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Immunogenicity Lessons Learned from the Clinical Development of Vatreptacog Alfa, A Recombinant Activated Factor VII Analog, in Hemophilia with Inhibitors

Kasper Lamberth, Karin Nana Weldingh, Silke Ehrenforth, Mette Ribel Chéhadé, and Henrik Østergaard

Abstract Hemophilia A is a rare bleeding disorder characterized by defective blood clotting due to diminished levels or absence of coagulation Factor VIII (FVIII). The preferred treatment option is FVIII replacement therapy. However, in 20–30% of the patients neutralizing (inhibitory) anti-FVIII antibodies develop rendering patients dependent on other treatment modalities such as the bypassing agent recombinant factor VIIa (rFVIIa). rFVIIa has a 20-year safety track record with no reports of immunogenicity in congenital hemophilia patients with inhibitors. To improve treatment efficacy of rFVIIa, the recombinant analog vatreptacog alpha was developed by Novo Nordisk A/S and taken into clinical development in 2006. Despite differing from rFVIIa by only three amino acid substitutions, results from the phase III trial demonstrated that some patients developed anti-drug antibodies. In this chapter, we give an introduction to hemophilia with focus on rFVIIa and the development of vatreptacog alfa. In addition, we summarize the findings from the clinical trials and characterization of the identified anti-drug antibodies. Finally, we show how various immunogenicity prediction tools have been used to investigate the immunogenicity risk of vatreptacog alfa leading to the identification of a potential new T-cell epitope that could contribute to the observed immunogenicity of the compound in humans.

Keywords Antidrug antibodies, FVIIa, Hemophilia, Immunogenicity, Inhibitors, Prediction, T-cell epitope, Vatreptacog alfa

K. Lamberth (✉), K.N. Weldingh, M.R. Chéhadé, and H. Østergaard
Novo Nordisk, Copenhagen, Denmark
e-mail: kplm@novonordisk.com

S. Ehrenforth
Novo Nordisk, Zurich, Switzerland

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1 Introduction

Vatreptacog alfa (Novo Nordisk, Bagsværd, Denmark) and BAY 86-6150 (Bayer, Leverkusen, Germany) were both recently in clinical development as bioengineered coagulation proteins designed to address the need for new treatment options in hemophilia with inhibitors [1–4]. These novel variants of activated coagulation factor VII (FVIIa) represent the vanguard of over a dozen bioengineered therapeutic coagulation proteins currently (or recently) in development [5–8]. Both vatreptacog alfa and BAY 86-6150 were shown to be safe and well tolerated in early clinical studies [2, 4]. However, development of both agents was discontinued when antidrug antibodies (ADAs) were identified in some treated patients during clinical trials [1, 9].

The vatreptacog alfa and BAY 86-6150 stories offer valuable lessons about the potential immunogenicity risks associated with bioengineered, sequence-modified proteins. These bioengineered analogs both triggered ADA development despite very modest changes in the primary sequence: vatreptacog alfa had three amino acid substitutions [1, 3], while BAY 86-6150 carried six substitutions [4, 7]. This

chapter presents a detailed discussion of the vatreptacog alfa case, which also offers a unique and valuable insight into the tools and methods used to predict the immunogenicity of protein therapeutics.

2 What Is Hemophilia?

Hemophilia is a congenital, X-linked bleeding disorder caused by deficiency or absence of specific coagulation factors [10–13]. The two forms of the disease are hemophilia A (deficiency or lack of coagulation factor VIII [FVIII]) and hemophilia B (deficiency or lack of coagulation factor IX [FIX]) [11, 12, 14]. The clotting factor deficiencies in hemophilia A and B result from a variety of mutations in the respective clotting factor genes [10, 13].

Hemophilia occurs in approximately 1 in 5,000 (hemophilia A) and 1 in 30,000–50,000 (hemophilia B) live male births [10, 12, 14]. Both hemophilia A and B are characterized by a lifelong bleeding tendency [13, 14]; the severity of this bleeding phenotype generally corresponds to residual clotting factor levels, such that severe disease is defined by FVIII or FIX levels of $\leq$1% [12–14]. Patients with severe hemophilia A or B suffer spontaneous bleeds into joints and muscles without any identifiable trauma or injury [13, 14]. If these bleeds are inadequately treated, over time they produce chronic pain, joint destruction, and disability [11, 14].

Standard treatment for hemophilia A and B involves infusion of the missing or deficient clotting factor [5, 13]. FVIII or FIX concentrates used in replacement therapy can be derived from fractionated human plasma or developed through recombinant DNA technology and can be administered on demand when a bleed occurs or as prophylaxis to prevent bleeding [5, 11]. Prophylaxis is the preferred clinical management strategy in developed countries.

3 Immune Responses in Hemophilia

Autoimmune responses to endogenous coagulation proteins are very rare, occurring in approximately one per million individuals per year [15, 16], and result in acquired hemophilia. However, as FVIII and FIX are biotherapeutic proteins, they trigger an immune response characterized by antidrug antibodies (ADAs) much more frequently when used therapeutically in hemophilia patients who lack the endogenous counterparts [17, 18]. Development of ADAs is a T cell-dependent process [17–21] resulting from a lack of central T-cell tolerance. Tolerance to self-proteins is a vital part of immune system development [18, 20, 22]; proteins (antigens) encountered later in life are usually recognized as foreign and may elicit an immune response [18] culminating in antibody production. In hemophilia A and B, mutations in the underlying FVIII and FIX genes, respectively, mean that the resulting factor protein is either nonfunctional, incomplete, or missing altogether.

As a result, tolerance to the complete, functional factor protein cannot be established during immunological development. When factor proteins are administered therapeutically, part of the protein molecule may therefore be recognized as foreign and may trigger an immune response [17, 18, 22]. Certain types of mutation in the FVIII and FIX genes are associated with a greater risk of inhibitor development [23, 24]. Even with small mutations in the FVIII or FIX gene, infused factor protein may trigger antibody development as one or more portions of the protein sequence will be foreign to the immune system [18].

ADAs do not always have a clinical manifestation, and such binding antibodies toward FVIII have been reported in healthy subjects as well as hemophilia patients who are well treated [25–27]. In some cases, however, they neutralize the target binding and thus the clinical efficacy of the infused protein [25, 26]. Clinically relevant neutralizing ADAs are known as inhibitors.

3.1 Inhibitor Development

The development of inhibitors against FVIII or FIX is the most serious and challenging treatment-related complication of hemophilia [2, 12, 13, 27–30]. Inhibitors are immunoglobulin G (IgG) antibodies that neutralize the infused clotting factor, rendering standard replacement therapy with FVIII or FIX ineffective [13, 29, 31].

The development of inhibitors in hemophilia depends on a complex interaction of numerous patient-related, clinical, and product-related factors [12, 28, 32]. In addition to the type of causative FVIII/FIX mutation, patient-related factors include polymorphisms of immune response genes, such as interleukin (IL)-10, tumor necrosis factor-alfa (TNFα), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), and major histocompatibility complex class II (MHC-II) – which is referred to as human leukocyte antigen (HLA) class II in humans [12]. The binding of peptides to MHC-II molecules on the surface of antigen-presenting cells in the immune system is a key factor in generating an immune response [20, 33]. The patient's HLA class II genotype governs which peptides can bind to HLA class II molecules and therefore influences the development of ADAs (including inhibitors) [34]. Clinical factors that may affect inhibitor development include, but are not limited to, the mode and route of administration, the length and type of exposure to product, and the indication for which the product is administered (e.g., surgery, severe bleeds) [12]. Potential product-related impact factors include product formulation, the presence and type of posttranslational modifications, level of similarity to endogenous protein, and the presence of T-cell epitopes.

4 Recombinant Activated Factor VII

FVIII- and FIX-bypassing agents are the only treatment options for hemophilia with high-titer inhibitors [35]. Recombinant FVIIa (rFVIIa; NovoSeven®; Novo Nordisk, Bagsværd, Denmark) is one of only two bypassing agents currently available and is the only recombinant one [35].

4.1 Rationale Behind rFVIIa Development

Hemostasis is the process of blood clot formation at the site of vessel injury. According to the cell-based model of hemostasis, three integrated phases are required to generate sufficient thrombin to form a fibrin clot [31, 36]. The initiation phase begins when endothelial damage allows tissue factor (TF) on the surface of TF-bearing cells to be exposed to the bloodstream. TF then binds and activates FVII, which facilitates the generation of a small amount of thrombin. In the amplification phase, thrombin activates platelets, leading to subsequent activation of FV, FVIII, and FXI on platelet surfaces. During the propagation phase, the formation of FVIIIa–FIXa (tenase) complexes activates FX and drives a "burst" of thrombin generation on activated platelets [36]. This leads to cleavage of fibrinogen to fibrin, yielding a stable, cross-linked fibrin clot.

The absence of either FVIII or FIX prevents assembly of FVIIIa–FIXa complexes on activated platelets, which prevents both FX activation and a full thrombin burst [31, 36]. The end result is a fibrin clot that is unstable and easily dissolved. Although most hemophilia patients can be treated with replacement FVIII or FIX products, patients with inhibitors require therapies that bypass the need for these factors. High-dose rFVIIa accomplishes this via TF-independent activation of FX on activated platelets in the absence of FVIIIa–FIXa complexes. This restores the thrombin burst and allows the formation of a stable clot [36].

4.2 rFVIIa in Hemophilia

rFVIIa has been available since 1996 in Europe and since 1999 in the USA and Canada. It has an amino acid sequence identical to that of endogenous FVIIa and has provided high levels of hemostatic efficacy and safety during clinical use in hemophilia patients with inhibitors for more than 20 years [37]. Hemostasis following treatment with rFVIIa 90 μg/kg has been rated effective after a mean of 2.2 injections in 92% (566/614) of bleeds [38], and successful outcomes were reported in patients with joint bleeds who injected single 270 μg/kg doses [39–41]. Data from hemophilia registries report that rFVIIa can be safely used at home and support the efficacy and safety of rFVIIa doses >200 μg/kg [42]. To date, there

have been no confirmed cases of neutralizing antibodies (inhibitors) against rFVIIa in congenital hemophilia patients [43].

Both currently available bypassing agents used to treat inhibitor patients are perceived to be less effective than regular factor replacement therapy in non-inhibitor patients [35]. As a result attempts have been made to develop improved rFVIIa variants with enhanced intrinsic procoagulant activity; one of these variants is vatreptacog alfa [3, 44].

