Imlifidase, a new option to optimize the management of patients with hemophilia A on emicizumab

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Title. IdeS, a new option to optimize the management of patients with hemophilia A on emicizumab

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Running head. Removal of emicizumab along with FVIII inhibitors by IdeS in hemophilia A

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Essentials section

- Acute bleed management is complicated in FVIII inhibitor-positive PwHA on emicizumab prophylaxis
- IdeS was tested in HA mice passively immunized with human anti-FVIII IgG and treated with emicizumab
- Removal of neutralizing anti-FVIII IgG by IdeS were not impaired by the presence of emicizumab
- IdeS should ease acute bleeds management in inhibitor-positive PwHA on emicizumab prophylaxis

Abstract

Background. Emicizumab is a bispecific chimeric humanized IgG4 that mimics the procoagulant activity of factor VIII (FVIII). Its long half-life and subcutaneous injection route have been life-changing in treating patients with hemophilia A (PwHA) with/without FVIII inhibitors. However, emicizumab only partially mimics FVIII activity; it prevents but does not treat acute bleeds. Emergency management is particularly complicated in patients with FVIII inhibitors on emicizumab prophylaxis, wherein exogenous FVIII is inefficient. We have shown recently that Imlifidase, a bacterial IgG-degrading enzyme (IdeS), efficiently eliminates human anti-FVIII IgG in a mouse model of severe HA with inhibitors and opens a therapeutic window for the administration of therapeutic FVIII.

Objectives. To investigate the impact of IdeS treatment in inhibitor-positive HA mice injected with emicizumab.

Methods. IdeS was injected to HA mice passively immunized with human neutralizing anti-FVIII IgG and treated with emicizumab.

Results. IdeS hydrolyzed emicizumab *in vitro* and *in vivo*, albeit at slower rates than another recombinant human monoclonal IgG4. While $F(ab')_2$ fragments were rapidly cleared from the circulation, thus leading to a rapid loss of emicizumab procoagulant activity, low amounts of single-cleaved intermediate IgG persisted for several days. Moreover, the IdeS-mediated elimination of the neutralizing anti-FVIII IgG and restoration of the hemostatic efficacy of exogenous FVIII were not impaired by the presence of emicizumab and polyclonal human IgG in inhibitor-positive HA mice.

Conclusions. Our results suggest that IdeS could be administered to inhibitor-positive PwHA under emicizumab prophylaxis to improve and ease the management of breakthrough bleeds or programmed major surgeries.

Keywords

Hemophilia A, factor VIII, Emicizumab, factor VIII inhibitors, IgG-degrading enzyme

Introduction

The treatment of hemophilia A (HA) by administration of exogenous factor VIII (FVIII) is complicated by the elevated cost, immunogenicity, and need for frequent intravenous injections. Alternatives to therapeutic FVIII have been developed in recent years to mimic the procoagulant activity of FVIII and enhance thrombin generation. Among these, emicizumab is an engineered humanized monoclonal IgG4 that bridges activated factor IX (FIXa) with its substrate [1], factor X (FX), and reduces the bleeding tendency in HA patients with and without anti-FVIII inhibitory antibodies [2,3]. However, emicizumab only partially mimics FVIII activity and requires concomitant treatment with FVIII or bypassing agents (BPAs) in case of breakthrough bleeds and surgeries. The use of rFVIIa has minimized the risk of thrombotic events, including thrombotic microangiopathy, initially described in PwHA with inhibitors concomitantly treated with activated prothrombin complex concentrates (aPCC) and emicizumab [4]. Yet, the management of such patients remains complex [6]. We recently documented the efficacy of Imlifidase, the IgG-degrading enzyme (IdeS) produced by *Streptococcus pyogenes* [7], in eliminating human neutralizing anti-FVIII IgG and opening a therapeutic window for FVIII replacement therapy in a mouse model of severe HA [8]. Here, we investigated the effect of IdeS on the concomitant elimination of emicizumab and FVIII inhibitors.

