TRUNCATED VON WILLEBRAND FACTOR POLYPEPTIDES FOR TREATING HEMOPHILIA

FIELD OF THE INVENTION

The present invention relates to products and methods for improving treatment of blood coagulation disorders.

BACKGROUND OF THE INVENTION

There are various bleeding disorders caused by deficiencies of blood coagulation factors. The most common disorders are hemophilia A and B, resulting from deficiencies of blood coagulation Factor VIII (FVIII) and IX, respectively. Another known bleeding disorder is von Willebrand's disease (VWD).

In plasma FVIII exists mostly as a noncovalent complex with von Willebrand Factor (VWF), and its coagulant function is to accelerate Factor IXa dependent conversion of Factor X to Xa.

Classic hemophilia or hemophilia A is an inherited bleeding disorder. It results from a chromosome X-linked deficiency of blood coagulation FVIII, and affects almost exclusively males with an incidence of between one and two individuals per 10,000. The X-chromosome defect is transmitted by female carriers who are not themselves hemophiliacs. The clinical manifestation of hemophilia A is an increased bleeding tendency.

In severe hemophilia A patients undergoing prophylactic treatment FVIII has to be administered intravenously (i.v.) about 3 times per week due to the short plasma half-life of FVIII of about 12 to 14 hours. Each i.v. administration is cumbersome, associated with pain and entails the risk of an infection especially as this is mostly done at home by the patients themselves or by the parents of children having been diagnosed for hemophilia A.

It would thus be highly desirable to increase the half-life of FVIII so that pharmaceutical compositions containing such FVIII would have to be administered less frequently.

Several attempts have been made to prolong the half-life of non-activated FVIII either by reducing its interaction with cellular receptors (WO 03/093313 A2, WO 02/060951 A2), by covalently attaching polymers to FVIII (WO 94/15625, WO 97/11957 and US 4970300), by encapsulation of FVIII (WO 99/55306), by introduction of novel metal binding sites (WO 97/03193), by covalently attaching the A2 domain to the A3 domain either by peptidic (WO 97/40145 and WO 03/087355) or disulfide linkage (WO 02/103024A2) or by covalently attaching the A1 domain to the A2 domain (WO2006/108590).

Another approach to enhance the functional half-life of FVIII or VWF is by PEGylation of FVIII (WO 2007/126808, WO 2006/053299, WO 2004/075923) or by PEGylation of VWF (WO 2006/071801) The increased half-life of pegylated VWF would indirectly also enhance the half-life of FVIII present in plasma. Also fusion proteins of FVIII have been described (WO 2004/101740, WO2008/077616 and WO 2009/156137).

VWF, which is missing, functionally defect or only available in reduced quantity in different forms of von Willebrand disease (VWD), is a multimeric adhesive glycoprotein present in the plasma of mammals, which has multiple physiological functions. During primary hemostasis VWF acts as a mediator between specific receptors on the platelet surface and components of the extracellular matrix such as collagen. Moreover, VWF serves as a carrier and stabilizing protein for procoagulant FVIII. VWF is synthesized in endothelial cells and megakaryocytes as a 2813 amino acid precursor molecule. The amino acid sequence and the cDNA sequence of wild-type VWF are disclosed in Collins et al. 1987, Proc. Natl. Acad. Sci. USA 84:4393–4397. The precursor polypeptide, pre-pro-VWF, consists of an N-terminal 22-residue signal peptide, followed by a 741-residue pro-peptide and the 2050-residue polypeptide found in mature plasma VWF (Fischer et al., FEBS Lett. 351: 345-348, 1994). After cleavage of the signal peptide in the endoplasmatic reticulum a C-terminal disulfide bridge is formed between two monomers of VWF. During further transport through the secretory pathway 12 N-linked and 10 O-linked carbohydrate side chains are added. More important, VWF dimers are multimerized via N-terminal disulfide bridges and the propeptide of 741 amino acids length is cleaved off by the enzyme PACE/furin in the late Golgi apparatus.

Once secreted into plasma the protease ADAMTS13 can cleave high-molecular weight VWF multimers within the A1 domain of VWF. Plasma VWF therefore consists of a whole range of multimers ranging from single dimers of 500 kDa to multimers consisting of up to more than 20 dimers of a molecular weight of over 10,000 kDa. The VWF-HMWM hereby having the strongest hemostatic activity, which can be measured in ristocetin cofactor activity (VWF:RCo). The higher the ratio of VWF:RCo/VWF antigen, the higher the relative amount of high molecular weight multimers.

In plasma FVIII binds with high affinity to VWF, which protects it from premature elimination and thus, plays in addition to its role in primary hemostasis a crucial role to stabilize FVIII, regulate plasma levels of FVIII and as a consequence is also a central factor to control secondary hemostasis. The half-life of non-activated FVIII bound to VWF is about 12 to 14 hours in plasma. In von Willebrand disease type 3, where no or almost no VWF is present, the half-life of FVIII is only about 2 to 6 hours, leading to symptoms of mild to moderate hemophilia A in such patients due to decreased concentrations of FVIII. The stabilizing effect of VWF on FVIII has also been used to aid recombinant expression of FVIII in CHO cells (Kaufman et al. 1989, Mol Cell Biol 9:1233-1242).

VWF-derived polypeptides, in particular VWF fragments, have been described to stabilize FVIII in vitro and in vivo. WO 2013/106787 A1 is directed at chimeric proteins comprising certain VWF fragments and a FVIII protein. Those chimeric hetero-dimers of FVIII and VWF-fragment do have a fixed molar ratio of VWF to FVIII of 1:1. WO 2014/198699 A2 and WO 2013/083858 A2 describe VWF fragments and their use in the treatment of hemophilia. It was found that bioavailability of FVIIIs may be significantly improved upon extravascular co-administration with similar molar amounts of VWF fragments. High molar excess of VWF over FVIII was said to be not desirable, and in experiments with VWF fragments co-administered s.c. with FVIII it was found that the VWF dose was not critical for FVIII bioavailability. Thus molar ratios of VWF fragments over FVIII were limited to maximally 50:1 and preferred ranges to maximally 1.5:1. WO 2011/060242 A2 discloses fusion polypeptides comprising certain VWF fragments and an antibody Fc region proposing specific molar ratios of VWF fragment over FVIII of up to 10:1. WO2013/093760 A2 describes a method for preparing a protein, comprising co-expressing FVIII or VWF polypeptides, including truncated forms of VWF, with a recombinant α-2,3-sialyltransferase. Yee et al. (2014) Blood 124(3):445-452 found that a VWF fragment containing the D'D3 domains is sufficient to stabilize Factor VIII in VWF-deficient mice. However, although a VWF D'D3-Fc fusion protein exhibited markedly prolonged survival

when transfused into FVIII-deficient mice, the VWF D'D3-Fc fusion protein did not prolong the survival of co-transfused FVIII. The prior art is silent as to the benefit of exceeding certain ratios of administered VWF fragments over endogenous VWF for the purpose of prolonging the *in vivo* half-life of co-administered FVIII.

There is an ongoing need for methods increasing the half-life of FVIII and FVIII products with reduced administration frequency.

SUMMARY OF THE INVENTION

It has been found by the inventors that the *in vivo* half-life of Factor VIII can be prolonged by co-administration of a high molar excess of a truncated VWF polypeptide (polypeptide of the invention). The truncated VWF preferably comprises a half-life extending moiety. The high molar excess may be relative to the concentration of co-administered Factor VIII or relative to the concentration of endogenous VWF present in the treated subject. Without wishing to be bound to any theory, it is believed that it is important to achieve a high surplus of the administered truncated VWF and preferably the half-life-extended truncated VWF to minimize the binding of the co-administered FVIII to the endogenous VWF which has a larger molecular structure probably leading to an increased catabolism as compared to the truncated VWF. This technical effect is even more pronounced if the truncated VWF is not only administered in a ratio above 50 over a co-administered Factor VIII but also comprises a half-life extending moiety.

The present invention therefore relates to the following embodiments [1] to [73]:

[1] A polypeptide comprising a truncated von Willebrand Factor (VWF) for use in the treatment of a blood coagulation disorder, said treatment comprising administering to a subject having endogenous VWF the polypeptide and a Factor VIII (FVIII), wherein the polypeptide is capable of binding to said FVIII, and wherein the molar ratio of the polypeptide to be administered to the FVIII to be administered is greater than 50.

[2] The polypeptide for use according to item [1], wherein the molar ratio of the polypeptide to be administered to the FVIII to be administered is greater than 100.

[3] The polypeptide for use according to item [1], wherein the molar ratio of the polypeptide to be administered to the FVIII to be administered is at least 200.

[4] The polypeptide for use according to item [1], wherein the molar ratio of the polypeptide to be administered to the FVIII to be administered is at least 300.

[5] The polypeptide for use according to item [1], wherein the molar ratio of the polypeptide to be administered to the FVIII to be administered is at least 400.

[6] The polypeptide for use according to item [1], wherein the molar ratio of the polypeptide to be administered to the FVIII to be administered is at least 500.

[7] The polypeptide for use according to any one of items [1] to [6], wherein the molar ratio of the polypeptide administered to the endogenous VWF is greater than 0.5.

[8] The polypeptide for use according to any one of items [1] to [6], wherein the molar ratio of the polypeptide administered to the endogenous VWF is greater than 1.

[9] The polypeptide for use according to any one of items [1] to [6], wherein the molar ratio of the polypeptide administered to the endogenous VWF is greater than 2.

[10] The polypeptide for use according to any one of items [1] to [6], wherein the molar ratio of the polypeptide administered to the endogenous VWF is greater than 4.

[11] The polypeptide for use according to any one of items [1] to [6], wherein the molar ratio of the polypeptide administered to the endogenous VWF is greater than 5.

[12] The polypeptide for use according to any one of items [1] to [6], wherein the molar ratio of the polypeptide administered to the endogenous VWF is greater than 7.

[13] The polypeptide for use according to any one of items [1] to [6], wherein the molar ratio of the polypeptide administered to the endogenous VWF is greater than 10.

[14] The polypeptide for use according to any one of items [1] to [6], wherein the molar ratio of the polypeptide administered to the endogenous VWF is greater than 25.

[15] The polypeptide for use according to any one of items [1] to [6], wherein the molar ratio of the polypeptide administered to the endogenous VWF is greater than 50.

[16] A polypeptide comprising a truncated von Willebrand Factor (VWF) for use in the treatment of a blood coagulation disorder, said treatment comprising administering to a subject having endogenous VWF the polypeptide and a Factor VIII (FVIII), wherein the

polypeptide is capable of binding to said FVIII, and wherein the molar ratio of the polypeptide administered to the endogenous VWF is greater than 0.5.

[17] The polypeptide for use according to item [16], wherein the molar ratio of the polypeptide to be administered to the FVIII to be administered is greater than 10.

[18] The polypeptide for use according to item [16], wherein the molar ratio of the polypeptide to be administered to the FVIII to be administered is greater than 20.

[19] The polypeptide for use according to item [16], wherein the molar ratio of the polypeptide to be administered to the FVIII to be administered is greater than 40.

[20] The polypeptide for use according to item [16], wherein the molar ratio of the polypeptide to be administered to the FVIII to be administered is greater than 50.

[21] The polypeptide for use according to item [16], wherein the molar ratio of the polypeptide to be administered to the FVIII to be administered is greater than 100.

[22] The polypeptide for use according to any one of the preceding items, which binds to the FVIII with an affinity characterized by a dissociation constant K_D of less than 1 nM.

[23] The polypeptide for use according to item [22], wherein the K_D ranges from 1 pM to 500 pM.

[24] The polypeptide for use according to item [22], wherein the K_D ranges from 10 pM to 200 pM.

[25] The polypeptide for use according to item [22], wherein the K_D ranges from 60 pM to 100 pM.

[26] The polypeptide for use according to any one of the preceding items, wherein the polypeptide is administered intravenously.

[27] The polypeptide for use according to any one of items [1] to [25], wherein the polypeptide is administered subcutaneously.

[28] The polypeptide for use according to any one of items [1] to [25], wherein the polypeptide is administered intramuscularly.

[29] The polypeptide for use according to any one of the preceding items, wherein the truncated VWF comprises (a) amino acids 776 to 805 of SEQ ID NO:4 or (b) an amino

acid sequence having a sequence identity of at least 90% to amino acids 776 to 805 of SEQ ID NO:4.

[30] The polypeptide for use according to any one of the preceding items, wherein the truncated VWF comprises (a) amino acids 766 to 864 of SEQ ID NO:4 or (b) an amino acid sequence having a sequence identity of at least 90% to amino acids 766 to 864 of SEQ ID NO:4.

[31] The polypeptide for use according to any one of the preceding items, wherein the truncated VWF comprises amino acids 764 to 1242 of SEQ ID NO:4.

[32] The polypeptide for use according to any one of the preceding items, wherein the truncated VWF consists of (a) amino acids 764 to 1242 of SEQ ID NO:4, (b) an amino acid sequence having a sequence identity of at least 90% to amino acids 764 to 1242 of SEQ ID NO:4, or (c) a fragment of (a) or (b).

[33] The polypeptide for use according to any one of the preceding items, wherein the truncated VWF lacks amino acids 1243 to 2813 of SEQ ID NO:4.

[34] The polypeptide for use according to any of the proceeding claims, wherein the polypeptide comprises a half-life extending moiety.

[35] The polypeptide for use according to item [34], wherein said half-life extending moiety is a heterologous amino acid sequence fused to the truncated VWF.

[36] The polypeptide for use according to item [35], wherein said heterologous amino acid sequence comprises or consists of a polypeptide selected from the group consisting of immunoglobulin constant regions and portions thereof, e.g. the Fc fragment, transferrin and fragments thereof, the C-terminal peptide of human chorionic gonadotropin, solvated random chains with large hydrodynamic volume known as XTEN, homo-amino acid repeats (HAP), proline-alanine-serine repeats (PAS), albumin, afamin, alpha-fetoprotein, Vitamin D binding protein, polypeptides capable of binding under physiological conditions to albumin or immunoglobulin constant regions, and combinations thereof.

[37] The polypeptide for use according to any one of items [34], wherein said half-life-extending moiety is conjugated to the polypeptide.

[38] The polypeptide for use according to item [37], wherein said half-life-extending moiety is selected from the group consisting of hydroxyethyl starch (HES), polyethylene

glycol (PEG), polysialic acids (PSAs), elastin-like polypeptides, heparosan polymers, hyaluronic acid and albumin binding ligands, e.g. fatty acid chains, and combinations thereof.

[39] The polypeptide for use according to any one of the preceding items, wherein the polypeptide is a glycoprotein comprising N-glycans, and wherein at least 75 % of said N-glycans comprise, on average, at least one sialic acid moiety.

[40] The polypeptide for use according to item [39], wherein at least 85 % of said N-glycans comprise, on average, at least one sialic acid moiety.

[41] The polypeptide for use according to items [39], wherein at least 95 % of said N-glycans comprise, on average, at least one sialic acid moiety.

[42] The polypeptide for use according to any one of the preceding items wherein the polypeptide comprises N-glycans, wherein less than 35%, preferably less than 34%, preferably less than 33%, preferably less than 32%, preferably less than 31%, preferably less than 30%, preferably less than 29%, preferably less than 28%, preferably less than 27% preferably less than 26%, preferably less than 25%, preferably less than 24%, preferably less than 23%, preferably less than 22%, preferably less than 21%, preferably less than 20%, preferably less than 19%, preferably less than 18%, preferably less than 17%, preferably less than 16%, preferably less than 15%, preferably less than 14%, preferably less than 13%, preferably less than 12%, preferably less than 11%, preferably less than 10%, preferably less than 9%, preferably less than 8%, preferably less than 7%, preferably less than 6% and preferably less than 5% of said N-glycans comprise, on average, two or more terminal and non-sialylated galactose residues.

[43] The polypeptide for use according to any one of the preceding items wherein the polypeptide comprises N-glycans, wherein less than 6%, preferably less than 5%, preferably less than 4%, preferably less than 3%, preferably less than 2%, and preferably less than 1% of said N-glycans comprise, on average, three or more terminal and non-sialylated galactose residues.

[44] The polypeptide for use according to any one of the preceding items, wherein the polypeptide is a dimer.

[45] The polypeptide for use according to item [44], wherein the two monomers forming the dimer are covalently linked to each other via one or more disulfide bridges formed by cysteine residues within the truncated VWF.

[46] The polypeptide for use according to item [45], wherein the cysteine residues forming the one or more disulfide bridges is/are selected from the group consisting of Cys-1099, Cys-1142, Cys-1222, Cys-1225, Cys-1227 and combinations thereof, wherein the amino acid numbering refers to SEQ ID NO:4.

[47] The polypeptide for use according to any one of items [44] to [46], wherein the affinity of said dimer to the FVIII is greater than the affinity of a monomeric polypeptide to said FVIII, said monomeric polypeptide having the same amino acid sequence as a monomeric subunit of the dimeric polypeptide.

[48] The polypeptide for use according to any one of the preceding items, wherein the mean residence time (MRT) and/or the terminal half-life of the FVIII is increased by the co-administration of the polypeptide as compared to a reference treatment, wherein said reference treatment is identical to said treatment, except that the polypeptide and the FVIII are administered in equimolar amounts in said reference treatment.

[49] The polypeptide for use according to item [48], wherein said increase in MRT and/or terminal half-life is at least 50%.

[50] The polypeptide for use according to any one of the preceding items, wherein the mean residence time (MRT) and/or terminal half-life of the FVIII is increased by the co-administration of the polypeptide as compared to a reference treatment with the FVIII alone.

