In vivo studies show synergistic effects of a procoagulant bispecific antibody and bypassing agents

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Essentials
- Patients with hemophilia A and inhibitors receiving emicizumab experience breakthrough bleeding.
- Safety concerns may exist when combining emicizumab with bypassing agents.
- Combined bypassing agent and bispecific antibody increased thrombin generation up to 17-fold.
- Thrombotic effects should be considered when combining emicizumab with plasma bypassing agent.

Summary. Background: Investigational non-factor products such as emicizumab offer a treatment option for patients with hemophilia and inhibitors. However, their mechanism of action raises questions regarding safety when they are combined with treatments for breakthrough bleeding. Objectives: To evaluate in vivo thrombin generation (TG) and clot formation for combinations of activated prothrombin complex concentrate (aPCC), recombinant activated factor VII (rFVIIa), and a sequence-identical analog of emicizumab (SIA). Methods: Therapeutic concentrations of SIA (20–600 nm) alone or with aPCC (0.05–1 U mL⁻¹), isolated aPCC components or rFVIIa (0.88–5.25 µg mL⁻¹) were tested for TG and compared with reference ranges for healthy donor plasma. Coagulation of FVIII-inhibited blood was determined with a widely established method, i.e. rotational thromboelastometry (ROTEM), and confirmed with the Total Thrombus-formation Analysis System. Results and conclusions: SIA (600 nm) or aPCC (0.5 U mL⁻¹) alone resulted in peak thrombin levels of 21.4 nM and 38.6 nM, respectively, both of which are lower than normal (83.7 ± 29.8 nM). SIA plus aPCC (0.5 U mL⁻¹) increased the peak thrombin level 17-fold over SIA alone, exceeding the reference plasma value by 4.2-fold. This hypercoagulable effect occurred with 600 nm SIA combined with as little as 0.25 U mL⁻¹ aPCC, confirmed by ROTEM. FIX was the main driver for enhanced TG. SIA plus rFVIIa (1.75 µg mL⁻¹) induced a 1.8-fold increase in the peak thrombin level in platelet-rich plasma, but it did not reach the normal range. These in vitro experiments demonstrate excessive TG after administration of a combination of aPCC and SIA at clinically relevant doses. Careful judgement may be required when breakthrough bleeding is treated in patients receiving emicizumab.

Keywords: activated partial thromboplastin time; emicizumab; FEIBA; hemophilia A; thrombotic microangiopathies.

Introduction
Hemophilia A (HA) treatment has improved to the extent that the life-expectancy of patients with access to factor VIII replacement therapy is now similar to that of the general population [1]. However, challenges remain in the treatment of severe HA (<1% endogenous FVIII activity), including the need for frequent injections for prophylaxis to prevent bleeding episodes and the development of neutralizing antibodies against FVIII (FVIII inhibitors), which occurs in ~30% of patients with severe HA [2]. FVIII inhibitors interfere with the hemostatic effect of FVIII replacement therapy, and thereby render the patient vulnerable to bleeding that is difficult to control. Efforts are underway to develop non-factor products that can augment coagulation in the presence of FVIII inhibitors [3].

Emerging non-factor products such as emicizumab (HEMLIBRA; emicizumab-kxwh; ACE910; Chugai Pharmaceutical Co., Tokyo, Japan), which has recently been licensed by the US Food and Drug Administration (FDA), offer an alternative approach for patients with HA and inhibitors [4]. Emicizumab is a bispecific antibody that is aimed at replacing the function of activated...
FVIII as the bridge between the activated FIX (FIXa) enzyme and the zymogen FX [5–7]. In the phase 3 HAVEN study of 102 patients with HA and FVIII inhibitors, emicizumab prophylaxis \( (n = 35) \) was associated with an 87% lower rate of bleeding events than in the group who received no prophylaxis \( (n = 18) \) [8]. However, emicizumab lacks the inherent activation and inactivation characteristics of FVIII and bypassing agents. The unregulated mechanisms of action of such non-factor molecules raise questions regarding safety in specific clinical situations, as demonstrated in the HAVEN trial, which reported three cases of thrombotic microangiopathy (TMA) and two cases of thrombosis in patients who received emicizumab prophylaxis and additional infusions of activated prothrombin complex concentrate (aPCC) (also known as FVIII-bypassing agent, FEIBA NF; Shire, Lexington, MA, USA) for breakthrough bleeding [8].

The current management of bleeding episodes in patients with HA and high-titer inhibitors involves treatment with a bypassing agent such as aPCC or recombinant activated FVII (rFVIIa) [epotacog alfa (activated)]; NovoSeven; Novo Nordisk, Plainsboro, NJ, USA) [9,10]. aPCC contains the zymogens FII, FVII, FIX, and FX, plus activated FVII (FVIIa); FVIII procoagulant antigen is also present, as are traces of FIXa and activated FX (FXa). Both bypassing agents have proven excellent safety and efficacy profiles and long-standing real-world use in the treatment of acute bleeding in patients with HA and hemophilia B with inhibitors [11,12].

In the event of breakthrough bleeding during emicizumab treatment, patients require treatment with a bypassing agent. However, because of the mechanism of action of emicizumab, there may be risks associated with combining it with additional procoagulants, in particular aPCC, which would increase the availability of FIX/FIXa and FX/FXa targets for the antibody [7]. Indeed, the emicizumab label includes a black-box warning of TMA and thromboembolism when aPCC is administered to patients receiving prophylaxis with emicizumab. The aPCC label also includes a black-box warning of thromboembolic events, particularly when aPCC is administered at high doses and/or in patients with thrombotic risk factors, and the rFVIIa label has a black-box warning of serious arterial and venous thrombotic events.

To evaluate the implications of combining treatment with an FIX(a)/FX(a) bispecific antibody molecule and a bypassing agent for patients experiencing breakthrough bleeding while receiving emicizumab prophylaxis, we studied the \textit{in vitro} thrombin generation (TG) profile produced when a sequence-identical analog of emicizumab (SIA) is combined with various combinations of aPCC or rFVIIa. Moreover, we assessed clot formation in FVIII-inhibited whole blood (WB) treated with combinations of aPCC and SIA. To further elucidate the effects of aPCC when it is combined with SIA, individual clotting factors present in aPCC were tested with SIA to delineate the components responsible for the synergistic hypercoagulatory effect.

