Published in final edited form as:

Thromb Res. 2012 December ; 130(6): 948–955. doi:10.1016/j.thromres.2012.08.299.

Effect of BAX499 aptamer on tissue factor pathway inhibitor function and thrombin generation in models of hemophilia

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Summary

Introduction—In hemophilia, thrombin generation is significantly suppressed due to decreased factor (F)X activation. Clinical studies and experiments with transgenic mice have suggested that the severity of hemophilia is substantially reduced by tissue factor pathway inhibitor (TFPI) deficiency.

Methods—We evaluated the effect of TFPI antagonist aptamer BAX499 (formerly ARC19499) on TFPI function in purified systems and on thrombin generation and clot formation in plasma and blood.

Results—BAX499 effectively neutralized TFPI inhibition of FXa and FXa dependent inhibition of TF/FVIIa by TFPI. BAX499 did not inhibit FXa or TF/FVIIa when used up to 500 nM. In the synthetic coagulation proteome with TFPI at its mean physiologic concentration, BAX499 at 1 – 10 nM increased thrombin generation triggered with 5 pM relipidated TF in a concentration-dependent manner. In severe hemophilia A or B models using the synthetic coagulation proteome, the addition of BAX499 at 5 nM increased thrombin generation to the levels observed in normal control. Thrombin generation measured in induced hemophilia B plasma required ~100 nM BAX499 to restore thrombin levels to those seen in untreated plasma. In induced hemophilia B whole blood, BAX499 repaired the clotting time but failed to appreciably impact the propagation phase of thrombin generation.

Conclusion—These data suggest that inhibition of TFPI by BAX499 may have potential for hemophilia treatment but requires further study in blood-based hemophilia systems.

Keywords

BAX499; blood coagulation; hemophilia; thrombin generation; tissue factor pathway inhibitor

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Portions of this work were presented at the XXIII Congress of the International Society of Thrombosis and Haemostasis (July 2011) in Kyoto, Japan.

Statement of Conflict of Interest

This work was supported by NIH grant P01 HL46703 Project 2 (Dr. Saulius Butenas) and Project 5 (Dr. Kathleen Brummel-Ziedins) and a grant from Archemix (Dr. Saulius Butenas).

Introduction

The most common blood coagulation defects leading to impaired hemostasis are the hemophilias, caused by factor (F)VIII (hemophilia A) or FIX (hemophilia B) deficiency. Both deficiencies lead to bleeding events, the severity of which is largely dependent upon the concentration of FVIII or FIX [1]. For many hemophilia patients, the standard treatment includes replacement of the missing protein. The most serious complication associated with this therapy is related to an immune response to the infused material, i.e. the generation of antibodies (often inhibitory) to FVIII or FIX [2]. As many as one-third of hemophilia A and 1.5 – 3% of hemophilia B patients develop such inhibitory antibodies toward the infused protein [2]. In attempts to circumvent this immune response, alternative prophylaxis strategies have been employed including recombinant FVIIa [3-6] or plasma-derived protein concentrates [2, 7, 8]. However, these alternatives often increase the already high cost of hemophilia treatment even further [9-11]. In response to these weaknesses of conventional treatments, alternative therapies have been proposed, including gene therapy [12], activated protein C inhibition [13], and, lately, tissue factor pathway inhibitor (TFPI) inhibition.

Tissue factor (TF) initiated blood coagulation is down regulated by several stoichiometric inhibitors including antithrombin, α_2 -macroglobulin, α_1 -antitrypsin and TFPI [14]. Of these, TFPI most efficiently prolongs the initiation phase of thrombin generation [15], by inhibiting both the free FXa as well as the TF/FVIIa/FXa product complex [16]. This impedes the ability of FXa to convert the initial trace amounts of prothrombin to thrombin [17], effectively delaying the propagation phase of thrombin generation, where prothrombin is activated at a very high rate [18].

Inhibition of TFPI as a therapy for hemophilia has shown some promise in several animal studies. Experiments using pentosan polysulfate or fucoidan as a blocking agent showed improved hemostasis in homozygous FVIII and FIX knockout mice [19]. Further work showed that oral administration of a select fucoidan (AV513) to severe hemophilia A dogs restored cuticle bleeding times to normal levels [20]. In addition, it has been demonstrated that an α -TFPI antibody shortens the cuticle bleeding time in rabbits with antibody induced hemophilia A [21].

