Expert Opinion

Enhancement of the efficacy of therapeutic proteins by formulation with PEGylated liposomes; a case of FVIII, FVIIa and G-CSF

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Importance of the field: Improving the pharmacodynamics of protein drugs has the potential to improve the care and the quality of life of patients suffering from a variety of diseases.
Areas covered in this review: Four approaches to improve protein drugs are described: PEGylation, amino acid substitution, fusion to carrier proteins and encapsulation. A new platform technology based on the binding of proteins/peptides to the outer surface of PEGylated liposomes (PEGLip) is then presented. Binding of proteins to PEGLip is non-covalent, highly specific and dependent on an amino acid consensus sequence within the proteins. Association of proteins with PEGLip results in substantial enhancement of the pharmacodynamic properties of proteins following administration. This has been demonstrated in preclinical studies and clinical trials with coagulation factors VIII and VIIa. It has also been demonstrated in preclinical studies with granulocyte colony-stimulating factor. A mechanism is presented that explains the improvements in hemostatic efficacy of PEGLip-formulated coagulation factors VIII and VIIa.
What the reader will gain: The reader will gain an understanding of the advantages and disadvantages of each of the approaches discussed.
Take home message: PEGLip formulation is an important new approach to improve the pharmacodynamics of protein drugs. This approach may be applied to further therapeutic proteins in the future.

Keywords: factor VIIa, factor VIII, G-CSF, PEGylated liposomes, pharmacodynamics, therapeutic proteins

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1. Introduction

Protein drugs are larger and much more structurally complex than small molecule drugs. For these drugs to function properly, they must be translated without error, must be appropriately modified post-translationally, and must fold and assemble properly. Following administration to the patient, protein drugs must bind with high affinity to their targets and induce the desired biological effects. Along the way, they may need to interact with multiple factors and function as part of multisubunit complexes and carefully balanced regulatory networks. Proteins must be stable enough to have lasting therapeutic effects but should not induce an immune response that may neutralize the treatment, complicate future treatments and endanger the health of the patient [1].

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Article highlights.

- Various approaches have been used in attempts to improve the pharmacodynamics of protein drugs. These include direct PEGylation, modification of amino acid sequence, fusion to carrier proteins and encapsulation in liposomes.
- PEGLip technology is based on the non-covalent binding of proteins to the outer surface of PEGylated liposomes.
- Binding of proteins to PEGLip is dependent on the presence of a conserved amino acid sequence in the protein and on PEG molecules on the liposome surface.
- Preclinical and clinical studies with FVIII and FVIIa showed that formulation of proteins with PEGLip resulted in improved pharmacodynamics and enhanced hemostatic efficacy.
- Preclinical studies with G-CSF showed that formulation with PEGLip improved mobilization of hematopoietic stem cells from the bone marrow to the peripheral blood.
- Several lines of evidence support the binding of liposomes to blood cells and the targeted delivery of formulated proteins to sites of action.
- PEGLip technology is an effective means of improving the pharmacodynamics of therapeutic proteins.
- Formulation of proteins with PEGLip is particularly attractive for large and sensitive proteins that may both lose activity and become increasingly immunogenic as a result of covalent modification.

This box summarises key points contained in the article.

Owing to the complexity of protein drugs and the competing demands placed on them, various approaches have been taken to improve these therapeutic agents. Some of these approaches have been directed at delivering the protein more conveniently (e.g., orally, transdermally, or by means of inhalation) while maintaining effectiveness [2-4]. Other work has focused on improving pharmacokinetics and pharmacodynamics following administration [1,5]. Approaches to improve effectiveness inside the body have focused primarily on overcoming one or more of the factors that limit the effectiveness of existing treatments. These limitations include poor stability or solubility, inefficient delivery to sites of action, toxicity, proteolytic degradation, cellular and renal clearance, and immunogenicity and neutralization by host antibodies [1].

In this review, four approaches to improve the effectiveness of protein drugs are discussed: PEGylation, modification of the amino acid sequence, fusion to carrier proteins and encapsulation in liposomes. PEGLip technology is then described, a new approach based on the non-covalent binding of proteins to the outer surface of PEGylated liposomes.

1.1 Polyethylene glycol conjugation

Covalent attachment of one or more chains of polyethylene glycol (PEG) to protein and peptide drugs has been shown to have substantial effects on protein pharmacokinetics. In most cases, PEG chains are attached by means of chemical linkers to reactive groups on amino acid side chains. These PEG chains may be of various lengths and shapes, may be connected to the protein through a variety of linkers, and may be attached at one or many sites [6,7]. In addition, attempts have been made to create forms of PEG conjugates with a degradable linker that releases the active protein inside the body [7,8].

PEGylation has been shown to improve physical and thermal stability and protect against enzymatic degradation, conferring prolonged circulating half-life [9,10]. Binding of water molecules to the ethylene oxide moieties enhances solubility, increases the protein's hydrodynamic volume and reduces renal clearance [6,11]. The PEG molecules may also mask immunogenic epitopes, thus interfering with antigen processing and presentation [12,13]. The first two Food and Drug Administration (FDA)-approved PEGylated drugs were PEGylated adenosine deaminase (Adagen®, Enzon Pharmaceuticals, Bridgewater, NJ, USA) for the treatment of severe combined immunodeficiency, approved in 1990 [14,15], and PEGylated L-asparaginase (Oncaspar®, Enzon Pharmaceuticals) for the treatment of pediatric acute lymphoblastic leukemia, approved in 1994 [16,17]. Since then, several more PEGylated protein drugs have been approved. These include PEG-interferon-α_{2b} (PegIntron®, Schering-Plough, North Wales, PA, USA), PEG-interferon-α_{2a} (Pegasys®, Roche, Basel, Switzerland), a PEGylated form of growth hormone receptor antagonist (Somavert®, Pfizer, New York, NY, USA), PEG-filgrastim (Neulasta®, Amgen, Thousand Oaks, CA, USA), methoxy PEG-epoetin-β (Mircera®, Roche) and a PEGylated recombinant humanized antibody Fab′ fragment that blocks human tumor necrosis factor-α (TNF-α) (Cimzia®, UCB, Brussels, Belgium) [6,8].

Although direct PEGylation has several desirable effects and has found widespread application and acceptance, it also has disadvantages. Therapeutic proteins frequently function as part of protein complexes or through interaction with receptors that reside on cell surfaces. PEG chains are generally quite flexible, but steric hindrance by PEG moieties may nonetheless interfere with binding of cofactors and interaction with receptors [6,8]. PEGylation may therefore lead to unexpected changes in function and specificity [11]. Moreover, whereas PEGylation of many proteins results in reduced immunogenicity, in rare cases PEGylation leads to increased antibody formation. This was demonstrated in preclinical studies with butyrylcholinesterase [18] and interferon-β_{1a} [19], in clinical trials with megakaryocyte growth and development factor [20], and by development of threshold levels of anti-FVIIa antibodies in a Phase I clinical study testing the pharmacokinetics and safety of PEG-FVIIa [21].

PEGylation must also be integrated into the production process. Depending on the characteristics of the protein, the linking chemistry and the type of PEG used, this may necessitate the addition of multiple steps. Furthermore, the PEGylation reaction must be carefully controlled (time, temperature, pH, protein concentration, protein:PEG ratio) so as to avoid

excessive or poorly placed modifications, to avoid crosslinking of proteins, and to avoid product polydispersity [22].

1.2 Amino acid substitution

In cases where a protein is produced recombinantly and there is an understanding of the relationship between structure and function, the amino acid sequence may be modified so as to improve the protein pharmacokinetics and pharmacodynamics. Insulin has been modified at specific amino acids in both the A and B chains to produce analogues that are either short acting or long acting. Short-acting analogues such as insulin lispro (Humalog®, Eli Lilly, Indianapolis, IN, USA), insulin aspart (NovoLog® and NovoRapid®, Novo Nordisk, Bagsvaerd, Denmark) and insulin glulisine (Apidra®, sanofi-aventis, Paris, France) are characterized by changes to protein charge or hydrophobicity that suppress dimerization and hexamerization and maintain the protein in a rapidly absorbed monomeric form [23,24]. Long-acting insulin analogues such as insulin glargine (Lantus®, Sanofi-Aventis) are designed to provide consistent basal levels of insulin. Insulin glargine molecules have an increased tendency to self-associate and precipitate in aqueous solutions at neutral pH and are absorbed slowly from subcutaneous injection sites [23,24].

Erythropoietin (EPO) has also been modified so as to change its glycosylation pattern. Aranesp® (Amgen) is an analogue of human EPO that differs from native human EPO at five amino acid positions. These modifications create two new carbohydrate addition sites and lead to the attachment of two extra N-linked oligosaccharides, an increase in protein size and a tripling of half-life relative to EPO-α [25,26].

Protein modification may also lead to an increase in specific activity. The NN1731 analogue of FVIIa (Novo Nordisk, Denmark) contains three amino acid substitutions and possesses increased intrinsic procoagulant activity independent of tissue factor, its natural counterpart. Improved efficacy compared with wild-type FVIIa was demonstrated both *in vitro* and *in vivo* [27-29]. Another analogue of FVIIa (named Bay7) with a modified glycosylation pattern showed superior *in vitro* clotting activity, greater circulation time and improved efficacy in preclinical experiments [30,31].

Although mutagenesis may increase protein activity, increasing activity to levels higher than those of the native protein or changing regulatory sequences may not be desirable. The FVIIa analogue NN1731 shortened clotting time in hemophilic blood so much that clotting occurred even faster than in normal blood [28]. Use of this analogue must therefore be limited to patients who do not respond well to conventional FVIIa and these patients must be monitored carefully for thrombosis.

Changes in the amino acid sequence of a protein may also induce an immune response. This may occur as a result of the modification rendering the protein enough unlike host proteins to induce a classical immune response [13]. Alternatively, an immune response may be elicited by sequence changes that lead to changes in post-translational modifications such as glycosylation. This may create or expose previously hidden epitopes and lead to increased immunogenicity [13].

