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ORIGINAL RESEARCH ARTICLE

Comparative Structure Activity Relationship Characterization of the Biosimilar BAT1806/BIIB800 to Reference Tocilizumab

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Abstract

Background BAT1806/BIIB800 (Tofidence™/tocilizumab-bavi), a biosimilar of tocilizumab, demonstrated a high degree of analytical and functional similarity to reference tocilizumab (TCZ) in a comprehensive comparative analytical assessment. Minor differences with respect to TCZ were observed for some attributes and this study assessed the potential impact of these differences through structure activity relationship characterization.

Methods Structure activity relationship studies were conducted to assess glycation, glycosylation, charge variants, hydrophobicity, oxidation, and deamidation differences, using a range of investigative techniques. Structure activity relationship studies were performed on one lot each of BAT1806/BIIB800 and TCZ (European Union sourced only) except for glycation, where additional lots sourced from China and the USA were used.

Results Average total glycated protein content of BAT1806/BIIB800 was higher than TCZ (10.08% vs 1.19%); however, biological activity, including target binding and functional potency, was unaffected. Stress-induced glycation of BAT1806/BIIB800 and TCZ also did not affect the biological activity of the products despite up to 60% total glycation content. Minor differences were observed between BAT1806/BIIB800 and TCZ in glycosylation, charge variants, hydrophobicity, oxidation, and deamidation without a relevant impact on interleukin-6 receptor binding, Fc-receptor binding, and effector functions. In forced degradation studies, oxidation and deamidation trends were comparable between the two products.

Conclusions Comparative structure activity relationship characterization of BAT1806/BIIB800 and TCZ indicated that there are no relevant differences in quality attributes between BAT1806/BIIB800 and reference TCZ. Observed differences between BAT1806/BIIB800 and TCZ had no functional impact on BAT1806/BIIB800. The results support the conclusion that BAT1806/BIIB800 is similar to TCZ.

1 Introduction

Tocilizumab (marketed as RoActemra®/Actemra®) is an anti-interleukin-6 receptor (IL-6R) humanized immunoglobulin G1 monoclonal antibody (mAb) whose primary mechanism of action is through fragment antigen binding–mediated inhibition of IL-6 signaling through competitive binding to both soluble and membrane-bound IL-6 receptors [1, 2]. Tocilizumab is used for the treatment of

Key Points

Structure activity relationship studies of the biosimilar BAT1806/BIIB800 and reference tocilizumab were conducted to assess the potential impact of observed differences in glycation, glycosylation, charge variants, hydrophobicity, oxidation, and deamidation, and the impact on biological activity with respect to target binding and functional potency.

Observed differences between BAT1806/BIIB800 and reference tocilizumab were not functionally relevant.

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rheumatoid arthritis and other immune-mediated diseases [3, 4]. While tocilizumab contains an N-linked glycosylation site at ASN298 and is able to bind to the fragment-crystallisable γIII receptor (FcγIII), it does not induce complement-dependent cytotoxicity (CDC) and/or antibody-dependent cell-mediated cytotoxicity (ADCC) [1, 2, 5].

BAT1806/BIIB800 (Tofidence™/tocilizumab-bavi) is a biosimilar of the reference tocilizumab (TCZ). Clinical studies have confirmed the pharmacokinetic equivalence of intravenous BAT1806/BIIB800 compared to TCZ, as well as equivalent efficacy, and comparative safety, pharmacokinetic, and immunogenicity profiles in patients with rheumatoid arthritis [6, 7]. Analytical studies demonstrated similarity of BAT1806/BIIB800 and TCZ in terms of structural and functional attributes and confirmed the absence of both ADCC and CDC [2]. BAT1806/BIIB800 recently received marketing authorizations in China (CN), the United States (US), and the European Union (EU). To meet regulatory requirements, biosimilars must demonstrate similarity with reference product(s) [RP(s)] in terms of structural, physicochemical, and biological properties (including potency), as well as general properties such as protein content [8–12]. It is common for biosimilars to exhibit differences in quality attributes compared with respective RP(s) because of their biological source, complexity, size of the active substance, and the manufacturing process [13]. Identification and comparison of relevant quality attributes from product characterization is a key factor in assessing whether the proposed product is highly similar to the RP in terms of potential clinical effect [12]. The implementation of a range of orthogonal analytical techniques using different physicochemical or biological principles for quantification of the same quality attribute offer independent data to demonstrate similarity of that quality attribute [14, 15]. Accordingly, a comprehensive comparative analytical assessment was conducted to determine the similarity of BAT1806/BIIB800 with CN-, EU-, and US-sourced TCZ [2]. BAT1806/BIIB800 and TCZ demonstrated high degrees of similarity for most quality attributes. Minor differences were observed in glycation, glycosylation, charge variants, hydrophobicity, oxidation, and deamidation [2]. To address the potential impact of these differences on biological activity, extensive structure activity relationship (SAR) studies were conducted using a range of sensitive and state-of-the-art investigative techniques. The objective of this study was to use SAR characterization to demonstrate that the differences observed between BAT1806/BIIB800 and TCZ do not affect the overall similarity assessment of BAT1806/BIIB800.

2 Materials and Methods

Analytical similarity acceptance criteria and calculation of the quality ranges for each attribute for BAT1806/BIIB800 to the RP are reported elsewhere [2]. Briefly, the quality range of 70–130% was based on the criticality of product quality attributes, method capability, and lot-to-lot variability of the RP. A tier system was implemented to classify each quality attribute. Tier 1 included critical quality attributes related to the primary mechanism of action; Tier 2 comprised quantitative assays measuring high-impact critical quality attributes; and Tier 3 covered quantitative assays measuring non-critical quality attributes and semi- or non-quantitative assays. Equivalence tests varied between tiers [2].

2.1 Materials

SAR studies were conducted for glycation, glycosylation, charge variants, hydrophobicity, oxidation, and deamidation using one lot each of BAT1806/BIIB800 and TCZ (one lot of EU-sourced only; SAR studies for glycation used one lot each of CN-, EU-, and US-sourced TCZ). Details regarding the BAT1806/BIIB800 and TCZ formulations are reported elsewhere [2]. The BAT1806/BIIB800 drug product was manufactured by Bio-Thera Solutions Ltd (Guangzhou, China) and the TCZ lots were purchased from the CN, EU, and US markets, and stored according to the manufacturers' instructions.

2.2 Glycation

2.2.1 Intact and Reduced Deglycosylated Mass Analysis by Liquid Chromatography-Mass Spectrometry

Intact mass liquid chromatography-mass spectrometry (LC–MS) can be used for qualitative and relative quantitative analysis of glycated and non-glycated antibodies and can distinguish mono- and poly-glycated antibodies. Samples of BAT1806/BIIB800 and TCZ were subjected to an intact mass LC–MS analysis to determine glycation content. Additionally, stress-glycated samples were analyzed by intact mass LC–MS to allow further assessment of the potential biological impacts of glycation modification of BAT1806/BIIB800 and TCZ. Control samples were incubated without glucose solution, and stress-glycated samples were incubated with 0, 50, and 200 mM glucose solutions, prepared with 1 M of glucose and 250 mM of ammonium bicarbonate solution (total volume of 100 µL; final concentration of 10 mg/mL). Samples were incubated at 37 °C for 24 h, then

purified in ultrapure water using 3-kD ultrafiltration tubes (Millipore, Burlington, MA, USA) and stored at −20 °C.

