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Research Article

Induction and Impact of Anti-Drug Responses Elicited by a Human Recombinant Coagulation Factor FXa^{I16L} in Preclinical Species

Chuenlei Parng,[1,5] Michael Bolt,[2] Debra D. Pittman,[3] Teresa Caiazzo,[4] Lisa Dyleski,[4] Boris Gorovits,[4] and Rob Webster[1]

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Abstract. This paper presents a systemic investigation of ADA development and ADA impact of a human coagulation factor in nonclinical species during drug development and provides insights into potential implications in human if a similar ADA occurs. FXa^{I16L}-induced ADA response was characterized in monkey, mouse, rat, and dog in different studies, and ADA effects on pharmacokinetic and/or pharmacodynamics of FXa^{I16L} were further examined in ADA-negative and ADA-positive animals. After repeated administrations, FXa^{I16L} elicited a dose and exposure day-dependent ADA response which ranged from no response to a transient or persistent response. Increase in exposure day and increase in dose generally enhanced ADA incidence except for a decrease in ADA incidence was observed in monkeys after repeated high-dose administrations. The observable ADA impact on pharmacokinetics was only found in some ADA+ animals and included decrease in clearance and increase in systemic exposure but no increase in half-life. In addition, no or limited effect on pharmacodynamics by ADA was observed. The earliest ADA response was observed after three exposure days, marked elevation of drug exposure was observed in some animals at log titer > 2.0, and the highest antibody titer excited was about 4 (Log10) in all species. A correlation between ADA induction and accumulative exposure after various repeat treatments in different species was found for FXa^{I16L}. In addition, potential immunogenicity risk and mitigation of ADA in clinics are discussed.

KEY WORDS: anti-drug antibody; coagulation factor; exposure day; FXa^{I16L}; hemophilia inhibitor; neutralizing antibody.

INTRODUCTION

Anti-drug antibody response (ADA) is one of the key challenges for biotherapeutics. ADAs are generated by B cells via T cell independent and dependent pathways in the host and the induction of ADA is affected by various factors including intrinsic properties of therapeutic protein, therapeutic target, target patient population, disease status, and/or environmental factors, etc. (1). ADA can potentially alter pharmacokinetics (PK), pharmacodynamics (PD), and/or safety profiles of biologics depending on the binding specificity, affinity, and titers of ADA (2). The ADA of non-antibody biologics can further develop cross-reactivity against endogenous proteins through T cell-mediated immune response and loss of central T cell tolerance for administrated agents after repeated exposure (3). It has been reported that recombinant erythropoietin and thrombopoietin therapeutics have resulted in cross-reactive ADAs which not only neutralized therapeutic protein but also inhibited functions of their endogenous counterparts (4,5). In addition, replacement coagulation factor VIII (FVIII) and IX (FIX) therapeutics had induced alloantibodies referred as inhibitors which neutralized replacement factors in 20–30% FVIII-deficient and 1–5% FIX-deficient hemophilia patients (6). The timing of FVIII inhibitor development in patients was observed after 10–15 exposure days and it reached plateau after 50–75 exposure days (7) (8). The exposure day is indicated as a unit of time one or more doses of replacement treatment is given to a

[1] *Present Address:* Pfizer Biomedicine Design, Pharmacokinetics Pharmacodynamics Metabolism, 610 Main St., Cambridge, Massachusetts 02139, USA.
[2] Pfizer Drug Safety Research and Development, Cambridge, Massachusetts 02139, USA.
[3] Pfizer Rare Disease Research Unit, Cambridge, Massachusetts 02139, USA.
[4] Pfizer Biomedicine Design, Andover, Massachusetts 01810, USA.
[5] To whom correspondence should be addressed. (e–mail: chuenlei.parng@pfizer.com)

Abbreviations *ADA*, Anti-drug antibody; *AUC*, Area under the curve; *Cmax* Maximum observed concentration; *EGRCK*, Glu-Gly-Arg-chloromethyl ketone; *FEIBA*, Factor eight inhibitor bypassing agent (anti-inhibitor coagulant complex); *FXa*, Active coagulation factor X; *nAb* Neutralizing antibody; *PK*, Pharmacokinetics; *PD* Pharmacodynamics

patient (7). After inhibitor development, many hemophilia patients do not respond to factor replacement therapy and need immune tolerance induction to eradicate inhibitors or receive bypassing therapies such as FEIBA (factor eight inhibitor bypassing agent) or activated factor VII (rFVIIa; NovoSeven, Novo Nordisk, Denmark) to achieve hemostasis. There was no report of ADA or nAb development against NovoSeven in FVIII- or FIX-deficient patients after treatments (9). On the other hand, clinical development of other bypassing FVIIa variants including BAY 86–6165 (Bayer, Leverkusen, Germany) and Vatreptacog alfa (Novo Nordisk) was discontinued due to the occurrence of ADA and nAb in some hemophilia A patients after multiple administrations (10,11). These FVIIa variants contain three or six amino acid modifications when compared to endogenous FVII and NovoSeven. In addition, both FVIIa variants were shown to be safe, well-tolerated, and had no ADA induction in phase I (single administration) and phase II (multiple administrations) clinical trials. However, development of ADA and nAb was observed in phase III trials with increasing exposure days and increasing subject numbers (10). These observations indicate that immunogenicity presents a significant challenge for clotting factor therapeutics development.

FXa^{I16L}, an active human coagulation factor X (FX) variant with a single amino acid mutation, has been in clinical development for treating acute intracerebral hemorrhages via a single intravenous administration and has potentials for treating other life-long bleeding disorders such Glanzmann's thrombasthenia (12). FXa^{I16L} contains a single amino acid substitution, isoleucine (I) to leucine (L), at position 16 based on chymotrypsin numbering compared to wild-type FXa and has an impaired conformational transition from the zymogen to active protease (13). We had previously reported that administrated FXa^{I16L} inactivated by serum protease exhibits via protein-protein interaction and had very short half-life that ranged from 2 to 6 min in preclinical species and a plasma residence time of 4 h for the total ^{125}I-FXa^{I16L} metabolites (14). For a single IV administration of FXa^{I16L} in non-factor deficient hemorrhagic stroke patients, the perceived clinical immunogenicity risk of FXa^{I16L} is predicted to be considerably lower than conventional life-long factor VIII and IX replacement therapies in hemophilia A and B patients. Whereas, the potential immunogenicity risk of FXa^{I16L} for other chronic treatments needs to be carefully assessed.

Recombinant human proteins, acting as xenogeneic proteins in preclinical species are likely to be immunogenic in nonclinical studies; therefore, the ADA results from preclinical studies cannot be used to predict the immunogenicity frequency in humans. However, the impact of anti-drug antibody on pharmacokinetics, pharmacodynamics, and safety in animals can not only provide additional insights into unexpected findings in pharmacology and toxicology studies but also provide information on the potential ADA consequence if ADA with similar reactivity occurs in human. To assist proper interpretation of preclinical efficacy and toxicology data, we assessed anti-drug antibody response and evaluated the ADA impact on pharmacokinetics and pharmacodynamics in different nonclinical toxicological and pharmacological studies with various FXa^{I16L} treatments. We report characteristics of dose- and time-dependent ADA responses in monkey, mouse, rat, and dog after intravenous administration of FXa^{I16L} and the ADA impact on pharmacokinetics and pharmacodynamics. In addition, we find a clear correlation between ADA incidence and cumulative exposure of FXa^{I16L} among species. Furthermore, we discuss potential impact on FXa^{I16L} if ADA occurs in clinics and its potential mitigations. This paper presents a systemic investigation of ADA development and ADA impact of a human coagulation factor in nonclinical species during drug discovery and provides insights into potential implications in human if a similar ADA occurs.