In this study, we evaluate BAX499, a polyethylene glycol conjugated aptamer that specifically binds to TFPI [22]. Recent studies have shown BAX499 treatment to shorten clot times [22-25] and increase thrombin generation in hemophilia A and hemophilia B plasma and to achieve substantial restoration of hemophilia A or B viscoelastic profiles to those of healthy controls [22, 25]. We extend these studies to a comprehensive biochemical analysis of this aptamer including its regulation of thrombin dynamics in the synthetic coagulation proteome and contact pathway inhibited whole blood, and its effects on clot stability using viscoelastic measurements.

Materials and Methods

Human subjects

Participation of all individuals was approved by the University of Vermont Human Studies Committee. All participants gave informed written consent.

Materials

BAX499 was provided by Archemix Corp. (Cambridge, MA). Human coagulation factors II, VII, IX and X were isolated from fresh frozen plasma using the methods of Bajaj *et al.* [26] and Jenny *et al.* [27]. The proteins were purged of trace contaminants and traces of active enzymes as described [28]. FX was activated to FXa in house [29]. Human FV [30] and

antithrombin [31] were isolated from freshly frozen citrated plasma. Recombinant FVIII and recombinant TF₁₋₂₄₃ (expressed in *E. coli*) were provided as gifts from Drs. Shu Len Liu and Roger Lundblad (Hyland division, Baxter Healthcare Corp, Duarte, CA). Recombinant human FVIIa was provided as a gift by Dr. Lars Pedersen (Novo Nordisk, Denmark). Recombinant full-length TFPI was a gift from Dr. K. Johnson (Chiron Corp., Emeryville, CA). 1,2-Dioleoyl-*sn*-Glycerol-3-Phospho-L-Serine (PS) and 1,2-Dioleoyl-*sn*-Glycerol-3-Phosphocholine (PC) were purchased from Avanti Polar Lipids (Alabaster, AL). TF was relipidated in PCPS (75% PC:25% PS) vesicles (1:2000 protein/lipid) by a previously described protocol [32, 33]. HEPES, sodium chloride, calcium chloride, polyethylene glycol (PEG) and EDTA were purchased from Fisher Scientific (Pittsburgh, PA). Corn trypsin inhibitor (CTI) was isolated as previously described [34]. Spectrozyme Xa and Spectrozyme TH were purchased from American Diagnostica (Stamford, CT). The monoclonal inhibitory antibody α -FIX-91 was produced in the Biochemistry Antibody Core Laboratory (University of Vermont) and characterized as previously described [35]. Single-chain t-PA was separated from a mixture of single-chain and two-chain t-PA (a gift from Genentech (San Francisco, CA)) as previously described [36]. D-Phe-Pro-Arg-CH₂Cl (FPRck) was produced in house [37]. The fluorogenic substrate Z-GGR-AMC was purchased from Bachem (Torrance, CA).

FXa inhibition by TFPI

The active concentration of TFPI was determined by a titration using FXa [38]. It was found to be 60% of the total protein mass present. All TFPI concentrations stated represent the active concentration.

Characterization of the reaction between TFPI and FXa in the presence or absence of BAX499 was performed as described by Baugh *et al.* [39]. TFPI (3 nM) was added to a mixture of human FXa (1 nM) and BAX499 (0 – 250 nM) in HEPES buffered saline (HBS)/0.1% PEG/2 mM CaCl₂ at 37 °C. At predetermined time intervals, aliquots were removed and added to 200 μ M Spectrozyme Xa. The reaction was diluted only 10% by the chromogenic substrate. The absorbance at 405 nm was read immediately in a THERMOMax microplate reader (Molecular Devices Corp., Menlo Park, CA). Initial velocity measurements were used to estimate the free or uninhibited concentration of FXa remaining at each sampled time point by reference to a calibration curve.

To estimate a K_d value for the interaction between TFPI and BAX499, it was assumed that the final distribution of species when TFPI, FXa and aptamer are mixed is defined by two competing reversible interactions involving 1 to 1 complexes between FXa and TFPI (equation 1) and TFPI and aptamer (equation 2).