1.3 Fusion to a carrier protein

Protein pharmacodynamics may also be modified by fusion of one protein to another. Proteins may be dimerized (e.g., EPO) [32] or fused to a carrier such as albumin or the Fc fragment of human immunoglobulin. All three of these approaches result in an increase in molecular mass and a reduction in renal clearance by glomerular filtration. Enbrel® (Amgen and Wyeth, Madison, NJ, USA) is an FDA-approved fusion protein that consists of the ectodomain of the TNF-α receptor fused to the Fc portion of immunoglobulin G1. This fusion protein blocks TNF-α and is being used for the treatment of autoimmune diseases [33]. Zalbin™ (Human Genome Sciences, Rockville, MD, USA) is a fusion of interferon-α_{2b} to human serum albumin. It is now being assessed in Phase III clinical trials for the treatment of hepatitis C and could become an alternative to PEGylated interferon [34,35]. Conjugation of albumin to the short-lived glucagon-like peptide 1 (GLP-1) improved its pharmacokinetics while preserving its antihyperglycemic activity [36].

The fusion of one protein to another causes a large structural change, but the effect of this change on activity and pharmacokinetics depends on where and how the proteins are joined, which residues are hidden as a result of the fusion, and how the fusion changes the structure of the residues that remain exposed [13]. Like genetically modified proteins, fusion proteins may be immunogenic [37]. A new form of interferon-α_{2b} fused to albumin showed increased immunogenicity in mice [38]. Furthermore, proper interaction between a fusion protein and one binding partner does not ensure proper interaction with other partners. Albumin-fused coagulation factor IX showed normal processing and γ-carboxylation but its interactions with activating proteases as well as with its cofactor FVIIIa were slow, resulting in a two- to threefold reduction in specific activity [39].

1.4 Liposome encapsulation

Encapsulation of therapeutic agents inside microparticle carriers such as liposomes, polymer microparticles [40] or cell ghosts (empty cell membranes) [41,42] is another way to improve their clinical properties. Liposomes have been used to encapsulate several small molecule anticancer drugs [43]. By making use of an effect known as 'enhanced permeability and retention' (EPR), drugs encapsulated in liposomes are selectively delivered to tumors by means of the leaky tumor vasculature [44]. At the same time, contact of these toxic agents with healthy tissues is minimized [45]. Doxorubicin encapsulated in PEGylated liposomes (Doxil®, Biotech, Horsham, PA, USA and Caelyx®, Schering-Plough) and in non-PEGylated liposomes (Myocet®, Cephalon Europe, Frazer, PA, USA) are approved by the FDA for the treatment of breast cancer, ovarian cancer and Kaposi's sarcoma [46,47].

Encapsulation of proteins in liposomes has also been shown to be beneficial. Liposome encapsulation of hemoglobin [48] and tissue plasminogen activator (tPA) [49] resulted in a prolongation of circulation half-life. Encapsulation of

interleukin-2 increased its half-life and efficacy [50,51], and encapsulation of TNF-α reduced its toxicity [52]. However, encapsulating proteins in liposomes is much more challenging than the encapsulation of small molecules. Proteins, especially enzymes, are sensitive to environmental perturbations and the proteins must maintain their structure throughout the encapsulation procedure to avoid loss of activity. Common encapsulation procedures involving the use of organic solvents or detergents often result in protein denaturation [53,54]. Entrapment of proteins by the dehydration–rehydration method usually preserves protein activity. This method entails freeze-drying a mixture of 'empty' small unilamellar vesicles and free proteins. On rehydration, large multilamellar vesicles that incorporate the protein are formed. The liposomes are then downsized to the desired final diameter [55]. The entrapment efficiency of the dehydration–rehydration method ranges from 5 to 50% [48,49,53,54,56], considerably less than the > 90% entrapment efficiency that can be achieved with small molecule drugs [46]. Moreover, encapsulation of large proteins has been shown to be less efficient than encapsulation of small proteins [57]. Efficient delivery can be achieved by incorporation of the proteins into the liposome bilayer, but this method is suitable only for transmembrane, anchored, or hydrophobic proteins [58,59]. Another challenge in developing liposome-encapsulated protein drugs is to control the release of the encapsulated proteins from the liposome following administration. This sometimes requires approaches such as ultrasound, as demonstrated in the release of tPA [56]. Owing to these difficulties and despite many attempts over the last two decades, still no liposomally encapsulated protein has been approved by the FDA and no such product is in advanced clinical trials.

2. Formulation of proteins with PEGylated liposomes (PEGLip technology)

PEGLip technology is based on the non-covalent, high-affinity binding of proteins and peptides that contain a specific amino acid sequence to the outer surface of PEGylated liposomes (PEGLip). This association enhances the pharmacodynamic properties of the formulated proteins. Unlike the approaches presented so far, PEGLip technology does not involve changes to amino acid sequence, does not involve covalent attachment of stabilizing agents, and does not involve encapsulation.

Formulation of a protein with PEGylated liposomes is gentle and easy. In the case of lyophilized proteins, the protein powder is simply reconstituted in liposome solution and allowed to dissolve fully. In the case of proteins in solution, the protein and PEGLip are mixed together and incubated at room temperature for a few minutes. Even large and sensitive proteins may be formulated without any change in the production process or the purification procedure. Formulation does not involve any covalent modification of the protein, does not change the protein's native structure, and therefore does not induce antibody production. The protein is fully active immediately after formulation [60-62] and it is free to interact with its normal binding partners [60]. Administration to patients remains, for the most part, unchanged, although dosage and frequency of injections may need to be adjusted to account for extended therapeutic activity.

The PEGLip that are typically produced are composed of a 97:3 molar ratio of 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC) to 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-[methoxy(polyethyleneglycol)-2000] (DSPE-PEG 2000). The liposomes are prepared as follows. Lipids are dissolved in *tert*-butanol and lyophilized. The resulting dry lipid powder is resuspended to 110 mM lipid in 50 mM sodium citrate, pH 6.7 to form liposomes. The liposomes are then downsized by extrusion through sequentially smaller polycarbonate filters until they reach a final diameter of 80 – 100 nm. The PEG molecules that extend outwards from the liposomes mediate binding of specific proteins to the liposome surface (**Figure 1**). The PEG molecules also limit uptake of the liposomes by the reticuloendothelial system, thereby extending half-life in the circulation [63-65].

Binding of liposomes to proteins may be measured in real time using surface plasmon resonance (SPR). A protein is bound to the surface of a chip and association is measured as a liposome solution flows over the chip. SPR measurements showed that PEGLip bind coagulation factor VIII (FVIII) [60,61], recombinant activated factor VII (FVIIa) [62], recombinant human granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF) and glucagon-like peptide 1 (GLP-1) [66]. Affinity constants for all of these interactions were in the low nanomolar range (0.4 – 12 nM). PEGLip did not bind to human serum albumin (HSA), human IgG, insulin, interferon-alpha 2a, interferon-alpha 2b, human growth hormone, or erythropoietin [66]. Analysis of protein sequences revealed a consensus sequence of eight amino acids (S/T–X–L/V–I/Q/S–S/T/Q–X–X–E) shared by the proteins that bind PEGLip but absent from the proteins that do not bind PEGLip (**Figure 1**). To assess the importance of this sequence, a peptide derived from amino acids 1783 – 1796 of FVIII (one of the two consensus sequences in FVIII) was synthesized and binding of PEGLip was measured. PEGLip bound the peptide with a K_d of 2.3 nM. Non-PEGylated POPC liposomes did not bind the peptide [66]. It was concluded that this consensus sequence is sufficient to mediate the binding of the proteins/peptides to PEGLip.

Analyses performed with liposomes with a variety of lipid compositions revealed that the interaction between PEGLip and proteins is mediated primarily by the PEG molecule and the carbamate group adjacent to the PEG molecule within DSPE-PEG 2000 [66]. When PEG molecules were not present on the liposome surface (as in the case of POPC liposomes) no binding occurred. Likewise, when proteins lacking the consensus sequence were assayed, no binding was observed. This indicates that binding is highly specific.

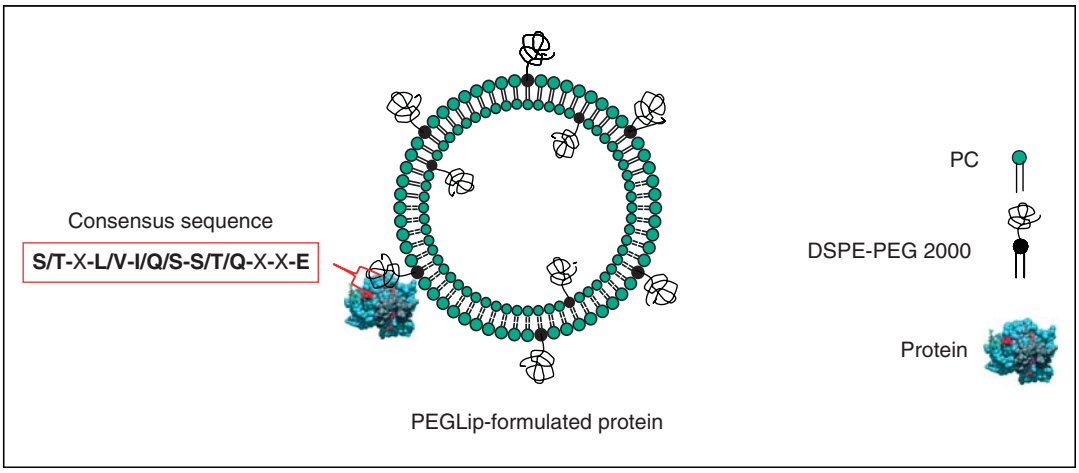

Figure 1. A schematic diagram showing a PEGLip-formulated protein. The protein is non-covalently bound to a polyethylene glycol moiety on the outer surface of a PEGylated liposome. Binding is mediated by an amino acid consensus sequence within the protein.

