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ORIGINAL ARTICLE

Clot formation and fibrinolysis assays reveal functional differences among hemostatic agents in hemophilia A plasma

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Abstract

Background: Measuring the activity of hemostatic agents used to treat hemophilia A often requires drug-specific assays. *In vitro* assays show hemophilic clots have abnormal characteristics, including prolonged clotting time and decreased resistance to fibrinolysis. The ability of certain agents to correct these parameters *in vitro* is associated with hemostatic efficacy *in vivo*.

Objectives: To compare effects of established and emerging hemostatic agents on clot formation and fibrinolysis in hemophilia A plasma.

Methods: Pooled and individual hemophilia A platelet-poor plasmas were spiked with replacement (recombinant factor VIII [rFVIII], PEGylated rFVIII, polysialylated rFVIII, and porcine rFVIII) or bypassing (emicizumab, rFVIIa, and activated prothrombin complex concentrate) products. Effects on tissue factor-initiated clot formation and fibrinolysis were measured by turbidity.

Results: Compared to normal pooled plasma, hemophilia-pooled plasma showed reduced clot formation and increased fibrinolysis, and all replacement agents improved these characteristics. rFVIII and PEGylated rFVIII produced similar effects at similar concentrations, whereas polysialylated rFVIII produced slightly higher and porcine rFVIII slightly lower effects at these concentrations. Bypassing agents enhanced clot formation and stability, but patterns differed from replacement agents. The clotting rate showed a concentration-response relationship for all agents. High concentrations of all products produced effects that exceeded the normal range in at least some parameters. Responses of individual donors varied, but all agents improved clot formation and stability in all donors tested.

Conclusion: Clotting and fibrinolysis assays reveal hemostatic effects of replacement and bypassing therapies at clinically relevant concentrations. These assays may help characterize hemostatic agents and optimize dosing.

KEYWORDS
clot, factor VIII, fibrinolysis, hemophilia, hemostasis

Essentials

- Hemostatic drugs with different mechanisms of action are measured by specialized clinical assays.
- Hemophilia A plasma has delayed clot formation and decreased resistance to fibrinolysis *in vitro*.
- Hemostatic drugs improve hemophilic clot formation at clinically relevant concentrations.
- *In vitro* clotting and fibrinolysis assays may facilitate comparisons between hemostatic agents.

1 | INTRODUCTION

Deficiency in factor VIII (FVIII) quantity or quality causes the bleeding disorder hemophilia A, which is associated with spontaneous joint and muscle bleeding and bleeding after dental extraction, surgery, and injury. FVIII replacement reduces bleeding events and is standard of care in children and many adults [1,2]. However, practical limitations, including the need for frequent intravenous infusions, reduce adherence. Inhibitor development is problematic, particularly in severe hemophilia [3], necessitating the use of bypassing agents in these individuals. Development of new hemostatic agents with improved safety and pharmacologic characteristics (extended circulating half-life, increased activity, and improved administration methods) and/or against novel targets that bypass conventional pathways is a high priority.

Multiple hemostatic products are currently in clinical use or development, and these have different pharmacologic features. Full-length recombinant FVIII (rFVIII [Advate]) is a third-generation replacement product expressed in a plasma/albumin-free cell culture method. rFVIII has an average half-life of ~12 hours and is given every other day. Other recombinant FVIII products based on the same manufacturing platform are PEGylated (Adynovate) or polysialylated (SHP656, formerly BAX-826 [4]) to increase their half-life up to ~1.5-fold versus rFVIII and reduce the need for frequent infusions [4]. A recombinant B-domainless FVIII construct based on the porcine FVIII sequence (Obizur) has low cross-reactivity with human FVIII inhibitors, which enables administration in patients with inhibitors [5]. Porcine rFVIII has a half-life of ~12 hours [6]. Conventional bypassing agents include plasma-derived, activated prothrombin complex concentrate (APCC, FVIII inhibitor bypassing activity) and rFVIIa (NovoSeven). APCC and rFVIIa therapies are well-established but require frequent dosing due to their relatively short half-lives (4-7 hours [7] and 2.5 hours [8], respectively). A humanized bispecific antibody that recognizes FIXa and FX simultaneously and recapitulates the tenase complex (emicizumab [Hemlibra]) is a more recent addition to the hemostatic arsenal. Advantages of the bispecific antibody, including its extended half-life (4 weeks) and the ability to administer subcutaneously, have substantially changed the landscape for bypassing therapies [9]. This myriad of hemostatic agents has expanded the therapeutic choices available to persons with hemophilia and enabled a more personalized approach to hemostatic therapy.

