Several members of the vascular endothelial growth factor (VEGF) family are potent regulators of vasculogenesis and angiogenesis. The VEGF family includes placental growth factor (PLGF) and VEGF-A, -B, -C, -D, and -E. 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.). VEGF-A was first identified in developmental systems and tumor cells as a vascular permeability factor and an angiogenic factor. Studies of tumor biology established that VEGF expression is regulated by tissue hypoxia. There are four major VEGF-A isoforms generated by alternative splicing. In humans, these isoforms are VEGF-A121, VEGF-A165, VEGF-A189, and VEGF-A206; 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. VEGF-A165, the predominant isoform expressed in humans, is most frequently implicated in pathologic angiogenesis and increased vascular permeability. VEGF-A can be upregulated by hypoxia in retinal cells in vitro and in vivo. These initial observations, together with the finding of increased VEGF-A levels in patients with diabetic retinopathy and other retinal diseases, led to pivotal studies establishing a causal link between VEGF, ocular neovascularization, and vascular leak. The demonstration that inhibition of VEGF using anti-VEGF antibodies, dominant negative VEGF receptors, or antisense oligonucleotides could block neovascularization in ocular models 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-A165; ranibizumab (Lucentis), a recombinant, humanized antibody antigen binding fragment (Fab) that neutralizes the soluble splice variants and proteolytic fragment of VEGF-A; 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). Bevacizumab, a humanized anti-VEGF monoclonal antibody
Abicipar: A Potent Anti-VEGF DARPin Therapeutic

Abicipar is an investigational DARPin molecule that has been shown to bind with high affinity and specificity to VEGF-A, and to be effective in treating vascular diseases.

The ANCHOR and MARINA registration studies with ranibizumab established monthly intravitreal injections of ranibizumab as the standard treatment for neovascular AMD; the size of these and RIDE studies similarly established monthly intravitreal injections of ranibizumab as the standard of care for diabetic macular edema. In an effort to reduce patient treatment burden, several 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. 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. The results of the VIEW 1 and VIEW 2 trials with aflibercept supported a bimonthly dosing regimen after three initial monthly doses. Although monthly or bimonthly dosing is recommended for current anti-VEGF therapies, a number of studies, including LUMIERE, AURA, and WAVE 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. 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. 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. 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. 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. DARPin molecules with binding affinities in the picomolar range are typical.

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. 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. 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. 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 (IC50) for at least 12 weeks. Here, we show that abicipar binds all soluble VEGF-A isoforms with high affinity, potently neutralizes VEGF-A165 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-A165 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-A165-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-A164 were obtained from R&D Systems (Minneapolis, MN, USA). Rabbit VEGF-A165 was produced at West Bioscience Research (Irvine, CA, USA). A biotin-SP-AffiPure goat anti-human IgG F(ab’)2 (Jackson ImmunoResearch, West Grove, PA, USA) was used to detect ranibizumab. Bevacizumab was detected using a biotin-SP-AffiPure goat anti-human IgG (H+L) (Jackson ImmunoResearch). Aflibercept was detected using a biotinylated-SP-conjugated affinity-purified goat anti-human immunoglobulin G (IgG) (Fc’2) (Jackson ImmunoResearch). Abicipar was detected using either a biotinylated antibody 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 Kd 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 = Kd × k on.
For $K_a$ 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 (5–600 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 µg/mL VEGF-A165 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.49

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-A165, 40 pM bevacizumab versus 82 pM VEGF-A165, and 200 pM abicipar versus 91 or 497 pM VEGF-A165. 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 model48 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,48 where the concentration of aflibercept (100 pM) and incubation time were held constant; VEGF-A165 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-A165. 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-A165 with or without preincubation with anti-VEGF molecules.

