Title: Molecular engineering of emicizumab to improve hemophilia A treatment

Authors: Yuri Teranishi-Ikawa[1]*†, Tetsuhiro Soeda[1]†, Hikaru Koga[1], Kazuki Yamaguchi[1], Kazuki Kato[1], Keiko Esaki[1], Kentaro Asanuma[1], Miho Funaki[1], Mina Ichiki[1], Yuri Ikuta[1], Shunsuke Ito[1], Eri Joyashiki[1], Shun-Ichiro Komatsu[1], Atsushi Muto[1], Kei Nishimura[1], Momoko Okuda[1], Hisakazu Sanada[1], Motohiko Sato[1], Norihito Shibahara[1], Tetsuya Wakabayashi[1], Koji Yamaguchi[1], Akiko Matsusaki[2], Zenjiro Sampei[1], Hirotake Shiraiwa[1], Hiroko Konishi[1], Yoshiki Kawabe[1], Kunihiro Hattori[1], Takehisa Kitazawa[1], and Tomoyuki Igawa[1,2]

Affiliations:

[1]Research Division, Chugai Pharmaceutical Co., Ltd.; 216 Totsuka, Yokohama, Kanagawa 244-8602, Japan.

[2]Translational Research Division, Chugai Pharmaceutical Co., Ltd.; 1-1 Nihonbashi-Muromachi, 2-Chome Chuo-ku, Tokyo 103-8324, Japan.

*Corresponding author. Email: teranishi.yuri36@chugai-pharm.co.jp

†These authors contributed equally to this work.

One Sentence Summary: Molecular improvement of emicizumab

Abstract: Emicizumab, a factor (F)VIIIa-function mimetic therapeutic bispecific antibody (BsAb) to FIXa and FX, has become an indispensable treatment for people with hemophilia A

(PwHA). However, non-clinical studies suggest that the maximum cofactor activity of emicizumab is lower than international standard activity (100 IU/dL of FVIII), leaving room for further improvement. Since not all PwHA experienced zero treated bleeds, increased cofactor activity would be beneficial for such patients. Here, we report NXT007, a BsAb against FIXa and FX developed through further engineering of emicizumab. While emicizumab has a common light chain, advances in antibody engineering enabled us to identify a more potent BsAb with two distinct new light chains, and following extensive mutational optimization of the two emicizumab-derived heavy chains and two light chains, the resulting NXT007 exerted in vitro thrombin generation (TG) activity in hemophilia A plasma corresponding to that at 100 IU/dL of FVIII when coagulation is triggered by tissue factor. NXT007 demonstrated potent hemostatic activity in an acquired hemophilia A model in non-human primates at much lower dosage than emicizumab, consistent with an around 30-fold dose shift in the in vitro TG activity between NXT007 and emicizumab. Moreover, together with Fc engineering that enhanced FcRn binding and reduced in vivo clearance, we demonstrate that NXT007 could be effective at much lower dosage with a longer dosing interval compared to emicizumab. These non-clinical results suggest that NXT007 is expected to maintain a non-hemophilic range of coagulation potential in PwHA and provides a rationale for its clinical testing.

Main Text:

INTRODUCTION

Congenital hemophilia A, which is a disease caused by the deficiency or malfunction of factor VIII (FVIII), is classified into three main forms: severe, moderate, and mild (*1*). About half of people with hemophilia A (PwHA) are classified with severe disease, which is defined as having

less than 1 IU/dL of FVIII activity in their plasma (i.e., <1% of the standard normal plasma) (*2*). Moderate and mild diseases show 1–5 IU/dL and 5–40 IU/dL of the activity, respectively (*1, 3*). PwHA with severe disease are at an especially high risk of bleeding, and would typically suffer frequent spontaneous bleedings into their joints and muscles without an appropriate bleeding-prophylactic treatment (*1*).

Prophylactic treatment for PwHA has been established by regular infusions of a FVIII agent, which was proven to effectively prevent clinical and subclinical bleeds if started in early childhood (*4, 5*). However, FVIII-prophylactic treatments still typically require two or three weekly intravenous (IV) administrations due to its short half-life and low subcutaneous bioavailability (*6, 7*), which impose a substantial treatment burden. Although extended half-life (EHL) products have been launched to reduce dosing frequency, the administration route has remained intravenous (*6*). More critically, a remaining challenge is that one third of PwHA with severe disease develop anti-FVIII alloantibodies, called FVIII-inhibitors, which render FVIII agents ineffective (*8*).

Emicizumab, launched in 2017, overcomes the shortcomings of FVIII agents. It is a therapeutic bispecific antibody (BsAb) to activated factor IX (FIXa) and factor X (FX) which mimics the cofactor activity of activated FVIII (FVIIIa) (*9, 10*). Owing to its long half-life and good bioavailability after subcutaneous administration, emicizumab became the first subcutaneously injectable agent for bleeding-prophylaxis. It achieved stably high cofactor activity with a markedly lower dosing frequency (once a week, once per 2 weeks or once per 4 weeks) than the FVIII agents, making it much easier to administer at home with less risk of treated bleeds. Moreover, emicizumab is effective even for PwHA who have FVIII-inhibitors (*11*) because it is not recognized by FVIII-inhibitors due to its different structure from FVIII.

However, previous in vivo studies showed that emicizumab cofactor activity did not reach the levels of 100 IU/dL of FVIII (equivalent to standard normal plasma) (*11*, *12*). Thus, there was still room to increase the cofactor activity of a FVIIIa-function mimetic BsAb, and that higher cofactor activity would be required for those PwHA who continue to experience bleeding episodes while receiving emicizumab.

Therefore, we aimed to create a new FVIIIa-function mimetic BsAb with higher cofactor activity. By enhancing the activity, we expected that non-hemophiliac ranges of coagulation potential (40–150 IU/dL of FVIII) could be constantly maintained in PwHA with a convenient dosing regimen. Since the binding epitopes in FIX(a) and FX of emicizumab have been clinically validated to be effective and safe in PwHA, we decided to further engineer emicizumab by utilizing the emicizumab's heavy chains as templates since the heavy chain mainly contributes to the binding specificity of antibodies. On the other hand, we speculated that use of the common light chain in emicizumab, which had been necessary at the time of emicizumab's discovery, limited the scope of antibody optimization; therefore, we decided to identify new pairs of light chains for each emicizumab heavy chain, then introduce mutations onto both heavy and light chains for further enhancing FVIIIa-mimetic cofactor activity. To enable the manufacturing of a four-chain asymmetric BsAb, we incorporated mutations at a C_H1/C_L interface to facilitate the correct pairing of heavy and light chains together with mutations at C_H3/C_H3 interface to promote heavy chain heterodimerization. In addition, Fc engineering to improve pharmacokinetics was performed by enhancing FcRn binding at acidic pH (*13*).

To identify which in vitro assay should be used in screening the next-generation BsAb, we first re-examined the previous non-clinical in vitro and in vivo studies of emicizumab since none of the in vitro assays can perfectly capture the actual coagulation process that occurs in the body.

Increasing the amount of FIX(a)–BsAb–FX ternary complex by strengthening the binding affinity to FIX(a) and/or FX would be one of the strategies for improving FVIIIa-mimetic cofactor activity. However, increased FIX(a)- or FX-occupancy would potentially suppress FIX(a) or FX function both up- and down-stream of the coagulation cascade. Therefore, during the screening of emicizumab variants, we carefully examined the undesirable inhibitory effect as well as the FVIIIa-mimetic cofactor activity. Using this screening process, we were able to identify a new BsAb, named NXT007. Non-clinical studies of NXT007 suggested that it is expected to maintain a non-hemophilic range of coagulation potential in PwHA and support its clinical testing.

