min); clot size after 60 min of the experiment. (C) Physiologic interpretation of the experimental model. Hemostatic fibrin clot formation occurs in a wound with relatively slow blood flow when compared with arteriolar or venous blood flow. Clot formation is stimulated by tissue factor localized on the edge of the wound and propagates further into the wound.

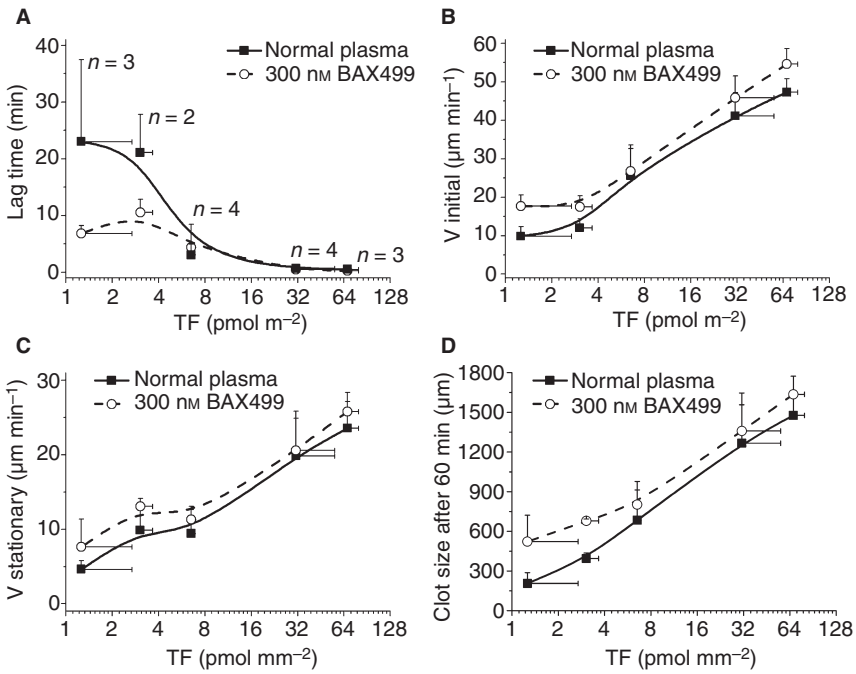

Fig. 2. Effect of BAX499 on clotting in normal pooled plasma as a function of the activation level. Clot formation parameters are plotted as a function of TF surface density: (A) lag time of clot growth, (B) initial velocity, (C) stationary velocity, (D) clot size at 60 min. The data are means ± SD (n is the number of experiments performed at a specific TF density; for TF density determination, $n = 2$). Each pool was prepared from the plasma of three donors.

was shortened by up to 2.5-fold upon BAX499 addition at TF densities of 1–3 pmole m^{-2}, but there was no effect of the aptamer at higher TF densities. The effect of BAX499 on initial velocity was also significant only at TF densities of 1–3 pmole m^{-2} (Fig. 2B). In contrast to lag time and initial velocity, BAX499 had little effect on stationary velocity. Inhibition of TFPI also affected clot size at 1–3 pmole m^{-2} of TF; the effect of BAX499 decreased as the TF density increased. Based on these results, we chose a low (1–2 pmol m^{-2}) and medium (10–20 pmol m^{-2}) TF density for further study of the effects of TFPI inhibition.

Specificity of the BAX499 effects

To gain insight into the BAX499 mechanism of action and regulation of spatial clotting by TFPI, experiments in reconstituted lyophilized TFPI-depleted plasma were performed. These experiments were performed in the absence and presence of 10 nM recombinant TFPI, with or without the addition of 300 nM BAX499. The addition of TFPI increased the lag time in TFPI-depleted plasma. Inhibition of TFPI by BAX499 returned the lag time back to that observed with no addition of TFPI; BAX499 had no effect on clotting in TFPI-depleted plasma without the addition of TFPI (Fig. 3A). Addition of BAX499, TFPI or a combination of the two did not affect initial clot growth velocity under these conditions (Fig. 3B). Due to spontaneous clotting throughout the reaction chamber, probably caused by contact pathway-induced activation during plasma immunodepletion or lyophilization, it was not possible to calculate stationary velocities and clot sizes at 60 min in experiments carried out in TFPI-depleted plasma.

Dose-dependence of BAX499 inhibition in normal plasma

To investigate the concentration dependence of the BAX499 effect in normal plasma, experiments at low and medium TF densities were performed with different BAX499 concentrations. At low TF density, the lag time decreased with increasing concentrations of BAX499 up to 30 nM, and then was stabilized at concentrations up to 1 μM (Fig. 3C). BAX499 increased the initial velocity (Fig. 3D) by 30% at concentrations at or above 30 nM, while the stationary velocity was not significantly affected by the aptamer. A detectable increase in clot size was also observed (Fig. S1). For all parameters, maximal effects of BAX499 were achieved by 300 nM, and the half-maximal effect concentration was < 10 nM. There was no significant effect of TFPI inhibition on any parameter at medium TF density (Fig. S2).