The relevant equations are:

$$K_d = \frac{[TFPI]_{free} * [FXa]_{free}}{[FXa-TFPI]} \quad 1$$

$$FXa_{total} = FXa_{free} + FXa-TFPI \quad 2$$

$$TFPI_{total} = TFPI_{free} + FXa-TFPI + BAX499-TFPI \quad 3$$

$$BAX499_{total} = BAX499_{free} + BAX499-TFPI \quad 4$$

$$K_d = \frac{[\text{TFPI}]_{\text{free}} [\text{BAX499}]_{\text{free}}}{[\text{BAX499-TFPI}]}$$

The time course of FXa inhibition for each aptamer concentration was analyzed using double exponential fits to estimate the free FXa concentration present at equilibrium. Using the measured $[\text{FXa}]_{\text{free}}$ and a K_d value of 0.4 nM [39], equations 1 and 2 were used to calculate $[\text{TFPI}]_{\text{free}}$, then equation 3 was used to calculate $[\text{BAX499-TFPI}]$, and equation 4 was used to calculate $[\text{BAX499}]_{\text{free}}$. The apparent K_d for the TFPI aptamer interaction at each concentration of aptamer was then calculated using equation 5.

FX activation

20 pM relipidated TF, 1 nM FVIIa, 20 μM PCPS, and aptamer (0 – 1000 nM) in HBS/0.1% PEG/2 mM CaCl_2 was incubated for 10 min at 37 °C. 250 nM FX and 3 nM TFPI were then added to the reaction mixture. Every minute, an aliquot was removed and quenched into 15 mM EDTA and 200 μM Spectrozyme Xa. Aliquots were diluted only 15% by the quenching solution. The absorbance at 405 nm was read immediately in a THERMOMax microplate reader. Initial rates of were calculated from a calibration curve constructed using dilutions of FXa.

Synthetic coagulation proteome

The procedure used is a modification of Lawson *et al.* [40] and van 't Veer *et al.* [28]. Relipidated TF at 5 pM and PCPS at 50 μM (final concentrations) were added to a mixture of procoagulant factors II, V, VII, VIIa, VIII, IX and X, as well as inhibitors TFPI and AT (all at mean physiologic concentrations) in HBS/0.1% PEG/2 mM CaCl_2 at 37 °C. When desired, BAX499 (0 – 10 nM) was added and FVIII or FIX was omitted. Every minute, an aliquot was removed and added to 20 mM EDTA and 200 μM Spectrozyme TH. The absorbance at 405 nm was read immediately in a THERMOMax microplate reader. The thrombin concentration was determined by a calibration curve constructed using dilutions of α -thrombin.

Thrombin generation assay (TGA)

TGAs were performed as previously described [41]. All assays utilized contact pathway inhibited (CTI, 0.1 mg/mL) citrated pooled normal plasma either in the absence or presence of 0.1 mg/mL α -FIX-91 to simulate hemophilia B. 80 μL of plasma (premixed with the desired concentration of BAX499) was mixed with 20 μL HBS containing 90 mM CaCl_2 and 2.5 mM fluorogenic substrate Z-GGR-AMC and incubated for 3 minutes at 37 °C. The reaction was initiated by the addition of 20 μL HBS containing 30 pM TF and 120 μM PCPS. Thrombin generation was monitored in the Synergy4 plate reader.

Whole blood clotting assay

The procedure used was a modification of Rand *et al.* [42]. Blood was collected by venipuncture and immediately placed into a beaker containing CTI (100 $\mu\text{g/mL}$) $\pm$ α -IX (100 $\mu\text{g/mL}$) $\pm$ BAX499 (100 nM). This blood was then aliquoted (1 mL) into rocking tubes at 37 °C containing TF (5 pM or 1 pM) relipidated in PCPS. Tubes were quenched at defined time intervals to 24 minutes with a 1 mL volume of a mix of inhibitors (50 mM EDTA, 20 mM benzamidine, 100 μM FPR-ck). All samples were centrifuged (4 °C, 1200 g, 30 min), and the soluble material was frozen at –80 °C until further analysis (in-house) for α -thrombinantithrombin (α -TAT) complex [43].

