



Biological Characterization of SB3, a Trastuzumab Biosimilar, and the Influence of Changes in Reference Product Characteristics on the Similarity Assessment

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Abstract

Background SB3 has been developed as a trastuzumab biosimilar, a therapeutic monoclonal antibody targeted to human epidermal growth factor receptor 2 (HER2), and approved by the European Commission and United States (US) Food and Drug Administration (FDA). During the developmental period of a biosimilar, setting an appropriate quality target is critical for assessing the similarity of the biosimilar product to the reference product. A stepwise approach should be taken to assessing similarity, beginning with extensive characterization of the reference product to establish the quality target.

Objective In this study, we evaluated the similarity of SB3 to the reference product and the impact of changes in the biological profile of the reference product on similarity assessment.

Methods Analytical similarity was assessed with defined test procedures in terms of critical quality attributes (CQAs) that could affect efficacy, potency, and safety, as well as for the non-CQAs that are related to process consistency. The quality target was established using up to 154 lots of European Union (EU)- and US-sourced Herceptin[®] (reference product), analyzed during the developmental period of SB3.

Results Trends of the EU- and US-sourced reference product showed that the biological profile exhibited two marked changes for Fc-related attributes, and then recovered to pre-change quality level. Since the similarity range set by pre-change lots was considered most relevant, the changed lots were excluded from establishing the similarity range, which resulted in tightened acceptance criteria. As shown in the results of similarity assessment using the stringent quality target ranges, SB3 exhibits highly similar functional activities compared to the reference product in terms of both CQAs and non-CQAs.

Conclusion SB3 has been developed as a trastuzumab biosimilar approved in the EU and USA, and its manufacturing process is deemed to be robust and well-controlled within stringent quality target ranges.

1 Introduction

A biosimilar is a biological medicinal product with structural and functional properties that are highly similar to an authorized and already approved original biological drug, and whose pharmacokinetics and clinical activity are shown to be similar in a comparative clinical trial [1]. The European Medicines Agency (EMA) and United States (US) Food and Drug Administration (FDA) have developed

Key Points

Up to 154 lots of Herceptin[®] (reference product) were analyzed for the characterization of the trastuzumab biosimilar SB3. After two sequential changes in the biological profile in both EU- and US-sourced reference product, the attributes recovered to pre-change levels.

Considering the influence of biological profile changes on the range, the quality target was tightened for similarity assessment by excluding the lots where the biological profile had changed.

SB3 demonstrated high similarity in critical biological quality attributes, including Fab-related biological activities, effector functions such as direct mechanism of action-related antibody-dependent cell-mediated cytotoxicity (ADCC) and Fcγ receptor IIIa binding, and neonatal Fc receptor (FcRn) binding that may affect its half-life.

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biosimilar guidelines, with a requirement to demonstrate the totality of evidence including analytical, non-clinical, and clinical similarity [2–7]. Analytical similarity in structural and functional properties is critical to expect the absence of any meaningful difference in clinical outcomes. Based on this fundamental assumption, a similarity assessment should be performed by a stepwise approach, starting with extensive physicochemical and biological characterization of both the biosimilar and the reference product [2, 3]. Studies for analytical similarity should be based on analytical data with defined test procedures and acceptance criteria for the reference product's critical quality attributes (CQAs) that could affect efficacy, potency, and safety, as well as for non-CQAs that are related to process consistency [4].

Human epidermal growth factor receptor 2 (HER2) is a membrane-bound receptor protein that promotes cell growth and division when activated by ligand binding. In approximately 20–25% of breast cancers, HER2 is overexpressed, associated with faster growth of the cancer and a higher recurrence rate [8]. Trastuzumab is a therapeutic monoclonal antibody (mAb) of the IgG1 subclass with a kappa light chain, specifically targeted to HER2. Since trastuzumab was developed by humanizing the parental murine antibody “4D5,” it contains a human framework region and the complementarity-determining regions of a murine antibody that binds to the domain IV region of HER2 [9]. HER2 is activated by dimerization, which leads to the activation of the Ras/Raf/mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K)/Akt, and phospholipase C γ (PLC γ)/protein kinase C pathways required for the cell proliferation [10, 11]. The mechanism of action (MoA) of trastuzumab is known to be its inhibition of proliferation of HER2-overexpressing cells by blocking MAPK and PI3K/Akt pathways [12, 13]. Another major mechanism of trastuzumab is to attract immune cells to HER2-overexpressing tumor sites, called antibody-dependent cell-mediated cytotoxicity (ADCC) [14, 15]. Upon antigen binding to mAbs, effector functions are mediated by their Fc part that binds to Fc γ receptor (Fc γ R) IIIa (Fc γ RIIIa, also known as CD16a). The *N*-glycosylation pattern of mAbs influences the binding affinity of its Fc part to Fc γ RIIIa. Trastuzumab also induces HER2 internalization and degradation through binding to HER2 [16].

Trastuzumab was approved by the US FDA in 1998 and by the European Commission (EC) in 2000 for the treatment of certain patients with breast cancers overexpressing HER2 under the trade name Herceptin[®] (hereafter called “reference product”) [17]. In addition, as a combination therapy, trastuzumab plus chemotherapy regimen has become the standard of care for the treatment of HER2-positive breast cancers, providing significant clinical benefit in adjuvant settings against early as well as advanced or metastatic breast cancers [18–20].

SB3 was developed as a trastuzumab biosimilar by Samsung Bioepis Co., Ltd. (Incheon, Republic of Korea), and approved by the EC in Nov 2017 and by the US FDA in Jan 2019, for the treatment of HER2-positive breast cancer and HER2-overexpressing gastric cancer. In the Republic of Korea and Australia, SB3 was approved by the Ministry of Food and Drug Safety and the Therapeutic Goods Administration in November 2017 and December 2018, respectively. In accordance with the International Conference on Harmonisation (ICH) Q8 (R2) guideline, a risk assessment was conducted to establish the CQAs for SB3 on the basis of the MoA for trastuzumab and the analytical capability of state-of-the-art methods. Risk priority number (RPN) is scored for the impact and uncertainty of attributes on efficacy and safety, and applied to evaluate the CQAs. The CQAs of SB3 were established based on impacts not only on HER2 binding and the subsequent anti-proliferation effect, but also on its effector functions associated with the Fc domain in general.

