



Analytical and Functional Similarity of Aflibercept Biosimilar ABP 938 with Aflibercept Reference Product

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ABSTRACT

Introduction: ABP 938 is being developed as a biosimilar candidate to aflibercept reference product (RP), a biologic used for certain angiogenic eye disorders. This study was designed to provide a comparative analytical assessment of the structural and functional attributes of ABP 938 and aflibercept RP sourced from the United States (US) and the European Union (EU).

Methods: Structural and functional characterization studies were performed using state-of-the-art analytical techniques that were appropriate to assess relevant quality attributes and capable of detecting qualitative and quantitative differences in primary structure, higher-

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order structure and biophysical properties, product-related substances and impurities, general properties, and biological activities.

Results: ABP 938 had the same amino acid sequence and exhibited similar secondary and tertiary structures, and biological activity as aflibercept RP. There were minor differences in a small number of biochemical attributes which are not expected to impact clinical performance. In addition, aflibercept RP sourced from the US and EU were analytically similar.

Conclusions: ABP 938 was structurally and functionally similar to aflibercept RP. Since aflibercept RP sourced from the US and EU were analytically similar, this allows for the development of a scientific bridge such that a single-source RP can be used in nonclinical and clinical studies.

PLAIN LANGUAGE SUMMARY

Eylea® (aflibercept) is a biologic medication approved for the treatment of patients with certain eye diseases that can result in low vision or blindness. Biosimilars are biologic medications that are highly similar to an existing approved biologic medication, often called a reference product. Biosimilars have the potential to reduce medication costs despite having no clinically significant differences in quality, efficacy, and safety from their reference

products. ABP 938 is currently being developed as a biosimilar to aflibercept reference product. We have conducted similarity studies to compare multiple batches of ABP 938 and aflibercept reference product sourced from both the United States and the European Union, using state-of-the-art analytical methods. The results demonstrated that ABP 938 had the same amino acid sequence and similar structural and biological activities as aflibercept reference product. Before biosimilars can be used as medicines, studies such as this one are required by the Food and Drug Administration and other regulatory authorities to ensure that biosimilars are as safe and effective as their reference products.

Keywords: ABP 938; Aflibercept; Analytical similarity; Structural and functional similarity; VEGF; Biosimilar pharmaceuticals; Eye diseases

Key Summary Points

ABP 938 is a biosimilar candidate of aflibercept reference product (RP).

This comparative similarity assessment employed orthogonal analytical techniques to detect potential structural and functional differences between ABP 938 and aflibercept RP, including primary structure, higher-order structure and biophysical properties, product-related substances and impurities, thermal stability and forced degradation, general properties, and biological activities.

The results demonstrated that ABP 938 is structurally and functionally similar to aflibercept RP.

leading causes of severe vision impairment around the world [1–3]. The discovery that vascular endothelial growth factor (VEGF) is a key mediator involved in the pathogenesis of angiogenic eye disorders [4] led to the introduction of anti-VEGF treatments. Commonly used anti-VEGF agents such as aflibercept have revolutionized the management of various retinal diseases and enhanced patient quality of life [5–8].

Aflibercept reference product (RP) is a recombinant fusion protein consisting of extracellular Domain 2 of human VEGF receptor-1 (VEGFR-1) and Domain 3 of VEGFR-2 fused to the fragment crystallizable (Fc) portion of human immunoglobulin G1. It acts as a soluble decoy receptor that binds VEGF-A and placental growth factor (PlGF), inhibiting the binding and activation of their cognate VEGF receptors and blocking the key signaling pathway responsible for angiogenesis and vascular leakage [9, 10]. ABP 938 is being developed as a biosimilar to aflibercept RP. To gain approval for a biosimilar, the United States (US) and European Union (EU) regulatory guidelines recommend a stepwise approach in developing the evidence needed to demonstrate biosimilarity, including structural and functional characterization, nonclinical evaluation, human pharmacokinetic and pharmacodynamic data, clinical immunogenicity, and comparative clinical efficacy and safety results [11–14]. The availability of biosimilars can potentially provide healthcare providers with additional treatment options, increase patient access to lower cost medications, and facilitate more equitable health outcomes [15].

This analytical similarity assessment was designed to characterize the structural and functional attributes of ABP 938 and aflibercept RP and to evaluate whether any differences have the potential to meaningfully impact clinical efficacy and safety.

INTRODUCTION

Retinal diseases such as neovascular (wet) age-related macular degeneration (nAMD) are

METHODS

Test Materials

ABP 938 was manufactured by Amgen Inc. Aflibercept RP was sourced from the US (Eylea[®], Regeneron Pharmaceuticals, Inc.) and the EU (Eylea[®], Bayer AG) as recommended in the regulatory guidance documents for biosimilar development [12, 14]. The RPs were stored and handled according to the manufacturer's instructions and tested as part of the analytical similarity assessment plan.

Methods Summary

The methods used for the analytical similarity assessments were selected on the basis of knowledge of the structure, function, and expected structural heterogeneity of aflibercept RP and ABP 938, including characteristics critical to biological activity, safety, and stability. The testing plan (Table 1) was designed to assess primary structure, higher-order structure (HOS) and biophysical properties, product-related substances and impurities, general properties, thermal stability and forced degradation, and biological activity. Details of experimental methods are included in the Supplementary Materials.

ABP 938 lots used in this study were acquired from development, clinical, and process validation production runs. Aflibercept RP lots acquired from the US and EU represented the product profile over multiple years to evaluate process variability during the course of ABP 938 development. Testing of aflibercept RP lots was performed shortly after receipt of the materials. For attributes expected to change as a function of time, aflibercept RP lots were tested again at or near the end of its shelf-life.

This article does not describe any studies with human participants or animals. All laboratory health and safety procedures have been complied with in the course of conducting this experimental work.

RESULTS

Physicochemical similarity assessment results for ABP 938 and aflibercept RP are presented in Table 2. Functional similarity assessment results are shown in Table 3.

Primary Structure

The similarity between ABP 938 and aflibercept RP primary structures was assessed using a number of state-of-the-art methods, including intact molecular mass by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS), reduced and deglycosylated molecular mass by electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS), and reduced peptide mapping with liquid chromatography–mass spectrometry (LC-MS). Aflibercept is a dimeric glycoprotein with a molecular mass of 115 kilodaltons (kDa) [13]. The intact mass analysis results demonstrated that ABP 938 and aflibercept RP had similar intact molecular masses (Table 2). Reduced and deglycosylated molecular mass analyses confirmed that ABP 938 and aflibercept RP had similar molecular masses that were within ± 100 parts per million (ppm) of the theoretical mass (Table 2). In addition, the deconvoluted mass profiles were similar and there were no new species observed for the reduced deglycosylated ABP 938 as compared to aflibercept RP (Fig. 1a). The major peaks corresponded to the expected theoretical masses, which suggested that the C-terminal lysine residues were processed and the conversion of five asparagine residues to aspartic acid residues as a result of removal of the five *N*-glycans by peptide:*N*-glycosidase F (PNGase F). There were no detectable levels of N-terminal variants observed in ABP 938; however, partially processed N-terminal signal peptides were detected in aflibercept RP (Table 2). This minor difference is not expected to impact clinical performance because both ABP 938 and aflibercept RP had similar biological activities (see “[Biological Activity](#)” section). Finally, the primary amino acid sequence was determined by overlapping peptide map with tandem MS. The results

