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Strategies towards a longer acting factor VIII

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Summary. The reduced mortality, improved joint outcomes and enhanced quality of life, which have been witnessed in the developed world for patients with haemophilia, have been an outstanding achievement. Advancements in biotechnology contributed significantly through the development of improved pathogen screening, viral inactivation techniques and the development of recombinant clotting factors. These were partnered with enhanced delivery of care through comprehensive haemophilia centres, adoption of home therapy and most recently effective prophylaxis. This came at great costs to governments, medical insurers and patients' families. In addition, barriers persist limiting the adoption and adherence of effective prophylactic therapy. Biotechnology has been successful at overcoming similar barriers in other disease states. Long-acting biological therapeutics are an incremental advance towards overcoming some of these barriers. Strategies that have been successful for other therapeutic proteins are now being applied to factor VIII (FVIII) and include modifications such as the addition of polyethylene glycol (PEG) polymers and polysialic acids and alternative formulation with PEG-modified liposomes. In addition, insight into FVIII structure and function has allowed targeted modifications of the protein to increase the duration of its cofactor activity and reduce its clearance *in vivo*. The potential advantages and disadvantages of these approaches will be discussed.

Keywords: bioengineering, clearance, factor VIII, half-life, polyethylene glycol, polysialic acids

Introduction

The last three decades of the recombinant technology era witnessed the cloning of the factor VIII (FVIII) gene, the development of recombinant FVIII (rFVIII) for infusion and furthered the progress towards a genetic cure. The resultant increased capacity for FVIII concentrate production coupled with advances in haemophilia comprehensive care, pathogen screening and viral inactivation technology has led to both exciting and sobering observations within the developed and developing countries worldwide. Within the United States, life expectancy is now approximately 65 years; serious blood-borne infections have not occurred since 1990 and joint disease has been eliminated for children under the age of 15 [1]. Home treatment and early initiation of prophylaxis have been the most significant advances to impact on joint disease prevention and quality of life [2]. This has of course come at great economic cost. The average cost of the treatment for a person with haemophilia in the United States in 2001 was $139 000, 72% of which was due to costs for factor concentrates [3]. This cost is not dissimilar to those for patients in the developed countries with socialized national health care systems such as the United Kingdom, Australia or Germany [4]. These kinds of costs are an insurmountable barrier for similar treatment in many developing countries. Although prophylaxis treatment strategies vary greatly, the typical regimen requires FVIII infused at a dose of 20–40 IU kg^{-1} three times per week. Despite the improved musculoskeletal outcomes and quality of life, enthusiasm for prophylaxis is hampered by suboptimal adherence to therapy [5] and frequent need for the placement of central venous access devices (CVAD) [6].

While we wait on an ultimate genetic cure, perhaps through gene therapy, the question is whether innovations in drug delivery or recombinant

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technology will be able to overcome these barriers (cost, compliance, need for CVAD) to continued effective prophylaxis. Certainly, the development of clotting factor delivery systems that did not rely on needle and syringe would be a major breakthrough, but even strategies to extend the functional half-life of FVIII could have a significant impact. There have been considerable advances in biological therapy in recent years in other disease states that have included non-invasive methods of delivering proteins and increasing their biological half-life. This review will explore successful strategies for other therapeutic proteins, the advantages and disadvantages of such strategies as applied to rFVIII delivery as well as new innovations in rFVIII therapy towards a longer acting FVIII protein.

Polyethylene glycol conjugation

Several *in vivo* factors can reduce the efficacy of biological therapeutic agents. These include poor solubility at physiological pH, neutralization by host antibodies, rapid renal clearance, cellular clearance and proteolysis [7]. Modification of proteins through conjugation with polyethylene glycol (PEG) polymers, or PEGylation, has been a successful strategy to overcome some of these efficacy limitations. PEG polymers are amphiphilic, non-toxic and immunologically inert. They consist of ethylene oxide subunits linked in a chain with a hydroxyl group at each terminal and may be linear (5–30 kDa) or branched (40–60 kDa) through connections produced by chemical linkers [7]. PEGylation was first described in the 1970s [8,9] and has resulted recently in several commercialized therapeutics. These modified biologics can have better efficacy, provided the parent protein's biological activity is retained.

