Complement Inhibition in Cynomolgus Monkeys by Anti-Factor D Antigen-Binding Fragment for the Treatment of an Advanced Form of Dry Age-Related Macular Degeneration

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d) **List of non-standard abbreviations**

AFD, anti-factor D; aHUS, atypical hemolytic uremic syndrome; AH50, 50% maximal hemolysis; AMD, age-related macular degeneration; AP, alternative complement pathway; BSA, bovine serum albumin; C<sub>max</sub>, maximum serum concentrations; CP, classical complement pathway; CS, normal cynomolgus monkey serum; Ea, sensitized sheep erythrocytes; ELISA, enzyme linked immunosorbent assay; Er, rabbit erythrocytes; Fab, fragment antigen binding; fB, factor B; FCs, flow cells; fD, factor D; GA, geographic atrophy; GN, glomerulonephritis; GVB, bovine skin gelatin in veronal buffer; GVB++, GVB supplemented with 0.05 mM CaCl<sub>2</sub> and 0.05 mM MgCl<sub>2</sub>; HBS-P buffer, 0.01 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 0.15 M sodium chloride, and 0.05% polysorbate 20; huC1s, human complement component 1s; IC50, half-maximal inhibition; IV, intravenous; IVT, intravitreal; k<sub>a</sub>, association constant; k<sub>d</sub>, dissociation constant; K<sub>D</sub>, dissociation equilibrium constant; mAb, monoclonal antibody; MS, multiple sclerosis; PBS, phosphate buffered saline; PBS-T, PBS containing 0.05% polysorbate 20; PD, pharmacodynamic; PK, pharmacokinetic; RA, rheumatoid arthritis; RPE, retinal pigment epithelium; RUs, response units; SA-HRP, streptavidin-horseradish peroxidase; SLE, systemic lupus erythematosus; SPR, surface plasmon resonance; S.D., standard deviation; Tmax, time to reach maximum concentrations; TMB, tetramethyl benzidine

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Abstract

Anti-factor D (AFD) (FCFD4514S, lampalizumab) is a humanized IgG Fab fragment directed against factor D (fD), a rate-limiting serine protease in the alternative complement pathway (AP). Evaluation of AFD as a potential intravitreal (IVT) therapeutic for dry age-related macular degeneration patients with geographic atrophy (GA) is ongoing. However, it is unclear whether IVT administration of AFD can affect systemic AP activation and potentially compromise host-immune responses. We characterized the pharmacological properties of AFD and assessed the effects of AFD administered IVT (2 or 20 mg) or IV (0.2, 2, or 20 mg) on systemic complement activity in cynomolgus monkeys. For the IVT groups, serum AP activity was reduced for the 20 mg dose group between 2–6 hours post-injection. For the IV groups, AFD inhibited systemic AP activity for periods of time ranging from 5 minutes (0.2 mg group) to 3 hours (20 mg group). Interestingly, the concentrations of total serum fD increased up to 10-fold relative to predose levels following administration of AFD. Furthermore, AFD was found to inhibit systemic AP activity only when the molar concentration of AFD exceeded that of fD. This occurred in cynomolgus monkeys at serum AFD levels ≥ 2 µg/mL, a concentration 8-fold greater than the serum C\text{max} observed following a single 10 mg IVT dose in a clinical investigation in patients with GA. Based on these findings, the low levels of serum AFD resulting from IVT administration of a clinically relevant dose are not expected to appreciably affect systemic AP activity.
Introduction

Age-related macular degeneration (AMD) is a leading cause of elderly blindness in industrialized nations and is estimated to affect nearly 6.5% of people over 40 years in the United States alone (Klein et al., 2011). There are two forms of AMD, termed exudative (wet) and nonexudative (dry). Dry AMD affects 85–90% of all AMD patients and is a chronic disease that can be a precursor to wet AMD and geographic atrophy (GA). In the early stages of AMD, insoluble extracellular aggregates called drusen, considered the hallmark lesion of dry AMD, accumulate in the retina (Bird, 2010). A late, advanced form of dry AMD, GA, is characterized by loss or atrophy of retinal pigment epithelium (RPE) and the associated fallout of underlying photoreceptors (Sarks et al., 1988; Jager et al., 2008). While treatment is available for wet AMD, there are no effective therapies for dry AMD; treatment for dry AMD is currently limited to prophylactic measures such as vitamin supplements, diet, and lifestyle modifications (Prasad et al., 2010).

The complement system assists the innate immune response by recognizing foreign antigens (Sarma and Ward, 2011). The alternative, classical, and lectin complement pathways consist of several plasma proteins that can be proteolytically activated to kill pathogens and promote inflammation. The rate-limiting initiator of the alternative complement pathway (AP) is complement factor D (fD), a chymotrypsin-like serine protease specific for factor B (fB) (Fig. 1A). When associated with C3 either in its hydrolyzed or cleaved (C3b) form, fB is a substrate for fD. Cleavage of C3-associated fB to its active form (fBb, another serine protease) yields the AP C3 convertase complex. This complex cleaves additional C3 molecules to generate even more C3 convertase,
greatly amplifying AP activation (Makrides, 1998). C5 associated with fBb and C3b yields the C5 convertase complex; this complex cleaves C5 to C5a and C5b. C5b plus C6-9 initiates membrane pore formation, contributing to tissue damage in several diseases including rheumatoid arthritis and glomerulonephritis (Ricklin et al., 2010; Carroll and Sim, 2011) (Fig. 1A). Because the AP functions in an “always on” mode, a host of negative regulators keep this pathway’s activity in check. Beyond its role in peripheral diseases, several lines of genetic and pathophysiological evidence implicate over-activation of AP in AMD (Anderson et al., 2002; Hageman et al., 2001; Johnson et al., 2001; Anderson et al., 2010; Loyet et al., 2012; Edwards et al., 2005; Yates et al., 2007; Montezuma et al., 2007; Francis et al., 2009; Ebrahimi et al., 2013; Fritsche et al., 2013). Preclinical and clinical evidence explicitly implicate fD in AMD. Mice lacking functional fD were protected against light-induced photoreceptor degeneration (Rohrer et al., 2007) in a photoreceptor injury model. In addition, circulating fD levels were found to be elevated in patients with AMD as compared to control individuals (Scholl et al., 2008; Stanton et al., 2011). In post-mortem vitreous fluid, fB activation was increased in advanced AMD patients compared to age-matched controls, although significant increases in fD levels were not seen in these samples (Loyet et al., 2012).

