Drug-drug interaction of the anti-TFPI aptamer BAX499 and factor VIII: Studies of spatial dynamics of fibrin clot formation in hemophilia A


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A B S T R A C T

Background: In recent years, a number of tissue factor pathway inhibitor (TFPI) antagonists have been developed to serve as bypassing agents to improve hemostasis in hemophilia A. Since TFPI antagonists and FVIII concentrates are procoagulants, their combined effect on spatial clot formation could be potentially pro-thrombotic.

Objective: To investigate the cooperative effect of TFPI inhibition and supplementation of FVIII in hemophilia A in a spatial, reaction-diffusion experiment in vitro.

Methods: Plasma was collected at different time points from hemophilia A patients undergoing prophylaxis and was supplemented in vitro with TFPI inhibitor BAX499 (formerly ARC19499) at concentrations from 0 up to 600 nM. Clotting propagation in recalci

Introduction

Widespread complications of anti-FVIII antibodies in hemophilia A patients generate the need of bypassing agents that improve hemostasis by acting in manners different from that of FVIII [1]. A promising possibility for bypassing therapy is inactivation of tissue factor pathway inhibitor (TFPI), the main regulator of the extrinsic fX-activating complex, to increase factor X generation during the activation phase of clotting [2]. In 1995, early experiments showed that inhibiting TFPI lead to the decrease in bleeding time in rabbits [3]. This line of research has led to the development of some TFPI inhibitors by several groups recently. It was shown quickly that different TFPI antagonists effectively improve hemostasis in normal and hemophilia plasmas [4–11]. Some TFPI antagonists were tested also in purified system [12]. The majority of mentioned studies were performed using homogeneous clotting tests, such as thrombin generation and thromboelastography assays. Our previous experiments proved that anti-TFPI aptamer BAX499 can improve also spatial fibrin clot formation initiated by immobilized TF [13]. After all these studies it is clear, the idea of improving hemostasis by inhibiting TFPI is a reality; however, there are still some unanswered questions. Since hemophiliacs usually have permanent treatment, there may be situations in medical practice when TFPI inhibitors could be administered to hemophilia patients simultaneously with factor VIII concentrates or at the moment, when FVIII has not been completely cleared from circulation. However, both types of these pharmaceuticals are procoagulants, which can make their combination potentially prothrombotic, especially if their effects are synergistic. In such cases it is very important to know the co-operative effect of TFPI inhibitors and factor VIII. The objective of this study was to determine the joint effects of TFPI inhibitors and FVIII on clotting.
Blood coagulation in vivo is a spatially heterogeneous process, where coagulation is initiated by tissue factor (TF) at the site of vascular damage and then propagates in space. It is also well known, that spatial fibrin clot formation significantly impaired in hemophilia [14,15]. This fact makes using spatial reaction-diffusion model, where activation occurs at the surface with immobilized TF, more justified for investigating hemophilia problems in vitro. Moreover, this experimental model and computer simulations employed in our previous study [13] suggests that TFPI inhibitors and factor VIII affect different stages of fibrin clot formation. So the spatial reaction-diffusion experimental model was used in this study again. Coagulation is activated in vitro by a surface with immobilized TF and then propagates into the bulk of plasma in order to mimic the role played by coagulation factor diffusion in vivo [1,15–17,26]. In previous studies it was shown that this model is sensitive to FVIII concentration [15,18] and showed good correlation in between spatial clot formation in vitro and clinical condition of patients with bleeding and thrombotic disorders [15,18,19]. For TFPI inhibiting we used the aptamer BAX499 [4,10,12,20] as a potent and well-characterized tool for investigating mechanisms of blood coagulation in vitro. In order to investigate the interaction of TFPI inhibitors and FVIII concentrates, we carried out experiments using plasma from severe hemophilia A patients. To move as close to the in vivo situation as possible, BAX499 was added in vitro to plasma samples from patients taking factor VIII concentrates at different times after FVIII administration.

Materials and methods

Reagents

The following materials were obtained from the sources shown in brackets: polyethylene imine (ICN Biomedicals, Irvine, CA, USA), glycine (Panreac Quimica S.A.U., Barcelona, Spain), thromboplastin (Renam, Moscow, Russia), factor X (Enzyme Research Laboratories, South Bend, IL, USA), factor Xa-specific chromogenic substrate S-2765 (Chromogenix, Lexington, MA, USA), CTI (Haematologic Technologies), Factor VIII deficient plasma, Pathromtin SL and lyophilized standard human plasma (SHP) were purchased at Dade Behring. All other reagents were from Sigma-Aldrich (St Louis, MO, USA). The TFPI antagonist aptamer BAX499 was synthesized as previously described [4,7,10].