[51] The polypeptide for use according to item [50], wherein said increase in MRT and/or terminal half-life is at least 50%.

[52] The polypeptide for use according to item [50], wherein said increase in MRT and/or terminal half-life is at least 100%.

[53] The polypeptide for use according to any one of the preceding items, wherein the clearance of the FVIII is decreased by the co-administration of the polypeptide as compared to a reference treatment with the FVIII alone.

[54] The polypeptide for use according to any one of the preceding items, wherein the clearance of the FVIII is decreased by the co-administration of the polypeptide as compared to a reference treatment, wherein said reference treatment is identical to said treatment, except that the polypeptide and the FVIII are administered in equimolar amounts in said reference treatment.

[55] The polypeptide for use according to item [53] or [54], wherein said decrease is at least 25%.

[56] The polypeptide for use according to item [53] or [54], wherein said decrease is at least 50%.

[57] The polypeptide for use according to item [53] or [54], wherein said decrease is at least 100%.

[58] The polypeptide for use according to any one of the preceding items, wherein the in vivo recovery of the FVIII is increased by the co-administration of the polypeptide as compared to a reference treatment with the FVIII alone.

[59] The polypeptide for use according to any one of the preceding items, wherein the frequency of administration of the FVIII is reduced as compared to a treatment with the FVIII alone.

[60] The polypeptide for use according to any one of the preceding items, wherein the MRT and/or plasma half-life of the polypeptide is greater than that of a reference polypeptide, wherein said reference polypeptide is endogenous VWF.

[61] The polypeptide for use according to any one of the preceding items, wherein the MRT and/or plasma half-life of the polypeptide is greater than that of a reference polypeptide that is identical to said polypeptide except that it lacks the half-life extending moiety.

[62] The polypeptide for use according to item [60] or [61], wherein the MRT and/or plasma half-life of the polypeptide is at least 25 % greater than that of the reference polypeptide.

[63] The polypeptide for use according to item [60] or [61], wherein the MRT and/or plasma half-life of the polypeptide is at least 50 % greater than that of the reference polypeptide.

[64] The polypeptide for use according to item [60] or [61], wherein the MRT and/or plasma half-life of the polypeptide is at least 75 % greater than that of the reference polypeptide.

[65] The polypeptide for use according to item [60] or [61], wherein the MRT and/or plasma half-life of the polypeptide is at least 100 % greater than that of the reference polypeptide.

[66] The polypeptide for use according to any one of the preceding items, wherein the subject is a human.

[67] A pharmaceutical composition comprising (i) a FVIII and (ii) a polypeptide as defined in any one of items [1] to [66], wherein the molar ratio of the polypeptide to the FVIII in the composition is greater than 50.

[68] A pharmaceutical composition comprising (i) a FVIII and (ii) a polypeptide as defined in any one of the items [1] to [66] for use in the treatment of a blood coagulation disorder, said treatment comprising administering to a subject having endogenous VWF the polypeptide and the FVIII, wherein the molar ratio of the polypeptide administered to the endogenous VWF is greater than 0.5.

[69] A pharmaceutical kit comprising (i) a FVIII and (ii) a polypeptide as defined in any one of items [1] to [66] for simultaneous, separate or sequential use in the treatment of a blood coagulation disorder, said treatment comprising administering to a subject having endogenous VWF the polypeptide and the FVIII, wherein the molar ratio of the polypeptide administered to the endogenous VWF is greater than 0.5.

[70] A pharmaceutical kit comprising (i) a FVIII and (ii) a polypeptide as defined in any one of items [1] to [66] for simultaneous, separate or sequential use in the treatment of a blood coagulation disorder, wherein the molar ratio of the polypeptide to be administered to the FVIII to be administered is greater than 50.

[71] The use of a polypeptide as defined in any one of items [1] to [66] for improving the plasma half-life of Factor VIII, and/or for reducing the frequency of administration of Factor VIII.

[72] A method of treating a blood coagulation disorder, comprising administering to a patient having endogenous VWF an effective amount of a polypeptide as defined in any one of items [1] to [66] and a FVIII, wherein the molar ratio of the polypeptide administered to the endogenous VWF immediately after administration of the polypeptide is greater than 0.5.

[73] A method of treating a blood coagulation disorder, comprising administering to a patient having endogenous VWF an effective amount of a polypeptide as defined in any one of items [1] to [66] and a FVIII, wherein the molar ratio of the polypeptide to be administered to the Factor VIII to be administered is greater than 50.

DESCRIPTION OF THE DRAWINGS

Fig. 1: Mean residence time, terminal half-life and clearance (mean) of D'D3-FP quantified by determining the albumin moiety in rats (Example 1).
Abbreviation: rVIII-SC: rVIII-SingleChain

Fig. 2: Mean residence time, terminal half-life and clearance (mean) of rVIII-SingleChain quantified by determining FVIII antigen in rats (Example 1).
Abbreviation: rVIII-SC: rVIII-SingleChain

Fig. 3: Mean residence time, terminal half-life and clearance (mean) of D'D3-FP quantified by determining the albumin moiety in rats (Example 2).
Abbreviation: rVIII-SC: rVIII-SingleChain

Fig. 4: Mean residence time, terminal half-life and clearance (mean) of rVIII-SingleChain quantified by determining FVIII antigen in rat (Example 2).
Abbreviation: rVIII-SC: rVIII-SingleChain

Fig. 5: Mean residence time, terminal half-life and clearance (individual animal data and mean) of D'D3-FP quantified by determining the albumin moiety in rabbits (Example 3).
Abbreviation: rVIII-SC: rVIII-SingleChain

Fig. 6: Mean residence time, terminal half-life and clearance (individual animal data and mean) of rVIII-SingleChain quantified by determining FVIII antigen in rabbits (Example 3).
Abbreviation: rVIII-SC: rVIII-SingleChain

Fig. 7: Mean residence time, terminal half-life and clearance (individual animal data and mean) of different recombinant FVIII products quantified by determining FVIII antigen in rabbits (Example 4).
Abbreviation: rVIII-SC: rVIII-SingleChain

Fig. 8: Changes of mean residence time, terminal half-life and clearance of FVIII in dependence of the molar ratio of D'D3-FP over rVIII-SingleChain (rVIII-SC given alone is defined as 1-fold change) in rats and rabbits.
Abbreviation: rVIII-SC: rVIII-SingleChain

Fig. 9: Changes of mean residence time, terminal half-life and clearance of FVIII in dependence of the molar ratio of D'D3-FP over endogenous VWF (rVIII-SC given alone is defined as 1-fold change) in rats and rabbits.

DETAILED DESCRIPTION

In a first aspect, the present invention relates to a polypeptide comprising a truncated von Willebrand Factor (VWF) for use in the treatment of a blood coagulation disorder, said treatment comprising administering to a subject having endogenous VWF the polypeptide and a Factor VIII (FVIII), wherein the polypeptide is capable of binding to said FVIII, and wherein the molar ratio of the polypeptide to be administered to the FVIII to be administered is greater than 50. In preferred embodiments the polypeptide comprises a half-life extending moiety.

In a second aspect, the present invention pertains to a polypeptide comprising a polypeptide comprising a truncated von Willebrand Factor (VWF), for use in the treatment of a blood coagulation disorder, said treatment comprising administering to a subject having endogenous VWF the polypeptide and a Factor VIII (FVIII), wherein the polypeptide is capable of binding to said FVIII, and wherein the molar ratio of the polypeptide administered to the endogenous VWF is greater than 0.5. In preferred embodiments the polypeptide comprises a half-life extending moiety.

The polypeptide comprising a truncated von Willebrand Factor (VWF) will be referred to herein as "polypeptide of the invention". The polypeptide of the invention preferably comprises a half-life extending moiety.

Ratios

As described in more detail below, the polypeptide of the invention may be a monomer, a dimer, or a mixture thereof. Any molar ratios according to the invention refer to a ratio of

the molar concentration of the monomeric subunit of the polypeptide of the invention, whether actually present as monomer or dimer. Ratios are formed either over the molar concentration of the co-administered FVIII or over the molar concentration of the endogenous VWF monomeric subunits. Any ratios of polypeptide of the invention over FVIII in this application refer to the amount of polypeptide of the invention to be administered (in mole) divided by the amount of FVIII to be administered (in mole), unless indicated otherwise. The endogenous VWF is the VWF which is naturally present in the plasma of the animal or human being to be dosed with the polypeptide of the invention and with the co-administered FVIII. It usually consists of a range of different oligomers of approximately 2 to 40 monomeric subunits of VWF. Unless indicated otherwise, any ratios of polypeptide of the invention over endogenous VWF in this application refer to the molar plasma concentration of polypeptide of the invention immediately after administration of the polypeptide of the invention, divided by the molar plasma concentration of endogenous VWF monomeric subunits (endogeneous VWF) The molar plasma concentration of the polypeptide of the invention immediately after administration of the polypeptide of the invention is calculated assuming a dilution of the polypeptide of the invention administered directly after administration in a plasma volume of 40 mL/kg. The amount of the polypeptide of the invention immediately after administration when administered intravenously is assumed for the purposes of the invention to be identical to the amount administered.

According to one aspect of the invention the molar ratio of the polypeptide of the invention to the endogenous VWF is greater than 0.5. The concentration of endogenous VWF in the plasma of the subject to be treated can be determined by an ELISA or and activity assay, e.g. as described in the Examples. Typically, the concentration measured will be given in U/mL. This value can be converted into a molarity as described in the following.

Normal human plasma (NHP) contains VWF in a concentration of 1 U/mL or 100% by definition. This corresponds to a protein concentration of approximately 10µg/mL (Haberichter S.L. and Montgomery R.R., Structure and function of von Willebrand factor; in: Hemostasis and Thrombosis, eds. Marder, Aird, Bennett, Schulman and White, Lippincott Williams & Wilkins 2013, pp 197-207). Based on this VWF concentration in NHP and a molecular weight of the mature VWF monomer of approximately 267,500 Da including 18-19% of glycosylation a molar plasma concentration of the VWF monomer unit of approximately 37 x 10^{-9} Mol/L can be calculated for NHP.

For calculation of the molar concentrations of rat or rabbit VWF subunits in normal rat or rabbit plasma, respectively, a molecular weight of the monomeric subunit comparable to human VWF was used (267,500 Da) together with an assumed comparable specific activity (100 U/mg) and the measured endogenous VWF activities in rat or rabbit plasma (refer also to examples).

The concentration of VWF in the human population varies from about 60% to about 200% of VWF concentration in NHP. In certain embodiments of the invention the concentration of endogenous VWF is defined as the concentration in NHP. In other embodiments the concentration of endogenous VWF is determined in the subject to be treated, and the dose of the polypeptide is based on this individual value.

The molar ratio of the polypeptide of the invention administered to the endogenous VWF is preferably at least 2, or at least 3, or at least 4, or at least 5, or at least 6, or at least 7, or at least 8, or at least 9, or at least 10, more preferably at least 15, or at least 20, or at least 25, or at least 30, most preferably at least 40, or at least 50, or at least 75.

The molar ratio of the polypeptide of the invention to be administered to the endogenous VWF may range from 0.5 to 1,000, or from 1 to 500, or from 2 to 400, or from 3 to 300, or from 4 to 250, or from 5 to 200, or from 6 to 150, or from 7 to 140, or from 8 to 130, or from 9 to 120, or from 10 to 110. Preferably, the molar ratio of the polypeptide of the invention administered to endogenous VWF ranges from 3 to 100, or from 4 to 90, or from 5 to 80, or from 6 to 75, or from 10 to 60.

The molar ratio of the polypeptide of the invention to be administered to FVIII to be administered is preferably at least 2, or at least 5, or at least 10, or at least 20, or at least 30, or at least 40, or at least 50, more preferably the ratio is greater than 50, or at least 75, at least 100, or greater than 100, or at least 200, most preferably at least 300, or at least 400, or at least 500, or at least 600, or at least 700, or at least 800, or at least 900, or at least 1,000, or at least 1,100, or at least 1,200, or at least 1,300, or at least 1,400, or at least 1,500, or at least 1,600, or at least 1,700, or at least 1,800, or at least 1,900, or at least 2,000, or at least 2,500, or at least 3,000 or at least 5,000, or at least 8,000 or up to 10,000.

The molar ratio of the polypeptide of the invention to be administered to FVIII to be administered may range from 2 to 10,000, or from 5 to 5,000, or from 10 to 4,000, or from 20 to 3,000, or from 30 to 2,000, or from 40 to 1,000. Preferably, the molar ratio of the

polypeptide of the invention to be administered to FVIII to be administered ranges from 60 to 2,500, or from 110 to 2,000, or from 150 to 1,500, or from 200 to 1,000.

Table 1 summarizes various embodiments of the treatment in accordance with this invention. In a given embodiment, both requirements of column 2 and 3, respectively, must be fulfilled.

Table 1:

Embodiment #	Molar ratio polypeptide of the invention : endogenous VWF	Molar ratio polypeptide of the invention : FVIII administered
1	at least 1	at least 2
2	at least 1	at least 5
3	at least 1	at least 10
4	at least 1	at least 40
5	at least 1	at least 50
6	at least 1	at least 80
7	at least 1	at least 100
6	at least 1	at least 150
7	at least 1	at least 250
8	at least 1	at least 400
9	at least 1	at least 800
10	at least 1	at least 1,000
11	at least 3	at least 2
12	at least 3	at least 5
13	at least 3	at least 10
14	at least 3	at least 40
15	at least 3	at least 50
16	at least 3	at least 80
17	at least 3	at least 100
18	at least 3	at least 150
19	at least 3	at least 250
20	at least 3	at least 400
21	at least 3	at least 800
22	at least 3	at least 1,000
23	at least 5	at least 2

Embodiment #	Molar ratio polypeptide of the invention : endogenous VWF	Molar ratio polypeptide of the invention : FVIII administered
24	at least 5	at least 5
25	at least 5	at least 10
26	at least 5	at least 40
27	at least 5	at least 50
28	at least 5	at least 80
29	at least 5	at least 100
30	at least 5	at least 150
31	at least 5	at least 250
32	at least 5	at least 400
33	at least 5	at least 800
34	at least 5	at least 1,000
35	at least 10	at least 2
36	at least 10	at least 5
37	at least 10	at least 10
38	at least 10	at least 40
39	at least 10	at least 50
40	at least 10	at least 80
41	at least 10	at least 100
42	at least 10	at least 150
43	at least 10	at least 250
44	at least 10	at least 400
45	at least 10	at least 800
46	at least 10	at least 1,000
47	at least 20	at least 2
48	at least 20	at least 5
49	at least 20	at least 10
50	at least 20	at least 40
51	at least 20	at least 50
52	at least 20	at least 80
53	at least 20	at least 100
54	at least 20	at least 150
55	at least 20	at least 250
56	at least 20	at least 400
57	at least 20	at least 800

Embodiment #	Molar ratio polypeptide of the invention : endogenous VWF	Molar ratio polypeptide of the invention : FVIII administered
58	at least 20	at least 1,000
59	at least 50	at least 2
60	at least 50	at least 5
61	at least 50	at least 10
62	at least 50	at least 40
63	at least 50	at least 50
64	at least 50	at least 80
65	at least 50	at least 100
66	at least 50	at least 150
67	at least 50	at least 250
68	at least 50	at least 400
69	at least 50	at least 800
70	at least 50	at least 1,000
71	at least 50	at least 2,000
72	at least 50	at least 4,000

Embodiments 1 to 72 shown in Table 1 can be combined with any other embodiment and aspect of the invention described herein. Further details of the treatment in accordance with the invention are described further below.

The truncated VWF

The term "von Willebrand Factor" (VWF) as used herein includes naturally occurring (native) VWF, but also variants thereof retaining at least the FVIII binding activity of naturally occurring VWF, e.g. sequence variants where one or more residues have been inserted, deleted or substituted. The FVIII binding activity is determined by a FVIII-VWF binding assay as described in Example 6.

The preferred VWF in accordance with this invention is human VWF represented by the amino acid sequence shown in SEQ ID NO:4. The cDNA encoding SEQ ID NO:4 is shown in SEQ ID NO:3.

The gene encoding human native VWF is transcribed into a 9 kb mRNA which is translated into a pre-propolypeptide of 2813 amino acids with an estimated molecular weight of 310,000 Da. The pre-propolypeptide contains an N-terminal 22 amino acids

signal peptide, followed by a 741 amino acid pro-polypeptide (amino acids 23-763 of SEQ ID NO:4) and the mature subunit (amino acids 764-2813 of SEQ ID NO:4). Cleavage of the 741 amino acids propolypeptide from the N-terminus results in mature VWF consisting of 2050 amino acids. The amino acid sequence of the human native VWF pre-propolypeptide is shown in SEQ ID NO:4. Unless indicated otherwise, the amino acid numbering of VWF residues in this application refers to SEQ ID NO:4, even if the VWF molecule does not comprise all residues of SEQ ID NO:4.

The propolypeptide of native VWF comprises multiple domains. Different domain annotations can be found in the literature (see, e.g. Zhou et al. (2012) Blood 120(2): 449-458). The following domain annotation of native pre-propolypeptide of VWF is applied in this application:

D1-D2-D'-D3-A1-A2-A3-D4-C1-C2-C3-C4-C5-C6-CK

With reference to SEQ ID NO:4, the D' domain consists of amino acids 764-865; and the D3 domain consists of amino acids 866-1242.

