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(54) Title: METHOD FOR IMPROVING THE STABILITY OF PURIFIED FACTOR VIII AFTER RECONSTITUTION

(57) Abstract: The present invention relates to a method for increasing the stability of a Factor VIII molecule after purification, lyophilization and reconstitution, comprising preventing proteolytic cleavage of the Factor VIII molecule into a first fragment comprising essentially the A1 domain and the A2 domain and a second fragment comprising essentially the A3 domain, the C1 domain and the C2 domain throughout manufacturing of the Factor VIII molecule. The invention further pertains to a method for improving the bioavailability of Factor VIII after intravenous and non- intravenous injection.

Method for improving the stability of purified Factor VIII after reconstitution

The present invention relates to a method for increasing the stability of a Factor VIII molecule after purification, lyophilization and reconstitution, comprising preventing proteolytic cleavage of the Factor VIII molecule into a first fragment comprising essentially the A1 domain and the A2 domain and a second fragment comprising essentially the A3 domain, the C1 domain and the C2 domain throughout manufacturing of the Factor VIII molecule. The invention further pertains to a method for improving the bioavailability of Factor VIII after intravenous and non-intravenous injection.

BACKGROUND OF THE INVENTION

Classic hemophilia or hemophilia A is an inherited bleeding disorder. It results from a chromosome X-linked deficiency of blood coagulation Factor VIII, and affects almost exclusively males with an incidence of between one and two individuals per 10,000. The X-chromosome defect is transmitted by female carriers who are not themselves hemophiliacs. The clinical manifestation of hemophilia A is an increased bleeding tendency. Before treatment with Factor VIII concentrates was introduced the mean life span for a person with severe hemophilia was less than 20 years. The use of concentrates of Factor VIII from plasma has considerably improved the situation for the hemophilia patients increasing the mean life span extensively, giving most of them the possibility to live a more or less normal life. However, there have been certain problems with the plasma derived concentrates and their use, the most serious of which have been the transmission of viruses. So far, viruses causing AIDS, hepatitis B, and non-A non-B hepatitis have hit the population seriously. Since then different virus inactivation methods and new highly

purified Factor VIII concentrates have recently been developed which established a very high safety standard also for plasma derived Factor VIII.

Several recombinant and plasma-derived, therapeutic polypeptides, e.g. blood coagulation factors, are commercially available for therapeutic and prophylactic use in humans. FVIII is a blood plasma glycoprotein of up to about 280 kDa molecular mass, produced in the liver of mammals. It is a critical component of the cascade of coagulation reactions that lead to blood clotting. Within this cascade is a step in which factor IXa (FIXa), in conjunction with activated factor VIII (FVIIIa), converts factor X (FX) to an activated form, FXa. FVIIIa acts as a cofactor at this step, being required together with calcium ions and phospholipids for maximizing the activity of FIXa.

An important advance in the treatment of hemophilia A has been the isolation of cDNA clones encoding the complete 2,351 amino acid sequence of human FVIII (United States Patent No. 4,757,006) and the provision of the human FVIII gene DNA sequence and recombinant methods for its production).

Factor VIII is synthesized as a single polypeptide chain with a molecular weight of about 280 kDa. The amino-terminal signal peptide is removed upon translocation of factor VIII into the endoplasmatic reticulum, and the mature (i.e. after the cleavage of the signal peptide) native Factor VIII molecule is then proteolytically cleaved after amino acid residues 1313 and 1648 in the course of its secretion. This results in the release of a heterodimer which consists of a C-terminal light chain of about 80 kDa in a metal ion-dependent association with an about 160-200 kDa N-terminal heavy chain fragment. (See review by Kaufman, Transfusion Med. Revs. 6:235 (1992)).

Physiological activation of the heterodimer occurs through proteolytic cleavage of the protein chains by thrombin. Thrombin cleaves the heavy chain to a 90 kDa protein, and then to 54 kDa and 44 kDa fragments. Thrombin also cleaves the 80 kDa light chain to a 72 kDa protein. It is the latter protein, and the two heavy chain

fragments (54 kDa and 44 kDa above), held together by calcium ions, that constitute active FVIII. Inactivation occurs when the 44 kDa A2 heavy chain fragment dissociates from the molecule or when the 72 kDa and 54 kDa proteins are further cleaved by thrombin, activated protein C or FXa. In plasma, FVIII is stabilized by association with a 50-fold molar excess of VWF protein ("VWF"), which appears to inhibit proteolytic destruction of FVIII as described above.

The amino acid sequence of FVIII is organized into three structural domains: a triplicated A domain of 330 amino acids, a single B domain of 980 amino acids, and a duplicated C domain of 150 amino acids. The B domain has no homology to other proteins and provides 18 of the 25 potential asparagine(N)-linked glycosylation sites of this protein. The B domain has apparently no function in coagulation and can be deleted with the B-domain deleted FVIII molecule still having procoagulatory activity.

The Factor VIII products on the market are currently presented as a lyophilized formulation of Factor VIII either produced by recombinant technology or purified from pooled plasma. The lyophilized product is reconstituted prior to administration. Once reconstituted, shelf-life of the Factor VIII is relatively short. Factor VIII is a relatively unstable protein, particularly in aqueous solutions. Stabilization during manufacturing and storage by complexing with other plasma proteins, particularly von Willebrand factor (vWF) and albumin, has been described. See, for example, US Patent No. 6,228,613. US Patent No. 5,565,427 discloses a stabilized formulation of Factor VIII comprising an amino acid or one of its salts or homologues and a detergent or an organic polymer such as polyethyleneglycol. US Patent No. 5,605,884 discloses stabilized formulations of Factor VIII in high ionic strength media based on histidine buffer in the presence of calcium chloride and a high concentration of sodium chloride or potassium chloride. Such compositions were shown to improve significantly the stability of Factor VIII in aqueous form following reconstitution. The importance of calcium ions in the formulations of Factor VIII is generally recognized. According to US Patent No. 6,599,724, the

presence of other divalent cations, namely Cu^{2+} and Zn^{2+}, optionally in the presence of Ca^{2+} ions or Mn^{2+} ions improves the stability of Factor VIII. Also WO 2011/027152 A1 describes stable aqueous Factor VIII compositions comprising various additives.

In view of the short shelf life of Factor VIII after reconstitution of a lyophilisate, there is a need for methods to increase the stability of reconstituted Factor VIII in aqueous solution. To provide a purified FVIII preparation with increased stability in the liquid phase is desirable for different reasons. First of all, it is of advantage to have a sufficient time span at ambient temperature to support manufacturing of the purified FVIII product at ambient temperature. In particular, the filling step necessitates some storage of a liquid bulk to increase flexibility in manufacturing. Secondly, an increased stability of the liquid purified FVIII would be of advantage for physician and patient if the product could not be applied directly after reconstitution. And finally, the use of FVIII under continuous infusion conditions e.g. upon surgery in hospitalized patients is depending on a preferably high product stability after reconstitution (Takedani H., Haemophilia 2010, 16: 740-746). A FVIII molecule with increased stability would also be an advantage for development of a FVIII preparation suitable for long term storage under liquid conditions.

The inventors of this application surprisingly found that the stability of purified Factor VIII after reconstitution of a lyophilisate is substantially enhanced in single-chain Factor VIII constructs. Such constructs can be obtained by preventing the proteolytic cleavage which typically occurs in the Golgi compartment prior to secretion of Factor VIII. The single-chain constructs exhibit a better stability in solution after purificationand/or a better bioavailability upon subcutaneous administration.

2012318292 07 Jul 2015

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present disclosure as it existed before the priority date of each claim of this application.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

SUMMARY OF THE INVENTION

In a first aspect the present invention provides a method for improving the plasma half-life of a Factor VIII molecule after intravenous or non-intravenous administration relative to human wild-type Factor VIII, comprising fusing a first amino acid selected from the amino acids at positions 740 to 1647 of the Factor VIII sequence with a second amino acid selected from the amino acids at positions 1649 to 1690 of the Factor VIII sequence, whereby the proteolytic cleavage site between Arg1648 and Glu1649, and, if present in the FVIII molecule, the cleavage site between Arg1313 and Ala1314 is inactivated.

In another aspect the present invention relates to a method for increasing the stability of a Factor VIII molecule after purification, lyophilization and reconstitution, comprising preventing proteolytic cleavage of the Factor VIII molecule into a first

2012318292 16 Apr 2015

fragment comprising essentially the A1 domain and the A2 domain and a second fragment comprising essentially the A3 domain, the C1 domain and the C2 domain.

A further aspect of the present invention encompasses a method for increasing the stability of a Factor VIII molecule after purification, lyophilization and reconstitution, comprising preventing proteolytic cleavage of the Factor VIII molecule into a first fragment comprising essentially the A1 domain and the A2 domain and a second fragment comprising essentially the A3 domain, the C1 domain and the C2 domain throughout manufacturing of the Factor VIII molecule.

A futher aspect of the present invention encompasses a method for increasing the stability of a Factor VIII molecule after purification, lyophilization and reconstitution, comprising preventing proteolytic cleavage of the Factor VIII molecule into a first fragment comprising essentially the A1 domain and the A2 domain and a second fragment comprising essentially the A3 domain, the C1 domain and the C2 domain prior to the purification of the Factor VIII molecule.

With regard to these methods according to the invention the terms “throughout manufacturing of the Factor VIII molecule” and “prior to the purification of the Factor VIII molecule” are intended to mean that the methods of the invention prevent the cleavage of Factor VIII into a first fragment comprising essentially the A1 domain and the A2 domain and a second fragment comprising essentially the A3 domain, the C1 domain and the C2 domain but the methods according to the invention do not prevent the activation cleavage of Factor VIII which may occur after administration of the reconstituted Factor VIII molecule. The Factor VIII molecules

generated by the methods of the invention can still be activated by thrombin which cleaves the Factor VIII molecule after Arg 372, Arg 740 and Arg 1689.

In a second aspect, the present invention relates to a method for increasing the stability of a Factor VIII molecule after purification, lyophilization and reconstitution, comprising inactivating the proteolytic cleavage sites which are cleaved during secretion of said Factor VIII molecule by the host cell expressing the Factor VIII molecule except the cleavage site between the signal sequence and the mature Factor VIII. Typically, at least 50% of the Factor VIII molecules expressed and secreted by the host cells are single-chain Factor VIII molecules. Preferably, at least 60%, or at least 70%, or at least 80%, or at least 90%, or at least 95% of the Factor VIII molecules expressed and secreted by the host cells are single-chain Factor VIII molecules.

Preferably, the method comprises inactivating the proteolytic cleavage site between Arg1648 and Glu1649 and, if present in the FVIII molecule, the proteolytic cleavage site between Arg1313 and Ala1314. The inactivation of the proteolytic cleavage site may be effected by deleting one or more residues of the protease recognition sequence. For example, inactivation step may comprise deleting at least Arg1648 from the Factor VIII sequence. In one embodiment, the inactivation step comprises deleting at least the amino acid sequence from Arg1313 to Arg1648 from the Factor VIII sequence.

In another embodiment of the first aspect of the invention the inactivation of the proteolytic cleavage site is effected by substituting one or more amino acid residues forming the protease recognition sequence.

In yet another embodiment (concerning those FVIII variants which retain the part of the B-domain comprising Arg1313) the method further comprises inactivating the proteolytic cleavage site between Arg1313 and Ala1314 by deletion or substitution of one or more residues forming the protease recognition sequence. In a

particularly preferred embodiment, the method comprises deleting at least a portion from the Factor VIII amino acid sequence which comprises both protease cleavage sites after residues Arg1313 and Arg1648.

It is further preferred that a first amino acid selected from the amino acids at positions 741 to 1647 of the Factor VIII sequence is fused with a second amino acid selected from the amino acids at positions 1649 to 1690 of the Factor VIII sequence, whereby the proteolytic cleavage site between Arg1648 and Glu1649 and, if present in the FVIII molecule, the cleavage site between Arg1313 and Ala1314 is inactivated.

In another preferred embodiment the Factor VIII molecule stabilized in accordance with the first or second aspect of the invention exhibits an increased stability in aqueous solution. The loss of activity of the modified Factor VIII molecule, in aqueous solution, after storage for 7 days at 25°C is preferably less than 15%.

In another preferred embodiment the Factor VIII molecule stabilized in accordance with the first or second aspect of the invention exhibits an increased stability in aqueous solution after reconstitution.

In yet another preferred embodiment the Factor VIII molecule stabilized in accordance with the first or second aspect of the invention exhibits an increased bioavailability after non-intravenous injection, as compared to the bioavailability of human wild type Factor VIII or as compared to a B-domain deleted Factor VIII molecule where Asn745 is fused to Pro1640, administered at the same dose and in the same manner. In yet another preferred embodiment the Factor VIII molecule stabilized in accordance with the first or second aspect of the invention exhibits an increased bioavailability after non-intravenous injection, as compared to the bioavailability of a B-domain deleted Factor VIII molecule where Asn745 is fused to Pro1640, administered at the same dose and in the same manner. The bioavailability of the modified FVIII is preferably increased by at least 25%, as

compared to the bioavailability of human wild type Factor VIII or of a B-domain deleted Factor VIII molecule where Asn745 is fused to Pro1640, each administered at the same dose and in the same manner. In another preferred embodiment the non-intravenous injection is subcutaneous, transdermal or intramuscular injection.

Another preferred embodiment is a method wherein (i) the Factor VIII exhibits improved plasma half-life after intravenous administration relative to human wild type Factor VIII; preferably wherein the plasma half-life is improved by at least 40% relative to human wild type Factor VIII, or (ii) wherein the Factor VIII exhibits a longer time period for the thrombin peak level as determined in a thrombin generation assay over time in hemophilia A mice to fall below 50 nM after intravenous administration relative to human wild type Factor VIII; preferably wherein this time period is prolonged by at least 10 hours relative to human wild type Factor VIII, or (iii) wherein the Factor VIII retains a higher activity as determined by a one-stage FVIII:C assay after having been incubated for 4 days in human plasma at 37°C relative to human wild type Factor VIII after having been incubated for 4 days in human plasma at 37°C; preferably wherein the retained activity of the Factor VIII is at least 10% higher relative to that of a human wild type Factor VIII after having been incubated for 4 days in human plasma at 37°C.

The methods may further comprise the steps of

(i) providing a nucleic acid encoding a modified Factor VIII molecule in which the proteolytic cleavage sites between Arg1648 and Glu1649, and between Arg1313 and Ala1314, are inactivated,

(ii) transforming a host cell with said nucleic acid,

(iii) culturing the transformed host cell under conditions such that the modified Factor VIII molecule is expressed, and

(iv) recovering the modified Factor VIII molecule from the host cells or from the culture medium.

In another aspect, the present invention relates to a method for improving the bioavailability of a Factor VIII molecule after non-intravenous administration, comprising inactivating the proteolytic cleavage site between Arg1648 and Glu1649 and, if present in the FVIII molecule, the proteolytic cleavage site between Arg1313 and Ala1314. Preferably, the non-intravenous injection is subcutaneous injection. The bioavailability after subcutaneous injection is preferably increased by at least 25% as compared to that of human wild type Factor VIII or of a B-domain deleted Factor VIII molecule where Asn745 is fused to Pro1640, each administered at the same dose and in the same manner.

In another aspect, the present invention relates to a method for improving the plasma half-life of a Factor VIII molecule after intravenous administration relative to human wild-type Factor VIII, comprising inactivating the proteolytic cleavage site between Arg1648 and Glu1649, and, if present in the FVIII molecule, the proteolytic cleavage site between Arg1313 and Ala1314.

In another aspect, the present invention relates to a method for prolonging the time period for the thrombin peak level as determined in a thrombin generation assay over time in hemophilia A mice to fall below 50 nM after intravenous administration of a Factor VIII molecule relative to human wild type Factor VIII, comprising inactivating the proteolytic cleavage site between Arg1648 and Glu1649, and, if present in the FVIII molecule, the proteolytic cleavage site between Arg1313 and Ala1314.

In another aspect, the present invention relates to a method for retaining a higher activity for a Factor VIII molecule as determined by a one-stage FVIII:C assay after having been incubated for 4 days in human plasma at 37°C relative to human wild type Factor VIII after having been incubated for 4 days in human plasma at 37°C, comprising inactivating the proteolytic cleavage site between Arg1648 and Glu1649, and, if present in the FVIII molecule, the proteolytic cleavage site between Arg1313 and Ala1314.

A preferred embodiment of the methods described above are methods wherein a first amino acid selected from the amino acids at positions 741 to 1647 of the Factor VIII sequence is fused with a second amino acid selected from the amino acids at positions 1649 to 1690 of the Factor VIII sequence, whereby the proteolytic cleavage site between Arg1648 and Glu1649, and, if present in the FVIII molecule, the proteolytic cleavage site between Arg1313 and Ala1314 is inactivated.

The preferred embodiments of the different aspects are applicable *mutatis mutandis*.

In yet another aspect, the present invention relates to a pharmaceutical preparation comprising a single chain Factor VIII molecule for use in the treatment or prophylaxis of a bleeding disorder, preferably hemophilia A, by (i) on the one hand non-intravenous administration, wherein the bioavailability of said single chain Factor VIII molecule is increased by at least 25% as compared to human wild type Factor VIII or as compared to a B-domain deleted human Factor VIII molecule where Asn745 is fused to Pro1640, administered at the same dose and in the same manner, or (ii) on the other hand by intravenous administration, wherein (a) the plasma half-life of said single chain Factor VIII molecule after intravenous administration is increased by at least 40%, relative to human wild type Factor VIII, administered at the same dose and in the same manner, or (b) the single chain Factor VIII molecule exhibits a time period prolonged by at least 10 hours for the thrombin peak level as determined in a thrombin generation assay over time in hemophilia A mice to fall below 50 nM after intravenous administration relative to human wild type Factor VIII, administered at the same dose and in the same manner.

In yet another aspect, the present invention relates to a pharmaceutical preparation comprising a single chain Factor VIII molecule for use in the treatment or prophylaxis of a bleeding disorder, preferably hemophilia A, wherein the single

chain Factor VIII molecule retains at least a 10% higher activity as determined by a one-stage FVIII:C assay after having been incubated for 4 days in human plasma at 37°C relative to human wild type Factor VIII after having been incubated for 4 days in human plasma at 37°C.

In yet another aspect, the present invention relates to a pharmaceutical preparation comprising a single chain Factor VIII molecule for use in the treatment or prophylaxis of a bleeding disorder, preferably hemophilia A, by non-intravenous administration, wherein the dose of said FVIII molecule can be decreased by at least 25% as compared to that of a B-domain deleted Factor VIII molecule where Asn745 is fused to Pro1640, administered at the same dose and in the same manner to achieve the same hemostatic activity in blood.

In another aspect, the present invention relates to the use of a single chain Factor VIII molecule for achieving an increased stability after reconstitution or a longer shelf life of a pharmaceutical preparation for treating a bleeding disorder, wherein (i) the Factor VIII activity of the pharmaceutical preparation comprising the single chain Factor VIII molecule, after reconstitution and storage at room temperature for 7 days after reconstitution is at least 10% higher than that of a pharmaceutical preparation comprising the same amount of a B-domain deleted Factor VIII molecule where Asn745 is fused to Pro1640, or (ii) wherein the single chain Factor VIII molecule retains at least a 10% higher activity as determined by a one-stage FVIII:C assay when incubated for 4 days in human plasma at 37°C relative to human wild type Factor VIII after having been incubated for 4 days in human plasma at 37°C at the same concentration.

DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the results of Example 1. Various Factor VIII molecules have been provided as aqueous solutions, and their stability has been monitored over a time period of seven days. The loss in activity after seven days of storage is much less for the single chain Factor VIII molecule as compared to heterodimeric (two-chain) full length Factor VIII molecules (Beriate® and Helixate®) and to heterodimeric (two-chain) B-domain deleted constructs (ReFacto®).