2.2.2 Reduced and Non-reduced Peptide Mapping by Liquid Chromatography–Tandem Mass Spectrometry

Reduced and non-reduced peptide mapping were performed as described elsewhere [2]. A site-specific post-translational modification (PTM) analysis was used to identify glycation modification sites and pyroglutamic acid at the N-terminus. Pre-treated samples were separated by liquid chromatography–tandem mass spectrometry (LC–MS/MS) and primary and secondary mass spectral signals of peptides were analyzed using Biopharmalynx software. Post-translational modification content of target peptides was calculated from the intensity of the primary mass spectral signal. Content of pyroglutamic acid and the N-terminus was calculated using the response value of the N-terminal peptides with pyroglutamic acid modifications/(the response value of the N-terminal peptide with pyroglutamic acid modification plus the response value of the N-terminal peptide without pyroglutamic acid modification).

2.3 Glycosylation

2.3.1 Sample Processing

Non-deglycosylated samples were prepared by adding 100 mM of Tris (pH 7.95) solution to 1 mg of BAT1806/BIIB800 or TCZ to give a final concentration of 10 mg/mL. Deglycosylated samples were initially prepared in the same manner (test samples diluted with 100 mM of Tris), then the PNGase F enzyme (New England Biolabs, Inc., Ipswich, MA, USA) was added (the mass:volume ratio of the PNGase F enzyme was 50 μg:1 μL) and incubated at 37 °C for 4 h.

2.3.2 Magnetic Bead Sample Purification

To allow measurement of the biological activity of deglycosylated BAT1806/BIIB800 and TCZ, samples underwent magnetic bead purification to remove PNGase F enzyme immediately after deglycosylation. Magnetic bead sample purification was performed according to the manufacturer's instructions for the magnetic bead kit (BeaverBeads™ Magrose Protein A; BEAVER, Suzhou, Jiangsu, China), and stored at 2–8 °C. The degree of deglycosylation (capillary electrophoresis sodium dodecyl sulfate under reducing conditions), sample purity, and the effect of enzyme removal (size exclusion chromatography-high performance liquid chromatography [HPLC]) were measured, as detailed elsewhere [2]. Briefly, for capillary electrophoresis sodium dodecyl sulfate, samples were treated with sample buffer and 2-mercaptoethanol and incubated at 70 °C for 10 min. Samples were then separated at −16.5 kV for 30 min in a capillary electrophoresis instrument (Agilent CE7100; Agilent Technologies, Santa Clara, CA, USA) coupled with a bare fused-silica capillary with a length of 24.5 cm (SCIEX, Framingham, MA, USA). For size exclusion chromatography-HPLC, diluted samples were injected directly onto an analytical column (TSK gel G30000 SW_{XL} [7.8 × 300 mm, 5 μm], Tosoh) with an HPLC (1260II; Agilent Technologies) flow rate of 0.5 mL/min.

2.3.3 Sample Ultrafiltration

Samples were further purified using ultrafiltration tubes and centrifugation. Briefly, 450 μL of ultrapure water was added to ultrafiltration tubes and centrifuged twice at 12,000 rpm for 6 min. Following this, 450 μL of the purified sample was added to the tube and centrifuged at 12,000 rpm for 6 min, then ultrapure water (450 μL) was added to each tube and centrifuged three times at 12,000 rpm for 6 min. Samples were concentrated to 100 μL, and the concentration was determined by a high-throughput microprotein concentration analyzer (Lunatic; Unchained Labs, LLC, Pleasanton, CA, USA).

2.4 Charge Variants

2.4.1 Charge Variant Identification Using Ion Exclusion Chromatography-High Performance Liquid Chromatography

Ion exclusion chromatography-HPLC (IEC-HPLC) is used to evaluate the charge variants of BAT1806/BIIB800 and TCZ samples with and without carboxypeptidase B (CpB) treatment, prepared as detailed elsewhere [2]. Samples were treated with CpB to remove C-terminal lysine residues and reduce lysine-induced charge heterogeneity between BAT1806/BIIB800 and TCZ. CpB-digested BAT1806/BIIB800 and TCZ samples were loaded onto a semipreparative strong cation exchange column (Proteomix SCX, 10 μm, 21.2 mm × 250 mm) on an Ultimate 3000 HPLC (Thermo Fisher Scientific, Waltham, MA, USA) and eluted at a flow rate of 10 mL/min to collect charge variant fractions. Samples were analyzed on an analytical column (Thermo MAbPac SCX-10, 10 μm, 4 × 250 mm). Sample charge

variant fractions were concentrated by low-temperature ultrafiltration using a 30-kDa ultrafiltration centrifuge tube and exchanged into BAT1806/BIIB800 or TCZ formulation buffer (pH 6.2; final concentration ≥ 2 mg/mL). Samples were stored at −60 °C.

2.5 Hydrophobicity

2.5.1 Hydrophobic Interaction Chromatography-High Performance Liquid Chromatography

Hydrophobic interaction chromatography-HPLC was used to monitor hydrophobic variants, per the methods detailed elsewhere [2]. The collection of hydrophobic variant fractions of BAT1806/BIIB800 and TCZ was performed on an Ultimate 3000 HPLC (Thermo Fisher Scientific, Waltham, MA, USA) using a phenyl sepharose high performance (GE HealthCare Technologies Inc., Chicago, IL, USA) packed preparative column. The antibody (150 mg) was loaded onto the column and eluted at a flow rate of 2.5 mL/min. Fractions were collected, concentrated by low-temperature ultrafiltration using a 30-kDa ultrafiltration centrifuge tube, and exchanged into BAT1806/BIIB800 formulation buffer (without polysorbate 80, pH 6.2; final concentration 2 mg/mL or higher). Samples were stored at −60 °C.

2.6 Oxidation and Deamidation

2.6.1 Forced Oxidation Sample Preparation

H_2O_2 was added to BAT1806/BIIB800 and TCZ samples to give final sample concentrations of 0.0005%, 0.001%, 0.002%, 0.005%, and 0.01% H_2O_2, respectively. Samples were incubated at 25 °C in the dark for 0, 1, and 2 days, respectively, and analyzed using LC–MS/MS.

2.6.2 Forced Deamidation Sample Preparation

Ammonium bicarbonate (250 mM) was added to BAT1806/BIIB800 and TCZ samples to give a final concentration of 50 mM of ammonium bicarbonate. Samples were incubated at 37 °C for 0, 2, 4, 6, and 8 days, respectively, and analyzed using LC–MS/MS.