2.1 PEGLip-formulated FVIII

Hemophilia A is a rare but serious inherited bleeding disorder caused by the lack or dysfunction of coagulation factor VIII (FVIII). FVIII is a large protein (2332 amino acids) that undergoes multiple processing steps before being released to the plasma where it circulates in association with von Willebrand factor (vWF). FVIII is then activated by thrombin, interacts with phospholipids and factor IXa, and catalyzes the activation of factor X in the clotting cascade. Severe hemophilia A patients (< 1% of normal FVIII activity) experience spontaneous, recurrent and potentially life-threatening bleeding episodes [67,68].

Prophylactic replacement therapy has been shown to provide hemophilia A patients with better care than on-demand treatment [69]. Prophylactic treatment reduces the frequency of hemorrhages and slows the development of long-term arthropathy. The half-life of human FVIII is ~ 10 – 12 h [70,71]. Three weekly infusions of 20 – 40 IU FVIII/kg are therefore required to maintain FVIII above levels at which spontaneous bleeding occurs. The need for frequent infusions may lead to problems with compliance, injection complications and a reduced quality of life. Central venous access devices are frequently required, especially among children, and these devices are plagued by recurrent infections and thrombosis [72].

A long-acting form of FVIII would provide extended protection against bleeding, fewer gaps in protection caused by drops in FVIII levels between injections, fewer injections, fewer complications and a better quality of life.

Several attempts have been made at generating long-acting forms of FVIII [73]. These have included mutagenesis of the FVIII molecule to increase its stability [74-76] and direct PEGylation of the FVIII protein [77-79]. Modifying a large and complex protein such as FVIII is a difficult undertaking, though. The integrity of multiple active sites must be maintained and protein conformation must be preserved. Development of modified forms of FVIII is still in the preclinical stage and the safety and efficacy of these approaches have yet to be demonstrated in humans [73].

Formulation of FVIII with PEGLip produces a long-acting form of FVIII while avoiding the pitfalls of mutagenesis and direct PEGylation. FVIII molecules are not covalently modified by PEGLip formulation and they maintain full activity after formulation [60,61]. Association of FVIII with PEGLip did not alter binding of several anti-FVIII antibodies to the protein [60], thus demonstrating that protein structure is preserved following PEGLip binding. Formulation of FVIII with PEGLip did not affect the *in vitro* binding of FVIII to its natural carrier vWF [60]. This suggests that FVIII is free to form complexes and interact with counterpart proteins after PEGLip formulation. Thus, binding of PEGLip to FVIII does not change the protein's biological properties.

PEGLip formulation of both recombinant and plasma-derived forms of FVIII resulted in extended hemostatic efficacy *in vivo*. Standard FVIII, PEGLip-FVIII, or saline were administered to hemophilic mice 24 h before transection of the left lateral tail vein. Mice that received PEGLip-FVIII bled less and survived significantly longer ($p < 0.05$) than mice that received standard FVIII or saline (**Figure 2A**) [60,61].

PEGLip-FVIII provided human subjects with extended protection from bleeding [80]. A single-arm, subject-blinded clinical trial was performed to assess the efficacy and safety of two levels of FVIII (25 or 35 IU/kg) with a fixed dose of liposomes (22 mg lipids/kg). Twenty-three severe hemophilia A patients were treated in three study segments. Standard FVIII was administered in the first study segment, whereas PEGLip-FVIII was provided in the second and third segments. Segments were separated by 4-day washout periods and each prophylactic infusion was administered while subjects were in a non-bleeding state. As a measure of efficacy, the time between each prophylactic infusion and the next

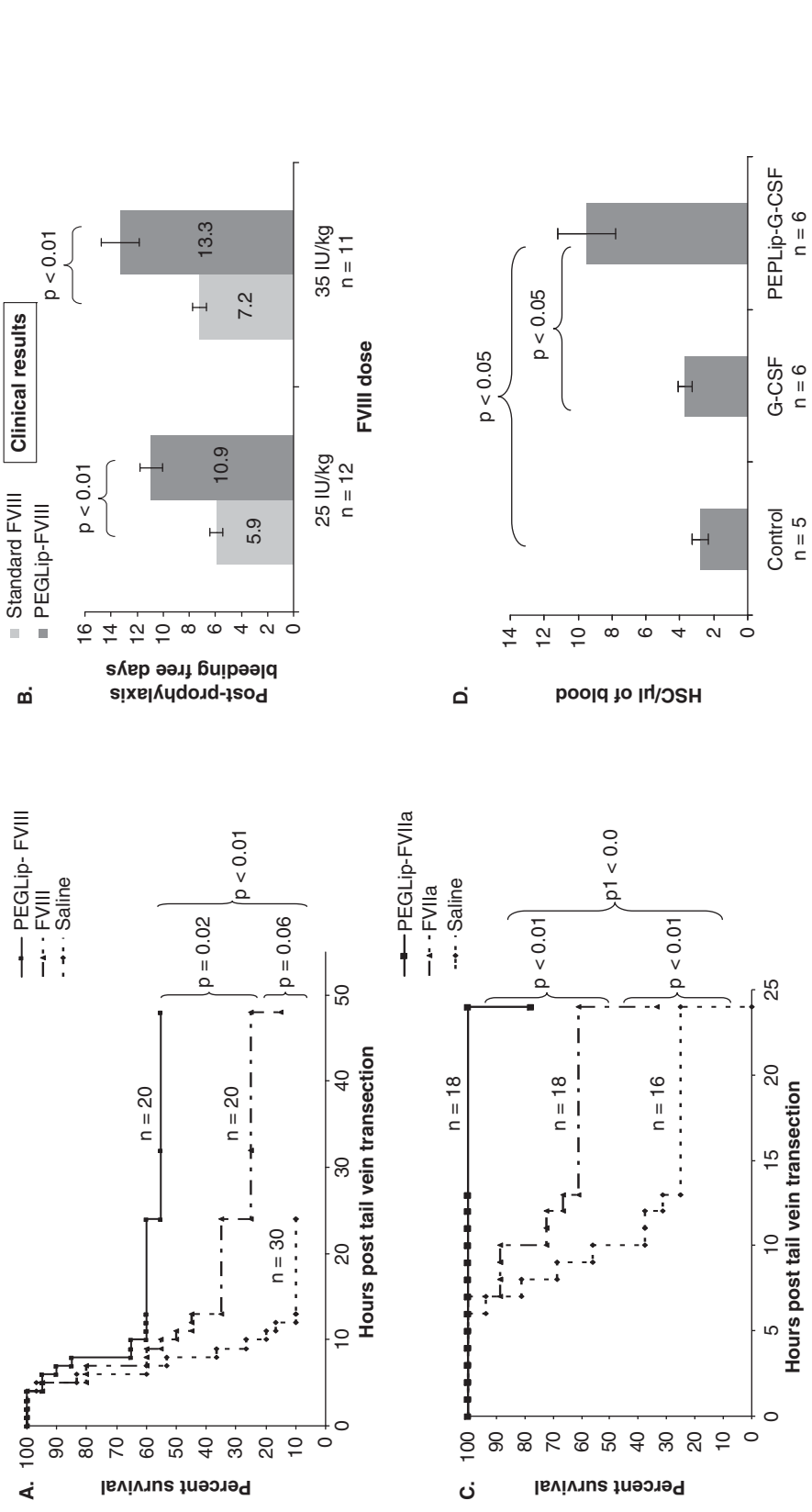

Figure 2. Efficacy of PEGLip-formulated proteins in preclinical experiments and a clinical trial. A. *In vivo* efficacy of PEGLip-FVIII. Hemophilic mice were injected into the tail vein with PEGLip-formulated FVIII, standard FVIII (both 0.1 IU/mouse), or saline. Twenty-four hours after injection, the left lateral tail vein of each mouse was cut and survival was scored. **B.** Number of bleeding-free days following PEGLip-FVIII or standard FVIII administration. Hemophilia A patients were given 25 or 35 IU/kg FVIII (standard or PEGLip-formulation of each dose) and the time between the prophylactic infusion and the next spontaneous bleed was recorded. Results are average ± SEM. **C.** *In vivo* efficacy of PEGLip-FVIIa. Hemophilic mice were injected with PEGLip-FVIIa, standard FVIIa (both 10 µg/mouse), or saline. The right and left lateral tail veins of each mouse were cut 15 min post-injection and survival was scored. **D.** *In vivo* efficacy of PEGLip-G-CSF. BALB/*c* mice were injected once a day for 3 days with PEGLip-G-CSF, standard G-CSF (both 300 µg/kg), or 5% dextrose and mobilization of hematopoietic stem cells (HSC) from the bone marrow to the peripheral blood was measured. Results are average ± SEM. p-Values in **A** and **C** were determined using a log-rank test. p-Values in **B** and **D** were determined using a homoscedastic t-test. **A**, **B** and **D** are based on previously published data [61,66,80].

spontaneous bleed was recorded. Prophylactic treatment with PEGLip-FVIII nearly doubled the length of time subjects were protected from spontaneous bleeding (**Figure 2B**). A single prophylactic injection of 25 IU/kg PEGLip-FVIII resulted in a mean bleed-free interval of 10.9 days compared with 5.9 days with standard FVIII. Similarly, injection of 35 IU/kg PEGLip-FVIII resulted in a mean bleed-free interval of 13.3 days compared with 7.2 days with standard FVIII. This difference was significant ($p < 0.05$) for both dose levels (**Figure 2B**) [80].

A subsequent randomized, subject-blinded, four-way crossover study involving 16 hemophilia A patients evaluated the efficacy and safety of prophylactic infusions of various PEGLip doses (4.2, 12.6, or 22.1 mg/kg) with a fixed FVIII dose (35 IU/kg). Mean number of bleeding-free days after each infusion increased from 7.8 days for 35 IU/ml of standard FVIII to 8.7, 10.8 and 10.9 days for 35 IU/ml of FVIII formulated in 4.2, 12.6 and 22.1 mg/kg of PEGLip, respectively [81]. The study showed a dose response to PEGLip that reached saturation at the highest dose level.

