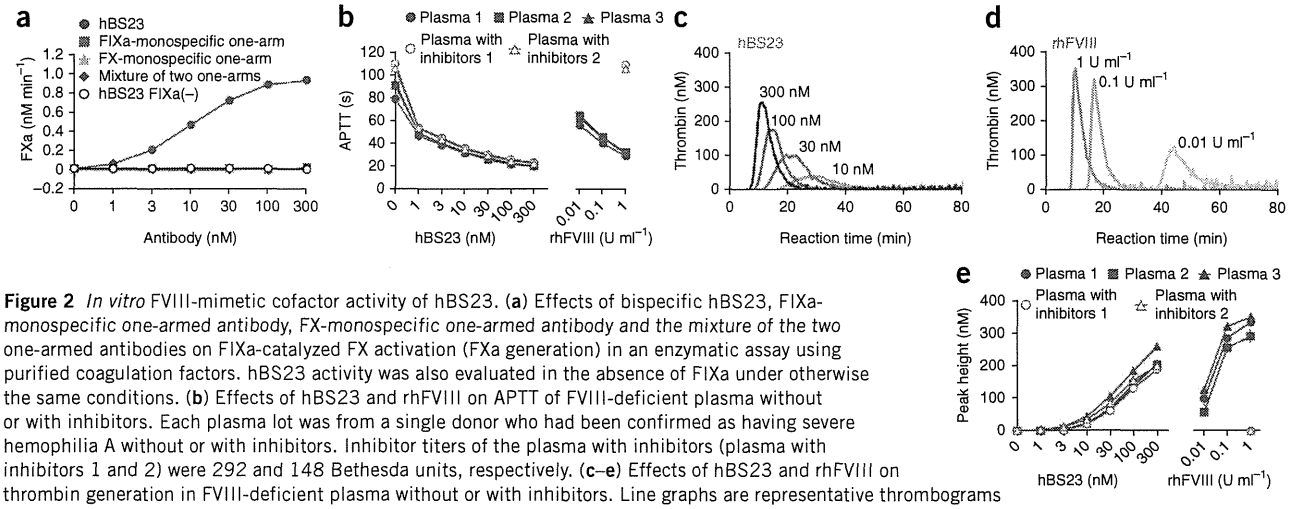

Figure 2 *In vitro* FVIII-mimetic cofactor activity of hBS23. (**a**) Effects of bispecific hBS23, FIXa-monospecific one-armed antibody, FX-monospecific one-armed antibody and the mixture of the two one-armed antibodies on FIXa-catalyzed FX activation (FXa generation) in an enzymatic assay using purified coagulation factors. hBS23 activity was also evaluated in the absence of FIXa under otherwise the same conditions. (**b**) Effects of hBS23 and rhFVIII on APTT of FVIII-deficient plasma without or with inhibitors. Each plasma lot was from a single donor who had been confirmed as having severe hemophilia A without or with inhibitors. Inhibitor titers of the plasma with inhibitors (plasma with inhibitors 1 and 2) were 292 and 148 Bethesda units, respectively. (**c**–**e**) Effects of hBS23 and rhFVIII on thrombin generation in FVIII-deficient plasma without or with inhibitors. Line graphs are representative thrombograms obtained from FVIII-deficient plasma without inhibitors (plasma 3) in the presence of hBS23 (**c**) or rhFVIII (**d**), indicating the time-course of changes in free thrombin concentration during the reaction triggered by factor XIa, phospholipid and Ca^{2+}. Effects of varying concentrations of hBS23 and rhFVIII on a thrombin generation parameter, peak height, is shown for each plasma lot (**e**). Data were collected in triplicate for each plasma lot and expressed as the means ± s.d., except for the representative thrombograms (in many cases, the bars depicting s.d. are shorter than the height of the symbols).

maintain an rpoFVIII activity of 0.01 U ml^{-1} (1%) or above, according to a simulation of multiple dose injections of rpoFVIII using pharmacokinetic parameters obtained from a single-dose injection study of rpoFVIII in cynomolgus monkeys (A.M., unpublished data). The mean plasma concentration of hBS23 was 5.8 μg ml^{-1} (40 nM) immediately after hBS23 administration and 2.5 μg ml^{-1} (18 nM) at day 3 (the end of the observation period). Neither prothrombin time prolongation nor a decrease in platelet numbers was observed in hBS23-treated monkeys (data not shown), suggesting that hBS23 did not induce consumptive coagulopathy.