Table 1 Testing plan and analytical methods for the physicochemical and functional similarity assessment of ABP 938 and aflibercept RP

Category	Attributes/activities by analytical techniques
Primary structure	Intact molecular mass by MALDI-TOF-MS
	Reduced and deglycosylated molecular mass by ESI-TOF-MS
	Amino acid sequence by reduced peptide map with LC-MS
	Extinction coefficient by amino acid analysis/UV spectroscopy
	Identity by anti-idiotypic ELISA
Higher-order structure and biophysical properties	Secondary structure by FTIR
	Tertiary structure by near-UV CD
	Thermal stability by DSC
	Submicron particles by DLS
	Size distribution by SV-AUC
	Molecular weight determination by SE-HPLC-LS
Product-related substances and impurities	Size variants by SE-UHPLC
	Size variants by rCE-SDS
	Size variants by nrCE-SDS
General properties	Protein concentration by UV absorbance
	Volume by gravimetric determination
Thermal stability and forced degradation studies	Accelerated condition at 30 °C; stressed condition at 40 °C; forced degradation at 45 °C and photostability at 25 °C by purity, peptide map, and functional assays
Biological activity	Inhibition of VEGF-A ₁₆₅ -mediated HUVEC proliferation
	VEGF-A ₁₆₅ /VEGFR-2 signaling inhibition in HUVEC
	VEGF-A ₁₆₅ /VEGFR-2 signaling inhibition in HEK293 cells
	VEGF-A ₁₆₅ /VEGFR-2 inhibition of binding by luminescence proximity assay
	Specificity against VEGF-C and VEGF-D by SPR
	PlGF-1/VEGFR-1 dimerization inhibition in U2OS cells
	PlGF-2/VEGFR-1 dimerization inhibition in U2OS cells
	PlGF-1/VEGFR-1 inhibition of binding by luminescence proximity assay

Table 1 continued

Category	Attributes/activities by analytical techniques
	PlGF-1 binding by SPR
	PlGF-2 binding by SPR
	Galectin-1 binding by SPR
	VEGF-B ₁₆₇ binding by ELISA
	FcRn binding by SPR
	C1q binding by ELISA
	FcγRIa binding by SPR
	FcRIIa-131H binding by SPR
	FcRIIb binding by SPR
	FcRIIIa-158V binding by SPR
	FcRIIIb binding by SPR
	Lack of ADCC activity in PBMC and SKOV3 cells
	Lack of ADCP activity in PBMC (CD14 ⁺) and SKOV3 cells
	Lack of CDC activity in SKOV3 cells with rabbit serum

ADCC antibody-dependent cell-mediated cytotoxicity, *ADCP* antibody-dependent cellular phagocytosis, *C1q* first sub-component of complement, *CDC* complement-dependent cytotoxicity, *DSC* differential scanning calorimetry, *ELISA* enzyme-linked immunosorbent assay, *ESI-TOF-MS* electrospray ionization time-of-flight mass spectrometry, *Fc* fragment crystallizable, *FcRn* neonatal Fc receptor, *FγR* Fc gamma receptor, *FTIR* Fourier transform infrared spectroscopy, *H* histidine, *HEK293 cells* human embryonic kidney 293 cells, *HUVEC* human umbilical vein endothelial cells, *LC-MS* liquid chromatography–tandem mass spectrometry, *LS* light scattering, *MALDI-TOF-MS* matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, *nrCE-SDS* non-reduced capillary electrophoresis-sodium dodecyl sulfate, *PBMC* peripheral blood mononuclear cells, *PlGF-1/2* placental growth factor 1 or 2, *rCE-SDS* reduced capillary electrophoresis-sodium dodecyl sulfate, *SE-UHPLC* size exclusion ultrahigh-performance liquid chromatography, *SPR* surface plasmon resonance, *SV-AUC* sedimentation velocity analytical ultracentrifugation *UV* ultraviolet, *UV CD* ultraviolet circular dichroism, *U2OS cells* human bone osteosarcoma epithelial cells, *V* valine, *VEGF-A, -B, -C, or -D* vascular endothelial growth factor A, B, C, or D, *VEGFR* vascular endothelial growth factor receptor

confirmed that ABP 938 and aflibercept RP have the same amino acid sequence. Moreover, the peptide map profiles were visually similar, and no new peaks above the integrity limit were detected in ABP 938 as compared to aflibercept RP (Fig. 1b).

ABP 938 and aflibercept RP displayed similar extinction coefficient values as determined by amino acid analysis using ultraviolet (UV) spectroscopy, and a similar identity as evaluated by a dual anti-idiotypic enzyme-linked immunosorbent assay (ELISA) (data not shown).

Higher-Order Structure and Biophysical Properties

The HOS and biophysical properties of ABP 938 and aflibercept RP were assessed using multiple techniques. The secondary structure was characterized by Fourier transform infrared (FTIR) spectroscopy and showed strong bands in the spectra at 1639 cm⁻¹ and 1690 cm⁻¹, indicative of a β-sheet secondary structure (Fig. 2a). The tertiary structure was characterized by near-UV circular dichroism (CD) spectra which contained signals from tryptophan, tyrosine, and phenylalanine superimposed on the broad

Table 2 Summary of physicochemical similarity assessment results on selected attributes for ABP 938 and aflibercept RP

Attributes	ABP 938 Range (<i>n</i>)	Aflibercept-US Range (<i>n</i>)	Aflibercept-EU Range (<i>n</i>)
Intact molecular mass (Da)	114,130–114,790 (6)	113,920–114,320 (6)	114,003–114,220 (6)
Reduced and deglycosylated molecular mass (48,463.6 Da) (difference, ppm)	0.4–10.3 (6)	14.4–17.1 (6)	7.8–16.5 (6)
Reduced and deglycosylated molecular mass with glycation (48,625.7 Da) (difference, ppm)	4.1–23.2 (6)	2.7–30.8 (6)	2.5–37.0 (6)
Reduced and deglycosylated molecular mass with N-terminal SG (48,607.7 Da) (difference, ppm)	ND (6)	0.0–28.6 (6)	2.1–18.5 (6)
Secondary structure by FTIR spectral similarity (%) ^a			
Aflibercept-US as reference	98.9–99.9 (6)	99.2–100.0 (6)	98.8–99.9 (6)
Aflibercept-EU as reference	99.3–99.9 (6)	98.2–99.9 (6)	99.2–100.0 (6)
Tertiary structure by near-UV CD spectral similarity (%) ^a			
Aflibercept (US) as reference	96.6–99.1 (6)	97.6–100.0 (6)	97.3–99.2 (6)
Aflibercept (EU) as reference	95.7–99.2 (6)	96.8–99.4 (6)	95.2–100.0 (6)
Thermal stability by DSC (°C)			
<i>T</i> _{m1}	68.2–68.5 (6)	68.1–68.3 (6)	68.2–68.2 (6)
<i>T</i> _{m2}	84.9–85.2 (6)	84.8–85.0 (6)	84.7–85.0 (6)
Molecular molar masses by SE-HPLC-LS (kDa)			
HMW peak	225–231 (6)	219–225 (6)	222–226 (6)
Main peak	111–113 (6)	111–112 (6)	112–113 (6)
SV-AUC, monomer (%)	98.0–100 (6)	97.4–99.4 (6)	97.0–99.7 (6)
Submicron particles by DLS	ND (6)	ND (6)	ND (6)
Protein concentration (mg/mL, vial)	38.5–42.1 (13)	39.3–40.6 (17)	39.5–40.9 (18)
Protein concentration (mg/mL, PFS)	39.5–41.3 (5)	39.4–40.7 (10)	39.6–40.9 (9)
Volume (mL)	0.263–0.293 (11)	0.263–0.285 (10)	0.257–0.274 (10)
Volume (mL, PFS)	0.150–0.162 (5)	0.154–0.172 (9)	0.146–0.163 (9)