MATERIALS AND METHODS

Protein Sequence Comparison

FX circulates as a zymogen and is converted to active FXa after proteolysis at a highly conserved site (arginine 15-isoleucine 16, based on chymotrypsin numbering) releasing the activation peptide (15). FX protein sequences from National Center for Biotechnology Information (NCBI) protein database for the following species, human (NP_000495), cynomolgus monkey (EHH58718.1), rat (AAH88151.1), CAA10933.1 (mouse), and dog (AAC26797.1). The FX sequences were aligned using a multiple sequence alignment program (ClustalW) and the identical and homologous amino acids were highlighted by a proteomics program, Boxshade. ClustalW and Boxshade programs are open access web tools provided by the Swiss Institute of Bioinformatics (SIB) Resource Portal (ExPASy).

Animal Treatment

All the animal experiments were conducted in full compliance with ethical and regulatory principles and local and national licensing regulations. The mouse, rat, and monkey repeated administration studies were performed in compliance with Good Laboratory Practice (GLP) Standards for Nonclinical Laboratory Studies regulations as set forth in the Code of Federal Regulations (21 CFR Part 58).

The dose vehicle contains 20 mM histidine, 4% mannitol, 1% sucrose, 0.9% sodium chloride, and 0.01% polysorbate 80, pH 6.5 in purified miliQ water; the same vehicle was used for all the animal studies.

Cynomolgus monkeys (Charles River Laboratories) (3–5 years old with mean body weight of 4.2 kg) were administrated with different intravenous (IV) treatments of FXa^{I16L} including the following: (1) a single dose treatment (0.5 mg/kg) ($n = 3$), (2) twice a day (BID) daily administration for 3 days (0.05, 0.1, and 0.5 mg/kg/day) ($n = 6$/group), (3) daily BID for 28 days (0.3, 1, and 3 mg/kg/day) ($n = 6$/group for 0.3 and 3 mg/kg/day, $n = 12$ for 1 mg/kg/day; 50% males and 50% females).

CD1 mice (Charles River Laboratories, Portage, MI, USA) (6–7 weeks old, and body weights ranged from 27 to 39 g) were treated with vehicle alone or different IV doses of FXa^{I16L}: (A) BID for 3 days (0.04, 0.12, and 0.4 mg/kg/day), (B) BID for 7 days (2.0 mg/kg/day), (C) BID for 28 days (0.2, 0.6). Plasma samples (sparse sampling) were collected (terminal bled, $n = 6$/time point, 3 males, 3 females) on the last treatment day; day 28 post the last administration ADA analysis.

Wistar-Han rats (Crl:WI (Han)) (Charles River Laboratories, Raleigh, NC, USA) (7–8 weeks old and body weights ranged from 170 to 300 g) were treated with IV doses of

FXa^{I16L}, BID for 3 days (0.03, 0.1, and 0.3 mg/kg/day) ($n = 6$/time point, sparse sampling, 3 males, 3 females).

Hemophilia dogs (University of North Carolina, NC, USA) were treated with three repeated dosing of FXa^{I16L} 0.005, 0.010, and 0.025 mg/kg (3 days apart between each administration) ($n = 3$/group) (serial sampling), and ADA samples were collected on days 8, 15, 29, and 45 after the first treatment.

The summarized treatment and ADA sampling time are described in Table I. QD indicated a once-a-day treatment. BID indicated twice-a-day treatment. All the BID treatments were conducted twice a day, 8 h apart. The blood was collected in tubes containing 3.2% sodium citrate solution, and the plasma was isolated by centrifugation at 3000*g* for 10 min and stored in − 80°C. The pharmacokinetic samples were processed as described before (14).

ADA Quantification

ADA samples were collected during and after treatment as described in Table I**.** Anti-FXa^{I16L} antibodies were measured in sodium citrate plasma samples using validated electrochemiluminescence (ECL) assays in monkeys, mice, and rats (in-house methods). In these assays, ADA was captured by biotinylated FXa^{I16L} and detected by ruthenium-labeled anti-FXa^{I16L}. Tripropylamine-containing buffer was used to generate chemiluminescent signals which were measured in relative light unit (RLU) on SECTOR Imager 6000 instrument (Meso Scale Diagnostics, Rockville, MD, USA). Data was acquired using Discovery WorkbenchTM 2006 MSD program (Meso Scale Diagnostics). Protein-A purified rabbit anti-FXa^{I16L} antibodies were used as a positive control (PC). Naïve plasma from untreated normal animals was used as a negative control (NC). The relative light units (RLU) of NC were used to calculate the plate cut point (discrimination point). Any sample from monkey, mouse, and rat generating signals greater than or equal to the plate cut point was reanalyzed further in a full dilution series to confirm the positive result and determine the anti-drug antibody titer. The antibody titer (log (base 10)) is defined as the reciprocal dilution of the sample that would generate an RLU equal to the cutpoint RLU. The estimated sensitivity of ADA assays was 21.9 ng/mL, 15.2 ng/mL, and 22 ng/mL for monkey, mouse, and rat respectively. The recovery of ADA detection in positive spike-in plasma was > 75%. Dog samples were only tested in an ADA screen assay and raw RLU was reported. In monkey, rat, and dog, the final assessment of immune response for each animal was based on the comparison of the pre-dose and post-dose sample results. In mouse, the group means were used for ADA comparison. The induction rate was determined as, (the number of ADA positive animals /total tested animals in the same treatment group) × 100%. A transient ADA response is defined as there was no detectable ADA at the last sampling time in the study. A persistent ADA response is defined as there was detectable ADA at the last sampling time.

Pharmacokinetics Assays

The Na-citrate plasma samples were collected 5, 15, 60, and 240 min after intravenous administration of FXa^{I16L}. Plasma concentration of active FXa^{I16L} was quantified using validated enzyme-linked immunosorbent assay (ELISA) as described before (14). A goat IgG antibody against human factor X (Cedarlane, ON, Canada) was used as the captured antibody for FXa^{I16L} and a biotin-labeled factor Xa inhibitor (EGRCK, Glu-Gly-Arg-chloromethyl ketone) (Haemtech, Essex Junction, VT, USA) was used for detection. This method only detected FXa^{I16L} with free active catalytic domain. One part of Na-citrated plasma samples was diluted with nine parts of assay buffer containing 1% bovine serum albumin, 0.05% proclin, and 0.05% polysorbate 20 in phosphate-buffered saline to a final concentration of 10%. Sample concentrations in the 10% plasma were determined by interpolation from the calibration curves that was fit using a 4-parameter logistic regression model with a weighting factor of $1/y^2$ using Watson laboratory information management system (Watson LIMS). The low limit of quantitation (LLQ) of PK assay was 0.781 ng/mL, 6.25 ng/mL, and 0.195 ng/mL for 10% monkey, mouse, and rat plasma samples respectively. Plasma pharmacokinetics was

Table I. Animal Treatment and ADA Sample Study Time

Summary of human recombinant FXa^{I16L} treatment in nonclinical species

Species	Treatment	Dose (mg/kg/day)	Exposure day	Date of ADA sample analysis
Monkey	QD[3]	0.5	1	Pretreatment, days 7, 14, 21, 28
	BID[4]	0.05, 0.1, 0.5	3	Pretreatment, days 18, 31
	BID	0.3, 1, 3	28	Pretreatment, days 14, 23, 28, 56
Mouse[1, 2]	BID	0.04, 0.12, 0.4	3	Vehicle treatment, days 17, 31
	BID	2	7	Vehicle treatment, days 9, 37
	BID	0.2, 0.6	28	Vehicle treatment, days 28, 56
Rat[1, 2]	QD	0.03, 0.1 and 0.3	3	Vehicle treatment, days 17, 31
Dog	QD	0.025	3	Pretreatment, days 8, 15, 22, 29, 45

[1] Spare sampling was used for mouse and rat, and a vehicle-treated group was used as the negative control
[2] ADA and PD were examined in different animals, group means were used for assessment
[3] QD: once a day treatment
[4] BID: twice a day treatment, 8 h apart between administrations
The daily dosing frequency, exposure day, dose, and ADA sample collection time used in monkeys, mice, rats, and dogs are listed. These studies were designed for pharmacological or toxicological studies and were not specific to ADA studies. Exposure day is defined as the number of days animals have received FXa^{I16L} treatment. 1, 3, 7, and 28 exposure days were used in the studies

analyzed using Phoenix WinNonlin 6.3 (Certara, NJ, USA). Pharmacokinetic parameters including the maximum observed concentration (Cmax) (ng/mL) and area under the curve (AUC) (ngX hr/mL) were estimated. The exposure day was defined as a day in which one or more replacement treatment was given as described previously (7). The cumulative exposure was calculated as (daily AUC × exposure day).

