Original Article

Comprehensive Analytical Characterization of the Proposed Biosimilar Trastuzumab

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ABSTRACT

Background: Biosimilars to monoclonal antibodies were developed to increase the accessibility of this drug and reduce the costs of this therapy without compromising on its quality, safety, and efficacy. The objective of this research is to describe the orthogonal analytical methods used for the physicochemical and biological characterization of the biosimilar proposed to trastuzumab, which is the basis of the biosimilar development program. **Methods:** The orthogonal analytical methods for characterization of the proposed biosimilar to trastuzumab were segregated based on the quality attributes of the product, such as the primary structure was evaluated using peptide mapping and high-end mass spectrometry, secondary and tertiary structure was evaluated using circular dichroism, fluorescence, and absorbance spectroscopy, identity and size heterogeneity were evaluated using Western blotting, size exclusion chromatography and capillary electrophoresis, charge heterogeneity, glycan heterogeneity and hydrophobic heterogeneity were evaluated using by weak cation exchange liquid chromatography, hydrophilic interaction chromatography and reverse phase liquid chromatography, antigen binding and Fc gamma receptor binding was evaluated using surface plasmon resonance, flow cytometry and cell-based enzyme linked immunosorbent assay, antiproliferation and antibody dependent cellular cytotoxicity were evaluated using *in vitro* cell-based bioassay. **Results and Conclusion:** The results of a comprehensive analytical characterization of the proposed biosimilar to the innovator trastuzumab confirm that the physicochemical and biological properties of the proposed biosimilar were comparable to those of the innovator molecule trastuzumab.

Key words: Therapeutic Monoclonal Antibody, Biosimilar, Critical Quality Attributes, Charge Variants, Aggregates, Fragments, Post-translational modifications, N-Linked Glycan and Antibody dependent cellular cytotoxicity.

INTRODUCTION

Overexpression of human epidermal growth factor receptor 2 (HER-2), also known as Receptor tyrosine-protein kinase erbB-2, has been observed in 20–30% of invasive breast cancers involving less chance of recovery and rapid relapse.[1,2] The cancer cells have been shown genetic alteration in expression of oncogenes for their proliferation; therefore, targeted therapies against the over-expressed oncoprotein can be successful even at the late-stage tumour. The studies which prove that anti-HER-2 antibodies inhibit proliferation of HER-2 overexpressing cancer cells were the most significant achievement in cancer therapy and have been the reason for the development of trastuzumab (Herceptin®), an anti-HER-2 recombinant humanized monoclonal antibody that binds to the extracellular domain of HER-2. Trastuzumab has shown better results in overall survival and progression-free survival for women with HER-2 positive breast cancer.[3,4] The main mechanism of action of trastuzumab to enhance the cytotoxicity effect of natural killer (NK) cells on tumours cells overexpressing HER-2. It has also been proposed that the binding of trastuzumab to its ligand leads to HER-2 internalization and degradation.[5]

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Trastuzumab is a monoclonal antibody produced by recombinant technology under the class of humanized immune globulin G (IgG) 1 kappa that targets the extracellular domain of HER-2.[5,6] It was developed by Genentech and gained United States Food and Drug Administration approval in 1998 under the brand name Herceptin®. Herceptin® was developed to bind and inhibit the functional property of the overexpressed HER-2 protein. The trastuzumab is a humanized monoclonal antibody derived from the human IgG amino acid sequence with murine complementarity–determining regions that targets human HER-2 with high specificity was expressed in suspension Chinese Hamster Ovary cell line. The drug product of trastuzumab was a lyophilized powder with white to pale yellow, preservative-free packed under sterile conditions used for intravenous administration. Recently, a new injectable (subcutaneous) formulation of Herceptin® was approved by the European Commission for the treatment of HER-2 positive breast cancer, an aggressive subtype of the disease.[7] Approval is for both the early and later stages of treatment.

Biosimilars or follow-on biologics are protein-based therapeutic products that are near-identical (similar), comparable and equivalent to the branded therapeutic product.[8] The major challenge in the development and manufacture of biosimilars lies in the molecule itself. Unlike small-molecule generics, biosimilars have been developed using recombinant deoxyribonucleic acid technology in living systems and extracted through complex purification techniques.[9-11] Therefore, the level of biosimilar characterization is not as simple as generic drugs.[11] Even minor changes in critical quality attributes of a biosimilar will impact the quality, safety, and efficacy of the product. The orthogonal high-end analytical characterizations are necessary for the biosimilar to match the innovator product in a clinical study.[12] The critical process parameters which affect the critical quality attributes of a biosimilar should be identified and controlled as it will impact immunogenicity, pharmacokinetics, safety and efficacy. The critical quality attributes like glycosylation, product-related substance or impurities will impact both the structural and functional properties of monoclonal antibodies.[13] Overall, the complete knowledge of the product, manufacturing process and orthogonal high-end analytical characterization are needed for biosimilar molecule development.

