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Research paper

Production of monoclonal antibodies for measuring Avastin and its biosimilar by Sandwich ELISA

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ABSTRACT

The development of Bevacizumab (Avastin) biosimilar products has grown rapidly over the last ten years as the original Avastin's patent will expire soon. The approval of Avastin biosimilars requires the demonstration of similarity between the biosimilar and the reference product. To support pre-clinical and clinical studies, pharmacokinetic (PK) assays are required to measure the biosimilar and Avastin with comparable precision and accuracy. The PK assay of Avastin employed by Genentech was a Sandwich ELISA which could detect the total drug concentration. However, it was developed in-house and not commercially available. Therefore, in most of the Avastin biosimilar pre-clinical studies, the antibody drug concentrations were measured using an indirect ELISA against coated VEGF, which could only measure the free instead of the total antibody drugs. It failed the essential requirement to develop the biosimilars. In this study, we reported the generation of mouse monoclonal antibodies (mAbs) that specifically recognize Avastin in a VEGF non-competitive manner. Using a pair of non-VEGF competing anti-Avastin mAbs, a Sandwich ELISA was developed with a lower limit of quantitation (LLOQ) at 400 ng/mL and upper limit of quantitation (ULOQ) at 12800 ng/mL. The assay validation was carried out with serum samples from monkey treated with Avastin biosimilar at seven different time points. Our data showed that the Sandwich ELISA kit we developed is sensitive, simple, reproducible and ready for use in human clinical trials.

1. Introduction

Bevacizumab (Avastin ®, Genentech, San Francisco, CA, USA) is a humanized mouse monoclonal antibody against the Vascular Endothelial Growth Factor (VEGF). Avastin can bind to VEGF, thus blocking the interaction between VEGF and VEGFR, inhibiting tumor angiogenesis and tumor growth (Santos et al., 2015). Avastin, approved by the FDA in February 2004, has been used for treating various types of metastatic cancers, namely breast cancers, metastatic colorectal cancer, advanced renal cell carcinoma, advanced non-small cell lung cancer (Montagnani et al., 2017; Burotto et al., 2017; Tan et al., 2017). As Avastin's patent is going to expire soon, many drug companies are developing its biosimilars all over the world. Just in China, there are > 20 Avastin biosimilars in different phases of human clinical trials (CFDA, 2015).

Although the primary amino acid sequence of the biosimilar is identical to the Avastin reference product, there can be many differences in glycosylation, de-amidation, oxidation, and 3D structure due to the variations in cell lines, culture conditions, etc. It is required by FDA to determine if the differences affect the functions of the antibody drugs including the PK properties. The concentration data is the foundation of the PK bioequivalence (BE) about the dose-response profile. It is recommended by FDA that PK assays must be equally precise, accurate and robust in the measurement of the Avastin biosimilar and the reference drug(DeSilva et al., 2003; US Department of Health and Human Services FDA CDER, 2001).

The PK profile of Avastin reference drug was assessed using a Sandwich ELISA that measured total serum Avastin concentrations (i.e.,

Abbreviations: LLOQ, lower limit of quantitation; mAb, monoclonal antibody; PK, pharmacokinetic; VEGF, Vascular Endothelial Growth Factor; ULOQ, upper limit of quantitation

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the assay did not distinguish between free Avastin and Avastin bound with VEGF ligand) (Avastin®[Bevacizumab], 2018). One of the key elements for this Sandwich ELISA was that those two mAbs' specific bindings to Bevacizumab could not be blocked by VEGF since there are various amounts of free VEGF in the sera of cancer patients. Unfortunately, such non-VEGF competing anti-Avastin mAbs were developed by Genentech and not commercially available, so in some of the pre-clinical studies of the Avastin biosimilars, the antibody drug concentrations in treated patients' sera were measured using an indirect ELISA against coated VEGF, which only can measure the free antibody drugs. The data would vary significantly since level of endogenous VEGF in the sera varies greatly from one patient to another. It is very important to develop a commercial Sandwich ELISA for measuring the total Avastin or its biosimilars.