**Materials and methods**

**Materials**

SIA (based on the amino acid sequence published for emicizumab [13]) was produced in HEK293 cells and purified from cell supernatant via cation exchange and size exclusion chromatography. For analysis, HPLC and SDS-PAGE were performed.

Normal reference plasma and HA inhibitor plasma (platelet-poor plasma [PPP]), which was obtained from individuals by plasmapheresis in FDA-licensed blood centers, were purchased as fresh frozen plasma from George King Bio-Medical (Overland Park, KS, USA) and HRF (Raleigh, NC, USA). Corn trypsin inhibitor (CTI) and purified human plasma-derived coagulation factors were from Hematologic Technologies (Essex Junction, VT, USA) or Enzyme Research Laboratories (South Bend, IN, USA). Human thrombin calibrator, tissue factor (TF) (PPP reagent LOW and platelet-rich plasma [PRP] reagent), MP reagent and FluCa reagent were from Thrombinscope BV (Maastricht, the Netherlands). Anti-human FVIII goat plasma, aPCC and rFVIIa were from Shire (Vienna, Austria).

Platelets and WB from healthy donors (Austrian Red Cross) were obtained with informed consent. Platelets were prepared by single-donor apheresis. WB was drawn by venipuncture and collected in citrate phosphate dextrose solution: 7.6 parts of blood were mixed with one part of sodium citrate (2.67%; final concentration 11.7 mM). Blood parameters, such as platelet count, were measured with the automated hematology analyzer pocH100i (Sysmex, Milton Keynes, UK). Reagents and application software used for the Total Thrombus-formation Analysis System (T-TAS) were from Fujimori Kogyo (Yokohama, Japan).

**Preparation of samples**

**Normal reference plasma** HNa-BSA buffer (25 mM HEPES, 175 mM NaCl, pH 7.35, 5 mg mL\(^{-1}\) bovine serum albumin) was added to normal healthy donor plasma as the control plasma.

**PPP** Fresh frozen plasma from four patients with HA and FVIII inhibitors was used. SIA and aPCC or rFVIIa were added in prediluted 5-µL aliquots to the PPP.

**PRP** Platelets from three healthy donors were added to PPP from a single HA donor to generate PRP. Platelets were washed in acid–citrate–dextrose buffer, resuspended in Tyrode’s buffer, and added to warmed (37 °C) plasma samples to achieve a platelet count of 150 × 10\(^3\) µL\(^{-1}\).
SIA and aPCC or rFVIIa were added in prediluted 5-μL aliquots to the PRP.

**Simulated hemophilic plasma** To test aPCC components combined with SIA in hemophilic conditions, heat-inactivated anti-human FVIII goat plasma (4488 Bethesda units [BU] mL⁻¹) [14] was added to pooled human plasma, resulting in an inhibitor concentration of 50 BU mL⁻¹. Purified coagulation factors equivalent to 0.5 U mL⁻¹ aPCC [11] or mixtures thereof were added as a 5-μL aliquot instead of aPCC.

**Simulated hemophilic WB** For the T-TAS [15], WB was prepared by incubating blood samples (430 μL) for 2 min with 50 μL of saline. To simulate hemophilic conditions, heat-inactivated anti-human FVIII goat plasma (4488 BU mL⁻¹) [14] was added to WB, resulting in inhibitor concentrations of 51 BU mL⁻¹ for rotational thromboelastometry (ROTEM) [16] and 200 BU mL⁻¹ for T-TAS experiments. The addition of rFVIIa (final concentration of 1.75 μg mL⁻¹ or 5.25 μg mL⁻¹), aPCC (0.05-0.5 U mL⁻¹) or SIA (600 nm) or rFVIIa–SIA and aPCC–SIA combinations to these WB samples was tested for clot formation.

**Calibrated automated thrombography (CAT) assay**

TG was evaluated with CAT as described by Hemker et al. [17], in two independent repeats.

Warmed (37 °C) PPP, PRP or simulated hemophilic plasma (80 μL) was added to each well of a 96-well microplate. TG in PPP was triggered by 10 μL of PPP reagent LOW containing recombinant human TF (rTF), a phospholipid mixture (48 μM) (MP reagent), and 62.5 μg mL⁻¹ CTI. A final TF concentration of 1 μM was selected to provide sensitivity to FVIII in the assay system in the presence of SIA, aPCC, rFVIIa, or combinations thereof, and to avoid overestimation of SIA-induced coagulation because of binding of excess FIXa. The final assay well volume was adjusted to 120 μL by addition of 10 μL of HNa-BSA buffer or samples (aPCC, rFVIIa, or SIA, or combinations).

TG in PRP was triggered by 5 μL of PRP reagent to achieve a final TF concentration of 1 μM. The final assay volume was adjusted to 125 μL by addition of 15 μL of HNa-BSA buffer or samples, and 5 μL of convulxin (2 μg mL⁻¹) for platelet activation.

The parameters of the resulting TG curves were calculated with THROMBINOSCOPE software [17]. The following TG parameters were assessed: peak thrombin level (nm), lag time, peak time, endogenous thrombin potential, and velocity index.

**Clot formation in WB determined by ROTEM**

Recordings were made with a ROTEM coagulation analyzer (Pentapharm, Munich, Germany) at 37 °C in the presence of CTI (final concentration: 53 μg mL⁻¹). To initiate coagulation, 300 μL of warmed FVIII-inhibited or normal WB were added to a cuvette containing 20 μL of 0.2 M CaCl₂, 10 μL of a 1:35 dilution of PRP reagent (12 pm rTF), and 20 μL of aPCC, rFVIIa, or SIA, or combinations thereof. The final concentration of rTF was 44 M; a minimal TF concentration is needed for sensitivity of the assay in FVIII-inhibited blood [14,18]. The ROTEM recording was started immediately and was allowed to proceed for at least 80 min. The following thromboelastometric parameters were assessed: clotting time (CT), clot formation time (CFT), and mean clot firmness (MCF). The thrombodynamic potential index (TPI) was calculated as described by Dimitrova-Karamfilova et al. [19].