RESULTS

Determination of conditions for screening FVIIIa-mimetic cofactor activity of emicizumab variants

While hemostatic activity of FVIII can be predictably described based on the international units, describing the potent hemostatic activity of emicizumab is challenging due to its unique mode of action. Previous non-clinical in vivo studies showed that the factor for converting emicizumab μg/mL to U/dL to describe in vivo equivalent-FVIII hemostatic activity was estimated to be around 0.3 (*11, 12*). We re-examined various non-clinical in vitro studies of emicizumab to determine which in vitro parameter could most appropriately reflect the in vivo hemostatic potential of emicizumab. Analyzing a series of in vitro datasets on emicizumab 50 μg/mL (*12, 14-16*), we found that the conversion factor from BsAb μg/mL to FVIII IU/dL determined by the peak height of plasma TG assays with low tissue factor-trigger (TF-trigger) was 0.28, which was

similar to the conversion factor (0.3) obtained from the previous in vivo study (Table S1). In addition, peak height is defined as the highest thrombin concentration in the course of the TG assay and can reliably indicate hyper- or hypo-coagulability (*17*). Also, the parameter clearly showed the concentration-dependency of recombinant human FVIII (rhFVIII) at 10–100 IU/dL, which was a suitable range to screen and evaluate emicizumab variants targeting 100 IU/dL activity. This also supported our rationale for using peak height as an in vitro guidepost.

Identification of novel non-common light chains respective to the heavy chains of emicizumab

When we originally created emicizumab, a common light chain format was selected to satisfy industrial manufacturing requirements (Fig.1A) (*10*). However, the use of a common light chain limited our freedom to identify the most potent light chains for the anti-FIXa arm and anti-FX arm. To overcome this limitation, we identified pairs of charge mutations in the C_H1/C_L interface (*18*) and the C_H3/C_H3 interface (*19*) that could electrostatically facilitate the correct pairing of heavy and light chains, and heavy chain heterodimerization to enable the industrial manufacturing of a non-common light chain BsAb (Fig.1B). We then screened novel light chains for each emicizumab heavy chain (anti-FIX(a) or anti-FX) using light-chain shuffled phage-displayed libraries; the common light chain of emicizumab was substituted using human naïve light chain libraries to form Fab-displayed phage libraries followed by panning against FIXa and FX (Fig. S1). Based on the binding property against each antigen and the screening results for the in vitro cofactor activity, we identified new light chains for each heavy chain, and selected NXT000 as a lead BsAb. NXT000 showed FVIIIa-mimetic cofactor activity comparable to that of emicizumab at a low concentration range in an enzymatic assay using purified coagulation

factor proteins (Fig, S2A). Similar data were also observed using a plasma TG assay with activated factor XI (FXIa)-triggering condition, which was selected due to higher sensitivity than TF-triggering condition. (Fig. S2B). Thus, we assumed that NXT000 has the potential to achieve higher activity than emicizumab through optimization.

Generation of the efficacy enhanced anti-FIXa/FX bispecific antibody NXT007

To optimize NXT000, we first aimed to improve the maximum FVIIIa-mimetic cofactor activity, especially the maximum equivalent-FVIII TG activity, so that it would be much more potent than emicizumab. To do this, we used the COSMO (Comprehensive substitution for multidimensional optimization) approach (*20*); each amino acid residue in all complementarity determining regions (CDRs) and in some positions in the framework regions of NXT000's heavy and light chains were replaced with a different natural amino acid except cysteine on a one-by-one basis. We selected mutations to combine which were able to increase FVIIIa-mimetic cofactor activity in the enzymatic assay compared to NXT000. Then, the effective combinations were further assessed using TG assays. In addition, to avoid undesirable interference with the innate reactions in the coagulation cascade, we simultaneously assessed inhibitory effect of the mutations in the anti-FX arm on FX function. We also introduced other mutations to achieve an isoelectric point (pI) difference between the two arms for purification processes, to decrease non-specific binding, to improve solubility and thermal stability, and to minimize immunogenicity in the same manner as emicizumab (*10, 21*). To improve pharmacokinetics and reduce antibody clearance, a set of mutations were also introduced into the C_H3 region to enhance the neonatal Fc receptor (FcRn)-binding at acidic pH (*13*). Consequently, we identified a clinical candidate which we named NXT007.

In vitro pharmacological profiles of NXT007

We then conducted a detailed analysis of the in vitro pharmacological profile of NXT007. In the enzymatic assay, NXT007 showed enhanced maximum FVIIIa-mimetic cofactor activity at a lower concentration compared with emicizumab (Fig 2A). In FVIII-deficient patient plasma, NXT007 also showed enhanced TG activity in the TF-triggering conditions (Fig 2B). In the TG assay, NXT007 showed comparable activity to emicizumab at approximately 30-fold lower concentration and showed greater activity than 100 IU/dL of rhFVIII at around 150 nM (Fig 2B). Notably, the maximum TG activity of NXT007 did not exceed rhFVIII 150 IU/dL, the upper level of the non-hemophilic range. In the FXIa-triggering condition, NXT007 also showed greater activity than emicizumab. A higher concentration of NXT007 was required to show the same equivalent FVIII activity in the FXIa-triggering condition; this was also seen with emicizumab (Fig.S3).

We determined that the K_D value of NXT007 to FIX/FIXa was approximately 1 μM, which is similar to that of emicizumab (*14*). The K_D value of NXT007 to FX/FXa was approximately 30 to 40-fold improved from emicizumab, and such enhancement of binding affinity can be explained by the improved k_a value rather than k_d (Fig 3A). The lower K_D value could potentially inhibit FX function since NXT007, having emicizumab's heavy chain variants, is considered to bind to epidermal growth factor-like domain 2 (EGF2 domain) of FX as emicizumab does, which may restrict the availability of FX/FXa for the other reactions in the coagulation cascade. Thus we carefully monitored the effect of BsAbs on FX function using prothrombin time (PT), which is able to reflect FVIIa/TF-catalyzed FX activation and FVa/FXa-catalyzed prothrombin activation. Consequently, PT was not clearly prolonged for NXT007 in FVIII-deficient patient

plasma at the concentration where the TG activity of NXT007 reaches 40 IU/dL of rhFVIII. (Fig. 3B).

Altogether, NXT007 showed the potential to achieve a non-hemophiliac range of TG activity in FVIII-deficient plasma in vitro while having a minimal inhibitory impact on FX(a)-function in plasma.

In vivo pharmacological profiles of NXT007

Prior to in vivo evaluation, we confirmed that NXT007 also dose-dependently enhanced TG activity in cynomolgus monkey plasma treated with anti-FVIII neutralizing antibody cyVIII-2236 (Fig. S5). In the experiments using monkey plasma, FXIa-triggering TG was selected due to the lack of detectability of recombinant porcine FVIII (rpoFVIII) activity, a positive control with no cross reactivity with cyVIII-2236, in the TF-triggering condition (*12*). Next, we evaluated the hemostatic activity of NXT007 using the artificially induced on-going bleeding model in acquired hemophilia A cynomolgus monkeys, which had been previously used to estimate the non-clinical equivalent-FVIII hemostatic activity of emicizumab (*12*, *14*). Briefly, in this model, a hemophilia A state was induced by the administration of cyVIII-2236. Bleeding was induced by muscle needling and subcutaneous exfoliation at abdomen, and then NXT007 was intravenously administered to evaluate its on-demand hemostatic activity. As a result, NXT007 dose-dependently suppressed bruised area on the skin and the reduction of blood hemoglobin observed in vehicle treated monkeys, suggesting the amelioration of bleeding symptoms (Fig. 4A, B). A single IV administration of 0.075 mg/kg NXT007 exerted a level of hemostatic activity similar to twice-daily administration of rpoFVIII 20 U/kg. At 0.075 mg/kg in

the NXT007 group, the plasma concentration ranged from 1.9 μg/mL (13 nM) at Day 0 to 0.75 μg/mL (5.2 nM) at Day 3 (Fig. 4C).

During the experimental period, one animal in vehicle group exhibited lethal bleeding at Day2 possibly due to the intensity of the bleeding induction method. The animal was excluded from the analysis, but this is not expected to affect the risk of overestimating NXT007 efficacy.

In vivo pharmacokinetic study and multiple dosing simulation of NXT007

Next, the pharmacokinetics (PK) of NXT007 were assessed in a single-dose study in non-human primates (n = 4). The clearance (CL) or CL/F of NXT007 was 2.46 mL/day/kg after intravenous (IV) administration at 2 mg/kg, and in the range of 2.68–3.37 mL/day/kg after a single subcutaneous (SC) administration at 0.02, 0.2, and 2 mg/kg, which is lower than that of typical monoclonal antibodies including emicizumab (*12, 22*), and the SC bioavailability was in the range of 82.0–92.2% (Fig 5B). The plasma elimination half-life of NXT007 was 22.1 days after IV administration at 2 mg/kg, and in the range of 19.6–24.4 days after a single SC administration at 0.02, 0.2, and 2 mg/kg (Fig. 5B). With SC administration, the maximum plasma concentration of NXT007 increased in an approximately linear dose-proportional manner (Fig 5A).