DLS dynamic light scattering, *DSC* differential scanning calorimetry, *EU* European Union, *FTIR* Fourier transform infrared spectroscopy, *HMW* high molecular weight, *n* lot numbers tested, *ND* not detected, *PFS* pre-filled syringe, *ppm* parts per million, *RP* reference product, *SE-HPLC-LS* size exclusion high-performance liquid chromatography with light scattering, *SG* serine glycine, *SV-AUC* sedimentation velocity analytical ultracentrifugation, *US* United States, *UV CD* ultraviolet circular dichroism

^aThe 100% similarity for the FTIR and near-UV CD similarity scores indicate that these particular datasets were used as the reference in the comparison

Table 3 Summary of biological activities of ABP 938 and aflibercept RP

Attributes	ABP 938 Range (n)	Aflibercept-US Range (n)	Aflibercept-EU Range (n)
Inhibition of VEGF-A ₁₆₅ -mediated HUVEC proliferation (%)	93–110 (12)	90–115 (18)	87–117 (19)
VEGF-A ₁₆₅ /VEGFR-2 signaling inhibition in HUVEC (%)	95–108 (12)	95–110 (12)	96–115 (12)
VEGF-A ₁₆₅ /VEGFR-2 signaling inhibition in HEK293 cells (%)	94–110 (12)	93–103 (12)	95–104 (12)
VEGF-A ₁₆₅ /VEGFR-2 inhibition of binding by luminescence proximity assay (%)	90–109 (13)	89–109 (18)	73–107 (19)
Specificity against VEGF-C and VEGF-D by SPR (RU)	No binding	No binding	No binding
PlGF-1/VEGFR-1 dimerization inhibition in U2OS cells (%)	89–112 (12)	91–111 (15)	84–116 (16)
PlGF-2/VEGFR-1 dimerization inhibition in U2OS cells (%)	89–111 (12)	92–110 (17)	90–110 (16)
PlGF-1/VEGFR-1 inhibition of binding by luminescence proximity assay (%)	89–108 (12)	100–113 (12)	102–116 (12)
PlGF-1 binding by SPR (%)	99–102 (12)	96–98 (11)	96–98 (12)
PlGF-2 binding by SPR (%)	98–102 (12)	94–100 (11)	95–100 (12)
Galectin-1 binding by SPR (%)	93–128 (12)	89–109 (17)	88–123 (16)
VEGF-B ₁₆₇ binding by ELISA (%)	98–114 (12)	92–116 (18)	89–112 (19)
C1q binding by ELISA (%)	89–112 (6)	94–114 (6)	102–117 (11)
FcRn binding by SPR (%)	76–106 (5)	95–105 (5)	101–106 (5)
FcγRI binding by SPR (%)	92–100 (3)	100–100 (3)	98–106 (3)
FcγRIIa-131H binding by SPR (%)	92–98 (3)	105–109 (3)	105–112 (3)
FcγRIIb binding by SPR (%)	100–104 (3)	113–119 (3)	112–117 (3)
FcγRIIIa-158V binding by SPR (%)	93–102 (5)	214–247 (5)	237–257 (5)
FcγRIIIb binding by SPR (%)	106–117 (3)	211–221 (3)	242–252 (3)
Lack of ADCC activity in PBMC and SKOV3 cells (luminescence, dead cells)	No ADCC	No ADCC	No ADCC
Lack of ADCP activity in PBMC (CD14 ⁺) and SKOV3 cells (CD14 ⁺ and encoded dye ⁺ for phagocytosis)	No ADCP	No ADCP	No ADCP
Lack of CDC activity in SKOV3 cells with rabbit serum (luminescence, live cells)	No CDC	No CDC	No CDC

ADCC antibody-dependent cell-mediated cytotoxicity, *ADCP* antibody-dependent cellular phagocytosis, *C1q* first sub-component of complement, *CDC* complement-dependent cytotoxicity, *ELISA* enzyme-linked immunosorbent assay, *EU* European Union, *Fc* fragment crystallizable, *FcγR* human leukocyte Fc gamma receptor, *FcRn* neonatal Fc receptor, *H* histidine, *HEK293 cells* human embryonic kidney 293 cells, *HUVEC* human umbilical vein endothelial cells, *n* lot numbers tested, *PBMC* peripheral blood mononuclear cells, *PlGF-1/2* placental growth factor 1 or 2, *RP* reference product, *RU* response unit, *SPR* surface plasmon resonance, *U2OS cells* human bone osteosarcoma epithelial cells, *US* United States, *V* valine, *VEGF-A, -B, -C, or -D* vascular endothelial growth factor A, B, C, or D, *VEGFR* vascular endothelial growth factor receptor