**Clot formation under flow determined with the T-TAS**

The atheroma chip (AR-chip) is coated with type I collagen (1.5 mg mL⁻¹) and tissue thromboplastin (0.1 mg mL⁻¹), and measures primary/secondary hemostasis attributable to simultaneous activation of platelets and the coagulation cascade at a shear rate of 240 s⁻¹ or 600 s⁻¹. Thrombus formation was analyzed by monitoring the flow pressure change. A minimum of three donors were analyzed per shear rate, with duplicate measurements. For each hemophilic WB sample, the onset of thrombus formation, i.e., the time at which the flow pressure increased by 10 kPa from baseline pressure (T10 kPa), and the occlusion of the capillary, i.e., the time at which the flow pressure increased by 80 kPa from baseline pressure (T80 kPa), were analyzed. For selected experiments, images were obtained at the end of the capillary to show clot formation at defined time intervals. Coagulation was started by addition of 20 μL of Ca²⁺-CTI to the sample (final concentrations: 12 mM Ca²⁺, and 50 μg mL⁻¹ CTI). The T-TAS parameters and images were derived with T-TAS PLUS application software (V 1.01.0).

**Statistical analysis**

A range for normal plasma in PPP was established by the use of plasma from healthy individuals (12 females and 16 males) in two independent TG assays. The normal plasma range in PRP was established by the use of plasma from 16 healthy individuals (five females and 11 males) in three independent TG assays. All data are presented as mean ± standard deviation (SD) or as median and ranges (minimum–maximum) when appropriate. Statistical comparisons of samples were performed with unpaired, two-tailed Student’s t-tests or one-way ANOVA with multiple comparison tests (Dunnett or Tukey’s), when appropriate. Differences were considered to be significant when the P-value was < 0.05. Analysis was performed with Microsoft Excel 2010 Version 14.0 and/or GRAPHPAD PRISM 7 Version 7.03.

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Results

The aPCC–SIA combination but not the rFVIIa–SIA combination resulted in TG above normal levels in PPP

Donor plasma from four patients with severe HA and FVIII inhibitors (inhibitor titers: 1, 3, 97 and 133 BU mL⁻¹) was used to determine the in vitro efficacy of SIA. TG was measured in the plasma of 28 healthy donors to establish a normal range for the peak thrombin level. The mean peak thrombin level was 84 ± 30 nm, with a range of 29–148 nm (Table 1). In HA inhibitor plasma, the addition of a therapeutic concentration of SIA (600 nm) [4] resulted in a modest increase in TG, with a peak thrombin level of 21 ± 13 nm (Fig. 1A). Thus, SIA did not restore TG to the normal plasma range.

Both aPCC (Fig. 1A; Table 2) and rFVIIa (Fig. 1B; Table 2) showed a concentration-dependent effect on TG in combination with SIA. The combination of clinically relevant doses of rFVIIa (1.75 µg mL⁻¹; correlated to 120 µg kg⁻¹ dose) [20] and SIA (600 nm) in inhibitor plasma resulted in a peak thrombin level of 39 ± 14 nm, which represents, on average, a 1.9-fold increase in the peak thrombin level over SIA alone and a lower TG than normal. In contrast, the aPCC–SIA combination (600 nm SIA + 0.5 U mL⁻¹ aPCC) resulted in a peak thrombin level of 353 ± 61 nm, representing an increase of up to 17-fold over SIA alone. This combination exceeded the normal plasma range by 3.5-fold to 4.9-fold for the four different donors (Fig. 1C).

Statistical analysis revealed that the difference in the peak thrombin level for aPCC 0.5 U mL⁻¹ versus SIA 600 nm was not significant (P = 0.9). In comparison, the difference in the peak thrombin level for aPCC 0.5 U mL⁻¹ versus aPCC 0.5 U mL⁻¹ plus SIA 600 nm was significant (P < 0.0001). In contrast, a difference in the peak thrombin level was not seen for SIA 600 nm versus rFVIIa 1.75 µg mL⁻¹ (P > 0.9) or rFVIIa 1.75 µg mL⁻¹ versus rFVIIa 1.75 µg mL⁻¹ plus SIA 600 nm (P = 0.8). aPCC alone at the highest concentration (1.0 U mL⁻¹) resulted in a peak thrombin level within the range for normal plasma (Fig. 1C). In contrast, the lowest tested aPCC concentration (0.05 U mL⁻¹) in combination with SIA resulted in a peak thrombin level within the normal range, whereas all other concentrations resulted in peak thrombin levels above normal (Table 1).

In order to provide a mechanistic explanation for the observed effect, selected single aPCC components – FII/ activated FII (FIIa), FIX, FIXa, FX, FXa [11] – at concentrations corresponding to 0.5 U mL⁻¹ aPCC were investigated in combination with SIA (600 nm) in FVIII-inhibited plasma (Fig. S1) and FVIII inhibitor plasma (Fig. 1D). SIA combined with FIX, and to a lesser extent with FIXa, made the largest contribution to the synergistic effect seen with aPCC–SIA combinations.