Figure 2 depicts the results of Example 2. Various lyophilized Factor VIII preparations were reconstituted to aqueous solutions, and their stability has been monitored over a time period of seven days. The loss in activity after seven days of storage is much less for the single chain Factor VIII molecule as compared to a heterodimeric (two-chain) full length Factor VIII molecule (Advate®) and to a heterodimeric (two-chain) and B-domain deleted construct (ReFacto®).

Figure 3 depicts the results of Example 3. Three different Factor VIII molecules have been injected subcutaneously in mice and their bioavailability has been determined as described in Example 2. The bioavailability of the single chain Factor VIII molecule is substantially higher than that of a two chain and full length Factor VIII (Advate®) or a heterodimeric (two chain) B-domain deleted construct (ReFacto®).

Figure 4 depicts the results of Example 4. The Factor VIII molecule of the invention and 2 commercially obtainable FVIII preparations were incubated at 37°C after purification, lyophilization and reconstitution. The FVIII- samples were incubated at 37°C for varying time periods (0, 0.25, 1, 2, 4 and 8 days) and the FVIII:C activity was determined by an one-stage-coagulation assay. The values shown represent the average and standard deviation of two samples (except 0.25 days only one sample).

Figure 5 depicts part of the results of Example 5. The pharmacokinetic (PK) profiles of scFVIII and full-length rFVIII (Advate®, Baxter Healthcare) were determined following a single I.V. injection to cynomolgus monkeys at a dose of 250 IU/kg.

Figure 6 depicts part of the results of Example 5. The pharmacokinetic (PK) profiles of full-length rFVIII (Advate®, Baxter Healthcare) were determined following a single I.V. injection to hemophilia A mice at a dose of 100 IU/kg.

Figure 7 depicts part of the results of Example 6. The average peak thrombin levels from days1-8 were determined after scFVIII or full-length rFVIII (Advate®, Baxter Healthcare) were administered to hemophilia A mice at a dose of 250 IU/kg.

Figure 8 depicts the results of Example 7. The pharmacokinetic (PK) profiles of full-length rFVIII (Advate®, Baxter Healthcare) and of a B-domain deleted Factor VIII (ReFacto®, Pfizer) was determined following a single I.V. injection to VWF deficient mice at a dose of 100 IU/kg.

DETAILED DESCRIPTION

The present invention relates to a method for increasing the stability of a Factor VIII molecule after purification, lyophilization and reconstitution, comprising preventing proteolytic cleavage of the Factor VIII molecule into a first fragment comprising essentially the A1 domain and the A2 domain and a second fragment comprising essentially the A3 domain, the C1 domain and the C2 domain.

This invention further pertains to a method for increasing the stability of a Factor VIII molecule after purification, lyophilization and reconstitution, comprising inactivating the proteolytic cleavage site between Arg1648 and Glu1649 and, optionally inactivating the proteolytic cleavage site between Arg1313 and Ala1314, if present in the Factor VIII molecule.

Factor VIII

The terms "blood coagulation Factor VIII", "Factor VIII" and FVIII" are used interchangeably herein. Mature human Factor VIII consists of 2332 amino acids which are arranged in the following domain structure:

A1:	residues 1-336,
A2:	residues 373-710,
B:	residues 741-1648,
A3.	residues 1690-2019,
C1:	residues 2020-2172,
and	
C2:	residues 2173-2332.

In addition, there are three acidic regions *a1* (337-372), *a2* (711-740), and *a3* (1649-1689). It is known that the acidic region *a3* is involved in the binding of the Factor VIII molecule to von Willebrand Factor (vWF) which plays an important role in blood coagulation. During secretion, the FVIII is cleaved between the B-domain and the a3 acidic region, resulting in a heterodimeric polypeptide The factor VIII heterodimer consists of a light chain (comprising A3, C1 and C2) and a variably sized heavy chain (comprising A1, A2 and B). The latter is heterogeneous due to limited proteolysis within the B-domain. In case of heterodimeric B-domain deleted constructs the "heavy chain" comprises A1 and A2 but lacks part or all of the B-domain.

The amino acid sequence of the mature wild type form of human blood coagulation Factor VIII is shown in SEQ ID NO:2. The reference to an amino acid position of a specific sequence means the position of said amino acid in the FVIII wild-type

protein and does not exclude the presence of mutations, e.g. deletions, insertions and/or substitutions at other positions in the sequence referred to. For example, a mutation in "Glu2004" referring to SEQ ID NO:2 does not exclude that in the modified homologue one or more amino acids at positions 1 through 2332 of SEQ ID NO:2 are missing. A DNA sequence encoding SEQ ID NO:2 is shown in SEQ ID NO:1.

"Blood coagulation Factor VIII" includes wild type blood coagulation Factor VIII as well as derivatives of wild type blood coagulation Factor VIII having the procoagulant activity of wild type blood coagulation Factor VIII. Derivatives may have deletions, insertions and/or additions compared with the amino acid sequence of wild type Factor VIII. Preferred derivatives are FVIII molecules in which all or part of the B-domain has been deleted. Amino acid positions indicated throughout this application always refer to the position of the respective amino acid in the full length mature (i.e. after signal peptide cleavage) wild-type FVIII.

The term "factor VIII" includes any factor VIII variants or mutants having at least 10%, preferably at least 25%, more preferably at least 50%, most preferably at least 75% of the biological activity of wild type factor VIII. A suitable test to determine the biological activity of Factor VIII is the one stage or the two stage coagulation assay (Rizza et al. 1982. Coagulation assay of FVIII:C and FIXa in Bloom ed. The Hemophilias. NY Churchchill Livingston 1992) or the chromogenic substrate FVIII:C assay (S. Rosen, 1984. Scand J Haematol 33: 139-145, suppl.). The content of these references is incorporated herein by reference.

As non-limiting examples, Factor VIII molecules include Factor VIII mutants preventing or reducing APC cleavage (Amano 1998. Thromb. Haemost. 79:557-563), albumin-fused FVIII molecules (WO 2011/020866 A2), FVIII-Fc fusion molecules (WO 04/101740 A), Factor VIII mutants further stabilizing the A2 domain (WO 97/40145), FVIII mutants resulting in increased expression (Swaroop et al. 1997. JBC 272:24121-24124), Factor VIII mutants with reduced immunogenicity

(Lollar 1999. Thromb. Haemost. 82:505-508), FVIII reconstituted from differently expressed heavy and light chains (Oh et al. 1999. Exp. Mol. Med. 31:95-100), FVIII mutants reducing binding to receptors leading to catabolism of FVIII like HSPG (heparan sulfate proteoglycans) and/or LRP (low density lipoprotein receptor related protein) (Ananyeva et al. 2001. TCM, 11:251-257), disulfide bond-stabilized FVIII variants (Gale et al., 2006. J. Thromb. Hemost. 4:1315-1322), FVIII mutants with improved secretion properties (Miao et al., 2004. Blood 103:3412-3419), FVIII mutants with increased cofactor specific activity (Wakabayashi et al., 2005. Biochemistry 44:10298-304), FVIII mutants with improved biosynthesis and secretion, reduced ER chaperone interaction, improved ER-Golgi transport, increased activation or resistance to inactivation and improved half-life (summarized by Pipe 2004. Sem. Thromb. Hemost. 30:227-237), and FVIII mutants having a deletion of all or part of the B-domain (see, e.g., WO 2004/067566 A1, WO 02/102850 A2, WO 00/24759 A1 and US patent No. 4,868,112). All of these factor VIII mutants and variants are incorporated herein by reference in their entirety.

The term "single-chain Factor VIII" refers to a Factor VIII molecule which has not been proteolytically cleaved into two chains (e.g. a heavy chain and a light chain) during secretion from the cells expressing said FVIII molecule and, accordingly, is present as a single polypeptide chain.

Preventing cleavage

The method of the invention comprises preventing proteolytic cleavage of the Factor VIII molecule into a first fragment comprising essentially the A1 domain and the A2 domain and a second fragment comprising essentially the A3 domain, the C1 domain and the C2 domain. The term "preventing proteolytic cleavage" includes partially preventing proteolytic cleavage and completely preventing proteolytic cleavage. It further includes the embodiment "reducing proteolytic cleavage". In other words, "preventing proteolytic cleavage of the Factor VIII molecule" does not

require completely abolishing any proteolytic cleavage such that substantially 100% of the Factor VIII molecules expressed and secreted by the host cells are single chain molecules (though this embodiment is encompassed by the method of the invention). Usually, the proteolytic cleavage of the Factor VIII molecule is prevented in a manner such that at least 50%, preferably at least 60%, more preferably at least 70%, more preferably at least 80%, more preferably at least 90%, most preferably at least 95% of the Factor VIII molecules expressed and secreted by the host cells are single chain molecules. The incomplete prevention of cleavage may, at least in part, be due to the fact that there can be some minor cleavage sites within the B domain which can lead to proteolytic cleavage of a small portion of the Factor VIII molecules even if the major cleavage sites (at R1313 and R1648) are absent. This minor cleavage may or may not be prevented in accordance with this invention.

The first fragment comprises essentially the A1 domain and the A2 domain of Factor VIII. The first fragment may comprise the A1 domain and the A2 domain, each domain having exactly the amino acid sequence indicated above. For example, the first fragment may comprise at least amino acids 1 to 740 of the amino acid sequence of SEQ ID NO:2. Alternatively, the first fragment may comprise a variant of this sequence, having amino acid deletions, substitutions and/or insertions which do not substantially affect the Factor VIII activity. The first fragment may additionally comprise an N-terminal part of the B domain of Factor VIII.

The second fragment comprises essentially the A3 domain, the C1 domain and the C2 domain. The second fragment may comprise the A3 domain, the C1 domain and the C2 domain, each domain having exactly the amino acid sequence indicated above. For example, the second fragment may comprise at least amino acids 1690 to 2332 of the amino acid sequence shown in SEQ ID NO:2. Alternatively, the second fragment may comprise a variant of this sequence, having amino acid deletions, substitutions and/or insertions which do not substantially affect the Factor

VIII activity. The second fragment may additionally comprise a C-terminal part of the acidic *a3* region.

The method of the invention comprises preventing the proteolytic cleavage during secretion of the recombinantly expressed FVIII molecule, which would result in a heterodimeric (two-chain) polypeptide. That is, the method includes obtaining a single-chain Factor VIIII molecule. This can be achieved in various ways, e.g. by inactivating the proteolytic cleavage sites involved in the intracellular processing of the mature, one-chain FVIII into the heterodimeric FVIII eventually secreted by the host cells.

In one embodiment the step of inactivating the proteolytic cleavage site between Arg1648 and Glu1649 comprises deleting one or more amino acids forming the protease recognition sequence. The cleavage site after residue 1648 is a furin-type cleavage site. The recognition sequence for the protease in the Factor VIII sequence is LKRHQR. Preferably, the inactivation step comprises deleting one, two, three, four, five or more of these amino acid residues forming the recognition sequence. Preferably, the inactivation step comprises deleting at least one basic amino acid within the recognition sequence, more preferably, the inactivation step comprises deleting at least the arginine at position 1648. Still more preferably, the inactivation step comprises deleting at least amino acids 1643 to 1648 of the Factor VIII sequence. If the respective FVIII derivative comprises Arg1313, the inactivation step comprises also deleting at least the arginine at position Arg 1313. Still preferably is deleting at least amino acids 1313 to 1648 of the Factor VIII sequence to inactivate both cleavage sites after 1313 and 1648, respectively.

Most preferably, the inactivation step comprises deleting at least the amino acid sequence from residues 800 to 1648 from the Factor VIII sequence, e.g. the amino acid sequence from residues 741 to 1648 from the Factor VIII sequence. In another preferred embodiment, a first amino acid selected from the amino acids at positions 741 to 1647 of the Factor VIII sequence is fused with a second amino acid selected

from the amino acids at positions 1649 to 1690 of the Factor VIII sequence, whereby the proteolytic cleavage during secretion is prevented. Preferred deletions are as follows:

- amino acid 740 is fused to amino acid 1650, whereby amino acids 741 to 1649 are deleted;
- amino acid 740 is fused to amino acid 1690, whereby amino acids 741 to 1689 are deleted;
- amino acid 740 is fused to amino acid 1669, whereby amino acids 741 to 1668 are deleted;
- amino acid 743 is fused to amino acid 1650, whereby amino acids 744 to 1649 are deleted;
- amino acid 764 is fused to amino acid 1650, whereby amino acids 765 to 1649 are deleted;
- amino acid 764 is fused to amino acid 1653, whereby amino acids 765 to 1652 are deleted;
- amino acid 764 is fused to amino acid 1656, whereby amino acids 765 to 1655 are deleted;
- amino acid 745 is fused to amino acid 1650, whereby amino acids 746 to 1649 are deleted;
- amino acid 745 is fused to amino acid 1653, whereby amino acids 746 to 1652 are deleted;
- amino acid 745 is fused to amino acid 1656, whereby amino acids 746 to 1655 are deleted;
- amino acid 757 is fused to amino acid 1650, whereby amino acids 758 to 1649 are deleted;
- amino acid 757 is fused to amino acid 1653, whereby amino acids 758 to 1652 are deleted;
- amino acid 757 is fused to amino acid 1656, whereby amino acids 758 to 1655 are deleted;

- amino acid 793 is fused to amino acid 1649, whereby amino acids 794 to 1648 are deleted;
- amino acid 793 is fused to amino acid 1690, whereby amino acids 794 to 1689 are deleted;
- amino acid 747 is fused to amino acid 1649, whereby amino acids 748 to 1648 are deleted;
- amino acid 751 is fused to amino acid 1649, whereby amino acids 752 to 1648 are deleted;
- amino acid 776 is fused to amino acid 1649, whereby amino acids 777 to 1648 are deleted;
- amino acid 770 is fused to amino acid 1667, whereby amino acids 771 to 1666 are deleted.

The molecules resulting from the deletion are usually obtained in the form of single chain Factor VIII molecules.

Preferred single chain FVIII molecules have a deletion of all or part of the B-domain and a deletion of all or a part of the acidic a3 region, so that the cleavage site at Arg1648 (which is usually cleaved during secretion) is deleted. Single chain FVIII molecules are disclosed in, e.g., WO 2004/067566 A1; US 2002/132306 A1; Krishnan et al. (1991) European Journal of Biochemistry vol. 195, no. 3, pages 637-644; Herlitschka et al. (1998) Journal of Biotechnology, vol. 61, no. 3, pages 165-173; Donath et al. (1995) Biochem. J., vol. 312, pages 49-55. These single-chain Factor VIII molecules described in these references are incorporated herein by reference.

The fusions referred to above may be direct fusions or indirect fusions. In the latter case, the deleted amino acids are replaced by a heterologous spacer. This embodiment is described in more detail hereinafter. It is possible that the deleted amino acids are replaced with a peptidic linker consisting of about 1 to about 500

amino acids, or about 2 to 250 amino acids, or about 3 to about 100 amino acids, or about 4 to about 50 amino acids, or about 5 to about 10 amino acids. The peptidic linker should be flexible and not immunogenic (Robinson et al.; PNAS (1998), Vol 95, p5929). The peptidic linkers may consist of Gly preceded N-terminally to said Gly by multimers of the amino acid sequence GlyGlySer or GlyGlySerSer or any combination thereof, in a specific embodiment the peptidic linker consists of 80 to 120 amino acids.

In an alternative embodiment, one or more amino acids which form the protease recognition site at residue 1313 and 1648 may be substituted with another amino acid such that the cleavage does not occur. For example, the basic amino acids may be replaced with hydrophobic amino acids.

Preparation of single-chain Factor VIII

The step of "preventing proteolytic cleavage" or "inactivating a proteolytic cleavage site" is carried out prior to the purification, lyophilisation and reconstitution of the Factor VIII. The step of "preventing proteolytic cleavage" or "inactivating a proteolytic cleavage site" is typically carried out during the preparation of the Factor VIII molecule. The method of the invention may include preventing the proteolytic cleavage during expression of the Factor VIII molecule (in host cells), or inactivating the proteolytic cleavage site at Arg1313 and/or Arg1648 during the preparation of the nucleic acid encoding the Factor VIII molecule.

These steps of "preventing proteolytic cleavage" or "inactivating a proteolytic cleavage site" may include removing, from a nucleic acid encoding Factor VIII, a portion encoding the proteolytic cleavage site at Arg1313 and/or Arg1648, in accordance with the embodiments described above. This typically results in a nucleic acid encoding single chain Factor VIII. Generally, the method of the

invention may further include providing a nucleic acid encoding the single-chain Factor VIII, e.g. in an expression plasmid or vector.

The nucleic acid, the expression vector or the expression plasmid may then be introduced into host cells, preferably mammalian host cells, for expression. The method of the invention may further comprise culturing the host cells under suitable conditions such that the modified Factor VIII molecule, e.g. the single chain Factor VIII molecule, is expressed; and optionally recovering (e.g. purifying) the modified Factor VIII molecule from the host cells or from the culture medium. Generally, techniques of manipulating the nucleic acid encoding Factor VIII, of culturing mammalian cells to allow expression of the Factor VIII, and of purifying Factor VIII from the cell culture medium are known in the art.

It is preferred to purify the single chain Factor VIII molecule to ≥ 80% purity, more preferably ≥ 95% purity and particularly preferred is a pharmaceutically pure state that is greater than 99.9% pure with respect to contaminating macromolecules, particularly other proteins or/and nucleic acids, and free of infectious and pyrogenic agents. Preferably, an isolated or purified modified Factor VIII molecule is substantially free of other polypeptides.

The methods of the invention may further comprise the steps of purifying, lyophilizing, and reconstituting the single chain Factor VIII. The reconstitution is preferably carried out by using water, e.g. "water for injection".

Stability

The Factor VIII molecules prepared in accordance with the present invention exhibit enhanced stability relative to full length Factor VIII and/or relative to a B-domain deleted Factor VIII molecule where Asn745 is fused to Pro1640 (i.e. a B-domain deleted Factor VIII molecule consisting essentially of amino acids 1-745 and 1640-2332 of SEQ ID NO:2).

As used herein, the term "stability" refers to stability in aqueous solution, preferably to stability in aqueous solution after reconstitution of a lyophilized Factor VIII preparation, e.g. by adding water to the lyophilized Factor VIII preparation. Typically, the lyophilized Factor VIII preparation is reconstituted with "water for injection".

The stability in aqueous solution can be determined by providing the Factor VIII molecule in aqueous solution and incubating it for a certain period of time. In a preferred embodiment, the conditions for determining the storage stability of the Factor VIII molecule are as follows:

The Factor VIII molecule is provided in aqueous solution having the following composition:

L-histidine	25 mM
NaCl	225 mM
calcium chloride	4 mM
Tween® 80	0.03% (w/w)
sucrose	2% (w/w)
D-mannitol	8% (w/w)
pH 7.0.	

This solution is referred to hereinafter as "Buffer A". The initial Factor VIII activity in the aqueous solution is preferably between 100 IU/ml and 1,500 IU/ml, preferably it is 100 IU/ml.

The so prepared Factor VIII solution can then be incubated at 25°C for at least 24 hours, preferably for at least two days, more preferably for at least five days, most preferably for seven or eight days. After the incubation period the stability is determined by measuring the Factor VIII activity in the solution, preferably by using a chromogenic substrate assay (e.g. Coamatic® Factor VIII, Chromogenix). The

lower the loss in activity relative to the initial activity, the higher is the stability of the Factor VIII molecule. Most preferably, the stability is determined as in Example 1 or 2 below.

According to the present invention the loss in Factor VIII activity of the single-chain Factor VIII after seven days of storage under the above-identified conditions is less than 15%, preferably less than 12%, most preferably less than 10%.