5 rFVIIa Analog Vatreptacog Alfa

Novo Nordisk developed vatreptacog alfa to provide a bypassing agent that offered more reliable and sustained resolution of acute bleeds in hemophilia patients with inhibitors [1, 2]. It is a genetically engineered variant of rFVIIa produced in a Chinese hamster ovary cell line cultured in a serum-free medium [1–3]. No raw materials or excipients of human or animal origin were used in its production.

5.1 Structure of Vatreptacog Alfa

Vatreptacog alfa has 99% amino acid identity to native FVIIa [1]. It is composed of a light and heavy chain connected by a disulfide bond (Fig. 1). The light chain includes one membrane-interactive domain and two epidermal growth factor-like domains; these domains are the same as in rFVIIa in order to maintain the clinically beneficial properties of rFVIIa [3]. The heavy chain comprises a single serine protease domain that is structurally similar to rFVIIa with the exception of three amino acid substitutions (V158D, E296V, and M298Q) [1–3]. These amino acid substitutions allow the N-terminal to dock efficiently and stabilize FVIIa in its active conformation without the need for TF [3, 45] and increase the molecule's TF-independent activity.

5.2 Functional Characterization of Vatreptacog Alfa

The procoagulant activity of vatreptacog alfa has been verified in various in vitro [46–48], ex vivo [49–52], and in vivo [53, 54] mice models of hemophilia. Together, these studies demonstrated that vatreptacog alfa provides hemostasis at lower concentrations than rFVIIa and that clot formation is faster and stronger, with increased stability against fibrinolytic degradation.

In a cell-based model of hemophilia, vatreptacog alfa generated 30-fold higher FXa levels than similar concentrations of rFVIIa [46]. It also resulted in four- to tenfold higher maximal thrombin generation rates than equal rFVIIa concentrations. Another cell-based model found that normalization of maximum thrombin

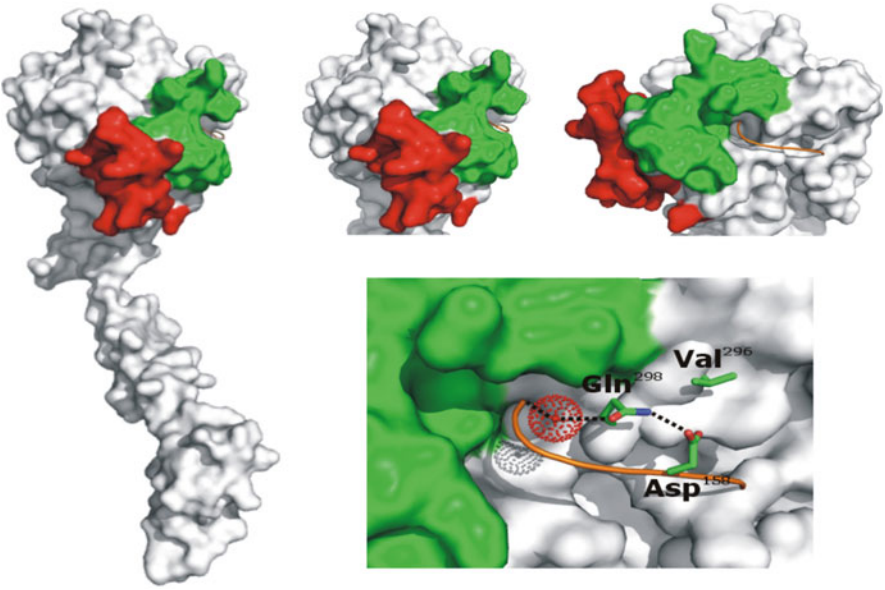

Fig. 1 The structure of vatreptacog alfa, showing the sites of the three amino acid substitutions *Left*: the entire molecule, with the protease domain at the *top*. The green area represents the activation domain, and the red area represents the tissue factor (TF)-interactive surface. *Top middle*: The protease domain in the same orientation as in the main figure to the *left*. *Top right*: The protease domain has been rotated 90° relative to the middle structure to clearly show the N-terminal tail (*orange*). *Bottom right*: Close-up of the region of the three amino acid substitutions. V158D is located in the tail, while E296V and M298Q are on the same β strand close to the activation domain. The *dotted lines* indicate hydrogen bonds that connect the N-terminus, a water molecule (*dotted red sphere*), M298Q and V158D. This hydrogen bond network does not exist in FVII. Reproduced from Persson et al. [3]

generation in severe hemophilia required 500 nM rFVIIa but only 25 nM vatreptacog alfa, while moderate/mild hemophilia required 25–100 nM rFVIIa but only 5 nM vatreptacog alfa [47]. Importantly, the generation of excess thrombin was confined to areas of damaged epithelium where hemostasis was required [48].

In assays using plasma from hemophilia patients, vatreptacog alfa shortened the clotting onset and increased the maximum rate of fibrin formation and fibrin network density in plasma clots at a concentration significantly lower than rFVIIa [52]. Vatreptacog alfa also resulted in a more pronounced, less variable hemostatic effect in blood samples from patients with varying degrees of hemophilia, producing a normalization of clotting parameters equivalent to values obtained in subjects with normal coagulation [50, 51]. In addition, increasing concentrations of vatreptacog alfa normalized platelet function, clot structure, and thrombin generation consistently in blood obtained from hemophilia patients with or without inhibitors [49].

The greater and faster thrombin burst for vatreptacog alfa relative to rFVIIa reported in the in vitro and ex vivo studies described above translated into greater efficacy and faster hemostatic effect in vivo. In murine models of hemophilia A,

vatreptacog alfa significantly shortened bleeding times and decreased blood loss in tail bleeding experiments, with a potency three to four times that of rFVIIa [53]. Vatreptacog alfa also showed greater efficacy and faster bleeding resolution than FVIII, rFVIIa, and pd-aPCC in a severe tail bleeding model [54]. No evidence of adverse events with vatreptacog alfa was found in these studies [53, 54].

5.3 *Mechanism of Action of Vatreptacog Alfa*

Since vatreptacog alfa and rFVIIa have the same membrane-binding domain and TF-interacting regions [3], the mechanism they employ to activate FX is the same, i.e., a TF-independent reaction localized to the platelet surface that bypasses the need for the FVIIIa–FIXa complex. The increased potency of vatreptacog alfa relative to rFVIIa can be explained by differing equilibria between the inactive and active conformations of each of the molecules: vatreptacog alfa exists predominantly in the active form, whereas rFVIIa exists predominantly in the inactive form [3]. As a result, a larger proportion of vatreptacog alfa molecules than rFVIIa molecules are active at any given time, resulting in an increased rate of vatreptacog alfa-mediated FX activation [55]. In addition, vatreptacog alfa binds to more sites on the surface of activated platelets than FVIIa, as assessed by flow cytometry (Fig. 2) [3, 36]. Together, these findings explain the larger and more rapid thrombin burst seen with vatreptacog alfa compared with rFVIIa (Fig. 3) [55].

Vatreptacog alfa, like rFVIIa, cannot directly activate platelets [3]. Its thrombin-generating effects require the presence of platelets that have already been activated and are therefore restricted to the injury site. As there is no reason to suspect that systemic thrombin generation occurs, there is consequently no reason to suspect an increased risk of thromboembolic events.

5.4 *Early Immunogenicity Risk Assessment*

Early assessment of the potential immunogenicity risk of vatreptacog alfa took place in the preclinical research phase using early in silico tools and in vivo animal models. At that time, in silico immunogenicity prediction tools that analyze peptide binding to MHC-II indicated a risk for generating a new potential T-cell epitope. However, these tools were still in their infancy and were not considered reliable. In rats neonatally tolerized to FVII, no difference in tolerance breakage was observed after administration of vatreptacog alfa compared with rFVIIa [56]. Furthermore, in immunization studies with rFVIIa and vatreptacog alfa in transgenic mice engineered to express human FVII, no significant difference in breaking of tolerance was found between the two molecules (unpublished data). Consequently, no difference in immunogenicity risk was found between wild-type FVIIa and vatreptacog alfa in these early animal studies.

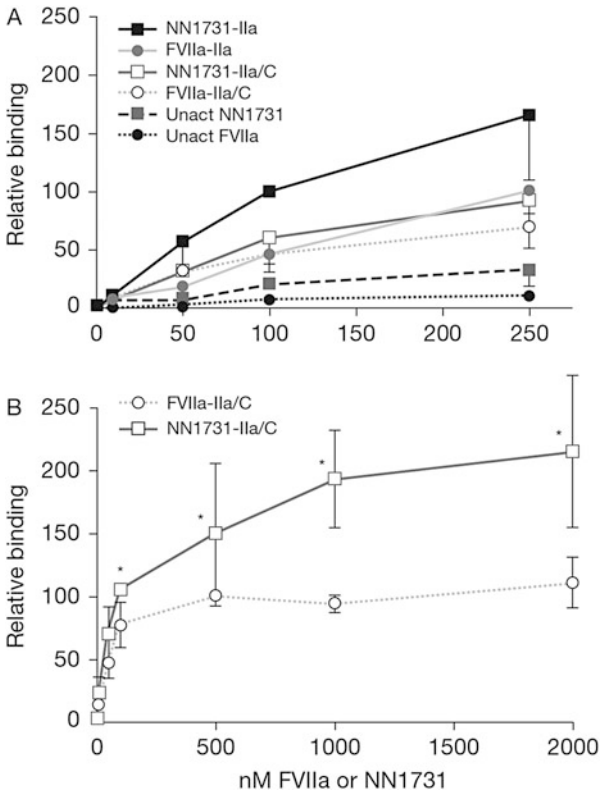

Fig. 2 Platelet binding of vatreptacog alfa (NN1731) and FVIIa. Unactivated (unact) platelets or platelets activated with thrombin (IIa) or thrombin + convulxin (IIa/C) were incubated with the indicated concentration of FVIIa or vatreptacog alfa and analyzed by flow cytometry. Binding was normalized to the binding of vatreptacog alfa at 100 nM in each experiment (=100%). *Upper panel*: Data shown are means and SD of data from four separate experiments on platelets from different donors. Binding of vatreptacog alfa to thrombin-activated platelets was slightly higher than for FVIIa ($p < 0.05$ by paired *t*-test at 250 nM), but binding of vatreptacog alfa to platelets activated by thrombin + convulxin was markedly higher than for FVIIa ($p < 0.01$ by paired *t*-test at 100 and 250 nM). *Lower panel*: Binding was characterized up to a concentration of 2 μM FVIIa or vatreptacog alfa on platelets activated by thrombin + convulxin. Data are from four experiments and were different from the experiments shown in the upper panel. Maximal binding of vatreptacog alfa to thrombin + convulxin-activated platelets was significantly greater than the maximal binding of FVIIa; therefore, vatreptacog alfa appears to bind to a greater number of sites per platelet. Binding of both molecules exhibited an apparent Kd of ~100 nM. *$p < 0.05$ by paired *t*-test. Reproduced from Hoffman et al. [97]