A challenge when evaluating the available products stems from their unique mechanisms of action, which produce different and sometimes unexpected effects in assays that employ different reagents and instruments [10–17]. The modified rFVIII products retain many of the biochemical properties of native FVIII but behave differently in the presence of different activators (eg, silica or kaolin-based products) [13]. Bypassing agents that include preactivated factors circumvent conventional initiation mechanisms and interfere with 1-stage assays (clotting times). Accordingly, discrepancies between 1-stage and 2-stage clotting or chromogenic assays complicate comparisons between, and postinfusion monitoring of, FVIII replacement products [10–13] and bispecific antibodies [14–17]. The use of product-specific reference standards enables quantification of individual agents but makes it difficult to assign FVIII-equivalent values, directly compare products during preclinical development, or measure therapeutic effects in the presence of endogenous factors or when more than 1 product is on board. These challenges complicate the operational workflow of clinical laboratories [10–13]. An assay that can detect dose-dependent, procoagulant effects of products with different mechanisms of action and be used to compare agents in preclinical and postclinical settings may facilitate product characterization. Moreover, the ability to measure activity in an individual's plasma milieu may help personalize therapy. This may be particularly important for FVIII-based products since elevated FVIII activity may paradoxically predispose individuals to thrombotic risk [18–23].

Compared to healthy individuals, plasma from persons with hemophilia exhibits a prolonged time to clot formation and increased susceptibility to fibrinolysis, and hemostatic agents that enhance clot formation, structure, and stability *in vitro* reduce bleeding *in vivo* [24–34]. Effects on clotting and clot stability are mediated through functional interactions between procoagulant and antifibrinolytic pathways that enable stable fibrin formation. Thus, assays that measure the pharmacodynamic effects of hemostatic therapy on clot formation and fibrinolysis may facilitate direct, functional comparisons between new and emerging hemostatic agents that have different mechanisms of action.

The goal of this study was to compare the effects of established and emerging hemostatic agents on plasma clot formation and stability.

2 | METHODS

2.1 | Materials

All pooled and individual platelet-poor plasmas were purchased from HRF, Inc. The hemophilic plasma pool (HPP; #21P3F8) included 6 male donors with severe congenital FVIII deficiency (<1% FVIII activity).

The normal plasma pool (NPP; #18P1NML, 59% FVIII [reference range, 56%-186%]) included 8 healthy donors (4 male, 4 female) with no history of bleeding or thrombotic events. The 11 individual male donors had severe congenital FVIII deficiency (<1% FVIII activity) and no inhibitors. rFVIII (Advate), PEGylated rFVIII (Adynovate), polysialylated rFVIII (SHP656), porcine rFVIII (Obizur), APCC (FVIII inhibitor bypassing activity), and rFVIIa (NovoSeven) were provided by Takeda. Recombinant tissue plasminogen activator (tPA; Alteplase) and emicizumab were acquired at the University of North Carolina at Chapel Hill. Stocks of each product were prepared in single-use aliquots, and a fresh aliquot was thawed each day. Dilutions were then made from the thawed aliquot on the day of the experiment. Supplementary Table S1 shows clinical dosing ranges and concentrations tested. Concentrations of each hemostatic agent were selected to span the plasma concentrations achieved following standard dosing strategies ([4,7,8,35–39] and indicated in Supplementary Table S1) and enable curve fitting to identify maximum responses, if possible. Human tissue factor (Dade Innovin) was from Siemens Healthcare Diagnostics. MP reagent (Stago Diagnostica) was used as a source of phospholipids.

2.2 | Clot formation and fibrinolysis reactions

Clot formation and fibrinolysis assays were performed as described previously [40]. Since not every agent could be tested on every plate, NPP, HPP, and HPP plus rFVIII, PEGylated rFVIII, polysialylated rFVIII, and porcine rFVIII were measured in 1 plate, and NPP, HPP, and HPP plus rFVIII, emicizumab, APCC, and rFVIIa were measured in a second plate (ie, NPP, HPP, and rFVIII were included on both plates). Briefly, plasma was thawed at 37 °C with gentle end-over-end mixing. Hemostatic agents (10 μL) were added to 85 μL of plasma and mixed gently. Reagents (4 μL phospholipids [4 μM final], 4 μL $CaCl_2$ [10 mM final], 4 μL tissue factor [1:15,000 dilution of Innovin, ~1 pM final tissue factor], and 4 or 8 μL HEPES-buffered saline (20 mM HEPES, pH 7.4, 150 mM NaCl) were added to a 96-well plate (Corning); each reagent was added to different parts of the well to prevent mixing. Fibrinolysis experiments were performed in the presence of tPA (4 μL, 0.5 μg/mL final). Plasma (80 μL) with the hemostatic agent was then added to the wells using a multichannel pipet. The reactions were gently but thoroughly mixed, avoiding air bubbles. Reactions were monitored for 2 hours by measuring the turbidity at 405 nm every 12 seconds using a SpectraMax 384Plus plate reader (Molecular Devices).