We performed a pharmacokinetic study of hBS23 in cynomolgus monkeys by bolus intravenous or subcutaneous injection at a dose of 0.3 mg per kg body weight (**Fig. 3d**). After intravenous injection, hBS23 was cleared from plasma in a biphasic manner (a distribution phase followed by an elimination phase) with a long half-life of 14 d, which is consistent with the general pharmacokinetics of IgG antibodies[27]. The subcutaneous bioavailability of hBS23 was 84%, denoting high subcutaneous bioavailability.

Our data show that a bispecific antibody to FIXa and FX can restore FVIII hemostatic activity by recruiting the two factors into appropriate proximity. We demonstrated that simultaneous recognition of FIXa and FX by a single antibody molecule was required to bring about their activity, as neither a FIXa- or FX-monospecific one-armed antibody, nor a mixture of them, could elicit detectable cofactor activity. Such activity was not detected in the absence of FIXa or phospholipid, indicating that hBS23 exerts its FVIII-mimetic activity only on a tertiary complex consisting of FIXa, FX and phospholipid. Kinetic analysis showed that hBS23 and FVIIIa improved k_{cat} and K_m quite differently: bispecific binding of FIXa and FX by hBS23 improves the interaction between these factors more efficiently than does binding by FVIIIa, but hBS23 has less potential to increase the turnover of the enzyme complex than does FVIIIa. hBS23 was calculated to have 1/14th the catalytic efficiency (k_{cat}/K_m) of FVIIIa. When designing the *in vivo* study, we considered that 0.3 mg per kg body weight of hBS23 would be expected to achieve a plasma concentration that could generate thrombin at a level equivalent to that of 0.01 U ml^{-1} (1%) FVIII *in vitro*. Accordingly, 0.3 mg per kg body weight of hBS23 showed an *in vivo* efficacy comparable to that achieved in a regimen designed to maintain 0.01 U ml^{-1} (1%) or above of FVIII activity, a level sufficient to control hemostasis in patients[3,8]. As the concentration-dependent activity of hBS23 in the TGA in FVIII-neutralized cynomolgus monkey plasma was comparable to that in human FVIII-deficient plasma (**Fig. 2e** and **Supplementary Figs. 3** and **4b,c**), we expect that this *in vivo* efficacy can be translated to the human clinical setting. Although we induced artificial bleeding in this study, the nonhuman primate model that we established has various clinical features of acquired hemophilia, including overt hemorrhage, anemia, bruising and muscle bleeds[30], supporting the extrapolation of this animal study into the clinical setting.

Single-dose pharmacokinetic analysis of hBS23 in cynomolgus monkeys revealed that its half-life (14 d) was slightly longer than that of other humanized or fully human IgG antibodies[31,32]. In addition, whereas the low subcutaneous bioavailability of FVIII necessitates venous access for its injection, hBS23 has high subcutaneous bioavailability (84%) in cynomolgus monkeys. Furthermore, we conducted a simulation study of multiple-dose injections of hBS23 in cynomolgus monkeys using the pharmacokinetic parameters that had been obtained from the pharmacokinetic study. This simulation indicated that once weekly subcutaneous administration of 1 mg per kg body weight of hBS23 would result in a plasma concentration that at any time point would greatly exceed 40 nM (K. Haraya and T. Tachibana, unpublished data), which is the initial plasma concentration in the *in vivo* hemostatic

Table 1 Effects of hBS23 on enzymatic kinetics of FIXa-catalyzed FX activation

Condition	K_m (μM)	V_{max} (nM min^{-1})	k_{cat} (min^{-1})	k_{cat}/K_m	Fold increase in k_{cat}/K_m
FIXa	0.0558 ± 0.00281	0.0442 ± 0.00332	0.00111	0.0199	—
+hBS23	0.00253 ± 0.000113	0.996 ± 0.0870	0.996	394	19,800
+FVIIIa	0.0249 ± 0.00247	135 ± 6.08	135	5,420	272,000

The K_m and the maximum velocity (V_{max}) are expressed as the means ± s.d. of three separate experiments. The k_{cat} and the k_{cat}/K_m are calculated from the mean values of K_m and V_{max}. The fold increase in the k_{cat}/K_m by hBS23 or FVIIIa compared to FIXa alone is also shown.