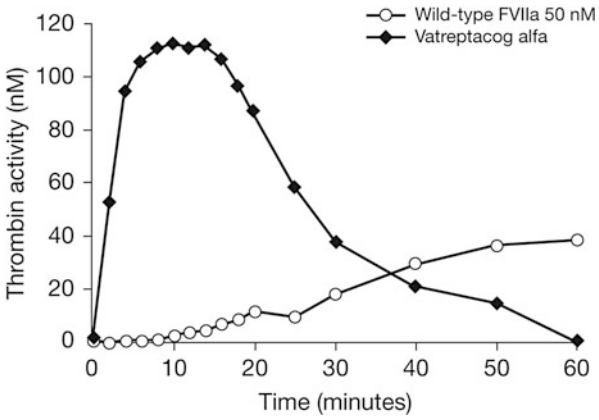

Fig. 3 Thrombin generation induced by vatreptacog alfa and other rFVIIa variants. Platelets were activated with the thrombin receptor agonist peptide SFLLRN, and then 50 nM of wild-type FVIIa (*open circle*) and vatreptacog alfa (*filled diamond*) were added along with protein mixture and calcium. Aliquots were removed and analyzed for thrombin amidolytic activity. Data shown are representative of three separate experiments. Reproduced from Persson et al. [55] Copyright (2001) National Academy of Sciences, USA

6 Clinical Trials with Vatreptacog Alfa: Design and Results

6.1 First Human Dose Trial [45]

The first clinical trial with vatreptacog alfa assessed the safety and pharmacokinetics (PK) of single doses in healthy male subjects. The trial had a randomized, double-blind, placebo-controlled, parallel-group, dose-escalation design; patients received vatreptacog alfa 5, 10, 20, and 30 μg/kg or placebo, with eight patients in each treatment group ($n = 32$). Progression to the next dose level depended on a blinded evaluation of safety data and PK.

Single doses of vatreptacog alfa $\leq$30 μg/kg were well tolerated. No serious or severe adverse events were reported, and there were no thromboembolic events. Three subjects (one in the placebo group) had a total of three five mild or moderate adverse events: headache [3], nausea, and vomiting. There were no clinically significant abnormal reports in any treatment group for laboratory safety values, vital signs, electrocardiogram, or physical examinations, and there were no local injection-site reactions. Based on FVIIa activity, vatreptacog alfa had a rapid initial distribution with a half-life of ~20 min, followed by a less rapid terminal phase with a half-life of ~3.1 h (Fig. 4). The total ($AUC_{0–24}$) and maximum (C_{5min}) exposure to vatreptacog alfa increased proportionally with dose. In addition, vatreptacog alfa was shown to be pharmacologically active based on coagulation-regulated parameters, suggesting the potential for a rapid induction of events leading to hemostasis and bleeding cessation.

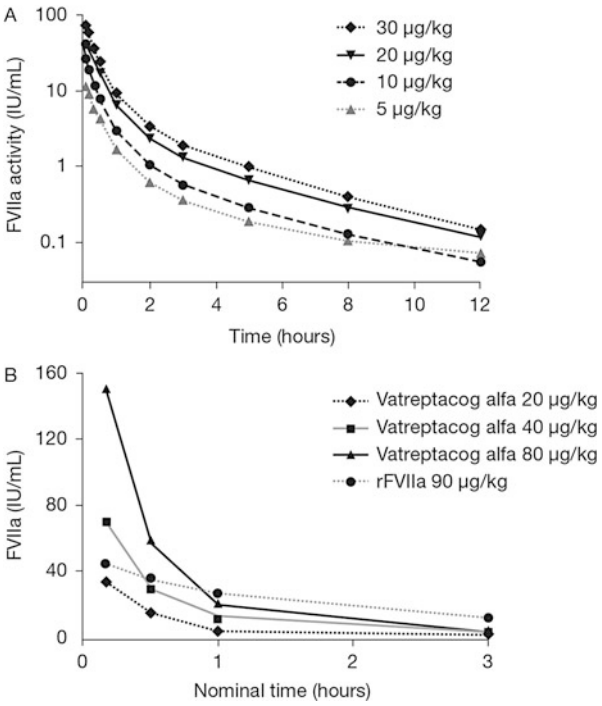

Fig. 4 Pharmacokinetic profiles (FVIIa activity) of vatreptacog alfa (**a**) mean single dose PK of vatreptacog alfa (5, 10, 20 or 30 µg/kg) in first human dose trial. Reproduced from Møss et al. [45]; (**b**) mean single dose PK of vatreptacog alfa (20, 40, or 80 µg/kg) and rFVIIa (90 µg/kg) in phase 2 trial. Reproduced from de Paula et al. [2]

Importantly, no antibodies to vatreptacog alfa were detected up to 3 months after a single dose in any of the vatreptacog alfa dose groups.

6.2 Phase 2 Dose-Escalation Trial (Adept™1) [2]

The phase 2, randomized, double-blind, active-controlled adept™1 trial evaluated the safety and preliminary efficacy of five escalating doses of vatreptacog alfa for the treatment of joint bleeds in hemophilia patients with inhibitors. Patients with at least two joint bleeds in the previous 6 months ($n = 51$) were randomized (4:1) to receive 1–3 doses per joint bleed (up to a maximum of five bleeds) of either vatreptacog alfa 5, 10, 20, 40, or 80 µg/kg or rFVIIa 90 µg/kg. The primary endpoint was frequency of adverse events; secondary endpoints included evaluations of immunogenicity, PK, and efficacy defined as the number of bleeds successfully controlled with a single treatment dose.

Results showed that vatreptacog alfa was well tolerated in the adept™1 trial, with a low frequency of adverse events in all dose groups. There were 15 serious adverse events (12 in the vatreptacog alfa groups) and one thrombotic event that were considered by investigators to be unrelated to treatment. The three adverse events considered to be related to vatreptacog alfa were mild. Laboratory

parameters revealed no safety concerns. The mean single-dose PK profiles of FVIIa activity versus time for vatreptacog alfa showed an exponential decline in activity following infusion (Fig. 4). The peak activity of vatreptacog alfa 80 μg/kg was three- to fourfold higher than that with rFVIIa 90 μg/kg, and vatreptacog alfa clearance was approximately three times faster than rFVIIa clearance. At 1 h post-dose, the mean plasma activities of all doses of vatreptacog alfa were below the level obtained following rFVIIa treatment.

A total of 95 joint bleeds were included in the analysis, 76 were treated with vatreptacog alfa and 19 with rFVIIa; 86 of 95 were controlled with 1–3 treatment doses. In a combined analysis of the vatreptacog alfa 20–80 μg/kg dose groups, 98% (41/42) of bleeds were controlled successfully with 1–3 doses, compared with 90% of bleeds treated with rFVIIa. The number of doses needed to control bleeding decreased with increasing dose of vatreptacog alfa, with 40% of bleeds effectively treated with a single 80 μg/kg dose.

The mean total number of vatreptacog alfa doses received during the trial was 3.6 and ranged from 1 to 15. Only one patient had >3 exposure days (EDs) to vatreptacog alfa. No antibody development was detected in any patients exposed to vatreptacog alfa in the trial for up to 28 days after the last treatment.

6.3 Phase 3 Adept™2 Trial [1]

The aim of the phase 3 adept™2 trial was to assess the efficacy and confirm the safety of vatreptacog alfa for treatment of bleeds in hemophilic patients with inhibitors. The randomized, double-blind, active-controlled, crossover trial enrolled 72 patients who had experienced at least five bleeds requiring treatment in the previous 12 months. Each bleeding episode was randomized to treatment with 1–3 doses of either vatreptacog alfa 80 μg/kg or rFVIIa 90 μg/kg, with 300 bleeds planned to be treated with vatreptacog alfa and 200 with rFVIIa. It was estimated that ≥15 patients had to have ≥10 days of exposure to vatreptacog alfa to sufficiently evaluate the potential risk of ADA development. The primary efficacy endpoint was effective bleeding control, and the main safety endpoint was immunogenicity.

Of 567 bleeds reported in the trial, 340 were treated with vatreptacog alfa. Both vatreptacog alfa and rFVIIa showed 93% efficacy in controlling bleeding with 1–3 doses at 12 h, including joint, mucocutaneous, muscle, soft tissue, and other bleeds. The mean number of doses administered to control bleeding within 9 h was significantly lower for vatreptacog alfa (2.42 doses) than for rFVIIa (2.52 doses; $p = 0.0304$).

Although the trial confirmed the efficacy of vatreptacog alfa, ADAs were detected in 8/72 patients (11%). The ADAs developed after <10 vatreptacog alfa EDs in seven of the eight patients and after 14–28 EDs in the remaining patient. This strong immunogenicity safety signal (discussed in detail in Sect. 7) was not

seen in previous clinical trials with vatreptacog alfa. Given the potential risks associated with ADAs, clinical development of vatreptacog alfa was discontinued.

7 Antidrug Antibody Development Observed in the Phase 3 Trial

In the phase 3 adept™2 trial, binding antibodies against vatreptacog alfa were detected in 8/72 (11%) exposed patients (Table 1). No antibody-positive patients had any concomitant medical conditions that would predispose to antibody development, and there were no differences in baseline characteristics between antibody-positive and antibody-negative patients [1]. Furthermore, when seven of the eight patients with anti-vatreptacog alfa antibodies were investigated by DNA analysis, none were found to have polymorphisms or mutations in the FVII gene (versus wild-type FVII), thus eliminating mutation in the endogenous protein as a causative factor underlying inhibitor development.

Four of the eight patients with ADAs against vatreptacog alfa, the ADAs developed low-titer cross-reactivity against rFVIIa; for one of these patients, the ADA also had in vitro neutralizing activity against vatreptacog alfa in a single blood sample taken at day 250 after first exposure [1]. There were no clinical manifestations of ADAs in any patient, including the patient who developed neutralizing antibodies, and all patients responded well to treatment with vatreptacog alfa and/or rFVIIa. Furthermore, all patients tested negative for neutralizing activity against endogenous FVIIa at all visits [1].

The immunogenicity findings were unexpected for two main reasons. First, vatreptacog alfa has 99% identity with rFVIIa, differing only by three amino acid substitutions, and rFVIIa is associated with a low risk of immunogenicity [1, 43]. Second, no immunogenic safety signals were detected in the animal immunogenicity studies or in previous clinical studies [1, 2, 45, 56]. Even so, most patients in the earlier phase 2 trial (adept™1) were only exposed to vatreptacog alfa once [2]; and consequently the overall exposure was markedly higher in the phase 3 adept™2 trial, though still limited to $\leq$10 EDs for most patients [1]. The longer exposure to vatreptacog alfa in adept™2 (1–28 EDs) versus adept™1 (1–5 EDs), together with the greater number of patients exposed in adept™2 ($n = 67$ versus $n = 46$ in adept™1), may explain why an immunogenicity signal was not apparent in the earlier trial [1].