Calculated parameters were onset (time to the inflection point before turbidity increase), maximum rate (slope of a line fitted to the maximum rate of turbidity increase using 5 to 10 points to determine the line; Softmax Pro v7.0, Molecular Devices), time to plateau/peak (time to the turbidity plateau [clot formation] or peak [fibrinolysis]; TTP), and turbidity change (maximum clot turbidity less the starting turbidity). The fibrinolysis analysis also included area under the curve (AUC) (calculated as the sum of trapezoids formed by turbidity curves − a baseline established by the lowest measurement recorded; calculated in GraphPad Prism v9.0.2 [GraphPad Software]). For samples that did not clot, parameters were recorded as 120 minutes (for onset and TTP) or 0 (for rate, peak turbidity change, and AUC). Representative clot formation and fibrinolysis curves are shown in Supplementary Figure S1.

2.3 | Statistical analysis

Descriptive statistics for clot formation, structure, and fibrinolysis parameters were summarized using means and SDs. Intraassay variation was the mean of the ratio of SD to mean of 3 to 4 replicates performed in a single experiment, averaged over 6 to 7 separate experiments. Interassay variation was the ratio of the mean of the SD to the mean of the means from 6 to 7 separate experiments. Interindividual variation was the ratio of SD to the mean from 9 to 11 individual hemophilic plasmas. Clot formation and fibrinolysis parameters were fit to a dose-response model using a variable slope in GraphPad Prism version 10.1.0 (GraphPad Software). Clot formation and fibrinolysis parameters from experiments with NPP and HPP were compared by *t*-tests or Wilcoxon tests, as appropriate for normally and nonnormally distributed data, respectively. For experiments testing concentration-response effects in HPP, drug concentrations producing supranormal responses were identified by Kruskal–Wallis test with Dunn's test, which were limited by comparing only responses identified by visual inspection as being shorter than (time parameters) or above (rate, turbidity, and AUC) the normal range. Effects of hemostatic agents on individual donor plasmas are presented descriptively.

3 | RESULTS

3.1 | Effects of hemostatic agents on clot formation in the hemophilic plasma pool

Interassay variability was less than 15% for all clot formation parameters (Supplementary Table S2). The mean clotting onset, TTP, and rate differed significantly ($P < .05$) between NPP and HPP (Figure 1, gray and red shaded areas, respectively) and were therefore used to compare effects of hemostatic agents. Increasing concentrations of each hemostatic agent generally approached maximum effects at clinically relevant doses except for the clot formation rate, for which maximum effects were not reached even at the highest concentrations of most agents tested (Figure 1). Compared to rFVIII, effects of PEGylated rFVIII were similar with respect to dose and impact, and both significantly exceeded the normal range for rate (65.8 ± 7.7 mOD/min) at 1 IU/mL (Figure 1). Polysialylated rFVIII had a more profound effect than rFVIII, significantly exceeding the normal range for TTP (25.2 ± 1.9 minutes) at 1 IU/mL and the normal range for rate at concentrations ≥0.125 IU/mL. Porcine rFVIII did not shorten the onset time but had similar effects on the TTP and rate as rFVIII (Figure 1). Compared to the replacement products, bypassing agents

