European Journal of Pharmaceutical Sciences 66 (2015) 157–162

Contents lists available at ScienceDirect

European Journal of Pharmaceutical Sciences

journal homepage: www.elsevier.com/locate/ejps

Intravenous administration of Factor VIII–O-Phospho-L-Serine (OPLS) complex reduces immunogenicity and preserves pharmacokinetics of the therapeutic protein

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ARTICLE INFO

Article history:
Received 10 June 2014
Received in revised form 28 August 2014
Accepted 10 October 2014
Available online 20 October 2014

Keywords:
Factor VIII
O-Phospho-L-Serine
Pharmacokinetics
Immunogenicity
T cell
Dendritic cell

ABSTRACT

Hemophilia A is a bleeding disorder caused by the deficiency of an important coagulation factor; Factor VIII (FVIII). Replacement therapy using exogenously administered recombinant FVIII is the most commonly used method of treatment. However, approximately 30% of Hemophilia A patients develop neutralizing antibodies (Nabs) against the recombinant protein. Nabs abolish FVIII activity and drastically influence efficacy of the protein. The immunogenic epitopes of FVIII reside predominantly in the C2 domain of FVIII. However, the C2 domain also contains a lipid binding region. O-Phospho-L-Serine (OPLS) which is the head-group moiety of phosphatidylserine, interacts with the lipid binding region of FVIII. Previous studies have shown that FVIII complexed with OPLS lowered Nab development against FVIII following subcutaneous administration. In dendritic cell–T-cell co-culture studies, OPLS treatment increased the secretion of immunosuppressive cytokines (Transforming Growth Factor-β and Interleukin-10), and simultaneously decreased pro-inflammatory IL-17 cytokine. Here, we investigated FVIII immune response and pharmacokinetics upon intravenous administration of FVIII–OPLS complex. We studied the effect of FVIII–OPLS complex on the interaction between a professional antigen presenting cell; dendritic cell and T-cell, and T-cell clonal expansion. Pharmacokinetics parameters were estimated following intravenous administration of FVIII and FVIII–OPLS. The results suggest that OPLS lowers FVIII immune response following intravenous administration. OPLS also hinders FVIII-specific T-cell clonal proliferation and preserves FVIII PK profile. Thus, the ease of protein-lipid complexation, preservation of FVIII activity and *in vivo* behavior, and improved *in vitro* FVIII stability, makes OPLS an attractive excipient in the preparation of next generation or biosimilar FVIII products with improved safety profile.

1. Introduction

Factor VIII is a multi-domain protein (Vehar et al., 1984) consisting of six domains viz. NH2-A1-A2-B-A3-C1-C2-COOH. It is an important co-factor in the blood clotting cascade (Foster and Zimmerman, 1989). Deficiency or lack of an active form of FVIII leads to a debilitating bleeding disorder called Hemophilia A. Although, recombinant human Factor VIII (FVIII) is commercially available as the option of choice to treat hemophiliacs, the protein is inflicted with several drawbacks including protein aggregation, short circulation half-life and adverse immune response. All these issues render the protein less efficacious and negatively impact patients' quality of life. The C2 domain of FVIII is critical for the protein *in vivo* activity (Pratt et al., 1999). Upon release into the systemic circulation, FVIII rapidly associates with its carrier protein called von Willebrand factor (vWF) Wise et al., 1991. Association with vWF prolongs FVIII plasma survival by protecting FVIII from degradation by circulating enzymes (Fay et al., 1991). Additionally, vWF also prevents FVIII endocytosis by dendritic cells (DC) Dasgupta et al., 2007. Studies have shown that regions within the C2 domain are involved in FVIII–vWF interaction (Saenko et al., 1994). FVIII clearance is mediated by low-density lipoprotein receptor related protein (LRP) Schwarz et al., 2000; Saenko et al., 1999, and C2 domain is involved in the interaction with LRP

Abbreviations: FVIII, Factor VIII; HA, Hemophilia A; OPLS, O-Phospho-L-Serine; PChg, Phosphocholine chloride calcium salt tetrahydrate; vWF, von Willebrand factor; APC, antigen presenting cell; DC, dendritic cell; I.V., intravenous; Nab, neutralizing antibody; PK, pharmacokinetics; aPTT, activated partial thromboplastin time; TGF-β, Transforming Growth Factor-beta; IL, Interleukin.