Table 1 Overview of ADA development in the phase 3 adept™2 trial [1, 57]

Patient	Binding ADAs		Neutralizing ADAs		Total EDs in trial		Vatreptacog alfa EDs before ADA development[b]	Peak anti-vatreptacog alfa ADA titer
	Vatreptacog alfa	rFVIIa	Vatreptacog alfa	FVIIa	Vatreptacog alfa	rFVIIa[a]		
A	Positive	Negative	Negative	Negative	11[c]	3	1–3[c]	16
B	Positive	Negative	Negative	Negative	5	3	1–5	4
C	Positive	Negative	Negative	Negative	4	1	4	4
D	Positive	Negative	Negative	Negative	4	2	1–4	1
E	Positive	Positive	Negative	Negative	11	4	4–8	256
F	Positive	Positive	Negative	Negative	7	3	1–4	256
G	Positive	Positive	Negative	Negative	28	16	14–28	64
H	Positive	Positive	Positive	Negative	12[c]	5	5–8[c]	64

ADA antidrug antibody, *EDs* exposure days, *FVIIa* activated factor VII, *rFVIIa* recombinant activated factor VII

[a]Some patients were exposed to rFVIIa before the adept™2 trial and therefore had a higher number of cumulative EDs

[b]Number of vatreptacog alfa EDs before ADA development is presented, a range is given if there was no antibody assessment between EDs

[c]Patients A and H were previously exposed to vatreptacog alfa in the phase 2 trial and therefore had a higher number of cumulative EDs

7.1 Characteristics and Clinical Course of Anti-vatreptacog Alfa Antibodies

7.1.1 Assessment of ADAs

Blood samples for ADA testing were taken before first drug exposure, before dosing at every scheduled visit during the trial (i.e., at least every 3 months), and at least 1 month after last trial product administration [1].

To assess the presence of binding ADAs against vatreptacog alfa and rFVIIa, all blood samples were incubated with ^{125}I-labeled vatreptacog alfa or rFVIIa and analyzed in two validated radioimmunoassays (RIA) including antibody confirmation assays [1]. All samples that were confirmed positive for vatreptacog alfa- or rFVIIa-binding ADAs were further analyzed for neutralizing activity in two in vitro clotting assays that measured neutralization of vatreptacog alfa or endogenous human FVII including FVIIa [1]. To detect anti-vatreptacog alfa-neutralizing activity, vatreptacog alfa was added to all pretrial and trial blood samples; clot formation was initiated by adding soluble truncated recombinant TF, and time to clot formation was measured. Samples were analyzed in parallel for neutralizing activity against endogenous FVII by initiating clot formation with full-length TF, which activates endogenous FVII to FVIIa. Assay sensitivity was ~160 ng/mL and 1,500–3,000 ng/mL antibody for the binding and neutralizing assays, respectively, and all assays had a false-positive rate of 0.1% [1, 57].

Antibody specificity was analyzed in patients with rFVIIa cross-reactive antibodies. For this analysis, blood samples were incubated with an excess of the unlabeled antigen of interest in a competition RIA [1]. Antibody binding to radioactively labeled rFVIIa was competed with unlabeled vatreptacog alfa, while labeled vatreptacog alfa competed for binding with unlabeled single-mutation analogs [57]. A significant reduction of antibody binding in the competition assay indicated a shared antibody epitope between either rFVIIa and vatreptacog alfa or the single-mutation analog and vatreptacog alfa [57].

7.1.2 Characterization of ADAs

The development of ADAs over time for each of the eight ADA-positive patients is shown in Fig. 5. All but one of these patients (88%) developed ADAs to vatreptacog alfa after 1–8 EDs, while the remaining patient first tested positive for ADAs between 14 and 28 EDs (Table 1) [57]. Peak antibody titers ranged from 1 to 256 (Table 1) [1, 57]. There was no further increase in ADA titer following additional vatreptacog alfa exposure in two patients (patients A and E), while two further patients showed an increase in ADA levels following additional exposure (patients F and H), suggesting that the maximum antibody titer had not yet been reached [57].

The four patients with the highest anti-vatreptacog alfa ADA titers (patients E–H) also developed cross-reactivity to rFVIIa (Table 1) [1, 57]. All anti-rFVIIa

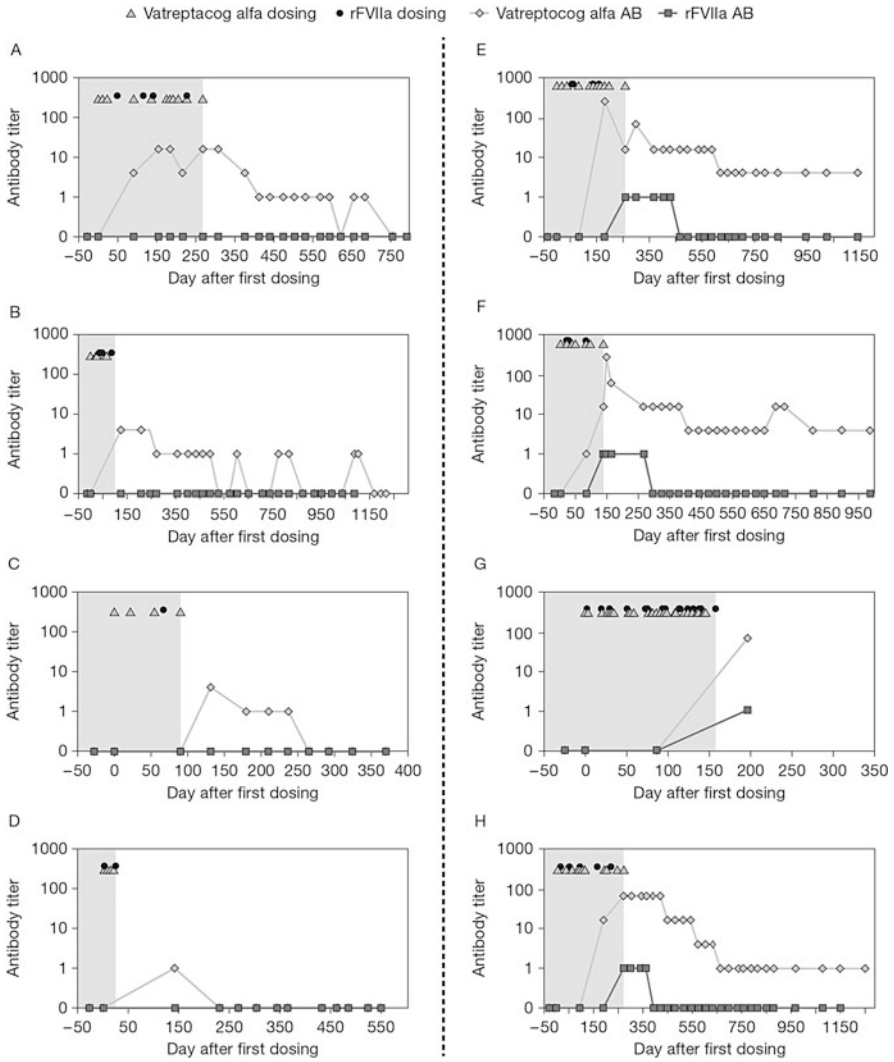

Fig. 5 The time course of ADA development in patients from the phase 3 adept™2 trial. (**a**–**d**) ADA development over time in four patients who did not develop rFVIIa cross-reactive antibodies. (**e**–**h**) ADA development over time in four patients who developed rFVIIa cross-reactive antibodies. Antibody titers for vatreptacog alfa and rFVIIa are shown during the trial dosing period (*gray-shaded area*) and during the post-dosing follow-up period (*white-unshaded area*). Treatment days on which patients received vatreptacog alfa and rFVIIa are also shown. rFVIIa, recombinant activated factor VII. Reproduced from Mahlangu et al. [57] with permission from John Wiley and Sons

antibody titers were low and developed later than anti-vatreptacog alfa antibodies, coinciding with the last vatreptacog alfa treatment in three patients (patients E, F, and H) and developing 1 month after the last vatreptacog alfa exposure in the fourth

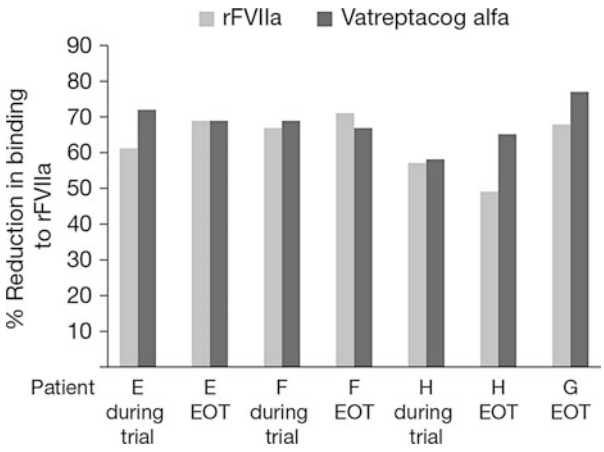

Fig. 6 Reduction of antibody binding to rFVIIa in a competition assay with vatreptacog alfa and rFVIIa in four patients with rFVIIa cross-reactive antibodies rFVIIa binding was competed with an excess of either unlabeled vatreptacog alfa or rFVIIa. The percentage reduction in binding to radiolabeled rFVIIa is presented. EOT, end of trial; rFVIIa, recombinant activated factor VII. Reproduced from Mahlangu et al. [57] with permission from John Wiley and Sons

(patient G). One reexposure to vatreptacog alfa after development of anti-rFVIIa ADAs in patients E, F, and H did not lead to an increase in rFVIIa antibody titer [57]. In all four patients with cross-reactive ADAs, the antibodies were reactive to structures shared between vatreptacog alfa and rFVIIa; there was no subpopulation of antibodies that were specific only to rFVIIa (Fig. 6) [57].

In vitro neutralizing activity against vatreptacog alfa was detected in one patient (patient H) in a single blood sample taken at day 250 after first vatreptacog alfa exposure [1, 57]. This sample neutralized 40% of the in vitro activity of 0.53 ng vatreptacog alfa present in the antibody assay which corresponds to neutralization of 0.2 ng vatreptacog alfa. The cut point for a positive neutralization result in this assay was 29% corresponding to neutralization of 0.15 ng vatreptacog alfa. However, all subsequent samples from this patient were negative for vatreptacog alfa-neutralizing antibodies [57].