| Condition | Peak thrombin level (nm) | Adjusted Peak time (min) | Lag time (min) | ETP (nm min⁻¹) | Velocity index (nm min⁻¹) | Fold increase in peak thrombin level from HA plasma |
|-----------|--------------------------|--------------------------|----------------|----------------|--------------------------|---------------------------------|
| Reference range | 84 ± 30 | 15 ± 3 | 6.2 ± 2.8 | 1279 ± 330 | 40.5 ± 34.6 | 12.9 |
| HA plasma | 7 ± 2 | 3 ± 5 | 9.8 ± 4.0 | 187 ± 44 | 0.3 ± 0.1 | 1.1 |
| SIA (20 nm) | 7 ± 2 | NS | 10.8 ± 3.6 | 223 ± 73 | 0.4 ± 0.1 | 1.1 |
| SIA (60 nm) | 8 ± 2 | NS | 10.3 ± 3.7 | 221 ± 69 | 0.4 ± 0.1 | 1.3 |
| SIA (200 nm) | 12 ± 5 | NS | 11.0 ± 3.4 | 315 ± 150 | 0.6 ± 0.3 | 1.9 |
| SIA (600 nm) | 21 ± 13 < 0.01 | 30 ± 3 | 12.4 ± 2.5 | 489 ± 285 | 1.2 ± 0.8 | 3.3 |
| aPCC (0.05 U mL⁻¹) | 10 ± 3 | NS | 7.8 ± 1.9 | 262 ± 81 | 0.6 ± 0.2 | 1.5 |
| aPCC (0.25 U mL⁻¹) | 23 ± 3 < 0.01 | 18 ± 1 | 5.7 ± 0.8 | 455 ± 99 | 1.9 ± 0.2 | 3.5 |
| aPCC (0.5 U mL⁻¹) | 39 ± 4 < 0.0001 | 16 ± 1 | 5.0 ± 0.5 | 648 ± 111 | 3.5 ± 0.3 | 6.0 |
| aPCC (1 U mL⁻¹) | 59 ± 8 < 0.0001 | 14 ± 2 | 4.7 ± 1.1 | 949 ± 32 | 6.5 ± 1.3 | 9.2 |
| aPCC (0.05 U mL⁻¹) + SIA (600 nm) | 82 ± 27 < 0.05 | 15 ± 1 | 5.7 ± 0.8 | 1202 ± 451 | 9.2 ± 2.7 | 12.7 |
| aPCC (0.25 U mL⁻¹) + SIA (600 nm) | 232 ± 63 < 0.0001 | 8 ± 0.5 | 3.6 ± 0.4 | 1991 ± 776 | 51.4 ± 15.2 | 36.0 |
| aPCC (0.5 U mL⁻¹) + SIA (600 nm) | 353 ± 61 < 0.0001 | 6 ± 0.4 | 3.0 ± 0.4 | 2504 ± 559 | 106.7 ± 21.9 | 54.7 |
| aPCC (1.0 U mL⁻¹) + SIA (600 nm) | 528 ± 39 < 0.0001 | 5 ± 0.5 | 2.3 ± 0.4 | 3627 (n = 1) | 176.9 ± 20.5 | 81.7 |

Table 1 Calibrated automated thromboigraphy (CAT) parameters for platelet-poor hemophilia A (HA) inhibitor plasma

aPCC, activated prothrombin complex concentrate; ETP, endogenous thrombin potential; NS, not significant; rFVIIa, recombinant activated factor VII; SIA, sequence-identical analog of emicizumab. CAT parameters are displayed as means ± standard deviation from four FVIII inhibitor plasma donors. Adjusted P-values represent the difference from HA plasma. The reference range was determined with 28 normal plasma donors.

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The aPCC–SIA combination resulted in TG above normal levels in PRP

For PRP, the normal mean peak thrombin level was 209 ± 22 nM, with a range of 177–267 nM (Table 3). It was established for 16 healthy individual plasmas supplemented with 150 × 10^3 μL^-1 platelets from three healthy donors. In HA inhibitor PRP (17 ± 1 nM peak thrombin), the addition of SIA (600 nM) resulted in a modest increase in TG, with a peak thrombin level of 38 ± 10 nM (Table 3; Fig. 1B), which is similar to the levels obtained with PPP.

Both rFVIIa (Fig. 1B) and aPCC showed effects on TG in combination with SIA in a concentration-dependent manner (Table 3). The rFVIIa–SIA combination (1.75 μg mL^-1 rFVIIa + 600 nM SIA) marginally increased peak thrombin levels to 58 ± 5 nM as compared with SIA alone in inhibitor plasma. In contrast, the aPCC–SIA combination (0.5 U mL^-1 aPCC + 600 nM SIA) resulted in peak thrombin levels above normal (259 ± 39 nM; Table 3). When the platelets were not stimulated with convulxin prior to TG, the overall TG levels were lower, but the overall outcome was not changed (data not shown).

ROTEM confirms the effect of the aPCC–SIA combination in WB

ROTEM was used to assess the effects of the aPCC–SIA and rFVIIa–SIA combinations in a WB matrix. FVIII inhibition of WB prolonged CT from 1075 ± 35 s to 2102 ± 250 s, and increased CFT from 183 ± 14 s to 1144 ± 293 s (Fig. 2; Table 4). SIA alone (600 nM) shortened CT (1875 ± 237 s) and CFT (520 ± 133 s), but did not restore clotting to normal levels.

Titration of aPCC in FVIII-inhibited WB improved CT and CFT in a concentration-dependent manner. aPCC (0.5 U mL^-1) reduced CT to 267 ± 11 s, whereas CFT was less affected, but was still reduced below the normal value to 79 ± 9 s. The aPCC–SIA combination
Table 2 Calibrated automated thrombography (CAT) parameters for activated prothrombin complex concentrate (aPCC) single components alone or in combination with sequence-identical analog of emicizumab (SIA) in hemophilia A (HA) inhibitor plasma

| Condition                        | Peak thrombin level (nm) | Lag time (min) | ETP (nm*min) | Velocity index (nm min⁻¹) |
|----------------------------------|--------------------------|----------------|--------------|---------------------------|
| Reference range                  | 84 ± 30.0                | 6.2 ± 2.8      | 1278.5 ± 329.7 | 40.5 ± 34.6               |
| HA plasma                        | 7.8 ± 0.5                | 12.2 ± 1.6     | 195 ± 26.6   | 0.4 ± 0.02                |
| SIA (600 nm)                     | 18.2 ± 7.4               | 14.5 ± 2.4     | 408 ± 197    | 1.1 ± 0.5                 |
| aPCC (0.5 U mL⁻¹)                | 41.5 ± 3.1               | 4.2 ± 0.7      | 628 ± 78     | 4.3 ± 0.02                |
| FX + SIA (600 nm)                | 26.3 ± 12.0              | 13.3 ± 2.4     | 558 ± 267    | 1.7 ± 0.8                 |
| FXa + SIA (600 nm)               | 18.3 ± 7.3               | 12.1 ± 1.6     | 408 ± 201    | 1.1 ± 0.5                 |
| FIX + SIA (600 nm)               | 163 ± 34                 | 4.4 ± 0.7      | 1196 ± 486   | 36.7 ± 2.4                |
| FIXa + SIA (600 nm)              | 66.1 ± 11.3              | 7.0 ± 0.9      | 926 ± 294    | 7.2 ± 0.5                 |
| Mix + SIA (600 nm)               | 202 ± 52                 | 3.8 ± 0.5      | 1240 ± 515   | 56.5 ± 9.8                |
| aPCC (0.5 U mL⁻¹) + SIA (600 nm) | 303 ± 67                 | 3.1 ± 0.4      | 1879 ± 719   | 92.2 ± 16.3               |

