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A comprehensive approach for evaluating charge heterogeneity in biosimilars

Zhiliang Xiao[a], Xiaohang Yin[a], Lina Han[a], Baiping Sun[a], Zhenduo Shen[a], Wanhui Liu[b], Fei Yu[b,*]

[a] Luye Pharmaceuticals, Inc., No.9 Baoyuan Road, Laishan District, Yantai 264003, China
[b] School of Pharmacy, Yantai University, 30, Qingquan RD, Laishan District, Yantai 264005, China

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ABSTRACT

Charge heterogeneity is often evaluated during biosimilar development as it is a universal feature of monoclonal antibodies (mAbs). A common approach in the industry is to develop a biosimilar product with a similar overall charge profile as the reference product. However, uncertainty remains with this approach as the same charge profile in two different products may be caused by different mechanisms. In this work, we present a comprehensive investigation of the charge variants of a therapeutic monoclonal antibody and its biosimilar candidate. Not only did the candidate show a similar charge profile as the reference product, our studies revealed that the same factors contributed to the charge variants of the reference product and the biosimilar candidate. We believe our cause-based approach mitigates the risks associated with the profile-based method and is a rational approach for the charge evaluation of biosimilars.

1. Introduction

The monoclonal antibody market is one of the fastest-growing segments of the world pharmaceutical industry. More than half of the top ten best-selling drugs in 2015 were mAbs or antibody-related therapeutics. With the upcoming patent cliff for many biological blockbusters, there has been a growing interest in biosimilar development (Ecker et al., 2015). Biosimilars are copies of already-authorized innovative biological products, with minor differences in clinically inactive components. In an effort to reduce healthcare costs and improve the accessibility of biologic products to patients, both the EU and US have established legal frameworks and regulatory pathways to push biosimilars to the market. Under the guidance of relevant policies and legislations, various biosimilars have been approved by the EMA and FDA. The approval of antibody biosimilars in the EU and US represents a major landmark for the biopharmaceutical industry, and paves the way for the registration of subsequent biosimilar products (Beck and Reichert, 2013).

The basic principle underlying the development of biosimilar biologics is to demonstrate that the proposed product is comparable to the reference product and verifying that the existing differences are not clinically important. Due to the complexity of protein molecules and their manufacturing process in nature, extensive physicochemical and functional characterization should be performed to evaluate the relevant quality attributes, especially those that define a product's safety and efficacy. Charge variation is one of the major quality attributes that should be closely monitored and tightly controlled during development. The commonly used approach for charge variant control is to develop the biosimilar with the same charge profile as the reference product, confirmed by either ion exchange chromatography or electrophoresis. Unless the biosimilar candidate shows a different charge profile compared to the reference, the causes for the charge variations typically remain uninvestigated. However, with this approach, some uncertainty remains as there is a chance that different factors causing charge heterogeneity may lead to the same charge profile and consequently gives false-positive results. To avoid this, we developed a comprehensive approach for evaluating the charge heterogeneity of biosimilars. Even if the biosimilar candidate shows a similar charge profile as the reference product, investigations are still conducted to identify the cause of each charge variant for both the biosimilar candidate and the reference product to demonstrate the biosimilarity at a causal level. In this study, we used an anti-VEGF monoclonal antibody and its biosimilar candidate to demonstrate our approach.

The cause of charge variation is complicated. Differences in primary structure, high order structure or lational modification (PTM) may alter the charge profile of a similar product, posing a great challenge to comparability study. In order to fully elucidate the charge variants of a product, the corresponding isoforms should be first isolated and purified to a certain extent (Neill et al., 2015; Ponniah et al., 2015; Hosken et al., 2016; Zhang et al., 2011; Dada et al., 2015). Once the charge

* Corresponding author.
E-mail address: yufei@luye.com (F. Yu).

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isoforms are fractionated and recovered, in-depth analytical characterization should be performed to detect the subtle differences between the isoforms and the main species. Advances in analytical technology have expanded our knowledge on the chemical nature of isoforms and their effect on the structure and function of the protein product (Khawli et al., 2010; Vlasak and Ionescu, 2008). Several PTMs have been reported to contribute to the formation of charge variants. The presence of C-terminal Lys (Dick et al., 2008), N-terminal Gln (Lyubarskaya et al., 2006) and C-terminal Pro amidation (Kaschak et al., 2011) are commonly observed in basic species, while sialylation, Asn deamidation (Khawli et al., 2010) and glycation (Quan et al., 2008) are major modifications identified in acidic species. In addition, there are modifications that do not alter the net charge directly, e.g. non-classical disulfide bonds, Fc glycosylation (Yang et al., 2014), oxidation, cysteinylation, aglycosylation, fragmentation and aggregation. However, in many cases, they do change the chromatographic behavior, and such modifications should be evaluated on a case by case basis.

2. Materials and Methods

Recombinant bevacizumab was produced through the standard CHO cell culture process and further purified to the purity required for pharmaceutical use. The reference product, Avastin, was purchased from Roche (Swiss). Acetonitrile was obtained from Merck (Germany). Sequencing-grade modified trypsin was purchased from Promega (USA). Dithiothreitol, iodoacetic acid, glucose, sodium chloride, trifluoroacetic acid, hydrogen peroxide, 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) and 2-(N-Morpholino) ethanesulfonic acid (MES) were purchased from Sigma Aldrich (USA). All other chemicals used were of reagent grade or higher.