Several study reports on biosimilars to trastuzumab have been published in literature.[14-16] The proposed biosimilar to trastuzumab (Herceptin® or Herclon®, Roche) has been developed using a mammalian expression system, and an efficient process has been standardized to express the proposed biosimilar and purify with higher recovery from fermentation broth.[14] The analytical comparison of the proposed biosimilar to the innovator product was executed in multiphase to assess structure, function and its potential variants. This current manuscript talks about in-depth analytical characterization data of the proposed biosimilar to that of the innovator molecule Herclon®.

MATERIALS AND METHODS

The following analytical methods like sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), western blot, reverse phase high performance liquid chromatography (RP-HPLC) of intact and reduced, size exclusion chromatography (SEC), Weak Cation Exchange-Liquid Chromatography (WCX-LC), N-Glycan analysis by hydrophilic interaction chromatography (HILIC), Flow cytometry analysis, surface plasmon resonance (SPR) binding kinetics, cell-based enzyme linked immunosorbent assay (ELISA) and anti-proliferation assay, which was previously described[14] was used for the characterization of the proposed biosimilar and the innovator trastuzumab.

Peptide mapping analysis by High Performance Liquid Chromatography

The samples were denatured with 5.7 M guanidine (Sigma-Aldrich) and 0.119 M Trizma base pH 7.8 (Sigma-Aldrich), reduction with 2 mM Cleland's reagent (Sigma-Aldrich), alkylation with 7.7 mM sodium iodoacetate (Sigma-Aldrich) and cleavage of specific peptide bonds with trypsin (Promega) in 1:25 enzyme-substrate mixture at 37°C for 18 hr. Before trypsin digestion, the samples were buffered with 100mM NH_4HCO_3 buffer pH 7.8, and after trypsin digestion; the samples were quenched with trifluoroacetic acid (Sigma-Aldrich) to a final concentration of 0.1% in sample. The peptides obtained from the treatment with trypsin endopeptidase were analyzed by the Waters Alliance high performance liquid chromatography system using an octadecylsilane column with particle size 5 µm, length 250 mm and internal diameter 2.1 mm. The mobile phase was 0.1% trifluoroacetic acid (Sigma- Aldrich) in filtered water for injection (WFI) and acetonitrile gradient (Merck). The eluted peptides were detected using 214 nm ultra-violet (UV) absorbance detector.[17]

Amino acid sequence analysis by Tandem Mass Spectrometry

The denatured, reduced, alkylated, and Trypsin-digested samples were separated on a 1260 Infinity Bioinert Quaternary liquid chromatography system (Agilent Technologies, India) using a Waters BEH C_{18} 2.1 × 150 mm reverse phase column connected to an Agilent 6550 electrospray ionization quadrupole time-of-flight mass spectrometry (MS) instrument. The amino acid sequence analysis was performed by monitoring the spectra in the range of 50–3200 m/z with a scanning time of 0.5 s in the positive ion mode. The capillary gas temperature/voltage and fragmentor voltage were set to 300°C, 4500 V, and 300 V, respectively. MS/MS-data were analysed manually using Agilent MassHunter BioConfirm data analysis software, which performed automated data extraction, sequence matching and sequence coverage calculation.[18]

Circular Dichroism Spectroscopy

The higher-order structural comparison of the proposed biosimilar to the innovator trastuzumab was assessed by far UV and near UV circular dichroism (CD) spectroscopy using a JASCO J-810 CD Spectrophotometer. Far-UV CD spectra (190-250 nm) were used to evaluate secondary structure similarity, and near-UV CD spectra (260-350 nm) were used to evaluate tertiary structure similarity. The path lengths for the far-UV CD spectra and near-UV CD spectra were 0.1 cm and 1.0 cm, respectively, with a sample temperature of 25°C in both analyses. The samples were diluted in filtered WFI to 0.1 mg/ml and 1.0 mg/ml for Far UV and Near UV CD spectroscopy, respectively.[19]

Fluorescence Spectroscopy and Absorbance Spectroscopy

The comparison of the tertiary structure of the innovator trastuzumab and the proposed biosimilar was evaluated using orthogonal techniques such as intrinsic and extrinsic fluorescence spectroscopy and absorbance spectroscopy techniques. The tertiary structure of trastuzumab innovator and proposed biosimilar (diluted to 0.5 mg/ml concentration) was evaluated by comparing the intrinsic fluorescence contributed by the indole ring of tryptophan. The samples were excited at 260 nm with a slit width of 1.0 nm, and the emission spectra were collected from 300 to 400 nm.

