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Development of a biosensor-based immunogenicity assay capable of blocking soluble drug target interference ☆

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ABSTRACT

As with other protein therapeutics, trebananib (AMG 386), an investigational peptide Fc-fusion protein ("peptibody") that inhibits angiogenesis by neutralizing the interaction of angiopoietin-1 (Ang1) and angiopoietin-2 (Ang2) with the Tie2 receptor, has the potential to trigger an immune response in cancer patients treated with the therapeutic. An electrochemiluminescence bridging anti-drug antibody (ADA) assay that was utilized to support early-phase clinical trials in the development of trebananib was found to lack adequate sensitivity and drug tolerance in later-phase clinical studies when higher doses of trebananib were administered. Therefore, we developed a surface plasmon resonance (SPR) immunoassay method utilizing a secondary confirmatory detector antibody (goat anti-human IgG F[ab′]2) known to cross-react with human IgG and IgM to better assess the potential impact of immunogenicity on the pharmacokinetics, pharmacodynamics, and toxicity of trebananib. The SPR method was more sensitive than the electrochemiluminescence bridging assay because of signal amplification from the confirmatory binding of the detector antibody; drug tolerance was improved since antibody binding avidity does not affect detection on this platform. Despite the inability of the confirmatory detector antibody to bind angiopoietins in protein-free buffer, false-positive ADA results were generated from patient serum samples containing Ang1 and Ang2 through an apparently specific binding between the angiopoietins and the confirmatory detector antibody, likely mediated by the interaction of the angiopoietins with serum immunoglobulins. Addition to the sample diluent of a human antibody that specifically binds to Ang1 and Ang2 with high affinity resulted in a complete block of angiopoietin interference without affecting ADA detection. This biosensor-based assay provides a reliable method for assessing immunogenicity in phase 3 clinical trials.

1. Introduction

Angiogenesis is the process of generating new blood vessels from the existing vasculature, and it plays a critical role in tumor growth and metastasis (Folkman, 1995; Fidler et al., 2001). A myriad of pro- and antiangiogenic factors are involved in angiogenesis, with numerous reports demonstrating associations between concentrations of proangiogenic factors or the density of microvasculature in tumors and patient prognosis (Leek, 2001; Mehta et al., 2001; Papamichael, 2001; Dvorak, 2002; Scappaticci, 2002; Reinmuth et al., 2003; Niedzwiecki et al., 2006; Wang et al.,

Abbreviations: ADA, anti-drug antibody; Ang1, angiopoietin-1; Ang2, angiopoietin-2; ECL, electrochemiluminescence; Ig, immunoglobulin; k_a, association rate constant; k_d, dissociation rate constant; K_D, affinity constant; MSD, Meso Scale Discovery; RU, response unit; S/N, signal-to-noise ratio; SPR, surface plasmon resonance.

☆ This manuscript is dedicated with gratitude to the memory of Dr. George Doellgast, who conducted the epitope mapping of the 12 anti-trebananib mouse monoclonal antibodies that contributed to the development and understanding of the surface plasmon resonance anti-drug antibody assay described in this publication.

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2007; Anargyrou et al., 2008; Sie et al., 2009). Antiangiogenic therapies targeting either the vascular endothelial growth factor or its receptors (as well as other tyrosine kinase receptors), have shown clinical efficacy in the treatment of a variety of solid tumors (Carmeliet and Jain, 2011). Angiopoietin-1 (Ang1) and angiopoietin-2 (Ang2) are key cytokines that regulate vascular and lymphatic vessel growth via their interaction with the receptor tyrosine kinase with immunoglobulin-like and epidermal growth factor-like domains 2 (Tie2) on vascular endothelial cells and hematopoietic cells (Yancopoulos et al., 2000; Yu et al., 2001; Sfiligoi et al., 2003; Peters et al., 2004; Scharpfenecker et al., 2005; Shim et al., 2007; Augustin et al., 2009; De Palma and Naldini, 2011). In some human tumors, higher levels of Ang2 have been associated with more advanced disease and with poorer prognosis (Bunone et al., 1999; Etoh et al., 2001; Leek, 2001; Mehta et al., 2001; Papamichael, 2001; Dvorak, 2002; Scappaticci, 2002; Wang et al., 2007; Anargyrou et al., 2008; Detjen et al., 2010). Hence, the angiopoietin-Tie2 axis represents another potential key therapeutic target in human cancers.

Trebananib (AMG 386) is an investigational nonglycosylated peptibody consisting of four copies of angiopoietin-binding peptides fused to an immunoglobulin G1 (IgG1) Fc fragment. The molecule exerts its antiangiogenic effect through sequestering Ang1 and Ang2, thereby neutralizing their interaction with Tie2. In preclinical studies, trebananib reduced proliferation of tumor endothelial cells and inhibited the growth of human tumor xenografts (Oliner et al., 2004). Importantly, studies with Colo205 tumor xenografts suggested that dual inhibition of Ang1 and Ang2 results in better efficacy than blocking Ang2 alone (Coxon et al., 2010). In clinical studies, trebananib has exhibited antitumor activity along with an acceptable toxicity profile across multiple tumor types (Herbst et al., 2009; Mita et al., 2010; Karlan et al., 2012; Rini et al., 2012). Several phase 3 studies are currently being conducted in ovarian cancer (ClinicalTrials.gov, NCT01204749; NCT01281254; NCT01493505).

As with other protein therapeutics, trebananib has the potential to elicit an immune response that might impact its toxicity profile and efficacy, depending on structural motif of the molecule, the mechanism of action, route of administration, and the immune status of the subjects. Therefore, the immunogenicity of trebananib has been evaluated early on in clinical studies using an acid-dissociation, electrochemiluminescence (ECL), bridging immunoassay (Zhong et al., 2010). Samples that were identified as positive for anti-trebananib antibodies were tested again using a receptor-binding assay for the ability of these antibodies to neutralize the biological activity of the drug. To date, the incidence of nonneutralizing, binding antibodies in clinical studies has been low, and treatment-emergent neutralizing antibodies have not been detected in phase 1 and 2 studies (Herbst et al., 2009; Mita et al., 2010; Karlan et al., 2012; Rini et al., 2012).