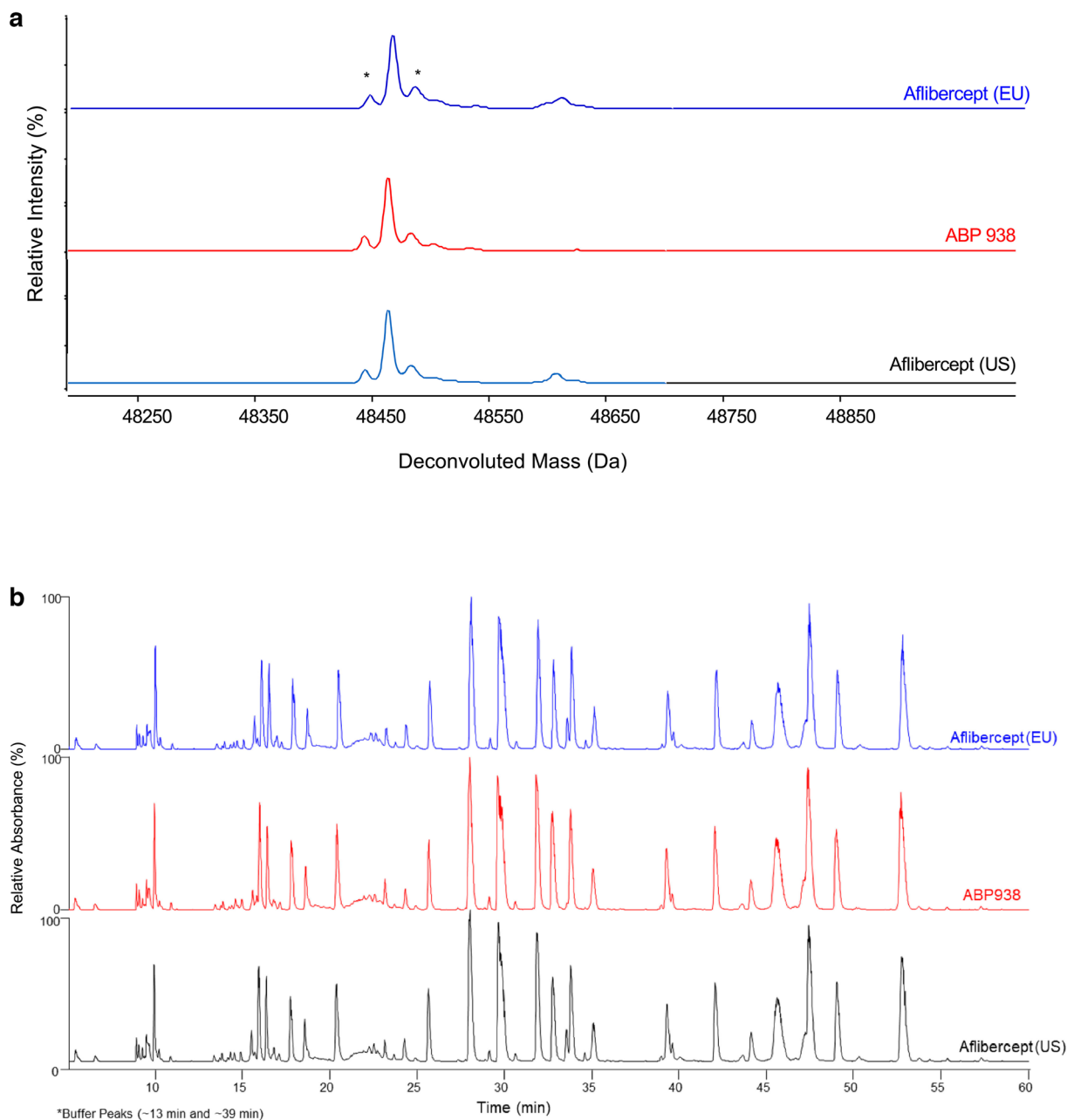


Fig. 1 Primary structure of afibercept (EU), ABP 938, and afibercept (US). **a** Reduced and deglycosylated mass profile by ESI-TOF-MS. *Adducts are artifacts of ionization. **b** Reduced peptide map by LC-MS. *ESI-TOF-MS*

electrospray ionization time-of-flight mass spectrometry, LC-MS liquid chromatography–mass spectrometry

disulfide signal from 240 to 350 nm (Fig. 2b). The spectral similarity value of ABP 938, afibercept (US), and afibercept (EU) met the pre-defined similarity assessment criteria of

$\geq 95\%$ for both FTIR and near-UV CD spectroscopy (Table 2).

Thermostability was characterized by differential scanning calorimetry (Fig. 2c). ABP 938 and afibercept RP displayed similar thermal

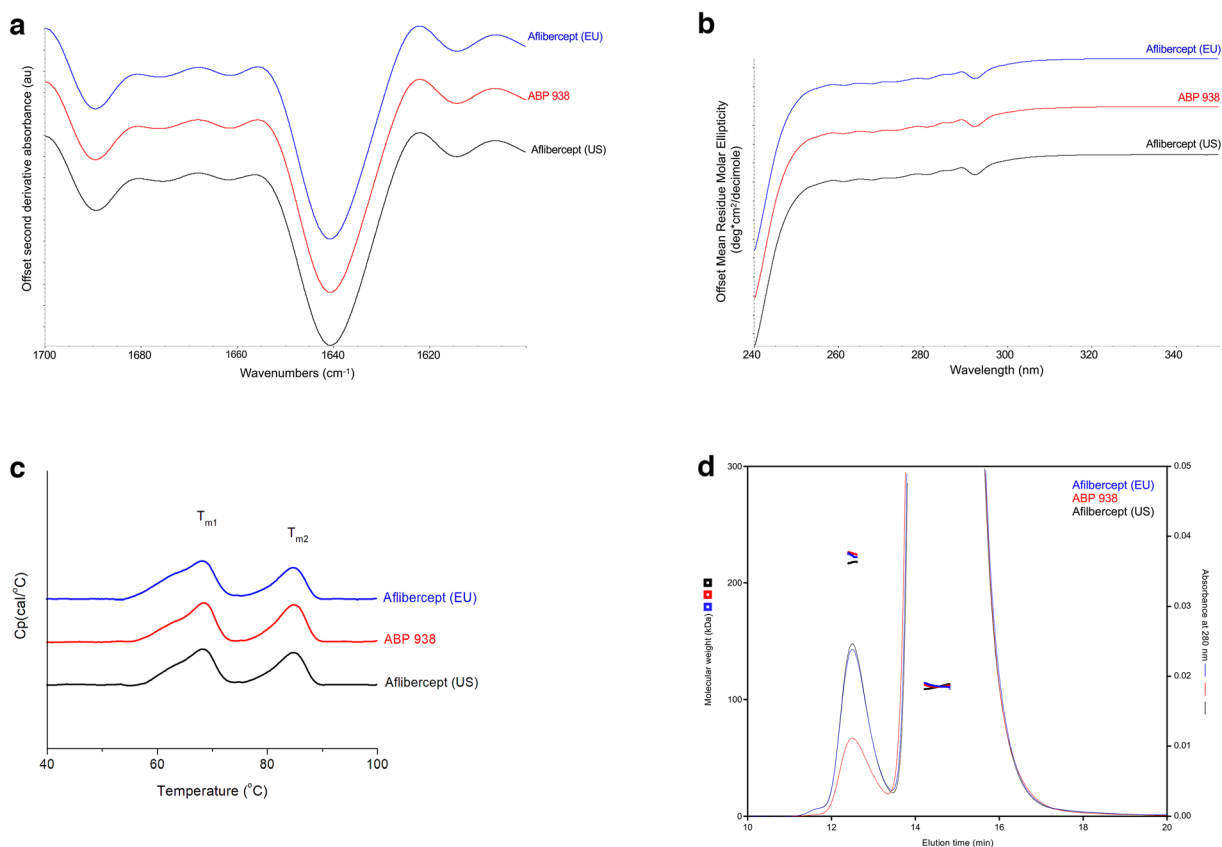


Fig. 2 Higher-order structure (HOS) and biophysical properties of aflibercept (EU), ABP 938, and aflibercept (US). **a** Secondary structure FTIR spectra. **b** Near-UV CD spectra. **c** DSC thermograms. **d** SE-HPLC-LS profiles. AU absorbance units, FTIR Fourier transform infrared

melting transition temperatures at approximately 68.1 °C for T_{m1} and 85.0 °C for T_{m2} (Table 2). An absence of submicron particles in ABP 938 and aflibercept RP was confirmed by dynamic light scattering (Table 2). In addition, ABP 938 and aflibercept RP had similar levels of monomer, with no detectable aggregates assessed by sedimentation velocity analytical ultracentrifugation (SV-AUC) (Table 2). Finally, ABP 938 and aflibercept RP had similar molar masses for the monomer and high molecular weight (HMW) species as evaluated by size exclusion high-performance liquid chromatography (SE-HPLC) coupled with light scattering detector (Fig. 2d). ABP 938 had a lower level of HMW species compared to aflibercept RP (Fig. 2d). The theoretical masses of aflibercept

spectroscopy, UVCD ultraviolet circular dichroism, DSC differential scanning calorimetry, SE-HPLC-LS size exclusion high-performance liquid chromatography-light scattering

