

# Functional Characterization of Abicipar-Pegol, an Anti-VEGF DARPIn Therapeutic That Potently Inhibits Angiogenesis and Vascular Permeability

Gerard A. Rodrigues, Matthew Mason, Lori-Ann Christie, Candice Hansen, Lisa M. Hernandez, James Burke, Keith A. Luhrs, and Thomas C. Hohman

Allergan plc, Irvine, California, United States

Correspondence: Gerard A. Rodrigues, Allergan, RD3-2D, 2525 Dupont Drive, Irvine, CA 92612, USA; [rodrigues\\_gerry@allergan.com](mailto:rodrigues_gerry@allergan.com).

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**PURPOSE.** DARPIn molecules are a novel class of small proteins that contain engineered ankyrin repeat domain(s) and bind to target proteins with high specificity and affinity. Abicipar-pegol (abicipar), a DARPIn molecule targeting vascular endothelial growth factor-A (VEGF-A), is currently under evaluation in patients with age-related macular degeneration. The pharmacodynamic properties of abicipar were characterized using in vivo and in vitro assays.

**METHODS.** The binding affinity of abicipar was assessed using a kinetic exclusion assay (KinExA). In vitro assays evaluated abicipar effects on VEGF-A<sub>165</sub>-induced calcium mobilization and tube formation in human umbilical vein endothelial cells. Abicipar was tested in vivo in a mouse model of corneal neovascularization and a rabbit model of chronic retinal neovascularization. The efficacies of abicipar and ranibizumab were compared in a rabbit model of VEGF-A<sub>165</sub>-induced retinal vasculopathy.

**RESULTS.** Abicipar has a high affinity for the soluble isoforms of VEGF-A; binding affinities for human VEGF-A<sub>165</sub> are approximately 100-fold greater than those of ranibizumab and bevacizumab and are similar for rat VEGF-A<sub>164</sub> but approximately 20-fold lower for rabbit VEGF-A<sub>165</sub>. Abicipar was effective in cell-based and in vivo models of angiogenesis and vascular leak, blocking neovascularization in a mouse model of corneal neovascularization and vascular permeability in a rabbit model of chronic neovascularization. In a rabbit model of VEGF-A<sub>165</sub>-induced vasculopathy, the duration of effect of abicipar was longer than ranibizumab when the two compounds were administered at molar-equivalent doses.

**CONCLUSIONS.** These data support the testing of abicipar as a treatment for retinal diseases characterized by neovascularization and vascular leak.

**Keywords:** abicipar, VEGF-A, DARPIn, wet AMD, age-related macular degeneration, angiogenesis, ophthalmology

Several members of the vascular endothelial growth factor (VEGF) family are potent regulators of vasculogenesis and angiogenesis.<sup>1</sup> The VEGF family includes placental growth factor (PlGF) and VEGF-A, -B, -C, -D, and -E.<sup>1</sup> A large body of evidence has established splice variants of VEGF-A as the primary mediators of both normal and pathologic angiogenesis (reviewed in Ferrara et al.<sup>1</sup>). VEGF-A was first identified in developmental systems and tumor cells as a vascular permeability factor and an angiogenic factor.<sup>2–7</sup> Studies of tumor biology established that VEGF expression is regulated by tissue hypoxia.<sup>7</sup> There are four major VEGF-A isoforms generated by alternative splicing. In humans, these isoforms are VEGF-A<sub>121</sub>, VEGF-A<sub>165</sub>, VEGF-A<sub>189</sub>, and VEGF-A<sub>206</sub>; the two smaller isoforms are soluble and freely diffusible, whereas the larger isoforms are bound to heparin sulfate and remain associated with cell surfaces or extracellular matrices.<sup>8</sup> VEGF-A<sub>165</sub>, the predominant isoform expressed in humans, is most frequently implicated in pathologic angiogenesis.<sup>1,9</sup> VEGF-A<sub>121</sub> and the degradation product VEGF-A<sub>110</sub> have mitogenic activity but are less potent than VEGF-A<sub>165</sub>.<sup>9,10</sup>

Retinal diseases, including age-related macular degeneration (AMD) and diabetic retinopathy, are characterized by neovas-

cularization and increased vascular permeability.<sup>11</sup> VEGF-A can be upregulated by hypoxia in retinal cells in vitro and in vivo.<sup>12–18</sup> These initial observations, together with the finding of increased VEGF-A levels in patients with diabetic retinopathy and other retinal diseases,<sup>14,19–21</sup> led to pivotal studies establishing a causal link between VEGF, ocular neovascularization, and vascular leakage. The demonstration that inhibition of VEGF using anti-VEGF antibodies, dominant negative VEGF receptors, or antisense oligonucleotides could block neovascularization in ocular models<sup>22–24</sup> paved the way for the development of anti-VEGF therapies for the treatment of retinal neovascular diseases.

There are three intravitreal anti-VEGF treatments currently approved for the treatment of neovascular AMD: pegaptanib (Macugen), a 28-base RNA aptamer that selectively binds and inhibits VEGF-A<sub>165</sub><sup>25</sup>; ranibizumab (Lucentis), a recombinant, humanized antibody antigen binding fragment (Fab) that neutralizes the soluble splice variants and proteolytic fragment of VEGF-A<sub>26</sub>; and aflibercept (Eylea), a chimeric protein consisting of portions of VEGF receptors 1 and 2 fused with the Fc portion of human immunoglobulin G1 (IgG1).<sup>27</sup> Bevacizumab, a humanized anti-VEGF monoclonal antibody



approved for cancer therapy, is widely used for this indication on an off-label basis.<sup>28,29</sup>

The ANCHOR<sup>30</sup> and MARINA<sup>31</sup> registration studies with ranibizumab established monthly intravitreal injections of ranibizumab as the standard treatment for neovascular AMD; the RISE and RIDE studies similarly established monthly intravitreal injections of ranibizumab as the standard of care for diabetic macular edema.<sup>32</sup> In an effort to reduce patient treatment burden, several more recent studies compared a fixed monthly treatment schedule with a pro re nata (PRN) treatment schedule. In the 12-month HARBOR study of patients with neovascular AMD, the mean improvement of best corrected visual acuity was 10.1 letters in the group receiving monthly injections versus 8.2 letters in the PRN treatment group. The group receiving monthly treatments also appeared to have a greater reduction in the choroidal neovascular lesion (CNV); the mean reduction in CNV lesion area was 2.14 disc areas (DA) compared with 1.74 DA in the PRN treatment group.<sup>33</sup> These results are similar to those of the CATT study, where switching from a monthly to a PRN regimen after the first year resulted in significantly worse visual outcomes.<sup>34</sup> The results of the VIEW 1 and VIEW 2 trials with aflibercept supported a bimonthly dosing regimen after three initial monthly doses.<sup>35</sup> Although monthly or bimonthly dosing is recommended for current anti-VEGF therapies, a number of studies, including LUMIERE,<sup>36</sup> AURA,<sup>37</sup> and WAVE,<sup>38</sup> have documented that in real-world experience, patients receive fewer treatments than those participating in clinical trials and, as a consequence, have poorer than expected visual outcomes. In addition to the significant treatment burden for patients and caregivers, frequent intravitreal injections increase the risk for complications, including endophthalmitis, cataracts, retinal detachment, and vitreous hemorrhage. Thus, achieving the benefit of monthly dosing with fewer intravitreal injections remains a significant unmet need.