In vivo toxicology profile of NXT007

Finally, we conducted a Good Laboratory Practice (GLP) toxicology study of NXT007. In the 6-month study (SC, every 2 weeks (Q2W)) in non-human primates, shortened APTT and prolonged PT were observed, which were considered to be a pharmacological effect and an exaggerated pharmacological effect. In 80 mg/kg, the prolonged PT was associated with

hemorrhage-related findings in pathology. Observed findings were considered reversible. No Observed Adverse Effect Level (NOAEL) for the 6-month GLP study in cynomolgus monkeys was 20 mg/kg SC Q2W.

DISCUSSION

The emicizumab maximum activity does not reach the level of the international standard activity (100 IU/dL of FVIII) in the TF-trigger TG assays. By developing a BsAb with higher maximum activity than emicizumab, we expect to suppress breakthrough bleedings more potently; we think this second-generation therapy can finally offer patients freedom from hemophilia, as they will no longer have to live their lives in constant fear of another bleed. In addition, advances in antibody technology (ie, production of a four-chain BsAb) have allowed to produce a superior FVIIIa-function mimetic BsAb, motivating us to engineer a second-generation emicizumab that achieves 100 IU/dL in vitro.

A common light chain format was selected in emicizumab since non-common light chain BsAbs were not technologically feasible at the time of emicizumab discovery research (*10*). However, it was difficult to find mutations which were effective for both the anti-FIXa and anti-FX arms because the common light chain format put limits on the scope of antibody optimization. Thus, we expected the non-common light chain format to increase the scope of antibody engineering for the next generation BsAb. Meanwhile, we aimed to target the same FIX(a) and FX epitopes targeted by emicizumab as they have already been clinically validated. Since the heavy chain is mainly responsible for the binding specificity (*23*), we decided to use variants of emicizumab's heavy chains for the next generation BsAb. These motivated us to use a human naïve light chain library to find novel pairs of light chains for each emicizumab heavy chain.

Thus, we used a phage library displaying Fabs consisting of emicizumab's heavy chain (anti-FIX(a) or anti-FX heavy chain) and a human naïve light chain library. Two distinct novel light chains were obtained, and following the multidimensional optimization of the two Fab domains, NXT007 was identified.

Increasing the amount of FIX(a)–BsAb–FX ternary complex would be one of the strategies for improving FVIIIa-mimetic cofactor activity. However, strengthening the binding affinity to FIX(a) and/or FX to increase the ternary complex would potentially affect the innate interactions of coagulation factors, in other words, increased FIX(a)- or FX-occupancy of emicizumab based-BsAb would suppresses FIX(a) or FX function both up- and down-stream of the coagulation cascade. Therefore, we applied a strategy to keep the K_D of anti-FIX(a) similar to that of emicizumab (micro molar range) while strengthening anti-FX K_D by increasing k_a rather than decreasing k_d, since our hypothesis was that faster association and dissociation to FX minimizes the coagulation inhibitory effect of the anti-FX arm while maintaining the turnover rate to promote FIX(a)–NXT007–FX ternary complex formation. We could examine the inhibitory effect of the anti-FX arm using PT, which is able to reflect FVIIa/TF-catalyzed FX activation and FVa/FXa-catalyzed prothrombin activation, whereas the inhibitory effect of anti-FIX(a) arm was difficult to evaluate in the plasma in vitro during the screening. Indeed, the PT prolongation in vitro plasma assay suggested that prolongation effect was less in NXT007 compared to emicizumab when equivalent-FVIII TG activity was the same. In the clinical study, emicizumab was safely administered to patients for several years with the highest concentrations in blood reaching 127 μg/mL (872 nM). This was calculated to be highest average steady-state peak value based on the average C_{min} and the ratio of C_{max}/C_{min} observed in clinical studies of emicizumab. (*24, 25*). In the PT prolongation in vitro assay, emicizumab 127 μg/mL (872 nM) corresponded

to NXT007 17 μg/mL (118 nM). This suggests that NXT007 can be safely administered if the clinical concentration is set below 118 nM. At higher concentrations (>69 nM) of NXT007, PT was prolonged, presumably due to increased FX(a)-occupancy, which had also been observed with emicizumab in the in vitro setting.

After confirming the promising in vitro profiles of NXT007, we then conducted in vivo studies to confirm whether its in vitro potency reflected its in vivo hemostatic potential in a similar manner to emicizumab. In the ongoing bleeding model of acquired hemophilia A using non-human primates, NXT007 dose-dependently ameliorated bleeding symptoms. Doses as low as 0.075 mg/kg demonstrated a significant hemostatic effect similar to twice daily doses of 20 U/kg rpoFVIII. In our previous study, a single IV administration of 3 mg/kg emicizumab exerted a hemostatic effect roughly comparable to twice daily doses of 10 U/kg rpoFVIII (*12*). Due to the ethical limitations on the use of non-human primates, an in vivo study directly comparing NXT007 and emicizumab could not be conducted. Between this study (NXT007) and the previous study (emicizumab), the mean values of hemoglobin decrease in the control groups were comparable. In addition, the hemostatic activity in the rpoFVIII groups was similar (Fig. S6). The doses of rpoFVIII were different; 20 or 10 U/kg twice daily in this study or the previous study, respectively. But we do not regard these outcomes to be contradictory, because both dosing regimens were set to achieve a substantial level of hemostatic activity and keep the disease severity mild. Therefore, we think that data from these two studies can be roughly compared if interpreted carefully.

Across studies, comparing the 0.075 mg/kg NXT007 group and the 3 mg/kg emicizumab group, the mean plasma concentration of NXT007 and emicizumab just after the single IV administration was 1.9 μg/mL (Fig. 4D) and 61 μg/mL, respectively (*12*). The difference in the

plasma BsAb concentrations was ~30-fold. Since NXT007 showed approximately 30-fold lower dose shift than emicizumab in the in vitro TG assay, these in vivo data are roughly consistent with the results of in vitro TG assays for NXT007 and emicizumab. In other words, the predictability of emicizumab's in vivo hemostatic activity using in vitro TG assays was reproduced for NXT007. However, this conclusion must be interpreted considering the environmental differences between the NXT007 and emicizumab studies, although we tried to minimize any potential impact. Also, the number of animals used in these two studies was somewhat small.

A single-dose pharmacokinetic study using cynomolgus monkeys demonstrated that the clearance of NXT007 was lower than that of emicizumab and typical monoclonal antibodies. This is likely due to the benefit of increased FcRn binding at acidic pH introduced by Fc engineering. The prediction of human pharmacokinetics for such Fc engineered antibodies is still challenging because very few have been tested in humans. However, since motavizumab-YTE, an Fc engineered antibody with similar clearance as NXT007 in cynomolgus monkeys (*26*), demonstrated a clearance of 43–68 mL/day (around 0.5–1.0 mL/day/kg) and a half-life of 70–100 days in humans (*27*), we expect NXT007 to have a similar pharmacokinetic profile in humans, which is longer-lasting than emicizumab, enabling highly convenient dosing for PwHA.

Based on the non-clinical efficacy, safety, and pharmacokinetic data, a simulation analysis was carried out to predict whether a non-hemophiliac range (40% or more equivalent-FVIII TG activity) could be safely maintained with a subcutaneous regimen of NXT007. This can be achieved by maintaining the NXT007 plasma trough concentration higher than 4.4 μg/mL (30 nM), which would exert 40% of equivalent-FVIII TG activity in the TF-triggering condition, and the maximum concentration lower than 17 μg/mL (118 nM), which is equivalent to 127 μg/mL

(872 nM) of emicizumab in prolonging PT. As shown in Fig.S7, there is a range of dosing intervals, such as every 4 weeks (Q4W), which can achieve a plasma concentration between 4.4 μg/mL and 17 μg/mL.

In the GLP-tox study, at Q2W SC dosing of 20 mg/kg NXT007, PT prolongation was observed without any pathological changes, while at 80 mg/kg, PT prolongation was accompanied by hemorrhage-related findings. C_{max} achieved in the 20 mg/kg SC Q2W study (approximately 1000 μg/mL; 6.9 μM) was more than 200-fold higher than the NXT007 concentration exerting 40% of equivalent-FVIII TG activity in the TF-triggering condition (4.4 μg/mL; 30 nM), and more than 40-fold higher than the NXT007 concentration (22.3 μg/mL; 154 nM) exerting 100% of equivalent-FVIII TG activity in TF-triggering condition.