Based on this evidence, local selective inhibition of the AP may represent a viable approach for the therapeutic treatment of AMD. Some components of the AP are poor candidates for inhibition due to high plasma concentrations and/or their dual role in both the alternative and classical complement pathways (CP). For example, protein C5 is crucial for both AP and CP activity. However, because fD has the lowest plasma concentration among the complement proteins (Volanakis et al., 1985), is specific to the
AP, and is the rate-limiting AP enzyme, it is deemed the most suitable AP therapeutic target.

AFD (FCFD4514S, lampalizumab), derived from murine parental monoclonal antibody (mAb) 166-32, is an antigen-binding fragment (Fab) of a humanized mAb directed against human fD (Tanhehco et al., 1999; Fung et al., 2001). mAb 166-32 was shown to reduce concentrations of cleaved complement components and leukocyte activation in a baboon model of cardiopulmonary bypass surgery (Undar et al., 2002). AFD is currently being investigated as a potential intravitreal (IVT) administered therapeutic for GA. In the studies presented here, we characterized the binding and pharmacological activities of AFD. It is highly desirable to inhibit AP only in the eye, sparing systemic AP activity as much as possible. Our studies in cynomolgus monkeys demonstrated that IVT administered AFD only transiently suppressed systemic AP activity. This is explained by the minimal systemic concentrations of AFD resulting from IVT administration combined with the progressive neutralization of AFD by a high steady-state production of fD.
Materials and Methods

Materials

AFD (produced at Genentech, Inc., South San Francisco, CA) was provided as a lyophilized powder at 70 mg/vial. Sterile water for injection, USP (Hospira, Inc., Lake Forest, IL), was used to reconstitute AFD to 100 mg/mL. Purified human fD, purified human fH, human C3a des Arg standard, C1q depleted human serum, factor B-depleted human serum were obtained from Complement Technologies (Tyler, TX). Recombinant cynomolgus fD, anti-NRP-1 Fab, anti-hu C5, and a mouse mAb to human fD (clone 4676) were produced at Genentech. Another mouse mAb to AFD (clone 242) developed at Genentech binds specifically to AFD and detects total AFD, regardless of whether it is free or bound to fD. Other materials include native activated huC1s (Calbiochem, EMD Millipore, Billerica, MA), human fD (positive control, Calbiochem), and rhuIFNα2 (negative control, Roche Pharmaceuticals, Nutley, NJ), bovine skin gelatin (Sigma, St. Louis, MO) veronal buffer (BioWhittaker, Walkersville, MD), anti-human C3a des Arg-specific mAb (Genetex, San Antonio, TX), biotinylated anti-human C3a alpha mAb (Fitzgerald Industries, Concord, MA), streptavidin-horseradish peroxidase (SA-HRP) (GE Healthcare, Piscataway, NJ), tetramethylbenzidine (TMB, KPL, Gaithersburg, MD), rabbit erythrocytes (Er) and sheep erythrocytes (Colorado Serum, Denver, CO), pooled male (n = 5) and female (n = 5) naïve Chinese origin cynomolgus monkey (Macaca fascicularis) serum (Valley BioSystems, Sacramento, CA), normal cynomolgus monkey serum (CS, Bioreclamation, Westbury, NY), goat anti-human IgG (H&L)-HRP (Bethyl Laboratories Inc., Montogomery, TX), and mouse IgG (Equitech-Bio, Inc., Kerrville, TX).
Measurement of Binding Affinity. Binding kinetics and affinities of AFD to native human fD and recombinant cynomolgus fD were characterized using a Biacore T100 instrument (GE Healthcare; Piscataway, NJ). AFD (10 μg/mL) was immobilized on flow cells (FCs), FC2 and FC4, of a Series S CM5 sensor chip (GE Healthcare) according to manufacturer’s instructions at approximately 80 response units (RUs). The two remaining FCs, FC1 and FC3, were used as in-line references. Various concentrations of fD diluted into HBS-P buffer (0.01 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 0.15 M sodium chloride, and 0.05% polysorbate 20) were injected into the four FCs at a flow rate of 100 μL/minute for 2.5 minutes followed by a 30 minute dissociation time. The sensor chip was then regenerated by consecutive injection of 4 M MgCl₂, at a flow rate of 30 μL/minute, for 100 seconds. The experiments were carried out at 37°C. Sensorgrams of the interaction between AFD and fD were generated for each experimental run after in-line reference cell correction followed by buffer sample subtraction. The dissociation rate constant (k_d) and association rate constant (k_a) were calculated with the BIA evaluation software (version 3.2; GE Healthcare) by fitting the sensorgrams with a 1:1 Langmuir binding model with mass transfer correction, and the dissociation equilibrium constant (K_D) was calculated by taking the ratio of k_d over k_a.

C3 Fluid Phase Convertase Assay. AFD was tested for its ability to inhibit the in vitro generation of C3a through the AP C3 convertase complex. Carboxypeptidase N in rabbit serum was used (Bokisch and Müller-Eberhard, 1970) to cleave C3a to its
inactive form, C3a des Arg. An antibody specific to the des Arg-containing neoepitope was then used in an ELISA to quantitate the amount of C3a generated by the C3 convertase complexe (Fig. 1A).

C3 purified from human serum (Wiesmann et al., 2006) was diluted to 0.2 µM in 0.5% bovine skin gelatin in veronal buffer (GVB). AFD was diluted in GVB. Equal volumes (25 µL each) of C3 and AFD or controls (fH as positive control, anti-NRP-1 Fab as negative control, and GVB alone as an untreated control) were combined in a 96-well round-bottom polypropylene plate and pre-incubated for 10 minutes at room temperature. Human fB and fD (Complement Technology) were combined at a concentration of 1.2 µM each in GVB containing 0.09 M EGTA and 0.1 M MgCl2; 25 µL was added to each well containing the C3/AFD mixture. After an additional 10 minute incubation at room temperature, the C3a produced by C3 convertase in the reaction mixtures was cleaved to C3a des Arg by adding rabbit serum (25 µL, diluted 1:2 with GVB); the reactions were quenched by adding 100 µL 0.25 M EDTA. C3a des Arg concentrations were subsequently measured by ELISA, as described below.