Typically, the initial Factor VIII activity at the start of the incubation period (t=0) is normalized to 100%. The remaining Factor VIII activity after 24 hours of storage in Buffer A at 25°C is preferably at least 95% of the initial Factor VIII activity. The remaining Factor VIII activity after 48 hours of storage in Buffer A at 25°C is preferably at least 95% of the initial Factor VIII activity. The remaining Factor VIII activity after 4 days of storage in Buffer A at 25°C is preferably at least 90%, more preferably at least 95% of the initial Factor VIII activity. The remaining Factor VIII activity after 7 days of storage in Buffer A at 25°C is preferably at least 85%, more preferably at least 90%, most preferably at least 95% of the initial Factor VIII activity. The remaining Factor VIII activity after 8 days of storage in Buffer A at 25°C is preferably at least 85%, more preferably at least 90%, most preferably at least 95% of the initial Factor VIII activity.

The remaining Factor VIII activity of the single chain Factor VIII is usually higher than that of two-chain Factor VIII molecules (assuming that both molecules have been incubated under identical conditions for the same period of time).

The term “human full length two-chain Factor VIII” is used herein interchangeably with the term “human wild-type Factor VIII”.

In one embodiment, the remaining Factor VIII activity of the single chain Factor VIII is higher than that of human full length two-chain Factor VIII. In another embodiment, the remaining Factor VIII activity of the single chain Factor VIII is

higher than that of a B-domain deleted Factor VIII molecule where Asn745 is fused to Pro1640 (i.e. a B-domain deleted Factor VIII molecule consisting essentially of amino acids 1-745 and 1640-2332 of SEQ ID NO:2).

Preferably, the remaining Factor VIII activity of the single chain Factor VIII after 48 hours of storage in Buffer A at 25°C exceeds the remaining Factor VIII activity of human full length two-chain Factor VIII by at least 4 percentage points. It is also preferred that the remaining Factor VIII activity of the single chain Factor VIII after 48 hours of storage in Buffer A at 25°C exceeds the remaining Factor VIII activity of a B-domain deleted Factor VIII molecule where Asn745 is fused to Pro1640 (i.e. a B-domain deleted Factor VIII molecule consisting essentially of amino acids 1-745 and 1640-2332 of SEQ ID NO:2) by at least 4 percentage points.

In another embodiment, the remaining Factor VIII activity of the single chain Factor VIII after 4 days of storage in Buffer A at 25°C exceeds the remaining Factor VIII activity of human full length two-chain Factor VIII by at least 5 percentage points. It is also preferred that the remaining Factor VIII activity of the single chain Factor VIII after 4 days of storage in Buffer A at 25°C exceeds the remaining Factor VIII activity of a B-domain deleted Factor VIII molecule where Asn745 is fused to Pro1640 (i.e. a B-domain deleted Factor VIII molecule consisting essentially of amino acids 1-745 and 1640-2332 of SEQ ID NO:2) by at least 5 percentage points.

In another embodiment, the remaining Factor VIII activity of the single chain Factor VIII after 7 days of storage in Buffer A at 25°C exceeds the remaining Factor VIII activity of human full length two-chain Factor VIII by at least 5, preferably by at least 10 percentage points. It is also preferred that the remaining Factor VIII activity of the single chain Factor VIII after 7 days of storage in Buffer A at 25°C exceeds the remaining Factor VIII activity of a B-domain deleted Factor VIII molecule where Asn745 is fused to Pro1640 (i.e. a B-domain deleted Factor VIII molecule consisting essentially of amino acids 1-745 and 1640-2332 of SEQ ID NO:2) by at least 5, preferably by at least 10 percentage points.

In another embodiment, the remaining Factor VIII activity of the single chain Factor VIII after 8 days of storage in Buffer A at 25°C exceeds the remaining Factor VIII activity of human full length two-chain Factor VIII by at least 5, preferably by at least 10 percentage points. It is also preferred that the remaining Factor VIII activity of the single chain Factor VIII after 8 days of storage in Buffer A at 25°C exceeds the remaining Factor VIII activity of a B-domain deleted Factor VIII molecule where Asn745 is fused to Pro1640 (i.e. a B-domain deleted Factor VIII molecule consisting essentially of amino acids 1-745 and 1640-2332 of SEQ ID NO:2) by at least 5, preferably by at least 10 percentage points.

Alternatively to Buffer A other buffers may also be used like, for example the buffer used in Example 2 of the present invention.

The preferred pH range for the buffers of the present invention is a pH range from 5.5 to 9.0, preferably a pH range from 6.0 to 8.5 and especially preferred a pH range from 6.5 to 8.0.

The activity of Factor VIII can be determined by a chromogenic or clotting assay, or any other bioassay. Preferably, the Factor VIII activity is determined as shown in Example 1 below.

Bioavailability

In another embodiment, the Factor VIII molecule stabilized in accordance with the present invention exhibits improved bioavailability after non-intravenous injection, as compared to two chain human wild type Factor VIII or compared to two chain human B-domain deleted Factor VIII. The non-intravenous injection is preferably subcutaneous, transdermal or intramuscular injection. Most preferably, the non-intravenous injection is subcutaneous injection.

The term „bioavailability", as used herein, refers to the proportion of an administered dose of a Factor VIII or a FVIII-related preparation that can be detected in plasma at predetermined times until a final time point after subcutaneous, intravenous or intradermal administration. Typically, bioavailability is measured in test animals by administering a dose of between 10 IU/kg and 1000 IU/kg of the preparation (e.g. 400 IU/kg body weight); obtaining plasma samples at pre-determined time points after administration; and determining the content of the Factor VIII or Factor VIII-related polypeptides in the samples using one or more of a chromogenic or clotting assay (or any bioassay), an immunoassay, or an equivalent thereof. The bioavailability is expressed as the area under the curve (AUC) of the concentration or activity of the coagulation factor in plasma on the y-axis and the time after administration on the x-axis until a predefined final time point after administration. Preferably, this predefined time point is 72 or 48 hours after administration. Most preferably, the bioavailability is determined as shown in Example 3 herein below. Relative bioavailability of a test preparation refers to the ratio between the AUC of the test preparation (here: single chain Factor VIII) and that of the reference preparation (e.g. full length recombinant two-chain Factor VIII or two-chain B-domain deleted Factor VIII) which is administered in the same dose and way (e.g. intravenous, subcutaneous or intradermal) as the test preparation.

According to the present invention, the bioavailability of the single chain Factor VIII after subcutaneous injection is higher than that of the two-chain human wild type Factor VIII or of two-chain human B-domain deleted Factor VIII. Preferably, the bioavailability (AUC over 72 hours after subcutaneous injection) is increased by at least 10%, more preferably by at least 25%, more preferably by at least 50%, most preferably by at least 75%, relative to wild type FVIII. In another embodiment, the bioavailability (AUC over 72 hours after subcutaneous injection) is increased by at least 10%, more preferably by at least 20%, more preferably by at least 30%, most preferably by at least 40%, relative to a B-domain deleted Factor VIII molecule where Asn745 is fused to Pro1640 (i.e. a B-domain deleted Factor VIII molecule consisting essentially of amino acids 1-745 and 1640-2332 of SEQ ID NO:2).

Improvement of plasma half-life (in –vivo)

In another embodiment, the Factor VIII molecule stabilized in accordance with the present invention exhibit increased pharmacokinetic (PK) parameters.

Factor VIII molecules of the invention can be tested by i.v. injection into different species like hemophilia A mice or cynomolgus monkeys e.g. at a dose of 100 IU/kg or 250 IU/kg respectively e.g. as determined in a chromogenic assay. Blood samples are drawn at various time points after administration e.g. until 72 hours (hrs) in hemophilia A mice and e.g. until 24 hrs in cynomolgus monkeys. Citrate plasma is prepared immediately and used for quantification of FVIII:C e.g. by a chromogenic assay system (FVIII:C) (Chromogenix - Instrumentation Laboratory SpA, Milan, Italy).

The AUC of the FVIII levels in plasma is calculated using the linear trapezoidal rule to calculate AUC_{last} : from t=0 to last observation. Terminal half-life ($t_{1/2\beta}$) is determined by a log-linear regression using the points of the terminal phase selected by the adjusted R2 criterion. AUC: from t=0 to infinity (extrapolated by using the regression model of the terminal phase).

The single chain FVIII molecules according to the invention show at least a 40%, preferably at least a 50%, even more preferably at least a 60% increased terminal half life as compared to the terminal half-life a human wild-type Factor VIII administered at the same dose and in the same manner.

Preferably the plasma half-life is determined as shown in Example 5.

Prolongation of efficacy as determined in a thrombin generation assay (in-vivo)

In another embodiment, the Factor VIII molecule stabilized in accordance with the present invention exhibit a longer time period for the thrombin peak level as determined in a thrombin generation assay over time in hemophilia A mice to fall below 50 nM after intravenous administration relative to human wild type Factor VIII. This test show that also the functionality of FVIII is stabilized in the molecules according to the invention.

FVIII molecules according to the invention can be tested by first administering the FVIII molecule of the invention at an equimolar dose (e.g. at 250 IU/kg) intravenously into hemophilia A mice. At different time points (e.g. daily from day 1 to 8) citrated blood is collected and a thrombin generation assay (TGA) is performed e.g. by calibrated thrombinography (CAT) (Thrombinoscope, Netherlands) after intrinsic activation in presence of Phospholipid (e.g. Rossix, Mölndal, Sweden) / Pathromtin® SL (Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany) (1:30). Thrombin peak levels are recorded. The average AUC of peak thrombin levels from days 1-8 is calculated by the linear trapezoidal rule. The AUC of the two Factor VIII products are compared using an approximate F-test for the difference in AUC in a linear model with variable variances per time-point and treatment group resulting in a estimated time until peak levels of thrombin drop below a defined limit ranging of 50 nM.

Preferably the efficacy in a thrombin generation assay is determined as shown in Example 6.

In hemophilia A mice scFVIII shows a favorable hemostatic activity compared to human wild-type Factor VIII. This translates into an averaged at least 10 hrs longer, preferably at least 15 hours longer and even more preferred at least 20 hours longer thrombin generation activity value for scFVIII versus full-length rFVIII before the thrombin peak level falls below a level of 50 nM.

Retaining higher FVIII:C activity in plasma (ex vivo)

In another embodiment, the Factor VIII molecule stabilized in accordance with the present invention retain a higher activity as determined by a one-stage FVIII:C assay after having been incubated for 4 days in human plasma at 37°C relative to human wild type Factor VIII after having been incubated for 4 days in human plasma at 37°C; preferably wherein the retained activity of the Factor VIII is at least 10% higher relative to that of a human wild type Factor VIII after having been incubated for 4 days in human plasma at 37°C.

Samples with Factor VIII molecules according to the invention can be tested by diluting them into with FVIII deficient plasma (e.g. from Siemens Healthcare Diagnostics) to 1 IU/mL FVIII:C (based on values determined by the chromogenic substrate assay). The FVIII- samples are then incubated at 37°C for varying time periods (e.g. for 0, 0.25, 1, 2, 4 and 8 days) in presence of 0.05% Na-azide. After each incubation period, FVIII:C is then determined by a one-stage-coagulation assay e.g. by using Pathromtin-SL (Siemens Healthcare Diagnostics) as activator, normalized to the value at t= 0 (% FVIII:C) and plotted versus the incubation time.

After a 4 day incubation at 37°C the Factor VIII molecule of the invention has retained at least a 10% higher FVIII:C activity, preferably at least 15% higher FVIII:C activity, preferably at least a 20% higher FVIII:C activity, preferably at least a 25% higher FVIII:C activity, preferably at least a 30% higher FVIII:C activity.

Preferably the activity in plasma is determined as shown in Example 4.

Treatment and prophylaxis

The single-chain Factor VIII constructs in accordance with the present invention having increased stability after reconstitution can be administered in the treatment or prophylaxis of bleeding disorders.

As used herein, the term “bleeding disorders” includes familial and acquired hemophilia A and B, familial or acquired von Willebrand disease, familial or acquired deficiency of any coagulation factor, all types of trauma, blunt or penetrating, leading to severe hemorrhage either from a single organ, a bone fraction or from polytrauma, bleeding during surgical procedures including peri- or postoperative haemorrhage, bleeding due to cardiac surgery including patients undergoing extracorporal circulation and hemodilution in pediatric cardiac surgery, intracerebral hemorrhage, subarachnoid hemorrhage, sub-or epidural bleeding, bleedings due to blood loss and hemodilution, by non-plasmatic volume substitution leading to reduced levels of coagulation factors in affected patients, bleedings due to disseminated intravascular coagulation (DIC) and a consumption coagulopathy, thrombocyte dysfunctions, depletion and coagulopathies, bleeding due to liver cirrhosis, liver dysfunction and fulminant liver failure, liver biopsy in patients with liver disease, bleeding after liver and other organ transplantations, bleeding from gastric varices and peptic ulcer bleeding, gynaecological bleedings as dysfunctional uterine bleeding (DUB), premature detachment of the placenta, periventricular haemorrhage in low birth weight children, post partum haemorrhage, fatal distress of newborns, bleeding associated with burns, bleeding associated with amyloidosis, hematopoietic stem cell transplantation associated with platelet disorder, bleedings associated with malignancies, infections with haemorrhaging viruses, bleeding associated with pancreatitis.

The components of the pharmaceutical preparation may be dissolved in conventional physiologically compatible aqueous buffer solutions to which there may be added, optionally, pharmaceutical excipients to provide the pharmaceutical preparation. The components of the pharmaceutical preparation may already contain all necessary pharmaceutical, physiologically compatible excipients and may be dissolved in water for injection to provide the pharmaceutical preparation.

Such pharmaceutical carriers and excipients as well as the preparation of suitable pharmaceutical formulations are well known in the art (see for example

"Pharmaceutical Formulation Development of Peptides and Proteins", Frokjaer et al., Taylor & Francis (2000) or "Handbook of Pharmaceutical Excipients", 3rd edition, Kibbe et al., Pharmaceutical Press (2000)). In certain embodiments, a pharmaceutical composition can comprise at least one additive such as a bulking agent, buffer, or stabilizer. Standard pharmaceutical formulation techniques are well known to persons skilled in the art (see, e.g., 2005 Physicians' Desk Reference®, Thomson Healthcare: Montvale, NJ, 2004; Remington: The Science and Practice of Pharmacy, 20th ed., Gennaro et al., Eds. Lippincott Williams & Wilkins: Philadelphia, PA, 2000). Suitable pharmaceutical additives include, e.g., sugars like mannitol, sorbitol, lactose, sucrose, trehalose, or others, amino acids like histidine, arginine, lysine, glycine, alanine, leucine, serine, threonine, glutamic acid, aspartic acid, glutamine, asparagine, phenylalanine, or others, additives to achieve isotonic conditions like sodium chloride or other salts, stabilizers like Polysorbate 80, Polysorbate 20, Polyethylene glycol, propylene glycol, calcium chloride, or others, physiological pH buffering agents like Tris(hydroxymethyl)aminomethan, and the like. In certain embodiments, the pharmaceutical compositions may contain pH buffering reagents and wetting or emulsifying agents. In further embodiments, the compositions may contain preservatives or stabilizers. In particular, the pharmaceutical preparation comprising the blood coagulation factor may be formulated in lyophilized or stable soluble form. The blood coagulation factor may be lyophilized by a variety of procedures known in the art. Lyophilized formulations are reconstituted prior to use by the addition of one or more pharmaceutically acceptable diluents such as sterile water for injection or sterile physiological saline solution or a suitable buffer solution.

The composition(s) contained in the pharmaceutical preparation of the invention may be delivered to the individual by any pharmaceutically suitable means. Various delivery systems are known and can be used to administer the composition by any convenient route. Preferably, the composition(s) contained in the pharmaceutical preparation of the invention are delivered to the individual by non-intravenous injection. More preferably, the composition(s) of the invention are formulated for

subcutaneous, intramuscular, intraperitoneal, intracerebral, intrapulmonar, intranasal, intradermal or transdermal adminstration, most preferably for subcutaneous, intramuscular or transdermal administration according to conventional methods. The formulations can be administered continuously by infusion or by bolus injection. Some formulations may encompass slow release systems.

The composition(s) of the pharmaceutical preparation of the present invention is/are administered to patients in a therapeutically effective dose, meaning a dose that is sufficient to produce the desired effects, preventing or lessening the severity or spread of the condition or indication being treated without reaching a dose which produces intolerable adverse side effects. The exact dose depends on many factors as e.g. the indication, formulation, mode of administration and has to be determined in preclinical and clinical trials for each respective indication.

In one embodiment of the invention, the plasma level of the coagulation factor in the treated subject is, during a period from 5 hours after injection to 8 hours after non-intravenous injection, continuously higher than 2%, preferably higher than 5%, more preferably higher than 8%, most preferably higher than 10%, of the normal plasma level of the coagulation factor in healthy subjects. The plasma level is to be determined as shown hereinafter in Example 3.

In one embodiment of the invention, the plasma level of the coagulation factor in the treated subject is, during a period from 4 hours after injection to 16 hours after non-intravenous injection, continuously higher than 2%, preferably higher than 5%, more preferably higher than 8%, most preferably higher than 10%, of the normal plasma level of the coagulation factor in healthy subjects.

In another embodiment of the invention, the plasma level of the coagulation factor in the treated subject is, during a period from 3 hours after injection to 24 hours after non-intravenous injection, continuously higher than 2%, preferably higher than

4%, more preferably higher than 6%, most preferably higher than 8%, of the normal plasma level of the coagulation factor in healthy subjects.

In another embodiment of the invention, the plasma level of the coagulation factor in the treated subject is, during a period from 2 hours after injection to 32 hours after non-intravenous injection, continuously higher than 2%, preferably higher than 3%, more preferably higher than 4%, most preferably higher than 5%, of the normal plasma level of the coagulation factor in healthy subjects.

Preferably, the dose of single-chain Factor VIII for one non-intravenous injection is less than 1,000 IU/kg body weight, or less than 800 IU/kg body weight, or less than 600 IU/kg body weight, or less than 400 IU/kg body weight, e.g. at a dose of from about 10 IU/kg body weight to about 1,000 IU/kg body weight, or from about 20 IU/kg body weight to about 800 IU/kg body weight, or from about 30 IU/kg body weight to about 700 IU/kg body weight, or from about 40 IU/kg body weight to about 600 IU/kg body weight, or from about 50 IU/kg body weight to about 500 IU/kg body weight, or from about 75 IU/kg body weight to about 400 IU/kg body weight, or from about 100 IU/kg body weight to about 300 IU/kg body weight, or from about 50 IU/kg body weight to about 1,000 IU/kg body weight, or from about 50 IU/kg body weight to about 800 IU/kg body weight, or from about 50 IU/kg body weight to about 700 IU/kg body weight, or from about 50 IU/kg body weight to about 600 IU/kg body weight, or from about 50 IU/kg body weight to about 500 IU/kg body weight, or from about 50 IU/kg body weight to about 400 IU/kg body weight, or from about 50 IU/kg body weight to about 300 IU/kg body weight, or about 50 IU/kg body weight to about 200 IU/kg body weight. The FVIII can be administered on its own, or as a complex with VWF.

The pharmaceutical composition(s) of the invention may be administered alone or in conjunction with other therapeutic agents. These agents may be incorporated as part of the same pharmaceutical.

Examples

Example 1: Stability of purified Factor VIII molecules after reconstitution

The following Factor VIII preparations were used in this Example:

Beriate®, a lyophilized human coagulation Factor VIII concentrate, was obtained from CSL Behring GmbH. Beriate® comprises plasma-derived Factor VIII in heterodimeric form.

Helixate®, a lyophilized, recombinant coagulation Factor VIII was obtained from CSL Behring GmbH. Helixate® contains recombinantly produced heterodimeric Factor VIII.

ReFacto® is a lyophilized Factor VIII preparation containing heterodimeric, B-domain-deleted Factor VIII produced by recombinant technology. It can be obtained from, e.g., Pfizer Pharma GmbH, Germany.

Beriate®, Helixate®, and ReFacto® are predominantly heterodimeric two-chain polypeptides.