RP monomer and dimer were approximately 115 kDa and 229 kDa, respectively, and the molar masses for monomer and HMW of ABP 938 and aflibercept RP were within the theoretical molar masses (Table 2). The molar masses of HMW observed in ABP 938 and aflibercept RP were consistent with that of dimers.

Product-Related Substances and Impurities

ABP 938 and aflibercept RP were evaluated for product-related substances and impurities. Size and charge variants are indicative of the stability of the products, which may change over time at the recommended storage conditions.

ABP 938 and aflibercept RP had visually similar HMW and main peak profiles with no new or missing peaks as assessed by size exclusion ultrahigh-performance liquid chromatography (SE-UHPLC) (Fig. 3a). The stability data demonstrated that under the recommended storage conditions, ABP 938 maintained a lower level of HMW species compared to aflibercept RP for up to 36 months (Fig. 3a inset).

Size variants were assessed by capillary electrophoresis–sodium dodecyl sulfate (CE-SDS) in reducing and denaturing conditions. This method is suitable for quantifying fragments and the purity of the samples. ABP 938 and aflibercept RP had visually similar total pre-peak (fragmentation) and main peak (purity) profiles, with no new or missing peaks as assessed by reduced CE-SDS (rCE-SDS) (Fig. 3b). The stability data demonstrated that the level of rCE-SDS main peaks for ABP 938 remained higher than aflibercept RP for up to 36 months (Fig. 3b inset). Size variants assessed by non-reduced CE-SDS (nrCE-SDS) showed that ABP 938 and aflibercept RP had visually similar profiles with no new or missing peaks (Fig. 3c). Size variants included fragments and partial molecules; however, partial molecule species were not observed in either ABP 938 or aflibercept RP samples. The pre-peaks observed are fragmented species, which were present at low levels. The stability data demonstrated that the levels of main peaks were similar for ABP 938 and aflibercept RP (Fig. 3c inset).

General Properties

Aflibercept RP is supplied in a single-dose vial and a single-dose pre-filled syringe (PFS) designed to deliver 0.05 mL at 40 mg/mL [16]. ABP 938 and aflibercept RP demonstrated similar product strength as assessed by protein concentration and volume in both vial and PFS presentations (Table 2).

Biological Activity

Aflibercept RP acts as a soluble decoy receptor that binds VEGF-A and PlGF, thereby inhibiting the binding and activation of its cognate VEGF

receptors [10, 16]. The VEGFR domain-mediated binding and cell-based functional studies demonstrated that ABP 938 was similar to aflibercept RP with respect to inhibition of VEGF-A₁₆₅-mediated proliferation in human umbilical vein endothelial cells (HUVEC), VEGF-A₁₆₅/VEGFR-2 signaling inhibition in HUVEC, VEGF-A₁₆₅/VEGFR-2 signaling inhibition in HEK293 cells, and VEGF-A₁₆₅/VEGFR-2 inhibition of binding (Fig. 4a–d). Additionally, the data suggested that ABP 938 and aflibercept RP were similar in PlGF-1/VEGFR-1 dimerization inhibition in human osteosarcoma U2OS cells, PlGF-2/VEGFR-1 dimerization inhibition in U2OS cells, PlGF-1 and PlGF-2 binding, and PlGF-1/VEGFR-1 inhibition of binding (Fig. 5a–e).

Aflibercept RP also binds to galectin-1 and VEGF-B but not to VEGF-C and VEGF-D [17, 18]. ABP 938 was similar to aflibercept RP with respect to galectin-1 and VEGF-B binding activity (Fig. 6a, b), and the absence of VEGF-C and VEGF-D binding activity (Table 3). Similar to aflibercept RP, ABP 938 showed a lack of antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), and complement-dependent cytotoxicity (CDC) activities (Table 3).

The studies evaluating Fc domain-mediated binding activity demonstrated that ABP 938 and aflibercept RP had similar levels of neonatal fragment crystallizable receptor (FcRn) and Fc-gamma receptor 1 (FcγRI) binding. While ABP 938 had lower levels of complement component 1q (C1q) and other FcγR binding compared to aflibercept RP (Table 3), the differences are not considered critical for clinical performance because both ABP 938 and aflibercept RP lacked ADCC, ADCP, and CDC activities (Table 3).

DISCUSSION

Comparative analytical similarity data from structural and functional studies of a proposed biosimilar and its RP provide a critical foundation for the assessment of biosimilarity [19]. This comparative similarity assessment employed orthogonal analytical techniques to