To demonstrate the similarity of SB3 to the reference product, an extensive analytical characterization was executed based on the quality target profile established during the developmental period. During the characterization study of SB3, up to 154 lots of reference product were analyzed, and two sequential changes in Fc-related attributes such as the sum value of %afucose + %high mannose, Fc γ RIIIa binding, ADCC, and %galactosylation (G1F + G2F) were observed in both EU- and US-sourced reference product expiring between August 2018 and December 2019 [21]. Herein, the analytical similarity of SB3 was assessed with the stringent quality target ranges set by excluding the lots of reference product demonstrating biological profile changes. The results demonstrate that SB3 is highly similar to the reference product in terms of biological quality attributes.

2 Materials and Methods

2.1 Materials

EU- and US-sourced reference product (up to 154 lots) were purchased from local distributors and then stored according to the manufacturer's instructions. The reference standard for the bioassays was prepared from EU-sourced reference product and used for characterization of the reference products and SB3. The CellTiter-Blue[®] and CytoTox-Glo[®] kits were obtained from Promega (Madison, WI, USA). The PNGase-F kit was obtained from New England Biolabs (NEB, Ipswich, MA, USA). The 2-aminobenzamide (2-AB) labelling kit for *N*-glycan analysis was obtained from Ludger (Oxfordshire, UK). Glutathione (GSH)-coated donor beads, human IgG-conjugated acceptor beads, and streptavidin-coated donor beads were obtained from PerkinElmer

(Waltham, MA, USA). The glutathione-*S*-transferase (GST)-tagged Fc γ RIIIa and HER2-Fc were obtained from Biogen (Cambridge, MA, USA). Biotin-labelled neonatal Fc receptor (FcRn) was obtained from Biogen (Cambridge, MA, USA). C1q protein was obtained from Quidel (San Diego, CA, USA). Anti-C1q horseradish peroxidase (HRP) conjugated antibody (catalog number AB46191) was obtained from Abcam (Cambridge, MA, USA). Statistical analysis and graphical comparison were performed with Minitab statistical software package (Leadtools Technologies Inc., version 18.1.0, Charlotte, NC, USA).

2.2 Cell Lines and Cell Culture

SKBR3 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and incubated at 37 °C, 5% CO₂ in McCoy's 5A (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA). BT474 cells were obtained from ATCC and incubated at 37 °C, 5% CO₂ in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12, Gibco) containing 10% FBS. NK92-CD16 cells were obtained from Biogen (Cambridge, MA, USA) and incubated at 37 °C, 5% CO₂ in Myelocult H5100 (Stemcell Technology, Vancouver, Canada) containing human interleukin-2 (Cell Signaling Technology, Beverly, MA, USA) and puromycin (Gibco).

2.3 Anti-proliferation Assay

The anti-proliferation potency was measured by CellTiter-Blue[®] cell viability assay kit (Promega) according to manufacturer's instruction. BT474 cells were incubated with ten serially diluted concentrations (0.008–1.0 µg/mL) of trastuzumab for 4 days at 37 °C, 5% CO₂ in DMEM/F12 containing 10% FBS. The relative number of viable cells was quantified by measuring the fluorescence (excitation at 560 nm and emission at 590 nm) using a SpectraMax[®] M3 (Molecular Devices, San Jose, CA, USA). Data were analyzed using Parallel Line Analysis 2.0 (PLA) software (Stegmann Systems GmbH, Rodgau, Germany) to calculate relative anti-proliferation potency.

2.4 ADCC Assay

The ADCC assay was performed to assess Fc-related biological activity of trastuzumab against a HER2-overexpressing human breast cancer cell line (SKBR3). NK92-CD16 cells, a human natural killer cell line expressing CD16, were used as effector cells. Experimentally, trastuzumab was incubated with SKBR3 and NK92-CD16 cells in flat-bottom 96-well plates for 4 h at 37 °C, 5% CO₂ in McCoy's 5A medium. After incubation, the SKBR3/NK92-CD16/sample mixture was incubated with a luminogenic peptide substrate

(alanyl-alanyl-phenylalanyl-aminoluciferin; AAF-Glo Substrate) as a part of the CytoTox-Glo[®] kit. The luminescence signals (dead cell signals) were quantified by an Envision[™] Multilabel Plate Reader (PerkinElmer). Data were analyzed by using PLA software to calculate relative ADCC potency.

2.5 HER2 Binding Assay

The competitive inhibitory HER2 binding assay was performed using time-resolved fluorescence resonance energy transfer (FRET) technology. When donor chromophore absorbs 340-nm wavelength light, it transfers 615-nm energy to an acceptor chromophore. Then, the acceptor chromophore emits longer wavelength light, and the detector accepts a 665-nm wavelength light. In this system, 12 concentrations (0.0488–100 µg/mL) of trastuzumab were incubated with a fixed volume of Europium chelate-labeled trastuzumab (donor chromophore) and Cy5-labeled HER2 (acceptor chromophore) in assay diluent [1 × phosphate-buffered saline (PBS), 0.1% bovine serum albumin (BSA), and pH 7.4]. The mixture was then incubated at 25 °C for 1 h with moderate agitation. After incubation, the fluorescent signal was obtained from the Envision[™] Multilabel Plate Reader (PerkinElmer) at a wavelength of 665 nm. Data were analyzed by using PLA software to calculate the relative binding activity.

2.6 C1q Binding Assay

The C1q binding assay was performed to assess Fc-related biological activity of trastuzumab using sandwich enzyme-linked immunosorbent assay (ELISA). In this system, 96-well plates were coated with seven concentrations (0.391–25 µg/mL) of trastuzumab and incubated at 37 °C for 1 h. Then, the plate was blocked using blocking buffer and incubated at room temperature for 1 h. After blocking, a fixed concentration of C1q (Quidel) solution was added to the plate and the plate was incubated at room temperature. After incubation, fixed concentration of C1q-HRP antibody (Abcam) was added to the plate and the plate was incubated at room temperature. Using 3,3',5,5'-tetramethylbenzidine (TMB) ELISA substrate (Sigma-Aldrich, St. Louis, MO, USA), C1q binding activity was quantified by measuring the absorbance at 450 nm using a SpectraMax[®] M3 (Molecular Devices). Data were analyzed by using PLA software to calculate the relative binding activity.