Patient selection and blood collection

Blood was collected from congenital hemophilia A patients with their informed consent under a protocol approved by the Center for Theoretical Problems of Physicochemical Pharmacology and National Research Center for Hematology Ethical Committees. A total of nine patients with severe hemophilia A having baseline FVIII concentrations less than 1% participated in this study. All patients (except patient #8) were under enduring therapy with different FVIII concentrates (Haemostin, Kogenate or Octanate), but they were claimed not receive FVIII concentrates for two-three days prior the experiment. Patient #8 was on an on-demand treatment. Eight of nine patients never had inhibitors. Patient 1 had inhibitor less than 1 BU/mL at period of 1996–1998, but then inhibitor disappeared and at the time of the experiments Patient 1 had no inhibitor. Inhibitor to FVIII was examined by Bethesda method. Blood samples for clot growth experiments were drawn before factor VIII administration (0 h) and at 1 and 24 h time points into 3.8% sodium citrate (pH 5.5) at a 9/1 blood/anticoagulant ratio, in the presence of CTI (0.1 mg/ml final concentration) to prevent any possible spontaneous contact activation. Additionally, samples at 4 and 48 hours (except Patients 7 and 8) were taken for measurements of FVIII activity and APTT. Additionally three more severe hemophilia A patients (FVIII concentration less than 1%) donated blood once for control experiments (without any FVIII concentrates administration).

Plasma characterization (APTT and FVIII:C level)

Activated partial thromboplastin time (aPTT) was measured with Pathromtin SL (Dade Behring). Clotting activities of factors VIII:C, were measured by a one stage clotting method, using factor VIII deficient plasma and Pathromtin SL. Calibration curves were produced with lyophilized standard human plasma (SHP). All measurements were performed at semi-automatic coagulometer Amelung KC-10.

Plasma preparation

Within 10 minutes of sample collection, blood was processed by centrifugation at 2,500 g for 15 min to obtain platelet-poor plasma, and then additionally centrifuged at 11,000 g for 5 min to obtain platelet-free plasma and frozen at −80 °C for further spatial coagulation assays.

Experimental design of the spatial model

The key property of the spatial experimental model is that plasma clotting is activated by a surface covered with immobilized TF and then propagates into the bulk of non-flowing plasma as shown in Fig. 1a. Clotting takes place in a thin flat chamber at 37 °C and is registered by light scattering from fibrin gel using dark field technique [15,21]. Images are captured every 15 s. The acquired series of images is then processed by computer and parameters of spatial dynamics of blood clotting are calculated. Thromboplastin was immobilized on a polystyrene surface by chemical sorption method essentially as described [16]. According to our previous experience [13], plasma was activated with low TF density (1.6 ± 0.5 pmole/m2, Mean ± SD, just a few TF molecules per squared micron). This is also consistent with study of another group showed that thrombin generation assay triggered with recalcification only is more sensitive to bypassing agents affecting extrinsic pathway [22].

Spatial clot formation assay

Before the experiment, plasma was thawed for 10 min under flowing water, and plasma pH was stabilized at 7.2–7.4 by lactic acid treatment as described [23]. BAX499 aliquots were thawed at room temperature for 30 min before the first experiment of the day. BAX499 was dissolved in PBS to achieve the necessary final concentration. At 15 min before each experiment, 300 μl of plasma was supplemented with 4.5 μl of BAX499 solution. In experiments with a final concentration of 0 nM of BAX499, plasma was supplemented with the same volume of vehicle PBS. Activator was placed into buffer (20 mM Hepes, 150 mM of NaCl, pH = 7.2–7.4) to reduce bubble formation near the activator during the experiment. The solution of 1 M CaCl2, buffer with activator and prepared plasma were incubated separately at 37 °C for 15 minutes. The experimental chamber was placed into the thermostat of the experimental device at 37 °C. Plasma was subsequently recalcified by addition of 6 μl 1 M CaCl2, quickly mixed and 300 μl of plasma was placed into the experimental chamber. The activator was taken out of the buffer, buffer excess was removed by blotter, and the activator was placed into the experimental chamber to start clotting. Spatial fibrin clot growth was recorded as described above.