Results and Discussion

We first validated that IdeS, which hydrolyzes all human IgG subclasses, cleaves emicizumab *in vitro*. As shown by SDS-PAGE (**Figure 1A**), IdeS hydrolyzed emicizumab in a time and dose-dependent manner. At a 10-fold molar excess of IdeS, emicizumab was completely hydrolyzed within 30 min, with the disappearance of the band corresponding to intact IgG and the appearance of major bands migrating at 100 kDa and 25 kDa, corresponding to the digested

$F(ab')_2$ and Fc fragments, respectively. IdeS cleaves IgG in two steps, a first fast cleavage (k_{cat} 10 s^{-1}) of one heavy chain followed by the slower hydrolysis (k_{cat} 0.1 s^{-1}) of the second heavy chain [9]. At a 1:0.1 IgG:IdeS molar ratio, single-cleaved intermediate IgG (scIgG, ~140 kDa, wherein only one heavy chain is proteolyzed) was detected even after incubation for 24 hours, indicating incomplete emicizumab hydrolysis. Accordingly, detecting the intact IgG4 or scIgG4 in a sandwich ELISA showed complete emicizumab hydrolysis at 6 hours only for IgG:IdeS molar ratios ≥1 (**Figure 1B**).

We recently showed that IdeS cleaves the human monoclonal anti-FVIII IgG, BO2C11, both in its IgG1 and IgG4 subclass formats [8]. Comparison of the proteolysis kinetics of emicizumab and BO2C11 IgG4 by IdeS (IgG:IdeS molar ratio of 12:1) revealed a slower digestion of emicizumab. Indeed, at 5 min, only about half of emicizumab was digested into scIgG, while most of BO2C11 was hydrolyzed (**Figure 1C**). Similarly, traces of emicizumab scIgG were still detected after 24 hours, while BO2C11 scIgG became undetectable after 1 hour. scIgG exhibit reduced binding to complement and Fcγ receptors and reduced engagement of complement and antibody-dependent cellular cytotoxicity or phagocytosis [10,11]. scIgG, however, retain essential properties of intact IgG. These include the ability to bind to their cognate antigen(s) and to the neonatal Fc receptor (FcRn), which endows them with a half-life of 3 weeks in humans and 9 days in mice.

Several point mutations have been introduced in the CH1, hinge, CH2 and CH3 domains of both emicizumab heavy chains. The introduction of the mutations improves its stabilization and downstream processing, reduces C-terminal heterogeneity and fosters heterodimerization of the heavy chains (**Figure 1D**) [12,13]. The introduced amino-acid changes could account for the reduced cleavage efficacy of emicizumab by IdeS. IdeS was recently shown to interact with the hinge regions and several loops of the CH2 domains of both heavy chains of a human monoclonal IgG1 [14]. Interestingly, the two mutations located in the hinge (S228P) and CH2

domains (F296Y) of emicizumab revert the corresponding IgG4 sequence to that of an IgG1, suggesting that they are not responsible for the lower cleavage efficacy of emicizumab by IdeS. Alternatively, the mutations introduced in the CH3 domain may affect the Fc regions of emicizumab involved in the interaction with IdeS. Indeed, IgG molecules exhibit long-distance structural coupling between their domains, and amino-acid replacements may induce structural/electrostatic modifications at distance from the mutated residue [15–19]. For instance, the introduction of mutations in the Fc portion at a distance from the FcRn contact surface may improve the binding affinity between the two molecules [20,21]. Conversely, a single mutation in the variable region of the broadly neutralizing antibody NIH45-46 increased by three folds the affinity of the IgG for FcRn [22]. We cannot rule out however the possibility that the nature of the Fab fragments plays a role in determining whether an IgG is a suitable substrate for IdeS. Indeed, therapeutic monoclonal antibodies that differ only in the sequence of their Fab domains and, hence, recognize different cognate antigens, but share identical Fc fragments, demonstrated different sensitivities to IdeS proteolysis *in vitro* [23].