Polyethylene glycol polymers incorporate many water molecules within their hydrophilic structure, so that their volume is disproportionately larger than that predicted by their molecular weight [10]. PEGylation increases the resultant size of the conjugated therapeutic protein above the limit for kidney filtration, thereby extending the circulating half-life [7]. The hydrated PEG chains are also highly mobile in aqueous medium potentially shielding antigenic determinants on the protein from access to immune-mediating cells [7]. PEGylation can reduce proteolysis by proteases through steric hindrance or site-specific conjugation that eliminates cleavage sites [10]. Some proteins are relatively insoluble with a tendency to aggregate. PEGylation increases the solubility of several proteins making it easier to formulate for administration [7]. All of these improved features could result in improved efficacy *in vivo* but may also contribute to greater patient adherence by reducing the frequency of administration and improving quality of life [7].

Commercialized PEGylated protein therapeutics have included PEG-asparaginase for the treatment of acute lymphoblastic leukaemia, PEG-adenosine deaminase for severe combined immunodeficiency disease, PEG-interferon for hepatitis C, PEG-granulocyte colony stimulating factor (G-CSF) for chemotherapy-induced neutropenia and PEG-growth hormone for the treatment of acromegaly. The PEGylation of G-CSF results in a molecule whose mode of elimination is almost entirely neutrophil-mediated clearance, thus the drug's elimination from plasma is dependent on the recovery of the patient's neutrophil count. UnPEGylated interferons require dosing three times per week, not too dissimilar from prophylactic FVIII administration regimens. PEGylation of interferon resulted in efficacious dosing schedules of once per week [11]. With regard to potential reduced immunogenicity from PEGylation, one study observed that some children who experienced anaphylaxis to unmodified asparaginase tolerated the PEGylated version without an allergic reaction [12].

Polyethylene glycolylation of FVIII would seem to be a natural progression for the advancement of haemophilia therapeutics. However, there are important drawbacks to consider with PEGylation as well as unique elimination properties for FVIII, which may limit this application. The main disadvantage of PEGylated protein formulations is that an improved pharmacokinetic profile can be accompanied by decreased specific activity [7]. Cross-linking PEG to proteins typically involves coupling of activated PEG molecules to amino groups of lysine or the protein's aminoterminal group [7,13]. Without the ability to discriminate where the PEG polymers conjugate to the protein, there may be decreased accessibility for key activating proteases or other protein–protein interactions integral to the therapeutic protein's biological activity. For example, PEGylated α-interferon retains only 7% of the antiviral activity of the native protein. However, *in vivo*, by virtue of its dramatically improved pharmacokinetics it still shows improved efficacy [14]. This degree of reduced biological activity would be detrimental to the efficacy of PEGylated FVIII for several reasons. FVIII is inactive in its native form, requiring proteolytic cleavage by thrombin for activation and interaction with the phospholipid (PL) surface, factor IXa (FIXa) and factor X (FX) in order to exert its cofactor function. PEGylation, depending