The construct termed "scFVIII" is a single-chain Factor VIII produced by recombinant expression in mammalian cell culture cells. The single-chain Factor VIII used in this Example was obtained by directly fusing Asn764 with Thr1653, and provided in lyophilized form after purification. That is, "scFVIII" is a single chain polypeptide consisting substantially of amino acids 1-764 and 1653-2332 of SEQ ID NO:2.

Beriate®, Helixate®, and ReFacto® were reconstituted according the manufacturer's instructions as given in the package insert. "scFVIII" was reconstituted by dissolving the purified and lyophilized FVIII preparation in water for injection resulting in a composition containing 25 mM L-histidine, 225 mM NaCl, 4 mM $CaCl_2$, 0.03 % Tween 80, 2% sucrose, 8% D-mannitol, pH 7.0.

The reconstituted FVIII products were incubated at 25°C. The FVIII activity of the products was determined in duplicates by a chromogenic substrate assay (Coamatic® Factor VIII, Chromogenix) at the following time points: 0 h, 6h, 1day, 2 days, 4 days, 7 days. Activity values were normalized to time point 0.

The results are shown in the following Table and in Figure 1.

Table 1: Factor VIII activity over time

Time (days)	0 d	0.25 d	1 d	2 d	4 d	7 d
Beriate®	100.0	103.8	92.7	97.9	90.0	82.7
Helixate®	100.0	101.4	103.3	95.5	85.0	78.6
ReFacto®	100.0	106.7	83.7	94.0	93.6	77.3
scFVIII	100.0	107.8	99.4	102.0	99.0	93.3

As can be seen, "scFVIII" shows the lowest loss in activity and, consequently, is the most stable Factor VIII molecule.

Example 2: Stability of purified Factor VIII molecules after reconstitution

The following Factor VIII preparations were used in this Example:

ReFacto® and "scFVIII" were the same as used in Example 1, with the difference that “scFVIII”, provided by CSL Behring GmbH, was applied in a formulation containing different excipients. Advate® is a full-length, heterodimeric, recombinant Factor VIII preparation which was purchased in lyophilized form from Baxter.

Advate® and ReFacto® were reconstituted according the manufacturer's instructions as given in the package insert. "scFVIII" was reconstituted in water for injection resulting in a composition containing 20 mM L-histidine, 280 mM NaCl, 3.4 mM $CaCl_2$, 0.02 % Tween 80, 0.6% sucrose, pH 7.0. The sample "scFVIII 001" had an initial FVIII activity of 100 IU/ml, the sample "scFVIII 0006" had an initial FVIII activity of 400 IU/ml. The reconstituted FVIII products were incubated at 25°C. The FVIII activity of the products was determined in duplicates by a chromogenic substrate assay (Coamatic® Factor VIII, Chromogenix) at the following time points: 0 h, 6h, 1day, 2 days, 4 days, 8 days. Activity values were normalized to time point 0.

The results are shown in the following Table and in Figure 2.

Table 2: Factor VIII activity over time

Time (days)	0 d	0.25 d	1 d	2 d	4 d	8 d
Advate	100.0	94.9	100.6	89.8	74.1	73.7
ReFacto	100.0	92.0	88.8	92.1	74.3	80.6
scFVIII 001	100.0	99.2	95.6	99.2	89.0	95.1
scFVIII 006	100.0	96.8	97.6	100.0	88.9	92.3

As can be seen, "scFVIII" shows the lowest loss in activity and, consequently, is the most stable Factor VIII molecule.

Example 3: Bioavailability of Factor VIII molecules

Advate®, ReFacto® and "scFVIII" were the same as used in Example 2 and reconstituted as described in Example 2.

Factor VIII knockout mice were used as animal model for hemophilia A. These mice lack exons 16 and 17 and thus do not express FVIII (Bi L. et al, Nature genetics,

1995, Vol 10(1), 119-121; Bi L. et al, Blood, 1996, Vol 88(9), 3446-3450). This allows the analysis of FVIII levels following treatment by quantification of FVIII activity in the plasma of the ko mice.

To assess whether extravascular injections might be an option for an improved therapy with human FVIII, subcutaneous injection was chosen. The design of the non-clinical pharmacokinetic study performed is detailed in table 3 below. Plasma levels of Factor VIII activity were determined following a single subcutaneous injection of the respective FVIII preparation (detailed treatment groups in table 3) to a hemophilia A model.

Corresponding groups were treated with the same dose of FVIII:chromogen activity. For a single application the Factor VIII was provided in a volume of 200 µL (identical volumes for all groups) prior to subcutaneous application to FVIII knockout (ko) mice weighing about 25 g. The treatment groups are summarized in table 3.

Under short term anesthesia, blood samples were drawn, anticoagulated using sodium citrate to 10 % citrate blood, processed to plasma and stored at –70°C for the determination of FVIII activity. The sampling time points are detailed in table 4. Quantification of FVIII activity in plasma was performed by a standard, aPTT based approach (Behring Coagulation Timer). The animals were kept at standard housing conditions.

Table 3: Treatment groups

No.	Treatment	FVIII:chromogen / Additive Dose	volume [mL/kg]	schedule	route	N
1	Advate®	400 IU/kg	8	single injection (t=0)	s.c.	25
2	ReFacto®	400 IU/kg	8	single injection (t=0)	s.c.	25
3	"scFVIII"	400 IU/kg	8	single injection (t=0)	s.c.	20

Results

The results are summarized in Table 4 and Figure 3.

Table 4:

timepoint [hours]	scFVIII			ReFacto®			Advate®		
	mean	SD	n	mean	SD	n	mean	SD	n
1	5.83	1.84	5	6.35	7.62	5	3.82	3.30	5
4	11.53	5.35	5	4.77	3.47	5	3.16	2.72	5
8	20.03	9.91	5	9.81	6.06	5	5.52	3.92	5
16	7.75	4.78	5	4.91	1.98	5	2.77	2.10	5
24	3.00	3.25	5	0.66	0.69	5	0.31	0.68	5
32	3.30	2.36	5	2.11	1.53	5	3.13	1.78	5
48	3.71	1.52	5	6.01	5.28	5	4.15	1.95	5
72	1.23	2.63	5	0.00	0.00	5	0.00	0.00	5
AUC_{0-72h} (h × % of the norm)	**383.9**			**275.1**			**195.1**		

Subcutaneous injection of 400 IU/kg single chain FVIII ("scFVIII") to FVIII ko mice resulted in a significant increase of FVIII activity in plasma level as compared to administration of heterodimeric full length FVIII (Advate®) or heterodimeric B-

domain-deleted FVIII (ReFacto®). That is, the single chain Factor VIII molecule shows the highest in vivo bioavailability after subcutaneous injection into mice. The two chain full length construct Advate®, as well as the two chain B-domain deleted preparation ReFacto® showed substantially lower bioavailability.

Example 4: Stability of Factor VIII molecules in plasma (in-vitro)

Different FVIII products (Advate®, ReFacto AF® and two lots of scFVIII as used in Example 2) were diluted with FVIII deficient plasma (Siemens Healthcare Diagnostics) to 1 IU/mL FVIII:C (based on values determined by the chromogenic substrate assay). The FVIII- samples were incubated at 37°C for varying time periods (0, 0.25, 1, 2, 4 and 8 days) in presence of 0.05% Na-azide. After each incubation period, FVIII:C was determined by one-stage-coagulation assay using Pathromtin-SL (Siemens Healthcare Diagnostics) as activator, normalized to the value at t= 0 (% FVIII:C) and plotted versus the incubation time. The values shown represent the average and standard deviation of two samples (except 0.25 days only one sample).

Table 5: Average % Activity Compared to Time 0

-	0 d	0,25 d	1 d	2 d	4 d	8 d
Advate	100,00	90,63	81,25	79,69	70,31	59,38
ReFacto	100,00	92,31	86,15	83,08	70,77	58,46
scFVIII 001	100,00	93,33	93,33	96,67	93,33	93,33
scFVIII 006	100,00	96,88	96,88	93,75	93,75	90,63

Example 5: Stability of Factor VIII molecules in plasma (in-vivo)

The pharmacokinetic (PK) profiles of scFVIII and full-length rFVIII (Advate®, Baxter Healthcare) was determined following a single I.V. injection to cynomolgus monkeys (Figure 5 and Table 6) and hemophilia A mice (Figure 6 and Table 7) at doses of 250 IU/kg and 100 IU/kg, respectively. Test items were dosed according to labeled activity for Advate® and chromogenic activity (FVIII:C) for scFVIII. Blood samples were drawn predose (monkeys only) and at various time points after administration until 72 hours (hrs) in hemophilia A mice and until 24 hrs in cynomolgus monkeys. Citrate plasma was prepared immediately and used for quantification of FVIII:C by a chromogenic assay system (FVIII:C) (Chromogenix - Instrumentation Laboratory SpA, Milan, Italy).

The AUC of the FVIII levels in plasma was calculated using the linear trapezoidal rule to calculate AUC_{last} : from t=0 to last observation. Terminal half-life ($t_{1/2\beta}$) was determined by a log-linear regression using the points of the terminal phase selected by the adjusted R2 criterion. AUC: from t=0 to infinity (extrapolated by using the regression model of the terminal phase).

In cynomolgous monkeys scFVIII showed a ~1.6 fold enhanced $AUC_{0\text{-}tlast}$ or $t_{1/2\beta}$ with a correspondingly ~2 fold lower clearance (CL), while FVIII activity peak levels (C_{max}), representative of in vivo recovery (IVR), and volume of distribution at steady state (V_{ss}) appeared more similar versus full-length rFVIII. These PK parameter results were obtained from n=10 animals after toxicokinetic data from 8 additional monkeys, when dosed during the GLP- toxicity studies with 250 IU/kg of scFVIII, were included (Table 6 and Figure 5).

In hemophilia A mice enhancement of $AUC_{0\text{-}tlast}$, of mean residence time (MRT), time until 5% FVIII activity trough levels, terminal half-life and a correspondingly lower CL ranged between 1.6-2 fold for scFVIII, while C_{max}, representative of IVR, and V_{ss} appeared similar versus full-length rFVIII. $AUC_{0\text{-}tlast}$ and $t_{1/2\beta}$ results

obtained after rVIII-SingleChain treatment were significantly better than for full-length rFVIII with an $AUC_{0\text{-}tlast}$ ratio of 1.97 (90% confidence interval (CI): 1.7-2.3; p-value(ratio=1):< 0.0001), and a $t_{1/2\beta}$ ratio of 1.65 (90% CI: 1.11-2.70; p-value(ratio=1): 0.036 (Table 7 and Figure 6).

Table 6: PK parameters of scFVIII and full-length rFVIII in cynomolgus monkeys

Parameters	scFVIII (n=10)	Full-length rFVIII (n=2)
$AUC_{0\text{-}tlast}$ (hrs·IU/mL)	78.4	49.1
C_{max} (IU/mL)	7.8	8.7
CL ((mL/hrs)/kg)	2.1	4.7
$t_{1/2\beta}$ (hrs)	11.0	6.8

Table 7: PK parameters of scFVIII and full-length rFVIII in hemophilia A mice

Parameters	scFVIII	Full-length rFVIII
$AUC_{0\text{-last}}$ (hrs·IU/mL)	35	18
C_{max} (IU/mL)	2.3	2.2
CL ((mL/hrs)/kg)	2.7	5.5
MRT (hrs)	18	10
V_{SS} (mL/kg)	50	57
$t_{1/2\beta}$ (hrs)	15.9	9.7
Time until 0.05 IU/mL (hrs)	73	39

Both sets of PK parameters reflect the increased stability of scFVIII after purification, lyophilization, reconstitution *in vivo* in plasma after administration to the two animal species tested.

Example 6: Thrombin generation assay in hemophilia A mice (ex vivo)

Citrate- (10% v/v) hemophilia A mouse blood was terminally collected under deep anesthesia at different time-points (days 1-8) when scFVIII or full-length rFVIII (Advate®), were dosed @ a level of 250 IU/kg. TGA was performed by calibrated thrombinography (CAT, Thrombinoscope, Netherlands) after intrinsic activation in presence of Phospholipid (Rossix, Mölndal, Sweden) / Pathromtin® SL (Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany) (1:30). Thrombin

peak levels were recorded. The average AUC of peak thrombin levels from days1-8 was calculated by the linear trapezoidal rule. The AUC of the two Factor VIII products were compared using an approximate F-test for the difference in AUC in a linear model with variable variances per time-point and treatment group resulting in estimated time until peak levels of thrombin drop below a defined limit ranging between 50-250 nM.

In hemophilia A mice scFVIII showed a favorable hemostatic activity compared to full-length rFVIII as indicated by the estimated time until peak levels of thrombin drop below a defined limit ranging from 50-250 nm peak level (Figure 8 and Table 8). This translated into an averaged 20 hrs longer thrombin generation activity value for scFVIII versus full-length rFVIII for the thrombin peak level interval between 50 and 250 nM. When assessing the area under the peak curve between days 1-8 the thrombin generation activity of scFVIII was significantly better with $p(AUC_{TGA\ Peak}$-ratio=1) = 0.0002 (estimated ratio 1.26, 90% CI: 1.14-1.39) compared to full-length FVIII, or in other words it took significantly longer for scFVIII to fall below a thrombin peak level of 50nm after administration than for the human wild-type Factor VIII Advate®.

These results again confirmed the increased functional stability of scFVIII after purification, lyophilization and reconstitution.

Table 8: Peak nm Thrombin

	CSL627 (N=8-14)		Advate (N=7-14)	
time [hrs]	mean	SD	mean	SD
0	0	0	0	0
24	325,5	40,2	343,4	40,41
32	294	102,8	305,2	72,13
48	277,1	22,44	279,6	30,92
72	283,1	42,88	163,6	79,87
96	115,4	29,63	75,44	35,28
120	91,85	57,63	55,17	30,89
144	45,6	36,15	24,61	13,52
168	8,453	12,66	14,13	19,44
192	3,901	9,384	4,71	8,1

Example 6: Stability of Factor VIII molecules in vWF deficient plasma (in-vivo)

scFVIII was reconstituted in 2.5 mL water for injection. ReFacto AF® and Advate® were reconstituted according to the description in the package insert. All test articles were aliquoted and stored immediately frozen at approximately -70°C. Prior to administration test articles were diluted with formulation buffer for CSL 627 to get a minimum practical volume ensuring a reliable administration.

12 VWF ko mice (6 female/6 male) per group received a single i.v. injection of 100 IU/kg of either scFVIII based on chromogenic FVIII activity and ReFacto AF® or Advate® based on the labeled FVIII activity into the lateral tail vein. Following administration of the different test items blood samples were drawn for determination of FVIII plasma levels at 0.083, 0.5, 1, 2, 4, 7, 16 and 24 hours from n= 2-3 mice per time point. Blood samples were processed to 10% citrate (3.13% w/v) plasma and subsequently subjected to FVIII plasma level analysis using the

chromogenic assays system The chromogenic FVIII activity was determined using the COAMATIC® FVIII test kit from Chromogenix, Italy.

The AUC of the FVIII levels in plasma was calculated using the linear trapezoidal rule to calculate AUClast : from t=0 to last observation.

Likewise to results obtained after i.v. administration to FVIII ko mice and normal monkeys as well as s.c. administration to FVIII ko mice analysis the exposure to CSL627 was higher compared to ReFacto AF® and Advate®. Since analysis of the AUC, the most relevant and representative PK parameter for systemic exposure yielded a 30 % higher AUC value after administration of CSL627 compared to both ReFacto AF® and Advate®. Again. these observations reflect the increased intrinsic stability of scFVIII after purification, lyophilization, reconstitution in vivo in plasma after administration to mice lacking systemic, circulating VWF, hence in absence of its shielding and protective effect for systemic, circulating FVIII.

Table 9: Plasma levels of scFVIII compared to Advate® and ReFacto AF® after administration to VWF deficient mice at a dose level of 100 IU/kg

	CSL627	Advate®	ReFacto AF®
Baseline	0,0	0,0	0,0
Total Area	2427	1654	1714
Total Peak Area	2427	1654	1714
Number of Peaks	1,000	1,000	1,000
Peak 1			
First X=	0,0830	0,0830	0,0830
Last X=	24,00	24,00	24,00
Peak X=	0,0830	0,0830	0,0830
Peak Y=	1770	1131	1248
Area under curve=	2427	1654	1714
%Area=	100,0	100,0	100,0

The claims defining the invention are as follows:

1. A method for improving the plasma half-life of a Factor VIII molecule after intravenous or non-intravenous administration relative to human wild-type Factor VIII, comprising fusing a first amino acid selected from the amino acids at positions 740 to 1647 of the Factor VIII sequence with a second amino acid selected from the amino acids at positions 1649 to 1690 of the Factor VIII sequence, whereby the proteolytic cleavage site between Arg1648 and Glu1649, and, if present in the FVIII molecule, the cleavage site between Arg1313 and Ala1314 is inactivated.

2. The method of claim 1, wherein the inactivation comprises deleting at least the amino acid sequence from Arg1313 to Arg1648 from the Factor VIII sequence.

