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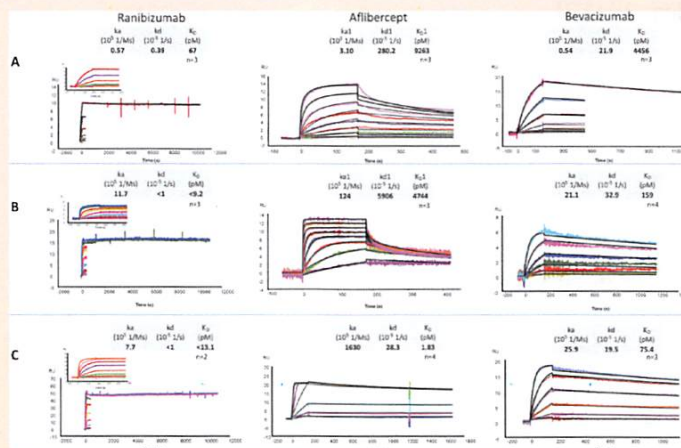
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## Comparison of Binding Characteristics and In Vitro Activities of Three Inhibitors of Vascular Endothelial Growth Factor A

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**ABSTRACT:** The objectives of this study were to evaluate the relative binding and potencies of three inhibitors of vascular endothelial growth factor A (VEGF), used to treat neovascular age-related macular degeneration, and assess their relevance in the context of clinical outcome. Ranibizumab is a 48 kDa antigen binding fragment, which lacks a fragment crystallizable (Fc) region and is rapidly cleared from systemic circulation. Aflibercept, a 110 kDa fusion protein, and bevacizumab, a 150 kDa monoclonal antibody, each contain an Fc region. Binding affinities were determined using Biacore analysis. Competitive binding by sedimentation velocity analytical ultracentrifugation (SV-AUC) was used to support the binding affinities determined by Biacore of ranibizumab and aflibercept to VEGF. A bovine retinal microvascular endothelial cell (BREC) proliferation assay was used to measure potency. Biacore measurements were format dependent, especially for aflibercept, suggesting that biologically relevant, true affinities of recombinant VEGF (rhVEGF) and its inhibitors are yet to be determined. Despite this assay format dependency, ranibizumab appeared to be a very tight VEGF binder in all three formats. The results are also very comparable to those reported previously.<sup>1–3</sup> At equivalent molar ratios, ranibizumab was able to displace aflibercept from preformed aflibercept/VEGF complexes in solution as assessed by SV-AUC, whereas aflibercept was not able to significantly displace ranibizumab from preformed ranibizumab/VEGF complexes. Ranibizumab, aflibercept, and bevacizumab showed dose-dependent inhibition of BREC proliferation induced by 6 ng/mL VEGF, with average  $IC_{50}$  values of  $0.088 \pm 0.032$ ,  $0.090 \pm 0.009$ , and  $0.500 \pm 0.091$  nM, respectively. Similar results were obtained with 3 ng/mL VEGF. In summary Biacore studies and SV-AUC solution studies show that aflibercept does not bind with higher affinity than ranibizumab to VEGF as recently reported,<sup>4</sup> and both inhibitors appeared to be equipotent with respect to their ability to inhibit VEGF function.

**KEYWORDS:** ranibizumab, aflibercept, bevacizumab, VEGF, affinity, analytical ultracentrifugation

### INTRODUCTION

The determination of binding affinity of a therapeutic protein to a target is an integral part of pharmaceutical development. A widely used methodology for assessing tight interactions is based on surface plasmon resonance (SPR) such as Biacore.<sup>5</sup> One caveat in this technology is that it requires ligand immobilization to a surface and it has been shown that orientation, method of binding, and which ligand is bound are

