Efficacy and Safety of a New-Class of Hemostatic Drug Candidate, AV513, in Hemophilia A Dogs

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Running title: EFFICACY AND SAFETY OF AV513 IN HEMOPHILIA A DOGS

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Abstract

AV513 is a select fucoidan, a sulfated polysaccharide of botanical origin, with procoagulant activity. It inhibits Tissue Factor Pathway Inhibitor (TFPI) activity and accelerates clotting of human hemophilia A and B plasma. In prior work, subcutaneous administration of AV513 to hemophilia A mice improved hemostasis. The current studies were designed to evaluate potential efficacy and safety in hemophilia A dogs with minimally increased hemostasis following Factor VIII gene transfer (AAV-FVIII) and in treatment-naïve severe hemophilia A dogs. AV513 administered subcutaneously to low-FVIII dogs over multiple weeks resulted in improved hemostasis as exhibited in thromboelastography (TEG) and cuticle bleeding time (CBT) tests. Moreover, AV513 administered orally in AAV-FVIII dogs and treatment-naïve severe hemophilia A dogs over a multi-week dose-escalating period yielded correction to normal ranges in both TEG and CBT end points at 5-15 mg/kg and 15-20 mg/kg dose levels, respectively. In all three separate studies, throughout their duration, AV513 was well-tolerated by the dogs without any adverse events. Additional pharmacological characterization of AV513 included intravenous pharmacokinetic analysis in rats. In summary, the combination of safety and efficacy in two global tests of hemostasis in the hemophilia A dog model indicate that further evaluation of AV513 as a hemostatic agent in hemophilia A patients is warranted.
Introduction

Hemophilia A patients have an increased bleeding tendency due to the absence or dysfunction of Factor VIII (FVIII). The current therapy for hemophilia A patients involves the intravenous administration of FVIII concentrates (either recombinant or plasma-derived). In addition to FVIII protein replacement, treatment with Desmopressin \(^1,2\) will increase FVIII levels in most mild hemophilia A patients, and the antifibrinolytic agents tranexamic acid \(^2\) and epsilon-aminocaproic acid \(^3\) can be effective adjunctive therapies. However, all of these therapies have limitations; for example, the potential for the development of functionally neutralizing anti-FVIII antibodies in approximately 25% of factor-treated patients. \(^4-8\) Given the high cost associated with recombinant factor therapy, there is a need to develop alternative treatments that are conveniently administered, effective, less costly, and safe. An added beneficial therapeutic characteristic would be hemostatic efficacy in multiple bleeding disorders.

AV513 is a plant polysaccharide, a fucoidan, derived from brown seaweed and described as a non-anticoagulant sulfated polysaccharide (NASP). \(^9\) When compared with other sulfated polysaccharides including heparin, pentosan polysulfate, N-acetyl heparin (NAH) and de-N-sulfated heparin (De-N-SH), AV513 demonstrated a superior non-anticoagulant activity in dilute prothrombin time (dPT) and activated partial thromboplastin time (aPTT) assays performed with human hemophilia A and B plasma. NASPs were originally conceived as a novel approach for improving coagulation based on their inhibition of physiological anticoagulation. Specifically, AV513 reversed the tissue factor pathway inhibitor (TFPI)-induced prolonged clotting time at nanomolar
concentrations and accelerated clotting in dPT assays with hemophilia A or B human plasma in the absence of added TFPI. 9

Fucoidans have been recognized to have various pharmacological activities in model systems in vitro or in vivo. In this context, several animal models of inflammation and hematopoietic progenitor cell mobilization have been used to assess the benefit of fucoidan by blocking selectin function expressed on the surface of activated circulating and vascular cells. 10-13 Low molecular weight fucoidan (8 kDa) has been shown to regulate neo-intimal formation and endothelial cell proliferation. 14-16 The observed effect on vascular cells is suggested to be due to the enhanced half-life of fibroblast growth factor through fucoidan binding. Unfractionated fucoidans, from various sources, are heterogeneous sulfated polysaccharides and may substantially differ in size, and also exhibit structural diversity. 17-20 As fucoidans have some structural similarities to heparin, anticoagulant activity has also been documented. Anticoagulation has been linked to multiple mechanisms; partially through heparin cofactor II, and by directly inhibiting thrombin activity 20,21 and potentially also via inhibition of the conversion of fibrinogen to fibrin by direct binding to fibrinogen.22,23