The tertiary structure contributed by the exposed hydrophobic pockets of standard and sample (diluted to 1.0 mg/ml concentration) was evaluated by incubating them with 2 µM concentration of ANS (8-Anilino-1-Naphthalene Sulfonate) dye and comparing the extrinsic fluorescence scans with an emission range from 410 to 600 nm. The excitation of the dye was performed at 380 nm.

The tertiary structure of trastuzumab innovator and proposed biosimilar (diluted to 0.5 mg/ml) was evaluated by comparing the UV absorption spectra scan contributed by the aromatic amino acids (tryptophan and tyrosine) by spectral scans in range from 250 to 400 nm.[20,21]

Capillary Electrophoresis Sodium Dodecyl Sulfate (Non-Reducing and Reducing)

The analysis was carried out using 50 µg of protein sample that was alkylated with iodoacetamide and heat denatured in the presence of SDS at 70°C for 10 m. In the case of Capillary electrophoresis sodium dodecyl sulfate (CE-SDS) Reducing analysis, 50 µg of protein sample was reduced with beta-mercaptoethanol and heat-denatured in the presence of SDS at 70°C for 10 m. Both non-reducing and reducing samples were analyzed using Beckman Coulter PA800 Plus instrument. A bare fused silica coated capillary with 50 cm x 30 µm with an effective length of 40 cm was used for analysis. Samples were injected electro-kinetically using reverse polarity and separated using -15 kV; absorbance was monitored at 220 nm.[22]

Isolation of Peripheral Blood Mononuclear Cells from Blood

The whole blood samples from healthy donors were collected. The whole blood was diluted with 1X DPBS (Invitrogen) in a 1:1 ratio. After dilution, peripheral blood mononuclear cells (PBMC) were isolated using Histopaque (Sigma, MO) in Sepmate tubes (Cell Technologies) according to the manufacturer's recommendations. PBMC (Buffy coat layer) were collected from the tube and rinsed with 1X DPBS for 5 m at 1000 rpm. Isolated PBMC were maintained in RPMI 1640 assay media.

Antibody Dependent Cellular Cytotoxicity assay using Peripheral Blood Mononuclear cells

BT-474 cells (target cells / breast cancer cells) were seeded at a density of 10,000 cells / well and 50 µl of varying concentrations of trastuzumab innovator and proposed biosimilar in the range of 5 pg/ml to 5 µg/ml were added to the cells. They were incubated at 37°C for 30 m in a CO_2 incubator. Thereafter, fresh/ frozen PBMC were added to the wells at a ratio of 1: 10 (target: effector ratio). The plate was further incubated for 6-7 hr in a CO_2 incubator at 37°C. After incubation with effector cells, cytotoxicity was measured using CytoTox-Glo™ kit (Promega) according to the

manufacturer's protocol. The Luminescence reading was taken using a Synergy HT microplate reader. Dose-response curves we reanalysed using Graph Pad Prism Software. Two controls were maintained in the assay: low cytotoxicity control containing target cells and PBMC and high control where the target cells-effector mixture was subjected to lysis using lysis reagent provided with the kit.[23]

All experiments were carried out in duplicate, and the average of the luminescence reading was considered for the calculation of cytotoxicity. The percentage of specific cytolysis was calculated using the formula: (sample luminescence reading - low control)/(high control - low control)*100.

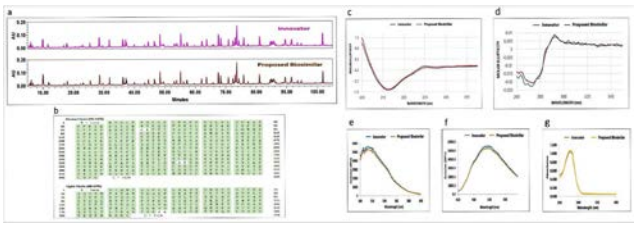

Figure 1: Structural characterization of the proposed biosimilar using trastuzumab innovator. (a) Overlapping Tryptic peptide mapping chromatogram of trastuzumab innovator and proposed biosimilar obtained from reverse phase chromatography, (b) Amino Acid sequence coverage of both heavy chain and light chain of proposed biosimilar by LC-MS/MS method, (c) Far UV CD spectra, (d) Near UV CD spectra, (e) Intrinsic Fluorescence spectra, (f) Extrinsic Fluorescence spectra and (g) UV Absorbance Spectra.