The feature "truncated" means that the polypeptide does not comprise the entire amino acid sequence of mature VWF (amino acids 764-2813 of SEQ ID NO:4). Typically, the truncated VWF does not comprise all amino acids 764-2813 of SEQ ID NO:4 but only a fragment thereof. A truncated VWF may also be referred to as a VWF fragment, or in the plural as VWF fragments.

Typically, the truncated VWF is capable of binding to a Factor VIII. Preferably, the truncated VWF is capable of binding to the mature form of human native Factor VIII. In another embodiment, the truncated VWF is capable of binding to the single-chain Factor VIII consisting of the amino acid sequence SEQ ID NO:5. Binding of the truncated VWF to Factor VIII can be determined by a FVIII-VWF binding assay as described in Example 6.

The truncated VWF of the present invention preferably comprises or consists of an amino acid sequence having a sequence identity of at least 90% to amino acids 776 to 805 of SEQ ID NO:4 and is capable of binding to FVIII. In preferred embodiments the truncated VWF comprises or consists of an amino acid sequence having a sequence identity of at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, to amino acids 776 to 805 of SEQ ID NO:4 and is capable of binding to FVIII. Most preferably, the truncated VWF comprises or consists of amino acids 776 to 805 of SEQ ID NO:4. Unless indicated

otherwise herein, sequence identities are determined over the entire length of the reference sequence (e.g. amino acids 776 to 805 of SEQ ID NO:4).

The truncated VWF of the present invention preferably comprises or consists of an amino acid sequence having a sequence identity of at least 90% to amino acids 766 to 864 of SEQ ID NO:4 and is capable of binding to FVIII. In preferred embodiments the truncated VWF comprises or consists of an amino acid sequence having a sequence identity of at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, to amino acids 766 to 864 of SEQ ID NO:4 and is capable of binding to FVIII. Most preferably, the truncated VWF comprises or consists of amino acids 766 to 864 of SEQ ID NO:4.

In another preferred embodiment, the truncated VWF consists of (a) an amino acid sequence having a sequence identity of at least 90% to amino acids 764 to 1242 of SEQ ID NO:4, or (b) a fragment thereof, provided that the truncated VWF is still capable of binding to FVIII. More preferably, the truncated VWF consists of (a) an amino acid sequence having a sequence identity of at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, to amino acids 764 to 1242 of SEQ ID NO:4, or (b) a fragment thereof, provided that the truncated VWF is still capable of binding to FVIII. Most preferably, the truncated VWF consists of (a) amino acids 764 to 1242 of SEQ ID NO:4, or (b) a fragment thereof, provided that the truncated VWF is still capable of binding to FVIII.

As described in more detail below, the polypeptide may be prepared by a method which uses cells comprising a nucleic acid encoding the polypeptide comprising the truncated VWF. The nucleic acid is introduced into suitable host cells by techniques that are known *per se*.

In a preferred embodiment, the nucleic acid in the host cell encodes (a) an amino acid sequence having a sequence identity of at least 90% to amino acids 1 to 1242 of SEQ ID NO:4, or (b) a fragment thereof, provided that the truncated mature VWF is still capable of binding to FVIII. More preferably, the nucleic acid encodes (a) an amino acid sequence having a sequence identity of at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, to amino acids 1 to 1242 of SEQ ID NO:4, or (b) a fragment thereof, provided that the truncated VWF is still capable of binding to FVIII. Most preferably, the nucleic acid encodes (a) amino acids 1 to 1242 of SEQ ID NO:4, or (b) a fragment thereof, provided that the truncated VWF is still capable of binding to FVIII. Especially if the polypeptide in accordance with this invention is a dimer, the nucleic acid will comprise a sequence

encoding amino acids 1 to 763 of VWF (e.g. SEQ ID NO:4), even if the truncated VWF in the polypeptide does not comprise amino acids 1 to 763 of VWF (e.g. SEQ ID NO:4).

In other embodiments the truncated VWF comprises or consists of one of the following amino acid sequences, each referring to SEQ ID NO:4:

776-805; 766-805; 764-805; 776-810; 766-810; 764-810; 776-815; 766-815; 764-815; 776-820; 766-820; 764-820; 776-825; 766-825; 764-825; 776-830; 766-830; 764-830; 776-835; 766-835; 764-835; 776-840; 766-840; 764-840; 776-845; 766-845; 764-845; 776-850; 766-850; 764-850; 776-855; 766-855; 764-855; 776-860; 766-860; 764-860; 776-864; 766-864; 764-864; 776-865; 766-865; 764-865; 776-870; 766-870; 764-870; 776-875; 766-875; 764-875; 776-880; 766-880; 764-880; 776-885; 766-885; 764-885; 776-890; 766-890; 764-890; 776-895; 766-895; 764-895; 776-900; 766-900; 764-900; 776-905; 766-905; 764-905; 776-910; 766-910; 764-910; 776-915; 766-915; 764-915; 776-920; 766-920; 764-920; 776-925; 766-925; 764-925; 776-930; 766-930; 764-930; 776-935; 766-935; 764-935; 776-940; 766-940; 764-940; 776-945; 766-945; 764-945; 776-950; 766-950; 764-950; 776-955; 766-955; 764-955; 776-960; 766-960; 764-960; 776-965; 766-965; 764-965; 776-970; 766-970; 764-970; 776-975; 766-975; 764-975; 776-980; 766-980; 764-980; 776-985; 766-985; 764-985; 776-990; 766-990; 764-990; 776-995; 766-995; 764-995; 776-1000; 766-1000; 764-1000; 776-1005; 766-1005; 764-1005; 776-1010; 766-1010; 764-1010; 776-1015; 766-1015; 764-1015; 776-1020; 766-1020; 764-1020; 776-1025; 766-1025; 764-1025; 776-1030; 766-1030; 764-1030; 776-1035; 766-1035; 764-1035; 776-1040; 766-1040; 764-1040; 776-1045; 766-1045; 764-1045; 776-1050; 766-1050; 764-1050; 776-1055; 766-1055; 764-1055; 776-1060; 766-1060; 764-1060; 776-1065; 766-1065; 764-1065; 776-1070; 766-1070; 764-1070; 776-1075; 766-1075; 764-1075; 776-1080; 766-1080; 764-1080; 776-1085; 766-1085; 764-1085; 776-1090; 766-1090; 764-1090; 776-1095; 766-1095; 764-1095; 776-1100; 766-1100; 764-1100; 776-1105; 766-1105; 764-1105; 776-1110; 766-1110; 764-1110; 776-1115; 766-1115; 764-1115; 776-1120; 766-1120; 764-1120; 776-1125; 766-1125; 764-1125; 776-1130; 766-1130; 764-1130; 776-1135; 766-1135; 764-1135; 776-1140; 766-1140; 764-1140; 776-1145; 766-1145; 764-1145; 776-1150; 766-1150; 764-1150; 776-1155; 766-1155; 764-1155; 776-1160; 766-1160; 764-1160; 776-1165; 766-1165; 764-1165; 776-1170; 766-1170; 764-1170; 776-1175; 766-1175; 764-1175; 776-1180; 766-1180; 764-1180; 776-1185; 766-1185; 764-1185; 776-1190; 766-1190; 764-1190; 776-1195; 766-1195; 764-1195; 776-1200; 766-1200; 764-1200; 776-1205; 766-1205; 764-1205; 776-1210; 766-1210; 764-1210; 776-1215; 766-1215; 764-1215; 776-1220; 766-1220; 764-1220; 776-1225; 766-1225; 764-1225; 776-1230; 766-1230; 764-1230; 776-1235; 766-1235; 764-1235; 776-1240; 766-1240; 764-1240; 776-1242; 766-1242; 764-1242; 764-1464; 764-1250; 764-1041; 764-828; 764-865; 764-1045; 764-1035; 764-1128; 764-1198; 764-1268; 764-1261; 764-1264; 764-1459; 764-1463; 764-1464; 764-1683; 764-1873; 764-1482; 764-1479; 764-1672; and 764-1874.

In certain embodiments the truncated VWF has an internal deletion relative to mature wild type VWF. For example, the A1, A2, A3, D4, C1, C2, C3, C4, C5, C6 domains or combinations thereof may be deleted, and the D' domain, the D3 domain and the CK domain is retained. In further embodiments the truncated VWF does not comprise the binding sites for platelet glycoprotein Ibα (GPIbα), collagen and/or integrin αIIbβIII (RGDS sequence within the C1 domain). In other embodiments, the truncated VWF does not comprise the cleavage site (Tyr1605-Met1606) for ADAMTS13 which is located at the central A2 domain of VWF. In yet another embodiment, the truncated VWF does not comprise the binding sites for GPIbα, and/or does not comprise the binding site for collagen, and/or does not comprise the binding site for integrin αIIbβIII, and/or it does not comprise the cleavage site (Tyr1605-Met1606) for ADAMTS13 which is located at the central A2 domain of VWF.

In other embodiments the truncated VWF comprises or consists of an amino acid sequence that has a sequence identity of at least 90%, or at least 91%, or at least 92%, or at least 93%, or at least 94%, or at least 95%, or at least 96%, or at least 97%, or at least 98%, or at least 99%, to one of the amino acid sequences recited in the preceding paragraph, provided that the truncated VWF is capable of binding to FVIII.

A polypeptide of the invention is termed a "dimer" in the present invention if two monomers of polypeptide of the invention are linked covalently. Preferably the two monomeric subunits are covalently linked via at least one disulfide bridge, e.g. by one, two, three or four disulfide bridges. The cysteine residues forming the at least one disulfide bridge are preferably located within the truncated VWF portion of the polypeptide of the invention. In one embodiment, these cysteine residues are Cys-1099, Cys-1142, Cys-1222, Cys-1225, or Cys-1227 or combinations thereof.

If the polypeptide of the invention is a dimer, the truncated VWF preferably comprises or consists of two polypeptides each with an amino acid sequence having a sequence identity of at least 90% to amino acids 764 to 1099, amino acids 764 to 1142, amino acids 764 to 1222, amino acids 764 to 1225, or amino acids 764 to 1227 of SEQ ID NO:4 and is capable of binding to FVIII. In preferred embodiments the truncated VWF comprises or consists of an amino acid sequence having a sequence identity of at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, to amino acids 764 to 1099, amino acids 764 to 1142, amino acids 764 to 1222, amino acids 764 to 1225, or amino acids 764 to 1227 of SEQ ID NO:4 and is capable of binding to FVIII. Most preferably, the truncated

VWF comprises or consists of amino acids 764 to 1099, amino acids 764 to 1142, amino acids 764 to 1222, amino acids 764 to 1225, or amino acids 764 to 1227 of SEQ ID NO:4.

The truncated VWF may be any one of the VWF fragments disclosed in WO 2013/106787 A1, WO 2014/198699 A2, WO 2011/060242 A2 or WO 2013/093760 A2, the disclosure of which is incorporated herein by reference.

The term "endogenous VWF" as used herein refers to monomeric subunits of VWF, independent of its degree of di- or oligomerization. For example in the determination of ratios according to the present invention a ratio formed by a certain number of molecules of a polypeptide of the invention divided by 1,000 molecules of decameric multimers of VWF would be the same as a ratio formed by the same number of molecules of polypeptides of the invention divided by 2,000 pentameric multimers of VWF.

Half-life extending moiety

In addition to the truncated VWF, the polypeptide of the invention may in certain preferred embodiments further comprise a half-life extending moiety. The half-life-extending moiety may be a heterologous amino acid sequence fused to the truncated VWF. Alternatively, the half-life-extending moiety may be chemically conjugated to the polypeptide comprising the truncated VWF by a covalent bond different from a peptide bond.

In certain embodiments of the invention, the half-life of the polypeptide of the invention is extended by chemical modification, e.g. attachment of a half-life extending moiety such as polyethylene glycol (PEGylation), glycosylated PEG, hydroxyl ethyl starch (HESylation), polysialic acids, elastin-like polypeptides, heparosan polymers or hyaluronic acid. In another embodiment, the polypeptide of the invention is conjugated to a HLEP such as albumin via a chemical linker. The principle of this conjugation technology has been described in an exemplary manner by Conjuchem LLC (see, e.g., US patent No. 7,256,253).

In other embodiments, the half-life-extending moiety is a half-life enhancing protein (HLEP). Preferably, the HLEP is an albumin or a fragment thereof. The N-terminus of the albumin may be fused to the C-terminus of the truncated VWF. Alternatively, the C-terminus of the albumin may be fused to the N-terminus of the truncated VWF. One or

more HLEPs may be fused to the N- or C-terminal part of VWF provided that they do not to interfere with or abolish the binding capability of the truncated VWF to FVIII.

In one embodiment the polypeptide has the following structure:

tVWF - L1 - H, [formula 1]

Wherein tVWF is the truncated VWF, L1 is a chemical bond or a linker sequence, and H is a HLEP.

L1 may be a chemical bond or a linker sequence consisting of one or more amino acids, e.g. of 1 to 50, 1 to 30, 1 to 20, 1 to 15, 1 to 10, 1 to 5 or 1 to 3 (e.g. 1 , 2 or 3) amino acids and which may be equal or different from each other. Usually, the linker sequences are not present at the corresponding position in the wild-type VWF. Examples of suitable amino acids present in L1 include Gly and Ser. The linker should be non-immunogenic and may be a non-cleavable or cleavable linker. Non-cleavable linkers may be comprised of alternating glycine and serine residues as exemplified in WO2007/090584. In another embodiment of the invention the peptidic linker between the truncated VWF moiety and the albumin moiety consists of peptide sequences, which serve as natural interdomain linkers in human proteins. Preferably such peptide sequences in their natural environment are located close to the protein surface and are accessible to the immune system so that one can assume a natural tolerance against this sequence. Examples are given in WO2007/090584. Cleavable linker sequences are described, e.g., in WO 2013/120939 A1.

Preferred HLEP sequences are described infra. Likewise encompassed by the invention are fusions to the exact "N-terminal amino acid" or to the exact "C-terminal amino acid" of the respective HLEP, or fusions to the "N-terminal part" or "C-terminal part" of the respective HLEP, which includes N-terminal deletions of one or more amino acids of the HLEP. The polypeptide may comprise more than one HLEP sequence, e.g. two or three HLEP sequences. These multiple HLEP sequences may be fused to the C-terminal part of VWF in tandem, e.g. as successive repeats.

Half-life enhancing polypeptides (HLEPs)

Preferably, the half-life extending moiety is a half-life extending polypeptide (HLEP), more preferably HLEP is selected from albumin or fragments thereof, immunoglobulin constant region and portions thereof, e.g. the Fc fragment, solvated random chains with large hydrodynamic volume (e.g. XTEN (Schellenberger et al. 2009; Nature Biotechnol. 27:1186-1190), homo-amino acid repeats (HAP) or proline-alanine-serine repeats (PAS), afamin, alpha-fetoprotein, Vitamin D binding protein, transferrin or variants thereof, carboxyl-terminal peptide (CTP) of human chorionic gonadotropin-ß subunit, polypeptides or lipids capable of binding under physiological conditions to albumin or immunoglobulin constant region.

A "half-life enhancing polypeptide" as used herein is preferably selected from the group consisting of albumin, a member of the albumin-family, the constant region of immunoglobulin G and fragments thereof, region and polypeptides capable of binding under physiological conditions to albumin, to members of the albumin family as well as to portions of an immunoglobulin constant region. It may be a full-length half-life-enhancing protein described herein (e.g. albumin, a member of the albumin-family or the constant region of immunoglobulin G) or one or more fragments thereof that are capable of stabilizing or prolonging the therapeutic activity or the biological activity of the coagulation factor. Such fragments may be of 10 or more amino acids in length or may include at least about 15, at least about 20, at least about 25, at least about 30, at least about 50, at least about 100, or more contiguous amino acids from the HLEP sequence or may include part or all of specific domains of the respective HLEP, as long as the HLEP fragment provides a functional half-life extension of at least 25% compared to the respective polypeptide without the HLEP.

The HLEP portion of the polypeptide of the invention may be a variant of a wild type HLEP. The term "variants" includes insertions, deletions and substitutions, either conservative or non-conservative, where such changes do not substantially alter the FVIII-binding activity of the truncated VWF.

In particular, the proposed VWF HLEP fusion constructs of the invention may include naturally occurring polymorphic variants of HLEPs and fragments of HLEPs. The HLEP may be derived from any vertebrate, especially any mammal, for example human, monkey, cow, sheep, or pig. Non-mammalian HLEPs include, but are not limited to, hen and salmon.

Albumin as HELP

The terms, "human serum albumin" (HSA) and "human albumin" (HA) and "albumin" (ALB) are used interchangeably in this application. The terms "albumin" and "serum albumin" are broader, and encompass human serum albumin (and fragments and variants thereof) as well as albumin from other species (and fragments and variants thereof).

As used herein, "albumin" refers collectively to albumin polypeptide or amino acid sequence, or an albumin fragment or variant, having one or more functional activities (e.g., biological activities) of albumin. In particular, "albumin" refers to human albumin or fragments thereof, especially the mature form of human albumin as shown in SEQ ID NO:6 herein or albumin from other vertebrates or fragments thereof, or analogs or variants of these molecules or fragments thereof.

In particular, the proposed polypeptides of the invention may include naturally occurring polymorphic variants of human albumin and fragments of human albumin. Generally speaking, an albumin fragment or variant will be at least 10, preferably at least 40, most preferably more than 70 amino acids long.

Preferred embodiments of the invention include albumin variants used as a HLEP of the polypeptide of the invention with enhanced binding to the FcRn receptor. Such albumin variants may lead to a longer plasma half-life of a truncated VWF albumin variant fusion protein as compared to a truncated VWF fusion with a wild-type albumin.