The anti-human C3a des Arg-specific mAb was diluted to 1 µg/mL in phosphate-buffered saline, pH 7.4 (PBS) and coated on 384-well ELISA plates (Nunc; Neptune, NJ) through an overnight incubation at 4°C. Plates were washed with PBS containing 0.05% polysorbate 20 (PBS-T) and blocked by a 2 hour incubation with PBS and 0.5% bovine serum albumin (BSA). This and all subsequent incubations were performed at room temperature with gentle agitation; washes with PBS-T were performed between each assay step. Human C3a des Arg standard and samples from the C3 convertase reaction were diluted in assay buffer (PBS-T containing 0.5% BSA), added to washed plates, and
incubated for 2 hours. Plate-bound C3a des Arg was detected following a 2 hour incubation with biotinylated anti-human C3a alpha mAb and a 30 minute incubation with SA-HRP. TMB was added, color was developed for 7–8 minutes, and the reaction was stopped using 1 M phosphoric acid. The plates were read at 450 nm with a 620 nm reference using a microplate reader (Multiscan Ascent, Thermo Fischer, Waltham, MA). The concentrations of C3a des Arg generated in the convertase reactions were calculated from a four-parameter fit of the C3a des Arg standard curve using in-house Excel-based software. The minimal quantifiable concentration (MQC) of C3a des Arg was 390 pg/mL.

**AP Hemolysis Assay.** As a more physiologically relevant *in vitro* assay, the ability of AFD to inhibit AP activity was evaluated in a hemolytic assay in which serum (either human or monkey) was combined with rabbit erythrocytes (Er) as designed and described by Pangburn (1988) and Katschke et al. (2009). To ensure complement activation did not occur through the CP, C1q depleted human serum was used, and the buffer included EGTA to chelate calcium, a cation essential for CP activity. Factor H (fH), a negative regulator of the AP, was used as a positive control in these studies and an irrelevant humanized Fab (anti-neuropillin-1 [NRP-1]) served as the negative control. C1q depleted human serum or pooled male (n = 5) and female (n = 5) naïve Chinese origin cynomolgus monkey (Macaca fascicularis) serum was used to activate the AP. The concentration of fD present in 10% C1q-depleted human serum was 9.6 nM in-well, a value in agreement with previously reported fD levels in serum (Barnum et al., 1984, Loyet et al., 2012).
CP Hemolysis Assay. To determine whether or not the inhibitory activity of AFD was specific for the AP, a CP mediated hemolysis assay was designed and performed as described by Mayer (1961) and Katschke et al., (2009) using sheep erythrocytes sensitized with rat anti-Forssman IgM antibody (Genentech; May and Frank, 1973) (Ea). To ensure complement activation proceeded through the CP only, factor B depleted human serum and buffer conditions designed to be optimal for CP activity were used. The positive control consisted of anti-human C5; the negative controls were fH and anti-NRP-1 Fab.

Pharmacokinetic/Pharmacodynamic Study in Cynomolgus Monkeys. AFD was administered by a single dose IVT or intravenous (IV) injection to male cynomolgus monkeys (Macaca fascicularis) of Chinese origin in order to assess the pharmacokinetics (PK) and pharmacodynamics (PD) of the molecule. The animals were aged 2.8 years ± 0.52 years (mean ± standard deviation; range 2.3 to 3.8 years) with an initial body weight of 2.4 kg ± 0.46 kg (mean ± standard deviation; range of 1.9 kg to 3.8 kg). This study was conducted at Covance Laboratories Inc., (Madison, WI). All procedures were conducted in compliance with the USDA Animal Welfare Act Regulations (9 CFR 3), the Guide for the Care and Use of Laboratory Animals, and the Office of Laboratory Animal Welfare.

The study consisted of five groups. For groups 1 and 2 (n = 10 each), AFD was administered to both eyes, in two 50 µL IVT doses, separated by 15 minutes. These animals therefore received 1 or 10 mg/eye, respectively, for a total of 2 or 20 mg/animal. Animals in groups 3, 4, and 5 (n = 3 each), received AFD administered at 0.2, 2, and 20 mg/animal, respectively, by a slow IV bolus injection via the saphenous vein.
Pre-dose and post-dose serum samples were collected from each animal via the femoral vein for PK and PD analyses. At each time point, whole blood was collected into serum separator tubes, allowed to clot at ambient temperature for at least 20 minutes, then centrifuged in a refrigerated centrifuge set at a temperature range of 2°C−8°C. The serum was harvested within 20 minutes of centrifugation and stored between -60°C and -80°C until analysis. For the 2 and 20 mg IVT groups, blood was collected pre-dose (day -2) and post-dose at the following time points: 45 minutes, and 2, 6, 10, 24, 34, 48, 96, 120, 154, 192, 288, and 384 hours. For the 0.2, 2, and 20 mg IV groups, blood samples were collected pre-dose (day -2) and post-dose at the following time points: 5 and 30 minutes, and 1, 2, 3, 5, 8, 24, 34, 96, and 168 hours. For IVT group animals only, after blood collections at 24, 48, 120, 192, and 384 hours, 2 animals per group were removed from the study and euthanized to collect ocular matrix; the ocular data are not shown in the current study.

**Determination of Inhibition of Systemic AP Activity in AFD-treated Cynomolgus Monkey Serum.** To evaluate the time course and dose-dependency of any potential inhibition of systemic AP activity subsequent to AFD dosing, an *ex vivo* assay similar to the *in vitro* AP hemolysis assay described above was performed. A key difference in this assay, however, was that instead of adding a dilution curve of exogenous AFD to the serum samples, the samples themselves were serially diluted, with any inhibition of hemolytic activity attributed to the injected dose of AFD.

Erythrocytes were prepared and the assay was performed as described above for the AP hemolysis assay with the following modifications. To determine the absorbance corresponding to maximum lysis, total lysis controls were prepared with sterile water
(80 µL/well) while GVB was added to all other wells (50 µL). Cynomolgus monkey serum samples were serially diluted 1:1.5 over six points and added along with a negative control (buffer only) to 96-well U-bottom polypropylene plates (30 µL/well). The total lysis controls represented maximum (100%) hemolysis. Data points were collected in triplicate and the mean percent maximum hemolysis was plotted against the reciprocal of the final serum dilution in the assay. AH50 values, defined as 50% maximal hemolysis, were determined by nonlinear regression analysis using a four-parameter fit model. For those curves that did not reach saturation, the AH50 was estimated using a curve fit in which the upper asymptote was fixed at 100%. The percent relative hemolysis was calculated for each individual time point as: \[
\frac{(\text{post-dose AH50 for the individual time point})}{(\text{pre-dose AH50})} \times 100
\]
The AH50 value for serum from each individual normal cynomolgus monkey can vary as much as 2-fold from the overall average of AH50 values. Therefore, the pre-dose and post-dose samples from each study animal were run on the same assay plate to ensure that post-dose changes in AP activity were directly compared to the individual animal’s baseline complement activity.

