(54) Title: COMPOUNDS SUITABLE FOR TREATMENT OF HAEMOPHILIA

(57) Abstract: The present invention relates to VWF compounds as well as compositions suitable for treatment of blood clotting diseases.

COMPOUNDS SUITABLE FOR TREATMENT OF HAEMOPHILIA

TECHNICAL FIELD

The present invention relates to treatment and/or prophylaxis of haemophilia.

BACKGROUND

Protein replacement therapy by intravenous administration of coagulation factors is currently used for treating patients suffering from haemophilia. For patient convenience and compliance, extravascular (e.g. subcutaneous (s.c.) or intradermal) administration would be preferable to the existing intravenous (i.v.) injections. There are furthermore potential safety advantages associated with extravascular administration, since many patients could avoid intravenous port surgery as well as the risk of infection and clots associated with insertion of such catheters.

S.c. administration of FVIII in FVIII deficient mice is disclosed in Shi et al, Haemophilia, 2012, DOI: 10.1 111/j. 1365-251 6.201 1.02735.x. The bioavailability of FVIII is herein reported to be low (about 1%).

S.c. administration of FVIII and VWF is furthermore disclosed in WO08151817 but no dose response relationship between the FVIII dose and the achieved circulating FVIII concentration is disclosed. In W0815817, the (Unit) ratio of VWF over FVIII was larger than 5:1 , corresponding to a 150-250 fold molar excess of the concentration of VWF protein as compared to that of FVIII. From a practical and economical pint of view, this type of ratios are, however, not desirable. In WO08151817, it is furthermore shown that the immunogenicity in mice of s.c. administered FVIII is significantly reduced when FVIII is co-formulated with VWF.

In W01 0062768, it is disclosed that PEGylation of FVIII can improve the bioavailability of FVIII in connection with subcutaneous injection into mice, whereas co-formulation with VWF does not improve the bioavailability of FVIII.

There is a need in the art for compounds and/or pharmaceutical compositions suitable for extravascular administration in treatment and/or prophylaxis of patients suffering from blood clotting diseases such as haemophilia A with or without inhibitors, and/or von Willebrand disease, as such administration forms would alleviate the burden of i.v. treatment both related to the infusion as such and also the risk of infections due to implanted portable catheters. Such compounds and compositions are preferably safe (i.e. have a low risk of immunogenicity) and/or have a high bioavailability and/or are preferably easy to handle in connection with production and formulation processes.

SUMMARY

The present invention relates to a recombinant VWF fragment comprising 1200 amino acids or less, such as e.g. the TIL' domain or the TIL7E' domain (Zhou et al. Blood 2012; 120(2): 449-458). The present invention furthermore relates to a pharmaceutical composition comprising: (i) a VWF fragment according to the invention and (ii) FVIII molecule (full-length/truncated B domain/conjugated). The present invention furthermore relates to use thereof for treatment of haemophilia, e.g. by extravascular administration. Such compounds and compositions will preferably result in a relatively high FVIII bioavailability and/or a relatively low risk of FVIII immunogenicity in connection with extravascular co-administration of FVIII.

DESCRIPTION

In one aspect of the invention, VWF fragments according to the invention co-administered with FVIII molecules having a prolonged *in vivo* circulatory half-life have a surprisingly high bioavailability in connection with extravascular (e.g. s.c.) administration thereof.

The inventors of the present invention have furthermore made the surprising observation that bioavailability of FVIII molecules may be significantly improved upon extravascular co-administration with similar molar amounts of VWF fragments according to the invention. Alternatively, high bioavailability may be achieved through extravascular co-administration of a pool of FVIII molecules, wherein the majority of said FVIII molecules are bound to VWF fragments according to the invention. Interestingly, full length VWF does not have a positive impact on the bioavailability of FVIII. Preferably, VWF should be in the form of a VWF fragment that comprise the TIL' or the TIL7E' domains. Compounds and compositions according to the present invention are thus useful for treatment and prophylaxis of haemophilia patients (in particular haemophilia A patients) with and without inhibitors, as well as for immune tolerance induction (ITI) of haemophilia patients with inhibitors.

Brief description of drawings

Figure 1: FVIII activity in plasma after subcutaneous administration of 10000 U/kg "N8-GP" with or without co-administration of **7.7** times the molar dose of VWF TIL7E7D3/A1 relatively to N8-GP. Data are mean and standard deviation of measurements from n=2 FVIII KO mice per time point. "N8-GP" is a glyco-PEGylated FVIII molecule produced as described in Examples 1+2 in WO20091 08806.

Figure 2: FVIII antigen in plasma after subcutaneous administration of 10000 U/kg N8-GP with or without co-administration of **7.7** times the molar dose of VWF TIL7E7D3/A1 relatively to N8-GP. Data are mean and standard deviation of measurements from n=2 FVIII KO mice per time point

Figure 3: FVIII activity in plasma after subcutaneous administration of 2500 U/kg N8-GP with or without co-administration of **7.7** times the molar dose of VWF TIL7E7D3/A1 relatively to N8-GP. Data are mean and standard deviation of measurements from n=2 FVIII KO mice per time point

Figure 4: FVIII antigen in plasma after subcutaneous administration of 2500 U/kg N8-GP with or without co-administration of **7.7** times the molar dose of VWF TIL7E7D3/A1 relatively to N8-GP. Data are mean and standard deviation of measurements from n=2 FVIII KO mice per time point.

Figure 5: FVIII activity in plasma after subcutaneous administration of 5000 or 20000 IU/kg wt FVIII (N8, turoctocog alfa) with or without co-administration of **7.7** times the molar dose of VWF TIL7E7D3/A1 relatively to FVIII, respectively. Data are mean and standard deviation of measurements from n=2 FVIII KO mice per time point. "N87"turocotog alfa" is a B domain truncated FVIII molecule produced as described in Example 1 in WO20091 08806.

Figure 6: FVIII antigen in plasma after subcutaneous administration of 5000 or 20000 IU/kg wt FVIII (N8, turoctocog alfa) with or without co-administration of **7.7** times the molar dose of VWF TIL7E7D3/A1 relatively to FVIII. Data are mean and standard deviation of measurements from n=2 FVIII KO mice per time point.

Figure 7: FVIII antigen in plasma after subcutaneous administration of 5000 IU/kg FVIII (N8, turoctocog alfa) with or without co-administration of **7.7** times the molar dose of VWF TIL7E7D3/A1 relatively to FVIII. Data are mean and standard deviation of measurements from n=2 FVIII KO mice per time point.

Figure 8: FVIII activity in plasma after subcutaneous administration of 5000 IU/kg FVIII (N8, turoctocog alfa) with or without co-administration of **7.7** times the molar dose of VWF TIL7E7D3/A1 relatively to FVIII. Data are mean and standard deviation of measurements from n=2 FVIII KO mice per time point.

Figure 9: VWF variant (764-865 SEQ ID NO 5) binding to FVIII (N8, turoctocog alfa) at 20 $^\circ$C. The upper panel shows raw data of heat released upon each titration. Lower panel shows binding isotherm obtained from integrating raw data. Data analysis shows that VWF variant (SEQ ID NO 5) binds to FVIII in an exothermic reaction with a stoichiometry of 1.14, ΔH of -5.82 kcal/mole, AS of 9.8 cal/mol/deg and a K_d of 0.33 μM. "F8/N8/turoctocog alfa" is

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a B domain truncated FVIII molecule produced as disclosed in Example 1 in
WO20091 08806.

Figure 10: s.c. administered N8-GP is haemostatic effective in vivo. The left panel
shows blood loss in FVIIIKO mice treated s.c. with N8-GP or vehicle 24 hr before tail
transection, or i.v. 5 min before tail transection. N8-GP" is a glyco-PEGylated FVIII molecule
produced as described in Examples 1+2 in WO20091 08806. The right panel shows clot
times in whole blood from the mice *ex vivo* using ROTEM.

Figure 11: SEC-UV (280 nm) chromatograms for FVIII, TIL7E7D3/A1 III, and a
mixture of FVIII and TIL7E7D3/A1 III in 155 mM NaCl, 10 mM Calciumacetat, 10 %
Isopropanol at 25 °C.

Figure 12:. SEC-UV (280 nm) chromatograms for FVIII, TIL7E7D3 II, and a mixture
of FVIII and TIL7E7D3 II in 155 mM NaCl, 10 mM Calciumacetat, 10 % Isopropanol at 25 °C.

Definitions

The term "treatment", as used herein, refers to the medical therapy of any human or
other vertebrate subject in need thereof. Said subject is expected to have undergone
physical examination by a medical practitioner, or a veterinary medical practitioner, who has
given a tentative or definitive diagnosis which would indicate that the use of said specific
treatment is beneficial to treating a disease in said human or other vertebrate. The timing and
purpose of said treatment may vary from one individual to another, according to the subject's
health. Thus, said treatment may be prophylactic, palliative, symptomatic and/or curative.

Mode of administration: Compounds and pharmaceutical compositions according to
the invention may be administered parenterally, such as e.g. intravenously or extravascularly
(such as e.g. intradermally, intramuscularly, subcutaneously, etc). Compounds and
pharmaceutical compositions according to the invention may be administered
prophylactically and/or therapeutically and/or on demand. According to the present invention,
several advantages are associated with extravascular administration of
compounds/pharmaceutical compositions according to the present invention. Extravascular
administration is easier, simpler, and associated with less pain, inconvenience, and
complications (and thus potentially resulting in better compliance) which is of potential benefit
to all patients but of particular benefit for children and small infants.

Combination treatments/co-administration: Combined administration of two or more
active compounds (e.g. FVIII and VWFA/WF fragments according to the invention having the
ability to bind to FVIII) may be achieved in a number of different ways. In one embodiment,
the two active compounds may be administered together in a single composition. In another

embodiment, the two active compounds may be administered in separate compositions as part of a combined therapy. For example, the first compound may be administered before, after, or concurrently with the second compound. In case FVIII and VWF fragment are administered extravascularly (e.g. subcutaneously) as two separate pharmaceutical

5   compositions, they are preferably administered in close proximity in order to benefit from the improved bioavailability that can be obtained when administering these two types of compounds together (i.e. the injection sites should be separated by no more than 5 cm, preferably no more than 4 cm, preferably no more than 3 cm, preferably no more than 2 cm, and most preferably no more than 1 cm). The two compounds should preferably also be

10   injected within about an hour, preferably within about 30 minutes, preferably within about 15 minutes, and most preferably within about 5 minutes.

Factor VIII: Factor VIII (FVIII) is a large, complex glycoprotein that is primarily produced by hepatocytes. Human FVIII comprises2351 amino acids, including a signal peptide, and contains several distinct domains as defined by homology. There are three A-

15   domains, a unique B-domain, and two C-domains. The domain order can be listed as NH2-A1-A2-B-A3-C1-C2-COOH. The chains are connected by bivalent metal ion-bindings. The A1-A2-B chain is termed the heavy chain (HC) while the A3-C1-C2 is termed the light chain (LC). Small acidic regions C-terminal of the A1 (the a1 region) and A2 (the a2 region) and N-terminal of the A3 domain (the a3 region) play important roles in its interaction with other

20   coagulation proteins, including thrombin and von Willebrand factor (VWF), the carrier protein for FVIII.

Endogenous FVIII molecules circulate *in vivo* as a pool of molecules with B domains of various sizes, the shortest having C-terminal at position 740, i.e. at the C-terminal of A2-a2, and thus contains no B domain. These FVIII molecules with B-domains of different length

25   all have full procoagulant activity. Upon activation with thrombin, FVIII is cleaved C-terminal of A1-a1 at position 372, C-terminal of A2-a2 at position 740, and between a3 and A3 at position 1689, the latter cleavage releasing the a3 region with concomitant loss of affinity for VWF. The activated FVIII molecule is termed FVIIIa. The activation allows interaction of FVIIIa with phospholipid surfaces like activated platelets and activated factor IX (FIXa), i.e.

30   the tenase complex is formed, allowing efficient activation of factor X (FX).

The terms "Factor VIII(a)" and "FVIII(a)" include both FVIII and FVIIIa. Similarly, the term "Factor VIII" and "FVIII" may include both FVIII and FVIIIa. "Factor VIII" or "FVIII" as used herein refers to a human plasma glycoprotein that is a member of the intrinsic coagulation pathway and is essential to blood coagulation. "Wildtype(wt)/native FVIM" is the

35   human FVIII molecule derived from the full length sequence as shown in **SEQ ID NO: 1**

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(amino acid 1-2332). "FVIII(a)" includes natural allelic variants of FVIII(a) that may exist and occur from one individual to another. FVIII(a) may be plasma-derived or recombinantly produced, using well known methods of production and purification. The degree and location of glycosylation, tyrosine sulfation and other post-translation modifications may vary, depending on the chosen host cell and its growth conditions.

Pharmaceutical compositions according to the present invention may comprise native or B domain-truncated FVIII molecules wherein the remaining domains correspond closely to the sequences as set forth in amino acid numbers 1-740 and 1649-2332 of SEQ ID NO: 3. In such molecules, as well as in FVIII comprising the full-length B domain amino acid sequence, mutations may be introduced. Amino acid modifications, such as substitutions, insertions, and deletions, may be introduced into the molecule in order to modify the binding capacity of FVIII with various other components such as low-density lipoprotein receptor-related protein (LRP) and related receptors, various other receptors, other coagulation factors, cell surfaces, introduction and/or abolishment of glycosylation sites, etc. Other mutations that do not abolish FVIII activity may also be accommodated in the FVIII molecules herein.

FVIII molecules herein (molecules/variants/derivatives/analogues/conjugates) are capable of functioning in the coagulation cascade in a manner that is functionally similar, or equivalent, to wt/endogenous FVIII, inducing the formation of FXa via interaction with FIXa on an activated platelet and supporting the formation of a blood clot. FVIII activity can be assessed *in vitro* using techniques well known in the art. Clot analyses, FX activation assays (often termed chromogenic assays), thrombin generation assays and whole blood thrombo-elastography are examples of such *in vitro* techniques. FVIII molecules according to the present invention have FVIII activity that is at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, 100% or even more than 100% of that of native human FVIII.

Endogenous full length FVIII is synthesized as a single-chain precursor molecule. Prior to secretion, the precursor is cleaved into the heavy chain and the light chain. Recombinant B domain-deleted or truncated FVIII can be produced by means of two different strategies. Either the heavy chain without the B-domain and the light chain are synthesized individually as two different polypeptide chains (two-chain strategy) or the B domain-deleted or truncated FVIII is synthesized as a single precursor polypeptide chain (single-chain strategy) that is cleaved into the heavy and light chains in the same way as the full-length FVIII precursor.

In a B domain-deleted or truncated FVIM precursor polypeptide, produced by the single-chain strategy, the heavy and light chain moieties are often separated by a linker. To minimize the risk of introducing immunogenic epitopes in the B domain-deleted FVIM, the sequence of the linker is preferably derived from the FVIM B-domain. In the B domain of full length FVIM, amino acid 1644-1648 constitutes this recognition site. The thrombin cleavage site leading to removal of the linker on activation of B domain-deleted FVIM is located in the heavy chain. Thus, the size and amino acid sequence of the linker is unlikely to influence its removal from the remaining FVIM molecule by thrombin activation. Deletion/truncation of the B domain is an advantage for production of FVIM. Nevertheless, parts of the B domain can be included in the linker without reducing the productivity. The negative effect of the B domain on productivity has not been attributed to any specific size or sequence of the B domain.

SEQ ID NO: 1: wt human FVIM (Ser750 residue shown in bold and underline)

ATRRYYLGAVELSWDYMQSDLGELPVDARFPPRVPKSFPFNTSWYKKTLFVEFT

DHLFNIAKPRPPWMGLLGPTIQAEVYDTVVITLKNMASHPVSLHAVGVSYWKASEGAEYDD

QTSQREKEDDKVFPGGSHTYVWQVLKENGPMASDPLCLTYSYLSHVDLVKDLNSGLIGALL

VCREGSLAKEKTQTLHKFILLFAVFDEGKSWHSETKNSLMQDRDAASARAWPKMHTVNGY

VNRSLPGLIGCHRKSVYWHVIGMGTTPEVHSIFLEGHTFLVRNHRQASLEISPITFLTAQTLL

MDLGQFLLFCHISSHQHDGMEAYVKVDSCPEEPQLRMKNNEEAEDYDDDLTDSEMDWRF

DDDNSPSFIQIRSVAKKHPKTWVHYIAAEEEDWDYAPLVLAPDDRSYKSQYLNNGPQRIGR

KYKKVRFMAYTDETFKTREAIQHESGILGPLLYGEVGDTLLIIFKNQASRPYNIYPHGITDVRP

LYSRRLPKGVKHLKDFPILPGEIFKYKWTVTVEDGPTKSDPRCLTRYYSSFVNMERDLASGLI

GPLLICYKESVDQRGNQIMSDKRNVILFSVFDENRSWYLTENIQRFLPNPAGVQLEDPEFQA

SNIMHSINGYVFDSLQLSVCLHEVAYWYILSIGAQTDFLSVFFSGYTFKHKMVYEDTLTLFPF

SGETVFMSMENPGLWILGCHNSDFRNRGMTALLKVSSCDKNTGDYYEDSYEDISAYLLSKN

NAIEPRSFSQNSRHPS<u>T</u>RQKQFNATTIPENDIEKTDPWFAHRTPMPKIQNVSSSDLLMLLRQ

SPTPHGLSLSDLQEAKYETFSDDPSPGAIDSNNSLSEMTHFRPQLHHSGDMVFTPESGLQL

RLNEKLGTTAATELKKLDFKVSSTSNNLISTIPSDNLAAGTDNTSSLGPPSMPVHYDSQLDTT

LFGKKSSPLTESGGPLSLSEENNDSKLLESGLMNSQESSWGKNVSSTESGRLFKGKRAHG

PALLTKDNALFKVSISLLKTNKTSNNSATNRKTHIDGPSLLIENSPSVWQNILESDTEFKKVTP

LIHDRMLMDKNATALRLNHMSNKTTSSKNMEMVQQKKEGPIPPDAQNPDMSFFKMLFLPES

ARWIQRTHGKNSLNSGQGPSPKQLVSLGPEKSVEGQNFLSEKNKVVVGKGEFTKDVGLKE

MVFPSSRNLFLTNLDNLHENNTHNQEKKIQEEIEKKETLIQENVVLPQIHTVTGTKNFMKNLF

LLSTRQNVEGSYDGAYAPVLQDFRSLNDSTNRTKKHTAHFSKKGEEENLEGLGNQTKQIVE

KYACTTRISPNTSQQNFVTQRSKRALKQFRLPLEETELEKRIIVDDTSTQWSKNMKHLTPSTL

8

TQIDYNEKEKGAITQSPLSDCLTRSHSIPQANRSPLPIAKVSSFPSIRPIYLTRVLFQDNSSHL

PAASYRKKDSGVQESSHFLQGAKKNNLSLAILTLEMTGDQREVGSLGTSATNSVTYKKVEN

TVLPKPDLPKTSGKVELLPKVHIYQKDLFPTETSNGSPGHLDLVEGSLLQGTEGAIKWNEAN

RPGKVPFLRVATESSAKTPSKLLDPLAWDNHYGTQIPKEEWKSQEKSPEKTAFKKKDTILSL

NACESNHAIAAINEGQNKPEIEVTWAKQGRTERLCSQNPPVLKRHQREITRTTLQSDQEEID

YDDTISVEMKKEDFDIYDEDENQSPRSFQKKTRHYFIAAVERLWDYGMSSSPHVLRNRAQS

GSVPQFKKVVFQEFTDGSFTQPLYRGELNEHLGLLGPYIRAEVEDNIMVTFRNQASRPYSFY

SSLISYEEDQRQGAEPRKNFVKPNETKTYFWKVQHHMAPTKDEFDCKAWAYFSDVDLEKD

VHSGLIGPLLVCHTNTLNPAHGRQVTVQEFALFFTIFDETKSWYFTENMERNCRAPCNIQME

DPTFKENYRFHAINGYIMDTLPGLVMAQDQRIRWYLLSMGSNENIHSIHFSGHVFTVRKKEE

YKMALYNLYPGVFETVEMLPSKAGIWRVECLIGEHLHAGMSTLFLVYSNKCQTPLGMASGHI

RDFQITASGQYGQWAPKLARLHYSGSINAWSTKEPFSWIKVDLLAPMIIHGIKTQGARQKFS

SLYISQFIIMYSLDGKKWQTYRGNSTGTLMVFFGNVDSSGIKHNIFNPPIIARYIRLHPTHYSIR

STLRMELMGCDLNSCSMPLGMESKAISDAQITASSYFTNMFATWSPSKARLHLQGRSNAW

RPQVNNPKEWLQVDFQKTMKVTGVTTQGVKSLLTSMYVKEFLISSSQDGHQWTLFFQNGK

VKVFQGNQDSFTPVVNSLDPPLLTRYLRIHPQSWVHQIALRMEVLGCEAQDLY

The B domain in FVIII spans amino acids 741-1648 of **SEQ ID NO: 1.** The B domain is cleaved at several different sites, generating large heterogeneity in circulating plasma FVIII molecules. The exact function of the heavily glycosylated B domain is unknown. What is known is that the B domain is dispensable for FVIII activity in the coagulation cascade. Recombinant FVIII is thus frequently produced in the form of B domain-deleted/truncated variants. In a preferred embodiment, the FVIII molecule is produced by an expression vector encoding a FVIII molecule comprising a 21 amino acid residue L (linker) sequence with the following sequence: **SEQ ID NO 2:** SFSQNSRHPS̲QNPPVLKRHQR (the O-glycan is attached to the underlined S). Alternative preferred B domain linker sequences may lack one or more of the amino acid residues set forth in SEQ ID NO 2, e.g. the C-terminal R in SEQ ID NO 2. Preferred FVIII molecules are B domain deleted/truncated variants comprising an O-glycan attached to the Ser 750 residue shown in SEQ ID NO 1 - optionally being conjugated to a polymeric (half life extending) moiety via this O-glycan.

The inventors of the present invention have made the surprising observation that B domain deleted FVIII molecules according to the invention having a B domain of a size from about 100 to about 400 amino acids ((preferably 150-650, more preferably 150-600, more preferably 150-550, more preferably 150-500, more preferably 150-450, more preferably 150-400, more preferably 150-350, more preferably 200-700, more preferably 200-600, more

preferably 200-500, more preferably 200-400, more preferably 200-300, and most preferably about 200 to 250) have a surprisingly high bioavailability in connection with extravascular (e.g. s.c.) administration compared to e.g. FVIII molecules having the entire B domain intact as well FVIII molecules having no or only a few amino acids (e.g. 15-30 amino acids) intact. Such molecules may or may not comprise the Ser750 residue according to SEQ ID NO 1. A simple and safe way of producing FVIII having improved bioavailability upon subcutaneous/intradermal administration is thus provided. It is plausible that the *in vivo* circulatory half-life of FVIII having B domains of 100 to about 400 amino acids may be prolonged by conjugating/fusing such variants with a half-life extending moiety. An example of a FVIII molecule comprising a 226 amino acid B domain is shown in **SEQ ID NO 3:**

    SEQ ID NO 3: (226 amino acid B domain variant):

    ATRRYYLGAVELSWDYMQSDLGELPVDARFPPRVPKSFPFNTSWYKKTLFVEFT
DHLFNIAKPRPPWMGLLGPTIQAEVYDTWITLKNMASHPVSLHAVGVSYWKASEGAEYDD
QTSQREKEDDKVFPGGSHTYVWQVLKENGPMASDPLCLTYSYLSHVDLVKDLNSGLIGALL
VCREGSLAKEKTQTLHKFILLFAVFDEGKSWHSETKNSLMQDRDAASARAWPKMHTVNGY
VNRSLPGLIGCHRKSVYWHVIGMGTTPEVHSIFLEGHTFLVRNHRQASLEISPITFLTAQTLL
MDLGQFLLFCHISSHQHDGMEAYVKVDSCPEEPQLRMKNNEEAEDYDDDLTDSEMDWRF
DDDNSPSFIQIRSVAKKHPKTWVHYIAAEEEDWDYAPLVLAPDDRSYKSQYLNNGPQRIGR
KYKKVRFMAYTDETFKTREAIQHESGILGPLLYGEVGDTLLIIFKNQASRPYNIYPHGITDVRP
LYSRRLPKGVKHLKDFPILPGEIFKYKWTVTVEDGPTKSDPRCLTRYYSSFVNMERDLASGLI
GPLLICYKESVDQRGNQIMSDKRNVILFSVFDENRSWYLTENIQRFLPNPAGVQLEDPEFQA
SNIMHSINGYVFDSLQLSVCLHEVAYWYILSIGAQTDFLSVFFSGYTFKHKMVYEDTLTLFPF
SGETVFMSMENPGLWILGCHNSDFRNRGMTALLKVSSCDKNTGDYYEDSYEDISAYLLSKN
NAIEPRSFSQNSRHPSTRQKQFNATTIPENDIEKTDPWFAHRTPMPKIQNVSSSDLLMLLRQ
SPTPHGLSLSDLQEAKYETFSDDPSPGAIDSNNSLSEMTHFRPQLHHSGDMVFTPESGLQL
RLNEKLGTTAATELKKLDFKVSSTSNNLISTIPSDNLAAGTDNTSSLGPPSMPVHYDSQLDTT
LFGKKSSPLTESGGPLSLSEENNDSKLLESGLMNSQESSWGKNVSHHHHHHSQNPPVLKR
HQREITRTTLQSDQEEIDYDDTISVEMKKEDFDIYDEDENQSPRSFQKKTRHYFIAAVERLW
DYGMSSSPHVLRNRAQSGSVPQFKKVVFQEFTDGSFTQPLYRGELNEHLGLLGPYIRAEVE
DNIMVTFRNQASRPYSFYSSLISYEEDQRQGAEPRKNFVKPNETKTYFWKVQHHMAPTKDE
FDCKAWAYFSDVDLEKDVHSGLIGPLLVCHTNTLNPAHGRQVTVQEFALFFTIFDETKSWYF
TENMERNCRAPCNIQMEDPTFKENYRFHAINGYIMDTLPGLVMAQDQRIRWYLLSMGSNEN
IHSIHFSGHVFTVRKKEEYKMALYNLYPGVFETVEMLPSKAGIWRVECLIGEHLHAGMSTLFL
VYSNKCQTPLGMASGHIRDFQITASGQYGQWAPKLARLHYSGSINAWSTKEPFSWIKVDLL

APMIIHGIKTQGARQKFSSLYISQFIIMYSLDGKKWQTYRGNSTGTLMVFFGNVDSSGIKHNIF

NPPIIARYIRLHPTHYSIRSTLRMELMGCDLNSCSMPLGMESKAISDAQITASSYFTNMFATW

SPSKARLHLQGRSNAWRPQVNNPKEWLQVDFQKTMKVTGVTTQGVKSLLTSMYVKEFLIS

SSQDGHQWTLFFQNGKVKVFQGNQDSFTPVVNSLDPPLLTRYLRIHPQSWVHQIALRMEVL

GCEAQDLY

Von Willebrand Factor (VWF) is a blood glycoprotein involved in hemostasis. It is deficient or defective in von Willebrand disease which is the most common hereditary bleeding disorder. VWF is a large multimeric glycoprotein present in blood plasma and produced constitutively in endothelium, megakaryocytes, and subendothelial connective tissue. The basic VWF monomer is a 2050 amino acid protein. Each monomer contains a number of specific domains with a specific function, including the TIL' or TIL7E' domain (Zhou et al. Blood 2012; 120(2): 449-458) which binds to FVIII. FVIII is bound to VWF while inactive in circulation and is released from VWF by the action of thrombin. FVIII(a) not bound to VWF is rapidly cleared and/or degraded. It is shown herein, that full-length VWF does not have the ability to significantly increase bioavailability of extra-vascularly co-administered FVIII despite of its inherent FVIII protective effects.

The full length VWF molecule is thus a very complex protein. The prepro VWF consists of 2813 amino acid residues (SEQ ID NO 22). During secretion, the signal peptide from amino acid residue 1 to 22 and the propeptide from amino acid residue 23 to 763 are cleaved off, leaving a mature VWF of 2050 amino acid residues. The amino acid numbering is thus often based on the prepro VWF and amino acid S764 is thus the first amino acid in the mature molecule. The mature molecule is believed to contain 12 Asn-linked and 10 Thr/Ser linked oligosaccharide side chains. Furthermore this molecule can form dimers, trimers etc. with multimer molecule weight of up to several million Daltons. Different allelic VWF variants are found in human beings and it is thus understood that VWF fragments according to the present invention can be derived from any one of these naturally occurring variants.

The glycosylation heterogeneity, together with the multimer forming properties, of the full length molecule makes it quite challenging to construct an expression system and a downstream purification procedure for a pharmaceutical composition of VWF.