ETP, endogenous thrombin potential; FIXa, activated factor IX; FXa, activated factor X. CAT parameters are displayed as means ± standard deviation from three FVIII inhibitor plasma donors. SIA (600 nm) was added alone or in combination with FIX (117 nm), FIXa (0.09 nm), FX (136 nm), FXa (0.05 nm), the mix of these components, or 0.5 U mL⁻¹ aPCC. The reference range was determined with 28 normal plasma donors.

Table 3 Calibrated automated thrombography (CAT) parameters for platelet-rich hemophilia A (HA) inhibitor plasma*

| Condition                        | Peak thrombin level (nm) | Adjusted Peak time (min) | Lag time (min) | ETP (nm min) | Velocity index (nm min⁻¹) | Fold increase in peak thrombin level from HA plasma |
|----------------------------------|--------------------------|--------------------------|----------------|--------------|---------------------------|---------------------------------------------------|
| Reference range                  | 209 ± 22                 | 11 ± 1                   | 5.7 ± 0.5      | 1738 ± 228   | 42.2 ± 5.2                | 12.5                                              |
| HA plasma                        | 17 ± 1                   | 42 ± 1                   | 7.8 ± 0.3      | 721 ± 58     | 0.5 ± 0.1                 | 1.0                                               |
| SIA (20 nm)                      | 17 ± 6 NS                | 42 ± 3                   | 7.5 ± 0.3      | 697 ± 248    | 0.5 ± 0.2                 | 1.0                                               |
| SIA (60 nm)                      | 19 ± 7 NS                | 42 ± 3                   | 8.0 ± 0.6      | 783 ± 304    | 0.6 ± 0.2                 | 1.1                                               |
| SIA (200 nm)                     | 27 ± 8 < 0.001           | 44 ± 4                   | 12.6 ± 2.4     | 932 ± 227    | 0.9 ± 0.3                 | 1.6                                               |
| SIA (600 nm)                     | 38 ± 10 < 0.0001         | 41 ± 4                   | 15.7 ± 2.5     | 1052 ± 191   | 1.5 ± 0.5                 | 2.3                                               |
| aPCC (0.05 U mL⁻¹)               | 20 ± 6 NS                | 38 ± 3                   | 7.3 ± 0.6      | 797 ± 32     | 0.7 ± 0.3                 | 1.2                                               |
| aPCC (0.25 U mL⁻¹)               | 41 ± 10 < 0.05           | 31 ± 4                   | 7.3 ± 1.0      | 1291 ± 271   | 1.8 ± 0.6                 | 2.4                                               |
| aPCC (0.5 U mL⁻¹)                | 68 ± 15 < 0.001          | 28 ± 4                   | 7.1 ± 1.2      | 1948 ± 296   | 3.4 ± 1.1                 | 4.1                                               |
| aPCC (1 U mL⁻¹)                  | 133 ± 26 < 0.0001        | 28 ± 4                   | 7.4 ± 1.3      | 4288 ± 283   | 6.7 ± 2.0                 | 8.0                                               |
| rFVIIa (0.88 µg mL⁻¹)            | 27 ± 7 < 0.0001          | 34 ± 4                   | 5.9 ± 0.4      | 1121 ± 286   | 1.0 ± 0.3                 | 1.6                                               |
| rFVIIa (1.75 µg mL⁻¹)            | 32 ± 7 < 0.0001          | 33 ± 5                   | 6.5 ± 1.2      | 1157 ± 169   | 1.2 ± 0.4                 | 1.9                                               |
| rFVIIa (5.25 µg mL⁻¹)            | 40 ± 7 < 0.0001          | 31 ± 4                   | 6.9 ± 1.1      | 1143 ± 113   | 1.7 ± 0.5                 | 2.4                                               |
| rFVIIa (0.88 µg mL⁻¹) + SIA (600 nm) | 51 ± 5                  | 33 ± 3                   | 10.1 ± 2.2    | 1219 ± 92    | 2.2 ± 0.3                 | 3.1                                               |
| rFVIIa (1.75 µg mL⁻¹) + SIA (600 nm) | 58 ± 5 < 0.0001         | 31 ± 4                   | 10.2 ± 2.9    | 1250 ± 7     | 2.8 ± 0.4                 | 3.4                                               |
| rFVIIa (5.25 µg mL⁻¹) + SIA (600 nm) | 63 ± 5 < 0.0001         | 28 ± 4                   | 9.8 ± 2.4     | 1170 ± 59    | 3.6 ± 0.6                 | 3.8                                               |

aPCC, activated prothrombin complex concentrate; ETP, endogenous thrombin potential; NS, not significant; rFVIIa, recombinant activated factor VII; SIA, sequence-identical analog of emicizumab. *Convulxin was added to activate platelets. Data are displayed as means ± standard deviation (SD) from a single FVIII inhibitor plasma donor analyzed in the presence of platelets from three different donors. Adjusted P-values represent the difference from HA plasma. The reference range was determined with 16 normal plasma donors in the presence of platelets from three different donors.

(0.5 U mL⁻¹ aPCC + 600 nm SIA) had a synergistic effect on CT and CFT, with measurements of 131 ± 6 s (two-fold faster than aPCC alone, P < 0.0001) and 62 ± 9 s (P = 0.028), respectively (Fig. 2). rFVIIa shortened both CT and CFT beyond the values seen with WB. This reduction was not affected by the addition of 600 nm SIA (Fig. 2; Table 4).