2.7 FcRn Binding Assay

The FcRn binding assay was performed to assess Fc-related biological activity of trastuzumab using Alphascreen[™] technology with active donor and acceptor beads. With excitation, a photosensitizer in a donor bead converts

ambient oxygen to reactive singlet oxygen. The singlet oxygen species reacts with thioxene compounds in the acceptor bead to generate a chemiluminescent signal that emits at 370 nm. In this system, 12 concentrations (0.021–200 µg/mL) of trastuzumab were incubated with a fixed volume of streptavidin-coated donor/human IgG-conjugated acceptor beads and biotin-labelled FcRn in assay diluent (20 mM 4-morpholineethanesulfonic acid, 150 mM NaCl, 0.1% BSA, pH 5.8). The mixture was incubated at 22 °C for 2 h with moderate agitation. After incubation, the fluorescent signal was obtained from the Envision™ Multilabel Plate Reader (PerkinElmer) with excitation at 680 nm and reading emission at 520–620 nm. Data were analyzed by using PLA software to calculate the relative binding activity.

2.8 FcγRIa Binding Assay

The competitive inhibitory FcγRIa binding assay was performed using time-resolved FRET technology. In this system, 12 concentrations (0.0257–150 µg/mL) of trastuzumab were incubated with a fixed volume of Europium chelate-labeled trastuzumab (donor chromophore) and Cy5-labeled FcγRIa (acceptor chromophore) in assay diluent (1 × PBS, 0.1% BSA, and pH 7.4). The mixture was then incubated at 25 °C for 1 h with moderate agitation. After incubation, the fluorescent signal was obtained from the Envision™ Multilabel Plate Reader (PerkinElmer) at a wavelength of 665 nm. Data were analyzed by using PLA software to calculate the relative binding activity.

2.9 FcγRIIa Binding Assay

The FcγRIIa binding assay was performed to assess Fc-related biological activity of trastuzumab using Alphascreen™ technology. In this system, 12 concentrations (0.26–166.67 µg/mL) of trastuzumab were incubated with a fixed volume of GSH-coated donor/human IgG-conjugated acceptor beads and GST-tagged FcγRIIa. The mixture was incubated at 22 °C for 2.0 h with moderate agitation. After incubation, the fluorescent signal was obtained from the Envision™ Multilabel Plate Reader (PerkinElmer) with excitation at 680 nm and reading emission at 520–620 nm. Data were analyzed by using PLA software to calculate the relative binding activity.

2.10 FcγRIIb Binding Assay

The FcγRIIb binding assay was performed to assess Fc-related biological activity of trastuzumab using Alphascreen™ technology. In this system, 12 concentrations (0.8–500 µg/mL) of trastuzumab were incubated with a fixed volume of GSH-coated donor/human IgG-conjugated acceptor beads and GST-tagged FcγRIIb. The mixture was

incubated at 22 °C for 2.0 h with moderate agitation. After incubation, the fluorescent signal was obtained from the Envision™ Multilabel Plate Reader (PerkinElmer) with excitation at 680 nm and reading emission at 520–620 nm. Data were analyzed by using PLA software to calculate the relative binding activity.

2.11 FcγRIIIa Binding Assay

The FcγRIIIa binding assay was performed to assess Fc-related biological activity of trastuzumab using Alphascreen™ technology. In this system, 12 concentrations (0.0488–100 µg/mL) of trastuzumab were incubated with a fixed volume of GSH-coated donor/human IgG-conjugated acceptor beads and GST-tagged FcγRIIIa. The mixture was incubated at 22 °C for 3.5 h with moderate agitation. After incubation, the fluorescent signal was obtained from the Envision™ Multilabel Plate Reader (PerkinElmer) with excitation at 680 nm and reading emission at 520–620 nm. Data were analyzed by using PLA software to calculate the relative binding activity.

2.12 Glycosylation Profile by 2-Aminobenzamide Labeling and HILIC-UPLC Analysis

N-glycan profiles were analyzed using a hydrophilic interaction liquid chromatography–ultra-performance liquid chromatography (HILIC-UPLC) system (Waters, Milford, MA, USA). For quantitative determination, 100 µg of sample was denatured using sodium dodecyl sulfate (SDS) and dithiothreitol (DTT) in the PNGase-F enzyme kit (NEB) at 100 °C for 10 min, and treated with PNGase F to release *N*-glycans. The released *N*-glycans were separated using protein precipitation with cold ethanol, and the supernatant was dried. The dried *N*-glycans were then labelled with 2-AB for 3 h using a LudgerTag™ 2-AB glycan labeling kit (Ludger). Samples were injected onto a UPLC BEH glycan column (2.1 mm × 150 mm, 1.7 µm). The labeled *N*-glycans were separated at a flow rate of 0.5 mL/min with mobile phase A (50 mM ammonium formate) and mobile phase B (100% acetonitrile). The signal was detected using a fluorescence detector (Waters) at the excitation wavelength of 330 nm and the emission wavelength of 420 nm. Empower™3 software (Waters) was used for data processing and calculating the relative peak area.

3 Results

3.1 Risk Assessment and CQA Classification

To evaluate the criticality of product quality attributes for SB3, a trastuzumab biosimilar functional risk assessment

was conducted. The potential impact of individual attributes on product potency, efficacy, and safety was scored as well as the uncertainty regarding the impact assessment of the attributes. Based on RPN values scored by impact and uncertainty categories, the quality attributes were categorized by CQA classification and summarized in Table 1. Nine analytical methods were used for the similarity assessment of SB3. Among them, five quality attributes met the criteria (RPN > 24) for CQA, in which Fab-related biological activities were included as the main MoA of trastuzumab. The effector-function-related attributes, especially ADCC and FcγRIIIa binding directly related to the MoA of trastuzumab, and FcRn binding that may affect the half-life were also categorized as a CQA.