In prior work, fucoidan selected for minimal anticoagulant activity, AV513 was shown to enhance the survival of hemophilia A mice following tail vein transection when the animals were treated by subcutaneous administration as a monotherapy. Moreover, in combination with rFVIII injection, enhanced efficacy was observed in the AV513 + rFVIII group compared with sub-optimal dose of rFVIII alone.9 In order to further explore the potential clinical utility of AV513, the drug candidate has now been evaluated in the well-characterized hemophilia A dog model 24 wherein both efficacy and safety
endpoints could be robustly evaluated. There is significant precedent for the use of the hemophilic dog model to perform pre-clinical evaluation of novel hemophilia treatments.\textsuperscript{25-28}

In this report, data is presented demonstrating dose-related cuticle bleeding time improvement and TEG correction in both minimally treated and naïve FVIII-deficient hemophilic dogs following either subcutaneous or oral administration of AV513. Studies were of both short-term and multi-week durations and co-monitored safety endpoints showed no adverse effects of AV513 therapy.
Materials and Methods

Fucoidans prepared from *F. Vesiculosus* (Sigma, St. Louis) and *L. Japonica* (*proprietary*) species of brown sea weed were used. Both preparations had similar potency in *in vitro* clotting studies. The molecular weight of fucoidans in the preparations range between 10–300 kDa in size as measured by laser light scattering system (Shodex OH pack column and Precision detectors PD2020 multi-detector light scattering system). The monosaccharide composition of AV513 preparations are mainly composed of Fucose, Xylose, Mannose, Glucose and Galactose as determined by GC/MS analysis.29

Animal procedures

The hemophilia A dogs were bred and maintained at Queen’s University, Kingston, Ontario, Canada. All animal procedures were in compliance with the Canadian Council for Animal Care, institutional Animal Care Committees, and with USDA Guide for the Care and Use of Laboratory Animals.

Dog cuticle bleeding time (CBT) assay

This *in vivo* test of haemostasis has been previously validated in the canine model in studies of novel pro-haemostatic agents and anticoagulants.24–27,30 Briefly, 10 minutes following anaesthetic induction, the dogs were placed in the lateral recumbency position, and all hair around the nail bed was carefully removed by clipping around the base of the claw to be used for the CBT. Silicone grease was applied to the claw to prevent blood tracking back underneath the nail. The apex of the cuticle was visualized, and the nail severed proximal to the dorsal nail groove using a spring-loaded sliding blade guillotine.
clipper. All CBTs were performed by the same experienced veterinary technologist. The animal’s paw was subsequently positioned over the edge of the operating table and blood from the severed cuticle allowed to fall freely. The number of blood drops in each of the subsequent 15 minutes was recorded and converted to a cumulative CBT score for the 15 minutes (Table 1). This cumulative CBT score for the 15 minute observation period for each dog is represented as the total CBT score. After 15 minutes observation, if the cuticle was still bleeding, the site of injury was cauterized by the topical application of silver nitrate. A single cuticle bleeding time was examined at the end of each dosing period and no cuticle was subjected to more than one injury in a single experiment in both AAV-FVIII dogs and severe hemophilia A dogs. The AAV-FVIII dog’s cuticles had been previously subjected to bleeding time measurements at the time of their gene transfer studies, while the cuticles of the naïve severe hemophilic dogs were injured for the first time during this study. Nevertheless, given the large number of CBTs performed during this study, repeat injuries of some cuticles was inevitable. Cuticle injuries on a single paw were never performed more than once weekly (as per the approved animal care protocol).

Table 1. Cuticle bleeding time scoring.

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Coagulation assays

Diluted PT (dPT) and aPTT assays were performed as described by Liu et al. 9 Whole blood clotting time was measured by incubating citrated whole blood at 37 °C in a glass test tube with continuous monitoring for clot formation.

TEG was performed with 340 μL of citrated whole blood from severe hemophilia A dogs or citrated, frozen platelet-poor plasma from AAV-hemophilia A dogs after pre-warming to 37 °C and transferring to a pre-warmed disposable TEG cup containing 20 μL of 0.2 M calcium chloride. The samples were gently mixed once by reverse pipetting and the real time dynamics of clot formation was monitored using a Haemoscope thromboelastograph (Haemoscope Corporation, Niles, IL). TEG R time (latency period for initial coagulation), angle α (rapidity of clot strengthening), and MA (maximum strength of the formed clot) were determined by the TEG® Analytical Software. For in vitro whole blood TEG studies, 330 μL of citrated whole blood was mixed with 10 μL of the appropriate AV513 concentration. The mixture was later added to the TEG cup containing 20 μL of 0.2 M calcium chloride.