The TPI was recently shown to be a sensitive parameter for distinguishing patients with thrombotic events. The TPI for normal blood was 0.7 ± 0.1 s⁻¹. TPI values in FVIII-inhibited blood (0.1 ± 0.1 s⁻¹) increased to 0.3 ± 0.1 s⁻¹ after the addition of 600 nm SIA, 2.2 ± 0.5 s⁻¹ after the addition of 0.5 U mL⁻¹ aPCC, and to 3.0 ± 0.9 s⁻¹ in the presence of the aPCC-SIA combination. However, large variation was observed between individual donors (range 2.21–4.17 s⁻¹).

Synergistic effect of SIA on aPCC-induced clot formation in FVIII-inhibited WB under flow conditions

The T-TAS is a microchip-based flow-chamber system that measures thrombus formation under physiological conditions.
blood flow conditions [21], and was used to confirm the effects of the aPCC–SIA and rFVIIa–SIA combinations. Shear rates of 600 s\(^{-1}\) and 240 s\(^{-1}\) on collagen/TF-coated AR-chips were used to represent average-sized arteries and veins, respectively [22–24].

The time of onset of thrombus formation (T10 kPa) in WB at a shear rate of 600 s\(^{-1}\) was 6.3 ± 1.0 min. Inhibition of FVIII in the WB samples resulted in a prolonged time to thrombus formation of 9.1 ± 1.4 min (Fig. 3A,B; Table 5). Addition of SIA (600 nm) to FVIII-inhibited WB did not improve the thrombus formation time at T10 kPa. With respect to the two bypassing agents, 0.5 U mL\(^{-1}\) aPCC reduced the thrombus formation time to levels seen in normal WB (6.3 ± 2.0 min), whereas rFVIIa (1.75 μg mL\(^{-1}\)) had no effect. The aPCC–SIA combination significantly shortened the thrombus formation time to 2.8 ± 0.5 min, which is 3.3-fold faster than in FVIII-inhibited WB and 2.3-fold faster than in normal WB (Fig. 3A,B; Table 5). In contrast, the rFVIIa–SIA combination showed no combinatorial effect in FVIII-inhibited WB.

Images collected in the capillary during thrombus formation over a period of 4.5 min confirmed the aPCC–SIA synergistic effect (Fig. 3C). There was no difference in thrombus formation between FVIII-inhibited WB alone and after the addition of SIA, whereas thrombus formation with aPCC was observed to start at 180 s. In contrast, the aPCC–SIA combination resulted in enhanced thrombus formation at 120 s, and clear increases in the number of platelets and the amount of fibrin that covered the AR-chip surface from 180 s (Fig. 3C).

Analysis at the time of capillary occlusion (T80 kPa) showed a similar effect to that seen at T10 kPa. The addition of the aPCC–SIA combination to FVIII-inhibited WB resulted in a 2.1-fold lower occlusion time (5.9 ± 1.3 min) than in FVIII-inhibited WB (12.2 ± 1.8 min) and a 1.4-fold lower occlusion time than in normal WB (8.4 ± 1.4 min) (Table 5). SIA, aPCC, rFVIIa or the rFVIIa–SIA combination did not affect T80 kPa of FVIII-inhibited WB (Fig. S1).

At the lower shear rate of 240 s\(^{-1}\), inhibition of FVIII delayed the onset of thrombus formation (T10 kPa) from 7.6 ± 0.6 min to 9.8 ± 0.9 min (Fig. 3A; Table 5). aPCC at this shear rate reduced the

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Table 4 Clotting parameters measured by rotational thromboelastometry (ROTEM)

| Condition          | ROTEM parameters | Adjusted P-value | Clot formation time (s) | Mean clot firmness (mm) | Thrombodynamic potential index (s\(^{-1}\)) |
|--------------------|------------------|------------------|-------------------------|------------------------|------------------------------------------|
| WB                 |                  |                  | 1075 ± 35               | 183 ± 14               | 57 ± 4                                   | 0.7 ± 0.1                              |
| FVIII-inhibited WB |                  |                  | 2102 ± 250             | 1144 ± 293             | 57 ± 4 (n = 3)                           | 0.1 ± 0.1                              |
| SIA (600 nm)       |                  |                  | 1875 ± 237             | 520 ± 134              | 60 ± 2                                   | 0.3 ± 0.1                              |
| aPCC (0.05 U mL\(^{-1}\)) |              |                  | 653 ± 30               | 174 ± 16               | 59 ± 4                                   | 0.8 ± 0.2                              |
| aPCC (0.125 U mL\(^{-1}\)) |             |                  | 432 ± 28               | 122 ± 20               | 60 ± 3                                   | 1.3 ± 0.3                              |
| aPCC (0.25 U mL\(^{-1}\)) |              |                  | 323 ± 20               | 93 ± 12                | 62 ± 3                                   | 1.8 ± 0.4                              |
| aPCC (0.5 U mL\(^{-1}\)) |              |                  | 267 ± 11               | 79 ± 9                 | 63 ± 3                                   | 2.2 ± 0.5                              |
| aPCC (0.05 U mL\(^{-1}\)) + SIA |            |                  | 296 ± 11               | 77 ± 9                 | 64 ± 2                                   | 2.4 ± 0.4                              |
| aPCC (0.125 U mL\(^{-1}\)) + SIA |             |                  | 215 ± 40               | 61 ± 6                 | 63 ± 2                                   | 2.9 ± 0.5                              |
| aPCC (0.25 U mL\(^{-1}\)) + SIA |              |                  | 208 ± 75               | 65 ± 16                | 64 ± 4                                   | 3.0 ± 1.2                              |
| aPCC (0.5 U mL\(^{-1}\)) + SIA |              |                  | 131 ± 6                | 62 ± 9                 | 64 ± 4                                   | 3.0 ± 0.9                              |
| rFVIIa (0.15 μg mL\(^{-1}\)) |             |                  | 585 ± 48               | 132 ± 19               | 61 ± 2                                   | 1.2 ± 0.3                              |
| rFVIIa (0.25 μg mL\(^{-1}\)) |              |                  | 465 ± 22               | 109 ± 14               | 61 ± 3                                   | 1.5 ± 0.4                              |
| rFVIIa (0.5 μg mL\(^{-1}\)) + SIA |            |                  | 623 ± 54               | 141 ± 24               | 58 ± 3                                   | 1.0 ± 0.3                              |
| rFVIIa (0.25 μg mL\(^{-1}\)) + SIA |            |                  | 502 ± 41               | 116 ± 16               | 60 ± 3                                   | 1.3 ± 0.3                              |

aPCC, activated prothrombin complex concentrate; NS, not significant; rFVIIa, recombinant activated factor VII; SIA, sequence-identical analog of emicizumab; WB, whole blood. ROTEM parameters n = 4 repeats. Adjusted P-values represent the difference from FVIII-inhibited WB.