2.6.3 Characterization of Oxidation and Deamidation by Liquid Chromatography–Tandem Mass Spectrometry

Liquid chromatography–tandem mass spectrometry was used to analyze site-specific PTMs for BAT1806/BIIB800 samples. Samples (5 µL) were denatured with 15 µL of 6 M of guanidine hydrochloride (100 mM Tris, pH 7.95), reduced with 0.5 µL of 0.5 M of dithiothreitol, and alkalized with 1 µL of 0.5 M of iodoacetamide. Following this, C-terminal lysine and arginine peptide bonds were hydrolyzed with a mass ratio of protein:trypsin of 1 µg:25 µg at 37 °C, pH 7.95. The reaction was then terminated with 5 µL of 5% formic acid. Separation and detection of fractions were performed using HPLC coupled with MS (Waters ACQUITY UPLC H-Class tandem with Xevo G2-S QToF). The chromatographic column (ACQUITY UPLC® Peptide BEH C18 Column [300Å, 1.7 µm, 2.1 mm×150 mm, 1/pkg]) was set at 60 °C, mobile phases A and B were 0.1% formic acid-water and 0.1% formic acid-acetonitrile, at a flow rate of 0.2 mL/min. The elution time was 2–122 min, 0.2–40% of phase B was used for effective elution, and the total gradient was 130 min. Data acquisition was performed using MassLynx software. Data were analyzed using BiopharmaLynx software.

2.7 Binding Affinity and Functional Assays

2.7.1 Binding Affinity to Fragment Crystallizable Receptors and a Complement Component 1q Binding Kinetic Analysis by Bio-layer Interferometry and Surface Plasmon Resonance

Binding affinity was tested to evaluate whether deglycosylation of BAT1806/BIIB800 and TCZ impacts binding affinity, and if test samples behave similarly. The affinity of BAT1806/BIIB800 and TCZ with FcγRIIIa (158F/V), FcγRIIa (131R/H), FcγRIIb, FcγRIa, neonatal FcR (FcRn), and complement component 1q (C1q) were determined by bio-layer interferometry using an Octet QKe platform (Sartorius, Göttingen, Germany) or surface plasmon resonance (SPR) using a Biacore™ T200 (GE HealthCare Technologies Inc., Chicago, IL, USA). Bio-layer interferometry and SPR methodological details are described in full elsewhere [2].

2.7.2 Antibody-Dependent Cell-Mediated Cytotoxicity and Complement-Dependent Cytotoxicity Analyses

A cell-based reporter gene assay was used to evaluate the ADCC effects of BAT1806/BIIB800 and TCZ. In the assay, TF-1 cells (human leukemic cells expressing membrane IL-6R) were used as target cells, and engineered Jurkat/NFAT-Luc+FcRIIIa-158V cells were used as effector cells.

A separate cell-based assay was used to evaluate the CDC effects of BAT1806/BIIB800 and TCZ, with TF-1 cells used as target cells, and human serum complement used to analyze CDC function. Ofatumumab (anti-CD20 mAb; lot number DM8H; Novartis Pharma GmbH, Nürnberg, Germany) was used as a positive control and CD20-expressing lymphoma (Raji) cells were used as positive cells. Pertuzumab

(anti-human epidermal growth factor receptor 2 mAb; lot number H0212H01; Roche, Munich, Germany) was used as a negative control.

2.7.3 Binding to the Soluble Interleukin-6 Receptor by an Enzyme-Linked Immunosorbent Assay

Binding to the soluble IL-6R by an enzyme-linked immunosorbent assay (ELISA) was performed as described elsewhere [2]. Results were reported as a percentage of the relative binding potency against the reference standard.

2.7.4 Binding Kinetics to the Interleukin-6 Receptor by Surface Plasmon Resonance

The IL-6R binding kinetics of BAT1806/BIIB800 and TCZ were determined by SPR methods. A carboxy-methylated sensor chip with pre-immobilized Protein A was used to capture the Fc region of each sample, then bound with serial concentration IL-6R. Results were reported as a percentage of the relative binding potency against the reference standard.

2.7.5 Inhibition of Interleukin-6-Mediated Proliferation in TF-1 Cells

The functional activity of BAT1806/BIIB800 and TCZ to inhibit IL-6-mediated cell proliferation was evaluated by a TF-1 cell-based assay. TF-1 cells were incubated with serial concentration of reference standard or test samples in the presence of IL-6. After incubation, a CellTiter-Glo® Luminescent Cell Viability Assay (Promega Corporation, Madison, WI, USA) solution was added, and luminescence measured with a microplate reader. Results were reported as a percentage of the relative binding potency against reference standard.

2.7.6 Interleukin-6 Blockage Activity by a Secreted Embryonic Alkaline Phosphatase Reporter Gene Assay

The functional activity of BAT1806/BIIB800 and TCZ to block IL-6-mediated signaling was evaluated with a secreted embryonic alkaline phosphatase (SEAP) reporter gene cell-based assay. HEK-Blue™ IL-6 cells (InvivoGen, Toulouse, France) expressing membrane IL-6R, signal transducer and activator of transcription 3 (STAT3) cDNA, and interferon-β response element-driven SEAP reporter genes were incubated with varying concentrations of reference standard or test samples in the presence of IL-6-mediated activation of STAT3. Downstream SEAP was measured by the addition of QUANTI-Blue™ (InvivoGen, Toulouse, France) solution and quantified with a SpectraMax M5e microplate reader (Molecular Devices, San Jose, CA, USA). Blockage activity was calculated against a dose–response curve of an internal reference standard by SoftMax Pro Software (Molecular Devices, San Jose, CA, USA) and reported as percent relative potency.

3 Results

3.1 Glycation

Protein glycation is a non-enzymatic glycosylation that primarily occurs in lysine residues [16]. Although typically mAb glycation does not appear to substantially affect antibody binding activities, it has been reported that it can affect antigen-binding affinity in certain molecules [16, 17]. Intact LC–MS demonstrated that the glycation content of BAT1806/BIIB800 is different from TCZ (Fig. 1). The glycation content of BAT1806/BIIB800 was composed of predominantly mono-glycated modifications and, to a lesser extent, di-glycated modifications; TCZ (CN, EU, and US) had mainly di-glycated modifications. By the semi-quantitative analysis, the average proportion of glycated protein relative to total protein content of BAT1806/BIIB800 was 10.08%, while the average proportion of glycated protein relative to total protein content of TCZ (CN, EU, and US) was 1.19% (Fig. 1).