In this study, we reported the generation of mouse monoclonal antibodies against Avastin, which will not compete with VEGF for binding. A Sandwich ELISA was developed for measuring the total Avastin or its biosimilars with a LLOQ at 400 ng/mL and ULOQ at 12800 ng/mL. The validation of assay was carried out with 7 samples from monkeys treated with Avastin biosimilar at different time points. Our data showed that the Sandwich ELISA kit we developed is sensitive, simple, reproducible and ready for use in human clinical trials of Avastin biosimilars.

2. Materials and methods

2.1. Materials

Avastin and its bio-similar were provided by Hualan Genetic Engineering Co., LTD (Henan, China). Freund's complete adjuvant (CFA), Freund's incomplete adjuvant (IFA), Polyethylene glycol 4000 (PEG4000), DMSO, TMB substrates were purchased from SIGMA (MO, USA). Female BALB/c mice (4–6 weeks-old) were obtained from Vital River Co. (Beijing, China). The Avastin-biosimilar treated monkey sera were provided by Joinin Lab (Beijing, China). DMEM and FBS were from HyClone (CA, USA). Goat anti-mouse IgG Fc secondary antibody is from Jackson Immun (MA, USA). Horseradish peroxidase (HRP) conjugation kit was purchased from Pierce (IL, USA).

2.2. Generation of mouse monoclonal antibodies

As previously described (Li et al., 2009), female BALB/c mice were first immunized with Avastin in Complete Freund's Adjuvant and boosted in Incomplete Freund's Adjuvant. Two to four weeks after the first immunization, tail bleeds were tested for titers by indirect ELISA.

The spleen cells from the mice with good titers were fused with the mouse myeloma cell line SP2/0. Culture supernatants from individual hybridoma clones were then screened by ELISA. To produce antibodies, the hybridoma clones were seeded in stationary bioreactors in DMEM with 10% low-IgG FBS. The bioreactor fluids were collected every 3 days, and IgG fractions were affinity-purified using protein G agarose columns (GE). The concentrations of purified IgGs were determined according to the absorbance at 280 nm.

2.3. Indirect ELISA

Each well of 96-well high binding EIA plates was coated with 1000 ng/mL of Avastin overnight at 4 °C in phosphate buffered saline (PBS). After two washes with PBS and blocking with 5% skim-milk in PBS for 1 h at room temperature, wells were incubated with either the sera or the culture supernatants or purified mAb in 5% skim-milk-PBS for another one hour at room temperature. After two washes with PBS, wells were then incubated with HRP-conjugated goat anti-mouse IgG Fc-specific secondary antibodies in 5% skim-milk-PBS for 1 h at room temperature. After five washes with PBS plus 0.1% Tween20 (PBST), HRP substrate 3, 3′, 5, 5′-tetramethylbenzidine (TMB) solution was added. The reaction was stopped with Stop solution (0.1 M H_2SO_4) after 30 min and absorbance was measured at 450 nm with a microplate reader.

2.4. Sandwich ELISA

Mouse anti-Avastin mAbs were conjugated with horseradish peroxidase according to the manufacturer's instructions. The specific activities of the conjugated antibodies were determined by the ratio of OD_{430}/OD_{280}.

Followed the protocols described previously (Ma et al., 2014), each well of 96-well high-binding ELISA plates was coated with different mouse non-VEGF competing anti-Avastin mAbs overnight at 4 °C in 0.05 M Na_2CO_3, pH 9.6. After two washes with PBS, the plates were blocked with 3% bovine serum albumin (BSA) in PBS, then air-dried and sealed in a plastic bag.

Monkey sera were diluted in 3% BSA-PBS and incubated in the wells for 1 h at room temperature. After two washes, wells were probed with the HRP-conjugated non-VEGF competing anti-Avastin mAb diluted in 3% BSA-PBS for another hour. After five washes with PBS-Tween 20 (PBST), chromogenic HRP substrate 3,3′,5,5′-tetramethylbenzidine (TMB) was added to the wells. The reaction was stopped with stop solution (0.1 M H_2SO_4) after 30 min incubation at room temperature and absorbance was measured at 450 nm with a plate reader.

