Haemophilia
The Official Journal of the World Federation of Hemophilia, European Association for Haemophilia and Allied Disorders and the Hemostasis & Thrombosis Research Society

Hybrid human-porcine factor VIII mutants escape the inhibitory effects of anti-factor VIII inhibitor alloantibodies with A2 or C2 epitopes

Journal:	*Haemophilia*
Manuscript ID	HAE-00187-2023
Manuscript Type:	Original Article
Date Submitted by the Author:	14-Jun-2023
Complete List of Authors:	Mizumachi, Kuniyoshi; Nara Medical University, Pediatrics Nakajima, Yuto; Nara Medical University, Pediatrics Shimonishi, Naruto; Nara Medical University, Pediatrics Furukawa, Shoko; Nara Medical University, Pediatrics Ogiwara, Kenichi; Nara Medical University, Pediatrics Takeyama, Masahiro; Nara Medical University, Pediatrics Nogami, Keiji; Nara Medical University, Pediatrics
Keywords:	inhibitor, porcine factor VIII, hemophilia A, epitope, neutralizing effect

To the *Haemophilia*

Dear Editor,

My colleague and I now submit a manuscript entitled as “Hybrid human-porcine factor VIII mutants escape the inhibitory effects of anti-factor VIII inhibitor alloantibodies with A2 or C2 epitopes”.

Porcine factor (pF)VIII has low cross-reactivity with anti-human (h)FVIII inhibitor alloantibodies whilst most FVIII inhibitors in human recognize the A2 and/or C2 domain(s). To evaluate the ability of human-porcine hybrid (hp)FVIII to limit the anti-FVIII activity of inhibitor alloantibodies, we created three hybrid mutants by substituting the A2, C2 domain or both in human hFVIII, with the corresponding domain of pFVIII (termed hp(A2), hp(C2), and hp(A2/C2), respectively). The reactivity of these mutants was assessed by one-stage clotting assays (OSA), thrombin generation assays (TGA), and rotational thromboelastometry (ROTEM). OSA demonstrated that mutation restricted the inhibitory effects of both anti-FVIII A2 or C2 monoclonal antibodies (mAb) and polyclonal inhibitor-antibodies (polyAb) from patients with hemophilia A and inhibitor (PwHA-I). TGA indicated that peak thrombin only with hp(A2) and hp(A2/C2) was not attenuated in the presence of anti-A2 polyAb. With anti-A2/C2 polyAb, the activity of hp(A2/C2) was unaffected. ROTEM demonstrated that the addition of hp(A2/C2) to anti-A2/C2 polyAb shortened clot times/clot formation times to a greater extent than the others. In conclusion, mutated human FVIII comprising porcine A2 and/or C2 sequences could escape the inhibitory effects, suggesting that hpFVIII provide novel treatments for PwHA-I.

Word counts: Abstract: 248; Text: 2,998; References: 40; Figures: 7; Tables: 1.

Our work is not previously published in any substantial part, and is not under consideration of publication elsewhere, and have submitted to *Haemophilia* for consideration of publication. We are very much grateful if this manuscript would be accepted in your journal.

Sincerely yours,

Yuto Nakajima

Yuto Nakajima, M.D., Ph.D.
Dept. Pediatrics, Nara Medical University,
840 Shijo-cho, Kashihara, Nara 634-8522, Japan
Tel: +81-744-39-8882, Fax: +81-744-24-9222,
e-mail; nakajima-yamanashi@naramed-u.ac.jp.

Hybrid human-porcine factor VIII mutants escape the inhibitory effects of anti-factor VIII inhibitor alloantibodies with A2 or C2 epitopes

Kuniyoshi Mizumachi,[1] Yuto Nakajima,[1,2] Naruto Shimonishi,[1,3] Shoko Furukawa,[1] Kenichi Ogiwara,[1] Masahiro Takeyama,[1] Keiji Nogami [1]

1. Department of Pediatrics, Nara Medical University, Kashihara, Nara, Japan
2. Advanced Medical Science of Thrombosis and Hemostasis, Nara Medical University, Kashihara, Nara, Japan
3. The Course of Thrombosis and Hemostasis Molecular Pathology, Nara Medical University, Kashihara, Nara, Japan

Running title: Hybrid FVIII unaffected by inhibitors

Type of article: Original Article

Statement of prior presentation: An account of this work was presented at the 64th Annual Meeting of American Society of Hematology 2022, in New Orleans, Louisiana.

Word counts: Abstract: 248; Text: 2,998; References: 40; Figures: 7; Tables: 1; Supplemental Figure: 1; Supplemental Table 1.

Keywords; inhibitor, porcine factor VIII, hemophilia A, epitope, neutralizing effect

Corresponding author:

Yuto Nakajima, MD, PhD

Department of Pediatrics, Nara Medical University,

840 Shijo-cho, Kashihara, Nara 634-8522, Japan

Tel: +81-744-29-8881, Fax: +81-744-24-9222

E-mail: nakajima-yamanashi@naramed-u.ac.jp

Abstract

Introduction: Porcine factor (pF)VIII has low cross-reactivity with anti-human (h)FVIII inhibitor alloantibodies. Clinical trials of pFVIII in congenital hemophilia A patients with inhibitor (PwHA-I) are in progress. Most FVIII inhibitors recognize the A2 and/or C2 domain(s), and recombinant human-porcine hybrid (hp)FVIII containing mutations within these domains may escape the neutralizing effects of these inhibitors.

Aim: To evaluate the ability of hpFVIII to limit the anti-FVIII activity of inhibitor alloantibodies.

Methods: Three hybrid mutants were created by substituting the A2, C2 domain or both in human hFVIII, with the corresponding domain of pFVIII (termed hp(A2), hp(C2), and hp(A2/C2), respectively). The reactivity of these mutants was assessed by one-stage clotting assays (OSA), thrombin generation assays (TGA), and rotational thromboelastometry (ROTEM) using FVIII-deficient samples.

Results: OSA demonstrated that mutation restricted the inhibitory effects of both anti-FVIII A2 or C2 monoclonal antibodies (mAb) and polyclonal inhibitor-antibodies (polyAb) from PwHA-I. TGA indicated that peak thrombin with hp(A2) and hp(A2/C2) was not attenuated in the presence of anti-A2 polyAb, but that with hFVIII and hp(C2) was suppressed to levels equivalent to those of FVIII-deficient plasma. With anti-A2/C2 polyAb, the activity of hp(A2/C2) was unaffected. ROTEM demonstrated that the addition of hp(A2) or hp(A2/C2) to anti-A2 polyAb shortened clot times/clot formation times, whilst hFVIII or hp(C2) were ineffective. Similarly with anti-A2/C2 polyAb, hp(A2/C2) restored coagulation potential to a greater extent than hp(A2) and hp(C2).

Conclusion: Hybrid FVIII mutations within the porcine A2 and/or C2 domain

corresponding to respective inhibitor-epitopes could support effective therapy in PwHA-I.

1. Introduction

Hemophilia A (HA) is characterized by a deficiency or defect of coagulation factor (F)VIII, and is commonly treated with on-demand or regular prophylactic therapy using FVIII agents [1]. However, in 20-30% of patients with severe HA (PwHA), the development of these neutralizing antibodies renders FVIII replacement ineffective [2-4]. Bypassing agents (BPAs) including recombinant activated FVII and activated prothrombin complex concentrates are utilized for bleeding episodes in PwHA with inhibitor (PwHA-I) [5,6]. Although emicizumab, a bispecific antibody to FIX/FIXa and FX/FXa, significantly reduces bleeding episodes in PwHA-I, additional BPA administration is needed for breakthrough bleeding [7-9]. The hemostatic improvement mediated by BPA therapy is inconsistent inter-individually[10], however, and is cumbersome to control, requiring complicated monitoring including global coagulation assays [11,12].

Porcine (p)FVIII is known to be highly homologous to human (h)FVIII in terms of protein structure [13], and has similar coagulation potential to hFVIII [13]. The cross-reactivity of autoantibodies against pFVIII has been shown to be significantly lower (<10%) in acquired PwHA [14-17]. Several reports have also indicated that inhibitor titers in congenital PwHA-I are lower when measured against pFVIII than with hFVIII [14-16,18,19], and clinical trials have demonstrated that pFVIII could be alternative agent to BPA for the treatment of bleeding events in PwHA-I [20,21].