The bridging immunoassay format has been successfully applied to detection of antibodies (anti-drug antibodies, ADA) against protein therapeutics in general and humanized monoclonal antibodies in particular, with good sensitivity and tolerance toward drug interference (Moxness et al., 2005; Mikulskis et al., 2011). However, this assay format is not without limitations. First, antibody detection in a bridging format depends on multivalent characteristics of serum ADAs to crosslink two drug molecules in the assay, which may make the bridging format prone to drug interference under certain circumstances. More importantly, certain epitopes may be underdetected for some structural motifs. Secondly, although the binding specificity is typically evaluated based on signal depletion by adding excess protein drug to the assay, additional laborious and time-consuming testing, such as protein G/L depletion, is required to verify whether the binding is of antibody origin (Sanchez et al., 2011). As a result, without an antibody confirmation step, the presence of a multimeric soluble drug target capable of competing with ADAs in the cross-linking of labeled drug molecules used as the assay reagent will lead to false-positive antibody detection in a bridging assay (Zhong et al., 2010; Mikulskis et al., 2011; Carrasco-Triguero et al., 2012). In contrast, the binding of a monomeric target to the protein drug can lead to false-negative antibody detection. Furthermore, the requirement in a bridging format for cross-linking two drug molecules would prevent monovalent and bispecific ADAs from being detected altogether. This is an important limitation because IgG4, which is primarily produced after prolonged chronic antigen exposure, can be functionally monovalent because of in vivo exchange of IgG half-molecules (Aalberse et al., 1983, 2009; Aalberse and Schuurman, 2002; Labrijn et al., 2009; Mytych et al., 2012). Consequently, formation of IgG4 ADAs may be underestimated in a bridging format (Aarden et al., 2008; Hart et al., 2011) among patients who are dosed with a therapeutic protein repeatedly for a prolonged period. Because IgG4 represents a major affinity-maturated antibody response, strong binding by it could potentially influence the pharmacokinetic profile and efficacy of a therapeutic. Hence, it is important that a given immunogenicity assay can detect the presence of IgG4.

Over the last decade, biosensor technology based on surface plasmon resonance (SPR) has become a vital tool for quantitative detection of antibody-antigen interactions in real time, for measurement of binding kinetics, and mapping of the epitopes of antibody response (Swanson, 2003; Mytych et al., 2009; Jawa et al., 2010). Other important characteristics of SPR-based biosensors for immunogenicity assessment include label-free antibody detection and the proven ability to detect low-affinity antibodies as well as all antibody isotypes and subclasses, including IgG4 (Swanson et al., 2004; Swanson, 2006; Liang et al., 2007; Mytych et al., 2009).

Considering the aforementioned limitations and the guidelines issued by the US Food and Drug Administration, which requires that a given immunogenicity assay is sensitive and detects all antibody isotypes and subclasses, the ECL bridging assay eventually proved inadequate for the reliable detection of anti-trebananib antibodies. Furthermore, in the currently ongoing clinical studies patients are receiving higher doses of trebananib than administered in earlier studies. However, the ECL assay showed only limited tolerance toward drug interference from high concentrations of circulating trebananib in patient serum. Herein, we report the development of a sensitive, SPR-based immunoassay for the detection of anti-trebananib antibodies in the presence of high drug concentration in the serum samples as it can be expected during prolonged

periods of treatment. We also describe the unusual interference of serum angiopoietins in this biosensor assay, due to their binding to anti-human IgG F(ab′)2 confirmatory detector antibody, despite exploitation of this anti-human antibody for ADA detection. The described biosensor assay has been validated and implemented to support immunogenicity assessments in ongoing phase 3 clinical studies of trebananib (ClinicalTrials.gov NCT01204749, NCT01281254, and NCT01493505).

2. Materials and methods

2.1. Equipment and associated reagents

Biacore® 3000 with GxP control software 4.1.2, Biacore® A100, A100 Evaluation Software 1.0, and the associated reagents (CM5 dextran Biacore® Chip CM5, amine-coupling kit, P-20 surfactant [P-20], and HBS-EP buffer) were purchased from GE Healthcare Company-Biacore® Group (Uppsala, Sweden). Tris base was purchased from Sigma-Aldrich (St Louis, MO). Meso Scale Discovery (MSD) Sector Imager 6000® and the associated reagents (MSD Read Buffer T and MSD Avidin High Bind plates) were acquired from MSD (Gaithersburg, MD).

2.2. Antibodies and assay reagents

For the Biacore® 3000 assay, a humanized κ light-chain anti-trebananib monoclonal antibody (Hu-κ-mAb), produced by Amgen Inc. (Thousand Oaks, CA), was utilized as the ADA positive control; a goat polyclonal antibody against human IgG F(ab′)2, purchased from Jackson ImmnoResearch (West Grove, PA), was used to detect human ADAs in a sample; a human IgG2 monoclonal antibody against Ang1 and Ang2 (Hu-Ang-mAb), produced by Amgen Inc. (Thousand Oaks, CA), was deployed to block the interference of Ang1 and Ang2. An affinity purified rabbit polyclonal antibody against trebananib, produced by Amgen Inc. (Thousand Oaks, CA), was used as the ADA positive control for the ECL bridging assay. Twelve mouse monoclonal antibodies against trebananib (Mu-mAb) 4A11, 7B4, 8B12, 13D6, 21G4, 22H8, 23G7, 28B8, 29A7, 30D5, 30E6, and 31A4 were used to evaluate the ability of the bridging assay and the biosensor-based platform to detect ADAs of various clones; these Mu-mAbs were produced by Amgen Inc. (Thousand Oaks, CA). A goat polyclonal antibody against mouse IgG Fc fragment, purchased from Jackson ImmunoResearch (West Grove, PA), was used to capture these Mu-mAbs for detection and affinity measurement on the SPR platform. A mouse monoclonal antibody (clone M94181) against human immunoglobulin M (IgM), purchased from Fitzgerald Industries International (Acton, MA), was used to characterize the ability of the SPR methodology to detect human IgM ADAs. Trebananib was produced by Amgen Inc. (Thousand Oaks, CA). Recombinant human Ang1 and Ang2 were purchased from R&D Systems (Minneapolis, MN). Pooled normal human serum and healthy donor serum samples were purchased from Bioreclamation, LLC (Oceanside, CA).