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http://dx.doi.org/10.1016/j.ejps.2014.10.010
0928-0987/

(Lenting et al., 1999). Additionally, it has been recognized that T-lymphocytes are essential for FVIII immune response (Reding et al., 2001; Reding et al., 1999). Upon interaction with professional antigen-presenting cells such as dendritic cells (DCs) that present immunogenic epitopes on their surface, activated antigen-specific T-cells subsequently interact with and activate B-cells which further differentiate into anti-drug antibody secreting plasma cells (Chirino et al., 2004; Reipert et al., 2010; Pratt and Thompson, 2009; Dasgupta et al., 2008; De Groot and Scott, 2007; Wu et al., 2001; Banchereau and Steinman, 1998). It was observed that most of the T-cell immune epitopes reside within the C2 domain of FVIII (Reding et al., 2003). Thus, the C2 domain of FVIII is not only involved in the protein activity, but also involved in FVIII aggregation (Ramani et al., 2005), clearance and immunogenicity (Meeks et al., 2007).

Hence, a clinical benefit would be with development of next generation FVIII replacement therapy that can overcome these aforementioned issues. Interestingly, the C2 domain also comprised of a lipid binding region (LBR). Phosphatidylserine; an endogenously present anionic lipid binds to the LBR via its headgroup O-Phospho-L-Serine (OPLS) Purohit and Balasubramanian, 2006; Gilbert and Drinkwater, 1993. Previous studies have investigated the utility of OPLS as an excipient in FVIII preparations. The results indicate that OPLS improved the physical stability to FVIII following FVIII–OPLS complexation, thereby reducing subcutaneously administered FVIII immune response in naïve HA mice (Miclea et al., 2007; Purohit et al., 2005). Moreover, co-culture of FVIII-specific splenic $CD4^+$ T-cells with DC pre-exposed to FVIII–OPLS resulted in the secretion of immunosuppressive Transforming Growth Factor (TGF)-β and Interleukin (IL)-10 cytokines and concomitant decrease in IL-17 pro-inflammatory cytokine level (Gaitonde et al., 2011). Additional studies were carried out to further our understanding of the beneficial effects of OPLS in FVIII therapy. Currently FVIII is administered via the intravenous (I.V.) route in the clinic. Therefore, here we investigated the effect of FVIII–OPLS on FVIII immune response following I.V. administration. As it is important to know the effect of OPLS on T-cells, we also studied OPLS effect on FVIII-specific $CD4^+$ T-cell clonal expansion *in vitro*. It is imperative that OPLS complexation do not cause any detrimental effect on FVIII pharmacokinetics (PK). Hence, we studied the effect of OPLS complexation on FVIII PK in naïve HA mice following I.V. administration.

The results indicate that FVIII–OPLS reduce FVIII immune response possibly due to its effect on reducing FVIII-specific T-cell activation and proliferation, as well as, due to the previously reported secretion of immunoregulatory cytokines in the cell-culture micro-environment. Additionally, complexation with OPLS maintained FVIII PK and retained FVIII *in vivo* activity. Thus, OPLS could be utilized as a next-generation excipient in FVIII preparations to minimize the undesired drawbacks associated with FVIII and improve FVIII therapy.