To further characterize the overall glycation rate and reveal potential additional glycation sites for both BAT1806/BIIB800 and TCZ, samples were stress-glycated [16]. Intact mass LC–MS demonstrated that there was no relevant effect of stress glycation on the glycation content of BAT1806/BIIB800 and TCZ (CN, EU, and US) samples according to a tunable ultraviolet peak type and retention time. There was also no apparent difference in the total ion current profiles between the stress-glycated BAT1806/BIIB800 and TCZ

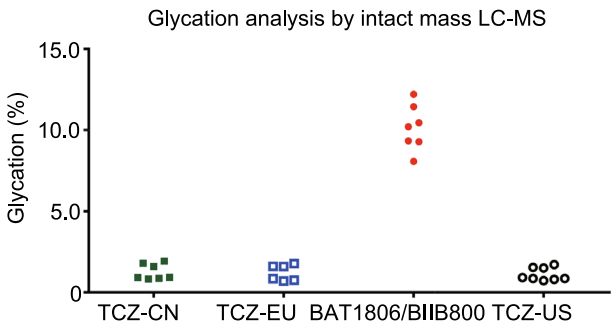

Fig. 1 Glycation content of BAT1806/BIIB800 and reference tocilizumab (TCZ). *CN* China, *EU* Europe, *LC–MS* liquid chromatography-mass spectrometry, *US* United States

samples, with three signals observed on all sample chromatograms (Fig. 1 of the ESM). Deconvolution, performed using the highest peak retention time, revealed that the total content of glycated protein of both BAT1806/BIIB800 and TCZ increased linearly with an increasing glucose concentration (Fig. 2 of the ESM).

Analysis of site-specific PTMs by peptide mapping on stress-glycated test samples revealed that the glycation modification content of the BAT1806/BIIB800 and TCZ samples increased with higher concentrations of glucose (50 mM and 200 mM). Glycation modification of BAT1806/BIIB800 could be detected at multiple sites, particularly LC K126, HC K135, and HC K276 (Table 1 of the ESM). When comparing glycation modification sites between BAT1806/BIIB800 and TCZ under 200-mM glucose stress treatment, the levels of glycation modification at the same lysine sites were similar; this was notable when comparing BAT1806/BIIB800 and all sources of TCZ. None of the main modification sites of the stress-glycated samples in either product was in the complementarity-determining region (CDR).

The impact of the stress-induced glycation modification of sites in the non-CDRs on the biological activity of both BAT1806/BIIB800 and TCZ samples was assessed with the IL-6R binding ELISA, the SEAP reporter gene assay, and the TF-1 cell proliferation potency assay. Stress-induced glycation of BAT1806/BIIB800 and TCZ did not affect the binding activity, biological activity, or functional potency, and all results met the quality standard of 70–130%, even with up to 60% total glycation content (Fig. 2).

3.2 Glycosylation

Glycosylation in the Fc domains of mAbs can affect FcR affinity and therefore effector functions, which may impact efficacy [18]. Although ADCC and CDC are not believed to contribute to the therapeutic effect of tocilizumab, the potential impact of deglycosylation on the biological function of BAT1806/BIIB800 and TCZ was evaluated to rule out any functional impact of glycosylation differences.

The polymeric formation of glycosylated and deglycosylated BAT1806/BIIB800 and TCZ samples was confirmed to be comparable using size exclusion chromatography-HPLC analyses. The polymeric content of deglycosylated BAT1806/BIIB800 samples was < 0.5% and the monomer content was > 98%. The size exclusion chromatography-HPLC analysis showed that the PNGase F enzyme used for deglycosylation was completely removed by the magnetic beads. The capillary electrophoresis sodium dodecyl sulfate analysis confirmed that 100% of the heavy chains were deglycosylated (data not shown).

Bio-layer interferometry and SPR analyses showed that deglycosylation reduced the affinity to all FcγRs and C1q, but not FcRn for both BAT1806/BIIB800 and TCZ (Table 1; Fig. 3 of the ESM). The magnitude of change in affinity to FcRs and C1q with deglycosylation was consistent between BAT1806/BIIB800 and TCZ. Although deglycosylation impacted affinity for FcγRs, neither glycosylated nor deglycosylated BAT1806/BIIB800 and TCZ demonstrated any measurable ADCC and CDC activity, confirming that glycosylation differences have no impact on Fc effector function activity (Fig. 3).

The soluble IL-6R binding analyses by ELISA indicated that the binding activity of deglycosylated BAT1806/BIIB800 and TCZ with IL-6R was 85% and 93%, respectively, which was within the specified quality range (70–130%) (Table 2). These data indicate that deglycosylation does not impact the IL-6R binding activity of BAT1806/BIIB800 and TCZ.

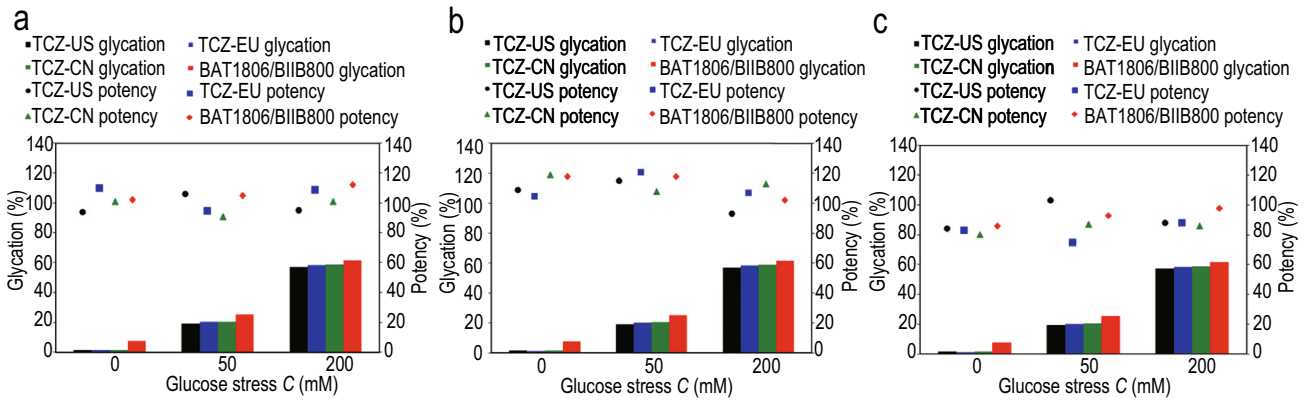

Fig. 2 Impact of total glycation content on binding activity (**a**), biological activity by a secreted embryonic alkaline phosphatase reporter gene assay (**b**), and biological activity by a TF-1 cell-based assay (**c**) of stress-glycated BAT1806/BIIB800 and reference tocilizumab (TCZ) samples. *CN* China, *EU* Europe, *US* United States

Table 1 Affinity of BAT1806/BIIB800 and TCZ with Fc receptors and C1q by bio-layer interferometry and surface plasmon resonance

Receptors	BAT1806/BIIB800	BAT1806/BIIB800 deglycosylated	TCZ	TCZ deglycosylated
FcγRIa	5.55E–11	3.84E–09	5.86E–11	3.51E–09
FcγRIIa (131R)	1.30E–06	N/D	1.30E–06	N/D
FcγRIIa (131H)	9.90E–07	N/D	1.00E–06	N/D
FcγRIIb	4.80E–06	N/D	3.50E–06	N/D
FcγRIIIa (158F)	1.82E–07	N/D	2.14E–07	N/D
FcγRIIIa (158V)	2.05E–07	N/D	2.18E–07	N/D
FcRn	2.41E–08	3.68E–08	1.97E–08	2.92E–08
C1q	2.60E–08	N/D	2.44E–08	N/D