In this study, our non-clinical findings on the efficacy, safety, and pharmacokinetics of NXT007 indicate that it will be able to safely maintain a non-hemophiliac range of coagulation potential in PwHA. A phase I/II clinical trial of NXT007 in PwHA is currently on-going.

MATERIALS AND METHODS

Study design

The main objective of our study was to evaluate the hemostatic activity, PK, and toxicity of the NXT007 bispecific antibody to FIXa and FX. The in vitro hemostatic activity was assessed in a TG assay using FVIII-deficient patient plasma in FXIa- and TF-triggering conditions. The in vitro FX(a)-inhibitory activity was assessed by PT. The in vivo hemostatic activity was assessed in a cynomolgus monkey bleeding model. After administration of NXT007 (n = 6 per group) and rpoFVIII (n = 6 per group), the bruised areas and blood hemoglobin level were measured. The

decrease in blood hemoglobin level was expressed as a percentage on Day 0 (Fig. 4A and 4B). The animals were sacrificed after the evaluation on Day 3. The toxicity of NXT007 was assessed using cynomolgus monkeys. The number of animals per group (n = 40 [20 per sex group]) was chosen according to the ICH-S4A guideline. For PK analysis, the data from animals that showed anti-drug antibody (ADA) production were excluded, regardless of the titer levels.

Materials

Emicizumab (ACE910), anti-primate FVIII neutralizing antibody (cyVIII-2236), and B domain-deleted recombinant porcine FVIII (rpoFVIII) were prepared as previously described (*28*).

The care and use of laboratory animals

We used 37 male cynomolgus monkeys for the in vivo hemostatic study (2.6–4.0 kg, 3–4 years; Shin Nippon Biomedical Laboratories, Ltd., Kagoshima, Japan), 16 males for the pharmacokinetic study (3.0–3.8 kg, 3 years; Shin Nippon Biomedical Laboratories, Ltd., Kagoshima, Japan) and 20 females and 20 males for the toxicology study (6.0–9.5 kg, 6–11 year old males and 2.8–5.9 kg, 4–9 year old females; Shin Nippon Biomedical Laboratories, Ltd., Kagoshima, Japan). All animal studies were approved by the Institutional Animal Care and Use Committee of Chugai Pharmaceutical Co., Ltd., and were conducted in accordance with the approved protocols and the Guidelines for the Care and Use of Laboratory Animals at the company. Shin Nippin Biomedical Laboratories and Chugai Pharmaceutical Co., Ltd. are fully accredited by The Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Identification of novel non-common light chains from phage displayed Fab library consisting of naïve light chain libraries and either one of the emicizumab heavy chains

The light chain shuffled Fab-displayed phage libraries were constructed in Chugai Pharmaceutical Co., Ltd. as follows: Human naïve kappa or lambda gene libraries derived from 43 healthy volunteers were combined with genes of either Q499 or J327 (both are the heavy chains of emicizumab), and then inserted into a phagemid vector to display Fab domains on phage. Phages were produced using E coli, followed by bio-panning using biotin-labelled human FIXa or FX as antigens. After several rounds of panning, phage ELISA was performed to check the binding against each antigen and select the pools which were then used for conversion of IgG expressible by mammalian cells such as Expi293 (ThermoFisher Scientific). Approximately 1000 IgGs were produced for each anti-FIX(a) or anti-FX antibody screening.

Expression and purification of bispecific antibodies

A series of BsAb variants consisting of two different heavy chains and two light chains were used. To generate these asymmetric BsAbs, each anti-FIXa and anti-FX antibody was produced separately by Expi293 expression system (ThermoFisher Scientific) then combined for screening. Proprietary electrostatic steering mutations were introduced into the C_H3 region of each heavy chain to promote heterodimerization (*19*). In producing NXT007, electrically charged mutation pairs were also introduced at each C_H1/C_L interface to form the correct pairing of heavy chain and light chain (*18*). Each gene was inserted into the Expi293 expression system (ThermoFisher Scientific) followed by purification using protein A and cation ion exchange

chromatography to remove mis-paired moieties. After purification, the BsAb ratio was approximately 95%.

Binding kinetics analysis by surface plasmon resonance (SPR)

The binding kinetics of NXT007 to human factor IX (hFIX), activated human factor IX (hFIXa), human factor X (hFX), activated human factor X (hFXa), cynomolgus monkey factor IX (cyFIX), or cynomolgus monkey factor X (cyFX) were assessed at pH 7.4 at 25°C using a Biacore T200 instrument (GE Healthcare). Each antibody was captured onto Sure Protein A (MabSelect SuRe) immobilized on a CM4 sensor chip (GE Healthcare), after which hFIX, hFIXa, hFX, hFXa (Enzyme Research Laboratories, Inc.), cyFIX, or cyFX (prepared in-house) were injected over the flow cells. To suppress the non-specific interaction of the analyte with the sensor chip, the surface negative charge of the sensor chip was reduced by the pre-treatment, described as follows. First, carboxyl groups on Flow Cell 1 (FC1) and Flow Cell 2 (FC2) were activated by injecting a mixed solution of equal volumes of NHS and EDC. Then, the active groups were blocked by injecting ethanolamine-HCl. The sensor chip surface was regenerated using 25mM NaOH. Kinetic parameters were determined by fitting the sensorgrams obtained by subtracting the FC1 sensorgram from the FC2 sensorgram with 1:1 binding model using Biacore T200 evaluation software, version 2.0 (GE Healthcare). The average values of kinetic parameters were calculated from three measurements.

Enzymatic assay for FIXa-catalyzed FX activation

We evaluated the conversion rate of FX to FXa in an enzymatic assay using purified coagulation factors as described previously with a slight modification (*9*). The assay system consisted of 1 nM human FIXa (Enzyme Research Laboratories), 140 nM human FX (Enzyme Research Laboratories), 4 μM phospholipid (10% phosphatidylserine, 60% phosphatidyl-choline, and 30% phosphatidylethanolamine), and bispecific antibodies. FXa generation was measured at room temperature for 1 min in TBS containing 1 mM $CaCl_2$, and 0.1% (wt/vol) BSA (pH 7.6). We stopped the reaction by adding EDTA. After adding S-2222 chromogenic substrate (Cromogenix), we measured absorbance at 405 nm to determine the rate of FXa generation. Data were collected in triplicate.

Thrombin generation (TG) assays

Thrombin generation (TG) in plasma sample was measured by Calibrated Automated Thrombography (CAT, Thrombinoscope BV) using a 96-well plate fluorometer (ThermoFisher Scientific) as described previously (*12, 14, 16*). Briefly, each well in a 96-well plate was dispensed with 80 μL of plasma sample, to which was then added 20 μL of triggering solution. For the evaluation of activity in human plasma, FVIII-deficient human plasma (George King Bio-Medical) containing BsAb or rhFVIII (Bayer AG) was used for plasma sample, and an intrinsic triggering solution (FXIa triggering solution) consisting of 31 pM human FXIa (Enzyme Research Laboratories) and 20 μM synthetic phospholipid (10% phosphatidylserine, 60% phosphatidyl-choline, and 30% phosphatidylethanolamine) or extrinsic triggering solution (TF triggering solution) using PPP-reagent low (Thrombinoscope BV) was used. For the evaluation of activity in cynomolgus monkey plasma, pooled citrated plasma of 6 cynomolgus male monkeys which contains 300 μg/mL of cyFVIII-2236, and BsAb or rpoFVIII was used for

plasma sample and intrinsic triggering solution consisting of 310 pM human FXIa and 20 μM synthetic phospholipid was used. For calibration, 20 μL of Thrombin Calibrator (Thrombinoscope BV) was added 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. We analyzed the thrombograms and peak height using the instrument's software. Data were collected in triplicate.

