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Review Article

TECHNICAL GUIDANCE ON THE PHYSICOCHEMICAL AND FUNCTIONAL COMPARABILITY EXERCISE FOR TRASTUZUMAB BIOSIMILARS

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ABSTRACT

The introduction of monoclonal antibodies (mAbs) into the field of oncology has revolutionized the treatment options available to clinicians for the treatment of several malignancies. Unlike their classical small molecule drug counterparts, mAbs are large complex biological molecules that are generated using recombinant engineering and produced through the use of living systems. The high complexity of these agents, combined with the complexity of their manufacturing process, poses significant challenges for the pharmaceutical industry in producing exact copies of the originator molecules. With several mAbs losing their patency in recent years, several pharmaceutical manufacturers are pursuing the development of mAb copies or what is known as biosimilars as generic copies to the originator mAbs. Developing a mAb biosimilar requires that the manufacturer performs an extensive comparability exercise between the originator mAb and its biosimilar to provide evidence that the biological copy is similar to the originator in regards to physicochemical and functional properties, non-clinical pharmacodynamics and immunogenicity, and finally, clinical trials to ensure the safety and efficacy of the biological molecule. The inability to perform a high-quality similarity exercise could generate inferior biological copies or what is known as intended copies. Trastuzumab is a humanized mAb that was designed to target HER 2 receptors which are highly expressed in a variety of tumors, including 25-30% of invasive breast carcinomas. The aim of this review is to provide technical guidance regarding the physicochemical and functional similarity exercise for pharmaceutical personnel working in the research and development of Trastuzumab biosimilars in addition to regulatory officer's worldwide reviewing biosimilars dossiers within public health authorities. This data will provide valuable information in detailing the main quality parameters needed to demonstrate the analytical similarity of any Trastuzumab biosimilar to its reference product.

Keywords: Trastuzumab, Biosimilars, Similarity, Biological comparability, Guidance

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INTRODUCTION

The introduction of monoclonal antibodies (mABs) to the field of oncology has revolutionized the therapeutic options available to clinicians for the treatment of cancer [1, 2]. Due to their high target selectivity, mAbs represent a significant advancement in the field of cancer therapeutics as they target specific molecular targets responsible for cancer pathogenesis without targeting normal cells and consequently having fewer side effects when compared with the classical cytotoxic chemotherapeutic counterparts [3, 4]. Trastuzumab is a humanized mAb that was designed to target HER 2 receptors which are highly expressed in a variety of tumors, including 25-30% of invasive breast carcinomas [5, 6]. HER2 signaling is involved in promoting cell proliferation and cell death and overexpression of the receptor enhances cell growth, tumor survival, angiogenesis and, metastasis [7, 8]. Trastuzumab binds to the extracellular domain of HER2 receptors inhibiting its tyrosine kinase-mediated intracellular cell signaling and activating antibody-dependent cellular cytotoxicity [9, 10]. Additionally, Trastuzumab inhibits HER2 dimerization and is responsible for cell cycle arrest and induction of apoptosis [11]. These events collectively inhibit HER2 overexpression and tumor proliferation leading to positive therapeutic outcomes in all clinical oncological parameters of breast cancer such as objective response, overall response, and time to disease progression [12, 13].

Trastuzumab belongs to the class of biopharmaceutical agents or what is known as biological therapeutics [14]. Unlike their small-scale classical chemical drug counterparts, biopharmaceuticals are generated using living cell systems and are more complex in nature [15]. The complexity is related to the nature of these molecules as they represent large proteins with intrinsic structural complexities related to their three-dimensional structure and their pharmacodynamic activity, which is highly dependent on the exact conformation and tertiary structure of the protein [16]. Additionally, the manufacturing process of biopharmaceuticals is highly complex with several critical process parameters that require high stringent monitoring, control, and consistency to ensure the quality of the biological therapeutics [17]. Trastuzumab (Herceptin®) is a mAb therapeutic that is developed, manufactured and produced using phage display technology and genetically modified Chinese hamster ovary cells (CHO) as the biological production platform for the upstream processing of the molecule [18]. The originator, Trastuzumab lost its patency in 2019 and consequently paved the way for any pharmaceutical manufacturer to develop its own generic copy of Trastuzumab or what is known as a biosimilar [19]. The regulatory pathway for the registration of mAb biosimilars is different from classic small-molecule generics. In principle, for a generic small molecule to be approved by regulatory authorities, it has to demonstrate that the molecule is pharmaceutically equivalent to the originator molecule in regards to the active ingredient's identity, purity, related substances, and degradation profile [20]. Additionally, the molecule has also to be bioequivalent to the originator molecule in relation to its pharmacokinetic properties without the need to demonstrate the efficacy or safety of the molecule using non-clinical or clinical validation methods [21]. This, of course, causes a dramatic decrease in regulatory development requirements of generic pharmaceuticals and consequently lowers the prices of these medications significantly. The situation for mAb biosimilars or biological generics is rather different as the complexity of the molecule and its production platform poses significant challenges in providing evidence that the biosimilar is clinically equivalent to its originator using standard analytical and bioequivalence analytical techniques [22]. Due to the urgent need of developing a pathway for biosimilar development and regulatory approval, the European Medicines Agency agency (EMA) and the U. S Food and Drug Administration (FDA) have set a specific technical, regulatory pathway for biosimilar registration and approval which resulted in the approval of EU's first biosimilar (Epoitein alpha) in 2007 and the approval of filgrastim in the US as the first biosimilar agent in 2015 [23]. Due to Trastuzumab's significant clinical benefits and positive outcomes in the treatment of breast cancer, several pharmaceutical companies have developed or/are currently