Thromboelastography (TEG)

Blood was collected by venipuncture and added to 100 µg/mL CTI ± 100 µg/mL α-FIX-91. 345 µL were added to a TEG cup containing relipidated TF (0.5 pM final), single chain t-PA (1 nM final) ± BAX499 (100 nM final). Analysis was carried out on each sample using a Thromboelastograph Haemostasis Analyzer Model 5000 (Haemoscope, Niles, IL) at 37 °C. TEG parameters were extracted using TEG V4 software (Haemoscope, Niles, IL, USA).

Results

Aptamer blockade of TFPI inhibition of FXa

At a concentration of up to 500 nM, BAX499 had no effect on FXa activity (data not shown) in the absence of TFPI. In the absence of BAX499, TFPI decreased the concentration of active FXa from 1 nM at zero time to 0.19 nM after 15 minutes (Figure 1A). Analysis of this binding isotherm indicated that the K_d for TFPI and FXa was ~0.4 nM, consistent with a previous report [39]. With increasing BAX499, the effect of TFPI was lessened in a concentration dependent manner. At 250 nM aptamer, 0.76 nM FXa remained after 15 minutes.

Using double exponential fits to analyze each binding isotherm, a dissociation constant was calculated for each concentration of aptamer (Figure 1B). Between 2.5 and 25 nM BAX499, the calculated K_d ranged between 1.2 and 1.8 nM. However, the calculated K_d increased at higher aptamer concentrations. At 100 nM aptamer, the calculated K_d was 5.8 nM. At 250 nM aptamer, the calculated K_d increased to 11.8 nM.

Aptamer blockade of TFPI inhibition of extrinsic FXase activity

At a concentration of 500 nM, BAX499 had no effect on FX activation by the TF/VIIa complex in the absence of TFPI (Figure 2, dotted line). In the absence of BAX499, the addition of 3 nM TFPI caused the FXa generation rate to be below the detection limit of our assay (<0.05 nM/min). With increasing BAX499 concentration, FXa generation increased. At 50 nM aptamer, about 50% of the FXase activity was recovered. However, saturating levels of BAX499 (~500 nM) only resulted in a FXa generation rate 75 – 80% of that observed in the absence of TFPI.

Aptamer modulation of TFPI activity in TF-initiated thrombin generation

Synthetic coagulation proteome—In the synthetic coagulation proteome model, the onset of thrombin generation in the control experiment (100% FVIII, 100% FIX) with no BAX499 occurred between 4 and 5 minutes (Figure 3A). Addition of 1 nM BAX499 decreased this time to 3 – 4 minutes. 5 nM BAX499 further decreased the onset to 2 – 3 minutes. The addition of 10 nM BAX499 resulted in a thrombin generation curve that was similar to that observed when TFPI was omitted from the system. The maximum level of thrombin generated similarly increased in a concentration dependent manner, from 270 nM with no aptamer to 380 nM in the presence of 10 nM aptamer.

BAX499 was then tested in hemophilia proteome models. In the absence of FVIII, the onset of thrombin generation occurred at 10 minutes and reached a maximum level of 30 nM (Figure 3B) compared to 4 – 5 minutes and 270 nM, respectively, in the control. The addition of 1 nM BAX499 decreased the onset time to 4 minutes and the maximum level of thrombin was increased to 110 nM. An increase to 5 nM BAX499 resulted in a faster onset of thrombin generation curve than in the control (100% FVIII), reaching a maximum level of 240 nM. A further increase to 10 nM BAX499 resulted in thrombin generation dynamics similar to that seen in the absence of TFPI. Comparable results were seen when BAX499 was evaluated in the proteome model of hemophilia B (absence of FIX; Figure 3C).

We then investigated BAX499 at FVIII concentrations seen in moderate to mild hemophilia. A titration without BAX499 is shown on Figure 4A. FVIII concentrations of less than 5% are characterized by slow thrombin generation (onset of 7 - 9 minutes) that has a maximum level of less than 50 nM. At 40% FVIII, the onset of thrombin generation occurs at 6 minutes, and the maximum level reaches 120 nM. The control experiment with 100% FVIII reaches a maximum level of approximately 250 nM and had an onset time of 5 minutes.