C1q complement component 1q, *FcRn* neonatal fragment crystallizable receptor, *N/D* no signal was detected; sample did not appear to bind to the receptor, *TCZ* reference tocilizumab

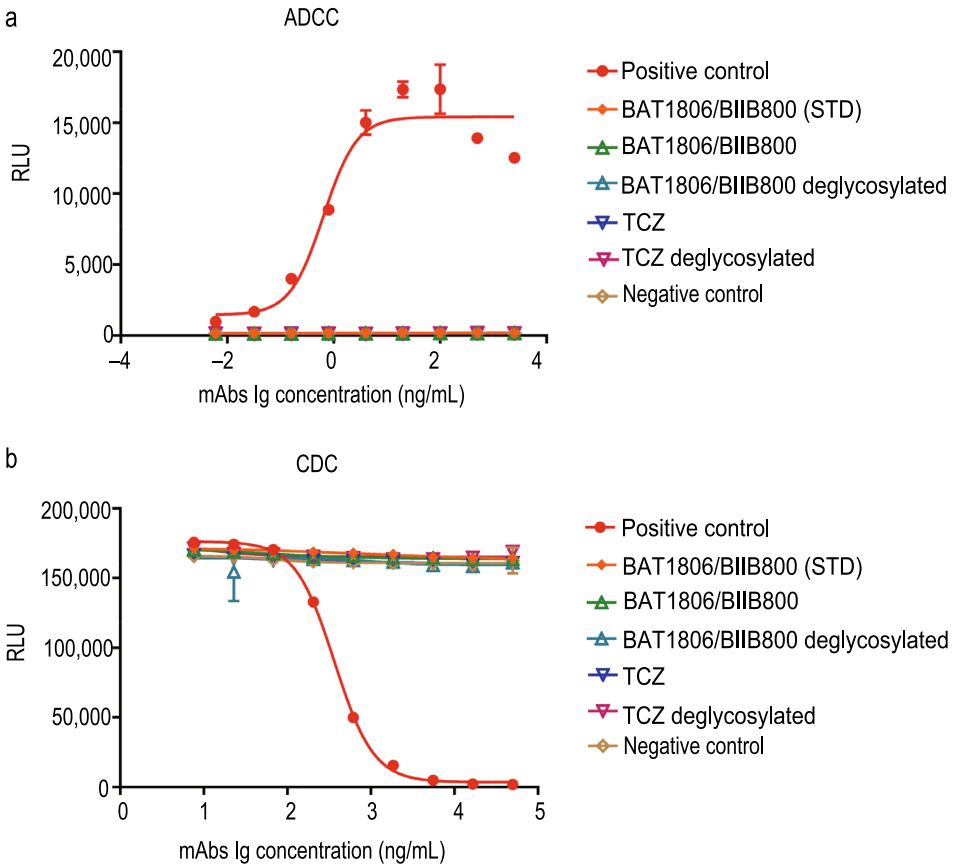

Fig. 3 Antibody-dependent cellular cytotoxicity [ADCC] (**a**) and complement-dependent cytotoxicity [CDC] (**b**) analysis of deglycosylated BAT1806/BIIB800 and reference tocilizumab (TCZ) in TF-1 cells. *Ig* logarithmic transformation, *mAb* monoclonal antibody, *RLU* relative light units, STD standard

To further characterize the binding characteristics of deglycosylated BAT1806/BIIB800 and TCZ samples with IL-6R antigen, SPR was used (Fig. 4 of the ESM). Results demonstrated that the binding rate constant, dissociation rate constant, and affinity of deglycosylated BAT1806/BIIB800 and TCZ samples were in the same order of magnitude with similar results observed between BAT1806/BIIB800 and TCZ. The relative affinity of deglycosylated BAT1806/BIIB800 and TCZ samples was 84% and 97%, respectively (Table 2 of the ESM).

To assess the relative functional potency of BAT1806/BIIB800 and TCZ, TF-1 cell-based proliferation assays were used. The assays demonstrated similarity between BAT1806/BIIB800 and TCZ samples before deglycosylation with relative potency of 121% and 113%, respectively. After deglycosylation, relative potency of BAT1806/BIIB800 and TCZ samples was

Table 2 The soluble IL-6R biological activity of deglycosylated BAT1806/BIIB800 and TCZ

Sample name	Relative potency for IL-6R binding (%)	Relative potency [TF-1 cell based assay] (%)
BAT1806/BIIB800	104	121
BAT1806/BIIB800 deglycosylated	85	109
TCZ	110	113
TCZ deglycosylated	93	86

IL-6R interleukin-6 receptor, *TCZ* reference tocilizumab

109% and 86%, respectively, indicating that biological activity remained similar between samples and within the specified quality range of 70–130% (Table 2).

3.3 Charge Variants

Deamidation, oxidation, isomerization, and fragmentation may give rise to charge variants that can alter the functional activity of a product, potentially elicit immunogenicity, and may cause further degradation [19]. BAT1806/BIIB800 and TCZ charge variant components were comparatively assessed to determine whether any differences between products impacted biological function. To assess charge variants, BAT1806/BIIB800 and TCZ samples were treated with CpB to remove clinically inactive C-terminal lysine residues, a well-known source of charge variation in mAbs. CpB-treated BAT1806/BIIB800 and TCZ samples had similar IEC-HPLC profiles, including the same peak shapes (Fig. 5 of the ESM). The main peak of BAT1806/BIIB800 was slightly higher (~ 3%) than TCZ and the basic peaks had lower fragments than TCZ (mean difference of 2.2–3.1%). There were no relevant differences in the content of acidic peaks between BAT1806/BIIB800 and TCZ. The acidic peaks and main peak of BAT1806/BIIB800 were similar to TCZ, but the BAT1806/BIIB800 basic peaks contained a higher aggregate content, a small number of fragments, a higher proportion of high mannose glycan, and amidated C-terminal proline.

PTMs were identified for the charge variants of BAT1806/BIIB800 and TCZ (Table 3 of the ESM). Comparison of the levels of PTMs for different charge variant components between BAT1806/BIIB800 and TCZ showed relative similarity. For both BAT1806/BIIB800 and TCZ, the acidic variants were mainly derived from asparagine deamidation, and the percentage of deamidation of heavy chain N386/N391 in acidic peaks 2 and 3 was higher than other components (Table 3 of the ESM). The basic variants of BAT1806/BIIB800 after CpB cleavage primarily occurred because of C-terminal proline amidation and N-terminal glutamine without cyclization, whereas the basic variants of TCZ originated from C-terminal proline amidation. The proportion of C-terminal proline amidation was higher for TCZ than BAT1806/BIIB800 (Table 3 of the ESM), which was consistent with the IEC-HPLC-CpB assay results. The difference is not expected to impact antibody effector function [20]. There were no differences in the level of PTMs analyzed at other sites.