developing Trastuzumab biosimilars with varying degrees of quality. This might introduce several biological copies with inferior quality to the originator Trastuzumab in what is known as biomimetics or intended copies [24, 25]. These agents do not meet the minimal quality standards that are needed to demonstrate the biological copy's similarity to the originator molecule and could pose a serious risk to the patient population receiving these medications due to their lack of efficacy or significant immunogenicity [26].

According to EMA and the U. S. FDA guidelines, the regulatory evidence needed to display a biological agent's similarity is based on a stepwise comparability exercise with the "reference biologic," starting with an extensive physicochemical, biological and, functional characterization followed by non-clinical *in vivo* and clinical evaluation (fig. 1) [27]. The extent of data required from the non-clinical and clinical studies depends mainly on the evidence generated from the initial physicochemical and biological head-to-head comparability exercise that is performed on the biosimilar and its reference biologic. Accordingly, the first step in the comparability exercise regarding the structural and physicochemical characterization of the biosimilar in addition to its *in vitro* biological activity is considered to be the basis of the comparability development program of any biosimilar agent.

The aim of this review is to discuss the main technical and analytical components of the first step in the biosimilarity exercise and that is, the physicochemical and biological analytical characterization of Trastuzumab biosimilars. This review will provide guidance for pharmaceutical personnel working in the research and development of Trastuzumab biosimilars in addition to regulatory officer's worldwide reviewing biosimilars dossiers within public health authorities. The review will detail the minimal threshold technical requirements and evidence needed to be included in step 1 of the exercise dossier to ensure high similarity between reference Trastuzumab and its biosimilars.

The data provided in this review article depends on studies and regulatory guidelines included in different databases, including pubmed, google scholar, Elsevier. Additionally, the authors have depended on different regulatory protocols detailed in both the Europeans Medicines Agency (EMA) and the U. S Food and Drug Administration FDA. The keywords used for the search criteria included: "Trastuzumab", "Biosimilars", "Primary structure", "higher-order structure", "Post-translational modifications", "Glycosylation", "Charge variants", "regulatory approval," and "functional comparability". The data and articles covered the years starting from 2007 until 2022.

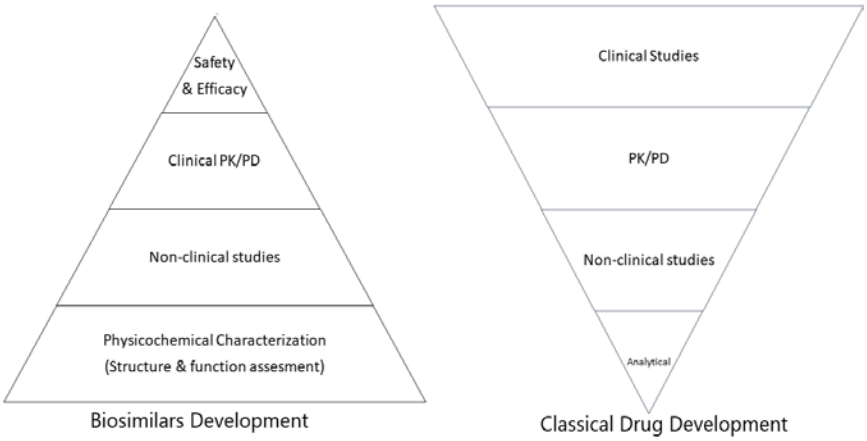

Fig. 1: The scientific and regulatory evidence based stepwise approach of the biosimilars regulatory exercise

