Abstract:

Title: HALF-LIFE PROLONGATION OF PROTEINS

Fig. 1

Inactivation

VWF

FVIII

Proteolytic non-proteolytic

Thrombin activation

FVIII

95-98%

2-5%

Clearance

Receptor mediated

Coagulation

95-98 %

95-98 %

2-5 %

(57) Abstract: Pharmaceutical preparation comprising: - a pharmaceutical active protein having at least one LRP-binding site and/or at least one HSPG binding site and - at least one ligand binding to the LRP-binding site and/or the HSPG binding site, the at least one ligand being selected from peptides, sugars, nucleic acids and small chemical compounds, said small chemical compounds having a molecular weight of 5 KDa or less.
**Half-life prolongation of proteins**

The present invention relates to methods for half-life prolongation of proteins.

The use of polypeptides such as proteins for therapeutic applications has expanded in recent years mainly due to advanced knowledge of the molecular biological principles underlying many diseases and the availability of improved recombinant expression and delivery systems for human polypeptides. Polypeptide therapeutics are mainly utilized in diseases where a certain natural polypeptide is defective or missing in the patient, in particular because of inherited gene defects.

For example, hemophilia is a disease caused by deficiency of a certain plasma protein. Hemophiliacs suffer from hemorrhagic morbidity caused by the disturbed function of protein components of the blood coagulation cascade. Depending on the affected clotting factor two types of hemophilia can be distinguished. Both have in common the inhibited conversion of soluble fibrinogen to an insoluble fibrin-clot. They are recessive X-chromosomally-linked genetic diseases affecting mainly the male population.

Hemophilia A affects 1-2 individuals per 10,000 males. It is caused by the deficiency or absence of factor VIII, a very large glycoprotein (Mw approximately 330 kDa (1), which represents an important element of the blood coagulation cascade. The polypeptide sequence can be subdivided into three regions, an N-terminal region consisting of the so-called A1 and A2 domains, a central B domain region and a C-terminal region composed of the A3, C1 and C2 domains. In the blood, coagulation factor VIII occurs as an inactive precursor. It is bound tightly and non-covalently to von Willebrand Factor (vWF), which acts as a stabilizing carrier protein. Proteolytic cleavage of factor VIII by thrombin at three specific positions (740, 372, 1689) leads to its dissociation from vWF and releases the procoagulant function within the cascade. In its active form, factor VIII functions as a cofactor for factor IXa, thereby accelerating the proteolytic activation of factor X by several orders of magnitude.
Hemophilia B occurs in about 1 of 25,000 males. It is characterized by the deficiency of the serine protease factor IX (Christmas factor). This 415 amino-acid polypeptide is synthesized in the liver as a 56 kDa glycoprotein. In order to attain its proper function a posttranslational carboxylation step is required which only occurs in the presence of vitamin K.

Treatment of both types of bleeding disorder traditionally involves infusion of human plasma-derived protein concentrates of factor VIII or factor IX. Although this method represents an efficient therapy for hemophiliacs, it carries the potential risk of transmission of various infectious agents, such as viruses causing hepatitis or AIDS, or thromboembolic factors. Alternatively, several recombinant DNA techniques for the production of clotting factors have been described. For this purpose, the corresponding cDNAs of wild type factor VIII and factor IX have been isolated and cloned into suitable expression vectors (EP-A-160457; WO-A-86/01961, U.S. Patents 4,770,999, 5,521,070 and 5,521,070).

However, polypeptide therapeutics such as factor VIII are associated with many drawbacks, including short circulating half-life and proteolytic degradation. For example, the half-life of the protein factor VIII in the human body is app. 12 hours whereas in severe von Willebrand disease (vWD) patients it is app. 2 hours. Nowadays prophylactic treatment represents the state of the art treatment of hemophilia patients in developed countries. Prophylactic treatment usually results in 2 to 4 infusions per week.

There are a number of further proteins which are used for therapeutic purposes for example erythropoietin, granulocyte-colony stimulating factor (GCSF), interferons, monoclonal antibodies and the like.

In many cases, it would be helpful to increase the half-life of the therapeutic proteins to increase efficiency or reduce the amount of therapeutic proteins and/or frequency of infusions applied to patient. This would also reduce the costs of the treatment.

In the prior art, the short circulating half-life of polypeptide therapeutics has been addressed by covalent attachment of a polymer to the polypeptide. For example, the attachment of polyethylene glycol (PEG), dextran, or hydroxyethyl starch (HES) has shown some improvement of the half-life of some polypeptides.

However, a number of problems have been observed with the attachment of polymers. For example, the attachment of polymers can lead to decreased drug activity. Furthermore, certain reagents used for coupling polymers to a protein are insufficiently reactive and therefore require long reaction times during which protein denaturation and/or inactivation can occur. Also, incomplete or non-uniform attachment leads to a mixed population of compounds having differing properties.
WO 2009/135888 discloses a complex comprising a target protein and at least one binding molecule wherein the binding molecule is bound to at least one water soluble polymer.

Because of the size of the water soluble polymer, the binding molecule has a high molecular weight.

It is the object of the invention to provide therapeutic proteins with increased half-life.

One embodiment of the invention is a pharmaceutical preparation comprising
- a pharmaceutically active protein having at least one LRP binding site and/or at least one HSPG binding site and
- at least one ligand binding to the LRP-binding site and/or the HSPG binding site,
the at least one ligand being selected from peptides, sugars, nucleic acids and small chemical compounds, said small chemical compounds having a molecular weight of 5 KDa or less.