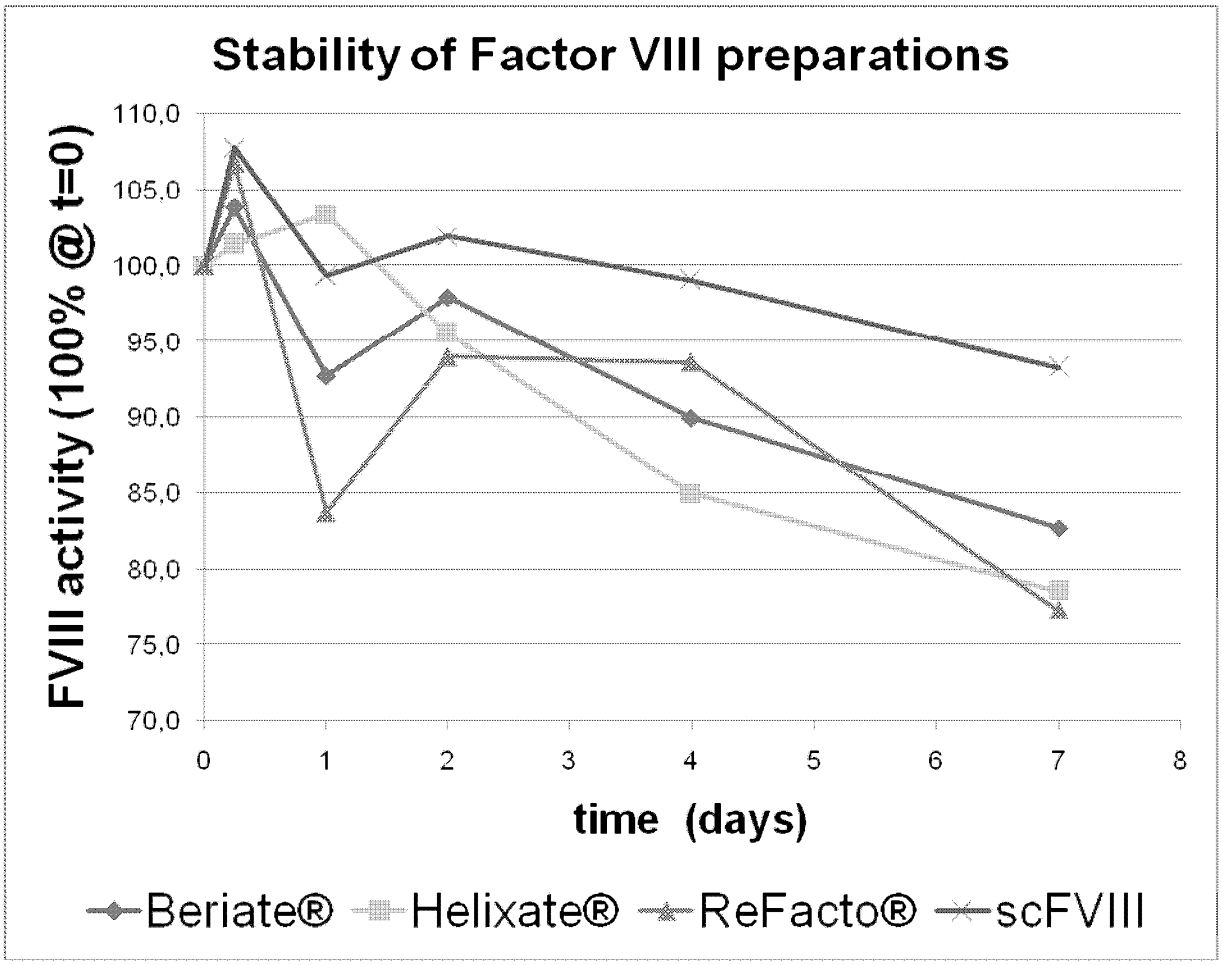
3. The method of claim 1 or claim 2, wherein
   - amino acid 740 is fused to amino acid 1650, whereby amino acids 741 to 1649 are deleted;
   - amino acid 740 is fused to amino acid 1690, whereby amino acids 741 to 1689 are deleted;
   - amino acid 740 is fused to amino acid 1669, whereby amino acids 741 to 1668 are deleted;
   - amino acid 743 is fused to amino acid 1650, whereby amino acids 744 to 1649 are deleted;
   - amino acid 764 is fused to amino acid 1650, whereby amino acids 765 to 1649 are deleted;
   - amino acid 764 is fused to amino acid 1653, whereby amino acids 765 to 1652 are deleted;
   - amino acid 764 is fused to amino acid 1656, whereby amino acids 765 to 1655 are deleted;
   - amino acid 745 is fused to amino acid 1650, whereby amino acids 746 to 1649 are deleted;
   - amino acid 745 is fused to amino acid 1653, whereby amino acids 746 to 1652 are deleted;
   - amino acid 745 is fused to amino acid 1656, whereby amino acids 746 to 1655 are deleted;

2012318292 07 Jul 2015

- amino acid 757 is fused to amino acid 1650, whereby amino acids 758 to 1649 are deleted;
- amino acid 757 is fused to amino acid 1653, whereby amino acids 758 to 1652 are deleted;
- amino acid 757 is fused to amino acid 1656, whereby amino acids 758 to 1655 are deleted;
- amino acid 793 is fused to amino acid 1649, whereby amino acids 794 to 1648 are deleted;
- amino acid 793 is fused to amino acid 1690, whereby amino acids 794 to 1689 are deleted;
- amino acid 747 is fused to amino acid 1649, whereby amino acids 748 to 1648 are deleted;
- amino acid 751 is fused to amino acid 1649, whereby amino acids 752 to 1648 are deleted;
- amino acid 776 is fused to amino acid 1649, whereby amino acids 777 to 1648 are deleted; or
- amino acid 770 is fused to amino acid 1667, whereby amino acids 771 to 1666 are deleted.

4. The method of any preceding claim, substantially as described herein.

Figure 1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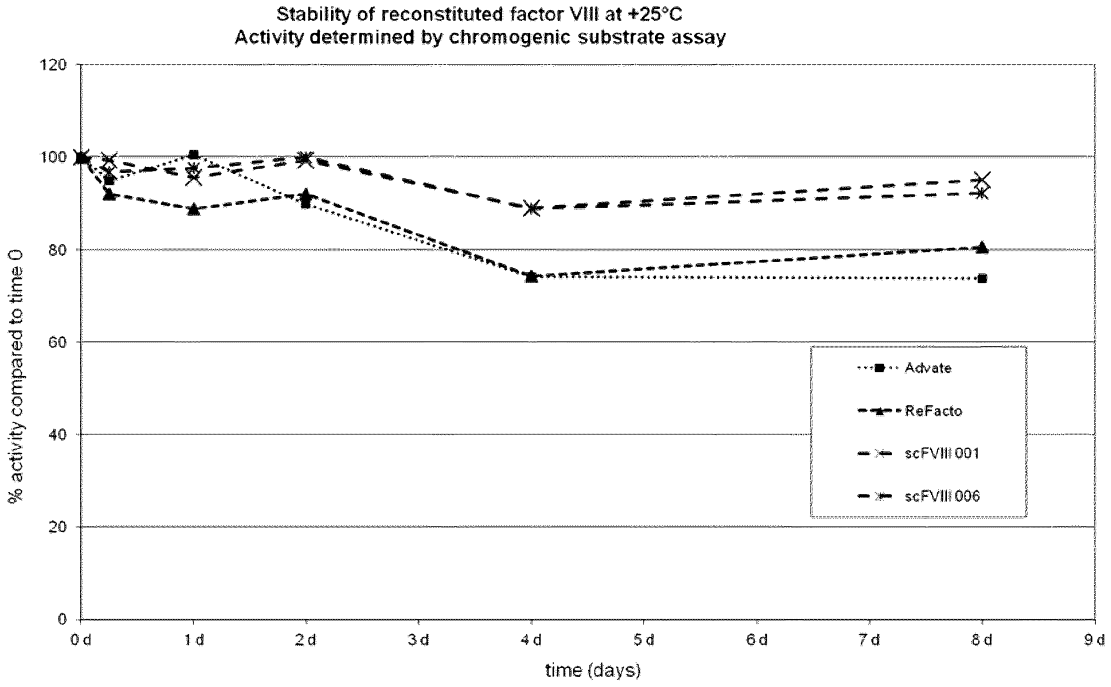
time (days)
Beriate®
Helixate®
ReFacto®
scFVIII

Figure 2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Advate
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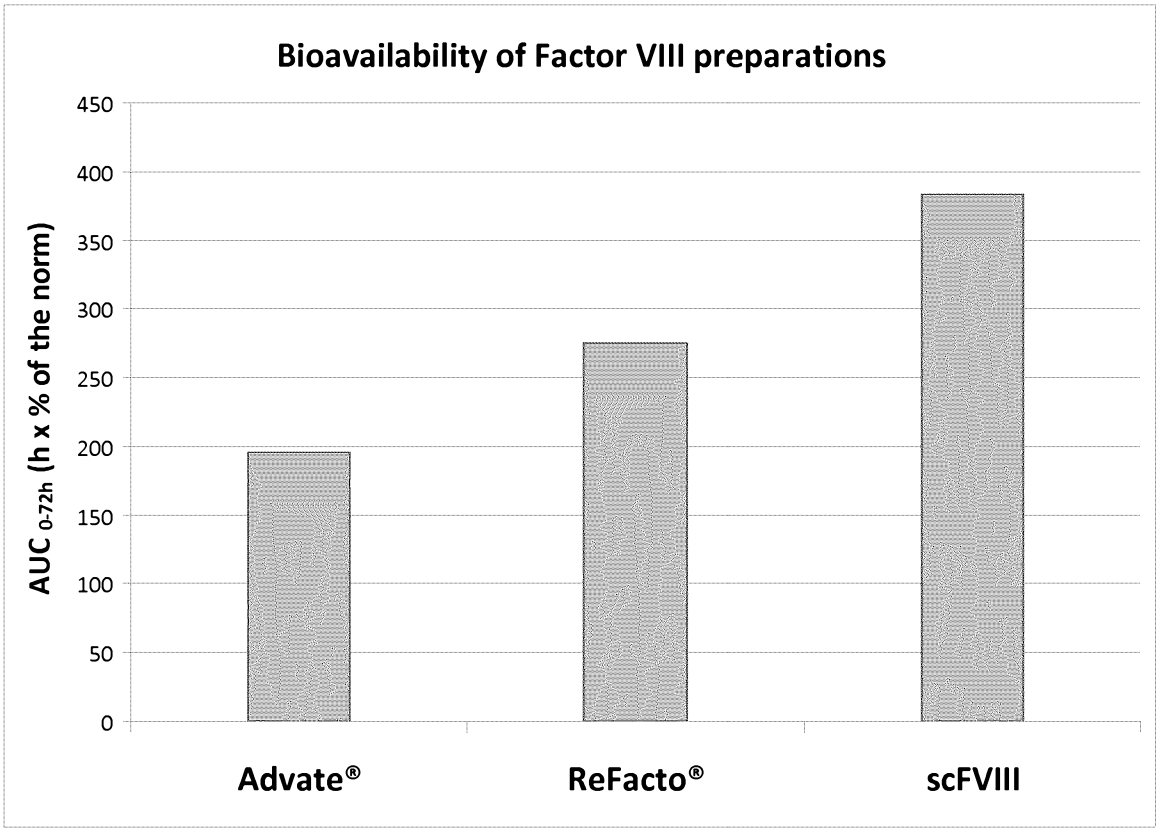
scFVIII 006

Figure 3


Bioavailability of Factor VIII preparations
AUC 0-72h (h x % of the norm)
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400
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300
250
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150
100
50
0
Advate®
ReFacto®
scFVIII

Figure 4

Stability of FVIII molecules in plasma (ex-vivo)

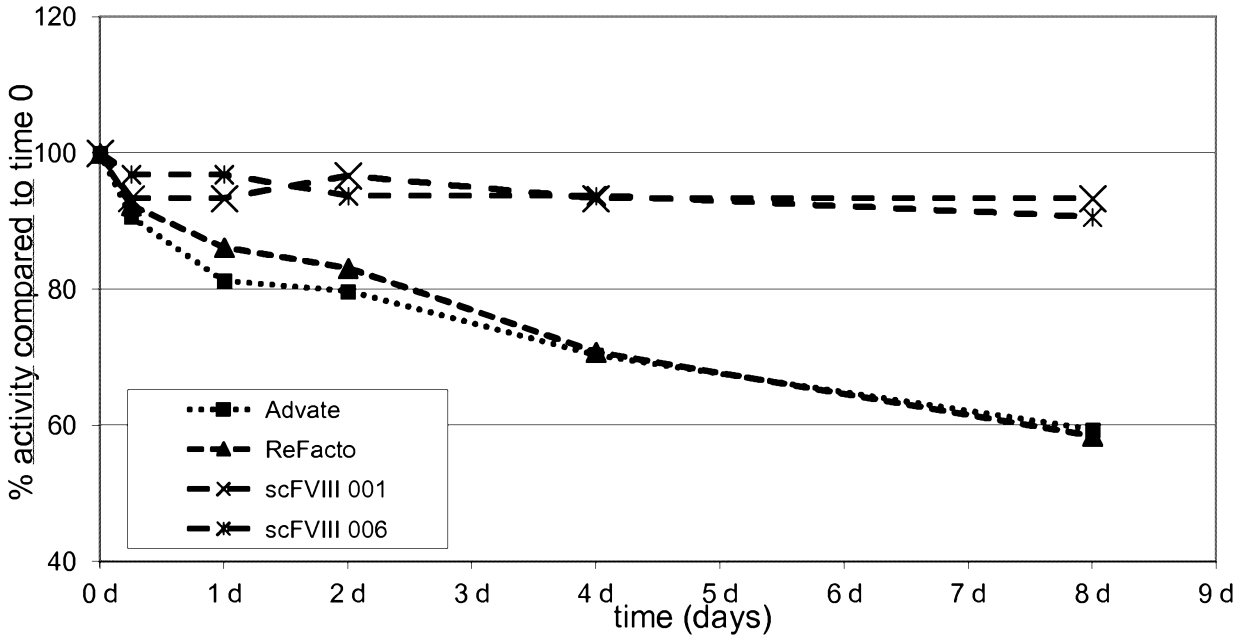


Figure 5

Stability of FVIII molecules in plasma (in-vivo) of cynomolgus monkeys (dosed at 250 IU/kg)

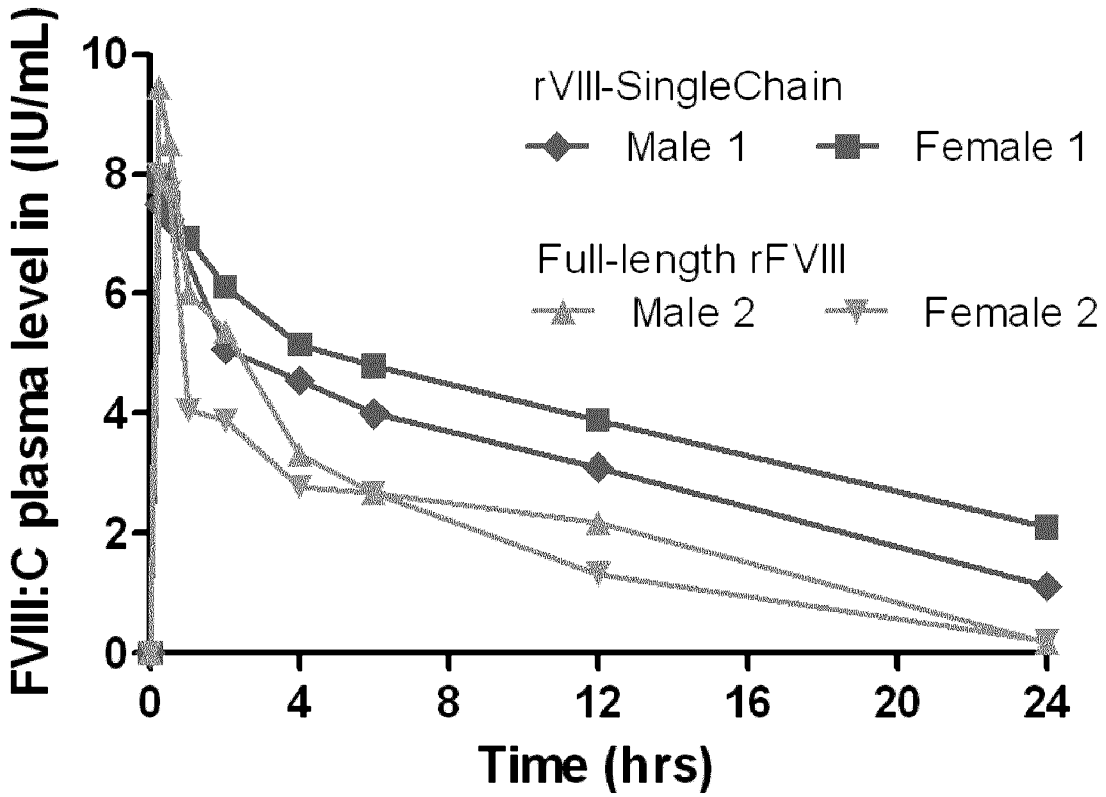


Figure 6

Stability of FVIII molecules in plasma (in-vivo) of in hemophilia A mice (dosed at 100 IU/kg)

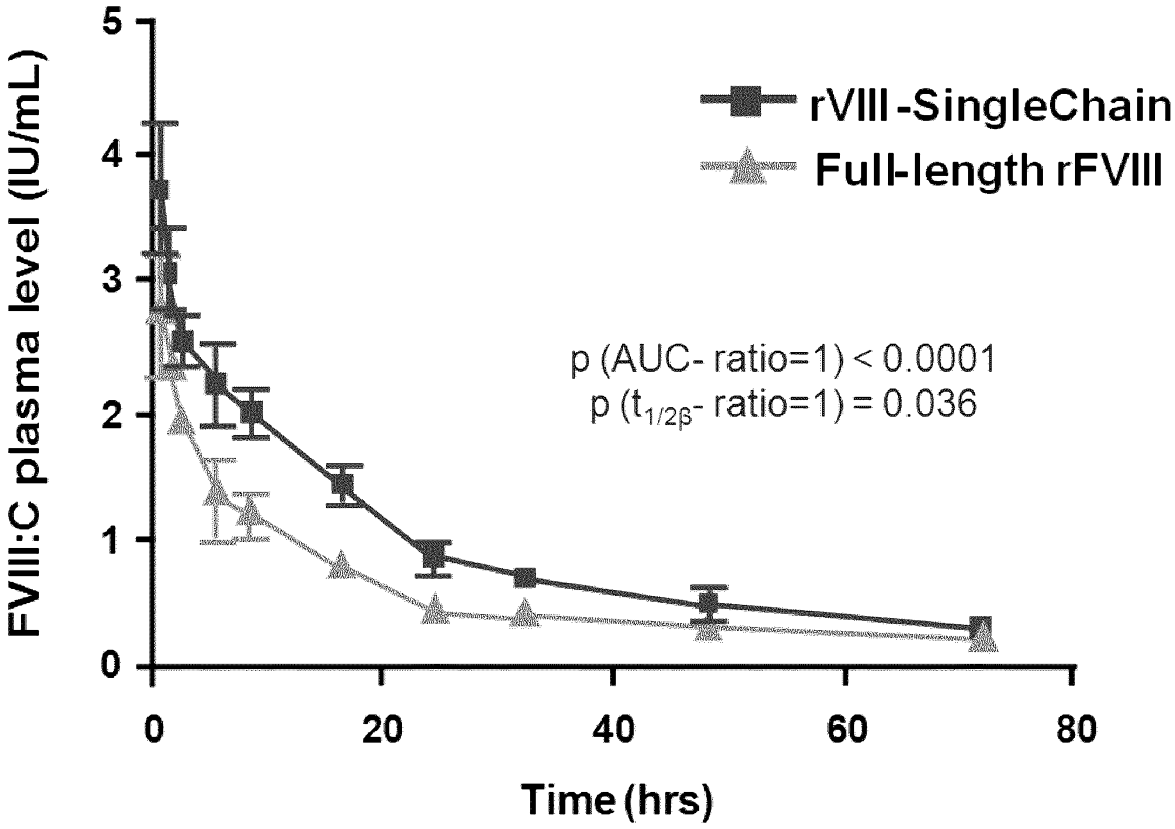


Figure 7

Thrombin generation in hemophilia A mice (ex vivo)