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on the number of PEG conjugates, would potentially interfere with all of these macromolecular interactions. Although reduced biological activity could be compensated by a dramatic improvement in pharmacokinetic properties, this may not be the case for a PEGylated form of FVIII. FVIII is already a large molecule and is not subject to renal elimination. Its plasma half-life is already significantly extended through its interaction with von Willebrand factor (VWF) *in vivo*. Whereas the plasma half-life of FVIII in the absence of VWF is ~2 h, it is ~12 h when associated with VWF [15]. PEGylation of FVIII risks interfering with VWF–FVIII affinity and actually compromising its plasma half-life. Although this may in turn be compensated by reduced interaction with cellular clearance mechanisms [16], *in vivo* studies are required to address this issue. Reduced biological activity of a PEGylated FVIII could be tolerated if the extended half-life resulted in a sustained biological action. However, the instability of activated FVIII (FVIIIa) may limit its utility in a PEGylated form. FVIII (domain structure A1-A2-B-A3-C1-C2) exists in plasma as an inactive heterodimer of a variably sized heavy chain (HC, subunits A1-A2-B, ~90–200 kDa) and a light chain (LC, subunits A3-C1-C2, ~80 kDa) associated via a copper (Cu+)-dependent interaction between the A1 and A3 subunits [17–19]. Upon activation with thrombin, proteolysis removes the B-domain and bisects the HC resulting in an FVIIIa heterotrimer (subunits A1/A2/A3-C1-C2) [20]. However, the FVIIIa heterotrimer is unstable and subject to spontaneous decay of its procoagulant activity attributable to first-order dissociation of its free A2 subunit, which occurs at physiological pH [21,22]. This type of proteolytic activation and inactivation does not occur with other commercialized PEGylated protein therapeutics. Merely improving the pharmacokinetics of the inactive FVIII heterodimer by PEGylation may have limited effect on *in vivo* efficacy, if the active form has significantly reduced the biological activity secondary to interference by the PEG conjugates as well as the inherent instability of FVIIIa.

Rostin *et al.* [23] published their experience with PEGylation of B-domain-deleted rFVIII (r-VIII SQ). They used several strategies for PEG conjugation. Using random PEG coupling at amino groups of lysines, they observed a significant decrease in the FVIII activity that was proportional to the degree of modification but was significant at even low degrees of modification; 1 PEG/r-VIII SQ reduced the specific activity to 50%. Using a less reactive PEG polymer, a larger excess of polymer was required to modify the protein. The reduced reactivity is thought to correlate with higher selectivity for conjugation. This resulted in some preservation of the FVIII activity; with 3 PEG/r-VIII SQ, the specific activity was 50%. Finally, they adsorbed the FVIII to an anionic exchange column in order to protect reactive lysines, presumably at the acidic regions of FVIII, from PEG conjugation. This had been successful in protecting the functional sites on target proteins in previous studies. This allowed the preservation of 50% of the FVIII activity with 4 PEG/r-VIII SQ. Western blot analyses suggested that modification sites were located on both the HCs and the LCs of FVIII. Their studies also suggested that the VWF-binding site was disturbed by PEGylation, as only 26–43% of the protein content was able to bind to VWF. The *in vivo* consequences of this perturbation on plasma half-life were not studied.

The success of PEGylation as a strategy to extend the half-life of FVIII may depend on new targeted methodologies for conjugation sites of PEG polymers. These strategies include specifically targeting non-essential functional groups on the surface of the protein such as free cysteines or oligosaccharides or even employing chemical strategies towards site-directed PEGylation [24]. One can also employ standard PEGylation while protecting active sites with an inhibitor or a substrate specific to the active site. This could be possible for FVIII but would require protection of multiple key sites; sites for thrombin recognition/cleavage, VWF interaction as well as interactive sites for FIXa and FX and PLs. Alternatively, one could employ chromatographic separation of isomers of PEGylated FVIII, selecting those with the most biological activity for evaluation. The B-domain of FVIII would make an attractive target for site-directed selective PEGylation. This large domain has a significant role in intracellular trafficking of FVIII within the secretion pathway, but when deleted FVIII still retains high specific activity and is efficacious therapeutically [25]. The B-domain does not play a significant role for interaction with VWF, FIXa or FX or the PL surface. Therefore, PEGylation in this region would be less likely to have a negative effect on VWF affinity or biological activity. In addition, upon thrombin activation the B-domain is proteolytically removed. Therefore, any potential steric hindrance of active sites by PEG would be abrogated after activation. Recent studies have demonstrated that the B-domain protein sequence can be modified significantly without impairing FVIII secretion [26]. Therefore, a strategy designed to introduce targeted sites for PEG modification (such as introducing cysteines to create thiol-reactive sites) has a high likelihood of success. Recent

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partnerships between technology leaders in targeted PEGylation strategies (Nektar Therapeutics, San Carlos, CA, USA) and manufacturers of rFVIII (Baxter International, Deerfield, IL, USA) suggest that there is ongoing investigation in this area towards a novel FVIII product [27].