DARPIn molecules are a novel class of binding proteins with the potential to overcome some limitations of antibody-based therapeutics. DARPIn molecules are small, highly stable proteins that contain engineered ankyrin repeat domain(s) and can be selected to bind any given target protein with high specificity and affinity.<sup>39</sup> They are derived from natural ankyrin repeat motifs, protein domains that mediate many protein-protein interactions. Starting with large, diverse ribosome display DARPIn libraries, and applying tools including error-prone polymerase chain reaction, off-rate and specificity selection schemes allow for the identification of molecules with high specificity and high affinity without some of the constraints of immunization-based technologies.<sup>40</sup> Additionally, the mode of target binding for DARPIn molecules is unique compared with prototypical antibody binding and likely also contributes to their high-affinity binding.<sup>39</sup> The rigid binding surface of DARPIn molecules minimizes free entropy losses upon binding, in contrast to antibody interactions, which typically involve induced fit of the flexible hypervariable loops making contact with the antigen.<sup>41</sup> Consistent with their consensus design, X-ray crystallography studies have revealed that the residues responsible for target binding are on the concave surface of the DARPIn molecule, forming an extended surface for interaction.<sup>39</sup> DARPIn molecules with binding affinities in the picomolar range are typical.<sup>42,43</sup>

In addition to their high affinity and selectivity, DARPIn molecules also display remarkable stability, as illustrated by melting temperatures routinely exceeding 80°C and in some cases even 100°C.<sup>44</sup> The molecular mass for a typical four- or five-repeat DARPIn molecule is about 14 to 18 kDa, which is approximately one-tenth the size of an antibody or one-third the size of a Fab fragment. This smaller size allows for higher dosing on a molar basis and has the potential to confer

improved tissue penetration. Moreover, DARPIn molecules can be engineered to modulate local or systemic pharmacokinetics through the attachment of additional DARPIn domains or through PEGylation. Thus, DARPIn molecules offer a number of advantages over existing antibodies or antibody fragments as potential therapeutics, including high affinity, stability, and small size.

Recently, several highly potent DARPIn molecules selected for their ability to neutralize VEGF-A have been described.<sup>45</sup> Here, we evaluate the properties of the anti-VEGF DARPIn molecule abicipar pegol (hereinafter referred to as abicipar), currently in phase III studies for the treatment of neovascular AMD. Abicipar is a 14-kDa recombinant protein coupled to a 20-kDa polyethylene glycol (PEG) moiety to yield a 34-kDa molecule. Previous phase I/II dose-escalation studies demonstrated the efficacy of abicipar for treatment of diabetic macular edema and AMD.<sup>46,47</sup> Additionally, pharmacokinetic data from the diabetic macular edema study showed that abicipar is present in the aqueous humor at levels several orders of magnitude above the half maximal inhibitory concentration (IC<sub>50</sub>) for at least 12 weeks.<sup>46</sup> Here, we show that abicipar binds all soluble VEGF-A isoforms with high affinity, potentially neutralizes VEGF-A<sub>165</sub> in a number of *vitro* assays, and effectively blocks both neovascularization and vascular leak in animal models. In a head-to-head comparison, abicipar showed affinity and potency for VEGF-A<sub>165</sub> similar to that of aflibercept but greater than bevacizumab and ranibizumab. Consistent with these observations, abicipar inhibited vascular leak for a significantly greater duration than ranibizumab in a rabbit model of VEGF-A<sub>165</sub>-induced vasculopathy. Together, these data establish the potential of abicipar to be clinically effective for a prolonged duration in patients with retinal neovascular diseases.

## MATERIALS AND METHODS

### VEGF Reagents

Ranibizumab (Lucentis) and bevacizumab (Avastin; Genentech, Inc., South San Francisco, CA, USA) as well as aflibercept (Eylea; Regeneron, Tarrytown, NY, USA) were purchased. Recombinant human VEGF-A (rhVEGF-A) proteins and recombinant rat VEGF<sub>164</sub> were obtained from R&D Systems (Minneapolis, MN, USA). Rabbit VEGF-A<sub>165</sub> was produced at West Bioscience Research (Irvine, CA, USA). A biotin-SP-AffiniPure goat anti-human IgG F(ab')<sub>2</sub> (Jackson ImmunoResearch, West Grove, PA, USA) was used to detect ranibizumab. Bevacizumab was detected using a biotin-SP-AffiniPure goat anti-human IgG (H+L) (Jackson ImmunoResearch). Aflibercept was detected using a biotinylated-SP-conjugated affinity-purified goat anti-human immunoglobulin G (IgG) (Fcγ) (Jackson ImmunoResearch). Abicipar was detected using either a biotinylated antibiologic antibody, followed by fluorescently labeled streptavidin (Jackson ImmunoResearch) or a fluorescently conjugated, affinity-purified mouse anti-PEG monoclonal IgM (ANP Tech, Newark, DE, USA).

### Binding Affinity Determination

A kinetic exclusion assay (KinExA) that measures the equilibrium binding affinity and kinetics between unmodified molecules in solution was used to assess the binding kinetics of abicipar. For affinity analysis, the equilibrium dissociation constant  $K_d$  and the rate of association  $k_{on}$  were experimentally determined, and the rate of dissociation  $k_{off}$  was calculated based on the following equation:  $k_{off} = K_d \cdot k_{on}$ .

For  $K_d$  measures, individual reactions containing ranibizumab (100–500 pM), bevacizumab (80–400 pM), aflibercept (422 fM–84.3 pM), or abicipar (500 fM–100 pM) versus a series of VEGF-A concentrations ranging from low femtomolar to nanomolar were allowed to bind to equilibrium (5–360 hours). The free fraction of each anti-VEGF inhibitor was then measured using the KinExA 3200 instrument (Sapidyne Instruments, Boise, ID, USA) and analyzed using the KinExA Pro software to calculate affinity. Azlactone beads (Sapidyne) coated with 10  $\mu\text{g/mL}$  VEGF-A<sub>165</sub> were used to capture free anti-VEGF drug, which was detected using either a biotinylated antibiologic antibody, followed by fluorescently labeled streptavidin, or anti-PEG antibody conjugated to Alexa 647. The dissociation constants were derived from fitting data to a 1:1 binding model.<sup>48</sup>

For  $k_{on}$  measurements, the free concentration of the anti-VEGF molecule was measured under pre-equilibrium conditions as a function of time for ranibizumab, bevacizumab, and abicipar using the “direct” method from reactions set up as follows: 500 pM ranibizumab versus 790 pM VEGF-A<sub>165</sub>, 40 pM bevacizumab versus 82 pM VEGF-A<sub>165</sub>, and 200 pM abicipar versus 91 or 497 pM VEGF-A<sub>165</sub>. As binding progressed toward equilibrium, free drug was captured and detected at multiple time points using the same VEGF-A-azlactone bead detection system. Pre-equilibrium sample draws were collected after incubation periods of 6, 12, or 18 minutes. Data were fitted to a direct pre-equilibrium binding model<sup>48</sup> to derive  $k_{on}$  using the KinExA Pro software;  $k_{off}$  was calculated from  $k_{on}$  and  $K_d$  as described above.

The  $k_{on}$  of aflibercept was measured using the “inject” method,<sup>48</sup> where the concentration of aflibercept (100 pM) and incubation time were held constant; VEGF-A<sub>165</sub> concentrations in the binding reactions ranged from 5.26 nM to 10.27 pM. Unbound aflibercept from each reaction was measured on the KinExA 3200 as above, and the resulting data were fitted and analyzed using the inject binding curve function of the KinExA Pro software.

### VEGF-Induced Calcium Mobilization Response (FLIPR Assay)

Human umbilical vein endothelial cells (HUVECs; Lonza Ltd, Basel, Switzerland) were plated at a density of 6,000 cells per well in fibronectin-coated glass bottom 384-well plates (Bio-MedTech, Tampa, FL, USA) in complete endothelium cell growth medium (EGM; Lonza Ltd) and incubated at 37°C overnight. Cells were then loaded with calcium-sensitive dye Fluo-4 AM (Invitrogen, Life Technologies, Grand Island, NY, USA) for 50 minutes in 20 mM HEPES, 2.5 mM probenecid, and 1% fetal bovine serum (pH 7.4); washed in the same solution without Fluo-4 AM; and incubated at 37°C for 5 minutes prior to stimulation with VEGF-A<sub>165</sub>. A FLIPR Tetra system (Molecular Devices, San Jose, CA, USA) was used to record calcium responses at emission and excitation wavelengths of 575 and 515 nm, respectively. Data were plotted and analyzed using peak fluorescence values from cells treated with VEGF-A<sub>165</sub> with or without preincubation with anti-VEGF molecules.