Activated partial thromboplastin time (APTT) and prothrombin time (PT)

We employed as Thrombocheck APTT-SLA (Sysmex) for the APTT reagent and RecombiPlasTin 2G (Instrumentation Laboratory) for PT reagent using CS-2000i (Sysmex) for measurement as the recommended protocol. We used FVIII-deficient human plasma (George King Bio-Medical) for APTT and PT assay, which were treated with each concentration of BsAb or rhFVIII (Bayer AG). Data were collected in triplicate. For the analysis of the correlation of equivalent-FVIII activity of NXT007 or emicizumab and PT value, equivalent-FVIII activity was calculated using the peak height of the TG assay with the TF triggering condition calibrated by the rhFVIII standard. The analysis was carried out below the concentration range which reached maximum thrombin generation activity of NXT007 or emicizumab.

In vivo hemostatic study in an acquired hemophilia A model

Since NXT007 is highly species specific in its FVIIIa-mimetic cofactor activity similar to its template, emicizumab, in vivo hemostatic activity was evaluated using the non-human primate bleeding model as previously described (*12*) with slight modification. On Day 0, to establish an

acquired hemophilia A state, the animals received an intravenous injection of cyVIII-2236 (10 mg/kg). Two hours thereafter, the animals were anesthetized by isoflurane inhalation. Then, the following 2 surgical procedures were performed: 1 cm-deep insertion of an 18G-needle into the muscle at 22 sites (2 sites in each upper arm, 3 sites in each forearm, 4 sites in each inside thigh, and 2 sites in each outside thigh), and subcutaneous exfoliation by 3 cm-long insertion of a tip of forceps beneath the abdominal skin at 2 sites. After administration of buprenorphine, an analgesic drug, the animals were recovered from the anesthesia. (The animals also received this analgesic treatment on the following days.) Although bleeding symptoms were not apparent at that time, visible hematoma at the sites of subcutaneous exfoliation became apparent about 6 to 8 hours later. The animals then received intravenous administration of NXT007 (0 (Vehicle), 0.0075, 0.025, 0.075 or 0.6 mg/kg; n = 7 for vehicle, and n = 6 for each NXT007 group), rpoFVIII (20 U /kg; n = 6). To the animals in the rpoFVIII group, rpoFVIII was intravenously administered once on Day 0 and twice daily on Days 1 and 2 (in the morning and evening; a total of 5 administrations). In the morning on Days 1, 2 and 3, the animals were anesthetized to measure the bruised areas. After the evaluation on Day 3, the animals were killed humanely. Citrated blood was collected before and 2 hours after the cyVIII-2236 injection, about 10 min after the test item administration on Day 0, and before measuring the bruised area on Days 1, 2, and 3. The decrease in blood hemoglobin level was expressed as a percentage on Day 0 (before initial NXT007 or rpoFVIII injection). During the experimental period, one animal in the vehicle group encountered lethal bleeding at Day 2 possibly due to the intensity of the bleeding induction method. The animal was excluded from the analysis. The plasma NXT007 concentration was determined by the method described in the Pharmacokinetic study and simulation section.

Pharmacokinetic study and simulation

NXT007 was administered intravenously (2 mg/kg; *n* = 4) or subcutaneously (0.02, 0.2 or 2 mg/kg; *n* = 4 for each group) to cynomolgus monkeys. For animals dosed intravenously, blood was sampled with a heparinized syringe at 5 min, 2, 8, 24, 48, 72, 96 hours, 7, 14, 21, 28, 35, 42, 49, 56, 63, 70, 77, and 84 days post dose. For animals dosed subcutaneously, blood was collected in the same way without sampling at 5 min. The collected blood was immediately centrifuged to separate the plasma, which was kept at −80°C until measurement. The concentration of NXT007 in monkey plasma was measured by ELISA assay. An anti-NXT007 rabbit IgG was dispensed onto a microplate and incubated for 1 hour at room temperature. After each plasma sample was added to the plates and removed, another anti-NXT007 mouse IgG was reacted with samples. Anti-mouse IgG with HRP label was used as detection antibody. The signal was detected by micro plate reader. Biotin labeled NXT007 and digoxigenin labeled NXT007 were sandwiched to detect anti-NXT007 antibodies. The signal was detected by micro plate reader in PK and ADA assays.

The pharmacokinetic parameters were analyzed by non-compartmental analysis with Phoenix Winnonlin (version 6.4, Certara LP) excluding ADA positive animals or sampling points. Multiple-dosing simulations were performed with SAAM II version 1.2 (SAAM Institute, Seattle, WA, USA) based on a 2-compartment model with first order absorption kinetics.

GLP-Tox study

The NXT007 drug substance was intermittently administered with SC injections at dose levels of 0 (control), 2, 20, and 80 mg/kg (dose volume: 0.96 mL/kg) to male and female cynomolgus monkeys (5 males and 5 females per group) once every 2 weeks for 6 months to evaluate toxicity. The reversibility of toxicity was assessed during a 5-month recovery period. Systemic exposure was also assessed. Vehicle (20 mmol/L histidine-aspartate buffer containing 150 mmol/L arginine, 20 mmol/L methionine, and 0.5 mg/mL polysorbate 80, pH 6.0) was intermittently administered to animals in the control group in the same manner as the test article. During the study, toxicological observations and examinations were conducted.

Statistical analysis

In the in vivo hemostatic study, data are presented as mean ± SE. Other data are presented as mean ± SD.

List of Supplementary Materials

Fig. S1. Schematic illustration of NXT000 identification.

Fig. S2. In vitro FVIIIa-mimetic cofactor activity of NXT000.

Fig. S3. In vitro FVIIIa-mimetic cofactor activity of NXT007.

Fig. S4. Clotting activity of NXT007 treated plasma.

Fig. S5. Thrombin generation activity of NXT007 in cynomolgus monkey plasma.

Fig. S6. Historical comparison of in vivo hemostatic activity of NXT007 and emicizumab.

Fig. S7. Pharmacokinetic simulation of NXT007

Table S1. Conversion factor of emicizumab to FVIII equivalent activity.

Table S2. Toxicokinetic parameters after administration of NXT007 in cynomolgus monkeys.

References and Notes

1. F. Peyvandi, I. Garagiola, G. Young, The past and future of haemophilia: diagnosis, treatments, and its complications. *Lancet* **388**, 187-197 (2016).
2. *Report on the annual global survey 2019* (World Federation of Hemophilia, Montreal, Canada, 1963). (2020).
3. M. Makris, J. Oldenburg, E. P. Mauser-Bunschoten, K. Peerlinck, G. Castaman, K. Fijnvandraat, The definition, diagnosis and management of mild hemophilia A: communication from the SSC of the ISTH. *J Thromb Haemost* **16**, 2530-2533 (2018).
4. M. J. Manco-Johnson, T. C. Abshire, A. D. Shapiro, B. Riske, M. R. Hacker, R. Kilcoyne, J. D. Ingram, M. L. Manco-Johnson, S. Funk, L. Jacobson, L. A. Valentino, W. K. Hoots, G. R. Buchanan, D. DiMichele, M. Recht, D. Brown, C. Leissinger, S. Bleak, A. Cohen, P. Mathew, A. Matsunaga, D. Medeiros, D. Nugent, G. A. Thomas, A. A. Thompson, K. McRedmond, J. M. Soucie, H. Austin, B. L. Evatt, Prophylaxis versus episodic treatment to prevent joint disease in boys with severe hemophilia. *N Engl J Med* **357**, 535-544 (2007).
5. M. M. Moreno, R. Cuesta-Barriuso, A history of prophylaxis in haemophilia. *Blood Coagul Fibrinolysis* **30**, S1-s3 (2019).
6. M. C. Pelland-Marcotte, M. D. Carcao, Hemophilia in a Changing Treatment Landscape. *Hematol Oncol Clin North Am* **33**, 409-423 (2019).

7. Y. Dargaud, M. Janbain, Clinical Utility of Subcutaneous Factor VIII Replacement Therapies in Hemophilia A: A Review of the Evidence. *J Blood Med* **12**, 1031-1036 (2021).

8. F. Peyvandi, P. M. Mannucci, I. Garagiola, A. El-Beshlawy, M. Elalfy, V. Ramanan, P. Eshghi, S. Hanagavadi, R. Varadarajan, M. Karimi, M. V. Manglani, C. Ross, G. Young, T. Seth, S. Apte, D. M. Nayak, E. Santagostino, M. E. Mancuso, A. C. Sandoval Gonzalez, J. N. Mahlangu, S. Bonanad Boix, M. Cerqueira, N. P. Ewing, C. Male, T. Owaidah, V. Soto Arellano, N. L. Kobrinsky, S. Majumdar, R. Perez Garrido, A. Sachdeva, M. Simpson, M. Thomas, E. Zanon, B. Antmen, K. Kavakli, M. J. Manco-Johnson, M. Martinez, E. Marzouka, M. G. Mazzucconi, D. Neme, A. Palomo Bravo, R. Paredes Aguilera, A. Prezotti, K. Schmitt, B. M. Wicklund, B. Zulfikar, F. R. Rosendaal, A Randomized Trial of Factor VIII and Neutralizing Antibodies in Hemophilia A. *N Engl J Med* **374**, 2054-2064 (2016).