RESULTS

Primary Structure Assessment

It is absolutely essential to have the same amino acid sequence to prove the identity in the primary structure of the molecule. The primary structures of the proposed biosimilar and the innovator trastuzumab were compared using the peptide mapping by the RP-HPLC method detected with UV absorbance showing similarity in the peptide peak profile between each other, which confirms that there is no major significant change in the primary structure of the proposed biosimilar. The complete coverage of the amino acid sequence for the proposed biosimilar against the theoretical amino acid sequence of the heavy chain trastuzumab and the light chain was performed by peptide mapping in combination with liquid chromatography mass spectrometry (LC/MS) and we concluded that more than 99% of the amino acid sequence was covered for both the heavy chain and the light chain. These results confirm that no significant differences in the primary structure were detected between the proposed biosimilar and the innovator trastuzumab (Figure 1a, 1b).

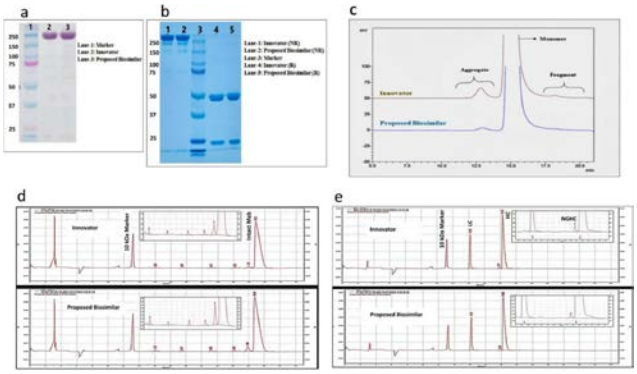

Figure 2: Electrophoretic and Size exclusion chromatographic analysis of trastuzumab innovator and proposed biosimilar for Identity and similarity based on size. (a) Western Blot Image, (b) SDS-PAGE (Non-reducing and Reducing conditions) image, (c) Chromatogram of Size exclusion chromatography, (d) Electropherogram of CE-SDS Non-reducing condition showing intact monoclonal antibody and (e) Electropherogram of CE-SDS Reducing condition showing light chain (LC), heavy chain (HC) and non-glycosylated heavy chain (NGHC).

Higher-Order Structure Assessment

The overall structure of trastuzumab defines the efficacy of the molecule. To determine the higher-order structure CD spectroscopy along with fluorescence and absorbance spectroscopy analysis was carried out. CD analysis of the proposed biosimilar at the far-UV range suggested the dominance of beta-sheet secondary structural conformation as the innovator due to single minima at 217nm in both. The estimated secondary structure in terms of Alpha helix and Beta sheets using K2D2 software (publicly accessible) for the proposed biosimilar, and the trastuzumab innovator was 3.28% and 3.25% containing alpha-helix and 42.71% and 42.72% containing beta-sheets, respectively, which confirms the secondary structure similarity of the proposed biosimilar compared to the trastuzumab innovator (Figure 1c).

The overlapping spectroscopic profiles of the proposed biosimilar to the trastuzumab innovator in the following techniques, such as near UV range CD spectroscopy, fluorescence (intrinsic and extrinsic) spectroscopy, and UV absorbance spectroscopy, indicate that the disulfide bonds and aromatic amino acids were in the correct conformation because of proper folding of the proteins, thereby confirming the tertiary structure similarity between the proposed biosimilar and the trastuzumab innovator (Figure 1d, 1e, 1f). In addition to the primary structure and higher order structure, the identity of the biosimilar proposed as trastuzumab was also confirmed using the Western blot technique (Figure 2a).

Size-based Purity Assessment

In a head-to-head biosimilarity exercise, the highly similar migration in Coomassie Blue-stained SDS-PAGE gel confirms the size similarity and absence of size variants in the proposed biosimilar and trastuzumab innovator under Non-Reduced and Reduced conditions (Figure 2b). Size exclusion chromatography is another powerful orthogonal technique for the detection of multimer proteins. The proposed biosimilar and the trastuzumab innovator were evaluated using SEC that showed a prominent monomer peak of around 99%, free of any aggregates. The reduced CE-SDS technique was used to estimate the non-glycosylated heavy chain percentage with the percentage of light chain and heavy chain in monoclonal antibodies. Similar percentages of non-glycosylated heavy chain, light chain, and heavy chain were observed in both the proposed biosimilar and the trastuzumab innovator (Table 1). The combination of SDS-PAGE (Non-Reduced and Reduced), CE-SDS (Non-Reduced and Reduced), and SEC techniques confirmed similarity in purity based on size between the proposed biosimilar and the trastuzumab innovator (Figure 2b, 2c, 2d, 2e).

Charge Variant and Hydrophobicity Analysis

The WCX-LC was carried out to compare the charge profile between that of the proposed biosimilar and the trastuzumab innovator. The chromatographic profile of WCX-LC analysis of trastuzumab innovator and the proposed biosimilar confirms that the proposed biosimilar has fewer charge variants in both acidic and basic forms compared to innovator (Table 1 and Figure 3a). The proposed biosimilar and the innovator trastuzumab shown an overlapping RP-HPLC chromatographic profile, which confirms the similarity in hydrophobicity between the proposed biosimilar and the innovator trastuzumab under reduced and intact conditions (Figure 3a, 3c, 3d).