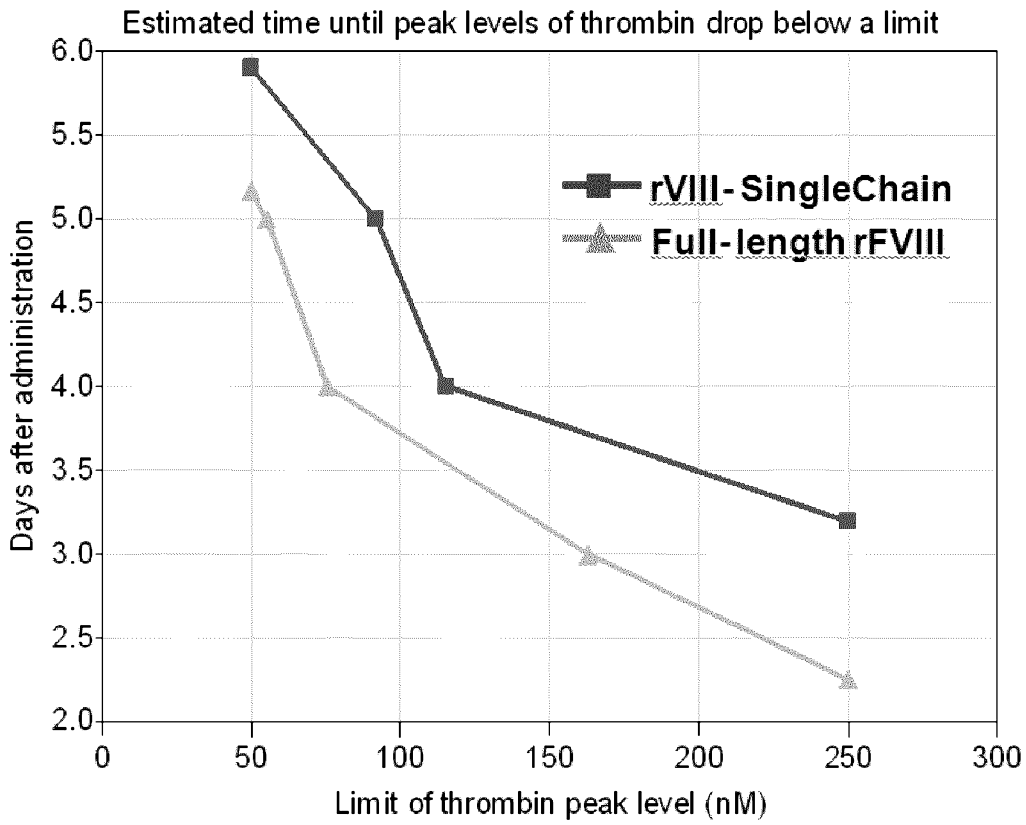
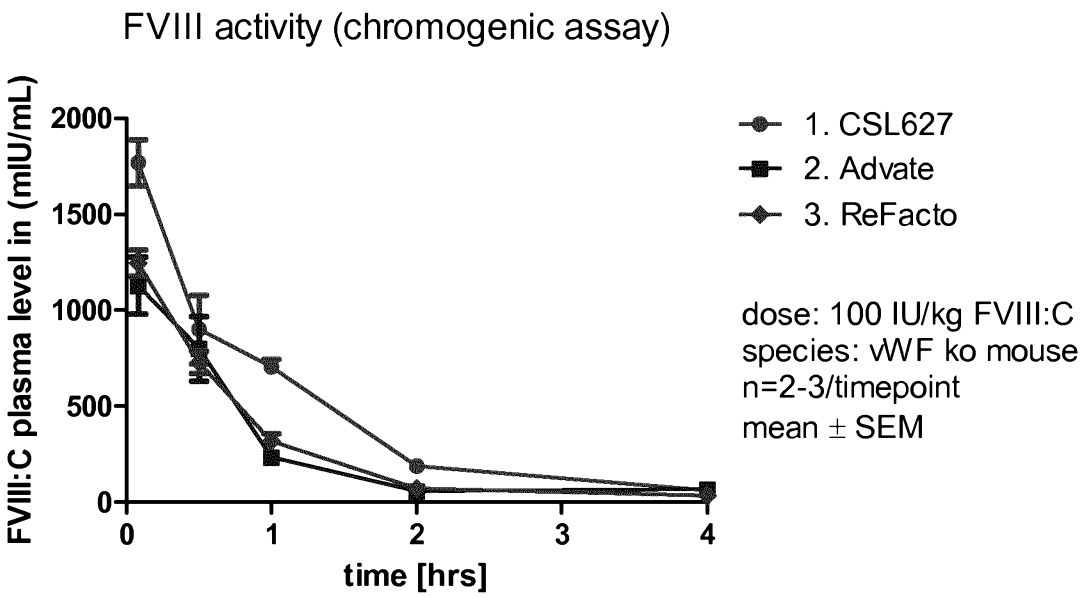


Figure 8

Stability of FVIII molecules in plasma (in-vivo) of vWF deficient mice



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<151> 2011-10-18

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            20                  25                  30

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Arg Val Pro Lys Ser Phe Pro Phe Asn Thr Ser Val Val Tyr Lys Lys
        35                  40                  45

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    50                  55                  60

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Arg Pro Pro Trp Met Gly Leu Leu Gly Pro Thr Ile Gln Ala Glu Val
65                  70                  75                  80

tat gat aca gtg gtc att aca ctt aag aac atg gct tcc cat cct gtc         288
Tyr Asp Thr Val Val Ile Thr Leu Lys Asn Met Ala Ser His Pro Val
                85                  90                  95

agt ctt cat gct gtt ggt gta tcc tac tgg aaa gct tct gag gga gct         336
Ser Leu His Ala Val Gly Val Ser Tyr Trp Lys Ala Ser Glu Gly Ala
            100                 105                 110

gaa tat gat gat cag acc agt caa agg gag aaa gaa gat gat aaa gtc         384
Glu Tyr Asp Asp Gln Thr Ser Gln Arg Glu Lys Glu Asp Asp Lys Val
        115                 120                 125

ttc cct ggt gga agc cat aca tat gtc tgg cag gtc ctg aaa gag aat         432
Phe Pro Gly Gly Ser His Thr Tyr Val Trp Gln Val Leu Lys Glu Asn
    130                 135                 140

ggt cca atg gcc tct gac cca ctg tgc ctt acc tac tca tat ctt tct         480
Gly Pro Met Ala Ser Asp Pro Leu Cys Leu Thr Tyr Ser Tyr Leu Ser
145                 150                 155                 160
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cat gtg gac ctg gta aaa gac ttg aat tca ggc ctc att gga gcc cta        528
His Val Asp Leu Val Lys Asp Leu Asn Ser Gly Leu Ile Gly Ala Leu
                165                 170                 175

cta gta tgt aga gaa ggg agt ctg gcc aag gaa aag aca cag acc ttg        576
Leu Val Cys Arg Glu Gly Ser Leu Ala Lys Glu Lys Thr Gln Thr Leu
            180                 185                 190

cac aaa ttt ata cta ctt ttt gct gta ttt gat gaa ggg aaa agt tgg        624
His Lys Phe Ile Leu Leu Phe Ala Val Phe Asp Glu Gly Lys Ser Trp
        195                 200                 205

cac tca gaa aca aag aac tcc ttg atg cag gat agg gat gct gca tct        672
His Ser Glu Thr Lys Asn Ser Leu Met Gln Asp Arg Asp Ala Ala Ser
    210                 215                 220

gct cgg gcc tgg cct aaa atg cac aca gtc aat ggt tat gta aac agg        720
Ala Arg Ala Trp Pro Lys Met His Thr Val Asn Gly Tyr Val Asn Arg
225                 230                 235                 240

tct ctg cca ggt ctg att gga tgc cac agg aaa tca gtc tat tgg cat        768
Ser Leu Pro Gly Leu Ile Gly Cys His Arg Lys Ser Val Tyr Trp His
                245                 250                 255

gtg att gga atg ggc acc act cct gaa gtg cac tca ata ttc ctc gaa        816
Val Ile Gly Met Gly Thr Thr Pro Glu Val His Ser Ile Phe Leu Glu
            260                 265                 270

ggt cac aca ttt ctt gtg agg aac cat cgc cag gcg tcc ttg gaa atc        864
Gly His Thr Phe Leu Val Arg Asn His Arg Gln Ala Ser Leu Glu Ile
        275                 280                 285

tcg cca ata act ttc ctt act gct caa aca ctc ttg atg gac ctt gga        912
Ser Pro Ile Thr Phe Leu Thr Ala Gln Thr Leu Leu Met Asp Leu Gly
    290                 295                 300

cag ttt cta ctg ttt tgt cat atc tct tcc cac caa cat gat ggc atg        960
Gln Phe Leu Leu Phe Cys His Ile Ser Ser His Gln His Asp Gly Met
305                 310                 315                 320

gaa gct tat gtc aaa gta gac agc tgt cca gag gaa ccc caa cta cga       1008
Glu Ala Tyr Val Lys Val Asp Ser Cys Pro Glu Glu Pro Gln Leu Arg
                325                 330                 335

atg aaa aat aat gaa gaa gcg gaa gac tat gat gat gat ctt act gat       1056
Met Lys Asn Asn Glu Glu Ala Glu Asp Tyr Asp Asp Asp Leu Thr Asp
            340                 345                 350

tct gaa atg gat gtg gtc agg ttt gat gat gac aac tct cct tcc ttt       1104
Ser Glu Met Asp Val Val Arg Phe Asp Asp Asp Asn Ser Pro Ser Phe
        355                 360                 365

atc caa att cgc tca gtt gcc aag aag cat cct aaa act tgg gta cat       1152
Ile Gln Ile Arg Ser Val Ala Lys Lys His Pro Lys Thr Trp Val His
    370                 375                 380

tac att gct gct gaa gag gag gac tgg gac tat gct ccc tta gtc ctc       1200
Tyr Ile Ala Ala Glu Glu Glu Asp Trp Asp Tyr Ala Pro Leu Val Leu
385                 390                 395                 400

gcc ccc gat gac aga agt tat aaa agt caa tat ttg aac aat ggc cct       1248
Ala Pro Asp Asp Arg Ser Tyr Lys Ser Gln Tyr Leu Asn Asn Gly Pro
                405                 410                 415

cag cgg att ggt agg aag tac aaa aaa gtc cga ttt atg gca tac aca       1296
Gln Arg Ile Gly Arg Lys Tyr Lys Lys Val Arg Phe Met Ala Tyr Thr
            420                 425                 430
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gat gaa acc ttt aag act cgt gaa gct att cag cat gaa tca gga atc       1344
Asp Glu Thr Phe Lys Thr Arg Glu Ala Ile Gln His Glu Ser Gly Ile
        435                 440                 445

ttg gga cct tta ctt tat ggg gaa gtt gga gac aca ctg ttg att ata       1392
Leu Gly Pro Leu Leu Tyr Gly Glu Val Gly Asp Thr Leu Leu Ile Ile
    450                 455                 460

ttt aag aat caa gca agc aga cca tat aac atc tac cct cac gga atc       1440
Phe Lys Asn Gln Ala Ser Arg Pro Tyr Asn Ile Tyr Pro His Gly Ile
465                 470                 475                 480

act gat gtc cgt cct ttg tat tca agg aga tta cca aaa ggt gta aaa       1488
Thr Asp Val Arg Pro Leu Tyr Ser Arg Arg Leu Pro Lys Gly Val Lys
                485                 490                 495

cat ttg aag gat ttt cca att ctg cca gga gaa ata ttc aaa tat aaa       1536
His Leu Lys Asp Phe Pro Ile Leu Pro Gly Glu Ile Phe Lys Tyr Lys
            500                 505                 510

tgg aca gtg act gta gaa gat ggg cca act aaa tca gat cct cgg tgc       1584
Trp Thr Val Thr Val Glu Asp Gly Pro Thr Lys Ser Asp Pro Arg Cys
        515                 520                 525

ctg acc cgc tat tac tct agt ttc gtt aat atg gag aga gat cta gct       1632
Leu Thr Arg Tyr Tyr Ser Ser Phe Val Asn Met Glu Arg Asp Leu Ala
    530                 535                 540

tca gga ctc att ggc cct ctc ctc atc tgc tac aaa gaa tct gta gat       1680
Ser Gly Leu Ile Gly Pro Leu Leu Ile Cys Tyr Lys Glu Ser Val Asp
545                 550                 555                 560

caa aga gga aac cag ata atg tca gac aag agg aat gtc atc ctg ttt       1728
Gln Arg Gly Asn Gln Ile Met Ser Asp Lys Arg Asn Val Ile Leu Phe
                565                 570                 575

tct gta ttt gat gag aac cga agc tgg tac ctc aca gag aat ata caa       1776
Ser Val Phe Asp Glu Asn Arg Ser Trp Tyr Leu Thr Glu Asn Ile Gln
            580                 585                 590

cgc ttt ctc ccc aat cca gct gga gtg cag ctt gag gat cca gag ttc       1824
Arg Phe Leu Pro Asn Pro Ala Gly Val Gln Leu Glu Asp Pro Glu Phe
        595                 600                 605

caa gcc tcc aac atc atg cac agc atc aat ggc tat gtt ttt gat agt       1872
Gln Ala Ser Asn Ile Met His Ser Ile Asn Gly Tyr Val Phe Asp Ser
    610                 615                 620

ttg cag ttg tca gtt tgt ttg cat gag gtg gca tac tgg tac att cta       1920
Leu Gln Leu Ser Val Cys Leu His Glu Val Ala Tyr Trp Tyr Ile Leu
625                 630                 635                 640

agc att gga gca cag act gac ttc ctt tct gtc ttc ttc tct gga tat       1968
Ser Ile Gly Ala Gln Thr Asp Phe Leu Ser Val Phe Phe Ser Gly Tyr
                645                 650                 655

acc ttc aaa cac aaa atg gtc tat gaa gac aca ctc acc cta ttc cca       2016
Thr Phe Lys His Lys Met Val Tyr Glu Asp Thr Leu Thr Leu Phe Pro
            660                 665                 670

ttc tca gga gaa act gtc ttc atg tcg atg gaa aac cca ggt cta tgg       2064
Phe Ser Gly Glu Thr Val Phe Met Ser Met Glu Asn Pro Gly Leu Trp
        675                 680                 685

att ctg ggg tgc cac aac tca gac ttt cgg aac aga ggc atg acc gcc       2112
Ile Leu Gly Cys His Asn Ser Asp Phe Arg Asn Arg Gly Met Thr Ala
    690                 695                 700
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tta ctg aag gtt tct agt tgt gac aag aac act ggt gat tat tac gag        2160
Leu Leu Lys Val Ser Ser Cys Asp Lys Asn Thr Gly Asp Tyr Tyr Glu
705                 710                 715                 720

gac agt tat gaa gat att tca gca tac ttg ctg agt aaa aac aat gcc        2208
Asp Ser Tyr Glu Asp Ile Ser Ala Tyr Leu Leu Ser Lys Asn Asn Ala
                725                 730                 735

att gaa cca aga agc ttc tcc cag aat tca aga cac cgt agc act agg        2256
Ile Glu Pro Arg Ser Phe Ser Gln Asn Ser Arg His Arg Ser Thr Arg
            740                 745                 750

caa aag caa ttt aat gcc acc aca att cca gaa aat gac ata gag aag        2304
Gln Lys Gln Phe Asn Ala Thr Thr Ile Pro Glu Asn Asp Ile Glu Lys
        755                 760                 765

act gac cct tgg ttt gca cac aga aca cct atg cct aaa ata caa aat        2352
Thr Asp Pro Trp Phe Ala His Arg Thr Pro Met Pro Lys Ile Gln Asn
    770                 775                 780

gtc tcc tct agt gat ttg ttg atg ctc ttg cga cag agt cct act cca        2400
Val Ser Ser Ser Asp Leu Leu Met Leu Leu Arg Gln Ser Pro Thr Pro
785                 790                 795                 800

cat ggg cta tcc tta tct gat ctc caa gaa gcc aaa tat gag act ttt        2448
His Gly Leu Ser Leu Ser Asp Leu Gln Glu Ala Lys Tyr Glu Thr Phe
                805                 810                 815

tct gat gat cca tca cct gga gca ata gac agt aat aac agc ctg tct        2496
Ser Asp Asp Pro Ser Pro Gly Ala Ile Asp Ser Asn Asn Ser Leu Ser
            820                 825                 830

gaa atg aca cac ttc agg cca cag ctc cat cac agt ggg gac atg gta        2544
Glu Met Thr His Phe Arg Pro Gln Leu His His Ser Gly Asp Met Val
        835                 840                 845

ttt acc cct gag tca ggc ctc caa tta aga tta aat gag aaa ctg ggg        2592
Phe Thr Pro Glu Ser Gly Leu Gln Leu Arg Leu Asn Glu Lys Leu Gly
    850                 855                 860

aca act gca gca aca gag ttg aag aaa ctt gat ttc aaa gtt tct agt        2640
Thr Thr Ala Ala Thr Glu Leu Lys Lys Leu Asp Phe Lys Val Ser Ser
865                 870                 875                 880

aca tca aat aat ctg att tca aca att cca tca gac aat ttg gca gca        2688
Thr Ser Asn Asn Leu Ile Ser Thr Ile Pro Ser Asp Asn Leu Ala Ala
                885                 890                 895

ggt act gat aat aca agt tcc tta gga ccc cca agt atg cca gtt cat        2736
Gly Thr Asp Asn Thr Ser Ser Leu Gly Pro Pro Ser Met Pro Val His
            900                 905                 910

tat gat agt caa tta gat acc act cta ttt ggc aaa aag tca tct ccc        2784
Tyr Asp Ser Gln Leu Asp Thr Thr Leu Phe Gly Lys Lys Ser Ser Pro
        915                 920                 925

ctt act gag tct ggt gga cct ctg agc ttg agt gaa gaa aat aat gat        2832
Leu Thr Glu Ser Gly Gly Pro Leu Ser Leu Ser Glu Glu Asn Asn Asp
    930                 935                 940

tca aag ttg tta gaa tca ggt tta atg aat agc caa gaa agt tca tgg        2880
Ser Lys Leu Leu Glu Ser Gly Leu Met Asn Ser Gln Glu Ser Ser Trp
945                 950                 955                 960

gga aaa aat gta tcg tca aca gag agt ggt agg tta ttt aaa ggg aaa        2928
Gly Lys Asn Val Ser Ser Thr Glu Ser Gly Arg Leu Phe Lys Gly Lys
                965                 970                 975
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aga gct cat gga cct gct ttg ttg act aaa gat aat gcc tta ttc aaa      2976
Arg Ala His Gly Pro Ala Leu Leu Thr Lys Asp Asn Ala Leu Phe Lys
            980                 985                 990

gtt agc atc tct ttg tta aag aca  aac aaa act tcc aat  aat tca gca      3024
Val Ser Ile Ser Leu Leu Lys Thr  Asn Lys Thr Ser Asn  Asn Ser Ala
        995                 1000                1005

act aat  aga aag act cac att  gat ggc cca tca tta  tta att gag      3069
Thr Asn  Arg Lys Thr His Ile  Asp Gly Pro Ser Leu  Leu Ile Glu
    1010                1015                1020

aat agt  cca tca gtc tgg caa  aat ata tta gaa agt  gac act gag      3114
Asn Ser  Pro Ser Val Trp Gln  Asn Ile Leu Glu Ser  Asp Thr Glu
    1025                1030                1035

ttt aaa  aaa gtg aca cct ttg  att cat gac aga atg  ctt atg gac      3159
Phe Lys  Lys Val Thr Pro Leu  Ile His Asp Arg Met  Leu Met Asp
    1040                1045                1050

aaa aat  gct aca gct ttg agg  cta aat cat atg tca  aat aaa act      3204
Lys Asn  Ala Thr Ala Leu Arg  Leu Asn His Met Ser  Asn Lys Thr
    1055                1060                1065

act tca  tca aaa aac atg gaa  atg gtc caa cag aaa  aaa gag ggc      3249
Thr Ser  Ser Lys Asn Met Glu  Met Val Gln Gln Lys  Lys Glu Gly
    1070                1075                1080

ccc att  cca cca gat gca caa  aat cca gat atg tcg  ttc ttt aag      3294
Pro Ile  Pro Pro Asp Ala Gln  Asn Pro Asp Met Ser  Phe Phe Lys
    1085                1090                1095

atg cta  ttc ttg cca gaa tca  gca agg tgg ata caa  agg act cat      3339
Met Leu  Phe Leu Pro Glu Ser  Ala Arg Trp Ile Gln  Arg Thr His
    1100                1105                1110

gga aag  aac tct ctg aac tct  ggg caa ggc ccc agt  cca aag caa      3384
Gly Lys  Asn Ser Leu Asn Ser  Gly Gln Gly Pro Ser  Pro Lys Gln
    1115                1120                1125

tta gta  tcc tta gga cca gaa  aaa tct gtg gaa ggt  cag aat ttc      3429
Leu Val  Ser Leu Gly Pro Glu  Lys Ser Val Glu Gly  Gln Asn Phe
    1130                1135                1140

ttg tct  gag aaa aac aaa gtg  gta gta gga aag ggt  gaa ttt aca      3474
Leu Ser  Glu Lys Asn Lys Val  Val Val Gly Lys Gly  Glu Phe Thr
    1145                1150                1155

aag gac  gta gga ctc aaa gag  atg gtt ttt cca agc  agc aga aac      3519
Lys Asp  Val Gly Leu Lys Glu  Met Val Phe Pro Ser  Ser Arg Asn
    1160                1165                1170

cta ttt  ctt act aac ttg gat  aat tta cat gaa aat  aat aca cac      3564
Leu Phe  Leu Thr Asn Leu Asp  Asn Leu His Glu Asn  Asn Thr His
    1175                1180                1185