In the experiments of Figure 4B, FVIII was titrated with 2.5 nM aptamer present in the proteome. When FVIII concentrations were 0%, 2% or 5% of the mean physiologic level, the presence of 2.5 nM BAX499 resulted in nearly identical thrombin generation curves. These profiles are characterized by an earlier onset of thrombin generation and maximum thrombin levels of 80 – 90% of that observed with the 100% FVIII control. At 40% FVIII with 2.5 nM BAX499, the onset of thrombin generation occurred at approximately 2 minutes and resulted in nearly an identical maximum level to the 100% FVIII control.

Thrombin generation assay (TGA)—Figure 5 presents an analysis of aptamer efficacy in normal and induced hemophilia B plasma. In the TGA, the control (no α -FIX-91, Figure 5A) experiment with no BAX499 reached a maximum of 85 nM, a concentration that was unchanged with the addition of 1 nM BAX499. An increase to 10 nM aptamer raised the maximum thrombin level to 105 nM. 100 nM BAX499 caused the maximum level to increase to 125 nM. A maximum of 150 nM thrombin was reached with 500 nM BAX499. An increase in aptamer concentration to 1 μ M did not increase the maximum thrombin level beyond 150 nM thrombin.

In induced hemophilia B plasma (Figure 5B), levels of BAX499 at or below 10 nM did not increase peak thrombin from 50 nM, the level that was observed in the absence of the aptamer (although the rate did increase). A maximum thrombin level of 75 nM was reached at 100 nM aptamer. At aptamer levels of 500 nM, the maximum level was 85 nM, nearly indistinguishable from the control (without α -FIX-91 or BAX499). An increase to 1 μ M BAX499 did not amplify thrombin generation further. In the plasma milieu, normalization of thrombin generation in hemophilia B required over 100 times more BAX499 compared to the buffer system.

Aptamer modulation of TFPI activity in TF-initiated coagulation in contact pathway inhibited whole blood

Thrombin generation—Initial experiments were conducted using a 5 pM TF stimulus in contact pathway inhibited whole blood. In contrast to proteome and plasma model systems, aptamer concentrations up to 1 μ M failed to improve thrombin generation (α -thrombin-antithrombin complex, α -TAT) in the induced hemophilia B model (data not shown). A subsequent TF titration in induced hemophilia B blood indicated that at lower TF concentrations (1 pM), the aptamer did affect thrombin dynamics, shifting clot times towards the control value (no α -FIX-91, data not shown). Subsequent studies using whole blood were conducted at TF concentrations 1 pM.

Figure 6 presents α -TAT formation in blood from two individuals in which whole blood clotting was initiated by 1 pM TF in the presence and absence of 100 nM BAX499, a concentration which when added to antibody treated whole blood had shown equivalence to untreated blood using TEG in a previous study [22]. The effect of this aptamer was tested in both normal and induced hemophilia B models. The addition of the aptamer to the control experiment decreased the clotting time from 8.3 to 6.7 minutes in subject 1 (Figure 6A) and from 10.6 to 8.5 minutes in subject 2 (Figure 6B). In neither subject did the effect of the aptamer increase the rate or extent of α -TAT formation.

In both individuals, aptamer addition to their induced hemophilia B blood reduced the clotting time. In subject 1, the clotting time was reduced from 12.8 to 8.1 minutes, which was similar to the control experiment (8.3 minutes). In subject 2, the aptamer reduced the clotting time from 14.0 to 12.1 minutes.

However, similar to results seen in control blood, the aptamer did not markedly improve α -TAT generation in the hemophilia setting. In subject 1, the 20 minute level of α -TAT for the control experiment was 310 nM. This value decreased to 60 nM in the presence of α -FIX-91, a level that was unchanged in the presence of 100 nM BAX499. The results for subject 2 similarly showed minimal improvement in α -TAT generation in the induced hemophilia B setting.