Activated Partial Thromboplastin Time Assay

Activated partial thromboplastin time (APTT) plasma samples were collected during pre-dose phase and approximately 30 min after compound administration. APTT assay was performed using C.K. PREST® reagent (Diagnostica Stago Inc. Parsippany, NJ) according to manufacturer's instruction. Fifty microliters of plasma sample was incubated with 50 μL of APTT for 3 min at 37°C. The APTT reaction was initiated immediately with the addition of 50 μL of 25 mM calcium chloride The time to clot was measured at 37°C using a STart®4 Coagulation Analyzer (Diagnostica Stago, Parsippany, NJ).

Prothrombin Time Measurement

Prothrombin time measurement (PT) plasma samples were collected during pre-dose phase and approximately 15 min after compound administration. For the PT test, 200 μl of the PT reagent (Dade® Innovin, Siemen Healthcare Diagnostics, Deerfield, IL) was added to 100ul citrate plasma and incubated at 37°C for 1 min. The time (in seconds) to clot was measured at 37°C using a STart®4 Coagulation Analyzer.

General Data Analysis

Plasma pharmacokinetics analysis was conducted using Phoenix WinNonlin 6.3 (Certara, Princeton, NJ, USA) as described before (14). Mean mouse PK parameters and individual monkey and rat mean PK parameters were determined by a noncompartment model. Model selection was based on goodness of fit, visual assessment of residual plots, and coefficient of variation. The maximum observed concentration (Cmax), area under the curve from time zero to the last observed concentration (AUClast), clearance (CL), and half-life ($T_{1/2}$) were described. The pharmacokinetics stimulation for a single treatment or 28-day repeat treatment was conducted using the estimated volume of distribution and clearance in monkey.

The statistical analysis was conducted to compare pharmacokinetics and pharmacodynamics between ADA− and ADA+ animals on day 28 (D28) and the results between day 1 (D1) and day 28 in the ADA+ or ADA− groups using unpaired Student's *t* test (GraphPad Prism, San Diego, CA, USA), significance was considered when $P < 0.05$.

The ADA titer data over time was analyzed using polynomial curve fit (GraphPad Prism, San Diego, CA, USA). The method was selected based on the goodness of fit and visual examination of data distribution. To achieve optimal fitting, a third order polynomial fit ($y = ax^3 + bx^2 + cx + d$) was used for monkey data and a second order polynomial fit ($y = ax^2 + bx + C$) was used for mouse and rat data.

RESULTS

FX Protein Sequence Homology Between Species

The protein sequence homology of FX preproprotein between preclinical species and human was compared based on their deduced amino acid sequences. The propeptide is cleaved prior to secretion and FX circulates as inactive zymogen, FX is converted to mature two-chain active FX (FXa) by the excision of tripeptide RKR (arginine-lysine-arginine). The mature FXa contains a light chain (AA41–179) and a heavy chain (AA183–488) linked by one or more disulfide bonds. The IVGGQ (isoleucine-valine-glycine-glycine-glutamic acid) (AA235–239) is the amino terminal for active FX heavy chain after cleavage (16). The amino acid sequence alignment for FX in human, monkey, mouse, rat, and dog (are shown in Fig. 1). A conservative sequence homology was observed for FX between species. It is estimated that approximately 94, 78, 76, and 77% amino acid identity was observed for human FX counterpart in monkey, mouse, rat, and dog respectively. One of the most diverse regions is the activation peptide between AA183–AA234 in the heavy chain that is excised off to generate active FX. The other diverse region is located in the carboxyl terminus of heavy chain (AA470–488) and is present in active FX. FXa^{I16L} has a single amino change on the AA235 position.

FXa^{I16L}-Induced ADA Responses in Monkeys

ADA induction time course by FXa^{I16L} was examined in monkeys after 1, 3, and 28 exposure days. No induction of ADA was observed in monkeys after one or three exposure days (data not shown). In contrast, ADA induction was observed in 28 exposure day group (Fig. 2). Three of six monkeys (50%) in 0.3 mg/kg group, eight of 12 monkeys (67%) in 1 mg/kg/day group, and two of six monkeys in 3 mg/kg/day group tested positive for ADA. In the 0.3 and 3 mg/kg/day groups, the titer decreased after treatment stopped and was undetectable on day 56 indicating a transient ADA response (Fig. 2a, c). A mixed titer response was observed for 1 mg/kg/day group, three/eight of these ADA-positive monkeys had transient ADA induction with an undetectable ADA on day 56, while, five/eight exhibited persistent ADA response with increasing ADA titers on day 56. A third order polynomial curve was used to fit the overall titer overtime data and the results are shown in Fig. 2d. Transient ADA responses were predicted after 28 exposure days and the maximum titer response was predicted to be around 40 days after these treatments in a trend analysis.

The pharmacokinetics of FXa^{I16L} has been reported previously, it follows a fast first-order clearance (14). Here, we examined if there was any difference on pharmacokinetic parameters between ADA− and ADA+ monkeys. The pharmacokinetic curves from both ADA− and ADA+ on day 1 and day 28 after 1 mg/kg/day treatment are shown in Fig. 3 a and b, and the pharmacokinetic parameters are summarized in Table II. No significant difference on PK between day 1 and day 28 from 1 mg/kg/day ADA− group