There is some concern as to the ultimate clearance mechanism for PEG as it is considered non-biodegradable. Although some potential pathways for PEG degradation have been described, they appear to occur at a low rate and are not considered normal detoxification mechanisms [28]. PEG would then end up in tissues participating in the uptake of PEGylated constructs where it would accumulate intralysosomally [29]. The potential adverse effects of long-term exposure to large amounts of PEG over an extended period of time (i.e. lifelong as would be predicted for haemophilia applications) are unknown.

PEGylated liposomes

Liposomes (lipid vesicles) are capable of encapsulating drugs either within their aqueous phase or within their lipid bilayer and have been utilized in a broad range of drug delivery applications [30]. Unfortunately, they are rapidly cleared by phagocytic cells of the reticuloendothelial system (RES). By incorporating PEGylated lipids onto the liposome surface, there is reduced RES clearance and prolonged circulation time. These modified liposomes are referred to as sterically stabilized liposomes (SSL) and have been successfully formulated with the chemotherapy drug doxorubicin and IL-2 [31,32]. Doxorubicin formulated with SSL resulted in prolonged circulation time and enhanced accumulation of the drug in the malignant exudates of cancer patients.

Acknowledging some of the disadvantages of direct modification of the FVIII protein by PEGylation, formulation of unmodified FVIII with SSL has garnered recent interest. Baru *et al.* [33] have published their preclinical characterization of a full-length rFVIII with SSL. In this formulation strategy, SSL were prepared containing distearoylphosphatidylethanolamine (DSPE) conjugated with PEG. They report that for this preparation (designated PEGLip-FVIII) FVIII is not encapsulated within the intraliposome aqueous phase or inserted into the lipid bilayer but rather is associated with the liposomes through high-affinity interaction with the PEG-DSPE. Binding studies using surface plasmon resonance (SPR) measurements demonstrated no hindrance to VWF binding and similar FVIII activity (measured by one-stage clotting assays and two-stage chromogenic assays) to FVIII formulated in the absence of SSL. Pharmacokinetic studies of PEGLip-FVIII compared with standard formulation FVIII in haemophilic mice (exon 16 FVIII knockout) demonstrated similar peak recovery after injection. Average half-life, area under the curve and mean residence time were 1.5–1.6 times higher for PEGLip-FVIII compared with the free FVIII. Significantly, the haemostatic efficacy of PEGLip-FVIII in haemophilia mice was prolonged following tail vein injection compared with standard formulation FVIII. This new formulation of FVIII is presently beginning Phase I trials at the University of California – Davis Medical Center in Sacramento and the Children's Hospital of Orange County in California through a partnership between Zilip-Pharma (Amsterdam, The Netherlands) and Bayer Biological Products (Research Triangle Park, NC, USA) [34].

Polysialic acid polymers

Polysialic acids (PSA) are considered nature's 'stealth' technology. PSA are linear polymers of *N*-acetylneuraminic acid (sialic acid) and are abundantly present on the surface of cells and many proteins [29]. PSA protect against invading bacteria by interfering with host complement activation and phagocytic activity. In addition, PSA modulate cell-to-cell inhibition, reducing the adherence of cancer cells and facilitating metastatic migration. Because of this insulating feature of PSA, it has been proposed that polysialylation of therapeutic molecules could improve their pharmacokinetics. The rationale would be that the extreme hydrophilicity of the PSA would form a 'watery cloud' around the therapeutic molecule protecting it from proteolytic enzymes, clearance receptors and even antibodies. Investigators have used PSA based on polysaccharides on *Escherichia coli* that are immunologically identical to PSA in the host; thus they are non-immunogenic even when conjugated to therapeutic proteins [29]. PSA are biodegradable giving them an advantage over PEGylation when applied to a therapeutic protein that will be delivered in large doses and over an extended period of time (as would be the case for haemophilia applications).