3.2 Determination of Quality Target for Biological Activity of SB3

A previously reported study demonstrated changes in the *N*-glycan profile, FcγRIIIa binding activity, and relative ADCC activity of EU- and US-sourced reference product [21]. The attributes for the *N*-glycan profile, FcγRIIIa binding activity, and relative ADCC activity showed two marked changes throughout the monitoring period. In the first instance, %galactose and %afucose were decreased. Hereafter, the level of %high mannose was remarkably elevated, which was defined as the second instance. Since *N*-glycan levels correlate with relative ADCC activity and FcγRIIIa binding activity is associated with effector binding in the Fc region during ADCC [22], the change in the *N*-glycan profile resulted in a downward shift of ADCC and FcγRIIIa binding activities in the reference product. Since publishing the data by Kim et al. [21], we have kept monitoring the biological profile trend of the reference product. In recent lots

(having an expiry date after February 2020), the biological profile of the reference product recovered to a status similar to that of pre-changed lots (Fig. 1). Therefore, it seems that the first and second changes in biological profile in certain periods were abnormal events; considering that ADCC is a major MoA, such changes in the biological profile have the potential to impact clinical efficacy of the reference product. Furthermore, in terms of similarity assessment, this change in the biological profile of the reference product brings about product variability and can affect the consistency of the target similarity range [mean ± 3SD(standard deviation)], as shown especially in Fc-related attributes (Fig. 2).

Accordingly, in this study, the target quality ranges were established by excluding the lots displaying these altered biological profiles and exclusively using pre-change lots in order to appropriately compare SB3 to the reference product.

3.3 Fab-Related Biological Attributes

Based on the MoA of trastuzumab, two sets of biological assays were performed to evaluate the Fab-related activities: anti-proliferation assays and HER2 binding assays. Trastuzumab binds to the extracellular domain IV region of HER2, and blocks proteolytic cleavage of the extracellular domain of HER2, leading to downregulation of the MAPK and PI3K/Akt pathways and inhibition of cell proliferation. HER2 binding activity was determined by a FRET-based competitive inhibition binding assay, and the result showed that SB3 is similar to EU- and US-sourced reference product. The mean values of %relative binding activity for SB3 and EU- and US-sourced reference product were 100%, 99%, and 98%, respectively. In addition, HER2 binding activities of all SB3 batches were within the target similarity range set by mean ± 3SD, demonstrating promising similarity in

Table 1 Quality attributes and analytical method by CQA classification

Category	Product quality attribute	Analytical method	Risk assessment		RPN	CQA classification
			Impact ^a	Uncertainty ^b		
Fab-related biological activity	Anti-proliferation activity	Cell-based assay	Very high	Moderate	60	CQA
	HER2 binding activity	FRET	Very high	Moderate	60	CQA
Fc-related biological activity	ADCC	Cell-based assay	High	Moderate	48	CQA
	FcγRIIIa binding activity	AlphaScreen [®]	High	Moderate	48	CQA
	FcγRIa binding activity	AlphaScreen [®]	Moderate	Low	24	Non-CQA
	FcγRIIa binding activity	AlphaScreen [®]	Moderate	Low	24	Non-CQA
	FcγRIIb binding activity	AlphaScreen [®]	Moderate	Low	24	Non-CQA
	FcRn binding	AlphaScreen [®]	High	Moderate	48	CQA
	C1q binding	ELISA	Moderate	Low	24	Non-CQA

ADCC antibody-dependent cell-mediated cytotoxicity, CQA critical quality attribute, ELISA enzyme-linked immunosorbent assay, FcRn neonatal Fc receptor, FRET fluorescence resonance energy transfer, HER2 human epidermal growth factor receptor 2, RPN risk priority number

^aImpact was scored by 5 categorizations as very low, low, moderate, high, and very high

^bUncertainty was scored by 4 categorizations as low, moderate, high, and very high

HER2 binding activity (Fig. 3a). For the anti-proliferation assay, the CellTiter-Blue[®] kit was used to indicate cell viability in the HER2-overexpressing human breast cancer cell line BT474. The mean values of %relative anti-proliferation

potency for SB3 and EU- and US-sourced reference product were 96%, 100%, and 100% respectively. All SB3 batches also showed relative potencies within the similarity range, demonstrating promising similarity in anti-proliferation