**Total AFD ELISA.** The PK of AFD subsequent to IVT or IV dosing was assessed in cynomolgus monkey serum samples using an ELISA that detects total (free and bound) AFD. Clone 242 was diluted to 1 µg/mL in coating buffer (0.05 M sodium carbonate buffer, pH 9.6), added to 96-well ELISA plates (Nunc MaxiSorp, Thermo Scientific; Rochester, NY), and coated overnight at 4°C. The plates were washed with PBS-T and blocked with assay buffer; the overall ELISA process was essentially the same as the C3a des Arg ELISA described above. The AFD standard curve was prepared by serially diluting AFD from 0.25 – 32 ng/mL in assay buffer containing 10% CS.
Assay controls and samples were diluted to a minimum of 1:10 in assay buffer and subsequent dilutions were made in assay buffer containing 10% CS. Plate-bound AFD was detected with goat anti-human IgG-HRP diluted with assay buffer containing 100 µg/mL mouse IgG. TMB substrate was added and the reaction was terminated with 1M phosphoric acid after an incubation of approximately 15 minutes. Plates were read as described above using a microplate reader (SpectraMax 190, Molecular Devices; Sunnyvale, CA), and AFD concentrations were calculated from a four-parameter fit of the standard curve on each plate (SoftMax Pro, Molecular Devices). Taking into account the minimum serum dilution (1:10), the effective standard curve concentration range for AFD was 2.5 – 320 ng/mL and the MQC was 15 ng/mL in cynomolgus monkey serum.

**Total fD ELISA.** Total fD was measured using an ELISA in which fD was captured on the plate, incubated with an excess of the AFD therapeutic to ensure complete saturation of available fD binding sites, and the fD/AFD complex was detected using the same AFD-specific mAb used above in the ELISA for total AFD.

Briefly, clone 4676 was diluted to 1 µg/mL in coating buffer and incubated overnight at 4°C on 96-well ELISA plates. The overall assay methodology was the same as described for the earlier ELISAs. The cynomolgus monkey fD standard curve was prepared by serially diluting fD from 0.04 - 5 ng/mL in sample buffer (assay buffer supplemented with 500 ng/mL of the AFD therapeutic, 50 µg/mL mouse IgG, and 1% fD-depleted CS). The assay controls and serum samples were diluted to a minimum of 1:100 in sample buffer. The diluted standards, controls, and samples were then incubated on the plates for 2 hours, and plate-bound fD/AFD complex was detected using biotin-conjugated mouse mAb to AFD (clone 242, 1µg/mL) followed by SA-HRP (3 ng/mL).
Color was developed, plates were read, and concentrations of fD were determined as described above. Taking into account the minimum serum dilution (1:100), the effective standard curve concentration range of fD was 4 - 500 ng/mL and the MQC in cynomolgus monkey serum was 5 ng/mL.
Results

**AFD binds to fD with high affinity and specifically inhibits human and cynomolgus AP activity.** SPR-based Biacore experiments demonstrated that AFD bound to human fD with an average $K_D$ of 20 pM (Table 1) under the tested conditions. The high association rate constant value ($k_a = 3.6 \times 10^7$ M$^{-1}$s$^{-1}$) indicated a very fast process, which might have approached or reached the detection limits of the Biacore T100 instrument (Onell and Anderson, 2005). However, no significant or systemic discrepancy was observed between the experimental data and the fitted curves (data not shown), suggesting the 1:1 binding model is adequate to describe the AFD and fD interaction.

AFD was next tested in two functional assays to determine whether it neutralized the ability of fD to initiate AP activation. The fluid phase C3 convertase assay tested the ability of AFD to inhibit fD-dependent activation of C3 convertase via quantitation of the amount of C3a des Arg produced (Fig. 1A). In this method, the reagent concentrations were optimized to yield an optimal signal rather than to adhere to physiological conditions, and fD was present at 400 nM in-well. Under these assay conditions, AFD completely inhibited C3a des Arg production at a concentration at and above 333 nM, while negligible inhibition was observed at 148 nM (Fig. 1B). In contrast, no inhibition of C3 convertase activity was detected with a negative control Fab tested at 1650 nM, while maximal inhibition was achieved with a positive control (fH, a specific inhibitor of the AP).
AFD was also tested in vitro in cell-based AP- and CP-dependent hemolysis assays. In the AP-dependent hemolysis assay, AFD displayed dose-dependent inhibition with an IC50 of 4.0 nM (Fig. 2A). AFD inhibited AP by 96% after background subtraction. In contrast, no inhibition was observed for the negative control Fab while fH caused a 37% inhibition of maximal hemolysis using the same assay method.

For the CP-driven hemolysis assay, AFD did not detectably inhibit CP activity (Fig. 2B) at a concentration of 500 nM. The two negative controls (anti-NRP-1 Fab and fH) likewise had no effect while the positive control, anti-C5 mAb, caused a 43% inhibition of CP activity.

SPR-based experiments demonstrated that the KD of AFD binding to cynomolgus monkey fD is very similar to that of human fD (average KD values of 12 and 20 pM, respectively; Table 1). The hemolysis assay was also performed using cynomolgus monkey serum to determine whether AFD would inhibit cynomolgus monkey AP. AFD caused dose-dependent AP inhibition with an IC50 of 3.9 nM (Fig. 3A). Although the IC50 values for AFD agreed closely for human and cynomolgus monkey serum, the maximal degree of inhibition by AFD was notably different. As opposed to maximal inhibition of 96% in human C1q-depleted serum, AFD had a maximal inhibition of 73% in cynomolgus monkey serum. Whereas a negative control Fab had no effect, the positive control, fH, inhibited AP activity by 72%.

When evaluated for its effect on CP activity in cynomolgus monkey serum, AFD (500 nM) caused no detectable inhibition (Fig. 3B), similar to the results observed for human fB-depleted serum. The positive control, anti-C5 mAb (500 nM), caused a 43%
inhibition of CP activity in cynomolgus monkey serum, equivalent to the inhibition seen in human serum (43%).