In a preferred embodiment the pharmaceutical active protein is Factor VIII, preferably human Factor VIII. Other therapeutic useful proteins having LRP binding sites include ApoE, Lipoprotein lipase, Hepatic lipase, tPA, uPA, Factor IXa, Factor VIII, Factor Villa, FactorVIIa/TFPI, MMP-13, MMP-9, Spingolipid activator protein (SAP), Pregnancy Zone Protein, a2 macroglobulin, Complement C3, PAI-1, CI inhibitor, Antithrombin III, TFPI, Heparin cofactor II, ch-Antitrypsin, APP, Thrombospondin-1, Thrombospondin-2, Pseudomonas exotoxin A, Lactoferrin, Rhinovirus, RAP, HSP-96, HIV-Tat protein.

Therapeutic proteins having HSPG binding sites include Albumin, Fibrinogen, Factor H, Fibronectin, Antithrombin III, Thrombin, Kininogen, vWF, FX, FXI, FIX, FVIII, PDGF, Platelet factor 4, VEGF, RANTES, MIP-la, bFGF, Apolipoprotein E3, Plasma protein C inhibitor.
In the embodiment using Factor VIII, Factor VIII can be derived from donor plasma or it can be recombinant Factor VIII. Included are also variants of Factor VIII having mutations or the like.

A very preferred embodiment is a B domain deleted Factor VIII.

FVIII as therapeutic protein used for hemophilia treatment is half-life prolonged by using small ligands binding with high affinity and high specificity to the HSPG- and/or LRP-receptor binding sites. The half-life prolonging effect is achieved by blocking a single site (for example the HSPG-site on the A2 domain) and/or by a combination of synergistically blocking several or all sites in parallel (for example synergistically blocking the HSPG- and LRPR-sites).

As ligands, peptides, sugars, small protein domains or fragments, nucleic acids (RNA, DNA) and derivatives thereof, or small chemical compounds can be used.

"Peptides" covers amino acid polymers. The term "peptides" as used in this application covers small peptides, oligopeptides, proteins.

"Sugars" are polymers of carbohydrates. The term covers saccharides of any size including oligo- and polysaccharides.

"Nucleic acids" covers polymers of nucleosides or nucleotides including oligo- and polynucleotides or -nucleosides.

"Small chemical compounds" are compounds typically prepared by organic chemical synthesis and have a molecular weight of 5 kDa or less, preferably 2 kDa or 1 kDa or less. The term includes all compounds having a molecular weight of 5 kDa or less, including natural compounds from animals or plants.

The ligands can be used as single molecules co-administered with FVIII and/or two or more ligands can be bridged covalently for blocking adjacent binding
sites (for example ligands blocking the HSPG-binding site and the nearby LRPR-binding site on A2 domain).

Peptide ligands can be linear or structured (for example cyclic peptides) and chemically modified (for example sulphated). Structured peptide ligands are constructed by taking into account available structural and sequence data about the targeted binding sites (for example the HSPG-binding site or the LRPR-binding site on A2 domain) as well as data about their physiological ligands (for example the LRPI-receptor, heparin), see Fig. 9 and 10. The structural 3-dimensional surface of the physiological ligands is thereby mimicked to produce a ligand with high affinity and high specificity (see Figure 10, A). The structural 3-dimensional surface of the target binding sites can be mimicked (for example the HSPG-site on A2 domain of FVIII) to design a binding site mimetic peptide which can be used for site-directed screening of ligands binding to this target site (see Figure 10, B).

These ligands can be further optimized by modifications of their amino acid sequence to vary: the length of the binding region (for example including the binding regions around a known binding site), the amino acid composition by varying the overall charge (by introducing or eliminating charged amino acids), the polarity (by exchanging hydrophilic and hydrophobic amino acids) or bulkiness (by exchanging amino acids with large side chains against those with smaller side chains or vice versa).

By using elaborated and defined screening methods, also random libraries of ligands (for example phage display libraries or synthetic peptide micro arrays) can be used to identify ligands specifically binding to the targeted HSPG- and/or LRP-receptor binding sites on FVIII. These screening includes the target protein and single domains or fragments thereof (for example FVIII, A2- or A3-domains), as well as physiological ligands and their surrogates (for example heparin or Clexane), their domains or fragments thereof (for example LRP-domains), monoclonal antibodies with binding epitopes overlapping or
surrounding the target binding site regions for competitions studies and constructed mimetic binding site peptides (see Figure 10, B).

In Factor VIII there are several LRP-binding sites. LRP binding sites are located in the A2 domain in residues 484-509, in the A3 domain residues 1811-1818, in the C2 domain residues 2173-2332 and in the CI domain residues 2065, 2092.

The numbering of the residues is in accordance with the numbering of SEQ ID NO. 1 (Figure 6).

A suitable HSPG binding site on Factor VIII is localized within the A2 domain residues 558-565 in accordance with SEQ ID NO. 1.

The ligand used in the pharmaceutical preparation has preferably a molecular weight below 150 KDa, preferably 100 KDa or less, 70 KDa or less, 50 KDa or less, 30 KDa or less, 10 KDa or less, 5 KDa or less, 2 KDa or less or 1 KDa or less.

Suitable ligands are selected from peptides, sugars, lipids, nucleic acids and variants thereof and chemically synthesized compounds.

In preferred embodiments, the peptide is a linear, cyclic, folded or a scaffold peptide.

In some embodiments, the ligand may comprise at least one sulphate group.

In preferred embodiments, the ligand does not comprise a polyalkylene glycol or derivative thereof like PEG, does not comprise a hydroxypropylmethacrylate HPMA group and its copolymers and does not comprise a starch like hydroxyalkyl starch.

A further embodiment of the invention is the method for testing a ligand’s ability to bind to at least one LRP-binding site and/or at least one HSPG binding site of FVIII comprising the steps of
- combining the ligand with FVIII to a FVIII-ligand complex
- measuring clearance of the FVIII-ligand complex in-vivo

wherein a reduced clearance indicates that the ligand binds to at least one LRP-binding site and/or at least one HSPG binding site of FVIII, the ligand being selected from peptides, sugars, nucleic acids and small chemical compounds, said small chemical compounds having a molecular weight of 5 KDa or less.