important variables that govern the determination of affinity

constants.<sup>6,7</sup>

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Alternatively, analytical ultracentrifugation (AUC) can be used as an orthogonal free-solution technique that circumvents the potential artifacts of matrix/stationary phase or chemical modifications associated with SPR. AUC has been widely used in the biophysical characterization of proteins to determine weight-average molecular mass, sedimentation coefficient, frictional coefficient associated with molecular shape, bimolecular interactions involving reversible associations or complex formation, self-association of glycosylated and nonglycosylated proteins, and competitive binding of anti-IgE antibodies to IgE and also as an orthogonal technique to size exclusion chromatography to determine the presence of aggregates.<sup>8</sup> In addition to being able to detect the presence of protein aggregates, AUC analysis allows measurements directly in the formulation buffer or condition of interest, thereby avoiding common size exclusion HPLC limitations of protein-resin interactions and significant dilution in the elution buffer that can potentially alter the size distribution of the self-associates and aggregates, as highlighted in the above studies. One aspect of this work is to assess AUC as an orthogonal technique to SPR in evaluating the binding of therapeutic proteins, highlighting that caution must be exercised while relying on SPR results. In addition, the recent development of fluorescence optics in the analytical ultracentrifuge<sup>9,10</sup> combined with the use of fluorescently labeled material can provide definitive information about the type of complex formed.

There have been several SPR studies and potency assessments of inhibitors of vascular endothelial growth factor A (VEGF),<sup>2–4,11,12</sup> a key driver of the vascular leakage and neovascularization seen in intraocular vascular diseases including age-related macular degeneration (wet AMD), retinal vein occlusion (RVO), and diabetic macular edema (DME).<sup>1</sup> VEGF inhibitors such as ranibizumab, aflibercept, and bevacizumab are used intravitreally in patients with wet AMD, RVO, and DME. The US Food and Drug Administration has approved ranibizumab for the treatment of wet AMD, RVO, and DME<sup>13</sup> and aflibercept for the treatment of wet AMD and central RVO.<sup>14</sup> Bevacizumab is used off-label. A recent SPR study concludes that aflibercept binds to VEGF with much higher affinity than ranibizumab.<sup>4</sup> Herein we report the results of the determination of affinity constants for binding of VEGF to ranibizumab, aflibercept, and bevacizumab by SPR using different assay formats as well as the potency of inhibition of VEGF. In addition, a novel solution based competitive analytical ultracentrifuge method is used to support our conclusions.

## MATERIALS AND METHODS

Lucentis (ranibizumab) and Avastin (bevacizumab) were obtained from Genentech, Inc., South San Francisco, CA. Eylea (aflibercept; Regeneron, Inc., Tarrytown, NY) was obtained commercially. Recombinant human VEGF<sub>165</sub> (rhVEGF) was expressed and purified at Genentech. Anti-Fab antibody was obtained from GE Healthcare (Pittsburgh, PA), and protein A was obtained from Thermo Fisher Scientific, Pierce Protein Biology Products (Rockford, IL).

**SPR Binding Assays.** Binding kinetics and affinities of inhibitors of rhVEGF were assessed using surface plasmon resonance technology on a Biacore T200 instrument (GE Healthcare, Pittsburgh, PA). A series of analyte concentrations were prepared in HBS-EP running buffer (0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, and 0.05% surfactant Polysorbate 20) and injected at a flow rate of 50  $\mu$ L/min for 3 min over flow

cells (FCs) of Series S CMS sensor chips immobilized with ligand molecules at various densities depending on assay formats.

In format 1, rhVEGF was the ligand immobilized directly onto FCs at  $\sim$ 20 resonance units (RU) density, while the inhibitors were the analytes. The dissociation of inhibitors from the immobilized rhVEGF was allowed to proceed for 5 min for all samples except for the ranibizumab and bevacizumab samples with the highest concentration (200 nM), in which dissociation proceeded for 3 h or 15 min, respectively. All experiments were carried out at 37  $^{\circ}$ C.