3. Results

3.1. Immunization of mice with Avastin

To generate antibody drug-specific mAbs, female BALB/c mice were immunized with full length Avastin with the help of adjuvants. On day 14 after multiple boosts, the mouse tail bleeds were collected and the titers of the sera (defined as the OD_{450} absorbance 2-times higher than the background) against Avastin were examined in indirect ELISA.

In general, the constant region (Fc) of humanized antibody IgG is very immunogenic in mice and this study's goal is to raise mAbs against the variable region (Fv) of Avastin. Thus, the task was very challenging. In order to identify the best mice which were producing polyclonal antibodies specific to the Fv of Avastin, the mouse tail bleeds were pre-incubated with 10 μg/mL of the total IgGs from whole human sera for one hour prior to the test to block out the mouse anti-human IgG signals. As you can see in Table 1, all mice except #1 had very good immune response (> 1:10,000), and mouse #2 had Avastin-specific titers at 1:50,000.

3.2. Generation and identification of non-VEGF competing anti-Avastin mAbs

To obtain Avastin-specific mAbs, two immunized mice (mice #2 and #5) were scarified on Day 17 and Day 22, and the spleen cells from them were used to fuse with mouse myeloma SP2/0 cells to generate

Table 1
Titers of tail bleeds against Avastin.

	1:500	1:1000	1:5000	1:10000	1:50000	NC
#1	0.374	0.213	0.110	0.071	0.077	0.083
#2	2.937	1.711	0.436	0.199	0.125	0.057
#3	1.982	0.791	0.212	0.117	0.075	0.062
#4	1.824	1.009	0.239	0.115	0.074	0.062
#5	1.714	1.494	0.261	0.126	0.075	0.042
#6	2.041	0.792	0.152	0.153	0.045	0.040

Wells coated with 1 μg/mL of Avastin were incubated with the mouse tail bleeds diluted at indicated folds in 3% BSA-PBS plus10 μg/mL of human IgGs, then probed with HRP-conjugated Goat anti-Mouse IgG (Fc 1 + 2a + 2b + 3). The values are expressed as mean calculated from duplicate samples.

Table 2
Specificity test of mouse anti-Avastin mAbs.

Coating material	1A11	1B1	1D7	1E4	1F2	1H3	5C6	5D3
Avastin.	> 3.0	> 3.0	> 3.0	> 3.0	> 3.0	> 3.0	2.054	> 3.0
Human IgG	0.097	0.198	0.098	0.124	0.095	0.206	0.106	0.149
	5D6	5D11	5E1	5E11	6B7	6D12	6F3	NC
Avastin.	2.663	2.921	> 3.0	1.651	> 3.0	1.134	> 3.0	0.159
Human IgG	0.161	0.108	0.769	0.073	0.155	0.204	0.204	0.109

Wells coated with 3 μg/mL of either Avastin or normal human IgGs were incubated with the culture supernatants from different clones, then probed with HRP-conjugated Goat anti-Mouse IgG (Fc 1 + 2a + 2b + 3).

hybridoma cell lines.

About 600 monoclonal hybridoma cell cultures were first screened against Avastin coated on 96-well plates, and 494 clones (85.7%) were found producing anti-Avastin monoclonal IgGs. At this point, it is not clear whether those monoclonals were Avastin-specific or just anti-human IgGs.

Since the PK assay will be used to measure the Avastin biosimilars in human patient's sera, which contain around 50 mg/mL of IgGs, the anti-Avastin mAbs to be selected have to show no cross reactivity towards human IgGs. We double-screened 40 anti-Avastin mAbs against both Avastin and total IgGs from whole human sera, and found that 32 (80%) of mAbs were Avastin-specific (example data shown in Table 2).

As mentioned before, to measure the total Avastin in human patient sera, it is essential to obtain a pair of non-VEGF competing anti-Avastin mAbs. To identify such mAbs, 17 of the Avastin-specific mAbs were examined for their binding to Avastin in the presence or absence of 3μg/mL of VEGF. As shown in Table 3, the bindings of mAbs 5C6, 5D6, 5D11, 5E1, 6B7, 6F3 and 6D12 to Avastin were not inhibited by the presence of VEGF, indicating they are non-VEGF competing anti-Avastin mAbs and suitable for the Sandwich ELISA development.