Canine efficacy studies

Subcutaneous administration of AV513: Three AAV-FVIII hemophilia A dogs; Angus, Gloria, and Morag treated several years ago with canine FVIII-expressing adeno-associated virus vector constructs express ≤1% plasma FVIII levels. In a dose escalation study, the AAV-FVIII hemophilia A dogs were subcutaneously injected with 0.03, 0.1, 0.5, 1.0, and 1.5 mg/kg dose of AV513 two times daily for five days per dose with a
seven day washout period after each dosing period. For whole blood clotting time and to prepare citrated plasma for TEG analysis, aPTT and dPT assays, blood was drawn into 1/10 volume of 3.8% tri-sodium citrate, two hours before and after the morning dose on the last day of each dose period.

**Oral administration of AV513:** Angus, Gloria, and Morag were orally administered with AV513 in # 0 capsules. In this continuous dose escalation study, animals were treated two times daily for 5 days at 3, 5 and 7.5 mg/kg and for 10 days at 15 mg/kg. For whole blood clotting time and to prepare citrated plasma for TEG analysis, aPTT and dPT assays, blood was drawn into 1/10 volume of 3.8% tri-sodium citrate two hours before and after the morning dose on the last day of each dose period.

**Multi-week dose escalation study:** Three treatment-naïve FVIII-deficient hemophilia A dogs: Bertha, Darla, and Wembley were treated orally with AV513 BID for seven days at 5, 7.5, 10 mg/kg and for ten days at 15 mg/kg. After a three-week wash out period, animals were treated with 20 mg/kg of AV513 for five days BID. Blood was drawn into 1/10 volume of 3.8% tri-sodium citrate for whole blood clotting time and TEG analysis two hours before and after the morning dose on the last day of each dose period except when animals were treated at 5 mg/kg, the analyses were performed on day five, before and after the morning dose. During the ten-day treatment at 15 mg/kg, blood was collected on the fifth day two hours after the morning dose to perform interim TEG analysis. Citrated plasma was prepared from each blood draw and frozen for aPTT and TEG assays.
In all dog studies, baseline analyses (TEG, aPTT, dPT, and whole blood clotting time) were performed seven days before starting the AV513 treatments. Clinical chemistry (urea, creatinine, alanine transaminase, bilirubin, creatine kinase) and hematology (hemoglobin, WBC, and platelet counts) assays were performed before and after the last dose of each dosing period. During the treatment period the dogs were closely monitored for 24 hours for cuticle re-bleeding after the CBT procedure and inspected daily for spontaneous bleeding and signs of discomfort.

*Pharmacokinetics of AV513*

Fluorescence labeling of AV513 was performed as previously described. Briefly, AV513 was activated by cyanogen bromide and derivatized with fluoresceinamine (Sigma, St. Louis, USA). Labeled AV513 (Fla-AV513) was purified on a G-50 sephadex (Sigma, St. Louis) gel column at a flow rate of 1.8 mL/min. Fractions were followed with a Hitachi L-2480 fluorescence detector at 490 nm. Fluorescence tag on AV513 was quantified using fluoresceinamine to derive a standard curve. The equation of this line was used to calculate the fluorescence concentration. Carbohydrate concentration was measured, using fucose as the standard, by the phenol-sulfuric acid method.

Four Sprague-Dawley rats (Harlan, Indianapolis, IN) housed for a week for acclimatization, were injected intravenously with Fla-AV513 at 5 mg/kg. Blood samples were collected at scheduled times through the tail vein into 1/10 volume of 3.8% trisodium citrate. Plasma was prepared by centrifuging whole blood at 6000 x g and read in a fluorescence reader at 490 nm. Plasma concentrations were determined from a standard
curve derived from known amount of Fla-AV513. Non-compartmental pharmacokinetic analysis was performed using the WinNonlin software program (Pharsight, Mountain View, CA).

