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Analytical and functional similarity of biosimilar Elizaria® with eculizumab reference product

Valentina Gusarova *, Maxim Degterev, Ivan Lyagoskin, Vladimir Simonov, Maxim Smolov, Sergey Taran, Rahim Shukurov

JSC "GENERIUM", 14 Vladimirskaya street, Volginskiy, Petushinskiy district, Vladimir Region, 601125 Russia

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ABSTRACT

A recombinant humanized monoclonal antibody (mAb) Eculizumab, C5-complement cascade inhibitor, is an important treatment of complement-based diseases recommended by international guidelines. Elizaria® Drug Product (DP), developed by IBC Generium, Russia, is the world's first registered biosimilar of eculizumab (Soliris®, Alexion Pharmaceuticals). Using sensitive state-of-the-art analytical techniques extensive similarity assessment has been conducted to demonstrate the structural and functional similarity of original Soliris® (Eculizumab Reference Product, RP) and the biosimilar Elizaria®, focusing on the physicochemical and biological quality attributes, including those known to affect the mechanisms of action. A multitude of analyses revealed that amino acid sequence is identical, the higher order structures, post-translational modifications, purity, and product variants are highly similar between Elizaria® DP and Eculizumab RP, except for minor differences in the relative abundance of the charge variants and glycan moieties considered not clinically significant. The results demonstrate that Elizaria® is highly analytically similar to Eculizumab RP in terms of physicochemical properties and biological activities.

1. Introduction

Elizaria® drug preparation, licensed in the Russian Federation in 2019 and Turkey in 2022, was developed as a biosimilar of Soliris® (eculizumab) by Alexion Pharmaceuticals Inc. Eculizumab approved by the by the European Medicines Agency (EMA) and US Food and Drug Administration (FDA) for the treatment of patients with paroxysmal nocturnal hemoglobinuria (PNH), atypical hemolytic uremic syndrome (aHUS), refractory generalized myasthenia gravis (gMG) and neuromyelitis optica spectrum disorder (NMOSD) in 2007, 2011, 2017 and 2019 respectively (*European Commission designations EU/3/03/166, EU/3/09/653, EU/3/14/1304, EU/3/13/1185*). Eculizumab inhibits the generation of C5a-metabolite formation of the membrane attack complex via C5b, due to its ability to block the cleavage of C5 during complement activation [1–3]. Amgen's eculizumab biosimilar, ABP 959, is currently in the clinical trial stages [4].

Structurally both Elizaria® (further Elizaria® DP) and Soliris® (further Eculizumab RP) are a recombinant humanized monoclonal antibody with human IgG2 CH1, hinge and a part of CH2 regions of the H-chain constant regions, human IgG4 CH3 and the rest part of CH2 region, while complementarity-determining regions (CDRs) are murine. The developed biosimilar Elizaria® DP has the same amino acid sequence as the Eculizumab RP, nevertheless, the structural and functional similarity largely depends on the production process, starting with the choice of the production cell line. Eculizumab RP is produced in mouse myeloma cell (NS0), while Chinese hamster ovary (CHO) cell line is used for manufacturing of Elizaria® DP, which can naturally affect the post-translational modifications of the compared products, particularly the glycosylation profile. Monoclonal antibodies from CHO usually contains more aglycosylated forms than from NS0 and Sp2/0 [5]. As for immunogenic oligosaccharides murine cells (NS0 and SP2/0) are known potentially attach not naturally found on human IgG for example, α-Gal epitope [6] or N-glycolylneuraminic acid (NGNA), that is the predominant sialic acid in the glycoproteins produced in murine cells but only trace amount usually found in CHO-produced glycoproteins [7,8].

During this research work, the Quality Target Product Profile (QTPP) based on a set of quantitative ranges for Critical Quality Attributes (CQAs) for Eculizumab RP that could affect product safety and efficacy, was defined. The analytical similarity assessment plan (Table 1) was designed to assess structural/physicochemical and functional similarity

* Corresponding author.
E-mail address: gusarova@ibcgenerium.ru (V. Gusarova).

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Table 1
Quality Attributes and methods used to evaluate physicochemical and biological properties of reference product Soliris® and eculizumab proposed biosimilar Elizaria®.

Characteristic	Quality Attribute and Test/ Assay	Risk Ranking	Tier Stat. Analysis
Primary Structure	Primary Amino Sequence by Peptide Mapping LC-MS and RP-HPLC	High	3*
	N- and C-ends Determination by LC-MS	High	3*
	Extinction coefficient by UV spectroscopy	Moderate	2
	Native and Reduced Disulfide Mapping by LC-MS	Moderate	3*
	N-linked Glycan Profiling by Hydrophilic interaction chromatography (HILIC)	Moderate	2
	Post-translational modifications (Deamidation, Oxidation, C-end Lysine Truncation, N-end Glutamine) by LC-MS Peptide Mapping	Moderate to Very Low	2
	Isoelectric point and profile by Isoelectric Focusing (IEF)	Moderate to Low	3*
Molecular mass	Molecular mass of Intact Protein by Intact-MS	Moderate to Low	2
	Molecular mass of Proteolytic Subunits by Middle-Up Approach (LC-MS)	Moderate to Low	2
	Molecular mass of Reduced HC and LC by LC-MS	Moderate to Low	2
Higher Order Structure	Secondary structure and profile by Far UV-CD and Fourier-transform infrared spectroscopy (FTIR)	Moderate	2
	Tertiary structure and profile by Near UV-CD and Intrinsic Fluorescence	Moderate	2,3
	Thermal Stability profile by Differential Scanning Calorimetry (DSC)	Moderate	2
Product-related substances and impurities	Size variants by SEC-HPLC, Non-reduced CE-SDS, HIC HPLC	Moderate	2
	Charge Variants Charge Variants by IEX-HPLC	Moderate	2
Biological activities	Anti-hemolytic Activity by Anti-hemolytic Assay (anti-HA)	Very High	1,2
	C5-component Binding by ELISA	Very High	1,2
	C5 and FcRn Binding by Surface Plasmon Resonance (SPR)	Very High Moderate to Low	1,2 2,3
	FcγRII(CD32) binding activity by SPR		
	Lack of FcγRI(CD64), FcγRIII (CD16) by SPR	Low	2,3
	Lack of C1q binding activities by ELISA	Low	2,3
General properties	Protein concentration by UV spectroscopy	Moderate to Low	2