2.3. Measurement of antibody affinity constants

The binding kinetics of 12 anti-trebananib Mu-mAbs were measured using a Biacore® A100 instrument. Specifically, goat anti-mouse IgG Fc was diluted to 30 μg/mL in 10 mM sodium acetate pH 5.0, and was covalently immobilized using amine-coupling chemistry on a carboxymethyl-dextran coated (CM5) sensor chip, aiming at a final density of 10,000 response units (RU). Mu-mAbs were diluted to 0.2 μg/mL in HEPES-buffered saline pH 7.4 containing 5 mM EDTA and 0.005% Tween 20 (HBS-EP). Diluted Mu-mAbs were captured on the chip by injecting (Quick Inject function of the Biacore® Method Wizard) the solution for 2 min across the ligand surface at 10 μL/min. Trebananib was serially diluted from 500 nM to 7.8 nM in HBS-EP. Ligand surface (Mu-mAb) binding was saturated by injecting diluted trebananib for 3 min across the ligand surface at 30 μL/min and allowing it to dissociate for 500 s. After injection of each sample, the chip surface was regenerated by injecting 150 μL of 50 mM glycine–HCl pH 1.4 at 30 μL/min. A flow cell containing only immobilized anti-mouse IgG Fc was used as a reference cell for background subtraction. The dissociation constant (K_D) for each Mu-mAb was determined by evaluating the respective, dilution series sensograms using the A100 Evaluation Software 1.0.

$$A + B \underset{k_d}{\overset{k_a}{\rightleftharpoons}} AB$$

Where k_a is the association rate constant and k_d is the dissociation rate constant

$$\text{Affinity constant } K_D = \frac{k_d}{k_a}$$

2.4. Isotyping for IgG and IgM of human anti-trebananib antibodies in clinical samples

An SPR-based Biacore® 3000 assay was used for the isotyping of human anti-trebananib antibodies in serum samples from cancer patients treated with trebananib, based on the binding of anti-human IgG and IgM to anti-trebananib antibodies that were captured by trebananib immobilized on the sensor chip surface. Serum samples were collected from patients receiving trebananib plus chemotherapy in phase 2 studies of colorectal (Peeters et al., 2013), gastric (Eatock et al., 2013), and clear cell renal cancer (Rini et al., 2012). Five milliliters of blood was collected in additive- and anti-coagulant-free tubes immediately prior to trebananib infusion at the protocol-specified time points. Samples coagulated for approximately 30 min, were centrifuged at 1500 ×*g* for 15 min at room temperature, and were stored at or below −10 °C. Serum samples were tested for the presence of anti-trebananib antibodies using a validated acid-dissociation, ECL, bridging immunoassay exactly as described previously (Zhong et al., 2010). The positive samples from the bridging immunoassay were characterized (isotype) using this SPR immunoassay method. In this assay, trebananib was diluted to 30 μg/mL in 10 mM sodium acetate pH 4.0 and was covalently immobilized on a CM5 sensor chip at a targeted density of 5000 RU using amine-coupling chemistry. Serum samples positive for anti-trebananib antibodies in the bridging assay were diluted

1:10 in sample diluent containing Tris-buffered saline pH 9.0 with 5 mM EDTA, 0.005% Tween 20, and 1% carboxymethyl dextran. Diluted samples were injected for 4 min across the ligand surface at 5 μL/min and antibodies were allowed to bind. Captured anti-trebananib antibodies were isotyped by injecting 10 μL of goat anti-human IgG, or murine anti-human IgM at 100 μg/mL in sample diluent at 5 μL/min. After each sample was isotyped, the chip surface was regenerated by injecting 10 μL of 100 mM HCl at 5 μL/min. Samples with a sample binding response value greater than 90 RU and an isotyping response value greater than 90 RU were considered positive for that specific isotype.

2.5. Biacore® 3000 assay for the detection of anti-trebananib antibodies in human serum

A Biacore® 3000 assay was developed for the direct detection of human anti-trebananib antibodies in serum samples from cancer patients treated with trebananib, based on the antibodies' ability to bind to trebananib immobilized on the sensor chip surface and confirmed by a goat anti-human IgG F(ab′)2 fragment-specific antibody. Trebananib was diluted to 30 μg/mL in 10 mM sodium acetate pH 4.0 and was covalently immobilized on a CM5 sensor chip targeting 5000 RU using amine-coupling chemistry using a flow rate of 5 μL/min. Serum samples were diluted 1:10 in sample diluent containing Tris-buffered saline pH 9.0 with 5 mM EDTA, 0.005% Tween 20, and 1% carboxymethyl dextran. To block potential interference resulting from the soluble drug targets, Ang1 and Ang2, present in the serum samples, the sample diluent was supplemented with Hu-Ang-mAb, which specifically binds to Ang1 and Ang2, at a final concentration of 10 μg/mL. Diluted samples containing Hu-Ang-mAb were injected for 4 min across the immobilized trebananib surface at 10 μL/min and allowed to bind. Captured human anti-trebananib antibodies were confirmed as antibodies by injecting 10 μL of goat anti-human IgG F(ab′)2 fragment-specific antibody diluted to 100 μg/mL in sample diluent, without adding Hu-Ang-mAb, at 10 μL/min. After injection of each sample and the confirmatory detector antibody, the chip surface was regenerated by injecting 5 μL of 100 mM HCl at 10 μL/min.

To determine the cut points, serum samples from 54 healthy donors were tested by two analysts over >2 days across 2 CM5 chip lots and 2 instruments. The screening cut points were calculated as the upper bound of a one-sided 95% prediction interval for the distribution of the sample responses in the assay. The confirmatory cut point was calculated as the upper bound of a one-sided 99% prediction interval for the distribution of the confirmatory response units of the goat anti-human IgG F(ab′)2 binding to ADAs captured on the biosensor chip. The calculations were based on industry recommendations (Mire-Sluis et al., 2004; Shankar et al., 2008) and regulatory guidelines (Committee for Medicinal Product for Human Use, 2007; US Food and Drug Administration, 2009).

The sensitivity of the assay was determined by measuring a dose–response curve of the Hu-κ-mAb ADA positive control at 20, 39, 78, 156, 313, and 625 ng/mL in pooled normal human serum on biosensor chip surfaces with a targeted immobilization density of 3500 to 6000 RU across 3 days. Additionally, pooled normal human serum samples containing 160 ng/mL and 500 ng/mL of Hu-κ-mAb were spiked with trebananib (1, 5, 10, and 20 μg/mL) and Ang1/Ang2 (v/v, 1:1; 63, 125, 250, 500, 667, and 1000 ng/mL). The resulting samples were tested to assess the interference of the drug and of Ang1 and Ang2 with the detection of ADA in human serum.

2.6. ECL bridging assay for detecting anti-trebananib Mu-mAbs

Pooled normal human serum was spiked with anti-trebananib Mu-mAbs and tested for antibody presence using a validated acid-dissociation, ECL bridging immunoassay exactly as described previously (Zhong et al., 2010). Briefly, the pooled normal human serum samples spiked with the anti-trebananib Mu-mAbs were diluted in acetic acid, followed by neutralization with Tris base in the presence of biotinylated and ruthenium-labeled trebananib. The resulting bridged complex was measured using an MSD Sector Imager 6000® instrument. The assay responses were recorded as ECL units, and the signal-to-noise ratios (S/N) were calculated by dividing the mean ECL value of each sample by the mean ECL value of pooled normal human serum (i.e., the negative control). The assay cut point of S/N 1.41 was calculated as the upper bound of a one-sided 95% prediction interval for the distribution of the S/N values of 247 samples from 64 healthy human serum donors, which were tested by two analysts over 2 days across 2 MSD instruments. This cut point was applied to the data analysis of the Mu-mAb-spiked human serum samples. An affinity-purified rabbit polyclonal antibody was used as the positive control in the assay.