An additional study tested the safety of PEGLip-FVIII and compared its pharmacokinetic profile with that of standard FVIII [82]. In this randomized double-blind study, 26 severe hemophilia A patients received a single injection of standard FVIII (35 IU/kg) followed by 12 observation days and a 2-day washout period. Patients then received a single injection of PEGLip-FVIII (35 IU/kg FVIII, 13 or 22 mg/kg PEGLip) followed once again by 12 days of observation. Pharmacokinetic analysis based on samples taken from patients during the trial showed no significant difference between standard FVIII and PEGLip-FVIII. This suggests that the increased protection from bleeding observed with PEGLip-FVIII did not result from a simple prolongation of FVIII half-life in the bloodstream.

Safety and tolerability of PEGLip-FVIII were assessed in another clinical trial involving 18 severe hemophilia A patients. This study was directed primarily at determining the optimal infusion rate for PEGLip-FVIII. The study showed that PEGLip-FVIII may be administered at an infusion rate similar to that of standard FVIII [83].

Production of inhibitory antibodies was not detected in any of the four studies described above, and no serious adverse events were reported. However, a few subjects experienced increased breathing frequency and flushing [82,83]. These symptoms resolved without medical intervention. This type of hypersensitivity reaction is known as complement activation-related pseudoallergy (CARPA) and has been described following administration of radiocontrast media, liposomal drugs such as Doxil, and micellar solvents. Unlike IgE-mediated reactions, CARPA reactions arise at first treatment and become milder or disappear on repeated exposure [83,84].

The combined results of the Phase I and II clinical studies described above indicate that PEGLip-FVIII is well tolerated and provides extended protection from bleeding following prophylactic administration. A large clinical trial involving 250 hemophilia A patients in > 70 hemophilia centers around the world is now being conducted to determine whether a once-a-week prophylactic treatment with 35 IU/kg PEGLip-FVIII is safe and can prevent bleeds in subjects with severe hemophilia A, similarly to control treatment of 3 weekly prophylactic injections of 25 IU/kg standard FVIII [85].

2.2 PEGLip-formulated FVIIa

Approximately 20 – 40% of hemophilia patients develop inhibitory antibodies against FVIII or factor IX (FIX) [86]. These patients must be treated with bypassing agents, proteins that induce clotting while avoiding neutralization by antibodies. Activated factor VII (FVIIa) has been shown to induce hemostasis in the absence of factor FVIII or FIX [87] and has been approved in many countries for the treatment of bleeding episodes in patients with congenital hemophilia and inhibitors to FVIII or FIX and patients with acquired hemophilia [88]. The half-life of FVIIa in the circulation is ~ 2.3 h [89,90]. In most patients, effective hemostasis is achieved only after 2 or 3 doses of 90 µg/kg given at 2 h intervals [91,92].

As in the case of FVIII, there is a clinical need for a more potent and long-acting form of FVIIa that would provide extended protection from bleeding while requiring fewer injections. Patients would also benefit from fewer dips in FVIIa levels, fewer complications and better control of bleeding episodes.

Several approaches have been used in continuing attempts to develop improved forms of FVIIa. These include substitution of one or more amino acids within the protein [27], PEGylation [93] and fusion of FVIIa to albumin [94]. These proteins showed promising results *in vitro* and in animal models, however the efficacy and safety of these approaches has yet to be demonstrated in humans.

SPR analyses showed that PEGLip bind coagulation FVIIa with high affinity and specificity. Separate experiments showed that formulation of FVIIa with PEGLip did not affect its *in vitro* activity [62]. The authors therefore set out to determine whether PEGLip formulation might improve the pharmacodynamics of FVIIa *in vivo*. Hemophilic mice were injected with standard FVIIa, PEGLip-FVIIa, or saline and tail vein transection assays were performed. It was found that mice injected with PEGLip-FVIIa survived significantly longer ($p < 0.05$) than mice injected with standard FVIIa (**Figure 2C**).

The safety and efficacy of PEGLip-FVIIa were tested in humans in an open label, exploratory, crossover, Phase I/II clinical study in six adult subjects with severe hemophilia A and inhibitory FVIII antibodies [95]. Each subject received 2 infusions: 1 infusion of 90 µg/kg standard FVIIa and 1 infusion of 90 µg/kg PEGLip-formulated FVIIa. The two infusions were separated by a 10-day washout period. Injection volume was kept constant whereas injection order was randomized.

Blood samples were collected from patients at various time points both before and after each infusion of FVIIa or PEGLip-FVIIa. Whole blood samples were then analyzed

by rotational thrombelastography to determine the kinetics of clot formation and the firmness of the clots. PEGLip-formulated FVIIa produced significantly ($p < 0.05$) shorter clotting times and higher clot firmness than standard FVIIa up to 5 h post-injection. Clotting time of standard FVIIa 1 h post-injection was the same as clotting time of PEGLip-FVIIa 3.2 h post-injection. Maximal clot firmness induced by standard FVIIa 0.5 h post-injection was as high as that induced by PEGLip-FVIIa ~ 2.7 h post-injection. Thrombin generation assays performed on samples collected in the clinical trial showed that thrombin was produced faster and more efficiently following infusion of PEGLip-FVIIa than following infusion of standard FVIIa. No significant differences were detected between the pharmacokinetics of PEGLip-FVIIa and standard FVIIa, suggesting that the increased efficacy of PEGLip-FVIIa was probably not the result of increased circulation time.

One of the subjects experienced transient hyperemia, increase in blood pressure and anxiety during the infusion of the first 1 ml of PEGLip-FVIIa. This non-IgE-mediated reaction (CARPA) is associated with PEGLip [84] and is not related to FVIIa. The reaction subsided within 1 h. In all other subjects, PEGLip-FVIIa was well tolerated and there were no serious adverse events, nor were there any significant changes in vital signs, clinical chemistry or hematological parameters. In addition, measurements of coagulation parameters indicated that there was no increase in thrombotic risk.

Together, the results of the clinical trial indicate that PEGLip-FVIIa provides about two more 'efficacy hours' than standard FVIIa. In treatment of bleeding episodes in hemophilia patients with inhibitors, one 90 μg/kg dose of PEGLip-FVIIa may be roughly equivalent to two infusions of standard FVIIa given at 2 h intervals.

2.3 PEGLip-formulated G-CSF

G-CSF is a cytokine that naturally regulates the survival, proliferation and differentiation of hematopoietic cells in the neutrophilic lineage and influences the function of mature neutrophils [96]. Recombinant human G-CSF is used to treat chronic neutropenia and to prevent post-chemotherapy febrile neutropenia by increasing the production of neutrophils. It is also used to induce mobilization of hematopoietic stem cells (HSC) from the bone marrow to the peripheral blood. Mobilized stem cells may then be collected and transplanted into patients whose hematopoietic systems have been damaged by cytotoxic therapy [97,98].

G-CSF is a short-lived protein, with a half-life of 3.5 h in humans [99]. Multiple injections are therefore required to achieve the desired therapeutic outcomes in both neutropenia and stem cell mobilization indications [100]. Furthermore, poor stem cell mobilization is observed in 10 – 20% of healthy donors [101,102] and in 35 – 70% of cancer patients who have undergone previous courses of chemotherapy [103,104]. There is thus a clinical need for a more effective form of G-CSF treatment.

A PEGylated form of recombinant human G-CSF (Neulasta, Amgen) with a half-life of 40 – 80 h is approved by the FDA for the treatment of neutropenia but not for the mobilization of HSC [100]. The authors found in SPR experiments that PEGLip bind G-CSF with high affinity and specificity. It was therefore tested whether G-CSF formulated with PEGLip might be more effective than standard G-CSF in inducing HSC mobilization.

An experiment was performed in which mice were given daily intravenous injections of standard G-CSF, PEGLip-G-CSF, or dextrose solution. Following 2 or 3 days of treatment, the mice were bled, leukocytes from each mouse were counted, and the number of HSC in the peripheral blood of each mouse was determined by flow cytometry. It was found that PEGLip-G-CSF induced mobilization of ~ 2.5 times as many HSC into the peripheral blood as standard G-CSF (**Figure 2D**) [66]. PEGLip-G-CSF has yet to be tested in humans.

3. Mechanism of action

There may be more than a single mechanism by which PEGLip formulation increases the efficacy of therapeutic proteins. In preclinical models, formulation of FVIII, FVIIa and G-CSF with PEGLip improved pharmacokinetic properties *in vivo* and increased circulation half-life [60,62,66]. However Phase I clinical experiments showed that there was no difference between the pharmacokinetic behavior of standard FVIII and PEGLip-formulated FVIII [82] and between standard FVIIa and PEGLip-formulated FVIIa [95]. This suggests that a mechanism other than extension of circulation half-life may be responsible for the increased hemostatic efficacy observed with PEGLip-formulated FVIII and FVIIa.

Liposomes of various compositions, both with and without PEG, have been shown to interact with blood cells *in vitro* and *in vivo* [105]. Platelets play a vital role in the coagulation process. Association of PEGLip with platelets may therefore help to explain how PEGLip formulation improves hemostatic efficacy. To test this hypothesis, PEGLip were fluorescently labeled by integrating lissamine rhodamine B-labeled phosphoethanolamine into the lipid bilayer of the liposomes. These fluorescent PEGLip were then incubated with human whole blood and their binding to platelets was measured by flow cytometry. It was found that PEGLip associated with platelets *in vitro* in a dose-related manner, with ~ 40% of the platelets showing PEGLip binding at a liposome concentration similar to that tested in clinical trials (data not shown). When fluorescent liposomes were injected into mice, association of fluorescent liposomes with platelets was also observed. Analysis of mouse platelet-rich plasma (PRP) by flow cytometry showed that the association between PEGLip and platelets was maintained up to 26 h post-injection (**Figure 3A**). Fluorescently labeled PEGLip thus interact with platelets both *in vitro* and *in vivo*.