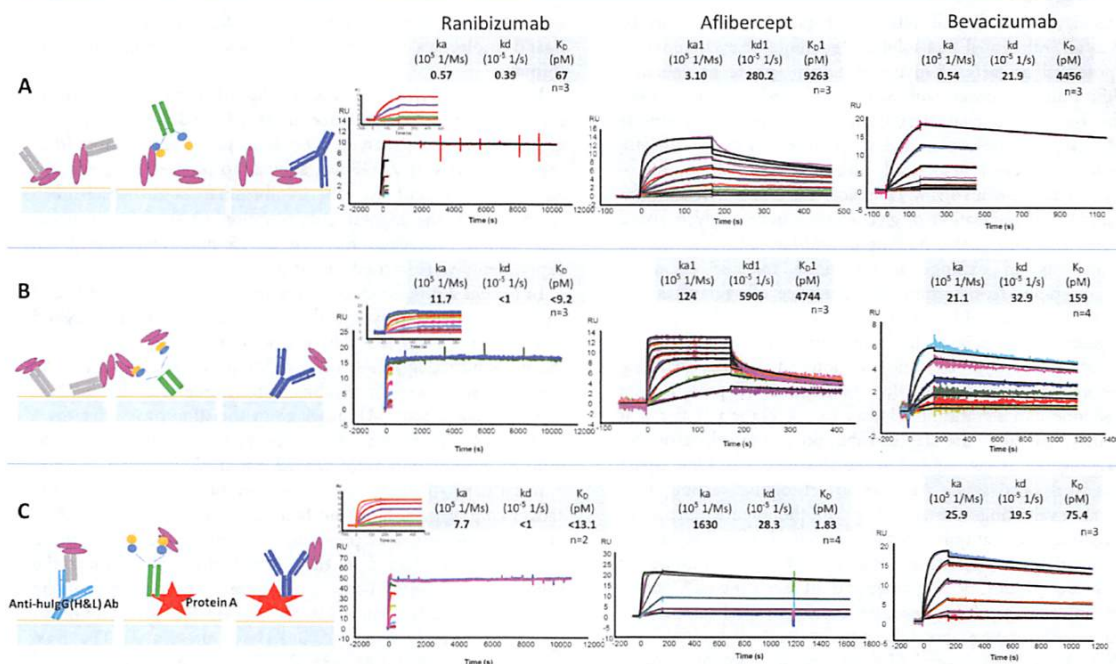
In format 2, the inhibitors were the immobilized ligands and rhVEGF was the analyte in the mobile phase. The final ligand density was 22–45 RU. The dissociation of the analytes from the immobilized ligand was allowed to proceed for 5 min for all samples except for the ranibizumab sample with the highest concentration (200 nM), in which dissociation proceeded for 3 h. The dissociation time for all bevacizumab samples was 20 min. All experiments were carried out at 37  $^{\circ}$ C.

In format 3, the inhibitors were immobilized indirectly to the sensor chip using anti-human IgG Fab antibody or protein A as capturing molecules as previously reported.<sup>4</sup> Rigorous surface testing was conducted in the current study to evaluate the validity of the method for all inhibitors. The densities of the capture molecules were  $\sim$ 11000 or 1000 RU for anti-human IgG Fab antibody and  $\sim$ 5500 RU for protein A. The final ligand density used was  $\sim$ 28 RU for the indirectly captured ranibizumab,  $\sim$ 40 RU for bevacizumab, and  $\sim$ 50 RU for aflibercept. The dissociation of the analytes from the immobilized ligand was allowed to proceed for 5 min for ranibizumab samples except for the sample with the highest concentration (200 nM), in which dissociation proceeded for 3 h. The dissociation time for all aflibercept and bevacizumab samples was 30 min. Experiments were carried out at 37  $^{\circ}$ C or 25  $^{\circ}$ C for this format.