BAT1806/BIIB800 and TCZ charge variants were also analyzed for glycosylation modifications and classified into neutral terminal galactosylated glycan (which may affect CDC), high mannose glycan (which may affect the

Table 3 Biological activity of BAT1806/BIIB800 and TCZ charge variants

Fractions	Sample name	Relative potency [soluble IL-6R binding assay] (%)	Relative potency [SEAP RGA] (%)	FcRn affinity, K_D(M)
Pre-collection	BAT1806/BIIB800	106	108	2.54E–08
	TCZ	100	99	2.58E–08
Acidic peak 1	BAT1806/BIIB800	97	94	2.84E–08
	TCZ	110	81	2.58E–08
Acidic peak 2	BAT1806/BIIB800	86	90	2.74E–08
	TCZ	86	94	2.64E–08
Acidic peak 3	BAT1806/BIIB800	89	103	2.69E–08
	TCZ	101	92	2.86E–08
Main peak	BAT1806/BIIB800	93	110	2.48E–08
	TCZ	105	100	2.59E–08
Basic peak 1	BAT1806/BIIB800	89	91	2.49E–08
	TCZ	96	94	2.50E–08
Basic peak 2	BAT1806/BIIB800	114	107	1.55E–08
	TCZ	93	101	1.46E–08

FcRn fragment crystallisable receptor (neonatal), *IL-6R* interleukin-6 receptor, *K_D(M)* affinity, *RGA* reporter gene assay, *SEAP* secreted embryonic alkaline phosphatase, *TCZ* reference tocilizumab

pharmacokinetics and ADCC), afucosylation glycan (which may affect ADCC), and sialylated glycan (which may affect the pharmacokinetics and immunogenicity). The pre-collection BAT1806/BIIB800 and TCZ samples were consistent in terms of glycan type, differing only on the content of specific individual glycans (Table 4 of the ESM). Compared with TCZ, BAT1806/BIIB800 showed lower levels of afucosylation glycan and high mannose glycan, as well as higher levels of terminal sialic acid (except in the main fraction where sialylation was the same for both BAT1806/BIIB800 and TCZ). BAT1806/BIIB800 and TCZ showed consistent differences between variant fractions; both had higher levels of sialic acid in the acidic fractions compared with the main or basic fractions. Liquid chromatography-mass spectrometry was used to determine the glycation content of charge variants from deglycosylated BAT1806/BIIB800 and TCZ samples. Deglycosylated BAT1806/BIIB800 and TCZ samples showed higher glycation content in the acidic peaks, particularly acidic peaks 1 and 2, compared with the pre-collection samples (Table 5 of the ESM). Although the level of glycation in deglycosylated BAT1806/BIIB800 was slightly higher than TCZ fractions, deglycosylation had no impact on the site of glycation.

To assess the biological activities of BAT1806/BIIB800 and TCZ charge variants, target binding, functional activity by SEAP reporter gene assay, and FcRn affinity were evaluated. The IL-6R binding and cellular potency activity, as assessed by the SEAP reporter gene assay, for both BAT1806/BIIB800 and TCZ charge variants were within the specified quality range of 70–130% (binding activity: BAT1806/BIIB800, 86–114%; TCZ, 86–110%; cellular activity: BAT1806/BIIB800, 90–110%; TCZ, 81–101%) (Table 3). Neonatal FcR receptor affinity was similar between BAT1806/BIIB800 and TCZ charge variants.

3.4 Hydrophobicity

Hydrophobicity is an important consideration for the stability, aggregation, solubility, and immunogenicity of mAbs [21]. To characterize the BAT1806/BIIB800 and TCZ hydrophobic fractions, and assess the impact of hydrophobicity on product functional activity, samples were fractionated and analyzed by hydrophobic interaction chromatography-HPLC. Hydrophobic interaction chromatography-HPLC demonstrated that BAT1806/BIIB800 was similar to TCZ with the same peak shape. The pre-collection fraction pre-peaks and main peak of BAT1806/BIIB800 showed slight differences (~ 2.0%) from TCZ (Table 6 of the ESM); post-peaks also showed minor differences (~ 1.0%). The hydrophobic fractions of BAT1806/BIIB800 were subsequently identified in terms of PTM quality attributes by orthogonal techniques. Overall, PTMs of the different hydrophobic variant fractions were similar between BAT1806/BIIB800 and TCZ (Table 7 of the ESM). For both BAT1806/BIIB800 and TCZ samples, the PTMs in the pre-peaks sites were mainly C-terminal Lys, oxidation, and deamidation. The level of C-terminal Lys modification of TCZ in the pre-peaks sites was higher than BAT1806/BIIB800. Post-translational modifications for most sites of the main peak and post-peaks samples were similar between the pre-collection BAT1806/BIIB800 and TCZ samples.

The hydrophobic fractions of BAT1806/BIIB800 and TCZ were analyzed for glycosylation modifications. Overall, the BAT1806/BIIB800 glycosylation pattern was consistent with TCZ; however, there were minor differences in the content of individual glycans between BAT1806/BIIB800 and TCZ. BAT1806/BIIB800 showed lower levels of afucosylation glycan and high mannose glycan, and slightly higher levels of sialylation compared with TCZ in a pre-collection sample (Table 8 of the ESM). BAT1806/BIIB800 and TCZ samples showed similar trends across variant fractions in terms of glycan content. Both BAT1806/BIIB800 and TCZ pre-peaks fractions had higher levels of high mannose and lower levels of galactosylation compared with the main and post-peaks fractions.

Molecular size variant analyses of the BAT1806/BIIB800 and TCZ hydrophobic fractions by SEC-HPLC showed that, in both products, low-molecular-weight content was more abundant in the pre-peaks component, and high-molecular-weight content was higher in post-peaks component as compared with the main peak component (Table 9 of the ESM).

To assess the impact of hydrophobic difference on the biological activity of BAT1806/BIIB800 and TCZ, target binding and cellular potency assays were conducted on the hydrophobic fractions. Binding and cellular potency, as measured in the SEAP reporter gene and TF-1 proliferation assays, were similar between BAT1806/BIIB800 and TCZ and within the specified quality range of 70–130% (binding activity: BAT1806/BIIB800, 102–112%; TCZ, 86–111%; cellular potency: BAT1806/BIIB800, 92–119%; TCZ, 101–115%) (Table 4). Neonatal FcR affinity was similar between BAT1806/BIIB800 and TCZ hydrophobic variants.

3.5 Oxidation and Deamidation

Oxidation and deamidation are common degradation processes for mAbs and can result in structural changes that impact antigen binding affinity and efficacy. [19] To characterize sites of oxidation, and the impact of oxidation and deamidation on BAT1806/BIIB800 and TCZ, forced oxidation and deamidation studies were conducted.