Physicochemical comparability exercise for trastuzumab biosimilars

Primary structure

The first step in the physicochemical comparability exercise is to demonstrate that the primary structure of both Trastuzumab's biosimilar and its reference product are identical [28, 29]. Determining the exact amino acid sequence of the biosimilar is essential in primary structure determination. Determination of the amino acid sequence employs peptide mapping where Trastuzumab's biosimilar and its reference product are selectively cleaved enzymatically using different tryptic enzymes and the resultant peptides digests are separated by liquid chromatography and characterized by mass spectrometry. Peptide mapping is considered an "identity test" as it allows detailed fingerprinting of the mAB being analyzed [30, 31]. Additionally, confirmatory peptide mapping of Trastuzumab can be performed through the use of Edman degradation, acting as an auxiliary method for the determination of the amino acid sequence of Trastuzumab in addition to determining the presence of any N and C terminal variants [32]. The primary structure comparability exercise for Trastuzumab should also include different analytical techniques for the determination of the intact and deglycosylated molecular mass of the mAB and these usually depend on Electrospray ionization mass spectrometry (ESI-MS) [33]. Table 1 List all the analytical techniques that are commonly employed for the determination of the primary structure of Trastuzumab and its biosimilar, including the acceptable similarity limits.

Table 1: Quality attributes parameters and analytical techniques required for Adalimumab's primary structure similarity exercise

Quality attribute	Specific quality parameter	Method/Tests	Acceptable limits	Reference
Primary structure	Amino acid sequence	LC/MS/MS–peptide mapping with bioinformatics	Identical amino acid sequence	[30-32]
		Peptide mapping/Edman degradation		[30-32]
	Intact molecular mass	LC-ESI-MS	Similar to reference	[33]
	Intact molecular mass (deglycosylated)	LC-ESI-MS	Similar to reference	[33]

Secondary and higher-order structure

Comparing Trastuzumab and its biosimilar in regard to the secondary and higher-order structure could prove to be a challenging quest as the impact of the minor changes in post translational modifications (PTMs) on the overall tertiary structure of Trastuzumab that results during the PTM stage during intracellular synthesis could have a significant impact on the overall structure of the protein [34]. Additionally, the higher-order structure of mAbs results from individual weak bonds and forces that collectively contribute to the conformation of the mAb. Any changes during the manufacture, including both the upstream and downstream processing of biopharmaceuticals can affect these forces or bonds without altering the primary structure of the protein [35]. The overall higher-order structure is crucial for the pharmacodynamic behavior of Trastuzumab and its functionality. However, there are currently several analytical techniques that can be employed to determine the similarity of biosimilars to their reference product covering their higher-order structure. One essential technique is the use of Circular dichroism in the near and far UV spectral regions as it provides significant insights into the secondary (a-helix, b-sheet, random coil) and tertiary structure, respectively [36]. Fourier-transform infrared spectroscopy (FTIR) could provide additional data needed for elucidation of the similarity of the secondary structure as it analyzes the vibrational state of mAbs with a focus on the Amide I and Amide II bands overlap between Trastuzumab biosimilar and its reference and provides confirmatory data on the secondary structure of the biosimilar that resulted from the Near and Far UV analysis [37]. For the tertiary structure, fluorescence microscopy could provide significant input on the tertiary structure of Trastuzumab and other mAbs in particular regarding Tryptophan's high sensitivity to conformational changes in proteins [38]. The excitation of Tryptophan at 295 nm for the biosimilar and the comparability of the fluorescence profile for Trastuzuamab biosimilar and the reference product is a valuable technique when understanding the overall tertiary structure of the biosimilar. Finally, the thermal stability of mAbs using differential scanning calorimetry and the heat denaturation profile of mAbs is also a strong indicator of the similarity of the higher-order structure of the antibody [39]. Table 2 lists all the analytical techniques that are commonly employed for the determination of the secondary and higher-order structure of Trastuzumab and its biosimilars, including the acceptable similarity limits.