2.1. Preparative Chromatography and Fraction Collection

The charge variants of the mAb were isolated using a Thermo Propac WCX-10 preparative column (22 × 250 mm) at a flow rate of 20.0 mL/min on a Waters 2545Q system. Mobile phase A contained 20 mM MES, pH 5.6 and 60 mM NaCl, while mobile phase B contained 20 mM MES, pH 5.6 and 240 mM NaCl. A generic linear gradient of 30% to 70% B over 35 min was used for the separation of different species, and charge isoforms were collected according to the retention time under UV detection at 214 nm. Each fraction was concentrated in a tangential flow filtration system (Millipore) to obtain a concentration higher than 0.5 mg/mL.

2.2. Analytical Cation Exchange Chromatography (CEX)

Analytical cation exchange HPLC was performed on an Agilent 1260 HPLC system using a Thermo Propac WCX-10 analytical column (22 × 250 mm). The flow rate was set to 0.8 mL/min. The mobile phase was composed of elution buffer A (20 mM MES, pH 5.6 and 60 mM NaCl) and elution buffer B (20 mM MES, pH 5.6 and 240 mM NaCl). Charge variants were separated with a linear gradient of 30% to 70% B over 35 min under UV detection at 214 nm. The UV trace was integrated to determine the relative percentage of each variant to the total mass.

2.3. Forced Degradation Study

2.3.1. Thermal and pH Stress

The biosimilar candidate was reconstituted in 100 mM Tris at pH 9.0 to a concentration of 1.0 mg/mL. Samples were then incubated at 37 °C for 1 d, 3 d, and 7 d respectively.

2.3.2. Oxidation Stress

The biosimilar candidate was diluted with 1% hydrogen peroxide to a concentration of 1.0 mg/mL. Samples were then incubated at 25 °C for 2 h.

2.3.3. Glycation Stress

The biosimilar candidate was reconstituted in 100 mM Tris at pH 7.5 combined with 100 mM glucose to a concentration of 1.0 mg/mL. Samples were then incubated at 37 °C for 7 days.

2.4. Tryptic Peptide Mapping by LC–MS

Protein samples were denatured and reduced using 6 M guanidine hydrochloride in 0.36 M Tris at pH 8.6 and 50 mM DTT at 37 °C for 30 min. The samples were alkylated using 50 mM iodoacetic acid for 30 min in the dark. Reduced and alkylated samples were buffer-exchanged to 20 mM Tris at pH 7.5 using a Bio-Rad Micro Bio-Spin 6 column. Digestion was initiated with the addition of sequencing-grade modified trypsin to achieve a 1:20 enzyme to substrate ratio. Digestion was carried out at 37 °C for 2 h, and the samples were stored at 4 °C until analysis.

An Agilent 1290 UHPLC system coupled to a 6530A Q-TOF MS instrument was employed to analyze tryptic peptides. Peptides were separated on a Waters UPLC CSH130 C-18 column (1.7 m, 2.1 mm × 150 mm). The mobile phase consisted of solution A (0.05% TFA in water) and solution B (0.05% TFA in ACN). The column temperature was maintained at 60 °C, and the flow rate was 0.3 mL/min. A linear gradient from 2% to 35% for solution B over 66 min was run for peptide elution. The mass spectrometer was operated in the positive scan mode with m/z in the range of 200–3200. The spectrometer was operated under the following conditions: spray voltage, 4000 V; drying gas, 8 L/min; gas temperature, 300 °C; and nebulizer, 30 psi. The MS/MS spectra of the peptides were obtained using a collision energy of 35 eV. Data collection was performed using the data-dependent acquisition mode.

2.5. N-glycan Profiling with HILIC

The N-glycan sample was prepared according to the instructions for the GlycoWorks *Rapi*Fluor-MS *N*-Glycan Kit (Waters). In brief, protein (15 g) was reconstituted in a 28.8 L solution of 1% (w/v) RapiGest surfactant. The solution was heated to 95 °C over 3 min, allowed to cool to 50 °C, and mixed with 1.2 L of PNGase F solution. Deglycosylation was completed by incubating the samples at 55 °C for 5 min. Deglycosylated proteins were allowed to cool to room temperature and then reacted with *Rapi*Fluor-MS tag for 5 min. Finally, fluorescence-labeled glycans were purified using HILIC based solid phase extraction columns.

The labeled N-glycans were separated on a Waters HILIC column (ACQUITY UPLC Glycan BEH Amide, 1.7 µm, 2.1 × 150 mm) using an Agilent 1290 UHPLC system with a fluorescence detector (excitation at 265 nm, emission at 425 nm). The mobile phase consisted of solution A (50 mM ammonium formate, pH 4.4) and another ACN. The column temperature was maintained at 60 °C, and the flow rate was 0.4 mL/min. A linear gradient from 25% to 40% solution A over 36 min was run for N-glycan elution.

2.6. Boronate Affinity Chromatography

A TSKgel Boronate-5PW column (10 µm, 7.5 mm × 75 mm, Tosoh) was used to determine glycation level. Affinity-based separation was performed on an Agilent 1260 system. The column temperature was maintained at 40 °C, the flow rate was 1.0 mL/min, and eluent absorbance was monitored at 280 nm. The flow of non-glycated proteins through the column was equilibrated with mobile phase A containing 100 mM HEPES, 200 mM NaCl, and 25 mM Tris at pH 8.6, then the glycated molecules were eluted from the column using the same buffer containing 0.5 M sorbitol.