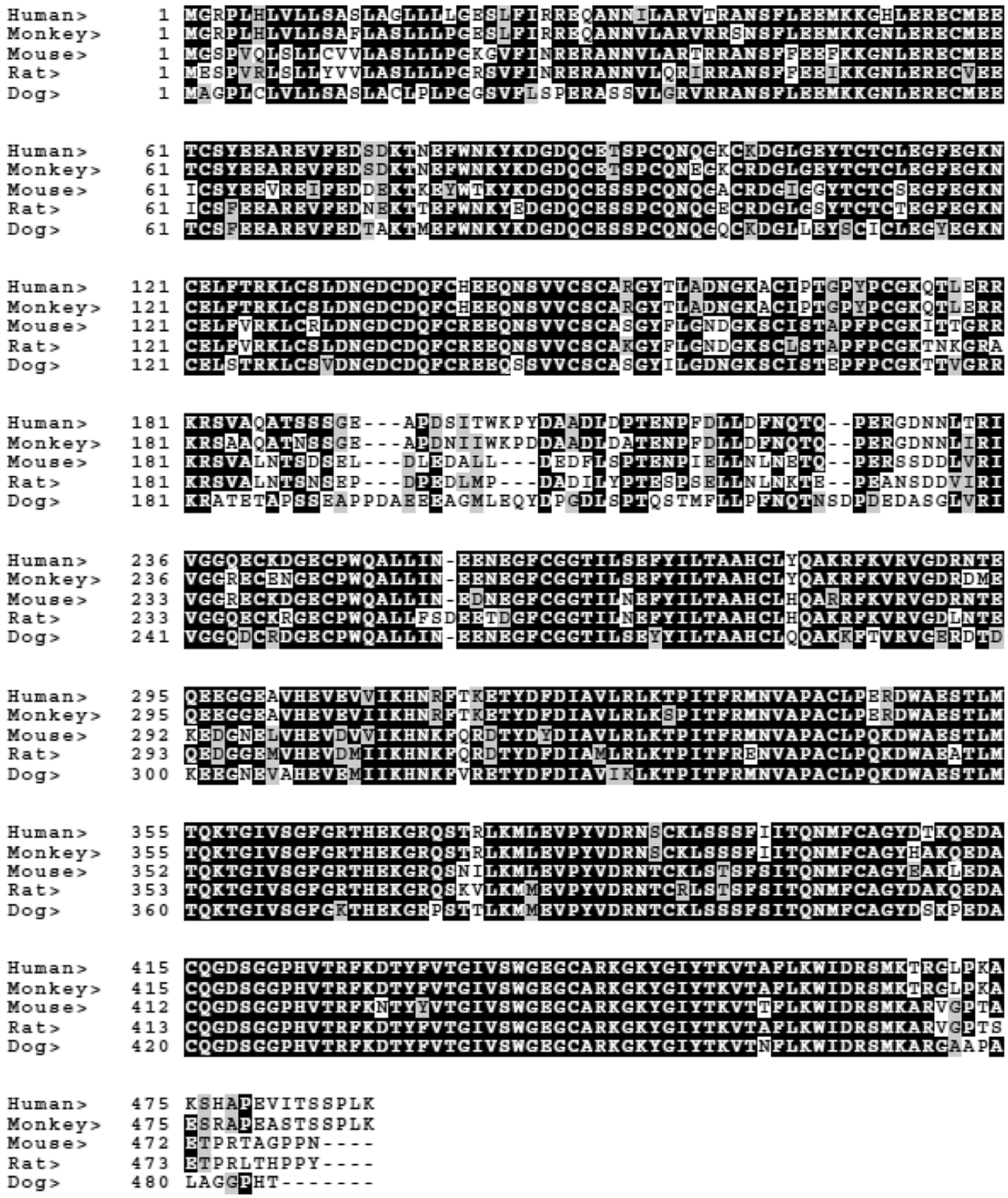

Fig. 1. The amino acid sequence alignment of FX from different species. The monkey, mouse, rat, and dog FX has 94, 78, 76, and 77% amino acid identity when compared to human FX. FX is converted to the active two-chain FXa by the excision of tripeptide RKR and the activation peptide

(Fig. 3a), however, increase in exposure was observed for most ADA+ animals (Fig. 3b). The ADA+ animals had significant increase in the systemic exposure (Cmax and AUC) on day 28 compared to their day 1 exposure or compared to day 28 exposure of ADA− animals ($P < 0.05$) (Cmax shown in Fig. 3c). The ADA+ animals also display significantly decreased clearance (CL) on day 28 when compared to day 0 but the decrease was not statistically significant when compared to the clearance from ADA− day 28. No significant ADA− effects on half-life were observed. The titer/Cmax relationship was further assessed in Fig. 3d. Increasing ADA titers (> 2) in high-dose groups resulted in a large increase in exposure. No significant effect on APTT or PT was observed in ADA+ monkeys after 0.3 mg/kg/day or 1 mg/kg/day treatment using group mean (Fig. 3e). However, when APT and PT were examined in individual monkeys (Fig. 4), marked shortening of APTT and PT were observed in some animals (5075, 5089, 5091) (Fig. 4f, g) and no effects

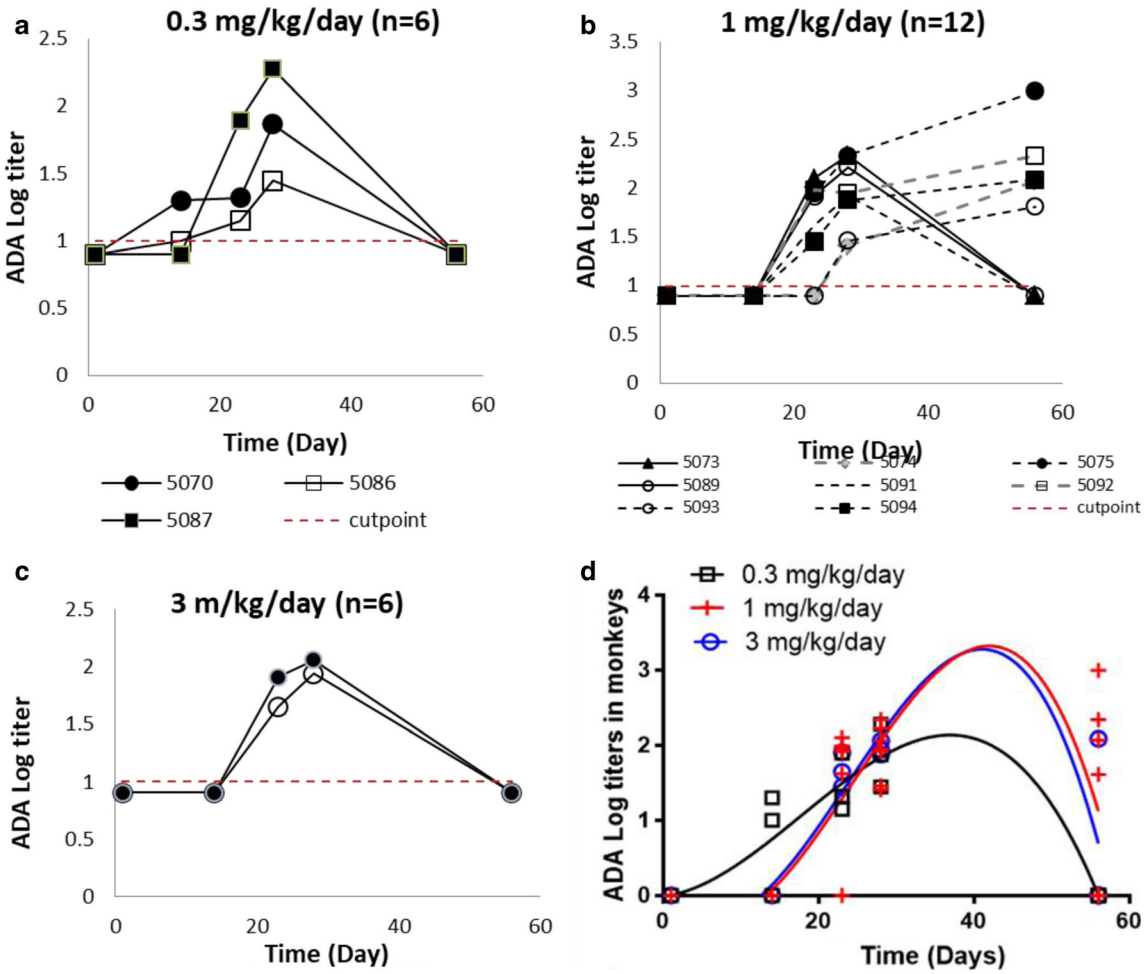

Fig. 2. ADA induction time course of FXa^{I16L} in monkeys during and after 28 exposure days. ADA was examined in monkeys during and after 28-daily BID treatment of FXa^{I16L} at **a** 0.3 ($n = 6$), **b** 1 ($n = 12$), and **c** 3 ($n = 6$) mg/kg/day for 28 days. Transient ADA response was observed in 0.3 and 3 mg/kg/day group. Both transient and persistent ADA responses were observed in 1 mg/kg/day group. The overall ADA induction rate was 50%, 67%, and 33% for 0.3, 1, and 3 mg/kg/day groups respectively. The ADA titers varied from 1 ~ 3. The highest titers were observed for persistent ADA during recovery phase in mid-dose group. **d** A polynomial curve fit was used to describe the data. The regression assessment for 0.3, 1, and 3 mg/kg group was 0.84, 0.58, and 0.74, respectively. The peak ADA titer was estimated to be around 40 days and all the ADA responses are transient

were observed in most ADA+ animals. The degree of effect was not dependent on the exposure or ADA titers indicating that these ADA may recognize different epitopes. The maximum degree of shortening effects was about 25% in the ADA+ monkeys.