**Statistical analyses**

Statistical significance among different groups was determined by the Student t test and error bars represent the standard deviation.
Results

The first studies were performed in three hemophilia A dogs that had previously received adeno-associated viral (AAV) vector-mediated canine FVIII gene therapy (AAV-FVIII). Following AAV gene transfer, these three AAV-FVIII dogs had undetectable levels of plasma FVIII (<1%) but showed shortening of their whole blood clot times and did not experience further spontaneous bleeding episodes for at least 3 years following treatment (normal frequency approximately 5/yr). We have regarded these dogs as showing partial phenotypic correction following AAV gene transfer. They best recapitulate the clinical picture documented in approximately 10% of severe hemophilia A patients (plasma FVIII <1%) who rarely bleed. Such a profile also mirrors the outcome of a low dose FVIII prophylaxis protocol.

In a multi-week dose escalation study, AV513 was administered subcutaneously twice daily to the AAV-FVIII hemophilia A dogs. CBT, plasma TEG assay, whole blood clotting time, dPT and aPTT assays performed on the last day of each dosing period served as efficacy endpoints. A dose-dependent decrease in cuticle bleeding time score was observed in all three dogs with an optimal activity at 1.0-1.5 mg/kg and a magnitude of efficacy within or very close to a normal dog CBT score of less than 10 (Table 2).

When citrated plasma from the three treated AAV-FVIII hemophilia A dogs was analyzed for changes in clot dynamics by TEG assay, a dose-dependent improvement in three important TEG parameters was observed. AV513-dependent reduction in clot initiation time (TEG R time), rate of fibrin generation (angle) and increased clot strength (MA) was evident. Significant improvement in clot initiation was observed when animals
were treated with AV513 at 1.0 and 1.5 mg/kg BID with TEG R time of 8.7 ± 2.4 and 9.6 ± 1.6 min, respectively, when compared to a baseline TEG R time of 24.4 ± 6.5 min (Figure 1A and 1B). Similar to the CBT scores, the slope of the TEG R time dose response curves varied between animals at low doses of AV513 (Fig.1A), although peak efficacy was observed at doses between 1.0 – 1.5 mg/kg where the TEG R time was corrected close to the normal dog plasma clot initiation time of <5 min (data not shown). Coincident with the decreased clot initiation time, a dose dependent rapid increase in angle (Fig. 1C) and clot strength (Fig. 1D) was observed at doses higher than 0.5 mg/kg, with the most effect observed at 1.5 mg/kg (angle; treated – 51 ± 4.9º vs baseline - 8.0 ± 7.0º, p =0.0009 and MA; treated – 25.5 ± 5.4 mm vs baseline – 7.4 ± 5.5 mm, p = 0.015). A representative dose-dependent improvement in clot dynamics is evident in Morag’s TEG tracings (Fig. 2A-F).

AV513 treatment did not affect other end points such as the whole blood clotting time, plasma dPT, and aPTT. Moreover, during the treatment period, there were no AV513-related changes in weekly-monitored hematology and clinical chemistry tests, no spontaneous bleeding episodes, and no behavioral or body weight changes.

Given the positive outcome with subcutaneous dosing, AV513 was next administered orally in the three AAV-FVIII hemophilia A dogs. In a multi-week dose escalation study, a single CBT analysis (Table 3) at the completion of 7.5 mg/kg dose and plasma TEG assay at the end of each dosing week was performed. Angus and Gloria had a substantial reduction in bleeding times with 79% and 68.5% reduction in CBT scores, respectively, while Morag had a slightly elevated score. Interestingly, when citrated plasma from the treated dogs was tested for clotting by TEG assay, a dose-dependent
decrease in the R time was observed with a maximum reduction in all three animals at 15 mg/kg (Figure 3).

As AV513 improved hemostasis in AAV-FVIII hemophilia A dogs, we next decided to evaluate its efficacy in severe, treatment-naïve hemophilia A dogs. The severe hemophilia A dogs in this colony have no detectable plasma levels of FVIII and have approximately five spontaneous bleeding episodes per year. The citrated plasma from these animals fails to clot in a TEG assay when stimulated with excess calcium. As a first step, the potency of AV513 to accelerate clot initiation was compared with citrated whole blood from AAV-FVIII hemophilia dogs and the severe hemophilia A dogs in an in vitro TEG assay. A dose-dependent reduction of TEG R time was observed in the citrated whole blood from treatment-naïve severe hemophilia A dogs and AAV-FVIII dogs (Figure 4) with an EC$_{50}$ of 2.8 µg/mL and 0.9 µg/mL, respectively.