Most FVIII inhibitors bind to epitopes located in A2, C2, and/or A3-C1 domains of FVIII [22-26], and mono-specific inhibitors associated with domains other than A2 and C2 are

infrequent [27]. The ability of pFVIII to resist the effects of human FVIII inhibitors might derive principally from differences in amino acid sequences between hFVIII and pFVIII, and in this context, Barrow et al. [28] pointed that hybrid human/porcine (hp)FVIII was less antigenic relative to pFVIII.

We hypothesized that substitution of only the A2 and C2 domains of hFVIII with corresponding pFVIII sequences could be sufficient to escape inhibitor reactions, and might be useful for the treatment of breakthrough bleeding in PwHA-I. Hybrid, hpFVIII mutants were prepared, therefore, and investigated for their abilities to resist inactivation using anti-FVIII monoclonal antibodies (mAbs) and polyclonal antibodies (polyAbs). In addition, the global coagulation potential of hpFVIII was examined using blood samples from congenital PwHA-I.

2. Materials and Methods

2.1- Reagents

FVIII-deficient plasma (FVIII-d; George King Bio-Medical Inc., Overland Park, KS, USA); recombinant human tissue factor (TF; Innovin®, Dade, Marburg, Germany); human α-thrombin, FIXa, FX (Haematologic Technologies Inc., Essex Junction, VT, USA); recombinant hirudin (Calbiochem, San Diego, CA, USA) and FXa substrate S-2222 (Chromogenix, Diapharma Inc., West Chester Township, OH, USA) were purchased from indicated vendors. Purified recombinant FVIII (Kogenate®FS) was obtained from Bayer Corp. Japan. Recombinant C2 protein was prepared as previously described [29].

Phospholipid vesicles containing 10% phosphatidylserine, 60% phosphatidylcholine, and 30% phosphatidylethanolamine (Sigma-Aldrich, St. Louis, MO, USA) were prepared as previously described [30]. Two anti-A2 mAbs, GMA-8015 and GMA-8021, two anti-C2 mAbs, GMA-8022 and GMA-8006, anti-A1 mAb GMA-8001 and anti-C1 mAb GMA-8011 were purchased from Green Mountain Antibodies (Burlington, VT, USA). Anti-emicizumab antibody [31] was generously gifted from Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan).

2.2 - Patients' whole blood and plasma samples

This study was approved by the Medical Research Ethics Committee of Nara Medical University (No. 2503), and blood samples were collected in accordance with the ethical guidelines of our university. Anticoagulated blood samples from patients and healthy individuals were collected into tubes containing 3.2 % sodium citrate at a 9:1 ratio. Platelet-poor plasma was recovered after centrifugation of citrated whole blood for 15 min at 1,500xg. Whole blood was obtained by venipuncture after informed consent following local ethical guidelines. IgG fractions from inhibitor plasma sample were prepared using Protein G-Sepharose® (Sigma-Aldrich).

2.3 - Mutagenesis, expression, and purification of hpFVIII

Recombinant human wild-type FVIII (WT) was prepared as B domain-deleted FVIII (lacking Q744-S1637), and was stably expressed in baby hamster kidney cells and purified [32]. Additionally, three mutated FVIII gene products were made by modifying the A2 domain and/or C2 domains of hFVIII with corresponding sequences from pFVIII.

Briefly, porcine A2 and/or C2 domain sequences were inserted into hFVIII digested with appropriate restriction endonuclease ligation. The mutated FVIII was expressed and purified as for WT (hFVIII), and termed hp(A2), hp(C2), and hp(A2/C2), respectively. Resultant FVIII forms were typically >90% pure as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and staining with GelCode™ Blue-Stain Reagent (Thermo Fisher Scientific; Waltham, MA, USA), with albumin representing the major contaminant. FVIII concentrations were measured by enzyme-linked immunosorbent assays (ELISA) using two anti-FVIII mAbs that recognize the A1 (GMA-8002) and C1 domain (GMA-8011), respectively. Samples were quick-frozen and stored at -80°C.

2.4 - Clotting assays of FVIII activity

FVIII activity (FVIII:C) was measured by one-stage clotting assays (OSA) using the STart®4 Hemostasis Analyzer (Diagnostica Stago, Asnieres, France). Activity was also assessed using chromogenic substrate assays (CSA) in a purified FXa generation system [33]. Time-course FVIII:C assays were assessed after adding FVIII (final concentration; f.c. 1 nM) to the FVIII deficient plasma (FVIII-d). Aliquots were removed from the mixtures at the predetermined times and FVIII:C was measured by OSA.

2.5 - Epitope mapping by electrophoresis and western blotting

The light chain and the C2 domain of hFVIII were isolated and purified as previously described [29,32]. Briefly, cDNA encoding the C2 domain with a four amino acid N-terminal extension (V2169-W2332) was constructed, transformed, and expressed in Pichia

pastoris cells, followed by purification. To determine inhibitor-epitopes from patient's plasmas, native FVIII, thrombin-cleaved FVIII, purified isolated light chain, and C2 domain were separated by SDS-PAGE using a 13% gel. For Western blotting, the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane at 100 V for 1 hour and probed with patient's plasma, followed by anti-human IgG peroxidase-conjugated secondary antibody. The signals were detected using enhanced chemiluminescence (PerkinElmer Life Science, Boston, MA, USA).

2.6 - Thrombin generation assays

Thrombin generation assays (TGA) were performed as previously described [34]. Briefly, plasma samples were preincubated for 10 min with trigger reagent containing PL and TF (f.c., 4 µM, and 1 pM, respectively). Fluorescent signals were monitored using a Fluoroskan Ascent™ microplate reader (Thermo Fisher Scientific) after the addition of reagent containing $CaCl_2$ and fluorogenic substrate. Data analyses were performed using the manufacturer's software to derive the standard parameters; peak thrombin and endogenous thrombin potential (ETP).

2.7 - Rotational thromboelastometry (ROTEM)

Rotational thromboelastometry (ROTEM) was performed 30 min after venipuncture using the Whole Blood Haemostasis Analyser (Pentapharm, Munich, Germany). Whole blood (300 µL) was preincubated at 37°C, mixed with 20 µL $CaCl_2$, and the viscoelasticity of clot formation monitored as previously described [35]. The coagulation process was assessed using clot time (CT; the time until detection of clot firmness at 2 mm amplitude)

and clot formation time (CFT; the time until detection of clot firmness of 20 mm amplitude). Normal parameters based on 20 healthy controls were CT [median 936 sec; 95% confidential interval (CI) 762-1,127 sec] and CFT (median 313 sec; 95% CI 207-511 sec) [35].

2.8 - Data analysis

Experiments were performed at least three times and the median and standard deviation are shown. Limited sample volume was available for the *ex vivo* assay, however, and only duplicate experiments were possible in this patient. Statistical analysis was performed by the Student's t-test. Significant differences between the groups were considered as $p < 0.05$.

3. Results

3.1 - Structure and specific activity of recombinant hpFVIII

A diagram of the hpFVIII domain structure is illustrated in **Figure 1.** The hFVIII WT and hybrid FVIII mutants with A2, C2 domain or both substituted with corresponding sequences of pFVIII were identified as hFVIII, hp(A2), hp(C2) and hp(A2/C2) respectively. Specific activities measured by OSA and CSA were expressed as a ratio of hFVIII (**supplemental Table 1**). Each hybrid mutant exhibited specific activity of approximately 60-90% (OSA) and 70% (CSA) relative to hFVIII. There was a slight difference between assays of hp(C2), but other mutants showed similar specific activities using each technique.

3.2 - Interactions of hpFVIII with FVIII antibodies in vitro assessed by OSA

The ability of hpFVIII to resist inactivation was first examined using two anti-A2 mAbs and two anti-C2 mAbs. Each mAb was incubated with FVIII-d at a final concentration of 2.7-4.4 BU/mL prior to the addition of hFVIII or hpFVIII at a concentration of 1 nM and monitoring FVIII:C. The FVIII:C was expressed as fold of the initial (no addition of mAb). Time-courses of FVIII:C using hFVIII and hpFVIII are shown in **Figure 2**.