9. T. Kitazawa, T. Igawa, Z. Sampei, A. Muto, T. Kojima, T. Soeda, K. Yoshihashi, Y. Okuyama-Nishida, H. Saito, H. Tsunoda, T. Suzuki, H. Adachi, T. Miyazaki, S. Ishii, M. Kamata-Sakurai, T. Iida, A. Harada, K. Esaki, M. Funaki, C. Moriyama, E. Tanaka, Y. Kikuchi, T. Wakabayashi, M. Wada, M. Goto, T. Toyoda, A. Ueyama, S. Suzuki, K. Haraya, T. Tachibana, Y. Kawabe, M. Shima, A. Yoshioka, K. Hattori, A bispecific antibody to factors IXa and X restores factor VIII hemostatic activity in a hemophilia A model. *Nat Med* **18**, 1570-1574 (2012).

10. Z. Sampei, T. Igawa, T. Soeda, Y. Okuyama-Nishida, C. Moriyama, T. Wakabayashi, E. Tanaka, A. Muto, T. Kojima, T. Kitazawa, K. Yoshihashi, A. Harada, M. Funaki, K. Haraya, T. Tachibana, S. Suzuki, K. Esaki, Y. Nabuchi, K. Hattori, Identification and

multidimensional optimization of an asymmetric bispecific IgG antibody mimicking the function of factor VIII cofactor activity. *PLoS One* **8**, e57479 (2013).

11. M. Shima, H. Hanabusa, M. Taki, T. Matsushita, T. Sato, K. Fukutake, N. Fukazawa, K. Yoneyama, H. Yoshida, K. Nogami, Factor VIII-Mimetic Function of Humanized Bispecific Antibody in Hemophilia A. *N Engl J Med* **374**, 2044-2053 (2016).
12. A. Muto, K. Yoshihashi, M. Takeda, T. Kitazawa, T. Soeda, T. Igawa, Y. Sakamoto, K. Haraya, Y. Kawabe, M. Shima, A. Yoshioka, K. Hattori, Anti-factor IXa/X bispecific antibody (ACE910): hemostatic potency against ongoing bleeds in a hemophilia A model and the possibility of routine supplementation. *J Thromb Haemost* **12**, 206-213 (2014).
13. A. Maeda, Y. Iwayanagi, K. Haraya, T. Tachibana, G. Nakamura, T. Nambu, K. Esaki, K. Hattori, T. Igawa, Identification of human IgG1 variant with enhanced FcRn binding and without increased binding to rheumatoid factor autoantibody. *MAbs* **9**, 844-853 (2017).
14. T. Kitazawa, K. Esaki, T. Tachibana, S. Ishii, T. Soeda, A. Muto, Y. Kawabe, T. Igawa, H. Tsunoda, K. Nogami, M. Shima, K. Hattori, Factor VIIIa-mimetic cofactor activity of a bispecific antibody to factors IX/IXa and X/Xa, emicizumab, depends on its ability to bridge the antigens. *Thromb Haemost* **117**, 1348-1357 (2017).
15. K. Nogami, T. Matsumoto, Y. Tabuchi, T. Soeda, N. Arai, T. Kitazawa, M. Shima, Modified clot waveform analysis to measure plasma coagulation potential in the presence of the anti-factor IXa/factor X bispecific antibody emicizumab. *J Thromb Haemost* **16**, 1078-1088 (2018).

16. T. Soeda, T. Kitazawa, A. Muto, Z. Sampei, T. Igawa, Y. Kawabe, M. Takeyama, K.Nogami, M. Shima, K. Hattori, poster presented at the WFH 2014 World Congress, Melbourne, 11 to 15 May 2014.

17. A. Tripodi, Usefulness of Thrombin Generation. *Hamostaseologie* **40**, 509-514 (2020).

18. Y. Teranishi, T. Soeda, H. Koga, Ka. Yamaguchi, K. Kato, A. Muto, K. Esaki, M. Okuda, M. Sato, N. Shibahara, Ko. Yamaguchi, H. Shiraiwa, T. Wakabayashi, H. Konishi, H. Kitamura, T. Kitazawa, and T. Igawa, poster presented at the ISTH 2020 congress, 12 to 14 July 2020.

19. T. Ishiguro, Y. Sano, S. I. Komatsu, M. Kamata-Sakurai, A. Kaneko, Y. Kinoshita, H. Shiraiwa, Y. Azuma, T. Tsunenari, Y. Kayukawa, Y. Sonobe, N. Ono, K. Sakata, T. Fujii, Y. Miyazaki, M. Noguchi, M. Endo, A. Harada, W. Frings, E. Fujii, E. Nanba, A. Narita, A. Sakamoto, T. Wakabayashi, H. Konishi, H. Segawa, T. Igawa, T. Tsushima, H. Mutoh, Y. Nishito, M. Takahashi, L. Stewart, E. ElGabry, Y. Kawabe, M. Ishigai, S. Chiba, M. Aoki, K. Hattori, J. Nezu, An anti-glypican 3/CD3 bispecific T cell-redirecting antibody for treatment of solid tumors. *Sci Transl Med* **9**, (2017).

20. Z. Sampei, K. Haraya, T. Tachibana, T. Fukuzawa, M. Shida-Kawazoe, S. W. Gan, Y. Shimizu, Y. Ruike, S. Feng, T. Kuramochi, M. Muraoka, T. Kitazawa, Y. Kawabe, T. Igawa, K. Hattori, J. Nezu, Antibody engineering to generate SKY59, a long-acting anti-C5 recycling antibody. *PLoS One* **13**, e0209509 (2018).

21. T. Kuramochi, T. Igawa, H. Tsunoda, K. Hattori, Humanization and Simultaneous Optimization of Monoclonal Antibody. *Methods Mol Biol* **1904**, 213-230 (2019).

22. N. L. Dirks, B. Meibohm, Population pharmacokinetics of therapeutic monoclonal antibodies. *Clin Pharmacokinet* **49**, 633-659 (2010).

23. J. L. Xu, M. M. Davis, Diversity in the CDR3 region of V(H) is sufficient for most antibody specificities. *Immunity* **13**, 37-45 (2000).

24. M. Shima, H. Hanabusa, M. Taki, T. Matsushita, T. Sato, K. Fukutake, R. Kasai, K. Yoneyama, H. Yoshida, K. Nogami, Long-term safety and efficacy of emicizumab in a phase 1/2 study in patients with hemophilia A with or without inhibitors. *Blood Adv* **1**, 1891-1899 (2017).

25. K. Yoneyama, C. Schmitt, N. Kotani, G. G. Levy, R. Kasai, S. Iida, M. Shima, T. Kawanishi, A Pharmacometric Approach to Substitute for a Conventional Dose-Finding Study in Rare Diseases: Example of Phase III Dose Selection for Emicizumab in Hemophilia A. *Clin Pharmacokinet* **57**, 1123-1134 (2018).

26. M. J. Borrok, Y. Wu, N. Beyaz, X. Q. Yu, V. Oganesyan, W. F. Dall'Acqua, P. Tsui, pH-dependent binding engineering reveals an FcRn affinity threshold that governs IgG recycling. *J Biol Chem* **290**, 4282-4290 (2015).

27. G. J. Robbie, R. Criste, W. F. Dall'acqua, K. Jensen, N. K. Patel, G. A. Losonsky, M. P. Griffin, A novel investigational Fc-modified humanized monoclonal antibody, motavizumab-YTE, has an extended half-life in healthy adults. *Antimicrob Agents Chemother* **57**, 6147-6153 (2013).

28. A. Muto, K. Yoshihashi, M. Takeda, T. Kitazawa, T. Soeda, T. Igawa, Z. Sampei, T. Kuramochi, A. Sakamoto, K. Haraya, K. Adachi, Y. Kawabe, K. Nogami, M. Shima, K. Hattori, Anti-factor IXa/X bispecific antibody ACE910 prevents joint bleeds in a long-term primate model of acquired hemophilia A. *Blood* **124**, 3165-3171 (2014).