Polysialylation has been applied to a wide range of therapeutics and has demonstrated important improvements over the parent molecule. For example, polysialylation of asparaginase protected it from proteolytic degradation in the presence of serum while its function was preserved [35]. Fully preserved function has also been observed for polysialylated forms of α2b-interferon and insulin even while their half-lives in circulation and areas under the curve

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were significantly increased [29]. There is also evidence of reduced immunological reactivity for polysialylated forms of asparaginase and insulin [36,37].

As applied to FVIII, polysialylation holds promise for extending the half-life of FVIII without compromising its functional activity. It may even reduce the immunogenicity of FVIII or protect it from neutralizing antibodies in patients with inhibitors. It remains to be demonstrated that polysialylated forms of FVIII can retain VWF affinity and still be effectively activated by thrombin. Recently announced collaborations between Lipoxen Technologies (London, UK) and Baxter International is the evidence of research interest in this area [27].

Stabilized forms of FVIIIa

Successful progress has been made in overcoming the inherent limitations of FVIII expression and biological function through techniques to improve rFVIII biosynthesis and secretion, functional activity, half-life and antigenicity/immunogenicity [38]. With regard to producing a longer acting FVIII molecule, FVIII bioengineered for resistance to inactivation holds promise.

The FVIIIa heterotrimer (A2/A2/A3-C1-C2) is unstable and susceptible to proteolytic inactivation by activated protein C (APC), FIXa or factor Xa. However, studies have also demonstrated that loss of procoagulant activity after thrombin activation results from a reversible dissociation of the A2 subunit from the heterotrimer and is another factor limiting the procoagulant activity of FVIIIa *in vivo*.

In order to address this limitation, an inactivation-resistant FVIII (IR8) was genetically engineered which is not susceptible to the dissociation of the A2-domain subunit and proteolytic inactivation by APC and therefore has a prolonged cofactor activity [39]. In designing IR8, B-domain residues 794–1689 were deleted and Arg740 was replaced by alanine, eliminating the thrombin cleavage sites at Arg740 and Arg1689. As a result, FVIII activation by thrombin occurs via a single cleavage after Arg372. This leads to the generation of an FVIIIa dimer that retains the A2-domain covalently attached to the LC, thus preventing its spontaneous dissociation. Additionally, missense mutations at APC inactivation cleavage sites provided resistance to further proteolysis of FVIIIa. The specific activity of IR8 proved to be markedly higher than that of wild-type FVIII (FVIII WT), and significant peak activity was retained for hours *in vitro*, whereas FVIII WT, under similar conditions was inactivated by thrombin after 10 min. The LC acidic region (amino acids 1648–1688) regulates FVIII cofactor activity by promoting high-affinity binding to VWF and reducing the affinity for PL. In order to create an IR8 through covalent linkage of the A2 subunit to the LC, the acidic region of the LC was deleted. The persistence of this epitope would have reduced the affinity for PL after activation. This, however, would predictably reduce the affinity of IR8 for VWF and compromise its circulating half-life. Indeed, in *in vitro* studies, IR8 exhibited a 20-fold reduced affinity for VWF but a 34-fold increased affinity for PL compared with FVIII WT [40]. A small amount of highly purified, endotoxin-free IR8 (500 U mL^{-1}) was prepared in collaboration with Genetics Institute (Cambridge, MA, USA). In collaboration with Dr. Timothy Nichols (Chapel Hill, NC, USA), two haemophilia A dogs were infused with IR8 (35 U kg^{-1}) [41]. After clearance of IR8, one dog was infused with rFVIII (Baxter International, 35 U kg^{-1}). Whole blood clotting times (WBCT) and cuticle bleeding times (CBT) were determined. No adverse reactions were observed following the infusions. There was no laboratory evidence of disseminated intravascular coagulation. (including no decrease in haematocrit or platelet counts). IR8 corrected the WBCT over time in a pattern that nearly paralleled the FVIII activity in a chromogenic assay and detection of FVIII antigen by ELISA. The measured plasma recovery (10–11.9 IU) and half-life (2–4 h) of IR8 was reduced compared with rFVIII (39.5 IU and 8–9 h). This is consistent with the reduced affinity for VWF, yet increased affinity for PL. Despite the significant differences in pharmacokinetics, IR8 still fully corrected the CBT performed 10 min postinfusion. The haemostatic efficacy of IR8 was also examined in the haemophilia A mouse model [42]. FVIII knockout mice were injected with FVIII WT or IR8 protein in physiological buffer to produce a corrected plasma FVIII activity from 1% to 100%. After 15 min, the mice underwent transection of the distal tail at a diameter of 2 mm. Blood was collected in a microfuge tube over 20 min and the blood volume was measured. Specific activity was determined by a one-stage aPTT clotting assay, and for the FVIII WT protein ranged from 320–2400 U mg^{-1} whereas the IR8 protein was 46 500 U mg^{-1}. Predicted plasma correction was based on the activity of the protein rather than the antigen; therefore, at least 20-fold less IR8 protein was infused compared with FVIII WT. IR8 exhibited superior control of blood loss in the 6–10% predicted plasma correction range compared with FVIII WT, and this persisted at higher correction ranges. This is especially significant considering that