Vatreptacog alfa ADAs were of the IgG subtype in seven of the eight patients, and isotyping results were inconclusive in the remaining patient (patient G) [57]. In patients F and H, the antibody maturation profile was consistent with the typical immune maturation pattern displayed by IgG antibodies: increased titer, cross-reactivity, and one sample of neutralizing in vitro activity. As binding ADAs can precede the development of neutralizing ADAs [26], it is possible that any further exposure to vatreptacog alfa in these patients may have triggered the development of high-titer antibodies that neutralized rFVIIa or endogenous FVIIa [57].

After completion of adept™2, 7/8 (88%) ADA-positive patients entered an ongoing follow-up study to monitor the course of the ADAs [57]. Blood samples are collected and tested on a monthly basis. Patients are followed up until two consecutive samples test negative for ADAs, or until further follow-up is deemed

unnecessary by the study sponsor or investigators. Anti-vatreptacog alfa ADA titer declined in all patients following discontinuation of vatreptacog alfa treatment (Fig. 5). At the time of writing, the ADA-positive patients have been followed for 3 years. Three of the seven patients are now negative for anti-vatreptacog alfa ADAs. One patient (patient B) has been fluctuating between being antibody negative and positive (titer 1). Cross-reactivity to rFVIIa disappeared in all three tested patients (E, F, and H) at 124, 156, and 208 days, respectively, after last vatreptacog alfa exposure, despite reexposure to FVII according to local practice [57]. One patient (patient G) did not participate in the follow-up trial, and so his ADA status remains unknown.

7.1.3 Pharmacokinetics of ADAs

To determine the potential impact of anti-vatreptacog alfa ADAs, PK profiles were assessed up to 8 h post-dose in four ADA-positive patients (patients A, E, F, and H). All PK measurements were taken after a single intravenous injection of vatreptacog alfa given to the patients in a non-bleeding state [57].

There was no indication of reduced recovery of FVIIa activity 10 min post-dose in any of the ADA-positive patients tested (Fig. 7). The reason for this lack of effect on in vivo recovery is not clear, but it could be attributable to the high sensitivity of the neutralizing antibody assay: a neutralization of 0.2 ng vatreptacog alfa may be masked at the activity levels evident in the patients tested (e.g., the post-dose FVIIa activity levels in patient H corresponded to a concentration of 596–253 ng/mL) [57].

Somewhat surprisingly, all four ADA-positive patients in whom PK was investigated showed prolonged elimination of vatreptacog alfa when compared with the mean PK profile of ADA-negative patients from the earlier phase 2 trial (adept™1 [2]) [57].

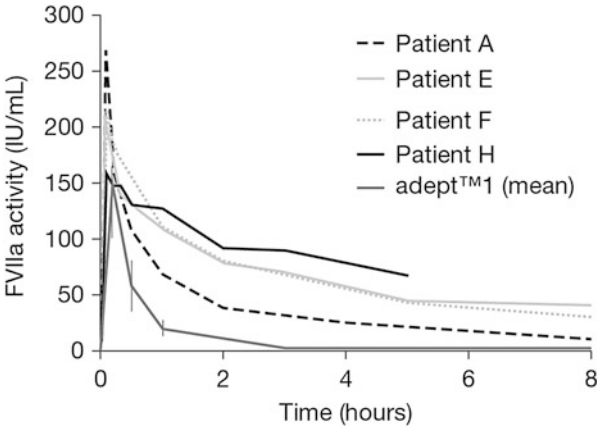

Fig. 7 PK profiles of four patients (patients A, E, F, H) with ADAs against vatreptacog alfa. Individual PK profiles are presented, together with the mean (±SD) FVIIa activity in ADA-negative patients in the phase 2 study (adept™1). Reproduced from Mahlangu et al. [57] with permission from John Wiley and Sons

8 Investigating the Immunogenicity of Vatreptacog Alfa Using Immunogenicity Prediction Tools

Since the only differences between rFVIIa and vatreptacog alfa are three amino acids, it is highly possible that the observed ADAs are due to the change in sequence. As the development of a mature ADA response is a T cell-dependent process [17–21], the assessment of vatreptacog alfa immunogenicity focused on the role of T-cell epitopes and their relationship with MHC-II molecules. Therefore, the role of T cells, T-cell epitopes, and MHC-II molecules in the generation of immune responses is briefly reviewed.

8.1 The Role of T Cells and T-Cell Epitopes in Immunogenicity

Several processes involving interaction between T-helper cells, B-cells, and antigen-presenting cells (APCs; e.g., dendritic cells) are necessary for antibody generation [18, 21]. If recognized by an APC, such as a dendritic cell, the infused therapeutic protein is taken inside the cell where it is processed and cleaved into peptides [18, 21]. These therapeutic protein-derived peptides then bind to MHC-II molecules, and the resulting peptide–MHC-II complexes are transported to the surface of the dendritic cell [18, 19, 21]. If antibodies are to be generated, the peptide must be presented as peptide–MHC-II complexes on dendritic cells and then be recognized by T-cell receptors (TCRs) on T-helper cells [18, 19, 21]. TCRs to self-proteins have generally been eliminated; thus, only peptides foreign to that individual will be recognized. Peptides that mediate a sustained association between dendritic cells and T cells are called T-cell epitopes [19], and T-cell epitope content is one of the major contributory factors to antigenicity [20]. The binding strength of a particular T-cell epitope to MHC-II molecules largely defines its immunogenicity, as epitopes with higher binding affinities are more likely to be presented on the surface of dendritic cells and thus recognized by TCRs [20]. Effective and sustained association between dendritic cells and T cells through formation of an MHC-II–peptide–TCR complex is necessary to induce stimulation and proliferation of T-helper cells [19, 21]; in turn, this leads to cytokine secretion and activation of B-cells that have also taken up the factor and presented the peptides on the same MHC-II molecules [19, 21]. A further series of steps leads to maturation of B-cells into factor-specific antibody-secreting plasma cells [18, 20, 21, 27].

Peptide binding to MHC-II molecules expressed by the host is one of the key determinants of whether the peptide will trigger an immune response [20, 33]. In this regard, the genotype of the highly polymorphic HLA determines the sequence of peptides that can be bound. As ADA development is thought to be influenced by a patient's HLA type [34], it is reasonable to expect that therapeutic proteins carrying promiscuous HLA class II-binding peptides (i.e., peptides that bind to

numerous HLA class II alleles with high affinity [58, 59]) have the potential to elicit an antibody response. These observations suggest that the affinity of mutant peptides for an individual's HLA class II repertoire may predict immunogenicity risk in that individual [60].

Immunogenicity prediction assessment of vatreptacog alfa sought to determine whether ADA development in adept™2 could have been elicited by presence of high-affinity mutant peptide sequences in vatreptacog alfa for individual patients' HLA class II profiles [98].

8.2 *Tools for Predicting T Cell-Dependent Immunogenicity*

Immunogenicity prediction typically involves more than one approach, as all methods have strengths and limitations [17] and possess different levels of predictive strength. There are currently no validated methods for predicting immunogenicity of therapeutic proteins in the clinic. However, in silico (i.e., using computers or computer simulations) tools and in vitro assays using recombinant proteins and living cells have been used to evaluate the T-cell epitope content, peptide–MHC-II interactions, and immunogenicity potential of therapeutic proteins [17, 22, 61]. Many of these tools have been developed and optimized relatively recently. As discussed in Sect. 5.4, early immunogenicity risk evaluation was performed for vatreptacog alfa using the tools available at that time; however, the unique case of vatreptacog alfa represents a valuable opportunity to evaluate the predictive value of the newer and different immunogenicity tools. An immunogenicity prediction strategy was developed for vatreptacog alfa using in silico, in vitro, and ex vivo tools to address the key processes of antigen presentation: (1) binding of the mutant peptides within the vatreptacog alfa molecule to MHC-II (in silico and in vitro), (2) protein and peptide processing and display by MHC-II on dendritic cells (in vitro), and (3) TCR recognition of the MHC-II–peptide complex (ex vivo).

8.3 *In Silico and In Vitro Peptide–MHC-II Binding Experiments*

In silico analysis involves the creation of computational models or simulations in order to make binding affinity predictions for specific peptide – HLA allele pairs [62]. Sequence analysis by in silico screening facilitates identification of potential T-cell epitopes [63] and is often used as a first step in the immunogenicity screening of therapeutic proteins [17]. Peptide–MHC-II binding is largely determined by a core residue of 9–10 amino acids within T-cell epitope sequences [17]. Therefore, it is computationally possible to predict T-cell epitopes based on peptide amino acid sequences if there is sufficient information available for peptides that are known to

bind to a particular MHC-II variant [17]. Databases such as the Immune Epitope Database and Analysis Resource (www.iedb.org) provide the basis for developing in silico T-cell epitope prediction tools [17].

In silico methods for immunogenicity prediction were still in their infancy during the early stages of vatreptacog alfa development (see Sect. 5.4). Since that time, a number of computational approaches to T-cell epitope prediction and immunogenicity risk assessment have been developed and optimized [64–74]. Such approaches are now well accepted in the field of vaccine design [63] and widely used to identify key epitopes that trigger autoimmunity [75]. In silico screening has also demonstrated predictive accuracy in the immunogenicity testing of protein therapeutics by showing an association between peptide–MHC-II binding and T cell-dependent immunogenicity in the clinic [17, 20, 70, 74]. These studies contribute to the growing body of evidence suggesting that in silico analysis predicts peptide–MHC-II interactions, serves as a first-line method to evaluate immunogenicity risk, and can even aid in the design of therapeutic proteins.

After defining putative T-cell epitopes with MHC-II binding affinity using in silico analysis, the results can be confirmed and validated using peptide/MHC-II binding assays. A number of different MHC-II binding assays can be used, including competition binding assays, real-time kinetic measurements, and direct binding assays [17].

8.3.1 Strengths and Limitations of In Silico and In Vitro Binding Experiments

In silico and in vitro peptide–MHC-II binding experiments focus on the contribution of T cells to ADA development. They are based on the binding of T-cell epitopes to HLA class II alleles, are cost-effective and relatively easy to use, and have a short time course that fits well into research and development programs for protein biotherapeutics [76–78]. Additionally, the in silico tools available today can rapidly screen and compare many different protein sequences and HLA alleles for putative T-cell epitopes [20, 76, 77] and can thus markedly reduce downstream in vitro testing [17].