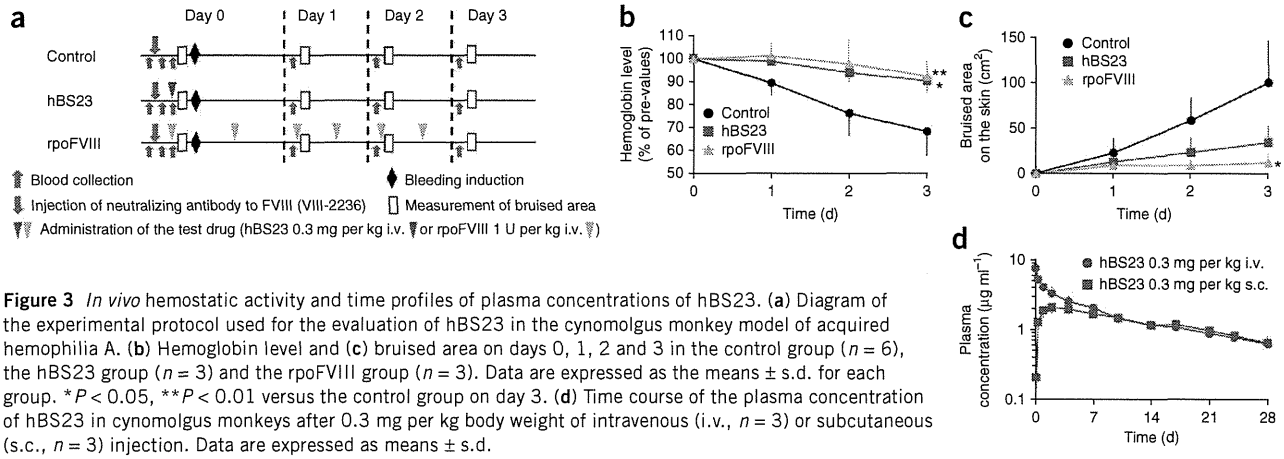

Figure 3 *In vivo* hemostatic activity and time profiles of plasma concentrations of hBS23. (**a**) Diagram of the experimental protocol used for the evaluation of hBS23 in the cynomolgus monkey model of acquired hemophilia A. (**b**) Hemoglobin level and (**c**) bruised area on days 0, 1, 2 and 3 in the control group ($n = 6$), the hBS23 group ($n = 3$) and the rpoFVIII group ($n = 3$). Data are expressed as the means ± s.d. for each group. $^*P < 0.05$, $^{**}P < 0.01$ versus the control group on day 3. (**d**) Time course of the plasma concentration of hBS23 in cynomolgus monkeys after 0.3 mg per kg body weight of intravenous (i.v., $n = 3$) or subcutaneous (s.c., $n = 3$) injection. Data are expressed as means ± s.d.

study. As the half-life of IgG antibodies in humans is generally longer than that in cynomolgus monkeys[33], hBS23 would be expected to have at least a 14-d half-life in humans, which is substantially longer than that of FVIII (0.5 d)[5]. We therefore expect that once weekly dosing of 1 mg per kg body weight of hBS23 would show a continuous hemostatic effect in humans. Effective prophylaxis by once-weekly subcutaneous administration would be an advantageous feature of our bispecific antibody in view of the frequent venous access required for FVIII treatment. Moreover, although maintenance of 1% of normal FVIII activity should be enough for prophylactic use, dosing of hBS23 above 0.3 mg per kg body weight would be expected to exert higher hemostatic activity, as indicated by the dose-dependent activity of hBS23 above 40 nM (the initial plasma concentration of hBS23 after dosing with 0.3 mg per kg body weight in the *in vivo* hemostatic study) in the TGA (at 300 nM, hBS23 had nearly 10% of normal FVIII activity).