In the presence of anti-A2 mAbs with different epitopes (**Figures 2A** and **2B**), FVIII:C of hFVIII and hp(C2) were markedly decreased, resulting in suppression below 20% of initial levels after 30 min incubation. In contrast, the FVIII:C of hp(A2) and hp(A2/C2) showed little decrease at 30 min. In the presence of two anti-C2 mAbs (**Figures 2C** and **2D**), FVIII:C of hFVIII and hp(A2) were markedly decreased, but were maintained at initial levels with hp(C2) and hp(A2/C2).

Similar experiments were repeated with purified anti-FVIII inhibitor IgGs derived from PwHA-I plasma samples. Inhibitor characteristics were confirmed by SDS-PAGE and Western blotting analyses (**Supplemental Figure 1**), and identified epitopes within domains of A1/A2/A3-C1 (**A**), A2 alone (**B**), and A2/C2 (**C** and **D**). In these experiments with the polyAb IgGs, the FVIII:C of both hFVIII and hp(C2) were depressed completely by the anti-A1/A2/A3-C1 inhibitor (**Figure 3A**) and the anti-A2 inhibitor within 10 min incubation (**Figure 3B**), whilst FVIII:C of hp(A2/C2) and hp(A2) were little or mildly decreased. Notably, higher levels of FVIII:C were maintained with hp(A2/C2) than with

hp(A2), likely reflecting the additional contribution of the C2 epitope in combination with the A2 epitope in these reactions. In mixtures containing the polyAb IgG with an A2/C2 epitope, FVIII:C of >60% at 30 min was maintained only with hp(A2/C2), whilst corresponding measurements with other mutants were decreased by approximately 80% **(Figure 3C**). Moreover, in parallel studies using the other polyAb IgG with an A2/C2 epitope together with hp(A2/C2) or hp(A2), FVIII:C was maintained at >45% and >25% of initial, respectively, whilst levels were almost undetectable with other mutants (**Figure 3D**). These data suggested that hybrid human FVIII containing the appropriate substituted porcine epitope-sequence could be resistant to inhibitory activity. Further investigations were devised, therefore, to assess global coagulation potential in the presence of the hybrid mutants.

3.3 - Interactions of hpFVIII with FVIII antibodies in vitro assessed by TGA

Each inhibitor IgG (anti-A1/A2/A3-C1 and anti-A2/C2) was fractionated from PwHA-I and mixed with FVIII-d as described above (*see* Figures 3A and 3C, respectively), prior to incubation with hFVIII or hpFVIII (2 nM) for 1.5 hours. Representative time-course reaction curves are illustrated in **Figure 4**, and corresponding data together with TGA parameters are shown in **Table 1**.

In the absence of inhibitor IgG (**Figure 4A-a**), the addition of each hybrid mutant to FVIII-d restored the TGA parameters (peak thrombin and ETP). In the presence of anti-A1/A2/A3-C1 inhibitor IgG (**Figure 4A-b**), the peak thrombin and ETP mediated by hp(A2) and hp(A2/C2) were at least 80% of those in the absence of inhibitor IgG. In contrast,

after the addition of hFVIII or hp(C2), these parameters were similar to those of non-spiked FVIII-d. These findings clearly indicated hp(A2) and hp(A2/C2) were not significantly inactivated by the anti-A1/A2/A3-C1 inhibitor IgG, unlike the native hFVIII and the hp(C2) mutant. Similarly, in the experiments with anti-A2/C2 inhibitor IgG (**Figure 4B-b**), the peak thrombin and ETP measurements in mixtures with hp(A2) and hp(C2) were attenuated by approximately 20-50% compared to those in the absence of inhibitor IgG (**Figure 4B-a**), whilst hp(A2/C2) was not inactivated by the inhibitor. Detailed analyses of these data are presented in **Table 1**, and demonstrate in particular that the recovery ratios (Inh+/Inh−) of hp(A2) and hp(A2/C2) were significantly higher than that with hFVIII.

These *ex-vivo* studies were then extended utilizing plasma from another PwHA-I (3.5 BU/mL anti-A2 inhibitor) treated with emicizumab prophylaxis. The potential effects of emicizumab in these experiments were neutralized using anti-emicizumab antibodies as described previously [31], before the addition of hFVIII or mutant hpFVIII to the patient's plasma at a concentration of 2 nM and incubation for 1.5 hours. Under these conditions, the TGA parameters were ameliorated with hp(A2) and hp(A2/C2) but not with hFVIII and hp(C2) (**Figure 5**). The findings confirmed, therefore, that hybrid FVIII composed of porcine A2 sequences escaped the inhibitory effects of corresponding inhibitors.

3.4 - Interactions of hpFVIII with FVIII antibodies in vitro assessed by ROTEM

ROTEM assays were performed utilizing whole blood collected from the PwHA-I with an anti-A2 inhibitor. The potential effects of emicizumab were neutralized effects as

described above (*see* Figure 5), and hFVIII or hpFVIII was added to the whole blood at a plasma concentration of 2 nM and incubated for 1.5-hour. **Figure 6** illustrates that marked improvements of CT and CFT were apparent in the experiments with hp(A2) **(Figure 6C)** and hp(A2/C2) **(Figure 6E)** in contrast to marginal or no improvements with either hFVIII **(Figure 6B)** or hp(C2) **(Figure 6D)**. Further ROTEM analyses were performed, therefore, to examine whether similar tendencies would be evident in the presence of anti-A2/C2 inhibitors (**Figure 7**). Whole blood samples were obtained from non-inhibitor PwHA, and mixed with anti-A2/C2 inhibitor IgG (*see* Figure 3C) prior to the addition of hFVIII or hpFVIII at a plasma concentration of 2 nM. The results indicated that, although a modest shortening of the CT was seen in the presence of hFVIII, the differences would be unlikely to influence clinical bleeding events. The mutant concentrates restored the ROTEM parameters significantly, however, especially the hp(A2/C2) product. (**Figure 7; *inset*).**

Overall, therefore, these experiments demonstrated that mutated hpFVIII provided the ability to escape the inhibitory effects of anti-FVIII inhibitor alloAbs with A2 and/or C2 epitopes, not only using plasma assays but also with global whole blood coagulation techniques.

4. Discussion

Previous clinical reports have demonstrated that recombinant (r)pFVIII offers an effective therapy for both congenital and acquired PwHA-I [20,21], and could provide a

useful alternative to BPA. Moreover, Barrow et al. [28] speculated that hybrid human-porcine FVIII (hpFVIII) could be less antigenic in PwHA-I but there was little detailed studies on the ability of hpFVIII mutants to escape the neutralizing effects of ant-FVIII inhibitor antibodies. Therefore, we examined the coagulation characteristics and inhibitor resistance properties of hFVIII hybridized with porcine A2 and/or C2 sequences. Our findings, utilizing three different plasma and whole blood techniques, OSA, TGA, and ROTEM, provided strong evidence that substitution of the human domains with corresponding porcine sequences substantially reduced the ability of anti-FVIII antibodies to neutralize FVIII activity.

Major inhibitor epitopes have been localized to A2 and/or C2 domains [22-26], and antibodies, which are mono-specific for domains other than A2 and C2 are scarce [27]. In addition, earlier studies suggested that clinically significant antibodies recognize epitopes restricted to the A2 domain (especially residues 484-508 [36]), the A3 acidic region, or A3/C2 sequences [22-26], and that anti-A1 and anti-C1 inhibitors have fewer clinical consequences [29]. Indeed, anti-A3 domain inhibitors were believed to be responsible for the inhibitory activity in some plasma samples [28,36], but anti-A3 antibodies appeared to be more common after administration of pFVIII in a mouse model of HA compared to administration of hFVIII [37]. In these circumstances, substitution of this region could cause another inhibitory antibodies against porcine A3 domain in hpFVIII.

In the experiments with the anti-A1/A2/A3-C1 IgG (*see* Figure 3A), the A1 and A3-C1

domains appeared to contribute little to inhibitory activity. The global coagulation assays (Figures 6 and 7) using purified IgG from the patient whose inhibitor recognize both A2 and C2 domain confirmed; however; that the specific mutant proteins; hp(A2) and hp(C2); could partially escape the inhibitory effects (Figure 3D, Figure 7). It seems likely that conformational changes of FVIII induced by partial substitutions may result in attenuation of inhibitory activity. Further investigations are required to clarify the detailed impact of variable molecular arrangements in these mechanisms.