In anticipation of a higher dose requirement for optimal efficacy in the treatment-naïve severe hemophilia A dogs, repeated 5 day oral dosing was initiated at 5 mg/kg BID and escalated to 20 mg/kg BID as the highest test dose. As end points, CBT analysis was performed at the end of 5, 7.5, and 15 mg/kg treatments, while TEG analysis was performed at the end of all dose periods with an additional interim analysis after 5 days when dogs received 15 mg/kg BID for 10 days. CBT scores from the treated dogs suggested a varied dose response in individual animals with a maximum efficacy observed at doses between 7.5 – 15 mg/kg (Table 4) with an optimal overall response at 15 mg/kg (Figure 5) in all three dogs. Interestingly, when whole blood TEG analysis was performed after each dose regimen, a 75% reduction in the R time (Figure 6A) was observed when dogs were treated with 7.5 mg/kg and 10 mg/kg and at 15 mg/kg BID for
5 days (interim). Surprisingly, when whole blood TEG analysis was performed after an additional 5 days of 15 mg/kg BID treatment, reduction of the TEG R time was not observed in all three dogs even though improvement in CBT scores was recorded (Table 4) in two of the three dogs. As we have seen no indications of attenuated efficacy with repeat daily dosing and as the CBT scores were clearly improved at this dose, we suspect an error in blood sampling and/or TEG analysis at this time point.

Given the endpoint variability at the end of 10 days of 15 mg/kg treatment of AV513, efficacy was assessed at a higher dose. After a three-week wash out period, the study was resumed with new baseline testing and then a seven-day BID regimen of 20 mg/kg of AV513. As seen in Table 4, two out of three dogs had bleeding times corrected to normal and, hence, a 90% reduction from baseline CBT scores. Darla had a peak response with 67% reduction in CBT score at 15 mg/kg and, surprisingly, did not respond at 20 mg/kg. The whole blood TEG analysis showed that all three dogs exhibited improved clotting dynamics two hours following the last treatment. The TEG analyses of blood samples drawn two hours before the last dose (fifteen hours after the previous dose) showed that all three animals clotted with an average TEG R time of 81 ± 24 min, while in the absence of AV513 no clotting was observed in two dogs (baseline >120 min) and in the third dog the baseline TEG R time was 112 min (Figure 6B). Hence, while AV513 efficacy is more optimal 2 hour post-dose, an effect 15 hours post-dose is still evident. Nonetheless, in a couple of assessments of efficacy at one or three week intervals following cessation of AV513 administration (s.c. or p.o.), CBT and TEG endpoints had returned to baseline levels (data not shown) indicating the absence of a sustained, cumulative effect.
As an additional component of pharmacological characterization, the pharmacokinetic properties of AV513 were examined with a fluorescent tagged AV513 (Fla-AV513) administered i.v. in rats. Fla-AV513 prepared with a labeling density of 2.36% (1 fluoresceine/ca. 42.4 fucose monomers), was injected intravenously into 4 rats and serial blood samples were collected at time points thereafter with analysis of plasma for Fla-AV513 levels. The plasma concentration vs time curve is presented in Figure 7. As determined by non-compartmental WIN NONLIN analysis, the elimination half-life was calculated to be 85 min, and other deduced parameters included a $C_{\text{max}}$ of 67 µg/mL, area under the curve (AUC$_\infty$ predicted) of 137 µg*hr/ml, $V_{ss}$ of 0.7 L/kg which may be indicative of low tissue penetration, and low plasma clearance of 36 mL/hr/kg.
Discussion

AV513, a fucoidan derived from brown seaweed is a branched sulfated polysaccharide of heterogeneous size. Fucoidans, present in more than fifty different species of brown seaweed have variable sulfation and possess fucose as the most abundant monosaccharide in the backbone but differ in the composition of other sugars such as xylose, mannose, galactose, and glucose. 10,34

The current results extend prior observations of in vitro and in vivo procoagulant efficacy by AV513. We previously reported 9 that AV513 inhibited exogenous TFPI activity and accelerated the clotting time of human hemophilia A and B plasma. In a murine tail transection model, fucoidan treatment improved the survival rate of hemophilia A mice. In this report, we have demonstrated, for the first time, the safety and efficacy of AV513 in minimally-treated hemophilia A dogs and in treatment-naïve severe hemophilia A dogs with undetectable levels of plasma FVIII.