Among the alternative therapeutic approaches for hemophilia A that are being pursued, the bispecific antibody described here has some notable advantages. Modified FVIII agents with longer half-lives than the native form of FVIII, including a FVIII Fc fusion protein ($t_{1/2}$ = 18.8 h), are currently being investigated in clinical trials, but although these modified FVIII agents may achieve a reduced injection frequency, they still require venous access[34,35]. Additionally, although gene therapy is in principle a very attractive therapeutic approach that has been actively explored for the past decade, many issues remain to be resolved before it can be used clinically[36].

A notable feature of our bispecific antibody is that it has the potential to overcome the most crucial drawback of treatment using injected FVIII, which would probably also apply to treatment with modified FVIII: the development of inhibitors. Such inhibitors affect not only 30% of the patients with severe disease but also a small percentage of the patients with moderate and mild disease[1,3]. Our experimental results showed that hBS23 activity was not affected by inhibitors. Moreover, considering the generally low immunogenicity of humanized antibodies[26], we expect the incidence of antibodies to hBS23 to be substantially lower than that of antibodies to FVIII. However, as humanized or fully human antibodies are immunogenic in rare cases[26,37], there is still a small risk for the generation of hBS23-specific antibodies in patients. Were hBS23 to become ineffective because of the generation of hBS23-specific antibodies, FVIII treatment could be a viable alternative as long as the hBS23-specific antibodies do not cross-react with FVIII. To estimate the risk of such cross-reactivity, we scanned the sequences of the variable regions of hBS23 to assess sequence similarity to FVIII. This alignment analysis found no similarity between the two molecules (T. Kitazawa, unpublished data); we therefore consider it unlikely that hBS23 treatment would result in the development of hBS23-specific antibodies cross-reactive to FVIII. Thus, we expect that hBS23 treatment would not lead to inhibitor development and could be used in patients who have already developed inhibitors.

In this study, we have generated an agent with the potential to overcome the two major drawbacks of current treatment modalities. Although hBS23 showed efficacy in an animal study, the molecular structure of hBS23 may require further optimization in several ways before the clinical use of such an agent in humans. The first is molecular engineering to enable large-scale manufacturing of the bispecific antibody at clinical grade. Although technological difficulties have prevented any recombinant bispecific antibody from yet reaching the market, various engineering technologies to overcome them have been reported[38,39]. The second is engineering to further reduce the immunogenicity of the humanized antibody. Even though humanized or fully human antibodies generally have low immunogenicity[26], they can be immunogenic if T cell epitopes are present in the complementarity-determining regions[37]. Various approaches to identify and remove such T cell epitopes have been recently reported[26,39]. The third is engineering to prolong the half-life and improve the physicochemical properties of the antibody, which would enable a subcutaneous formulation with an even longer dosing interval[39,40]. The fourth is engineering to further increase the FVIII-mimetic activity of the bispecific antibody. Mutations in the complementarity-determining regions increased the activity of hBS23 over that of the lead chimeric bispecific antibody (data not shown), and the activity of hBS23 could be improved in a same manner. Although hBS23 may require further optimization, this type of bispecific antibody may be able to overcome current limitations and reduce the burden of care for the treatment of hemophilia A.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

T. Kitazawa and T. Igawa led the pharmacological studies and the optimization of the bispecific antibody, respectively, in the program and wrote the manuscript. Z.S. designed the lead chimeric bispecific antibody and hBS23. T. Kojima led the lead identification. H.T. provided ideas on bispecific antibody engineering. T. Suzuki, H.A., T.M., S.I., M.K.-S. and T. Iida generated FVIII-, FIXa- and FX-specific antibodies. T. Soeda, Y.O.-N., A.H., M.F., C.M., E.T., T. Toyoda and A.U. performed the *in vitro* experiments. K.E. and S.S. performed the affinity analyses. Y. Kikuchi, T.W., M.W. and M.G. purified the bispecific antibody and the coagulation factor. A.M. and K.Y. performed the *in vivo* pharmacological study. K. Haraya and T. Tachibana performed the pharmacokinetic study. H.S. and Y. Kawabe provided direction and guidance for the various functional areas. M.S. and A.Y. provided advice on the program from the viewpoints of their medical expertise in hemophilia. K. Hattori provided the hypothesis and directed and organized the program.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

The care and use of laboratory animals. Cynomolgus monkey, mouse, rat and rabbit studies were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals at Chugai Pharmaceutical under the approval of the company's Institutional Animal Care and Use Committee and also in compliance with the "Act on Welfare and Management of Animals" in Japan. The company is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International, a nonprofit organization that promotes the humane treatment of animals in science through voluntary accreditation and assessment programs (http://www.aaalac.org/). We conducted the cynomolgus monkey research adhering to the principles stated in the US National Research Council's Guide for the Care and Use of Laboratory Animals.