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20-fold less IR8 protein was infused providing the first evidence that a stabilized FVIIIa can provide superior haemostasis.

Gale and colleagues hypothesized that a disulphide bond (DSB) engineered between the A2- and A3-domains could also stabilize FVIIIa by preventing A2 subunit dissociation following thrombin activation. They used a three-dimensional (3D) homology model of FVIII A-domains [43] to identify two sets of residues (amino acids 664–1826 and 662–1828) as potential DSB pairs. A similar strategy had previously allowed successful engineering of a DSB within factor V [44]. The homology model suggested that cysteines substituted at either of these residue pairs within FVIII would result in a DSB at the edge of the interface between the two A-domains very near the solvent-exposed surfaces of the two domains. Thus, the resulting DSB should not significantly disrupt the structure of the A-domain interface. A B-domain-deleted-rFVIII mutant containing Cys664 and Cys1826 was produced and purified [45]. This FVIII variant (C664–C1826) exhibited normal procoagulant specific activity. The DSB linking the A2-domain to the A3-domain was confirmed by Western blotting with anti-FVIII A2-domain antibody. Reduction of the protein with dithiothreitol eliminated the new cross-link, confirming that it was a DSB. Following thrombin activation of the C664–C1826 rFVIII, the mutant lost only 20% of its peak activity after 40 min at 22°C as measured by one-stage clotting assay. During this same incubation time, wild-type FVIIIa had lost more than 99% of its activity. Radtke *et al.* [46] recently examined these DSB proteins by rotational thromboelastogram (ROTEG). They observed an approximately 10-fold increased potency of C664–C1826 and C662–C1828 compared with a wild-type B-domain-deleted FVIII, as measured by several ROTEG parameters of clot strength, probably reflecting increased thrombin-generating capacity.

Factor VIII proteins with prolonged activity following thrombin activation have the potential of increasing the efficacy of FVIII in plasma. It is possible that they may correct haemostasis *in vivo* at lower expression of protein, prolonging the activity of FVIIIa even when FVIII in plasma is present in levels usually ineffective for haemostasis. This may allow for a longer interval between therapeutic infusions to maintain effective haemostatic control.

Modification of receptor-mediated catabolism of FVIII

One of the mechanisms regulating the level of FVIII in the circulation is its receptor-mediated clearance. According to the current concept, based on several lines of evidence, catabolism of FVIII is mediated by two receptors from the low-density lipoprotein receptor family – low-density lipoprotein receptor-related protein (LRP) and low-density lipoprotein receptor (LDLR), which cooperate in this process [47–52]. LRP is predominantly expressed in the liver on hepatocytes and is also present in the lung, placenta and brain [53]. LDLR is ubiquitously expressed and, similar to LRP, is most prominent in the liver supporting the concept of their cooperation. While LRP has over 35 structurally and functionally distinct ligands, LDLR has restricted specificity and represents a classical receptor for the binding and cellular uptake of ApoB- and ApoE-containing lipoproteins [52].