Prolonged APTT and PT were observed on day 28 in two ADA+ monkeys treated with 3 mg/kg/day of FXa^{I16L}. When the overall plasma levels of fibrinogen and coagulation factors were examined in these two monkeys with prolonged clotting time, they had declined ADA titers, no/or very low fibrinogen, no/or low levels of coagulation factors, and excess bleeding occurred (data not shown) indicating occurrence of coagulopathy. Similarly, pharmacology-induced coagulopathy was also observed in some ADA− animals on day 7 after 3 mg/kg/day dosing, hence, this prolongation of blood clotting was considered as exaggerated pharmacological effects from repeated daily high-dose treatment and it was not ADA-related. No further neutralizing antibody testing for cross-reactivity against endogenous FXa was conducted.

FXa^{I16L}-Induced ADA Responses in Mice

The ADA induction time course in mice was examined for 3, 7, and 28 exposure days and the changes of ADA titers over time are shown in Fig. 4. Mice were treated with vehicle or BID dosing of FXa^{I16L} at 0.04, 0.12, and 0.4 mg/kg/day for 3 days, the development of ADA was examined 2 weeks (day 18) and 4 weeks (day 32) later after treatment ($n = 6$ per time point). After three exposure days, no ADA was detected in the vehicle group and most of the treatment group; the only ADA detected was in one of the animals (1/24; 4%) with 0.4 mg/kg/day treatment (Fig. 5a). After nine exposure days, 10 of 24 mice (42%) had ADA induction (2 mg/kg/day) and the mean ADA titer increased during recovery period. After 28 exposure days, the ADA induction was 6/11 (55%) and 11/12 (92%) in the 0.2 and 0.6 mg/kg/day groups, and the ADA titers increased during recovery (Fig. 5b). A second-order polynomial fit (quadratic) was conducted for these titer-time data, and the R-square for goodness of fit was ranged from 0.71–0.92. Based on the fitting

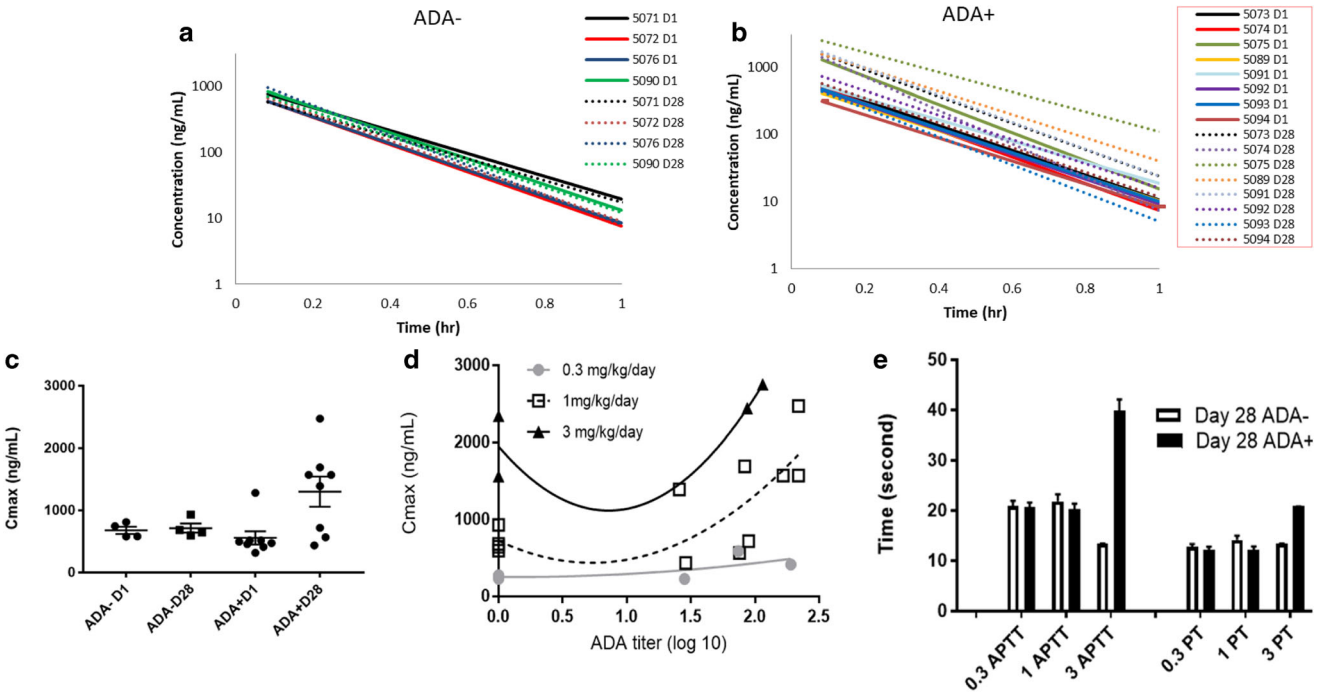

Fig. 3. Mean ADA effects on pharmacokinetics and pharmacodynamics of FXa^{I16L} in monkeys on day 1 and day 28. The pharmacokinetic curves for individual ADA− and ADA+ monkeys on day 1 and day 28 are shown in (**a**, **b**). ADA+ animals had higher drug concentration on day 28 when compared to day 1. **c** ADA effects on Cmax. Some marked changes in individual Cmax for ADA+ monkeys on day 28 were observed. **d** The relationship between ADA titer and exposure. Greater ADA titer (>2) animals had markedly increased in Cmax. **e** The ADA effects on blood clotting. The mean shortening of clotting times including APTT and PT were not changed by ADA in the majority of monkeys. Except for two monkeys at 3 mg/kg/day group had prolonged APTT and PT

curves, transient ADA responses are predicted for three and nine exposure day `groups, and also the 28 exposure day group with 0.2 g/kg/day treatment. No ADA saturation was observed for the 0.6 g/kg/day group. When the mean drug exposure was examined on day 1 and day 28 for the 0.6 mg/kg/day group and a significant increase in Cmax was observed on day 28 when ADA presented (Fig. 5c). No significant difference was observed in the clotting time in the 0.6 mg/kg ADA+ group compared to vehicle-treated mice at day 28 or day 56 (Fig. 5d).