Image processing and data analysis

For each experiment, parameters of clot growth were determined on the basis of image series. First, background image was subtracted from each image of the series, and the resulting images were analyzed. A perpendicular to the activator was drawn and clot profiles, plots of mean light scattering (based on pixel intensity) versus distance from the activator were calculated. For each profile, the clot size was determined as a coordinate where the light-scattering intensity was 50% of...
the maximal one, which corresponds to half-maximal fibrinogen conversion into fibrin [21]. As shown in Fig. 1c, based on clot size versus time plots, the following parameters were calculated: lag time (delay between contact of plasma with activator and beginning of clot formation), initial velocity of clot growth (mean slope of the clot size versus time curve over the first 10 min after the lag time), spatial velocity of clot growth (mean slope over the next 30 min), clot size after 60 min of the experiment. For each experiment, four perpendiculars to the activator surface were drawn at different areas of activator. Profiles of clot growing were analyzed and four values of each clotting parameter were calculated and then averaged to obtain means.

Statistical analysis

Averaging, SD and SE calculations were done in OriginPro v.8 (from OriginLab Corporation). For statistical analysis of differences between groups, pair-sample t-test or Student t-test was used with significance level $p = 0.05$.

Mathematical modeling

Computer simulations of blood clotting were carried out using a detailed mechanism-driven mathematical model [17] of clotting in a reaction-diffusion system with minor modifications [13].

Results

Characterization of hemophilia A patients

Experiments were performed using plasma samples from nine patients listed in Table S1 in supplementary material. All patients were diagnosed with congenital severe hemophilia A (FVIII:C < 1%) without inhibitors, were on regular prophylaxis except patient 8, and claimed not having used factor VIII concentrates for two-three days prior to the experiment. Blood samples for spatial clotting experiments were collected before the administration of factor VIII (time point 0 h), and at 1 and 24 hours after it. For additional information some more samples were collected for all patients (usually at 4 and 48 hours) to show factor VIII clearance. For these samples only factor VIII level and APTT (Table S1) were measured.

Factor VIII interaction with BAX499 at different pharmacokinetics time points

Plasma of nine hemophilia A patients were used at different time points (see M&M). For each blood sample a number experiments in vitro with BAX499 concentration ranging from 0 to 600 nM were performed. Fig. 1 illustrates typical image series at different time points (Fig. 1a) and clot size as a function of time for the same experiments (Fig. 1b-d). For all patients the data for the same time and BAX499 concentrations then was averaged. Fig. 2 illustrates statistical dose-dependence for BAX499 for each measured parameter at different

Specifically, addition of BAX499 decreased lag time and increased clot growth velocity. This led to a significant increase in the integral parameter of total clot size. Lag time decreased and clot size statistically increased until 100 nM of BAX499 for every time point with saturation at 30-100 nM. Initial and stationary velocity steadily increased within the whole range of BAX499. The relative effect of BAX499 (at saturating concentrations 300-600 nM to base level 0 nM) on clot size decreased with fVIII concentration increasing (Fig. 2). However, for two out of the nine patients (patient 2 and 9) the relative effect of BAX499 was similar for different time points of prophylaxis. The effect of BAX499 and fVIII on clot size for all patients shown at Fig. S1.

For further statistical analysis only the data from experiments with 0 and 100 nM (saturating concentration) of BAX499 were used. For each chosen BAX499 concentration the data was rearranged and sorted by fVIII levels in the samples, independently at what time data was observed. Three ranges of fVIII activity were allocated: below 5%, in between 5% and 30%, above 30% (NOTE that these ranges does not strictly correspond to data observed at time points of 0, 1 and 24 hours). Clotting parameters were averaged within each range. Clotting parameters with/without 100 nM of BAX499 were plotted against fVIII activity (Fig. 3). In the presence of less than 30% of fVIII:C, BAX499 significantly decreased the lag time 2-fold. In presence of more than 30% of factor VIII activity, effect of BAX499 was not significant. 100 nM of BAX499 increased spatial clot growth velocities only 1.2- to 1.4-fold in whole range of fVIII activity. For initial velocity the effect was significant in range 0-30% of fVIII; for spatial velocity significant difference was found only below 5%. The most pronounced effect was observed for clot size. In the ranges of less than 5%, 5-30%, and more than 30% of factor VIII activity, the clot size increased by 200%, 70% and 30% respectively. Therefore, as factor VIII concentration increased, the BAX499 efficiency decrease was observed, although fibrin formation improvement remained significant for the whole range of concentrations. From the position of fVIII, it affects mostly clot growth velocities in lack of BAX499. Increasing fVIII activity from 3 to 70% reduce lag time only by 30%, but augment velocities by 100% and clot size by 250% (Fig. 3).