**AFD transiently inhibits systemic hemolytic activity *ex vivo* in a cynomolgus monkey PK study.** To evaluate the effect of circulating AFD on serum AP activity in cynomolgus monkeys, an *ex vivo* AP-specific hemolytic activity assay was used to assess the degree of post-dose AP inhibition, expressed as a decrease in serum hemolytic activity relative to that observed in pre-dose serum. The results of the *ex vivo* hemolytic assay show little effect of AFD on serum AP activity in the 2 mg/animal IVT group (Fig. 4A). In contrast, for the 20 mg/animal IVT group, AP inhibition begins from 2 - 6 hours after IVT dosing. Activity returns to normal (~80% average relative hemolysis) for 8 of 10 cynomolgus monkeys by 10 hours and for all by 24 hours post-dose (Fig. 4B). For the IV groups, inhibition in the 0.2 mg IV group was observed for 2 of 3 animals at the 5 minute time point only (Fig. 5A), while for the 2 mg IV group, all animals showed AP inhibition up to 1 hour, then a return to normal AP activity at 2 hours post-dose (Fig. 5B). All animals in the 20 mg IV group showed inhibition of AP activity up to 3 hours post-dose, followed by a return to baseline for 2 of 3 animals at 5 hours (Fig. 5C) with the third animal showing some residual inhibition of approximately 40-60% until 168 hours. The maximal *ex vivo* systemic AP inhibition by AFD for monkeys in groups 2 – 5 ranged from 41 – 99% with a mean maximal inhibition of 75%. This is quite comparable to the 73% maximal inhibition of AFD observed for the *in vitro* hemolysis assay (Fig. 3), which used a cynomolgus monkey serum pool from a different cohort of animals (n = 10).
Measurement of total AFD and total fD in AFD treated cynomolgus monkeys.

The serum concentrations of AFD were measured using an assay capable of detecting total Fab (free and fD-bound). The concentration profiles showed that in the IVT groups, the time to reach maximum AFD concentrations ($T_{\text{max}}$) occurred gradually at 6–10 hours post dose (Fig. 6A) whereas the $T_{\text{max}}$ was at the first post-injection time point (5 minutes) for the IV groups (Fig. 6B).

The average baseline (pre-dose) serum fD concentrations were ~20 nM to 30 nM (Fig. 6). For the IVT groups, as serum total AFD concentrations rose or fell, so did serum total fD concentrations in a temporally concurrent fashion. Although the serum AFD $C_{\text{max}}$ was 30 nM at 6 hours post-dose (IVT), the average fD concentration had increased to 57 nM at this time point, nearly double the AFD average molar concentration (Fig. 6A). At no point in time did the AFD concentration exceed the fD concentration, suggesting that sufficient fD was present in an active, unbound form to initiate the AP in the ex vivo hemolytic activity assay; this is in agreement with the ~100% (baseline) AP activity seen at all time points for the animals dosed IVT with 2 mg AFD (Fig. 4A).

Similarly, at the earliest time point for the 20 mg/animal IVT group (45 minutes), the average total fD concentration was 33 nM. This was more than three-fold higher than the concentration of AFD at this time point (9 nM); hence no systemic AP inhibition was seen for the 20 mg/animal IVT group at 45 minutes post-dose (Fig. 4B). However, at 2 hours, the average AFD concentration had increased to 126 nM, while there was a lower average fD concentration (92 nM); thus there was significant inhibition of AP
activity for most animals. The average serum C\text{max} for AFD was 260 nM at 6 hours post-dose, at which time the average total fD concentration had increased almost 10-fold from baseline to 221 nM. At 10 hours post-dose, the average total fD C\text{max} (247 nM) was nearly equimolar to that of the average total serum AFD concentration (251 nM), suggesting fD predominantly existed as a bound complex form. Furthermore, the AP activity of all animals did not return to within ~10% of baseline until 96 hours post-dose (Fig. 4B), presumably due to neutralization of AFD by increased total fD concentrations, which was sustained through the last few time points (> 100 hours) when the average molar AFD again dropped lower than the average molar fD (Fig. 6A).

In contrast to the IVT groups where the AFD and fD T\text{max} occurred during the same time range (6-10 hours post-dose), the T\text{max} of fD was delayed relative to the T\text{max} of AFD in the IV dosing groups (Fig 6B). At 0.1 hour, the average serum C\text{max} levels of AFD for the 0.2 mg, 2 mg, and 20 mg IV groups were 41, 459, and 5158 nM, respectively. These average AFD concentrations were higher than the average fD concentration (20 – 30 nM), therefore there was AP inhibition at 0.1 hours for all IV groups (Fig. 5). For these same groups, the average total fD serum C\text{max} levels were 47 nM at 0.5 hour, 138 nM at 2 hours, and 215 nM at 5 hours, respectively. At these respective time points, as the average serum AFD concentration decreased to 31 nM, 109 nM, and 226 nM, concentrations which were below or nearly equimolar to the fD C\text{max}, the AP activity returned to baseline levels, which was sustained for the rest of the study. For the 20 mg IV group, the serum fD C\text{max} (215 nM) was almost 10-fold greater than the baseline serum fD concentration (26 - 33 nM) (Fig. 6B).
Activity-concentration relationship in AFD treated cynomolgus monkeys. In order to understand the activity-concentration relationship of IVT-injected AFD, we evaluated the association between systemic AP inhibition measured by percent relative hemolysis and serum concentrations of total AFD and total fD. All data points for all animals at all time points, for both IVT and IV routes of administration, were plotted together (Fig. 7) as well as separately for each group (Supplementary Fig.1A-E). Systemic AP inhibition (relative hemolysis < 60%) was observed at serum AFD concentrations of 40-5198 nM (2-242 μg/mL). The lowest AFD concentrations causing > 40% inhibition were 40 nM for the 0.2 mg IV group (Supplementary Fig. 1C); 140 nM for the 2 mg IV group (Supplementary Fig. 1D); and 200 nM for the 20 mg IV group (Supplementary Fig. 1E) if one animal was excluded (cynomolgus monkey #29, which showed a partial inhibition of hemolysis through the 96 hour time point; Fig. 5A). As the molar serum concentration of AFD approached and exceeded that of fD (fD/AFD < 1, green and blue symbols), the percent relative hemolysis decreased. For IVT-dosed animals (Supplementary Fig. 1A-B), AP inhibition of 40% or more was seen in samples from the higher IVT dose group (20 mg/animal), but not in samples from the lower IVT dose group (2 mg/animal). Both the 20 mg IVT and IV groups contained samples with a wide range of AP inhibition (0 – 80% AP inhibition) despite a narrow AFD or fD concentration range (~150 nM – 350 nM) at fD/AFD of ~1 (Supplementary Fig. 1B). For those animals given the lowest IV dose (0.2 mg), all samples demonstrated < 20% AP inhibition with the exception of those at 5 minutes post-dose wherein up to 80% AP inhibition was observed (Fig. 5A, Supplementary Fig. 1C) since molar concentrations of fD were lower than those of AFD (fD/AFD < 1, AFD C\text{max} of 40 nM (2 μg/mL)). This is
in contrast to the 2 mg IVT group that had an AFD $C_{\text{max}}$ of 35 nM (1.75 μg/mL), fD/AFD $> 1$ at 6 hours post-dose (Fig. 6A), and did not show AP inhibition (Fig. 4A) since molar concentrations of AFD were lower than those of fD.
Discussion