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**Fig. 2.** Clot formation with the combination of activated prothrombin complex concentrate (aPCC) and sequence-identical analog to emicizumab (SIA) in FVIII-inhibited whole blood (WB) measured by rotational thromboelastometry. Clotting in WB from a representative donor shows the change in clot elasticity over time. WB (gray dashed line) was incubated with FVIII inhibitors, resulting in FVIII-inhibited WB (gray). FVIII-inhibited WB was treated with 600 nm SIA (pink), 0.5 U mL\(^{-1}\) aPCC (blue), the aPCC–SIA combination (red), 1.75 μg mL\(^{-1}\) recombinant activated factor VII (rFVIIa) (dark green), or the rFVIIa–SIA combination (light green).
Fig. 3. Clot formation under flow measured with the Total Thrombus-formation Analysis System in FVIII-inhibited whole blood (WB) with a combination of bypassing agents and a sequence-identical analog to emicizumab (SIA). WB from healthy donors was analyzed on collagen/tissue factor-coated atheroma chips. FVIII was inhibited by the addition of anti-FVIII goat plasma, resulting in delayed clot formation. (A) Flow pressure graph from a representative donor at 600 s⁻¹ showing the change in pressure from the start of the experiment to occlusion of the capillary. WB (black dashed line) was incubated with anti-FVIII, resulting in FVIII-inhibited WB (black). FVIII-inhibited WB was treated with 600 nM SIA (pink), 0.5 U mL⁻¹ activated prothrombin complex concentrate (aPCC; blue), or the aPCC–SIA combination (red). The dashed gray line indicates the point at which T10 kPa is reached. (B) Scatter plots show the time to onset of thrombus formation (T10 kPa) at 600 s⁻¹ (left) and 240 s⁻¹ (right) for WB, FVIII-inhibited WB, and FVIII-inhibited WB after addition of SIA (600 nm), aPCC (0.5 U mL⁻¹), recombinant activated factor VII (rFVIIa) (1.75 µg mL⁻¹), a combination of aPCC and SIA, or a combination of rFVIIa and SIA. Each dot represents a donor and is the average of duplicate measurements. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. (C) Example of clot formation over a period of 4.5 min for the different treatments of FVIII-inhibited WB. The lighter color demonstrates formation of a clot, which initiates from the capillary wall (outer border) and develops towards the middle to fully occlude the vessel. Arrows indicate clot formation areas of interest. NS, not significant.
thrombus formation time to 2.9 ± 0.9 min. The aPCC–SIA combination also lowered the thrombus formation time, but to the same extent as aPCC alone, with a mean of 5.2 ± 5.9 min. SIA treatment alone had no effect, consistent with results from experiments at 600 s⁻¹. rFVIIa improved the thrombus formation time to WB levels of 6.9 ± 2.4 min. No additional effect of SIA when combined with rFVIIa was observed. With regard to occlusion time (T80 kPa), only rFVIIa had a significant effect as compared with FVIII-inhibited WB (Fig. S2).

**Discussion**

Bispecific antibodies such as emicizumab represent an advance in the treatment of patients with HA and inhibitors by reducing bleeding events when administered prophylactically. However, because non-factor products such as emicizumab are in a constitutively active state, and are not subject to the same regulatory mechanisms and proteolytic activation and inactivation as FVIII [25], there are potential risks that need to be addressed for non-factor therapies to be considered to be well tolerated in all clinical settings. Emicizumab prophylaxis significantly reduced the annual bleeding rate of patients with HA and inhibitors, but breakthrough bleeds still occurred.

During the phase 3 HAVEN 1 trial, TMA and thrombotic events were reported among some patients who received concomitant emicizumab prophylaxis and additional infusions of aPCC [8]. Our *in vitro* experiments demonstrate that a sequence-identical analog of emicizumab, at clinically relevant doses in combination with an aPCC, resulted in thrombin levels exceeding the normal physiological range.

We hypothesize that this excessive thrombin-generating activity may indicate that patients receiving bispecific procoagulant antibody prophylaxis may be at increased risk of thrombosis when subsequently treated with aPCC for breakthrough bleeding. Further support for this hypothesis comes from clot formation analysis data obtained by employing T-TAS and ROTEM technology, which showed that the aPCC–SIA combination resulted in CFTs two-fold to eight-fold lower than in WB from healthy controls.

The T-TAS is a novel research tool that measures thrombus formation in WB under flow conditions, and has been used for hemophilic mouse studies [26] and for the analysis of other bleeding disorders, such as von Willebrand disease [27]. We used this technology to confirm the exaggerated clotting observed with the aPCC–SIA combination by using established methods such as CAT and ROTEM. In addition, we were interested in clot formation under blood flow conditions. The T-TAS data clearly showed the synergistic effect of the aPCC–SIA combination in producing clotting times lower than in normal WB.