Bleeding episodes in PwHA-I are treated with high-dose FVIII or BPA, and are commonly dependent on the latest inhibitor titer [5,6]. Neutralizing therapy using FVIII product can temporarily restore normal coagulation activity, but the response might be refractory and fails to adequately control bleeding due to an elevation of Bethesda titer caused by immunologic stimulation [1]. Moreover, the response to BPA is highly variable, and routine coagulation assays may not monitor treatment efficacy reliably. Non-factor agents, especially emicizumab are administered to an increasing number of PwHA-I including children, and dramatically improve clinical outcomes [38,39], but alternative FVIII agents or BPAs are needed in breakthrough bleeding events [7-9]. The present studies provide evidence that hpFVIII could offer advantages over the current recombinant hFVIII agents or BPAs in that the mutants could escape inhibition, retain FVIII:C efficiently, and be monitored by OSA or CSA.

We acknowledge that there are several limitations in this study. Firstly, eligible PwHA with inhibitors linked to the FVIII C2 domain alone was not identified. Nevertheless, we

demonstrated that hp(C2) completely escaped the inhibitory activity of anti-C2 mAbs (Figure 2C and 2D), and that hp(A2/C2) was resistant to inhibitors that recognized the A2 and C2 domain. These findings confirmed that substitution of the C2 domain was effective against anti-C2-related inhibitors. Secondly, recombinant pFVIII is not available in Japan at present, and the correlation between the inhibitory activity of rFVIII and hpFVIII was not determined. Lastly, several reports have indicated that some PwHA-I produced anti-pFVIII inhibitor after administration of (r)pFVIII, and the possibility of producing new immunogenic inhibitors against hpFVIII remains a concern. Approximately one third of those PwHA-I could be treated with pFVIII on multiple occasions, however, without becoming refractory to this therapy [16,18,40]. Further studies utilizing animal models of HA would be useful to clarify the efficacy and immunogenicity of hpFVIII *in vivo*.

5. Conclusion

Mutant human FVIII comprising porcine A2 and/or C2 sequences substituted in the corresponding human domains was shown to escape the adverse functional effects of respective inhibitors utilizing OSA, TGA and ROTEM. The findings suggest that hybrid FVIII products resistant to inhibitor activity could provide novel treatments for PwHA-I as well as porcine FVIII product. Hence, even in the 'post-emicizumab era', improvements with therapeutic FVIII concentrates and advances in strategies for treating PwHA-I warrant further study.

Acknowledgments

We are especially grateful to Ms. Keiko Nishimura for her technical assistance.

Financial support: This research was supported by the Japan Agency for Medical Research and Development (AMED) under Grant Number 21fk0410037, supported by a Grant-in-Aid for Scientific Research (KAKENHI) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) to KN (21K07804) and YN (22K15928), and conducted by a part of collaborative research with Takeda Pharmaceuticals Limited.

Authorships

K.M carried out the experiments, analyzed the data, interpreted the data, wrote the manuscript, and created the figures and tables; **Y.N** designed the study, analyzed and interpreted the data, wrote the manuscript and approved the final version for publication; **N.S, S.F, K.O** and **M.T** interpreted the data; **K.N** designed the study, analyzed the data, interpreted the data, wrote the manuscript, and edited the manuscript.

Conflicts of interest

K.M has no conflict of interest to disclose.

Y.N has received research grant from Takeda Pharmaceutical Co..

N.S teaches a course endowed by CSL Behring.

S.F taught a course endowed by CSL Behring, and has received personal fees from Chugai Pharmaceutical Co., Ltd., CSL Behring, and Takeda Pharmaceutical Co..

K.O taught a course endowed by CSL Behring and has received personal fees from

Chugai Pharmaceutical Co., Ltd. and CSL Behring.

M.T has received personal fees from Chugai Pharmaceutical Co., Ltd. KM Biologics Co., Bayer AG, and CSL Behring.

K.N has received grants, personal fees, and non-financial support from Chugai Pharmaceutical Co., Ltd., personal fees from F. Hofmann-La Roche Ltd., grants, personal fees, and non-financial support from Sysmex Co. and Sekisui Medical, grants and personal fees from Takeda Pharmaceutical Co., grants and personal fees from Sanofi S.A, personal fees from CSL Behring Co., grants and personal fees from KM Biologics Co., grants and personal fees from Novo Nordisk A/S, grants and personal fees from Bayer AG, grants and personal fees from Bioverativ Inc., and grants and personal fees from Shire Plc, personal fees from Fujimoto Seiyaku and is an inventor of the patents relating to emicizumab.

Data Availability Statement;

The full data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

References

1. Srivastava A, Brewer AK, Mauser-Bunschoten EP, et al. Guidelines for the management of hemophilia. *Haemophilia.* 2013;19(1):e1-47.
2. Blanchette VS, Manco-Johnson MJ. Meeting unmet needs in inhibitor patients. *Haemophilia.* 2010;16 Suppl 3:46-51.
3. Kempton CL, Meeks SM. Toward optimal therapy for inhibitors in hemophilia. *Blood.* 2014;124(23):3365-3372.
4. Ljung R. Aspects of prophylactic treatment of hemophilia. *Thromb J.* 2016;14(Suppl 1):30.
5. Hedner U, Lee CA. First 20 years with recombinant FVIIa (NovoSeven). *Haemophilia.* 2011;17(1):e172-182.
6. Collins PW, Chalmers E, Hart DP, et al. Diagnosis and treatment of factor VIII and IX inhibitors in congenital haemophilia: (4th edition). UK Haemophilia Centre Doctors Organization. *Br J Haematol.* 2013;160(2):153-170.
7. Nakajima Y, Mizumachi K, Shimonishi N, et al. Comparisons of global coagulation potential and bleeding episodes in emicizumab-treated hemophilia A patients and mild hemophilia A patients. *Int J Hematol.* 2022;115(4):489-498.
8. Barg AA, Livnat T, Budnik I, et al. Emicizumab treatment and monitoring in a paediatric cohort: real-world data. *Br J Haematol.* 2020;191(2):282-290.
9. Peyvandi F, Mahlangu JN, Pipe SW, et al. Application of a hemophilia mortality framework to the Emicizumab Global Safety Database. *J Thromb Haemost.* 2021;19 Suppl 1(Suppl 1):32-41.
10. Berntorp E. Differential response to bypassing agents complicates treatment in patients with haemophilia and inhibitors. *Haemophilia.* 2009;15(1):3-10.
11. Fosbury E, Drebes A, Riddell A, Chowdary P. Review of recombinant anti-haemophilic porcine sequence factor VIII in adults with acquired haemophilia A. *Ther Adv Hematol.* 2017;8(9):263-272.
12. Tarantino MD, Cuker A, Hardesty B, Roberts JC, Sholzberg M. Recombinant porcine sequence factor VIII (rpFVIII) for acquired haemophilia A: practical clinical experience of its use in seven patients. *Haemophilia.* 2017;23(1):25-32.
13. Mosesson MW, Fass DN, Lollar P, et al. Structural model of porcine factor VIII and factor VIIIa molecules based on scanning transmission electron microscope (STEM) images and STEM mass analysis. *J Clin Invest.* 1990;85(6):1983-1990.
14. Kernoff PB, Thomas ND, Lilley PA, Matthews KB, Goldman E, Tuddenham EG. Clinical experience with polyelectrolyte-fractionated porcine factor VIII concentrate in the treatment of hemophiliacs with antibodies to factor VIII. *Blood.* 1984;63(1):31-41.
15. Gatti L, Mannucci PM. Use of porcine factor VIII in the management of seventeen patients with factor VIII antibodies. *Thromb Haemost.* 1984;51(3):379-384.
16. Brettler DB, Forsberg AD, Levine PH, et al. The use of porcine factor VIII concentrate (Hyate:C) in the treatment of patients with inhibitor antibodies to factor VIII. A

multicenter US experience. *Arch Intern Med.* 1989;149(6):1381-1385.