A role of fD in the pathogenesis of AMD is supported by a murine model in which a genetic fD deficiency provided protection against oxidative-stress-mediated photoreceptor degeneration (Rohrer et al., 2007). In addition, increased systemic activation of complement, including fD, has been detected in the serum of AMD patients versus controls, suggesting that AMD may be a systemic disease with local manifestations in the aging macula (Scholl et al., 2008). Many complement pathway inhibitors are currently in clinical or preclinical development for AMD (Ricklin and Lambris, 2007); AFD is being evaluated as a potential IVT-administered therapeutic.

*In vitro* studies were performed to characterize the binding and activity of AFD in human and cynomolgus serum. Since animal studies are necessary as a precursor to clinical trials, it was important to determine the cross-reactivity of AFD with cynomolgus monkey fD to establish the appropriateness of this species for *in vivo* testing. AFD was shown to bind to native human fD (20 pM) and recombinant cynomolgus fD (12 pM) with high affinity. AFD specifically inhibited fD activity in the C3 convertase assay. In addition, *in vitro* serum-based RBC hemolysis assays were used as a more physiologically relevant system; the results demonstrated that AFD potently inhibited the cynomolgus monkey (IC50, 3.9 nM) and human (IC50, 4.0 nM) AP but did not inhibit the CP in these species.

The reason for the higher baseline activity (Fig 3A) remaining after AFD treatment of cynomolgus monkey serum was unclear, but it was consistent across all assays that were performed using this particular cynomolgus monkey serum pool. Unlike
the human AP hemolysis assay, the cynomolgus monkey serum was not depleted of C1q. It is therefore possible that CP activation could account for the higher baseline activity. However, this possibility is lessened by including EGTA in the assay buffer to chelate calcium, an essential factor for CP activity. Differences in the maximal activity of human fH in human versus cynomolgus monkey serum could be due to a species-dependent difference in C3b concentration or fH binding to C3b, however, this has not been tested.

Slight differences in AFD affinities for human and cynomolgus fD may be explained by a single amino acid difference in human versus cynomolgus fD (Glu-178 versus Gln-178) in a region near the AFD binding interface (Katschke et al., 2012). The AP hemolysis assays require nM fD concentrations to achieve adequate hemolysis. Given the 1:1 stoichiometry of AFD:fD, nM AFD concentrations are required for inhibition in these assays despite the pM affinities of AFD binding to fD shown by SPR. The results of the AP hemolysis assay were consistent with the C3 convertase assay in terms of a complete inhibition of AP activity by AFD. Although a difference in AFD potency was observed in the two assay systems, this was expected given the different fD concentrations (9.6 nM versus 400 nM in-well for the AP hemolysis and C3 convertase assays, respectively). The results overall indicate that AFD inhibits the AP by blocking fD proteolytic activity. In confirmation, crystallographic studies have shown that AFD inhibits fD by binding to the surface loops on fD that form the exosite, thereby blocking macromolecular substrate access (Katschke et al., 2012).
Together, the \textit{in vitro} results demonstrate a specific interaction of AFD with the AP in both human and cynomolgus monkey serum; show a lack of effect on the CP in either species; and support the use of cynomolgus monkeys as a relevant preclinical model for evaluating the PK and PD of AFD. Evaluating the activity of AFD in cynomolgus monkeys was limited to assessing its effects on serum complement activity due to the absence of a non-human primate model of GA. In addition, although laser-induced choroidal neovascularization (CNV) is used as a primate model for wet AMD, our in-house studies did not generate conclusive evidence for the involvement of complement in this model. Lastly, based on poor AP inhibition by AFD in dogs or sheep and no AP inhibition by AFD in rabbits, rats, mice, guinea pigs, hamsters, or pigs (US Patent Publication 2002-0081293 A1), these species were not considered appropriate or relevant for the \textit{in vivo} evaluation of AFD activity. For these reasons, we were not able to perform studies evaluating the efficacy of AFD in relevant preclinical animal models.

A single-dose cynomolgus monkey study evaluated the AFD PK and PD after IVT and IV administration. The vitreous elimination half-life was approximately 2.4 days in monkeys, consistent with ranibizumab, and the systemic elimination half-life was approximately 2.2 days following IVT administration and 7 hours to 20 hours following IV administration (Do et al., 2014). The difference in systemic half-life following IVT compared to IV administration suggests that the AFD kinetics following IVT administration was absorption rate-limited. The lowest observed AFD serum concentration causing transient AP inhibition was 40 nM (2 \( \mu \)g/mL). However, the serum AP activity assay results for both IV and IVT administered AFD demonstrated that the effect of systemic concentrations of AFD on the AP activity was not directly related
to the serum concentration of AFD at all time points in cynomolgus monkeys. The wide range of AP inhibition (0–80%) within ~ 150 nM to 350 nM AFD concentration range (Fig. 7) is not surprising given that the fD and AFD concentrations determined by ELISA can be expected to deviate from the ‘true’ concentrations by up to ± 20%, as is typical and widely accepted for ELISAs. Even this level of error can potentially affect the hemolysis assay results since the AP can be activated by serum containing 30 nM fD (Biesma et al, 2001).