A further embodiment of the invention is a method for reducing clearance of FVIII comprising the step of

- combining FVIII with a ligand binding to the LRP-binding site and/or the HSPG binding site of FVIII to form a FVIII-ligand complex
- administering said FVIII-ligand complex to a patient in need of FVIII, the ligand being selected from peptides, sugars, nucleic acids and small chemical compounds, said small chemical compounds having a molecular weight of 5 KDa or less.

A further embodiment of the invention is a ligand binding with high affinity and high selectivity to or in the region of LRP and/or HSPG in order to block the mentioned sites.

Receptor-mediated clearance, proteolytic or non-proteolytic inactivation mechanisms are responsible for the "loss" of proteins from the blood stream.

This is explained in more detail for Factor VIII.

After its synthesis and secretion into the blood, 95-98% of FVIII is captured by vonWillebrandFactor, which protects FVIII from clearance and inactivation by binding tightly to its C2 domain, thus circulating in the bloodstream as a tight complex with FVIII. 2 to 5% of FVIII in the blood stream however remain vWF-unbound and are subjected to receptor-mediated clearance processes.
Activation of FVIII by thrombin-cleavage leads to the loss of vWF binding and therefore to the loss of its protecting properties. Activated FVIII (FVIIIa) is also subjected to receptor mediated clearance and proteolytic degradation, as well as non-proteolytic inactivation mechanisms.

Receptor-mediated clearance of FVIII is mainly mediated by low density lipoprotein related protein receptor 1 (LRPR-1) and heparan sulphate proteoglycans (HSPGs) (for references see (2;3)).

Heparan sulphate proteoglycans (HSPGs) are cell surface sugar structures capable of capturing proteins comprising a HSPG-binding site. They therefore enrich the local concentration of these captured proteins two-dimensionally at the cell surface, thus facilitating receptor-mediated endocytosis by cell surface receptors (see (4-6) for reference). HSPG-mediated FVIII clearance takes place in cooperation with the LRPI-receptor (LRPR-1). However, there is evidence for HSPG-mediated FVIII clearance together with other yet unidentified cell surface receptors (5).

LRPR-1 belongs to the low density lipoprotein receptor family, also comprising LDLR (low density lipoprotein receptor), VLDLR (very low density lipoprotein receptor) and megalin-receptor, which all solely play an inferior role in FVIII-clearance (4-6). LRPR-1 mainly mediates the receptor-mediated FVIII clearance either self-sufficiently or in cooperation with HSPGs (5). The effect of LRPR-1 on FVIII clearance has been extensively determined and elucidated (5;7-14):

It has been shown in vitro that the dissociation constant for LRPR-1 and FVIII purified proteins is of high affinity ($K_D = 25-100$ nM or 116 nM, respectively, see (8;12).

In cell-based assays it could be demonstrated that LRPR-1-deficient cells showed 50% less FVIII clearance, and blocking LRPR-1 with RAP (receptor associated protein, a LRPR-I-inhibitor) also resulted in a 50% reduced FVIII
clearance \((8;11;12)\). *In vivo* LRPR-l-knockout mice showed increased FVIII plasma levels, the half-life of intravenously administered FVIII was increased from 2.5 to 4 hours corresponding to a half-life prolongation of factor 1.5 \((5;7)\). Furthermore, clearance studies in mice, in which LRPR-1 was inhibited by RAP showed a half-life prolongation of factor 3.5 \((5)\). There also exist a lot of data about LRPR-l-polymorphisms in humans leading to 20% increased FVIII plasma levels \((9; 10; 13; 14)\).

Sarafanov et al. \((5)\) proved the effect of synergistically blocking HSPGs and LRPR-1 on FVIII clearance in mice (see Figure 2). In a first set-up they blocked HSPGs using its antagonist protamin \((1\) in Figure 2) resulting in a half-life prolonging effect for FVIII of factor 1.6. In a second set-up LRP-receptors were blocked by RAP \((2\) in Figure 2), resulting in a half-life prolonging effect of factor 3.5 for FVIII. In a third set-up both, HSPGs and LRP-receptors, were blocked synergistically, yielding a FVIII half-life prolongation of factor 5.5.

Prior art has tried to block the receptor to influence receptor mediated clearance. The present invention is directed to a method for influencing receptor mediated clearance by modifying the therapeutic proteins by blocking them non-covalently via specific binding of a small ligand or covalently by attaching a small ligand. FVIII has various binding sites for proteins involved in Tenase complex formation, FVIII-clearance and proteolytic inactivation. These binding sites partially or totally overlap (for example the binding sites for LRP-receptor/HSPG and FIXa) due to their importance during different phases within the FVIII life cycle.

FVIII comprises five different so far identified binding sites for LRP-receptor and one clearly characterized binding site for HSPG, which will be described in more detail in the following.

FVIII features one clearly identified and well characterized HSPG binding site located within the A2 domain, comprising the amino acids 558-565 (see Figure 3). This site reveals high affinity for heparin and analogues \((25.8 \text{ nM})\).
used as surrogates for HSPGs (5) and overlaps with the binding region for FIXa. However, there is evidence for a second low-affinity \( (K_D = 652 \text{ nM}) \) HSPG-binding site elsewhere on FVIII (5).

FVIII features five characterized binding sites for LRP-receptor located within the A2, A3, C1 and C2 domains (see Figure 4). Two of them are high-affinity sites: the LRP-receptor binding sites in the A2 (484-509) and in the A3 domain (1811-1818), revealing dissociation constants (for purified proteins) of 25-116 nM \( (8;12) \). Both high-affinity LRP-binding sites overlap with the binding region for FIXa involved in Tenase complex formation. The LRP-receptor binding sites within the C2 and C1 domains are of low affinity and shielded by vWF, thus playing only an inferior role in receptor-mediated FVIII clearance.