3. Results

3.1. Characterization of anti-trebananib Mu-mAbs

We first evaluated whether the ECL bridging assay, which has been used as the immunogenicity assay of choice in early trebananib clinical studies, was capable of detecting a variety of trebananib ADAs of various clones with differing characteristics. To that end, we determined the kinetic constants of 12 mouse anti-trebananib Mu-mAbs, which were known to bind to the angiopoietin-binding region of trebananib, using an SPR immunoassay (Biacore® A100). A goat anti-mouse IgG Fc polyclonal antibody was immobilized on the biosensor chip to capture the Mu-mAbs for kinetic measurement. All Mu-mAbs exhibited high binding affinities to trebananib that was injected across the biosensor chip surface, ranging from 9.08×10^{-9} M to greater than 1.00×10^{-13} M (Table 1). Two antibody clones, 28B8 and 30E6, had fast association rate constants ($k_a > 1 \times 10^7$) and slow dissociation rate constants ($k_d < 1 \times 10^{-6}$), respectively, that were outside the acceptable ranges, and therefore, their K_D could not be reliably determined with the A100 kinetics software. Additionally, the K_D of five Mu-mAbs (8B12, 4A11, 7B4, 23G7, and 13D6) may be lower than the estimated values because these antibodies had fast association rate constants just above the acceptable range (10^7) according to the A100 evaluation software. The data indicated that all 12 Mu-mAb clones against trebananib (with varying affinities for the drug) could be detected on the SPR platform.

Table 1
Kinetic constants of anti-trebananib mouse monoclonal antibodies (Mu-mAbs).

Mu-mAb clone designation	K_D (M)	k_a (M^{-1} s^{-1})	k_d (s^{-1})
28B8	1.00×10^{-13}	1.00×10^{7}	1.00×10^{-6}
30E6	1.00×10^{-13}	1.00×10^{7}	1.00×10^{-6}
8B12	3.01×10^{-11}	9.95×10^{7}	2.99×10^{-3}
4A11	4.16×10^{-11}	4.20×10^{7}	1.75×10^{-3}
7B4	5.21×10^{-11}	8.81×10^{7}	4.60×10^{-3}
23G7	2.05×10^{-10}	7.03×10^{7}	1.44×10^{-2}
13D6	2.27×10^{-10}	4.85×10^{7}	1.10×10^{-2}
29A7	3.46×10^{-10}	1.82×10^{6}	6.31×10^{-4}
30D5	1.50×10^{-9}	5.12×10^{5}	7.66×10^{-4}
22H8	2.89×10^{-9}	4.21×10^{5}	1.22×10^{-3}
31A4	$2.97E \times 10^{-9}$	6.71×10^{5}	1.99×10^{-3}
21G4	9.08×10^{-9}	6.34×10^{4}	5.76×10^{-4}

3.2. Detection of anti-trebananib Mu-mAbs in human serum using the ECL bridging assay

Based on the K_D values obtained with the Biacore® A100 assay, each of the 12 anti-trebananib Mu-mAbs tested should be reliably detectable using the validated ECL bridging assay. To evaluate this, pooled normal human serum samples were individually spiked with each of these 12 Mu-mAbs and tested in the MSD assay. However, applying the calculated assay cut point of 1.41 (S/N ratio) for healthy human serum samples, only one Mu-mAb clone (31A4) out of 12 was detectable at a concentration of 1000 ng/mL (Fig. 1). At a concentration of 5000 ng/mL, five additional Mu-mAb clones (4A11, 8B12, 21G4, 22H8, and 30D5) were detectable. In summary, the ECL bridging assay did not possess adequate sensitivity to detect 12 Mu-mAbs known to bind to the angiopoietin-binding region of trebananib, in spite of their high affinities to the drug as determined on the SPR platform.

3.3. Isotyping of human anti-trebananib antibodies in clinical samples using the Biacore® 3000 assay

We tested the Biacore® 3000 assay for its ability to capture both IgM and IgG by determining the antibody response of seven cancer patients with suspected ADA who received trebananib in three unblinded phase 2 studies across multiple tumor types (see Section 2.4 for description of samples). Using the Biacore® 3000 assay, anti-trebananib antibodies present in serum samples from the patients were captured and isotyped using anti-human IgG and anti-human IgM antibodies. Two samples had both detectable IgG and IgM (IgG and IgM bindings > 90 RU), while samples from the remaining five patients had detectable IgG only (Table 2).

3.4. Development of a Biacore® 3000 clinical anti-trebananib antibody immunoassay

A Biacore® 3000 clinical immunoassay was developed for detection of anti-trebananib antibodies (referred to as "ADA") in