There is a limited capacity to maintain concentrations of free (and therefore active) AFD at a level high enough to cause prolonged inhibition of AP activity in the systemic circulation following IVT administration. Metabolism and excretion of systemic AFD, combined with neutralization of AFD by target binding and a high rate of fD synthesis, contribute to rapidly decreasing serum concentrations of free AFD. The substantial increases in serum levels of total fD seen after administering AFD (Fig. 6) can potentially be explained by less efficient filtering of the higher molecular weight AFD-fD complexes into the extravascular space and/or slower elimination of the AFD-fD complexes relative to free fD. However, an increase in the total (free and bound) concentration of target following drug administration is often seen for therapeutic antibodies that bind soluble endogenous targets, especially when the antibody affinity is high and the target turnover is fast (Davda and Hanson, 2010; Hayashi et al., 2007). The high rate of fD synthesis, estimated to be 1.33 mg/kg/day in humans (Pascual et al., 1988), was substantiated by the persistently elevated total fD concentrations, even while total AFD decreased, as seen in the 2 and 20 mg IV groups. Factor D is primarily expressed in adipose tissue (Cook et al., 1985), although it is also expressed in several other tissues.
and cell types at a much lower level. Expressed sequence tag distribution data indicate
fD expression in the eye is ~40-fold lower than in adipose tissue (http://research-
public.gene.com/Research/genentech/genehub-gepis/index.html), and the neural retina
contains relatively few fD transcripts compared to peripheral tissues (Anderson et al.,
2010). Factor D protein levels are higher in the systemic circulation than in the eye. In
cynomolgus monkeys, 20–31 nM fD was measured in serum as compared to 0.52 nM in
vitreous fluid (38-fold lower, average of 8 naïve cynomolgus monkeys). Similarly, fD
concentrations from post-mortem human vitreous samples were lower than in serum
(Loyet et al., 2012). Therefore, local inhibition of fD in the eye can be achieved at lower
doses of AFD than would inhibit fD systemically, and the low systemic concentrations of
AFD resulting from IVT dosing are expected to be well below the threshold
concentration required to affect systemic fD activity.

A single ascending dose Phase Ia study with IVT administration of 0.1 to 10 mg
AFD per eye demonstrated that AFD (FCFD4514S) was safe and well tolerated in
patients with GA secondary to AMD (Do et al., 2014). The serum AFD C\text{max} following a
single 10 mg IVT dose ranged from 4 to 5 nM. These levels were 5- to 6-fold lower than
the baseline molar concentrations of serum fD seen in this preclinical study (20–30 nM),
suggesting that systemic AP activity would remain unaffected. In addition, the highest
AFD C\text{max} of 257 ng/mL (5 nM) was still approximately eight-fold lower than 2 µg/mL
(40 nM), the lowest concentration causing a temporary effect on systemic AP activity in
cynomolgus monkeys. Evaluating the clinical samples using the AH50 assay
demonstrated that the systemic concentrations of IVT administered AFD had no observed
adverse effects on the serum AP activity in these GA patients.
A Phase Ib/II evidence of activity study (ClinicalTrials.gov Identifier: NCT01229215) showed the potential clinical benefits of AFD in patients with GA (Yaspan, 2014). The in vitro and in vivo data presented here suggest that IVT administered AFD has the potential to be an important novel therapeutic for patients with GA and intermediate dry AMD.

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Authorship contributions

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Conducted experiments: Loyet, Good, Davancaze, Sturgeon, Wang

Contributed new reagents, assays, or analytic tools: Wong, Hass

Performed data analysis: Loyet, Good, Davancaze, Sturgeon, Wang, Yang, Le, Morimoto

Wrote or contributed to the writing of the manuscript: Loyet, Good, Wang, Yang, Le, van Lookeren Campagne, Morimoto, Damico-Beyer, DeForge
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Yaspan B (2014) A common SNP at the CFI locus is associated with rapid progression of geographic atrophy. ARVO Abstract 2234.
Footnotes

a) This study was funded by Genentech, Inc. Support for third-party writing assistance by AnshinBioSolutions Services, was provided by Genentech, Inc.

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**Figure Legends**

**Fig. 1.** AFD inhibited fD-dependent formation of C3 convertase as determined via measurement of C3a des Arg generation. (A) A schematic of the AP is shown. The C3 convertase reaction with the assay read-out is highlighted in yellow. Highlighted in blue is the C5 convertase reaction that results in membrane attack complex (MAC) formation, which can contribute to tissue damage in several diseases. In the C3 convertase assay, purified protein components, C3, factor B (fB), and fD were combined to spontaneously form the C3 convertase, which cleaves C3 into C3a and C3b. C3a was converted to C3a des Arg by the addition of rabbit serum, which provided a source of carboxypeptidase N. (B) C3a des Arg was quantified by ELISA and plotted as C3 convertase activity, % of untreated control. Negative control, humanized anti-NRP-1 Fab, and positive control, fH, were used at 1650 nM. Mean and S.D. values from three independent experiments are presented. Data points were collected in triplicate and analyzed for C3a des Arg in duplicate. The mean % maximum C3 convertase activity for three independent experiments is plotted. The untreated control represented maximum (100%) C3 convertase activity and consisted of C3 in the absence of inhibitors.

aHUS, atypical hemolytic uremic syndrome; GN, glomerulonephritis; MS, multiple sclerosis; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus

**Fig. 2.** AFD demonstrated dose-dependent inhibition of the AP (A) in C1q-depleted human serum but did not detectably inhibit activity of the CP (B) when used with fB depleted human serum in *in vitro* hemolytic cell-based assays. For the AP
hemolysis assay, the negative control was humanized anti-NRP-1 Fab (500nM); the positive control was fH (500 nM). For the CP hemolysis assay, the negative controls were humanized anti-NRP-1 Fab (500 nM) and fH (500 nM); the positive control was anti-C5 antibody; AFD was used at 500 nM. Mean (average background subtracted) and S.D. values from three independent experiments are presented; data points were collected in triplicate in each experiment. The untreated control represented maximum (100%) hemolytic activity and consisted of C1q-depleted human serum (A) or factor B-depleted human serum (B) in the absence of inhibitors.