Having shown that PEGLip bind FVIII and FVIIa [60,62] and also bind platelets, the authors hypothesized that

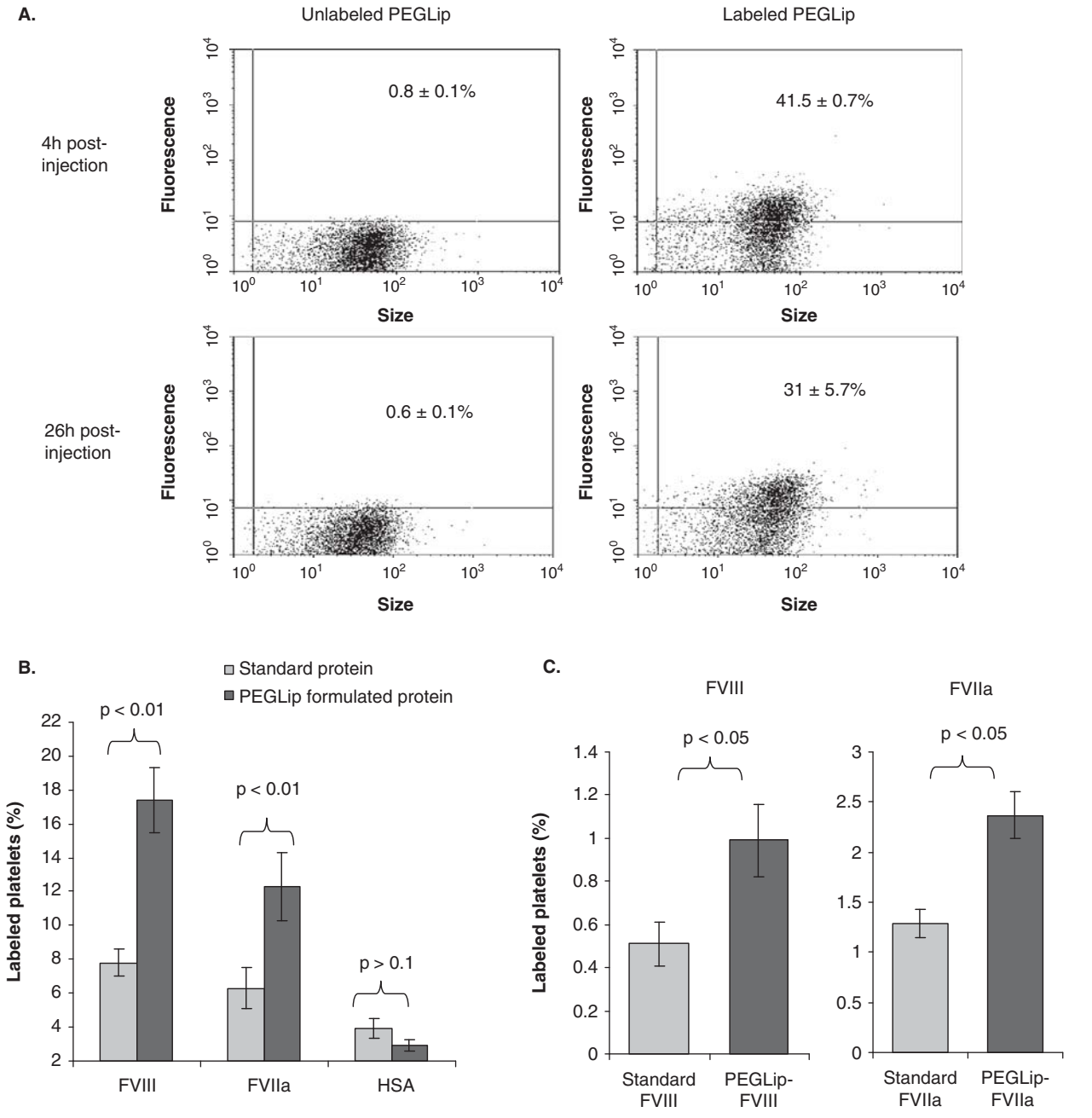

Figure 3. Interaction of PEGLip with platelets. A. *In vivo* binding of PEGLip to mouse platelets. Unlabeled PEGLip or fluorescently labeled PEGLip were injected into hemophilic mice and the percentage of platelets labeled by the fluorescent liposomes was determined by flow cytometry. Dot plots from a representative mouse in each group are shown with average percentages ± SEM (n = 4) written above. **B.** *In vitro* binding of standard or PEGLip-formulated proteins to human platelets. Factor VIII (n = 4), FVIIa (n = 4) and HSA (n = 4) were fluorescently labeled, formulated in buffer or PEGLip, and incubated for 2 h at 37°C with washed human platelets. The percentage of platelets associated with the fluorescent proteins was determined by flow cytometry (average ± SEM). **C.** *In vivo* binding of standard or PEGLip-formulated proteins to mouse platelets. Fluorescently labeled FVIII (n = 11) and FVIIa (n = 9) were formulated in buffer or PEGLip, and injected to hemophilic mice. Blood was drawn 1 h after injection and the percentage of platelets associated with the fluorescent proteins was determined by flow cytometry (average ± SEM). p-Values in **B** were determined using a paired t-test. p-Values in **C** were determined using a homoscedastic t-test.

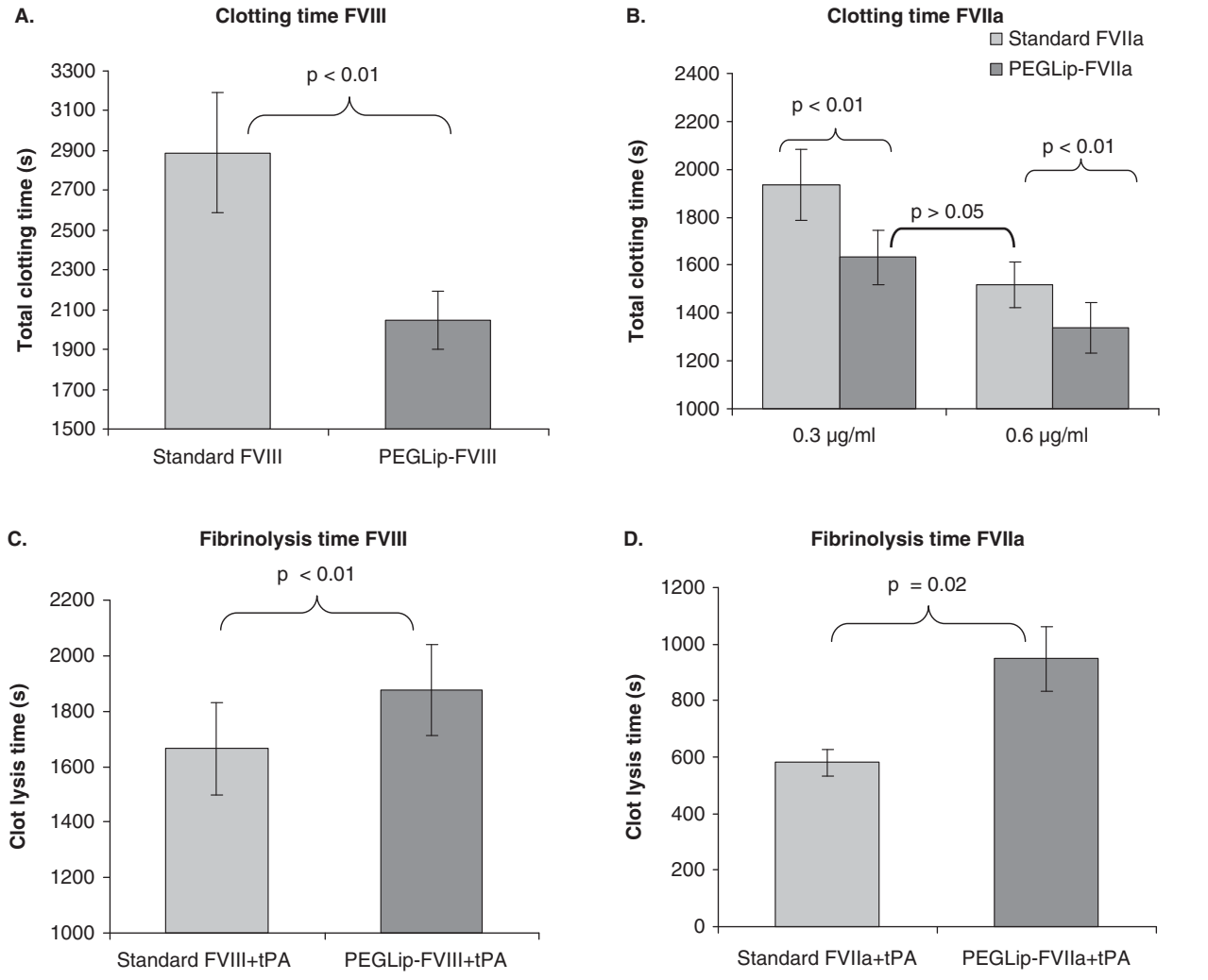

Figure 4. Effect of PEGLip formulation of FVIII and FVIIa on clot formation and lysis as measured by rotational thrombelstography using a ROTEG 5 instrument. A. Total clotting time in severe hemophilic whole blood spiked with standard FVIII or PEGLip-FVIII (both 0.01 IU/ml), n = 10. **B.** Total clotting time in severe hemophilic platelet-rich plasma (SH-PRP) containing anti-FVIII inhibitory antibodies spiked with standard FVIIa or PEGLip-FVIIa (0.3 or 0.6 µg/ml), n = 6. **C.** Fibrinolysis time in SH-PRP spiked with standard FVIII or PEGLip-FVIII (both 0.025 IU/ml) and tissue plasminogen activator (tPA, 225 IU/ml), n = 4. **D.** Fibrinolysis time in SH-PRP containing anti-FVIII inhibitory antibodies spiked with standard FVIIa or PEGLip-FVIIa (both 0.3 µg/ml) and tPA (225 IU/ml), n = 4. Results are average ± SEM p-Values were determined using a paired t-test. **B** and **D** are based on previously published data [62].

PEGLip may link protein drugs to platelets. This hypothesis was tested in experiments in which proteins were fluorescently labeled, formulated with unlabeled PEGLip, and then assayed for binding to washed human platelets *in vitro* by flow cytometry. Both fluorescent FVIII and fluorescent FVIIa that had been formulated with PEGLip showed significantly enhanced binding to human platelets (**Figure 3B**). Fluorescently labeled HSA, which does not bind to PEGLip, did not show increased binding to platelets following formulation with PEGLip. This indicates that association with platelets is dependent on proteins first binding to PEGLip. When fluorescently labeled FVIII and FVIIa were formulated with PEGLip and injected into hemophilic mice, significantly enhanced binding of the fluorescent proteins to platelets was also observed (**Figure 3C**).