Dose dependence of the BAX499 and rVIIa effects in hemophilia A plasma

To study the effect of TFPI inhibition on clot formation in hemophilia A plasma, experiments with different concentrations of BAX499 (0–1000 nM) were performed. For comparison, rVIIa (0–1000 nM) was also evaluated. These studies were

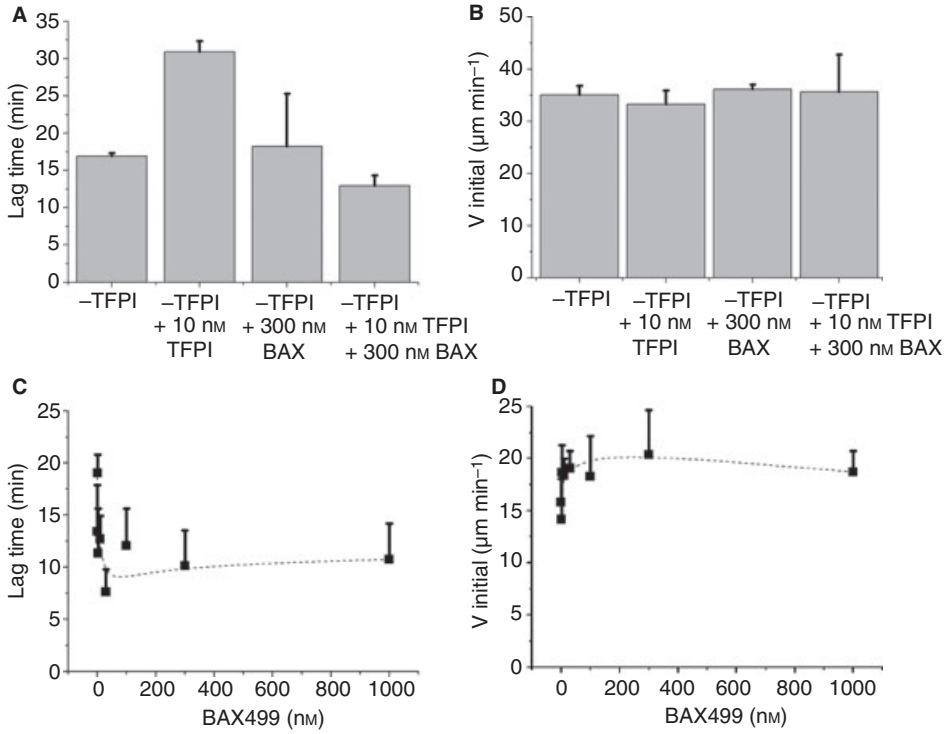

Fig. 3. Specificity and dose-dependence of BAX499. (A) Lag time and (B) initial velocity for spatial clotting in TFPI-depleted plasma supplemented with or without TFPI (10 nM) and BAX499 (300 nM). Activation is by TF at 2 pmole m^{-2}. The data are means ± SE, $n = 2$. (C) Lag time and (D) initial velocity for spatial clotting in normal pooled plasma activated by TF at 2 pmole m^{-2}. The data are means ± SE, the number of experiments with different plasma pools is $n = 3$.

conducted using plasma from six patients (Table S1). All patients were diagnosed with severe hemophilia A (FVIII:C < 1%), were on prophylaxis (25–30 IU kg^{-1}), and did not take FVIII concentrates for 3 days prior to the experiment.

Clotting parameters from experiments in plasma from the six patients were averaged and normalized to baseline (Fig. 4); the raw data for a typical patient are shown in Fig. S3. The lag time decreased by up to 2-fold with increasing concentrations of BAX499, with a half-maximal effect at 0.7 ± 0.04 nM. For rVIIa, the lag time decreased 3.7-fold at 3 nM, with a half-maximal effect at 0.51 ± 0.04 nM (Fig. 4A). BAX499 increased the initial velocity 2-fold at concentrations at or above 10 nM (Fig. 4B); rVIIa increased this parameter 3-fold. BAX499 had no effect on stationary velocity (Fig. 4C), while addition of rVIIa increased this parameter 5–8-fold. The clot size after 60 min was also impacted by BAX499 and rVIIa. The clot size was increased by 50–100% with BAX499 concentrations up to 30 nM, and did not increase further at concentrations up to 1000 nM. In the range of 0–30 nM, rVIIa increased clot size by up to 9-fold. BAX499 had little effect on clotting parameters in the presence of medium density TF, while rVIIa decreased the lag time and increased the spatial velocity with medium density TF (Fig. S4).