Receptor-mediated clearance of FVIII is facilitated by heparan sulphate proteoglycans (HSPGs) of extracellular matrix, which provide the initial binding of FVIII to the cell surface [54]. The role of HSPGs in FVIII clearance *in vivo* was demonstrated in mice when the simultaneous blocking of LRP and HSPGs by receptor-associated protein and protamine, respectively, led to a more significant prolongation of FVIII half-life (fivefold) than the blocking of LRP alone (threefold) [54].

Interaction of FVIII with LRP involves multiple, at least three, FVIII-binding sites: within the A2-domain of HC [47] and the A3- and C2-domains of LC [48,55]. The *high-affinity* LRP-binding site of HC was localized *within the A2-domain* and mapped to the region R484–F509 [47]. The *high-affinity* LRP-binding site within LC has been localized to *the A3-domain* and mapped to the residues 1811–1818 [55]. In contrast to the A2- and A3-domains, the isolated recombinant C2-domain was reported to exhibit *a low-affinity* interaction with LRP [48,55]. Thus, the A2 and A3 sites are the major contributors to the FVIII/LRP interaction. However, other surface-exposed structural elements within the A3-C1 fragment may also contribute to the binding to LRP. The HSPG-binding site was mapped to the A2 region 558–565 and is distinct from the LRP-binding site within the A2-domain [54].

Establishment of the role of LRP and LDLR as receptors mediating removal of FVIII from the circulation *in vivo*; establishment of the role of HSPGs in facilitating this process and identification of LRP-binding sites within FVIII have led to strategies to prolong FVIII lifetime in the circulation by interfering with clearance. This could be achieved through site-directed mutagenesis of LRP (LDLR, HSPGs)-binding sites within the isolated FVIII fragments as a first step and subsequent verification of

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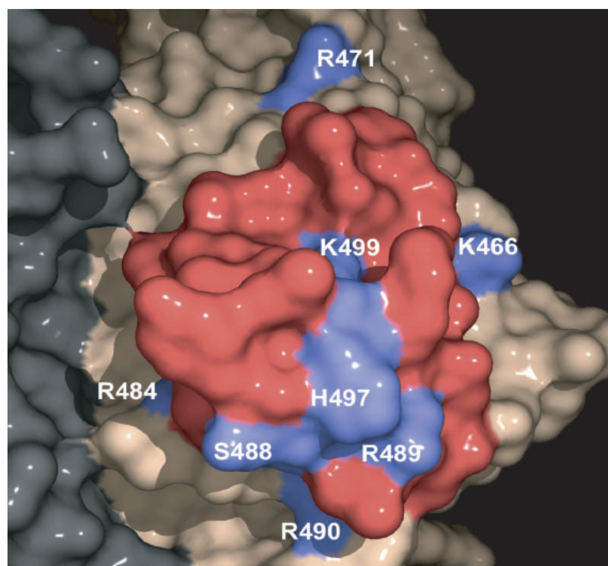

Fig. 1. Three-dimensional presentation of LRP-binding site within the A2-domain of FVIII. Location of the LRP-binding region 484–509 (red) and A2 residues critical for interaction with LRP (blue) on the molecular surface of A2 is shown. Other residues of the A2-domain and the residues of the A1-domain are coloured in beige and grey, respectively. The figure was generated based on the 3D model of A2 (Stoilova-McPhie *et al.*, 2002 [58]) using PyMOL program (http://www.pymol.org). Reproduced from Sarafanov et al., 2006 [59].

the inhibitory effects of mutations of critical residues on the binding of full-length FVIII to these receptors. Considering that the A3 region 1811–1818 represents a high-affinity FIXa-binding site [56] and the A2 region 484–509 contributes to FVIIIa cofactor activity [57], the challenge is to select mutations that will maximally reduce the interaction of FVIII with LRP but will not affect the functional properties of FVIII.