Current in silico analyses offer a reasonable level of accuracy when predicting peptide–HLA class II-binding affinities for a large number of HLA alleles. However, the major limitation of both in silico and in vitro peptide–MHC-II binding analyses is their tendency to overpredict the number of T-cell epitopes [77], as they do not account for other important biological factors that limit the number of true functional T-cell epitopes [76]. These additional factors include protein and peptide processing in the APC, T-cell phenotype, TCR affinity for the peptide–MHC-II complex, and induction of T-cell tolerance to non-germline peptides [17]. Furthermore, neither approach proves that a peptide predicted to bind to MHC-II molecules will actually initiate a T-cell response [77, 78]. However, the protein needs to contain T-cell epitopes in order to induce a high affinity ADA response. These epitopes can be

identified by in silico or in vitro peptide–MHC-II binding analyses with a certain level of false positives and a very low number of false negatives.

8.3.2 In Silico and In Vitro Assessment of Vatreptacog Alfa

For vatreptacog alfa, the aim of in silico peptide–MHC-II binding experiments was to identify potential neo-T-cell epitopes within the mutant peptides, using the wild-type FVIIa as a reference sequence to exclude endogenous, tolerized epitopes. The in silico predictions were made using two different algorithms: NetMHCIIpan v2.1 [79] and NetMHCII v2.2 [80]. While NetMHCIIpan v2.1 evaluates the binding affinity of peptides to all HLA-DRB1 alleles, NetMHCII v2.2 evaluates peptide binding affinity to the HLA-DRB1 alleles as well as the most frequent HLA-DP and HLA-DQ alleles. As peptide–MHC-II binding affinity is governed mainly by a core sequence of 9–10 amino acids within potential T-cell epitopes [17] and partly by the three flanking amino acid residues on each side of the binding core sequence, the in silico analyses were conducted using the default peptide length (15 amino acids with a nine amino-acid binding core sequence; [98]).

In silico analysis generated a heat map in which colors correspond to the binding affinity of a given peptide/HLA allele pair [98]. After subtracting the HLA class II-binding peptides that contained only wild-type FVIIa sequences, various neo-epitopes that were predicted to bind to HLA-DR, HLA-DP, and HLA-DQ alleles were identified for vatreptacog alfa. The heat map of predicted peptide–MHC-II affinities showed that peptides with the E296V and M298Q mutations bind to several HLA class II alleles with high affinity and therefore contain potential T-cell epitopes; in contrast, the E158D mutation does not give rise to HLA class II-binding peptides (Fig. 8).

The in silico findings were then confirmed using an in vitro direct peptide binding assay, which assessed the binding affinities of vatreptacog alfa peptides for ten HLA variants representing the major HLA class II molecules. The assay chosen for this purpose was the luminescent oxygen channeling immunoassay (LOCI), a two-bead assay system [81], performed by Professor Søren Buus at Copenhagen University. Donor beads were coated with streptavidin, a capture reagent that binds various biotinylated HLA-DRB1 alleles [81], and acceptor beads were coated with monoclonal antibodies specific to fully folded HLA-DRB1. Donor and acceptor beads formed pairs in the presence of vatreptacog alfa peptides that bind to the HLA class II variants, and photochemically triggered chemiluminescence allowed detection of these bead pairs [81–83]. This in vitro analysis validated the in silico findings.

While peptide–MHC-II binding is a necessary step in ADA development, it is not sufficient to induce an immune response on its own: recognition of the peptide–MHC-II complex on dendritic cells by TCRs, followed by T-cell activation, is also required. Therefore, the immunogenicity of vatreptacog alfa was tested further using human cell-based assays that assess peptide presentation and T-cell responses.

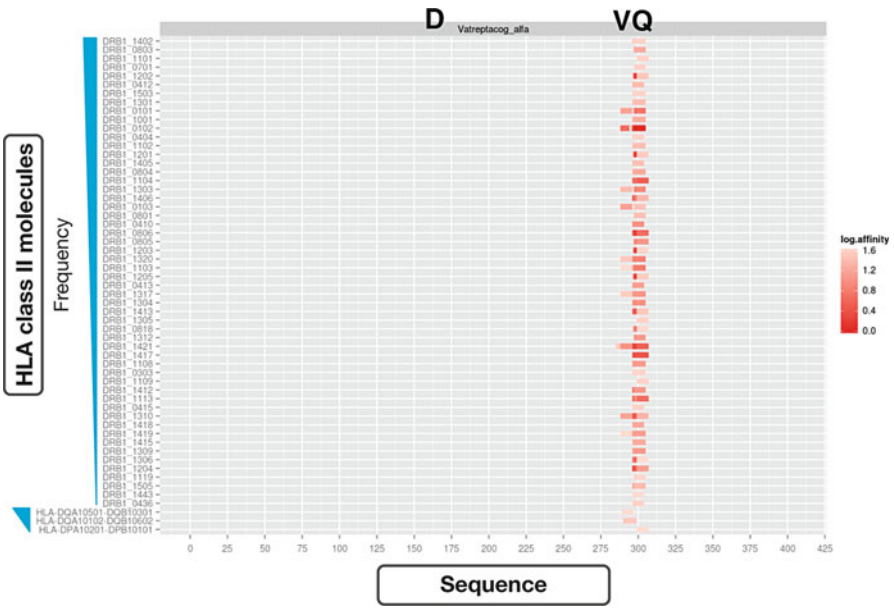

Fig. 8 In silico predicted HLA class II-binding peptides for vatreptacog alfa. Graphic representation of the predicted HLA class II-binding peptides in vatreptacog alfa and their binding affinities to the tested HLA-DR, HLA-DP, and HLA-DQ alleles. Each *red bar* represents a HLA class II-binding peptide with its position in the vatreptacog alfa sequence (*x* axis) and its binding affinity to a given HLA allele (*y* axis). Native FVIIa has been used as reference sequence, thus only novel peptides binding to HLA molecules are identified

8.4 Human Cell-Based Peptide Processing, Presentation, and Activation Assays

While in silico analysis can predict putative T-cell epitopes and reveal their binding affinities for MHC-II molecules, cell-based assays that use T cells and APCs derived from the peripheral blood (peripheral blood mononuclear cells [PBMCs]) of human donors address several processes relevant to T-cell activation: APC antigen uptake and processing, the stability of peptide–HLA class II binding, identification of a T-cell repertoire toward the peptide in question, and subsequent T-cell activation resulting in proliferation and cytokine release.

8.4.1 Strengths and Limitations of Human Cell-Based Assays

Although more technically demanding than in silico analysis, human cell-based assays provide more biologically relevant measurements of T-cell epitopes, predict the relative immunogenicity potential of the epitopes, and, in some cases, correlate

well to immunogenicity seen in the clinic [17, 77, 78]. As a result, they offer a greater level of predictive accuracy and reliability than in silico analyses in immunogenicity risk assessments.

However, cell-based assays are not without limitations. For example, to avoid overestimating the T-cell response to the peptides under investigation, PBMC-based T-cell activation assays measuring cytokine release must be fine-tuned to negate the effects of other cytokine-secreting cells present in the PBMC preparation [17]. Indeed, a major challenge with these assays is the need to find an appropriate balance between minimizing irrelevant immune responses and supporting the immune response of interest [17]. A large number of individual blood donors and large blood volumes are required to perform a dendritic cell presentation assay or a T-cell activation assay [17]. This can be very cost prohibitive for many laboratories and is also time-consuming.

Despite these challenges, however, and despite the improvements that are still needed, human cell-based assays effectively strengthen the predictive value of immunogenicity analyses by evaluating the final impact of the potential T-cell epitopes identified in silico [17].

8.4.2 In Vitro Dendritic Cell Presentation Assay

In vitro profiling of vatreptacog alfa- and FVIIa-derived peptides was performed to determine whether mutant peptides in vatreptacog alfa are presented on dendritic cells and therefore have the potential to induce T-cell activation. This was achieved using human cell-based antigen presentation assays, which investigate peptide processing in the dendritic cells and presentation of peptides by HLA class II molecules on the dendritic cell surface [76]. One of the aspects of peptide processing reflected in the assay is intracellular peptide trimming. MHC-II-binding peptides usually range between 12 and 25 amino acids in length and can be represented as linear sequences including the core binding sequence of 9–10 amino acids. This core binding sequence fits into a binding groove on the MHC-II molecule; however, additional amino acids flank the core binding sequence outside the groove at each end [84, 85]. The in vitro dendritic cell presentation assay addresses intracellular peptide trimming and allows clusters of peptides to be defined that share a common stretch but differ in the lengths of the amino acid sequences that lie outside the MHC-II binding groove.

The assay used human monocyte-derived dendritic cells, which are widely considered to be the major APC in human immune responses [86, 87]. Immature dendritic cells are present in peripheral tissues, where they effectively take up both foreign and “self”-proteins and process them intracellularly into small peptides for antigen presentation via MHC-II to the adaptive immune system [88–90]. When dendritic cells capture antigens in the presence of “danger signals” [87, 88], they switch to a mature state, following which they become very efficient in presenting

protein-derived peptides to naïve T-helper cells in the context of their MHC-II repertoire [67, 87, 88, 91].

Peripheral blood samples obtained from healthy donors of unknown HLA type were processed to generate human monocyte-derived dendritic cells. Human monocytes were incubated in vitro with IL-4 and granulocyte–macrophage colony-stimulating factor (GM-CSF) for 5 days, to obtain dendritic cells, which were then pulsed with antigen. Exposure of the dendritic cell to (and subsequent uptake of) vatreptacog alfa was followed by overnight lipopolysaccharide-induced maturation of the dendritic cell. Peptide processing and binding to HLA class II in the mature dendritic cell led to cell surface presentation of peptide–HLA class II complexes, after which the membrane proteins were solubilized using cell lysis. Peptide–HLA class II complexes were then immunocaptured using HLA-DR-coated beads. HLA class II-binding peptide sequences were eluted and characterized by mass spectrometry (MS). The eluted HLA class II-binding peptide sequences had undergone intracellular peptide trimming and thus represented clusters of peptides sharing a common stretch but differing in the lengths of their amino- and carboxy-terminal extensions.

The results showed that HLA-DR-displayed peptides could be grouped into distinct clusters distributed throughout the vatreptacog alfa heavy chain, with one cluster overlapping the predicted HLA class II-binding peptides from the initial in silico and in vitro analyses (data not shown). These results thus correlated with the in silico findings and demonstrate that the peptides predicted to bind HLA class II were in fact processed by the dendritic cells. Importantly, the data showed that peptides spanning positions 296 and 298 were presented on the cell surface of APCs. Therefore, this analysis substantiates the hypothesis that the mutations in these positions could be the trigger of the ADAs that developed in some patients.