The albumin portion of the polypeptides of the invention may comprise at least one subdomain or domain of HA or conservative modifications thereof.

Immunoglobulins as HLEPs

Immunoglobulin G (IgG) constant regions (Fc) are known in the art to increase the half-life of therapeutic proteins (Dumont J A et al. 2006. BioDrugs 20:151-160). The IgG constant region of the heavy chain consists of 3 domains (CH1-CH3) and a hinge region. The immunoglobulin sequence may be derived from any mammal, or from subclasses IgG1, IgG2, IgG3 or IgG4, respectively. IgG and IgG fragments without an antigen-binding domain may also be used as HLEPs. The therapeutic polypeptide portion is connected to the IgG or the IgG fragments preferably via the hinge region of the antibody or a peptidic

linker, which may even be cleavable. Several patents and patent applications describe the fusion of therapeutic proteins to immunoglobulin constant regions to enhance the therapeutic protein's in vivo half-lives. US 2004/0087778 and WO 2005/001025 describe fusion proteins of Fc domains or at least portions of immunoglobulin constant regions with biologically active peptides that increase the half-life of the peptide, which otherwise would be quickly eliminated in vivo. Fc-IFN-β fusion proteins were described that achieved enhanced biological activity, prolonged circulating half-life and greater solubility (WO 2006/000448). Fc-EPO proteins with a prolonged serum half-life and increased in vivo potency were disclosed (WO 2005/063808) as well as Fc fusions with G-CSF (WO 2003/076567), glucagon-like peptide-1 (WO 2005/000892), clotting factors (WO 2004/101740) and interleukin-10 (U.S. Pat. No. 6,403,077), all with half-life enhancing properties.

Various HLEPs which can be used in accordance with this invention are described in detail in WO 2013/120939 A1.

N-Glycans and Sialylation of the polypeptide of the invention

The polypeptide of the invention preferably comprises N-glycans, and at least 75%, preferably at least 85%, more preferably at least 90% of said N-glycans comprise, on average, at least one sialic acid moiety. In preferred embodiments, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, of said N-glycans comprise, on average, at least one sialic acid moiety. The inventors found that polypeptides comprising highly sialylated VWF fragments not only have a prolonged half-life themselves, but are also capable to extend the half-life of co-administered FVIII. In other words, administration of the polypeptide of the invention leads to an extended half-life and/or to a reduced clearance of co-administered FVIII.

The polypeptide of the invention preferably comprises N-glycans, and at least 50% of the sialyl groups of the N-glycans of the glycoproteins are α-2,6-linked sialyl groups. In general, terminal sialyl groups can be attached to the galactose groups via a α-2,3- or via a α-2,6-linkage. Typically, N-glycans of the polypeptide of the invention comprise more α-2,6-linked sialyl groups than α-2,3-linked sialyl groups. Preferably, at least 60%, or at least 70%, or at least 80%, or at least 90% of the sialyl groups of the N-glycans are α-2,6-linked sialyl groups. These embodiments can be obtained by, e.g., co-expressing human α-2,6-sialyltransferase in mammalian cells.

In one embodiment, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, of the N-glycans of the polypeptide of the invention comprise at least one sialic acid group. In another embodiment, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, of the N-glycans of the polypeptide of the invention comprise at least one sialic acid group.

In another embodiment, less than 15%, less than 12%, less than 10%, or less than 8%, or less than 6%, or less than 5%, or less than 4%, or less than 3%, or less than 2% or even less than 1% of the N-glycans of the polypeptide of the invention are asialo-N-glycans, i.e. they are N-glycans lacking a sialic acid group. In another embodiment, less than 15%, less than 12%, less than 10%, or less than 8%, or less than 6%, or less than 5%, or less than 4%, or less than 3%, or less than 2% or even less than 1% of the N-glycans of the polypeptide of the invention are asialo-N-glycans, i.e. they do not have a sialic acid group.

Other embodiments of the invention comprise a truncated von Willebrand Factor (VWF), wherein said truncated VWF is capable of binding to a Factor VIII (FVIII), and wherein said glycoprotein comprises N-glycans, wherein less than 35%, preferably less than 34%, preferably less than 33%, preferably less than 32%, preferably less than 31%, preferably less than 30%, preferably less than 29%, preferably less than 28%, preferably less than 27% preferably less than 26%, preferably less than 25%, preferably less than 24%, preferably less than 23%, preferably less than 22%, preferably less than 21%, preferably less than 20%, preferably less than 19%, preferably less than 18%, preferably less than 17%, preferably less than 16%, preferably less than 15%, preferably less than 14%, preferably less than 13%, preferably less than 12%, preferably less than 11%, preferably less than 10%, preferably less than 9%, preferably less than 8%, preferably less than 7%, preferably less than 6% and preferably less than 5% of said N-glycans comprise, on average, two or more terminal and non-sialylated galactose residues.

Still other embodiments of the invention comprise a truncated von Willebrand Factor (VWF), wherein said truncated VWF is capable of binding to a Factor VIII (FVIII), and wherein said truncated VWF comprises N-glycans, wherein less than 6%, preferably less than 5%, preferably less than 4%, preferably less than 3%, preferably less than 2%, and preferably less than 1% of said N-glycans comprise, on average, three or more terminal and non-sialylated galactose residues.

The above-described embodiments can be combined with each other. Any percentages of N-glycans mentioned above, or any indications of the degree of sialylation, are to be understood as average percentages or degrees, i.e. they refer to a population of

molecules, not to a single molecule. It is clear that the glycosylation or sialylation of the individual glycoprotein molecules within a population of glycoproteins will show some heterogeneity.

Dimers

It has further been found that the polypeptides of this invention have a high proportion of dimers. The polypeptide of the invention is therefore preferably present as dimer. In one embodiment, at least 50%, or at least 60%, or at least 70%, or at least 80%, or at least 90%, or at least 95% or about 100% of the polypeptides are present as dimers. In another embodiment, the ratio dimer : monomer of the polypeptide of the invention is at least 1.5, preferably at least 2, more preferably at least 2.5 or at least 3. Most preferably all polypeptides of the invention are present as dimers. The use of dimers is favorable, as the dimer has an improved affinity to Factor VIII as compared to the monomer. The dimer content, and the ratio of dimer to monomer of the polypeptide of the invention can be determined as described in Example 1.

In one embodiment, the affinity of the polypeptide of the invention to Factor VIII is greater than that of human native VWF to the same Factor VIII molecule. The factor VIII affinity may refer to human native Factor VIII, or to the Factor VIII molecule characterized by SEQ ID NO:5.

It has been found that preparations of the polypeptide of this invention with a high proportion of dimers do have an increased affinity to Factor VIII. Such increased affinity to Factor VIII does lead to an enhanced stabilization of Factor VIII by the polypeptides of the present invention. Alternatively to or in combination with an increased dimer proportion also polypeptides in accordance with the invention with mutations within the Factor VIII binding domain which do increase the affinity to Factor VIII are preferred embodiments of the invention. Suitable mutations are disclosed, e.g., in WO 2013/120939 A1.

Preparation of the polypeptide

The nucleic acid encoding the polypeptide of the invention can be prepared according to methods known in the art. Based on the cDNA sequence of VWF (SEQ ID NO:3), recombinant DNA encoding the above-mentioned truncated VWF constructs or polypeptides of the invention can be designed and generated.

Even if the polypeptide which is secreted by the host cells does not comprise amino acids 1 to 763 of VWF, it is preferred that the nucleic acid (e.g. the DNA) encoding the intracellular precursor of the polypeptide comprises a nucleotide sequence encoding an amino acid sequence having a sequence identity of at least 95%, at least 96%, at least 97%, at least 98%, or at least 99%, to amino acids 23 to 763 or preferably to amino acids 1 to 763 of SEQ ID NO:4. Most preferably, the nucleic acid (e.g. the DNA) encoding the intracellular precursor of the polypeptide comprises a nucleotide sequence encoding amino acids 23 to 763 of SEQ ID NO:4, or amino acids 1 to 763 of SEQ ID NO:4.

Constructs in which the DNA contains the entire open reading frame inserted in the correct orientation into an expression plasmid may be used for protein expression. Typical expression vectors contain promoters that direct the synthesis of large amounts of mRNA corresponding to the inserted nucleic acid in the plasmid-bearing cells. They may also include an origin of replication sequence allowing for their autonomous replication within the host organism, and sequences that increase the efficiency with which the synthesized mRNA is translated. Stable long-term vectors may be maintained as freely replicating entities by using regulatory elements of, for example, viruses (e.g., the OriP sequences from the Epstein Barr Virus genome). Cell lines may also be produced that have integrated the vector into the genomic DNA, and in this manner the gene product is produced on a continuous basis.

Typically, the cells to be provided are obtained by introducing the nucleic acid encoding a polypeptide of the invention into mammalian host cells.

Any host cell susceptible to cell culture, and to expression of glycoproteins, may be utilized in accordance with the present invention. In certain embodiments, a host cell is mammalian. Non-limiting examples of mammalian cells that may be used in accordance with the present invention include BALB/c mouse myeloma line (NSO/ 1, ECACC No: 85110503); human retinoblasts (PER.C6 (CruCell, Leiden, The Netherlands)); monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., J. Gen Virol., 36:59, 1977); baby hamster kidney cells (BHK, ATCC CCL10); Chinese hamster ovary cells +/-DHFR (CHO, Urlaub and Chasin, Proc. Natl. Acad. Sci. USA, 77:4216, 1980); mouse sertoli cells (TM4, Mather, Biol. Reprod., 23:243 251, 1980); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1 587); human cervical carcinoma cells (HeLa, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human

lung cells (W138, ATCC CCL 75); human liver cells (HepG2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., Annals NY. Acad. Sci., 383:44-68, 1982); MRC 5 cells; PS4 cells; human amniocyte cells (CAP); and a human hepatoma line (Hep G2). Preferably, the cell line is a rodent cell line, especially a hamster cell line such as CHO or BHK.

Methods suitable for introducing nucleic acids sufficient to achieve expression of a glycoprotein of interest into mammalian host cells are known in the art. See, for example, Gething et al., Nature, 293:620-625, 1981; Mantei et al., Nature, 281:40-46, 1979; Levinson et al. EP 117,060; and EP 117,058. For mammalian cells, common methods of introducing genetic material into mammalian cells include the calcium phosphate precipitation method of Graham and van der Erb (Virology, 52:456-457, 1978) or the lipofectamine™ (Gibco BRL) Method of Hawley-Nelson (Focus 15:73, 1993). General aspects of mammalian cell host system transformations have been described by Axel in US. Pat. No. 4,399,216. For various techniques for introducing genetic material into mammalian cells, see Keown et al., Methods in Enzymology, 1989, Keown et al., Methods in Enzymology, 185:527-537, 1990, and Mansour et al., Nature, 336:348-352, 1988.

The cells are cultured under conditions that allow expression of the polypeptide. The polypeptide can be recovered and purified using methods that are known to the skilled artisan.

Terminal half-life, MRT and Clearance

Another aspect of the invention is the use of a polypeptide as defined hereinabove for increasing the terminal half-life or mean residence time (MRT) or reducing the clearance of Factor VIII. For evaluation of the pharmacokinetic data a linear pharmacokinetics model (compound elimination via the central compartment) was applied. Accordingly, any pharmacokinetic parameters used herein are based on a linear pharmacokinetics model (compound elimination via the central compartment), unless indicated otherwise.

The "half-life" T1/2(t) at a certain time t is the time it takes to halve the plasma concentration C(t) that is present at time t, i.e. $C [t + T1/2(t)] = C(t)/2$. The "terminal half-life" is the limit of T1/2(t) when t tends to infinity.

The terminal half-life of administered FVIII is increased by at least 25%, preferably by at least 50%, more preferably by at least 75%, more preferably by at least 100%, most preferably by at least 150%, if an effective amount of the polypeptide of the present

invention is co-administered, relative to administration of the FVIII alone. Another aspect of the invention is the use of a polypeptide as defined hereinabove for increasing the terminal half-life of Factor VIII.

The term "MRT", as used herein, means the average time a drug molecule (e.g. the polypeptide of the invention or a FVIII) resides in the body. In a linear pharmacokinetic system with constant clearance MRT can be calculated as the area under the first moment curve (AUMC) divided by the area under the plasma concentration-time curve (AUC). The first moment curve is time multiplied by plasma concentration at that time.

The MRT of administered FVIII is increased by at least 25%, preferably by at least 50%, more preferably by at least 75%, more preferably by at least 100%, most preferably by at least 150%, if an effective amount of the polypeptide of the present invention is co-administered, relative to administration of the FVIII alone. Another aspect of the invention is the use of a polypeptide as defined hereinabove for increasing the terminal half-life or mean residence time (MRT) or reducing the clearance of Factor VIII.

The term "clearance", as used herein, refers to the rate at which plasma is cleared of drug. Specifically, it is the current elimination rate of a drug divided by its current plasma concentration. In a linear pharmacokinetic system after a single intravenous administration the clearance can be calculated as the ratio of dose over the area under the plasma concentration-time curve (AUC), provided the clearance is constant. The lower the clearance the longer it takes until the plasma is cleared of the drug.

The clearance of administered FVIII is reduced by at least 10%, preferably by at least 25%, more preferably by at least 50%, more preferably by at least 60%, most preferably by at least 70%, if an effective amount of the polypeptide of the present invention is co-administered, relative to administration of the FVIII alone.

The invention further relates to a method of increasing the MRT or half-life, or to a method of reducing the clearance of Factor VIII *in vivo*, comprising administering to a subject an effective amount of a polypeptide as defined hereinabove.

The term "in vivo recovery of FVIII", as used herein, is defined as the percentage of FVIII, which is in the circulation extrapolated to t=0 in relation to the total amount of FVIII administered. As a basis for calculation of the expected FVIII concentration in the circulation a plasma volume of 40 mL per kg is assumed in general.

The invention further relates to the use of a polypeptide as defined hereinabove, e.g. but not limited to embodiments [01] to [72] detailed in Table 1 above, for increasing the in vivo recovery of FVIII. The in vivo recovery of FVIII is increased by at least 5%, preferably by at least 10%, more preferably by at least 15%, 18%, 20%, 25%, even more preferably by at least 26%, 27%, 28%, 29%, or 30%, most preferably by more than 35% or even more than 40% relative to the FVIII recovery without administration of the polypeptide.

A further aspect of this invention is a method of treating a blood coagulation disorder, comprising administering to a patient in need thereof an effective amount of a polypeptide as defined hereinabove.

A further aspect is the use of a polypeptide as defined hereinabove, e.g. by any of but not limited to embodiments [01] to [72] in Table 1 above, for reducing the frequency of administration of FVIII in a treatment of hemophilia A. The frequency of intravenous or subcutaneous administration of FVIII may be reduced to twice per week. Alternatively, the frequency of intravenous or subcutaneous administration of FVIII may be reduced to once per week, or even lower, e.g. to once per 10 days or once per 14 days. The FVIII may be administered twice weekly, every 5 days, once weekly, every 10 days, every two weeks, every three weeks, every four weeks or once a month, or in any range between any two of the foregoing values, for example from every four days to every month, from every 10 days to every two weeks, or from two to three times a week, etc.

Another aspect is the use of a polypeptide as defined hereinabove for reducing the dose of FVIII to be administered in a treatment of hemophilia A.

Treatment of coagulation disorder

The polypeptides of the invention are useful for treating coagulation disorders including hemophilia A. The term "hemophilia A" refers to a deficiency in functional coagulation FVIII, which is usually inherited.

Treatment of a disease encompasses the treatment of patients already diagnosed as having any form of the disease at any clinical stage or manifestation; the delay of the onset or evolution or aggravation or deterioration of the symptoms or signs of the disease; and/or preventing and/or reducing the severity of the disease.

A "subject" or "patient" to whom a polypeptide of the invention is administered preferably is a human. In certain aspects, the human is a pediatric patient. In other aspects, the human is an adult patient.

Compositions comprising a polypeptide of the invention and, optionally FVIII, are described herein. The compositions typically are supplied as part of a sterile, pharmaceutical composition that includes a pharmaceutically acceptable carrier. This composition can be in any suitable form (depending upon the desired method of administering it to a patient).

The term "Factor VIII" and "FVIII" are used interchangeably herein and encompass both plasma derived FVIII and recombinant FVIII. Recombinant FVIII encompasses without limitation full-length FVIII as well as two-chain B-domain deleted or truncated variants as well as single-chain B-domain deleted or truncated variants for example those described in WO 2004/067566 and other FVIII variants with mutations outside the B-domain but having the biological activity of FVIII.

The polypeptide of the invention can be administered to a patient by a variety of routes such as orally, transdermally, subcutaneously, intranasally, intravenously, intraperitoneally, intramuscularly, topically or locally. The most suitable route for administration in any given case will depend on the particular polypeptide, the subject, and the nature and severity of the disease and the physical condition of the subject. Typically, a polypeptide of the invention will be administered intravenously.

The polypeptide and the FVIII are preferably administered intravenously or subcutaneously.

In a first embodiment, both the polypeptide and the FVIII are administered intravenously. In a second embodiment, both the polypeptide and the FVIII are administered subcutaneously.

In another embodiment, the FVIII is administered intravenously, and the polypeptide is administered via a different route. In further embodiments, the polypeptide is administered subcutaneously, and the FVIII is administered via a different route. For example, the polypeptide may be administered subcutaneously, and the FVIII may be administered intravenously.