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

rhVEGF-A165, 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-A165 in 384-well plates, shaken (600 RPM) for 5 minutes, and then incubated at 30°C for 4 hours. These mixtures of VEGF-A165 (20 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.49 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 µL of a 20 mg/mL fibrinogen solution and added in 1 mL EGM-2 medium to a 12-well plate containing 10 µL thrombin (0.06 units/µL). 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 × 10^5 cells/well with 2.5 ng/mL of rhVEGF-A165 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 µm) 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/group) were anesthetized with an intraperitoneal (i.p.) injection of a cocktail of 100 mg/kg ketamine and ≤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/group) were injected with 100 to 250 μL 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 ≤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 CD51 conjugated to FITC (Invitrogen, Carlsbad, CA, USA) and α-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 20X.

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 μg (50 μL of a 8-mg/mL solution) of d-alpha-aminooacidic peptide (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 ≥3 on a 0 to 4 scale, were selected for the study. Four rabbits received a single intravitreal injection of 700 μg of abicipar. Following the application of topical anesthesia, 50 μL of abicipar was delivered by intravitreal injection to the midretina using a 31-gauge 0.3-μL 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 compositized, 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 (μm²) 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 ± 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 μg (50 μL) of abicipar into each eye. A second cohort of 18 rabbits received a single intravitreal injection of 170 μg (50 μL) of ranibizumab into each eye. A third cohort of 18 rabbits received a single intravitreal injection of vehicle (50 μL sterile saline) into each eye. Intravitreal injections were positioned midvitreous using a 0.5-cc syringe attached to a 28G 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 μL containing 1 μg rhVEGF-A165 in PBS with 0.1% BSA; the VEGF-A165 was injected into the midvitreous of both eyes using a 0.5-cc syringe with a 28G 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-A165 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 (Kd) was 394 fM for human VEGF-A165. The binding affinities for other splice variants of human VEGF-A, including VEGF-A189, VEGF-A121, and VEGF-A110, 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-A165 were measured. Although the binding affinity of abicipar for rat VEGF-A165 was similar to that for human VEGF-A165, the affinity for rabbit VEGF-A165 was 20-fold lower than for human VEGF-A165 (Table 1). Thus, abicipar demonstrates high affinity binding to VEGF-A from multiple species.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Affinity (Kd)</th>
<th>95% CI (Kd)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human VEGF-A165</td>
<td>394 fM</td>
<td>264–552 fM</td>
</tr>
<tr>
<td>Human VEGF-A189</td>
<td>745 fM</td>
<td>0.75–1.66 pM</td>
</tr>
<tr>
<td>Human VEGF-A121</td>
<td>789 fM</td>
<td>0.43–1.38 pM</td>
</tr>
<tr>
<td>Human VEGF-A111</td>
<td>806 fM</td>
<td>23.50–168 fM</td>
</tr>
<tr>
<td>Rat VEGF-A165</td>
<td>380 fM</td>
<td>260–556 fM</td>
</tr>
<tr>
<td>Rabbit VEGF-A165</td>
<td>8.49 pM</td>
<td>4.51–15.50 pM</td>
</tr>
</tbody>
</table>

CI, confidence interval.
Abicipar is a DARPin therapeutic that shows promise in the treatment of AMD. This study evaluated its binding affinity and neutralization potency compared to standard therapies like ranibizumab and bevacizumab.

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

The VEGF-A165 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-A165 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-A165 association rates (k on) for abicipar were 7.94 \times 10^6 M^{-1}s^{-1}, and the k off value for aflibercept was 9.02 \times 10^6 M^{-1}s^{-1}. Both abicipar and aflibercept bind VEGF-A165 with 30- to 100-fold faster on-rates than those observed with ranibizumab (8.25 \times 10^5 M^{-1}s^{-1}) and bevacizumab (2.55 \times 10^5 M^{-1}s^{-1}) (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-A165-induced calcium mobilization. Published data indicated that preincubation time with VEGF-A165 has a significant effect on the apparent potency of some anti-VEGF molecules with slower VEGF-binding on-rates. Thus, we selected 26 pM VEGF-A165 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-A165. Under these conditions, mean potencies (IC50 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 (IC50 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.