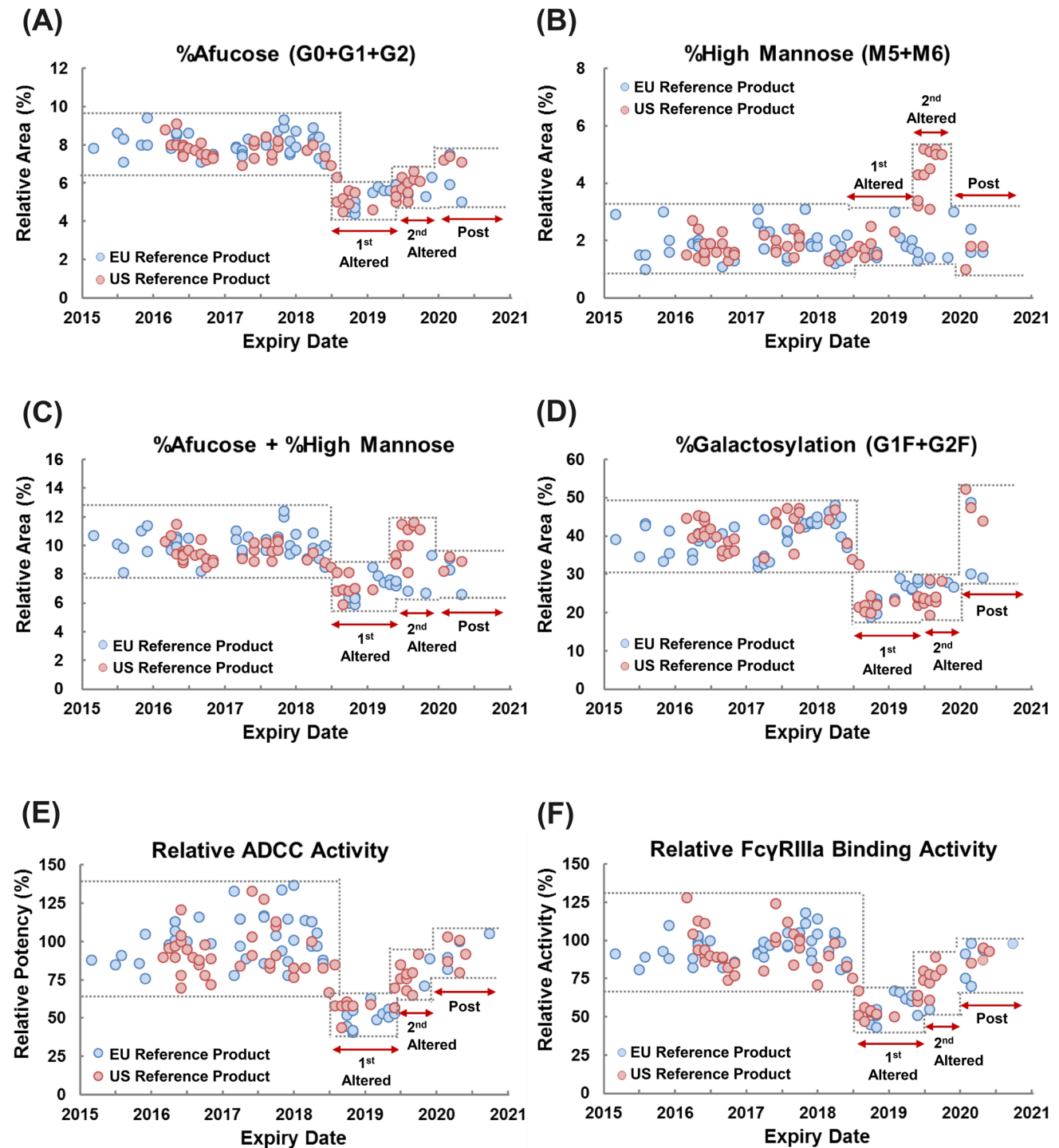
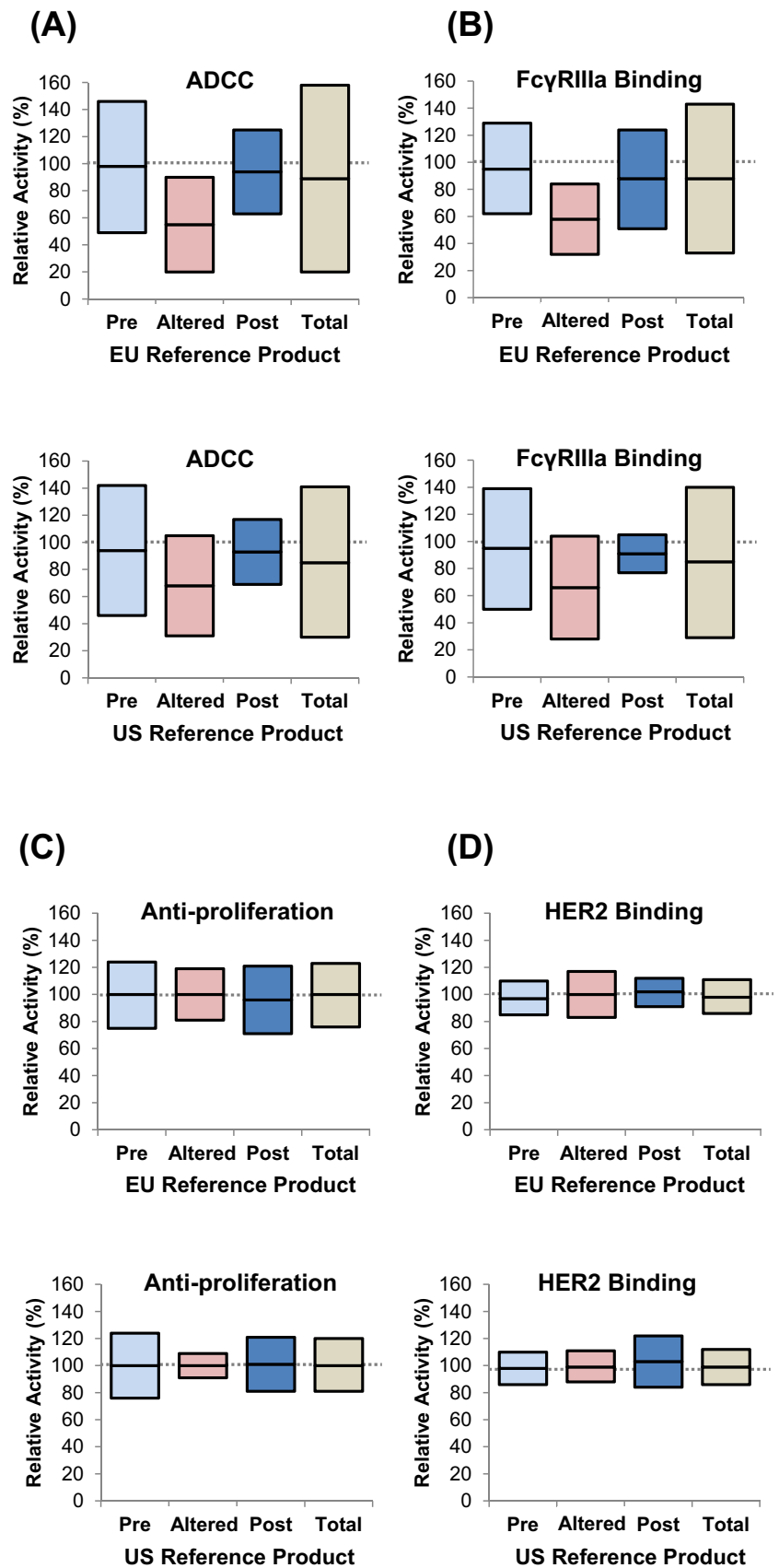


Fig. 1 Trends of Fc-related biological attributes of the reference product. Dotted line shows the min–max range of expiry date before August 2018, 1st alteration, 2nd alteration, and post-alteration. **a** %Afucose, **b** %high mannose, **c** %afucose + %high mannose, **d**