### Assessment of Inhibition of the VEGF-A-Induced Calcium Mobilization Response

rhVEGF-A<sub>165</sub>, ranibizumab, bevacizumab, aflibercept, and abicipar were diluted to 2× the final top concentrations in FLIPR assay buffer. Dilutions of the anti-VEGF molecules were conducted in FLIPR assay buffer to give final concentrations between 6.51 nM and 100 fM, were mixed with equal volumes of VEGF-A<sub>165</sub> in 384-well plates, shaken (600 RPM) for 5 minutes, and then incubated at 30°C for 4 hours. These

mixtures of VEGF-A<sub>165</sub> (26 pM) and test agents were added to HUVECs, and the FLIPR assay as described above was used to measure inhibition of the VEGF-A-induced calcium response. Each agent was tested in duplicate, and the dose responses of all four anti-VEGF molecules were evaluated in six independent experiments following the same procedures. Statistical significance was determined with an adjusted alpha level using Bonferroni correction.

### Three-Dimensional Tube Formation Assays

Three-dimensional tube formation assays were conducted as previously described.<sup>49</sup> To facilitate visualization and quantitation of sprouts, HUVECs were prelabeled with the fluorescent dye CellTracker Red CMTPX (Life Technologies, Grand Island, NY, USA) diluted 1:1000 in cell culture media at 37°C for 30 minutes. Following the removal of the staining solution, HUVECs were absorbed onto dextran-coated cytodex 3 beads (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) at a concentration of 400 cells/bead for 4 hours and then transferred to a T25 flask in EGM-2 and incubated overnight. The following day, 200 HUVEC-coated beads were collected and mixed with 100  $\mu\text{L}$  of a 20 mg/mL fibrinogen solution and added in 1 mL EGM-2 medium to a 12-well plate containing 10  $\mu\text{L}$  thrombin (0.06 units/ $\mu\text{L}$ ). Plates were left to polymerize at room temperature for 5 minutes and then transferred to 37°C; EGM-2 medium was added and incubated for 20 minutes at 37°C. The medium was removed and Detroit 551 human skin fibroblasts (ATCC, Manassas, VA, USA) were added to the top of the gel at a concentration of  $4.8 \times 10^5$  cells/well with 2.5 ng/mL of rhVEGF-A<sub>165</sub> and 2 ng/mL of hepatocyte growth factor in 1 mL EGM-2 with or without abicipar. The cultures were incubated at 37°C and monitored daily for vessel sprouting. Medium containing drug was changed every 2 days. Two weeks after plating, multiple images of the cultures were collected using a Keyence BZ-9000E fluorescence microscope (Keyence, Itasca, IL, USA). Image settings were standardized and maintained across all treatment groups, and images were saved as TIFF files for quantitative analysis. For each dilution, the number of sprouts on 10 beads taken from multiple fields were counted and expressed as the mean number of sprouts per bead. The length of a sprout had to exceed one bead diameter (175  $\mu\text{m}$ ) to be counted.

### Animal Welfare

Animals were treated in accordance with United States Department of Agriculture (USDA) guidelines and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. This study complied with all requirements of the USDA; all regulations issued by the USDA implementing the Animal Welfare Act, 9 CFR, Parts 1, 2, and 3; the Guide for the Care and Use of Laboratory Animals; and all requirements for approval by Allergan's Animal Care and Use Committee.

### Mouse Corneal Neovascularization Model

Mice ( $n = 8/\text{group}$ ) were anesthetized with an intraperitoneal (i.p.) injection of a cocktail of 100 mg/kg ketamine and  $\leq 10$  mg/kg xylazine. One to two drops of proparacaine hydrochloride were placed in the right eye prior to the debridement procedure. Three microliters of a 0.15-mM NaOH solution were applied to the corneal surface and, after 30 seconds, were removed with a saline wash. The corneal and limbal epithelium were then removed using a number 21 surgical blade. Mice were dosed daily with 8 mg/kg abicipar i.p. for 11 days in prevention mode (day 1 [D1] to D9) or 10 days (D14 to D23) in intervention mode. Mice in the control group received an

equal volume of vehicle (i.e., 0.2 mL PBS) injected i.p. At termination of the experiment, anesthetized mice ( $n = 5/\text{group}$ ) were injected with 100 to 250  $\mu\text{L}$  of 2000 kDa FITC-dextran (25 mg/mL) via the tail vein and euthanized by cervical dislocation 10 minutes later. Right and left eyes were enucleated and placed in 4% paraformaldehyde for 1 hour at 4°C. Corneas were dissected, flat-mounted, and photographed using a Keyence BZ-9000E fluorescence microscope (Keyence, Itasca, IL, USA). A masked experimenter used Image J to trace the area of neovascularization and quantified the percentage of corneal area occupied by neovessels. A Student's *t*-test was used for pairwise comparisons between abicipar and the corresponding vehicle group, and a *P* value of  $\leq 0.05$  was considered statistically significant. The remaining three mice from each experimental group were euthanized by cervical dislocation and the right and left eyes enucleated and placed in 4% paraformaldehyde for 1 hour at 4°C. Corneas were double stained with CD31 conjugated to FITC (Invitrogen, Carlsbad, CA, USA) and  $\alpha\text{SMA-Cy3}$  (Sigma-Aldrich Corp., St. Louis, MO, USA) in PBS with 0.5% Triton X-100, incubated at room temperature overnight, and flat-mounted on a glass slide. Images were captured using a Zeiss LSM 710 confocal microscope at 20 $\times$ .

### Rabbit Model of Chronic Retinal Neovascularization

Unilateral (OD) retinal neovascularization was induced in Dutch Belted rabbits (Covance, San Diego, CA, USA) by intravitreal injection of 400  $\mu\text{g}$  (50  $\mu\text{L}$  of a 8-mg/mL solution) of dl-alpha-aminoadipic acid (DL-AAA). Angiographic leak was monitored for 2 months following DL-AAA treatment. DL-AAA-treated rabbits, with stable angiographic leak area of at least 2 disc diameters and leak severity of  $\geq 3$  on a 0 to 4 scale, were selected for the study. Four rabbits received a single intravitreal injection of 700  $\mu\text{g}$  of abicipar. Following the application of topical anesthesia, 50  $\mu\text{L}$  of abicipar was delivered by intravitreal injection to the midvitreal using a 31-gauge 0.3-mL insulin syringe. The entry point was in the superior sclera on the temporal side of the superior rectus muscle, 4 mm from the limbus. Animals were examined using fluorescein angiography at baseline (maximum of 2 weeks prior to dosing of abicipar); weeks 2, 4, and 6; and then weekly thereafter out to 10 weeks. High-resolution fluorescein angiograms were taken with a Heidelberg retinal angiographic system (HRA2) fitted with a 55 degree lens following an intravenous injection of 0.5 mL 10% sodium fluorescein into the marginal ear vein. Late-phase angiograms of the experimental eye and the contralateral normal eye were obtained. Angiograms were then composited, including each region of the eye that was captured (i.e., nasal, temporal, and central retinal vessels). The “scaling” or focus at which the images were captured was noted, and this number was used for calculating the area of leak ( $\text{mm}^2$ ) from the number of pixels that were present per micron in the selected region of leak in the composite image. A masked experimenter traced the area of leakage and quantified the area of encompassed pixels normalized relative to baseline for each eye and expressed this value as a percentage. Summary data are presented as the mean  $\pm$  the standard deviation.