Table 1: Results of size variants (from SEC and CE-SDS) and charge variants (from WCX-LC) between trastuzumab innovator and proposed biosimilar which was obtained from the average value of duplicate injections were reported.

Analytical Methods	Quality Attributes	trastuzumab innovator	proposed biosimilar
SEC	% Aggregates	0.49	0.10
	% Monomer	99.41	99.85
	% Fragments	0.10	0.05
CE-SDS (Reduced)	% Light Chain	32.48	32.43
	% NGHC	0.69	0.18
	% Heavy Chain	66.83	67.39
CE-SDS (Non-Reduced)	% LMW	2.13	5.54
	% Intact	97.87	94.46
WCX-LC	% Acidic Variants	30.49	9.66
	% Main Peak	57.86	83.86
	% Basic Variants	11.66	6.49

NGHC: *Non-glycosylated heavy chain;* ***LMW:*** *Low molecular weight.*

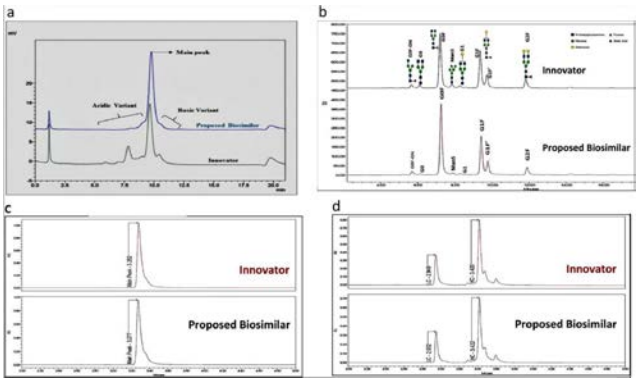

Figure 3: Charge based heterogeneity, hydrophobic heterogeneity and released glycan characterization of trastuzumab innovator and proposed biosimilar. (a) Chromatogram of acidic, main and basic variant analysis by Weak cation exchange liquid chromatography, (b) Chromatogram of released glycan profiling by Hydrophilic interaction chromatography, (c) Chromatogram of Reverse phase liquid chromatography Non-reducing condition, (d) Chromatogram of Reverse phase liquid chromatography Reducing condition.

N-Linked Glycosylation Analysis

Glycan analysis: Glycans play a critical role in determining the efficacy, immunogenicity, and pharmacokinetics of monoclonal antibody. The enzyme N-glycosidase F was used to release the N-glycans from the monoclonal antibody labeled with fluorophore and was analysed using a HILIC column. Only the major glycan variants like G0F-GN, G0, G0F, Man-5, G1, G1F, G1F' and G2F were monitored as other minor glyco-forms were relatively low and had no significant impact on the product. The total galactose-containing glycans determined from the major glyco-forms in the proposed biosimilar and the innovator trastuzumab were 45.9% and 51.4% respectively. Similarly, the total afucosylated glycans determined from the major glyco-forms in the proposed biosimilar and the innovator trastuzumab were 2.6% and 5.6%, respectively (Table 2). These minor differences have no significant impact on binding to HER-2 as well as the antiproliferative activity of trastuzumab and binding to CD16a as well as the antibody dependent cellular cytotoxicity (ADCC) assay of trastuzumab which did not impact the function of the antibody (Figure 3b).

Table 2: Results of Major Glycan variants between trastuzumab innovator and proposed biosimilar obtained from the average value of duplicate injections were reported.

% Major Glycan Variants	trastuzumab innovator	proposed biosimilar
G0F-GN	2.47	2.57
G0	2.33	1.04
G0F	40.05	48.50
Man5	3.76	1.95
G1	2.41	1.16
G1'	0.83	0.44
G1F	29.09	27.27
G1F'	11.14	11.28
G2F	7.92	5.80
Afucosylation (G0+G1+G1')	**5.6**	**2.6**
Galactosylation (G1+G1'+G1F+G1F'+G2F)	**51.4**	**45.9**

Table 3: Results of HER-2 and CD16a (V158) binding affinity (K_D) by SPR between trastuzumab innovator and proposed biosimilar was obtained from five different concentrations of the respective sample.