%galactosylation (%G1F + %G2F), **e** ADCC activity, and **f** FcγRIIIa binding activity. ADCC antibody-dependent cell-mediated cytotoxicity, FcγR Fcγ receptor, G1F FA2[3]G1 or FA2[6]G1, G2F FA2G2, M5 Mannose5, Man5, M6 Mannose6, Man6

Fig. 2 Change of target ranges depending on the biological profile change periods of reference product. Boxplots show the mean \pm 3SD ranges of EU- and US-sourced reference product for pre-alteration, altered, and post periods. **a** ADCC activity, **b** Fc γ RIIIa binding activity, **c** anti-proliferation activity, and **d** HER2 binding activity. ADCC antibody-dependent cell-mediated cytotoxicity, HER2 human epidermal growth factor receptor 2, Fc γ R Fc γ receptor, SD standard deviation



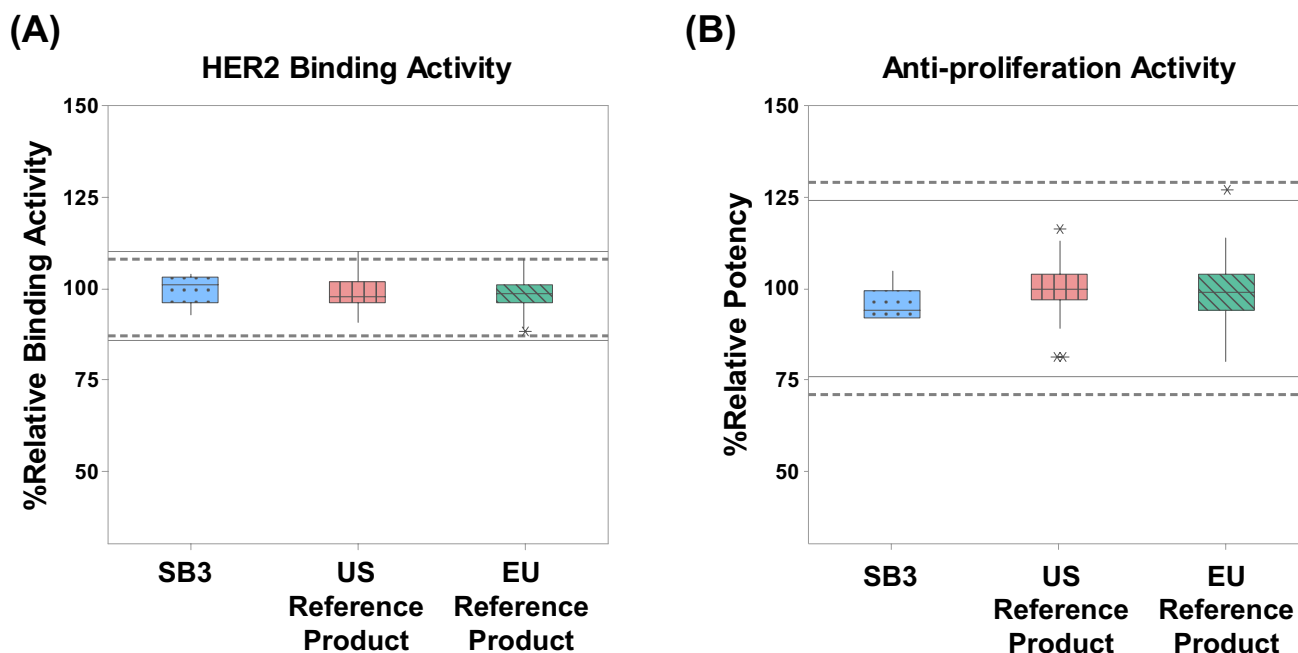


Fig. 3 Comparison of the Fab-related biological activities of SB3 and reference product. Dotted line shows the similarity range (mean \pm 3SD) of pre-altered EU-sourced reference product. Solid line shows the mean \pm 3SD range including pre-altered and altered

EU-sourced reference product. **a** HER2 binding activity and **b** anti-proliferation activity. *HER2* human epidermal growth factor receptor 2, *SD* standard deviation

potency (Fig. 3b). Therefore, these results indicate that SB3 is evidently similar to the reference product in terms of Fab-related biological attributes.

3.4 Fc-Related Biological Attributes

The Fc region of trastuzumab binds to Fc γ Rs, FcRn, and C1q (a complement protein) for most of its effector functions and in vivo stability [23]. The interaction of the Fc region in the target-bound antibody with Fc γ RIIIa (a member of Fc γ Rs) on the effector cell is the key mechanism of ADCC for trastuzumab [24–26]. As CQAs of Fc-related biological attributes, ADCC, Fc γ RIIIa binding, and FcRn binding activities were assessed to demonstrate the similarity of SB3. The mean values of relative ADCC potency for SB3 and EU- and US-sourced reference product were 98%, 85%, and 89%, respectively, and those of relative Fc γ RIIIa binding activities were 98%, 84%, and 88%, respectively. These mean differences for ADCC and Fc γ RIIIa binding activities are considered to result from the reference products with altered biological profiles. To verify this hypothesis, the similarity of SB3 was re-assessed with only the mean values and the similarity ranges excluding the reference products with altered biological profiles. As a result, the mean value of SB3 was similar to the quality of pre-altered reference

products, and all data for independent batches fell within the tightened target similarity range (Fig. 4).

Affinity binding to FcRn is critical to extend the half-life of mAb by recycling and exocytosis at the plasma membrane [27]. The mean values of relative FcRn binding activity for SB3 and EU- and US-sourced reference product were 96%, 88%, and 93%, respectively (Fig. 4). Furthermore, all SB3 batches were within the similarity range, demonstrating similarity in FcRn binding activity.