### Rabbit Retinal Vasculopathy Model

Fifty-four Dutch Belted rabbits (Covance) were anesthetized with 5 mg/kg ketamine and 1.5 mg/kg xylazine i.v. Eighteen rabbits received a single intravitreal injection of 120  $\mu\text{g}$  (50  $\mu\text{L}$ ) of abicipar into each eye. A second cohort of 18 rabbits received a single intravitreal injection of 170  $\mu\text{g}$  (50  $\mu\text{L}$ ) of

**TABLE 1.** Binding Affinity of Abicipar for VEGF-A Isoforms From Different Species Determined by a KinExA

| Ligand                       | Affinity ( $K_d$ ) | 95% CI ( $K_d$ ) |
|------------------------------|--------------------|------------------|
| Human VEGF-A <sub>165</sub>  | 394 fM             | 264–552 fM       |
| Human VEGF-A <sub>189</sub>  | 745 fM             | 0.75–1.66 pM     |
| Human VEGF-A <sub>121</sub>  | 789 fM             | 0.43–1.38 pM     |
| Human VEGF-A <sub>111</sub>  | 80.6 fM            | 23.50–168 fM     |
| Rat VEGF-A <sub>164</sub>    | 386 fM             | 260–556 fM       |
| Rabbit VEGF-A <sub>165</sub> | 8.49 pM            | 4.51–15.50 pM    |

CI, confidence interval.

ranibizumab into each eye. A third cohort of 18 rabbits received a single intravitreal injection of vehicle (50  $\mu\text{L}$  sterile saline) into each eye. Intravitreal injections were positioned midvitreal using a 0.5-cc syringe attached to a 28G  $\times$  1/2-inch needle inserted superotemporal through the pars plana approximately 3 mm posterior to the limbus. Two, 4, and 6 weeks after the intravitreal injection of abicipar, ranibizumab, or vehicle, rabbits received a single intravitreal injection of 100  $\mu\text{L}$  containing 1  $\mu\text{g}$  rhVEGF-A<sub>165</sub> in PBS with 0.1% BSA; the VEGF-A<sub>165</sub> was injected into the midvitreal of both eyes using a 0.5-cc syringe with a 28G  $\times$  1/2-inch needle. The needle was inserted superonasal into the pars plana approximately 3 mm posterior to the limbus. Seventy-two hours post-VEGF-A<sub>165</sub> treatment, pupils were dilated and the rabbits were anesthetized. Using a Zeiss FF450 retinal camera, color fundus images of the retinal vasculature were captured and late-phase angiograms were acquired following intravenous injection of 10% sodium fluorescein (10 mg/kg). Examiners masked to the identity of the treatment groups reviewed fundus images and fluorescein angiograms from both nasal and temporal retinal vascular beds. Each fundus image received a combined score for both vascular tortuosity and caliber (scale of normal = 1 to severe = 5) from each examiner. Each fluorescein angiogram was scored for the severity of retinal leakage (scale of no leakage = 1 to severe leakage = 5). Standard images representative of scores for both fundus and angiography grading scales were used for reference to ensure scoring consistency. The nasal and temporal image scores from three examiners were averaged for each eye ( $n = 11$  or 12 eyes per treatment or vehicle control group per time point). Statistical significance between the treatment groups and the vehicle control group was determined using the nonparametric two-tailed Kruskal-Wallis ANOVA test followed by Dunn's multiple comparison test.

## RESULTS

### Binding Affinity of Abicipar for VEGF Isoforms From Different Species Determined Using a KinExA

A KinExA was used to determine the binding affinities of abicipar for the various isoforms of VEGF-A. The abicipar binding affinity ( $K_d$ ) was 394 fM for human VEGF-A<sub>165</sub>. The binding affinities for other splice variants of human VEGF-A, including VEGF-A<sub>189</sub>, VEGF-A<sub>121</sub>, and VEGF-A<sub>110</sub>, were all subpicomolar (Table 1). To determine whether rodent and rabbit models could be used to test the in vivo efficacy of abicipar, the binding affinities for rat and rabbit VEGF-A<sub>165</sub> were measured. Although the binding affinity of abicipar for rat VEGF-A<sub>164</sub> was similar to that for human VEGF-A<sub>165</sub>, the affinity for rabbit VEGF-A<sub>165</sub> was 20-fold lower than for human VEGF-A<sub>165</sub> (Table 1). Thus, abicipar demonstrates high affinity binding to VEGF-A from multiple species.

TABLE 2. Human VEGF-A<sub>165</sub>-Binding Affinity of Ranibizumab, Bevacizumab, Aflibercept, and Abicipar Evaluated in Solution by a KinExA

| VEGF Inhibitor | K <sub>d</sub> | K <sub>d</sub> 95% CI | k <sub>on</sub> (M <sup>-1</sup> s <sup>-1</sup> ) | k <sub>on</sub> 95% CI (M <sup>-1</sup> s <sup>-1</sup> ) | k <sub>off</sub> (s <sup>-1</sup> ) |
|----------------|----------------|-----------------------|----------------------------------------------------|-----------------------------------------------------------|-------------------------------------|
| Ranibizumab    | 42.5 pM        | 14.3 pM–103 pM        | 8.25 × 10 <sup>4</sup>                             | 7.17 × 10 <sup>4</sup> –9.43 × 10 <sup>4</sup>            | 3.46 × 10 <sup>-6</sup>             |
| Bevacizumab    | 30.3 pM        | 11.5 pM–81.3 pM       | 2.55 × 10 <sup>5</sup>                             | 2.02 × 10 <sup>5</sup> –3.13 × 10 <sup>5</sup>            | 7.72 × 10 <sup>-6</sup>             |
| Aflibercept    | 200 fM         | 100 fM–392 fM         | 9.02 × 10 <sup>6</sup>                             | 7.64 × 10 <sup>6</sup> –1.05 × 10 <sup>7</sup>            | 1.80 × 10 <sup>-6</sup>             |
| Abicipar       | 486 fM         | 150 fM–1.17 pM        | 7.94 × 10 <sup>6</sup>                             | 6.48 × 10 <sup>6</sup> –9.61 × 10 <sup>6</sup>            | 3.86 × 10 <sup>-6</sup>             |

**VEGF-Binding Affinity of Ranibizumab, Bevacizumab, Aflibercept, and Abicipar Evaluated in Solution by KinExA**

The VEGF-A<sub>165</sub> binding affinities and binding kinetics of abicipar were compared with those for other anti-VEGF biologics currently in use for the treatment of AMD. The binding affinity of abicipar for human VEGF-A<sub>165</sub> was similar to that of aflibercept, 486 fM and 200 fM, respectively. The values for both compounds are within the 95% confidence intervals of either compound. In contrast, these affinities are approximately 100-fold greater than those of ranibizumab and bevacizumab (Table 2). VEGF-A<sub>165</sub> association rates (k<sub>on</sub>) for abicipar were

also evaluated and compared with those for aflibercept, ranibizumab, and bevacizumab. The VEGF-A<sub>165</sub> k<sub>on</sub> for abicipar was 7.94 × 10<sup>6</sup> M<sup>-1</sup>s<sup>-1</sup>, and the k<sub>on</sub> value for aflibercept was 9.02 × 10<sup>6</sup> M<sup>-1</sup>s<sup>-1</sup>. Both abicipar and aflibercept bind VEGF-A<sub>165</sub> with 30- to 100-fold faster on-rates than those observed with ranibizumab (8.25 × 10<sup>4</sup> M<sup>-1</sup>s<sup>-1</sup>) and bevacizumab (2.55 × 10<sup>5</sup> M<sup>-1</sup>s<sup>-1</sup>) (Table 2). Thus, differences in binding affinity between either abicipar or aflibercept and ranibizumab or bevacizumab are largely attributable to differences in on-rates between the compounds.