HER-2 Binding Kinetics by SPR			CD16a (V158) Binding Kinetics by SPR		
Kinetic Parameters	trastuzumab innovator	proposed biosimilar	Kinetic Parameters	trastuzumab innovator	proposed biosimilar
k_a ($10^5 M^{-1}s^{-1}$)	8.6	9.7	k_a ($10^4 M^{-1}s^{-1}$)	5.3	1.9
k_d ($10^{-5}s^{-1}$)	1.1	1.5	k_d ($10^{-3}s^{-1}$)	9.8	5.8
R_{max} (RU)	130	126	Rmax (RU)	58	41
K_D (10^{-12}M)	**12**	**15**	**K_D (10^{-6}M)**	**0.19**	**0.31**
Chi^2 (RU^2)	13.8	18.5	Chi^2 (RU^2)	0.6	0.4

Functional Properties Assessment

A method to assess the kinetics of the proposed biosimilar and innovator trastuzumab to HER-2 binding has successfully been developed and qualified using BIACORE instrument from GE. The association and dissociation rate constants ka (on rate) and kd (off rate) were measured for the binding kinetics of anti-HER-2 monoclonal antibody towards the recombinant extracellular domain of HER-2 antigen using Biacore. The ratio between the dissociation and association rate constants was calculated to determine the equilibrium dissociation constant (K_D). Both samples demonstrated very strong binding to HER-2 and the affinity was observed to be 12 picomolar and 15 picomolar for trastuzumab innovator and proposed biosimilar respectively (Table 3).

The ADCC mechanism of action will occur when the tumour cells and immune cells were come into contact through trastuzumab Fab binding to HER-2 of tumour cells and Fc binding to Fcγ receptors expressed on all immune cells. The affinity between the Fcγ receptors and the trastuzumab has a significant impact on ADCC activity. Therefore, binding to receptors Fcγ CD16a was compared between the proposed biosimilar and innovator trastuzumab using Biacore has similar binding constants in 0.31 micromolar and 0.19 micromolar respectively (Table 3). The Biacore data for HER-2 and CD16a binding are shown in Figure 4a, 4b. The ligand binding affinity of HER-2 receptors expressed on BT474 was also carried out through a flow cytometry-based assay in which cancer cells were incubated with biotinylated proposed biosimilar and the trastuzumab innovator. The level of receptor binding was assessed by probing streptavidin tagged with fluorescein isothiocyanate (FITC) and measuring the fluorescence level using a flow cytometer. A clear shift in the peak observed for the proposed biosimilar and innovator trastuzumab in comparison to both cell and negative control (where rituximab was used as biotinylated substrate), indicating the binding specificity of the proposed biosimilar and innovator trastuzumab to HER-2 receptors. The overlay of the histogram data for proposed biosimilar and innovator trastuzumab along with similar peak shift and peak intensity, indicated that the proposed biosimilar had comparable binding efficacy to that of the innovator trastuzumab (Figure 4c). Comparable binding of the proposed biosimilar with that of innovator trastuzumab was further confirmed by performing Cell-based ELISA where the plate was coated with BT474 cell line and a dose-response curve was observed (Figure 4d). The relative binding potency of the proposed biosimilar with respect to innovator trastuzumab was 95.5% which confirms the similarity by BT474 Cell based ELISA. The proliferation of BT474 breast cancer cell line was inhibited by trastuzumab by preventing the dimerization of HER-2 surface protein expressed on BT474 breast cancer cell line. A comparable potency was observed in the proposed biosimilar with that of the innovator trastuzumab, which showed dose-dependent inhibition of the BT474 breast cancer cell line (Figure 4e). The relative potency of the anti-proliferation assay

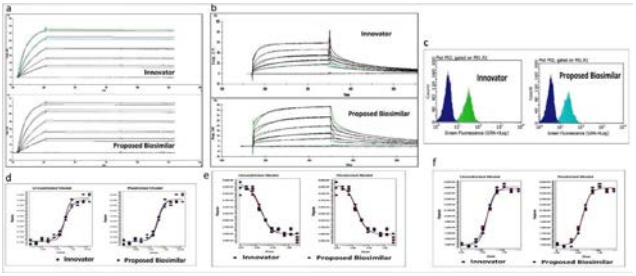

Figure 4: HER-2, CD16a (V158) binding analysis and functional assay of trastuzumab innovator and proposed biosimilar using orthogonal analytical techniques. (a) SPR Sensogram of HER-2 binding kinetic analysis, (b) SPR Sensogram of CD16a (V158) binding kinetic analysis, (c) BT-474 binding analysis by Flow Cytometry, (d) BT-474 Cell based binding ELISA dose response curve. (e) BT-474 antiproliferation assay dose response curve, (f) BT474 Antibody dependent cellular cytotoxicity assay using PBMC effector cell dose response curve.

of the proposed biosimilar with respect to innovator trastuzumab was 103.4% which confirms the similarity in anti-proliferation of BT474 cells. Trastuzumab lyses the target cell through an ADCC mechanism. The Fab part of trastuzumab binds to HER-2, which is expressed on tumour cells and the Fc region of trastuzumab activates effector cells (NK cells) through CD16a (Fc gamma RIIIa) cell surface receptor, activated NK cells kill the tumour cells by releasing perforins and granzymes. A similar cytotoxic potency was observed between the proposed biosimilar and the innovator trastuzumab when it was added to premixed BT474 cells and PBMC in a dose-dependent manner (Figure 4f). The relative potency in ADCC assay of the proposed biosimilar with respect to innovator trastuzumab was 93.8% which confirms the similarity in the antibody dependent cellular cytotoxicity of BT474 cells using effector cells.