Binding activities for other Fc γ Rs (Fc γ RIa, Fc γ RIIa, and Fc γ RIIb) were also tested as part of the similarity assessment. All Fc γ Rs belong to the immunoglobulin superfamily and are the most important Fc receptors for inducing phagocytosis of microbes. This superfamily includes several members such as Fc γ RI (CD64), Fc γ RII (CD32), and Fc γ RIII (CD16), which differ in their affinities for antibodies and in their functions [28]. In the case of trastuzumab, ADCC would be affected by Fc γ RIIIa rather than Fc γ RIa, Fc γ RIIa, and Fc γ RIIb [29]. Therefore, the other Fc γ Rs were categorized as non-CQA even though they should be controlled within the range to demonstrate biosimilarity. The mean values of relative binding activities for other Fc γ Rs of SB3 were similar to reference product, summarized in Fig. 5, including the similarity ranges. In the same manner, SB3 demonstrated similarity to the reference product for

Fig. 4 Comparison of the Fc-related biological activities of SB3 and reference product. Dotted line shows the similarity range (mean \pm 3SD) of pre-altered EU-sourced reference product. Solid line shows the mean \pm 3SD range including pre-altered and altered EU-sourced reference product. **a** ADCC activity, **b** Fc γ RIIIa binding activity, and **c** FcRn binding activity. ADCC antibody-dependent cell-mediated cytotoxicity, Fc γ R Fc γ receptor, FcRn neonatal Fc receptor, SD standard deviation

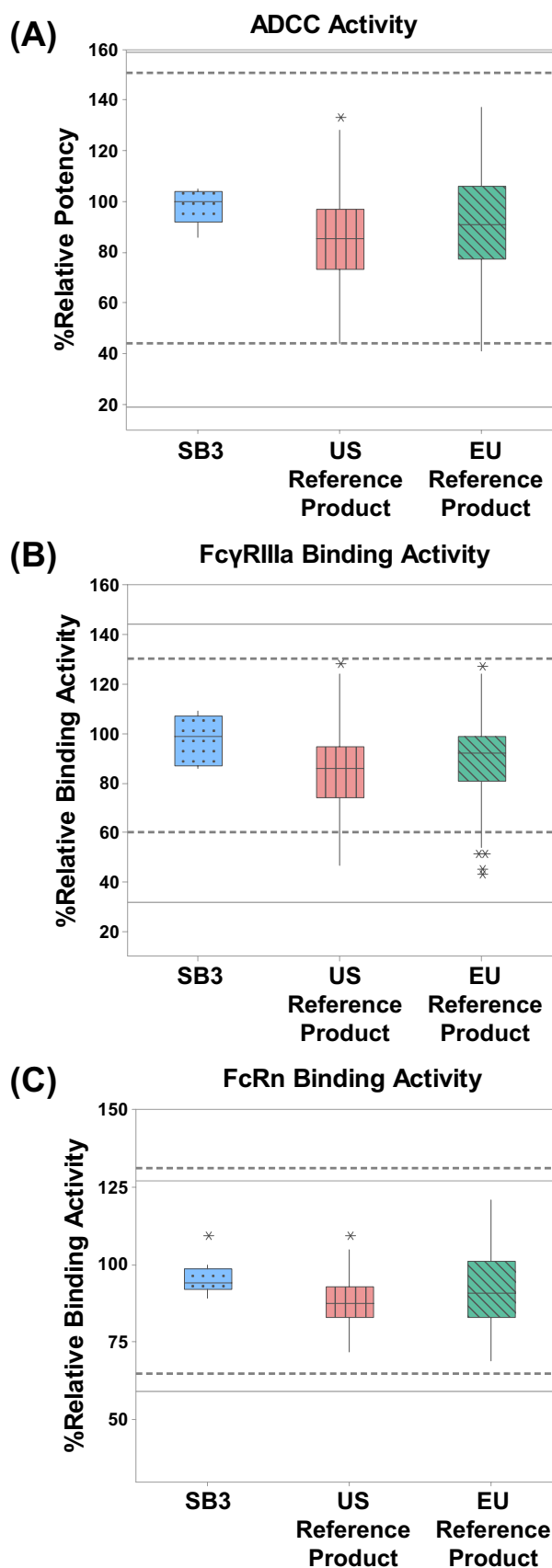
C1q binding activity that induces complement-dependent cytotoxicity (CDC) (Fig. 5).

Taken together, in all the SB3 batches tested, each of the Fc-related biological activities fell within the similarity range. These data strongly support the similarity of SB3 to the reference product in Fc-related as well as Fab-related attributes.

4 Discussion

Extensive analytical characterization is a key component for demonstrating similarity of a proposed therapeutic protein product as a biosimilar to a reference product. To understand the biological quality attributes of trastuzumab, we characterized up to 154 lots of EU- and US-sourced reference product and established reliable similarity acceptance criteria. Based on information obtained about these attributes, SB3 was developed as a trastuzumab biosimilar and approved by the EC as the first trastuzumab biosimilar in the EU. Although the analytical similarity of SB3 was assessed with quality attributes covering a wide spectrum of structural/physicochemical and functional characteristics, this study only includes functional characteristics. Briefly, the majority of biological attributes categorized as CQAs met established similarity acceptance criteria. SB3 was similar to both EU- and US-sourced reference product in Fab-related functional activities, including HER2 binding and anti-proliferation. The similarity of SB3 was also demonstrated in Fc-related attributes including ADCC, Fc γ RIIIa binding and FcRn binding activities. Additionally, other Fc γ R binding activities conformed to the similarity range set by the reference products.

ADCC is an important MoA of trastuzumab and is significantly correlated with the level of the sum of afucosylated glycan and high mannose, as well as Fc γ RIIIa binding activity [7]. By binding of the Fc part of IgG to Fc γ RIIIa (CD16) expressed on natural killer cells, target cell-bound mAbs induce ADCC. Since the affinity of Fc γ R to the Fc part is influenced by *N*-glycosylation, ADCC is enhanced for mAbs with lower fucosylation. The glycosylation pattern has inherent variation from one batch to another, but can also be changed in manufacturing processes [30]. Among the various features of *N*-glycans, the levels of the afucosyl and high-mannose forms (labeled %afucose and %high mannose,