**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. 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-A165, robust sprouting is observed within the fibrin matrix (Fig. 2A, left and middle panels). Abicipar inhibited VEGF-A165-mediated tube formation in a dose-dependent fashion with an IC50 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...
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 2](image1)

**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\( \times \) magnification). **Middle panel** is a fluorescent image of HUVECs labeled with CellTracker Red dye and treated with VEGF (20\( \times \) magnification). **Right panel** is a fluorescent image of HUVECs labeled with CellTracker Red dye and treated with VEGF plus 100 nM abicipar (10\( \times \) magnification). (B) The graph shows the number of sprouts/bead and demonstrates a dose-dependent inhibition of vessel sprouting. Values represent the means ± SD.

![Figure 3](image2)

**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 ± 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\( \times \).
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\(^{51,52}\) (Li Y, et al. IOVS 2010;51:ARVO E-Abstract 5327). Shen et al.\(^{53}\) previously reported that subretinal injection of DL-AAA selectively disrupts retinal Müller cells in rat, which, in turn, induces vascular telangiectasia, 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 dilation, 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.\(^{51}\) observed that after the lesion achieved a stable morphology, the leakage persisted for at least 65 weeks in this model.\(^{51}\) More recently, Li et al.\(^{55}\) 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 out to 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\(_{A165}\) compared with human VEGF\(_{A165}\). Thus, we used a rabbit model of retinal vasculopathy induced by intravitreal injection of rhVEGF\(_{A165}\) for the assessment.\(^{54}\) 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\(_{A165}\) and retinal fluorescein angiograms were acquired 72 hours later. Both eyes were drug or vehicle treated and then challenged with rhVEGF\(_{A165}\) 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\(_{A165}\)-induced vascular leak 2 weeks following a single injection and significantly inhibited VEGF\(_{A165}\)-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 vasoadjiation 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\(_{A165}\)-induced vascular leak at 2...
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-Aisoforms 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-Aisoforms 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 kinetics (in particular, with abicipar) and the ability to measure affinities for abicipar with VEGF-A165 using SPR that were also reported calculated affinities for ranibizumab, bevacizumab, and aflibercept binding to VEGF-A165 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. A more recent publication comparing the affinities of ranibizumab, bevacizumab, and aflibercept for VEGF-A165 was able to define dissociation rates and reported good alignment between SPR and KinExA measurements ($\sim 10^8$ $\text{M}^{-1} \text{s}^{-1}$), which can exceed the practical limits of SPR-based off-rate detection and make lower bounds difficult to define.

Although previous publications describe VEGF-A165 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. and Lowe et al. evaluated the binding affinity of ranibizumab for VEGF-A165 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. A more recent publication comparing the affinities of ranibizumab, bevacizumab, and aflibercept for VEGF-A165 was able to define dissociation rates and reported good alignment between SPR and KinExA measurements ($\sim 10^8$ $\text{M}^{-1} \text{s}^{-1}$), which can exceed the practical limits of SPR-based off-rate detection and make lower bounds difficult to define.

In summary, abicipar is a potent inhibitor of angiogenesis 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. 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-A165-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-µg dose of ranibizumab chosen for this study is equivalent to the 500-µg dose currently used clinically when scaled for the rabbit vitreal volume. However, the molar equivalent dose of 120 µg 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-µg dose of exogenous VEGF used to induce permeability is significantly higher than in human vitreous even under pathologic conditions. Thus, we would anticipate a longer duration for abicipar when tested at clinically equivalent doses. Consistent with this, we observed that 700 µg 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-A165 compared with human VEGF-A165 (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.

Acknowledgments

Sponsored by Allergan plc, Dublin, Ireland.

Disclosure: G.A. Rodrigues, Allergan (E); M. Mason, Allergan (E); L.-A. Christie, Allergan (E); C. Hansen, Allergan (E); L.M.
References

18. Stone J, Chan-Ling T, Pe’er J, Itin A, Gnessin H, Keshet E. Roles of vascular endothelial growth factor and astrocyte degener-