Rotational thrombelastography showed that binding of FVIIa and FVIII via PEGLip to platelets resulted in faster kinetics of clot formation and higher clot firmness. In one set of experiments, clotting time was measured in human hemophilic whole blood spiked *in vitro* with standard FVIII or PEGLip-FVIII (**Figure 4A**). In similar experiments, clotting time was measured in hemophilic PRP spiked with standard FVIIa or PEGLip-FVIIa (**Figure 4B**) [62]. In both cases, clotting occurred significantly faster when coagulation was induced by PEGLip-FVIII or PEGLip-FVIIa than when it was induced by similar concentrations of standard FVIII or FVIIa. Such

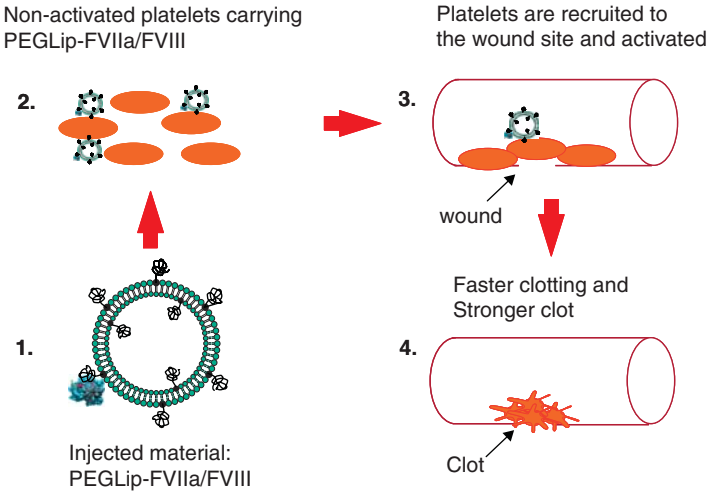

Figure 5. Mechanism of action of PEGLip-formulated FVIII and FVIIa. (1) Formulation of FVIII or FVIIa with PEGLip leads to non-covalent binding of the protein to the outer surface of the PEGylated liposomes. (2) The liposomes are injected into the bloodstream where they associate with non-activated platelets. (3) Platelets are recruited to sites of injury where they adhere to the damaged vessel wall. They carry FVIII and FVIIa with them. (4) Platelets are activated at the wound site and coagulation complexes form on the surface of the activated platelets. As FVIII and FVIIa are already present on the platelets before activation, the coagulation cascade is more efficient. Clots form faster and the clots are more stable.

enhancement was not detected in platelet-poor plasma, indicating that platelets must be present for PEGLip to enhance efficacy. Moreover, clotting time measured in PRP for PEGLip-FVIIa at 0.3 µg/ml was very similar ($p > 0.05$) to the clotting time measured for standard FVIIa at 0.6 µg/ml (**Figure 4B**). This suggests that PEGLip formulation almost doubled the effective concentration. Clots not only formed faster with PEGLip-FVIII and PEGLip-FVIIa, but they were also more resistant to fibrinolysis. Clots induced by PEGLip-FVIII or PEGLip-FVIIa in the presence of tissue plasminogen activator had longer fibrinolysis times than clots induced by standard FVIII and FVIIa (**Figure 4C,D**) [62]. PEGLip on their own had no effect on clotting.

Platelets play a central role in hemostasis. Adhesion of platelets to the damaged vascular wall is followed by platelet activation, a process that exposes negatively charged phospholipids on the platelet surface. Factor VIII, FIX, FX and several cofactors have high affinity for this charged surface [106]. Assembly of coagulation factor complexes on the platelet surface leads to generation of thrombin, cleavage of fibrinogen to fibrin, polymerization of fibrin, and formation of a hemostatic plug [107]. Interaction of FVIII with platelets via PEGLip before activation may lead to more rapid assembly of complexes containing FVIII, FIXa and FX. This may lead to faster clotting when and where it is needed. Similarly, the effect of PEGLip formulation on FVIIa may be related to platelet binding. At pharmacological doses, FVIIa activity is dependent on binding to activated platelets [108]. Here again, PEGLip-dependent binding of FVIIa to platelets before activation may accelerate interaction with FX and formation of the coagulation complex (**Figure 5**). This may lead to faster generation of fibrin clots even when the overall concentration of these coagulation factors in the circulation is low. In independent studies, expression of low levels of FVIII in platelets was shown to be effective at inducing hemostasis, even though FVIII protein expression did not exceed 1% of normal levels. This was probably because of local release of FVIII from activated platelets at the wound site [109].

4. Conclusion

Various approaches have been used in attempts to improve the pharmacodynamics of protein drugs. These include direct PEGylation, modification of amino acid sequence, fusion to carrier proteins and encapsulation in liposomes. Each of these approaches has advantages and disadvantages and what works for one protein may not necessarily work for others. The design of an improved therapeutic protein must be based on an understanding of the physical and chemical properties of the protein and its mechanism of action inside the body.

PEGLip technology is based on the non-covalent binding of proteins to the outer surface of PEGylated liposomes. This specific binding is dependent on the presence of a conserved amino acid sequence within the protein and on PEG molecules on the liposome surface. Preclinical and clinical studies with FVIII and FVIIa and preclinical studies with G-CSF showed that formulation of proteins with PEGLip resulted in improved pharmacodynamics. PEGLip were shown to be safe and well tolerated by patients and they did not pose a risk of induction of antibodies against the formulated protein. Several lines of evidence support the binding of liposomes to blood cells and the targeted delivery of formulated proteins to sites of action. This mechanism helps explain the improved efficacy of PEGLip-formulated proteins even in the absence

of changes to pharmacokinetics. PEGLip formulation is a platform technology that has the potential to improve the pharmacodynamics of therapeutic proteins while avoiding the complications that have plagued other approaches.

5. Expert opinion

In recent years, various technologies have emerged for improvement of the pharmacokinetics and efficacy of protein drugs. For several small proteins, mainly cytokines, these efforts have led to the generation of more potent and patient-friendly drugs. Attempts to apply these technologies to large and complex proteins have produced limited results, though. For example, FVIII variants with genetic modifications and PEGylated forms of FVIII have not yet reached clinical trials.

In existing approaches to enhance protein pharmacodynamics, the immunological effects of a formulation or modification remain largely unknown until the protein reaches advanced clinical trials. This introduces a considerable element of risk to the drug development process. One of the primary advantages of PEGLip formulation is that pharmacodynamics is improved while the structure of the therapeutic protein remains unchanged and the risk of induction of antibodies does not increase. Owing to the large size and complexity of the FVIII protein and the sensitivity of hemophilia A patients to even small changes in the protein [110], FVIII serves as a worst-case scenario for increased immunogenicity. Extensive experience with PEGLip-FVIII in several clinical studies has shown no increase in antibody generation. PEGLip formulation thus avoids one of the major pitfalls of the drug development process.

So far, an enhancement of pharmacodynamics as a result of PEGLip formulation has been demonstrated with three clinically important proteins: FVIII, FVIIa and G-CSF. PEGLip technology has its limitations, though. For a protein to be formulated with PEGLip, it must contain a sequence capable of binding the outer surface of the liposomes. That being said, if a protein is found to be suitable, PEGLip formulation is very straightforward.

In the coming years, the authors expect PEGLip technology to take its place among other approaches to improve the properties of protein drugs. The technology is particularly attractive for large and sensitive proteins that may both lose activity and become increasingly immunogenic as a result of modification.

Declaration of interest

The authors are employees of Omri Laboratories Ltd.

Bibliography

Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.

1. Banga AK. Therapeutic peptides and proteins: formulation, processing, and delivery systems. 2nd edition. Boca Raton, FL: CRC/Taylor & Francis; 2006
2. Gomez-Orellana I. Strategies to improve oral drug bioavailability. Expert Opin Drug Deliv 2005;2(3):419-33
3. Schuetz YB, Naik A, Guy RH, et al. Emerging strategies for the transdermal delivery of peptide and protein drugs. Expert Opin Drug Deliv 2005;2(3):533-48
4. Siekmeier R, Scheuch G. Systemic treatment by inhalation of macromolecules–principles, problems, and examples. J Physiol Pharmacol 2008;59:(Suppl 6):53-79
5. Stolnik S, Shakesheff K. Formulations for delivery of therapeutic proteins. Biotechnol Lett 2009;31(1):1-11
6. Bailon P, Won CY. PEG-modified biopharmaceuticals. Expert Opin Drug Deliv 2009;6(1):1-16
   • **A general review of PEGylation and its effect on biomolecules.**
7. Khandare J, Minko T. Polymer-drug conjugates: progress in polymeric prodrugs. Prog Polym Sci 2006;31:359-97
8. Ryan SM, Mantovani G, Wang X, et al. Advances in PEGylation of important biotech molecules: delivery aspects. Expert Opin Drug Deliv 2008;5(4):371-83
9. Harris JM, Martin NE, Modi M. Pegylation: a novel process for modifying pharmacokinetics. Clin Pharmacokinet 2001;40(7):539-51
10. Harris JM, Chess RB. Effect of pegylation on pharmaceuticals. Nat Rev Drug Discov 2003;2(3):214-21
11. Caliceti P, Veronese FM. Pharmacokinetic and biodistribution properties of poly (ethylene glycol)-protein conjugates. Adv Drug Deliv Rev 2003;55(10):1261-77
12. Basu A, Yang K, Wang M, et al. Structure-function engineering of interferon-beta-1b for improving stability, solubility, potency, immunogenicity, and pharmacokinetic properties by site-selective mono-PEGylation. Bioconjug Chem 2006;17(3):618-30
13. De Groot AS, Scott DW. Immunogenicity of protein therapeutics. Trends Immunol 2007;28(11):482-90
14. Hershfield MS, Buckley RH, Greenberg ML, et al. Treatment of adenosine deaminase deficiency with polyethylene glycol-modified adenosine deaminase. N Engl J Med 1987;316(10):589-96
15. Chakravarti VS, Borns P, Lobell J, et al. Chondroosseous dysplasia in severe combined immunodeficiency due to adenosine deaminase deficiency (chondroosseous dysplasia in ADA deficiency SCID). Pediatr Radiol 1991;21(6):447-8
16. Avramis VI, Sencer S, Periclou AP, et al. A randomized comparison of native Escherichia coli asparaginase and polyethylene glycol conjugated asparaginase for treatment of children with newly diagnosed standard-risk acute lymphoblastic leukemia: a Children's