3.3. Development of the Sandwich ELISA for measuring total Avastin

The assay format is exactly same as the one developed by Genentech for measuring the total Avastin in sera. Briefly, one non-VEGF competing anti-Avastin mAb is used to coat the surface of the wells of 96-well plates to capture the biosimilar and reference product from sera, and a second HRP-labeled VEGF-non-competing anti-Avastin mAb for detection of the bound Avastin or its biosimilar (Fig. 1).

To develop the Sandwich ELISA, seven different non-VEGF competing anti-Avastin mAbs were coated to pair with two different HRP-conjugated non-VEGF competing anti-Avastin mAbs respectively. The best manufacturing and testing key parameters obtained are 1) coating with 20 μg/mL of non-VEGF competing anti-Avastin mAb 5D11 at 4 °C overnight, 2) diluting the samples with 20% calf serum (CS)-0.1% Proclin 300 in PBS, 3) detecting with 0.2 μg/mL of HRP-conjugated non-VEGF competing anti-Avastin mAb 5E1 in 3% BSA-0.1% Proclin 300 in PBS, and 4) final washing with PBS-Tween. Negative control human sera were prepared by pooling human sera from 50 different individuals to be used as the assay matrix. The PK standards were prepared by spiking various amounts of Avastin (25,600, 12,800, 6400, 3200, 1600, 800, 400, 200, 100, 0 ng/mL) in negative control human sera. As shown in Fig. 2, plotted using 4-P Fit method, the Sandwich ELISA for measuring Avastin has a linear range of 100–25,600 ng/mL with lower limit of detection (LOD) at 100 ng/mL, the lower limit of quantitation (LLOQ) at 400 ng/mL, and the upper limit of quantitation (ULOQ) at 12,800 ng/mL.

Avastin has been used at 1-20 mg/kg to treat cancer patients (Avastin®[Bevacizumab], 2018), the Cmax of Avastin in the cancer patients' sera was in the range between 50,000 and 200,000 ng/mL. The Sandwich ELISA developed here is sensitive enough for measuring the concentrations of total Avastin in the cancer patients' samples

3.4. Validation of Assay for measuring Avastin in human sera

To fulfill the requirements, more than two batches of the Avastin Sandwich ELISA kits were manufactured at different times. To prepare the standards and QC samples, the Avastin were spiked into negative control human sera. The five QC samples that spanned the linear range of the method were ULOQ at 12,800 ng/mL, high quality control (QCH) at 10,000 ng/mL, medium quality control (QCM) at 5000 ng/mL, low quality control (QCL) at 1000 ng/mL, and LLOQ at 400 ng/mL.

The tests were carried out by three different technicians at two different days. The inter- and intra-assay precision and accuracy of standards and QC samples were determined. For each QC sample the back-calculated concentration, standard deviation (SD), coefficient of variation (%CV) and the recovery rate were determined.

Summary of method validation and sample analysis were shown in Table 4. The accuracies of 5 quality controls were between 81.68 and 119.68%, with the precisions between 1.19 and 11.75% respectively. All of them met the requirements of PK assay validation.

3.5. Comparison of standard curves using both Avastin and biosimilar

Development of PK assays for quantitative measurement of the biosimilar and reference products in serum matrix is a critical component to the in vivo characterization of the test products, and the concentration data serves as the foundation of the PK bioequivalence (BE) assessment of the dose-response profiles. FDA requires that the PK methods that are employed must be equally precise, accurate and robust in the measurement of the biosimilar and reference product.

In this study, a comparison of analytical standard curves prepared with either the Avastin biosimilar or FDA-licensed Avastin reference product was carried out. As shown in Fig. 3, there were no distinguishable differences in the standard curve profiles, demonstrating the Sandwich ELISA kit can measure the concentrations of both the Biosimilar and reference products comparably.

3.6. Measurement of Avastin biosimilar concentrations in treated monkeys

In the pre-clinical study, serum samples were collected from one monkey injected with Avastin biosimilar. Total of 7 sera were collected

Table 3
Identification of non-VEGF competing anti-Avastin mAbs.