Statistical: comparison of the BAX499 and rVIIa effects

To estimate the extent of clotting normalization at low TF density by BAX499 and rVIIa, statistical averaging for all patients was done at saturating BAX499 (300 nM) and rVIIa (30 nM) concentrations (Fig. 5). The effects of BAX499 on lag time, initial velocity and clot size were statistically significant in hemophilia A plasma. BAX499 shortened the lag time below the normal level, normalized the initial velocity, and increased the clot size at 60 min, but had no effect on the spatial velocity. RVIIa decreased the lag time and increased the initial velocity approximately 4-fold; the stationary velocity was increased to an even greater extent, such that final clot sizes were 2–3-fold larger than clots in normal plasma. To evaluate the difference in BAX499 effects in normal vs. hemophilia A plasma, we plotted ratios of different clotting parameters, with and without BAX499, in FVIII-deficient and normal plasmas (Fig. 6A). The effect of BAX499 in hemophilia A plasma was greater than that in normal plasma with respect to both the initial velocity and clot size parameters. Additionally, at low TF density, BAX499 doubled the clot size in hemophilia A plasma, resulting in a clot size increase from 30% to 60% of that observed with normal plasma (Fig. 6B).

Computer simulations of TFPI antagonism

To study whether the effects of BAX499 on spatial clotting propagation observed experimentally are consistent with our current knowledge of the coagulation network regulation, we performed mathematical modeling for four different scenarios: normal plasma, normal plasma with inactivated TFPI, hemophilia A plasma (no FVIII), and hemophilia A plasma with inactivated TFPI. At a TF density of 5 pmole m^{-2}, the computer simulation predicted that inactivation of TFPI would lead to a shortened lag time, and spatial propagation far from the activator would not be affected (Fig. 7A). Computer simulations with 100 pmole m^{-2} TF density showed almost non-detectable improvements in clot formation in the absence of TFPI (Fig. 7B). Mathematical simulations predicted that TFPI inhibition would affect the initial clot formation phase at low TF concentration, predominantly

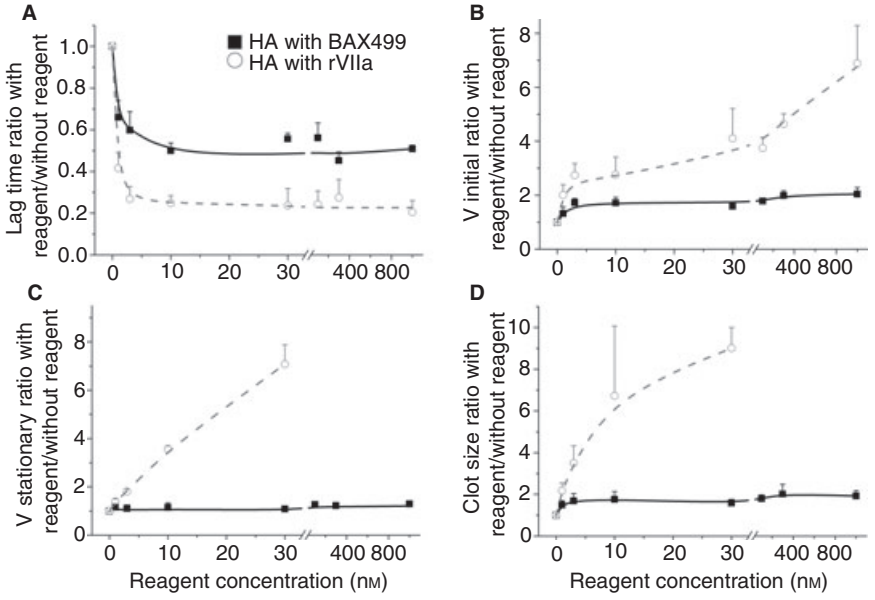

Fig. 4. Effects of BAX499 and rVIIa on clotting in hemophilia A plasma. The panels show averaged and normalized (relative to the baseline value) clotting parameters for plasmas supplemented with BAX499 or rVIIa: (A) lag time, (B) initial velocity, (C) stationary velocity, (D) clot size at 60 min. Activation is by TF at 2 pmole m^{-2}. The data are means ± SE, the number of experiments with plasmas from different patients is $n = 3$.