While site-directed mutagenesis within the A3 LRP-binding site and the A2 HSPG-binding site has not yet been performed, a comprehensive site-directed mutagenesis of the A2 LRP-binding region 484–509 and residues spatially close to this region suggested by a 3D model of A2 [58] has been accomplished. In competition and SPR-based assays, affinities of A2 mutants K466A, R471A, R484A, S488A, R489A, R490A, H497A and K499A for LRP were found decreased by several fold [59]. This correlated with a decrease in LRP-mediated internalization of the mutants in cell culture. Importantly, combining these mutations into pairs (K466A/R489A, R471A/R484A, S488A/R499A and R490A/H497A) led to accumulative effects, i.e. approximately 10-fold decrease in the affinity for LRP of A2 double-point mutants and approximately twofold decrease in their LRP-mediated internalization in cell

Table 1. Summary of strategies for longer-acting FVIII.

Strategy	Expected outcome	Potential advantages	Potential disadvantages
PEG polymer conjugation of FVIII	Prolonged circulation time	Demonstrated efficacy with other protein therapeutics Reduced immunological reactivity	Reduced specific activity Reduced VWF affinity Unknown long-term adverse effects of PEG exposure
Sterically stabilized (PEGylated) liposomes	Prolonged circulation time	Demonstrated efficacy with other drugs No FVIII protein modification	Pharmacokinetic impact reduced compared with direct modification of FVIII
Polysialic acid modification of FVIII	Prolonged circulation time	Demonstrated efficacy with other protein therapeutics Reduced immunological reactivity	Reduced VWF affinity
Stabilized FVIIIa	Inactivation resistance Prolonged cofactor activity		
IR8		High specific activity Resistance to activated protein C	Reduced VWF affinity Possible increased immunological reactivity Possible increased thrombogenicity
DSB-FVIII		Normal activity and VWF affinity	Possible increased immunological reactivity Possible increased thrombogenicity
LRP-binding site mutagenesis	Reduced receptor mediated plasma clearance	Retained VWF affinity Direct targeting of receptor-mediated clearance	Possible reduced specific activity Possible increased immunological reactivity

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culture [59]. Based on these results, the A2 residues K466, R471, R484, S488, R489, R490, H497 and K499 were identified as major determinants in the formation of the A2-binding site for LRP. The computer modelling of the 3D structure of this region reveals that four positively charged residues K466, R471, R489, R490 and hydrophilic residues Y487, S488, presumably, form a frame of the binding epitope for LRP (Fig. 1). Importantly, mutations R484A, S488A, R489A, R490A and H497A yielded proteins with reduced affinity for LRP, yet retaining cofactor activity in FVIIIa molecules reconstituted from the A2 mutants and A1/A3-C1-C2 heterodimer [59]. These mutations may be good candidates for introduction into full-length FVIII and may lead to a clinically significant extension of the half-life of FVIII in circulation.

Conclusions

Despite the benefits of prophylaxis and recommendations that it should be considered optimal therapy for children with severe haemophilia, only up to 50% of boys with haemophilia in the United States receive such therapy [60]. A longer acting FVIII therapeutic (strategies summarized in Table 1) holds promise to overcome some of the barriers to adoption and adherence to prophylaxis. The time-consuming nature of prophylaxis is frequently mentioned as a significant challenge [5]. A FVIII therapeutic that provided effective prophylaxis with once per week dosing would certainly be a breakthrough. Unfortunately, it is unlikely that this would be associated with a significant reduction in costs. Experience with other long-acting biologics is such that the 'course of therapy' costs (e.g. G-CSF daily administration vs. PEG-G-CSF single dose for chemotherapy-induced neutropenia) are similar. More complex pharmacoeconomic analysis would likely be required to show that improvements in the adoption and adherence from a long-acting FVIII, resulting in reduced joint bleeding and better joint outcomes, would justify the cost of therapy.

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