The understanding of the organization and the boundaries of domains in VWF is not yet complete. Only the so-called A domains are well characterized and their crystal structures determined. The chemical assignments of di-sulfides within VWF are limited. However, recent studies on homologies of domains in VWF to domains in and other proteins

suggest that several disulfide bonds may be formed. The domain definition of VWF described in Zhou et al. Blood 2012; **120,** 449-458 is used herein.

The present invention relates to VWF fragments that are preferably easier to produce than the full length molecule. VWF fragments according to the invention furthermore

5   preferably have the ability to increase bioavailability of s.c. co-administered FVIII. VWF fragments according to the present invention comprise the at least the 15 N-terminal amino acids of the **TIL'** domain/subdomain (spanning amino acids 764-778 of SEQ ID NO 22) or the **TIL'** domain/subdomain (spanning amino acids 764-828 of SEQ ID NO 22 or amino acids 764-829 of SEQ ID NO 22) or the **TIL7E'** domain/sub-domains (spanning amino acids 764-

10   865 of SEQ ID NO 22) and have a size of less than 1500 amino acids, preferably less than 1400 amino acids, preferably less than 1300 amino acids, preferably less than 1200 amino acids, preferably less than 1100 amino acids, preferably less than 1000 amino acids, preferably less than 900 amino acids, preferably less than 800 amino acids, preferably less than 700 amino acids, preferably less than 600 amino acids, preferably less than 500 amino

15   acids, preferably less than 400 amino acids, preferably less than 300 amino acids, preferably less than 275 amino acids, preferably less than 250 amino acids, preferably less than 225 amino acids preferably less than 200 amino acids, preferably less than 175 amino acids, preferably less than 150 amino acids, preferably less than 125 amino acids, preferably less than 100 amino acids, preferably less than 95 amino acids, preferably less than 90 amino

20   acids, preferably less than 85 amino acids, or preferably less than 80 amino acids, or preferably less than 75 amino acids, or preferably less than 70 amino acids, or preferably less than 65 amino acids, or preferably less than 60 amino acids, or preferably less than 55 amino acids, or preferably less than 50 amino acids, or preferably less than 45 amino acids, or preferably less than 40 amino acids, or preferably less than 35 amino acids, or preferably

25   less than 30 amino acids, or preferably less than 25 amino acids, or preferably less than 20 amino acids, or preferably less than 15 amino acids. VWF fragments according to the invention preferably comprise the TIL7E7D3 domains (where D3 is divided into subdomains VWD3-C8-3-TIL-3-E3) spanning amino acids 764-1250 or amino acids 764-1261 or amino acids 764-1268 of SEQ ID NO 22 of SEQ ID NO 22 .VWF fragments according to the

30   invention preferably comprise at least the 15 N-terminal amino acids of TIL', TIL' or TIL7E' domains (amino acids 764-778, 764-828 or amino acids 764-865 of SEQ ID NO 22). VWF fragments according to the invention may furthermore contain fewer potentially antigenic regions. The molecular weight of VWF fragment dimers according to the present invention may - naturally - be about twice as high as for the monomeric fragments (Dimers according

to the present invention may thus comprise up to about 2400 amino acids if the monomer size is 1200 amino acids).

Preferably, the VWF fragments according to the present invention comprise at least amino acids 764-828 (SEQ ID NO 4), or at least amino acids 764-865 (SEQ ID NO 5), or at least amino acids 764-1035 (SEQ ID NO 6), or at least amino acids 764-1041 (SEQ ID NO 7), or at least amino acids 764-1045 (SEQ ID NO 8), or at least amino acids 764-1 128 (SEQ ID NO 9), or at least amino acids 764-1 198 (SEQ ID no 10), or at least amino acids 764-1250 (SEQ ID NO 11), or at least amino acids 764-1261 (SEQ ID NO 14), or at least amino acids 764-1268 (SEQ ID NO 22).

In an embodiment, the C1099 and/or the C 1142 cysteines may be mutated in the VWF fragments according to the present invention. These cysteine residues are believed to be responsible for the oligomerization/dimerization of the VWF protein. VWF fragments with both cysteines intact may form dimers and homo-oligomers. Modifying both of these cysteines may lead to a product composed of monomer VWF fragments, whereas deletion of one or the other may lead to dimer VWF fragments or potentially to oligomer VWF fragments. Both of the above scenarios may lead to a simpler product purification procedure as compared to the full-length protein.

In another embodiment, both of the C1099 and C 1142 cysteines are kept intact which may lead to a preferentially dimeric VWF fragment. There may be a safety advantage associated with the native sequences incl. the C1099 and the C 1142 cysteines.

Surprisingly, co-formulation of FVIII and VWF fragments according to the invention demonstrate improved bioavailability compared to co-formulation of FVIII with a full length VWF molecule. The co-formulations according to the invention show increased bioavailability of Factor VIII when injected subcutaneously. VWF fragments according to the present invention comprise the D' domain (spanning amino acids 764-865/866 of SEQ ID NO: 22) which is thought to be the primary FVIII binding site where FVIII may dock onto D' by electrostatic dipole-dipole like interactions. VWF fragments according to the invention preferably comprise the D' domain and/or the D3-domain (the D3 domain spans amino acids 865/866-1250/1261/1268 of SEQ ID NO: 15). Based on the findings herein, it is possible that both the D' and the D'D3 domains have the ability to bind to FVIII. VWF fragments according to the invention do not to any significant degree (i.e. preferably less than 5%, more preferably less than 4%, preferably less than 3%, preferably less than 2%, more preferably less than 1%) form multimers (i.e., having more than two units, such as e.g. oligomers) because the cysteines (C1099 and C 1142) essential for multimer assembly are not present or have been mutated/substituted. Some VWF fragments according to the present invention do

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furthermore not form dimers to any significant degree- in particular those wherein the C1099 and/or C1142 cysteines are not present.

In some cases, VWF fragments forming dimers may, however, also be useful in connection with the present invention - the TIL7E7D3/A1 dimer has e.g. been shown to have a higher FVIII affinity than the monomer. VWF fragment dimers may furthermore be a relatively homogenous product that can be produced relatively easily.

One advantage of the VWF fragments according to the invention is that it is easier to produce such compounds on an industrial scale as a relatively homogenous product due to the low degree of multimerization and due to the fact that the compounds are smaller compounds with fewer posttranslational modifications compared to full length VWF. This means that a high expression level is easier to obtain and/or purification will be less complex due to a less complex molecule. Also, production of recombinant peptides and proteins in simple organisms such as e.g. yeast is a faster and more inexpensive production method compared to production in mammalian cell lines - some VWF fragments according to the present invention can be produced in yeast.

VWF fragments according to the present invention can be in the form of one single VWF fragment (such as e.g. the entire TIL7E7D3/A1 region spanning amino acids 764-1459 in SEQ ID NO 22) or alternatively in the form of multiple groups of sequential amino acids from VWF fused together and thus deleting intermediary fragments (such as e.g. a "fusion" of the TIL' and the TIL7E' domain spanning amino acids 764-828+764-865 in SEQ ID NO 22). Another example could be amino acids 764-828+1 127-1 197 in SEQ ID NO 22. VWF fragments according to the invention may alternatively be in the form of the repetitive elements. Homologous or heterologous "spacer" sequences may be introduced between the fused VWF fragments/elements (such as e.g. a multiple fusion of TIL7E' domains such as e.g. TIL7ETIL7ETIL7E'). VWF fragments according to the invention may also comprise one or more amino acid alternations (e.g. substitutions, deletions, additions) in the VWF derived sequence(s).

Bioavailability of FVIII in connection with extravascular co-administration of FVIII and VWF fragments according to the invention may be further improved by conjugating FVIII with at least one half life extending moiety. It thus follows, that extra-vascular co-administration of VWF fragments comprising the TIL' and/or the TIL7E' domains with a FVIII molecule conjugated with at least one half life extending moiety is associated with a relatively high FVIII bioavailability.

Examples of VWF fragments according to the present invention (using the domain annotation from Zhou et al.) are shown below in SEQ ID NOs 4-21 . TIL7E7VWD3 I,

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TIL7E7VWD3 II and TIL7E7VWD3 III denote three versions (different lengths) of
TIL7E7VWD3.

SEQ ID NO 4 : amino acids 764-828 (TIL'):

5   SLSCRPPMVKLVCPADNLRAEGLECTKTCQNYDLECMSMGCVSGCLCPPGMVRHENRCV
ALERCP

SEQ ID NO 5 : amino acids 764-865 (TIL7E'):

SLSCRPPMVKLVCPADNLRAEGLECTKTCQNYDLECMSMGCVSGCLCPPGMVRHENRCV

10   ALERCPCFHQG KEYAPGETVK IGCNTCVCQDRKWNCTDHVCDA

V

SEQ ID NO 6 : amino acids 764-1035 (TIL7E7VWD3 I):

SLSCRPPMVKLVCPADNLRAEGLECTKTCQNYDLECMSMGCVSGCLCPPGMVRHENRCV

ALERCPCFHQGKEYAPGETVKIGCNTCVCQDRKWNCTDHVCDATCSTIGMAHYLTFDGLK

15   YLFPGECQYVLVQDYCGSNPGTFRILVGNKGCSHPSVKCKK RVTILVEGGEIELFDGEVNV

KRPMKDETHFEVVESGRYII LLLGKALSVVWDRHLSISWLKQTYQEKVCGLCGNFDGIQ

NNDLTSSNLQ VEEDPVDFGN SWKVSSQCADTR

SEQ ID NO 7 : amino acids 764-1041 (TIL7E7VWD3 II):

20   SLSCRPPMVKLVCPADNLRAEGLECTKTCQNYDLECMSMGCVSGCLCPPGMVRHENRCV

ALERCPCFHQGKEYAPGETVKIGCNTCVCQDRKWNCTDHVCDATCSTIGMAHYLTFDGLK

YLFPGECQYVLVQDYCGSNPGTFRILVGNKGCSHPSVKCKK RVTILVEGGEIELFDGEVNV

KRPMKDETHFEVVESGRYII LLLGKALSVVWDRHLSISWLKQTYQEKVCGLCGNFDGIQ

NNDLTSSNLQ VEEDPVDFGN SWKVSSQCADTRKVPLDS

25

SEQ ID NO 8 : amino acids 764-1045 (TIL7E7VWD3 III):

SLSCRPPMVKLVCPADNLRAEGLECTKTCQNYDLECMSMGCVSGCLCPPGMVRHENRCV

ALERCPCFHQGKEYAPGETVKIGCNTCVCQDRKWNCTDHVCDATCSTIGMAHYLTFDGLK

YLFPGECQYVLVQDYCGSNPGTFRILVGNKGCSHPSVKCKK RVTILVEGGEIELFDGEVNV

30   KRPMKDETHFEVVESGRYII LLLGKALSVVWDRHLSISWLKQTYQEKVCGLCGNFDGIQ

NNDLTSSNLQ VEEDPVDFGN SWKVSSQCADTRKVPLDSSPAT

SEQ ID NO 9 : amino acids 764-1 128 (TIL7E'/VWD3/C8-3) - Cysteine 1099 is marked with bold. This cysteine can be substituted to another amino acid, e.g. Ser:
SLSCRPPMVKLVCPADNLRAEGLECTKTCQNYDLECMSMGCVSGCLCPPGMVRHENRCV
ALERCPCFHQGKEYAPGETVKIGCNTCVCQDRKWNCTDHVCDATCSTIGMAHYLTFDGLK
YLFPGECQYVLVQDYCGSNPGTFRILVGNKGCSHPSVKCKKRVTILVEGGEIELFDGEVNVK
RPMKDETHFEWESGRYII LLLGKALSWWDRHLSISWLKQTYQEKVCGLCGNFDGIQ
NNDLTSSNLQVEEDPVDFGNSWKVSSQCADTRKVPLDSSPATCHNNIMKQTMVDSSCRILT
SDVFQDCNKLVDPEPYLDVCIYDTCSCESIGDCACFCDTIAAYAHVCAQHGKWTWRTATL
CPQ

SEQ ID NO 10: amino acids 764-1198 (TIL7E'/VWD3/C8-3/TIL-3) - Cysteines 1099 and 1142 are marked with bold. One or both of these cysteines can be substituted to another amino acid, e.g. Ser:
SLSCRPPMVKLVCPADNLRAEGLECTKTCQNYDLECMSMGCVSGCLCPPGMVRHENRCV
ALERCPCFHQGKEYAPGETVKIGCNTCVCQDRKWNCTDHVCDATCSTIGMAHYLTFDGLK
YLFPGECQYVLVQDYCGSNPGTFRILVGNKGCSHPSVKCKK RVTILVEGGEIELFDGEVNV
KRPMKDETHFEWESGRYII LLLGKALSWWDRHLSISVVLKQTYQEKVCGLCGNFDGIQ
NNDLTSSNLQVEEDPVDFGN SWKVSSQCADTRKVPLDSSPATCHNNIMKQTMVDSSCRIL
TSDVFQDCNKLVDPEPYLDVCIYDTCSCESIGDCACFCDTIAAYAHVCAQHGKVVTWRTA
TLCPQSCEERNLRENGYECEWRYNSCAPACQVTCQHPEPLACPVQCVEGCHAHCPPGKIL
DELLQTCVDPEDCPV

SEQ ID NO 11: amino acids 764-1250 (TIL7E7D3 I) - Cysteines 1099 and 1142 are marked with bold. One or both of these cysteines can be substituted to another amino acid, e.g. Ser:
SLSCRPPMVKLVCPADNLRAEGLECTKTCQNYDLECMSMGCVSGCLCPPGMVRHENRCV
ALERCPCFHQGKEYAPGETVKIGCNTCVCQDRKWNCTDHVCDATCSTIGMAHYLTFDGLK
YLFPGECQYVLVQDYCGSNPGTFRILVGNKGCSHPSVKCKK RVTILVEGGEIELFDGEVNV
KRPMKDETHFEWESGRYII LLLGKALSVVWDRHLSISWLKQTYQEKVCGLCGNFDGIQ
NNDLTSSNLQVEEDPVDFGN SWKVSSQCADTRKVPLDSSPATCHNNIMKQTMVDSSCRIL
TSDVFQDCNKLVDPEPYLDVCIYDTCSCESIGDCACFCDTIAAYAHVCAQHGKVVTWRTA
TLCPQSCEERNLRENGYECEWRYNSCAPACQVTCQHPEPLACPVQCVEGCHAHCPPGKIL
DELLQTCVDPEDCPVCEVAGRRFASGKKVTLNPSDPEHCQ ICHCDWNLTCEACQEPGGL
VVPPTDA

SEQ ID NO 12: amino acids 864-1250 (D3 I)- Cysteines 1099 and 1142 are marked with bold. One or both of these cysteines can be substituted to another amino acid, e.g. Ser:

ATCSTIGMAHYLTFDGLKYLFPGECQYVLVQDYCGSNPGTFRILVGNKGCSHPSVKCKK
5   RVTILVEGGEIELFDGEVNVKRPMKDETHFEWESGRYIILLLGKALSVVWDRHLSISVVLKQT
YQEKVCGLCGNFDGIQNNDLTSSNLQVEEDPVDFGNSWKVSSQCADTRKVPLDSSPATCH
NNIMKQTMVDSSCRILTSDVFQDCNKLVDPEPYLDVCIYDTCSCESIGDCACFCDTIAAYAHV
CAQHGKWTWRTATLCPQSCEERNLRENGYECEWRYNSCAPACQVTCQHPEPLACPVQC
VEGCHAHCPPGKILDELLQTCVDPEDCPVCEVAGRRFASGKKVTLNPSDPEHCQICHCDVV
10   NLTCEACQEPGGL WPPTDA

SEQ ID NO 13: amino acids 864-1268 (D3 II) - Cysteines 1099 and 1142 are marked with bold. One or both of these cysteines can be substituted to another amino acid, e.g. Ser:

15   ATCSTIGMAHYLTFDGLKYLFPGECQYVLVQDYCGSNPGTFRILVGNKGCSHPSVKCKK
RVTILVEGGEIELFDGEVNVKRPMKDETHFEWESGRYIILLLGKALSVVWDRHLSISVVLKQT
YQEKVCGLCGNFDGIQNNDLTSSNLQVEEDPVDFGNSWKVSSQCADTRKVPLDSSPATCH
NNIMKQTMVDSSCRILTSDVFQDCNKLVDPEPYLDVCIYDTCSCESIGDCACFCDTIAAYAHV
CAQHGKWTWRTATLCPQSCEERNLRENGYECEWRYNSCAPACQVTCQHPEPLACPVQC
20   VEGCHAHCPPGKILDELLQTCVDPEDCPVCEVAGRRFASGKKVTLNPSDPEHCQICHCDVV
NLTCEACQEPGGL WPPTDAPVSPTTLYVEDISEPPLHD

SEQ ID NO 14: amino acids 764-1 261 (TIL7E7D3 II) - Cysteines 1099 and 1142 are marked with bold. One or both of these cysteines can be substituted to another amino acid, e.g. Ser:

25   SLSCRPPMVKLVCPADNLRAEGLECTKTCQNYDLECMSMGCVSGCLCPPGMVRHENRCV
ALERCPCFHQGKEYAPGETVKIGCNTCVCQDRKWNCTDHVCDATCSTIGMAHYLTFDGLK
YLFPGECQYVLVQDYCGSNPGTFRILVGNKGCSHPSVKCKK RVTILVEGGEIELFDGEVNV
KRPMKDETHFEVVESGRYII LLLGKALSVVWDRHLSISWLKQTYQEKVCGLCGNFDGIQ
30   NNDLTSSNLQVEEDPVDFGN SWKVSSQCADTRKVPLDSSPATCHNNIMKQTMVDSSCRIL
TSDVFQDCNKLVDPEPYLDVCIYDTCSCESIGDCACFCDTIAAYAHVCAQHGKWTWRTA
TLCPQSCEERNLRENGYECEWRYNSCAPACQVTCQHPEPLACPVQCVEGCHAHCPPGKIL
DELLQTCVDPEDCPVCEVAGRRFASGKKVTLNPSDPEHCQ ICHCDWNLTCEACQEPGGL
VVPPTDAPVSPTTLYVED

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SEQ ID NO 15: amino acids 764-1264 (TIL7E7D3 III) - Cysteines 1099 and 1142 are marked with bold. One or both of these cysteines can be substituted to another amino acid, e.g. Ser:

SLSCRPPMVKLVCPADNLRAEGLECTKTCQNYDLECMSMGCVSGCLCPPGMVRHENRCV
ALERCPCFHQGKEYAPGETVKIGCNTCVCQDRKWNCTDHVCDATCSTIGMAHYLTFDGLK
YLFPGECQYVLVQDYCGSNPGTFRILVGNKGCSHPSVKCKK RVTILVEGGEIELFDGEVNV
KRPMKDETHFEVVESGRYII LLLGKALSVVWDRHLSISWLKQTYQEKVCGLCGNFDGIQ
NNDLTSSNLQVEEDPVDFGN SWKVSSQCADTRKVPLDSSPATCHNNIMKQTMVDSSCRIL
TSDVFQDCNKLVDPEPYLDVCIYDTCSCESIGDCACFCDTIAAYAHVCAQHGKWTWRTA
TLCPQSCEERNLRENGYECEWRYNSCAPACQVTCQHPEPLACPVQCVEGCHAHCPPGKIL
DELLQTCVDPEDCPVCEVAGRRFASGKKVTLNPSDPEHCQ ICHCDWNLTCEACQEPGGL
VVPPTDAPVSPTTLYVEDISEP

SEQ ID NO 16: amino acids 764-1268 (TIL7E7D3 IV) - Cysteines 1099 and 1142 are marked with bold. One or both of these cysteines can be substituted to another amino acid, e.g. Ser:

SLSCRPPMVKLVCPADNLRAEGLECTKTCQNYDLECMSMGCVSGCLCPPGMVRHENRCV
ALERCPCFHQGKEYAPGETVKIGCNTCVCQDRKWNCTDHVCDATCSTIGMAHYLTFDGLK
YLFPGECQYVLVQDYCGSNPGTFRILVGNKGCSHPSVKCKK RVTILVEGGEIELFDGEVNV
KRPMKDETHFEVVESGRYII LLLGKALSVVWDRHLSISVVLKQTYQEKVCGLCGNFDGIQ
NNDLTSSNLQVEEDPVDFGN SWKVSSQCADTRKVPLDSSPATCHNNIMKQTMVDSSCRIL
TSDVFQDCNKLVDPEPYLDVCIYDTCSCESIGDCACFCDTIAAYAHVCAQHGKWTWRTA
TLCPQSCEERNLRENGYECEWRYNSCAPACQVTCQHPEPLACPVQCVEGCHAHCPPGKIL
DELLQTCVDPEDCPVCEVAGRRFASGKKVTLNPSDPEHCQ ICHCDWNLTCEACQEPGGL
VVPPTDAPVSPTTLYVEDISEPPLHD

SEQ ID NO 17: amino acids 764-1459 (TIL7E7D3/A1 I) - Cysteines 1099 and 1142 are marked with bold. One or both of these cysteines can be substituted to another amino acid, e.g. Ser:

SLSCRPPMVKLVCPADNLRAEGLECTKTCQNYDLECMSMGCVSGCLCPPGMVRHENRCV
ALERCPCFHQGKEYAPGETVKIGCNTCVCQDRKWNCTDHVCDATCSTIGMAHYLTFDGLK
YLFPGECQYVLVQDYCGSNPGTFRILVGNKGCSHPSVKCKK RVTILVEGGEIELFDGEVNV
KRPMKDETHFEVVESGRYII LLLGKALSVVWDRHLSISWLKQTYQEKVCGLCGNFDGIQ
NNDLTSSNLQVEEDPVDFGN SWKVSSQCADTRKVPLDSSPATCHNNIMKQTMVDSSCRIL
TSDVFQDCNKLVDPEPYLDVCIYDTCSCESIGDCACFCDTIAAYAHVCAQHGKWTWRTA

18

TLCPQSCEERNLRENGYECEWRYNSCAPACQVTCQHPEPLACPVQCVEGCHAHCPPGKIL
DELLQTCVDPEDCPVCEVAGRRFASGKKVTLNPSDPEHCQ ICHCDWNLTCEACQEPGGL
VVPPTDAPVSPTTLYVEDISEPPLHDFYCS RLLDLVFLLD GSSRLSEAEF EVLKAFWDM
MERLRISQKWVRVAVVEYHDGSHAYIGLKDRKRPSELRRI ASQVKYAGSQVASTSEVLKY
5   TLFQIFSKIDRPEASRITLLLMASQEPQRMSRNFVRYVQGLKKKKVIVIPVGIGPHANLK
QIRLIEKQAPENKAFVLSSVDELEQQRDEI VSYLCD

**SEQ ID NO 18: amino acids 764-1463 (TIL7E7D3/A1 II) - Cysteines 1099 and 1142 are
marked with bold. One or both of these cysteines can be substituted to another amino**
10   **acid, e.g. Ser:**
SLSCRPPMVKLVCPADNLRAEGLECTKTCQNYDLECMSMGCVSGCLCPPGMVRHENRCV
ALERCPCFHQGKEYAPGETVKIGCNTCVCQDRKWNCTDHVCDATCSTIGMAHYLTFDGLK
YLFPGECQYVLVQDYCGSNPGTFRILVGNKGCSHPSVKCKK RVTILVEGGEIELFDGEVNV
KRPMKDETHFEVVESGRYII LLLGKALSVVWDRHLSISWLKQTYQEKVCGLCGNFDGIQ
15   NNDLTSSNLQVEEDPVDFGN SWKVSSQCADTRKVPLDSSPATCHNNIMKQTMVDSSCRIL
TSDVFQDCNKLVDPEPYLDVCIYDTCSCESIGDCACFCDTIAAYAHVCAQHGKWTWRTA
TLCPQSCEERNLRENGYECEWRYNSCAPACQVTCQHPEPLACPVQCVEGCHAHCPPGKIL
DELLQTCVDPEDCPVCEVAGRRFASGKKVTLNPSDPEHCQ ICHCDWNLTCEACQEPGGL
VVPPTDAPVSPTTLYVEDISEPPLHDFYCS RLLDLVFLLD GSSRLSEAEF EVLKAFWDM
20   MERLRISQKWVRVAVVEYHDGSHAYIGLKDRKRPSELRRI ASQVKYAGSQVASTSEVLKY
TLFQIFSKIDRPEASRITLLLMASQEPQRMSRNFVRYVQGLKKKKVIVIPVGIGPHANLK
QIRLIEKQAPENKAFVLSSVDELEQQRDEI VSYLCDLAPE

SEQ ID NO 19: amino acids 764-1464 (TIL7E7D3/A1 III) - Cysteines 1099 and 1142 are
25   **marked with bold. One or both of these cysteines can be substituted to another amino
acid, e.g. Ser:**
SLSCRPPMVKLVCPADNLRAEGLECTKTCQNYDLECMSMGCVSGCLCPPGMVRHENRCV
ALERCPCFHQGKEYAPGETVKIGCNTCVCQDRKWNCTDHVCDATCSTIGMAHYLTFDGLK
YLFPGECQYVLVQDYCGSNPGTFRILVGNKGCSHPSVKCKK RVTILVEGGEIELFDGEVNV
30   KRPMKDETHFEVVESGRYII LLLGKALSVVWDRHLSISWLKQTYQEKVCGLCGNFDGIQ
NNDLTSSNLQVEEDPVDFGN SWKVSSQCADTRKVPLDSSPATCHNNIMKQTMVDSSCRIL
TSDVFQDCNKLVDPEPYLDVCIYDTCSCESIGDCACFCDTIAAYAHVCAQHGKWTWRTA
TLCPQSCEERNLRENGYECEWRYNSCAPACQVTCQHPEPLACPVQCVEGCHAHCPPGKIL
DELLQTCVDPEDCPVCEVAGRRFASGKKVTLNPSDPEHCQ ICHCDWNLTCEACQEPGGL
35   VVPPTDAPVSPTTLYVEDISEPPLHDFYCS RLLDLVFLLD GSSRLSEAEF EVLKAFWDM

MERLRISQKWVRVAVVEYHDGSHAYIGLKDRKRPSELRRI ASQVKYAGSQVASTSEVLKY
TLFQIFSKIDRPEASRITLLLMASQEPQRMSRNFVRYVQGLKKKKVIVIPVGIGPHANLK
QIRLIEKQAPENKAFVLSSVDELEQQRDEI VSYLCDLAPEA

SEQ ID NO 20: amino acids 764-1683 (TIL7E7D3/A1/A2) - Cysteines 1099 and 1142 are marked with bold. One or both of these cysteines can be substituted to another amino acid, e.g. Ser:

SLSCRPPMVKLVCPADNLRAEGLECTKTCQNYDLECMSMGCVSGCLCPPGMVRHENRCV
ALERCPCFHQGKEYAPGETVKIGCNTCVCQDRKWNCTDHVCDATCSTIGMAHYLTFDGLK
YLFPGECQYVLVQDYCGSNPGTFRILVGNKGCSHPSVKCKK RVTILVEGGEIELFDGEVNV
KRPMKDETHFEVVESGRYII LLLGKALSVVWDRHLSISWLKQTYQEKVCGLCGNFDGIQ
NNDLTSSNLQVEEDPVDFGN SWKVSSQCADTRKVPLDSSPATCHNNIMKQTMVDSSCRIL
TSDVFQDCNKLVDPEPYLDVCIYDTCSCESIGDCACFCDTIAAYAHVCAQHGKWTWRTA
TLCPQSCEERNLRENGYECEWRYNSCAPACQVTCQHPEPLACPVQCVEGCHAHCPPGKIL
DELLQTCVDPEDCPVCEVAGRRFASGKKVTLNPSDPEHCQ ICHCDWNLTCEACQEPGGL
VVPPTDAPVSPTTLYVEDISEPPLHDFYCS RLLDLVFLLD GSSRLSEAEF EVLKAFWDM
MERLRISQKWVRVAVVEYHDGSHAYIGLKDRKRPSELRRI ASQVKYAGSQVASTSEVLKY
TLFQIFSKIDRPEASRITLLLMASQEPQRMSRNFVRYVQGLKKKKVIVIPVGIGPHANLK
QIRLIEKQAPENKAFVLSSVDELEQQRDEIVSYLCDLAPEAPPPTLPPDMAQVTVGPGLLGV
STLGPKRNSMVLDVAFVLEGSDKIGEADFNRSKEFMEEVIQRMDVGQDSIHVTVLQYSYMV
TVEYPFSEAQSKGDILQRVREIRYQGGNRTNTGLALRYLSDHSFLVSQGDREQAPNLVYMV
TGNPASDEIKRLPGDIQWPIGVGPNANVQELERIGWPNAPILIQDFETLPREAPDLVLQRCC
SGE GLQIPTLSPA

SEQ ID NO 21: amino acids 764-1873 (TIL7E7D3/A1/A2/A3) - Cysteines 1099 and 1142 are marked with bold. One or both of these cysteines can be substituted to another amino acid, e.g. Ser:

SLSCRPPMVKLVCPADNLRAEGLECTKTCQNYDLECMSMGCVSGCLCPPGMVRHENRCV
ALERCPCFHQGKEYAPGETVKIGCNTCVCQDRKWNCTDHVCDATCSTIGMAHYLTFDGLK
YLFPGECQYVLVQDYCGSNPGTFRILVGNKGCSHPSVKCKK RVTILVEGGEIELFDGEVNV
KRPMKDETHFEVVESGRYII LLLGKALSVVWDRHLSISWLKQTYQEKVCGLCGNFDGIQ
NNDLTSSNLQVEEDPVDFGN SWKVSSQCADTRKVPLDSSPATCHNNIMKQTMVDSSCRIL
TSDVFQDCNKLVDPEPYLDVCIYDTCSCESIGDCACFCDTIAAYAHVCAQHGKWTWRTA
TLCPQSCEERNLRENGYECEWRYNSCAPACQVTCQHPEPLACPVQCVEGCHAHCPPGKIL

DELLQTCVDPEDCPVCEVAGRRFASGKKVTLNPSDPEHCQ ICHCDWNLTCEACQEPGGL
VVPPTDAPVSPTTLYVEDISEPPLHDFYCS RLLDLVFLLD GSSRLSEAEF EVLKAFWDM
MERLRISQKWVRVAVVEYHDGSHAYIGLKDRKRPSELRRIASQVKYAGSQVASTSEVLKY
TLFQIFSKIDRPEASRITLLLMASQEPQRMSRNFVRYVQGLKKKKVIVIPVGIGPHANLKQIRLI
EKQAPENKAFVLSSVDELEQQRDEIVSYLCDLAPEAPPPTLPPDMAQVTVGPGLLGVSTLG
PKRNSMVLDVAFVLEGSDKIGEADFNRSKEFMEEVIQRMDVGQDSIHVTVLQYSYMVTVEY
PFSEAQSKGDILQRVREIRYQGGNRTNTGLALRYLSDHSFLVSQGDREQAPNLVYMVTGNP
ASDEIKRLPGDIQVVPIGVGPNANVQELERIGWPNAPILIQDFETLPREAPDLVLQRCCSGEG
LQIPTLSPAPDCSQPLDVILLLDGSSSFPASYFDEMKSFAKAFISKANIGPRLTQVSVL
QYGSITTIDVPWNWPEKAHLLSLVDVMQREGGPSQIGDALGFAVRYLTSEMHGARPGAS
KAVVILVTDVSVDSVDAAADAARSNRVTVFPIGIGDRYDAAQLRILAGPAGDSNWKLQRIED
LPTMVTLGNSFLHKLCS

**SEQ ID NO 22: wild-type human VWF according to the UniProtKB/Swiss-Prot database
(entry P04275) - cysteine residues at positions 1099 and 1142 are marked with bold:**
MIPARFAGVLLALALILPGTLCAEGTRGRSSTARCSLFGSDFVNTFDGSMYSFAGYCSYLLA
GGCQKRSFSIIGDFQNGKRVSLSVYLGEFFDIHLFVNGTVTQGDQRVSMPYASKGLYLETEA
GYYKLSGEAYGFVARIDGSGNFQVLLSDRYFNKTCGLCGNFNIFAEDDFMTQEGTLTSDPY
DFANSWALSSGEQWCERASPPSSSCNISSGEMQKGLWEQCQLLKSTSVFARCHPLVDPE
PFVALCEKTLCECAGGLECACPALLEYARTCAQEGMVLYGWTDHSACSPVCPAGMEYRQC
VSPCARTCQSLHINEMCQERCVDGCSCPEGQLLDEGLCVESTECPCVHSGKRYPPGTSLS
RDCNTCICRNSQWICSNEECPGECLVTGQSHFKSFDNRYFTFSGICQYLLARDCQDHSFSI
VIETVQCADDRDAVCTRSVTVRLPGLHNSLVKLKHGAGVAMDGQDVQLPLLKGDLRIQHTV
TASVRLSYGEDLQMDWDGRGRLLVKLSPVYAGKTCGLCGNYNGNQGDDFLTPSGLAEPR
VEDFGNAWKLHGDCQDLQKQHSDPCALNPRMTRFSEEACAVLTSPTFEACHRAVSPLPYL
RNCRYDVCSCSDGRECLCGALASYAAACAGRGVRVAWREPGRCELNCPKGQVYLQCGTP
CNLTCRSLSYPDEECNEACLEGCFCPPGLYMDERGDCVPKAQCPCYYDGEIFQPEDIFSDH
HTMCYCEDGFMHCTMSGVPGSLLPDAVLSSPLSHRSKRSLSCRPPMVKLVCPADNLRAEG
LECTKTCQNYDLECMSMGCVSGCLCPPGMVRHENRCVALERCPCFHQGKEYAPGETVKI
GCNTCVCQDRKWNCTDHVCDATCSTIGMAHYLTFDGLKYLFPGECQYVLVQDYCGSNPGT
FRILVGNKGCSHPSVKCKKRVTILVEGGEIELFDGEVNVKRPMKDETHFEVVESGRYIILLLG
KALSVVWDRHLSISVVLKQTYQEKVCGLCGNFDGIQNNDLTSSNLQVEEDPVDFGNSWKVS
SQCADTRKVPLDSSPATCHNNIMKQTMVDSSCRILTSDVFQDCNKLVDPEPYLDVCIYDTCS
CESIGDCACFCDTIAAYAHVCAQHGKWTWRTATLCPQSCEERNLRENGYECEWRYNSCA
PACQVTCQHPEPLACPVQCVEGCHAHCPPGKILDELLQTCVDPEDCPVCEVAGRRFASGK

KVTLNPSDPEHCQICHCDVVNLTCEACQEPGGLVVPPTDAPVSPTTLYVEDISEPPLHDFYC
SRLLDLVFLLDGSSRLSEAEFEVLKAFWDMMERLRISQKWVRVAVVEYHDGSHAYIGLKDR
KRPSELRRIASQVKYAGSQVASTSEVLKYTLFQIFSKIDRPEASRITLLLMASQEPQRMSRNF
VRYVQGLKKKKVIVIPVGIGPHANLKQIRLIEKQAPENKAFVLSSVDELEQQRDEIVSYLCDLA

5   PEAPPPTLPPDMAQVTVGPGLLGVSTLGPKRNSMVLDVAFVLEGSDKIGEADFNRSKEFME
EVIQRMDVGQDSIHVTVLQYSYMVTVEYPFSEAQSKGDILQRVREIRYQGGNRTNTGLALR
YLSDHSFLVSQGDREQAPNLVYMVTGNPASDEIKRLPGDIQVVPIGVGPNANVQELERIGW
PNAPILIQDFETLPREAPDLVLQRCCSGEGLQIPTLSPAPDCSQPLDVILLLDGSSSFPASYFD
EMKSFAKAFISKANIGPRLTQVSVLQYGSITTIDVPWNVVPEKAHLLSLVDVMQREGGPSQIG

10   DALGFAVRYLTSEMHGARPGASKAVVILVTDVSVDSVDAAADAARSNRVTVFPIGIGDRYDA
AQLRILAGPAGDSNVVKLQRIEDLPTMVTLGNSFLHKLCSGFVRICMDEDGNEKRPGDVWT
LPDQCHTVTCQPDGQTLLKSHRVNCDRGLRPSCPNSQSPVKVEETCGCRWTCPCVCTGS
STRHIVTFDGQNFKLTGSCSYVLFQNKEQDLEVILHNGACSPGARQGCMKSIEVKHSALSVE
LHSDMEVTVNGRLVSVPYVGGNMEVNVYGAIMHEVRFNHLGHIFTFTPQNNEFQLQLSPKT

15   FASKTYGLCGICDENGANDFMLRDGTVTTDWKTLVQEWTVQRPGQTCQPILEEQCLVPDS
SHCQVLLLPLFAECHKVLAPATFYAICQQDSCHQEQVCEVIASYAHLCRTNGVCVDWRTPD
FCAMSCPPSLVYNHCEHGCPRHCDGNVSSCGDHPSEGCFCPPDKVMLEGSCVPEEACTQ
CIGEDGVQHQFLEAWVPDHQPCQICTCLSGRKVNCTTQPCPTAKAPTCGLCEVARLRQNA
DQCCPEYECVCDPVSCDLPPVPHCERGLQPTLTNPGECRPNFTCACRKEECKRVSPPSCP

20   PHRLPTLRKTQCCDEYECACNCVNSTVSCPLGYLASTATNDCGCTTTTCLPDKVCVHRSTI
YPVGQFWEEGCDVCTCTDMEDAVMGLRVAQCSQKPCEDSCRSGFTYVLHEGECCGRCL
PSACEVVTGSPRGDSQSSWKSVGSQWASPENPCLINECVRVKEEVFIQQRNVSCPQLEVP
VCPSGFQLSCKTSACCPSCRCERMEACMLNGTVIGPGKTVMIDVCTTCRCMVQVGVISGF
KLECRKTTCNPCPLGYKEENNTGECCGRCLPTACTIQLRGGQIMTLKRDETLQDGCDTHFC

25   KVNERGEYFWEKRVTGCPPFDEHKCLAEGGKIMKIPGTCCDTCEEPECNDITARLQYVKVG
SCKSEVEVDIHYCQGKCASKAMYSIDINDVQDQCSCCSPTRTEPMQVALHCTNGSWYHEV
LNAMECKCSPRKCSK

       FVIII molecules/variants/derivatives/analogues: The term "FVIN" as used herein, is

30   intended to designate any FVIII molecule having FVIII activity, incl. wt FVIII, B domain
deleted/truncated FVIII molecules, variants of FVIII exhibiting substantially the same or
improved biological activity relative to wt FVIII and FVIII-related polypeptides, in which one or
more of the amino acids of the parent peptide have been chemically modified, e.g. by
protein:protein fusion, alkylation, PEGylation, HESylation, PASylation, PSAylation, acylation,

35   ester formation or amide formation or the like (conjugated to a half-life extending moiety).

Half-life extending moieties/ protractive groups: The term "half-life extending moieties" is herein understood to refer to one or more chemical groups, e.g. a hydrophilic polymer, such as e.g. PEG and/or a polysaccharide covalently attached to FVIII via e.g. -SH, -OH, -COOH, -CONH2, -NH2, or one or more N- and/or O-glycan structures that can

5   increase *in vivo* circulatory half life when conjugated to these proteins. Examples of protractive groups/half-life extending moieties suitable for being conjugated to FVIII in connection with the present invention include: Biocompatible fatty acids and derivatives thereof, Hydroxy Alkyl Starch (HAS) e.g. Hydroxy Ethyl Starch (HES), Poly Ethylene Glycol (PEG), Poly (Glyx-Sery)n (HAP), Hyaluronic acid (HA), Heparosan polymers (HEP),

10   Phosphorylcholine-based polymers (PC polymer), Fleximers, Dextran, Poly-sialic acids (PSA), an Fc domain, an Fc receptor, Transferrin, Albumin, Elastin like peptides, XTEN polymers, Albumin binding peptides, a CTP peptide, and any combination thereof. In general, conjugation of FVIII with one or more half-life extending moieties (such as e.g. hydrophilic polymers) generally have a better bioavailability in connection with s.c./intradermal co-

15   administration with VWF fragments according to the invention as compared with FVIII with no half life extending moieties.

PEGylated FVIII molecules in connection with the present invention may have one or more polyethylene glycol (PEG) molecules attached to any part of the FVIII protein including any amino acid residue or carbohydrate moiety. Chemical and/or enzymatic

20   methods can be employed for conjugating PEG or other polymeric groups (half life extending moieties) to a glycan on FVIII. An example of an enzymatic conjugation process is described e.g. in WO03031464. The glycan may be naturally occurring or it may be inserted via e.g. insertion of an N-linked and/or O-linked glycan using methods well known in the art. "Cysteine-PEGylated FVIII" according to the present invention have one or more PEG

25   molecules conjugated to a sulfhydryl group of a cysteine present in FVIII. "Cysteine-acylated FVIII" according to the present invention have one or more hydrophobic half-life extending moieties (e.g. fatty acids) conjugated to a sulfhydryl group of a cysteine in FVIII - this cysteine residue may be introduced by genetic engineering or a part of the native amino acid sequence. It is furthermore possible to link half-life extending moieties to other amino acid

30   residues.

Fusion proteins: Fusion proteins according to the present invention are proteins created through the in-frame joining of two or more DNA sequences which originally encoded FVIII and the fusion partner. Translation of the fusion protein DNA sequence will result in a single protein sequence which may have functional properties derived from each of the

35   original proteins or peptides. DNA sequences encoding fusion proteins may be created

artificially by standard molecular biology methods such as overlapping PCR or DNA ligation and the assembly is performed excluding the stop codon in the first 5'-end DNA sequence while retaining the stop codon in the 3'end DNA sequence. The resulting fusion protein DNA sequence may be inserted into an appropriate expression vector that supports the

5   heterologous fusion protein expression in a standard host organism.

Fusion proteins may contain a linker or spacer peptide sequence that separates the protein or peptide parts which define the fusion protein. The linker or spacer peptide sequence may facilitate the correct folding of the individual protein or peptide parts and may make it more likely for the individual protein or peptide parts to retain their individual

10   functional properties. Linker or spacer peptide sequences may be inserted into fusion protein DNA sequences during the in frame assembly of the individual DNA fragments that make up the complete fusion protein DNA sequence i.e. during overlapping PCR or DNA ligation. Examples of fusion proteins comprising FVIII and a fusion partner are shown in WO201 1101284.

15   Fc fusion protein: The term "Fc fusion protein" is herein meant to encompass FVIII fused to an Fc domain that can be derived from any antibody isotype. An IgG Fc domain will often be preferred due to the relatively long circulatory half-life of IgG antibodies. The Fc domain may furthermore be modified in order to modulate certain effector functions such as e.g. complement binding and/or binding to certain Fc receptors. Fusion of FVIII with an Fc

20   domain, which has the capacity to bind to FcRn receptors, will generally result in a prolonged *in vivo* circulatory half-life. Mutations in positions 234, 235 and 237 in an IgG Fc domain will generally result in reduced binding to the FcγRI receptor and possibly also the FcγRIIa and the FcγRIII receptors. These mutations do not alter binding to the FcRn receptor, which promotes a long circulatory *in vivo* half-life by an endocytic recycling pathway. Preferably, a

25   modified IgG Fc domain of a fusion protein according to the invention comprises one or more of the following mutations that will result in decreased affinity to certain Fc receptors (L234A, L235E, and G237A) and in reduced C1q-mediated complement fixation (A330S and P331S), respectively. Alternatively, the Fc domain may be an IgG4 Fc domain, preferably comprising the S241 P/S228P mutation.

30   Bioavailability (of FVIII): The term "Bioavailability" describes the percentage of compound absorbed to the blood after extravascular is calculated as the Area under the concentration time curves after extravascular dosing of the compound. This is calculated from the Area under the concentration curves of FVIII after s.c. administration divided by the dose, relatively to the area under the concentrations curve divided by the dose of the same

35   FVIII compound, dosed i.v. According to the present invention, the bioavailability of FVIII

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molecules (in connection with subcutaneous/intradermal co-administration of FVIII and VWF fragments according to the invention) is at least 3%, preferably at least 5%, preferably at least 6%, preferably at least 7%, preferably at least 8%, preferably at least 9%, preferably at least 10%, preferably at least 11%, preferably at least 12%, preferably at least 13%,

5   preferably at least 14%, preferably at least 15%, preferably at least 16%, preferably at least 17%, preferably at least 18%, preferably at least 19%, preferably at least 20%, preferably at least 2 1%, preferably at least 22%, preferably at least 23%, preferably at least 24%, preferably at least 25%, preferably at least 26%, preferably at least 27%, preferably at least 28%, preferably at least 29%, preferably at least 30%, preferably at least 3 1%, preferably at

10   least 32%, preferably at least 33%, preferably at least 34%, preferably at least 35%, preferably at least 36%, preferably at least 37%, preferably at least 38%, preferably at least 39%, preferably at least 40%, preferably at least 4 1%, preferably at least 42%, preferably at least 43%, preferably at least 44%, preferably at least 45%, preferably at least 46%, preferably at least 47%, preferably at least 48%, preferably at least 49%, preferably at least

15   50%, preferably at least 55%, preferably at least 60%, preferably at least 65%, preferably at least 70%, and most preferably at least 75%. Bioavailability can be measured as described herein. Preferably, the FVIII bioavailability (FVIII antigen and/or activity) of formulations according to the invention will be high enough to exert prophylactic effects under conditions with normal activity when such formulations are administered extravascularly (e.g.

20   subcutaneously or intra-dermally) e.g. once or twice a day or once, twice or three times a week. Preferably, FVIII dosages are comparable with those used in connection with I.V. administration of FVIII, preferably twice as high, and more preferably three times as high, more preferably four times as high, more preferably about 10 times as high, more preferably about 15 times as high, more preferably about 20 times as high, and most preferably about

25   25 times as high. Safety and cost considerations may be considered in connection with dosage determinations.

          Saturation of FVIII with VWF fragments according to the invention: saturation of FVIII with VWF fragment/ the relative amount of FVIII bound to or in complex with VWF/the amount of FVIII bound to VWF divided by the total amount of FVIII. This calculation is based

30   on the KD value of the binding between FVIII and the protein. For FVIII binding to VWF fragments, the measured KI values are used as KD.

          The following (quadratic) equations can be used to calculate the concentration of bound FVIII (A) to another protein (B) from the total concentrations [A]t [B]t.

5

$$K_D = \frac{[A] \times [B]}{[AB]}$$

$$[A] = [A]_t - [AB]$$

$$[B] = [B]_t - [AB]$$

$$[AB]^2 - (K_D + [A]_t + [B]_t) \times [AB] + [A]_t \times [B]_t = 0$$

$$\alpha \times [AB]^2 + \beta \times [AB] + \delta = 0$$

$$\alpha = 1, \beta = -(K_D + [A]_t + [B]_t), \delta = [A]_t \times [B]_t$$

10

$$[AB] = \frac{-\beta \pm \sqrt{\beta^2 - 4 \times \alpha \times \delta}}{2 \times \alpha}$$

Pharmaceutical compositions: The present invention provides compositions comprising VWF fragments and preferably also FVIII. Accordingly, one object of the invention is to provide a pharmaceutical composition comprising a FVIII molecule present in a

15   concentration from 40 IU/ml to 25,000 IU/ml, and wherein said composition has a pH from 2.0 to 10.0. In a preferred embodiment, the FVIII molecules are co-administered together with VWF fragments. Pharmaceutical compositions according to the invention may thus comprise FVIII in a concentration of from 40 IU/ml to 25,000 IU/ml, such as e.g. from 50-25,000 IU/ml, 100-25,000 IU/ml, 250-25,000 IU/ml, 500-25,000 IU/ml, 1000-25,000 IU/ml,

20   2000-25,000 IU/ml, 3000-25,000 IU/ml, 4000-25,000 IU/ml, 5000-25,000 IU/ml, 6000-25,000, 7000-25,000, 8000-25,000, 9000-25,000, 10,000-25,000 IU/ml, 50-20,000 IU/ml, 100-20,000 IU/ml, 250-20,000 IU/ml, 500-20,000 IU/ml, 1000-20,000 IU/ml, 2000-20,000 IU/ml, 3000-20,000 IU/ml, 4000-20,000 IU/ml, 5000-20,000 IU/ml, 6000-20,000 IU/ml, 7000-20,000 IU/ml, 8000-20,000 IU/ml, 9000-20,000 IU/ml, 10,000-20,000 IU/ml, 50-15,000 IU/ml, 100-15,000

25   IU/ml, 250-15,000 IU/ml, 500-15,000 IU/ml, 1000-15,000 IU/ml, 2000-15,000 IU/ml, 3000-15,000 IU/ml, 4000-15,000 IU/ml, 5000-15,000 IU/ml, 6000-15,000 IU/ml, 7000-15,000 IU/ml, 8000-15,000 IU/ml, 9000-15,000 IU/ml, 10,000-15,000 IU/ml, 50-10,000 IU/ml, 100-10,000 IU/ml, 250-10,000 IU/ml, 500-10,000 IU/ml, 1000-10,000 IU/ml, 2000-10,000 IU/ml, 3000-10,000 IU/ml, 4000-10,000 IU/ml, 5000-10,000 IU/ml, 50-5000 IU/ml, 100-5000 IU/ml, 250-

30   5000 IU/ml, 500-5000 IU/ml, and 1000-5000 IU/ml. Compositions according to the invention may further comprise one or more pharmaceutically acceptable excipients such as e.g. a buffer system, a preservative, a tonicity agent, a chelating agent, a stabilizer, or a surfactant, as well as various combinations thereof. The use of preservatives, isotonic agents, chelating agents, stabilizers and surfactants in pharmaceutical compositions is well-known to the

skilled person. Reference may be made to Remington: The Science and Practice of Pharmacy, 19th edition, 1995.

In one embodiment, the pharmaceutical composition is an aqueous composition. Such a composition is typically a solution or a suspension, but may also include colloids, dispersions, emulsions, and multi-phase materials. The term "aqueous composition" is defined as a composition comprising at least 50% w/w water. Likewise, the term "aqueous solution" is defined as a solution comprising at least 50 % w/w water, and the term "aqueous suspension" is defined as a suspension comprising at least 50 %w/w water.

In another embodiment, the pharmaceutical composition is a freeze-dried composition, to which the physician or the patient adds solvents and/or diluents prior to use.

In a further aspect, the pharmaceutical composition comprises an aqueous solution of such an antibody, and a buffer, wherein the antibody is present in a concentration from 1 mg/ml or above, and wherein said composition has a pH from about 2.0 to about 10.0.

Pharmaceutical compositions according to the present invention are preferably suitable for extravascular administration (e.g. s.c. or intradermal administration) in prophylactic/therapeutic treatment of blood clotting diseases.

"Ratio of FVIIhVWF": According to the present invention, preferred ratios of FVIII and VWFA/WF fragment include FVIIIA/WF ratios (molar ratios) from 0.5:1 to 1:50, such as e.g. 1:1 to 1:50, such as e.g. 1:1 to 1:25, such as e.g. 1:1 to 1:20, or 1:1 to 1:15, or 1:1 to 1:10, or 1:1 to 1:7,5, or 1:7 to 1:8, or 1:6 to 1:8, or 1:6 to 1:9, or 1:5 to 1:10. Preferred ratios thus include: 1:1, 1:2, 1:3, 1:4, 1:5, 1:5,5; 1:6; 1:6,5, 1:7; 1:7,1 ; 1:7,2; 1:7,3; 1:7,4; 1:7,5; 1:7,6; 1:7,7; 1:7,8; 1:7,9, 1:8, 1:9, 1:10, 1:15, 1:20, 1:25, 1:30, 1:35, 1:40, 1:45, and 1:50. Preferred ratios include: 0.5:1 ; 0.6:1 ; 0.7:1 ; 0.8:1 ; 0.9:1 ; 1:1; 1.1:1; 1.2:1 ; 1.3:3; 1.4:1 , and 1.5:1 . A molar ratio close to 1:1 generally has the advantage of minimizing the required amount of active substance. The optimal ratio between FVIII and VWF fragment in a co-formulation mixture may be determined by calculating the amount of bound FVIIhVWF at certain protein concentrations based on the binding affinity to the VWF variant for the FVIII species in question. The binding affinity can be determined e.g. by ELISA, SPR or by ITC.

"Haemophilia": Haemophilia/hemophilia/blood clotting diseases is a group of hereditary genetic disorders that impair the body's ability to control blood clotting or coagulation ("bleeding disorders"), which is used to stop bleeding when a blood vessel is broken. Haemophilia A (clotting factor VIII deficiency) is the most common form of the disorder, present in about 1 in 5,000-10,000 male births. In connection with the present invention, the term "haemophilia" encompasses von Willebrand disease.

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List of embodiments:

1. A VWF fragment comprising up to 1500, 1400, 1300, or 1200, wherein said VWF fragment comprises the TIL' domain. Said fragment may comprise different or repetitive VWF sequences joined by peptide bonds.

2. A VWF fragment according to the invention, wherein said fragment comprises the TIL' and the E' domains.

3. A VWF fragment consisting of the TIL' or the TIL7E' domains.

4. A VWF fragment (according to the invention), wherein said fragment comprises the amino acid sequence according to any one of SEQ ID NO 4, 5, 6, 7, 8. 9. 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or 21.

5. A VWF fragment according to the invention, wherein said VWF fragment does not comprise cysteine residues at position(-s) 1099 and/or 1142 of SEQ ID NO 22. These cysteine residue(-s) can be deleted by amino acid substitution and/or deletion.

6. A VWF fragment according to the invention, wherein said fragment comprises SEQ ID NO 9, wherein the 1099 Cysteine residue is substituted with another amino acid, such as e.g. Histidine, Alanine, Isoleucine Arginine, Leucine, Asparagine, Lysine, Aspartic acid, Methionine, Phenylalanine, Glutamic acid, Threonine, Glutamine, Tryptophan, Glycine, Valine, Proline, Serine, Taurine, and Tyrosine.

7. A VWF fragment according to the invention, wherein the 1099 cysteine residue is substituted with Serine.

8. A VWF fragment according to the invention, wherein said fragment comprises an amino acid sequence selected from the list consisting of: SEQ ID NO 10, SEQ ID NO 11, SEQ ID NO 12, SEQ ID NO 13, SEQ ID NO 14, SEQ ID NO 15, SEQ ID NO 16, SEQ ID NO 17, SEQ ID NO 18, SEQ ID NO 19, SEQ ID NO 20 and SEQ ID NO 21, wherein the 1099 and the 1142 cysteine residues are substituted with another amino acid, such as e.g. Histidine, Alanine, Isoleucine Arginine, Leucine, Asparagine, Lysine, Aspartic acid, Methionine, Phenylalanine, Glutamic acid, Threonine, Glutamine, Tryptophan, Glycine, Valine, Proline, Serine, Taurine, and/or Tyrosine.

9. A VWF fragment according to the invention, wherein the 1099 and the 1142 cysteine residues are substituted with serine.

10. A pharmaceutical composition comprising a VWF fragment according to the invention, wherein less than 10%, preferably less than 9%, preferably less than

8%, preferably less than 7%, preferably less than 6%, preferably less than 5%, preferably less than 4%, preferably less than 3%, preferably less than 2%, preferably less than 1% of said VWF fragment are in the form of oligomers and/or multimers.

11. A VWF fragment according to the invention, wherein said VWF fragment is part of a dimer. The percentage of dimer formation may be at least 5%, preferably at least 10%, preferably at least 15%, preferably at least 20%, preferably at least 25%, preferably at least 30%, preferably at least 35%, preferably at least 40%, preferably at least 45%, preferably at least 50%, preferably at least 55%, preferably at least 60%, preferably at least 65%, preferably at least 70%, preferably at least 75%, preferably at least 80%, preferably at least 85%, preferably at least 90%, most preferably at least 95%.

12. A pharmaceutical composition comprising FVIII and a VWF fragment, wherein FVIII bioavailability is at least 5% following extravascular (e.g. sub-cutaneous/intradermal) administration of said pharmaceutical formulation.

13. A pharmaceutical composition comprising FVIII and a VWF fragment, wherein FVIII bioavailability is at least 5% following extravascular (e.g. sub-cutaneous/intra-dermal) administration of said pharmaceutical formulation, wherein the ratio of FVIII and VWF fragment is about 0.5:1 - 1:50. Preferably said ratio is about 0.5:1, 1:1, or 1:2.

14. A VWF fragment, wherein the amino acid sequence of said VWF fragment comprises or consists of an amino acid sequence selected from the list consisting of: SEQ ID NO 4, SEQ ID NO 5, SEQ ID NO 6, SEQ ID NO 7, SEQ ID NO 8, SEQ ID NO 9, SEQ ID NO 10, SEQ ID NO 11 SEQ ID NO 12, SEq ID NO 13, , SEQ ID NO 14, SEQ ID NO 15, SEQ ID NO 16, SEQ ID NO 17, SEQ ID NO 18, SEQ ID NO 19, SEQ ID NO 20, and SEQ ID NO 21.

15. A pharmaceutical composition comprising: (i) a VWF fragment according to the invention; and (ii) FVIII, preferably recombinant FVIII. Alternatively, said composition may comprise two, three, four, five or more different VWF fragments according to the invention and/or two, three, four, or five different FVIII molecules.

16. A pharmaceutical composition according to the invention, wherein said FVIII molecule comprises a truncated B domain at a size of 5-700 amino acids, such as e.g. 5-500, 5-400, 5-300, 5-200, 5-100, 5-50, 5-40, 5-30, 5-25, 5-20, 10-700, 10-500, 10-400, 10-300, 10-200, 10-100, 10-50, 10-40, 10-30, 10-20, 20-700,

20-500, 20-400, 20-300, 20-200, 20-100, 20-50, 20-25, 50-700, 50-500, 50-400, 50-300, 50-200, 50-100, 100-700, 100-500, 100-400, 100-300, 100-200, 5, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 45, 50, 75, or 100 amino acids

17. A pharmaceutical composition according to the invention, wherein the amino acid sequence of said truncated B domain is derived from the wt FVIII B domain amino acid sequence.