Our concept for non-covalent half-life prolongation (HLP) of proteins like FVIII is based on the assumption, that blocking of certain binding sites on the surface of FVIII by small ligands (peptides, sugars, small proteins, nucleic acids, chemical compounds) will be sufficient to inhibit/decelerate receptor-mediated clearance and proteolytic inactivation mechanisms of FVIII, thus prolonging its plasma half-life.

In general the target site can be any region on the FVIII surface whose blocking by a small binding ligand results in a half-life prolonging effect of the target protein.

For this approach the A2 and A3 domains of FVIII are targeted for the following reasons:

- The A2 domain harbours a high-affinity binding site for the LRP-receptor as well as for HSPGs which are essentially involved in receptor-mediated clearance of FVIII.
- The A3 domain, in addition, features a second high-affinity binding site for the LRP-receptor.

Thus, preferred target sites for HLP of FVIII are (see Figure 5):
- the A2 LRP-receptor binding site,
- the A2 HSPG-binding site and
- the A3 LRP-receptor binding site

It has been demonstrated by Sarafanov et al., (5) that blocking HSPGs and LRPI-receptor on cell surfaces results in effective half-life prolongation for FVIII. However, only blocking both the HSPGs and the LRPI-receptor simultaneously yielded the maximal HLP-effect (factor 5.5), whereas single inhibition of HSPGs or LRPI-receptor showed smaller FVIII-HLP (factor 1.6 for blocking HSPGs and factor 3.5 for blocking LRPI-receptors).

According to the invention, site-specific blocking of the above listed target sites on FVIII by small, highly affine and specific ligands will prolong the plasma half-life of FVIII. At which combination the target sites have to be blocked and if all target sites must be blocked in parallel to yield the maximal HLP-effect needs to be determined.

Ligands found binding to or around the HSPG- or LRPI-receptor binding sites on FVIII during the screening procedure can be further optimized regarding their binding affinity and/or specificity.

For FVIII the amino acid sequence is known, several structural data are available and the HSPG- and LRPI-receptor binding sites are characterized. The LRP-receptor sequence is also available, as well as structural information of LRPR-subdomains involved in direct binding of FVIII A2 and A3 domains. Additionally, various information about heparin and heparin-analogs is available.

In case of binding peptide ligands, the ligand sequence is known and the structure could be solved for example by nuclear magnetic resonance (NMR), if needed.
Taking into account all the available information, the binding affinity and/or specificity can be enhanced by varying the amino acid sequence of the binding region of the ligand (changing charge, polarity or bulkyness of one or more amino acid positions) and/or the overall length of the ligand.

Ligands can be unstructured or of structured nature, comprising a specific folding.

Unstructured, linear peptide or sugar ligands for example will adopt different flexible conformations in solution. Therefore, the binding to the target protein might be more probable and of high affinity, but maybe of low specificity.

As mentioned, an optimal ligand for non-covalent half-life prolongation should bind with high affinity and specificity. It should be stable and soluble in solution, non-immunogenic and non-toxic, and should not influence the biological activity of the target protein.

Structured, folded ligands (for examples cyclic scaffold peptides, small proteins or protein domains) comprise the advantages of a stable folding and therefore a defined conformation in solution. This folding corresponds to a specific surface of this ligand, thus rendering a binding event to be specific. The ligand folding is also responsible for a better stability in solution.

As stated above a ligand for a non-covalent half-life prolongation approach should fulfill the following properties:

- The binding event should be of high affinity ("tight binding").
- The ligand should bind with high specificity to the target protein and should not cross-react with other binding partners (no "promiscuity").

The following section deals with ligand categories useful for this approach.

Linear peptides of variable length and sequence are used as binding partners for non-covalent half-life prolongation of FVIII. Their conformation is flexible in
solution, thus they can adopt various conformations upon a binding event. Linear peptides binding with high affinity and specificity to FVIII are identified by screening of huge random libraries.

Linear sulphated peptides are used as HSPG-mimetics and targeted to the HSPG-site on the A2 domain on FVIII. There are some sulphated heparin-mimetic peptides known to bind to fibroblast growth factor 1 (FGF1) or vascular endothelial growth factor (VEGF), both being HSPG or heparin-binding proteins (15;16). Although this targeted approach is promising in terms of binding, the synthesis of such poly-sulphated peptides is highly complicated and time consuming, as the intermediates and products may not be stable.

Structured peptides can be any peptide showing a distinct folding or structure. These peptides can be mono-, bi-, tri- or polycyclic. Their cyclic structure can be achieved for example by introduction of cysteines and subsequent disulfide bond formation.

Structured peptides comprise the advantages of a higher stability due to their 3-dimensional structure. They have a defined surface, which renders the binding to a potential target protein much more specific than for example linear peptides would achieve. With structured peptides it is possible to mimic known binding ligands or binding surfaces (see Figure 9 and 10).

A continuation of the idea using peptides as small binding ligands is the idea to use small proteins or protein domains with a specific modified binding region for specifically binding to the targeted sites on the target protein. The function of the overall protein folding is to stabilize the specific binding region (see Figure 7).

A different substance class applicable as specific binding ligands for half-life prolongation are sugars. As an example, heparin-mimetic sulphated sugars targeted to the HSPG-binding site of FVIII can be used. In addition sugars
binding to the LRP-receptor binding site on FVIII can be used as LRP-mimetic sugar ligands.

Sugars provide the advantage of low immunogenicity. However their designed synthesis can be very complex, cost and time consuming.