Cancer Group study. Blood 2002;99(6):1986-94

17. Graham ML. Pegaspargase: a review of clinical studies. Adv Drug Deliv Rev 2003;55(10):1293-302
18. Chilukuri N, Sun W, Parikh K, et al. A repeated injection of polyethyleneglycol-conjugated recombinant human butyrylcholinesterase elicits immune response in mice. Toxicol Appl Pharmacol 2008;231(3):423-9
19. Pepinsky RB, LePage DJ, Gill A, et al. Improved pharmacokinetic properties of a polyethylene glycol-modified form of interferon-beta-1a with preserved in vitro bioactivity. J Pharmacol Exp Ther 2001;297(3):1059-66
20. Basser RL, O'Flaherty E, Green M, et al. Development of pancytopenia with neutralizing antibodies to thrombopoietin after multicycle chemotherapy supported by megakaryocyte growth and development factor. Blood 2002;99(7):2599-602
21. Moss J, Rosholm A, Laurén A. Safety and pharmacokinetics of a long-acting glycopegylated rFVIIa derivative: a first human dose trial in healthy subjects. J Thromb Haemost 2009;7:OC-WE-055
22. Roberts MJ, Bentley MD, Harris JM. Chemistry for peptide and protein PEGylation. Adv Drug Deliv Rev 2002;54(4):459-76
23. Hirsch IB. Insulin analogues. N Engl J Med 2005;352(2):174-83
24. Sheldon B, Russell-Jones D, Wright J. Insulin analogues: an example of applied medical science. Diabetes Obes Metab 2009;11(1):5-19
25. Macdougall IC. An overview of the efficacy and safety of novel erythropoiesis stimulating protein (NESP). Nephrol Dial Transplant 2001;16:(Suppl 3):14-21
26. Egrie JC, Browne JK. Development and characterization of novel erythropoiesis stimulating protein (NESP). Br J Cancer 2001;84:(Suppl 1):3-10
27. Allen GA, Persson E, Campbell RA, et al. A variant of recombinant factor VIIa with enhanced procoagulant and antifibrinolytic activities in an in vitro model of hemophilia. Arterioscler Thromb Vasc Biol 2007;27(3):683-9
28. Sorensen B, Persson E, Ingerslev J. Factor VIIa analogue (V158D/E296V/M298Q-FVIIa) normalises clot formation in whole blood from patients with severe haemophilia A. Br J Haematol 2007;137(2):158-65
29. Brophy DF, Martin EJ, Nolte ME, et al. Effect of recombinant factor VIIa variant (NN1731) on platelet function, clot structure and force onset time in whole blood from healthy volunteers and haemophilia patients. Haemophilia 2007;13(5):533-41
30. Liu T, Zhang X, Pan J, et al. Enhanced and prolonged efficacy of a novel recombinant FVIIa variant (BAY86-6150) for acute and prophylactic treatments in hemophilia A (HemA) mice. J Thromb Haemost 2009;7(2):OC-WE-057
31. Pan J, Kim J, Zhu D, et al. Binding to activated platelets and enhanced clotting properties of the recombinant FVIIa analogue Bay7. J Thromb Haemost 2009;7(2):OC-WE-059
32. Sytkowski AJ, Lunn ED, Risinger MA, et al. An erythropoietin fusion protein comprised of identical repeating domains exhibits enhanced biological properties. J Biol Chem 1999;274(35):24773-8
33. Alonso-Ruiz A, Pijoan JI, Ansuategui E, et al. Tumor necrosis factor alpha drugs in rheumatoid arthritis: systematic review and metaanalysis of efficacy and safety. BMC Musculoskelet Disord 2008;9:52
34. Kratz F. Albumin as a drug carrier: design of prodrugs, drug conjugates and nanoparticles. J Control Release 2008;132(3):171-83
35. Rustgi VK. Albinterferon alfa-2b, a novel fusion protein of human albumin and human interferon alfa-2b, for chronic hepatitis C. Curr Med Res Opin 2009;25(4):991-1002
36. Gao Z, Bai G, Chen J, et al. Development, characterization, and evaluation of a fusion protein of a novel glucagon-like peptide-1 (GLP-1) analog and human serum albumin in Pichia pastoris. Biosci Biotechnol Biochem 2009;73(3):688-94
37. Schmidt SR. Fusion-proteins as biopharmaceuticals–applications and challenges. Curr Opin Drug Discov Devel 2009;12(2):284-95
38. Zhao HL, Xue C, Wang Y, et al. Elimination of the free sulfhydryl group in the human serum albumin (HSA) moiety of human interferon-alpha2b and HSA fusion protein increases its stability against mechanical and thermal stresses. Eur J Pharm Biopharm 2009;72(2):405-11
39. Sheffield WP, Mamdani A, Hortelano G, et al. Effects of genetic fusion of factor IX to albumin on in vivo clearance in mice and rabbits. Br J Haematol 2004;126(4):565-73
40. Singh R, Lillard JW Jr. Nanoparticle-based targeted drug delivery. Exp Mol Pathol 2009;86(3):215-23
41. Paukner S, Stiedl T, Kudela P, et al. Bacterial ghosts as a novel advanced targeting system for drug and DNA delivery. Expert Opin Drug Deliv 2006;3(1):11-22
42. Rossi L, Serafini S, Pierige F, et al. Erythrocyte-based drug delivery. Expert Opin Drug Deliv 2005;2(2):311-22
43. Zamboni WC. Concept and clinical evaluation of carrier-mediated anticancer agents. Oncologist 2008;13(3):248-60
44. Matsumura Y, Maeda H. A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of tumoritropic accumulation of proteins and the antitumor agent smancs. Cancer Res 1986;46(12 Pt 1):6387-92
45. Park JW. Liposome-based drug delivery in breast cancer treatment. Breast Cancer Res 2002;4(3):95-9
46. Gabizon A, Shmeeda H, Barenholz Y. Pharmacokinetics of pegylated liposomal Doxorubicin: review of animal and human studies. Clin Pharmacokinet 2003;42(5):419-36
47. Immordino ML, Dosio F, Cattel L. Stealth liposomes: review of the basic science, rationale, and clinical applications, existing and potential. Int J Nanomed 2006;1(3):297-315
48. Brandl M, Gregoriadis G. Entrapment of haemoglobin into liposomes by the dehydration-rehydration method: vesicle characterization and in vivo behaviour. Biochim Biophys Acta 1994;1196(1):65-75
49. Kim JY, Kim JK, Park JS, et al. The use of PEGylated liposomes to prolong circulation lifetimes of tissue plasminogen activator. Biomaterials 2009;30(29):5751-6

50. Kedar E, Braun E, Rutkowski Y, et al. Delivery of cytokines by liposomes. II. Interleukin-2 encapsulated in long-circulating sterically stabilized liposomes: immunomodulatory and anti-tumor activity in mice. J Immunother Emphasis Tumor Immunol 1994;16(2):115-24

51. Kedar E, Gur H, Babai I, et al. Delivery of cytokines by liposomes: hematopoietic and immunomodulatory activity of interleukin-2 encapsulated in conventional liposomes and in long-circulating liposomes. J Immunother 2000;23(1):131-45

52. Debs RJ, Fuchs HJ, Philip R, et al. Immunomodulatory and toxic effects of free and liposome-encapsulated tumor necrosis factor alpha in rats. Cancer Res 1990;50(2):375-80

53. Walde P, Ichikawa S. Enzymes inside lipid vesicles: preparation, reactivity and applications. Biomol Eng 2001;18(4):143-77

- **A review describing and comparing various methods for encapsulating enzymes in liposomes.**

54. Colletier JP, Chaize B, Winterhalter M, et al. Protein encapsulation in liposomes: efficiency depends on interactions between protein and phospholipid bilayer. BMC Biotechnol 2002;2:9

55. Kirby CJ, Gregoriadis G. Preparation of liposomes containing factor VIII for oral treatment of haemophilia. J Microencapsul 1984;1(1):33-45

56. Tiukinhoy-Laing SD, Huang S, Klegerman M, et al. Ultrasound-facilitated thrombolysis using tissue-plasminogen activator-loaded echogenic liposomes. Thromb Res 2007;119(6):777-84

57. Adrian G, Huang L. Entrapment of proteins in phosphatidylcholine vesicles. Biochemistry 1979;18(25):5610-4

58. Kedar E, Rutkowski Y, Braun E, et al. Delivery of cytokines by liposomes. I. Preparation and characterization of interleukin-2 encapsulated in long-circulating sterically stabilized liposomes. J Immunother Emphasis Tumor Immunol 1994;16(1):47-59

59. Elbayoumi TA, Torchilin VP. Liposomes for targeted delivery of antithrombotic drugs. Expert Opin Drug Deliv 2008;5(11):1185-98

60. Baru M, Carmel-Goren L, Barenholz Y, et al. Factor VIII efficient and specific non-covalent binding to PEGylated liposomes enables prolongation of its circulation time and haemostatic efficacy. Thromb Haemost 2005;93(6):1061-8

- **The first preclinical study showing binding of PEGLip to FVIII and enhancement of hemostatic efficacy in mice.**

61. Dayan I, Robinson M, Baru M. Enhancement of haemostatic efficacy of plasma-derived FVIII by formulation with PEGylated liposomes. Haemophilia 2009;15(5):1006-13

62. Yatuv R, Dayan I, Carmel-Goren L, et al. Enhancement of factor VIIa haemostatic efficacy by formulation with PEGylated liposomes. Haemophilia 2008;14(3):476-83