**VEGF Neutralization Comparison of Ranibizumab, Bevacizumab, Aflibercept, and Abicipar**

We evaluated the ability of ranibizumab, bevacizumab, aflibercept, and abicipar to neutralize ligand activation of VEGF receptors in a cellular assay measuring VEGF-A<sub>165</sub>-induced calcium mobilization. Published data indicated that preincubation time with VEGF-A<sub>165</sub> has a significant effect on the apparent potency of some anti-VEGF molecules with slower VEGF-binding on-rates.<sup>50</sup> Thus, we selected 26 pM VEGF-A<sub>165</sub> and 4 hours of preincubation at 30°C conditions to minimize the effect of slower on-rates on the potency assessments while still preserving the activity of VEGF-A<sub>165</sub>. Under these conditions, mean potencies (IC<sub>50</sub> values) were 101.9 pM for ranibizumab, 97.4 pM for bevacizumab, 13.8 pM for aflibercept, and 24.8 pM for abicipar. Figure 1A shows representative fitted dose-response curves from one of the six experiments with these anti-VEGF agents. Figure 1B summarizes the data (IC<sub>50</sub> values) from all six experiments. Significant differences (*P* < 0.0001) were observed between abicipar and both ranibizumab and bevacizumab but not between abicipar and aflibercept.

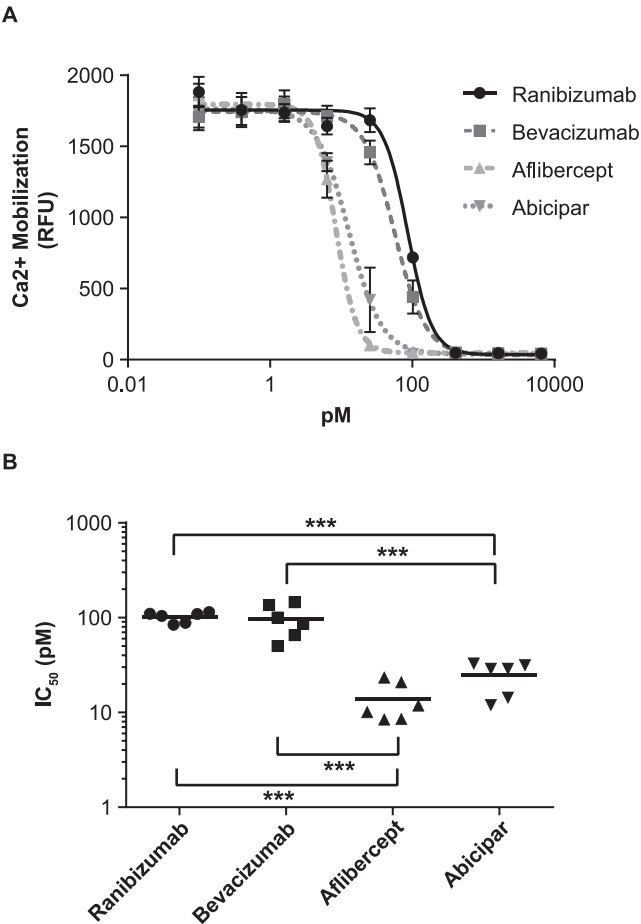


FIGURE 1. VEGF neutralization comparison of ranibizumab, bevacizumab, aflibercept, and abicipar in the calcium mobilization assay. Shown are representative dose-response curves from one of six experiments (A). Error bars represent  $\pm$  SEM (*n* = 4 replicates). Graph of VEGF-A<sub>165</sub> neutralization potencies of ranibizumab, bevacizumab, aflibercept, and abicipar in the calcium mobilization assay is shown (B). Each data point represents the mean IC<sub>50</sub> calculated from duplicates from one experiment. The line indicates the mean IC<sub>50</sub> from six experiments for that molecule. \*\*\**P* < 0.0001 versus ranibizumab and bevacizumab.

**Effects of Abicipar on Angiogenesis in a Cell-Based Assay for Vascular Tube Formation**

To test the ability of abicipar to inhibit angiogenesis in vitro, a three-dimensional in vitro model of vessel sprouting was employed.<sup>49</sup> In this assay HUVECs, supported by a feeder layer of fibroblasts, are grown on cytodex beads embedded in a fibrin gel. Representative images are shown in Figure 2A. In the presence of VEGF-A<sub>165</sub>, robust sprouting is observed within the fibrin matrix (Fig. 2A, left and middle panels). Abicipar inhibited VEGF-A<sub>165</sub>-mediated tube formation in a dose-dependent fashion with an IC<sub>50</sub> of 1.1 nM (Fig. 2B). These results demonstrate the ability of abicipar to potently block angiogenesis in vitro.

**Abicipar Inhibits Neovascularization in a Corneal Angiogenesis Model**

To test its ability to inhibit angiogenesis in vivo, abicipar was tested in a mouse model of corneal neovascularization induced by limbal and epithelial debridement. Mice were dosed once per day with 8 mg/kg abicipar from D1 to D9 postsurgery. The primary endpoint was the percent area of cornea occupied by neovessels, as assessed by FITC-dextran

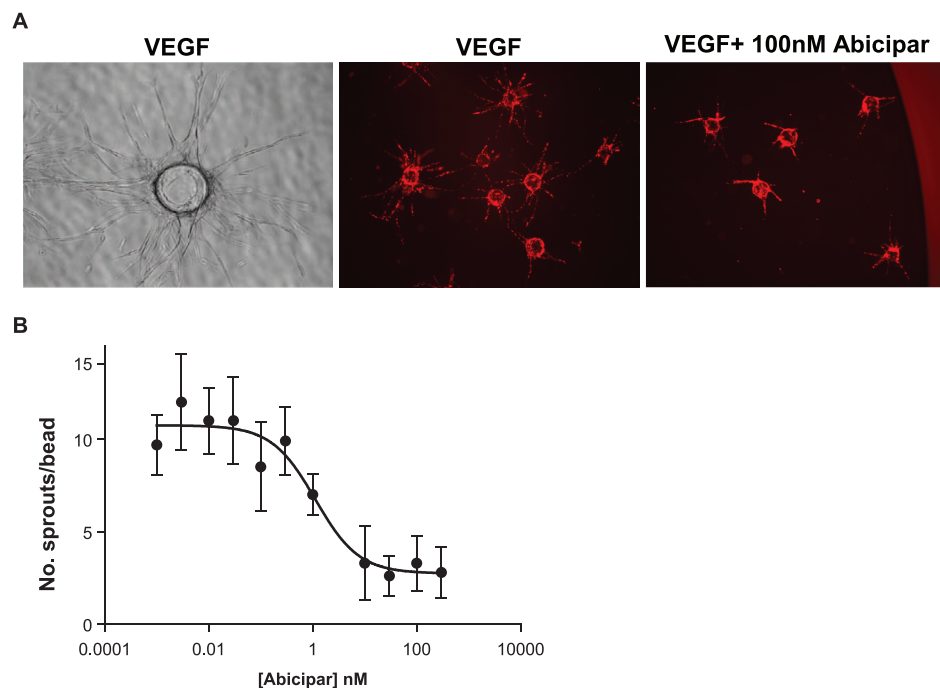


**FIGURE 2.** Effects on VEGF with and without abicipar on tube formation using a three-dimensional in vitro model of vessel sprouting. (A) *Left panel* is a phase contrast image showing extensive stimulation of tube formation in the presence of 2.5 ng/mL VEGF (20× magnification). *Middle panel* is a fluorescent image of HUVECs labeled with CellTracker Red dye and treated with VEGF (20× magnification). *Right panel* is a fluorescent image of HUVECs labeled with CellTracker Red dye and treated with VEGF plus 100 nM abicipar (10× magnification). (B) The graph shows the number of sprouts/bead and demonstrates a dose-dependent inhibition of vessel sprouting. Values represent the means  $\pm$  SD.