18. A pharmaceutical composition according to the invention, wherein said FVIII molecule is a B domain truncated FVIII molecule, wherein said B domain comprises an O-glycan linked to the Ser 750 amino acid residue as set forth in SEQ ID NO 1. Preferably, said FVIII molecule comprises one O-linked glycan in the truncated B domain, wherein said O-linked glycan is attached to the Ser 750 residue as set forth in SEQ ID NO 1.

19. A pharmaceutical composition according to the invention, wherein said FVIII molecule comprises a B domain having the amino acid sequence as set forth in SEQ ID NO 2. Alternatively, one or more amino acids in the B domain are deleted from SEQ ID NO 2, such as e.g. the N-terminal Ser residue and/or the C-terminal Arg residue.

20. A pharmaceutical composition according to the invention, wherein the amino acid sequence of the FVIII B domain comprises or consists of an amino acid sequence selected from the group consisting of: amino acids 741-857 + 1637-1648; amino acids 741-914 + 1637-1648; amino acids 741-954 + 1637-1648; amino acids 741-965 + 1637-1648; amino acids 741-965 + 1637-1648; amino acids 741-1003 + 1637-1648; amino acids 741-1003 + 1637-1648; amino acids 741-1020 + 1637-1648; amino acids 741-1079 + 1637-1648; amino acids 741-1206 + 1637-1648; amino acids 741-1261 + 1637-1648; amino acids 741-1309 + 1637-1648; amino acids 741-914 + 1637-1648; amino acids 741-954 + 1637-1648; amino acids 741-968 + 1637-1648; amino acids 741-1003 + 1637-1648; amino acids 741-1018 + 1637-1648; amino acids 741-1070 + 1637-1648; amino acids 741-1230 + 1637-1648; amino acids 741-1301 + 1637-1648; amino acids 741-965 + 1637-1648; amino acids 741-965 + 1637-1648; amino acids 741-965 + 1637-1648; and amino acids 741-965 + 1637-1648 as set forth in SEQ ID NO 1.

21. A pharmaceutical composition according to the invention, wherein said FVIII molecule is conjugated with at least one half-life extending moiety. Preferably,

said half life extending moiety is a water soluble polymer. Preferably a PEG and/or a polysaccharide.

22. A pharmaceutical composition according to the invention, wherein at least one water soluble polymer is covalently attached to a glycan present in the B domain, preferably an O-glycan, preferably an O-glycan attached to the Ser750 amino acid residue as set forth in SEQ ID NO 1.

23. A pharmaceutical composition according to the invention, wherein said water soluble polymer is selected from the group consisting of: PEG, PSA, HES, HEP and HSA.

24. A pharmaceutical composition according to the invention, wherein said FVIII molecule is produced using an expression vector encoding a FVIII molecule comprising the FVIII B domain is as set forth in SEQ ID NO 2.

25. A pharmaceutical composition according to the invention, wherein the bioavailability of said FVIII molecule is at least 2, 3, 4, 5, 6, 7, 8, 9, or 10%. Preferably, the bioavailability is measured as the area under the curve of the plasma levels of FVIII after subcutaneous administration using either an antigen assay or a clotting assay.

26. A pharmaceutical composition according to the invention, wherein the ratio between FVIII and VWF is 1:50, 1:34, 1:25, 1:20: 1:15, 1:10, 1:7,5, preferably 0.5:1 , 1:1, or 1:2.

27. A pharmaceutical formulation according to the invention, wherein the concentration of FVIII is at least about 100, 150, 200, 250, 300, 350, 400, 500, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, 10,000, 11,000, 12,000, 13,000, 14,000, 15,000, 16,000, 17,000, 18,000, 19,000, 20,000, 20,000, 2 1,000, 22,000, 23,000, 24,000, 25,000, 26,000, 27,000, 28,000, 29,000, or 30,000 IU/ml.

28. A pharmaceutical formulation according to the invention, wherein the amount of FVIII bound to VWF fragment is at least 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, or 95% of the total amount of FVIII in said formulation.

29. Use of a compound according to the invention, or a pharmaceutical composition according to the invention, for treatment of haemophilia by extravascular, preferably subcutaneous, administration. The pharmaceutical composition according to the invention can also be administered by intradermal administration. The pharmaceutical composition according to the invention can furthermore be administered by intravenous administration.

30. A method of treatment of a haemophilia, wherein said method comprises subcutaneous administration of a therapeutically effective amount of a compound according to the present invention, or a pharmaceutical composition according to the present invention, to a patient in need thereof.

31. A method of increasing bioavailability of FVIII, wherein said method comprises a step of extravascular (e.g. subcutaneous/intradermal) co-administration of FVIII and a VWF fragment according to the invention, wherein the ratio of said FVIII and said VWF fragment is about 1:1 - 1:50, preferably 0.5:1, 1:1, 1:2, 1:10, 1:20 or 1:34.

32. A DNA molecule encoding a VWF fragment according to the invention.

33. An expression vector comprising a DNA molecule according to the invention.

34. A host cell comprising an expression vector according to the invention.

35. A method for making a VWF fragment according to the invention, wherein said method comprises incubation of a host cell in a suitable medium under suitable conditions and subsequently recovering said recombinant VWF fragment.

36. A pharmaceutical composition according to the invention, wherein said composition comprises one or more VWF fragments according to the invention.

37. .A pharmaceutical composition comprising one or more VWF fragments according to the invention.

38. A method of treatment of von willebrand disease, wherein said method comprises extravascular (e.g. subcutaneous) administration of a therapeutically effective amount of a pharmaceutical composition according to the present invention, to a patient in need thereof

39. A VWF fragment or VWF-like polypeptide comprising the 15 N terminal amino acids of the TIL' sequence 764-778, or more. Relatively small VWF fragments according to the present invention may form part of, or be "embedded" in or grafted onto a scaffold polypeptide sequence of any origin, including non-VWF origin.

40. A VWF fragment according to the invention, wherein said VWF fragment interacts with/binds to residues C1858-Q1874, S2063-D2074 AND V2125-A2146 of the FVIII amino acid sequence as set forth in SEQ ID NO 1.

41. A VWF fragment according to the invention, wherein said fragment is conjugated with a half life extending moiety.

42. A VWF fragment according to the invention, wherein said fragment is conjugated with a half life extending moiety via a N- and/or O-linked glycan.

43. A VWF fragment according to the invention, wherein said VWF fragment reduced uptake of FVIII by antigen presenting cells in connection with binding of said VWF fragment to FVIII..

It is understood that all aspects and embodiments of the invention can be combined and that they are not to be understood in any limiting way.

EXAMPLES

While certain features of the invention have been illustrated and described herein, many modifications, substitutions, changes, and equivalents will now occur to those of ordinary skill in the art. It is, therefore, to be understood that the appended claims are intended to cover all such modifications and changes as fall within the true spirit of the invention.

Example 1:

Subcutaneous administration in FVIII knockout mice (1):

Two test compounds were prepared:

a) GlycoPEGylated FVIII, i.e. "N8-GP" (prepared essentially as disclosed in example 1+2 in WO20091 08806) 2000 U FVIII/ml determined by chromogenic activity equivalent to 1.2 μM based on protein content.

b) GlycoPEGylated FVIII i.e. N8-GP (2000 U FVIII/ml or 1.2 μM, co-formulated with 0.74 mg/ml VWF fragment TIL7E7D3/A1 (equivalent to 9.3 μM) Both test compounds were formulated in 18 mg/ml NaCl, 3 mg/ml saccharose, 1.5 mg/ml L-histidine, 0.1 mg/ml polysorbate 80, 0.25 mg/ml CaCl$_2$, pH 7.3

12 FVIII KO mice, exon 16 knock-out in a mixed background of C57Bl/6 and SV129, bred at Taconic M&B (B6.129S4-F8tm1 Kaz/J) with an approximate weight of 22 g were dosed subcutaneously in the flank with 10000IU/kg FVIII or FVMIA/WF, 6 mice with each test compound.

Blood was sampled at 1, 3, 7, 17, 24, 30, 48, 72 and 96 h post administration. The mice were anaesthetized by Isoflurane/0$_2$/N$_2$0 prior to blood sampling via the retroorbital plexus. Three samples were taken from each mouse. Blood (45 μl) was stabilised with 5 μl of sodium-citrate (0.13 M) and added 200 μl FVIII coatest SP buffer (50mM TRIS-HCl, 1% BSA, Ciprofloxacin 10 mg/L, pH 7.3). After centrifugation at 4000 g for 5 minutes at room

temperature, the supernatants were immediately frozen on dry ice before storage at -80°C prior to analysis.

Samples were analysed with regards to FVIII activity in a chromogenic assay as described by Oviisen K et al. J. Thromb. Haemost, 2008, 6: 969-975 and by FVIII antigen analysis using two FVIII light chain antibodies (4F45 and 4F1 1) in a FVIII LOCI assay (Luminescence oxygen channelling immunoassay).

Mean plasma concentration versus time data were analysed by non-compartmental analysis using WinNonlin Phoenix (Pharsight Corporation) estimating the given pharmacokinetic parameters. The bioavailability was estimated using a previous i.v. pharmacokinetic study of N8-GP in FVIII KO mice.

The circulating profiles of FVIII activity are shown graphically in Fig. 1, the circulating concentrations of FVIII antigen are shown in Fig. 2.

In this experiment, the bioavailability of GlycoPEGylated FVIII alone was calculated to be 27% based on activity and 19% based on antigen. The co-formulation with VWF increased the bioavailability to 40 and 47%, respectively.

Example 2:

Subcutaneous administration in FVIII knockout mice (2):

Two test compounds were prepared:

a) GlycoPEGylated FVIII (500 IU FVIII/ml determined by chromogenic activity equivalent to 0.3 μM)

b) GlycoPEGylated FVIII (500 IU FVIII/ml or 0.3 μM, co-formulated with 0.185 mg/ml VWF fragment TIL7E7D3/A1 (equivalent to 2.3 μM)

Based on a measured IC50 of 1.5 nM of the VWF fragment to FVIII and assuming that the measured IC50 equals K_d, 99% of the FVIII should be bound to VWF in this composition.

Both test compounds were formulated in 18 mg/ml NaCl, 3 mg/ml saccharose, 1.5 mg/ml L-histidine, 0.1 mg/ml polysorbate 80, 0.25 mg/ml CaCl2, pH -7.3

12 FVIII KO mice, exon 16 knock-out in a mixed background of C57Bl/6 and SV129, bred at Taconic M&B (B6.129S4-F8tm1Kaz/J) with an approximate weight of 22 g were dosed subcutaneously in the flank with 2500 IU/kg FVIII or FVMIA/WF, 6 mice with each test compound.

Blood was sampled at 1, 3, 7, 17, 24, 30, 48, 72 and 96 h post administration. The mice were anaesthetized by Isoflurane/0$_2$/N$_2$0 prior to blood sampling via the retroorbital

plexus. Three samples were taken from each mouse. 45 µl of blood was stabilised with 5 µl of sodium-citrate (0.13 M) and added 200 µl FVIII coatest SP buffer (50mM TRIS-HCl, 1% BSA, Ciprofloxacin 10 mg/L, pH 7.3). After centrifugation at 4000 g for 5 minutes at room temperature, the samples were immediately frozen on dry ice before storage at -80°C prior to analysis.

Samples were analysed with regards to FVIII activity in a chromogenic assay as described by Ovlisen K et al. J. Thromb. Haemost, 2008, 6: 969-975 and by FVIII antigen analysis using two FVIII light chain antibodies (4F45 and 4F1 1) in a FVIII LOCI assay (Luminescence oxygen channelling immunoassay).

Mean plasma concentration versus time data were analysed by non-compartmental analysis using WinNonlin Phonix (Pharsight Corporaton) estimating the given pharmacokinetic parameters. The bioavailability was estimated using a previous i.v. pharmacokinetic study of N8-GP in FVIII KO mice.

The circulating profiles of FVIII activity are shown graphically in Fig. 3, the circulating concentrations of FVIII antigen are shown in Fig. 4.

In this experiment, the bioavailability of GlycoPEGylated FVIII alone was calculated to be 29% based on activity and 14% based on antigen. The co-formulation with VWF increased the bioavailability to 36% (antigen measurement).

Example 3:

Haemostatic efficacy ofs.c. administrated co-formulations of FVIII compounds with VWF compounds:

Study outline:

Animals: FVIII k/o mice, 8-18 weeks old, male and females

Tail bleeding: n=6-12 per timepoint / group

Thrombo-elastography: n=2-4 per timepoint/group

Administration route: s.c. in the neck or flank (i.v. in the tail vein for control groups)

Dose volumes 1-10ml/kg

Groups:

Vehicle controls dosed 24hr prior to injury

i.v. controls dosed 5 min prior to injury

FVIII compounds co-formulated with VWF compounds dosed s.c. 5min, 1, 3, 5, 12, 24, 48, 72, 96, 120, 144 or 168hr prior to injury.

Procedures:

35

Compounds of interest are prepared in buffer (10 mM L-Histidine, 8.8 mM Sucrose, 0.01 % Polysorbate 80, 308 mM NaCl, 1.7 mM CaCl2 (dihydrate), 0.37 mM L-Methionine, pH 6.9) to a concentration between 40 and 10000U/ml and stored at -80C until use.

Before tail transection, the mice are anaesthetised with isoflurane and placed on a heating pad

The tails are placed in pre-heated saline at 37 °C for 10 min

I.v. controls are injected 5 min, 24 or 48hr prior to injury

The tail is transected 4 mm from the tip

Immediately before tail cut a 20 µl blood sample is drawn from the peri-orbital plexus for FVIII determination

Blood is collected over 30 min and the haemoglobin concentration determined by spectrophotometry at 550 nm

Parallel animals are used for blood sampling and subsequent analysis of their clotting parameters (ex vivo efficacy).

Results:

The prophylactic effect of the co-formulation is determined from comparing the blood loss during the 30min study period at a certain time after s.c. administration (5min until 168hr) to that of 1, a vehicle control and 2, an i.v. control group with FVIII or glycoPEGylated FVIII. Fig 10 shows that glycoPEGylated FVIII are haemostatic effective 24 hr after s.c. administration of 2500 U/kg as shown by reduction of blood loss and shortening of clot time ex vivo. Similar effect is seen for FVIII co-formulated with a VWF fragment.

Example 4:

Evaluation of bioavailability of FVIII:

Bioavailability of co-compositions of FVIII and VWFA/WF fragments according to the invention can be determined from evaluations of the effect on bioavailability in PK experiments as those described in examples 1 and 2 as well as evaluations of the prophylactic effect as described in example 3.

The bioavailability of a FVIII compound co-formulated with a concentration of VWF fragment that enables the majority of FVIII to be bound to a VWF fragment compound in the injection composition can be determined from the concentration of FVIII compound in the composition and from experiments evaluating the binding affinity of the VWF fragment compound to the FVIII compound such as e.g. surface plasmon resonance experiments.

Example 5:

Titration ofdosis of FVIII:VWF co-composition:

Dose titration can be carried out as disclosed in examples 1-3. Briefly, plasma concentration of FVIII will be evaluated after s.c. administration of doses of 70, 100, 150, 280, 500, 1000 and 2500IU/kg (FVIII units) alone or together with a VWF fragment in FVIII k/o mice.

Example 6:

Titration of ratio between FVIII compound and VWF compound:

Titration of ratios between FVIII and VWF can be carried out as disclosed in experiments similar to that in examples 1 and 2 as well as that described in example 3.

For PK evaluation, doses of 280, 500, 1000 or 2500IU/kg FVIII compound will be co-formulated with VWF fragments at a molar ratio of 1:1, 1:1.5, 1:2, 1:3, 1:4, 1:5, 1:7.7 or up to 1:100 (FVIII to VWF fragment) and plasma concentration of FVIII evaluated in FVIII k/o mice after s.c. administration. The maximum molar surplus of VWF fragment to FVIII will be determined from binding affinities of the fragment to the FVIII compound in question; the highest molar surplus used will be the one that should result in at least 99% of the FVIII used bound to a VWF fragment.

For prophylactic effect, the candidate compositions from the PK experiments will be evaluated in efficacy models, such as the tail bleeding described in example 3.

Example 7:

Effect of VWF on immunogenicity of FVIII

The immuno-modulatory effect of VWF co-formulated with a FVIII compound is evaluated in comparison to wild type FVIII and FVIII compounds alone.

In vivo, the relative immunogenicity is evaluated from the titer of FVIII binding antibodies and the determination of the level of neutralizing antibodies (inhibitors) at certain time points after administration. The assay for detection of FVIII binding antibodies is a radioimmunoassay (RIA). Briefly, anti-FVIII antibodies from a sample bind to radioactive ^{125}I-labelled rFVIII. Immunoglobulin and immune complexes bind to protein G-sepharose and is precipitated by centrifugation. The radioactivity in the precipitate is measured and this is proportional to the amount of anti-FVIII antibodies in the sample. The result is expressed in per cent of the total amount of added radioactivity. i.e. as % bound/total (%B/T).

Samples positive for anti-FVIII antibodies are analysed for the presence of FVIII neutralizing antibodies using a chromogenic assay. Briefly, samples are incubated with 1 IU/ml FVIII for 1 hr. The remaining FVIII activity is determined by addition of FIX, FX, thrombin, $CaCl_2$ and phospholipids. After incubation the amount of generated FXa is determined by addition of the chromogenic substrate S-2760 and the change in optical density (OD) is measured. The OD change is proportional to FVIII activity in the samples, and is compared to samples containing a known amount of FVIII and no inhibitors. The % remaining activity of the test sample is calculated compared to the reference samples without inhibitors/anti-FVIII antibodies added. Furthermore, the presence of anti-VWF antibodies is measured by ELISA using monoclonal or polyclonal anti-human VWF antibodies which does not cross react with murine VWF. If a strong anti-VWF response is detected, this can be expected to interfere with the binding of VWF to FVIII and the in vivo analysis is repeated using murine VWF fragments.

The appearance of anti-drug antibodies is evaluated after repeated (e.g. once weekly for 4 weeks or once daily for three weeks) s.c. administration of the compounds in naive mice, in FVIII k/o mice as well as in mice tolerized to human FVIII. The readout is the ratio of animals with positive titres at certain time points after the first and/or the last administration (e.g. 1, 2, 3, 4, 5, 6, 7 or 8 weeks). FVIII k/o mice are injected weekly e.g. with 1000IU/kg FVIII alone or in combination with VWF in a molar ratio ensuring that at least e.g. 87% of FVIII is bound to VWF. For daily administration, the FVIII dose is lower and based upon the bioavailability of the FVIII-VWF complex. Mice tolerized to hFVIII are injected weekly for e.g. eight weeks s.c. with e.g. 1000 IU/kg FVIII with or without VWF and in some

experiments including additional challenge with complete Freund's adjuvant (CFA) for the first injection followed by weekly challenges by incomplete Freund's adjuvant (IFA).

Relative immunogenicity of VWF versus VWF fragments and of wild type FVIII versus a FVIII compound co-formulated with VWF is furthermore evaluated in vitro in a

5   human CD4+ T-cell assay. This is done using peripheral blood mononuclear cells (PBMCs) depleted of CD8+ T-cells. FVIII is added to the cell culture e.g. for eight days. T-cell proliferation is evaluated during the course of the assay by pulsing for e.g. 18h with ^{3}H-thymidine in sub-samples from the cultures and subsequently measuring ^{3}H-thymidine incorporation. Interleukin 2 production is measured at the end of the assay using an

10   ELISPOT IL-2 kit e.g. from R&D Systems, following the manufacturer's instructions. The data obtained in the assays are converted to a "stimulation index" describing the ratio between compound-stimulated versus un-stimulated cells.

The HLA-binding capacity of VWF has been evaluated using in silico analysis of HLA-binding properties. Strong binding to a sequence in a modified VWF may indicate novel

15   T-cell epitopes, although the in silico analysis tool is predicting epitopes that may not be processed by the naturally occurring proteases. In order to predict if the Cys->Ser mutation will induce a risk of induced immunogenicity in the VWF-mutants, the VWF protein sequences are applied to an in silico peptide/HLA-ll binding prediction software. The peptide/HLA-ll binding prediction software is based on two different algorithms, NetMHCMpan

20   2.1 (NetMHCIIpan-2.0 - Improved pan-specific HLA-DR predictions using a novel concurrent alignment and weight optimization training procedure. Nielsen M, Lundegaard C, Justesen S, Lund O, and Buus S. Immunome Res. 2010 Nov 13;6(1):9) performing pan-specific HLA-DR predictions - and NetMHCII 2.0 (NN-align - A neural network-based alignment algorithm for MHC class II peptide binding prediction. Nielsen M and Lund O. BMC Bioinformatics. 2009

25   Sep 18;1 0:296) performing HLA-DP/DQ predictions.

Twenty-three amino acid long peptides with the point of mutation in position 12 are used as input to the algorithms. The optimal processed peptide is assumed to be a 15'mer peptide with a nine amino acid core peptide binding to the HLA-II. The output is 15 amino acid long peptides with 9 amino acid long core peptides (in contact with HLA-II) and the

30   predicted binding affinities in nanomolar.

The predicted binding affinities of the VWF mutant peptides are in the same range as the binding affinities of the wild type sequences (data not shown) - and because the peptides are predicted to bind with relatively poor affinity to the HLA-II molecules, the risk of inducing novel CD4+ T-cell epitopes is considered to be very low.

Of note, the in silico peptide/HLA-II binding predictions are based on experimental peptide/HLA-II binding data where it is very challenging to test cysteine-rich peptides (due to the nature of the peptides). Thus, cysteine-rich peptides are underrepresented in data sets used to train the different prediction algorithms. Therefore, the peptide/HLA-II binding predictions of these cysteine-rich VWF peptides are uncertain and should be analysed further using other immunogenicity prediction platforms (etc. in vitro peptide/HLA-II binding assays or ex vivo T-cell assays).

Example 8:

Subcutaneous administration in FVIII knockout mice (3):

Two test compounds were prepared:

a) B-domain truncated FVIII ("turoctocog alfa"/"N8" - produced essentially as disclosed in example 1 in WO2009108806) (4000 IU FVIII/ml determined by chromogenic activity assay and equivalent to 2.4 μM)

b) B-domain truncated FVIII (turoctocog alfa) (1000 IU FVIII/ml determined by chromogenic activity assay and equivalent to 0.6 μM) co-formulated with 0.37 mg/ml VWF fragment TIL7E7D3/A1 (equivalent to 4.6 μM)

Based on a measured binding affinity of 1.5nM of the VWF fragment to FVIII, 99% of the FVIII should be bound to VWF in this composition.

Both test compounds were formulated in 18 mg/ml NaCl, 3 mg/ml saccharose, 1.5 mg/ml L-histidine, 0.1 mg/ml polysorbate 80, 0.25 mg/ml $CaCl_2$, pH -7.3

12 FVIII KO mice, exon 16 knock-out in a mixed background of C57Bl/6 and SV129, bred at Taconic M&B (B6.129S4-F8tm1Kaz/J) with an approximate weight of 22 g were dosed subcutaneously in the flank with 10000 IU/kg FVIII or FVIIIA/WF, 6 mice with each test compound.

Blood was sampled at 1, 3, 7, 17, 24, 30, 48, 72 and 96 h post administration. The mice were anaesthetized by Isoflurane/0$_2$/N$_2$0 prior to blood sampling via the retroorbital plexus. Three samples were taken from each mouse. 45 μl of blood was stabilised with 5 μl of sodium-citrate (0.13 M) and added 200 μl FVIII coatest SP buffer (50mM TRIS-HCl, 1% BSA, Ciprofloxacin 10 mg/L, pH 7.3). After centrifugation at 4000 g for 5 minutes at room temperature, the supernatants were immediately frozen on dry ice before storage at -80°C prior to analysis.

Samples were analysed with regards to FVIII activity in a chromogenic assay as described by Ovlisen K et al. J. Thromb. Haemost, 2008, 6: 969-975 and by FVIII antigen

analysis using two FVIII light chain antibodies (4F45 and 4F1 1) in a FVIII LOCI assay (Luminescence oxygen channelling immunoassay).

Mean plasma concentration versus time data were analysed by non-compartmental analysis using WinNonlin Phoenix (Pharsight Corporaton) estimating the given

5   pharmacokinetic parameters. The bioavailability was estimated using a previous i.v. pharmacokinetic study of N8-GP in FVIII KO mice.

The circulating profiles of FVIII activity are shown graphically in Fig. 5 and antigen levels are shown in fig. 6.

In this experiment, the bioavailability of B-domain truncated FVIII alone was

10   calculated to be 0,9% based on activity. The co-formulation with the VWF fragment increased the bioavailability to 11%.

Example 9:

Subcutaneous administration in FVIII knockout mice (4):

15   Two test compounds were prepared:

a) 226 amino acid B domain variant (1000 IU FVIII/ml determined by chromogenic activity assay and equivalent to 2.4 μM)

b) 226 amino acid B domain variant (1000 IU FVIII/ml determined by chromogenic activity assay and equivalent to 0.6 μM) co-formulated with

20   0.37 mg/ml VWF fragment TIL7E7D3/A1 (equivalent to 4.6 μM)

Based on a measured binding affinity of 1.5nM of the VWF fragment to FVIII, 99% of the FVIII should be bound to VWF in this composition.

Both test compounds were formulated in 18 mg/ml NaCl, 3 mg/ml saccharose, 1.5 mg/ml L-histidine, 0.1 mg/ml polysorbate 80, 0.25 mg/ml

25   $CaCl_2$, pH -7.3

12 FVIII KO mice, exon 16 knock-out in a mixed background of C57Bl/6 and SV129, bred at Taconic M&B (B6.129S4-F8tm1Kaz/J) with an approximate weight of 22 g were dosed subcutaneously in the flank with 10000 IU/kg FVIII or FVMIA/WF, 6 mice with each test compound.

30   Blood was sampled at 1, 3, 7, 17, 24, 30, 48, 72 and 96 h post administration. The mice were anaesthetized by Isoflurane/0 $_2$/N$_2$0 prior to blood sampling via the retro-orbital plexus. Three samples were taken from each mouse. 45 μl of blood was stabilised with 5 μl of sodium-citrate (0.13 M) and added 200 μl FVIII coatest SP buffer (50mM TRIS-HCl, 1% BSA, Ciprofloxacin 10 mg/L, pH 7.3). After centrifugation at 4000 g for 5 minutes at room

temperature, the supernatants were immediately frozen on dry ice before storage at -80°C prior to analysis.

Samples were analysed with regards to FVIII activity in a chromogenic assay as described by Ovlisen K et al. J. Thromb. Haemost, 2008, 6: 969-975 and by FVIII antigen analysis using two FVIII light chain antibodies (4F45 and 4F1 1) in a FVIII LOCI assay (Luminescence oxygen channelling immunoassay).

Mean plasma concentration versus time data were analysed by non-compartmental analysis using WinNonlin Phonix (Pharsight Corporaton) estimating the given pharmacokinetic parameters. The bioavailability was estimated using a previous i.v. pharmacokinetic study of N8-GP in FVIII KO mice.

In this experiment, the bioavailability of the 226 amino acid B domain FVIII variant alone was similar to that obtained with co-formulation with VWF. Hence, for this variant with a longer B-domain, VWF did not increase the bioavailability.

Example 10

Construction of expression vectors encoding FVIII molecules

Plasmid with insert encoding the F8-500 FVIII molecule (F8-500 equals turoctocog alfa/N8 encoding sequence) was used for production of FVIII. Starting at the N-terminus, the F8-500 vector encodes the FVIII heavy chain without the B domain (amino acids 1-740), a 21 amino acid linker (SFSQNSRHPSQNPPVLKRHQR - **SEQ ID NO 2),** and the FVIII light chain (amino acids 1649-2332 of full-length wild-type human FVIII). The sequence of the 21 amino acid linker is derived from the FVIII B domain and consists of amino acids 741-750 and 1638-1648 of full length wild-type human FVIII. Fragments of FVIII cDNA were amplified from full length FVIII cDNA and inserted into F8-500 coding plasmid giving rise to DNA constructs encoding the BDD FVIII.

Contructs encoding F8-500D-HIS-C2-linked-(GGGS)6-hFc(IgG1), F8-500D-HIS-C2-Nnked-(GGGS)6-mFc(IgG2A), and F8-500D-HIS-C2-linked-(GGGS)6-albumin were established as described in the following. The internal BamHI site (aa 604-606) in F8-500 coding DNA was eliminated by site-directed mutagenesis and DNA encoding the flexible $(GGGS)_6$ linker was inserted 3' to the coding region. A new BamHI site was introduced in the 3' end of the linker-coding DNA in order to ease cloning of C-terminal fusion partners between BamHI and NotI sites. Thus, a construct encoding F8-500-C2-linked-(GGGS)6 was generated. DNA encoding human Fc (IgG1), mouse Fc (IgG2a), and human serum albumin was amplified.