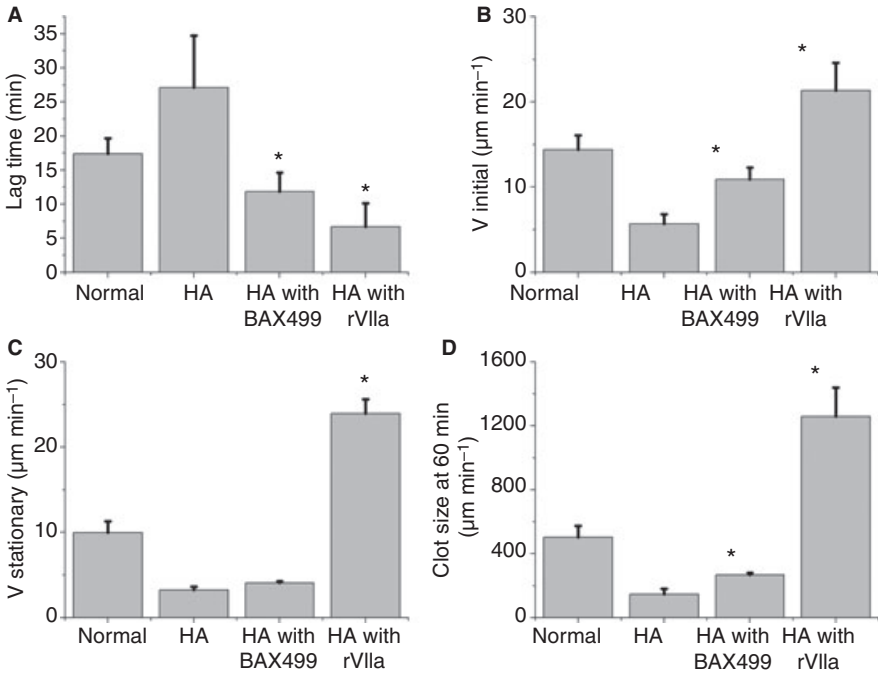

Fig. 5. Maximal effects of BAX499 and rVIIa on clotting in hemophilia A: statistics and comparison with normal plasma. The panels show averaged spatial clotting parameters: (A) lag time, (B) initial velocity, (C) stationary velocity, (D) clot size at 60 min. The columns in each panel display parameters for: normal plasma, hemophilia A plasma, and hemophilia A plasma with BAX499 (300 nM) or rVIIa (30 nM). The data are from the experiments depicted in Figs 2–4. Data are means ± SE, $n = 3$–6. Clot growth was initiated by TF at 2 pmole m^{-2}. Asterisks show significant differences for BAX499 or rVIIa addition when compared with hemophilia A plasma alone, as determined by an unpaired *t*-test.

decreasing the lag time (Fig. 7C) and increasing the initial clot growth velocity, consistent with our experimental observations described above.

To understand relative roles of TFPI and FVIII in spatial clot formation and to predict possible drug-drug interaction effects for TFPI antagonism and FVIII supplementation, computer simulations were also performed for ranges of FVIII (0–0.7 nM) and TFPI (0–2.5 nM) concentrations (Fig. 8). The model predicted that lower TFPI levels would influence clot initiation time by shortening the lag time, while the effect of FVIII would be predominantly on the clot propagation velocity. The relative increase of clot size upon TFPI removal was greater without FVIII than in the presence of normal FVIII concentrations.

Discussion

This study evaluated the effect of the TFPI antagonist aptamer BAX499 on plasma coagulation in a spatial *in vitro* experimental model mimicking the process of clotting in a wound. We found that BAX499 significantly improved spatial fibrin clot formation in normal and hemophilia A plasma initiated with low-density TF (up to ~10 pmole m^{-2}) in a TFPI-dependent manner, manifested by a lag time shortening and an increase in clot size with a ~0.7 nM half-maximal effect concentration.

The role of TFPI in clotting regulation has been extensively investigated in homogeneous experimental models [4,14–16]. However, hemostasis *in vivo* is usually initiated heterogeneously, by the contact of blood with extravascular TF-bearing cells. Inhibition of FIXa and FXa by plasma inhibitors limits their effective diffusion distance [5,6] and, consequently, the role of TFPI in spatial clotting propagation. How far does the influence of TFPI extend from the TF-bearing cells? What parameters of spatial thrombus propagation are controlled by TFPI? Is it possible to improve defective spatial propagation of the clotting process by inactivating TFPI? To our knowledge, this study is the first attempt to answer such questions.

Inactivation of TFPI by BAX499 significantly improves spatial clot formation in normal and hemophilia A plasma at low TF density (1–2 pmole m^{-2}). The main effect of FVIII deficiency in hemophilia is decrease of spatial velocity, as demonstrated by previous results [6,8,10,11]; the major effects of TFPI inhibition are shortening of the lag time and acceleration of the initial spatial propagation. TFPI inhibition has little effect on spatial propagation velocity. Thus, in agreement with homogeneous studies using synthetic plasma mixture [14] and with our computer simulations based on our current knowledge of the coagulation system, TFPI predominantly affects clotting initiation. The region of its influence does not extend beyond 100–200 μm from the TF-bearing surface (Fig. 1). However, this acceleration of initiation is sufficient to increase the final clot size in hemophilia A plasma by up to 2-fold, bringing it close in size to that observed with normal plasma (Fig. 5).