Acknowledgments: We thank the donors and patients who consented to the use of their cells for the studies. We also thank to colleagues in Chugai Pharmacutical Co., Ltd. and Chugai Research Institute for Medical Science Inc; S. Naoi, S. Nagatomo, Y. Wakahara, K. Komura, Y. Kaneta, S. Komatsu, B. Juan, Y. Ikeda, N. Sato, M. Uematsu, A. Ohba, Y. Oyama, A. Maeno, K. Matsunaga, S. Hiranabe, T. Kato for antibody preparation and characterization; S. Kadono, T. Torizawa, J. Okude for structural analysis; F. Isomura, Y. Ochiai for in vitro experiments; R. Saito, M. Namba, T. Hasegawa, M. Fukazawa, H. Azabu, M. Hiranuma, H. Arakawa, R. Takemoto, Y. Miura, T. Koike, T. Nakatogawa, H. Tai for in vivo experiments.

Funding: This study was funded and supported by Chugai Pharmaceutical Co., Ltd.

Author contributions: Y.T.-I., T.S., H. Koga, Kazuki Y., H. Sanada, N.S., A. Matsusaki, and T.K. wrote the manuscript; Y.T.-I, H. Koga, K.K., K.E., M.F., M.I., Y.I., M.O., M.S., and T.W. designed, produced, and characterized the antibodies; T.S., Kazuki Y., E.J., A. Muto, and K.N. executed the pharmacological studies and analyzed the assay data; K.A., S.I., S.i.-K., H. Sanada, N.S., and Koji Y. executed the PK and toxicological studies and analyzed the assay data; Z.S., H. Shiraiwa, H. Konishi, Y.K., K.H., T.K., and T.I. designed and supervised the studies.

Competing interests: Chugai Pharmaceutical Co., Ltd. is developing NXT007 as a clinical compound. Y.T.-I, T.S., H. Koga, Kazuki Y., K.K., K.E., K.A., M.F., M.I., Y.I., S.I., E.J., S.i.-K., A. Muto, K.N., M.O., H. Sanada, M.S., N.S., T.W., Koji Y., A. Matsusaki, H. Z.S., Shiraiwa, H. Konishi, Y.K., K.H., T.K., and T.I. are employees of Chugai Pharmaceutical Co., Ltd.; Chugai Pharmaceutical Co., Ltd. has filed patent applications related to this work for NXT007. Y.I.-T, T.S., H. Koga, Kazuki Y., K.K., and T.I. are inventors on patent application

(WO/2019/065795) submitted by Chugai Pharmaceutical Co., Ltd. that covers the NXT007 molecule.

Data and materials availability: Materials are available from Chugai Pharmaceutical Co., Ltd. under a material transfer agreement.

Figures

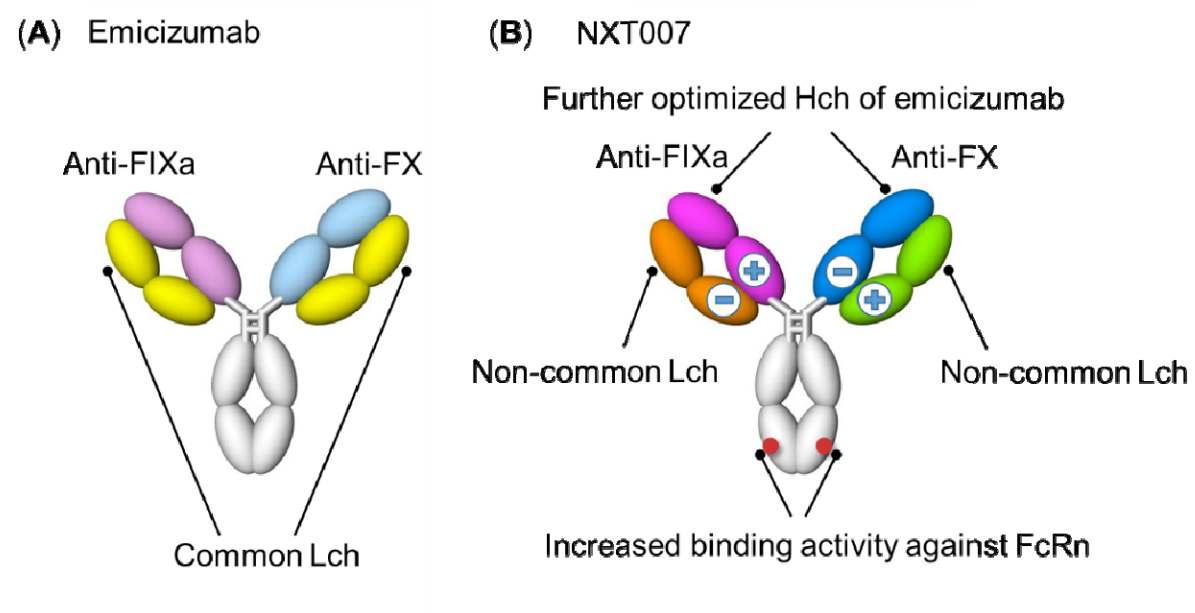


Fig. 1. Molecular features of NXT007 and emicizumab

(A) Schematic illustration of emicizumab structure. The two Fab arms share a common light chain (Lch), depicted in yellow.

(B) Schematic illustration of NXT007 structure and the introduced mutations. Optimized emicizumab-derived heavy chains and two distinct light chains from human naïve antibody libraries. Charged residue mutations are inserted into the C_H1/C_L interface for correct paring of heavy chain (Hch) and light chain (Lch).

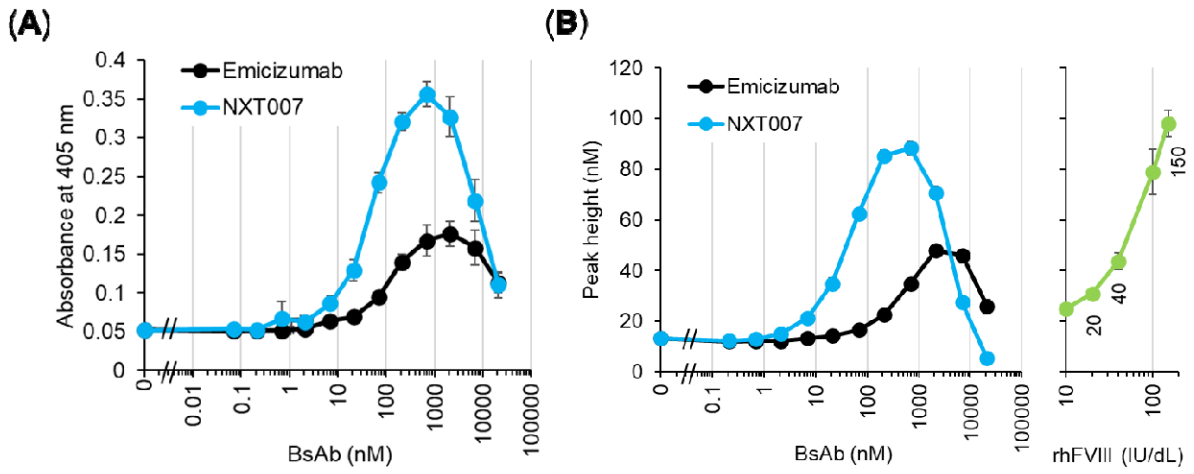


Fig. 2. In vitro FVIIIa-mimetic cofactor activity of NXT007

(A) Effect of NXT007 or emicizumab on FIXa-catalyzed FX activation in an enzymatic assay using purified coagulation factors. Data are expressed as mean ± SD (n = 3)

(B) Effect of NXT007, emicizumab or rhFVIII on thrombin generation using FVIII-deficient patient plasma. The reaction was triggered by TF. Data are expressed as mean ± SD (n = 3)

(A)

Analyte	k_a(/Ms)	k_d(/s)	K_D (μM)
hFIX	1.50×10^4	1.62×10^{-2}	1.08
hFIXa	1.83×10^4	1.33×10^{-2}	0.728
hFX	3.10×10^5	1.66×10^{-2}	0.0538
hFXa	4.08×10^5	9.42×10^{-3}	0.0231
cyFIX	2.67×10^4	9.09×10^{-2}	3.51
cyFX	6.79×10^4	2.25×10^{-2}	0.331

(B)

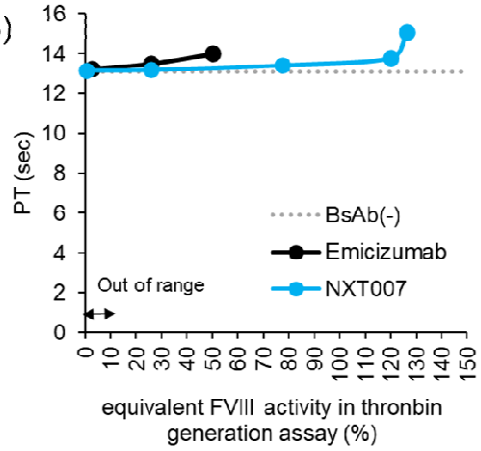


Fig. 3. Antigen binding affinities and effect on prothrombin time of NXT007

(A) Antigen binding affinity of NXT007 to hFIX/hFIXa, hFX/hFXa, cyFIX and cyFX using surface plasmon resonance (SPR). The k_a, k_d and K_D values are expressed as average of three experiments. The affinity of emicizumab was previously reported (*14*).