63. Klibanov AL, Maruyama K, Torchilin VP, et al. Amphipathic polyethyleneglycols effectively prolong the circulation time of liposomes. FEBS Lett 1990;268(1):235-7

64. Gabizon AA, Barenholz Y, Bialer M. Prolongation of the circulation time of doxorubicin encapsulated in liposomes containing a polyethylene glycol-derivatized phospholipid: pharmacokinetic studies in rodents and dogs. Pharm Res 1993;10(5):703-8

65. Gabizon A, Catane R, Uziely B, et al. Prolonged circulation time and enhanced accumulation in malignant exudates of doxorubicin encapsulated in polyethylene-glycol coated liposomes. Cancer Res 1994;54(4):987-92

66. Yatuv R, Carmel-Goren L, Dayan I, et al. Binding of proteins to PEGylated liposomes and improvement of G-CSF efficacy in mobilization of hematopoietic stem cells. J Control Release 2009;135(1):44-50

- **A preclinical study showing binding of PEGLip to G-CSF and other proteins, improved stem cell mobilibization in mice, and identification of the molecular regions in proteins and on liposomes responsible for PEGLip binding.**

67. Kaufman RJ, Anthonorakis SE, Fay PJ. Hemostasis and thrombosis: basic principles and clinical practice. 4th edition. Philadelphia: Lippincott Williams & Wilkins; 2001

68. Roosendaal G, Mauser-Bunschoten EP, De Kleijn P, et al. Synovium in haemophilic arthropathy. Haemophilia 1998;4(4):502-5

69. Hoots WK, Nugent DJ. Evidence for the benefits of prophylaxis in the management of hemophilia A. Thromb Haemost 2006;96(4):433-40

- **A review describing the advantages of prophylactic rather than on-demand treatment for hemophilia A.**

70. Fijnvandraat K, Berntorp E, ten Cate JW, et al. Recombinant, B-domain deleted factor VIII (r-VIII SQ): pharmacokinetics and initial safety aspects in hemophilia A patients. Thromb Haemost 1997;77(2):298-302

71. Weiss HJ, Sussman II, Hoyer LW. Stabilization of factor VIII in plasma by the von Willebrand factor. Studies on posttransfusion and dissociated factor VIII and in patients with von Willebrand's disease. J Clin Invest 1977;60(2):390-404

72. Saenko EL, Ananyeva NM, Moayeri M, et al. Development of improved factor VIII molecules and new gene transfer approaches for hemophilia A. Curr Gene Ther 2003;3(1):27-41

73. Saenko EL, Pipe SW. Strategies towards a longer acting factor VIII. Haemophilia 2006;12:(Suppl 3):42-51

74. Pipe SW, Kaufman RJ. Characterization of a genetically engineered inactivation-resistant coagulation factor VIIIa. Proc Natl Acad Sci USA 1997;94(22):11851-6

75. Gale AJ, Pellequer JL. An engineered interdomain disulfide bond stabilizes human blood coagulation factor VIIIa. J Thromb Haemost 2003;1(9):1966-71

76. Gale AJ, Radtke KP, Cunningham MA, et al. Intrinsic stability and functional properties of disulfide bond-stabilized coagulation factor VIIIa variants. J Thromb Haemost 2006;4(6):1315-22

77. Murphy JE, Pan C, Barnett T, et al. Site-specific PEGylation of rFVIII results in prolonged in vivo efficacy. J Thromb Haemost 2007;5(2):P-T-022

78. Regan LM, Jiang X, Ramsey P, et al. Biological activity of PEGylated factor VIII. J Thromb Haemost 2007;5(2):P-T-026

79. Tang L, Pan C, Atwal H, et al. PEGylation protects factor VIII from the inhibition of antibody inhibitors. J Thromb Haemost 2007;5(2):P-T-036

80. Spira J, Plyushch OP, Andreeva TA, et al. Prolonged bleeding-free period following prophylactic infusion of recombinant

factor VIII reconstituted with pegylated liposomes. Blood 2006;108(12):3668-73

- **The first study showing an improvement in the hemostatic efficacy of PEGLip-FVIII in humans.**

81. Spira J, Plyushch OP, Andreeva TA, et al. Evaluation of liposomal dose in recombinant factor VIII reconstituted with pegylated liposomes for the treatment of patients with severe haemophilia A. Thromb Haemost 2008;100(3):429-34
82. Powell JS, Nugent DJ, Harrison JA, et al. Safety and pharmacokinetics of a recombinant factor VIII with pegylated liposomes in severe hemophilia A. J Thromb Haemost 2008;6(2):277-83
83. Martinowitz U, Lalezari S, Luboshitz J, et al. Infusion rates of recombinant FVIII-FS with PEGylated liposomes in haemophilia A. Haemophilia 2008;14(5):1122-4
84. Szebeni J. Complement activation-related pseudoallergy: a new class of drug-induced acute immune toxicity. Toxicology 2005;216(2-3):106-21
85. Available from: http://clinicaltrials.gov/ct2/show/NCT00623727. [Last accessed November 2009]
86. Mathew P. Current opinion on inhibitor treatment options. Semin Hematol 2006;43(2 Suppl 4):S8-13
87. Lusher JM, Roberts HR, Davignon G, et al. A randomized, double-blind comparison of two dosage levels of recombinant factor VIIa in the treatment of joint, muscle and mucocutaneous haemorrhages in persons with haemophilia A and B, with and without inhibitors. rFVIIa Study Group. Haemophilia 1998;4(6):790-8
88. Ingerslev J. Efficacy and safety of recombinant factor VIIa in the prophylaxis of bleeding in various surgical procedures in hemophilic patients with factor VIII and factor IX inhibitors. Semin Thromb Hemost 2000;26(4):425-32
89. Lindley CM, Sawyer WT, Macik BG, et al. Pharmacokinetics and pharmacodynamics of recombinant factor VIIa. Clin Pharmacol Ther 1994;55(6):638-48
90. Fridberg MJ, Hedner U, Roberts HR, et al. A study of the pharmacokinetics and safety of recombinant activated factor VII in healthy Caucasian and Japanese subjects. Blood Coagul Fibrinolysis 2005;16(4):259-66
91. Ingerslev J, Thykjaer H, Kudsk Jensen O, et al. Home treatment with recombinant activated factor VII: results from one centre. Blood Coagul Fibrinolysis 1998;9:(Suppl 1):S107-10
92. Key NS, Aledort LM, Beardsley D, et al. Home treatment of mild to moderate bleeding episodes using recombinant factor VIIa (Novoseven) in haemophiliacs with inhibitors. Thromb Haemost 1998;80(6):912-8
93. Stennicke HR, Ostergaard H, Bayer RJ, et al. Generation and biochemical characterization of glycoPEGylated factor VIIa derivatives. Thromb Haemost 2008;100(5):920-8
94. Schulte S. Use of albumin fusion technology to prolong the half-life of recombinant factor VIIa. Thromb Res 2008;122:(Suppl 4):S14-19
95. Baru M, Spira J, Plyushch OP, et al. Safety, pharmacokinetics and efficacy of factor VIIa formulated with PEGylated liposomes in hemophilia A patients with inhibitors to factor VIII. J Thromb Haemost 2009;7(2):PP-WE-608
96. Roberts AW. G-CSF: a key regulator of neutrophil production, but that's not all! Growth Factors 2005;23(1):33-41
97. Anderlini P, Korbling M. The use of mobilized peripheral blood stem cells from normal donors for allografting. Stem Cells 1997;15(1):9-17
98. Jansen J, Hanks S, Thompson JM, et al. Transplantation of hematopoietic stem cells from the peripheral blood. J Cell Mol Med 2005;9(1):37-50
99. Layton JE, Hockman H, Sheridan WP, et al. Evidence for a novel in vivo control mechanism of granulopoiesis: mature cell-related control of a regulatory growth factor. Blood 1989;74(4):1303-7
100. Smith TJ, Khatcheressian J, Lyman GH, et al. 2006 update of recommendations for the use of white blood cell growth factors: an evidence-based clinical practice guideline. J Clin Oncol 2006;24(19):3187-205
101. Anderlini P, Przepiorka D, Seong C, et al. Factors affecting mobilization of CD34+ cells in normal donors treated with filgrastim. Transfusion 1997;37(5):507-12
102. Holm M. Not all healthy donors mobilize hematopoietic progenitor cells sufficiently after G-CSF administration to allow for subsequent CD34 purification of the leukapheresis product. J Hematother 1998;7(2):111-3
103. Tricot G, Jagannath S, Vesole D, et al. Peripheral blood stem cell transplants for multiple myeloma: identification of favorable variables for rapid engraftment in 225 patients. Blood 1995;85(2):588-96
104. Stiff P, Gingrich R, Luger S, et al. A randomized phase 2 study of PBPC mobilization by stem cell factor and filgrastim in heavily pretreated patients with Hodgkin's disease or non-Hodgkin's lymphoma. Bone Marrow Transplant 2000;26(5):471-81
105. Constantinescu I, Levin E, Gyongyossy-Issa M. Liposomes and blood cells: a flow cytometric study. Artif Cells Blood Substit Immobil Biotechnol 2003;31(4):395-424
106. Ahmad SS, Scandura JM, Walsh PN. Structural and functional characterization of platelet receptor-mediated factor VIII binding. J Biol Chem 2000;275(17):13071-81
107. Dahlback B. Blood coagulation and its regulation by anticoagulant pathways: genetic pathogenesis of bleeding and thrombotic diseases. J Intern Med 2005;257(3):209-23

- **A general review on coagulation that details the importance of platelets in the formation of coagulation protein complexes.**

108. Hedner U. Mechanism of action of factor VIIa in the treatment of coagulopathies. Semin Thromb Hemost 2006;32:(Suppl 1):77-85

- **An article describing the importance of platelets in the function of FVIIa.**

109. High KA. The leak stops here: platelets as delivery vehicles for coagulation factors. J Clin Invest 2006;116(7):1840-2
110. Raut S, Di Giambattista M, Bevan SA, et al. Modification of factor VIII in therapeutic concentrates after virus inactivation by solvent-detergent and pasteurisation. Thromb Haemost 1998;80(4):624-31

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