perfusion. In addition, immunohistochemical staining of corneal flatmounts was conducted using anti-CD31 and anti- $\alpha$ SMA antibodies. Figure 3A shows the angiostatic efficacy of abicipar on mouse corneal neovascularization in prevention mode. Abicipar inhibited vessel growth by 84% compared

with the PBS-treated group. Similarly, abicipar inhibited vessel growth when administered using an intervention mode (Fig. 3B). Representative images of corneal flatmounts stained with anti-CD31 and anti- $\alpha$ SMA antibodies are shown in Figure 3 and illustrate the inhibition of vessel growth by abicipar. These

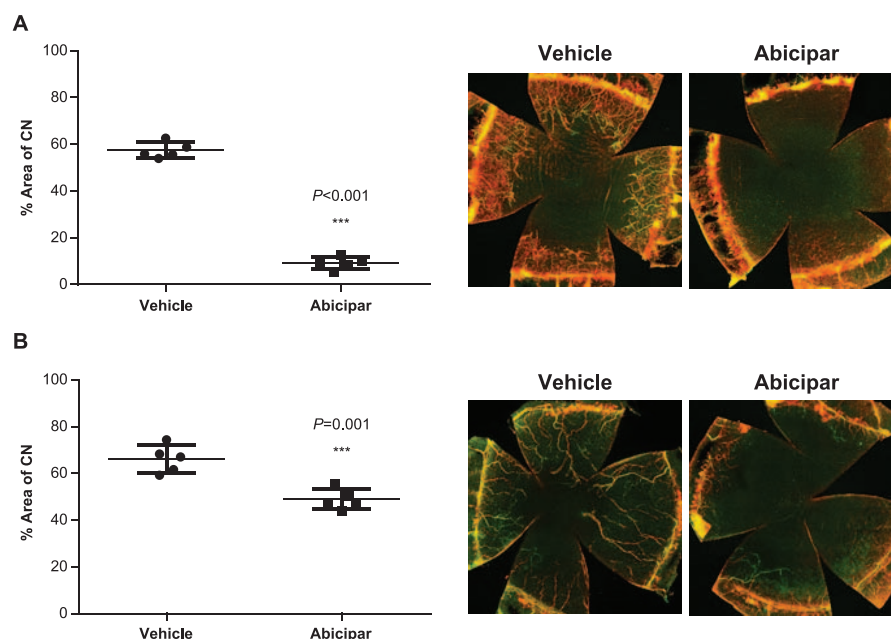


**FIGURE 3.** Angiostatic efficacy of abicipar on mouse corneal neovascularization in prevention mode (A) or intervention mode (B). Percentage of cornea occupied by neovessels is shown. Percentages were derived from tracing the FITC-dextran flatmounts. Symbols represent individual animal percentages, and the horizontal line represents the group median  $\pm$  SD. Abicipar showed a statistically significant effect in preventing vessel growth compared with the vehicle control in both modes. Representative corneal flatmounts stained with CD31 to label endothelial cells (green) and  $\alpha$ SMA to label pericytes (red) are shown. Images were taken on a confocal microscope at 20×.

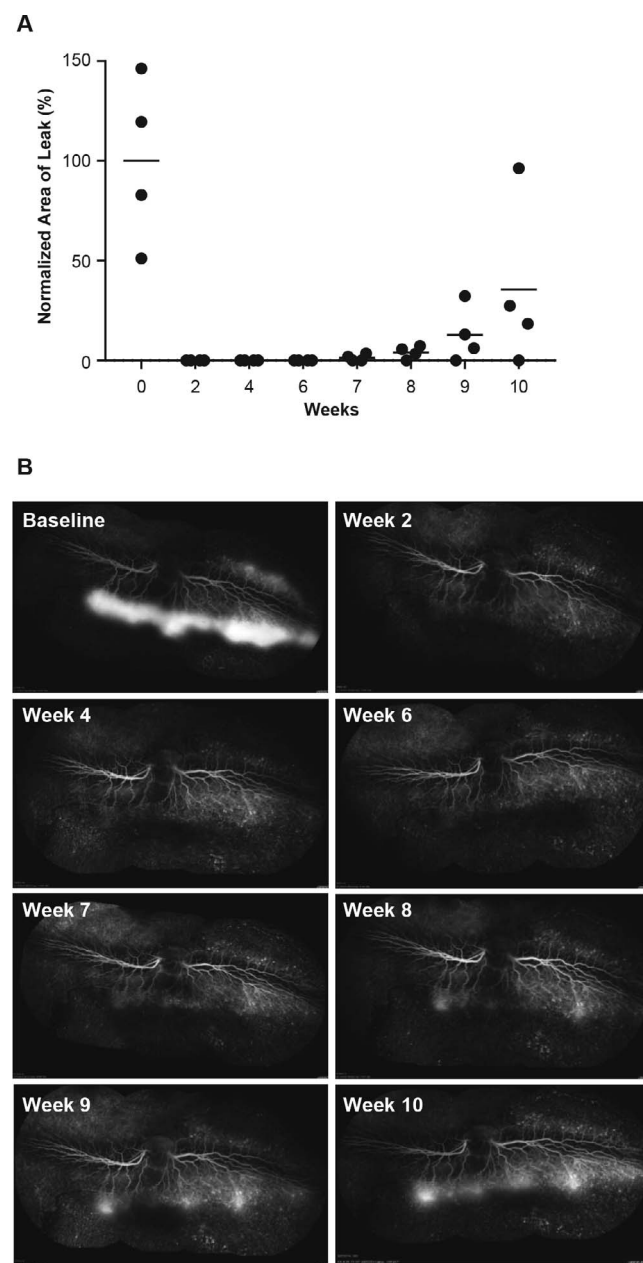


**FIGURE 4.** Assessment of inhibition of vascular leak in a model of chronic retinal neovascularization. (A) A dose of 700  $\mu$ g abicipar was administered intravitreally to four rabbits in which a chronic retinal neovascularization and permeability phenotype was induced by intravitreal DL-AAA administration 12 weeks prior. The extent of vascular leak was assessed by fluorescein angiography every 2 weeks for the first 6 weeks and weekly thereafter out to week 10 posttreatment. Area of leak was normalized relative to baseline for each eye and expressed as a percent. Symbols indicate individual data points, and mean values are shown by lines. (B) Representative images of angiograms showing the effects of a single intravitreal injection of 700  $\mu$ g of abicipar on vascular leak.

results demonstrate that abicipar is effective at blocking neovascularization in vivo.

### Abicipar Inhibits Retinal Vascular Leak in a Rabbit Model of Chronic Retinal Neovascularization

To assess duration of action, abicipar was tested in a model of chronic retinal neovascularization in rabbit<sup>51,52</sup> (Li Y, et al. *IOVS*

2010;51:ARVO E-Abstract 5327). Shen et al.<sup>53</sup> previously reported that subretinal injection of DL-AAA selectively disrupts retinal Müller cells in rat, which, in turn, induces vascular telangiectasis, increases the expression of VEGF, increases vascular permeability, and reduces the expression of the tight junction protein claudin-5. Based on this work, we developed a model of persistent retinal neovascularization in the rabbit by intravitreal administration of DL-AAA (Li Y, et al. *IOVS* 2010;51:ARVO E-Abstract 5327). Vessel dilatation, tortuosity, and angiographic leak at the distal end of retinal vessels were observed within 2 days of DL-AAA injection. This was followed by a neovascular response, which continued to develop until about 8 weeks following the injection, when the lesions appeared to stabilize. Angiographic leak from these neovessels was detectable at 1 week postinjection and persisted through 18 months, the latest time point evaluated (Li Y, et al. *IOVS* 2010;51:ARVO E-Abstract 5327). Similarly, Cao et al.<sup>51</sup> observed that after the lesion achieved a stable morphology, the leakage persisted for at least 65 weeks in this model.<sup>51</sup> More recently, Li et al.<sup>52</sup> described the natural history of this model and noted that persistent vascular leak was observed up to 48 weeks. To assess the duration of action of abicipar in this model, four rabbits with stable angiogenic lesions received a single unilateral intravitreal injection of 700  $\mu$ g of abicipar at D0 and the extent of vascular leak was assessed by fluorescein angiography every 2 weeks for the first 6 weeks and weekly thereafter through week 10. Angiograms from a representative rabbit showing the effects of a single intravitreal injection of abicipar on vascular leak at various time points are shown in Figure 4B. A summary of the results is shown in Figure 4A. Retinal vascular leak was completely suppressed out to 8 weeks in all animals. At week 9, the mean percent leak versus baseline (preabicipar treatment) was 11%. At 10 weeks, three of four animals still had leak values less than 50% of baseline. The mean leak value observed at this time point was 28% of baseline.