In further embodiments, the FVIII is administered subcutaneously, and the polypeptide is administered via a different route. In further embodiments, the polypeptide is administered intravenously, and the FVIII is administered via a different route. For example, the polypeptide may be administered intravenously, and the FVIII may be administered subcutaneously.

Determination of the total number of doses, and length of treatment with a polypeptide of the invention is well within the capabilities of those skilled in the art. The dosage of the polypeptide of the invention to be administered depends on the concentrations of the FVIII to be administered, the concentration of endogenous VWF in the patient to be treated, or both. An effective dosage based on the ratios defined by the inventors of this application can be determined by the skilled person, taking into account the molecular weight of the polypeptide of the invention. Typical dosages for FVIII may range from about 20 U/kg body weight to about 100 U/kg body weight.

In accordance with this invention, the patient being treated with the polypeptide of the invention is also treated with blood coagulation Factor VIII. The polypeptide of the invention and the Factor VIII may be administered simultaneously or in a sequential fashion both modes of administration being encompassed by the term "combination therapy" and "co-administration". The polypeptide of the invention and the Factor VIII may be administered as a mixture, i.e. within the same composition, or separately, i.e. as separate compositions.

The concentration of Factor VIII in the composition used is typically in the range of 10-10,000 IU/mL. In different embodiments, the concentration of FVIII in the compositions of the invention is in the range of 10-8,000 IU/mL, or 10-5,000 IU/mL, or 20-3,000 IU/mL, or 50-1,500 IU/mL, or 3,000 IU/mL, or 2,500 IU/mL, or 2,000 IU/mL, or 1,500 IU/mL, or 1,200 IU/mL, or 1,000 IU/mL, or 800 IU/mL, or 750 IU/mL, or 600 IU/mL, or 500 IU/mL, or 400 IU/mL, or 300 IU/mL, or 250 IU/mL, or 200 IU/mL, or 150 IU/mL, or 125 IU/mL, or 100 IU/mL, or 62.5 IU/mL, or 50 IU/mL, provided the requirements regarding the ratio with respect to the VWF polypeptide of the invention as defined herein are fulfilled.

"International Unit," or "IU," is a unit of measurement of the blood coagulation activity (potency) of FVIII as measured by a FVIII activity assay such as a one stage clotting assay or a chromogenic substrate FVIII activity assay using a standard calibrated against

an international standard preparation calibrated in "IU". One stage clotting assays are known to the art, such as that described in N Lee, Martin L, et al., An Effect of Predilution on Potency Assays of FVIII Concentrates, Thrombosis Research (Pergamon Press Ltd.) 30, 511 519 (1983). Principle of the one stage assay: The test is executed as a modified version of the activated Partial Thromboplastin Time (aPTT)-assay: Incubation of plasma with phospholipids and a surface activator leads to the activation of factors of the intrinsic coagulation system. Addition of calcium ions triggers the coagulation cascade. The time to formation of a measurable fibrin clot is determined. The assay is executed in the presence of Factor VIII deficient plasma. The coagulation capability of the deficient plasma is restored by Coagulation Factor VIII included in the sample to be tested. The shortening of coagulation time is proportional to the amount of Factor VIII present in the sample. The activity of Coagulation Factor VIII is quantified by direct comparison to a standard preparation with a known activity of Factor VIII in International Units.

Another standard assay is a chromogenic substrate assay. Chromogenic substrate assays may be purchased commercially, such as the coamatic FVIII test kit (Chromogenix-Instrumentation Laboratory SpA V. le Monza 338 - 20128 Milano, Italy). Principle of the chromogenic assay: In the presence of calcium and phospholipid, Factor X is activated by Factor IXa to Factor Xa. This reaction is stimulated by Factor VIIIa as cofactor. FVIIIa is formed by low amounts of thrombin in the reaction mixture from FVIII in the sample to be measured. When using the optimum concentrations of Ca2+, phospholipid and Factor IXa and an excess quantity of Factor X, activation of Factor X is proportional to the potency of Factor VIII. Activated Factor X releases the chromophore pNA from the chromogenic substrate S-2765. The release of pNA, measured at 405 nm, is therefore proportional to the amount of FXa formed, and, therefore, also to the Factor VIII activity of the sample.

Pharmaceutical compositions

Therapeutic formulations of the polypeptide of the invention suitable in the methods described herein can be prepared for storage as lyophilized formulations or aqueous solutions by mixing the polypeptide having the desired degree of purity with optional pharmaceutically-acceptable carriers, excipients or stabilizers typically employed in the art (all of which are referred to herein as "carriers"), i.e., buffering agents, stabilizing agents, preservatives, isotonifiers, non-ionic detergents, antioxidants, and other miscellaneous

additives. See, Remington's Pharmaceutical Sciences, 16th edition (Osol, ed. 1980). Such additives must be nontoxic to the recipients at the dosages and concentrations employed.

Buffering agents help to maintain the pH in the range which approximates physiological conditions. They can present at concentration ranging from about 2 mM to about 50 mM. Suitable buffering agents include both organic and inorganic acids and salts thereof such as citrate buffers (e.g., monosodium citrate-disodium citrate mixture, citric acid-trisodium citrate mixture, citric acid-monosodium citrate mixture, etc.), succinate buffers (e.g., succinic acid- monosodium succinate mixture, succinic acid-sodium hydroxide mixture, succinic acid- disodium succinate mixture, etc.), tartrate buffers (e.g., tartaric acid-sodium tartrate mixture, tartaric acid-potassium tartrate mixture, tartaric acid-sodium hydroxide mixture, etc.), fumarate buffers (e.g., fumaric acid-monosodium fumarate mixture, fumaric acid-disodium fumarate mixture, monosodium fumarate-disodium fumarate mixture, etc.), gluconate buffers (e.g., gluconic acid-sodium glyconate mixture, gluconic acid-sodium hydroxide mixture, gluconic acid-potassium gluconate mixture, etc.), oxalate buffer (e.g., oxalic acid-sodium oxalate mixture, oxalic acid-sodium hydroxide mixture, oxalic acid-potassium oxalate mixture, etc), lactate buffers (e.g., lactic acid-sodium lactate mixture, lactic acid-sodium hydroxide mixture, lactic acid-potassium lactate mixture, etc.) and acetate buffers (e.g., acetic acid-sodium acetate mixture, acetic acid-sodium hydroxide mixture, etc.). Additionally, phosphate buffers, histidine buffers and trimethylamine salts such as Tris can be used.

Preservatives can be added to retard microbial growth, and can be added in amounts ranging from 0.2%- 1% (w/v). Suitable preservatives include phenol, benzyl alcohol, meta-cresol, methyl paraben, propyl paraben, octadecyldimethylbenzyl ammonium chloride, benzalconium halides (e.g., chloride, bromide, and iodide), hexamethonium chloride, and alkyl parabens such as methyl or propyl paraben, catechol, resorcinol, cyclohexanol, and 3-pentanol. Isotonicifiers sometimes known as "stabilizers" can be added to ensure isotonicity of liquid compositions and include polhydric sugar alcohols, preferably trihydric or higher sugar alcohols, such as glycerin, erythritol, arabitol, xylitol, sorbitol and mannitol. Stabilizers refer to a broad category of excipients which can range in function from a bulking agent to an additive which solubilizes the therapeutic agent or helps to prevent denaturation or adherence to the container wall. Typical stabilizers can be polyhydric sugar alcohols (enumerated above); amino acids such as arginine, lysine, glycine, glutamine, asparagine, histidine, alanine, ornithine, L-leucine, 2-phenylalanine, glutamic acid, threonine, etc., organic sugars or sugar alcohols, such as lactose, trehalose,

stachyose, mannitol, sorbitol, xylitol, ribitol, myoinisitol, galactitol, glycerol and the like, including cyclitols such as inositol; polyethylene glycol; amino acid polymers; sulfur containing reducing agents, such as urea, glutathione, thioctic acid, sodium thioglycolate, thioglycerol, α-monothioglycerol and sodium thio sulfate; low molecular weight polypeptides (e.g., peptides of 10 residues or fewer); proteins such as human serum albumin, bovine serum albumin, gelatin or immunoglobulins; hydrophylic polymers, such as polyvinylpyrrolidone monosaccharides, such as xylose, mannose, fructose, glucose; disaccharides such as lactose, maltose, sucrose and trisaccacharides such as raffinose; and polysaccharides such as dextran. Stabilizers can be present in the range from 0.1 to 10,000 weights per part of weight active protein.

Non-ionic surfactants or detergents (also known as "wetting agents") can be added to help solubilize the therapeutic agent as well as to protect the therapeutic protein against agitation-induced aggregation, which also permits the formulation to be exposed to shear surface stressed without causing denaturation of the protein. Suitable non-ionic surfactants include polysorbates (20, 80, etc.), polyoxamers (184, 188 etc.), Pluronic polyols, polyoxyethylene sorbitan monoethers (TWEEN®-20, TWEEN®-80, etc.). Non-ionic surfactants can be present in a range of about 0.05 mg/ml to about 1.0 mg/ml, or in a range of about 0.07 mg/ml to about 0.2 mg/ml.

Additional miscellaneous excipients include bulking agents (e.g., starch), chelating agents (e.g., EDTA), antioxidants (e.g., ascorbic acid, methionine, vitamin E), and cosolvents.

The formulation herein can also contain a second therapeutic agent in addition to a polypeptide of the invention. Examples of suitable second therapeutic agents are provided below.

The dosing schedule can vary from once a month to daily depending on a number of clinical factors, including the type of disease, severity of disease, and the patient's sensitivity to the polypeptide of the invention. In specific embodiments, a polypeptide of the invention is administered, twice weekly, every 5 days, once weekly, every 10 days, every two weeks, every three weeks, every four weeks or once a month, or in any range between any two of the foregoing values, for example from every four weeks to every month, from every 10 days to every two weeks, or from two to three times a week, etc.

The dosage of a polypeptide of the invention to be administered will vary according to the particular polypeptide, the subject, and the nature and severity of the disease, the physical condition of the subject, the therapeutic regimen (e.g., whether a second therapeutic agent is used), and the selected route of administration; the appropriate dosage can be readily determined by a person skilled in the art.

It will be recognized by one of skill in the art that the optimal quantity and spacing of individual dosages of a polypeptide of the invention will be determined by the nature and extent of the condition being treated, the form, route and site of administration, and the age and condition of the particular subject being treated, and that a physician will ultimately determine appropriate dosages to be used. This dosage can be repeated as often as appropriate. If side effects develop the amount and/or frequency of the dosage can be altered or reduced, in accordance with normal clinical practice.

The nucleotide and amino acid sequences shown in the sequence listing are summarized in the Table 2.

Table 2:

SEQ ID NO:	Remarks
1	DNA sequence encoding a polypeptide comprising acids 1 to 1242 of human VWF, a glycine/serine linker and human albumin; nucleotide positions (nt): nt 1-6: EcoRI restriction enzyme cleavage site nt 32 – 3757: coding sequence for VWF amino acids 1 to 1242 nt 3758 – 3850: coding sequence for glycine/serine linker nt 3851 – 5608: coding sequence for human albumin nt 5609 – 5616: NotI restriction enzyme cleavage site
2	Amino acid sequence encoded by SEQ ID NO:1 (mature form): amino acid positions (aa): aa 1 – 479: VWF D´D3 region (VWF amino acids 764 – 1242) aa 480 – 510: glycine/serine linker aa 511 – 1195: human albumin
3	DNA sequence encoding the pre-pro form of human native VWF
4	Amino acid sequence encoded by SEQ ID NO:3
5	Amino acid sequence of a single chain Factor VIII molecule
6	Amino acid sequence of mature human serum albumin
7	Amino acid sequence of D´D3-His8 aa 1 – 479: VWF D´D3 region (VWF amino acids 764 – 1242) aa 480 – 511: glycine/serine linker aa 512 – 519: polyhistidine tag
8	Amino acid sequence of D´D3-CTP aa 1 – 479: VWF D´D3 region (VWF amino acids 764 – 1242) aa 480 – 511: glycine/serine linker aa 512 – 576: C-terminal peptide of human chorionic gonadotropin-ß subunit aa 577 – 584: polyhistidine tag

The following Examples illustrate the invention but should not be construed as limiting the present invention to the specific embodiments described herein below.

EXAMPLES

Aim

We aimed at investigating the impact of the ratio of a polypeptide comprising a truncated VWF and a half-life-extending moiety ("polypeptide of the invention") to FVIII and endogenous VWF, respectively, on pharmacokinetics (PK) of the fragment and FVIII.
An overview of the experiments is given in the following table.

Table 3:

Ex. #.	Animal	polypeptide of the invention dose	FVIII dose	Dose ratio polypeptide of the invention : FVIII	Dose ratio polypeptide of the invention : endogenous VWF
1	rat	Increasing (D´D3-FP)	constant	increasing	increasing
2	rat	Increasing (D´D3-FP)	increasing	constant	increasing
3	rabbit	Increasing (D´D3-FP)	constant	increasing	increasing
4	rabbit	Single dose with different recombinant FVIII products			
5	rat	D´D3 without half-life extending moiety and D´D3-CTP at a fixed ratio			

In the pharmacokinetic examples, different molar ratios were calculated. Therefore, the following assumptions were made:

- The drugs are diluted in 40 mL plasma per kg body weight after their administration
- Molecular weight of the polypeptide of the invention used: D'D3-FP molecular weight of monomeric subunit (including glycosylation): 127,000 Da (HLEM = human albumin)
- Molecular weight of FVIII used: rVIII-SingleChain molecular weight (with glycosylation): 180,000 Da and specific activity: 11,000 IU/mg
- Molecular weight of albumin as part of the D'D3-FP: 66,000 Da
- Same molar FVIII activity of the recombinant FVIII products was used (rVIII-SingleChain, Advate®, NovoEight®), calculating for identical molar ratios when administered at same activity doses

- Endogenous human, rat or rabbit VWF monomer molecular weight (with 18-19% glycosylation): 267,500 Da
- Endogenous rat or rabbit VWF is assumed to have the same specific activity as human VWF, i.e. one U/mL (or 100% of the norm) is assumed to be 10 µg/mL (published human concentration relating to one U/mL or 100 % of the norm)
- Endogenous VWF activity in rat plasma measured as 0.946±0.181 U/mL and calculated to 35.36 * 10^{-9} mol/L from 12 CD rats using the INNOVANCE® VWF Ac kit (Siemens Healthcare Diagnostics GmbH, Eschborn, Germany) calibrated against standard human plasma (calibrated against the respective WHO standard, Siemens Healthcare Diagnostics GmbH)
- Endogenous VWF activity in rabbit plasma measured as 0.242±0.056 U/mL and calculated to 9.05 * 10^{-9} mol/L from 5 rabbits from example 3 using the INNOVANCE® VWF Ac kit (Siemens Healthcare Diagnostics GmbH, Eschborn, Germany) calibrated against standard human plasma (calibrated against the respective WHO standard, Siemens Healthcare Diagnostics GmbH).

Example 1: Prolongation of pharmacokinetics of FVIII by co-administration of rVIII-SingleChain (constant dose) and increasing D'D3-FP doses in the rat

Material and Methods

Generation of D´D3 albumin fusion protein (D´D3-FP):

The expression cassette for D´D3-FP consisting of cDNA encoding VWF amino acids 1 to 1242, a glycine/serine linker and the cDNA of human albumin was prepared by custom gene synthesis (Eurofins Genomics, Ebersberg, Germany). Through flanking restriction sites (EcoRI, NotI) the expression cassette was excised from the cloning vector supplied and inserted into a pIRESneo3 vector (BD Biosciences, Franklin Lakes, NJ, USA) linearized with EcoRI and NotI. The resulting expression plasmid contained nucleotide sequences encoding the VWF propeptide, D´ and D3 (VWF amino acids 1 to 1242 of SEQ ID NO:4) fused to the albumin coding sequence through a short linker coding sequence under CMV promoter control. The nucleotide sequence of the coding sequence is displayed as SEQ ID NO:1, the amino acid sequence of the mature D´D3-FP is shown as SEQ ID NO:2.

A similar approach was used to generate an expression plasmid for a His-tagged D´D3 protein (D´D3 and His8 linked by a glycine/serine linker) and a D´D3 fusion protein to the C-terminal peptide of human chorionic gonadotropin-ß subunit, also linked via a glycine/serine linker and tagged by 8 histidines at the C-terminus of the fusion protein. The amino acid sequence of the mature D´D3-His8 is shown as SEQ ID NO: 7 and the amino acid sequence of the mature D´D3-CTP is shown as SEQ ID NO: 8.

The expression plasmids as described above were grown up in XL10 Gold (Agilent Technologies) and purified using standard protocols (Qiagen, Hilden, Germany).

CHO K1 cells were transfected using the Lipofectamine 2000 reagent (Invitrogen) and grown up in serum-free medium (CD-CHO, Invitrogen) in the presence of 500-1000 µg/ml Geneticin. An expression plasmid encoding PACE/furin (pFu-797) as described in WO2007/144173 was cotransfected to maximize propeptide cleavage efficacy. Single cell derived clones were grown up and selected according to their D´D3-FP expression yield as quantified by an albumin specific enzyme immunoassay (see below). The cell line finally selected for D´D3-FP fermentation was called T2050-CL3.

Production of D´D3-FP was carried out in bioreactors applying a fermentation process in perfusion mode. The fermentation process for the production of D´D3-containing polypeptides started with the thaw of cell line T2050-CL3 followed by cell expansion in shake flasks and finally a fermentation process in perfusion mode using the Sartorius BioStat B-DCU 5 L bioreactor and the BioStat STR 50L single-use bioreactors. The BioSeps 10L or 200L (Applikon), respectively, were used as cell retention devices. Cell culture media were either PowerCHO3 (Lonza BESP1204) with 8 mM L-glutamine and 1 µM $CuSO_4$ or ProCHO5 (Lonza BESP1072) with 10 mM L-glutamine and 1 µM $CuSO_4$.