DISCUSSION

In order to prove biosimilarity to the innovator product, it is essential to use effective orthogonal analytical tools which will determine the structural properties of the target molecule and the functional properties of the molecule. The structure and function of trastuzumab determine the *in vivo* physiological response and must therefore be controlled. Critical molecular attributes (commonly termed as "quality attributes") that are responsible for the *in vivo* physiological response to trastuzumab are (a) the amino acid sequence (identity) for correct folding required for trastuzumab HER-2 interaction, (b) high-molecular weight variants / aggregates (purity), (c) the overall higher order structure which serves as the scaffold for correct positioning of the binding sites, (d) receptor binding reflecting the integrity of the overall structure and the binding sites, (e) *in vitro* proliferation assay (potency) for demonstrating activation of downstream signalling cascades as a result of full structural integrity of the molecule, and (f) antibody dependent cellular cytotoxicity (potency) for demonstrating NK cell-mediated cytotoxicity of target cell that is bound by trastuzumab. In designing analytical methods, an emphasis was laid on evaluating the quality attributes that are known to have clinical relevance, as outlined in Table 4. The analytical comparison of proposed biosimilar to the innovator product trastuzumab was executed in multiphase to assess structure, function and its potential variants. The analysis was carried out using the drug product of the proposed biosimilar. As a first step, a comparison of the structure of proposed biosimilar to the trastuzumab innovator product was evaluated to match the amino acid sequence and protein folding patterns. Once the structural similarity was proved, functional activities were evaluated to establish that target antigen binding and Fc receptor binding properties and efficacy are also similar.

The overall size of a protein and its size variants can be assessed by the use of multiple complementary techniques, including size exclusion chromatography, CE-SDS (Non-Reduced and Reduced) and SDS-PAGE (Non-Reduced and Reduced). Examples of size variants are dimers, oligomers, aggregates, non-glycosylated heavy chain and lower molecular weight fragments generated during synthesis in the cell. Aggregates are known to cause immunogenicity and are therefore tightly controlled in protein-based therapeutics. The non-Reduced CE-SDS method is one of the best tools to estimate and identify lower molecular weight impurities in monoclonal antibodies. The results of non-Reduced CE-SDS showed a minor increase in low molecular impurities in the proposed biosimilar compared to the trastuzumab innovator with no additional impurities compared to each other. This slight increase in the low molecular impurities of proposed biosimilar does not affect the binding property and the efficacy of functional assays in the following characterization proves that it is well within the limit of similarity.

Charge heterogeneity was an inherent property of monoclonal antibodies. These heterogeneities of monoclonal antibodies were generated either inside the cell or outside the cell during the purification process and storage stability. The charge heterogeneity occurred either enzymatic and non-enzymatic, which includes disulfide bonds mismatch, glycosylation, N-terminal glutamine cyclization, C-terminal lysine

Table 4: Overview of quality attributes assessment and analytical methods selection strategy performed for biosimilar characterization.

Quality Attributes	Critically	Relevant for	Methods used
Amino acid sequence	Very high	Efficacy, safety and immunogenicity	Edman, Peptide Mapping by LC-MS
Potency	Very high	Efficacy, safety	Antiproliferation assay against the human breast cancer cell line
Effector Function	Very high	Efficacy, safety	SPR assay for CD16a binding, ADCC assay against human breast cancer cell line (PBMC effector cells)
Target binding	Very high	Efficacy, safety	SPR assay for HER-2 binding, Human breast cancer cell line binding ELISA, and flow cytometry analysis.
Protein concentration	Very high	Efficacy	Absorbance 280 nm
Protein structure	High	Efficacy, immunogenicity	UV, CD, DSC, Fluorescence spectroscopy
Aggregates and Fragments	High	Efficacy, immunogenicity	Size exclusion chromatography and CE-SDS (NR)
Charge Heterogeneity (Glycation, Deamidation, Isomerization)	Very High	Efficacy	WCX-LC, CE-IEF
Glycosylation	Very High	Efficacy, immunogenicity, clearance	Released glycan analysis by NP-UPLC, CE-SDS (R) for determination of non-glycosylated heavy chain.

processing, deamidation, oxidation, glycation, and peptide bond cleavage. Charge heterogeneity has been shown to affect the efficacy and stability of monoclonal antibodies. The carboxypeptidase-B enzyme was used in this process to remove C-terminal lysine variants in the proposed biosimilar product and the clearance of carboxypeptidase-B was also confirmed using a kit-based assay. The RP-HPLC analysis of IgG1 monoclonal antibody will have a single major peak under intact conditions and a light chain and heavy chain peak under reduced conditions.