With the exception of this novel information about spatial effects of BAX499 and particularly spatial localization of its action to the site of activation, the effects observed in the

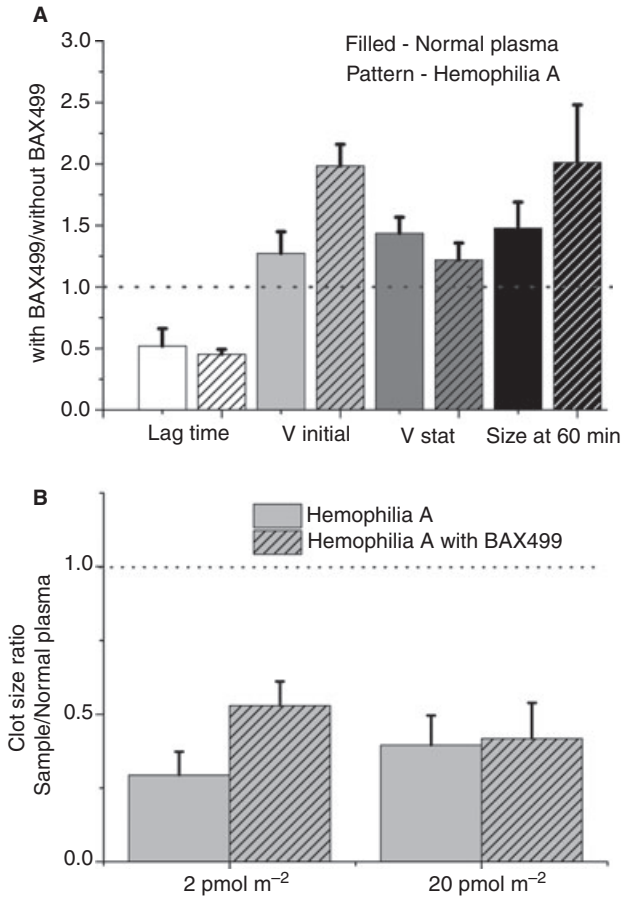

Fig. 6. Comparative efficiency of BAX499 in normal and hemophilia A plasma. (A) Ratios of clotting parameters with and without BAX499 (300 nM) in normal and hemophilia A plasma. Shown are means ± SEM, $n = 3$. (B) Ratios of clot size in hemophilia A plasma, with or without BAX499, and normal plasma initiated by TF at 2 pmole m^{-2} or 20 pmole m^{-2}. Shown are means ± SEM, $n = 3$–6.