The seed trains in shake flasks were performed at 37°C, 7.5% CO_2 at a shaker speed of 160 rpm.

The 5L bioreactor was inoculated with a target VCD of 2.5×10^5 cells/mL. The cells were cultivated in PowerCHO3 with 8 mM L-glutamine and 1 µM $CuSO_4$ at a temperature of +37.0°C, a pH of 7.00, and at 30 % oxygen saturation. A temperature shift to +34.0 °C (evaluated range +31°C to +35°C) was performed after initial harvests from the bioreactor run at +37°C had been taken. The pH was controlled using CO_2 sparged as acid and $NaHCO_3$ as base. The overlay air flow rate was set to 0.5 L/min. A ring sparger was used

as a sparging unit. The agitation rate was 150 rpm with a 2fold pitch blade impeller in down pull mode.

The 50L bioreactor was inoculated with a target VCD of 3.0 x 10^5 cells/mL. The cells were cultivated in ProCHO5 medium with 10 mM L-glutamine and 1 µM $CuSO_4$ at a temperature of +37.0°C, a pH of 6.90, and at 30 % oxygen saturation. A temperature shift to +34.0 °C was performed after the initial one or two harvests. PH control as above, the overlay air flow rate was set to 2 L/min. A micro sparger was used as a sparging unit. The agitation rate was 90 rpm with a 2fold pitch blade impeller in down pull mode.

The perfusion was initiated when the VCD in the bioreactor was ≥1.0 x 10^6 cells/mL. The perfusion rate was set to 1.0 volume/volume/day. The BioSep was operated in back flush mode with 5 (10) minutes runtime and 10 seconds back flush at a power input of 7 (30) W (numbers in brackets refer to the 50L bioreactor). The perfusate and the bleed were filtered inline and collected in bags over 48 hours at +2 to +8°C. The VCD was controlled by active bleeding using a turbidity probe using glucose consumption as parameter with a target of 2 g/L glucose. Harvest and bleed were filtered inline, the harvest system consisting of a disposable filter and disposable bag was changed every second day.

To prepare material for the PK analyses described below D´D3 albumin fusion protein harvests were purified by affinity and size exclusion chromatography. . Briefly, the cell-free harvest from the bioreactor was concentrated 30-fold using a TFF system (e.g. Pall Centramate 500 S) with a 30 kD membrane (e.g Pall Centramate OS030T12). That concentrate was spiked with NaCl and EDTA to a final concentration of 0.75 M NaCl and 5 mM EDTA and loaded overnight on a CaptureSelect Human Albumin column (Life Technologies) which was pre-equilibrated with 20 mM Tris buffer pH 7.4. After washing the column with equilibration buffer D'D3-FP was eluted with elution buffer (20 mM Tris, 2 M $MgCl_2$, pH 7.4). The eluate was then 10-fold concentrated and dialyzed against 50 mM Tris, 150 mM NaCl, pH 7.4 using Ultra Centrifugal Filters with a 30 kD cut-off (e.g. Amicon. UFC903024). To separate the D´D3-FP dimer from the monomer portion that material was loaded on a Superdex 200 pg column (GE Healthcare Code: 17-1069-01) pre-equilibrated with 50 mM Tris, 150 mM NaCl, pH 7.4 and the peak fractions containing the D'D3-FP dimer were pooled. The area under the curve for the dimer and monomer peak fractions were used to calculate dimer to monomer ratio. Dimer preparations of D'D3-FP were used for the pharmacokinetic experiments in Examples 1-4.

His-tagged D´D3 proteins were purified by Ni-chelate affinity and size exclusion chromatography. Briefly, TFF concentrated cell-free bioreactor harvest (see above for details) was loaded on a preequilibrated (20mM sodium phosphate / 500 mM NaCl, pH 7.4) Ni-Sepharose column (HisTrap™, GE Healthcare) over night. After washing the column with 20mM sodium phosphate / 500 mM NaCl / 30 mM Imidazol, pH 7.4 the protein was eluted with 20mM sodium phosphate + 500 mM NaCl + 500 mM Imidazol, pH 7.4. The eluate was then concentrated and dialysed (TBS, pH7.4) using an Amicon Ultra Centrifugal Filter (see above). The final product was then loaded onto a SEC column (see above), the peak fractions containing the dimer were pooled and concentrated to about 7 mg/mL $OD_{280-320}$.

Animals:

Female Crl:CD (Sprague Dawley) rats in a weight range of 240-300 g were bred at Charles River Laboratories (Sulzfeld, Germany). In house, the animals were kept at standard housing conditions, i.e. at 21-22°C under a 12 h/12 h light-darkness cycle. Animals were fed ad libitum with standard rat diet (Ssniff-Versuchsdiäten, Soest, Germany). Tap water was supplied ad libitum. Animal husbandry and study procedures complied with the German Animal Welfare law and European Union regulations. The group size was n=6, divided in 2 cohorts. Thus, n=3 animals per time-point were used.

Laboratory evaluations:

The test articles were administered i.v. by a single injection into the lateral tail vein at a total volume of 3 mL/kg. D'D3-FP preparations were applied at dose levels from 50 to 10,000 µg/kg based on human albumin values, and co-administered with 200 IU/kg rVIII-SingleChain (rVIII-SingleChain, chromogenic activity) after incubating for approximately 30 minutes at +37°C. Animals receiving only rVIII-SingleChain served as control (Table 4). This led to administered molar ratios of D'D3-FP (calculated as monomer) over rVIII-SingleChain ranging from 7.5 to 1500 as well as a molar ratio of D'D3-FP over endogenous rat VWF (both as monomers to adjust for FVIII binding sites) from 0.54 to 107.1.

Blood samples were taken retroorbitally under short term anaesthesia at 5 min, 3, 8, 24, 32, 48, 56 and 72 h after intravenous bolus injection using an alternating sampling scheme. The PK profile was taken from two cohorts of rats per group. Blood samples

were anticoagulated using sodium citrate (2 parts sodium citrate 3.13% + 8 parts blood), processed to plasma and stored at –20°C for the determination of FVIII antigen and/or albumin.

D'D3-FP exposure was determined by measurement of the albumin part of the construct using a human albumin ELISA. Accordingly, any amount or dose of D'D3-FP is indicated as the amount or dose of albumin. Other D´D3-containing polypeptides were detected with a D´D3 specific Elisa. FVIII:Ag plasma levels were detected with the FVIII Asserachrom ELISA testkit from Stago, S.A.S., France.

D´D3-albumin fusions were measured in plasma samples in an Elisa with a commercial polyclonal antibody preparation. For capture and detection the same antibodies were used, except that the detection antibodies were POD labelled. Briefly, each well of a 96-well plate (Nunc Immuno-Plate Maxisorp, product number 449824) was coated with 100 µL of coating solution (goat anti-human Albumin-IgG, Cat. No. A80-129A, Bethyl Laboratories, diluted to 2 µg/mL in 50 mM coating buffer (Carbonate-Bicarbonate Capsules, Sigma product No.: C-3041)) and incubated at +21°C for 2-20 hours. The coating procedure was followed by 3 wash steps using wash buffer (phosphate buffered saline with tween 20, Sigma product No.: P3563). Subsequent blocking was performed for 1.5 h at +21°C, with blocking solution (Candor Biosience, Cat.No. 110500) followed by another 3 wash steps. Subsequently 100 µl of each sample were applied to the plate and incubated for 1 h at +37°C. As standards the respective injection solutions of the substances tested were diluted with sample buffer (Low Cross Buffer, Candor Biosience, Cat. No. 100500) to concentrations from 50 ng/mL to 0.78 ng/mL in 2 step decrements. The citrated plasma samples, taken from the animals were also diluted with sample buffer at least 1:30, sample buffer was also applied as blank. After another four washing steps, 100 µL of detection solution (POD labelled anti human albumin-IgG, Cat. No. A80-129P, Bethyl Laboratories, diluted 1:40000 in blocking solution solution) was applied to each well for 45 min at +37°C. After another four washing steps, 100 µL per well of chromogenic substrate (TMB, Siemens Healthcare product OUVF, OUVG) was applied for 20 min, followed by addition of another 100 µl stop solution (OSFA, Siemens Healthcare) per well. The plates were measured in a plate reader at 450/650nm.

D´D3-containing polypeptides without albumin were measured in plasma samples with an anti-D'D3 sandwich ELISA with proprietary anti-D´D3 antibodies. Briefly, each well of a 96-well plate (Nunc Immuno-Plate Maxisorp, product number 449824) was coated with 100 µL of coating solution (D'D3 capture antibody diluted to 1 µg/mL in 50 mM coating

buffer (Carbonate-Bicarbonate Capsules, Sigma product No.: C-3041)) and incubated at +21°C for 16 hours. The coating procedure was followed by 3 wash steps using wash buffer (Tris buffer saline with tween 20, Sigma product No.: T9039). Blocking was performed for 1.5 h at +21°C, with blocking solution (Candor Bioscience, Cat. No. 110500), followed by another 3 wash steps. Subsequently 100 µl each of each sample were applied to the plate and incubated for 1.5 h at +21°C. As standards the respective injection solutions of the substances tested were diluted with sample buffer (Tris buffer saline with tween 20, Sigma product No.: T9039) to concentrations from 70 ng/mL to 1.1 ng/mL in 2 step decrements. The citrated plasma samples were also diluted with sample buffer at least 1:30, sample buffer was also applied as blank. After another three washing steps, 100 µL of detection solution (POD labelled anti D'D3 detection antibody (proprietary research grade preparation), diluted to 0.2 µg/mL in sample buffer) was applied to each well and incubated for 1 h at +21°C. After another three washing steps, 100µL per well of chromogenic substrate (TMB, Siemens Healthcare product No.: OUVF, OUVG) was applied for 30 min, followed by another 100 µl stop solution (OSFA, Siemens Healthcare) per well. The plates were measured in a plate reader at 450/650nm.

Estimation of mean residence time (MRT), clearance (CL) and terminal half-life (t1/2) was done by non-compartmental methods.

Table 4: Treatment groups

No.	Treatment	D'D3-FP dose [mg albumin/kg]	FVIII dose [IU FVIII:C/kg]	Molar ratio D'D3-FP * : rVIII-SC	Molar ratio D'D3-FP* over rat VWF**
1	rVIII-SC	-	200	-	-
3	D'D3-FP & rVIII-SC	0.05	200	7.5	0.54
4	D'D3-FP & rVIII-SC	0.10	200	15	1.07
5	D'D3-FP & rVIII-SC	0.20	200	30	2.14
6	D'D3-FP & rVIII-SC	0.50	200	75	5.36
7	D'D3-FP & rVIII-SC	1.00	200	150	10.7
8	D'D3-FP & rVIII-SC	2.00	200	300	21.4
9	D'D3-FP & rVIII-SC	5.00	200	750	53.6
10	D'D3-FP & rVIII-SC	10.00	200	1500	107.1

FVIII:C = chromogenic FVIII activity; rVIII-SC = rVIII-SingleChain

* D'D3-FP calculated as monomer

** D'D3-FP calculated as monomer over endogenous VWF monomer to compare same numbers of FVIII binding sites. For calculation of the endogenous rat VWF the same specific activity as human VWF was assumed, i.e. one U/mL (or 100% of the norm) is assumed to be 10 µg/mL (measured rat plasma concentration 0.946 U/mL)

Results

D'D3-FP was quantified by determining the albumin component, followed by calculation of the D'D3-FP concentration, and all measured data (up to 72 h p.a.) were well above the detection limit of the assay. The ELISA used specifically detected human albumin. Mean residence time (MRT) and clearance of D'D3-FP were not affected by the given dose in a range from 0.05 to 10 mg/kg i.v. (Fig. 1 and Table 5).

The exposure of the co-administered FVIII (200 IU/kg chromogenic FVIII activity), quantified as FVIII:Ag via ELISA, was prolonged in the presence of D'D3-FP (Fig. 2 and Table 5). Thus, in the rVIII-SingleChain group, FVIII:Ag reached the detection limit of 117 mIU/mL at 24-32h p.a., while the D'D3-FP co-treated groups had dose-dependently increased plasma concentrations and did not reach the detection limit up to 72h p.a. in the groups co-treated with 5 and 10 mg/kg.

Already the lowest dose of 0.05 mg/kg i.v. of D´D3-FP increased MRT and terminal half-life and slightly reduced clearance of rVIII-SingleChain. Up to 0.2 mg/kg only minor changes were seen on MRT, terminal half-life and clearance, and from 0.5 to 10 mg/kg a dose-dependent increase was seen for MRT and terminal half-life or decrease for clearance. It is thus concluded that in this experimental setting an about 75-fold excess of D'D3-FP (the 0.5 mg/kg dose) over rVIII-SingleChain is needed to relevantly prolong FVIII half-life.

It is assumed that at lower doses of D'D3-FP, the endogenous rat VWF competes with D'D3-FP for binding to the co-administered FVIII more relevantly than at higher doses, thereby explaining the dose-dependence of D'D3-FP dose on FVIII PK.

Calculation of increases of MRT and terminal half-life and decreases of clearance as compared to rVIII-SingleChain given alone is presented in Table 5. Co-administration of rVIII-SingleChain with 0.05-0.2 mg/kg D'D3-FP reduced clearance and prolonged MRT and terminal half-life by less than a factor of 2. Starting from 0.5 mg/kg (molar ratio D'D3-FP over rVIII-SingleChain = 75; molar ratio over endogenous rat VWF = 5.36), a dose-dependent reduction of clearance and increase of MRT and terminal half-life can be seen. Even at a dose of 10 mg/kg D'D3-FP (molar ratio D'D3-FP over rVIII-SingleChain = 1500; molar ratio over endogenous rat VWF = 107.1), a further change over the lower dose was seen, suggesting that the plateau of the effect was not yet reached.

Table 5: Pharmacokinetic parameters of D'D3-FP and FVIII:Ag after co-administration of rVIII-SingleChain and D'D3-FP in rats (non-compartmental analysis)
Dose D'D3-FP 0.05 to 10 mg/kg, dose rVIII-SingleChain 200 IU/kg

No.	Treatment*	C_{max}, extrap.	Clearance	MRT	Half-life, terminal
		µg/mL (D'D3-FP [c]) IU/mL (FVIII:Ag)	mL/kg/h	h	h
		D'D3-FP			
3	D'D3-FP (0.05) & rVIII-SC	1.27	1.00	56.6	40.2
4	D'D3-FP (0.1) & rVIII-SC	2.33	1.09	59.5	42.4
5	D'D3-FP (0.2) & rVIII-SC	5.15	1.07	55.4	39.7
6	D'D3-FP (0.5) & rVIII-SC	9.47	1.11	64.1	45.5
7	D'D3-FP (1) & rVIII-SC	23.80	1.29	47.7	34.0
8	D'D3-FP (2) & rVIII-SC	36.24	1.22	56.4	39.3
9	D'D3-FP (5)& rVIII-SC	115.17	1.28	46.8	33.8
10	D'D3-FP (10) & rVIII-SC	221.84	1.25	54.0	38.6
		FVIII:Ag			
1	rVIII-SC	3.36	8.25	7.4	5.2
3	D'D3-FP (0.05) & rVIII-SC	4.41	4.93 (1.7fold[a])	10.5 (1.4fold[b])	7.1 (1.4fold[b])
4	D'D3-FP (0.1) & rVIII-SC	4.52	4.72 (1.7fold[a])	10.8 (1.5fold[b])	7.7 (1.5fold[b])
5	D'D3-FP (0.2) & rVIII-SC	4.35	4.72 (1.7fold[a])	11.9 (1.6fold[b])	8.4 (1.6fold[b])
6	D'D3-FP (0.5) & rVIII-SC	3.93	3.78 (2.2fold[a])	15.1 (2.0fold[b])	10.5 (2.0fold[b])
7	D'D3-FP (1) & rVIII-SC	4.28	2.83 (2.9fold[a])	16.7 (2.3fold[b])	11.0 (2.1fold[b])
8	D'D3-FP (2) & rVIII-SC	4.62	2.18 (3.8fold[a])	19.9 (2.7fold[b])	13.9 (2.7fold[b])
9	D'D3-FP (5)& rVIII-SC	4.82	1.85 (4.5fold[a])	25.8 (3.5fold[b])	17.6 (3.4fold[b])
10	D'D3-FP (10) & rVIII-SC	4.63	1.68 (4.9fold[a])	29.6 (4.0fold[b])	20.3 (3.9fold[b])

rVIII-SC = rVIII-SingleChain

[a] fold decrease over data from rVIII-SingleChain given alone

[b] fold increase over data from rVIII-SingleChain given alone

[c] determination of albumin in µg/mL

Example 2: Prolongation of pharmacokinetics of FVIII by co-administration of rVIII-SingleChain and D'D3-FP at different doses (constant in D'D3-FP : rVIII-SingleChain ratio) in the rat

Material and Methods

Animals: Female Crl:CD (Sprague Dawley) rats in a weight range of 220-270 g were bred at Charles River Laboratories (Sulzfeld, Germany). In house, the animals were kept at standard housing conditions, i.e. at 21-22°C under a 12 h/12 h light-darkness cycle. Animals were fed ad libitum with standard rat diet (Ssniff-Versuchsdiäten, Soest, Germany). Tap water was supplied ad libitum. Animal husbandry and study procedures complied with the German Animal Welfare law and European Union regulations.