Thromboelastography—Hemophilia blood can display prolonged R values (clot time) and reduced angle parameter values (rate of clot growth) relative to normal blood when analyzed by thromboelastography [44, 45]. The inclusion of t-PA in TEG assays has been used to quantify differences in clot stability in hemophilia in response to various hemostatic agents [46-48]. Figure 7 presents the results for three individuals of a viscoelastic analysis of aptamer effects on clot stability. Coagulation in contact pathway inhibited blood from 3 healthy subjects was initiated with 0.5 pM TF in the presence of 1 nM t-PA in normal or induced hemophilia B systems. The TF concentration was chosen to maximize the differences seen between control and induced hemophilia blood. Table 1 summarizes the resulting TEG parameters.

In all three subjects, the addition of 100 nM BAX499 shortened the R time in control (no α -FIX-91 + 1 nM t-PA) blood by an average of 6 minutes, while maximum amplitude (MA) values were relatively unchanged (Figure 7, Table 1). R times in induced hemophilia B blood (+ 1 nM t-PA) were increased relative to the control, with subjects 3 (Figure 7A) and 4 (Figure 7B) having an undefined R time (>120 minutes). All subjects' MA values were strongly decreased (or undefined) in hemophilia blood relative to their controls.

With the addition of 100 nM BAX499 to induced hemophilia B blood, two of the three subjects displayed a normalized R time. Subject 4's undefined R time remained undefined despite the presence 100 nM BAX499. The effect of BAX499 on the MA parameter in induced hemophilia B blood varied among these subjects, with only subject 5 (Figure 7C) showing a value approaching that of the untreated control.

In two subjects (subjects 3 and 5, Figures 7A and 7C), the addition of 100 nM BAX499 generated a clot in induced hemophilia blood faster than without the aptamer. However, the clots did not appear to have lasting strength. In both cases, the clots were solubilized faster in induced hemophilia blood with BAX499 than in the hemophilia control.

Discussion

TFPI primarily influences the initiation phase of thrombin generation [15, 28], simultaneously neutralizing the initiating TF stimulus and suppressing the steady state levels of FXa. This interval, spanning from the time of injury to clot time, is only moderately impacted by FVIII or FIX deficiency, because the level of extrinsic FXase activity can be sufficient to produce enough FXa to generate the relatively low amounts (~2 nM) of α -thrombin required for a clot to occur [49]. The primary defect in hemophilia is diminished levels of prothrombinase, caused by the deficiency in formation of the intrinsic FXase complex, resulting in impaired post-clot thrombin formation. This prevents the major amplification of thrombin generation that is required for proper wound sealing [50]. Previous work from our laboratory [51] and others [52-54] have shown that individuals with

hemophilia have an impaired conversion of fibrinogen to fibrin, including slower rates of FXIII activation, impaired TAFI activation [55, 56] and fibrinopeptide release compared to healthy individuals. These quantitative defects result in more soluble, thus weaker, clots. Work from the laboratories of Wolberg [57] and Weisel [58] have detailed the importance of the interplay of thrombin generation and the fibrinolytic process on clot stability. Thus the restoration of both the clotting time and the propagation phase parameters will be required if BAX499 is to effectively neutralize the overall consequences of FVIII and FIX deficiencies.

The potential of treating hemophilia A individuals with BAX499, a specific TFPI antagonist, has been explored by several groups [22-25]. In this study we have presented analyses of BAX499 mechanism, specificity and potency in experimental systems with purified components, in contact pathway-inhibited citrate plasma and in contact pathway-inhibited whole blood. In entirely synthetic systems of purified proteins, the aptamer BAX499 specifically and effectively neutralizes TFPI function, including direct TFPI inhibition of FXa, and its FXa dependent inhibition of TF/FVIIa. Results from these studies are consistent with previous reports [22-25], including the observation of incomplete blockade of TFPI inhibition of the extrinsic tenase [24]. However, one unexpected finding was the increase in calculated apparent K_d for the BAX499/TFPI interaction with increasing aptamer concentration. We hypothesized that aptamer dimerization/multimerization could be occurring, however light scattering measurements with up to 1.5 μM BAX499 showed no evidence of aggregation at the concentrations used in our assays (data not shown).