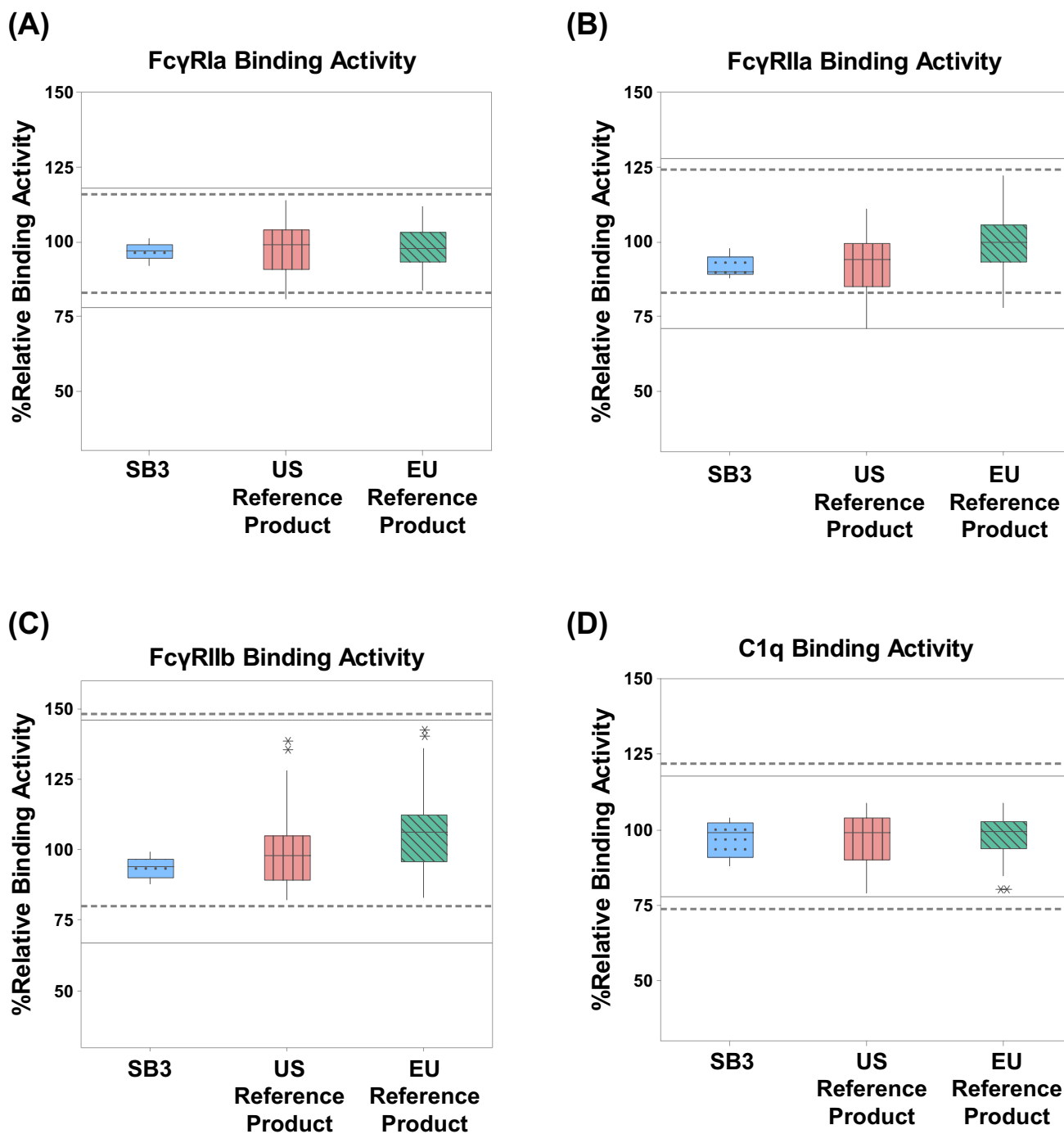


Fig. 5 Binding activities for FcγRs and C1q of SB3 and reference product. Dotted line shows the similarity range ($\text{mean} \pm 3\text{SD}$) of pre-altered EU-sourced reference product. Solid line shows the $\text{mean} \pm 3\text{SD}$ range including pre-altered and altered EU-sourced ref-

erence product. **a** FcγRIa binding activity, **b** FcγRIIa binding activity, **c** FcγRIIb binding activity, and **d** C1q binding activity. FcγR Fcγ receptor, SD standard deviation

respectively) correlate with ADCC and FcγRIIIa binding activities, and the level of galactosylation correlates with CDC and C1q binding activities. As discussed in Kim et al., fluctuations were observed in biological attributes including %afucose + %high mannose, FcγRIIIa binding, and ADCC

in certain lots of reference products. In addition, %galactosylation was also altered, which might affect C1q binding and CDC activities. However, the C1q binding activity of the reference product was not altered during that period, which indicated that the influence of %galactosylation change is

not enough to affect functional activity [21]. Incorporating these fluctuations in setting the quality targets for a similarity assessment could result in broader similarity acceptance criteria. Therefore, the data from these lots displaying changed biological profiles were excluded from establishing the similarity acceptance criteria, resulting in more conservative target quality ranges for SB3. Even with the narrower quality target, the results indicated that SB3 is highly similar to the reference product in the respective Fc-related quality attributes including %afucose + %high mannose, ADCC, and FcγRIIIa binding activity (Figure 5).

Based on the reference product, the recent lots showed biological profile at the pre-change level, which means that even though the cause of the biological profile change is still unknown, the change in biological profiles was transient. The abrupt changes in biological profile also occurred in other marketed products, Rituxan® and Enbrel®, which showed alterations of N-glycan and ADCC activity as well as the reference product. According to the literature, these observed biological profile changes in the products might be caused by changes in the manufacturing processes such as cell production, growth conditions, and purification sequence [30]. However, in terms of the relevance to clinical efficacy, the influence of these alterations in biological profiles in product lots cannot be confirmed without additional clinical studies. Nevertheless, it is critical for assessing the similarity of a biosimilar product that the quality target is established without any meaningful difference from the originally approved molecular entity. As in the case of SB3, any incidental changes in the biological profile of a reference product could become a hurdle with regard to establishing the appropriate quality target, with the potential for a clinically meaningful difference in a developed biosimilar.

In conclusion, SB3 is deemed to show similar efficacy and safety to the reference product in proven indications.

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Compliance with Ethical Standards

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Conflict of interest The authors, Jae Hee Lee, Kyungyeol Paek, Jae Hyon Moon, Sunyoung Ham, Jinsu Song, and Seokkyun Kim, are employees of Samsung Bioepis, and declare that they have no conflict of interest.

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