The group size was n=6, divided in 2 cohorts. Thus, n=3 animals per time-point were used.

Laboratory evaluations: The test articles were administered i.v. by a single injection into the lateral tail vein at a total volume of 4.5 mL/kg. D'D3-FP preparations were applied at dose levels from 1 to 10 mg/kg based on human albumin values, and co-administered with 100 to 1000 IU/kg rVIII-SingleChain (rVIII-SingleChain, chromogenic activity) at constant D'D3-FP to rVIII-SingleChain ratios after incubating for approximately 30 minutes at +37°C. Animals receiving only rVIII-SingleChain served as control (Table 6).

Blood samples were taken retro-orbitally under short term anaesthesia at 5 min, 3, 8, 24, 32, 48, 56 and 72 h after intravenous bolus injection using an alternating sampling scheme. The PK profile was taken from two cohorts of rats per group. Blood samples were anticoagulated using sodium citrate (2 parts sodium citrate 3.13% + 8 parts blood), processed to plasma and stored at –20°C for the determination of FVIII antigen and/or albumin.

D'D3-FP exposure was determined by measurement of the albumin part of the construct using a human albumin ELISA. Accordingly, any amount or dose of D'D3-FP is indicated as the amount or dose of albumin. FVIII:Ag plasma levels were detected with the FVIII Asserachrom ELISA testkit from Stago, S.A.S., France.

Estimation of mean residence time (MRT), clearance (CL) and terminal half-life (t1/2) was done by non-compartmental methods.

Table 6: Treatment groups

No	Treatment	D'D3-FP dose [mg albumin/kg]	FVIII dose [IU FVIII:C/kg]	Molar ratio D'D3-FP* over rVIII-SC	Molar ratio D'D3-FP* over rat VWF **
1	rVIII-SC (100)	-	100	-	-
2	D'D3-FP (1) & rVIII-SC (100)	1	100	300	10.7
3	rVIII-SC (300)	-	300	-	-
4	D'D3-FP (3) & rVIII-SC (300)	3	300	300	32.1
5	rVIII-SC (1000)	-	1000	-	-
6	D'D3-FP (10) & rVIII-SC (1000)	10	1000	300	107.1

FVIII:C = chromogenic FVIII activity; rVIII-SC = rVIII-SingleChain

* D'D3-FP calculated as monomer

** D'D3-FP calculated as monomer over endogenous VWF monomer to compare same numbers of FVIII binding sites. For calculation of the endogenous rat VWF the same specific activity as human VWF was assumed, i.e. one U/mL (or 100% of the norm) is assumed to be 10 µg/mL (measured rat plasma concentration 0.946 U/mL)

Results

D'D3-FP was quantified via its albumin component, and all measured data (up to 72 h p.a.) were well above the detection limit of the assay. Mean residence time (MRT) and clearance of D'D3-FP were not affected by the given dose in the range from 1 to 10 mg/kg i.v. (Fig. 3 and Table 7, compare Fig. 1 and Table 5).

The exposure of the co-administered FVIII (100 to 1000 IU/kg chromogenic FVIII activity), quantified as FVIII:Ag via ELISA, was prolonged in the presence of D'D3-FP (Fig. 4 and Table 7). With the dose-dependency of FVIII exposure, the low dose rVIII-SingleChain group reached the FVIII:Ag detection limit of 117 mIU/mL already at 24h p.a., while higher doses reached the detection limit later, and longest profiles were observed with co-treatment with D'D3-FP, especially both high dose D'D3-FP co-treated groups did not reach the detection limit up to 72h p.a.

Pharmacokinetic characteristics of FVIII were not affected by the FVIII dose. As stated before, D'D3-FP pharmacokinetics were also not affected by the D'D3-FP dose.

Table 7: Pharmacokinetic parameters of D'D3-FP and FVIII:Ag after co-administration of rVIII-SingleChain and D'D3-FP in rats (non-compartmental analysis)
Dose D'D3-FP 1 to 10 mg/kg, dose rVIII-SingleChain 100 to 1000 IU/kg, constant D'D3-FP to rVIII-SingleChain ratio.

Treatment*	**C_{max}, extrap.**	**Clearance**	**MRT**	**Half-life, terminal**
	µg/mL (D'D3-FP [c]) IU/mL (FVIII:Ag)	mL/kg/h	h	h
	D'D3-FP			
D'D3-FP (1 mg/kg) & rVIII-SC (100 IU/kg)	18.0	1.26	67.1	48.0
D'D3-FP (3 mg/kg) & rVIII-SC (300 IU/kg)	67.3	1.22	52.8	37.7
D'D3-FP (10 mg/kg) & rVIII-SC (1000 IU/kg)	234.6	1.09	58.4	42.3
	FVIII:Ag			
rVIII-SC (100 IU/kg)	1.50	9.99	6.4	4.5
D'D3-FP (1 mg/kg) & rVIII-SC (100 IU/kg)	2.03	3.42 (2.9fold[a])	15.7 (2.5fold[b])	10.8 (2.4fold[b])
rVIII-SC (300 IU/kg)	5.45	8.78	7.9	5.8
D'D3-FP (3 mg/kg) & rVIII-SC (300 IU/kg)	6.04	2.41 (3.6fold[a])	23.0 (2.9fold[b])	15.7 (2.7fold[b])
rVIII-SC (1000 IU/kg)	19.03	12.42	6.8	5.2
D'D3-FP (10 mg/kg) & rVIII-SC (1000 IU/kg)	24.46	1.64 (7.6fold[a])	30.2 (4.4fold[b])	20.5 (3.9fold[b])

rVIII-SC = rVIII-SingleChain

[a] fold decrease over data from rVIII-SingleChain given alone

[b] fold increase over data from rVIII-SingleChain given alone

[c] determination of albumin in µg/mL

Example 3: Prolongation of pharmacokinetics of FVIII by co-administration of rVIII-SingleChain (constant dose) and increasing D'D3-FP doses in the rabbit

Material and Methods

Animals: Female CHB rabbits in a weight range of 2.2-2.8 kg (Bauer, Neuental, Germany) were housed one per cage in wire-steel cages at standard housing conditions, i.e. at 21-23°C and 50% relative humidity under a 12 h/12 h light-darkness cycle. The animals were provided tap water ad libitum and fed rabbit pellets (Deukanin®, Deutsche Tiernahrung Cremer GmbH & Co. KG, Düsseldorf, Germany). Animal husbandry and study procedures complied with the German Animal Welfare law and European Union regulations.

Laboratory evaluations: The test articles were administered i.v. by a single injection into the lateral ear vein, with a group size of n=3 animals per group. D'D3-FP preparations were applied at a dose level of 0.5 to 3 mg/kg based on human albumin values, and co-administered with 150 IU/kg rVIII-SingleChain (rVIII-SingleChain, chromogenic activity) after incubating for approximately 30 minutes at +37°C. Animals receiving only rVIII-SingleChain served as control (Table 8).

Blood samples were taken from the ear artery at pre-dose, 5 and 30 min, 1, 2, 4, 6, 8, 24, 32, 48, 72 and 96 h (rVIII-SingleChain) or pre-dose, 5 min, 1, 4, 8, 24, 32, 48, 72, 96, 120, 144 and 168 h (rVIII-SingleChain co-treated with D'D3-FP) after intravenous bolus injection. Blood samples were anticoagulated using sodium citrate (2 parts sodium citrate 3.13% + 8 parts blood), processed to plasma and stored at –20°C for the determination of FVIII antigen and/or D'D3-FP. D'D3-FP exposure was determined by measurement of the albumin part of the construct using a human albumin-specific ELISA. Accordingly, any amount or dose of D'D3-FP is indicated as the amount or dose of albumin. FVIII:Ag plasma levels were detected by ELISA (Asserachrom Stago, S.A.S., France).

Estimation of mean residence time (MRT), clearance (CL) and terminal half-life (t1/2) was done by non-compartmental methods.

Table 8: Treatment groups

Treatment*	D'D3-FP dose [mg albumin/kg]	FVIII dose [IU FVIII:C/kg]	Volume [mL/kg]	Molar ratio D'D3-FP* over rVIII-SC	Molar ratio D'D3-FP* over rabbit VWF**
rVIII-SC	-	150	0.434	-	-
D'D3-FP & rVIII-SC	0.5	150	0.519	100	20.9
D'D3-FP & rVIII-SC	1.5	150	0.688	300	62.8
D'D3-FP & rVIII-SC	3.0	150	0.943	600	125.6

FVIII:C = chromogenic FVIII activity; rVIII-SC = rVIII-SingleChain

* D'D3-FP calculated as monomer

** D'D3-FP calculated as monomer over endogenous VWF monomer to compare same numbers of FVIII binding sites. For calculation of the endogenous rabbit VWF the same specific activity as human VWF was assumed, i.e. one U/mL (or 100% of the norm) is assumed to be 10 µg/mL (measured rabbit plasma concentration 0.242 U/mL)

Results

In general, results in rats and rabbits are very comparable. The following observations were made in detail:

D'D3-FP was quantified via its albumin component; and measurements were well above the detection limit of the assay up to the measured 168 h p.a. The increase in dose in the range from 0.5 to 3 mg/kg did not affect MRT and clearance (Fig. 5) or terminal half-life (Table 9).

The exposure of the co-administered FVIII (150 IU/kg chromogenic FVIII activity), quantified as FVIII:Ag via ELISA, was relevantly prolonged in the presence of D'D3-FP (Fig. 6). In the rabbit, plasma levels of FVIII:Ag could be measured maximally up to 48 h p.a. when rVIII-SingleChain was given alone (detection limit of 117 mIU/mL), and maximally up to the last time-point of 168 h p.a. after co-treatment with 3 mg/kg D'D3-FP. Like in the rat, this prolongation of PK was dose-dependent.

Table 9: Pharmacokinetic parameters of D'D3-FP and FVIII:Ag after co-administration of rVIII-SingleChain and D'D3-FP in rabbits (non-compartmental analysis)
Dose D'D3-FP 0.5-3 mg/kg, dose rVIII-SingleChain 150 IU/kg

Treatment*	**Cmax, extrap.**	**Clearance**	**MRT**	**Half-life, terminal**
	µg/mL (D'D3-FP [c]) IU/mL (FVIII:Ag)	mL/kg/h	h	h
		D'D3-FP		
D'D3-FP (0.5 mg/kg) & rVIII-SC	7.0	0.516	181	126
D'D3-FP (1.5 mg/kg) & rVIII-SC	22.0	0.638	154	107
D'D3-FP (3 mg/kg) & rVIII-SC	48.2	0.493	150	104
		FVIII:Ag		
rVIII-SC	2.51	3.71	23.2	17.3
D'D3-FP (0.5 mg/kg) & rVIII-SC	2.59	1.79 (2.1fold[a])	33.0 (1.4fold[b])	21.2 (1.2fold[b])
D'D3-FP (1.5 mg/kg) & rVIII-SC	2.84	1.37 (2.7fold[a])	47.3 (2.0fold[b])	32.6 (1.9fold[b])
D'D3-FP (3 mg/kg) & rVIII-SC	2.38	0.98 (3.8fold[a])	64.2 (2.8fold[b])	43.9 (2.5fold[b])

rVIII-SC = rVIII-SingleChain

[a] fold decrease over data from rVIII-SingleChain given alone

[b] fold increase over data from rVIII-SingleChain given alone

[c] determination of albumin in µg/mL

Calculation of increases of clearance, MRT and terminal half-life of FVIII:Ag is presented in Table 9. The lowest co-administered dose of 0.5 mg/kg (molar ratio D'D3-FP over rVIII-SingleChain = 100) already relevantly prolong FVIII:Ag exposure. The increase in D'D3-FP dose to 3 mg/kg (molar ratio D'D3-FP over rVIII-SingleChain = 600) led to an at least 2.5fold prolongation of FVIII:Ag MRT and terminal half-life and a 2.5 fold reduction of clearance of FVIII:Ag.

Example 4: Prolongation of pharmacokinetics of FVIII by co-administration of different recombinant FVIII products with D'D3-FP in the rabbit

Material and Methods

Animals: Female CHB rabbits in a weight range of 2.2-2.8 kg (Bauer, Neuental, Germany) were housed one per cage in wire-steel cages at standard housing conditions, i.e. at 21-23°C and 50% relative humidity under a 12 h/12 h light-darkness cycle. The animals were provided tap water ad libitum and fed rabbit pellets (Deukanin®, Deutsche Tiernahrung Cremer GmbH & Co. KG, Düsseldorf, Germany). Animal husbandry and study procedures complied with the German Animal Welfare law and European Union regulations.

Laboratory evaluations: The test articles were administered i.v. by a single injection into the lateral ear vein, with a group size of n=3 animals per group. D'D3-FP preparations were applied at a dose level of 1.5 mg/kg based on human albumin values, and co-administered with 150 IU/kg rVIII-SingleChain (rVIII-SingleChain, dosed according to measured chromogenic activity), 150 IU/kg Advate® (recombinant full length FVIII, dosed according to label) or 150 IU/kg NovoEight® (recombinant B-domain-deleted FVIII, dosed according to label) after incubating for approximately 30 minutes at +37°C. Animals receiving only 150 IU/kg rVIII-SingleChain, Advate® or NovoEight® served as control (Table 10).

Blood samples were taken from the ear artery at pre-dose, 5 and 30 min, 1, 2, 4, 6, 8, 24, 32, 48, 72 and 96 h (recombinant FVIII products alone) or pre-dose, 5 min, 1, 4, 8, 24, 32, 48, 72, 96, 120, 144 and 168 h (recombinant FVIII products co-treated with D'D3-FP) after intravenous bolus injection. Blood samples were anticoagulated using sodium citrate (2 parts sodium citrate 3.13% + 8 parts blood), processed to plasma and stored at –20°C for the determination of FVIII antigen and/or D'D3-FP. D'D3-FP exposure was determined by measurement of the albumin part of the construct using a human albumin ELISA. Accordingly, any amount or dose of D'D3-FP is indicated as the amount or dose of albumin. FVIII:Ag plasma levels were detected by ELISA (Asserachrom Stago, S.A.S., France).

Estimation of mean residence time (MRT), clearance (CL) and terminal half-life (t1/2) was done by non-compartmental methods.

Table 10: Treatment groups

Treatment*	D'D3-FP dose [mg albumin/kg]	FVIII dose [IU/kg]	Volume [mL/kg]	Molar ratio D'D3-FP* over rFVIII	Molar ratio D'D3-FP* over rabbit VWF**
rVIII-SC	-	150	0.434	-	-
Advate®	-	150	1.920	-	-
NovoEight®	-	150	1.536	-	-
D'D3-FP & rVIII-SC	1.5	150	0.688	300	62.8
D'D3-FP & Advate®	1.5	150	2.174	300	62.8
D'D3-FP & NovoEight®	1.5	150	1.790	300	62.8

rFVIII = recombinant factor VIII; rVIII-SC = rVIII-SingleChain

* D'D3-FP calculated as monomer

** D'D3-FP calculated as monomer over endogenous VWF monomer to compare same numbers of FVIII binding sites. For calculation of the endogenous rabbit VWF the same specific activity as human VWF was assumed, i.e. one U/mL (or 100% of the norm) is assumed to be 10 µg/mL (measured rabbit plasma concentration 0.242 U/mL)

Assumption: same specific activity of all products, thus calculating for identical molar ratios

Results

In general, results between the different recombinant FVIII products are very comparable. The following observations regarding the prolongation of FVIII in plasma due to co-administration of D'D3-FP were made in detail:

The exposure of the co-administered FVIII (150 IU/kg chromogenic FVIII activity), quantified as FVIII:Ag via ELISA, was relevantly prolonged in the presence of D'D3-FP (Fig. 7). Plasma levels of FVIII:Ag could be measured maximally up to 32 h p.a. (Advate®) or 48 h p.a. (rVIII-SingleChain and NovoEight®) when the recombinant FVIII products were given alone (detection limit of 117 mIU/mL), and maximally up to the last time-point of 96 h p.a. (Advate®) or 120 h p.a. (rVIII-SingleChain and NovoEight®) after co-treatment with D'D3-FP.

Table 11: Pharmacokinetic parameters of D'D3-FP and FVIII:Ag after co-administration of different rFVIII products and D'D3-FP in rabbits (non-compartmental analysis) Dose D'D3-FP 1.5 mg/kg, dose rFVIII 150 IU/kg

Treatment*	**Cmax, extrap.**	**Clearance**	**MRT**	**Half-life, terminal**
	µg/ml (D'D3-FP [c]) IU/mL (FVIII:Ag)	mL/kg/h	h	h
D'D3-FP				
D'D3-FP & rVIII-SC	22.0	0.638	154	107
D'D3-FP & Advate®	22.2	0.615	126	86
D'D3-FP & NovoEight®	20.2	0.643	137	94
FVIII:Ag				
rVIII-SC	2.51	3.71	23.2	17.3
Advate®	1.52	9.93	17.8	13.4
NovoEight®	2.48	4.42	23.0	18.5
D'D3-FP & rVIII-SC	2.84	1.37 (2.7fold[a])	47.3 (2.0fold[b])	32.6 (1.9fold[b])
D'D3-FP & Advate®	1.34	3.82 (2.6fold[a])	57.5 (3.2fold[b])	40.5 (3.0fold[b])
D'D3-FP & NovoEight®	2.22	1.88 (2.4fold[a])	44.1 (1.9fold[b])	31.1 (1.7fold[b])

rVIII-SC = rVIII-SingleChain

[a] fold decrease over data from each rFVIII product given alone

[b]fold increase over data from each rFVIII product given alone

[c] determination of albumin in µg/mL

Calculation of decreases of clearance, as well as increases in MRT and terminal half-life of FVIII:Ag is presented in Table 11. Decreases in clearance were very comparable for all three products, i.e. between 2.4fold to 2.7fold changes. Based on the difference in t1/2 and MRT of the three recombinant FVIII products alone, the relative increase in MRT and t1/2 showed higher variability and ranged from 3.0-3.2fold for Advate®, which has the shortest pharmacokinetics when given alone to 1.7-2.0fold for rVIII-SingleChain and NovoEight®, which showed roughly comparable pharmacokinetics when given alone. Taken together and with major sight on relative decreases in clearance, the prolongation of pharmacokinetics of the three recombinant FVIII products was very comparable.