8.4.3 Ex Vivo T-Cell Activation Assays

Ex vivo T-cell activation assays were conducted to explore potential T-cell responses to the mutations in the modified sequences of vatreptacog alfa. These assays are based on human PBMCs containing CD4+ T cells and APCs from healthy blood donors or the desired patient population [76, 77]; T-cell activation is typically evaluated by T-cell proliferation (as measured by incorporation of radiolabeled thymidine) and/or cytokine (e.g., IL-2) release [67, 77, 78, 92]. Immunogenic potential can be assessed based on the magnitude and frequency of donor T-cell responses to the investigated protein or peptide sequences [77, 78] and typically includes a panel of healthy donors representing various HLA types.

The ex vivo T-cell activation assays for vatreptacog alfa assessment were performed at Antitope Ltd (Cambridge, UK). All assays were performed using peripheral blood leucocytes from 50 healthy donors who had been HLA typed and

selected to provide a proportional representation of HLA alleles found in the world population. Donor cells were incubated with full-length FVIIa and vatreptacog alfa peptides; T-cell proliferation and IL-2 secretion – both markers of T-cell activation – were then estimated using a tritiated thymidine uptake assay and an IL-2 ELISpot assay, respectively [93]. The percentage of responders to the full-length proteins or peptides was then determined. According to Antitope, a frequency of <10% is associated with low or no risk for immunogenicity in the clinic [94].

Full-length wild-type FVIIa and vatreptacog alfa induced a T-cell response in <10% of the donor cohort; thus, there was no significant difference in responders between full-length vatreptacog alfa and full-length wild-type FVIIa. This suggests that ex vivo T-cell activation assays were not sensitive enough to predict the immunogenicity of vatreptacog alfa when full-length molecules were used.

In order to increase the sensitivity of the assay, short (15 amino acids) and long (23–25 amino acids) vatreptacog alfa peptides spanning the protein sequences of interest were used. This more diverse set of mutant peptides was designed to fit two different versions of the T-cell assay (a time course assay and a peptide assay) (Table 2). The long peptides were designed to allow the point of mutation to be located in any of the nine amino acid positions in the binding core sequence with a three-amino-acid flanking sequence at each end of the peptide. The long peptides were tested in the time course assay to allow for antigen uptake and processing of the peptides. This approach facilitates enhanced presentation of mutant peptides by HLA class II molecules when donor PBMCs are incubated with the wild-type or

Table 2 Short (15 amino acids) and long (23–25 amino acids) peptides used in T-cell activation assays

Peptide number	Peptide name	Amino acid sequence
Time course assay		
1	158_V	SKPQGRIVGGKVCPKGECPWQVL
2	158_D	SKPQGRIVGGKDCPKGECPWQVL
3	296_E	GQLLDRGATALELMVLNVPRLMT
4	296_V	GQLLDRGATALVLMVLNVPRLMT
5	298_M	LLDRGATALELMVLNVPRLMTQD
6	298_Q	LLDRGATALELQVLNVPRLMTQD
7	298_F	LLDRGATALELFVLNVPRLMTQD
8	296_E_298_M	GQLLDRGATALELMVLNVPRLMTQD
9	296_V_298_Q	GQLLDRGATALVLQVLNVPRLMTQD
Peptide assay		
10	296_E_298_M	ATALELMVLNVPRLM
11	296_V_298_M	ATALVLMVLNVPRLM
12	296_E_298_Q	ATALELQVLNVPRLM
13	296_E_298_F	ATALELFVLNVPRLM
14	296_V_298_Q	ATALVLQVLNVPRLM
15	HA307-319 (pos. ctl.)	PKYVKQNTLKLAT

mutant peptides. The 15-amino-acid peptides that were identified and based on the in silico algorithm were tested in the peptide assay where the peptides are loaded directly onto the HLA class II on the cell surface of the APCs. In turn, these approaches increase the density of the mutant peptide in question on the dendritic cell surface and therefore increase the chance and strength of T-cell activation if the peptide is immunogenic.

In the second analysis, three 15-amino-acid mutant peptides from vatreptacog alfa were found to trigger T-cell activation: the E296V, M298Q, and double E296V/M298Q mutants produced a higher T-cell response than wild-type FVIIa, while the E158D mutant did not (Fig. 9). This difference was more pronounced when longer peptides were used. These results are consistent with the heat map generated by in silico analysis (Fig. 8) and confirm that the E296V and M298Q mutant peptides bind with high affinity to several HLA class II alleles, while the E158D mutant does not. According to the in silico prediction analysis, peptides with the 298F mutation bind with an increased affinity compared to 298Q. Therefore peptides with the 298F mutation were added to the assays to explore how this high affinity would translate in the T-cell assays. Actually, the peptides with the 298F mutation had a slightly increased T-cell response compared to the peptides with the 298Q mutation.

8.5 HLA Typing of ADA-Positive Patients

Following discontinuation of the adept™2 trial, seven of the eight patients who developed ADAs against vatreptacog alfa and who participated in the follow-up study were HLA typed [57]. Saliva samples were obtained from all seven patients, and HLA typing was performed using polymerase chain reaction sequence–specific oligonucleotide probe (PCR–SSOP) to resolve major allele groups into four digits [57]. The purified genomic DNA was amplified using PCR and incubated with a panel of different oligonucleotide probes that have distinctive reactivity with different HLA types [57]. The HLA class II loci of interest were DRB1, DRB3/DRB4/DRB5, DPA1, DPB1, DQA1, and DQB1.

As expected, due to the promiscuity of the predicted T-cell epitopes, ADA-positive patients did not share a common HLA class II allele, suggesting that no single allele was responsible for ADA development (Table 3). However, all ADA-positive patients had at least one HLA class II allele that binds to a vatreptacog alfa peptide with high affinity. HLA class II alleles identified in six of the seven patients were represented in the healthy donor cohort used for the T-cell activation assays; also, as expected, HLA class II alleles that occur frequently in the population (e.g., DRB1*0701) also appeared in the ADA-positive patients. Interestingly, according to the in silico analysis, these alleles did not bind with high affinity to vatreptacog alfa. However, the double-mutant E296V/M298Q peptides from vatreptacog alfa bound with high affinity to at least one HLA class II

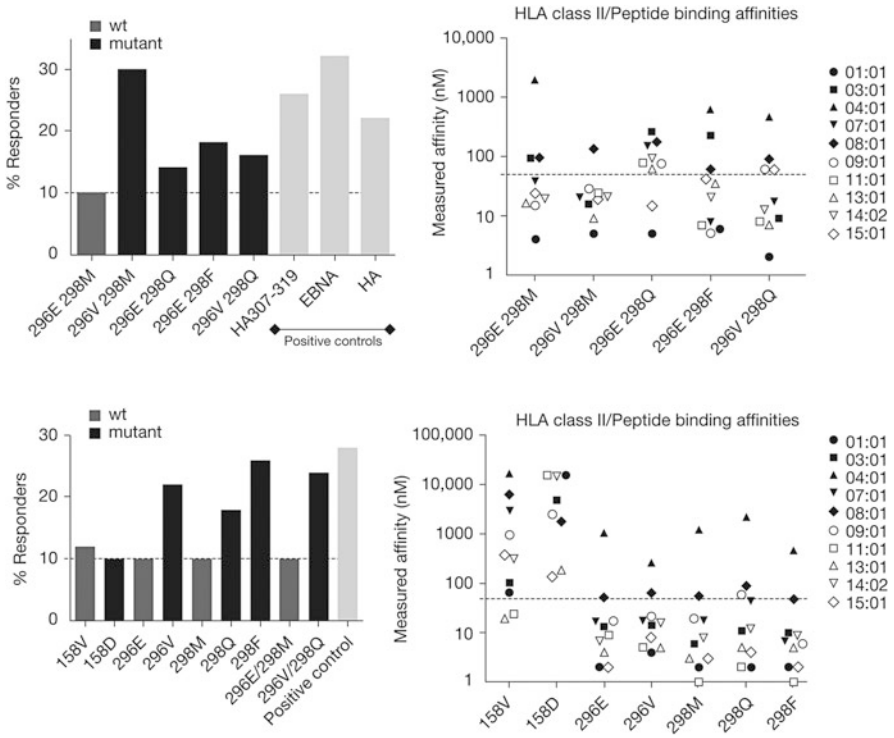

Fig. 9 Ex vivo T-cell activation assays using (**a**) 15-amino-acid peptides and (**b**) long peptides (23–25 amino acids). *Left panels*: Percentage of responders in a T-cell proliferation assay in a cohort of 50 healthy donors. Wild-type peptides all induced a response in ~10% of the cohort, and this was used as a threshold. The E296V, M298Q, and double E296V/M298Q mutants showed an increase in the percentage of responders (versus wild-type FVIIa), whereas the E158D mutant did not. This increase in responders was evident for 15-amino-acid mutant peptides (**a**) but much more pronounced for long peptides (**b**). Right panels: Peptide–HLA class II binding affinities. (**a**) The first peptide on the *x* axis is wild-type FVIIa; the others are different mutant peptides derived from vatreptacog alfa. Wild-type and all mutant peptides bind with similar affinity to the different HLA class II molecules. (**b**) On the *x* axis, peptides 158V, 296E, and 298M are wild-type FVIIa; the others are different mutant peptides derived from vatreptacog alfa. Wild type and mutants spanning the amino acid positions 296–298 bind with very high affinity to the different HLA class II molecules, while peptides spanning position 158 show a lack of HLA binding affinity

allele in all ADA-positive patients. One limitation of this analysis is that only ADA-positive patients from adept™2 were HLA typed. Therefore, it is not possible to conclude whether there is a set of HLA class II alleles that occur more frequently in ADA-positive (versus ADA-negative) patients.