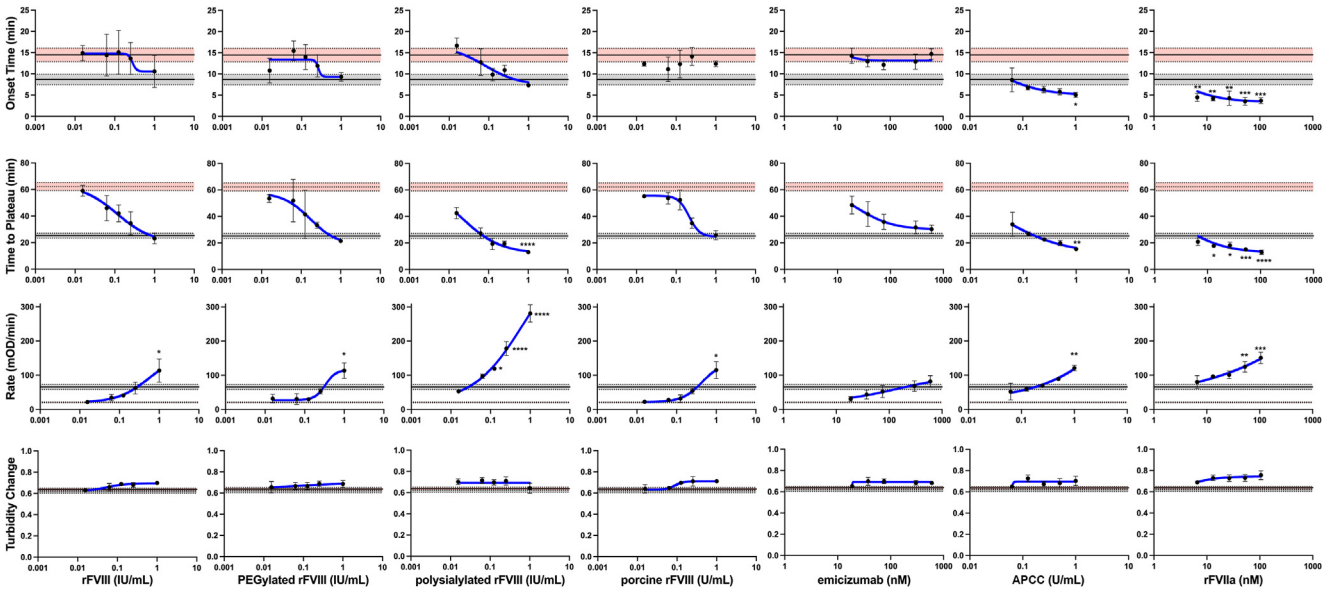

FIGURE 1 Effect of hemostatic agents on clot formation of hemophilia A pooled plasma. Each hemostatic agent was tested in hemophilia-pooled plasma in 3 to 6 separate experiments. Dots and error bars show the mean ± SD for each concentration of agent tested. The data were fit to a dose-response model (heavy blue line). The data for onset time for porcine recombinant factor VIII (rFVIII) could not be fit. Gray shaded area indicates the mean ± SD from normal pooled plasma (N = 5-6 separate experiments). Red shaded area indicates the mean ± SD from untreated hemophilia-pooled plasma (N = 3-6 separate experiments). *$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$. APCC, activated prothrombin complex concentrate; rFVIIa, recombinant factor VIIa.

showed different shapes of the fitted curves, likely reflecting their different mechanisms of action. Emicizumab did not alter the onset time but shortened the TTP and increased the clot formation rate (Figure 1). Both APCC and rFVIIa shortened the onset time, and even low concentrations of rFVIIa (6.6 nM) significantly shortened the onset and TTP to shorter than normal. Both APCC and rFVIIa increased the clot formation rate in a concentration-dependent fashion, significantly surpassing the normal range at 1 IU/mL and 52.5 nM, respectively (Figure 1).

3.2 | Effects of hemostatic agents on clot formation in hemophilia plasma from individual donors

As anticipated, baseline clotting parameters in plasmas from different individual donors were highly variable (Supplementary Table S2 and Figure S2, Figure 2). All hemostatic agents showed procoagulant effects in all individual plasmas tested, but there was considerable heterogeneity in response to different agents between donors (Figure 2). Based on the analysis of pooled plasmas (Figure 1) and the wide range in the onset time among individual plasmas, only the TTP and clot formation rate were used to compare effects of hemostatic agents. As seen in experiments with HPP, rFVIII and PEGylated rFVIII had similar effects with respect to dose and extent in all individual donor plasmas tested (Figure 2). Similar to that seen in HPP, ≥0.25 IU/mL polysialylated rFVIII shortened the TTP to shorter than normal in all plasmas tested. Polysialylated rFVIII also increased the clot formation rate for all donors tested; 0.25 IU/mL enhanced the clotting rate in some, but not all donors and 1 IU/mL produced a rate that exceeded the normal range for all donors tested (Figure 2). Porcine rFVIII had less effect on the onset or TTP but increased the clot formation rate similarly to that seen with rFVIII, exceeding the normal range in only 1 donor. Likewise, emicizumab had little effect on the TTP for most donors tested but enhanced the clot formation rate similar to that seen with rFVIII. Higher concentrations of APCC and rFVIIa (≥0.5 U/mL and ≥100 nM, respectively) shortened the TTP to shorter than normal and increased the rate to faster than normal for all donors tested (Figure 2).

3.3 | Effects of hemostatic agents on fibrinolysis in hemophilic plasma pool

Interassay variability was higher in fibrinolysis than in clot formation assays (Supplementary Table S3). The TTP, clot formation rate, turbidity change, and AUC differed significantly ($P < .05$) between NPP and HPP (Figure 3, gray and red shaded areas, respectively) and were used to compare effects of hemostatic agents. All agents increased the rate, turbidity change, and AUC, demonstrating clot-stabilizing effects in the presence of a fibrinolytic challenge. Increasing concentrations of most agents approached maximum effects within the range tested, except the rate for which maximum concentration-dependent effects were not reached even at the highest concentrations of most agents tested (Figure 3). Among replacement agents, rFVIII and PEGylated rFVIII