Other ligands for the covalent or non-covalent HLP-approach can comprise any substance fitting the above mentioned ligand properties needed. These can include for example DNA or RNA-molecules (structured) (e.g. using the aptamer technology) or antibody-derived domains, any non-covalent, specific binding substance irrespective of its exact chemical nature, including peptides, DNA, RNA, sugars, oligosaccharides, or any other chemical substance.

**Brief description of the figures**

Figure 1 is a schematic overview of clearance and inactivation mechanisms for FVIII and FVIIIa.

Figure 2 is a schematic overview of clearance studies in mice performed by Sarafanov et al., (5).

Figure 3 shows B-domain deleted FVIII and its binding sites for Tenase complex formation, clearance receptors and proteolytic inactivation. Capital letters indicate the domain structure; numbers give the amino acid positions of the binding sites.

Figure 4 shows an overview of binding sites for HSPG-and LRP-receptor on B-domain deleted FVIII. Capital letters give the FVIII domain structure; numbers give the amino acid positions of the corresponding binding site.

Figure 5 shows: FVIII and preferred target sites for half-life prolongation.

Figure 6 shows the full length mature FVIII protein sequence (w.o. signal peptide).

Figure 7 is a schematic representation of the non-covalent binding approach using small proteins or domains with specific, designed binding regions stabilized by the overall protein folding.
Figure 8 shows the relative half-life of FVIII with bound low molecular weight heparin Clexane derived by a pharmacokinetic mouse study where wild-type mice were administered with exogenous FVIII with and without Clexane. Clexane binds to the HSPG-binding site of FVIII and therefore blocks the interaction with cell surface HSPGs partially responsible for FVIII clearance. The plasma half-life was prolonged by factor 2. All data were statistically evaluated and passed the significance test (P-values < 0.05: P(FVIII) = 0.0414 and P(FVIII+Clexane) = 0.0363).

Figure 9 is a schematic representation of potential binding sites on a protein, their structure and possibilities to mimic these binding sites using peptides: linear, cyclic or poly-cyclic peptides; circles: single amino acids, yellow connected circles indicate cysteines bridged by sulfhydryl groups.

Figure 10 is a schematic representation of the design of ligands for half-life prolongation of FVIII (A) and construction of binding site mimetics (B). A: the ligand structures and sequence information are used to generate peptide ligands resembling and mimicking the physiological ligand (for example LRP1-receptor); B: the FVIII structures and sequence information can be used to generate binding site mimetics mimicking the FVIII HSPG- and/or LRP-receptor binding sites and can be used as screening tools for finding ligands targeted to these binding sites.

**Examples**

**Sulphated sugars**

Sulphated sugars are mimick HSPGs or heparin and are therefore likely to bind to the HSPG- or heparin binding sites of proteins. Different heparin-mimetic sugars varying in length and sulphation pattern are used to find sugar-ligands binding with high affinity and specificity to the HSPG-binding site on the A2 domain of FVIII to block the clearance interaction with cell surface HSPGs.
The effect of a heparin-mimetic sugar bound to the HSPG-site on FVIII on its plasma half-life by pharmacokinetic animal study using wild-type mice is demonstrated (see Figure 8).

These were administered with exogenous recombinant FVIII with bound Clexane. Clexane is a low molecular weight (LMW) heparin (Mw = 4.5 kDa), which binds to the HSPG-binding site on the A2 domain of FVIII and therefore blocks the interaction with cell surface HSPGs partially responsible for FVIII-c Clearance. Binding Clexane resulted in an around 2-fold longer plasma half-life of FVIII.

This clearly indicates that a small ligand (like Clexane) is able to sufficiently block an important clearance site of FVIII, thus directly resulting in a prolonged plasma half-life. The results are shown in Figure 8.

These findings are also in line with the results of Sarafanov et al. (5) who performed FVIII-c Clearance studies in mice and found a half-life prolonging effect for FVIII by factor 1.6 via direct blocking of the HSPGs by Protamin on cell surfaces.

**Peptides**

a) Octet-Screening

Screening and kinetic measurements have been carried out using the Octet Red system. The principle is based on the optical measurement of the thickness of a protein layer on a biosensor tip. Streptavidin sensors are used for screening and kinetic experiments of biotinylated peptides with recombinant FVIII, recombinant FVIII domain A2. As a negative control, the C2 domain of FVIII and recombinant FIX were used and showed no binding to the ligands. The biosensor is coated with a ligand. Then the immobilized ligand is exposed to an analyte. The thickness of the resulting ligand-analyte complex/layer is observed during association and dissociation. Thereof the binding affinity between ligand and analyte is computed.
Measurement took place in a 96-well plate with eight biosensors analyzing up to eight samples in parallel. The concentrations of the ligands for screening experiments have been optimized to 5 pg/rnl for peptides and antibodies. Experiments with streptavidin sensors are conducted with 10 pg/rnl biotin as quenching agent.

Screening random peptides, the following binding kinetics could be determined for SEQ ID NO 2 to 23:

<table>
<thead>
<tr>
<th>Ligand-ID</th>
<th>Sequence</th>
<th>rhFVIII $K_D$</th>
<th>A2-domain $K_D$</th>
</tr>
</thead>
<tbody>
<tr>
<td>r18</td>
<td>TTWGMRIRLGERQAR</td>
<td>4.9 nM</td>
<td>5.1 nM</td>
</tr>
<tr>
<td>r19</td>
<td>RMRMYSSLRAWKESA</td>
<td>6.7 nM</td>
<td>1.7 nM</td>
</tr>
<tr>
<td>r26</td>
<td>PIRIMILISLMNRPT</td>
<td>4.4 nM</td>
<td>3.6 nM</td>
</tr>
<tr>
<td>r27</td>
<td>SPSWVRPSIMEWRMN</td>
<td>5.1 nM</td>
<td>1.4 nM</td>
</tr>
<tr>
<td>r40</td>
<td>NKLTSGDRPGLWFMV</td>
<td>4.6 nM</td>
<td>-</td>
</tr>
<tr>
<td>r43</td>
<td>RDERMKLIRNFSPIM</td>
<td>1.0 nM</td>
<td>-</td>
</tr>
<tr>
<td>r44</td>
<td>EPLMTMKMIRVMRKL</td>
<td>1.5 nM</td>
<td>1.8 nM</td>
</tr>
<tr>
<td>r45</td>
<td>TERTQMIHKIFALMSG</td>
<td>16.5 nM</td>
<td>-</td>
</tr>
<tr>
<td>r47</td>
<td>EMMKPNYADLMRKR</td>
<td>0.7 nM</td>
<td>-</td>
</tr>
<tr>
<td>r49</td>
<td>LSGKNGPWVKMIMWG</td>
<td>8.6 nM</td>
<td>10 nM</td>
</tr>
<tr>
<td>r55</td>
<td>WKHHMRRKAIKYEGK</td>
<td>2.2 nM</td>
<td>25 nM</td>
</tr>
<tr>
<td>r57</td>
<td>DMDKSVQRQMMSRGR</td>
<td>1.33 nM</td>
<td>484 nM</td>
</tr>
<tr>
<td>r58</td>
<td>QRIKGLHFATSMGYA</td>
<td>0.5 nM</td>
<td>760 nM</td>
</tr>
<tr>
<td>r82</td>
<td>SPWSWQRQMKWTPQ</td>
<td>4.34 nM</td>
<td>-</td>
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<tr>
<td>r89</td>
<td>AYRSTSREHWRRHW</td>
<td>35 nM</td>
<td>15.6 nM</td>
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<tr>
<td>r94</td>
<td>KKRDARTNYRMSYS</td>
<td>15.2 nM</td>
<td>7.91 nM</td>
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<tr>
<td>r106</td>
<td>ISRRLMMGKKSHQHD</td>
<td>3.77 nM</td>
<td>-</td>
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<tr>
<td>FIX-2 linear</td>
<td>iadkeytnifkfgsvyss</td>
<td>22.9 nM</td>
<td>1.95 nM</td>
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<td>FIX-4 linear</td>
<td>rvpvdratcrstkiyynn</td>
<td>5.57 nM</td>
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<tr>
<td>FIX-6 linear</td>
<td>iadkeytnifkfgvsvlvdratcrstkiyynn</td>
<td>1.41 nM</td>
<td>4.56 nM</td>
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<tr>
<td>P2</td>
<td>EYISWEYEC</td>
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<td>-</td>
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<tr>
<td>FIX3-loops2</td>
<td>wcpfgefgknceldvtnikggrc</td>
<td>1.2 nM</td>
<td>-</td>
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</table>

"-" indicates no detectable binding

Peptides showing binding to recombinant FVIII, but not to the A2 domain, bind through a different binding site.
Phage-Display

Starting from a random library, phages were selected for their ability to bind to recombinant FVIII or recombinant FVIII domains (for example A2).

An ELISA was conducted to quantify interaction between the binding ligands and FVIII or A2. The following ligands (SEQ ID NO 24 to 25) were identified:

<table>
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<tr>
<th>Sequence</th>
<th>rhFVIII [OD]</th>
<th>A2-domain [OD]</th>
</tr>
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<tbody>
<tr>
<td>HHHHNRHQWLWTDTNVKVTS</td>
<td>0,426</td>
<td>0,384</td>
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<tr>
<td>QVQLLESGGGLVQPGRSLCAASGFTVS</td>
<td>0,558</td>
<td>0,656</td>
</tr>
<tr>
<td>PNMSWVRQPAGKGLEWVSISSITVPNGSTYAD</td>
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</tr>
<tr>
<td>SVKGRFRDLNSKNTLYLQMNSLRAEDTAVYYCAGYSPWYDNFRYWGQGTLVTVSS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Helper Phage</td>
<td>0,096</td>
<td>0,085</td>
</tr>
</tbody>
</table>

Macroarray-Screening

Intavis-(384)-peptide arrays containing FIX, LRP-II and LRP-IV sequences were used for detecting specific protein-peptide interactions on the peptide array in order to identify potential ligands for half-life prolongation of FVIII. The peptide array was incubated with recombinant FVIII or the A2 domain.

<table>
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<tr>
<th>Ligand-ID</th>
<th>Sequence</th>
<th>rhFVIII</th>
<th>A2_domain</th>
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<tr>
<td>384-1</td>
<td>IRIIPHH</td>
<td>+</td>
<td>+</td>
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<tr>
<td>384-2</td>
<td>TKVSRYY</td>
<td>+</td>
<td>+</td>
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<tr>
<td>384-3</td>
<td>TQFTCNNGRCI</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>384-4</td>
<td>TQFTCNNGRCINIW</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>384-5</td>
<td>QCRLDGLCIPILRWRC</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>384-6</td>
<td>HTEQKRNVIRIIPHHNYNAIINK</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>384-7</td>
<td>MKGKYGIYTKVSVYVWNIKEKTK</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>384-8</td>
<td>ADKEYTNIFLKFGSGYVSGWGRVFHKGRSAL</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Ligand 384-1 to ligand 384-8 (SEQ ID NO 26 to 33) show binding to both FVIII and the A2 domain.
References


19. Dolmer, K., Huang, W., and Gettins, P. G. (1998) Characterization of the calcium site in two complement-like domains from the low-density lipoprotein receptor-related protein (LRP) and comparison with a repeat from the low-density lipoprotein receptor, Biochemistry 37, 17016-17023.