An alternative explanation is that the TFPI-aptamer complex is itself an inhibitor of fXa, but with lower affinity. Thus as the TFPI-aptamer complex concentration increases during titration experiments, a new phase of fXa inhibition emerges blocking complete restoration of fXa activity, thus resulting in apparently increasing K_d values for the aptamer TFPI interaction. Chang *et al.* [24] have previously suggested, on the basis of a modeling analysis of their kinetic data, that the TFPI-aptamer complex inhibits fXa. This mechanism could also explain the apparent “resistance” of the extrinsic fXase to complete restoration of function by aptamer binding of TFPI. However, a definite resolution would require direct binding studies testing the TFPI-aptamer complex binding to fXa.

In hemophilia models directly measuring thrombin generation after TF initiation, BAX499 proved effective in restoring the defect in thrombin generation. In the synthetic coagulation proteome models of hemophilia A and B, just 1 – 5 nM BAX499 is sufficient to mitigate the delayed onset and suppressed thrombin generation associated with hemophilias A and B. Compared to the buffer-based coagulation proteome, contact pathway inhibited plasma thrombin generation assays required a higher concentration of BAX499 (~100 nM) to restore the induced hemophilia B system to control levels. Previous reports [22, 25] have shown a similar concentration dependence in this model system and have indicated differences in aptamer efficacy can derive from individual variation in the TFPI concentration [25]. Interpretation of these data in light of the titration data from the synthetic coagulation proteome experiments suggests that the effective aptamer concentration in plasma is substantially reduced, presumably reflecting non-specific association of the aptamer with plasma proteins or the influence of interactions between protein S and TFPI [59].

BAX499 efficacy in whole blood models of hemophilia appeared less straightforward. In our whole blood system of thrombin generation, aptamer efficacy had a pronounced sensitivity to TF concentration. At 5 pM TF, neither the clot time nor the level of thrombin generation were improved by concentrations of BAX499 up to 1 μM . When initiated with 1 pM TF, clot times were shortened in both control and induced hemophilia B blood but in both models the presence of the aptamer did not alter the propagation phase parameters of

thrombin generation (maximum rate and level of TAT formation). Similarly, Parunov *et al.* [23] demonstrated in their hemophilia A plasma-based spatial diffusion experimental model that the parameter most responsive to BAX499 was the clot time parameter, while improvement of other model parameters required higher BAX499 concentrations and did not result in full normalization of the parameters to control levels.

In our modified TEG assay with t-PA [48], we noticed significant individual variation. This assay is designed to provide a more comprehensive assessment of a hemophilia individual's capacity to form a stable clot by simultaneously challenging each individual's procoagulant and fibrinolytic systems. Assayed in this way, our three subjects showed three different levels of response to the α -FIX-91 antibody and to BAX499. However, in only one case did the aptamer restore hemophilia profiles close to the state of its corresponding control. Consistent with this level of variation, Waters *et al.* [22] reported significant variability in BAX499 efficacy in correcting bleeding times in a nonhuman primate model of induced hemophilia A, with some individuals not responding to BAX499. Effective clot formation in this *in situ* model presumably reflects the overall balance between procoagulant and fibrinolytic processes. Together these data suggest that variation between individual blood donors can contribute to inconsistent outcomes with BAX499, with its effects on clot time restoration more universal than its capacity to normalize thrombin generation and clot stability. Because the primary defect in hemophilia stems from an impaired propagation phase [50], the blockade of TFPI function may not be sufficient to normalize hemostasis in some individuals. Additional experiments will be required to first confirm our observations and those of Waters *et al.* [22] on significant individual variation in the response to BAX499 and then to understand the mechanistic origins of this variable response.

Acknowledgments

The authors would like to thank Maya Aleshnick, Matthew Whelihan and Vicentios Zachary for technical assistance and Dr. Kenneth G. Mann for the opportunity to use his laboratory facilities. This work was supported by NIH grant P01 HL46703 Project 2 (Dr. Saulius Butenas) and Project 5 (Dr. Kathleen Brummel-Ziedins) and a grant from Archemix (Dr. Saulius Butenas).