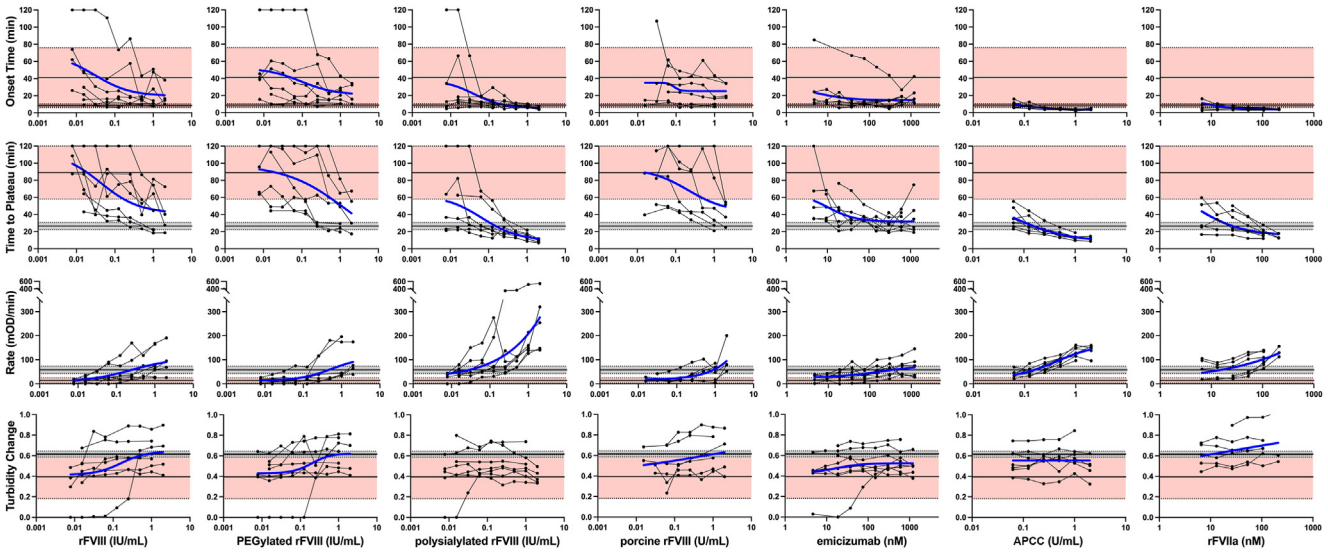

FIGURE 2 Effect of hemostatic agents on clot formation of individual hemophilia A plasmas. Plasma from 10 different individuals was tested; each hemostatic agent was tested on a subset of donors (*N* = 7-8 donors per agent) that were randomized to each treatment. Dots show measurements for each donor and concentration of agent, and lines connect measurements from individual donors. Collective data from all donors were fit to a dose-response model (heavy blue line). The data for turbidity change for polysialylated recombinant factor VIII (rFVIII) could not fit. Gray shaded area indicates the mean ± SD from normal pooled plasma (*N* = 5-6 separate experiments). Red shaded area indicates the mean ± SD of all of the individual hemophilia A plasmas. APCC, activated prothrombin complex concentrate; rFVIIa, recombinant factor VIIa.

produced similar effects, whereas polysialylated rFVIII had higher activity and significantly exceeded the normal range for rate (51.6 ± 7.0 mOD/min) at concentrations ≥0.25 IU/mL. Responses to emicizumab fell largely between baseline and normal parameters (Figure 3). High APCC concentrations (1 U/mL) produced effects that significantly exceeded the normal range for all parameters. Likewise, higher