We then investigated the elimination kinetics of emicizumab by IdeS in a mouse model of severe HA. FVIII-deficient mice were first injected intravenously with different doses of emicizumab, and the plasma concentration of emicizumab was followed for up to 24 hours (**Figure 2A**). Irrespective of the dose administered, the circulating concentration of emicizumab stabilized 6 hours following injection (**Figure 2B**). The concentration of emicizumab was almost linearly correlated with the injected dose, both 5 min and 24 hours after injection (**Figure 2C**), as reported [24]. Thus, a dose of 3 mg/kg yielded a plasma concentration of 32.4±10.2 µg/ml 6 hours later, which is in the same order of magnitude as trough levels measured in PwHA dosed once-weekly with 1.5 mg/kg (i.e., 45 µg/ml) [4].

Next, HA mice were treated with PBS or with one or two injections of equimolar or 10-fold molar excess IdeS within the first 9 hours that followed the injection of 3 mg/kg emicizumab

(**Figure 3A**). Treatment with IdeS of mice injected with emicizumab 6 hours or 6 and 9 hours earlier resulted in the hydrolysis of emicizumab (**Figure 3B**). Cleaved Fc fragments were detectable as early as 3 hours following the first IdeS treatment. $F(ab')_2$ fragments were systematically undetectable within 18 hours of IdeS treatment, irrespective of the dose or number of IdeS administrations. This suggests a faster clearance of emicizumab $F(ab')_2$ fragments compared to the typical ~12-hour long half-life of human $F(ab')_2$ fragments in mice [25,26]. In stark contrast, scIgG, that retain binding capacity to FcRn and, hence, an unmodified *in vivo* half-life [10], were still detected at 72 hours (**Figure 3B**). Whether the rat-mouse hybrid nature of the VH and VL domains of emicizumab [27], that are chimerized with a human IgG4 heavy chain or kappa light chain, explains such a rapid clearance of emicizumab $F(ab')_2$ fragments is not clear. Alternatively, we cannot completely exclude the presence of low-affinity cross-reactivity of the anti-FIX and/or anti-FX variable regions of emicizumab with endogenous murine FIX or FX, which might accelerate the catabolism of the $F(ab')_2$ fragments. Plasma levels of intact IgG or scIgG were further quantified by ELISA at different time points. The levels of emicizumab were reduced by 70.5% 72 hours after a set of 2 injections of equimolar amounts of IdeS (i.e., 63 hours after the second injection) (**Figure 3C**). The decrease was enhanced when a 10-fold excess of IdeS was injected once or twice (by 86.9% and 90.3%, respectively), indicating that a 10-fold molar excess of the enzyme compensates for the lesser susceptibility of emicizumab to IdeS-mediated hydrolysis. Interestingly, 3 hours after the first injection of IdeS, the circulating levels of total IgG or scIgG were reduced by 27.2%, 69.7% and 71.0% (**Figure 3C**) when IdeS was injected twice at an equimolar IdeS:IgG ratio, once at a 10-fold molar IdeS:IgG ratio or twice at a 10-fold molar ratio, respectively.

Circulating levels of emicizumab $F(ab')_2$ fragments showed a rapid decrease within 18 hours after IdeS injection (**Figure 3D**). The decrease culminated when IdeS was injected a second time at a 10-fold molar excess over the concentration of emicizumab. Our ELISA setup does

not discriminate between cleaved $F(ab')_2$ fragments and scIgG. However, the absence of $F(ab')_2$ fragments shown in Western blot from the 24-hour time point onwards (**Figure 3B**), together with the steady signal detected by ELISA over this time period (**Figure 3D**), indicate that the emicizumab isoform that remains is the scIgG. The estimated procoagulant activity of emicizumab confirmed the almost complete elimination of the functionally active molecule (**Figure 3E**): the reduction in emicizumab procoagulant activity ranged between 23.4% and 44.2% after a first injection of IdeS, depending on the amounts of IdeS injected, and peaked at 88.1% when IdeS was injected twice in 10-fold molar excess. Our data also show that a double injection of a 10-fold excess of IdeS reduced the circulating concentration of emicizumab from 35.4±3.9 µg/ml to 10.1±1.3 µg/ml in 3 hours and to 3.6±0.7 µg/ml over 21 hours (**Figure 3C**). At these concentrations, emicizumab still shortens the activated partial thromboplastin clotting time, at least *in vitro* in human plasma (data not shown).