17. Morrison AE, Ludlam CA, Kessler C. Use of porcine factor VIII in the treatment of patients with acquired hemophilia. *Blood.* 1993;81(6):1513-1520.
18. Lozier JN, Santagostino E, Kasper CK, Teitel JM, Hay CR. Use of porcine factor VIII for surgical procedures in hemophilia A patients with inhibitors. *Semin Hematol.* 1993;30(2 Suppl 1):10-21.
19. Ciavarella N, Antoncecchi S, Ranieri P. Efficacy of porcine factor VIII in the management of haemophiliacs with inhibitors. *Br J Haematol.* 1984;58(4):641-648.
20. Kempton CL, Abshire TC, Deveras RA, et al. Pharmacokinetics and safety of OBI-1, a recombinant B domain-deleted porcine factor VIII, in subjects with haemophilia A. *Haemophilia.* 2012;18(5):798-804.
21. Mahlangu JN, Andreeva TA, Macfarlane DE, Walsh C, Key NS. Recombinant B-domain-deleted porcine sequence factor VIII (r-pFVIII) for the treatment of bleeding in patients with congenital haemophilia A and inhibitors. *Haemophilia.* 2017;23(1):33-41.
22. Fulcher CA, de Graaf Mahoney S, Roberts JR, Kasper CK, Zimmerman TS. Localization of human factor FVIII inhibitor epitopes to two polypeptide fragments. *Proc Natl Acad Sci U S A.* 1985;82(22):7728-7732.
23. Scandella D, DeGraaf Mahoney S, Mattingly M, Roeder D, Timmons L, Fulcher CA. Epitope mapping of human factor VIII inhibitor antibodies by deletion analysis of factor VIII fragments expressed in Escherichia coli. *Proc Natl Acad Sci U S A.* 1988;85(16):6152-6156.
24. Scandella D, Mattingly M, de Graaf S, Fulcher CA. Localization of epitopes for human factor VIII inhibitor antibodies by immunoblotting and antibody neutralization. *Blood.* 1989;74(5):1618-1626.
25. Scandella D, Timmons L, Mattingly M, Trabold N, Hoyer LW. A soluble recombinant factor VIII fragment containing the A2 domain binds to some human anti-factor VIII antibodies that are not detected by immunoblotting. *Thromb Haemost.* 1992;67(6):665-671.
26. Scandella D, Mattingly M, Prescott R. A recombinant factor VIII A2 domain polypeptide quantitatively neutralizes human inhibitor antibodies that bind to A2. *Blood.* 1993;82(6):1767-1775.
27. Prescott R, Nakai H, Saenko EL, et al. The inhibitor antibody response is more complex in hemophilia a patients than in most nonhemophiliacs with factor VIII autoantibodies *Blood.* 1997;89(10):3663-3671.
28. Barrow RT, Healey JF, Gailani D, Scandella D, Lollar P. Reduction of the antigenicity of factor VIII toward complex inhibitory antibody plasmas using multiply-substituted hybrid human/porcine factor VIII molecules. *Blood.* 2000;95(2):564-568.
29. Takeshima K, Smith C, Tait J, Fujikawa K. The preparation and phospholipid binding property of the C2 domain of human factor VIII. *Thromb Haemost.* 2003;89(5):788-794.
30. Mimms LT, Zampighi G, Nozaki Y, Tanford C, Reynolds JA. Phospholipid vesicle

formation and transmembrane protein incorporation using octyl glucoside. *Biochemistry.* 1981;20(4):833-840.

31. Nogami K, Soeda T, Matsumoto T, Kawabe Y, Kitazawa T, Shima M. Routine measurements of factor VIII activity and inhibitor titer in the presence of emicizumab utilizing anti-idiotype monoclonal antibodies. *J Thromb Haemost.* 2018;16(7):1383-1390.
32. Nogami K, Zhou Q, Wakabayashi H, Fay PJ. Thrombin-catalyzed activation of factor VIII with His substituted for Arg372 at the P1 site. *Blood.* 2005;105(11):4362-4368.
33. Lollar P, Fay PJ, Fass DN. Factor VIII and factor VIIIa. *Methods Enzymol.* 1993;222:128-143.
34. Hemker HC, Giesen P, Al Dieri R, et al. Calibrated automated thrombin generation measurement in clotting plasma. *Pathophysiol Haemost Thromb.* 2003;33(1):4-15.
35. Nogami K. The utility of thromboelastography in inherited and acquired bleeding disorders. *Br J Haematol.* 2016;174(4):503-514.
36. Healey JF, Lubin IM, Nakai H, et al. Residues 484-508 contain a major determinant of the inhibitory epitope in the A2 domain of human factor VIII. *J Biol Chem.* 1995;270(24):14505-14509.
37. Healey JF, Parker ET, Barrow RT, Langley TJ, Church WR, Lollar P. The comparative immunogenicity of human and porcine factor VIII in haemophilia A mice. *Thromb Haemost.* 2009;102(1):35-41.
38. Oldenburg J, Mahlangu JN, Kim B, et al. Emicizumab Prophylaxis in Hemophilia A with Inhibitors. *N Engl J Med.* 2017;377(9):809-818.
39. Young G, Liesner R, Chang T, et al. A multicenter, open-label phase 3 study of emicizumab prophylaxis in children with hemophilia A with inhibitors. *Blood.* 2019;134(24):2127-2138.
40. Hay C, Lozier JN. Porcine factor VIII therapy in patients with factor VIII inhibitors. *Adv Exp Med Biol.* 1995;386:143-151.

Figure Legends

Figure 1. Structure of human/porcine hybrid mutants

The white boxes illustrate the domains of human (h)FVIII and the gray boxes show alternative sequences in porcine (p)FVIII. Chimeric FVIII is illustrated with human A2, C2, or both A2 and C2 domains recombined with respective porcine sequences.

hp(A2); hFVIII A2 domain hybridized with the corresponding sequence of pFVIII: hp(C2); hFVIII C2 domain hybridized with the corresponding sequence of pFVIII: hp(A2/C2); hFVIII A2 and C2 domains hybridized with the corresponding sequences of pFVIII.

Figure 2. The ability of human-porcine hybrid mutants to escape from the inhibitory activity of commercially available anti-FVIII mAbs

hFVIII, hp(A2), hp(C2), or hp(A2/C2) was added to FVIII-deficient plasma and incubated together with commercial anti-A2 inhibitor mAbs or anti-C2 inhibitor mAbs prior to measuring FVIII:C at the indicated times in one-stage clotting assays as described in Methods. *Panels* (**A** and **B**) show the data in the presence of anti-A2 inhibitor mAbs (2.7 and 3.3 BU/mL, respectively). *Panels* (**C** and **D**) show the data in the presence of anti-C2 inhibitor mAbs (4.1 and 8.2 BU/mL, respectively). The initial FVIII:C of hFVIII (*closed circles*), hp(A2) (*closed squares*), hp(C2) (*open triangles*) and hp(A2/C2) (*open circles*) were 51.6, 48.6, 61.5, and 35.6 IU/dL at time zero, respectively. The horizontal axes show the time (min) and vertical axes show the relative changes in initial FVIII:C. All experiments were performed in duplicate on two separate occasions and the average results were calculated. The error bars indicate standard deviation.

hFVIII; B domain deleted human FVIII, hp(A2); hybrid FVIII with hFVIII A2 domain recombined with the corresponding sequence of pFVIII, hp(C2); hybrid FVIII with hFVIII

C2 domain recombined with the corresponding sequence of pFVIII, hp(A2/C2); hybrid FVIII with hFVIII A2 and C2 domains substituted with the corresponding sequences of pFVIII.

Figure 3. The ability of human-porcine hybrid mutants to escape the inhibitory activity of purified inhibitor IgG obtained from PwHA-I

hFVIII, hp(A2), hp(C2) or hp(A2/C2) was added to FVIII-deficient plasma together with purified anti-A2 inhibitor or anti-C2 inhibitor IgG obtained from PwHA-I prior to measuring FVIII:C at the indicated times in one-stage clotting assays as described Material and Methods. *Panel* **(A)** show the data in the presence of anti-A1/A2/A3C1 inhibitor IgG (4.8 BU/mL). *Panel* **(B)** show the data in the presence of anti-A2 inhibitor IgG (6.2 BU/mL). *Panels* (**C** and **D**) show the data in the presence of anti-A2/C2 inhibitor IgG (5.8 and 3.4 BU/mL, respectively). The initial FVIII:C of hFVIII (*closed circles*), hp(A2) (*closed squares*), hp(C2) (*open triangles*), and hp(A2/C2) (*open circles*) were 98.0, 56.4, 73.8, and 32.0 IU/dL at time zero, respectively. The horizontal axes show the time (min) and vertical axes show the relative changes in initial FVIII:C. All experiments were performed in duplicate on two separate occasions and the average results were calculated. The error bars indicate standard deviation.

hFVIII; B domain deleted human FVIII, hp(A2); hybrid FVIII with hFVIII A2 domain recombined with the corresponding sequence of pFVIII, hp(C2); hybrid FVIII with hFVIII C2 domain recombined with the corresponding sequence of pFVIII, hp(A2/C2); hybrid FVIII with hFVIII A2 and C2 domains substituted with the corresponding sequences of pFVIII.