FXa^{I16L}-Induced ADA Responses in Rats

The ADA induction time course in rats after three exposure days was examined and the change of ADA titers over time are shown in Fig. 6. Rats were treated with vehicle or a single dose of FXa^{I16L} at 0.03 (A), 0.1 (B), and 0.3 (C) mg/kg/day for 3 days, the development of ADA was examined 2 weeks (day 18) and 4 weeks (day 32) after the last exposure day ($n = 6$ per time point). No ADA was detected in vehicle group. One of six rats in 0.03 mg/kg/day group had detectable ADA on day 32. One of the six rats (17%) in 0.1 mg/kg/day group had detectable ADA on both day 18 and day 32. However, five of six rats in 0.3 mg/kg/day group had detectable ADA on day 18 (titer 2.6–3.12) and six of six had detectable ADA on day 32 (titer 2.29–4.06); the induction rate for 0.3 mg/kg/day group was 100%. When a second-order polynomial fit was used to assess the ADA titer-time data, transient ADA responses were predicted for 0.1 and 0.3 mg/kg/day group (Fig. 6a), no ADA saturation was predicted for 0.03 mg/kg/day group within 56 days. The

Table II. Pharmacokinetic Comparison Between ADA− and ADA+ Monkeys

1 mg/kg/day	Cmax (ng/mL)	AUC (ng*hr./mL)	T1/2 (hr)	CL (mL/h/kg)
ADA− D1	679 ± 96	288 ± 43	0.15 ± 0.02	3531 ± 573
ADA− D28	715 ± 314	310 ± 125	0.15 ± 0.04	3262 ± 1745
ADA+ D1	558 ± 299	262 ± 126	0.16 ± 0.01	4279 ± 1392
ADA+ D28	1302 ± 686$^{*\#}$	644 ± 370$^{*\#}$	0.15 ± 0.03	2286 ± 1678*

ADA−, ADA negative during entire treatment; *ADA+*, ADA positive was detected at day 28; *D1*, day 1; *D28*, day 28; *Cmax,* maximum observed concentration; *AUC*, area under curve; *T1/2*, half-life; *CL,* clearance

$^{*}P < 0.05$ when compared to ADA+ D1

$^{\#}P < 0.05$ when compared to ADA− D28

Pharmacokinetics of 1 mg/kg/day treatment group was conducted using a noncompartmental analysis. The observed maximum concentration (Cmax), area under the curve (AUC), clearance (CL), and half-life ($T_{1/2}$) are presented in the table. Statistically significant increase in Cmax, AUC, and decrease in clearance were observed for ADA+ animal on day 28

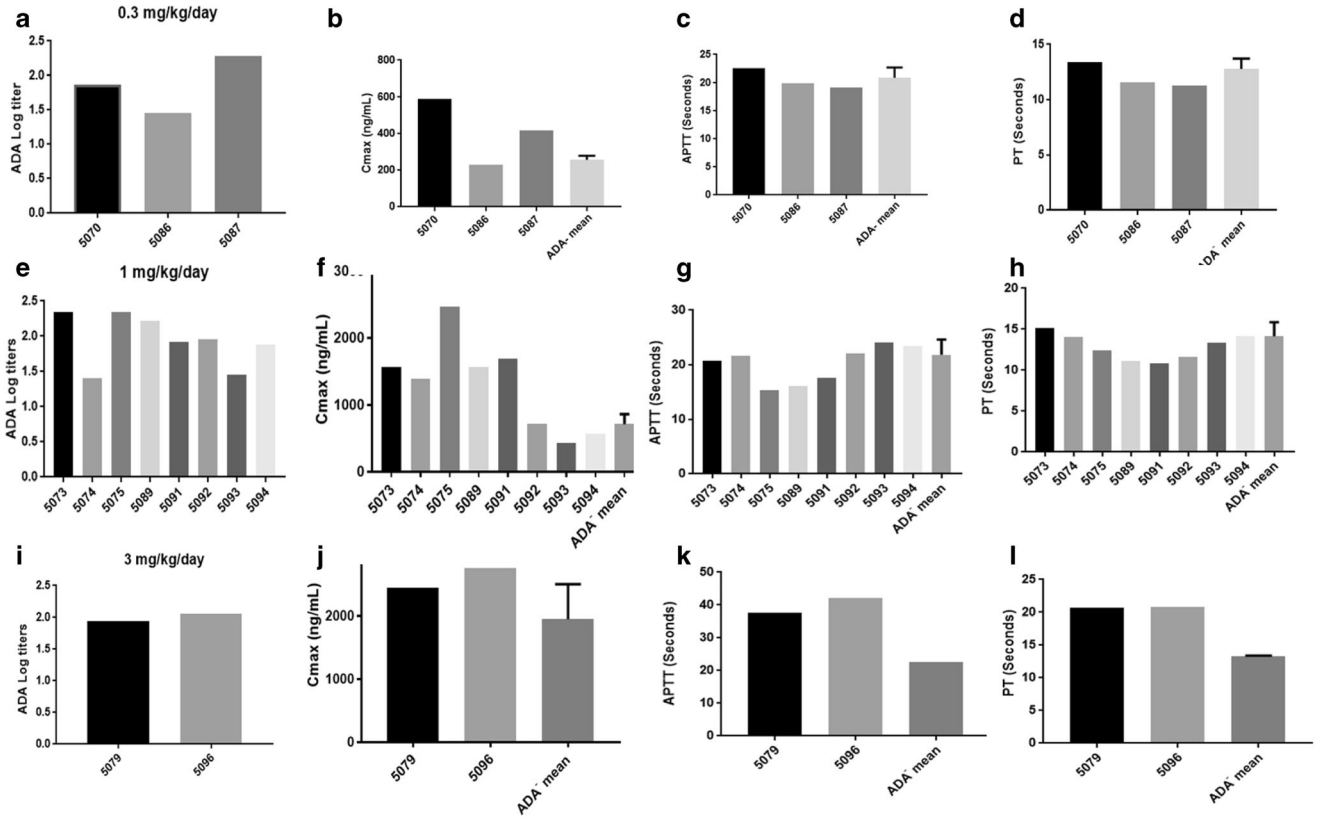

Fig. 4. Individual ADA effects on pharmacokinetics and pharmacodynamics of FXa^{I16L} in monkeys on day 1 and day 28. All dose groups induced a similar range of ADA titer (1.5–2.4), but had different ADA effects on PK and PD. **a**, **b** In the 0.3 mg/kg/day group, two monkeys (#5079, #5087) displayed 2–3× increase in exposure whereas, the other monkey (#5086) had no effect on the exposure, and all the monkeys had similar ADA titers. **c**, **d** The three ADA+ monkeys in the low-dose group showed no marked effect on hemostatic PD markers, APTT and PT. **e**, **f** In the 1 mg/kg/day group, ADA increased drug exposure (1–3×) in five ADA+ animals and had no effect in three ADA+ animals (5092, 5093, and 5094). **g**, **h** A slight shortening of APTT and PT were observed in three of the eight ADA+ animals (5075, 5089, and 5091). **i**, **j** In the 3 mg/kg/day group, no marked increase in exposure was observed in the two ADA+ animals, however, **k**, **l** marked prolongation in both of APTT and PT were observed in ADA+ animals

pharmacodynamics was further examined in the 0.3 mg/kg/day group and no difference in APTT and PT was observed in 0.3 mg/kg/day group between day 4 (no ADA induction) and day 32 (100% ADA+) (Fig. 6b).

FXa^{I16L}-Induced ADA Responses in Dogs

ADAs were examined for three hemophilia A dogs on days 8, 15, 22, 29, and 45 after three exposure days and the raw data expressed as RLU are shown in Fig. 7a. The highest response was observed in one of the three hemophilia dogs on day 15 and the titer declined during the recovery period indicating a transient ADA response.

Exposure/ADA Relationship

To compare results between species, the data in this study are summarized in Table III. In general, ADA incidence increased with increasing FXa^{I16L} exposure day. To investigate potential effect of drug exposure on ADA, the relationship between ADA incidence and cumulative exposure was determined and shown in Fig. 7b. The cumulative exposure is estimated based on the exposure and exposure days, the daily exposure is equivalent to steady state exposure due to the short compound half-life. Higher exposure of FXa^{I16L} was observed in monkey studies when compared to exposure observed in mouse, rat, and dog studies. Monkeys had cumulative exposure ranging approximately from 70 to 23,000 ng·hr/mL in the study. No ADA induction was observed in monkeys at the low cumulative exposure (< 1000 ng·hr/mL), and ADA was induced with increasing cumulative exposure (> 1000 ng·hr/mL) and reached a plateau around 7600 ng·hr/mL. In contrast, ADA was induced in mice, rats, and dogs at a lower cumulative exposure range (3–400 ng hr/mL).