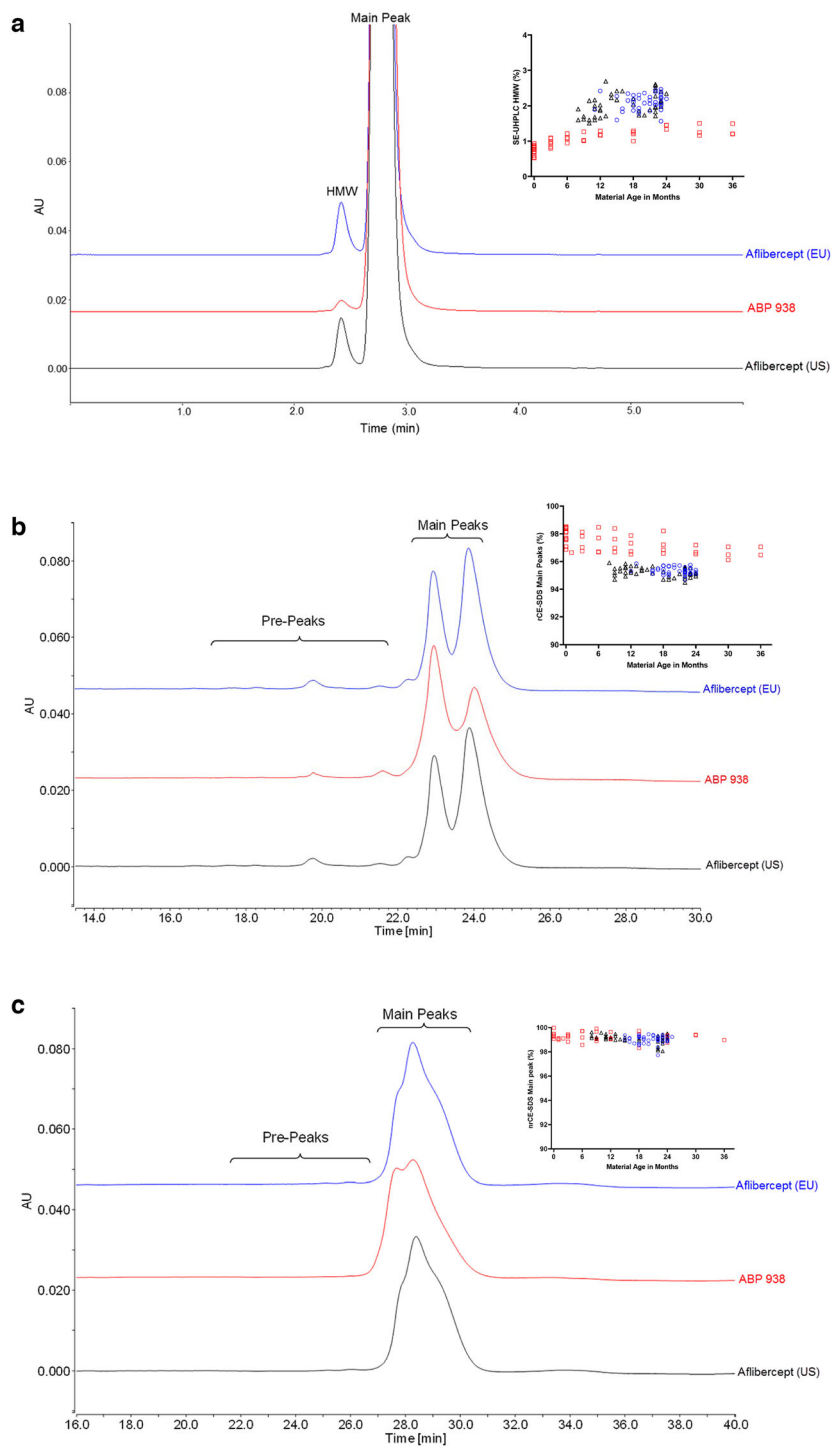


Fig. 3 Product-related substances and impurities of aflibercept (EU), ABP 938, and aflibercept (US). **a** SE-UHPLC profiles; SE-UHPLC HMW stability at recommended storage condition (inset). **b** rCE-SDS profiles; rCE-SDS main peak stability at recommended storage condition (inset). **c** nrCE-SDS profile; nrCE-SDS main peak stability at

recommended storage condition (inset). *AU* absorbance units, *HMW* high molecular weight, *kDa* kilodaltons, *nrCE-SDS* non-reduced capillary electrophoresis–sodium dodecyl sulfate, *rCE-SDS* reduced capillary electrophoresis–sodium dodecyl sulfate, *SE-UHPLC* size exclusion ultrahigh-performance liquid chromatography

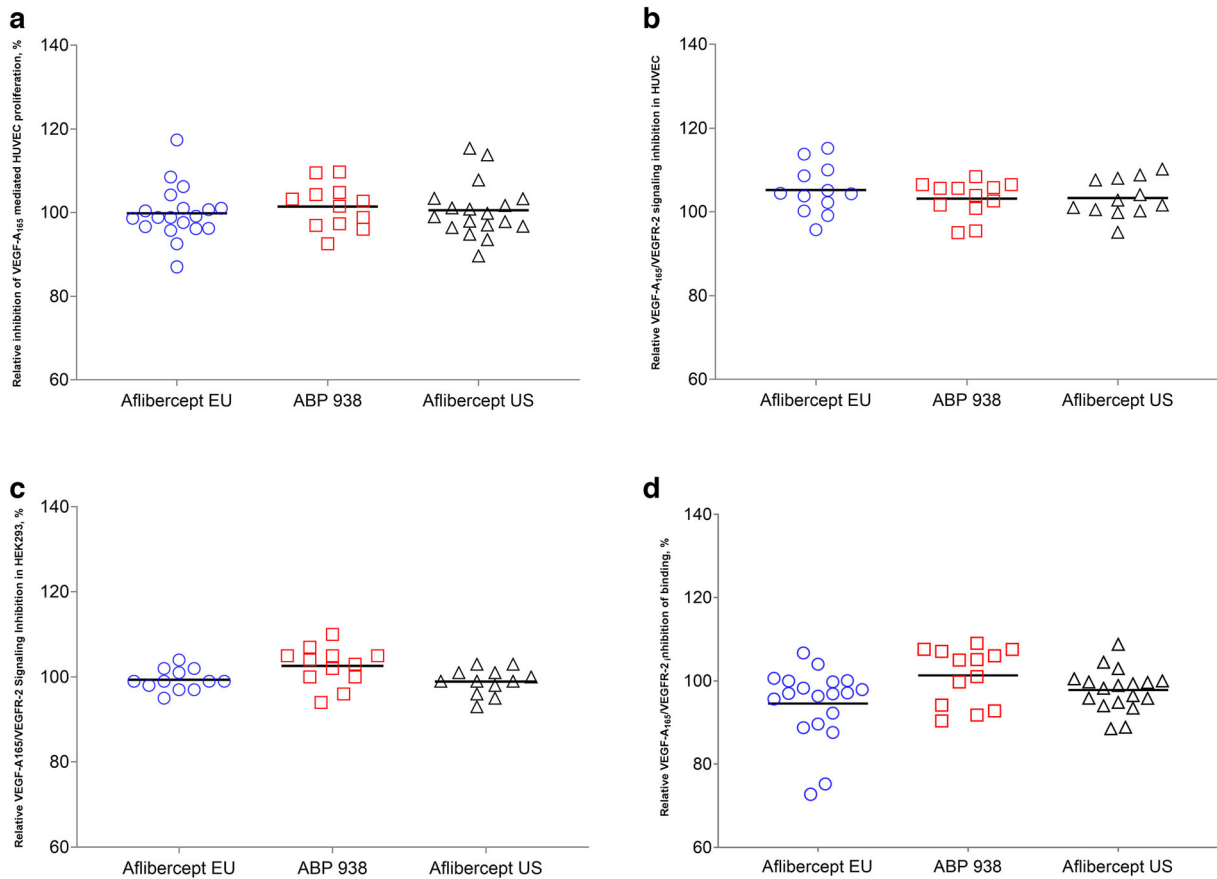


Fig. 4 Inhibition of VEGF-A-mediated biological activities of afibercept (EU), ABP 938, and afibercept (US). **a** Relative inhibition of VEGF-A₁₆₅-mediated HUVEC proliferation. **b** Relative VEGF-A₁₆₅/VEGFR-2 signaling inhibition in HUVEC. **c** Relative VEGF-A₁₆₅/VEGFR-2 signaling inhibition in HEK293 cells. **d** Relative inhibition

of VEGF-A₁₆₅/VEGFR-2 binding. HEK293 human embryonic kidney 293 cells, *HUVEC* human umbilical vein endothelial cells, *VEGF-A* vascular endothelial growth factor A, *VEGFA*₁₆₅ vascular endothelial growth factor A165 isoform, *VEGFR-2* vascular endothelial growth factor receptor 2

evaluate physicochemical properties and functional activities to detect potential differences between ABP 938 and afibercept RP.