Possible mechanism behind the factor VIII-BAX499 interaction effects

In order to gain insight into the mechanism of interaction of these two drugs, we performed computer simulation experiments of spatial fibrin clot formation using a mathematical model [17]. Factor VIII and BAX499 are known to promote coagulation via two distinct pathways of factor X activation. Factor VIII acts as a co-factor for factor IXa and improves factor Xa formation via acceleration of factor IXa-dependent catalysis (i.e. intrinsic tenase); while BAX499 accelerated factor X activation by the factor VIIa-TF complex (i.e. extrinsic tenase) by preventing its inactivation by TFPI. To separate these contributions, we calculated concentration profiles of factor Xa produced by extrinsic and intrinsic tenases in our mathematical model (Fig. 4). This was done for four cases: normal plasma (100% fVIII:C) with and without TFPI (2.5 nM or 0), and severe hemophilia A plasma (0% fVIII:C) with or without TFPI.

In agreement with the experiments of this study and with our previous computer simulations [13], the mathematical model showed that the relative effect of TFPI removal should be smaller for high factor VIII concentrations (Fig. 4A). The data on factor Xa production by two different enzymes in the clotting system (Fig. 4B) allow us to suggest why this is so. Under normal conditions, factor Xa produced by extrinsic and intrinsic tenases in our mathematical model (Fig. 4). This was done for four cases: normal plasma (100% fVIII:C) with and without TFPI (2.5 nM or 0), and severe hemophilia A plasma (0% fVIII:C) with or without TFPI.

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Removal of TFPI greatly boosts near-activator factor Xa production by extrinsic tenase by an order of magnitude (Fig. 4B, rows 3 and 4; notice a different concentration scale), but this factor Xa cannot rapidly get far from the activator because of its inhibition by plasma inhibitors [17].

These data suggest a possible explanation of the fact that the absolute effects of BAX499 and factor VIII are more or less independent, which lead to the great mutual decrease of the relative effect: this is caused by their action on two different, spatially separated stages of fibrin clot formation that are controlled by two different modules of the coagulation network [24].

Discussion

The present study evaluated the effect of TFPI inhibitor (BAX499) on in-vitro spatial blood coagulation in the presence of different concentrations of factor VIII in a spatial in vitro experimental model. The most significant practical conclusion from the result is BAX499 has little effect on clotting in hemophilia A plasma at high factor VIII concentrations and vice versa. We could expect the same effects for other TFPI inhibitors, since the mechanisms of clotting remain the same; however effects could be more or less pronounced because of different activities against TFPI. This finding suggests a possibility of TFPI inhibitor administration independently of factor VIII levels, as well as normal use of factor VIII concentrates in combination with anti-TFPI agonists.

Importantly, there were two exceptions to this: in two patients, the relative effects of BAX499 addition (in concentration 600 nM) were similar for very different factor VIII concentration. In one of them (Fig. S1b), however, these effects were relatively small (~100% increase in clot size in contrast to ~300-700% effects observed in others at low factor VIII concentrations). This patient, therefore, does not influence the conclusion. In another (Fig. S1i), the relative effects were even less pronounced, approximately 25%, may be because of high initial fVIII activity (37%). It seems that patient #9 failed to comply with the schedule of fVIII infusion. However, this patient also does not influence final conclusion.

In agreement with our previous findings [13], BAX499 predominantly improved coagulation by acting on the initial stages of spatial fibrin clot formation, in contrast to factor VIII that improves spatial propagation [15,17,21]. Our computer simulations (Fig. 4) showed that this can be the major factor behind the phenomena of such drug-drug interaction. TFPI removal promoted factor Xa production by extrinsic tenase by an order of magnitude, but this factor could not get far from the activator because of plasma inhibitors, and thus cannot significantly influence spatial propagation. This data is consistent with findings that shows TFPI rapidly inhibits factor Xa generation by extrinsic tenase and addition of BAX499 prolonged factor Xa generation [4]. In other words, there is little that TFPI inhibitors can do in order to "assist" factor VIII directly. Because of their participation at two different, spatially and temporally distributed stages of coagulation process, the relative effect of one is smaller in the presence of another. The experiment suggests this to an even greater extent: clot size increase by saturating aptamer concentration was ~400 μm for fVIII:C < 5%, and only ~200 μm for fVIII:C > 30%. In this study hemophilia A plasma without inhibitors were used. However, since anti-TFPI agonists could also be used in the future for inhibitory hemophilia treatment, it would be useful to know how they will interact with much higher concentrations of factor VIII in that case. We could expect that the results will be the same for most of the patients for whom the relative effect of fVIII and anti-TFPI aptamer on clot formation appear to be independent of one
Fig. 4. The mechanism of drug-drug interaction: relative contribution of extrinsic and intrinsic tenases to clotting. (A) Clot size as a function of time for four combinations of factor VIII and TFPI deficiencies. (B) Concentration of factor Xa produced by either intrinsic or extrinsic tenase as a function of space and time for the same experiments. The data were obtained from computer simulations using a mathematical model of clotting initiated by immobilized TF at 10 pmole/m².