DISCUSSION

Because human coagulation factor Xa plays a critical role in regulation of the coagulation cascade, the neutralization of endogenous counterpart would cause devastating consequence in patients receiving treatment. Development of ADA against FXa^{I16L} or FXa in preclinical species was warranted due to the foreign amino acid sequence in human proteins that are aberrant from the endogenous FX and FXa in preclinical species. The number of foreign epitopes in FXa^{I16L} plays a role in determining the compound exposure requirement for ADA induction in the host. This notion is supported by the observations that the monkey with highest protein homology of human FXa^{I16L} required higher FXa^{I16L} exposure to elicit ADA response. In addition, administered FXa^{I16L} which presented

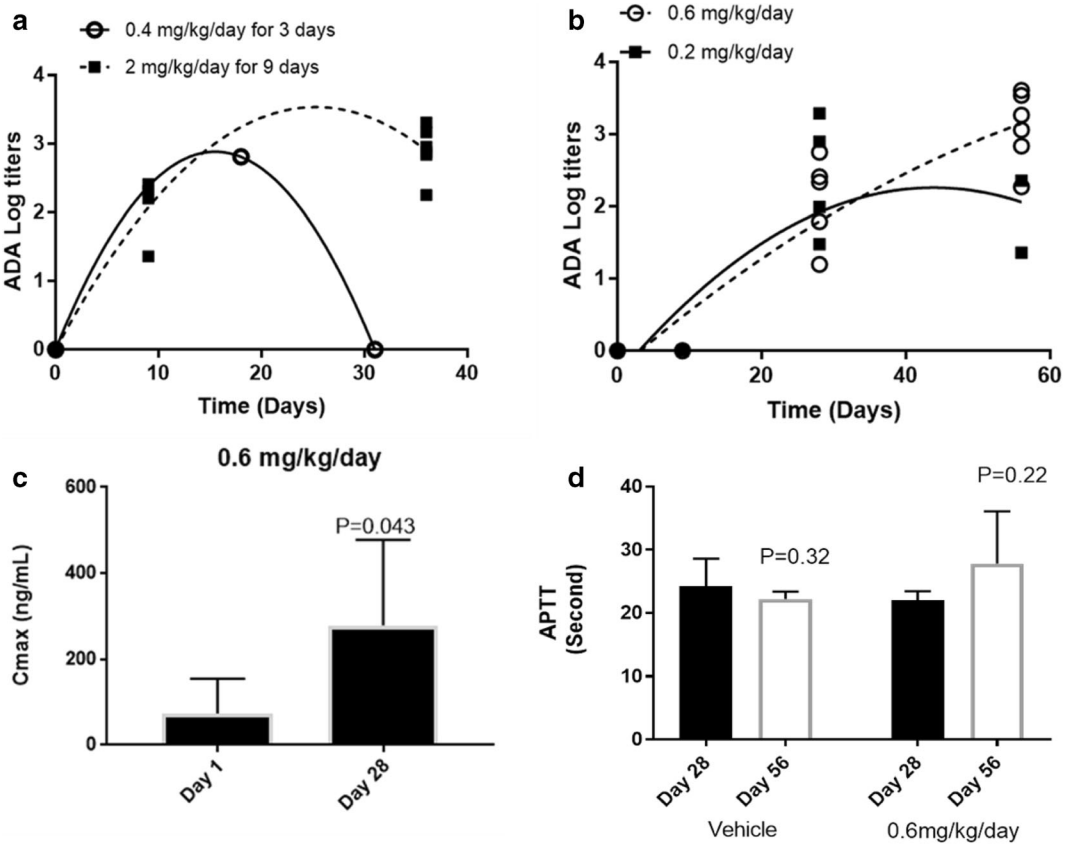

Fig. 5. ADA induction time course and effects on pharmacokinetics and pharmacodynamics of FXa^{I16L} in mice. The ADA induction time course was characterized in mice ($n = 6$) after 3 or 28 exposure days and during recovery (day 56) at different doses: **a** 0.4 mg/kg/day for 3 days, 2 mg/kg/day for 9 days (R-square = 0.95), and **b** 0.2 and 0.6 mg/kg/day for 28 days (R-square = 0.85 and 0.91) using a second-order polynomial fit. The ADA effects on **c** Cmax and **d** APTT in 0.6 mg/kg/day group were examined. The ADA+ mice had increasing Cmax but no effects on APTT when compared to ADA− mice

similar numbers of foreign amino acids in similar regions in mice, rats, and dogs, induced similar ADA responses at comparable cumulative exposure in this study.

Effects of FXa^{I16L}-induced ADA varied between animals. Some animals with higher tiers of ADA had no effects on PK or PD. Increased Cmax, AUC, and decreased clearance were observed in some ADA+ monkeys indicating that ADA-FXa^{I16L} complex may increase FXa^{I16L} exposure. Interestingly, although antibody has much longer half-life when compared to FXa (less than 15 min), no significant increase in half-life of FXa^{I16L} was observed in ADA+ animals indicating that the overall elimination of FXa^{I16L}-ADA complex is similar to free FXa^{I16L} and different from antibody. It is known that FXa^{I16L} can be eliminated through

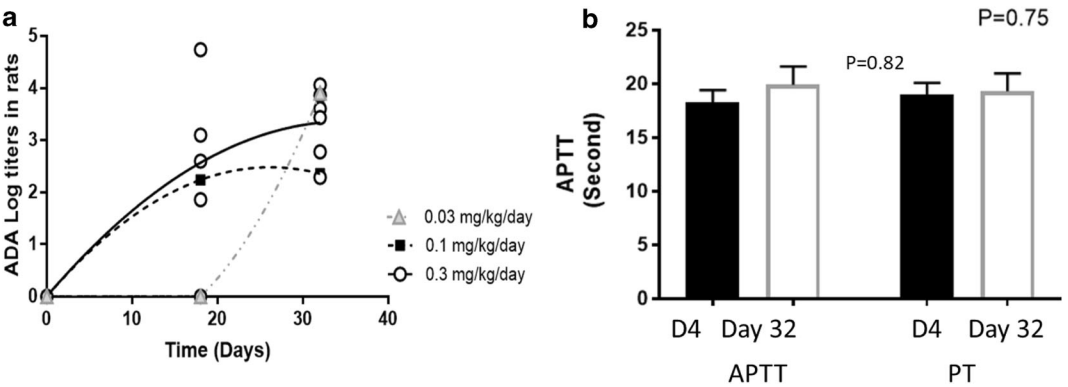

Fig. 6. ADA induction time course and effects on pharmacokinetics and pharmacodynamics of FXa^{I16L} in rats. **a** The ADA induction time course were characterized in rats ($n = 6$) after three exposure days with 0.03, 0.1, and 0.3 mg/kg/day dosing of FXa^{I16L} using a second-order polynomial fit (R-square = 0.71). The ADA incidence increased with increasing doses; 0.3 mg/kg/day group were 100% ADA+. **b** The ADA effects on APTT in 0.3 mg/kg/day group were examined. ADA was present on day 32 but not day 4 and it did not affect APTT or PT