We have recently reported that IdeS efficiently eliminates inhibitory anti-FVIII IgG in a mouse model of inhibitor-positive severe HA [25]. In contrast to emicizumab, the elimination of the $F(ab')_2$ fragments of the hydrolyzed anti-FVIII IgG required 12 to 72 hours to reach a low titer (<5 BU/ml), depending on the starting inhibitory titer [25]. To further mimic the conditions in PwHA with inhibitors on emicizumab, we studied the kinetics of elimination of emicizumab by IdeS *in vivo*, in the presence of anti-FVIII IgG and/or of physiological levels of polyclonal human IgG. HA mice were injected with emicizumab and a pool of human neutralizing monoclonal anti-FVIII IgG1, alone or in combination with polyclonal human IgG (IVIg). Mice were then treated twice with a 10-fold molar excess of IdeS over emicizumab, or with PBS (**Figure 4A**). IdeS injection resulted in an 84% and 97% decrease in total IgG levels within 96 hours, in the absence or presence of IVIg, respectively (**Figure 4B**). The improved catabolism of IgG in the presence of IVIg reflects the increased proteolytic efficiency of IdeS at non-limiting substrate concentrations. IdeS efficiently removed neutralizing anti-FVIII IgG,

irrespective of the presence of IVIg, as depicted by the complete FVIII recovery performed 96 hours after IdeS injection (**Figure 4C**). In parallel, in the absence of IVIg, the procoagulant activity of emicizumab diminished by 84% 96 hours after IdeS injection (**Figure 4D**). Of note, the presence of IVIg accelerated the spontaneous catabolism of emicizumab, which is compatible with a competition between IVIg and emicizumab for binding to the recycling FcRn [28]. Treatment with IdeS, however, increased the kinetics of emicizumab clearance in the presence of IVIg. Taken together, these observations indicate that IdeS efficiently eliminates FVIII inhibitors, as well as emicizumab, even in the presence of close to physiological circulating IgG levels. We also confirmed that IdeS does not interfere with the standard coagulation assays, including aPTT, PT, fibrinogen, one- or two-stage FVIII:C assays, and emicizumab concentration measurements *in vitro* in human plasma spiked with increasing doses of IdeS (data not shown).

The present data highlight the potential of IdeS in inhibitor-positive PwHA on emicizumab prophylaxis. In the latter patients, IdeS would remove both emicizumab and FVIII inhibitors, thus restoring the therapeutic efficacy of exogenous FVIII in the context of programmed surgeries with high hemorrhagic risks or breakthrough bleeds in patients unresponsive to rFVIIa. Importantly, the rapid clearance of IdeS (half-life of 4.9±2.8 hours in humans) [29] should allow to quickly resume emicizumab treatment and reach hemostatic potency in the post-surgical period or as soon as the severe acute bleeds have been resolved. A potential limitation of IdeS treatment could be the reported immunogenicity of the molecule in humans, which would preclude its repeated administration [29]. Whether preexisting or induced anti-IdeS antibodies impair IdeS proteolytic efficacy remains to be deciphered, however. At term, the use of IdeS should alleviate the need for bypassing agents to treat major bleeds in inhibitor-positive patients, thus facilitating the management of inhibitor-positive PwHA and monitoring of hemostasis.

Authors' contributions.

MBJ, SD, ClDe, ODC, CVD, PJL, VP and SLD designed the research;

MBJ, AM, SD, IP, LC and VD performed experiments;

MBJ, SD, IP, ClDe, ODC, CVD, PJL, VP and SLD analyzed the results and made the figures;

MBJ, JDD, VP and SLD wrote the paper.