Studies in FVIII deficient mice indicated that the maximum benefit was achieved when mice received fucoidan along with low dose intravenous infusion of rFVIII. The superior activity of fucoidan in the presence of very low FVIII levels prompted us to first evaluate the efficacy of subcutaneously administered AV513 in hemophilia A dogs that had received AAV-mediated FVIII gene therapy and had <1% of normal FVIII levels28 but had not experienced spontaneous bleeding post-gene transfer and exhibited shortened whole blood clot times. Coagulation parameters such as cuticle bleeding time, plasma and/or whole blood TEG analysis, aPTT, dPT and whole blood clotting were measured as efficacy end points. In a multi-week dose escalation study, AV513 was found to be
efficacious in all three dogs with a individualized dose response at twice daily s.c. doses 0.5-1.5 mg/kg with decreased CBT scores and accelerated clotting as observed in plasma TEG analysis. When AV513 was orally administered in the same dogs, plasma TEG R times were greatly improved at doses \( \geq 5 \) mg/kg. A single CBT analysis done at the end of 7.5 mg/kg treatment showed improved CBT scores in two of the three animals.

Given the demonstrated efficacy in the FVIII-AAV dogs, AV513 was then tested in treatment-naïve severe hemophilia A dogs with no detectable levels of FVIII. Oral administration of AV513 in these dogs also showed improvement in the cuticle bleeding time and TEG clotting analyses at higher doses. Improvement of the cuticle bleeding time score was observed mainly at 15 mg/kg and 20 mg/kg, while in whole blood TEG analysis, acceleration of clotting was observed at doses higher than 7.5 mg/kg. The requirement of higher doses of AV513 in the severe hemophilia A dogs for optimal activity is evident in the \textit{in vitro} whole blood TEG assay (Figure 4). To achieve a similar TEG R time, a three-fold higher concentration of AV513 is added to severe hemophilia A blood when compared to AAV-FVIII dogs. However, concentrations higher than 5 \( \mu g/mL \) of AV513 have similar potency in the absence or presence of low FVIII levels. These results further substantiate that AV513 can be more effective at low concentrations in the presence of sub-optimal levels of FVIII and at higher concentrations it has the potential to compensate for severe FVIII deficiency and function as a stand-alone procoagulant.

While an overall improvement from baseline was achieved at several dose levels, the individual animal optimal dose level(s) varied. AAV-FVIII dogs, Gloria and Morag, had a peak response when treated at 1.0 mg/kg and Angus at 1.5 mg/kg, as determined by
the CBT score in the subcutaneous administration study. Similarly, when the treatment-naïve severe hemophilia A dogs were treated orally, Darla had a maximum reduction in CBT score at 15 mg/kg but had no response with the 20 mg/kg regimen. Considering that AV513 at high concentrations can potentially become anticoagulant \(^9\), dogs were carefully monitored for dPT and aPTT changes or treatment-related bleeding episodes. The aPTT values of all dogs at every point in the study were slightly reduced or unchanged relative to baseline or washout values (data not shown). Platelet count and hematocrit values were likewise not affected by AV513 treatment. AAV-FVIII hemophilia A dogs, Gloria and Morag and severe hemophilia A dog, Darla, had a maximum reduction in the TEG R time when treated orally with 1.5 mg/kg s.c. and 20 mg/kg orally, respectively. The ex-vivo clotting of plasma and whole blood in these dogs suggests that at these treatment doses, AV513 promoted clot initiation. In contrast, the lack of CBT correction in these dogs at these AV513 doses could simply be due to low and variable circulating drug levels that can support the formation of a clot \textit{in vitro} but cannot maintain the clot \textit{in vivo} where other factors such as hemodynamic forces play a major role in clot stability. In addition, variability of CBT outcomes due to local vascular factors (ie. differences in the size of the injured vessel) and minor variations in the depth of incision cannot be ruled out despite the fact that well controlled procedures were performed.