Generation of hBS23, a humanized bispecific antibody to FIXa and FX. We prepared the genes of the variable regions of FIXa- and FX-specific antibodies from 46 female mice, 34 female rats and 12 female rabbits that had been immunized with human FIXa or FX (Enzyme Research Laboratories) and inserted the genes into an expression vector containing the constant region of human IgG. The constant region of all the expressed bispecific antibodies had a S238P (EU numbering) substitution, intended to improve hinge stabilization[41], and 'knobs-into-holes' substitutions, intended to increase the desired bispecific combination by promoting heterodimerization efficiency of the two heavy chains[38]. To express a series of bispecific antibodies in HEK 293 cells, we co-transfected a mixture of four expression vectors encoding the heavy and light chains of FIXa- and FX-specific antibodies. After culturing the transfectants, we individually collected and incubated each of the culture supernatants for 2 h with 10 nM FIXa, 10 nM FX and 62.5 μM synthetic phospholipid in 50 mM Tris-buffered saline (TBS) containing 5 mM $CaCl_2$ and 1 mM $MgCl_2$ (pH 7.6). The phospholipid consisted of 10% phosphatidylserine, 60% phosphatidylcholine and 30% phosphatidylethanolamine (Avanti Polar Lipids) and was prepared as previously described[42]. After stopping the reaction by the addition of EDTA, we added a chromogenic substrate specific to FXa (S-2222, Chromogenix) and evaluated the ability of each culture supernatant to promote FXa generation by measuring the absorbance at 405 nm. From approximately 40,000 bispecific combinations, we selected a lead chimeric bispecific antibody based on its ability to generate FXa and the feasibility of using only one light chain, the so-called common light chain. The combination of two heavy chains and two light chains results in ten possible combinations, of which only one yields the desired bispecific antibody; however, use of a common light chain for the two arms of the bispecific antibody reduces the number of combinations of heavy and light chains to three[38]. To generate hBS23, we further humanized the lead chimeric bispecific antibody by complementarity-determining region grafting[43] and engineered it to have stronger FVIII-mimetic activity and better pharmaceutical properties. We transiently expressed the one light chain and two heavy chains of hBS23 in HEK 293 cells and purified it with recombinant Protein A and gel permeation chromatography. We also prepared in the same way one-armed monospecific antibodies[28], each of which have one arm containing either the FIXa- or the FX-specific variable region of hBS23, as well as two-armed monospecific antibodies, in which both arms contain the FIXa- or FX-specific variable region of hBS23.

Characterization of antigen-antibody interaction of hBS23 using surface plasmon resonance. We analyzed the interactions of FIX, FIXa, FX and FXa with the corresponding variable regions of hBS23 by surface plasmon resonance using a Biacore T200 system (GE Healthcare). First, we immobilized MabSelect SuRe Ligand (recombinant Protein A; GE Healthcare) onto a CM4 sensor chip (GE Healthcare) that had been preactivated with NHS/EDC and predeactivated with ethanolamine (Amine Coupling Kit; GE Healthcare). To capture the test antibodies on the sensor chip, we injected each of the FIXa- or the FX-monospecific two-armed IgG antibodies into flow cell 2 and natalizumab (Biogen-Idec) as control human IgG_4 antibody into flow cell 1. We next injected each analyte (0 as baseline, 80, 160, 320, 640, or 1,280 nM human FIX, FIXa, FX, or FXa), which had been dissolved in running buffer (10 mM HEPES, 150 mM NaCl, 0.05% (vol/vol) Surfactant P20, 2.5 mM $CaCl_2$ (pH 7.4); GE Healthcare), into both flow cells on the sensor surface at a flow rate of 30 μl min^{-1} to monitor the association phase for 120 s and the dissociation phase for 30 s. The data were analyzed by the 1:1 binding model in the Biacore T200 Evaluation software (version 1.0, GE Healthcare).