Forced oxidation (0.01% H_2O_2) revealed oxidative modifications at five sites (HC-M70, 106, 254, 430, and HC-W279) for both BAT1806/BIIB800 and TCZ (Table 10 of the ESM). Under forced oxidation conditions, the rate of oxidation of the acidic peaks was slightly lower (~1%) for BAT1806/BIIB800 versus TCZ (Table 11 of the ESM).

Table 4 Biological activity for BAT1806/BIIB800 and TCZ hydrophobic variants

Fractions	Sample name	Relative potency [soluble IL-6R binding assay] (%)	Relative potency [TF-1 cell-based assay] (%)	FcRn affinity K_D(M)
Pre-collection	BAT1806/BIIB800	107	92	2.10E–08
	TCZ	101	115	2.30E–08
Pre-peaks	BAT1806/BIIB800	112	106	2.45E–08
	TCZ	100	105	2.61E–08
Main peak	BAT1806/BIIB800	102	115	2.14E–08
	TCZ	111	101	2.46E–08
Post-peaks	BAT1806/BIIB800	102	119	2.09E–08
	TCZ	86	112	2.53E–08

FcRn fragment crystallizable receptor (neonatal), *IL-6R* interleukin-6 receptor, $K_D(M)$ affinity, *TCZ* reference tocilizumab

Similarly, the proportion of oxidized sites for the main peak was higher (~5%) for BAT1806/BIIB800 than TCZ. Despite this, the difference in total oxidation level did not exceed 1% between BAT1806/BIIB800 and TCZ samples. With forced oxidation, biological activity of BAT1806/BIIB800 and TCZ, as measured by the SEAP reporter gene assay, rapidly decreased (Table 5). Oxidation of HC-M70, HC-M106, and HC-M254 correlated with a substantial impact on the biological activity of BAT1806/BIIB800 and TCZ. In particular, oxidation of HC-M106, which is located in the CDR, indicated that an oxidation level of approximately 95% reduced both the SEAP reporter gene and TF-1 proliferation biological activity of BAT1806/BIIB800 and TCZ to below 50% (Fig. 4). However, the cellular potency activity of BAT1806/BIIB800 and TCZ, as measured by the TF-1 proliferation assay, was within the specified quality range standard with an HC-M106 oxidation level up to approximately 10%, which was significantly higher than the levels of oxidation under physiological conditions (<1%).

Under forced deamidation conditions (pH 9.0), IEC-HPLC showed that the acidic peaks content of BAT1806/BIIB800 had a rapid ~30% increase with longer incubation periods, whereas the main peak showed a ~30% decrease over the same time period (Table 12 of the ESM). The trend and degree of change were similar between BAT1806/BIIB800 and TCZ; the aggregate degradation degree for BAT1806/BIIB800 was lower than TCZ, whereas the degradation degree in the acidic peaks was higher than TCZ. Binding activity, cellular potency activity, and FcRn affinity were similar between deamidated BAT1806/BIIB800 and TCZ samples and were within the specified quality range of 70–130% (Table 6).

4 Discussion

Biosimilars are expected to have structural or analytical differences when compared with their RPs because of the inherent variability in the manufacturing process, parameters for cell culture (cell lines/culture medium), and storage conditions [16]. BAT1806/BIIB800 and TCZ demonstrated a high degree of similarity for quality attributes in a comprehensive comparative analytical assessment [2]; however, using highly sensitive and state-of-the-art analytics, some differences were observed in glycation, glycosylation, charge variants, hydrophobicity, oxidation, and deamidation. Observed structural or analytical differences between the biosimilar and RP must not impact clinical safety or efficacy [8, 10–12]. Here, several SAR studies were conducted to further characterize the observed differences between BAT1806/BIIB800 and TCZ. We demonstrate that residual structural differences in glycation, glycosylation, charge variants, hydrophobicity, oxidation, and deamidation between BAT1806/BIIB800 and TCZ had no relevant functional impact. In conjunction with the available clinical evidence, these results provide adequate assurance that the observed physicochemical differences have no clinical relevance.

The effect of glycation on antibody function depends on the site that is modified [16]. Glycation modifications located in the CDR of the antibody could affect the binding activity of the antibody to the antigen [22]. Stress glycation of BAT1806/BIIB800 and TCZ (CN, EU, and US) samples indicated that there was no effect on the biophysical behavior of BAT1806/BIIB800 and TCZ according to the tunable ultraviolet peak type and retention time and total ion current profiles. Analysis of deconvolution signal intensity to determine the percentage of total glycation content suggested that both antibodies have similar glycation modification trends, with linear increases respective to increased glucose concentrations. Site-specific PTM analyses demonstrated BAT1806/BIIB800 samples had similar glycation sites and site modification proportions relative to TCZ.

Table 5 Biological activity for forced oxidation BAT1806/BIIB800 and TCZ

Sample name	H_2O_2 concentration (%)	Incubation time (days)	Relative potency [SEAP RGA] (%)	Relative potency [TF-1 cell-based assay] (%)	Relative FcRn affinity (%)
BAT1806/BIIB800	0.0005	1	95	103	89
		2	90	88	90
	0.001	1	86	93	87
		2	75	84	86
	0.002	1	63[a]	76	73
		2	53[a]	72	74
	0.005	1	18[a]	46[a]	42
		2	13[a]	49[a]	35
	0.01	1	8[a]	31[a]	19
		2	7[a]	25[a]	32
TCZ	0.0005	1	66[a]	96	86
		2	64[a]	107	87
	0.001	1	68[a]	82	82
		2	60[a]	113	82
	0.002	1	39[a]	70	72
		2	28[a]	70	71
	0.005	1	15[a]	49[a]	47
		2	8[a]	36[a]	44
	0.01	1	8[a]	29[a]	32
		2	8[a]	21[a]	31

FcRn fragment crystallizable receptor (neonatal), *H_2O_2* hydrogen peroxide, *RGA* reporter gene assay, *SEAP* secreted embryonic alkaline phosphatase, *TCZ* reference tocilizumab

[a]Beyond the specified quality range (70–130%)