another. However, the synergetic reaction for the second group of patients could be more complicated and need to be investigated separately.

The method used in this study allows spatially separating extrinsic and intrinsic pathways of coagulation. Reactions of extrinsic pathway occur at the activator surface, while clot propagates in space like it happens in vivo. Therefore in spatial diffusion model clot growth is driven by diffusion of activated factors in a bulk of plasma in contrast to the homogeneous models, which take into account only activities of factors and inhibitors. However, our experiments in platelet free plasma don’t take into account flow, blood cells and endothelium cells with inhibitors. It makes experiments simple but nevertheless far from physiological. For instance, according computer simulation [27] TFPI may play even more critical role in clot formation in presence of flow. This result allows us to speculate that procoagulant effects of TFPI inhibitors may be more pronounced in vitro in presence of flow and in vivo.

It was previously shown that used experimental model is sensitive to hemophilia abnormalities [15,17,18,21]. However, obtained results were quite noisy in this study. Relatively high errors were caused by individuality of patients (VIII level and pharmacokinetics). Moreover, if-TFPI level is a determinant of global hemostatic parameters in CAT and ROTEM assays in plasma with inhibited VIII [6,25]; and its level may vary in wide range (from 0.1 nM to 0.4 nM - 4 fold differences) [6,25]. Unfortunately, if-TFPI level was not measured in this study to estimate its impact. Considerable contribution to increasing of errors and decreasing of sensitivity of method was TF variability: the system is highly sensitive to TF at such extremely low density range [13].

In what position do these findings put TFPI inhibitors compared with other procoagulant anti-hemophilia bypassing agents? Previous thrombin generation assay-based studies reported that activated prothrombin complex concentrates are likely to interact with factor VIII synergistically [28,29], while activated factor VII was found to work with factor VIII in an additive fashion [28]. The combination of Vlla and activated prothrombin complex concentrate was found to be either additive [28], or synergistic [29], or one of the two depending on the patient [30]. Although the pharmacokinetics-based ex vivo design of our experiments and use of qualitatively different coagulation assay does not allow direct comparison of the results, our data indicate that the interaction of BAX499 and factor VIII are somewhere between “additive” and "somewhat less than additive" for most cases; only two patients showed something resembling synergetic reaction. We showed that BAX499 improves clotting at all factor VIII concentrations, and vice versa so their combined addition can potentially improve the coagulation response. However, the mutual decrease of their effects appears to be a significant factor for the majority of the patients suggesting a potential for their use in combination, systematically or upon emergencies, without monitoring their levels.

Addendum

Author contributions are: LA. Parunov, J.C. Gilbert, R.G. Schaub, F.I. Ataullakhano, M.A. Panteleev designed experiments; LA. Parunov, A.N. Balandina, N.P. Soshitova performed experiments; O.A. Fadeeva prepared and characterized activators with immobilized TF; K.G. Kopylov, and M.A. Kumskova recruited and characterized patients; J.C. Gilbert, R.G. Schaub, K.E. McGinnis contributed critically important reagent (BAX499) and conceived the study; LA. Parunov, J.C. Gilbert, R.G.Schaub, K.E. McGinnis, F.I. Ataullakhano, M.A. Panteleev analyzed the data; LA. Parunov and M.A. Panteleev wrote the paper with contributions from all authors.

Conflict of interest statement

J.C.G. and R.G.S. were employees of Archemix Corp. K.E.M. was an employee of Archemix Corp and Baxter Healthcare Corporation. The work reported by L.A.P., O.A.F., A.N.B., N.P.S., K.G.K., M.A.K., M.A.B., M.A.P. was supported by grants from Archemix.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jthroms.2013.10.036.

References