Figure 4. Coagulation potentials of human-porcine hybrid mutants in FVIII-deficient plasma mixed with purified inhibitor IgG obtained from PwHA-I

hFVIII, hp(A2), hp(C2), or hp(A2/C2) (2 nM) was added to FVIII-deficient plasma with purified anti-A2 inhibitor or anti-A2/C2 inhibitor IgG obtained from PwHA-I as described above. Global coagulation potential was determined by thrombin generation assays as described in methods. *Panels* (**A-a** and **B-a; *left***) show representative data in the absence of inhibitor. *Panels* (**A-b** and **B-b; *right***) show representative data in the presence of anti-A1/A2/A3C1 inhibitor or anti-A2/C2 inhibitor (4.1 and 2.8 BU/mL, respectively). Experiments were performed three separate times, and the mean values are shown. Temporal thrombin generation curves are shown.

black; FVIII-deficient plasma; *green*; hFVIII; *red*; hp(A2); *light blue*; hp(C2); *purple*; hp(A2/C2).

hFVIII; B domain deleted human FVIII, hp(A2); hybrid FVIII with hFVIII A2 domain recombined with the corresponding sequence of pFVIII, hp(C2); hybrid FVIII with hFVIII C2 domain recombined with the corresponding sequence of pFVIII, hp(A2/C2); hybrid FVIII with hFVIII A2 and C2 domains substituted with the corresponding sequences of pFVIII.

Figure 5. Coagulation potentials of human-porcine hybrid mutants in plasmas obtained from PwHA with anti-A2 inhibitor

Anti-emicizumab antibody was added to the patient's plasma to neutralize the potentially effects of emicizumab treatment. hFVIII, hp(A2), hp(C2), or hp(A2/C2) (2 nM) was added to the anti-A2 inhibitor plasma (3.5 BU/mL), and global coagulation potential was assessed by TGA as described in *Method*. Temporal thrombin generation curves are

shown.

black; FVIII-deficient plasma; *green*; hFVIII; *red*; hp(A2); *light blue*; hp(C2); *purple*; hp(A2/C2). hFVIII; B domain deleted human FVIII, hp(A2); hybrid FVIII with hFVIII A2 domain recombined with the corresponding sequence of pFVIII, hp(C2); hybrid FVIII with hFVIII C2 domain recombined with the corresponding sequence of pFVIII, hp(A2/C2); hybrid FVIII with hFVIII A2 and C2 domains substituted with the corresponding sequences of pFVIII.

Figure 6. Coagulation potentials of human-porcine hybrid mutants in whole blood samples obtained from PwHA with anti-A2 inhibitor

Anti-emicizumab antibody was added to the patient's whole blood to neutralize the potentially effects of emicizumab treatment. hFVIII, hp(A2), hp(C2), or hp(A2/C2) was added to the anti-A2 inhibitor plasma (5.0 BU/mL) and potential assessed by $CaCl_2$-triggered ROTEM as described in methods. Represent thromboelastograms are shown.

ROTEM; Rotation thromboelastometry, hFVIII; B domain deleted human FVIII, hp(A2); hybrid FVIII with hFVIII A2 domain recombined with the corresponding sequence of pFVIII, hp(C2); hybrid FVIII with hFVIII C2 domain recombined with the corresponding sequence of pFVIII, hp(A2/C2); hybrid FVIII with hFVIII A2 and C2 domains substituted with the corresponding sequences of pFVIII.

Figure 7. Coagulation potentials of human-porcine hybrid mutants in PwHA whole blood samples with anti-A2/C2 inhibitor added *in vitro*

Purified anti-A2/C2 inhibitor (4.7 BU/mL) was added whole blood collected from a severe PwHA without inhibitor to simulate severe PwHA with anti-A2/C2 inhibitor. hFVIII,

hp(A2), hp(C2) or hp(A2/C2) (1 nM) was added to the simulated whole blood samples and global coagulation potential assessed by $CaCl_2$-triggered ROTEM as above. Each box plot represents the interquartile range (IQR) with median values (*horizontal line*) and mean values (*cross mark*) in CT. Comparison between hFVIII and no FVIII or each hpFVIII was performed by the Student's t-test, followed by comparison between each mutant in *inlet*. Significant differences between the groups were considered as $p < 0.05$.

ROTEM; Rotation thromboelastometry, CT; clot time: CFT; clot formation time, hFVIII; B domain deleted human FVIII, hp(A2); hybrid FVIII with hFVIII A2 domain recombined with the corresponding sequence of pFVIII, hp(C2); hybrid FVIII with hFVIII C2 domain recombined with the corresponding sequence of pFVIII, hp(A2/C2); hybrid FVIII with hFVIII A2 and C2 domains substituted with the corresponding sequences of pFVIII.

Table 1. Comparisons of TGA parameters in the absence and presence of inhibitor IgG prepared from PwHA-I

The peak thrombin and ETP (endogenous thrombin potential) in TGA with purified patient's inhibitor IgG (Figure 4) are shown. The inhibitor epitope recognized the A1/A2/A3-C1 domain (**A**) or A2/C2 domain (**B**). The parameters are represented as median ± standard deviation. The experiments are carried out on two separate occasions in duplicate. Comparison between human FVIII and each hybrid FVIII was performed by the Student's t-test. P values of < 0.05 were considered to be statistically significant.

(A) Anti-A1/A2/A3-C1 inhibitor IgG

FVIII protein	Inhibitor absent		Inhibitor present		Present/Absent ratio	
	Peak thrombin	ETP	Peak thrombin	ETP	Peak thrombin	ETP
no FVIII	30.1 ± 1.3	571.8 ± 21.9	27.3 ± 1.9	521.3 ± 43.8	0.94 ± 0.07	0.92 ± 0.01
hFVIII	89.6 ± 2.9	1,313.6 ± 142.6	30.0 ± 1.3	577.3 ± 35.9	0.33 ± 0.01	0.43 ± 0.03
hp(A2)	72.2 ± 1.4	1,111.8 ± 34.9	67.4 ± 8.1	1,205.8 ± 181.6	0.81 ± 0.11**	1.08 ± 0.13**
hp(C2)	111.1 ± 9.5	1,639.8 ± 111.1	43.0 ± 15.9	851.9 ± 354.2	0.39 ± 0.18	0.50 ± 0.24
hp(A2/C2)	78.7 ± 6.0	1,156.0 ± 43.1	74.8 ± 28.9	1605.1 ± 585.8	0.95 ± 0.32*	1.39 ± 0.48*

* p <0.05, ** p <0.01

(B) Anti-A2/C2 inhibitor IgG

FVIII protein	Inhibitor absent		Inhibitor present		Present/Absent ratio	
	Peak thrombin	ETP	Peak thrombin	ETP	Peak thrombin	ETP
no FVIII	25.2 ± 2.0	470.7 ± 30.9	26.6 ± 3.8	514.1 ± 73.8	1.00 ± 0.17	1.07 ± 0.18
hFVIII	87.1 ± 11.6	1,303.9 ± 156.8	49.4 ± 5.1	910.4 ± 115.6	0.52 ± 0.07	0.64 ± 0.10
hp(A2)	67.5 ± 9.4	1,048.9 ± 110.3	49.1 ± 1.6	838.7 ± 26.9	0.71 ± 0.08*	0.81 ± 0.07
hp(C2)	117.7 ± 7.3	1,692.4 ± 62.5	69.8 ± 7.6	1,116.7 ± 140.6	0.56 ± 0.07	0.56 ± 0.07
hp(A2/C2)	80.5 ± 4.7	1,190.7 ± 72.9	73.9 ± 14.2	1,158.0 ± 153.4	0.92 ± 0.12**	0.98 ± 0.07**