aat caa  gaa aaa aaa att cag  gaa gaa ata gaa aag  aag gaa aca      3609
Asn Gln  Glu Lys Lys Ile Gln  Glu Glu Ile Glu Lys  Lys Glu Thr
    1190                1195                1200

tta atc  caa gag aat gta gtt  ttg cct cag ata cat  aca gtg act      3654
Leu Ile  Gln Glu Asn Val Val  Leu Pro Gln Ile His  Thr Val Thr
    1205                1210                1215

ggc act  aag aat ttc atg aag  aac ctt ttc tta ctg  agc act agg      3699
Gly Thr  Lys Asn Phe Met Lys  Asn Leu Phe Leu Leu  Ser Thr Arg
    1220                1225                1230
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caa aat  gta gaa ggt tca tat  gac ggg gca tat gct  cca gta ctt        3744
Gln Asn  Val Glu Gly Ser Tyr  Asp Gly Ala Tyr Ala  Pro Val Leu
    1235                 1240                 1245

caa gat  ttt agg tca tta aat  gat tca aca aat aga  aca aag aaa        3789
Gln Asp  Phe Arg Ser Leu Asn  Asp Ser Thr Asn Arg  Thr Lys Lys
    1250                 1255                 1260

cac aca  gct cat ttc tca aaa  aaa ggg gag gaa gaa  aac ttg gaa        3834
His Thr  Ala His Phe Ser Lys  Lys Gly Glu Glu Glu  Asn Leu Glu
    1265                 1270                 1275

ggc ttg  gga aat caa acc aag  caa att gta gag aaa  tat gca tgc        3879
Gly Leu  Gly Asn Gln Thr Lys  Gln Ile Val Glu Lys  Tyr Ala Cys
    1280                 1285                 1290

acc aca  agg ata tct cct aat  aca agc cag cag aat  ttt gtc acg        3924
Thr Thr  Arg Ile Ser Pro Asn  Thr Ser Gln Gln Asn  Phe Val Thr
    1295                 1300                 1305

caa cgt  agt aag aga gct ttg  aaa caa ttc aga ctc  cca cta gaa        3969
Gln Arg  Ser Lys Arg Ala Leu  Lys Gln Phe Arg Leu  Pro Leu Glu
    1310                 1315                 1320

gaa aca  gaa ctt gaa aaa agg  ata att gtg gat gac  acc tca acc        4014
Glu Thr  Glu Leu Glu Lys Arg  Ile Ile Val Asp Asp  Thr Ser Thr
    1325                 1330                 1335

cag tgg  tcc aaa aac atg aaa  cat ttg acc ccg agc  acc ctc aca        4059
Gln Trp  Ser Lys Asn Met Lys  His Leu Thr Pro Ser  Thr Leu Thr
    1340                 1345                 1350

cag ata  gac tac aat gag aag  gag aaa ggg gcc att  act cag tct        4104
Gln Ile  Asp Tyr Asn Glu Lys  Glu Lys Gly Ala Ile  Thr Gln Ser
    1355                 1360                 1365

ccc tta  tca gat tgc ctt acg  agg agt cat agc atc  cct caa gca        4149
Pro Leu  Ser Asp Cys Leu Thr  Arg Ser His Ser Ile  Pro Gln Ala
    1370                 1375                 1380

aat aga  tct cca tta ccc att  gca aag gta tca tca  ttt cca tct        4194
Asn Arg  Ser Pro Leu Pro Ile  Ala Lys Val Ser Ser  Phe Pro Ser
    1385                 1390                 1395

att aga  cct ata tat ctg acc  agg gtc cta ttc caa  gac aac tct        4239
Ile Arg  Pro Ile Tyr Leu Thr  Arg Val Leu Phe Gln  Asp Asn Ser
    1400                 1405                 1410

tct cat  ctt cca gca gca tct  tat aga aag aaa gat  tct ggg gtc        4284
Ser His  Leu Pro Ala Ala Ser  Tyr Arg Lys Lys Asp  Ser Gly Val
    1415                 1420                 1425

caa gaa  agc agt cat ttc tta  caa gga gcc aaa aaa  aat aac ctt        4329
Gln Glu  Ser Ser His Phe Leu  Gln Gly Ala Lys Lys  Asn Asn Leu
    1430                 1435                 1440

tct tta  gcc att cta acc ttg  gag atg act ggt gat  caa aga gag        4374
Ser Leu  Ala Ile Leu Thr Leu  Glu Met Thr Gly Asp  Gln Arg Glu
    1445                 1450                 1455

gtt ggc  tcc ctg ggg aca agt  gcc aca aat tca gtc  aca tac aag        4419
Val Gly  Ser Leu Gly Thr Ser  Ala Thr Asn Ser Val  Thr Tyr Lys
    1460                 1465                 1470

aaa gtt  gag aac act gtt ctc  ccg aaa cca gac ttg  ccc aaa aca        4464
Lys Val  Glu Asn Thr Val Leu  Pro Lys Pro Asp Leu  Pro Lys Thr
    1475                 1480                 1485
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tct ggc  aaa gtt gaa ttg ctt  cca aaa gtt cac att  tat cag aag        4509
Ser Gly  Lys Val Glu Leu Leu  Pro Lys Val His Ile  Tyr Gln Lys
    1490                 1495                 1500

gac cta  ttc cct acg gaa act  agc aat ggg tct cct  ggc cat ctg        4554
Asp Leu  Phe Pro Thr Glu Thr  Ser Asn Gly Ser Pro  Gly His Leu
    1505                 1510                 1515

gat ctc  gtg gaa ggg agc ctt  ctt cag gga aca gag  gga gcg att        4599
Asp Leu  Val Glu Gly Ser Leu  Leu Gln Gly Thr Glu  Gly Ala Ile
    1520                 1525                 1530

aag tgg  aat gaa gca aac aga  cct gga aaa gtt ccc  ttt ctg aga        4644
Lys Trp  Asn Glu Ala Asn Arg  Pro Gly Lys Val Pro  Phe Leu Arg
    1535                 1540                 1545

gta gca  aca gaa agc tct gca  aag act ccc tcc aag  cta ttg gat        4689
Val Ala  Thr Glu Ser Ser Ala  Lys Thr Pro Ser Lys  Leu Leu Asp
    1550                 1555                 1560

cct ctt  gct tgg gat aac cac  tat ggt act cag ata  cca aaa gaa        4734
Pro Leu  Ala Trp Asp Asn His  Tyr Gly Thr Gln Ile  Pro Lys Glu
    1565                 1570                 1575

gag tgg  aaa tcc caa gag aag  tca cca gaa aaa aca  gct ttt aag        4779
Glu Trp  Lys Ser Gln Glu Lys  Ser Pro Glu Lys Thr  Ala Phe Lys
    1580                 1585                 1590

aaa aag  gat acc att ttg tcc  ctg aac gct tgt gaa  agc aat cat        4824
Lys Lys  Asp Thr Ile Leu Ser  Leu Asn Ala Cys Glu  Ser Asn His
    1595                 1600                 1605

gca ata  gca gca ata aat gag  gga caa aat aag ccc  gaa ata gaa        4869
Ala Ile  Ala Ala Ile Asn Glu  Gly Gln Asn Lys Pro  Glu Ile Glu
    1610                 1615                 1620

gtc acc  tgg gca aag caa ggt  agg act gaa agg ctg  tgc tct caa        4914
Val Thr  Trp Ala Lys Gln Gly  Arg Thr Glu Arg Leu  Cys Ser Gln
    1625                 1630                 1635

aac cca  cca gtc ttg aaa cgc  cat caa cgg gaa ata  act cgt act        4959
Asn Pro  Pro Val Leu Lys Arg  His Gln Arg Glu Ile  Thr Arg Thr
    1640                 1645                 1650

act ctt  cag tca gat caa gag  gaa att gac tat gat  gat acc ata        5004
Thr Leu  Gln Ser Asp Gln Glu  Glu Ile Asp Tyr Asp  Asp Thr Ile
    1655                 1660                 1665

tca gtt  gaa atg aag aag gaa  gat ttt gac att tat  gat gag gat        5049
Ser Val  Glu Met Lys Lys Glu  Asp Phe Asp Ile Tyr  Asp Glu Asp
    1670                 1675                 1680

gaa aat  cag agc ccc cgc agc  ttt caa aag aaa aca  cga cac tat        5094
Glu Asn  Gln Ser Pro Arg Ser  Phe Gln Lys Lys Thr  Arg His Tyr
    1685                 1690                 1695

ttt att  gct gca gtg gag agg  ctc tgg gat tat ggg  atg agt agc        5139
Phe Ile  Ala Ala Val Glu Arg  Leu Trp Asp Tyr Gly  Met Ser Ser
    1700                 1705                 1710

tcc cca  cat gtt cta aga aac  agg gct cag agt ggc  agt gtc cct        5184
Ser Pro  His Val Leu Arg Asn  Arg Ala Gln Ser Gly  Ser Val Pro
    1715                 1720                 1725

cag ttc  aag aaa gtt gtt ttc  cag gaa ttt act gat  ggc tcc ttt        5229
Gln Phe  Lys Lys Val Val Phe  Gln Glu Phe Thr Asp  Gly Ser Phe
    1730                 1735                 1740
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act cag  ccc tta tac cgt gga  gaa cta aat gaa cat  ttg gga ctc        5274
Thr Gln  Pro Leu Tyr Arg Gly  Glu Leu Asn Glu His  Leu Gly Leu
    1745                 1750                 1755

ctg ggg  cca tat ata aga gca  gaa gtt gaa gat aat  atc atg gta        5319
Leu Gly  Pro Tyr Ile Arg Ala  Glu Val Glu Asp Asn  Ile Met Val
    1760                 1765                 1770

act ttc  aga aat cag gcc tct  cgt ccc tat tcc ttc  tat tct agc        5364
Thr Phe  Arg Asn Gln Ala Ser  Arg Pro Tyr Ser Phe  Tyr Ser Ser
    1775                 1780                 1785

ctt att  tct tat gag gaa gat  cag agg caa gga gca  gaa cct aga        5409
Leu Ile  Ser Tyr Glu Glu Asp  Gln Arg Gln Gly Ala  Glu Pro Arg
    1790                 1795                 1800

aaa aac  ttt gtc aag cct aat  gaa acc aaa act tac  ttt tgg aaa        5454
Lys Asn  Phe Val Lys Pro Asn  Glu Thr Lys Thr Tyr  Phe Trp Lys
    1805                 1810                 1815

gtg caa  cat cat atg gca ccc  act aaa gat gag ttt  gac tgc aaa        5499
Val Gln  His His Met Ala Pro  Thr Lys Asp Glu Phe  Asp Cys Lys
    1820                 1825                 1830

gcc tgg  gct tat ttc tct gat  gtt gac ctg gaa aaa  gat gtg cac        5544
Ala Trp  Ala Tyr Phe Ser Asp  Val Asp Leu Glu Lys  Asp Val His
    1835                 1840                 1845

tca ggc  ctg att gga ccc ctt  ctg gtc tgc cac act  aac aca ctg        5589
Ser Gly  Leu Ile Gly Pro Leu  Leu Val Cys His Thr  Asn Thr Leu
    1850                 1855                 1860

aac cct  gct cat ggg aga caa  gtg aca gta cag gaa  ttt gct ctg        5634
Asn Pro  Ala His Gly Arg Gln  Val Thr Val Gln Glu  Phe Ala Leu
    1865                 1870                 1875

ttt ttc  acc atc ttt gat gag  acc aaa agc tgg tac  ttc act gaa        5679
Phe Phe  Thr Ile Phe Asp Glu  Thr Lys Ser Trp Tyr  Phe Thr Glu
    1880                 1885                 1890

aat atg  gaa aga aac tgc agg  gct ccc tgc aat atc  cag atg gaa        5724
Asn Met  Glu Arg Asn Cys Arg  Ala Pro Cys Asn Ile  Gln Met Glu
    1895                 1900                 1905

gat ccc  act ttt aaa gag aat  tat cgc ttc cat gca  atc aat ggc        5769
Asp Pro  Thr Phe Lys Glu Asn  Tyr Arg Phe His Ala  Ile Asn Gly
    1910                 1915                 1920

tac ata  atg gat aca cta cct  ggc tta gta atg gct  cag gat caa        5814
Tyr Ile  Met Asp Thr Leu Pro  Gly Leu Val Met Ala  Gln Asp Gln
    1925                 1930                 1935

agg att  cga tgg tat ctg ctc  agc atg ggc agc aat  gaa aac atc        5859
Arg Ile  Arg Trp Tyr Leu Leu  Ser Met Gly Ser Asn  Glu Asn Ile
    1940                 1945                 1950

cat tct  att cat ttc agt gga  cat gtg ttc act gta  cga aaa aaa        5904
His Ser  Ile His Phe Ser Gly  His Val Phe Thr Val  Arg Lys Lys
    1955                 1960                 1965

gag gag  tat aaa atg gca ctg  tac aat ctc tat cca  ggt gtt ttt        5949
Glu Glu  Tyr Lys Met Ala Leu  Tyr Asn Leu Tyr Pro  Gly Val Phe
    1970                 1975                 1980

gag aca  gtg gaa atg tta cca  tcc aaa gct gga att  tgg cgg gtg        5994
Glu Thr  Val Glu Met Leu Pro  Ser Lys Ala Gly Ile  Trp Arg Val
    1985                 1990                 1995
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gaa tgc  ctt att ggc gag cat  cta cat gct ggg atg  agc aca ctt        6039
Glu Cys  Leu Ile Gly Glu His  Leu His Ala Gly Met  Ser Thr Leu
    2000                 2005                 2010

ttt ctg  gtg tac agc aat aag  tgt cag act ccc ctg  gga atg gct        6084
Phe Leu  Val Tyr Ser Asn Lys  Cys Gln Thr Pro Leu  Gly Met Ala
    2015                 2020                 2025

tct gga  cac att aga gat ttt  cag att aca gct tca  gga caa tat        6129
Ser Gly  His Ile Arg Asp Phe  Gln Ile Thr Ala Ser  Gly Gln Tyr
    2030                 2035                 2040

gga cag  tgg gcc cca aag ctg  gcc aga ctt cat tat  tcc gga tca        6174
Gly Gln  Trp Ala Pro Lys Leu  Ala Arg Leu His Tyr  Ser Gly Ser
    2045                 2050                 2055

atc aat  gcc tgg agc acc aag  gag ccc ttt tct tgg  atc aag gtg        6219
Ile Asn  Ala Trp Ser Thr Lys  Glu Pro Phe Ser Trp  Ile Lys Val
    2060                 2065                 2070

gat ctg  ttg gca cca atg att  att cac ggc atc aag  acc cag ggt        6264
Asp Leu  Leu Ala Pro Met Ile  Ile His Gly Ile Lys  Thr Gln Gly
    2075                 2080                 2085

gcc cgt  cag aag ttc tcc agc  ctc tac atc tct cag  ttt atc atc        6309
Ala Arg  Gln Lys Phe Ser Ser  Leu Tyr Ile Ser Gln  Phe Ile Ile
    2090                 2095                 2100

atg tat  agt ctt gat ggg aag  aag tgg cag act tat  cga gga aat        6354
Met Tyr  Ser Leu Asp Gly Lys  Lys Trp Gln Thr Tyr  Arg Gly Asn
    2105                 2110                 2115

tcc act  gga acc tta atg gtc  ttc ttt ggc aat gtg  gat tca tct        6399
Ser Thr  Gly Thr Leu Met Val  Phe Phe Gly Asn Val  Asp Ser Ser
    2120                 2125                 2130

ggg ata  aaa cac aat att ttt  aac cct cca att att  gct cga tac        6444
Gly Ile  Lys His Asn Ile Phe  Asn Pro Pro Ile Ile  Ala Arg Tyr
    2135                 2140                 2145

atc cgt  ttg cac cca act cat  tat agc att cgc agc  act ctt cgc        6489
Ile Arg  Leu His Pro Thr His  Tyr Ser Ile Arg Ser  Thr Leu Arg
    2150                 2155                 2160

atg gag  ttg atg ggc tgt gat  tta aat agt tgc agc  atg cca ttg        6534
Met Glu  Leu Met Gly Cys Asp  Leu Asn Ser Cys Ser  Met Pro Leu
    2165                 2170                 2175

gga atg  gag agt aaa gca ata  tca gat gca cag att  act gct tca        6579
Gly Met  Glu Ser Lys Ala Ile  Ser Asp Ala Gln Ile  Thr Ala Ser
    2180                 2185                 2190

tcc tac  ttt acc aat atg ttt  gcc acc tgg tct cct  tca aaa gct        6624
Ser Tyr  Phe Thr Asn Met Phe  Ala Thr Trp Ser Pro  Ser Lys Ala
    2195                 2200                 2205

cga ctt  cac ctc caa ggg agg  agt aat gcc tgg aga  cct cag gtg        6669
Arg Leu  His Leu Gln Gly Arg  Ser Asn Ala Trp Arg  Pro Gln Val
    2210                 2215                 2220

aat aat  cca aaa gag tgg ctg  caa gtg gac ttc cag  aag aca atg        6714
Asn Asn  Pro Lys Glu Trp Leu  Gln Val Asp Phe Gln  Lys Thr Met
    2225                 2230                 2235