(B) Effect of NXT007 or emicizumab on prothrombin time (PT) using FVIII-deficient hemophilia A plasma. Equivalent-FVIII activity of NXT007 and emicizumab was calculated using the peak height of the TG assay with the TF-triggering condition calibrated by the rhFVIII standard. The analysis was carried out below the concentration range which reached maximum thrombin generation activity of NXT007 or emicizumab. Data are expressed as mean ± SD. (n = 3)

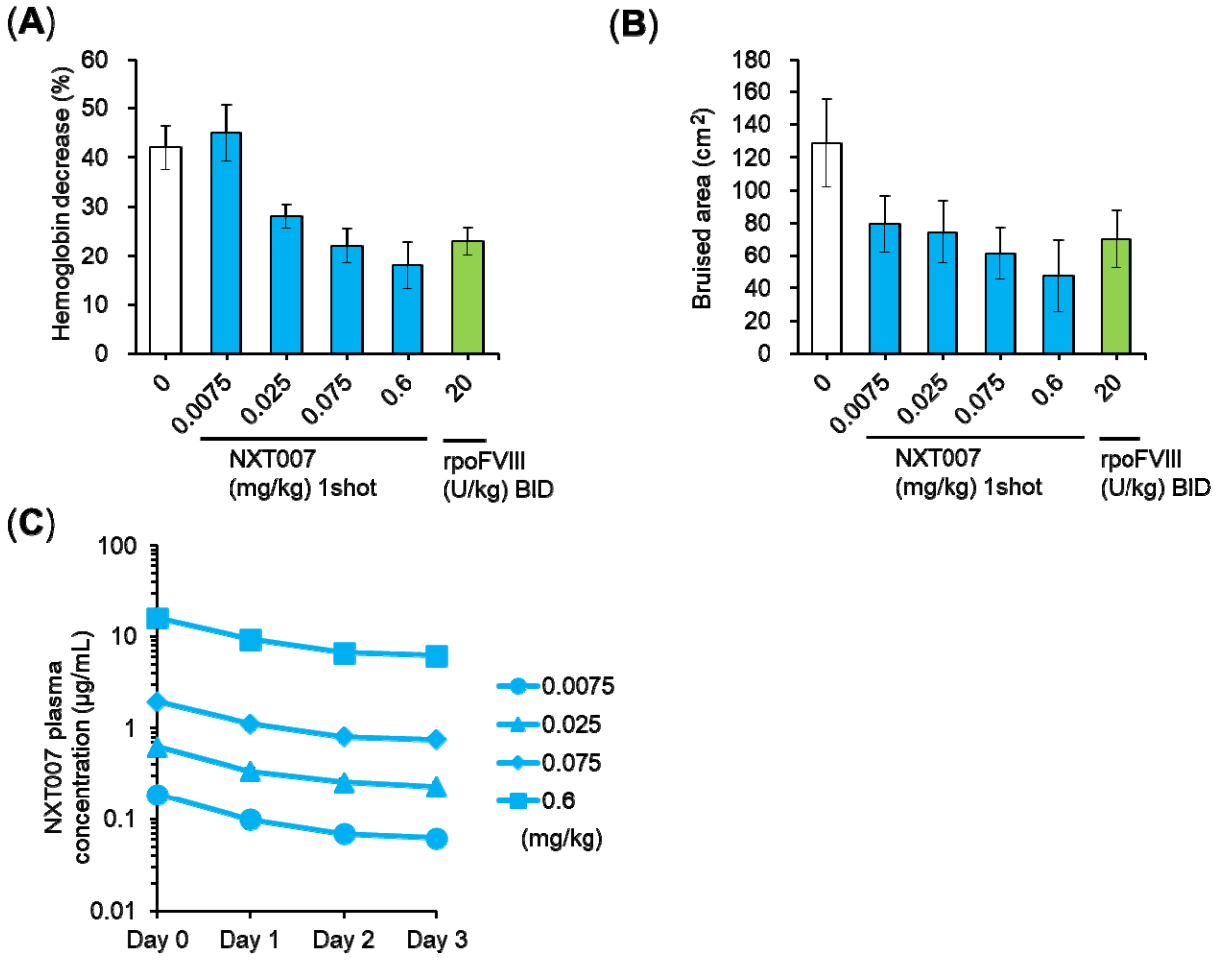
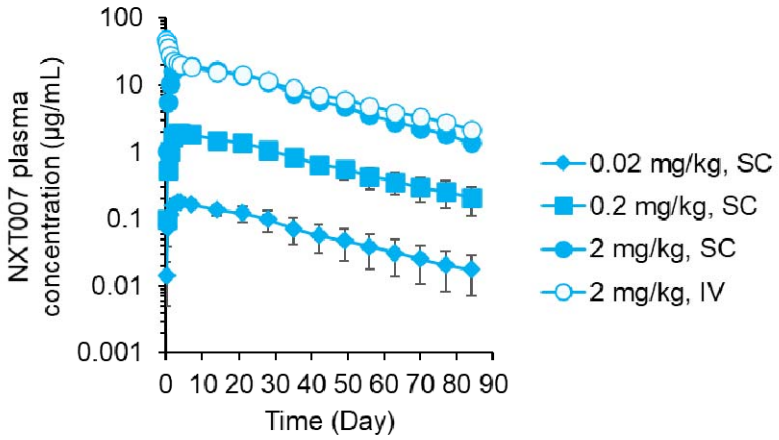


Fig. 4. In vivo hemostatic activity of NXT007

(A-C) Hemostatic activity of NXT007 or rpoFVIII on acquired hemophilia A bleeding cynomolgus monkey model. Decrease of blood hemoglobin level (A) and bruised area on the skin (B) on Day 3 are shown. FVIII neutralization by cyVIII-2236 injection and bleeding induction are conducted on Day 0. Single administration for vehicle (n = 7, one animal was excluded from analysis due to lethal bleeding at Day2) and NXT007 groups (n = 6, each group), and twice daily administration for rpoFVIII groups (n = 6). Data are expressed as mean ± SE. **(C)** Time course of plasma NXT007 levels by ELISA. Data are expressed as mean ± SD. (n = 6)

(A)



(B)

	Dose (mg/kg)	$t_{1/2}$ (day)	C_{max} (μg/mL)	AUC_{inf} (μg・day/mL)	CL or CL/F (mL/day/kg)	F (%)
SC	0.02	21.9	0.181	6.84	3.37	82.0
SC	0.2	24.4	1.95	76.9	2.68	92.2
SC	2	19.6	21.1	704	2.85	84.4
IV	2	22.1	-	834	2.46	-

Fig. 5. Pharmacokinetic profile of NXT007

(A-B) Pharmacokinetic profile of NXT007. (A) Time course of plasma concentration of NXT007 in cynomolgus monkey after 0.02-2 mg/kg intravenous (IV) or 2 mg/kg subcutaneous (SC) injection. Data are expressed as mean ± SD. (n = 4, excluded ADA-positive samples) (B) Pharmacokinetics parameters. PK parameters were calculated after excluding sampling points (n = 4, 0.02, 0.2 mg/kg, n = 2, 2 mg/kg IV and SC). －: Not applicable