Claims

1. Pharmaceutical preparation comprising
   - a pharmaceutically active protein having at least one LRP-binding site
     and/or at least one HSPG binding site and
   - at least one ligand binding to the LRP-binding site and/or the HSPG
     binding site, the at least one ligand being selected from peptides,
     sugars, nucleic acids and small chemical compounds, said small
     chemical compounds having a molecular weight of 5 kDa or less.

2. Pharmaceutical preparation according to claim 1 wherein the
   pharmaceutically active protein is Factor VIII, preferably human FVIII.

3. Pharmaceutical preparation according to claim 2 wherein FVIII is
   recombinant FVIII.

4. Pharmaceutical preparation according to at least one of claims 2 to 3,
   wherein FVIII is B domain deleted FVIII.

5. Pharmaceutical preparation according to at least one of claims 2 to 4,
   wherein the FVIII LRP binding site is localized within the A2 domain
   residues 484-509 and/or the A3 domain residues 1811-1818 C2 domain
   residues 2173-2332 and/or CI domain residues 2065, 2092 in
   accordance with SEQ ID NO. 1.

6. Pharmaceutical preparation according to at least one of claims 2 to 5,
   wherein the FVIII HSPG binding site is localized within the A2 domain
   residues 558-565 in accordance with SEQ ID NO. 1.

7. Pharmaceutical preparation according to at least one of claims 1 to 6,
   wherein the ligand has a molecular weight below 5 kDa.

8. Pharmaceutical preparation according to at least one of claims 1 to 7,
   wherein the ligand is selected from peptides, sugars, lipids, nucleic acids
   and variants thereof and chemically synthesized compounds.
9. Pharmaceutical preparation according to claim 8, wherein the peptide is a linear, cyclic, folded or a scaffold peptide.

10. Pharmaceutical preparation according to at least one of claims 1 to 9, wherein the ligand comprises at least one sulphate group.

11. Pharmaceutical preparation of claims 1 to 10, wherein the ligand is selected from peptides comprising SEQ ID No. 2 to 33.

12. A pharmaceutical preparation comprising
   - a pharmaceutically active protein having at least one LRP-binding site and/or at least one HSPG binding site and
   - at least one molecule binding to the LRP-binding site and/or the HSPG binding site, the at least one molecule comprising two or more ligands covalently bridged for blocking adjacent binding sites.

13. The pharmaceutical preparation of claim 12 wherein one of the binding sites is a HSPG-binding sites and the other binding site is a LRP binding site.

14. A method for testing a ligand's ability to bind to at least one LRP binding site and/or at least one HSPG binding site of FVIII comprising the steps of
   - combining the ligand with FVIII to a FVIII-ligand complex
   - measuring clearance of the FVIII-ligand complex in-vivo
   wherein a reduced clearance indicates that the ligand binds to at least one LRP-binding site and/or at least one HSPG binding site of FVIII, the ligand being selected from peptides, sugars, nucleic acids and small chemical compounds, said small chemical compounds having a molecular weight of 5 KDa or less.
15. A method for reducing clearance of FVIII comprising the step of
- combining FVIII with at least one ligand binding to the LRP-binding site
  or the HSPG binding site of FVIII to form a FVIII-ligand complex
- administering said FVIII-ligand complex to a patient in need of FVIII,
  the ligand being selected from peptides, sugars, nucleic acids and
  small chemical compounds, said small chemical compounds having a
  molecular weight of 5 KDa or less.