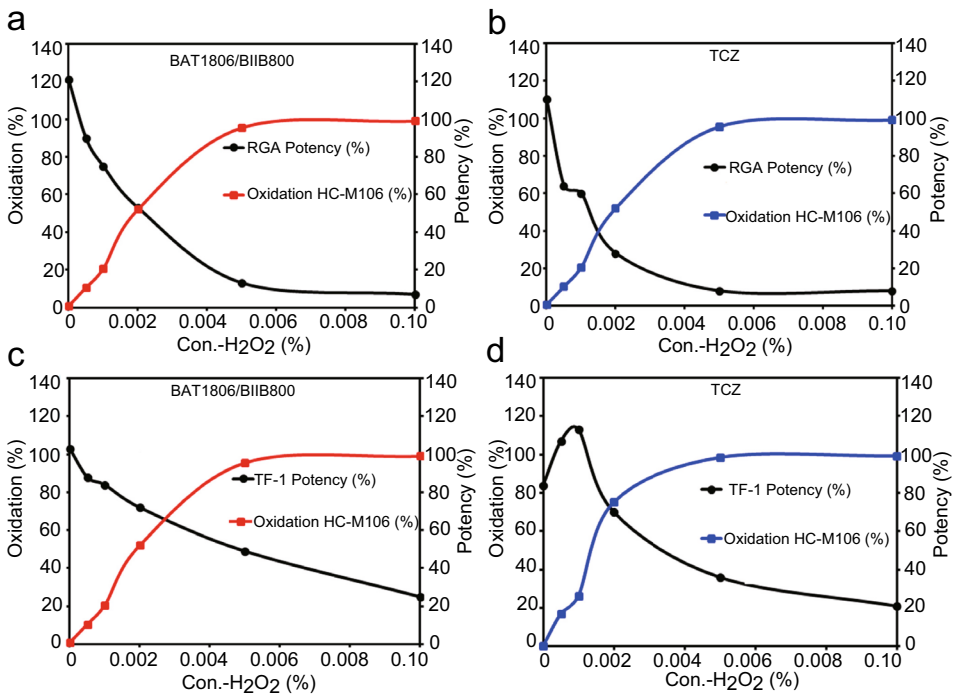

Fig. 4 Biological activity by a secreted embryonic alkaline phosphatase reporter gene assay and HC-M106 oxidation levels (**a**, **b**); biological activity by a TF-1 cell-based assay and HC-M106 oxidation levels (**c**, **d**) for BAT1806/BIIB800 and reference tocilizumab (TCZ). *H_2O_2* hydrogen peroxide, *RGA* reporter gene assay

Table 6 Biological activity for forced deamidation BAT1806/BIIB800 and TCZ

Sample name	Incubation time (days)	Relative potency [SEAP RGA] (%)	Relative potency [TF-1 cell-based assay] (%)	Relative FcRn affinity (%)
BAT1806/BIIB800	0	120	116	104
	2	115	112	97
	4	98	92	113
	6	96	109	101
	8	101	108	90
TCZ	0	101	115	96
	2	106	112	100
	4	104	105	86
	6	107	118	90
	8	100	100	91

FcRn fragment crystallizable receptor (neonatal), *RGA* reporter gene assay, *SEAP* secreted embryonic alkaline phosphatase, *TCZ* reference tocilizumab

The main modification sites of the stress-glycated samples were outside the CDR, suggesting that glycation will not affect the binding and biological activity of either BAT1806/BIIB800 and TCZ. Additionally, based on stress glycation studies, glycation in the non-CDRs (assessed using the target binding assay, SEAP reporter gene assay, and TF-1 proliferation inhibition potency assay) did not affect the binding activity, biological activity, or cellular potency activity of either BAT1806/BIIB800 or TCZ. Therefore, the SAR data adequately justify that the observed difference in glycation content between BAT1806/BIIB800 and TCZ has no impact on the safety and efficacy profile of BAT1806/BIIB800. In support of this conclusion, a study investigating the rates and impact of human antibody glycation in vivo demonstrated that glycation occurs in approximately 14% of circulating antibodies in healthy humans [23]. This level of glycation was not associated with changes in FcRn, FcγRIIIa, or protein A binding for the antibodies investigated [23]. Additionally, the study reported that even under severe glycation conditions with a glucose content over 200 times that observed in vivo, there was little functional change observed for the antibodies analyzed [23]. Similarly, SAR studies evaluating another tocilizumab biosimilar (MSB11456 [Tyenne®]) reported that while the glycation content for MSB11456 was significantly higher than TCZ, biological activity between the products remained similar and glycation did not impact the efficacy of MSB11456 [24].

The impact of deglycosylation on the biological activity of BAT1806/BIIB800 and TCZ was assessed. Deglycosylation had no impact on target binding activity. The binding affinity to most FcRs and C1q decreased with deglycosylated BAT1806/BIIB800 and TCZ, while the binding affinity to FcRn was largely unaffected, consistent with results reported in the literature [25]. Additionally, as anticipated per the biosimilar and RP mechanism of action, both intact and deglycosylated BAT1806/BIIB800 and TCZ showed no ADCC or CDC activity in TF-1 cells, indicating that deglycosylation did not impact the Fc-mediated effector functions of BAT1806/BIIB800 or TCZ. This was consistent with another study evaluating the tocilizumab biosimilar MSB11456, which reported similar glycosylation profiles between products and the absence of CDC and ADCC [24]. Functional potency of deglycosylated BAT1806/BIIB800 and TCZ with respect to non-deglycosylated BAT1806/BIIB800 and TCZ, as measured by the inhibition of TF-1 cell proliferation, was also unaffected (relative potency range, 86–121%).

Charge variant studies previously demonstrated differences between BAT1806/BIIB800 and TCZ, both before and after CpB enzyme treatment [2]. However, the peak subsequent fraction analysis showed similarity in the SAR study with the exception of some differences in aggregate content, number of fragments, proportion of high mannose glycan, and proportion of amidated C-terminal proline in the basic peaks following CpB treatment. While there were minor differences between the BAT1806/BIIB800 and TCZ charge variants, the target binding and functional potency of the BAT1806/BIIB800 basic peaks was similar to that of the main peak, indicating that the differences between the BAT1806/BIIB800 and TCZ basic peaks are unlikely to have a clinical impact. This conclusion is further supported by data from pre-clinical and clinical studies showing that BAT1806/BIIB800 and TCZ behaved similarly in terms of pharmacokinetics [6, 7, 26].

Evaluation of hydrophobicity identified slight differences between BAT1806/BIIB800 and TCZ pre-, main, and post-peaks. However, no differences were observed with target binding activity, functional potency, and FcRn receptor

affinity. Therefore, these small differences in hydrophobicity are unlikely to impact the clinical efficacy and/or safety of BAT1806/BIIB800.

Forced degradation is not often observed under physiological conditions [27]; however, SAR studies were conducted to assess forced degradation similarities between BAT1806/BIIB800 and TCZ. The biological activity of both BAT1806/BIIB800 and TCZ was negatively correlated with increased oxidation levels of the CDR. The level of deamidation of BAT1806/BIIB800 and TCZ in forced degradation studies showed similar trends, but had no impact on biological activity or FcRn affinity.

5 Conclusions

Structure activity relationship studies were conducted to investigate the potential impact of observed differences in quality attributes between BAT1806/BIIB800 and TCZ. The observed differences in glycation between BAT1806/BIIB800 and TCZ did not impact the binding or biological activities. Minor differences in glycosylation, charge variants, hydrophobicity, oxidation, and deamidation were also shown to have no impact on functional activities at the levels observed. Overall, these results indicate that there are no functionally relevant differences between BAT1806/BIIB800 and TCZ. Together these data support the conclusion that BAT1806/BIIB800 is similar to TCZ.

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Declarations

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