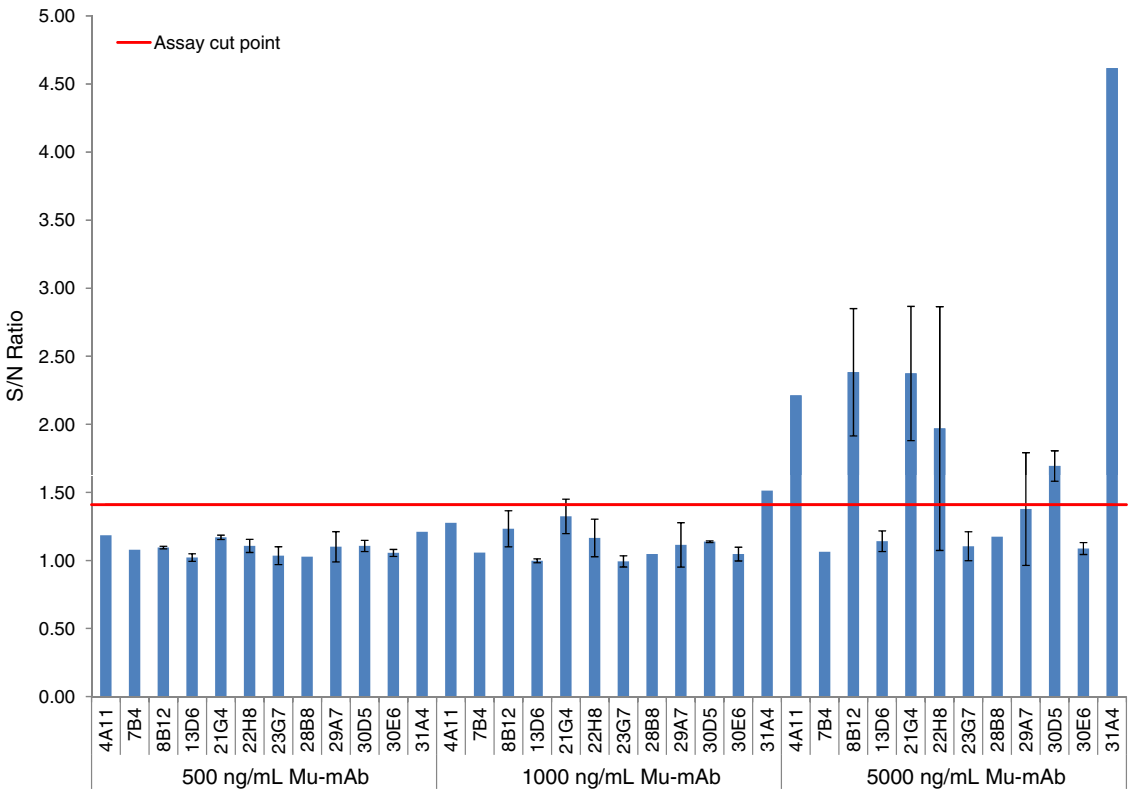

Fig. 1. Detection of mouse anti-trebananib monocloncal antibody (Mu-mAb) clones using the validated chemiluminescence bridging assay. S/N, signal-to-noise.

Table 2
Detection of anti-trebananib IgG and IgM antibodies in serum samples from cancer patients who were treated with trebananib.

Patient no	Sample binding (RU)[a]	IgG binding (RU)[b]	IgM binding (RU)[c]	Isotype
1	113.1	183.4	NA	IgG
2	189.3	259.1	113.5	IgM, IgG
3	115.2	156.5	35.9	IgG
3	65	128.3	5.9	Weak IgG
4	166.2	233.1	NA	IgG
5	160.1	219.3	90.3	IgM, IgG
6	103.6	122.7	−17	IgG
7	186.2	364.1	57.1	IgG

[a] Sample binding: response of serum antibody captured by trebananib immobilized on the sensor chip surface.
[b] IgG binding: binding response of goat anti-human IgG(Fab′)2 antibody to serum antibody.
[c] IgM binding: binding response of mouse anti-human IgM to serum antibody.

human serum samples diluted to 10% (Fig. 2). To facilitate detection of the human ADA by the goat anti-human IgG F(ab′)2 antibody in the assay, a humanized monoclonal, Hu-κ-mAb against trebananib, which binds to the angiopoietin-binding sequence, was used as the ADA positive control in this SPR method. The assay consists of two binding phases: the binding of anti-trebananib antibodies in the serum sample to trebananib that is immobilized on the biosensor CM5 chip surface, a reaction that results in the sample response; and the confirmatory binding of the goat anti-human IgG F(ab′)2 antibody to the serum ADA captured on the biosensor chip surface, which provides the confirmatory response. The sample response was evaluated first to determine the screening assay cut point, which was found to be 35 RU. Applying this tentative screening cut point, adding 1 μg/mL of trebananib would have significantly suppressed the antibody detection in pooled normal human serum samples containing trebananib and the humanized ADA positive control (Fig. 3).

To improve antibody detection in the presence of high drug levels in the serum, as it can be expected in patients who had repeated and prolonged exposure to trebananib, the combined response unit (defined as the sum of sample response and confirmatory response), was used to re-calculate the screening cut point, which was thus determined to be 67 RU for healthy human serum. Based on this screening cut point determined from the combined response, 500 ng/mL of the ADA positive control can be detected in the presence of as high as 10 μg/mL trebananib (Fig. 3). A sample with a combined response value above the screening cut point has to be evaluated in the subsequent confirmation analysis for antibody detection. To confirm the antibody response, a confirmatory cut point of 42 Ru was derived from the confirmatory response for healthy human serum.

The assay sensitivity was defined as the concentration of the Hu-κ-mAb ADA positive control at the screening cut point based on the combined response. A full titration curve was generated by analyzing pooled normal human serum samples spiked with the humanized ADA positive control (Fig. 4). In the absence of trebananib, the assay was estimated to be able to detect 20 ng/mL of the ADA positive control, based on the screening cut point of 67 RU in normal human serum.

3.5. Angiopoietin interference

In order to assess the potential cross-reactivity and interference of Ang1 and Ang2, an angiopoietin dose–response curve was generated by spiking Ang1 and Ang2 in pooled normal human serum as well as in the protein-free assay buffer. Fig. 5 shows a proportional increase of both combined response and confirmatory response with the increase of Ang1

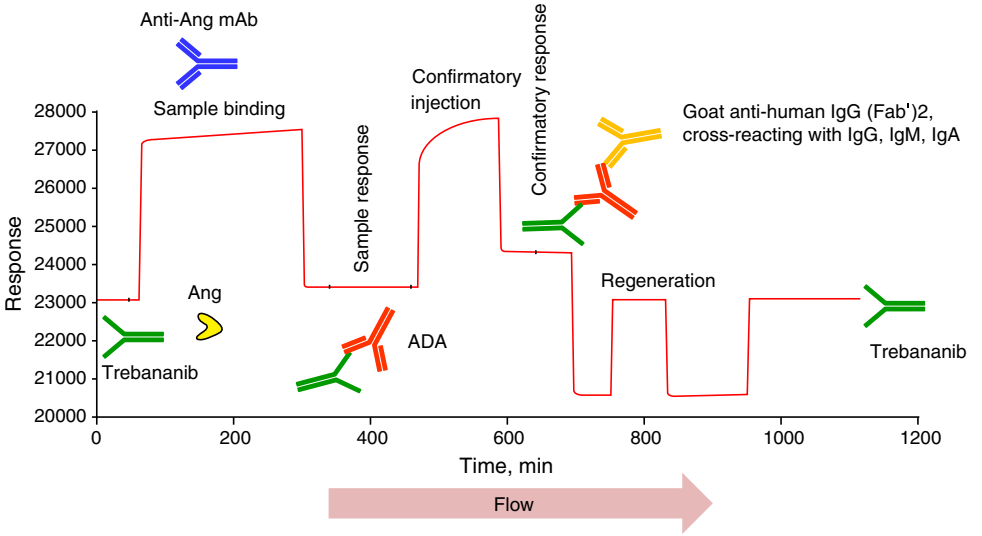

Fig. 2. Schematic display of the surface plasmon resonance immunoassay that was developed for the detection of human anti-trebananib antibodies (ADAs) in serum samples from patients who receive the drug. ADA, anti-drug antibody; Ang, angiopoietin.