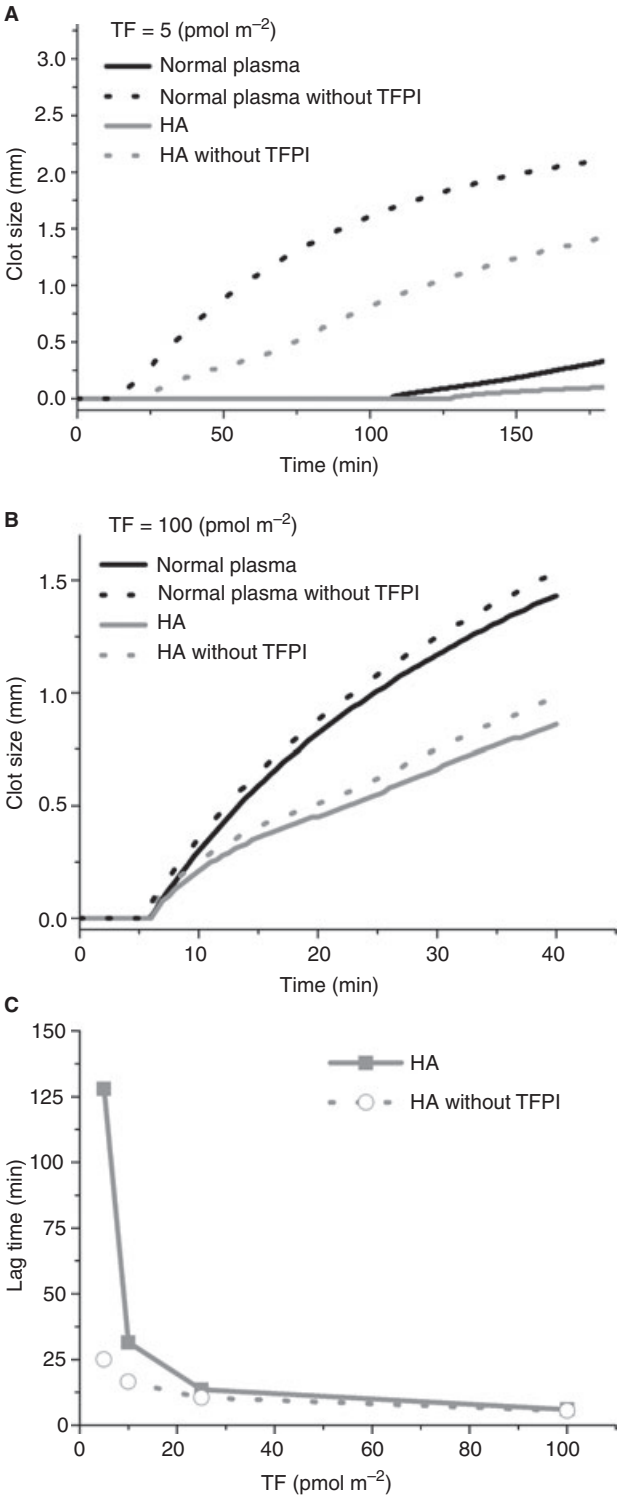

Fig. 7. Computer simulations of spatial clotting: effects of TFPI and FVIII depletion. Panels (A) and (B) show clot size vs. time plots for clotting activation with TF at 5 or 100 pmole m^{-2}, respectively. (C) Lag time in hemophilia A plasma with and without TFPI as a function of TF surface density.

experimental model of the present study are similar in magnitude, specificity and dose dependence to those obtained previously in other global coagulation assays: BAX499 was found to shorten the lag time in the thrombin generation assay and thrombelastography by a factor of two, and to increase the peak thrombin and endogeneous thrombin potential several-fold [4]. The typical BAX499 concentrations required to achieve these effects (1–10 nM) are also in good agreement with our data. Finally, in the spatial assay, as in thrombin generation and thrombelastography, the effects of BAX499 in hemophilia A plasma are more pronounced than in normal plasma. These experimental data are consistent with our knowledge of the molecular mechanisms of clotting reactions, as indicated by their comparison with the results of computer simulations.

Interestingly, TFPI inhibition appears to improve clotting exactly where it is needed in hemophilia (i.e. at low TF concentrations). The effects of FVIII and TFPI in our experiments were found to be activation dependent: clotting in hemophilia A was more impaired and BAX499 had a larger effect at lower TF densities (Fig. 6). Although there is little information regarding estimation of the TF surface densities during injuries *in vivo*, the values of < 20 pmole m^{-2} can be

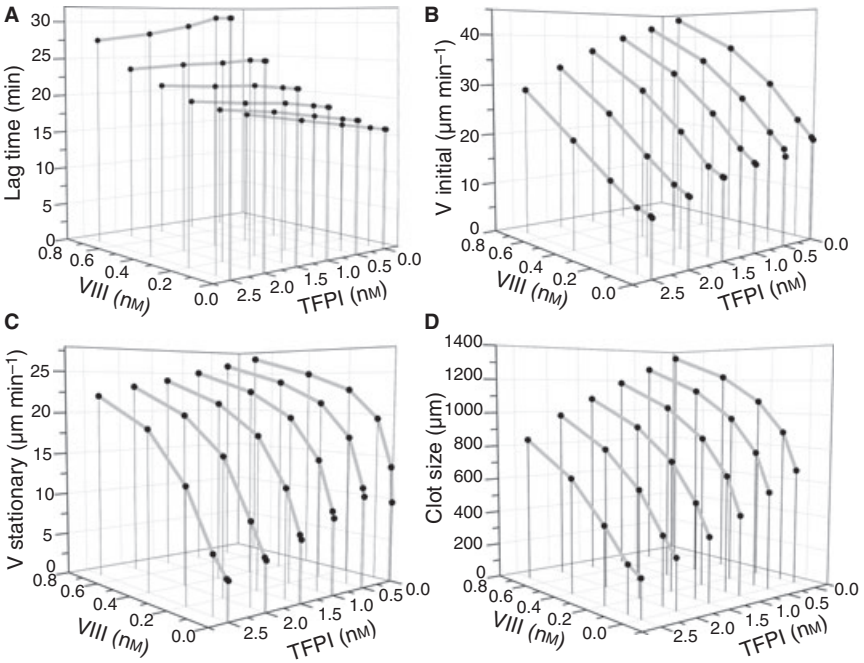

Fig. 8. Computer simulation of clot formation as a function of TFPI and FVIII: prediction of drug-drug interaction of TFPI antagonism and FVIII infusion using computer simulations. Panels show the dependence of clotting parameters (A) lag time, (B) initial velocity, (C) stationary velocity and (D) clot size on TFPI and FVIII concentrations. Clotting activation is with TF at 10 pmole m^{-2}.