2. Materials and methods

Excipient-free, full-length, recombinant human Factor VIII was a generous gift from the Western New York Hemophilia Foundation. O-Phospho-L-Serine (OPLS) and Phosphocholine chloride calcium salt tetrahydrate (PChg) was purchased from Sigma Aldrich (St. Louis, MO). Sterile water for injection, sterile syringes, needles, isoflurane and other surgical items were purchased from Henry Schein Inc. (Melville, NY). Sterile 0.22 μm syringe filters were procured from Millipore (Billerica, MA). Buffer salts were purchased from Fischer Scientific (Fair Lawn, NJ). Sterile cell strainers, petri dishes, NUNC maxisorb ELISA plates and tissue culture microwell plates were purchased from VWR Inc. (Bridgeport, NJ). aPTT reagents were obtained from T-coag Ireland Ltd. (Ireland). ESH8 standard antibody was purchased from American Diagnostica Inc. (Stamford, CT) and Goat anti-mouse IgG detection antibody was purchased from Southern Biotech (Birmingham, AL). PNPP substrate reagent kit was purchased from KPL Inc. (Gaithersburg, MD). Recombinant murine Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) was purchased from Peprotech Inc. (Rocky Hill, NJ). Sterile cell culture media, sterile distilled water and Dynabeads $CD4^+$ negative isolation kit were obtained from Invitrogen Inc. (Carlsbad, CA). Sterile, heat-inactivated fetal bovine serum was obtained from Lonza Ltd. (Switzerland). Chromogenix Coamatic FVIII activity determination kit was purchased from DiaPharma Group Inc., (West Chester, OH).

2.1. Animals

C57BL/6J strain of mice with target deletion of Exon 16 of the f8 gene (HA mice) was used for the studies. All animal handling and experiments were performed according to an approved protocol by the Institutional Animal Care and Use Committee (IACUC) of University at Buffalo, NY.

2.2.1. Preparation of OPLS or Phosphocholine solution

Tris buffer was prepared using sterile water for injection. Solubility of OPLS (mol.wt. 185.07) and PChg (mol.wt. 329.73) in water is 50 mg/mL and 100 mg/mL respectively. 10 mM OPLS or Phosphocholine chloride calcium salt tetrahydrate (henceforth referred to as OPLS/PChg) solution was prepared by adding appropriately weighed quantity of OPLS/PChg to 10 mL of Tris buffer. pH was adjusted to 7.0 and the resulting solution was sterile filtered through 0.22 μm syringe filter under aseptic conditions.

2.2.2. Preparation of FVIII–OPLS/PChg complexes

Required quantity of FVIII was added to 10 mM OPLS/PChg solution and complexed by incubating for 30 min at room temperature as previously described (Purohit et al., 2005). This incubation condition is found to be optimal for complexation.

2.2.3. Endotoxin test

The prepared complexes were analyzed for endotoxin level by using Endosafe® Endochrome-K endotoxin assay kit (Charles River Inc., MA). Any endotoxin, if present, was below the detection limit of the assay kit. The samples were subsequently used for further studies.

2.3. FVIII or FVIII–OPLS/PChg immune response

Naïve HA male mice were used for the study. The animals were divided into three groups with 6 animals in each group and were administered four consecutive once-a-week I.V. doses of FVIII or FVIII–OPLS/PChg (2 μg FVIII /animal /injection) via the penile vein. During the course of study, one animal in the FVIII group died for unknown reason. On the sixth week, all remaining animals were sacrificed and blood was collected by cardiac puncture in 10% acid citrate dextrose (ACD) anti-coagulant buffer. Plasma was isolated by centrifugation of blood samples at 5000 g at 4 °C for 5 min. The plasma samples were stored at −80 °C until further analysis.

2.4. Anti-FVIII Nab titer determination

Level of neutralizing antibodies (Nabs) against FVIII was determined by activated partial thromboplastin time (aPTT) FVIII residual activity assay following Nijmegen's modification (Verbruggen et al., 1995). Plasma samples were serially diluted using FVIII-deficient human plasma. The resulting diluted samples were incubated with normal pooled human plasma in 1:1 ratio and