Sensorgrams of ranibizumab, aflibercept, and bevacizumab binding to rhVEGF using all three formats were analyzed to obtain kinetic data and affinities using Biacore T200 Evaluation Software (version 2.0.1; GE Healthcare). Because of the dimeric nature of rhVEGF and the presence of two potential binding sites in all inhibitors except ranibizumab, definitive monovalent binding affinities for rhVEGF and its inhibitors can be challenging to obtain. Very low immobilization densities were used to encourage monovalent binding, and the presence of such interactions were evaluated using a 1:1 Langmuir binding model. In all but two conditions tested, the 1:1 binding model was sufficient to describe interactions between rhVEGF and its inhibitors. The dissociation rate constant ( $k_d$ ) and association rate constant ( $k_a$ ) were obtained via kinetic fitting, and the equilibrium dissociation constant ( $K_D$ ) was derived by taking the ratio of  $k_d$  over  $k_a$  calculated using the simplest 1:1 binding model. Only in cases where aflibercept was evaluated in formats 1 and 2 was the 1:1 binding model insufficient to describe interactions between the inhibitor and rhVEGF. In those cases a bivalent analyte binding model was used and the first equilibrium dissociation constant ( $K_{D1}$ ), first dissociation rate constant ( $k_{d1}$ ), and first association rate constant ( $k_{a1}$ ) were reported.

**Competitive Binding Assessed by Sedimentation Velocity Analytical Ultracentrifugation (SV-AUC).** Each molecule individually and preformed complexes between ranibizumab and VEGF and aflibercept and VEGF were first evaluated to obtain their sedimentation coefficients. After this,





**Figure 1.** Binding of rhVEGF–anti-VEGF inhibitor molecules in Biacore assays. (A) rhVEGF is the ligand immobilized directly onto the flow cell (FC), while the inhibitors were the analytes injected over the FC at varying concentrations (format 1). (B) VEGF inhibitors immobilized as ligand with VEGF in the mobile phase as analyte (format 2). (C) Inhibitors immobilized indirectly to the sensor chip with VEGF in the mobile phase as analyte (format 3). Note: limit of  $k_d$  that can be accurately measured by the instrument is  $\sim 5 \times 10^{-6} \text{ s}^{-1}$ . To be conservative, a  $k_d < 10^{-5} \text{ s}^{-1}$  was chosen.

competition experiments were conducted using a preformed inhibitor/VEGF complex challenged with a different VEGF inhibitor to assess whether the previously reported  $\sim 100$ -fold higher affinity of aflibercept to rhVEGF compared with ranibizumab<sup>4</sup> is valid in free solution, i.e., no binding to a surface as in SPR measurements.

Experiments were performed at room temperature in PBS, pH 7.2 (137 mM NaCl, 27 mM KCl, 8 mM  $\text{Na}_2\text{HPO}_4$ , and 1.5 mM  $\text{KH}_2\text{PO}_4$ ). Alexa Fluor 488 protein labeling kits were purchased from Molecular Probes (Eugene, OR). All chemicals used were reagent grade or higher. Alexa Fluor 488 labeled ranibizumab (denoted as ranibizumab\*) was produced as recommended by the manufacturer.

Sedimentation velocity experiments were performed in an Optima XL-I analytical ultracentrifuge equipped with absorbance optics, interference optics (Beckman Coulter, Fullerton, CA), and fluorescence optics (Aviv Biomedical) in centrifuge cells with 12 mm graphite-filled Epon centerpieces (Spin Analytical, Durham, NH) at 20 °C and rotor speed of 40000 rpm. Quartz windows were used when using the absorbance optics, and the scans were acquired at a wavelength of 230 nm at 30  $\mu\text{m}$  radial increments. When using the fluorescence optics, sapphire windows were used, and the data were acquired at 20  $\mu\text{m}$  radial increments averaging five revolutions per scan. The sedimentation boundaries were analyzed with SEDFIT, version 11.3 and 11.72c.<sup>15</sup> The resulting continuous,  $c(s)$ , distribution with 70% confidence level was calculated after optimizing baseline, meniscus, and cell bottom positions by nonlinear regression. All  $s$  values obtained with the  $c(s)$  distribution in

PBS were converted to  $s_{20,w}$  with SEDNTERP (version 1.09) using the measured density and viscosity of PBS.

**Bovine Retinal Microvascular Endothelial Cell (BREC) Proliferation Assay.** A BREC assay was used because bovine microvascular endothelial cells are well established as a cell type that is highly responsive to growth factors such as VEGF and bFGF.<sup>16</sup> Unlike another cell line that has been used to assess potency, HUVEC, which is derived from a large vessel, BREC is a more physiologically relevant cell type to investigate angiogenesis.