Loss of AV513 activity at higher doses \textit{in vivo} can also be due to its potential anti-coagulant activity. While no increase in aPTT was observed at higher doses in the dogs, our studies in normal rats have shown that AV513 oral doses >100 mg/kg demonstrate little or no increase in aPTT (data not shown). In addition, the safety of
fucoidan has also been reported in animal toxicology and human clinical trials. In rats, a toxicology study with fucoidan derived from *Laminaria Japonica* was described wherein doubling of clotting time after oral dosing of male and female rats with a high dose of 2500 mg/kg/day was observed. Notably, over 6 months of treatment at this high dose, there were no adverse changes in hematology, serum chemistry and body weight. 35

The dual activity of AV513 as pro- and anti-coagulant does call for careful monitoring during human testing. Interestingly, safe outcomes were reported over multi-day and multi-week time at dose levels up to 3 g/day in human clinical studies with fucoidan derived from *Undaria Pinnatifida* orally administered to Herpes patients, 36 oncology patients and normal volunteers. 37 An apparently broad window of efficacy and safety for AV513 was affirmed in the current hemophilia A dog studies wherein three series of multi-week studies with six dogs were completed with no adverse events in behavior or clinical pathology endpoints. Thus, our results along with the published findings suggest that fucoidans appear well-tolerated and that safe hemostasis can be achieved.

In the development of AV513, one would ideally correlate efficacy with plasma or serum drug levels. However, with a botanical drug candidate like fucoidan, it is very challenging to analytically measure plasma levels of AV513 because of the heterogeneous molecular weight, branched structure, and similarity in monosaccharide composition to mammalian polysaccharides. Although there are limitations to labeling fucoidan due to its chemical structure, methods have been developed to achieve low density fluorescent labeling. 31 With 2.6% of fluorescent moieties on AV513, a labeling density that reduces detection sensitivity to 10 µg/mL, we determined the i.v. AV513
pharmacokinetics in rats. The heterogeneous species of Fla-AV513 exhibited an apparent elimination $t_{1/2}$ of 1.25 hours with a relatively low clearance rate of 36 mL/kg/hr. These PK parameters are superior to the properties reported for LMW fucoidan $^{14}$ which had a shorter half-life and was eliminated faster from circulation. Fucoidan is known to interact with L- and P-selectins $^{10,38-40}$ expressed on hematopoietic and vascular cells. It is possible that, aside from its interaction with coagulation protein target(s), AV513 may bind to vascular cells, given the potential for selectin interactions affecting its true half life.

AV513 administered either subcutaneously or orally, appears to provide therapeutically significant hemostatic improvement in low, or totally deficient, FVIII subjects (mice and dogs). Clearly, the current results underscore a need for individualized dose optimization in bleeding disorder patients. The combined efficacy and safety of AV513 by two routes of administration over extended time periods in two groups of hemophilia A dogs, supports the assessment of this drug candidate in humans. One objective of future studies will be to ascertain whether AV513 is best suited to use as an oral hemostatic adjunct to factor replacement (e.g. to complement prophylactic regimens) or whether its hemostatic efficacy is sufficient to act as a stand-alone procoagulant agent in some patients.

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KWJ, SP1 and DL designed the studies, analyzed the data and wrote the paper. AL, TK, EB, SP2, SP1 and SK performed the studies. Conflict-of interest; KWJ, SP1 and SK are employees of Avigen and interested in developing therapy for bleeding disorders. At the time these studies were performed, DL had no conflicts of interest. Subsequent to completion of the studies, DL has assumed a limited financial interest in Avigen.
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Table 2: Effect of subcutaneous injections of AV513 on the cuticle bleeding time (CBT) in AAV-FVIII Hemophilia A dogs. In a dose escalation study, three AAV-FVIII dogs received different doses of AV513 BID s.c. for 5 days. After each dosing period, dogs were evaluated for the cuticle bleeding time as described in methods. The number of blood drops/min was converted to a bleeding score and a total bleeding score was calculated for a 15 minute bleeding time evaluation at each time point. * normal range = \leq 10.

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Figure 1: Subcutaneous injections of AV513 improve the clot dynamics in AAV-hemophilia A dogs. In a dose-escalation study, plasma prepared from AAV-hemophilia A dogs before dosing and at the end of each dosing period were evaluated for clotting in a TEG assay. TEG R time represents the time required to initiate a 2 mm clot. A. Progressive change in TEG R times of individual animals during the treatment period is plotted. B. A TEG R time averaged from three dogs at each dose is represented as a bar graph with the standard deviation. (*p= 0.017, **p= 0.019 compared to baseline values) C. TEG angle represents the rate of fibrin formation, p values at 0.5, 1.0, and 1.5 mg/kg dose are 0.04, 0.002, and 0.0009 respectively D. TEG MA represents clot strength, p
values at 0.5, 1.0, and 1.5 mg/kg dose are 0.03, 0.02, and 0.015 respectively. Squares – Gloria, triangles – Morag and open rhombus – Angus. The boxed area in the graphs represents the clot dynamics values for normal plasma.