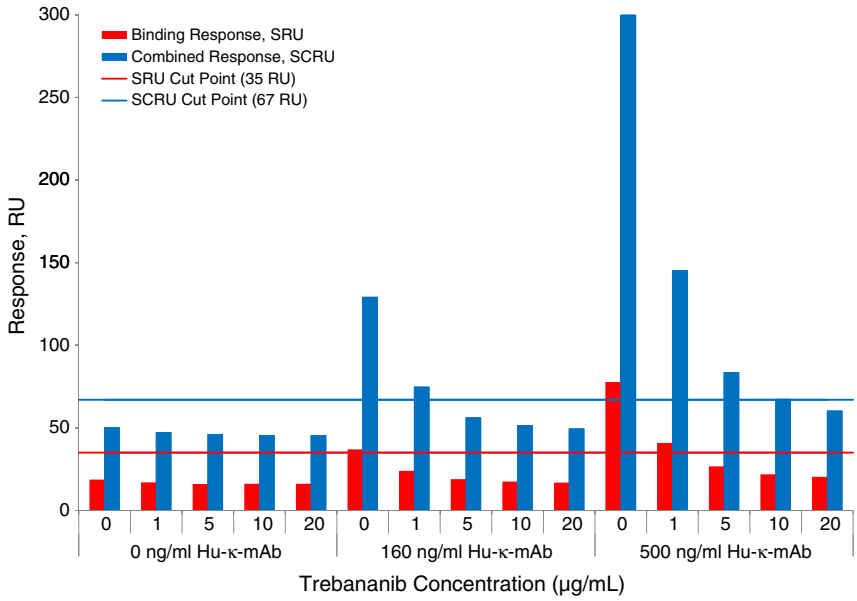

Fig. 3. Evaluation of the drug tolerance of the surface plasmon resonance immunoassay. ADA, anti-drug antibody. RU, response unit.

and Ang2 concentrations in the serum. Although the sample containing Ang1 and Ang2 at a combined concentration of 63 ng/mL yielded a combined response greater than the screening cut point of 67 RU, the confirmatory response was below the confirmatory cut point and the sample was not confirmed as positive by the detector antibody. As Ang1 and Ang2 reached a total concentration of 125 ng/mL in human serum, the binding of the detector antibody resulted in a confirmatory response greater than the confirmatory cut point of 42 RU, leading to a false-positive antibody result, even though the magnitude of the confirmatory binding was only about 50% of the binding to the captured ADA (Fig. 6).

By comparison, although the addition of Ang1 and Ang2 to the protein-free assay buffer at a combined concentration of 2000 ng/mL in a ratio of 1:1 resulted in an assay response of 987 RU in the sample binding phase, there was no binding (7.1 RU) observed during the confirmatory detection phase using the anti-human IgG F(ab′)2 antibody, demonstrating that Ang1 and Ang2 had no cross-reactivity with the anti-human IgG F(ab′)2 in the assay buffer. To better understand this unusual indirect interference, serum samples were diluted in a diluent containing a human IgG2 mAb (Hu-Ang-mAb) that specifically binds to angiopoietin-1 and -2 with high affinity. Upon treatment with 10 μg/mL of Hu-Ang-mAb in the sample diluent, the binding response of angiopoietins was abrogated in the negative control serum samples that were spiked with Ang1 and Ang2 (Fig. 7). Addition of Ang1 and Ang2 led to higher assay responses in samples containing 250 ng/mL and 500 ng/mL of the humanized ADA positive control (Fig. 7). Additionally, in the presence of the anti-angiopoietin Hu-Ang-mAb, the combined assay response value at each ADA level was comparable regardless of the angiopoietin concentrations. Validation experiments further confirmed that the addition of 10 μg/mL of Hu-Ang-mAb to the sample diluents effectively blocked the interference of angiopoietins at a total concentration as high as 1000 ng/mL, without impacting detection of the humanized ADA positive control.

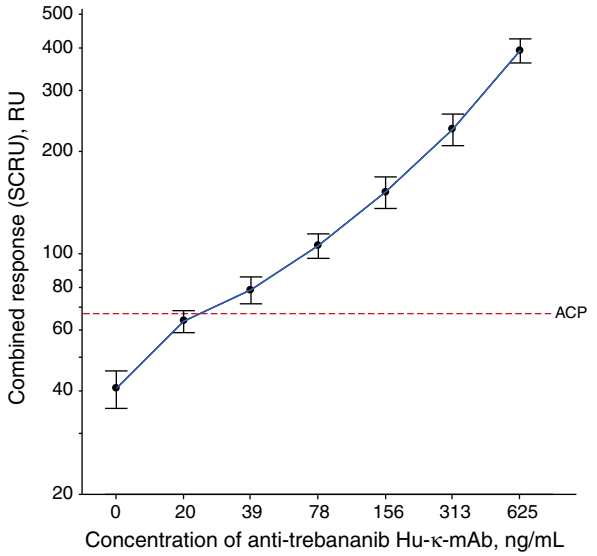

Fig. 4. Titration curve of anti-trebananib Hu-κ-mAb ADA positive control. Error bars extend one standard deviation from the mean. ACP, screening assay cut point; CRU, confirmatory binding response unit; RU, response unit; SCRU, combined response unit (CRU + SRU); SRU, sample binding response unit.

4. Discussion

In the early development phase of trebananib, an ECL bridging immunoassay was used for the detection of ADA in patients' serum samples (Zhong et al., 2010). However, the assay had a number of limitations, some of which could be

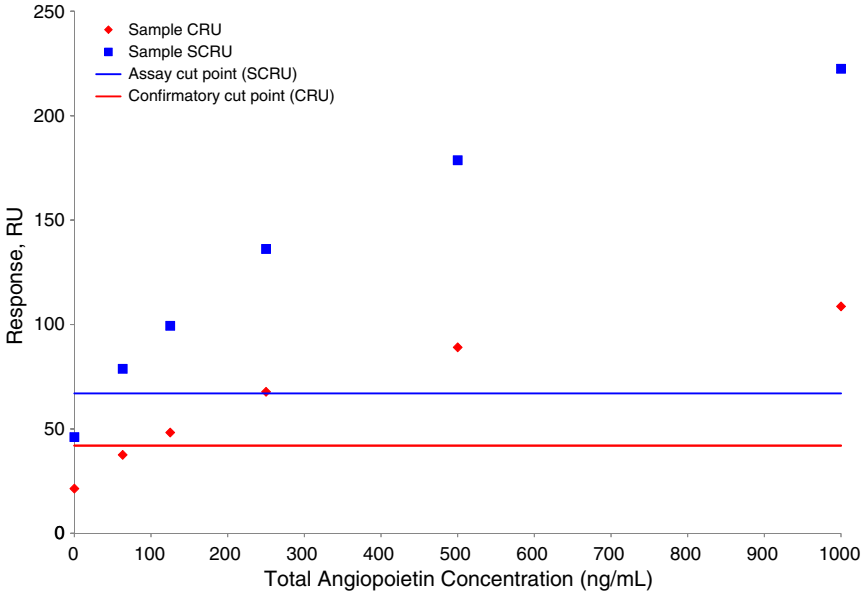

Fig. 5. Interference of angiopoietins in 10% human serum in the surface plasmon resonance immunoassay. ACP, screening assay cut point; CCP, confirmatory cut point; CRU, confirmatory binding response unit; SCRU, combined response unit (CRU + SRU); RU, response unit; SRU, sample binding response unit.