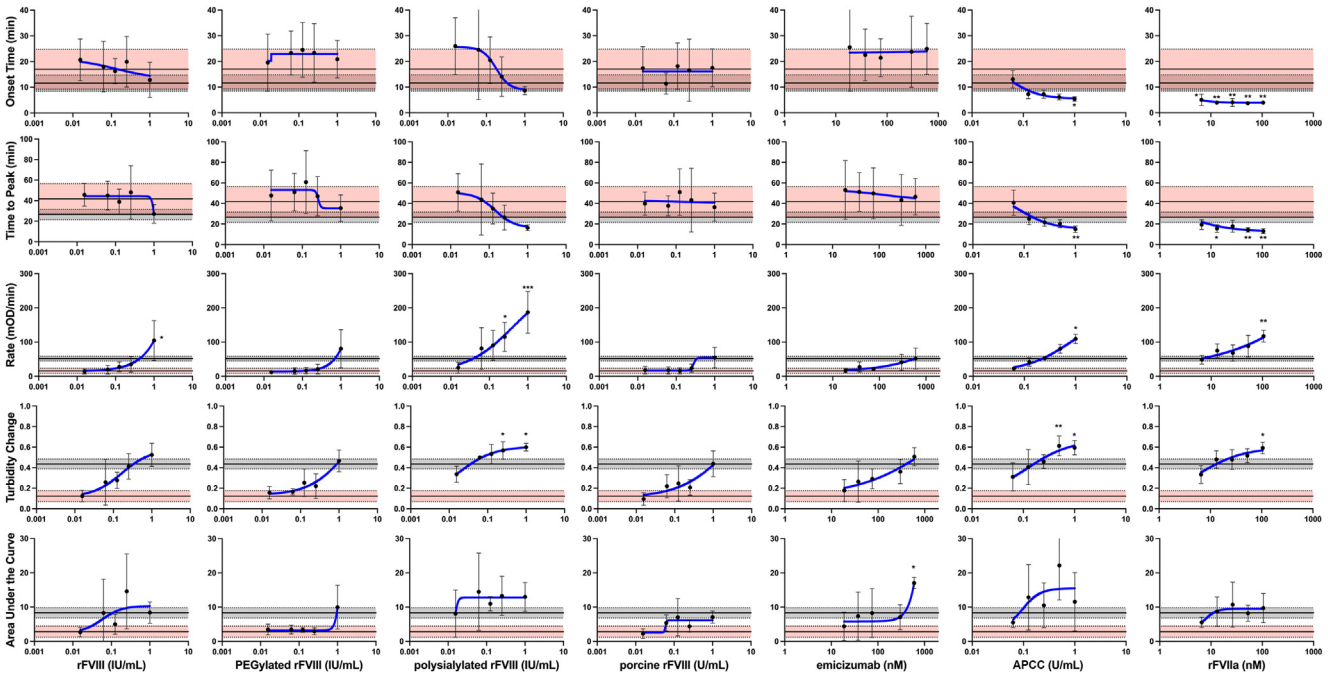

FIGURE 3 Effect of hemostatic agents on fibrinolysis of hemophilia A pooled plasma. Each hemostatic agent was tested in hemophilia-pooled plasma in 3 to 5 separate experiments. Dots and error bars show mean ± SD for each concentration of agent tested. The data were fit to a dose-response model (heavy blue line). Gray shaded area indicates the mean ± SD from normal pooled plasma (*N* = 5-6 separate experiments). Red shaded area indicates the mean ± SD from untreated hemophilia-pooled plasma (*N* = 3-5 separate experiments). $^{*}P < .05$, $^{**}P < .01$. APCC, activated prothrombin complex concentrate; rFVIII, recombinant factor VIII; rFVIIa, recombinant factor VIIa.

concentrations of rFVIIa (≥13.1 nM, 105 nM, and 105 nM) significantly shortened the TTP and increased the rate and turbidity change beyond the normal range.

3.4 | Effects of hemostatic agents on fibrinolysis in hemophilia plasma from individual donors

As anticipated from the clot formation assays, baseline fibrinolysis parameters from different individual donors were highly variable (Supplementary Table S3, Figure 4). As seen in the clot formation assays, all agents produced hemostatic effects in all plasmas tested, but there was heterogeneity in the response between donors (Figure 4). Based on the analysis of pooled plasmas (Figure 3), but lack of separation between individual hemophilia donors and NPP in AUC, only the TTP, clot formation rate, and turbidity change were compared between agents. Consistent with HPP, rFVIII, PEGylated rFVIII, and porcine rFVIII had generally similar procoagulant effects on the TTP, rate, and turbidity change in all plasmas tested (Figure 4). Compared to rFVIII, polysialylated rFVIII had slightly greater ability to enhance the clot formation rate, and ≥0.25 IU/mL exceeded the normal range (59.7 ± 20.6 mOD/min) for half of the donors tested. Emicizumab had a modest effect on the TTP and rate in most plasmas tested (Figure 4). Both APCC and rFVIIa shortened the TTP and increased the rate and turbidity change for all donors tested; high concentrations of APCC (≥1 U/mL) and rFVIIa (≥210 nM) shortened the TTP and increased the rate to greater than normal for all plasmas tested.

4 | DISCUSSION

The variable performance of hemostatic drugs with differing mechanisms of action in clinical hemostasis assays complicates efforts to compare agents in preclinical settings, monitor postinfusion pharmacologic responses, and optimize dosing for efficacy and safety. Compared to normal plasma, hemophilia plasma delays clot formation and reduces stability in tissue factor-triggered assays, and products that enhance hemostasis improve these characteristics. Here, we compared effects of conventional and emerging hemostatic agents in plasma clot formation and fibrinolysis assays and observed that 1) using clot formation and fibrinolysis parameters that differ significantly between normal and hemophilic plasmas, we detected procoagulant effects of all of the established and new replacement and bypassing agents tested, 2) concentration relationships and effects differed between drugs, showing these assays are sensitive to distinct mechanisms of action, and 3) all agents produced procoagulant effects within clinically relevant plasma concentrations, but effects of some drugs on assay parameters exceeded the normal range and did so variably between individuals. Collectively, these observations suggest *in vitro* clot formation, and fibrinolysis assays may help define hemostatic effects among products with different mechanisms of action and reveal individual pharmacodynamic responses for personalized therapy.