Competition	1A11	1B1	6F6	1B7	1D7	1E4	1F2	1F12	2E11	5C6	5D6
VEGF	0.384	0.558	0.39	0.198	0.917	0.328	0.332	0.253	0.649	0.563	1.042
No VEGF	1.447	1.95	0.285	0.288	2.704	0.834	0.839	0.484	0.363	0.562	0.898
	5D11	5E11	6B7	6B8	6D12	6F3	1H3	5D3	5E1	NC	
VEGF	1.154	0.199	1.006	0.447	0.644	1.14	0.18	0.231	1.608	0.048	
No VEGF	0.962	0.781	1.393	0.514	0.865	1.777	0.707	0.367	1.743	0.051	

Wells coated with Avastin were pre-incubated with or without 3 μg/mL of VEGF, then the culture supernatants of selected hybridomas were added to each well. After washes, wells were then probed with HRP-conjugated Goat anti-Mouse IgG (Fc 1 + 2a + 2b + 3).

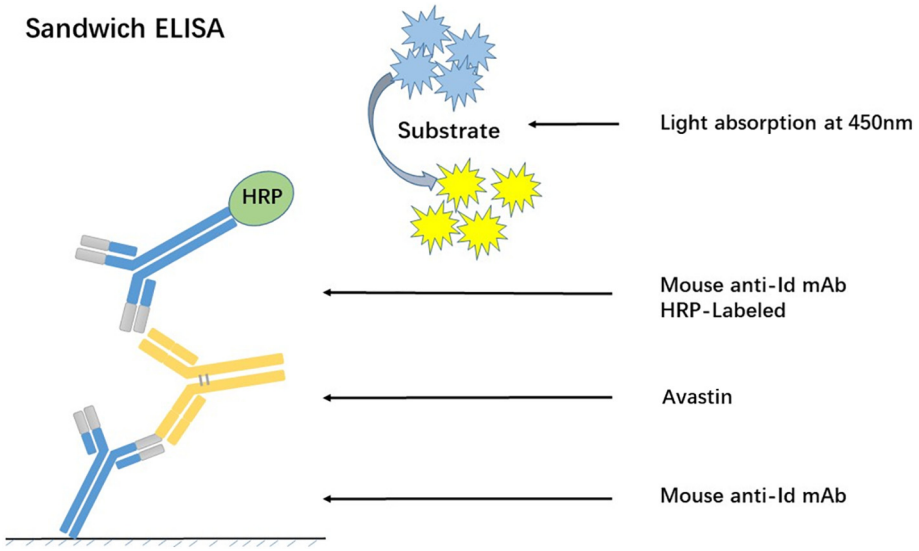

Fig. 1. Sandwich ELISA for measuring total Avastin.
A schematic illustration of sandwich ELISA. Plate was coated with one non-VEGF competing anti-Avastin mAb and probe was HRP conjugated another non –VEGF competing anti-Avastin mAb.

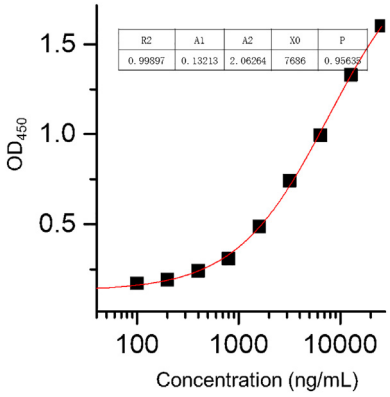

Fig. 2. Standard curve of Sandwich ELISA for Avastin.
Standard curve of sandwich assay for Avastin. Wells coated with 2 μg of anti-Avastin mAb 5D11 were incubated with Avastin standards (0, 100, 200, 400, 800, 1600, 3200, 6400, 12,800, 25,600 ng/mL), then probed with HRP-conjugated anti-Avastin mAb 5E1. Results was plotted using 4-Parameters method with the equation: y = A2 + (A1 − A2)/(1 + (X/X0)ˆP)

Table 4
Evaluation of the accuracy and precision of assay.