Figure 2. Improved clot dynamics in Morag’s plasma TEG tracings after s.c. AV513 treatment. In a dose-escalation study, plasma prepared from Morag 2 hours after the last dose was evaluated for clotting. Dose dependent reduction in TEG R time, improved angle and enhanced clot strength is recorded in the TEG tracings. A - Baseline, B - 0.03 mg/kg, C - 0.1 mg/kg, D – 0.5 mg/kg, E – 1.0 mg/kg, F – 1.5 mg/kg.
Table 3: Effect of orally administered AV513 on the cuticle bleeding time (CBT) in AAV-FVIII Hemophilia A dogs. Three AAV-FVIII dogs received an oral dose of 7.5 mg/kg of AV513 BID for 5 days. After the last dose, the cuticle bleeding time was measured and total CBT score was assessed. (* within normal range)
Fig 3: Oral administration of AV513 to AAV-FVIII hemophilia A dogs accelerates the plasma clotting time. Plasma prepared from AAV-hemophilia A dogs before and at the end of each dosing period were evaluated for clotting in a TEG assay. A TEG R time averaged from three dogs after each dose is represented as a bar graph with standard deviation. (*p = 0.05, **p = 0.01 compared to baseline values).
Fig 4: AV513 has different potency in AAV-FVIII and severe hemophilia A dog whole blood TEG assays. TEG R times were determined in citrated whole blood from dogs with low Factor VIII (AAV-FVIII) or severe, treatment-naive hemophilia A in the presence or absence of added AV513. TEG R times for each dose were averaged from three dogs/group.
Table 4: Effect of orally administered AV513 on the cuticle bleeding time (CBT) in severe hemophilia A dogs. Three treatment-naïve severe hemophilia A dogs received BID, indicated doses of AV513 by mouth for different periods of time. After the last treatment of each regimen, dogs were evaluated for cuticle bleeding time and a total CBT score was assessed. * Within normal range.

<table>
<thead>
<tr>
<th>AV513 dose (mg/kg)</th>
<th>Total CBT score</th>
<th>Bertha</th>
<th>Darla</th>
<th>Wembley</th>
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<tr>
<td>Baseline</td>
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<td>7.5 (7 days)</td>
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<td>29</td>
<td>11*</td>
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<td>Wash out (3 weeks)</td>
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<td>49</td>
<td>54</td>
<td>59</td>
</tr>
<tr>
<td>20.0 (7 days)</td>
<td></td>
<td>3*</td>
<td>58</td>
<td>2*</td>
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</table>
Figure 5: Oral administration of AV513 improves the cuticle bleeding in treatment-naïve severe hemophilia A dogs. Cuticle bleeding scores obtained after each dosing period were averaged from three dogs. (*p = 0.04 compared to baseline).
Figure 6: Improved clotting in treatment-naïve severe hemophilia A dogs following oral AV513 administration. A. Citrated whole blood from severe hemophilia A dogs prepared before and at the end of each dosing period was evaluated for clot formation in the TEG assay. A. TEG R time averaged from three dogs after each dose with the standard deviation value is represented as a bar graph. (*p= 0.001 compared to baseline).

B. Citrated whole blood from severe hemophilia A dogs prepared before and after the last dose of 20mg/kg were evaluated for clotting in the TEG assay. TEG R times were averaged from three dogs and are represented as a bar graph with the standard deviation. (*p= 0.002 compared to baseline). The 2 hours- pre analyses were performed with blood drawn 2 hours before the last dose (ie. 15 hours after the previous dose), while the 2 hours-post analyses were performed with blood drawn 2 hours after the last dose.
Figure 7: AV513 plasma concentrations following intravenous administration of fluorescent labeled AV513 (Fla-AV513) in rats. Four adult rats were injected intravenously with 5 mg/kg of Fla-AV513. At indicated time intervals post-dosing, blood was drawn for plasma preparation and Fla-AV513 was quantified by measuring fluorescence at 485/538 nm.
Efficacy and safety of a new-class of hemostatic drug candidate, AV513, in hemophilia A dogs

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