BREC proliferation assays were performed as previously described.<sup>12</sup> Cells were seeded in 96-well plates in low glucose DMEM (supplemented with 10% heat-inactivated calf serum, 2 mM glutamine, and antibiotics) at a density of 500 cells/well. Ranibizumab and aflibercept were tested from 0.004 to 10 nM, while bevacizumab was tested from 0.04 to 90 nM. Twenty minutes after addition of inhibitors, VEGF was added to a final concentration of 6 ng/mL (0.15 nM) or 3 ng/mL (0.075 nM). After 6 days, cell growth was assayed with the use of alamarBlue (BioSource). Fluorescence was measured at 530 nm excitation wavelength and 590 nm emission wavelength.  $\text{IC}_{50}$  values were calculated using KaleidaGraph. For statistical analysis, one-way ANOVA was used, followed by the Tukey–Kramer HSD test comparing all pairs.

## RESULTS

**Binding Affinities and Kinetics of VEGF Inhibitors Using SPR Technology.** *Format 1.* rhVEGF, an antiparallel homodimer, was the immobilized ligand with inhibitors in the mobile phase as analytes (Figure 1A). Binding of ranibizumab

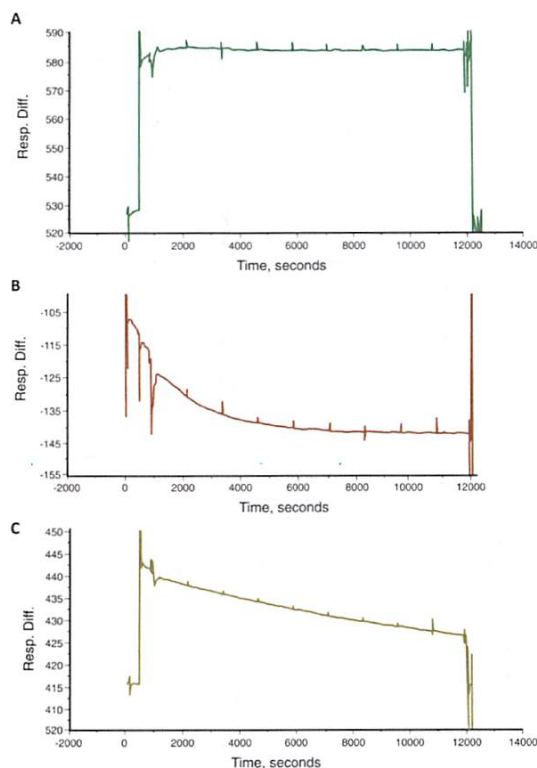


followed a simple monovalent (1:1) analyte binding model as expected because the Fab molecule has only one VEGF binding site. This was clearly shown by the closeness of the fit to the experimental data (Figure 1A). Aflibercept and bevacizumab each contained two VEGF-binding sites. Using very low rhVEGF immobilization levels, binding of these two inhibitors to rhVEGF was encouraged to favor monovalent binding.<sup>17,18</sup> This approach worked for bevacizumab since the 1:1 binding model sufficiently described the interactions between the inhibitor and rhVEGF (Figure 1A). However, in the case of aflibercept, even the lowest immobilization level of rhVEGF was insufficient to completely shift the interaction to monovalent binding, and attempts to fit the data with a 1:1 Langmuir binding model failed (data not shown). Therefore, a bivalent analyte binding model was used considering each aflibercept molecule has two potential VEGF binding sites. Although the curve fits still deviated from the experimentally obtained results (Figure 1A), the overall quality of the fit was much improved over that obtained from using a 1:1 binding model (data not shown). The challenge in fitting the aflibercept binding curves may be due to the global fit bivalent model that allows individual bulk effect correction to accommodate baseline drift,<sup>18</sup> although other factors such as binding induced conformational change cannot be ruled out. Because the second step in the bivalent analyte binding model involves intramolecular binding on a sensor chip without an increase in mass, only two-dimensional kinetics for the second step are obtained. The first step kinetics from a bivalent analyte binding model are most relevant in understanding the binding kinetics and strength between an analyte and a ligand. Therefore, only first kinetic parameters ( $k_{a1}$ ,  $k_{d1}$ ) and first  $K_{D1}$  were shown for aflibercept binding to rhVEGF. Although the (first) association rate constants for all three inhibitors were similar, ranibizumab had a much slower dissociation rate constant ( $0.39 \times 10^{-5} \text{ s}^{-1}$ ) than aflibercept and bevacizumab ( $280.2 \times 10^{-5}$  and  $21.9 \times 10^{-5} \text{ s}^{-1}$ , respectively). As a result, ranibizumab showed a lower  $K_D$  value (67 pM) than aflibercept (9263 pM) or bevacizumab (4456 pM) (Figure 1A, insets).