The combined results of both intact glycosylated molecular mass analysis and reduced and deglycosylated molecular mass analysis provided evidence that ABP 938 and afibercept RP are dimeric glycoproteins with a protein molecular weight of 97 kDa and contain glycosylation, resulting in a total molecular mass of 115 kDa. The polypeptide compositions were similar and consistent with the theoretical masses of ABP 938 and afibercept RP. The reduced peptide map demonstrated that ABP 938 and afibercept RP had the same

primary amino acid sequence and similarly low levels of post-translational modifications, indicating that they have a similar primary structure. The HOS and biophysical properties are important for confirming structural integrity. The analysis of the HOS confirmed that ABP 938 and afibercept RP have similar secondary and tertiary structures. Additionally, ABP 938 and afibercept RP demonstrated similar thermal stability profiles. Furthermore, ABP 938 and afibercept RP were similar for the molar masses of the monomer and HMW species.

Product-related substances and impurities of ABP 938 and afibercept RP were assessed using an array of separation methods that evaluate

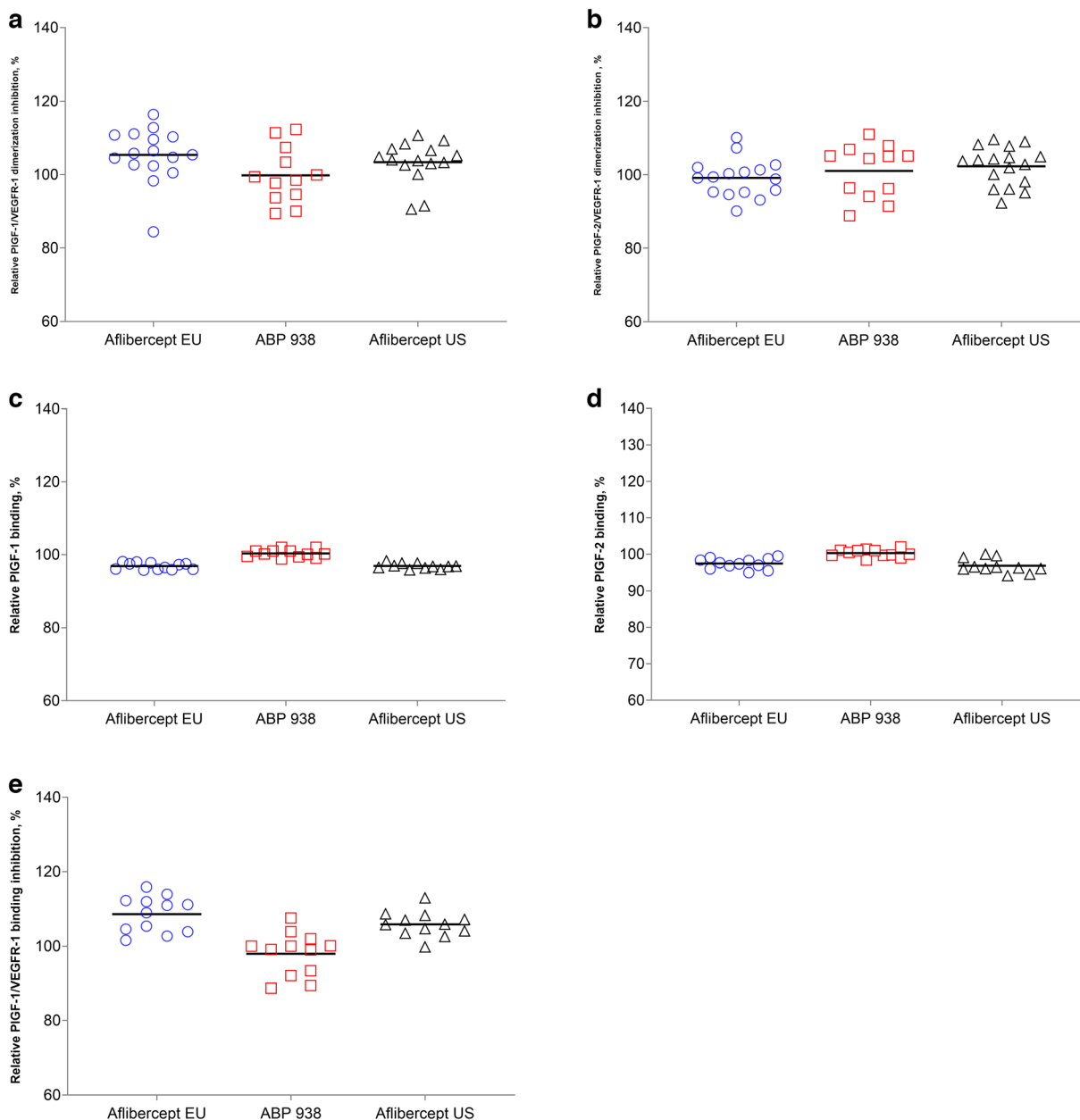


Fig. 5 Inhibition of PlGF-mediated biological activities of aflibercept (EU), ABP 938, and aflibercept (US). **a** Relative PlGF-1/VEGFR-1 dimerization inhibition in U2OS cells. **b** Relative PlGF-2/VEGFR-1 dimerization inhibition in

U2OS cells. **c** Relative PlGF-1 binding. **d** Relative PlGF-2 binding. **e** Relative inhibition of PlGF-1/VEGFR-1 of binding. *PlGF-1 and -2* placental growth factor 1 and 2, *VEGFR-1* vascular endothelial growth factor receptor 1

size and charge variants. The SE-UHPLC profiles were similar with no new or missing peaks observed for ABP 938 and aflibercept RP. ABP 938 had a lower level of HMW species compared to aflibercept RP, and the levels of

HMW for ABP 938 remained below those of aflibercept RP throughout the study. HMW species can have a potential impact on clinical safety and immunogenicity; however, the HMW observed in ABP 938 and aflibercept RP

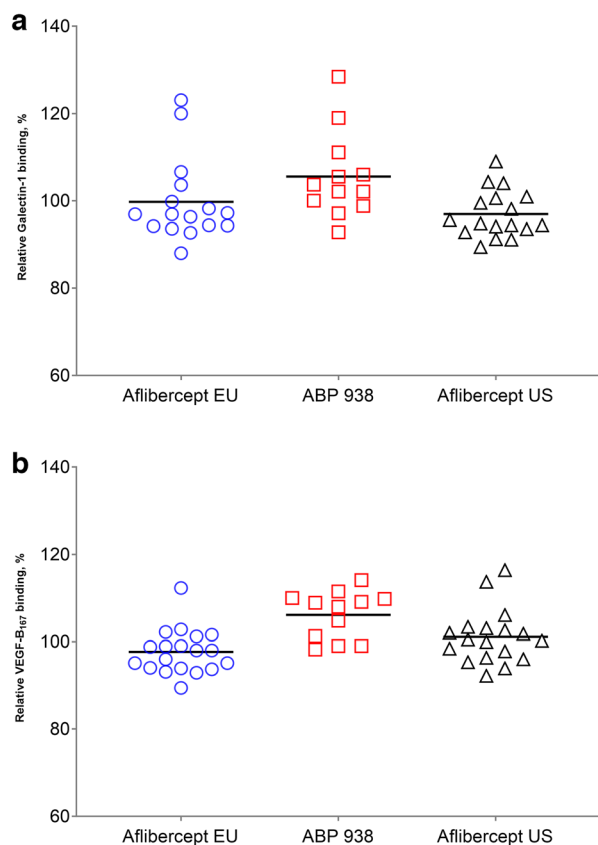


Fig. 6 Galectin-1 and VEGF-B binding activities of aflibercept (EU), ABP 938, and aflibercept (US). **a** Relative galectin-1 binding. **b** Relative VEGF-B₁₆₇ binding. *VEGF-B*₁₆₇ vascular endothelial growth factor B167 isoform