Binding affinities between trastuzumab and HER-2 reflect the structural integrity of the molecule as well as the correct three-dimensional conformation generated by the amino acid residues within the target binding regions.

Overall, the similarity in functional assays between the proposed biosimilar and the innovator trastuzumab has been demonstrated through orthogonal analytical methods, which further indicates that the proposed biosimilar was structurally intact and in the required conformation.

CONCLUSION

The developed Proposed Biosimilar was well characterized against the innovator trastuzumab with regard to its structural and functional characteristics, using orthogonal high-end analytical methods, and confirms the similarity in an in-depth comparability exercise. Overall, the quality of the proposed biosimilar was considered to be in line with the quality of the innovator trastuzumab. The comprehensive analytical program that has been conducted confirms that the proposed biosimilar was similar to innovator trastuzumab (Herceptin) with regard to the sequence and folding structure. Through different orthogonal efficacy analyses, it has also been confirmed that the proposed biosimilar functions in the same pathway as that of the innovator. These structural and functional similarity data give the totality of evidence to conduct non-clinical and clinical studies without any uncertainty and exhibits similar pharmacological profiles.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

ABBREVIATIONS

ADCC: Antibody dependent cellular cytotoxicity; **ANS:** 8-Anilinonaphthalene-1-sulfonic acid; **CD:** Circular dichroism; **CE-SDS:** Capillary electrophoresis sodium dodecyl sulfate; **ELISA:** enzyme-linked immunosorbent assay; **HER-2:** Human epidermal growth factor receptor 2; **HILIC:** Hydrophilic interaction liquid chromatography; **HPLC:** High Performance Liquid Chromatography; **MS:** Mass spectrometry; **PBMC:** peripheral blood mononuclear cells; **SDS-PAGE:** sodium dodecyl sulphate polyacrylamide gel electrophoresis; **SEC:** Size-exclusion chromatography; **SPR:** Surface plasmon resonance; **UV:** Ultraviolet; **WCX-LC**: Weak cation exchange liquid chromatography.

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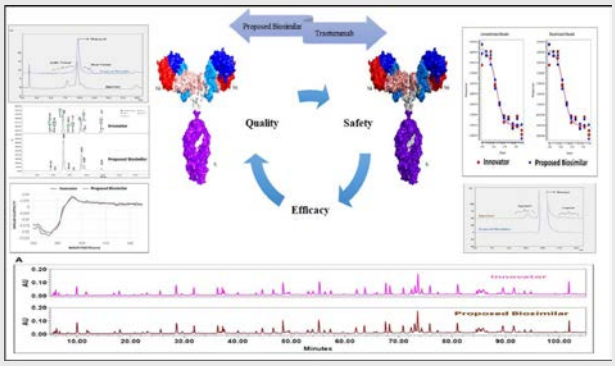
PICTORIAL ABSTRACT



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SUMMARY

The biosimilar market has grown tremendously in the last decade, and it is estimated that a major part of biosimilars in development are monoclonal antibodies. Manufacturing a monoclonal antibody is a complex and sophisticated process, so orthogonal analytical characterization is vital for the development of biosimilar monoclonal antibodies. The main aim of the analytical characterization is to evaluate the physicochemical and biological characteristics of the biosimilar comparison with the innovator. Here, the proposed biosimilar to Herceptin® (Trastuzumab), which was developed in accordance with all regulatory guidelines, was characterized by comprehensive analytical methods for its structural and functional similarity. The analytical comparison of the proposed biosimilar to the innovator product was executed in multiphase to assess its structure, function, and potential variants. As a first step, a comparison of the structure of biosimilar to the innovator product was evaluated to match the amino acid sequence and protein folding patterns. Once the structural similarity was proved, functional activities were evaluated to establish that target antigen binding and Fc receptor binding properties and efficacy are also similar. Several orthogonal *in vitro* potency assays were performed, including antiproliferation, ADCC, and HER-2 binding ELISA to assess the functional properties of trastuzumab. The comprehensive analytical program confirmed that the proposed biosimilar was similar to the innovator with regard to the sequence and folding structure. Through different orthogonal efficacy analyses, it was also verified that the proposed biosimilar functions in the same pathway as that of the innovator molecule.

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