overcome while others remained unresolved. For example, false positive ADA detection, due to binding of the soluble drug targets, Ang1 and Ang2, could be prevented by blocking the drug target interference with a monoclonal antibody against angiopoietins. In contrast, as the trebananib program progressed to phase 3, when patients were treated with a higher dose of the drug and therefore, higher concentrations of circulating trebananib were present in the serum, the assay's tolerance toward drug interference was no longer adequate, as demonstrated during assay validation. Attempts to improve sensitivity of the bridging assay in the presence of the drug were unsuccessful. Such a lack of drug tolerance is likely due to the formation of a 1:1 immune complex of trebananib with the ADA through the interaction of the latter with the drug's four angiopoietin-binding peptide sequences, thus effectively preventing the serum antibodies from cross-linking two drug molecules (the prerequisite for detecting ADA in a bridging assay format). More importantly, and as demonstrated herein, further evaluation of the ECL bridging assay also revealed that it did not appear to have adequate sensitivity for detecting serum antibodies of various clones even in the absence of drug. When using 12 anti-trebananib mouse monoclonal antibodies that bind to the angiopoietin-binding peptide sequence of the molecule (Amgen Inc., data on file), the ECL bridging assay detected only one at 1000 ng/mL even though all 12 antibodies were shown to bind to trebananib with high affinities, as assessed by a kinetic binding study on the SPR platform. Regulatory agencies require that an immunogenicity assay be sensitive (detecting ADAs at concentrations of 250 to 500 ng/mL) and able to detect all antibody classes and subclasses with various affinities (Committee for Medicinal Product for Human Use, 2007; US Food and Drug Administration, 2009). Considering that the aforementioned limitations of the ECL bridging assay suggested it did not represent an adequate bioanalytical tool for monitoring the immunogenicity of trebananib, we therefore developed a biosensor-based SPR immunoassay method equipped with a detection antibody against human immunoglobulins for the detection of ADAs in serum samples from patients treated with trebananib.

We first evaluated the ability of the biosensor chip to capture both anti-trebananib IgM and IgG in serum samples from patients who had received trebananib in clinical studies using a mouse monoclonal antibody against human IgM and a goat polyclonal antibody against human IgG F(ab′)2, respectively. The data indicated that both IgG and IgM antibodies against trebananib were detectable, and that both isotypes were sufficiently captured for detection on the biosensor chip surface. Subsequently, a single flow-cell Biacore® 3000 assay was developed (Fig. 2). In this assay, ADA present in serum samples was captured by trebananib immobilized on the sensor chip surface followed by confirmatory binding of the serum ADA by the goat anti-human IgG F(ab′)2 antibody known to cross-react with human IgG, IgM, and IgA (data not shown).

Drug interference is a critical concern for all immunogenicity assays, especially for those based on therapeutic proteins that have long half-lives and are administered at high doses (Wang et al., 2012). High levels of a therapeutic protein in patient serum can mask ADAs that may have developed by forming a drug–ADA immune complex, which in turn may lead to false-negative results in the assay. Various approaches to this problem have been recommended by the industry (Mire-Sluis et al., 2004; Swanson, 2006; Koren et al., 2008) and in regulatory guidelines (Committee for Medicinal Product for Human Use, 2007; US Food and Drug Administration, 2009). In addition to collecting patient samples at a time when the drug concentration is sufficiently low in the serum, better assay tolerance toward drug interference can be achieved by using different technology

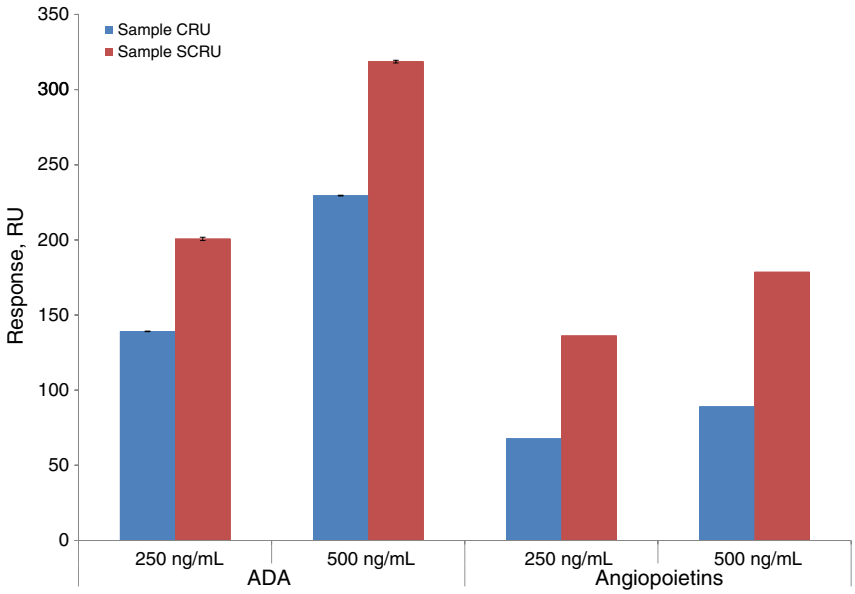

Fig. 6. Binding of anti-Human IgG F(ab′)2 antibody to anti-drug antibodies (ADA) and angiopoietins in the surface plasmon resonance immunoassay. CRU, confirmatory binding response unit; RU, response unit; SCRU, combined response unit (CRU + SRU).