Conventional endpoint assays (eg, activated partial thromboplastin time) provide information on the initial events leading to early thrombin generation and fibrin fiber formation. However, additional thrombin generation and fibrin formation, maturation of the fibrin network, and generation of antifibrinolytic mechanisms that protect the clot from premature degradation occur after the clotting time [41]. A strength of the *in vitro* clot formation and fibrinolysis assays tested here is that they can detect both the clotting onset as well as additional parameters that can differentiate normal and hemophilic clots. Consequently, these assays enabled us to study products with conventional effects on clotting, as well as nontraditional agents that do not alter onset time-based assays but enhance subsequent coagulation events that promote hemostasis. Both replacement and bypassing agents improve multiple parameters in these assays, enabling several means for comparing products. It is currently unclear whether one or more of these parameters can serve as a "universal readout" for these comparisons. The onset and TTP were only concentration-responsive for the human FVIII-based products. However, the clot formation rate in both assays and turbidity change (maximum fibrin formation) in the fibrinolysis assay appeared concentration-responsive for all products and donors tested, suggesting these parameters have potential application for multiple hemostatic agents. Since the clotting assay is less variable than the fibrinolysis assay, the clotting assay may be more readily adapted for translational and clinical use.

Effects detected in these clot formation and fibrinolysis assays may provide information on each agent's mechanism of action, individually and in tandem with additional products. Breakthrough bleeding is often treated with combinations of products, and there is considerable interest in learning whether these combinations function in additive or synergistic manners [32,42–45]. The importance of this question is underscored by the thrombotic events seen in a subset of persons with hemophilia treated with emicizumab and APCCs [46]. Other products (eg, tranexamic acid [47]) exert hemostatic benefit through thrombin-independent mechanisms and can only be assessed through assays that invoke fibrinolysis. Currently, it is difficult to quantify potential interactions between these drugs, which requires an assay that is sensitive to each of the agents being evaluated. Global assays like the thrombin generation assay coupled with these clotting and fibrinolysis assays that provide additional data on fibrin formation and stability may help define the operant biochemistry and inform the choice of hemostatic agents in these situations.

Administration of hemostatic agents, even to persons with congenital hypocoagulability, is accompanied by a small but significant risk of thrombosis. Risk is particularly evident for high levels of FVIII, an established prothrombotic risk factor [18–23]. Although current clinical assays can identify bleeding risk, methods to identify thrombotic risk have lagged behind. Development of an assay that can identify not only hemostatic efficacy but also safety would be a major advance [48]. Elevated FVIII enhances thrombin generation and accelerates the production of abnormal clots that resist fibrinolysis [18,19,49]. It is, therefore, notable that higher concentrations of several agents (most prominently polysialylated rFVIII, APCC, and rFVIIa) produced clotting and fibrinolysis parameters that exceeded