	Accuracy (%Recovery)	Precision (%CV)
ULOQ	84.39–119.68	1.20–9.84
QCH	87.60–111.32	1.19–11.75
QCM	81.68–103.81	1.49–11.63
QCL	96.13–99.52	2.08–8.92
LLOQ	86.40–101.71	2.51–6.27

Accuracy: (observed/nominal) X 100%. Precision: (SD/mean concentration) X 100%

at 7 different time points including prior to injection (−1 h), right-after injection (0 h), and different times after the injection (1 h, 6 h, 24 h, 48 h, 72 h). As shown in Fig. 4, there was a time-dependent change of Avastin biosimilar concentrations in the monkey sera. A further study with human patient samples will be carried out soon.

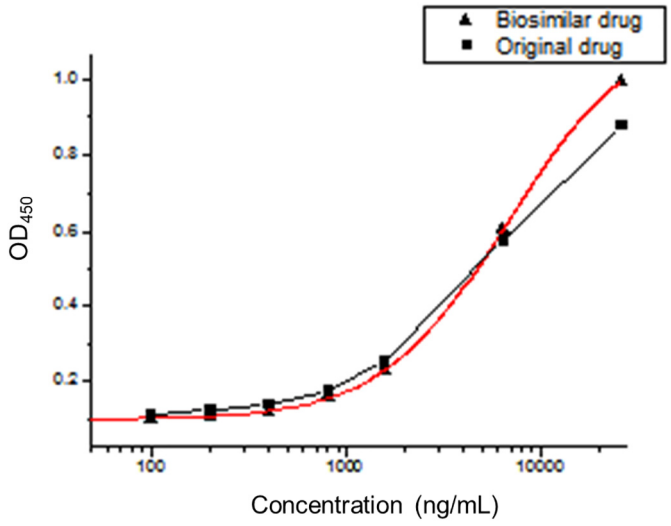

Fig. 3. Comparison of standard curves between Avastin and its biosimilar.
Standard curves of sandwich assay for Avastin and its biosimilar. Wells coated with 2 μg of anti-Avastin mAb 5D11 were incubated with various amounts of either Avastin or its biosimilar (0, 100, 200, 400, 800, 1600, 3200, 6400, 12,800, 25,600 ng/mL), then probed with HRP-conjugated anti-Avastin mAb 5E1.

4. Discussion

In the past, due to the lack of non-VEGF competing anti-Avastin mAb from commercial source, the PK studies of many Avastin biosimilars were carried out using indirect ELISA against VEGF. Such assay was only capable to detect the free Avastin, not the total serum Avastin. Now with the non-VEGF competing anti-Avastin mAbs generated here, a Sandwich ELISA kit for measuring the total concentrations of Avastin or its biosimilars was developed. The method has been shown to be equally precise, accurate and robust in the measurement of the Avastin biosimilar and reference product in serum samples. It enables many more drug developers to speed up their development of Avastin biosimilars, making the anti-VEGF biotherapy more affordable to millions more cancer patients worldwide.

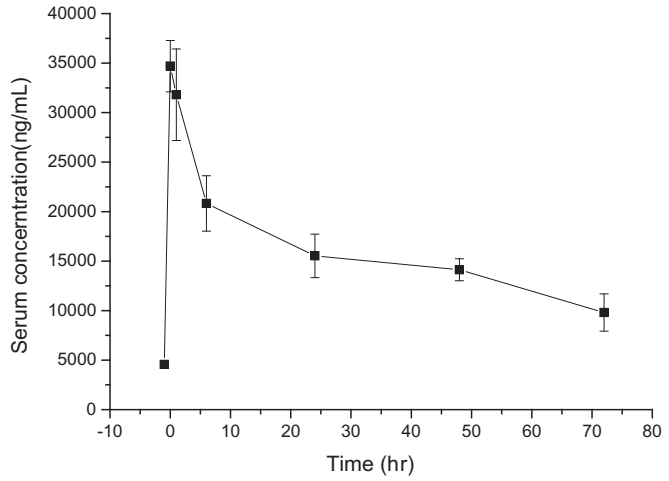

Fig. 4. Measurement of Avastin biosimilar in monkey sera.
Concentrations of Avastin biosimilar in monkey sera. Wells coated with 2 μg of anti-Avastin mAb 5D11 were incubated with monkey sera collected at different time points prior or post injection of Avastin biosimliar, then probed with HRP-conjugated anti-Avastin mAb 5E1. Results were the mean of duplicates.

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