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Conflict of interest. SLD and JDD are inventors on patent EP18305971.6 related to the use of IdeS in the context of AAV-mediated gene therapy. Other authors declare no competing financial interests.

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Legends to Figures

Figure 1. *In vitro* cleavage of emicizumab by IdeS. Panel A. Emicizumab was incubated with IdeS at different IgG:IdeS molar ratios (1:0, 1:0.01, 1:0.1, 1:1, 1:10) for 30 minutes, 6 hours, and 24 hours at 37°C in phosphate saline buffer (PBS). Samples (1.2 µg/lane) were separated by 4-12% SDS-PAGE under non-reducing conditions. Molecular weight markers and the predicted molecular weights of intact IgG, scIgG, $F(ab')_2$ and Fc fragments are shown on the left and right of the gel, respectively. **Panel B.** The levels of intact and/or scIgG4 were determined in samples obtained after 6 hours of incubation with different IgG:IdeS molar ratios by ELISA. Plates were coated with a goat anti-human Ig kappa chain antibody (Southern Biotech), blocked with PBS-3% bovine serum albumin (BSA), and the Fc was recognized using an HRP-labeled mouse monoclonal antibody specific for human Fcγ (Southern Biotech) and the 3,3',5,5'-Tetramethylbenzidine (TMB) substrate. Results are expressed in arbitrary units (AU) from optical density measured at 450 nm. **Panel C.** Kinetics of cleavage of emicizumab and of a monoclonal $IgG4_k$ (BO2C11) by IdeS (12 IgG:1 IdeS molar ratios) were compared over an incubation time of 24 hours at 37°C. Migration profiles (3 µg/lane) were analyzed by 4-12% non-reducing SDS-PAGE. **Panel D.** Clustal alignment of the CH1 (black), hinge (bold), CH2 (orange) and CH3 (blue) domains of the heavy chains of a human IgG4, of the two heavy chains of emicizumab (HCFIXa and HCFX) and of a human IgG1. Mutations introduced in HCFIXa and HCFX are highlighted: S228P (purple) stabilizes the core hinge region of the IgG4; K196Q, F296Y, E356K and H435R (yellow) reduce undesirable bio-analytical and bioprocessing behaviors; R409K (green) avoids Fab-arm exchange; L445P/G446>del/K447>del (cyan) reduce IgG4 C-terminal heterogeneity; E356K and K438E (bold underlined) allow heterodimerization of the heavy chains.

Figure 2. Half-life of emicizumab in FVIII-deficient mice. Panel A. C57BL/6 exon 16 FVIII-deficient mice (n=4-11) were injected with emicizumab at different doses (3.0, 6.0, 9.0, and 12.5 mg/kg). **Panel B.** Levels of emicizumab (µg/ml) in mouse plasma were measured over time using an ELISA. Plates were coated with a goat anti-human Ig kappa chain antibody (Southern Biotech), blocked with 3% PBS-BSA and Fc-containing emicizumab fragments revealed with an HRP-labeled mouse monoclonal antibody specific for human Fcɣ (Southern Biotech) and the TMB substrate. **Panel C.** Correlation of the plasma levels of emicizumab measured 5 min and 24 hours post-injection with the dose of emicizumab injected to the mice.