Table 2: Quality attributes parameters and analytical techniques required for Adalimumab's primary structure similarity exercise

Quality attribute	Specific quality parameter	Method/Tests	Acceptable limits	Reference
Secondary and higher order structure	Secondary structure	Far UV CD Spectroscopy	Similar secondary structure	[36]
		Near UV CD Spectroscopy		[36]
		FTIR Spectroscopy	Similar Amide I and II band analysis	[37]
	Tertiary structure	Intrinsic fluorescence spectroscopy	Similar tertiary structure	
		Thermal denaturation by DSC	Similar thermal stability of higher-order structure	[39]

Posttranslational modifications

Posttranslational modification (PTM) is one of the essential critical quality parameters that should be closely evaluated when performing the comparability exercise for Trastuzumab biosimilars [40]. PTMs and in particular any alterations in the glycosylation (galactosylation, galactosylation, fucosylation, high mannose derivatives, and sialylation) pattern of the mAb could have a significant impact on the protein's activity [41]. These changes can also alter the cohesive bonding forces that manifest in the formation of the protein's three-dimensional structure leading to protein aggregation, which could pose serious immunogenicity issues for the biosimilar [42]. The major glycan structures and glycan profile of Trastuzumab can be obtained through (N-Linked glycosylation) which includes enzymatic removal of the major glycan species from Trastuzumab and separating these structures using chromatographic techniques such as high-performance anion exchange chromatography or normal phase liquid chromatography [43, 44]. Additionally, the total afucosylation and terminal galactosylation of the N-terminal glycans can be analyzed by separating the fragments using normal chromatography and labeling the released species with fluorescent probes for detection.

The glycan species of both the reference Trastuzumab and its biosimilar can be analyzed for any major differences in their glycan profile or the type of sugars present using ESI-MS. Any major changes in the glycosylation profile between the Trastuzumab biosimilar and its reference should not alter the biological and functional activity of the mAb or its safety profile. Accordingly, when analytical studies display changes in the glycosylation profile of a mAb and its biosimilar, further non-clinical *in vitro* and *in vivo* studies should be performed to exclude any major deviations in the biological activity or safety profile of the biosimilar [45].

Other PTMs of importance are Disulfide bonding, deamidation and oxidation [46]. Disulfide bond pairings are evaluated using a native peptide map with MS detection, and the number of sulfhydryl groups per protein may be determined by Ellman's assay [47]. Deamidation and, oxidation sites are often evaluated using peptide mapping. A summary of the main parameters and analytical techniques needed to demonstrate Trastuzumab biosimilarity in terms of posttranslational modifications are listed in table 3.

Charge heterogeneity

Charge heterogeneity of mAbs is a phenomenon that can arise due to several manufacturing processes and at several stages of mAb production [48]. The heterogeneity could arise during PTMs affecting the net charge of the mAb or inducing individual amino acid changes that could alter the conformation and the three-dimensional structure of the protein [49]. Additionally, charge heterogeneity could arise during production and in particular, during the upstream manufacturing processing of mAbs where terminal lysine residue could be cleaved or through glycan linking with the reduced sugars of glycan chains forming covalent bonds with lysines [50]. Finally, modifications in the charge that result from mAb degradation and the process of deamidation could have a significant impact on the overall charge consistency and homogeneity of the mAb product. Charge heterogeneity and charge variants of the same mAb could have significant effects on the structure and biological activity of the protein [51]. These changes could alter the mAb's biological function, kinetics, drug stability and, immunogenicity. The charge heterogenicity of Trastuzumab and its biosimilars should be compared using size-exclusion high-performance liquid chromatography (CEX-HPLC) and isoelectric capillary electrophoresis (cIEF) as both methods separate the proteins based on their charge into acidic, main and basic species [52]. Additionally, both methods look for the comparability of the isoform species between reference Trastuzumab and its biosimilars. Table 4 List all the analytical techniques that are commonly employed for the determination of charge heterogeneity of Trastuzumab and its biosimilar including the acceptable similarity limits.

Table 3: Quality attributes, parameters and analytical techniques required for identifying the similarity of Trastuzumab and its biosimilars in regards to posttranslational modifications

Quality attribute	Specific quality parameter	Method/Tests	Acceptable limits	Reference
Posttranslational modifications	N-linked glycan distribution profile, structure and composition	Exoglycosidase digestion/HILIC	Similar N-linked glycan structural assignments and glycosidic linkages	[43, 44]
		HILIC/MS	Similar relative proportions of major level N-linked glycans	
	Total a fucosylation	HILIC with fluorescence detection	Similar ranges of terminal galactosylation	[45]
	Terminal galactosylation	HILIC with fluorescence detection	Similar ranges of terminal galactosylation	[45]

Table 4: Quality attributes, parameters and analytical techniques required for identifying the similarity of trastuzumab and its biosimilars in regards to charge heterogeneity

Quality attribute	Specific quality parameter	Method/Tests	Acceptable limits	Reference
Charge heterogeneity	Acid and basic isoforms	Cation exchange chromatography (CEX-HPLC)	Similar levels of acidic and basic species	[52]
	Charge isoforms	Isoelectric capillary electrophoresis (cIEF)	Similar levels of major and minor charge isoforms	
		CEX-HPLC followed by MS characterization	Similar levels of major and minor charge isoforms	[52]