16. A ligand selected from peptides having one of the SEQ. ID. No 2 to 33.
Fig. 1

Fig. 2

Protamin = Heparin-antagonist
RAP (Receptor associated protein) = LRP receptor inhibitor
ATRRYLYGAVELSWDYMQSDLGELPVDARFPVRPVPSFPFNTSUVYKKTTLFVEFTDLHFN
IAKPRPWPWMLGGLGTAQFQEVYDTVITLKNMASHVPLSLHAVGSYVWKASEGAEYDDQTSQ
REKEDDKVFPGGSHYVWQVLYQNGPMASPDCLTLYSLSHLVHDNLSEGALLOWLYCER
EGSLAKEYEQTLHFKFILLFAVDEGKSWHSETKSNMQRDAASARAPKTMHTNVGYVR
SLPGLJGCHRKSUYHVHJNHGMTPPESIFLEHHTLVRHNRQASLIESIPITLAQTLL
MDLGQFLLLFCIHSSHQHDGMEAYVKVDSCEPEPQLRMRKNNREEAYDDDLTDDSEMVDVRF
DDDDNPSFQIIRSVAHKHPWTWYHIAAEEEDWDYAYPLVAPLDDRSYSQYLNNGPQIRG
RYKYMVRAYMAYTFDCTREAQHESQILGPLYGEVGDGTYIIYFNKQASRPYNJYHPG
IDTVRPLYRRLPKVYKLKDFPILPGEIFKYKWVTVEDEGTPKSDCRPLTRYSVSSNFME
RDLASLIGPLICYKESVDQRSQHNIQMSDKXVILFSFDENSWYLTIENIQRFPLNPAG
VQLEDPEQASONIMHINGSNYFGDQSLVSCLVIHEAVYWYILISAGAQTFDLSFVSSFGFHYK
KMYVEDTTLTPFSGETUVFSMENPGPLVLGCHNSDFRNGMTALKVSSCDKNTGDYYE
DSYEDIAYLLSKNAIEPSRHQSNSHPRSTRQKFQFNATTIPENDITKDDPWFARHPTMP
KIQNVSSSDLMLLLQSPTHGPLSLSLQAEKYETFSDDPSPAIDSNNSLEMMTHFRPQ
LHSGDMVFEPESGLQLRLNEKLGTTAATELKLDFKVSSTSNLSTIPSNDLAACTDQ
TSSLGPPSMVPVHYSQDLTTLFKKSSPLTESGGLPLSLEEENSDKLLEGSMLNSQESSW
GKNSVSTESGRLFQGKRAHPALLTKDNALFKVSSLKTNKSNTSANTRKTHIDGSP
LIENSPSVWQNILEDTEFKKVKVTLHDIRMLDKNATALRNHMSTSKNNMEMVQQK
KEGPIPPDAQPNMDDSFKKFMLEPSARWUIRTHKGKNSLNSQQPSKPQKQSVLPGKESV
EGQNLSELKENVVVKGKGTEFTDKVGMFVMPSRNLNLFLNLNLDNLHENTHNQIEKKIEE
KETLQENVLPQIHVTGKTNKMNLFLSLTRQNVEGYSYDGAAPQLVQDFRSLNLDSTNR
TKKHTAHFSDKGKEEENLEGLGGNOQTQIVKYEYACTTRISPNTSQNFTVQSKRALKQFR
PLEETELEKRIIVDDSTQWSKNMHHLTPTSTLQIDYNEKEKGAQTIPSLSDLCRSHSI
PQANRSPPLIAKVSSPSFSPRPIYLTRLFQDNSHLPAAASYRKDSGVQESSHFLQGAKK
NNLSLAILTLTMGTGDQREVGLQSTANSVTYKVENTVLPKDLPKTSKQVLPHVVHI
YQKDFLPFTETSSNGPSGHLDEVLQEQTEGAIKWNENEAPGPVKVFPLRVALTESSAKTPSK
LDDPLAWDNHYGTQIPKKEEWKSQKPEKTKTKDILTNLACSENHAIAIAINENQKPK
EIEVTWAKQGRTERLCQSNNPVLRRHRQEITLRLQDSEEIDITSVEMKCEDFIY
DENEQPRSFQKXTRHYTIALAVERLWDYGMSSSHPHLRERQSGPSVPQFKVQVFQETTD
GSFTQPLYRLNENLHGLPQDLQAEVEDNIMTVFNQASPVFSLISYEDQROQGA
EPKKNFVKPETKTYWFKVQHMAPTKDFECDKAWASYDVEKDHVSLQGILPLLVCVT
NTLNPAPHRQTVQFESAFLITFIDETKSTYFENEMERNCRPCNIQMEDPTFKEHYRFA
INGYIMDTLPVLVMAQDRIRWLYLSSGSMENHISIFGSGHVFTVRKEEYKMLYNLQP
GVFETVEMPSKAIWVRJCEGLHAGMSTMLFLVYSNCQPLTMSAGHIRDQITAS
GQYGQWAPKLRALHYSGSINDWSTKEPSWIKVDLAPMIHIGKTQGARQKFSSLYISQ
FIMYSLDGKWKQRTYGNSGTGLMVFNNVDSSGKIHNNINPPIARYIRLHPTHYIRS
TLMRLMGCNLNSCMLPSGLMESISKAISDAIQATASSYTNMFATWSPSKARLHQGRSNW
RPVNNPKEWLQVDFQKTMKVGTVTQGVKSSLNTMVKFELISSQDSGQHWTLLFQNGKVN
KVDFQGQNSDFTPVNLSLPPPLTRYRHIPQSWSHVQIALRMEVLGCIEAQDLY

Fig. 6
Fig. 7

Fig. 8
Fig. 9
Fig. 10
A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K38/37 A61K47/48 G01N33/50 A61P7/02
ADD.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K G01N

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal , BIOSIS, Sequence Search , EMBASE, CHEM ABS Data, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Date of the actual completion of the international search
21 June 2011

Date of mailing of the international search report
06/07/2011

Name and mailing address of the ISA
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer
Grei f, Gabri e l a
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<td>DATABASE BIOSIS, [Online] 16 November 2003 (2003-11-16), MERTENS KOEN ET AL: &quot;The endocytic receptors megalin and low-density lipoprotein receptor-related proteins share binding to coagulant anti on factor VIII&quot;, XP002589830, retrieved from BIOSIS Database access no. PREV200400172501 abstract</td>
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<td>FAY P J ET AL: &quot;Mutati ng factor VIII: lessons from structure to function&quot;, BLOOD REVIEWS, CHURCHILL LIVINGSTONE LNKD-D01:10.1016/ J. BLDREV 2004.02.003, vol. 19, no. 1, 1 January 2005 (2005-01-01), pages 15-27, XP004661269, abstract page 19, left-hand column, paragraph 3 - right-hand column, paragraph 1; figure 1 page 22, left-hand column, paragraph 2 - page 24, right-hand column, paragraph 2</td>
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<td>SCHWARZ HANS PETER ET AL: &quot;Invol vement of low-density lipoprotein receptor-related protein LRP in the clearance of factor VIII in von Willebrand factor-deficient mice&quot;, BLOOD, vol. 95, no. 5, 1 March 2000 (2000-03-01), pages 1703-1708, XP002589828, ISSN: 0006-4971 the whole document</td>
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<td>ANANYEVA N M ET AL: &quot;Catabolism of the coagulati on factor VIII: can we prolong lifetime of VII in circulation?&quot;. TRENDS IN CARDIOVASCULAR MEDICINE, ELSEVIER SCIENCE, NEW YORK, NY, US LNKD-DOI: 10.1016/51050-1738(01)00124-4, vol. 11, no. 6, 1 August 2001 (2001-08-01), pages 251-257, XP002320945, ISSN: 1050-1738, the whole document</td>
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<td>LENTING P J ET AL: &quot;The light chain of factor VIII comprises a binding site for low density lipoprotein receptor-related protein&quot;. JOURNAL OF BIOLOGICAL CHEMISTRY, THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, INC., BALTIMORE, MD, US, vol. 274, no. 34, 1 August 1999 (1999-08-01), pages 23743-23749, XP000882874, ISSN: 0021-9258, DOI: DOI: 10.1074/JBC.274.34.23734, the whole document</td>
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