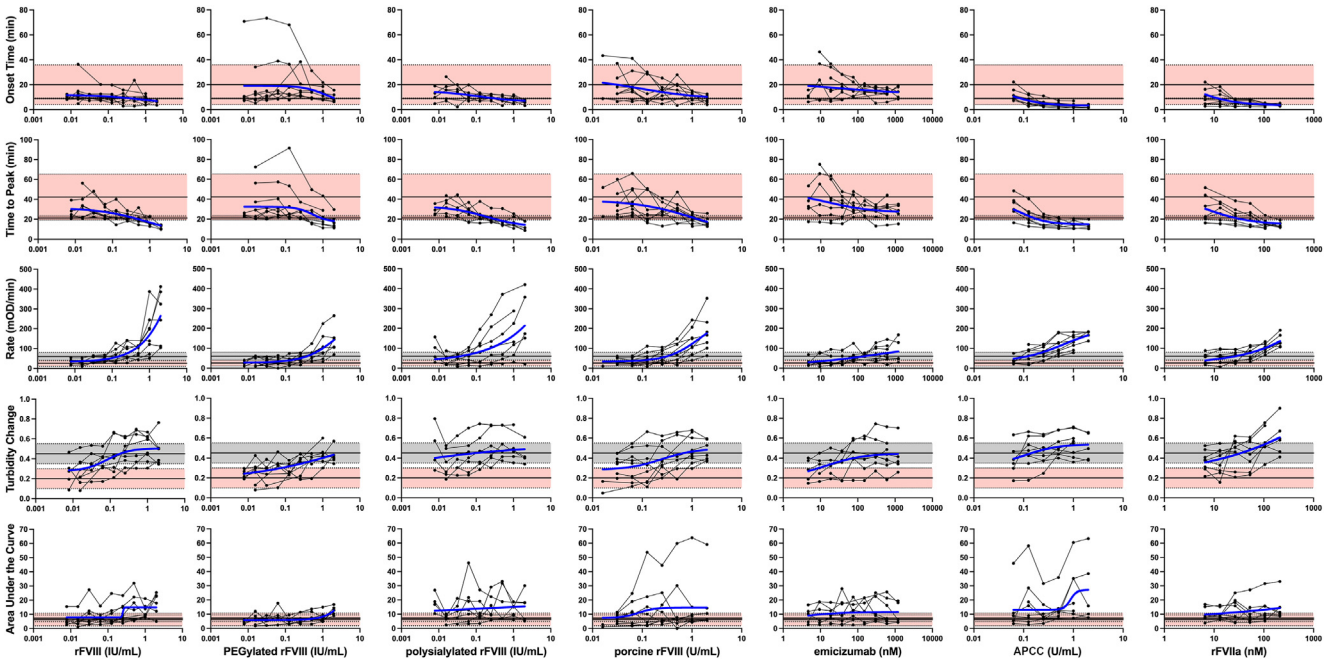

FIGURE 4 Effect of hemostatic agents on fibrinolysis of individual hemophilia A plasmas. Plasma from 9 different individuals was tested; each hemostatic agent was tested on a subset of donors (*N* = 7-8 donors per agent) that were randomized to each treatment. Dots show measurements for each donor and concentration of agent, and lines connect measurements from individual donors. Collective data from all donors were fit to a dose-response model (heavy blue line). Gray shaded area indicates the mean ± SD from normal pooled plasma (*N* = 3 separate experiments). Red shaded area indicates the mean ± SD of all of the individual hemophilia A plasmas. APCC, activated prothrombin complex concentrate; rFVIII, recombinant factor VIII; rFVIIa, recombinant factor VIIa.

the normal range. Analysis of individual plasmas shows this effect was donor-specific, suggesting that an individual's plasma composition is a strong determinant of a product's impact on coagulation. Interindividual variability in response to hemostatic therapy has been seen in human and animal models of hemophilia [50–52] and underscores the potential value of global assays in a personalized setting. Although the present data do not directly associate these drug levels or parameters with thrombosis, these findings suggest that *in vitro* clotting and fibrinolysis assays may help identify potentially unsafe dosing regimens not only across a population but also for individuals prior to initiating therapy or during dose optimization.

Our study has limitations. First, we tested a relatively small number of plasmas from individuals with severe hemophilia A without inhibitors. The performance of these agents may differ in moderate hemophilia or in the presence of inhibitors where low levels of FVIII and/or FVIII:inhibitor complexes may produce additive or synergistic effects in the presence of the hemostatic drugs. Future studies are needed to test this possibility. Moreover, since information on race and ethnicity was not available, we were unable to infer socio-cultural determinants of health in the studied population. Second, whereas all persons with hemophilia A were male, the normal range was established using a pool of 4 male and 4 female donors. However, use of this commercial pool from the same source as the individual plasmas enabled us to standardize phlebotomy and plasma preparation, which can affect plasma assays [53]. Third, the assays tested here have not been standardized for clinical use. Concentrations of tissue factor, lipids, calcium, and tPA used in the present study distinguish normal and hemophilia clots but may benefit from optimization. Moreover, although the clot formation and fibrinolysis assays are simple in design, they require some technical proficiency to limit interassay variability, which may stem from pipetting and/or experimental (dilution) strategies employed. Currently, these assays are only performed in a research setting. However, observations that these assays may reveal clinically relevant information not accessible through other methods motivate efforts to standardize these assays for clinical use. Fourth, although assays with platelet-poor plasma enable the use of frozen samples, they do not incorporate contributions of platelets or other vascular cells. For example, high-dose rFVIIa activity is mediated by its binding to the platelet surface and local FXa generation [54]. Accordingly, hemostatic effects of rFVIIa in thrombin generation assays are best assessed in the presence of platelets [55]. Although high phospholipid concentrations may compensate for the lack of platelets [28], we used a standardized lipid concentration (4 μM) to enable comparison with established assay protocols, including calibrated automated thrombography. Finally, the assays performed here involved *in vitro* spiking of hemostatic agents. Kizilocak et al. [56] observed that when spiked *in vitro*, clinically relevant concentrations of APCC produce excessive thrombin generation; however, *in vivo* administration of APCC produces normal thrombin generation at these same doses when measured *ex vivo*. Thus, our *in vitro* data that show supranormal effects of hemostatic agents may overestimate the pharmacodynamic potential of these drugs when administered *in vivo*.

As expected, individual responses to the clot formation and fibrinolysis assays were variable. However, most clinically relevant concentrations of replacement and bypassing agents produced effects in most donors that fell within the normal range. This observation is consistent with the established utility of these agents in hemophilia and suggests clot formation and fibrinolysis assays may inform dose-finding studies in preclinical pipelines and help characterize therapeutic responses in individual persons with hemophilia.

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AUTHOR CONTRIBUTIONS

L.A.H. and J.C.P. designed and performed experiments and analyzed data. P.L.T. provided critical input, interpreted data, and edited the manuscript. A.S.W. designed the research, analyzed and interpreted data, and wrote the manuscript. All authors read, edited, and approved the final version of the manuscript.

RELATIONSHIP DISCLOSURE

A.S.W. received funding from Takeda to perform the study. P.L.T. is an employee of Baxalta Innovations GmbH, a Takeda company, and holds relevant Takeda patents and Takeda stocks.

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SUPPLEMENTARY MATERIAL

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