The PCR products were inserted between the BamHI and Not I sites of the F8-500-

C2-linked-(GGGS)6 coding vector giving rise to constructs encoding F8-500-C2-linked-(GGGS)6-hFc(IgG1), F8-500-C2-linked-(GGGS)6-mFc(IgG2A), and F8-500-C2-linked-(GGGS)6-albumin. A SphI/ClaI restriction fragment from the latter constructs were transferred to a F8-500D-His coding constructs in order to generate F8-500D-HIS-C2-linked-

5   (GGGS)6-hFc(IgG1)-, F8-500D-HIS-C2-linked-(GGGS)6-mFc(IgG2A)-, and F8-500D-HIS-C2-linked-(GGGS)6-albumin coding constructs.

For transient expression as described in Example 11, DNA constructs consisting of the mammalian expression vector pTT5 with insert encoding BDD FVIII were utilized. For generation of stable cell lines producing BDD FVIII, the vector pTSV7 is utilized. This vector

10   encodes dihydrofolate reductase allowing selection of transfected cells with the dihydrofolate reductase system. A SpeI/AgeI restriction fragment from a pTT5-derived vector encoding F8-500D-His was transferred to a pTSV7-derived vector encoding F8-500 leading to construct #1917 consisting of pTSV7 with insert encoding F8-500D-His.

15   **Example 11**

Transient expression of FVIII

HKB1 1 cells at a density of 0.9 - 1.1 x 10^6 were transfected with a complex of plasmid (0.7 mg/l or 1.0 mg/l) and the transfection agent, 293Fectin (Invitrogen) (1.0 ml/l or 1.4 ml/l). The transfection complex was prepared by diluting the plasmid and the transfection

20   separately, mixing the two solutions, and incubating the mixture at room temperature for 20 minutes. The complex mixture was added to the cell suspension and the suspension was incubated in shaker incubator for 4 or 5 days at 36.5 °C or 37 °C and at 5 % or 8 % CO_2. Cell culture harvests were analysed by chromogenic FVIII assay as described in Example 14 and/or filtered through a 0,22 μm membrane filter and utilized for purification of FVIII as

25   described in Example 13.

Example 12

Stable cell line expressing FVIII

Serum-free adapted CHO-DUKX-B1 1 cells were transfected with the expression

30   plasmid construct #1917 described in Example 10 and encoding the FVIII F8-500D-His. Transfected cells were selected with the dihydrofolate reductase system and cloned by limiting dilution. Clones were screened for FVIII production by ELISA and chromogenic activity assay. The clone GedT019A was selected for upscaling. The cells were transferred to a bioreactor. The F8-500D-His protein was purified from cell culture harvests as described

35   in Example 13

43

Example 13

Purification of FVIII

A column was packed with the resin VIMSelect (GE Healthcare), with the dimensions

5   1.6cm in diameter and 4cm in bed height giving 8ml_, and was equilibrated with 20mM

Imidazole + 10mM $CaCl_2$ + 0.01 % Tween80+250mM NaCl, pH7.3 at 500cm/h. The culture

filtrate prepared as described in Example 3 was applied to the column, and the column was

subsequently washed with first equilibration buffer and then 20mM Imidazole + 10mM $CaCl_2$

+ 0.01 % Tween80+1 .5M NaCl, pH7.3. The bound FVIII was eluted isocratic at 90cm/h with

10   20mM Imidazole + 10mM $CaCl_2$ + 0.01% Tween80 + 1M Ammoniumacetate + 6.5M

Propylenglycol, pH7.3. The fractions containing FVIII were pooled and diluted 1:10 with

20mM Imidazole + 10mM $CaCl_2$ + 0.01 % Tween80, pH7.3 and applied to a column packed

with F25-Sepharose (Thim *et al.,* Haemophilia, 2009). The column dimension was 1.6cm in

diameter and 2cm in bed height giving 4ml_ in column volume. The column was equilibrated

15   at 180cm/h with 20mM Imidazole + 10mM $CaCl_2$ + 0.01% Tween80 + 150mM NaCl + 1M

Glycerol, pH7.3 prior to application. After application the column was washed first with

equilibration buffer and then 20mM Imidazole + 10mM $CaCl_2$ + 0.01% Tween80 + 650mM

NaCl, pH7.3. The bound FVIII was isocratic eluted with 20mM Imidazole + 10mM $CaCl_2$ +

0.01% Tween80 + 2.5M NaCl + 50%(v/v) Ethylenglycol, pH7.3 at 30cm/h. The fractions

20   containing FVIII were pooled and diluted 1:15 with 20mM Imidazole + 10mM $CaCl_2$ + 0.01 %

Tween80, pH7.3, except FVIII-variants with deletions of the a3 domain which were diluted

1:45 in the same buffer. The diluted pool was applied to a column packed with Poros 50HQ

(PerSeptive Biosystem), with the column dimensions 0.5cm in diameter and 5cm in bed

height giving 1ml_ in column volume. The column was equilibrated at 300cm/h with 20mM

25   Imidazole + 10mM $CaCl_2$ + 0.01% Tween80 + 50mM NaCl + 1M Glycerol, pH7.3 prior to

application. The column was washed with equilibration buffer before the elution using a linear

gradient over 5 column volumes from equilibration buffer to 20mM Imidazole + 10mM $CaCl_2$

+ 0.01% Tween80 + 1M NaCl + 1M Glycerol, pH7.3. The fractions containing FVIII were

pooled and the pool was stored at -80° until use.b

30   The FVIII molecules with HIS-tag were purified essentially as described above,

however the second purification step (F25-sepharose) was exchanged to Chelating

Sepharose FF (GE Healtcare) charged with 2 column volumes of 1M $NiSO_4$. The column

dimension was 0.5cm in diameter and 5cm bed height giving 1mL column volume. The

column was equilibrated with 30mM Imidazole + 10mM $CaCl_2$ + 0.01 % Tween80 + 1.5M

35   NaCl, pH7.3 at 180cm/h prior to application. After application the column was washed with

44

30 column volumes of equilibration buffer prior to elution using a linear gradient over 5 column volumes to 250mM Imidazole + 10mM $CaCl_2$ + 0.01% Tween80 + 1.5M NaCl, pH7.3. The fractions containing FVIII were pooled and diluted 1:30 with 20mM Imidazole + 10mM $CaCl_2$ + 0.01% Tween80, pH7.3. The final purification step (Poros 50HQ) was performed as described above.

Example 14

FVIII activity in cell culture harvests measured by chromogenic assay

The FVIII activity (FVIIhC) of the rFVIII compound was evaluated in a chromogenic FVIII assay using Coatest SP reagents (Chromogenix) as follows: rFVIII samples and a FVIII standard (Coagulation reference, Technoclone) were diluted in Coatest assay buffer (50 mM Tris, 150 mM NaCl, 1 % BSA, pH 7.3, with preservative). Fifty µl of samples, standards, and buffer negative control were added to 96-well microtiter plates (Spectraplates MB, Perkin Elmer). All samples were tested diluted 1:100, 1:400, 1:1600, and 1:6400. The factor IXa/factor X reagent, the phospholipid reagent and $CaCl_2$ from the Coatest SP kit were mixed 5:1:3 (vol:vol:vol) and 75 µl of this added to the wells. After 15 min incubation at room temperature, 50 µl of the factor Xa substrate S-2765/thrombin inhibitor 1-2581 mix was added and the reactions were incubated 5 min at room temperature before 25 µl 1 M citric acid, pH 3, was added. The absorbance at 405 nm was measured on an Envision microtiter plate reader (Perkin Elmer) with absorbance at 620 nm used as reference wavelength. The value for the negative control was subtracted from all samples and a calibration curve prepared by linear regression of the absorbance values plotted vs. FVIII concentration. The yields of the present FVIII relative to that of the F8-500 protein are shown in Table 1.

Example 15

FVIIIactivity in purified samples measured by chromogenic assay

The FVIII activity (FVIIhC) of the rFVIII compound was evaluated in a chromogenic FVIII assay using Coatest SP reagents (Chromogenix) as follows: rFVIII samples and a FVIII standard (e.g. purified wild-type rFVIII calibrated against the 7th international FVIII standard from NIBSC) were diluted in Coatest assay buffer (50 mM Tris, 150 mM NaCl, 1 % BSA, pH 7.3, with preservative). Fifty µl of samples, standards, and buffer negative control were added to 96-well microtiter plates (Nunc) in duplicates. The factor IXa/factor X reagent, the phospholipid reagent and $CaCl_2$ from the Coatest SP kit were mixed 5:1:3 (vol:vol:vol) and 75 µl of this added to the wells. After 15 min incubation at room temperature 50 µl of the factor Xa substrate S-2765/thrombin inhibitor 1-2581 mix was added and the reactions incubated 10

45

min at room temperature before 25 µl 1 M citric acid, pH 3, was added. The absorbance at 415 nm was measured on a Spectramax microtiter plate reader (Molecular Devices) with absorbance at 620 nm used as reference wavelength. The value for the negative control was subtracted from all samples and a calibration curve prepared by linear regression of the
5   absorbance values plotted vs. FVIII concentration. The specific activity was calculated by dividing the activity of the samples with the protein concentration determined by HPLC. For HPLC, the concentration of the sample was determined by integrating the area under the peak in the chromatogram corresponding to the light chain and compare with the area of the same peak in a parallel analysis of a wild-type rFVIII, where the concentration was
10   determined by amino acid analyses. The results are shown in Table 1.

Example 16

FVIII activity in purified samples measured by one-stage clot assay

FVIII activity (FVIIhC) of the rFVIII compounds was further evaluated in a one-stage
15   FVIII clot assay as follows: rFVIII samples and a FVIII standard (e.g. purified wild-type rFVIII calibrated against the 7th international FVIII standard from NIBSC) were diluted in HBS/BSA buffer (20 mM hepes, 150 mM NaCl, pH 7.4 with 1 % BSA) to approximately 10 U/ml followed by 10-fold dilution in FVIII-deficient plasma containing VWF (Dade Behring or Siemens). The samples were subsequently diluted in HBS/BSA buffer. The APTT clot time
20   was measured on an ACL300R or an ACL9000 instrument (Instrumentation Laboratory) using the single factor program. FVIII-deficient plasma with VWF (Dade Behring or Siemens) was used as assay plasma and SynthASil, (HemosIL™, Instrumentation Laboratory) as aPTT reagent. In the clot instrument, the diluted sample or standard is mixed with FVIII-deficient plasma, aPTT reagents at 37°C. Calcium chloride is assed and time until clot formation is
25   determined by turbidity. The FVIII activity in the sample is calculated based on a standard curve of the clot formation times of the dilutions of the FVIII standard. The results are shown in table 1.

Table 1: Yields and specific activities of different BDD FVIM molecules ("His-tagged" for easier purification).

Compound	B domain amino acids	Yield by transient transfection (relative to F8-500)	Specific activity measured by chromogenic assay (IU/mg)	Specific activity measured by one-stage clot assay (IU/mg)
F8-500E-His	741-857 + 1637-1648	0.7	10501	9122
F8-500L-His	741-914 + 1637-1648	0.6	10330	8282
F8-500M-His	741-954 + 1637-1648	0.6	12404	10259
F8-500D-His	741-965 + 1637-1648	0.3	9015	9579
F8-500G-His	741-965 + 1637-1648 Amino acid replacements: N757Q- N784Q-N828Q-N900Q-N943Q-N963Q	0.7	11507	9822
F8-500N-His	741-1003 + 1637-1648	0.4	-	-
F8-500H-His	741-1020 + 1637-1648	0.7	10027	10541
F8-500I-His	741-1079 + 1637-1648	0.7	-	-
F8-500J-His	741-1206 + 1637-1648	0.6	-	-
F8-500F-His	741-1261 + 1637-1648	0.3	5691	4855
F8-500K-His	741-1309 + 1637-1648	0.4	-	-
F8-500-His2-4N	741-914 + 1637-1648	0.6	-	-
F8-500-His2-5N	741-954 + 1637-1648	0.7	-	-
F8-500-His2-6N	741-968 + 1637-1648	0.6	14088	12784
F8-500-His2-7N	741-1003 + 1637-1648	0.5	7211	7542
F8-500-His2-8N	741-1018 + 1637-1648	0.7	8664	7481
F8-500-His2-10N	741-1070 + 1637-1648	0.6	12391	8253
F8-500-His2-11N	741-1230 + 1637-1648	0.5	-	-
F8-500-His2-15N	741-1301 + 1637-1648	0.4	-	-
F8-500D-His-D519V-E1984A	741-965 + 1637-1648	0.5	15282	9729
F8-500D-His-C2	741-965 + 1637-	0.6	-	-

linked-(GGGS)6-hFc(IgG1)	1648			
F8-500D-His-C2 linked-(GGGS)6-mFc(IgG2a)	741-965 + 1637-1648	0.6	13509	8858
F8-500D-His-C2 linked-(GGGS)6-albumin	741-965 + 1637-1648	0.7	12226	5852

Example 17.

Construction of expression vectors encoding VWF fragments

DNA fragments encoding the VWF signal peptide, followed by different C-terminally truncated versions, the VWF D' domain and the VWF D3 domain, an Ala-Leu-Ala spacer and a HPC4 tag were generated by polymerase chain reaction (PCR) using plasmid pLC095 as template (Plasmid pLLC095 is described in Example 26. The primer JP1000 was used as forward primer in all PCR reactions in combination with the reverse primers JP1001- JP1008 shown in **Table 2.**

Table 2

Forward primer	Forward primer Sequence (5'-3')
JP1000 VWF-HindIII S	CTAAGCGT<u>AAGCTT</u>GCCACC**ATG**ATTCCTGCCAGATTTGCCGG **(SEQ ID NO 23)**
Reverse primer	**Reverse primer Sequence (5'-3')**
JP1001 VWF 764-828	TGGTCCTCA<u>GCTAGC</u>GCGGGACACCTTTCCAGGGCCACAC **(SEQ ID NO 24)**
JP1002 VWF 764-865	TGGTCCTCA<u>GCTAGC</u>GCGGCATCACACACATGGTCTGTGC **(SEQ ID NO 25)**
JP1003 VWF 764-1035	TGGTCCTCA<u>GCTAGC</u>GCTCTGGTGTCAGCACACTGCGAGCTC **(SEQ ID NO 26)**
JP1004 VWF 764-1041	TGGTCCTCA<u>GCTAGC</u>GCTGAGTCCAGAGGCACTTTTCTGG **(SEQ ID NO 27)**
JP1005 VWF 764-1045	TGGTCCTCA<u>GCTAGC</u>GCGGTGGCAGGGGATGAGTCCAGAG **(SEQ ID NO 28)**
JP1006 VWF 764-1250	TGGTCCTCA<u>GCTAGC</u>GCGGCATCTGTGGGAGGCACCACC **(SEQ ID NO 29)**
JP1007 VWF 764-1261	TGGTCCTCA<u>GCTAGC</u>GCGTCCTCCACATACAGAGTGGTG **(SEQ ID NO 30)**
JP1008 VWF 764-1268	TGGTCCTCA<u>GCTAGC</u>GCATCGTGCAACGGCGGTTCCGAG **(SEQ ID NO 31)**

The PCR products were digested with HindIII and NheI and were subsequently cloned into a HindIII and NheI digested pJSV164 vector using Rapid DNA Ligation kit (Roche Diagnostics GmbH, Mannheim, Germany). pJSV164 is a pTT5 based expression vector (Yves Durocher, CNRC, Montreal, Canada) containing a CD33 signal peptide and a HPC4

tag. Digestion of pJSV164 with HindIII and NheI removes the CD33 signal peptide and allows cloning of the gene of interest in frame with the HPC4 tag to generate an expression cassette encoding a C-terminally HPC4 tagged gene of interest in which the gene of interest and the HPC4 tag is separated by an Ala-Leu-Ala linker peptide. The ligation reactions were

5   transformed into Top10 cells (Life Technologies, Carlsbad, CA, USA).

        The resulting eight plasmids were named as shown in Table 3. The amino acid sequences of the generated proteins are outlined in SEQ ID NO 4, 5, 6, 7, 8, 11 and 16.

10   **Table 3**

Vector name	Insert
pJSV343	VWF 764-828-HPC4 (SEQ ID NO 4)
pJSV344	VWF 764-865-HPC4 (SEQ ID NO 5)
pJSV345	VWF 764-1035-HPC4 (SEQ ID NO 6)
pJSV346	VWF 764-1041-HPC4 (SEQ ID NO 7)
pJSV347	VWF 764-1045-HPC4 (SEQ ID NO 8)
pJSV348	VWF 764-1250-C1099/1142S-HPC4 (SEQ ID NO 11)
pJSV349	VWF 764-1261-C1099/1142S-HPC4 (SEQ ID NO 14)
pJSV350	VWF 764-1268-C1099/1142S-HPC4 (SEQ ID NO 15)

        Example 18:

Construction of expression vectors encoding VWF fragments (2)

        Three additional HPC4 tagged, truncated variants of VWF were generated by

15   Ligation independent cloning (LIC) using pJSV348 (see **Example 17)** as template. Three independent PCR reactions were set-up on pJSV438 using the primers shown in **Table 4.**

        Table 4

Fragment	Primer name	Primer sequence (5'-3')
VWF(864-1250)-HPC4 (SEQ ID NO 12)	VWF(864-1250)-HPC4 S	GGGACCCTTTGTGATGCCACGTGCTCCACGATCG G **(SEQ ID NO 32)**

	VWF(864-1250)-HPC4 AS	GCACGTGGCATCACAAAGGGTCCCTGGCAAAATG AG **(SEQ ID NO 33)**
VWF(764-1128)-HPC4 (SEQ ID NO 9)	VWF(764-1 128)-HPC4 S	TTGTGCCCCCAGGAGGACCAAGTAGATCCGCGGC TC **(SEQ ID NO 33)**
	VWF(764-1 129)-HPC4 AS	TACTTGGTCCTCCTGGGGGCACAATGTGGCCGTC **(SEQ ID NO 34)**
VWF(764-1198)-HPC4 (SEQ ID NO 10)	VWF(764-1 198)-HPC4 S	GACTGTCCAGTGGAGGACCAAGTAGATCCGCGG **(SEQ ID NO 35)**
	VWF(764-1 198)-HPC4 AS	TTGGTCCTCCACTGGACAGTCTTCAGGGTCAA **(SEQ ID NO 36)**

The three PCR fragments VWF(864-1250)-HPC4, VWF(764-1 128)-HPC4 and VWF(764-1 198)-HPC4 were 5685/5610/5817 bp in size respectively. The PCR fragments were DpnI treated to remove methylated template DNA. The PCR fragments were
5   subsequently purified from gel and were self-ligated by LIC using the In-Fusion HD Cloning Kit (Clontech, Mountain View, CA,

USA) to generate circular DNA fragments and subsequently transformed into Top10 cells (Life Technologies, Carlsbad, CA, USA).

10   The resulting three plasmids were named as shown in **Table 5.** The amino acid sequences of the generated proteins are outlined in **SEQ ID NOs 12, 9, and 10.**

Table 5

Vector name	Insert
pJSV405	VWF(864-1250)-C1099/1142S-HPC4 monomer (SEQ ID NO 12)
pJSV406	VWF(764-1128)-C1099S-HPC4 monomer (SEQ ID NO 9)
pJSV407	VWF(764-1198)-C1099/1142S-HPC4 monomer (SEQ ID NO 10)

15   **Example 19:**

Transient expression of VWF fragments

Human embryonic kidney 293 6E suspension cells at a density of 0.9 - 1.1 x 10^6 cells/ml were transfected with a complex of VWF fragment coding plasmid (0.7 mg/l or 1.0 mg/l) and the transfection agent 293Fectin (Invitrogen) (1.0 ml/l or 1.4 ml/l). The transfection

50

complex was prepared by diluting the plasmid and the transfection separately, mixing the two solutions, and incubating the mixture at room temperature for 20 minutes. The complex mixture was added to the cell suspension and the suspension was incubated in shaker incubator for 5 days at 36.5 °C or 37 °C and at 5 % or 8 % CO_2. Cell culture harvests were

5   filtered through a 0,22 μιη membrane filter and utilized for purification of VWF fragment as described in Example 22.

Example 20:

Preparation of dimer forms of VWF fragments

10   In the native full length VWF molecule (SEQ ID NO 22) two cysteine residues in the N-terminal part of the molecule are supposed to participate in the dimerization and/or multimerization of VWF: Cys1099 and Cys1 142.

In all of the monomeric fragments of the sequences (SEQ ID NO 10, SEQ ID NO 11, SEQ ID NO 12, SEQ ID NO 13, SEQ ID NO 14, SEQ ID NO 15, SEQ ID NO 16, SEQ ID NO

15   17, SEQ ID NO 18, SEQ ID NO 19, SEQ ID NO 20, and SEQ ID NO 21) two cysteine residues (Cys1099 and Cys1 142) are mutated to other amino acid residues so that the expressed molecule is not able to form dimers/multimers. A monomeric fragment of SEQ ID NO 9 is generated by mutating Cys 1099 to another amino acid residue.

In some cases, a dimeric form of the VWF fragments is wanted. This can be

20   accomplished in several ways:

One method to accomplish dimer formation is to keep the two residues at position 1099 and position 1142 as cysteines. In order to make a recombinant dimeric molecule, the cDNA encoding the desired VWF fragment is including the presequence of VWF e.g the D1D2 sequence of VWF (amino acid residues 23-763 of SEQ ID NO 22). This will, during

25   processing in the golgi apparatus align two monomers of a given VWF fragment in a configuration allowing a dimeric molecule to be formed with two disulphide bonds in which Cys1099 in monomer 1 is connected to a Cys1099 in monomer 2 and Cys1 142 in monomer 1 is connected to Cys1 142 in monomer 2.

Another method to accomplish dimer formation is to avoid the inclusion of the

30   presequence (amino acid residues 23-763 of SEQ ID NO 22) and simply let a recombinant VWF fragment with Cys in position 1099 and 1142 form a dimeric molecule. This can in principle result in a series of different dimers e.g.:

Cys1099-Cys1 142/Cys1 142-Cys1 142 (two disulphide bonds - like above)

Cys1099-Cys1 142/Cys1099-Cys1 142 (two disulphide bonds)

35   Cys1 099-Cys1 099 (one disulphide bond)

51

Cys1 142-Cys1 142 (one disulphide bond)

Cys1 099-Cys1 142 (one disulphide bond)

Yet another method to accomplish dimer formation may be toto replace one of the cysteine residues 1099 or 1142 with other amino acid residues (e.g. Serine, Arginine).

5   If Cys1 099 is replaced with a non-Cysteine residue, the molecule may form a dimer by establishment of a disulphide bond between Cys1 142 in monomer 1 with Cys1 142 in monomer 2.

If Cys1 142 is replaced with a non-Cysteine residue, the molecule may form a dimer by establishment of a disulphide bond between Cys1099 in monomer 1 with Cys1099 in
10   monomer 2.

The dimeric forms mentioned above may be constructed either with or without the D1D2 presequence of VWF (amino acid residues 23-763 of SEQ ID NO 22).

The different monomeric and dimeric forms will have different properties with regards to their binding to FVIII, their ease of production and their effect on bioavailability of
15   FVIII when injected subcutaneously as a co-formulation.

Example 21:

Evaluation of binding of VWF and VWF fragments to FVIII using a competition ELISA

20   In order to investigate the binding of the different VWF fragments to FVIII the following method is used. Briefly, human VWF is coated in a microtiterplate and incubated overnight at 4°C. After blocking, a solution with pre-incubated FVIII (1nM) and VWFA/WF-fragment is added to the plate, followed by detection with biotinylated anti FVIII antibody and streptavidin-peroxidase S-POD (1:20000). The absorbance is measured at 450/620nm. The
25   IC50 values are shown in Table 6.

Table 6:

Compound number	Domain/comment	VWF fragment sequence	Derived from SEQ ID NO	IC50 (Ki)
2304	TIL'E'	VWF(764-865)-ALA-HPC4 monomer	5	2.0 μM
2306	TIL7E7VWD3 II	VWF(764-1 041)-ALA-HPC4 monomer	7	2.2 μM
2307	TIL7E7VWD3 III	VWF(764-1 045)-ALA-HPC4 monomer	8	2.0 μM
2308	TIL7E7D3 I	VWF(764-1 250)-C1 099/1 142S-ALA-HPC4 monomer	11	12 nM
2309	TIL7E7D3 II	VWF(764-1 261)-C1 099/1 142S-ALA-HPC4 monomer	14	10 mM
2310	TIL7E7D3 III	VWF(764-1 268)-C1 099/1 142S-ALA-HPC4 monomer	16	15 nM
0170	TIL7E7D3/A1 III	VWF(764-1464)-C1 099/1 142S-HPC4 monomer	19	12 nM
0194	TIL7E7D3/A1 III	VWF(764-1464)-C1 099S-HPC4 monomer	19	8.0 nM

0240	TIL7E7D3/A1 IIdimer	VWF(764-1464)-HPC4 dimer	19	0.7 nM
0001	D3 I	VWF(864-1 250)-C1 099/1 142S-ALA-HPC4 monomer	12	20 μM
0003	TIL7E7VWD3/C8-3/TIL-3	VWF(764-1 198)-C1 099/1 142S-ALA-HPC4 monomer	10	28 nM
0314	Plasma derived full length VWF	VWF (764-281 3)	22	1.1 nM

These differences in FVIII binding between different fragments could indicate different effects in a subcutaneously administered FVIII co-formulation. The IC50 values are also being used to determine the optimal VWF and FVIII concentrations in the co-formulation mixtures.

Example 22:

Purification and characterisation of HPC4-tagged VWF fragments

Some VWF fragments are cloned and expressed with a C-terminal HPC4 tag: EDQVDPRLIDGK **(SEQ ID NO 37).** Sometimes an additional linker with the sequence of ALA is introduced between the VWF fragment and the HPC4 tag. After cloning, expression and cell culturing the cell media is added $CaCl_2$ to a final concentration of 1mM. The media is passed over an anti-HPC4 column. The column is equilibrated with 20mM HEPES, 100mM NaCl, 1mM $CaCl_2$, pH=7.5. After application of the cell media, the column is washed with 20mM HEPES, 1M NaCl, 1mM $CaCl_2$, pH=7.5 and the HPC4-tagged VWF fragment is subsequently eluted with 20mM HEPES, "IOOmM NaCl, 5mM EDTA, pH=7.5. The pool from the anti-HPC4 column is added 3 volumes of water to reduce the conductivity and applied onto a Mono Q column. Prior to the application the Mono Q column is equilibrated with 20mM HEPES, "IOOmM NaCl, 5mM EDTA, pH=7.5. The Mono Q column is washed with 20mM HEPES, 10OmM NaCl, pH=7.5 and the VWF fragment is eluted with a gradient from 10OmM NaCl to 2M NaCl in 20mM HEPES, 10mM $CaCl_2$, pH=7.5.

The purified protein is characterised by 1) SDS-gel electrophoreses, 2) analytical HPLC and 3) amino acid sequence analysis.

Purification and characterisation of non-tagged VWF fragments.

After cloning, expression and cell culturing the cell media is passed over an anti-VWF column. The anti-VWF antibody recognise amino acid residue number 764-865 of VWF (SEQ ID NO 5).The column is equilibrated with 20mM HEPES, "IOOmM NaCl, pH=7.5. After application of the cell media, the column is washed with 20mM HEPES, 1M NaCl, pH=7.5 and the VWF fragment is subsequently eluted with 50mM acetic acid, 100mM NaCl, pH=4.0.

53

The pool from the anti-VWF column is adjusted to pH=7.5 and applied onto a Mono Q column . Prior to the application the Mono Q column is equilibrated with 20mM HEPES, 1OOmM NaCl, pH=7.5. The Mono Q column is washed with 20mM HEPES, 1OOmM NaCl, pH=7.5 and the VWF fragment is eluted with a gradient from 100mM NaCl to 2M NaCl in
5   20mM HEPES, pH=7.5.

The purified VWF fragment is characterised by 1) SDS-gel electrophoreses, 2) analytical HPLC and 3) amino acid sequence analysis.

Example 23:
10   *Evaluation of VWF fragments binding to FVIII by using Isothermal titration*
calorimetry

All protein samples are dialyzed in 50 mM Hepes pH 7.4, 150 mM NaCl, 10 mM $CaCl_2$ buffer. Each iTC experiment involves filling the iTC cell with FVIII (approximately 250 μL) and the syringe with VWF variants (approximately 40 μL). Temperature is set as required
15   and the protein sample is allowed to equilibrate under given experimental conditions (approximately 10 minutes). Typically 17 - 20 injections (of 2 - 2.5 μL) of VWF variants into cell, containing FVIII, are performed . The first injection is always of 0.2 μL and is discarded from the final data analysis in order to account for diffusion during equilibration step. Stirring speed is set between 700 - 1000 rpm. Filter period for data collection is 5 sec with a high
20   feedback mode setting. Each titration is spaced by 120 sec. Appropriate control experiments are performed . Raw data is processed to set baseline and integrated to obtain a final isotherm. This binding isotherm is fit to a single-site model to yield K_d, stoichiometry (n), ΔH, and AS values to complete characterization of VWF variant binding to FVIII. An example binding isotherm is shown in fig. 9. These data are being used for determining the optimal
25   concentrations of the FVIII and the VWF fragment in co-formulations intended for subcutaneous administrations.