* $p < 0.05$, ** $p < 0.01$

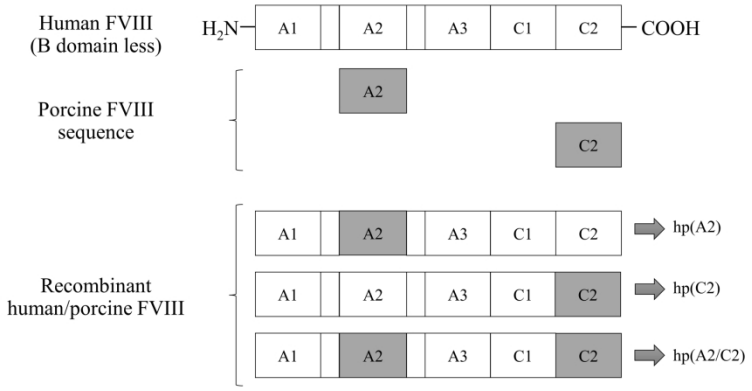


Figure 1

Figure 1

254x190mm (300 x 300 DPI)

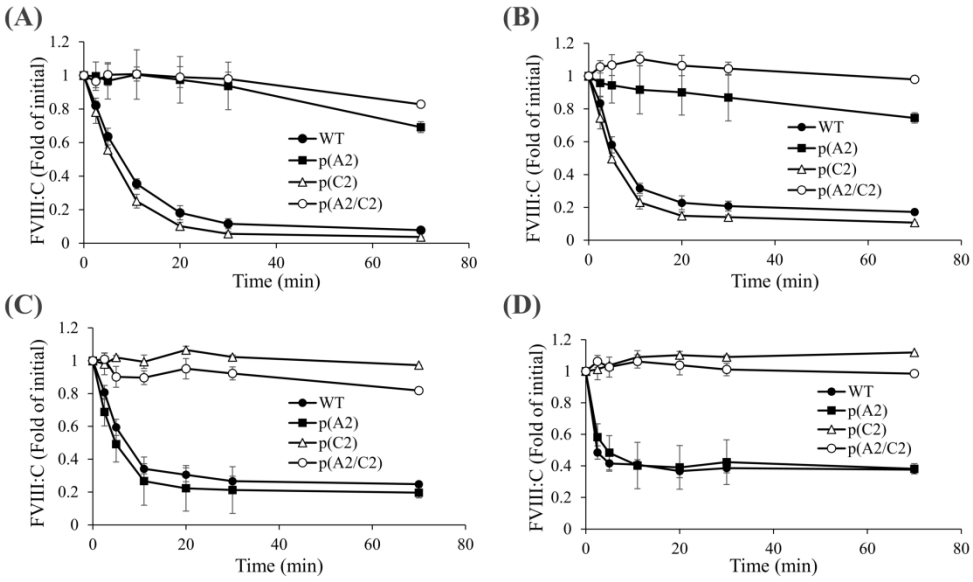


Figure 2

Figure 2

254x190mm (300 x 300 DPI)

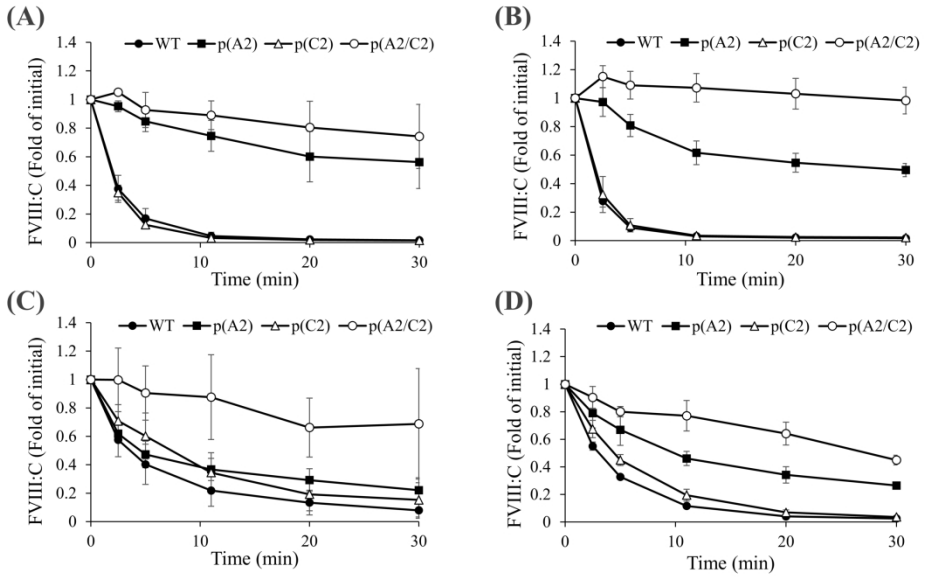


Figure 3

Figure 3

254x190mm (300 x 300 DPI)

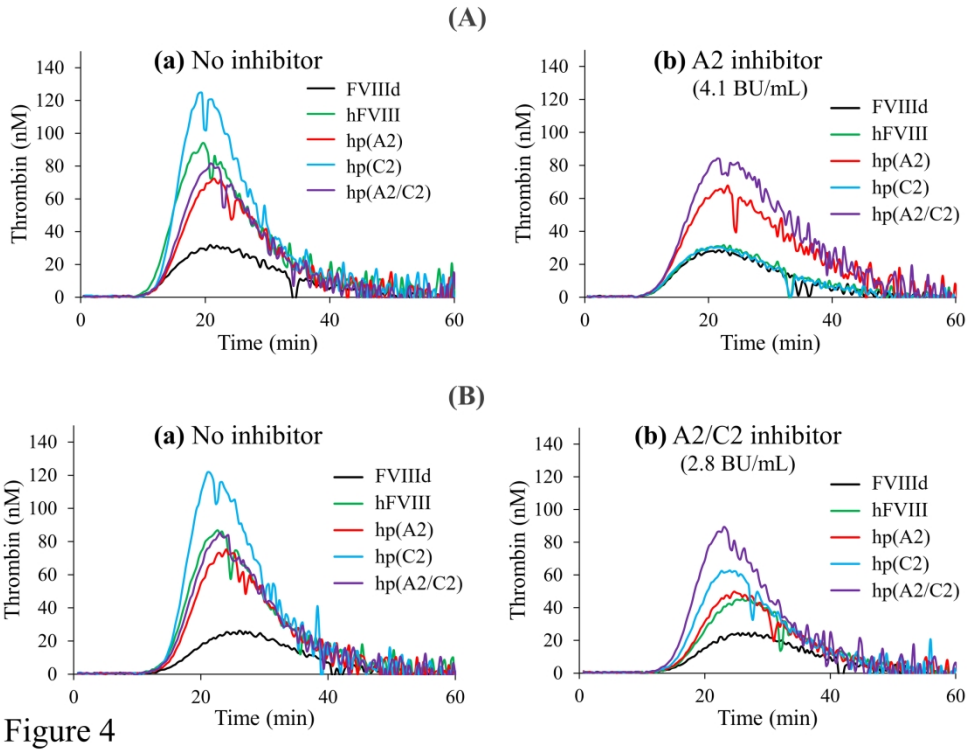


Figure 4

Figure 4

254x190mm (300 x 300 DPI)

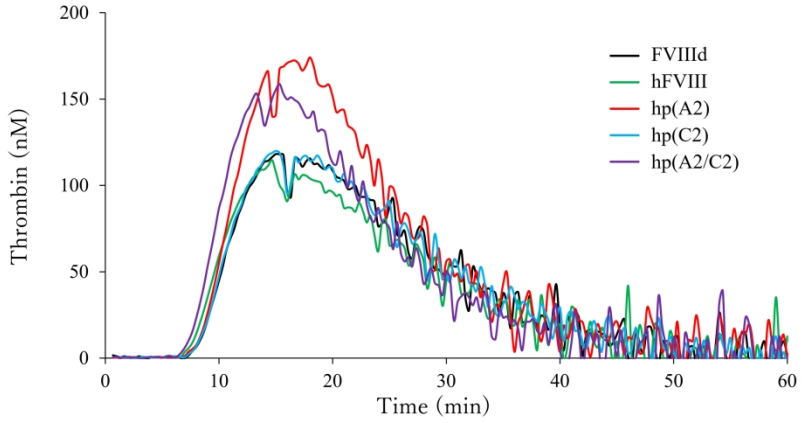


Figure 5

Figure 5

254x190mm (300 x 300 DPI)