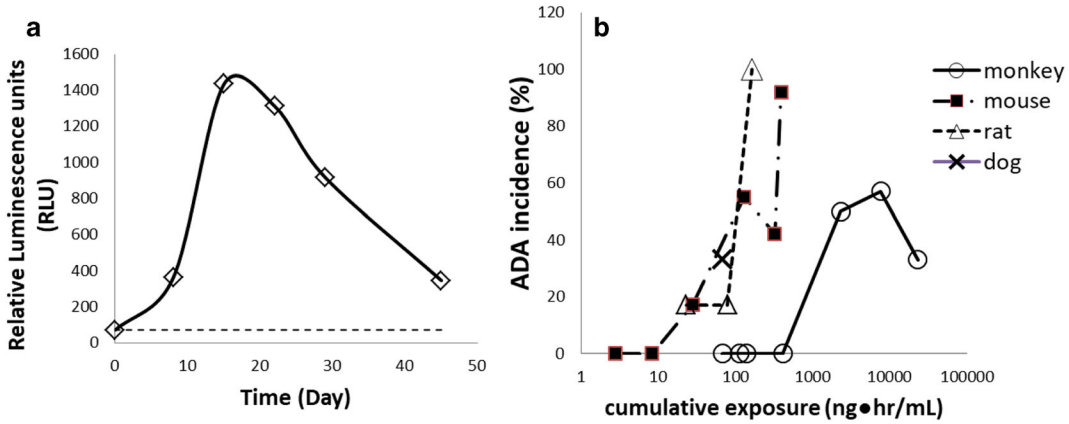

Fig. 7. **a** ADA induction time course in dogs. The ADA induction time course was characterized in dogs ($n = 3$) after three exposure days with 0.025 mg/kg of FXa^{I16L} and during recovery days. A transient ADA response was observed after treatment. **b** The relationship between ADA incidence and cumulative exposure was characterized using data collected from monkey, mouse, rat, and dog studies. The ADA induction occurred in similar exposure levels for mouse, rat, and dog. A higher cumulative exposure was needed to induce ADA response in monkeys

different protein-protein interactions and serine protease (14). It is likely that anti-drug antibody only prevents interaction of FXa^{I16L} to minor clearance machinery and the major clearance pathway is still active. Overall, less than twofold decrease in mean clearance and less than twofold increase in mean AUC was observed in ADA+ monkeys. The most significant difference was observed for Cmax. In addition, the pharmacological function of FXa^{I16L} (shortening of clotting time) has been shown to be mainly driven by Cmax (15,16), therefore Cmax was used to correlate ADA effects on exposure. The PK assay conducted for the study was using a conjugated active-site probe of FXa; no observed ADA-induced decrease in PK indicates that ADA did not interact with catalytic site of FXa^{I16L}. In addition, the potential formation of nAb was assessed by ADA effects on APTT and PT based on the notion that nAb of FXa^{I16L} would decrease FXa^{I16L} activity and prolong clotting time. No changes or slightly increase in clotting activity were observed in most ADA+ animals indicating that no neutralizing activity was observed and ADA has the potential to increase clotting activity. Except in two ADA-affected monkeys that had excessive bleeding and prolonged APTT and PT on day 28 and for the meantime, they had undetectable level of coagulation factors and cofactors which were characterized as pharmacological-related coagulopathy after daily repeated administration. Similar exaggerated pharmacology-induced coagulopathy was observed in two of the ADA− monkeys in high-dose group on day 7. No neutralizing antibody assay for cross-reactivity against endogenous FXa was conducted in this study.

Various PK effect models had been tested to fit the ADA-drug concentration and ADA-time data, however, it was challenging to obtain reasonable fitting between species and between doses using the same model. The polynomial

Table III. Summary of FXa^{I16L}-Induced ADA Response and ADA Consequence in Nonclinical Species

Summary of human recombinant FXa^{I16L} -induced ADA response in nonclinical species				
Exposure day	Species	Overall ADA incidence %	Impact on exposure	Impact on clotting activity
1	Monkey	0		
3	Monkey	0		
	Mouse	17 (0.4, T)[1]		
	Rat	17 (0.03, T), 17 (0.1, P), 100 (0.3, P)	ND	NO
	Dog	33 (0.025, T)	No	No
7	Mouse	42 (2, P)		
28	Monkey	50 (0.3, T), 67 (1 T&P), 33 (3, T)[2]	No or increase	No or decrease
	Mouse	55 (0.2, P), 92 (0.6, P)	Increase	No

ND, not determined; *No*, no change
[1] The number in the parentheses is the IV dose (mg/kg/day)
*P*Indicates persistent ADA
*T*Indicates a transient ADA
*T&P*Indicates mix of transient and persistent ADA observed in the group
[2] Decrease in clotting activity was observed in monkey 3 mg/kg/day group which had coagulopathy with no detectable fibrinogen and very low platelet count and they were identified to be not caused by nAb
Overall ADA incidence after of FXa^{I16L} treatment and ADA impact on FXa^{I16L} *exposure and clotting activity are summarized in Table II. After three exposure days, rats had the highest ADA incidence. Monkeys exhibited both transient and persistent ADA responses against* FXa^{I16L} *but rodents had only persistent ADA at the time point examined. Some ADA positive animals had increasing* FXa^{I16L} *exposure and had increasing clotting activity*

data fit was presented based on its capacity to describe the ADA-time data at mean R-square value of 0.85 in mouse and rat and at mean R-square value of 0.72 in monkey. This type of curve fit predicted that transient ADA responses were induced by FXa^{I16L} in monkeys and rodents.

Dose level and exposure time have played critical roles in mediating ADA development against FXa^{I16L} in nonclinical species consistent with previous observations for FVIII products. No ADA induction occurred after a single exposure day or three exposure days in monkeys and a transient ADA response occurred in dogs after three exposure days. Rodents had persistent ADA responses after daily dosing for 3, 7, or 28 days. Increase in exposure days was associated with stronger ADA responses supporting the notion that T cell activation is required to enhance production and affinity maturation of antibody by B cells (17). An increase in dose was generally associated with higher ADA incidences for FXa^{I16L}. The only exception was that monkeys in the high-dose group after 28 exposure days had decreased ADA incidence and titers during recovery period. Due to the short plasma half-life of FXa^{I16L}, there was no FXa^{I16L} present in the circulation during recovery to cause false-negative ADA. The decrease in ADA can be explained by the occurrence of a potential immune tolerance that diminishes ADA in monkeys after repeated administration of FXa^{I16L} at 3 mg/kg/day. Compared to FVIII products in which almost 100% ADA response was observed in animals (data not shown), the induction of ADA incidences by FXa^{I16L} was relatively lower. This may be caused by low accumulative exposure and a shorter half-life for FXa^{I16L} (2–15 min) when compared to FVIII (1–3 h).

A correlation between accumulative exposure and ADA induction was established in the study. No ADA was induced in monkeys at the cumulative exposure was less than 1000 ng·hr/mL. In human, FXa^{I16L} induced a low titer transient clinical ADA response in one of the subjects from phase I trial (3 μg/kg cohort) after a single administration (12). The long-term effects on immunogenicity after repeated dosing of FXa^{I16L} have not been investigated. Shortening of dosing intervals has been implicated to suppress formation of ADA for infliximab (18). High-dose immune tolerance induction is being used in hemophilia patients with inhibitors (6). On the other hand, low dose and longer dosing interval have been also reported to reduce inhibitor formation in hemophilia prophylaxis (19). To mitigate the immunogenicity risk of FXa^{I16L} in the chronic treatment, changes in dose and dosing interval may be considered. A potential immunal tolerance was observed in monkeys after daily high-dose treatment, in addition, transient ADA was observed after recovery periods with low-dose treatments. Based on high potency of FXa (~ 1 ng/mL) and FXa^{I16L} (1 ~ 5 μg/kg) in the formation of blood clots, the use of low does treatment with drug holidays may be applicable. Additional efficacy data of FXa^{I16L} in clinics are needed to further assess the potential use of FXa^{I16L} in chronic treatments.

CONCLUSIONS

The ADA development time course and potential consequence of ADA induced by FXa^{I16L} were investigated in this study. The potential effects of ADA on PK included decreased clearance and increased exposure. ADA did not increase half-life of FXa^{I16L} and had no or limited increase in pharmacodynamics. No catalytic site binding ADA and no neutralizing activity on clotting function were observed. A correlation between ADA induction and accumulative exposure after various repeat treatments in different species was found for FXa^{I16L}. The learning of ADA consequence in preclinical species may be relevant to human if similar ADA occurs.

ACKNOWLEDGEMENTS

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