were consistent with that of dimers, and a lower level of HMW in ABP 938 would not be expected to impact clinical safety. The rCE-SDS profiles were similar between ABP 938 and aflibercept RP. ABP 938 showed a higher level of main peaks (i.e., purity), and conversely, a lower level of pre-peaks (i.e., fragmentation), when compared to aflibercept RP throughout the study. The nrCE-SDS profiles were visually similar. A low level of pre-peaks observed were likely fragmented species. ABP 938 and aflibercept RP showed similar levels of main peak over time under the recommended storage conditions.

VEGF family members, including VEGF-A, VEGF-B, VEGF-C, VEGF-D, and PlGF, are key contributors to vasculogenesis and angiogenesis

and therefore crucial for embryonic development and vessel repair [20, 21]. Aflibercept is known to bind to VEGF-A, PlGF, and VEGF-B, but not to the VEGF-C or VEGF-D family members [22]. Among the VEGF family, VEGF-A is the most potent mitogen of angiogenesis [20]. Proliferation of vascular endothelial cells and angiogenesis is induced predominantly through VEGF-A-mediated VEGFR-2 activation [23]. Endothelial cell response to VEGF-A leads to neovascularization and vascular permeability that contributes to disease pathology [10]. At least nine VEGF-A isoforms have been identified. The VEGF-A₁₁₁, VEGF-A₁₂₁, VEGF-A₁₄₅, VEGF-A₁₆₅, VEGF-A₁₈₉, and VEGF-A₂₀₆ isoforms are the most commonly expressed transcripts [24, 25]. All VEGF-A isoforms bind both VEGFR-1 and VEGFR-2 through conserved VEGFR binding domains [26–29]. Because VEGF-A₁₆₅ is the most extensively investigated and representative VEGF-A isoform, it was used to assess the functional activities of ABP 938 and aflibercept RP. ABP 938 and aflibercept RP demonstrated similar biological activities related to the mechanism of action in various assays (Fig. 4a–d).

PlGF acts as a paracrine factor upon activation of VEGFR-1, leading to proliferation, migration, and survival responses in endothelial cells [30, 31]. Although the contribution of PlGF in pathological ocular neovascularization in humans has not been fully elucidated [32], its expression is elevated in patients with angiogenic eye disorders [33]. In humans, PlGF exists as four splice isoforms that bind VEGFR-1 through conserved receptor-binding domains [34, 35]. The most relevant isoforms involved in ocular diseases are PlGF-1 and PlGF-2 [32, 36], whereas expression of PlGF-3 and PlGF-4 is primarily in the placenta and umbilical vein endothelial cells [37]. Therefore, PlGF-1 and PlGF-2 were used in orthogonal assays to demonstrate that ABP 938 and aflibercept RP have similar biological activities (Fig. 5a–e).

Galectin-1 directly binds to N-glycans on VEGFR-2 [16] and has been identified in neovascular eye tissues surgically excised from patients with angiogenic retinal disorders. Aflibercept blocks galectin-1-induced VEGFR-2 phosphorylation in human retinal

microvascular endothelial cells [33]; however, the biological role of galectin-1 in the pathophysiology of eye disease remains to be determined. ABP 938 and aflibercept RP demonstrated similar galectin-1 binding activity.

VEGF-B binding is a known property of aflibercept RP [14]; however, VEGF-B has not been implicated in the pathogenesis of angiogenic eye disorders. It exists as two isoforms which bind to VEGFR-1 through conserved receptor binding domains [38]. VEGF-B₁₆₇ is the predominant isoform expressed in most tissues and organs, accounting for more than 80% of the total VEGF-B transcripts, while VEGF-B₁₈₆ is expressed at lower levels and in a limited number of tissues [39]. Therefore, VEGF-B₁₆₇ was used to demonstrate that ABP 938 and aflibercept RP were similar in VEGF-B binding activity.

ABP 938 showed similar binding of FcRn and Fc γ RI and lower binding of C1q, Fc γ RIIa, Fc γ RIIb, Fc γ RIIIa, and Fc γ RIIIb as compared to aflibercept RP. However, the lower binding activities are not considered clinically meaningful because both ABP 938 and aflibercept had no Fc domain-mediated effector functions. The biological functions of aflibercept RP are primarily mediated through its VEGFR domains. The Fc domain of the molecule does not induce effector functions, as members of the VEGF protein family exist as predominantly soluble proteins [22]. Overall, the functional activity results indicate that ABP 938 is highly similar to aflibercept RP. It should be noted that the exhaustive structural and functional comparisons of ABP 938 with aflibercept RP that have been presented here are a first step in the development of ABP 938 as a biosimilar to aflibercept and provide the scientific basis for its further development in the clinical setting. Indeed, a comparative clinical study (NCT04270747) of ABP 938 and aflibercept RP in patients with nAMD has recently been completed. Publication of the results of this study is awaited and should provide evidence related to the clinical efficacy and safety of ABP 938. Taken together, these results should provide the totality of evidence for biosimilarity and form the basis for clinical decisions.

CONCLUSIONS

Biosimilars have the potential to provide patients with access to more affordable treatments for retinal diseases. Structural and functional characterization of a candidate biosimilar is an important step in generating the totality of evidence necessary for regulatory approval. In this comparative analytical similarity assessment, we investigated critical attributes of ABP 938 and aflibercept RP, including the primary structure, HOS, product-related substances and impurities, general properties, thermal stability and forced degradation, and biological activities. The results demonstrated that ABP 938 is structurally and functionally similar to aflibercept RP. Since aflibercept RP sourced from the US and EU are analytically similar, this allows for the development of a scientific bridge such that a single-source RP can be used in nonclinical and clinical studies.

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Data Availability. The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of Interest. Neungseon Seo, Xiaoyan Guan, Tian Wang, Hyo S. Helen Chung, Mats Wikström, Rupa Padaki, Kevin Kalenian, Scott Kuhns, Kelli Matthies, Jill Crouse-Zeineddini, Helen Y. Wong, Michael Ng, Ian N. Foltz, Shawn Cao, and Jennifer Liu are employees and stockholders of Amgen, Inc.

Ethical Approval. This article does not describe any studies with human participants or animals. All laboratory health and safety procedures have been complied with in the course of conducting this experimental work.

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