**Format 2.** The inhibitors were the immobilized ligands with rhVEGF in mobile phase as the analyte (Figure 1B). Since each rhVEGF has two potential binding sites for the immobilized inhibitor molecules, experimental conditions were again optimized to encourage monovalent interactions by using low ligand immobilization levels. Similar to format 1, the fits using the 1:1 binding model for both ranibizumab and bevacizumab showed reasonable agreement to the experimentally obtained results (Figure 1B); for aflibercept, much discrepancy was once again observed between the experimental data and the fitted curves using the 1:1 binding model (data not shown). Therefore, the bivalent analyte binding model was used, and first kinetic parameters and dissociation equilibrium constant were summarized in Figure 1B, inset. Ranibizumab again dissociated much more slowly than the other two inhibitors, and a very conservative limit ( $1 \times 10^{-5} \text{ s}^{-1}$ ) of  $k_d$  was used in order to confidently assess the upper limit of  $K_D$  value. Even with this conservative approach, ranibizumab showed a higher binding affinity than aflibercept and bevacizumab, with  $K_D$  ( $K_{D1}$ ) values of <9.2, 4744, and 159 pM, respectively (Figure 1B, insets). Comparing results obtained from format 2 and format 1 revealed some interesting observations: the (first) association rate constants  $k_a$  for all three inhibitors were higher using format 2 than format 1. While it is challenging to know exactly how different the  $k_d$  values were for ranibizumab, both

formats showed very slow dissociation. The  $k_d$  ( $k_{d1}$ ) values obtained from both formats were very similar for bevacizumab but not for aflibercept (Figure 1A,B, insets).

**Format 3.** The inhibitors were immobilized indirectly to the sensor chip using anti-human IgG Fab antibody or protein A as capturing molecules following surface testing to evaluate the validity of the method for all VEGF inhibitors (Figure 1C). While the levels of captured aflibercept and bevacizumab (not shown) stayed almost the same during the time needed for kinetic analysis (Figure 2A), a significant amount of



**Figure 2.** Levels of aflibercept and ranibizumab captured by an anti-Fab antibody or protein A. (A) Level of aflibercept captured by protein A over 3 h in an indirect capturing format at 37 °C. Level of protein A immobilized was approximately 5500 RU. Aflibercept was indirectly captured at approximately 50 RU. (B) Level of ranibizumab captured by anti-Fab antibody over 3 h in an indirect capturing format at 37 °C. Level of anti-Fab antibody immobilized on the sensor chip was approximately 11000 RU. Ranibizumab was indirectly captured at approximately 28 RU. (C) Level of ranibizumab captured by the anti-Fab antibody over 3 h in an indirect capturing format at 25 °C. Level of anti-Fab antibody immobilized on the sensor chip was approximately 1000 RU. Ranibizumab was indirectly captured at approximately 27 RU.

ranibizumab dissociated from the anti-Fab antibody capture molecule: signal decreased nearly 100% when anti-Fab antibody was immobilized at 11000 RU at 37 °C (Figure 2B) and more than 50% when anti-Fab antibody was immobilized at 1000 RU at 25 °C (Figure 2C), a condition reported in the literature.<sup>4</sup> These results indicated that this anti-Fab antibody is not suitable to capture ranibizumab for affinity measurements. We

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