Enzymatic assays. We evaluated the conversion rate of FX to FXa in an enzymatic assay using purified coagulation factors. The assay system consisted of 1 nM human FIXa, 140 nM human FX, 20 μM phospholipid and either hBS23, FIXa- or FX-monospecific one-armed antibodies, or a mixture of two monospecific one-armed antibodies, and measured FXa generation at room temperature for 2 min in TBS containing 5 mM $CaCl_2$ and 0.1% (wt/vol) BSA. We also performed the experiments in the absence of FIXa or phospholipid under otherwise the same conditions. We stopped the reaction by adding EDTA at various time points. After adding S-2222 chromogenic substrate, we measured absorbance at 405 nm to determine the rate of FXa generation and quantified it by extrapolation from a standard curve prepared using known amounts of human FXa (Enzyme Research Laboratories). Data were collected in triplicate.

Kinetic analyses. We monitored the rate of FIXa-catalyzed FX activation by incubating 1 nM human FIXa, various concentrations of human FX and 20 μM phospholipid with either 300 nM hBS23 or 30 U ml^{-1} rhFVIII (Bayer Healthcare) that had been preactivated by thrombin and converted into FVIIIa in TBS containing 5 mM $CaCl_2$ and 0.1% (wt/vol) BSA (pH 7.6) at room temperature. We also performed the experiments in the absence of hBS23 and FVIIIa under the same conditions, except that we altered the FIXa concentration to 40 nM. We stopped the FXa generation reaction by adding EDTA at various time points. The rates of FXa generation were determined in the same way as described in the previous section. The values of K_m and V_{max} were calculated using a nonlinear regression data analysis program (GraphPad PRISM version 5.04).

Preparation of rpoFVIII. We expressed a B domain–deleted form of rpoFVIII in Chinese hamster ovary cells by stable transfection. The vector employed for the transfection was designed on the basis of a previously reported sequence of the *F8* (*Sus scrofa*) gene (GenBank: NM_214167) and the sequence of B domain–deleted human FVIII (refs. 44,45). After diluting the supernatant of rpoFVIII-containing medium with 20 mM Tris-HCl and 5 mM $CaCl_2$ (pH 7.6), we loaded it onto a Q Sepharose Fast Flow column (GE Healthcare) equilibrated with the same buffer. We then eluted rpoFVIII with a linear 0 to 1 M NaCl gradient in the same buffer. We pooled and concentrated the fractions containing rpoFVIII and then subjected them to gel permeation chromatography on a HiLoad 26/60 Superdex200pg column (GE Healthcare) equilibrated in 150 mM NaCl, 10 mM histidine-HCl, 5 mM $CaCl_2$, 0.1% (wt/vol) sucrose and 0.02% (wt/vol) polysorbate 80 (Junsei Chemical) (pH 7.0). We again pooled the fractions containing rpoFVIII and aseptically filtered the mixture. We assessed the FVIII activity (U ml^{-1}) of the purified rpoFVIII in a one-stage coagulation assay, one of the standard assays for determining FVIII activity, using normal human plasma (Siemens Healthcare) as the standard.

Generation of VIII-2236, a neutralizing antibody against FVIII. We established hybridomas producing an antibody against human FVIII from eight female mice that had been immunized with rhFVIII. Using APTT measurements and the TGA, we identified VIII-2236 as a neutralizing antibody against FVIII with cross-reactivity to cynomolgus monkey FVIII but not to porcine FVIII.

APTT measurements. We measured APTT with standard techniques using Thrombocheck APTT-SLA (Sysmex). We employed three lots of commercially available human FVIII-deficient plasma without inhibitors (<1% normal FVIII activity) and two lots with inhibitors (George King Bio-Medical). Each lot contained frozen fresh plasma from a single donor with severe hemophilia A. We also used the pooled citrated plasma of nine male cynomolgus monkeys, which contained 300 μg ml^{-1} VIII-2236 (2.6% by volume for VIII-2236 solution). Data were collected in triplicate.