### Pharmacodynamic Comparison of Abicipar With Ranibizumab in a Rabbit Model of VEGF-Induced Retinal Vasculopathy

The results in the chronic retinal neovascularization model suggested that abicipar may have a significantly longer duration of action than has been reported for ranibizumab. To confirm this, we conducted a head-to-head pharmacodynamic study comparing equimolar doses of the two compounds. Both ranibizumab and abicipar have reduced affinity for rabbit VEGF-A<sub>165</sub> compared with human VEGF-A<sub>165</sub>. Thus, we used a rabbit model of retinal vasculopathy induced by intravitreal injection of rhVEGF-A<sub>165</sub> for the assessment.<sup>54</sup> At 2, 4, and 6 weeks following a single intravitreal injection of 170  $\mu$ g (the human clinical dose scaled for the rabbit vitreal volume) of ranibizumab or an equimolar dose of abicipar (120  $\mu$ g) or vehicle, the eyes were challenged with rhVEGF-A<sub>165</sub>, and retinal fluorescein angiograms were acquired 72 hours later. Both eyes were drug or vehicle treated and then challenged with rhVEGF-A<sub>165</sub> and, thus, were considered independent observations ( $n = 12$  per treatment group per time point). A total of 120  $\mu$ g of abicipar completely suppressed VEGF-A<sub>165</sub>-induced vascular leak 2 weeks following a single injection and significantly inhibited VEGF-A<sub>165</sub>-induced vascular leak 4 weeks ( $P < 0.05$ ) after administration (Fig. 5A). Severity scores for retinal vascular tortuosity and caliber were assessed from color fundus images. Compared with vehicle-treated eyes, 120  $\mu$ g of abicipar significantly reduced tortuosity and vasodilation of the retinal vasculature at 2 and 4 weeks ( $P < 0.05$ ) but not at week 6 (Fig. 5B). In contrast, 170  $\mu$ g of ranibizumab significantly inhibited VEGF-A<sub>165</sub>-induced vascular leak at 2

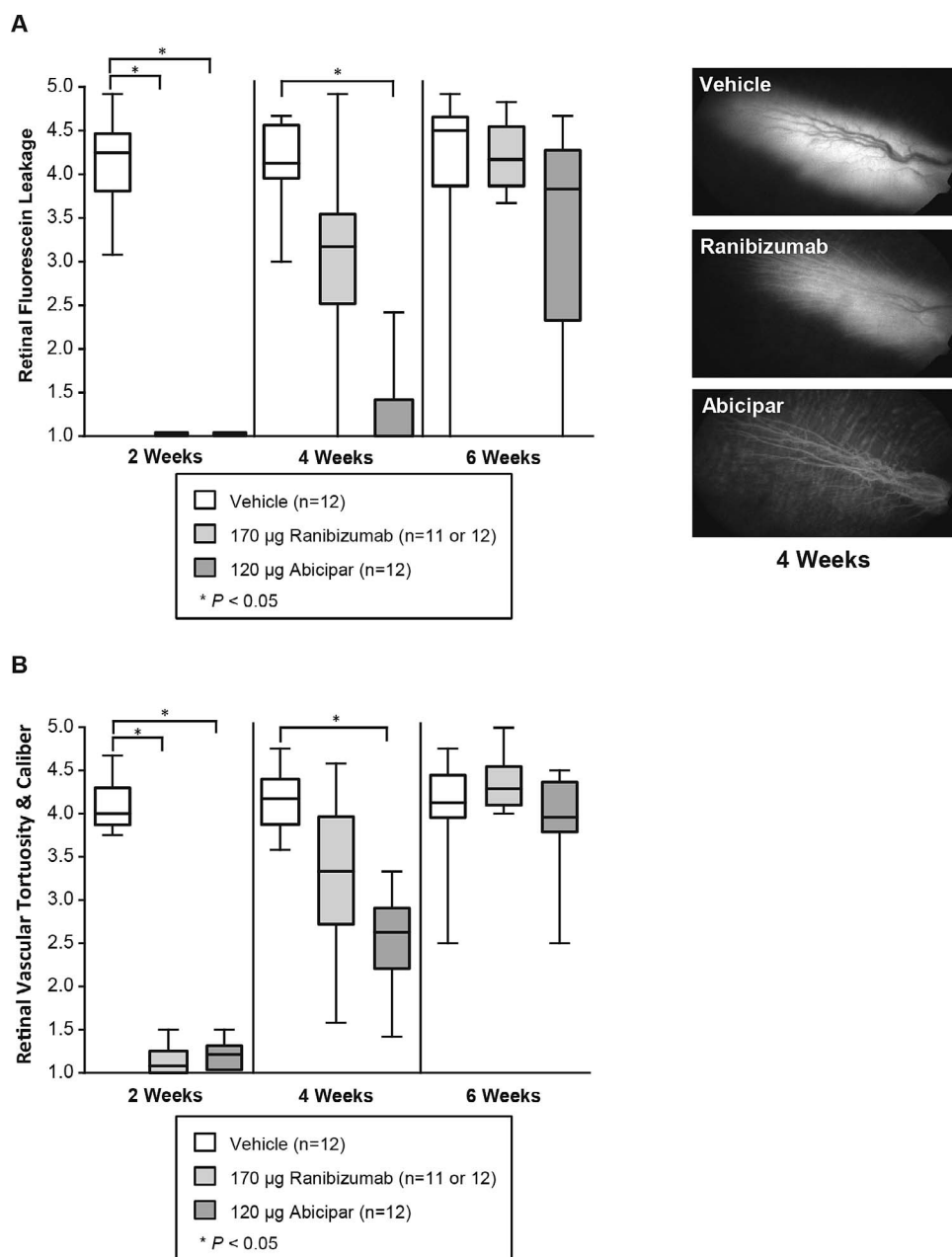


**FIGURE 5.** Evaluation of abicipar in the rabbit model of vasculopathy induced by rhVEGF-A<sub>165</sub>. Effects of abicipar on vascular leakage (**A**). Severity scores of vascular fluorescein leakage were evaluated from retinal fluorescein angiograms 2, 4, and 6 weeks after the single intravitreal injection of 120 µg abicipar, 170 µg ranibizumab, or vehicle. The ends of the box are the upper and lower quartiles, and the median is marked by a horizontal line inside the box. Representative images of angiograms taken at 4 weeks postabicipar treatment are shown in the right panel. Effects of abicipar on vascular tortuosity and caliber in the rabbit VEGF-A<sub>165</sub> model (**B**). Severity scores of retinal vascular tortuosity and caliber were assessed from color fundus images 2, 4, and 6 weeks after the single intravitreal injection of 120 µg abicipar, 170 µg ranibizumab, or vehicle. The ends of the box are the upper and lower quartiles, and the median is marked by a horizontal line inside the box.

weeks ( $P < 0.05$ ) but not at 4 weeks (Fig. 5A). Similarly, ranibizumab suppression of retinal vasculature tortuosity and vasodilation was observed at 2 weeks but not at week 4 (Fig. 5B). Thus, at an equimolar dose, abicipar provides a longer duration of effect in this model than ranibizumab.