Example 24
Subcutaneous administration in FVIII knockout mice
30   Test compounds were prepared as follows: Test compounds were formulated in 18 mg/ml NaCl, 3 mg/ml saccharose, 1.5 mg/ml L-histidine, 0.1 mg/ml polysorbate 80, 0.25 mg/ml CaCl2, pH -7.3. For test formulations containing VWF or VWF fragments the % FVIII bound by VWF in the co-formulation was calculated using the available IC50 (Ki) values as described above in example 2 1 (table 6) assuming Ki=K_d or the K_d values obtained as
35   described in example 23.

54

FVIII KO mice, exon 16 knock-out in a mixed background of C57Bl/6 and SV129, bred at Taconic M&B (B6.129S4-F8tm1Kaz/J) with an approximate weight of 22 g were dosed subcutaneously in the flank with FVIII in combination with various proteins, 6-9 mice with each test compound. The dose volume was 5ml/kg or 0.25ml/kg if indicated in table 7.

5   Blood was sampled at 9 time points from 0-96 h, n= 2-3 mice/time point, 3 blood samples from each mice in a sparse sampling regime. The mice were anaesthetized by Isoflurane/0 $_2$/N$_2$0 prior to blood sampling via the retroorbital plexus. 45 µl of blood was stabilised with 5 µl of sodium-citrate (0.13 M) and added 200 µl FVIII Coatest SP buffer (50mM TRIS-HCl, 1% BSA, Ciprofloxacin 10 mg/L, pH 7.3). After centrifugation at 4000 g for

10   5 minutes at room temperature, the supernatants were immediately frozen on dry ice before storage at -80°C prior to analysis.

Samples were analysed with regards to FVIII chromogenic activity as described by Ovlisen K et al. J. Thromb. Haemost, 2008, 6: 969-975 and by FVIII antigen analysis using two FVIII light chain antibodies (4F45 and 4F1 1) in a FVIII LOCI assay (Luminescence

15   oxygen channelling immunoassay).

Mean plasma concentration versus time data were analysed by non-compartmental analysis using WinNonlin Phoenix (Pharsight Corporaton) estimating the given pharmacokinetic parameters. The bioavailability was estimated using a previous i.v. pharmacokinetic study of N8 or N8-GP in the FVIII KO mouse strain.

20   The s.c. FVIII bioavailabilities of the test compounds are shown in table 7 below and in figur 7 and 8.

Table 7. FVIII Bioavailability values of a series of different FVIII molecules and FVIII/VWF fragment co-formulations obtained with s.c. administration in FVIII k/o mice. The

25   left column "FVIII" denotes the FVIII compound used in the experiment. The column labelled "FVIII dose" denotes the FVIII dose (IU/kg) used in the experiment, the column labelled "co-formulation protein" denotes the co-formulated protein (if any) used in the experiment. The column labelled "Molar ratio" denotes the molar ratio to FVIII of the protein in the co-formulation. The column labelled "FVIII Saturation" denotes the calculated fraction of FVIII

30   that is binding the co-formulated protein at the concentrations used in the experiment. The column labelled "F%" denotes the bioavailability of FVIII obtained in the experiment.

FVIII	FVIII Dose	Co-Formulation Protein	Molar Ratio	FVIII Saturation	F%
Turoctocog alfa	5000	(764-1464) monomer VWF	1	87%	7.3
rFVIII derived from the full-	2500	(764-1464) Dimer VWF	1	82%	7.4

length sequence (Kogenate®)					
Turoctocog alfa	2500	(764-1250) Monomer VWF	1	82%	7.6
Turoctocog alfa	2500	(764-1041) Monomer VWF	34	82%	7.8
Turoctocog alfa	2500	(764-828) Monomer VWF	1	12%	1.4
Turoctocog alfa	2500	(764-865) Monomer VWF	1	12%	2.7
Turoctocog alfa	2500	(764-1045) Monomer VWF	1	12%	2.0
Turoctocog alfa	2500	(764-865) Monomer VWF	34	83.3%	4.3
Turoctocog alfa	2500/ 0.25 ml/kg	(764-1041) Monomer VWF	3x	85.5%	5.03
Turoctocog alfa	2500/ 0.25 ml/kg	(764-865) Monomer VWF	3x	86.5%	1.9
Turoctocog alfa	2500/ 0.25 ml/kg	(764-1464) Dimer VWF	1x	99%	8.4
Turoctocog alfa	2500	(764-1464) Murine monomer VWF	1	82%	5.6
Turoctocog alfa	2500	Human serum Albumin	6 1 1	Not applicable	3.7
Turoctocog alfa	2500	plasma derived full length VWF	1	99%	0.0
Turoctocog alfa	5000	(764-1464) monomer VWF	7.7	99%	8.2
Turoctocog alfa	5000	(764-1464) monomer VWF	3	99%	6.7
Turoctocog alfa	5000	(764-1464) monomer VWF	1	87%	7.3
Turoctocog alfa	5000	None	Not applicable	Not applicable	2.3
FVIII with a 226 aa B domain	5000	None	Not applicable	Not applicable	4.3
FVIII with a - 226 aa B domain	5000	(764-1464) monomer VWF	7.7	0.99	7.0
N8-GP	2500	(764-1464) monomer VWF	1	0.82	27
N8-GP	10000	(764-1464) monomer VWF	7.7	0.99	47
N8-GP	2500	(764-1464) monomer VWF	7.7	0.99	36
N8-GP	2500	(764-1464) Dimer VWF	1	0.99	33
FVIII-K1804-Hep157	2500	(764-1464) monomer VWF	1	0.82	50
FVIII-K1804-Hep157	2500	None	Not applicable	Not applicable	27

PSA40Kd-O-Glycan-N8	2500	(764-1464) monomer VWF	1	0.82	8.8
PSA40Kd-O-Glycan-N8	2500	None	Not applicable	Not applicable	6.1
40kDa-PEG-FVIII-K2092A+F2093A	10000	None	Not applicable	Not applicable	20
N8-GP	10000	4F30 FVIII reduced uptake antibody	5	0.99	11
N8-GP	1000	Hirudin	0.5mg/kg	Not applicable	7.6
N8-GP	10000	Hyaluronidase	0.5 activity ratio	Not applicable	8.4
N8-GP	20000	None	Not applicable	Not applicable	28
N8-GP	10000	None	Not applicable	Not applicable	19
N8-GP	2500	None	Not applicable	Not applicable	14
N8-GP	1000	None	Not applicable	Not applicable	17

The s.c. bioavailability of FVIII co-formulated with a VWF fragment appear to depend on the saturation of the FVIII VWF binding sites in the co-formulation rather than on the VWF fragment length. The shortest VWF fragment, wherein a >80% saturation of FVIII was achieved, was 764-865 - this formulation displayed a FVIII bioavailability of 4.3% (34 molar excess of N8/turoctocog alfa over VWF fragment). The longest VWF fragment tested, under similar conditions with respect to saturation, was the 764-1464 fragment which resulted in a FVIII bioavailability of 7.3%. The dimer form of the 764-1464 dosed in a lower volume of 0.25ml/kg resulted in a FVIII bioavailability of 8.4%.

Fragments shorter than 764-1250, which do not contain the entire D3 region, bind FVIII with a higher IC50 (K_i) than longer fragments. Thus, 1 to 1 molar formulation of FVIII and VWF fragments shorter than 764-1250 displayed lower FVIII bioavailabilities, i.e. less than 4%.

The s.c. FVIII bioavailability-improving effect of VWF fragments according to the invention may thus be obtained by saturation of the FVIII VWF binding sites with VWF-fragment. Short VWF fragments with relatively low FVIII binding affinity should thus be used in higher ratios compared to longer VWF fragments with better binding FVIII binding properties in order to obtain a high degree of bioavailability.

FVIII derived from the full-length sequence (Kogenate®) displayed the same degree of bioavailability as FVIII with a truncated B domain (turoctocog alfa/N8) when co-formulated

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with the 764-1464 VWF fragment. This indicates that high FVIII bioavailability is not dependent on co-formulation with turoctocog alfa/N8 but is dependent on presence of the VWF fragment.

Co-formulation of FVIII (turoctocog alfa/N8) with full-length plasma-derived human VWF resulted in FVIII bioavailability of about 0% thus demonstrating that only fragments of VWF are able to enhance bioavailability of FVIII. The reason for the lack of effect of the full-length VWF may be due to the presence of collagen binding site in the A3 domain which may result in binding and entrapment of. Preferrred VWF fragments according to the present do thus not comprise the A3 domain. Alternatively or additionally, the multimerisation capabilities of full-length VWF produces large multimers that restricts systemic absorption due to size of the complex. The data indicates that also longer VWF fragments (prefeably without the A3 domain) than those tested in table 7 will have the same beneficial effect on FVIII bioavailability.

Serum albumin did not improve the s.c. bioavailability of FVIII (turoctocog alfa/N8). Thus, presence of additional protein in a FVIII formulation does not appear to increase the s.c. bioavailability of FVIII - unless this protein is a VWF fragment according to the present invention.

VWF dose was not critical for FVIII s.c. bioavailability as seen for molar ratios between 1:1 and 1:7.7 of FVIIhVWF fragment. The critical factor for achieving a high FVIII bioavailability thus appear to be a high degree of FVIII saturation (binding) with VWF fragment. All compositions in these experiments comprising a calculated saturation of N8 of at least 86.8% thus resulted in similar bioavailabilities. VWF fragments according to the invention may thus protect FVIII at the s.c. injection site.

FVIII with a 226 amino acid (aa) B domain (SEQ ID NO 3), displayed a higher s.c. FVIII bioavailability than turoctocog alfa/N8. However, bioavailability of this FVIII with a 226 aa B-domain was comparable to turoctocog alfa/N8 in connection with s.c. co-administration with the VWF-fragment 764-1464 (TIL7E7D3/A1) monomer. It may thus be speculated that the additional amino acids in the 226 aa B-domain (compared to turoctocog alfa/N8) may protect clearance sites of FVIII in connection with extravascular administration thereof, meaning that such FVIII molecules might be used for s.c. administration with or without VWF according to the present invention.

FVIIIK1804C-HEP157, displayed a bioavailability of 50% dosed in co-administration with the VWF-fragment 764-1464 (TIL7E7D3/A1) monomer and a bioavailability of 27% dosed alone. PSA40Kd-O-Glycan-N8, displayed a bioavailability of 8.8% dosed in co-administration with the VWF-fragment 764-1464 (TIL7E7D3/A1) monomer and 6.1 1% dosed

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alone. It may thus be speculated that conjugation of FVIII molecules with Heparosan polymers and/or Polysialic acid polymers either protects FVIII against breakdown/uptake in the sub cutis or enhances s.c. absorption. Heparosan appear to be more effective than Sialic acid polymers in enhancing the s.c. bioavailability. Both FVIII variants displayed higher

5   bioavailability's when dosed together with VWF fragment.

N8-GP and FVIIIK1804C-HEP157 + 764-1464 (TIL7E7D3/A1) monomer and dimer, resulted in the highest bioavailability obtained. Bioavailability of N8-GP may thus be increased by increasing the dose or the concentration in the co-formulation. Dose volume was 5ml/kg in all dosing's, thus the N8-GP concentration in the dosing solution was 2 times

10   higher in the 20000 IU/kg dosing than in the 10000IU/kg dosing. This resulted in 28% and 19% bioavailability respectively.

The 764-1464 dimer VWF fragment does not contain any mutations. The 764-1464 dimer VWF fragment binds stronger to Turoctocog alfa and N8-GP (table 6) but result in a similar bioavailability of FVIII as the monomer version of the fragment. This indicates that

15   substituting Cys1099 and/or Cys1142 in the VWF fragments according to the invention does not influence the bioavailability of FVIII. Also, the binding affinity of VWF fragments to N8-GP does not influence the effect on bioavailability of N8-GP as long as more than 80% of the FVIII molecules are in complex with VWF fragment in co-formulation. Additionally, since the dimer version of VWF fragment 764-1464 improves the bioavailability, the maximum

20   molecular weight of a desired VWF fragment may be equal to or larger than 158.8 KDa.

Co-formulation of N8-GP with hyaluronidase did not increase the FVIII bioavailability, indicating that the Hyaluron network in the extracellular matrix in the subcutis is not hindering the passage of FVIII into the bloodstream. Likewise, Hirudin dosed to a level that inhibits thrombin activity in vivo did not affect bioavailability of N8-GP. Thrombin

25   activation of FVIII does thus not appear to affect s.c. FVIII bioavailability.

The antibody 4F30 (further characterised in WO2012035050), which bind to C1 and inhibits cellular uptake of FVIII, did not improve the bioavailability of N8-GP. In this formulation, 2000 IU/ml N8-GP was co-formulated with 1 mg/ml of 4F30 which means that 99.6% of FVIII was bound to the mAb also after in vivo dilution assuming a K_d of 0.6 nM, an

30   in vivo dilution of 20x, a molecular weight for FVIII (turoctocog alfa/N8) of 170000 g/mol, a specific activity of 10000 IU/mg for turoctocog alfa/N8, and a molecular weight for 4F30 of 150000 g/mol. Also, the PEGylated FVIII with K2092A+F2093A mutations displayed decreased uptake in cells but the mutations did not improve the bioavailability compared to N8-GP. Inhibition of cellular FVIII uptake does thus not appear to be the mechanism by

35   which co-formulated VWF fragments result in increased s.c. bioavailability of FVIII.

Example 25:

Subcutaneous administration in New Zealand White Rabbits

Test compounds were formulated in 18 mg/ml NaCl, 3 mg/ml saccharose, 1.5 mg/ml L-histidine, 0.1 mg/ml polysorbate 80, 0.25 mg/ml $CaCl_2$, pH -7.3. For test formulations containing VWF or VWF fragments the % FVIII bound by VWF was calculated using the available IC50 values (table 6) assuming IC50=Ki=K_d.

Female New Zealand white rabbits weighing approximately 2-3 kg were used for the study. The animals were allowed free access to feed and water. The rabbits were dosed subcutaneously over the thigh with FVIII in combination with various proteins, 4-5 rabbits with each test compound. The dose volume was 0.2ml/kg or 1ml/kg.

Blood was sampled at 11 time points from 0 to 96 h with n= 4-5 rabbits/time point. At each sampling time point, 1ml blood was sampled from an ear artery by use of a 21G needle and EDTA coated tubes. The tubes were centrifuged within 10 minutes after blood drawing at 4000 G for 5 minutes and plasma separatedThe samples were immediately frozen on dry ice before storage at -80°C prior to analysis. The samples were analysed by FVIII antigen analysis using two FVIII light chain antibodies (4F45 and 4F11) in a FVIII LOCI assay (Luminescence oxygen channelling immunoassay).

Mean plasma concentration versus time data were analysed by non-compartmental analysis using WinNonlin Phoenix (Pharsight Corporation) estimating the given pharmacokinetic parameters. The bioavailability was estimated using pharmacokinetics of FVIII (turoctocog alfa/N8) and N8-GP administered i.v. to rabbits.

The obtained bioavailabilities are shown in table 8.

Table 8:

FVIII	FVIII Dose/dose volume	co formulation protein	Molar ratio co-formulation protein:FVIII	Saturation FVIII with co-formulated protein (%)	F%
FVIII (turoctocog alfa/N8) +VWF	2000/0.2ml/kg	TIL'/E'/D3/A1	3	99	6.2
N8-GP	700/0.2ml/kg	-	-	-	40
N8-GP+VWF	700/0.2ml/kg	TIL'/E'/D3/A1	3	99	59
N8-GP+VWF	500/ 1ml/kg	TIL'/E'/D3/A1	3	82	34

The s.c. bioavailability in rabbits of N8-GP and N8-GP co-formulated with VWF fragment TIL7E7D3/A1 dosed in a dosing volume of 0.2ml/kg was 40 and 59%, respectively. The bioavailability of N8-GP + VWF dosed in a dosing volume of 1 ml/kg was 34%. The

bioavailability of N8-GP may thus be influenced either by the species or by the differences in dosing volumes (5ml/kg in mice and 0.2ml/kg or 1ml/kg in rabbits). 0.2 ml/kg is closest to a dosing volume relevant for humans. FVIII (turoctocog alfa/N8) dosed together with VWF fragment TIL7E7D3/A1 displayed a similar bioavailability in rabbits compared to mice despite

5   the higher dosing concentration.

Example 26:

Construction of expression vectors encoding VWF fragments

        Plasmid #796 consisting of the pZEMHygro vector with insert consisting of wild-type

10   human VWF cDNA was utilized as the starting point for generating DNA constructs for the expression of truncated human VWF proteins.

        DNA encoding the VWF signal peptide, followed by the VWF TIL'E' domain, the VWF D3 domain, the VWF A1 domain, and a HPC4 tag was generated by polymerase chain reaction (PCR) using plasmid #796 as template, forward primer ol_LC089 VWF forward, and

15   reverse primer ol_LC092 VWF A1 HPC4 reverse. These primers contain a Nhe I and a Not I restriction site, respectively. The resulting PCR product was inserted into the pCR2.1-TOPO vector (Invitrogen). From here the VWF(TIL7E7D3/A1)-HPC4 coding DNA was excised with the Nhe I and a Not I restriction enzymes and inserted into pZEM219b digested with the same restriction enzymes. Thus, the pl_LC089 construct was established consisting of

20   pZEM219b with insert encoding VWF(TIL7E7D3/A1)-HPC4.

        Nucleotide substitutions leading to the amino acid replacements C1099/1142S in the VWF VWF(TIL7E7D3/A1)-HPC4 protein encoded by pLLC089 were introduced by site-directed mutagenesis of pLCC089 using the QuikChange XL Site-directed Mutagenesis kit (Stratagene) and the oLLC101-f, oLLC102-r, oLLC103-f, and oLLC104-r mutagenesis

25   primers. The site.directed mutagenesis gave rise to the pLLC095 vector consisting of pZEM219b with insert encoding VWF (TIL7E7D3/A1)C1099/1142S-HPC4.

        Table 9: Oligonucleotide primers used for generating VWF fragment coding DNA contructs

Primer name	Primer sequence (5'-3')
OLLC089 VWF forward	CCGCTAGCCCATGATTCCTGCCAGATTTGCCGGGGTGCTGCTTGCTCT GGCCCTCATTTTGCCAGGGACCCTTTGTAGCCTATCCTGTCGGCCCCCC ATG **(SEQ ID NO 38)**
OLLC092 VWF A1 HPC4 reverse	GATGCGGCCGCCTACTACTATTTGCCATCAATCAGACGCGGATCCACCT GATCTTCGGCTTCAGGGGCAAGGTCACAGAGGTAGC **(SEQ ID NO 39)**

oLLC101-f	CATTGGGGACTGCGCCTCCTTCTGCGACACCATTGCTGCC **(SEQ ID NO 40)**
oLLC102-r	GGCAGCAATGGTGTCGCAGAAGGAGGCGCAGTCCCCAATG **(SEQ ID NO 41)**
oLLC103-f	CGGGAGAACGGGTATGAGTCTGAGTGGCGCTATAACAGCTGTGC **(SEQ ID NO 42)**
oLLC104-r	GCACAGCTGTTATAGCGCCACTCAGACTCATACCCGTTCTCCCG **(SEQ ID NO 43)**

Example 27:

Stable cell lines expressing VWF fragments

Baby hamster kidney (BHK) cells grown in Dulbecco's modified Eagle's medium with 10 % fetal calf serum were transfected with pLL095 using Genejuice transfection reagent (Merck). A pool of transfected cells was generated by selection with 1.5 DM methotrexate giving rise to a non-clonal BHK cell line producing VWF (TIL7E7D3/A1)C1099/1 142S-HPC4. The cells were seeded in a biofermentor and the VWF (TIL7E7D3/A1)C1099/1 142S-HPC4 protein was purified from the cell culture supernatant as described in Example 22.

CHO-DUKX-B1 1 suspension cells grown in suspension were transfected with pLLC095 by electroporation. A pool of transfected cells was generated by adaptation to growth in medium without nucleosides. Subsequently, the pool was adapted to growth in the presence of 100 mM methotrexate giving rise to the VWF (TIL7E7D3/A1)C1099/1 142S-HPC4 producing non-clonal CHO-DUKX-B1 1 cell line MBML001. The cells were seeded in a biofermentor and the VWF (TIL7E7D3/A1)C1099/1 142S-HPC4 protein was purified from the cell culture supernatant as described in Example 22.

Example 28:

VWF fragments protects FVIII against cellular uptake

The effect of plasma-derived (pd) VWF and fragments of VWF on FVIII cellular uptake is evaluated in human monocyte-derived macrophages or dendritic cells, which both are antigen presenting cells, or U87 MG cells. U87 MG cells are obtained from ATCC (HTB-14). The cells are cultured in fibronectin-coated 24-well plates for 48 hours in EMEM supplemented with 10% heat inactivated FCS at 37°C in 5% CO_2 The cells are carefully washed with buffer A (10 mM HEPES, 150 mM NaCl, 4 KCl, 11 mM Glucose, pH 7.4) and incubated for 15 min with buffer B (buffer A supplemented with 5 mM $CaCl_2$ and 1 mg/ml

BSA). Radioactively labelled FVIII (^{125}I-FVIII, final concentration 1 nM) is incubated alone or premixed with different concentrations of pdVWF (American Diagnostica, final concentration 0.001 nM - 50 nM based on monomer content) or TIL7E7D3/A1 (final concentration 0.25 nM - 500 nM or 1000 nM) 10 min prior to addition to the U87 MG cells and incubated with the

5   cells 1 hour at 37 °C to allow binding and internalization. Cells are subsequently washed three times with ice-cold buffer B. Surface bound proteins are cleaved off by incubating the cells in PBS containing 100 μg/ml trypsin, 50 μg/ml proteinase K, 5 mM EDTA (pH 7.4) for 1 hour on ice. The detached cells are transferred to tubes and centrifuged to pellet the cells. The supernatant representing the cell bound FVIII is transferred to new tubes. The

10   radioactivity in tubes with the supernatants (bound FVIII) and cell pellets (internalized FVIII) are quantified in a gamma counter, and values calculated in FVIII concentration by using a standard curve based on ^{125}I-FVIII. Bound ^{125}I-FVIII in the absence of VWF are set to 100%.

Dendritic cells and macrophages are differentiated from monocytes isolated from buffy coats by magnetic separation using magnetic anti-CD14-beads (Miltenyi Biotec) and a

15   MACS column (Miltenyi Biotec) according to the manufactures instructions. Monocytes (0.5×10^6 cells/ml) are seeded in T-75 tissue culture flasks and cultured in IMDM media (GIBCO) containing 10% FBS, 1% penicillin/streptomycin and 3.3 ng/ml M-CSF (R&D Systems) in order to differentiate the cells into macrophages. Additional 3.3 ng/ml M-CSF is added after three days of culturing. The monocytes can alternatively be differentiated into

20   dendritic cells by stimulating with 40 ng/ml GM-CSF (R&D Systems) and 40 ng/ml IL-4 for five days. Dendritic cells are washed in buffer B and transferred to low binding Nunc tubes with 0.5×10^6 cells/tube. Fluorescently labelled FVIII, e.g. Oregon-Green FVIII (e.g. 30 and 100 nM) are added and incubated 1 hour at 37°C. Cells are washed once and analysed by flow cytometry using a LRS Fortessa instrument (BD). The macrophages are after six days

25   culturing washed with PBS and incubated 10-20 min at 4°C with 2.5 mM EDTA in PBS with 5% FCS to detach cells. Macrophages (7×10^5/well) are seeded on fibronectin-coated 96-well glass bottom tissue culture plates (Perkin Elmer ViewPlate Black). 24 hours post seeding the cells are washed once with buffer B before addition of 30 nM fluorescently-labelled FVIII (e.g. OregonGreen-FVIII) alone or in the presence of increasing concentrations (15-240 nM) of

30   pdVWF (American Diagnostica) or TIL7E7D3/A1. Macrophages are incubated for 1 hour at 37°C. Subsequently, cells are washed twice with buffer B to remove non-internalized material before addition of PBS containing 2.5 μg/ml Hoechst33342 (Molecular Probes) to visualize the cell nuclei. The plate is then immediately imaged on the Operetta® High Content Screening system (Perkin Elmer, Hamburg) in widefield fluorescence mode using the 20X

35   high NA objective. Ten fields per well are imaged and analysed. The approach to image

analysis in the Harmony® software is based on counting nuclei (Hoechst channel), followed by texture analysis (FVIII channel) using the "find particle'" method to detect vesicular FVIII. Dead or apoptotic cells are excluded from the analysis based on nuclei fragmentation and/or excessive binding of FVIII to the plasma membrane. In order to quantify the internalized FVIII the integrated fluorescent intensity of the vesicular FVIII signal is calculated and plotted against time.

IC50 values for inhibition of FVIII binding and internalization in U87 MG cells and macrophages are shown in table 10. Both pdVWF and TIL7E7D3/A1 are able to inhibit FVIII cell binding/uptake in both cell types providing sufficient high concentrations are used.

As uptake in antigen presenting cells is the initial step in presenting FVIII to the immune system, the data may indicate that a reduced immune response can be achieved upon co-formulation of FVIII with a VWF fragment.

Table 10. Effect of pdVWF and TIL7E7D3/A1 fragment on FVIII binding and internalization in U87 MG cells and uptake in macrophages.

Cell type	IC50 (nM)		Maximal inhibition (%)	
	pdVWF	TIL'/E'/D3/A1	pdVWF	TIL'/E'/D3/A1
U87 (n=3-4) Binding	1.2±0.9	17.6±13.0	34.3±4.2	39.8±7.8
U87 (n= 3-4) Internalization	1.3±1.2	22.1±19.2	32.2±7.0	41.2±11.5
Macrophages (n=3)	15.6±3.5	31.5±6.1	32.6±11.4	47.2±11.7

Example 29:

Efficacy of FVIII compounds co-formulated with VWF variants after subcutaneous dosing:

FVIII deficient, FVIII-KO mice, 12-16 weeks old, male and females are divided into 3 groups of 12 animals. In each group, eight animals are subjected to tail bleeding and 4 animals are used in parallel for ex vivo efficacy testing using ROTEM analysis.

GlycoPEGylated FVIII or vehicle is dosed s.c. 24hr prior to tail transection. As a positive control glycoPEGylated FVIII is dosed i.v. 5 min prior to injury. The s.c injection is performed in the neck and the i.v. injection in a lateral tail vein. The dose volume is 5 ml/kg.

GlycoPEGylated FVIII is prepared in buffer (10 mM L-Histidine, 8.8 mM Sucrose, 0.01 % Polysorbate 80, 308 mM NaCl, 1.7 mM $CaCl_2$ (dihydrate), 0.01 % Polysorbate 80 0.1 mg/ml, pH 6.9) to a concentration of 40 and 500 U/ml and stored at -80 °C until use.

Before tail transection, the mice are anaesthetised with isoflurane and placed on a heating pad. The tails are placed in pre-heated saline at 37 °C for 10 min. The tail is transected 4 mm from the tip.

Immediately before tail transection a 20 µl blood sample is drawn from the peri-

5   orbital plexus for FVIII determination.

Blood is collected over 30 min and the haemoglobin concentration determined by spectrophotometry at 550 nm.

Parallel animals are used for blood sampling and subsequent analysis of their clotting parameters (ex vivo efficacy). A blood sample is taken from the peri-orbital plexus

10   with 20 µL capillary tubes without additive. The blood sample is diluted 1:10 in 0.13M sodium citrate and carefully mixed and stored at rum temperature for immediate thromboelastography by ROTEM. The blood sample is re-calcified by adding 7 µL CaCl$_2$ to a mini curvet (StarTEM). Thereafter, 105 µL of blood is added to the mini curvet and mixed. The analysis is performed until the maximum amplitude is reached.

15

Results:

The prophylactic effect of s.c. administered FVIII is determined by comparing the blood loss during the 30min study period at 24hr after s.c. administration to that of 1) a vehicle control group and 2) an i.v. control group with glycoPEGylated FVIII. The blood loss

20   in the group dosed s.c. with glycoPEGylated FVIII is comparable to the blood loss in the group dosed i.v. (figure 10, left panel). The blood loss data are supported by the *ex vivo* efficacy parallel study of the examined clotting parameters, e.g. clot time (figure 10, right panel).

In conclusion, subcutaneously administered FVIII appear to be hemostatically active

25   based on the PK profile and the results from the *ex vivo* activity. Therefore, subcutaneously administered FVIII co-formulated with a VWF fragment is also believed to be hemostatically active as can be predicted from its pharmacokinetic profile.

Example 30:

30   *Effect of s.c. administered FVIII ± VWF fragments in FVIII-deficient mice.*

Test compounds : Test compounds are prepared in 10 mM L-Histidine (1.55 mg/ml), 8.8 mM Sucrose (3.0 mg/ml), 308 mM NaCl (18 mg/ml), 1.7 mM CaCl2 dihydrate (0.25 mg/ml), 0.01 % Polysorbate 80 (0.1 mg/ml), pH 7.3.