Thrombin generation assays. We obtained thrombograms by calibrated automated thrombography[46] using a 96-well plate fluorometer (Thermo Fisher

Scientific Instruments) equipped with a 390/460 filter set, a dispenser and analyzing software (Thrombinoscope software version 3.0.0.29, Thrombinoscope BV). Briefly, into each well we dispensed 80 µl of a plasma solution containing hBS23, rhFVIII or rpoFVIII added to the human FVIII-deficient plasma or the FVIII-neutralized pooled cynomolgus monkey plasma (<1% by volume). We then added 20 µl of the triggering solution containing 0.47 nM human factor XIa (Enzyme Research Laboratories) and 20 µM phospholipid but no Ca^{2+}. For calibration, we added 20 µl of Thrombin Calibrator (Thrombinoscope BV) instead of the triggering solution. To initiate the reaction, 20 µl of FluCa reagent prepared from FluCa kit (Thrombinoscope BV) was dispensed by the instrument as programmed. The instrument's software analyzed the thrombograms, peak height and ETP. Data were collected in triplicate.

***In vivo* experiments in the acquired hemophilia A model.** We used 12 male cynomolgus monkeys (2.50–3.60 kg and 3–4 years of age), housed individually, which were divided into three groups, receiving hBS23 (n = 3), rpoFVIII (n = 3) or neither agent (control group; n = 6). The experimental protocol is diagrammed in **Figure 3a**. On day 0, each monkey intravenously received an adjusted dose of VIII-2236 that would result in an APTT twice as long as the baseline APTT for that monkey. The means ± s.d. of the adjusted VIII-2236 dose was 3.6 ± 3.8 mg per kg body weight in the control group, 3.3 ± 2.3 mg per kg body weight in the hBS23 group and 4.5 ± 1.9 mg per kg body weight in the rpoFVIII group. Two hours thereafter, we anesthetized each monkey by isoflurane inhalation and measured the area of purple-colored bruising on the skin of the entire body of monkeys whose pelage had been closely cropped. We next intravenously administered the test drug to each monkey in the hBS23 or the rpoFVIII group at 0.3 mg per kg or 1 U per kg body weight, respectively. We then induced bleeding in the monkeys by performing an injury procedure: 1-cm-deep insertions of an 18-G needle into muscles at 16 sites (four sites in each thigh, two sites in each upper arm and two sites in each forearm). We maintained the anesthesia until the completion of this injury procedure. In our previous experience, more severe injury procedures did not decrease hemoglobin levels in normal monkeys, so we did not expect this procedure to decrease hemoglobin in the absence of FVIII neutralization. On days 1, 2 and 3, we again anesthetized the monkeys and measured the bruised area. After completing the evaluation of the bruised area on day 3, the monkeys were killed humanely. To each of the monkeys in the rpoFVIII group, we administered additional intravenous injections of rpoFVIII (1 U per kg body weight) in the mornings of days 1 and 2 just before bruise measurement and also in the evenings of days 0, 1 and 2 (twice a day, total of six administrations of rpoFVIII). We collected blood samples before and after injecting VIII-2236, after the first administration of the test drug on day 0 and before measuring the bruised area on days 1, 2 and 3 to confirm the neutralizing effect of VIII-2236 against endogenous FVIII and to determine the concentration of hBS23 and the hemoglobin level. We expressed the change of hemoglobin level as a percentage of the value on day 0 (after the injection of VIII-2236) for the respective monkey.

Pharmacokinetic study. We intravenously or subcutaneously administered a single dose of 0.3 mg per kg body weight of hBS23 to male cynomolgus monkeys (2.57–3.10 kg and 2–3 years of age, n = 3 for the intravenous administration group and n = 3 for the subcutaneous administration group). We collected blood samples before the administration and 0.5 h (intravenous administration only), 2 h (subcutaneous administration only), 8 h, 1 d, 2 d, 4 d, 7 d, 10 d, 14 d, 17 d, 21 d, 24 d and 28 d after the administration. Pharmacokinetic parameters were calculated by WinNonlin Professional software (version 4.0.1, Pharsight). The subcutaneous bioavailability was calculated by dividing the AUC_{inf} for the subcutaneous administration by the AUC_{inf} for intravenous administration.

Statistical analyses. Data are presented as means ± s.d. In the *in vivo* experiments employing the acquired hemophilia A model, significant differences in the data of day 3 were determined by the parametric Dunnett's multiple comparison test (two-tailed) compared to the control group (SAS preclinical package version 5.00, SAS Institute Japan).

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