Fig. 3. AFD demonstrated dose-dependent inhibition of the AP (A) but did not detectably inhibit activity of the CP (B) in cynomolgus monkey serum in in vitro hemolytic cell-based assays. For the AP hemolysis assay, the negative control was humanized anti-NRP-1 Fab (500nM); the positive control was fH (500 nM). For the CP hemolysis assay, the negative controls were humanized anti-NRP-1 Fab (500 nM) and fH (500 nM); the positive control was anti-C5 antibody; AFD was used at 500 nM. Mean (average background subtracted) and S.D values from three independent experiments are presented; data points were collected in triplicate in each experiment. The untreated control represented maximum (100%) hemolytic activity and consisted of a cynomolgus monkey serum pool in the absence of inhibitors.
**Fig. 4.** *In vivo* inhibition of the AP pathway following IVT dosing in cynomolgus monkeys was observed in the highest dose group, but was transient and returned to normal by 10-24 hours post AFD administration. The % relative inhibition, normalized for each individual predose sample (100% relative hemolysis) is shown for all animals (n=10/group) dosed IVT with 1 mg/eye (2 mg total) AFD (A) and 10 mg/eye (20 mg total) AFD (B). % relative hemolysis = (AH50 postdose / AH50 predose time point) x 100; baseline (●) = 100% relative hemolysis. Note: Data for all time points unavailable for some animals as they were removed from the study and used for collecting ocular samples.

**Fig. 5.** *In vivo* inhibition of the AP pathway following IV dosing in cynomolgus monkeys was observed in all dose groups, but was transient and returned to normal by 45 minutes to 10 hours post AFD administration. The % relative inhibition, normalized for each individual predose sample (100% relative hemolysis) is shown for all animals (n=3/group) dosed IV with 0.2 mg (A), 2 mg (B), or 20 mg (C) of AFD. % relative hemolysis = (AH50 postdose / AH50 predose time point) x 100; baseline (●) = 100% relative hemolysis.

**Fig. 6.** The total fD concentrations in cynomolgus monkey serum increased following an increase in serum total AFD concentrations. The average total fD concentrations (●) are shown on the left y-axis while the total AFD concentrations (●) are shown on the right y-axis, both versus time (hours, x-axis). For the 2 mg/animal (●) and 20 mg/animal (●) IVT groups, data points represent mean ± S.D., n = 2-10 animals.
per time point (A). For the 0.2 (■), 2 (●), and 20 mg (▲) IV-dosed groups, data points represent mean ± S.D., n = 3 animals per time point (B). A plot on linear X and log Y axis demonstrate non-linear PK for IVT administered AFD (C) vis-à-vis a linear PK with IV dosing (D).

Fig. 7. *In vivo* inhibition of the AP pathway was generally observed when the serum concentration of AFD was both greater than 40 nM (2 µg/mL) and fD/AFD ratio was less than 1. The % relative hemolysis data (normalized to individual animals’ predose % relative hemolysis, which was set at 100%) from all time points (n=263) are shown plotted versus serum AFD concentrations for all animals (n=29). Data points are color-coded for the ratio of fD versus AFD concentrations, with fD/AFD values greater than 1 increasing from greenish-yellow to yellow, orange, and red, and with fD/AFD values less than 1 decreasing from green to turquoise, indigo, and blue. % relative hemolysis = (AH50 predose / AH50 postdose time point) x 100; baseline (—) = 100% relative hemolysis; AP inhibition (---) < 60% relative hemolysis.
Table 1: Kinetic analysis and binding affinity of human and recombinant cynomolgus monkey fD to AFD.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>$k_a$ (107M$^{-1}$ s$^{-1}$)</th>
<th>$k_d$ (10$^{-4}$s$^{-1}$)</th>
<th>$K_D$ (pM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human fD</td>
<td>3.6±1.0</td>
<td>7.0±1.1</td>
<td>19.7±2.9</td>
</tr>
<tr>
<td>r cyno fD</td>
<td>20.8±10.0</td>
<td>23.3±9.0</td>
<td>11.7±1.6</td>
</tr>
</tbody>
</table>

$k_a$ = association rate constant; $k_d$ = dissociation rate constant; $K_D$ = dissociation equilibrium constant.

The data shown are mean±S.D. Mean and S.D. were calculated based on four independent experimental runs for human fD binding to AFD; and five independent experimental runs for cynomolgus monkey fD binding to AFD.
Figure 1

A

*In vitro* addition of carboxypeptidase converts C3a to C3a des Arg, which can be measured by ELISA.

†Uncontrolled activation of AP can contribute to tissue damage in diseases such as AMD, GN, RA, SLE, aHUS, MS, Myocarditis, and others.

B

![Graph showing C3 Convertase Activity](image)
Figure 2

(A) Human Alternative Pathway Hemolysis (% Hemolysis of Untreated Serum Control) as a function of AFD concentration. Different markers represent different treatments: Anti-NRP-1 Fab, fH, and No serum.

(B) Human Classical Pathway Hemolysis (% Hemolysis of Untreated Serum Control) for different test molecules at 500 nM: Anti-C5, AFD, Anti-NRP-1 Fab, and fH.
Complement Inhibition in Cynomolgus Monkey by Anti-Factor D Antigen-Binding Fragment for the Treatment of an Advanced Form of Dry Age-Related Macular Degeneration

Kelly M. Loyet, Jeremy Good, Teresa Davancaze, Lizette Sturgeon, Xiangdan Wang, Jihong Yang, Kha Le, Maureen Wong, Philip E. Hass, Menno van Lookeren Campagne, Peter Haughney, Alyssa Morimoto, Lisa A. Damico-Beyer, Laura E. DeForge

Supplementary Fig. 1:

In vivo inhibition of the AP pathway was generally observed when the serum concentration of AFD was both greater than 40 nM (2 µg/mL) and fD/AFD ratio was less than 1. The % relative hemolysis data (normalized to individual animals’ predose % relative hemolysis, which was set at 100%) from all time points are shown plotted versus serum AFD concentrations for: (A) 2 mg, IVT group (●) (n=10 animals, 86 data points); (B) 20 mg, IVT group (★) (n=10 animals, 88 data points); (C) 0.2 mg, IV group (♦) (n=3 animals, 26 data points); (D) 2 mg, IV group (■) (n=3 animals, 30 data points); (E) and 20 mg, IV group (▼) (n=3 animals, 33 data points). Data points are color-coded for the ratio of fD versus AFD concentrations, with fD/AFD values greater than 1 increasing from greenish-yellow to yellow, orange, and red, and with fD/AFD values less than 1 decreasing from green to turquoise, indigo, and blue. % relative hemolysis = (AH50 predose / AH50 postdose time point) x 100; baseline (—) = 100% relative hemolysis; AP inhibition (---) < 60% relative hemolysis.