platforms as well as through sample dilution, drug removal, and sample pretreatment with acid in an effort to dissociate any drug–ADA complexes that may be present (Patton et al., 2005; Bourdage et al., 2007; Lofgren et al., 2007; Smith et al., 2007; Neubert et al., 2008; Sickert et al., 2008; Spengler et al., 2009; van Schouwenburg et al., 2010). However, caution is necessary to preserve the immunoreactivity of the ADAs during sample pretreatment. In a biosensor-based assay, the use of anti-human antibody detection confirms antibody production and amplifies the antibody-binding response—potentially representing another approach to achieving greater drug tolerance via better assay sensitivity. Therefore, we evaluated for our assay two potential screening cut points, statistically derived from sample binding response alone and the combined assay response of the sample binding and the detector antibody binding, for their impact on the tolerance to drug interference. The data indicated that the chosen biosensor assay displayed greater drug tolerance using the screening cut point calculated from the total response, likely because of greater assay sensitivity which was achieved

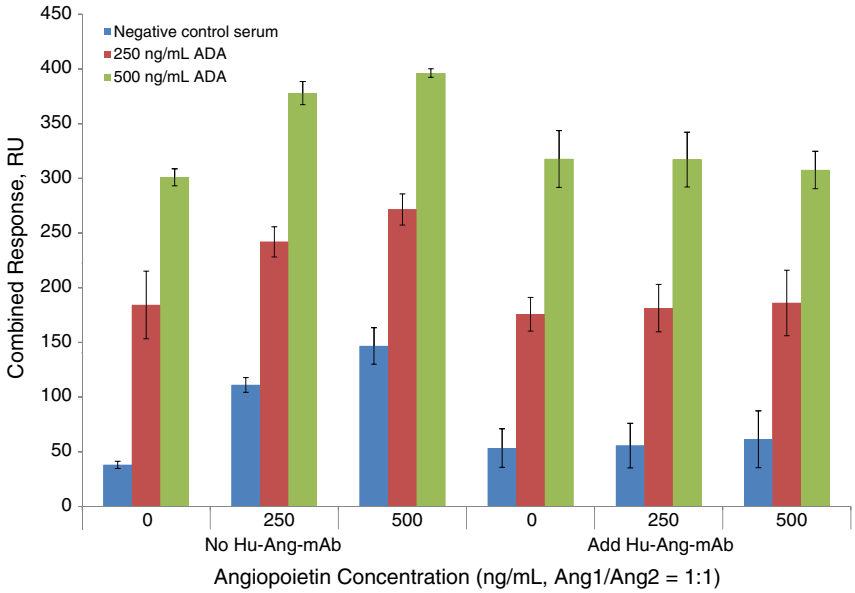

Fig. 7. Blocking of angiopoietin binding to immobilized trebananib using a human anti-angiopoietin monoclonal antibody in the surface plasmon resonance immunoassay. ADA, anti-drug antibody; Ang1, Ang2, angiopoietin-1 and angiopoietin-2; RU, response unit; SRU, sample binding response unit; CRU, confirmatory binding response unit; SCRU, combined response unit (CRU + SRU).

through signal augmentation by secondary binding of the anti-human IgG F(ab′)2. It is noteworthy that no additional sample treatment, such as acid preprocessing, was needed to achieve the superior sensitivity and drug tolerance, which allowed for the preservation of ADA that may be prone to degradation under harsh sample treatment conditions.

Ang1 and Ang2 have been shown to consist of C-terminal fibrinogen-like domain binding to the Tie2 receptor, a coiled-coil region dimerizing the fibrinogen-like domains, and a super-clustering motif facilitating higher order multimerization. Multimerization in vivo is required to initiate binding to the Tie2 receptor and subsequent downstream signaling (Davis et al., 2003; Kim et al., 2005; Barton et al., 2006). The ECL bridging assay format has been shown to have propensity for false-positive ADA detection (Zhong et al., 2010), resulting from cross-linking of two labeled trebananib molecules by multimeric angiopoietin species that are present in serum from patients who have increased concentrations of Ang1 and Ang2 following treatment with trebananib. In contrast, the SPR immunoassay relies on the detection of human antibodies by the anti-human IgG F(ab′)2 antibody which is not anticipated to bind to Ang1 and Ang2, thereby eliminating potential interference from the soluble drug targets. Indeed, although adding Ang1 and Ang2 at a total concentration of 2000 ng/mL to the protein-free assay buffer resulted in a high binding response with trebananib, the anti-human IgG F(ab′)2 showed no cross-reactivity to the Ang1 and Ang2 molecules that were captured by the sensor chip surface immobilized with trebananib. Surprisingly, though, when the angiopoietins were added to pooled normal human serum, we observed a dose–response relationship between the angiopoietin concentration and the confirmatory binding of the anti-human IgG F(ab′)2. The presence of low levels of angiopoietins (e.g., total concentration of 63 ng/mL) in serum did not appear to lead to false-positive antibody detection caused by the binding of the anti-human IgG F(ab′)2 at the confirmatory binding phase, in spite of the combined response value being greater than the screening cut point at the binding phase. However, as the angiopoietin concentration increased above 125 ng/mL, the binding of the anti-human IgG F(ab′)2 to the captured angiopoietins would lead to a false-positive antibody confirmation. Additionally, the interaction between angiopoietins and the detector antibody was effectively disrupted by adding a human IgG2 antibody that specifically binds to both Ang1 and Ang2 with high affinity (Hu-Ang-mAb) in excess to the assay reaction. Hence, the cross-reactivity was likely mediated by the interaction between angiopoietins and serum immunoglobulins, which were subsequently detected by the anti-human IgG F(ab′)2. Hu-Ang-mAb has a unique protein sequence (data on file) different from the angiopoietin-binding sequence of trebananib, which is an IgG1 Fc fusion protein. Our results showed that Hu-Ang-mAb effectively blocked the interference of Ang1 and Ang2 without impacting detection of ADAs against trebananib.

In conclusion, we developed a sensitive biosensor-based immunoassay with drug tolerance and sensitivity superior to that of the ECL bridging assay previously used to determine the immunogenicity of trebananib, through signal augmentation of a confirmatory detector antibody against human immunoglobulins. Furthermore, the biosensor-based immunoassay has been shown to detect both IgG and IgM antibodies against trebananib. Despite the use of a specific anti-human IgG F(ab′) as a secondary detector antibody for confirming ADA formation, the soluble drug targets (angiopoietins) interfered with the detection of ADAs, leading to false positive ADA detection, likely via their interaction with human immunoglobulins in serum. This cross-reactivity can be effectively blocked by adding to the assay an angiopoietin-specific Hu-Ang-mAb without affecting ADA detection. The biosensor-based immunoassay is presently being used to evaluate the immune response of patients who receive trebananib at higher doses in ongoing clinical trials, such as the pivotal phase 3 studies in ovarian cancer (ClinicalTrials.gov identifiers: TRINOVA-1, NCT01204749; TRINOVA-2, NCT01281254; TRINOVA-3, NCT01493505).

Disclosure statement

All authors are employees of and stockholders in Amgen Inc.

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