Functional comparability exercise for trastuzumab biosimilars

Following the physicochemical comparability exercise between Trastuzumab and its biosimilar, the next step in the comparability exercise would involve performing extensive pharmacodynamic non-clinical *in vitro* assays that are reflective of the mechanism of action of Trastuzumab and would ensure that the biosimilar is binding to its receptors and exerting its pharmacological post-binding activity efficiently [ref]. Trastuzumab binding to its receptor inhibits HER-2 mediated signaling within HER-2 overexpressed breast cancer cells and prevents the cleavage of the extracellular HER domain, which is considered a crucial step in HER 2 mediated activation of intracellular cell signaling, proliferation and, tumorigenesis [53]. Additionally, the Fc region of Trastuzumab upon binding with its target HER 2 receptor, plays a major role in activating Antibody-dependent cell cytotoxicity (ADCC) and thus attracts a significant number of macrophages that would eventually engulf the HER 2 overexpressing breast cancer cells. Accordingly, the functional biosimilarity exercise for Trastuzumab biosimilars should focus on identifying the similarity between reference Trastuzumab and its biosimilar in regards to HER 2 binding (affinity, potency and, kinetics) in addition to ADCC activity [54]. The *in vitro* HER 2 binding activity studies within the similarity exercise usually employ SKBr3 cell lines with the relative potency and kinetics measured and identified using either flow cytometry or surface Plasmon resonance (SPR). Moreover, cell proliferation assays employing SKBr3 cell lines can be used to determine the reference Trastuzumab and its biosimilar capability of inhibiting cell growth as a result of blocking HER 2 mediated cell growth and proliferation [55].

The ability of the reference product and its biosimilar to bind the neonatal FC receptor (FcnR) and the Fc gamma receptors (FcγR) is also included in the biological similarity assay. The studies usually focus on the ability of the antibody to adhere to these receptors and are usually measured and quantified using SPR. Finally, and to obtain confirmatory data on the functional activity of Trastuzumab and its biosimilar through nonclinical *in vitro* studies, performing comparative complementary dependent cytotoxicity studies could be included in the functional and biological exercise [56]. Table 5 lists all the functional and biological *in vitro* assays included in Trastuzumab's biosimilarity exercise.

Table 5: Quality attributes, parameters and analytical techniques required for structural and biological similarity exercise of trastuzumab and its biosimilars

Quality attribute	Specific parameter	Methods/Tests	Analytical similarity summary	Reference
Binding assays	HER2 binding affinity	Surface plasmon resonance (SPR)	Similar binding affinity and kinetics	[55]
		Flow cytometry for surface antigen	Similar dose-response curves	[55]
	FcγRIIIa binding affinity	Flow cytometry	Comparable binding activity	[56]
	FcRn binding affinity	SPR	Similar binding affinity and kinetics	[56]
***In vitro* bioassays**	ADCC	$SKBr_3$ based ADCC assay	Comparable activity	[56]
	Induction of Apoptosis	$SKBr_3$ based apoptotic assay	Similar low-level induction of apoptosis	[55, 56]
	CDC	Cell based assay	Comparable activity	[55, 56]
	Inhibition of proliferation	$SKBr_3$ cell based assay	Comparable activity	[55, 56]
	Macrophage induction	Cell based assay	Comparable activity	[55, 56]
	C1q binding	C1q binding assay	Similar dose-response curve	[55, 56]

CONCLUSION

Trastuzumab has proved to be one of the highly successful mAbs in the field of oncology and specifically for the treatment of HER2+positive breast cancer [57]. The pursuit of developing Trastuzumab biosimilars will continue to grow in the upcoming years due to the popularity of the molecule in the treatment of breast cancer in addition to its total sales potential. In this review, we have listed in detail the technical requirements for performing what is s known as the physicochemical and functional biosimilarity exercise for Trastuzumab biosimilars. This exercise constitutes the main pillar of the overall comparability exercise required for Trastuzumab regulatory approval and is the basis for the other following clinical studies needed to demonstrate Trastuzumab's similarity to the originator reference product. We aimed in this review to discuss the minimal technical parameters and orthogonal analytical methods that should be performed for Trastuzumab's biosimilarity exercise; this information could prove to be valuable for scientists working in the research and development divisions of manufacturers currently working on the development of Trastuzumab biosimilars. Moreover, the technical requirements and parameters are of essential value for personnel operating in the

public regulatory sector responsible for the review and approval of biosimilars worldwide.

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICTS OF INTERESTS

The authors declare no conflict of interest.

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