Example 5: Prolongation of pharmacokinetics of FVIII by co-administration of rVIII-SingleChain with D'D3-His8 as well as D´D3-CTP fusion protein in the rat

Material and Methods

Animals: Female Crl:CD (Sprague Dawley) rats in a weight range of about 230-300 g were bred at Charles River Laboratories (Sulzfeld, Germany). In house, the animals were kept at standard housing conditions, i.e. at 21-22°C under a 12 h/12 h light-darkness cycle. Animals were fed ad libitum with standard rat diet (Ssniff-Versuchsdiäten, Soest, Germany). Tap water was supplied ad libitum. Animal husbandry and study procedures complied with the German Animal Welfare law and European Union regulations.

The group size was n=6, divided in 2 cohorts. Thus, n=3 animals per time-point were used.

Laboratory evaluations: The test articles were administered i.v. by a single injection into the lateral tail vein at a total volume of 3.0 mL/kg. D'D3 comprising proteins were applied at a dose level of 1 mg/kg based on OD values and co-administered with 200 IU/kg rVIII-SingleChain (rVIII-SingleChain, chromogenic activity) after incubating for approximately 30 minutes at +37°C. This leads to the D'D3 comprising protein ratios over rVIII-SingleChain of 78 and over endogenous VWF of 5.6 (Table 12); animals receiving only rVIII-SingleChain served as control.

Blood samples were taken retro-orbitally under short term anaesthesia at 5 min, 3, 8, 24, 32, 48, 56 and 72 h after intravenous bolus injection using an alternating sampling scheme. The PK profile was taken from two cohorts of rats per group. Blood samples were anticoagulated using sodium citrate (2 parts sodium citrate 3.13% + 8 parts blood), processed to plasma and stored at –20°C for the determination of FVIII antigen and/or D'D3 fusion protein.

D'D3-His8 and D´D3-CTP fusion protein exposure was determined by an ELISA method using D'D3-FP as a standard (see above). FVIII:Ag plasma levels were detected with the FVIII Asserachrom ELISA testkit from Stago, S.A.S., France.

Estimation of mean residence time (MRT), clearance (CL) and terminal half-life (t1/2) was done by one-compartmental methods.

Table 12: Treatment groups

No	Treatment	D'D3-His8 and D´D3-CTP protein dose [mg/kg]*	FVIII dose [IU FVIII:C/kg]	Molar ratio D'D3-His8 and D´D3-CTP protein** over rVIII-SC	Molar ratio D'D3-His8 and D´D3-CTP protein** over rat VWF ***
1	rVIII-SC	-	200	-	-
2	D'D3-His8 & rVIII-SC	1	200	78	5.6
4	D'D3-CTP & rVIII-SC	1	200	78	5.6

FVIII:C = chromogenic FVIII activity; rVIII-SC = rVIII-SingleChain

* D'D3-His8 and D´D3-CTP protein dose based on OD

** D'D3-His8 and D´D3-CTP protein dose corrected by molecular weight

*** D'D3-His8 and D´D3-CTP calculated as monomer over endogenous VWF monomer to compare same numbers of FVIII binding sites. For calculation of the endogenous rat VWF the same specific activity as human VWF was assumed, i.e. one U/mL (or 100% of the norm) is assumed to be 10 µg/mL (measured rat plasma concentration 0.946 U/mL)

Results

All measured data (up to 72 h p.a.) were well above the detection limit of the assay. MRT, t1/2 and clearance suggest that D'D3-CTP had a longer half-life and mean residence time as well as reduced clearance compared to D'D3-8His but even D´D3-8His showed slightly longer MRT and half-life as well as reduced clearance (Table 13).

The pharmacokinetic parameters of the co-administered FVIII (200 IU/kg chromogenic FVIII activity), quantified as FVIII:Ag via ELISA, were improved in the presence of both D'D3 proteins (Table 13). The difference in exposure of the D'D3 comprising proteins translates to the difference in FVIII:Ag exposure, i.e., D'D3-CTP led to a longer FVIII:Ag exposure than D'D3-8His.

Table 13: Pharmacokinetic parameters of D'D3 comprising proteins and FVIII:Ag after co-administration of rVIII-SingleChain and D'D3 proteins in rats (non-compartmental analysis). Dose D'D3 comprising protein 1 mg/kg, dose rVIII-SingleChain 200 IU/kg.

Treatment*	**C_{max}, extrap.**	**Clearance**	**MRT**	**Half-life, terminal**
	µg/mL (D´D3 fusion protein [c]) IU/mL (FVIII:Ag)	mL/kg/h	h	h
	D'D3 fusion protein			
D'D3-His8 & rVIII-SC	31.6	6.80	10.3	7.2
D'D3-CTP & rVIII-SC	19.7	2.43	34.9	26.9
	FVIII:Ag			
rVIII-SC	3.02	6.45	10.3	7.1
D'D3-His8 & rVIII-SC	3.84	4.61 (1.4fold[a])	11.3 (1.1fold[b])	7.8 (1.1fold[b])
D'D3-CTP & rVIII-SC	3.35	3.58 (1.8fold[a])	16.7 (1.6fold[b])	11.6 (1.6fold[b])

rVIII-SC = rVIII-SingleChain

[a] fold decrease over data from rVIII-SingleChain given alone

[b] fold increase over data from rVIII-SingleChain given alone

[c] determination based on OD

Conclusion from PK study results

These studies demonstrated that co-administration of D'D3-FP with FVIII products prolonged FVIII:Ag plasma MRT and half-life and decreased FVIII:Ag clearance independent of whether the FVIII molecule does contain the B domain is B-domain deleted or is present as a single- or two-chain product. This prolongation solely was dependent on the ratio of D'D3-FP over co-administered FVIII as well as over endogenous VWF.

Effects in the rat tend to be stronger than in the rabbit, when the increase in terminal half-life and MRT or reduction of clearance is depicted over the molar ratio of D'D3-FP over administered rVIII-Single-Chain (Fig. 8) as well as over endogenous VWF (Fig. 9). Regarding the molar ratio over rVIII-SingleChain, in the rat $\leq$30-fold ratios lead to smaller than twofold changes, while more favourable effects can be achieved at >50fold ratios, and equal to or more than 2.7 fold improvement can be achieved starting at about 300 fold ratios. Even higher ratios above 750 or preferably 1000 or more preferably 1250 or even more preferably1500 lead to a still higher improvement of terminal half-life and MRT or reduction of clearance.

Regarding ratios over endogenous VWF, species-specific differences are larger, suggesting that a 2 fold improvement may-be achieved in the rat (and in humans assuming similar endogenous VWF levels as in rats) at about ratios $\geq$5 and in the rabbit at about >60. 3fold improvements were achieved in the rat at ratios of about >50.

Surprisingly it could be shown that D´D3-His8 having no half-life extending moiety also prolonged FVIII pharmacokinetic parameters slightly when administered at a ratio of 78 over co-administered FVIII and/or a ratio of 5.6 over endogenous VWF. Even better prolongation of FVIII half-life and mean residence time and reduced clearance was obtained at these ratios with a D'D3-CTP fusion protein which comprises the C-terminal peptide of human chorionic gonadotropin-ß subunit as a half-life extending moiety instead of the albumin comprised in D´D3-FP.

Since in the rat and the rabbit (in contrast to human haemophilia A patients) human administered and endogenous FVIII compete with D'D3-FP and VWF binding sites, it may be expected that the effect on FVIII in the human haemophilia A patient is even stronger.

Example 6: Determination of FVIII affinity to VWF fragment dimer and monomer

A VWF fragment (1-1242) albumin fusion (D´D3-FP) was expressed in a bioreactor; after purification as described above and isolation of monomer and dimer, the affinity of FVIII to these preparations was assessed through surface plasmon resonance via a Biacore instrument (T200, GE Healthcare).

An anti-albumin antibody (MA1-20124, Thermo Scientific) was covalently coupled via its N-terminus to an activated CM 3 chip by NHS (N-Hydroxysuccinimide) and EDC (Ethanolamine hydrochloride), both contained in the amine coupling kit (BR1000-50) from GE Healthcare. For immobilization 3 µg/mL of the antibody were diluted in sodium acetate buffer (10 mM, pH 5.0) and the antibody solution was flown over the chip for 7 min. at a flow rate of 10 µL/min. After the immobilization procedure non-coupled dextran filaments were saturated by flowing ethanolamine solution (1 M, pH 8.3) over the chip for 5 min (at a flow rate of 10 µL/min). The aim of saturating the flow cell was to minimize unspecific binding of the analytes to the chip. A reference flow cell was set up by saturating an empty flow cell with ethanolamine by using the same procedure as above.

Dimeric and monomeric D´D3-FP proteins, respectively, were immobilized to the covalently coupled anti-albumin antibody by a flow of the D´D3-FP proteins (5 µg/mL) over the chip for 3 min (flow rate of 10 µL/min). The captured mass of dimeric D'D3-FP was 335 RU and for monomeric D'D3-FP 147 RU, assuming one binding site both on the monomer and on the dimer D'D3-FP for FVIII.

To create binding curves for FVIII, each D´D3-FP protein preparation was diluted in running buffer (HBS-P+: 0.1 M HEPES, 1.5 M NaCl and 0.5% v/v Surfactant P20, pH 7.4; product code BR100671, GE Healthcare) to concentrations of 0.25 nM, 0.5 nM, 1 nM, 3nM and 4 nM. By performing a single cycle kinetic, samples with ascending concentrations of each dilution were flown over the chip for 2 min (flow rate 30µL/min.), followed by a dissociation time of 10 min. with running buffer HBS-P+. All measurements were performed twice. The temperature for the measuring procedure was adjusted to +25°C.

Binding parameters were calculated using BiaEvaluation Software. The curve fitting methods were based on Langmuir equations. The input data for calculations were the molar mass of the analyte FVIII (rVIII-SingleChain), other parameters like max. RU and slopes were automatically extracted out of the fitted association and dissociation curves. The outputs of BiaEvaluation Software are the association rate constants and the dissociation rate

constants, from which the affinity constants were calculated. The results are shown in Table 12.

Table 12: FVIII affinity data for D´D3-FP dimer and monomer

D'D3-FP preparation	**ka [1/Ms]**	**kd [1/s]**	**KD [M]**
D'D3-FP Dimer	2,33E+07	1,37E-03	5,90E-11
D'D3-FP Monomer	4,41E+07	3,96E-03	8,99E-11

The association rate constant was slightly increased for rVIII-SingleChain to the monomeric D'D3-FP, while the dissociation rate constant of rVIII-SingleChain to D'D3-FP dimer was three times slower than to the monomer. The quotient of the dissociation rate constant and the association rate constant indicates the affinity of rVIII-SingleChain to D'D3-FP. The dimeric D'D3-FP hence shows an increased affinity to FVIII compared to the D'D3-FP monomer.

Claims

1. A polypeptide comprising a truncated von Willebrand Factor (VWF) for use in the treatment of a blood coagulation disorder, said treatment comprising administering to a subject having endogenous VWF the polypeptide and a Factor VIII (FVIII), wherein the polypeptide is capable of binding to said FVIII, and wherein the molar ratio of the polypeptide to be administered to the FVIII to be administered is greater than 50.

2. A polypeptide for use according to claim 1, wherein the polypeptide comprises a half-life extending moiety.

3. A polypeptide comprising a truncated von Willebrand Factor (VWF) for use in the treatment of a blood coagulation disorder, said treatment comprising administering to a subject having endogenous VWF the polypeptide and a Factor VIII (FVIII), wherein the polypeptide is capable of binding to said FVIII, wherein the molar ratio of the polypeptide administered to the endogenous VWF is greater than 0.5, and wherein said molar ratio is the molar plasma concentration of the polypeptide of the treated subject immediately after administration of the polypeptide, divided by the molar plasma concentration of endogenous VWF of the treated subject.

4. A polypeptide for use according to claim 3, wherein the polypeptide comprises a half-life extending moiety.

5. The polypeptide for use according to claims 3 and 4, wherein the concentration of the endogenous VWF is defined as the concentration in normal human plasma (NHP).

6. The polypeptide for use according to any one of claims 1 to 5, wherein the subject is a human.

7. The polypeptide for use according to any one of claims 1 to 6, wherein the polypeptide is administered intravenously.

8. The polypeptide for use according to any one of the preceding claims, wherein the truncated VWF comprises amino acids 764 to 1242 of SEQ ID NO:4.

9. The polypeptide for use according to any one of the preceding claims, wherein the truncated VWF lacks amino acids 1243 to 2813 of SEQ ID NO:4.

10. The polypeptide for use according to any one of the preceding claims, wherein the truncated VWF consists of (a) amino acids 764 to 1242 of SEQ ID NO:4, (b) an amino acid sequence having a sequence identity of at least 90% to amino acids 764 to 1242 of SEQ ID NO:4, or (c) a fragment of (a) or (b).

11. The polypeptide for use according to claim 2 and to claims 4 to 10, wherein the half-life extending moiety is a heterologous amino acid sequence fused to the truncated VWF.

12. The polypeptide for use according to claim 11, wherein said heterologous amino acid sequence comprises or consists of a polypeptide selected from the group consisting of immunoglobulin constant regions and portions thereof, e.g. the Fc fragment, transferrin and fragments thereof, the C-terminal peptide of human chorionic gonadotropin, solvated random chains with large hydrodynamic volume known as XTEN, homo-amino acid repeats (HAP), proline-alanine-serine repeats (PAS), albumin, afamin, alpha-fetoprotein, Vitamin D binding protein, polypeptides capable of binding under physiological conditions to albumin or immunoglobulin constant regions, and combinations thereof.

13. The polypeptide for use according to claim 2 and to claims 4 to 10, wherein the half-life extending moiety is conjugated to the polypeptide.

14. The polypeptide for use according to claim 13, wherein said half-life-extending moiety is selected from the group consisting of hydroxyethyl starch (HES), polyethylene glycol (PEG), polysialic acids (PSAs), elastin-like polypeptides,

heparosan polymers, hyaluronic acid and albumin binding ligands, e.g. fatty acid chains, and combinations thereof.

15. The polypeptide for use according to any one of the preceding claims, wherein the polypeptide is a glycoprotein comprising N-glycans, and wherein at least 75 %, preferably at least 85 % of said N-glycans comprise, on average, at least one sialic acid moiety.

16. The polypeptide for use according to any one of the preceding claims, wherein the polypeptide is a dimer.

17. The polypeptide for use according to any one of the preceding claims, wherein the mean residence time (MRT) of the FVIII is increased by the co-administration of the polypeptide, as compared to a reference treatment, wherein said reference treatment is identical to said treatment, except that the polypeptide and the FVIII are administered in equimolar amounts in said reference treatment.

18. The polypeptide for use according to any one of the preceding claims, wherein the frequency of administration of the FVIII is reduced as compared to a treatment with the FVIII alone.

19. The polypeptide for use according to any one of the preceding claims, wherein the plasma half-life of the polypeptide is greater than that of endogenous VWF.

20. The polypeptide for use according to claim 17, wherein the plasma half-life of the polypeptide is at least 25 % greater than that of the endogenous VWF.

21. A pharmaceutical composition comprising (i) a FVIII and (ii) a polypeptide as defined in any one of claims 1 to 20, wherein the molar ratio of the polypeptide to the FVIII in the composition is greater than 50.

22. A pharmaceutical kit comprising (i) a FVIII and (ii) a polypeptide as defined in any one of claims 1 to 20 for simultaneous, separate or sequential use in the treatment of a blood coagulation disorder, said treatment comprising administering to a subject having endogenous VWF the polypeptide and the FVIII, wherein

(i) the molar ratio of the polypeptide administered to the endogenous VWF is greater than 0.5, wherein said molar ratio is the molar plasma concentration of the polypeptide of the treated subject immediately after administration of the polypeptide, divided by the molar plasma concentration of the endogenous VWF of the treated subject, and/or

(ii) the molar ratio of the polypeptide to be administered to the FVIII to be administered is greater than 50.

23. The use of a polypeptide as defined in any one of claims 1 to 20 for improving the plasma half-life of FVIII, and/or for reducing the frequency of administration of FVIII.

24. A method of treating a blood coagulation disorder, comprising administering to a patient having endogenous VWF an effective amount of a polypeptide as defined in any one of claims 1 to 20 and a FVIII, wherein

(i) the molar ratio of the polypeptide administered to the endogenous VWF is greater than 0.5, wherein said molar ratio is the molar plasma concentration of the polypeptide of the treated subject immediately after administration of the polypeptide, divided by the molar plasma concentration of the endogenous VWF of the treated subject, and/or

(ii) wherein the molar ratio of the polypeptide to be administered to the FVIII to be administered is greater than 50.