expected to be found in many cases [17–20]; a monolayer of fibroblasts has TF surface density as high as 50–100 pmole m^{-2} [17,18]. In view of the prediction that the role of TFPI would be greatly increased by the presence of blood flow [21], it is reasonable to speculate that flow would modulate the effects of TFPI antagonism as well. It is also likely that the experimental model of the present study significantly undervalues the effects of TFPI antagonism by disregarding cell-surface-associated TFPI. The same caution should be exercised in comparing the effects of rVIIa, BAX499 and FVIII: while the conditions of the current experimental model mimic those in a wound, the contribution of blood flow, thrombomodulin or heparin sulfate on the endothelium can also complicate inferences made about intravessel clotting.

Subnanomolar effective concentrations of BAX499, together with lack of the effect in TFPI-depleted plasma, are indicators of high specificity and affinity of TFPI inhibition by BAX499, consistent with previous studies [4]. In addition to this, the effects of BAX499 are maximal and stable from 10 nM up to at least 1000 nM in normal and hemophilia A plasma. These data suggest that possible safety and stability of TFPI antagonism could be expected *in vivo*.

The spatial experimental model was also able to reveal several interesting differences in the mode of action of TFPI antagonism and extrinsic pathway stimulation by rVIIa. In homogeneous assays such as thrombin generation, both these molecules act similarly by increasing peak thrombin and endogeneous thrombin potential and shortening the lag time [4,22,23]. In contrast, the action of BAX499 in the spatial system is strictly localized to the TF-covered surface, while that of rVIIa is, to a significant extent, independent of TF. Even at low concentrations, when there was no clotting outside of the TF-bound clot, rVIIa increased the spatial velocity of clot propagation at all distances from the activator. Our previous report on the effect of rVIIa on spatial clot propagation [23] suggested that both this acceleration and TF-independent clotting are most likely caused by direct activation of FX by FVIIa on platelet microparticles [22]; a more detailed understanding of this phenomenon will require a more physiological experimental model that includes blood flow and endothelium. Interestingly, while the BAX499 effect is TF dependent, rVIIa significantly improves clotting at the TF surface density of 20 pmole m^{-2}, as well as at 2 pmole m^{-2}. The BAX499 effect is rapidly saturated with increasing concentration, while the effects of rVIIa are concentration-dependent throughout the concentration range tested.

These mechanisms of action for TFPI and BAX499 in the spatial system derived from the data in this study are schematically represented in Fig. 9. In a healthy person, the extrinsic tenase (TF-FVIIa) is inhibited by TFPI after some FXa has been produced, thus preventing further activation of FX and FIX. This is normally compensated for by the intrinsic tenase (FVIIIa-FIXa) activity, which is inhibited by antithrombin III (ATIII). In contrast to FXa, FIXa is inhibited by ATIII slowly and can efficiently diffuse [6], bind to FVIIIa, and produce FXa far from the activator (Fig. 9A). This intrinsic pathway does not function in hemophilia A plasma (Fig. 9B), resulting in normal clotting initiation but impaired spatial propagation, as demonstrated previously in the experimental system employed in the current study [6,10]. Inhibition of TFPI