Table 3 HLA typing of patients with anti-vatreptacog alfa ADAs in the adept™2 trial

Patient	HLA-DRB1	HLA-DRB3	HLA-DRB4	HLA-DRB5	HLA-DQB1	HLA-DPB1	HLA-DQA1	HLA-DPA1
A	*01:02/43/46	NT	*01:01/03/06/08	NT	*05:01/12/18	*01:01	*01:01/04/05	*01:03
	*04:04/23/108/118/120N/121/149	NT	NT	NT	*04:02	*18:01	*03:01/02/03	*02:01
B	*04:06/144	NT	*01:03	NT	*03:02P	*02:01P	*01:03/10	*01:03P
	*08:03P	NT	NT	NT	*06:01/43	*05:01:01G	*03:01:01G	*02:02/05
C	*11:04	*02:02/27/28/29N	*01:01/03/06	NT	*02:02	*04:01	*02:01	*01:03
	*07:01	NT	NT	NT	*03:01/27/28/29/35/42/47	*10:01	*05:05/09/11	*02:01
D	*13:01/105/112/117/148/153	*02:02/27/28/29N	*01:01/03/06	NT	*06:03/41	*01:01	*01:03/10	*02:01
	*07:01	NT	NT	NT	*02:02	*13:01/107:01	*02:01	NT
E	*13:01/105/109/112/117/138/143/146/147/148/153	*02:02/27/28/29N	NT	NT	*06:02	*01:01	*01:03/10	*02:01
	*08:04	NT	NT	NT	*04:02	*14:01	*04:01	*02:02/05
F	*03:01/50/68N/82/83/86	*01:01/11	NT	NT	*02:01	*01:01	*05:01/05/09/11	*01:03
	*11:01/95/97/100/117/133/134/140/141	*02:02/27/28/29N	NT	NT	*03:01/27/28/29/35/42/47	*04:01	NT	*02:01
H	*15:02/80N	*03:01	NT	*01:02/08N	*05:01/12/18	*05:01	*01:01/04/05	*02:01
	*12:02/37	NT	NT	NT	*03:01/27/28/29/35/42/47	*13:01/107:01	*06:01	*02:02/05

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9 Vatreptacog Alfa and Immunogenicity: What Have We Learned?

ADAs developed against vatreptacog alfa in 8/72 (11%) treated patients in the phase 3 adept™2 trial. Of these eight patients, the antibodies in four developed cross-reactivity against rFVIIa, of which one developed in vitro neutralizing activity against vatreptacog alfa [1, 57]. No effects of the ADAs on clinical efficacy were manifest in any patients, and ADA titer declined in all cases following discontinuation of vatreptacog alfa treatment. Cross-reactivity to rFVIIa also disappeared after last exposure to vatreptacog alfa, despite reexposure to rFVIIa according to local standard care. Vatreptacog alfa ADAs were of the IgG subtype in all patients who had conclusive immunoglobulin isotype determination, and all ADAs were specific for vatreptacog alfa rather than rFVIIa [57]. PK assessment of ADA-positive patients suggested prolonged elimination of vatreptacog alfa but gave no indication of reduced FVIIa levels [57].

Vatreptacog alfa ADAs did not appear to be caused by FVII polymorphisms or mutations.

The binding of peptides to HLA class II molecules is a key factor in determining whether the peptides will trigger an immune response [20, 33]; ADA development is therefore influenced by an individual's HLA class II repertoire [34], and immunogenicity risk may be predicted by peptide–HLA class II binding affinity [60, 98]. Post hoc immunogenicity prediction studies using in silico, in vitro, and ex vivo tools were undertaken to investigate whether ADA development in the eight patients from adept™2 could have been triggered by the affinity of the mutant peptide sequences in vatreptacog alfa for individual patients' HLA class II profiles [98]. The results of these studies confirmed that vatreptacog alfa peptides with the E158D mutation lack the necessary condition (i.e., high-affinity peptide–HLA class II binding) to elicit an immune response. However, the E296V and M298Q mutant analogs do bind with high affinity to several HLA class II variants. Furthermore, all ADA-positive patients expressed at least one of the HLA class II variants shown to bind a vatreptacog alfa peptide with high affinity [98]. Finally, mutant peptides carrying the E296V and M298Q mutations were detected on HLA class II proteins isolated from dendritic cells. Together, these findings indicate that the E296V and M298Q mutations can result in peptide sequences that act as strong T-cell epitopes in patients with one or more of these "high-risk" alleles [98].

A bioengineered analog of rFVIIa, vatreptacog alfa, has >99% sequence identity to native FVIIa, with only three amino acid substitutions. However, the development of ADAs in treated patients, together with the results of the immunogenicity prediction studies, points to the greatest lesson learned from the vatreptacog alfa case, namely, that even modest sequence changes can create new T-cell epitopes and significantly alter the immunogenicity profile of a therapeutic protein.

10 Immunogenicity Prediction in the Future

As the vatreptacog alfa case shows, considerable challenges may hinder the clinical development of bioengineered therapeutic coagulation proteins, especially when dealing with sequence-modified analogs [1]. One of the greatest challenges lies in developing a protein that offers improved clinical efficacy without introducing an increased immunogenicity risk. As highlighted by the vatreptacog alfa clinical trial program, a primary challenge is the need to include a sufficient number of patients and exposures in phase 2 trials to allow reliable detection of an immunogenic signal [1].

However, a growing area of interest in immunogenicity assessment lies in the development of accurate strategies for predicting the immunogenicity profiles of therapeutic proteins [17]. Animal models are useful for evaluating some of the factors that influence immunogenicity (e.g., product formulation, dosing regimen, and other non-sequence-related affects) [78]; however, as most therapeutic proteins show species differences in amino acid sequence, they are often recognized by animals as foreign [78, 95]. Consequently animal models have restricted predictive value for evaluating mutations in amino acid sequences. The animal and human MHC-II repertoires are not equivalent, as they differ at the amino acid level; therefore, the results of in vivo prediction studies conducted in animals that are not transgenic for HLA class II should be interpreted with great caution [67, 92]. The challenges inherent in using animal models to predict immunogenicity are evident in the vatreptacog alfa case: two studies using rats [56] and mice [98] failed to detect the increased immunogenicity of vatreptacog alfa versus rFVIIa. To overcome some of these challenges, specialized mouse models are being developed [78].

Improved immunogenicity prediction methods and models are clearly needed [57]. Since the early immunogenicity risk assessment for vatreptacog alfa was performed, more than a decade ago, the landscape of immunogenicity prediction has changed considerably, and the tools available today offer a greater level of reliability and prediction accuracy. There was very good concordance between the different immunogenicity prediction tools used for the post hoc assessment of vatreptacog alfa. However, no single tool used alone can accurately predict immunogenicity or address all questions relating to immunogenicity [17, 77]. If the proper sample format is used, T-cell proliferation assays are better predictors of immunogenicity risk than peptide–HLA class II-binding affinities but are more useful in estimating risk in the population as a whole than in individuals. Therefore, strategic combination of multiple predictive approaches is needed. When used in combination, in silico peptide–HLA class II binding predictions, in vitro antigen presentation assays, and ex vivo T-cell proliferation assays provide useful indications of the immunogenicity risk posed by specific neo-sequences in bioengineered therapeutic proteins [67, 77]. Today, a number of drug developers [3] are incorporating this combined approach into preclinical development programs [17, 77]. Such methods are also recommended by both the European Medicines

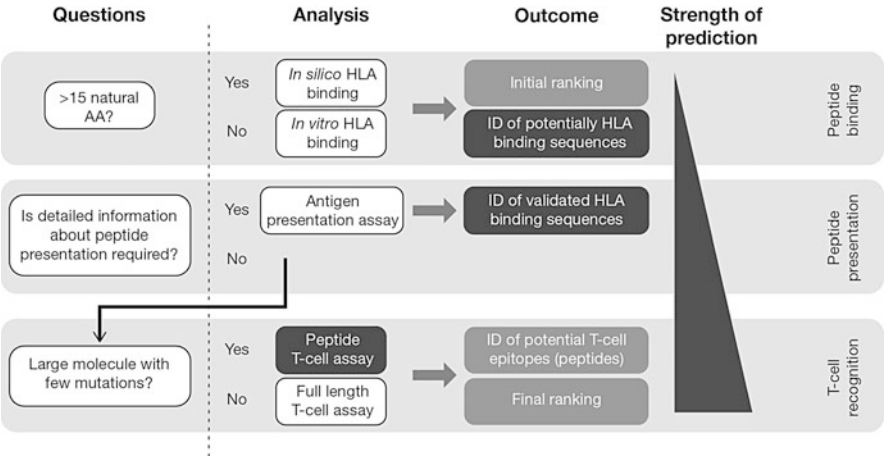

Fig. 10 Suggested pathway for the use of immunogenicity prediction tools. This multistep approach evaluates the immunogenic potential of a therapeutic protein. The successive steps evaluate, in turn, peptide binding, peptide presentation, and T-cell recognition; each step provides a greater predictive strength and thus validates results from the previous step. The first step is in silico prediction of peptide–HLA class II binding, followed by a confirmatory in vitro peptide binding assay, to identify putative HLA-binding peptide sequences ranked in order of potential immunogenicity. The second step is an antigen presentation assay, which evaluates antigen processing and peptide presentation by HLA class II molecules on the surface of antigen-presenting (e.g., dendritic) cells. This step validates the putative HLA class II-binding sequences identified in the first step. The third step is a T-cell proliferation assay, which identifies potential T-cell epitopes, assesses T-cell response, and establishes a final ranking of peptides according to immunogenic potential

Agency (EMA) and the United States (US) Food and Drug Administration (FDA) in their current guidelines for preclinical immunogenicity risk assessment [95, 96]. Indeed, the EMA guidelines now indicate a move away from preclinical animal studies toward combining these non in vivo tools in strategies to perform early immunogenicity risk assessment [95].

A suggested early immunogenicity risk assessment strategy – based on lessons learned from the vatreptacog alfa case – is presented in Fig. 10. If the sequence of interest consists of 15 or more natural amino acids, it is possible to perform an in silico analysis to identify HLA-binding peptides. If the sequence is less than 15 amino acids long or includes unnatural amino acids, the in silico analysis cannot be used, so the in vitro peptide binding assay should be performed instead. The next suggested step is to identify which peptides are presented on the dendritic cell surface by using the DC presentation assay. There are then two sample format options to investigate whether or not there is a T-cell repertoire toward the peptide–HLA complex. If the molecule of interest is large with few mutations (i.e., low degree of foreignness), peptides may be used as samples, designed using information from the in silico or in vitro DC presentation assays. Alternatively, small molecules with more mutations (i.e., high degree of foreignness) may require a format that assays the full length of the molecule.

It is hoped that guidelines for standardizing immunogenicity prediction testing will emerge from the increasing and continued implementation of these novel tools in the early stages of drug discovery and development [17]. Unquestionably, the overriding short-term aim of all these endeavors is to reduce immunogenicity risk in the clinic. A longer-term goal is to improve the prediction of clinical immunogenicity. This will require validation of the prediction tools but will in turn enable more efficient development of bioengineered protein drugs that carry great efficacy potential. This validation will be based on clinical data and the use of the tools to test molecules that are known to be immunogenic. An even longer-term goal is to address the question of what will be required to establish immunogenicity prediction tools as mandatory aspects of the development process for biopharmaceuticals. If these goals can all be met with satisfaction, then the development of "personalized" pharmaceuticals to target patients with low immunogenicity risk becomes a feasible – though still distant – possibility.

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