aaa gtc  aca gga gta act act  cag gga gta aaa tct  ctg ctt acc        6759
Lys Val  Thr Gly Val Thr Thr  Gln Gly Val Lys Ser  Leu Leu Thr
    2240                 2245                 2250
```

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agc atg  tat gtg aag gag ttc  ctc atc tcc agc agt  caa gat ggc        6804
Ser Met  Tyr Val Lys Glu Phe  Leu Ile Ser Ser Ser  Gln Asp Gly
    2255                 2260                 2265

cat cag  tgg act ctc ttt ttt  cag aat ggc aaa gta  aag gtt ttt        6849
His Gln  Trp Thr Leu Phe Phe  Gln Asn Gly Lys Val  Lys Val Phe
    2270                 2275                 2280

cag gga  aat caa gac tcc ttc  aca cct gtg gtg aac  tct cta gac        6894
Gln Gly  Asn Gln Asp Ser Phe  Thr Pro Val Val Asn  Ser Leu Asp
    2285                 2290                 2295

cca ccg  tta ctg act cgc tac  ctt cga att cac ccc  cag agt tgg        6939
Pro Pro  Leu Leu Thr Arg Tyr  Leu Arg Ile His Pro  Gln Ser Trp
    2300                 2305                 2310

gtg cac  cag att gcc ctg agg  atg gag gtt ctg ggc  tgc gag gca        6984
Val His  Gln Ile Ala Leu Arg  Met Glu Val Leu Gly  Cys Glu Ala
    2315                 2320                 2325

cag gac  ctc tac                                                    6996
Gln Asp  Leu Tyr
    2330
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<210> 2
<211> 2332
<212> PRT
<213> homo sapiens

<400> 2

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Ala Thr Arg Arg Tyr Tyr Leu Gly Ala Val Glu Leu Ser Trp Asp Tyr
1               5                  10                  15

Met Gln Ser Asp Leu Gly Glu Leu Pro Val Asp Ala Arg Phe Pro Pro
            20                  25                  30

Arg Val Pro Lys Ser Phe Pro Phe Asn Thr Ser Val Val Tyr Lys Lys
        35                  40                  45

Thr Leu Phe Val Glu Phe Thr Asp His Leu Phe Asn Ile Ala Lys Pro
    50                  55                  60

Arg Pro Pro Trp Met Gly Leu Leu Gly Pro Thr Ile Gln Ala Glu Val
65                  70                  75                  80

Tyr Asp Thr Val Val Ile Thr Leu Lys Asn Met Ala Ser His Pro Val
                85                  90                  95

Ser Leu His Ala Val Gly Val Ser Tyr Trp Lys Ala Ser Glu Gly Ala
            100                 105                 110

Glu Tyr Asp Asp Gln Thr Ser Gln Arg Glu Lys Glu Asp Asp Lys Val
        115                 120                 125

Phe Pro Gly Gly Ser His Thr Tyr Val Trp Gln Val Leu Lys Glu Asn
    130                 135                 140
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Gly Pro Met Ala Ser Asp Pro Leu Cys Leu Thr Tyr Ser Tyr Leu Ser
145                 150                 155                 160

His Val Asp Leu Val Lys Asp Leu Asn Ser Gly Leu Ile Gly Ala Leu
                165                 170                 175

Leu Val Cys Arg Glu Gly Ser Leu Ala Lys Glu Lys Thr Gln Thr Leu
            180                 185                 190

His Lys Phe Ile Leu Leu Phe Ala Val Phe Asp Glu Gly Lys Ser Trp
        195                 200                 205

His Ser Glu Thr Lys Asn Ser Leu Met Gln Asp Arg Asp Ala Ala Ser
    210                 215                 220

Ala Arg Ala Trp Pro Lys Met His Thr Val Asn Gly Tyr Val Asn Arg
225                 230                 235                 240

Ser Leu Pro Gly Leu Ile Gly Cys His Arg Lys Ser Val Tyr Trp His
                245                 250                 255

Val Ile Gly Met Gly Thr Thr Pro Glu Val His Ser Ile Phe Leu Glu
            260                 265                 270

Gly His Thr Phe Leu Val Arg Asn His Arg Gln Ala Ser Leu Glu Ile
        275                 280                 285

Ser Pro Ile Thr Phe Leu Thr Ala Gln Thr Leu Leu Met Asp Leu Gly
    290                 295                 300

Gln Phe Leu Leu Phe Cys His Ile Ser Ser His Gln His Asp Gly Met
305                 310                 315                 320

Glu Ala Tyr Val Lys Val Asp Ser Cys Pro Glu Glu Pro Gln Leu Arg
                325                 330                 335

Met Lys Asn Asn Glu Glu Ala Glu Asp Tyr Asp Asp Asp Leu Thr Asp
            340                 345                 350

Ser Glu Met Asp Val Val Arg Phe Asp Asp Asp Asn Ser Pro Ser Phe
        355                 360                 365

Ile Gln Ile Arg Ser Val Ala Lys Lys His Pro Lys Thr Trp Val His
    370                 375                 380

Tyr Ile Ala Ala Glu Glu Glu Asp Trp Asp Tyr Ala Pro Leu Val Leu
385                 390                 395                 400

Ala Pro Asp Asp Arg Ser Tyr Lys Ser Gln Tyr Leu Asn Asn Gly Pro
                405                 410                 415
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Gln Arg Ile Gly Arg Lys Tyr Lys Lys Val Arg Phe Met Ala Tyr Thr
            420                 425                 430

Asp Glu Thr Phe Lys Thr Arg Glu Ala Ile Gln His Glu Ser Gly Ile
        435                 440                 445

Leu Gly Pro Leu Leu Tyr Gly Glu Val Gly Asp Thr Leu Leu Ile Ile
    450                 455                 460

Phe Lys Asn Gln Ala Ser Arg Pro Tyr Asn Ile Tyr Pro His Gly Ile
465                 470                 475                 480

Thr Asp Val Arg Pro Leu Tyr Ser Arg Arg Leu Pro Lys Gly Val Lys
                485                 490                 495

His Leu Lys Asp Phe Pro Ile Leu Pro Gly Glu Ile Phe Lys Tyr Lys
            500                 505                 510

Trp Thr Val Thr Val Glu Asp Gly Pro Thr Lys Ser Asp Pro Arg Cys
        515                 520                 525

Leu Thr Arg Tyr Tyr Ser Ser Phe Val Asn Met Glu Arg Asp Leu Ala
    530                 535                 540

Ser Gly Leu Ile Gly Pro Leu Leu Ile Cys Tyr Lys Glu Ser Val Asp
545                 550                 555                 560

Gln Arg Gly Asn Gln Ile Met Ser Asp Lys Arg Asn Val Ile Leu Phe
                565                 570                 575

Ser Val Phe Asp Glu Asn Arg Ser Trp Tyr Leu Thr Glu Asn Ile Gln
            580                 585                 590

Arg Phe Leu Pro Asn Pro Ala Gly Val Gln Leu Glu Asp Pro Glu Phe
        595                 600                 605

Gln Ala Ser Asn Ile Met His Ser Ile Asn Gly Tyr Val Phe Asp Ser
    610                 615                 620

Leu Gln Leu Ser Val Cys Leu His Glu Val Ala Tyr Trp Tyr Ile Leu
625                 630                 635                 640

Ser Ile Gly Ala Gln Thr Asp Phe Leu Ser Val Phe Phe Ser Gly Tyr
                645                 650                 655

Thr Phe Lys His Lys Met Val Tyr Glu Asp Thr Leu Thr Leu Phe Pro
            660                 665                 670

Phe Ser Gly Glu Thr Val Phe Met Ser Met Glu Asn Pro Gly Leu Trp
        675                 680                 685
```

```
Ile Leu Gly Cys His Asn Ser Asp Phe Arg Asn Arg Gly Met Thr Ala
    690                 695                 700

Leu Leu Lys Val Ser Ser Cys Asp Lys Asn Thr Gly Asp Tyr Tyr Glu
705                 710                 715                 720

Asp Ser Tyr Glu Asp Ile Ser Ala Tyr Leu Leu Ser Lys Asn Asn Ala
                725                 730                 735

Ile Glu Pro Arg Ser Phe Ser Gln Asn Ser Arg His Arg Ser Thr Arg
            740                 745                 750

Gln Lys Gln Phe Asn Ala Thr Thr Ile Pro Glu Asn Asp Ile Glu Lys
        755                 760                 765

Thr Asp Pro Trp Phe Ala His Arg Thr Pro Met Pro Lys Ile Gln Asn
    770                 775                 780

Val Ser Ser Ser Asp Leu Leu Met Leu Leu Arg Gln Ser Pro Thr Pro
785                 790                 795                 800

His Gly Leu Ser Leu Ser Asp Leu Gln Glu Ala Lys Tyr Glu Thr Phe
                805                 810                 815

Ser Asp Asp Pro Ser Pro Gly Ala Ile Asp Ser Asn Asn Ser Leu Ser
            820                 825                 830

Glu Met Thr His Phe Arg Pro Gln Leu His His Ser Gly Asp Met Val
        835                 840                 845

Phe Thr Pro Glu Ser Gly Leu Gln Leu Arg Leu Asn Glu Lys Leu Gly
    850                 855                 860

Thr Thr Ala Ala Thr Glu Leu Lys Lys Leu Asp Phe Lys Val Ser Ser
865                 870                 875                 880

Thr Ser Asn Asn Leu Ile Ser Thr Ile Pro Ser Asp Asn Leu Ala Ala
                885                 890                 895

Gly Thr Asp Asn Thr Ser Ser Leu Gly Pro Pro Ser Met Pro Val His
            900                 905                 910

Tyr Asp Ser Gln Leu Asp Thr Thr Leu Phe Gly Lys Lys Ser Ser Pro
        915                 920                 925

Leu Thr Glu Ser Gly Gly Pro Leu Ser Leu Ser Glu Glu Asn Asn Asp
    930                 935                 940

Ser Lys Leu Leu Glu Ser Gly Leu Met Asn Ser Gln Glu Ser Ser Trp
945                 950                 955                 960
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```
Gly Lys Asn Val Ser Ser Thr Glu Ser Gly Arg Leu Phe Lys Gly Lys
                965                 970                 975

Arg Ala His Gly Pro Ala Leu Leu Thr Lys Asp Asn Ala Leu Phe Lys
            980                 985                 990

Val Ser Ile Ser Leu Leu Lys Thr Asn Lys Thr Ser Asn Asn Ser Ala
        995                 1000                1005

Thr Asn Arg Lys Thr His Ile Asp Gly Pro Ser Leu Leu Ile Glu
    1010                1015                1020

Asn Ser Pro Ser Val Trp Gln Asn Ile Leu Glu Ser Asp Thr Glu
    1025                1030                1035

Phe Lys Lys Val Thr Pro Leu Ile His Asp Arg Met Leu Met Asp
    1040                1045                1050

Lys Asn Ala Thr Ala Leu Arg Leu Asn His Met Ser Asn Lys Thr
    1055                1060                1065

Thr Ser Ser Lys Asn Met Glu Met Val Gln Gln Lys Lys Glu Gly
    1070                1075                1080

Pro Ile Pro Pro Asp Ala Gln Asn Pro Asp Met Ser Phe Phe Lys
    1085                1090                1095

Met Leu Phe Leu Pro Glu Ser Ala Arg Trp Ile Gln Arg Thr His
    1100                1105                1110

Gly Lys Asn Ser Leu Asn Ser Gly Gln Gly Pro Ser Pro Lys Gln
    1115                1120                1125

Leu Val Ser Leu Gly Pro Glu Lys Ser Val Glu Gly Gln Asn Phe
    1130                1135                1140

Leu Ser Glu Lys Asn Lys Val Val Val Gly Lys Gly Glu Phe Thr
    1145                1150                1155

Lys Asp Val Gly Leu Lys Glu Met Val Phe Pro Ser Ser Arg Asn
    1160                1165                1170

Leu Phe Leu Thr Asn Leu Asp Asn Leu His Glu Asn Asn Thr His
    1175                1180                1185

Asn Gln Glu Lys Lys Ile Gln Glu Glu Ile Glu Lys Lys Glu Thr
    1190                1195                1200

Leu Ile Gln Glu Asn Val Val Leu Pro Gln Ile His Thr Val Thr
    1205                1210                1215
```

```
Gly Thr Lys Asn Phe Met Lys Asn Leu Phe Leu Leu Ser Thr Arg
    1220                1225                1230

Gln Asn Val Glu Gly Ser Tyr Asp Gly Ala Tyr Ala Pro Val Leu
    1235                1240                1245

Gln Asp Phe Arg Ser Leu Asn Asp Ser Thr Asn Arg Thr Lys Lys
    1250                1255                1260

His Thr Ala His Phe Ser Lys Lys Gly Glu Glu Glu Asn Leu Glu
    1265                1270                1275

Gly Leu Gly Asn Gln Thr Lys Gln Ile Val Glu Lys Tyr Ala Cys
    1280                1285                1290

Thr Thr Arg Ile Ser Pro Asn Thr Ser Gln Gln Asn Phe Val Thr
    1295                1300                1305

Gln Arg Ser Lys Arg Ala Leu Lys Gln Phe Arg Leu Pro Leu Glu
    1310                1315                1320

Glu Thr Glu Leu Glu Lys Arg Ile Ile Val Asp Asp Thr Ser Thr
    1325                1330                1335

Gln Trp Ser Lys Asn Met Lys His Leu Thr Pro Ser Thr Leu Thr
    1340                1345                1350

Gln Ile Asp Tyr Asn Glu Lys Glu Lys Gly Ala Ile Thr Gln Ser
    1355                1360                1365

Pro Leu Ser Asp Cys Leu Thr Arg Ser His Ser Ile Pro Gln Ala
    1370                1375                1380

Asn Arg Ser Pro Leu Pro Ile Ala Lys Val Ser Ser Phe Pro Ser
    1385                1390                1395

Ile Arg Pro Ile Tyr Leu Thr Arg Val Leu Phe Gln Asp Asn Ser
    1400                1405                1410

Ser His Leu Pro Ala Ala Ser Tyr Arg Lys Lys Asp Ser Gly Val
    1415                1420                1425

Gln Glu Ser Ser His Phe Leu Gln Gly Ala Lys Lys Asn Asn Leu
    1430                1435                1440

Ser Leu Ala Ile Leu Thr Leu Glu Met Thr Gly Asp Gln Arg Glu
    1445                1450                1455

Val Gly Ser Leu Gly Thr Ser Ala Thr Asn Ser Val Thr Tyr Lys
    1460                1465                1470
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Lys Val  Glu Asn Thr Val Leu  Pro Lys Pro Asp Leu  Pro Lys Thr
    1475                 1480                 1485

Ser Gly  Lys Val Glu Leu Leu  Pro Lys Val His Ile  Tyr Gln Lys
    1490                 1495                 1500

Asp Leu  Phe Pro Thr Glu Thr  Ser Asn Gly Ser Pro  Gly His Leu
    1505                 1510                 1515

Asp Leu  Val Glu Gly Ser Leu  Leu Gln Gly Thr Glu  Gly Ala Ile
    1520                 1525                 1530

Lys Trp  Asn Glu Ala Asn Arg  Pro Gly Lys Val Pro  Phe Leu Arg
    1535                 1540                 1545

Val Ala  Thr Glu Ser Ser Ala  Lys Thr Pro Ser Lys  Leu Leu Asp
    1550                 1555                 1560

Pro Leu  Ala Trp Asp Asn His  Tyr Gly Thr Gln Ile  Pro Lys Glu
    1565                 1570                 1575

Glu Trp  Lys Ser Gln Glu Lys  Ser Pro Glu Lys Thr  Ala Phe Lys
    1580                 1585                 1590

Lys Lys  Asp Thr Ile Leu Ser  Leu Asn Ala Cys Glu  Ser Asn His
    1595                 1600                 1605

Ala Ile  Ala Ala Ile Asn Glu  Gly Gln Asn Lys Pro  Glu Ile Glu
    1610                 1615                 1620

Val Thr  Trp Ala Lys Gln Gly  Arg Thr Glu Arg Leu  Cys Ser Gln
    1625                 1630                 1635

Asn Pro  Pro Val Leu Lys Arg  His Gln Arg Glu Ile  Thr Arg Thr
    1640                 1645                 1650

Thr Leu  Gln Ser Asp Gln Glu  Glu Ile Asp Tyr Asp  Asp Thr Ile
    1655                 1660                 1665

Ser Val  Glu Met Lys Lys Glu  Asp Phe Asp Ile Tyr  Asp Glu Asp
    1670                 1675                 1680

Glu Asn  Gln Ser Pro Arg Ser  Phe Gln Lys Lys Thr  Arg His Tyr
    1685                 1690                 1695

Phe Ile  Ala Ala Val Glu Arg  Leu Trp Asp Tyr Gly  Met Ser Ser
    1700                 1705                 1710

Ser Pro  His Val Leu Arg Asn  Arg Ala Gln Ser Gly  Ser Val Pro
    1715                 1720                 1725
```

```
Gln Phe Lys Lys Val Val Phe Gln Glu Phe Thr Asp Gly Ser Phe
1730                1735                1740

Thr Gln Pro Leu Tyr Arg Gly Glu Leu Asn Glu His Leu Gly Leu
1745                1750                1755

Leu Gly Pro Tyr Ile Arg Ala Glu Val Glu Asp Asn Ile Met Val
1760                1765                1770

Thr Phe Arg Asn Gln Ala Ser Arg Pro Tyr Ser Phe Tyr Ser Ser
1775                1780                1785

Leu Ile Ser Tyr Glu Glu Asp Gln Arg Gln Gly Ala Glu Pro Arg
1790                1795                1800

Lys Asn Phe Val Lys Pro Asn Glu Thr Lys Thr Tyr Phe Trp Lys
1805                1810                1815

Val Gln His His Met Ala Pro Thr Lys Asp Glu Phe Asp Cys Lys
1820                1825                1830

Ala Trp Ala Tyr Phe Ser Asp Val Asp Leu Glu Lys Asp Val His
1835                1840                1845

Ser Gly Leu Ile Gly Pro Leu Leu Val Cys His Thr Asn Thr Leu
1850                1855                1860

Asn Pro Ala His Gly Arg Gln Val Thr Val Gln Glu Phe Ala Leu
1865                1870                1875

Phe Phe Thr Ile Phe Asp Glu Thr Lys Ser Trp Tyr Phe Thr Glu
1880                1885                1890

Asn Met Glu Arg Asn Cys Arg Ala Pro Cys Asn Ile Gln Met Glu
1895                1900                1905

Asp Pro Thr Phe Lys Glu Asn Tyr Arg Phe His Ala Ile Asn Gly
1910                1915                1920

Tyr Ile Met Asp Thr Leu Pro Gly Leu Val Met Ala Gln Asp Gln
1925                1930                1935

Arg Ile Arg Trp Tyr Leu Leu Ser Met Gly Ser Asn Glu Asn Ile
1940                1945                1950

His Ser Ile His Phe Ser Gly His Val Phe Thr Val Arg Lys Lys
1955                1960                1965

Glu Glu Tyr Lys Met Ala Leu Tyr Asn Leu Tyr Pro Gly Val Phe
1970                1975                1980
```

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Glu Thr  Val Glu Met Leu Pro  Ser Lys Ala Gly Ile  Trp Arg Val
    1985                 1990                 1995

Glu Cys  Leu Ile Gly Glu His  Leu His Ala Gly Met  Ser Thr Leu
    2000                 2005                 2010

Phe Leu  Val Tyr Ser Asn Lys  Cys Gln Thr Pro Leu  Gly Met Ala
    2015                 2020                 2025

Ser Gly  His Ile Arg Asp Phe  Gln Ile Thr Ala Ser  Gly Gln Tyr
    2030                 2035                 2040

Gly Gln  Trp Ala Pro Lys Leu  Ala Arg Leu His Tyr  Ser Gly Ser
    2045                 2050                 2055

Ile Asn  Ala Trp Ser Thr Lys  Glu Pro Phe Ser Trp  Ile Lys Val
    2060                 2065                 2070

Asp Leu  Leu Ala Pro Met Ile  Ile His Gly Ile Lys  Thr Gln Gly
    2075                 2080                 2085

Ala Arg  Gln Lys Phe Ser Ser  Leu Tyr Ile Ser Gln  Phe Ile Ile
    2090                 2095                 2100

Met Tyr  Ser Leu Asp Gly Lys  Lys Trp Gln Thr Tyr  Arg Gly Asn
    2105                 2110                 2115

Ser Thr  Gly Thr Leu Met Val  Phe Phe Gly Asn Val  Asp Ser Ser
    2120                 2125                 2130

Gly Ile  Lys His Asn Ile Phe  Asn Pro Pro Ile Ile  Ala Arg Tyr
    2135                 2140                 2145

Ile Arg  Leu His Pro Thr His  Tyr Ser Ile Arg Ser  Thr Leu Arg
    2150                 2155                 2160

Met Glu  Leu Met Gly Cys Asp  Leu Asn Ser Cys Ser  Met Pro Leu
    2165                 2170                 2175

Gly Met  Glu Ser Lys Ala Ile  Ser Asp Ala Gln Ile  Thr Ala Ser
    2180                 2185                 2190

Ser Tyr  Phe Thr Asn Met Phe  Ala Thr Trp Ser Pro  Ser Lys Ala
    2195                 2200                 2205

Arg Leu  His Leu Gln Gly Arg  Ser Asn Ala Trp Arg  Pro Gln Val
    2210                 2215                 2220

Asn Asn  Pro Lys Glu Trp Leu  Gln Val Asp Phe Gln  Lys Thr Met
    2225                 2230                 2235
```

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Lys Val  Thr Gly Val Thr Thr  Gln Gly Val Lys Ser  Leu Leu Thr
    2240                2245                2250

Ser Met  Tyr Val Lys Glu Phe  Leu Ile Ser Ser Ser  Gln Asp Gly
    2255                2260                2265

His Gln  Trp Thr Leu Phe Phe  Gln Asn Gly Lys Val  Lys Val Phe
    2270                2275                2280

Gln Gly  Asn Gln Asp Ser Phe  Thr Pro Val Val Asn  Ser Leu Asp
    2285                2290                2295

Pro Pro  Leu Leu Thr Arg Tyr  Leu Arg Ile His Pro  Gln Ser Trp
    2300                2305                2310

Val His  Gln Ile Ala Leu Arg  Met Glu Val Leu Gly  Cys Glu Ala
    2315                2320                2325

Gln Asp  Leu Tyr
    2330
```