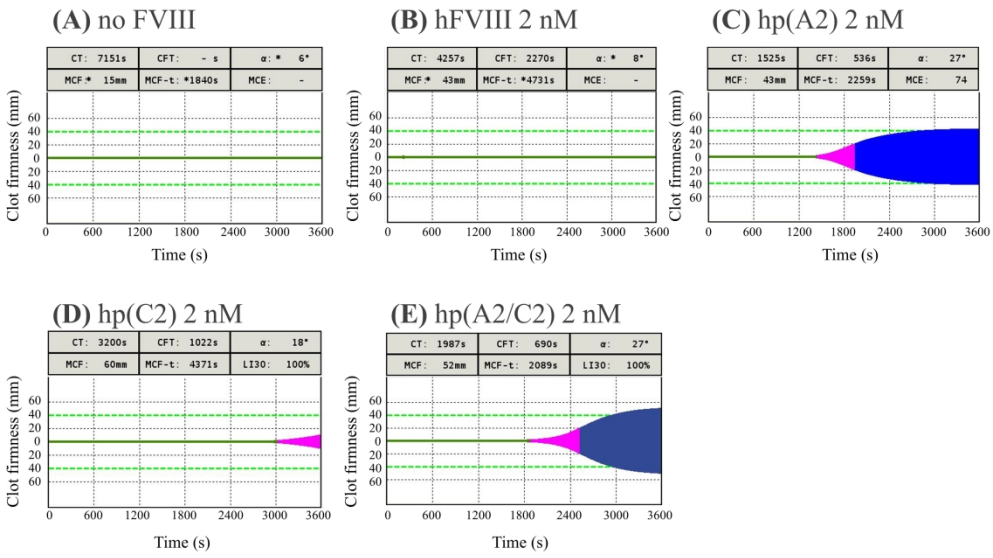


Figure 6

Figure 6

254x190mm (300 x 300 DPI)

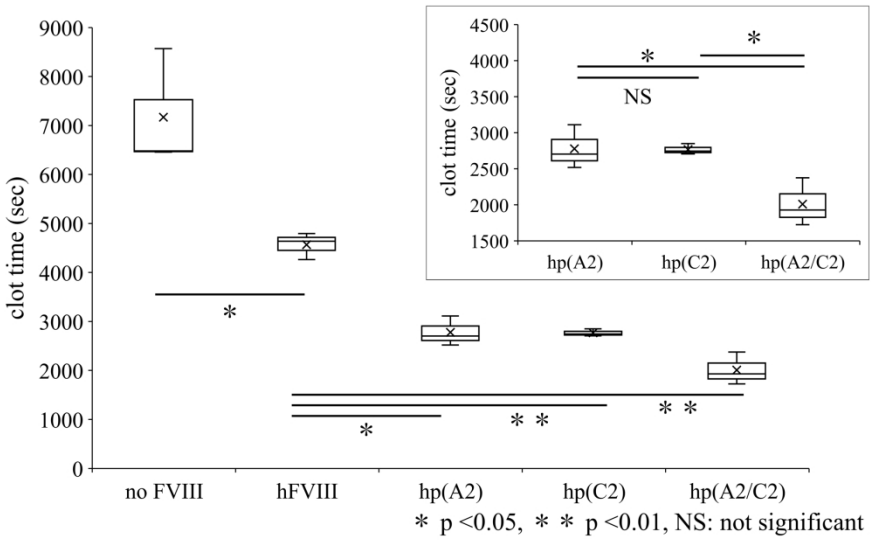
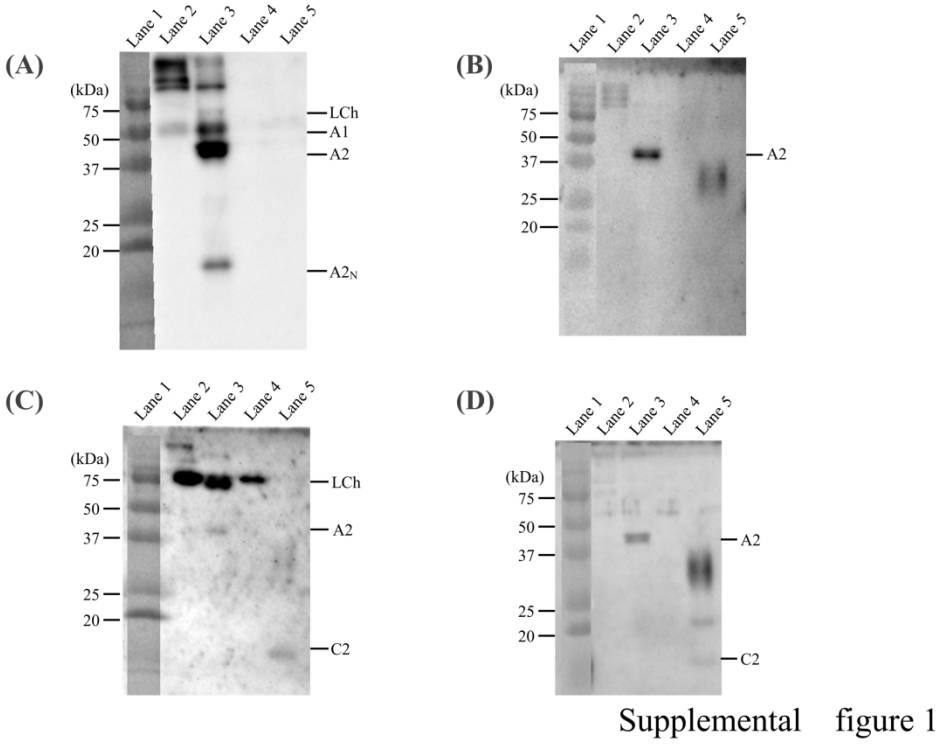


Figure 7

Figure 7

254x190mm (300 x 300 DPI)



Supplemental figure 1

254x190mm (300 x 300 DPI)

Supplemental data

Supplemental Table 1. Specific activities of human-porcine hybrid FVIII mutants

The characteristics of each mutant were determined utilizing OSA and CSA as described in Material and Methods. Specific activity was calculated as average ± standard deviation of the ratio to hFVIII in separate experiments at least three times.

FVIII protein	FVIII:C -OSA (ratio to hFVIII)	FVIII:C -CSA (ratio to hFVIII)
hFVIII	1.00	1.00
hp(A2)	0.76 ± 0.08	0.76 ± 0.10
hp(C2)	0.89 ± 0.06	0.71 ± 0.11
hp(A2/C2)	0.62 ± 0.03	0.70 ± 0.12

hFVIII; B domain deleted human FVIII: hp(A2); human-porcine hybrid FVIII substituted hFVIII A2 domain for corresponding sequence of pFVIII: hp(C2); human-porcine hybrid FVIII substituted hFVIII C2 domain for corresponding sequence of pFVIII: hp(A2/C2); human-porcine hybrid FVIII substituted hFVIII A2 and C2 domains for corresponding sequence of pFVIII, FVIII:C; FVIII activity: OSA; one-stage assay: CSA; chromogenic substrate assay

Supplemental Figure 1. Inhibitor epitope analysis of patients' plasma by western blotting

Analyses of recombinant native hFVIII (*Lane* 2), thrombin-cleaved hFVIII (*lane* 3), purified isolated light chain (LCh *Lane* 4) and recombinant C2 domain (*Lane* 5) are illustrated. Molecular weight marker was applied to *Lane 1.* SDS-PAGE was performed using 13% gels at 150 V for 1 hour, and the separated proteins were transferred to a polyvinylidene difluoride membrane at 100 V for 1 hour for Western blotting. After blocking, the membrane was pre-incubated with individual patient's plasma as primary antibody, and washed with phosphate buffered saline buffer. Binding to hFVIII and respective fragments were detected by further incubation with anti-human peroxidase-linked secondary antibody. The marker signals were integrated with blot signals utilizing dedicated software after filming. Patients (**A-D**) correspond to those illustrated in Figure 3. The obscure smear in *Lane* 5 (between 37-25 kDa) was considered to be due to a non-specific reaction.

SDS-PAGE; sodium dodecyl sulfate polyacrylamide gel electrophoresis, hFVIII; B domain deleted human FVIII.