Animals: Experiments are performed using groups of *F8* knockout (FVIII k/o) mice

35   (129/C57BL/6 or C57BL/6, exon 16 disrupted). Animals are included in experiments when

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12-1 8 weeks old at which time they are weighing roughly 18 - 25 grams. Twelve to 15 animals are included per group.

Administration of test compounds: Test compounds are administered subcutaneously (or intravenously for controls) using a dose volume of maximally 10 ml/kg (or
5   5 ml/kg for controls).

Bleeding model: A tail vein transection (TVT) bleeding model is conducted with the mice under full isoflurane anaesthesia. Briefly, following anaesthesia the bleeding challenge comprises a template-guided transection of a lateral tail vein at a tail diameter of 2.7 mm. The tail is immersed in saline at 37 °C allowing visual recording of the bleeding for 60 min,
10   where after the blood is isolated and the blood loss determined by measuring the haemoglobin concentration as described in "Example 3". When feasible and justified, blood is sampled for assessment of FVIII activity (FVIIhC) in plasma as described above.

Dose response: Different doses of FVIII or FVIII co-formulated with VWF fragments (e.g. N8-GPA/WF) are injected subcutaneously at defined time point(s) prior to TVT. Vehicle
15   and intravenous control/treatment groups are included for no effect and maximal effect, respectively.

Duration of action: FVIII or FVIIIA/WF is injected s.c. to identify prolonged effect, i.e. improved bleeding phenotype after treatment. TVT is performed at several time points, e.g. 24, 48, 72, 96, after dosing.

20   Repeated dose: FVIII or FVIIIA/WF fragment is dosed s.c. once daily for several days. TVT is performed at different time points to assess any improvement in the bleeding phenotype.

Data processing and analyses: Data are physically recorded throughout the experiment. Hereafter, data are aggregated for analysis using MS Excel (Microsoft, WA,
25   USA) before being analysed in GraphPad Prism version 5 (GraphPad Software, Inc, CA, USA).

Example 31:

Effect of s.c. FVIII ± VWF fragments in other FVIII-deficient species.

Additional pharmacodynamic experiments are conducted in other species to verify effect after subcutaneous administration in non-murine animal models of haemophilia A, e.g. rat and dog. FVIII or FVIIIA/WF are injected subcutaneously before assessing ex vivo effect, before inducing a bleeding challenge, or as a means to treat or prevent spontaneous bleeds.

Test compounds : Test compounds are prepared in 10 mM L-Histidine (1.55 mg/ml), 8.8 mM Sucrose (3.0 mg/ml), 308 mM NaCl (18 mg/ml), 1.7 mM CaCl2 dihydrate (0.25 mg/ml), 0.01% Polysorbate 80 (0.1 mg/ml), pH 7.3.

Animals: Experiments are performed in adolescent rats (-12 weeks old) or dogs (6+ months old) with haemophilia A.

Administration of test compounds: Test compounds are administered subcutaneously (or intravenously for controls) using a dose volume of maximally 10 ml/kg (or 5 ml/kg for controls).

Dog effect model: In dogs with haemophilia A the effect is assessed ex vivo using surrogate markers, e.g. thrombelastography as previously described (Knudsen et al, 201 1; Haemophilia, 17, 962-970), or in vivo, e.g. using a standardized bleeding challenge monitored by acoustic force radiation force impulse (ARFI) ultrasound as described (Scola et al, 201 1; Ultrasound in Med. & Biol., 37(12), 2126-2132). Capacity allowing, test compound are administered to treat spontaneously bleeding dogs. Effect is monitored by assessing the resolution of clinical manifestation in comparison with historic data on i.v. treatment.

Rat effect model: In rats with haemophilia A the effect is assessed ex vivo using surrogate markers, e.g. thrombelastography as described above for mice and dogs, or in vivo, e.g. using a standardized bleeding challenge as described for mice. Capacity allowing, test compound are administered to treat spontaneously bleeding rats. Effect is monitored by assessing the resolution of clinical manifestation in comparison with historic data on i.v. treatment.

Additional pharmacodynamic experiments are conducted in other species to verify effect after subcutaneous administration in non-murine animal models of haemophilia A, e.g. rat and dog.

Example 32:

Construction of expression vectors encoding VWF fragments

A nucleotide substitution leading to the amino acid replacement S1142C in the VWF(764-1 250)-C1 099/1 142S-ALA-HPC4 protein encoded by pJSV348 described in

Example 17 was introduced by PCR-based site-directed mutagenesis using the VWF 1099C S and VWF 1099C AS primers (Table P). This gave rise to the pGB237 vector consisting of pTT5 with insert encoding VWF(764-1250)-C1099S-ALA-HPC4 (SEQ ID NO 11). The cysteine at position 1142 allows dimerization of the protein as described in Example 20.

Likewise, a nucleotide substitution leading to the amino acid replacement S1099C in the VWF(764-1 250)-C1 099/1 142S-ALA-HPC4 protein encoded by pJSV348 described in Example 17 was introduced by PCR-based site-directed mutagenesis using the VWF 1142C S and VWF 1142C AS primers (Table P). This gave rise to the pGB238 vector consisting of pTT5 with insert encoding VWF(764-1250)-C1 142S-ALA-HPC4 (SEQ ID NO 11). The cysteine at position 1099 allows dimerization of the protein as described in Example 20.

In a similar manner, the S1099C amino acid replacement was introduced in the VWF(764-1 128)-C1099S-HPC4 protein encoded by pJSV406 described in Example 18, giving rise to the pGB249 vector consisting of pTT5 with insert encoding VWF(764-1 128)-HPC4 (SEQ ID NO 9). The cysteine at position 1099 allows dimerization of the protein as described in Example 20.

cDNA encoding amino acid 1-1250 of human VWF was amplified by PCR using plasmid #796 (described in Example 26) as template, forward primer JP1 000 VWF-HindIII S (Table 2), and reverse primer JP1 006 VWF764-1250 (Table 2). Primer JP1 006 VWF764-1250 contains a Nhe I site. The resulting PCR product was inserted into the pCR4BLUNT-TOPO vector (Invitrogen) downstream of Pme I restriction site. From here, the vWF(1-1250) coding DNA was excised with the Pme I and a Nhe I restriction enzymes and inserted into pJSV164 described in Example 17 generating the pGB242 vector consisting of pTT5 with insert encoding vWF(1-1250)-ALA-HPC4. The cysteines at position 1099 and 1142 allow dimerization of the protein as described in Example 20, and proteolytic removal of the presequence will generate vWF(764-1250)-ALA-HPC4 (SEQ ID NO 11).

DNA sequences of pJSV348 (described in Example 17) and construct #796 (described in Example 26) were inverse amplified by PCR using overlapping primers. The pJSV348 sequence was amplified using primer 2764pJSV348 and 1202pJSV348R (Table P), while the construct #796 sequence was amplified using primer 221#796F and 3537#796R (Table P). The amplification products from pJSV348 (recipient) and construct #796 (donor) were excised from an agarose gel and joined by ligation independent cloning (LIC) using the In-Fusion HD Cloning Kit (Clontech) to generate circular DNA and subsequently transformed into Stellar competent cells (Clontech). The resulting expression vector, named pGB252 consists of PTT5 with insert encoding VWF(1-1 128)-ALA-HPC4. The cystein at position 1099 allows dimerization of the protein as described in Example 20, and

proteolytic removal of the presequence will generate vWF(764-1 128)-ALA-HPC4 (SEQ ID NO 9).

Likewise, amplification using pJSV348 (described in Example 17) as template with the primers 2764pJSV348 and 1202pJSV348R (Table P) and amplification using #796
5   (described in Example 26) as template with the primers 221#796F and 3747#796R (Table P) generated pJSV348 (recipient) and construct #796 (donor) amplification products that were also excised from an agarose gel and joined by ligation independent cloning (LIC) using the In-Fusion HD Cloning Kit (Clontech) to generate circular DNA and subsequently transformed into Stellar competent cells (Clontech). The resulting expression vector, named pGB253
10 consists of PTT5 with insert encoding VWF(1 - 1 198)-ALA-HPC4. The cysteines at position 1099 and 1142 allow dimerization of the protein as described in Example 20, and proteolytic removal of the presequence will generate vWF(764-1 198)-ALA-HPC4 (SEQ ID NO 10).

In a similar manner, DNA sequences of pJSV348 (described in Example 17) and construct #796 (described in Example 26) were inverse amplified by PCR using overlapping
15 primers. The pJSV348 sequence was amplified using primer 2764pJSV348 and 2420pJSV348R (Table 11), while the construct #796 sequence was amplified using primer 3666#796F and 5203#796R (Table P). The amplification products from pJSV348 (recipient) and construct #796 (donor) were excised from an agarose gel and joined by ligation independent cloning (LIC) using the In-Fusion HD Cloning Kit (Clontech) to generate circular
20 DNA and subsequently transformed into Stellar competent cells (Clontech). The resulting expression vector, named pGB250 consists of PTT5 with insert encoding VWF(764-1873)-C 1099/1 142C-ALA-HPC4 (SEQ ID NO 20).

Human VWF cDNA sequences amplified from construct #796 (described in Example 26) were combined generating the pLLC122 vector consisting of pZEM219b with insert
25 encoding vWF (1-1464)-HPC4. The cysteines at position 1099 and 1142 allow dimerization of the protein as described in Example 20, and proteolytic removal of the presequence will generate vWF(764-1464)-HPC4 (SEQ ID NO 19).

Table 11: Oligonucleotide primers used for generating VWF fragment coding DNA
30 constructs

Primer name	Primer sequence (5'-3')
VWF 1099C S	GGGGACTGCGCCTGCTTCTGCGACACC **(SEQ ID NO 44)**
VWF 1099C AS	GGTGTCGCAGAAGCAGGCGCAGTCCCC **(SEQ ID NO 45)**
VWF 1142C S	GAACGGGTATGAGTGTGAGTGGCGCTATA **(SEQ ID NO 46)**
VWF 1142C AS	TATAGCGCCACTCACACTCATACCCGTTC **(SEQ ID NO 47)**

2764pJSV348F	GCGCTAGCTGAGGACCAAGTAGATCCGCGGCTCATTGATGGG **(SEQ ID NO 48)**
1202pJSV348R	GGGCCAGAGCAAGCAGCACCCCGGCAAATCTGGCAGG **(SEQ ID NO 49)**
221 #796 F	CCTGCCAGATTTGCCGGGGTGCTGCTTGCTCTGGCCC **(SEQ ID NO 50)**
3537#796R	TACTTGGTCCTCAGCTAGCGCCTGGGGGCACAATGTGGCCGTCCTCC **(SEQ ID NO 51)**
3747#796R	TACTTGGTCCTCAGCTAGCGCCACTGGACAGTCTTCAGGGTCAACGC **(SEQ ID NO 52)**
2420pJSV348R	GGCTCAGGGTGCTGACACGTGACTTGACAGGCAGGTGC **(SEQ ID NO 53)**
3666#796F	GCACCTGCCTGTCAAGTCACGTGTCAGCACCCTGAGCC **(SEQ ID NO 54)**
5203#796R	TACTTGGTCCTCAGCTAGCGCTGCAGGGGAGAGGGTGGGGATCTGC **(SEQ ID NO 55)**

Example 33.

VWF fragments inhibit FVIII uptake by human dendritic cells.

Human monocyte-derived dendritic cells were prepared as described in example 28.
Expression of the dendritic cell markers CD209 and CD86 were controlled by flow cytometry
using a LRS Fortessa instrument (BD). Fluorescent labelled FVIII (Oregon green- FVIII, 30
nM final concentration) was premixed with different concentrations of plasma-derived VWF or
VWF fragments before incubating 1 h at 37°C with dendritic cells. Live/Dead cell kit
(Invitrogen # L101 19, APC-Cy7) was used for gating on live dendritic cells, and FVIII uptake
within this cell population was quantified. Data was normalized for each individual
experiment. The signal in samples without VWF was defined as 100% FVIII uptake, and the
signal in the sample with the highest concentration of plasma-derived VWF (240 nM based
on monomer content) was defined as 0%. Values from 3-5 experiments were combined and
IC50 values calculated using non- linear regression in Prism software (log(inhibitor) vs.
response - Variable slope (four parameters)). The resulting IC50 values are shown in table
12. The data show that all tested VWF fragments were able to inhibit FVIII uptake by the
dendritic cells provided sufficiently high concentrations are used. As FVIII uptake by antigen-
presenting cells is the initial step in presenting FVIII to the immune system the data suggests
that co-formulation of FVIII with sufficiently high concentration of VWF fragment may have a
potential in reducing immunogenicity of FVIII.

70

Table 12. Effect of plasma derived VWF and VWF fragments on FVIM uptake in dendritic cells.

Domain/comment	VWF fragment sequence	IC50 (nM)*
TIL'/E'/VWD3	VWF(764-1041)-ALA-HPC4 monomer	570 (400-820)
TIL'/E'/D3	VWF(764-1250)-C1099/1142S-ALA-HPC4 monomer	31 (25-39)
TIL'E'/D3/A1 monomer	VWF(764-1464)-C1099/1142S-HPC4 monomer	31 (18-52)
TIL'E'/D3/A1 dimer	VWF(764-1464)-HPC4 dimer**	16 (11-22)
Plasma-derived VWF	VWF (764-2813)	9.8 (7.6-13)

*) Best fit value and 95% confidence intervals of data from 3-5 experiments

**) IC50 value based on molar concentration of the dimer, i.e. multiply IC50 with 2 to
5   reflect IC50 value based on content of VWF monomer fragment.

Example 34:

Effect of s.c. FVIII ± VWF fragments in animals with inhibiting antibodies against FVIII.

10   The objective is to evaluate the potential of pharmaceutical compositions to treat
haemophilia A patients with inhibitors against FVIII. We dose FVIII alone or co-formulated
with VWF-fragments subcutaneously to naive FVIII-KO mice or FVIII-KO mice where
inhibitors are induced by repeated subcutaneous or intravenous administrations of FVIII prior
to treatment with the compositions, or by injecting a polyclonal or monoclonal anti-FVIII
15   antibody. The effect of the treatments is evaluated in anaesthetized mice after transection of
a lateral tail vein. The tail is placed in pre-warmed saline at 37 °C and the bleeding is
observed for 60 minutes. The blood loss during the experiment is a measure of the effect of
the composition.

20   **Example 35:**

Administration of VWF fragments to VWF knockout mice:

Test compound:

Murine VWF fragment TIL7E7D3/A1 1.829 nmol/ml, 0.01 5mg/ml

The test compound was formulated in 20mM imidazol 150mM NaCl, 0.02% Tween
25   80, 1.1M Glycerol, 10 mM CaCl2, pH 7.3

6 VWF knockout mice, with an approximate weight of 25 g were dosed intravenously
in the tail with 9.48 nmol/kg Murine VWF fragment TIL7E7D3/A1 .

Blood was sampled pre-dose and at 0.08, 0.33, 0.5, 1, 2, 4, 7, 18 and 24 h post administration in a sparse sample design with 2 mice sampled per time point. The mice were anaesthetized by Isoflurane/02/N20   prior to blood sampling via the retroorbital plexus. Three samples were taken from each mouse. Blood (45 μl) was stabilised with 5 μl of sodium-

5   citrate (0.13 M) and added 200 μl FVIII coatest SP buffer (50mM TRIS-HCl, 1% BSA, Ciprofloxacin 10 mg/L, pH 7.3). After centrifugation at 4000 g for 5 minutes at room temperature, the supernatants were immediately frozen on dry ice before storage at -80°C prior to analysis.

Samples were analysed with regards to FVIII concentration in an antigen LOCI

10   assay (Luminescence oxygen channelling immunoassay).

Mean plasma concentration versus time data were analysed relatively to the predose values.

The relative mean FVIII concentration in time after dosing is shown in table 13

15   *Table 13.* *Effect of Murine D'D3A1 IV on FVIII blood concentration in VWF KO mice.*

Time (h)	FVIII increase (% of predose)
0.08	174
0.33	190
0.5	176
1	163
2	274
4	250
7	330
18	225
24	207

FVIII concentration increased gradually in time after dosing of VWF fragment intravenously with a Tmax after 7 hours. This finding supports the potential for VWF fragments for the treatment of VWF disease as well as haemophilic disorders.

20

Example 36:

Interaction mapping by HX-MS of vWF fragments TIL7E7D3/A1, TIL7E7D3, TILE, and TIL7E7VWD3 on Turoctocog alfa (FVIII) and Turoctocog alfa (FVIII) on vWF fragment TIL7E7D3/A1

5   *Introduction to HX-MS*

The HX-MS technology exploits that hydrogen exchange (HX) of a protein can readily be followed by mass spectrometry (MS). By replacing the aqueous solvent containing hydrogen with aqueous solvent containing deuterium, incorporation of a deuterium atom at a given site in a protein will give rise to an increase in mass of 1 Da. This mass increase can

10   be monitored as a function of time by mass spectrometry in quenched samples of the exchange reaction. The deuterium labelling information can be sub-localized to regions in the protein by pepsin digestion under quench conditions and following the mass increase of the resulting peptides.

One use of HX-MS is to probe for sites involved in molecular interactions by

15   identifying regions of reduced hydrogen exchange upon protein-protein complex formation. Usually, binding interfaces will be revealed by marked reductions in hydrogen exchange due to steric exclusion of solvent. Protein-protein complex formation may be detected by HX-MS simply by measuring the total amount of deuterium incorporated in either protein members in the presence and absence of the respective binding partner as a function of time. The HX-

20   MS technique uses the native components, i.e., protein and antibody or Fab fragment, and is performed in solution. Thus HX-MS provides the possibility for mimicking the *in vivo* conditions (for a recent review on the HX-MS technology, see Wales and Engen, Mass Spectrom. Rev. 25, 158 (2006)).

25   *Materials*
          Protein batches used were:
          FVIII protein batches used were:
          FVIII (N8, Turoctocog alfa, SEQ ID NO 2) Batch 0155-0000-0004-37A

30   *vWF fragments*
          D'D3A1 (SEQ ID NO 19; Cys1099Ser; Cys1 142Ser) Batch 0129-0000-01 70-6B; 2304 (SEQ ID NO 5) Batch 0 129-0000-2304-1 B; 2307 (SEQ ID NO 8) Batch 0129-0000-2307-1 B; 2308 (SEQ ID NO 11) Batch 0129-0000-2308 2B.

All proteins were buffer exchanged into 20 mM Imidazole, 500 mM NaCl, 10 mM CaCl2, adjusted to pH 7.3 before experiments.

Methods: HX-MS experiments

5   *Instrumentation and data recording*

The HX experiments were performed on a nanoACQUITY UPLC System with HDX Technology (Waters Inc.) coupled to a Synapt G2 mass spectrometer (Waters Inc.). The Waters HDX system contained a Leap robot (H/D-x PAL; Waters Inc.) operated by the LeapShell software (Leap Technologies Inc/Waters Inc.), which performed initiation of the

10   deuterium exchange reaction, reaction time control, quench reaction, injection onto the UPLC system and digestion time control. The Leap robot was equipped with two temperature controlled stacks maintained at 20 °C for buffer storage and HX reactions and maintained at 2 °C for storage of protein and quench solution, respectively. The Waters HDX system furthermore contained a temperature controlled chamber holding the pre- and analytical

15   columns, and the LC tubing and switching valves at 1 °C. A separately temperature controlled chamber holds the pepsin column at 25 °C. For the inline pepsin digestion, 100 µL quenched sample containing 100 pmol hIL-21 was loaded and passed over a Poroszyme® Immobilized Pepsin Cartridge (2.1 × 30 mm (Applied Biosystems)) placed at 25°C using a isocratic flow rate of 100 µL/min (0.1 % formic acid:CH_3CN 95:5). The resulting peptides were

20   trapped and desalted on a VanGuard pre-column BEH C18 1.7 µm (2.1 × 5 mm (Waters Inc.)). Subsequently, the valves were switched to place the pre-column in-line with the analytical column, UPLC-BEH C18 1.7 µm (1 × 100 mm (Waters Inc.)), and the peptides separated using a 8 min gradient of 8-45% B delivered at 120 µl/min from the nanoAQUITY UPLC system (Waters Inc.). The mobile phases consisted of A: 0.1% formic acid and B:

25   0.1 % formic acid in CH_3CN. The ESI MS data and the separate elevated energy (MS[E]) experiments were acquired in positive ion mode using a Synapt G2 mass spectrometer (Waters Inc.). Leucine-enkephalin was used as the lock mass ([M+H][+] ion at *m/z* 556.2771) and data was collected in continuum mode (For further description, see Andersen and Faber, Int. J. Mass Spec, 302, 139-148(201 1)).

30

Data analysis

Peptic peptides were identified in separate experiments using standard MS[E] methods where the peptides and fragments are further aligned utilizing the ion mobility properties of the Synapt G2 (Waters Inc.). MS[E] data were processed using ProteinLynx

35   Global Server version version 2.5 (Waters Inc.). The HX-MS raw data files were processed in

the DynamX software (Waters Inc.). DynamX automatically performs the lock mass-correction and deuterium incorporation determination, i.e., centroid determination of deuterated peptides. Furthermore, all peptides were inspected manually to ensure correct peak and deuteration assignment by the software.

5

Epitope mapping experiment

Amide hydrogen/deuterium exchange (HX) was initiated by a 10-fold dilution of FVIII in the presence or absence of vWF fragment, i.e., D'D3A1, 2308, 2307, or -2304 at time 0 into 20 mM Imidazole, 150 mM NaCl, 10 mM CaCl2, pH 7.3 (uncorrected value) at later time

10   points into the corresponding deuterated buffer *(i.e.* 20 mM Imidazole, 150 mM NaCl, 10 mM CaCl2 prepared in D_2O , 98% D_2O final, pH 7.3 (uncorrected value)). All HX reactions were carried out at 20°C and contained 3 μM FVIII in the absence or presence of 4.5 μM vWF fragment thus giving a 1.5 fold molar excess of vWF fragment binding partner. At appropriate time intervals ranging from 10 sec to 240 sec, 50 μl aliquots of the HX reaction were

15   quenched by 50 μl ice-cold quenching buffer (1.36 M TCEP, 2 M urea) resulting in a final pH of 2.5 (uncorrected value).

Results and Discussion

20   *Interaction mapping of 2304 and 2307 on FVIII*

The HX time-course of 191 peptides, covering 83% of the primary sequence of FVIII were monitored in the absence or presence of the vWF fragments 2304 or 2307 for i.e., 10, 20, 30, 40, 60, 120, and 240 sec.

25   The vWF fragments 2304 and 2307 both induce identical alterations in the exchange profile of FVIII and will be described together here. The observed exchange pattern in the time points (i.e., 10, 20, 30, 40, 60, 120, and 240 sec) in the presence or absence of 2304/2307 can be divided into different groups: One group of peptides display an exchange pattern that is unaffected by the binding of 2304/2307. In contrast, another group of peptides

30   in FVIII show protection from exchange upon 2304/2307 binding.

The regions displaying protection upon 2304/2307 binding encompass peptides covering residues 1855-1875, 1857-1875, 2062-2070, 2125-2147, 2125-2148, 2127-2147, 2275-2291 , 2275-2302, 2275-2305, 2292-2305, and 2293-2312 (Table 14). However, by comparing the relative amounts of exchange protection within each peptide upon binding

35   2304/2307 and the lack of epitope effects in overlapping and adjacent peptides in these

regions, the regions that display reduced deuterium incorporation can be narrowed to residues 1862-1875, 2062-2070, 2125-2147, and 2285-2299.

Interaction mapping of D'D3A1 and 2308 on FVIII

5   The HX time-course of 185 peptides, covering 79% of the primary sequence of FVIII were monitored in the absence or presence of the vWF fragments D'D3A1 or 2308 for 10, 20, 30, 40, 60, 120, and 240 sec.

The vWF fragments D'D3A1 and 2308 both induce identical alterations in the exchange profile of FVIII and will be described together here.

10   The regions displaying protection upon D'D3A1 or 2308 binding encompass peptides covering residues 1669-1680, 1738-1765, 1743-1765, 1856-1869, 1870-1874, 2061-2074, 2063-2074, 2123-2146, and 2260-2280 (Table 15).

However, by comparing the relative amounts of exchange protection within each peptide upon binding of D'D3A1 or 2308 and the lack of epitope effects in overlapping and
15   adjacent peptides in these regions, the regions that display reduced deuterium incorporation can be narrowed to residues 1671-1680, 1745-1754, 1858-1874, 2063-2074, 2125-2146, 2262-2280.

Interaction mapping of FVIII on DV3A1

20   The HX time-course of 82 peptides, covering 58% of the primary sequence of vWF fragment D'D3A1 were monitored in the absence or presence of FVIII for 10, 20, 40, 60, 120, and 240 sec.

The region displaying exchange protection upon FVIII binding encompass the peptide covering residues 768-778 (Table 16).
25   However, by comparing the relative amounts of exchange protection within each peptide upon binding FVIII and the lack of epitope effects in overlapping and adjacent peptides in these regions, the regions that display reduced deuterium incorporation can be narrowed to residues 770-778.

30   ### Conclusion

Upon binding of either 2304 or 2307 all regions of FVIII showed similar responses. The same group of peptides were affected by vWF fragment binding in the early time-points.

Furthermore, these affected regions identified for 2304/2307 binding were found to show overlap with affected regions upon binding to DO3A1/2308 within domain A3 and C1 of
35   FVIII.

Due to lacking sequence coverage of the peptic peptide map conducted to the HX-MS time course of 2304/2307 binding it was not possible to exchange characteristics for residues 1671-1680. Thus it was not possible to verify if 2304/2307 binding induces

5   exchange protection to this region as it was identified upon DO3A1/2308 binding.

Upon binding of FVIII the regions covering residues 770-778 of D'D3A1 showed exchange protection. The obtained sequence coverage of 58% of D'D3A1 afforded by the peptic peptides conducted to HXMS analysis of FVIII binding, does not allow to leave out that

10   more interaction site are present within DO3A1/2308.

Conclusion

The identified regions of FVIII showing protection upon binding to vWF fragments D'D3A1, 2308, 2304, or 2307 are structurally situated at remote distances when mapping on

15   to the crystal structure PDB: 2R7E. This makes it highly unlikely that they can all be assigned to protection induced by binding interface between FVIII and the vWF fragments DO3A1, 2308, 2304, or 2307. The HX-MS analysis is unable to distinguish between exchange protection induced by binding interface with exchange protections induced by rapid conformational changes.

20   Thus it is plausible that the observed regions showing exchange protection upon binding to vWF fragments D'D3A1, 2308, 2304, or 2307 are induced by both binding interface and conformational changes of FVIII.

The HXMS study of FVIII binding to vWF fragments D'D3A1, 2308, 2304, or 2307 revealed overlapping regions within domains A3 and C1, and therefore the complex binding

25   to this part of FVIII is identical for the vWF fragments investigated.

The observed discrepancy in domain C2 hints that this part of FVIII undergoes conformational changes upon complex formation with the vWF-fragments. Furthermore, the obtained results hint that the truncation differences between DO3A1/2308 and 2304/2307 induces different conformational changes of domain C2. In contrast the truncation difference

30   between 2304 and 2307 does not seem to affect the conformational orientation of C2, since identical exchange profiles of domain C2 were observed for binding to these vWF-fragment species.

It is well known that the domains C1 and C2 are essential for the membrane binding affinity of FVIII. It can be speculated that conformational changes of these part of FVIII will

35   reduce the membrane binding ability of FVIII. The conformational position of domains C1 and

C2 of FVIII complex bound to the vWF fragments might be unfavourable for membrane binding affinity of FVIII. Furthermore, it is highly likely that the fragments in complex with FVIII will shield for the membrane binding affinity of FVIII as it has been established for the membrane binding characteristics of FVIII complex bound to endogenous vWF. A reduced membrane binding affinity of FVIII complex bound to the vWF fragments in comparison to free FVIII would lead to a reduced binding of FVIII to cell membranes of the immune system, e.g. antigen presenting cells. This could decrease presentation of FVIII-derived peptides on MHC class II and it can therefore be speculated that FVIII complex bound to vWF fragments will be less immunogenic than free FVIII.

Table 14: HXMS analysis of FVIII (Turoctocog alfa; seq. no. using wt FVIII) (SEQ ID 2) binding to the vWF fragments 2304 (SEQ ID 5) or 2307 (SEQ ID 8). After deuterium exchange reaction. FVIII is digested with pepsin yielding the present peptic peptides identified to show exchange protection in the presence of 2304 or 2307.

Sequence	Domain	2304	2307
L1855-E1875	A3	EX	EX
V1857-E1875	A3	EX	EX
W2062-W2070	A3	EX	EX
V2125-R2147	C1	EX	EX
V2125-Y2148	C1	EX	EX
F2127-R2147	C1	EX	EX
F2275-T2291	C2	EX	EX
F2275-L2302	C2	EX	EX
F2275-Y2305	C2	EX	EX
P2292-Y2305	C2	EX	EX
V2293-S2312	C2	EX	EX

EX: exchange protection of FVIII residues upon 2304 or 2307 binding indicating interaction region (40 sec incubation in D20, > 0.4 Da).