Abbreviations

CTI	corn trypsin inhibitor
F	factor
FPRck	D-Phe-Pro-Arg-CH ₂ Cl
HBS	HEPES buffered saline
PEG	polyethylene glycol
PC	1,2-Dioleoyl- <i>sn</i> -Glycero-3-Phosphocholine
PS	1,2-Dioleoyl- <i>sn</i> -Glycero-3-Phospho-L-Serine
PCPS	phospholipid vesicles composed of 75% PC and 25% PS
TF	tissue factor
TFPI	tissue factor pathway inhibitor

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Figure 1. BAX499 regulation of TFPI inhibition of FXa

A. Time courses of active FXa remaining after addition of 3 nM TFPI to 1 nM FXa in the presence of BAX499. ◆, no aptamer; ▲, 2.5 nM aptamer; ●, 5 nM aptamer; ■, 10 nM aptamer; △, 25 nM aptamer; □, 100 nM aptamer; *, 250 nM aptamer. B. Dissociation constant (K_d) calculated for each concentration of aptamer.

Figure 2. BAX499 regulation of TFPI inhibition of the extrinsic FXase

Initial rates of FXa generation in the presence of 20 pM TF/FVIIa, 250 nM FX, 20 μ M phospholipid and 3 nM TFPI at varying aptamer concentrations (8.33, 25, 50, 200, 500, 1000 nM). The dotted horizontal line gives the rate for the experiment in the absence of TFPI ($\pm$ aptamer).

Figure 3. BAX499 modulation of thrombin generation in the synthetic coagulation proteome model of severe hemophilia A and B

5 pM TF-initiated thrombin generation in a purified system at varying BAX499 concentrations. A. Control. B. Hemophilia A. C. Hemophilia B. ◇ no aptamer (0% FVIII (panel B) or 0% FIX (panel C)); ◆, no aptamer (100% FVIII and FIX); ▲, 1 nM aptamer; ●, 5 nM aptamer; ■, 10 nM aptamer; +, no TFPI, no aptamer.

Figure 4. BAX499 modulation of thrombin generation in synthetic coagulation proteome models of mild, moderate and severe hemophilia A

Thrombin generation was initiated with 5 pM TF in the absence (panel A) and presence (panel B) of 2.5 nM BAX499. ◆, 100% FVIII (No aptamer); ×, 100% FVIII (2.5 nM aptamer); ■, 40% FVIII; ●, 5% FVIII; ▲, 2% FVIII; ◇ 0% FVIII.

Figure 5. BAX499 modulation of thrombin generation in contact pathway inhibited plasma

A. Normal plasma B. Induced hemophilia B plasma. Solid black line, no BAX499; broken black line, 1 nM BAX499; dotted black line 10 nM BAX499; solid gray line, 100 nM BAX499; broken gray line, 500 nM BAX499; dotted gray line 1000 nM BAX499.

Figure 6. BAX499 effects on thrombin generation in contact pathway inhibited whole blood
A. Subject 1. B. Subject 2. Arrows indicate visual clotting times. ◆, Control; ▲, Control + 100 nM aptamer; ◇, Induced hemophilia B; △, Induced hemophilia B + 100 nM aptamer.

Figure 7. Thromboelastographic analysis of BAX499 effects on clot stability

A-C. Subjects 3 – 5. Each subject's contact pathway inhibited blood supplemented with 1 nM t-PA was analyzed in the presence (gray lines) or absence (black lines) of 100 nM BAX499. Both control (outer lines) and induced hemophilia B (inner lines) are shown.

Table 1**Thromboelastographic analysis of BAX499**

Each subject's contact pathway inhibited blood supplemented with 1 nM t-PA was analyzed in the presence or absence of 100 nM BAX499 and in the presence or absence of inhibitory antibody α -FIX-91 (n=2 for all measurements).

Subject	Control				Induced Hemophilia B			
	-100 nM BAX499		+100 nM BAX499		-100 nM BAX499		+100 nM BAX499	
	R	MA	R	MA	R	MA	R	MA
3	26 $\pm$ 4	21 $\pm$ 5	20 $\pm$ 0	23 $\pm$ 6	UD	UD	26 $\pm$ 1	7 $\pm$ 1
4	25 $\pm$ 1	26 $\pm$ 2	20 $\pm$ 0	30 $\pm$ 3	UD	UD	UD	UD
5	23 $\pm$ 1	28 $\pm$ 3	17 $\pm$ 0	37 $\pm$ 2	35 $\pm$ 1	8 $\pm$ 2	21 $\pm$ 1	20 $\pm$ 5

R – clot time

MA – maximum amplitude

UD – undefined