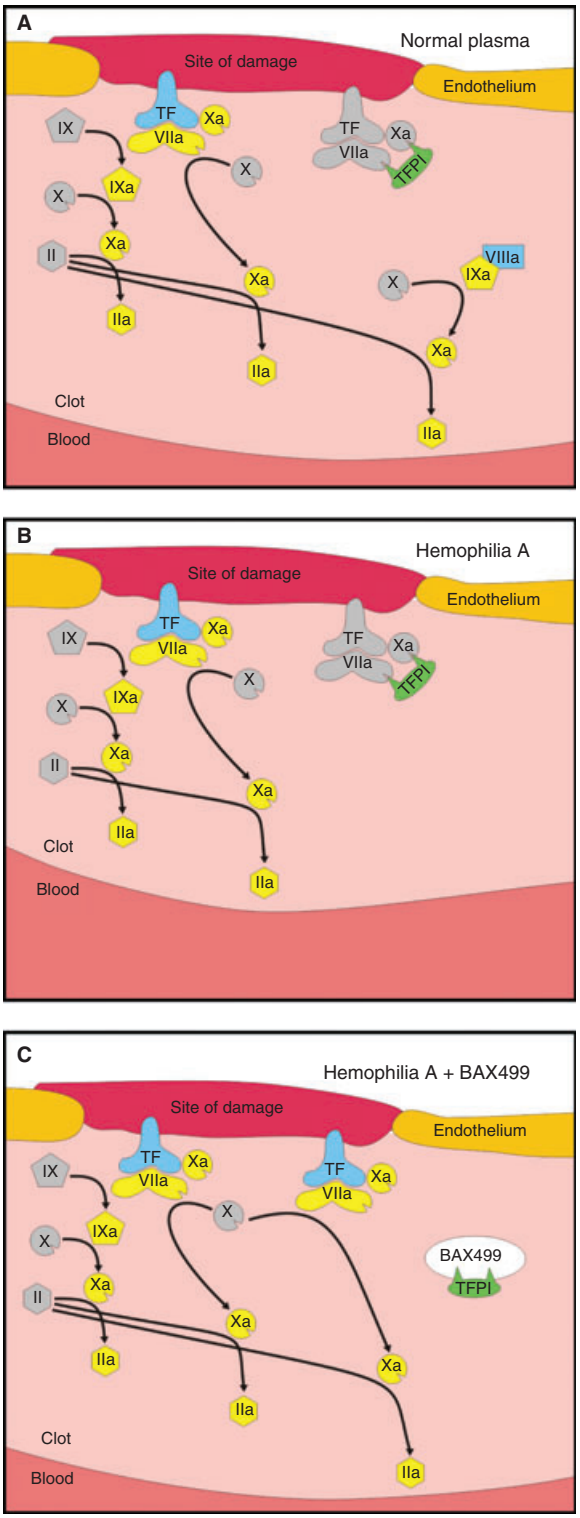

Fig. 9. Role of TFPI in spatial clot formation and consequences of its inactivation. (A) Under normal conditions, TFPI rapidly inhibits the extrinsic tenase in an FXa-dependent manner; this does not stop fibrin clot spatial propagation because FX is activated by the intrinsic tenase. (B) Clot size in hemophilia A plasma is significantly impaired because the absence of FVIIIa prevents spatial propagation. (C) Addition of BAX499 to hemophilia A plasma inhibits TFPI, allowing coagulation initiation by the extrinsic pathway to continue, resulting in normal fibrin clot size.

by BAX499 in hemophilia A plasma prevents inhibition of the membrane-bound FVIIa-TF complex. Higher production of FXa and FIXa leads to an increase of thrombin levels and accelerates the initial phase of clot formation (Fig. 9C) but not the defective spatial propagation phase. To summarize, inactivation of TFPI with BAX499 does not normalize impaired spatial clot propagation in hemophilia A plasma, but rather accelerates clotting initiation, ultimately resulting in a final clot size similar to that observed in normal plasma.

The possibility of hemophilia treatment by TFPI antagonism has remained a subject of interest over the last two decades [2,3,24,25]. The results of the present study obtained with BAX499 in a spatial experimental model, in combination with results reported previously [4], favor this concept. Our data suggest that significant improvement in clotting due to TFPI antagonism is combined with rapid saturation and spatial 'boundedness' of the effects. In contrast to bypassing agents that have independent procoagulant activity, such as rVIIa, TFPI antagonists are likely to be difficult to overdose because the magnitude of their effect will be limited by the available TFPI. Additionally, a TFPI antagonist would not be expected to cause undesired coagulation, because its procoagulant effects occur only at sites of TF exposure.

Addendum

Author contributions: L. A. Parunov, J. C. Gilbert, R. G. Schaub, F. I. Ataullakhanov and M. A. Panteleev designed the experiments; L. A. Parunov, A. N. Balandina and N. P. Soshitova performed the spatial assay experiments; O. A. Fadeeva prepared and characterized activators with immobilized TF; L. A. Parunov and A. N. Balandina performed computer simulations; K. G. Kopylov and M. A. Kumskova recruited and characterized patients; R. G. Schaub and K. E. McGinness contributed a critically important reagent (BAX499); L. A. Parunov, J. C. Gilbert, R. G. Schaub, K. E. McGinness, F. I. Ataullakhanov and M. A. Panteleev analyzed the data; L. A. Parunov and M. A. Panteleev wrote the paper with contributions from all authors.

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Disclosure of Conflict of Interests

J. C. Gilbert, R. G. Schaub and K.E. McGinness were employees of Archemix Corp. during the time when this work was performed. K. E. McGinness is an employee of Baxter Healthcare Corporation. The work reported by L. A. Parunov, O. A. Fadeeva, A. N. Balandina, N. P. Soshitova, K. G. Kopylov, M. A. Kumskova, F. I. Ataullakhanov and M. A. Panteleev was supported by grants from Archemix Corp.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. BAX499 dose-dependence at low TF density.
Figure S2. BAX499 dose-dependence at medium TF density.
Figure S3. Dose-dependence of BAX499 and rVIIa at low TF density in plasma from a hemophilia A patient.
Figure S4. Dose-dependence of BAX499 and rVIIa at medium TF density in plasma from a hemophilia A patient.
Table S1. Hemophilia A patients.

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