## DISCUSSION

The work presented here provides a characterization of abicipar, a novel, highly potent anti-VEGF biologic based on the DARPIn platform. Abicipar binds VEGF-A<sub>165</sub> isoforms with

affinity and potency similar to aflibercept but higher than that of ranibizumab and bevacizumab. Additionally, abicipar effectively blocks both angiogenesis and vascular permeability and provides a longer duration of action than ranibizumab at an equivalent molar dose. Together, these data support the use of abicipar as a treatment for retinal diseases characterized by neovascularization and vascular leak.

The equilibrium binding affinities of abicipar as well as other anti-VEGF biologics for human VEGF-A<sub>165</sub> were determined using KinExA, which measures solution phase binding between unmodified molecules at equilibrium. Measuring

equilibrium binding by kinetic exclusion affords a number of benefits over surface plasma resonance (SPR)/Biacore methodologies, including a direct measurement of interactions at equilibrium rather than calculating affinities from independently observed on- and off-rates. Furthermore, solution binding has the potential to more accurately approximate native interactions and avoid artifacts and limitations common to solid phase-based kinetics.<sup>48</sup> Finally, the high affinity biologics investigated here exhibit slow binding off-rates ( $<1 \times 10^{-6} \text{ s}^{-1}$ ), which can exceed the practical limits of SPR-based off-rate detection and make lower bounds difficult to define.<sup>26,55</sup> Although previous publications describe VEGF-A<sub>165</sub> binding properties for at least one VEGF binding biologic, data from comparative analysis or from assays using solution phase methodology, such as KinExA, are limited. For example, Chen et al.<sup>26</sup> and Lowe et al.<sup>55</sup> evaluated the binding affinity of ranibizumab for VEGF-A<sub>165</sub> using SPR. In both cases the dissociation kinetics were too slow to be accurately measured, so only estimates of the upper limits of the  $K_d$  are reported.<sup>26,55</sup> A more recent publication comparing the affinities of ranibizumab, bevacizumab, and aflibercept for VEGF-A<sub>165</sub> was able to define dissociation rates and reported good alignment between SPR and KinExA measurements (490 fM and 660 fM, respectively) as well as with the results reported here (394 fM).<sup>56</sup> Interestingly, another recent study also reported calculated affinities for ranibizumab, bevacizumab, and aflibercept binding to VEGF-A<sub>165</sub> using SPR that were consistent with data from Papadopoulos et al.<sup>56</sup> but only when employing a similar immobilization strategy. These findings highlight the need to carefully consider the limitations of solid phase methods, such as SPR, when characterizing high-affinity interactions.<sup>57</sup> Thus, SPR data notwithstanding, the current study results are largely in agreement with published data for the molecules examined.

Consistent with differences in binding affinity, abicipar and aflibercept were significantly more potent in the HUVEC calcium mobilization assay than ranibizumab and bevacizumab (Fig. 1B). Similarly, Papadopoulos et al.<sup>56</sup> demonstrated that aflibercept was more potent than ranibizumab and bevacizumab in a HUVEC calcium mobilization assay. However, in that study, the anti-VEGF compounds were preincubated with VEGF-A<sub>165</sub> for 1 hour. Previous results with ranibizumab and bevacizumab, molecules with slower VEGF-A<sub>165</sub>-binding on-rates, demonstrated that potency measurements can be modulated by preincubating these inhibitors with VEGF-A<sub>165</sub> for different lengths of time prior to assessing potency.<sup>50</sup> In the studies presented here, we used a 4-hour preincubation to minimize the effect of differences in the association constants of various inhibitors when comparing their VEGF-A<sub>165</sub> neutralization potencies. Under these conditions, the potencies of abicipar and aflibercept were still significantly higher than those of ranibizumab and bevacizumab. These results differ from those reported by Yu et al.<sup>50,57</sup> In their experiments measuring effects on MAP kinase phosphorylation, HUVEC migration, and bovine retinal endothelial cell (BREC) proliferation, no differences were observed between ranibizumab and aflibercept. However, in contrast to the experiments presented here as well as those of Papadopoulos et al.,<sup>56</sup> the concentrations of VEGF used to drive cellular responses in those experiments (1.25 nM for MAP kinase phosphorylation, 500 pM for HUVEC migration, and 150 pM for BREC proliferation) far exceeded the  $K_d$  values for the compounds. Thus, the efficacy observed may largely reflect stoichiometric effects rather than differences in potency. More recently, Yang et al.<sup>57</sup> compared the potencies of ranibizumab, bevacizumab, and aflibercept in a BREC proliferation assay using 75 pM VEGF-A<sub>165</sub> and similarly did

not detect differences between ranibizumab and aflibercept. The discrepancy between these results and ours, as well as those of Papadopoulos et al., could again reflect the concentrations of VEGF-A<sub>165</sub> used (75 pM versus 26 pM in the results presented here and 20 pM in the study by Papadopoulos et al.) and/or the extended assay times required to measure cellular proliferation (6 days).

Abicipar effectively inhibited angiogenesis *in vitro*, which was measured using a three-dimensional *in vitro* model of vessel sprouting (Fig. 2). In contrast to other sprouting assays, this model recapitulates all the *in vivo* stages of angiogenesis, including degradation of the basement membrane, proliferation and migration of endothelial cells, and alignment of endothelial cells into tubes. Moreover, these tubes have distinct lumen formation and undergo branching and anastomosis.<sup>58</sup> Thus, activity in this assay is expected to be a good predictor of *in vivo* efficacy. Consistent with this premise, abicipar was effective in animal models of neovascularization and vascular leak. In the mouse cornea neovascularization model, abicipar significantly inhibited neovascularization. This model is VEGF dependent and includes a sustained neovascular response (up to 4 weeks) and, as such, represents a reliable model for testing angiostatic compounds.<sup>59</sup> Although there was significant inhibition of neovascularization (Fig. 3B) in intervention mode, we cannot determine whether vessels had undergone regression or if abicipar simply inhibited further growth of the vessels. In support of the latter, the extent of inhibition and/or regression observed in the intervention mode was less than that seen in prevention mode (Fig. 3B). In the rabbit model of VEGF-A<sub>165</sub>-induced vasculopathy, the duration of the anti-vascular permeability effect of abicipar was longer than that of ranibizumab when the two compounds were administered at molar equivalent doses. The effects of the two compounds were comparable at the 2-week time point. However, vascular leakage as well as vascular tortuosity and caliber were significantly inhibited in the eyes of animals treated with abicipar but not in the eyes of animals treated with ranibizumab at the 4-week time point. The 170- $\mu\text{g}$  dose of ranibizumab chosen for this study is equivalent to the 500- $\mu\text{g}$  dose currently used clinically when scaled for the rabbit vitreal volume. However, the molar equivalent dose of 120  $\mu\text{g}$  for abicipar used in this study is significantly lower than the dose currently being used in clinical trials, even accounting for differences in vitreal volume. In addition, the 1- $\mu\text{g}$  dose of exogenous VEGF used to induce permeability is significantly higher than in human vitreous even under pathologic conditions.<sup>20</sup> Thus, we would anticipate a longer duration for abicipar when tested at clinically equivalent doses. Consistent with this, we observed that 700  $\mu\text{g}$  of abicipar gave 2 to 3 months of duration of inhibition in the rabbit model of chronic retinal neovascularization, a model in which endogenous VEGF levels induce the retinal vascular leak. This extended duration was observed despite a 20-fold lower affinity of abicipar for rabbit VEGF-A<sub>165</sub> compared with human VEGF-A<sub>165</sub> (Table 1).

In summary, abicipar is a potent inhibitor of angiogenesis and vascular leak, with a higher affinity, greater potency, and longer duration of action than ranibizumab and bevacizumab. Thus, abicipar has the potential to be clinically effective with a sustained duration in patients with retinal neovascular diseases.

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