Figure 3. *In vivo* elimination of emicizumab upon hydrolysis by IdeS in FVIII-deficient mice. Panel A. C57BL/6 FVIII-deficient mice (n=4-5/group) were injected with emicizumab (3.0 mg/kg) and treated with IdeS (0.7 mg/kg or 7 mg/kg) at the indicated molar ratios, or with PBS, once after 6 hr or twice after 6 and 9 hours. Blood was collected 5 minutes, 6, 9, 24, 48 and 72 hours following emicizumab injection. **Panel B.** The presence of emicizumab IgG, scIgG, $F(ab')_2$ fragments and Fc fragments was detected in mouse plasma by Western blot. Plasma (12 µl of 1/5 diluted plasma) was loaded on a 4-12% non-reducing SDS-PAGE. Proteins were transferred onto nitrocellulose membranes. IgG, scIgG and $F(ab')_2$ fragments, or Fc fragments, were revealed using a polyclonal goat anti-human $F(ab')_2$ fragment-specific antibody (Invitrogen) or a polyclonal rabbit anti-human Fcɣ-specific antibody (Sigma-Aldrich), respectively. Bound antibodies were revealed using an HRP-coupled rabbit anti-goat IgG (R&D System) or an HRP-coupled goat anti-rabbit IgG (Cell signaling) and the Pierce™ ECL Western Blotting Substrate and iBright™ FL1000 Imaging System (Thermo Scientific). **Panel C.** The levels of intact IgG and/or scIgG in mouse plasma were measured over time in an ELISA using a goat anti-human Ig kappa chain antibody (Southern Biotech) as a capture antibody, an HRP-labeled mouse monoclonal antibody specific for human Fcɣ (Southern Biotech) as detection

antibody a PBS-3% BSA blocking buffer (mean±SD). **Panel D.** The levels of scIgG and/or F(ab')$_2$ fragments in mouse plasma were measured over time in an ELISA using a goat anti-human Ig kappa chain antibody (Southern Biotech) as a capture antibody, and an HRP-labeled goat anti-human IgG F(ab')$_2$ fragment secondary antibody (Thermo Scientific) as detection antibody, diluted in PBS-3% BSA blocking buffer (mean±SD). Data are representative of two independent experiments. **Panel E.** The estimated procoagulant activity of emicizumab in mouse plasma was measured using a FVIII:C chromogenic assay with human FIXa and FX (Biophen FVIII:C, Hyphen Biomed). The data (mean±SD) represent the % of procoagulant activity of emicizumab as a function of the activity measured in plasma just before the first injection of IdeS (at 6 hours: T6). Statistical significance was assessed using a 2-way ANOVA with Geisser-Greenhouse correction on the 9 to 72-hour time points (*: $P<0.05$; ***: $P<0.001$; ****: $P<0.0001$; ns: not significant).

Figure 4. Efficacy of therapeutic FVIII in IdeS-treated inhibitor-positive HA mice on emicizumab. Panel A. C57BL/6 FVIII-deficient mice (6 mice per group) were injected with emicizumab (3.0 mg/kg) and a pool of four anti-FVIII IgG [8] (3170 BU/kg) in the presence or absence of human polyclonal IgG (IVIg, 390 mg/kg). Mice were then treated with two doses of 10 molar excess IdeS over emicizumab, or with PBS at 6 and 9 hours. Blood was collected 5 minutes, 6, 9, 24 and 96 hours following emicizumab injection and 2 hours after the injection of 200 IU/kg therapeutic FVIII (Helixate®, Bayer). **Panel B.** The levels of intact IgG and/or scIgG in mouse plasma that received or not IVIg were measured over time in a sandwich ELISA using a goat anti-human Ig kappa chain antibody (Southern Biotech) as a capture antibody, an HRP-labeled mouse monoclonal antibody specific for human Fcγ (Southern Biotech) as detection antibody (mean±SD). **Panel C.** FVIII (200 IU/kg) was injected to mice at 96 hours. Residual FVIII:C was measured in plasma in a chromogenic assay with bovine FIXa and FX 2

hours later (FVIII:C Siemens). In the graph, the horizontal bars represent means±SD and each symbol depicts an individual mouse. The grey area represents the FVIII levels (mean±SD) measured in FVIII-treated HA mice without FVIII inhibitors. Statistical differences were assessed using the non-parametric Kruskal-Wallis test. **Panel D.** The estimated procoagulant activity of emicizumab in mouse plasma was measured using a FVIII:C chromogenic assay with human FIXa and FX (Biophen FVIII:C, Hyphen Biomed). The graph (mean±SD) depicts the % of procoagulant activity of emicizumab as a function of the activity measured in plasma just before the first injection of IdeS (T6). Statistical significance was assessed using the 2-way ANOVA with Geisser-Greenhouse correction (GraphPad Prism 9).