Table 15: HXMS analysis of FVIII (Turoctocog alfa; seq. no. using wt FVIII) (SEQ ID 2) binding to the vWF fragments DO3A1 (SEQ ID 19; Cys1099Ser; Cys1 142Ser) or 2308 (SEQ ID 11; Cys1099Ser; Cys1 142Ser). After deuterium exchange reaction. FVIII is digested with pepsin yielding the present peptic peptides identified to show exchange protection in the presence of D'D3A1 or 2308.

Sequence	Domain	D'D3A1	2308
S1669-Y1680	a3	EX	EX
F1738-E1765	A3	EX	EX
F1743-E1765	A3	EX	EX
L1856-R1869	A3	EX	EX
Q1870-Q1874	A3	EX	EX
A2061-D2074	C1	EX	EX
S2063-D2074	C1	EX	EX
L2123-A2146	C1	EX	EX
F2260-V2280	C2	EX	EX

EX: exchange protection of FVIII residues upon D'D3A1 or 2308 binding indicating interaction region (40 sec incubation in D20, > 0.4 Da).

Table 16: HXMS analysis of vWF fragment DO3A1 (SEQ ID 19; Cys1099Ser; Cys1 142Ser) binding to the FVIII (Turoctocog alfa (SEQ ID 2). After deuterium exchange reaction. D'D3A1 is digested with pepsin yielding the present peptic peptide identified to show exchange protection in the presence of FVIII.

Sequence	Domain	FVIII
R768-A778	D'	EX

EX: exchange protection of D'D3A1 residues upon FVIII binding indicating interaction region (40 sec incubation in D20, > 0.4 Da).

Example 37

Complex formation of FVIII (SEQ ID 2) with TIL7E7D3/A 1 1II (SEQ ID 19; Cys1099Ser; Cys1142Ser) and of FVIII (SEQ ID 2) with TIL7E7D3 II (SEQ ID 14; Cys1099Ser; Cys1142Ser) analysed by SEC-UV

79

Materials

Protein batches used were:

FVIII protein batches used were:

FVIII (N8, Turoctocog alfa, SEQ ID NO 2) Batch 0155-0000-0004-37A; TIL7E7D3/A1
5   III (SEQ ID NO 19; Cys1099Ser; Cys1 142Ser) Batch 0129-0000-01 70-6B; TIL7E7D3 II (SEQ
ID 14; Cys1099Ser; Cys1 142Ser) Batch 0129-0000-2309-1 B.

Methods

Size-exclusion chromatography was performed on a Waters Biosuite , 4.6 X 300mm
10   column using a flow rate of 0.3 ml/min and a running buffer of 155 mM NaCl, 10 mM
Calciumacetat, 10 % Isopropanol at 25 °C. The absorbance of the effluent was monitored by
a UV detector at 280 nm. SEC-UV characterization were performed of FVIII, TIL7E7D3/A1 III,
TIL7E7D3 II, and 1:2 complexes of FVIII - TIL7E7D3/A1 III and of FVIII - TIL7E7D3 II.
Samples of FVIII 10 μM, TIL7E7D3/A1 III 20 μM, TIL7E7D3 II 20 μM, and in complex were
15   prepared and 15 μL were loaded on to the column.

Results and Conclusion

SEC-UV of the mixtures of FVIII - TIL7E7D3/A1 III and FVIII - TIL7E7D3 II showed
20   significant fractions of the complex to elute intact from the column. The complex would be
expected to elute a little earlier than FVIII; this was also observed in both cases.

CLAIMS

1. A VWF fragment comprising up to 1200 amino acids, wherein said VWF fragment comprises the TIL' domain.

2. A VWF fragment according to claim 1, wherein said fragment comprises the TIL' and the E' domains.

3. A VWF fragment according to any one of the preceding claims, wherein said VWF fragment comprises one or two amino acid substitution(-s) of the 1099 and/or 1142 cysteine(s-).

4. A VWF fragment according to any one of the preceding claims, wherein less than 5% of said VWF fragment are in the form of oligomers and/or multimers.

5. A VWF fragment according to any one of the preceding claims, wherein said VWF fragment is part of a dimer.

6. A VWF fragment according to any of the preceding claims, wherein said VWF fragment is a monomer.

7. A VWF fragment according to any one of the preceding claims, wherein said fragment comprises an amino acid sequence selected from the list consisting of: SEQ ID NO 4, SEQ ID NO 5, SEQ ID NO 6, SEQ ID NO 7, SEQ ID NO 8, SEQ ID NO 9, SEQ ID NO 10, SEQ ID NO 11, SEQ ID NO 12, SEQ ID NO 13, SEQ ID NO 14, SEQ ID NO 15, SEQ ID NO 16, SEQ ID NO 17, SEQ ID NO 18, SEQ ID NO 19, SEQ ID NO 20 and SEQ ID NO 21.

8. A VWF fragment according to any one of the preceding claims, wherein said fragment comprises SEQ ID NO 9, wherein the 1099 cysteine residue is substituted with another amino acid.

9. A VWF fragment according to claim 8, wherein the 1099 cysteine residue is substituted with serine.

10. A VWF fragment according to any one of claims 1-7, wherein said fragment comprises an amino acid sequence selected from the list consisting of: SEQ ID NO 10, SEQ ID NO 11, SEQ ID NO 12, SEQ ID NO 13, SEQ ID NO 14, SEQ ID NO 15, SEQ ID NO 16, SEQ ID NO 17, SEQ ID NO 18, SEQ ID NO 19, SEQ ID NO 20 and SEQ ID NO 2 1, wherein the 1099 and the 1142 cysteine residues are substituted with another amino acid.

11. A VWF fragment according to claim 10, wherein the 1099 and the 1142 cysteine residues are substituted with serine.

12. A pharmaceutical composition comprising: (i) a VWF fragment according to any one of claims 1-1 1; and (ii) a FVIII molecule.

13. A pharmaceutical composition according to claim 12, wherein said FVIII molecule comprises a truncated B domain at a size of 5-700 amino acids.

14. A pharmaceutical composition according to any one of claims 12-13, wherein FVIII is a B domain truncated variant, wherein the amino acid sequence of said truncated B domain is derived from the wt FVIII B domain amino acid sequence as set forth in SEQ ID NO 1.

15. A pharmaceutical composition according to claim 14, wherein said B domain comprises an O-glycan linked to the Ser 750 amino acid residue as set forth in SEQ ID NO 1.

16. A pharmaceutical composition according to any one of claims 12-15, wherein said FVIII molecule is conjugated with at least one half-life extending moiety.

17. A pharmaceutical composition according to any one of claims 12-16, wherein at least one half life extending moiety is covalently attached to an O-glycan present in the FVIII B domain.

18. A pharmaceutical composition according to any one of claims 12-17, wherein the bioavailability of said FVIII molecule is at least 5%.following subcutaneous administration.

19. A pharmaceutical composition according to any one of claims 12-18, wherein the molar ratio between FVIII and VWF is 1:1.

20. A pharmaceutical formulation according to any one of claims 12-19, wherein the concentration of FVIII is at least 500 IU/ml.

21. A pharmaceutical formulation according to any one of claims 12-20, wherein the amount of FVIII bound to VWF fragment is at least 70% of the total amount of FVIII in said formulation.

22. A pharmaceutical composition according to any one of claims 12-21 for use in treating haemophilia, wherein said pharmaceutical composition is for subcutaneous administration.

23. A pharmaceutical composition wherein said composition comprises a VWF fragment, wherein the amino acid sequence of said VWF fragment is selected from the list consisting of: SEQ ID NO 4, SEQ ID NO 5, SEQ ID NO 6, SEQ ID NO 7, SEQ ID NO 8, SEQ ID NO 9, SEQ ID NO 10, SEQ ID NO 11, SEQ ID NO 12, SEQ ID NO 13, SEQ ID NO 14, SEQ ID NO 15, SEQ ID NO 16, SEQ ID NO 17, SEQ ID NO 18, SEQ ID NO 19, SEQ ID NO 20 and SEQ ID NO 21.

24. A pharmaceutical composition according to claim 23 for use in treatment of von willebrand disease by intravenous or subcutaneous administration.
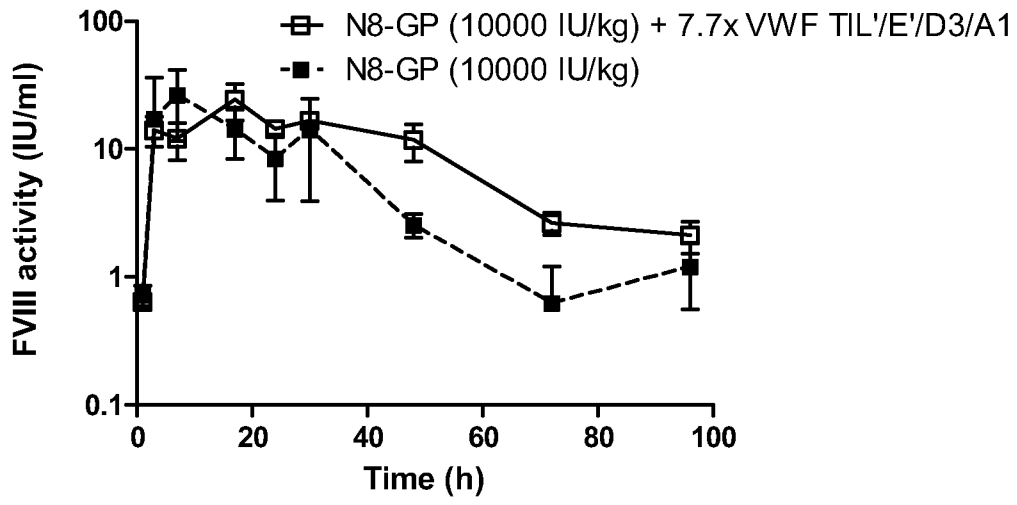
Fig. 1
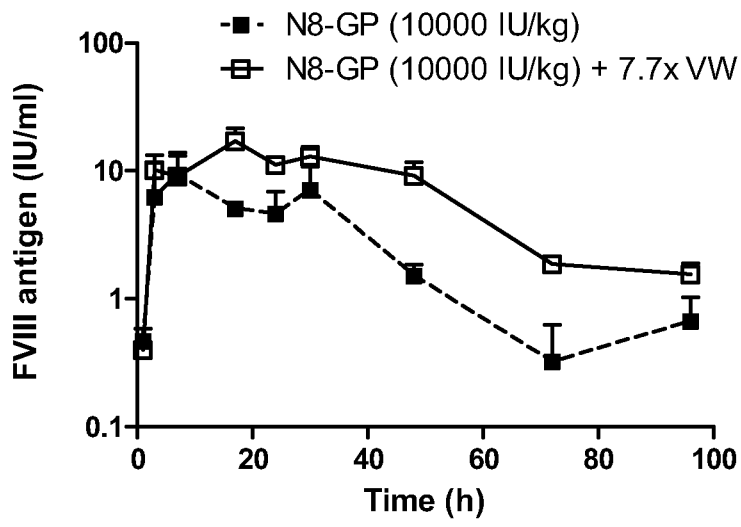
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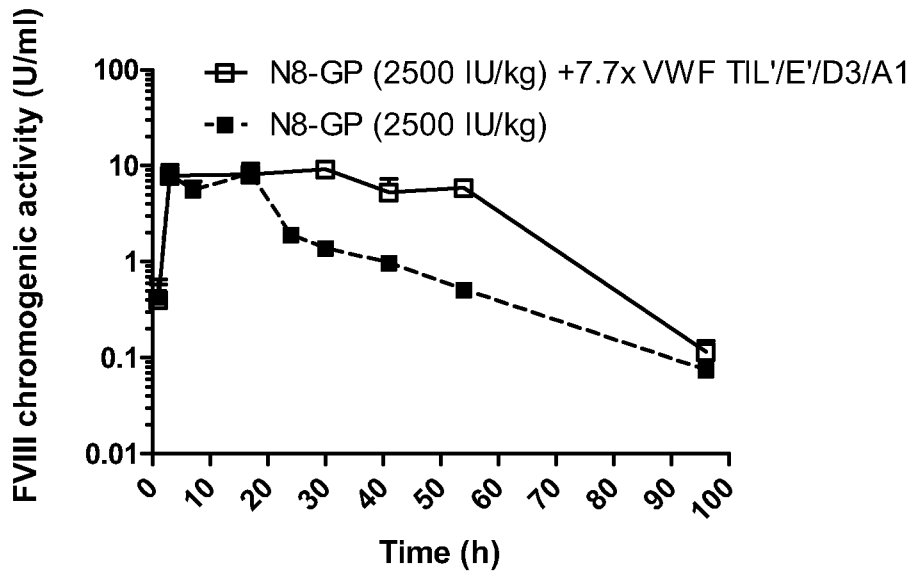
Fig. 3
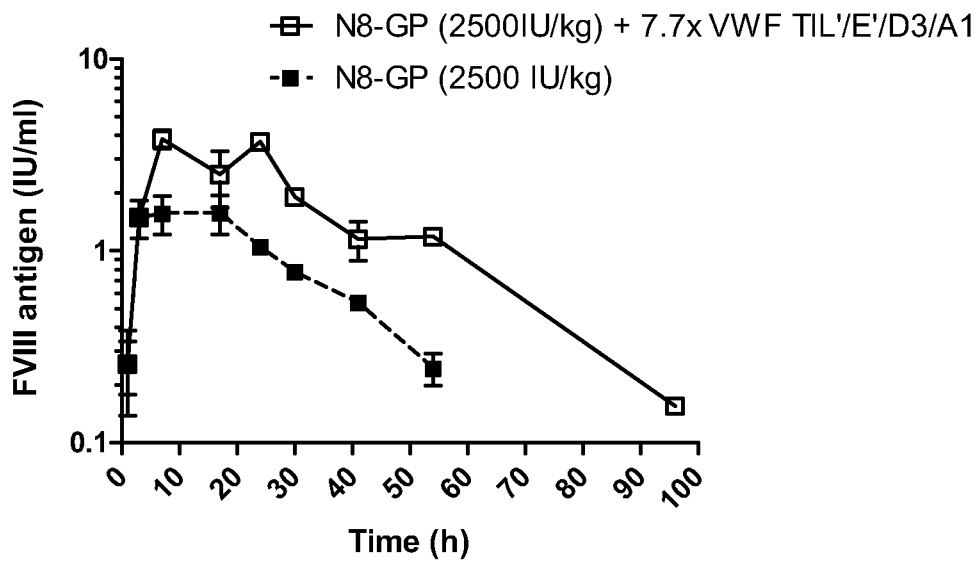
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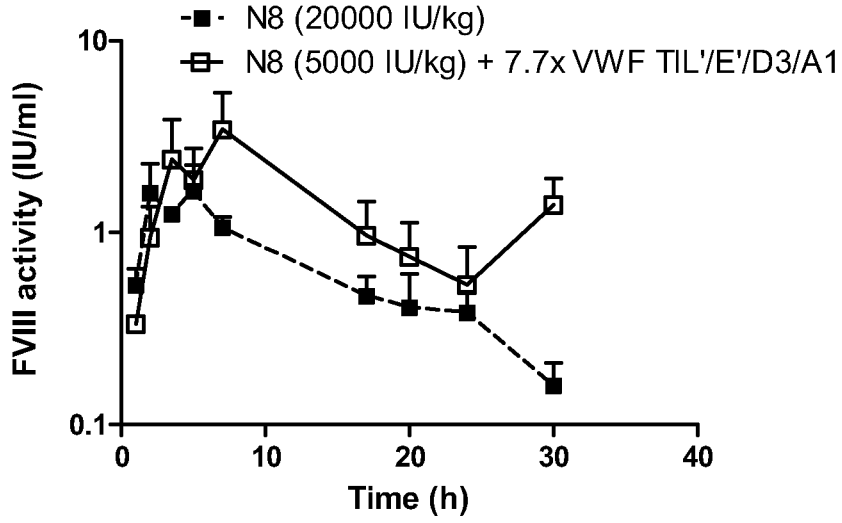
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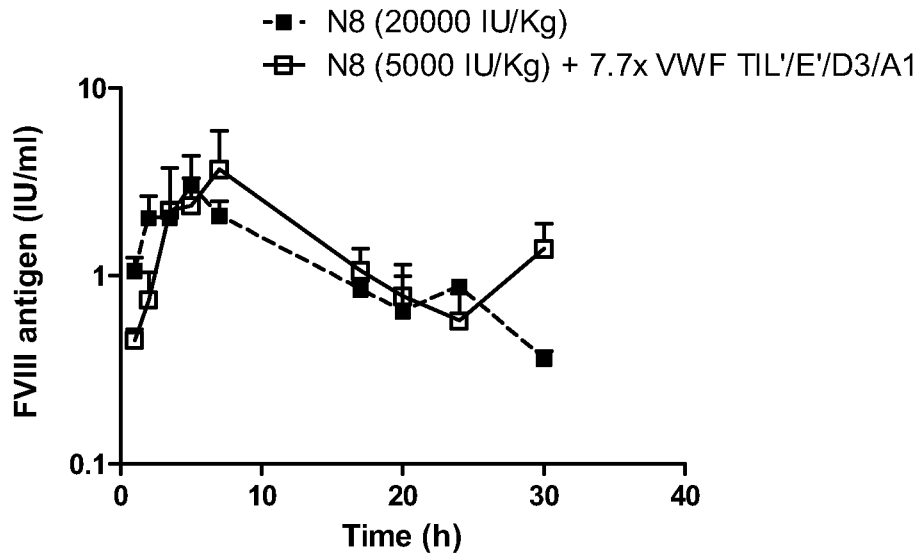
Fig. 6
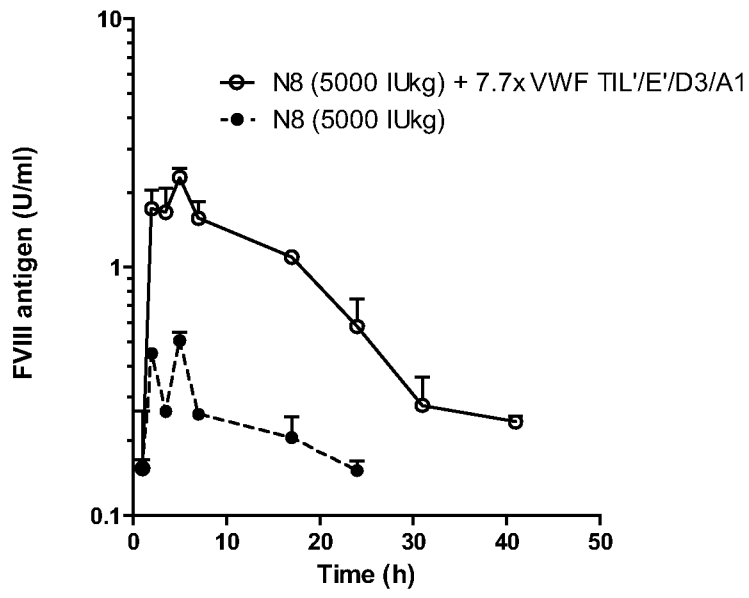
Fig 7
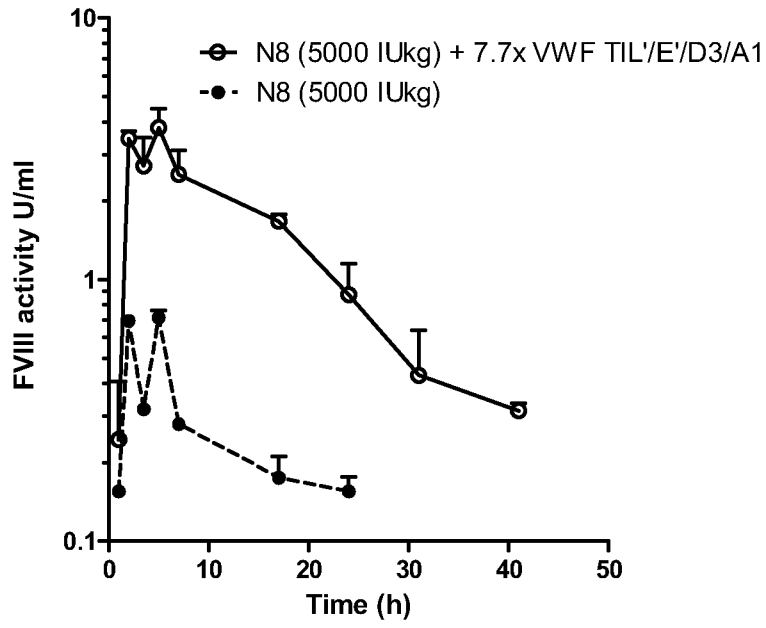
Fig. 8

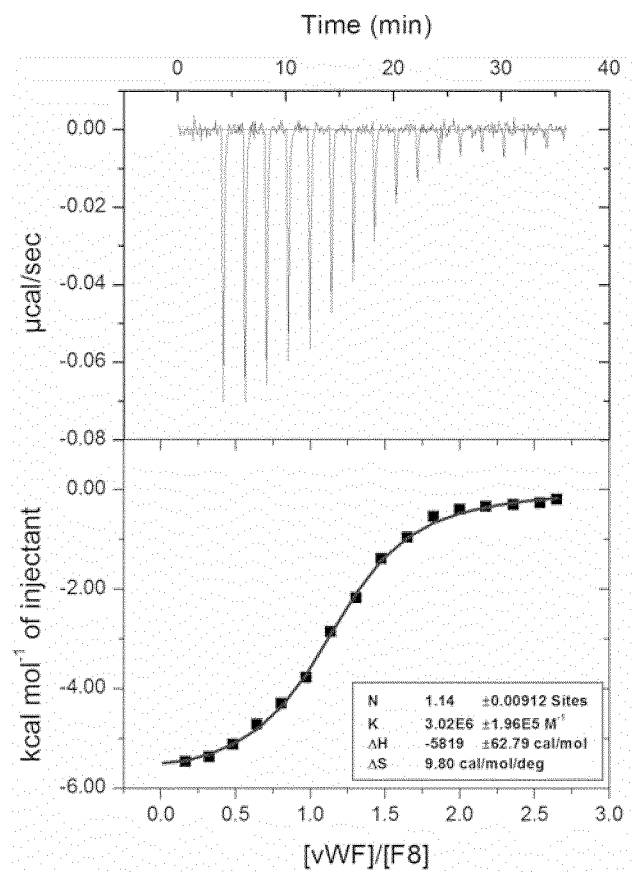Fig. 9
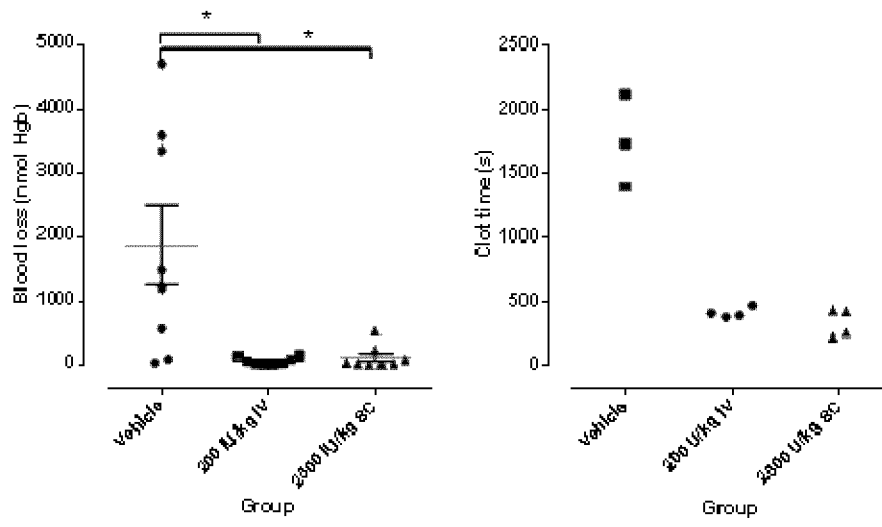
Fig. 10

Fig. 11

Fig. 12
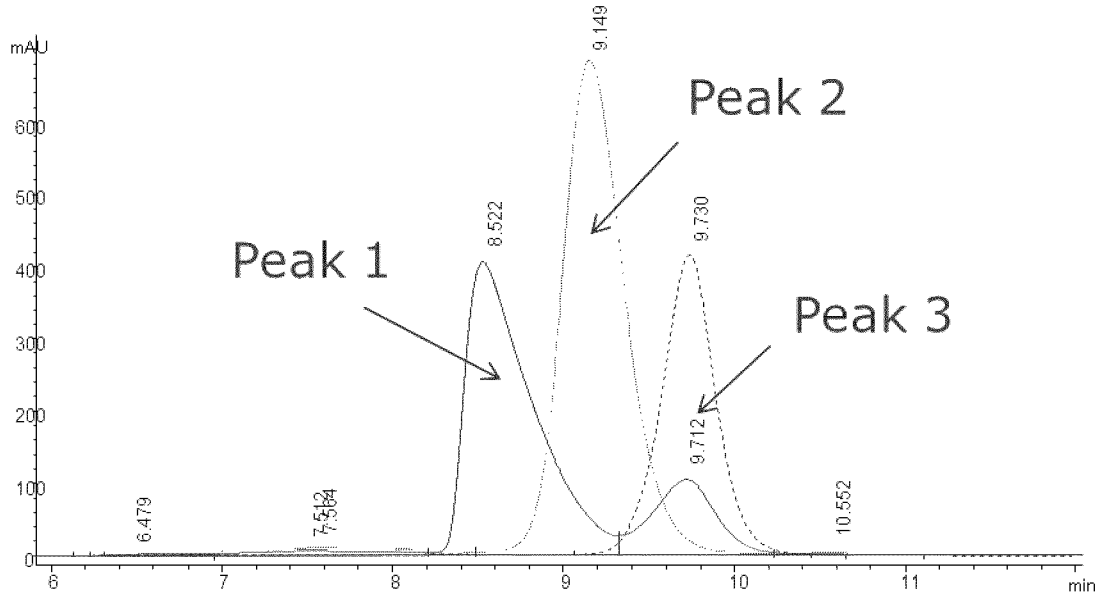
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

INV. C07K14/755 A61K38/37
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal , EMBASE, BIOSIS, WPI Data, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2010/183556 AI (CHOI SANG YON [KR] ET AL) 22 July 2010 (2010-07-22) paragraph [0034]; claim 18; figure 5A; sequences 2,4,6,8 -----	1,2,7, 12,14,19
X	WO 97/41220 AI (FRACTIONNEMENT ET DES BIOT LAB [FR]; MAZURIER CLAUDINE [FR]; JORIEUX S) 6 November 1997 (1997-11-06) page 2, line 28 - line 34; sequence 1 ----- -/- ·	1,2,4,6, 7

☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
3 May 2013	21/05/2013

Name and mailing address of the ISA/	Authorized officer
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C(Continuation).	DOCUMENTS CONSIDERED TO BE RELEVANT	
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A	figures 2,6; sequences 1,4,7 page 34, line 4 - line 22 -----	12-14, 16, 18
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Y	the whole document -----	23,24
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A	THIM L ET AL: "Purification and characterization of a new recombinant factor VIII (N8)", HAEMOPHILIA, BLACKWELL SCIENCE, OXFORD, GB, vol. 16, no. 2, 1 March 2010 (2010-03-01), pages 349-359, XP002583862, ISSN: 1351-8216, DOI: 10.1111/J.1365-2516.2009.02135.X [retrieved on 2009-11-11] the whole document -----	13-15
A	DENIS C V ET AL: "Von Willebrand factor's clearance", HEMATOLOGIE 200602 FR, vol. 12, no. 1, February 2006 (2006-02), pages 34-43, XP002683523, ISSN: 1264-7527 figures 1,3 -----	1-11
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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	V. TERRAUBE ET AL: "Factor VIII and von Willebrand factor interaction: biological, clinical and therapeutic importance", HAEMOPHILIA, vol. 16, no. 1, 1 January 2010 (2010-01-01), pages 3-13, XP055011708, ISSN: 1351-8216, DOI: 10.1111/j.1365-2516.2009.02005.x abstract page 6, column 2, paragraph 1 page 7, column 1, paragraph 2 -----	12, 19
X	A. R. PURVIS ET AL: "Two Cys residues essential for von Willebrand factor multimer assembly in the Golgi", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, vol. 104, no. 40, 2 October 2007 (2007-10-02), pages 15647-15652, XP55061797, ISSN: 0027-8424, DOI: 10.1073/pnas.0705175104 abstract page 15651, column 1, last paragraph -----	1-11
Y	WO 2009/108806 A1 (NOVO NORDISK AS [DK]; DEFREES SHAWN [US]) 3 September 2009 (2009-09-03) cited in the application claim 1; examples 1,2 -----	17

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