Potential limitations of the study include the *in vitro* nature of the assays and the caution that needs to be exercised when extrapolating the data to clinical characteristics. Additionally, plasma from a small number of volunteers was used in the study, which is mitigated by the wide range of plasma samples analyzed, covering a range of BU titers from 1 IU mL⁻¹ to 133 IU mL⁻¹, achieving similar TG curves and responses to procoagulant stimuli. Although the T-TAS was previously utilized in studies on antiplatelet and antithrombotic therapies [28,29], the parameters used in our study were different, and thrombogenicity cannot be inferred on the basis of a direct comparison with published results. However, the T-TAS

### Table 5 Onset of clot formation (T10 kPa) and occlusion of capillary (T80 kPa) measurements obtained with the Total Thrombus-formation Analysis System (T-TAS)

| Condition                  | T10 kPa (min)  | T80 kPa (min)  |
|----------------------------|----------------|----------------|
|                            | 600 s⁻¹         | 240 s⁻¹         | 600 s⁻¹         | 240 s⁻¹         |
| WB                         |                |                |                |                |
| FVIII-inhibited WB         | 6.3 ± 1.0 (4.9–7.9)†† | 7.6 ± 0.6 (6.9–8.6) NS† | 8.4 ± 1.4 (6.3–10.5)†† | 11.1 ± 1.1 (10.1–12.8) NS†† |
| SIA (600 nm)               | 9.1 ± 1.4 (6.7–10.8)†† | 9.8 ± 0.9 (8.8–11.3) NS† | 12.2 ± 1.8 (9.4–14.7)†† | 16.0 ± 2.3 (13.8–20.3) NS†† |
| aPCC (0.5 U mL⁻¹) + SIA (600 nm) | 9.0 ± 2.2 (6.6–12.2) NS‡ | 10.6 ± 1.8 (8.8–13.4) NS‡ | 11.7 ± 2.2 (9.2–14.6) NS‡ | 15.5 ± 2.9 (12.8–20.4) NS‡ |
| rFVIIa (1.75 µg mL⁻¹) + SIA (600 nm) | 6.3 ± 2.0 (3.8–9.3) NS§ | 2.9 ± 0.9 (2.4–4.0) NS§ | 12.2 ± 4.4 (6.9–16.4) NS§ | 15.7 ± 4.6 (12.4–18.9) NS§ |
| rFVIIa (1.75 µg mL⁻¹) + SIA (600 nm) | 2.7 ± 0.5 (2.1–3.2) NS****†† | 5.2 ± 5.9 (1.8–12.0)* | 5.9 ± 1.3 (4.9–7.7) NS** | 16.3 ± 4.4 (12.8–21.2) NS |

aPCC, activated prothrombin complex concentrate; NS, not significant; rFVIIa, recombinant activated factor VII; SIA, sequence-identical analog of emicizumab; WB, whole blood. Normal healthy blood was treated with anti-FVIIH goat plasma. †n = 7. ‡n = 6. §n = 5. ¶n = 2. ††n = 4. When no symbol is present, n = 3. Adjusted- values represent the difference from FVIII-inhibited WB. *P < 0.05, **P < 0.01, ****P < 0.0001. As the sample numbers used in these analysis were small, P-values should be viewed with caution.
was mainly utilized to corroborate results from ROTEM and the TG assay [30], which, in turn, have been shown to correlate with thrombotic events in a variety of disease states [19,31–33]. For example, CFT and MCF in ROTEM were upregulated in patients with thromboembolic complications, whereas an increase in the TPI indicated a hypercoagulable state [19,33]. In addition, TG assay parameters have demonstrated correlation with some thrombotic complications [34]. There are also data to support the use of the TG assay as a laboratory tool to measure the risk of recurrent venous thromboembolism and other hypercoagulation disorders [32].

Studies using isolated single components of aPCC (FII, FIIa, FIX, FIXa, FX, and FXa) revealed the theoretical mechanism of aPCC–SIA synergistic TG. SIA in combination with FIX, and to a lesser extent FIXa, made the largest contribution to the synergistic effect seen with the aPCC–SIA combination. Thus, we hypothesize that SIA can more readily form the FX activation complex in the presence of aPCC. This feature, coupled with increased TG, drives the highly reduced CTs that we observed in the ROTEM and T-TAS assays.

Interestingly, when SIA was combined with rFVIIa, the result was an additive increase in TG that did not reach the normal physiological range of TG. However, the effect of combining SIA with aPCC was synergistic to a level beyond the normal physiological range of TG. Statistical analysis showed that there was no difference between the single reagents 600 nM SIA and 0.5 U mL−1 aPCC or 1.75 μg mL−1 rFVIIa, or for the rFVIIa–SIA combination, in all four test systems. Then only significant difference seen was for the aPCC–SIA combination as compared with aPCC alone. There may be several factors affecting the activity resulting from these combinations, one of which is the mechanism of action of SIA and the composition of aPCC, as described above. Concomitant use of aPCC and SIA causes increased levels of enzyme (FIXa) and substrate (FX).

The results from our in vitro model, coupled with adverse events emerging from the emicizumab clinical trial, suggest a critical need for additional studies to define the potential safety risks associated with the use of non-factor therapies such as emicizumab in the presence of bypassing agents. At the time of writing, there were reports of two cases of thromboembolic complications and three cases of TMA in 35 emicizumab-treated patients who were then treated for breakthrough bleeding with a bypassing agent during the phase 3 clinical trial (NCT02622321) [35]. For the use of aPCC alone, a rate of 0.005% thromboembolic events and no TMAs were reported in postmarketing reports. In the absence of more detailed data on these events in the phase 3 trial and the lack of knowledge about the relationships between aPCC use, dose, frequency, and timing, and the clinical condition of the patients, it is not possible to assess the risk of TMA associated with aPCC in combination with emicizumab at this time. Therefore, careful judgement will be required when breakthrough bleeding is treated in patients receiving emicizumab. For the development of any non-factor procoagulant therapy, as stated by Stanulovic et al., companies seeking marketing authorization, as well as prescribers, clinical trial sponsors, investigators, and regulators, should make an effort to extrapolate from the observed adverse events to predict further safety issues [36].

Addendum

R. Hartmann contributed to the design and conception of the study, and analysis and interpretation of the results. T. Feenstra contributed to the execution of the study, and analysis and interpretation of the results. L. Valentino, M. Dockal, and F. Scheiflinger contributed to the conception and design of the study, and interpretation of the results. All authors revised the work critically for important intellectual content, and all authors approved the final version.

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Disclosure of Conflict of Interests

R. Hartmann, M. Dockal and F. Scheiflinger are employees of Shire and may hold stock in the company. F. Scheiflinger has patent US 7,033,590 issued. L. Valentino and T. Feenstra are former employee of Shire and were employed by Shire at the time when the work was conducted for this article. L. Valentino may hold stock in the company.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article:

Fig. S1. TG of single aPCC components in FVIII-inhibited plasma.
Fig. S2. Capillary occlusion measured by T-TAS in FVIII-inhibited WB with a combination of bypassing agents and SIA.

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