anti-HA anti-hemolytic Assay, *CE-SDS* capillary electrophoresis-sodium dodecyl sulftate, *DSC* Differential Scanning Calorimetry, *ELISA* enzyme-linked immunosorbent assay, *IEF* isoelectric focusing, *IEX-HPLC* ion-exchange high-performance liquid chromatography, *Far UV-CD* far ultraviolet circular dichroism, *FTIR* Fourier-transform infrared spectroscopy, *HC* heavy chain, *HIC-HPLC* hydrophobic interaction high-performance liquid chromatography, *HILIC* hydrophilic interaction liquid chromatography, *LC-MS* liquid chromatography-mass spectrometry, *LC* light chain, *Near UV-CD* near ultraviolet circular dichroism, *RP-HPLC* reversed-phase high-performance liquid chromatography, *SEC-HPLC* size-exclusion high-performance liquid chromatography, *SPR* surface plasmon resonance

* Tier 3 was assigned because nature of the assays is qualitative despite of "high" or "moderate" risk ranking.

and ensure the understanding of whether any differences between Elizaria® DP and Eculizumab RP had the potential to impact clinical performance, consistent with current ICH, FDA and EMA regulatory documents for the development of biosimilar products [9–12].

Because of biosimilarity is assessed in terms of totality-of-evidence, and statistical evaluation for the assay is critical, for an adequate statistical assessment of the potential effect of the quality attribute on the clinical performance, the FDA-recommended risk-based approach was applied [10] in accordance with the general principles of risk ranking determination described in case study on the development of a monoclonal antibody [13]. In general, biological activities were given greater weight on the risk ranking than physicochemical properties since they directly measure activities linked to mechanisms of action, activity, efficacy, safety and immunogenicity of the product (Table 1). For example, risk ranking for anti-hemolytic activity and C5-binding affinity were determined to be "very high" since they are key mechanisms of action of eculizumab for its clinical efficacy as a treatment for PNH and aHUS. Other clinically relevant biological activities were ranked either "high" or "moderate" depending on their significance on clinical efficacy. Other biological activities such as C1q-binding affinity were ranked "low" since they have little role in the anti-hemolytic activity of eculizumab. With regards to the physicochemical properties, primary amino acid sequence that is directly related to efficacy and safety of the product were ranked "high". Purity related attributes such as aggregates and fragments were ranked "moderate" considering they may cause reduced biological activity and immunogenicity and so on.

2. Materials and methods

2.1. Samples for analysis

The comparison included Elizaria® DP batches manufactured by JSC GENERIUM with the pilot- and industrial-scale processes, some critical quality parameters (anti-hemolytic activity, C5-binding, higher order structure) were investigated using batches involved in preclinical and clinical studies. The total number of batches of EU-sourced Eculizumab RP (Soliris®, Alexion Pharmaceuticals) studied at different stages of development is currently 12 batches, acquired over a time frame that spans expiration dates of several years, which complies with the WHO and FDA recommendations [9,10].

2.2. Chemicals and reagents

Carboxypeptidase B (≥125 Units/mg of protein) (CpB), proteomics grade peptide-N-glycosidase F (PNGase F) were obtained from SigmaAldrich (Burlington, MA, USA). Human complement-containing serum, human complement C1q protein, human complement protein C5 were supplied by Quidel Corporation (San Diego, CA, USA) and horseradish peroxidase-labeled antibody specific to human IgG4 Fc-fragment by Abcam (Cambridge, UK). Anti-C1q-specific sheep IgG antibodies conjugated with horseradish peroxidase was obtained from Bio-Rad (Hercules, CA, USA). IdeS enzyme and sequencing grade modified trypsin were purchased from Promega (Madison, WI, USA), IEF CleanGel and Pharmalyte® 3–10 for IEF – from GE Healthcare (Chicago, IL, USA) and polyacrylamide PA 800 plus SDS MW gel – from Beckman Coulter, Inc (Brea, CA, USA). LC-MS grade acetonitrile was purchased from Biosolve BV (Valkenswaard, the Netherlands). MS-grade trifluoroacetic acid and difluoroacetic acid were obtained from Waters (Milford, MA, USA). All other reagents were of analytical grade and purchased from standard commercial supplier. Ultra-pure water was prepared in-house using MilliPore Advantage A10 System (Merck Millipore, Burlington, MA, USA).

2.3. HPLC-MS tryptic peptide mapping

The test samples were deglycosylated with PNGase F, reduced with

dithiothreitol (DTT) followed by alkylation with iodoacetic acid. The sample aliquots intended for disulfide bond testing have not been treated either with DTT or iodoacetic acid. Excess reagents were removed by means of Slide-A-Lyzer Mini devices (Thermo Scientific, Waltham, MA, USA) prior to specific proteolysis with trypsin at 37 °C for 16 h. Resulting peptides were separated using a high-performance nanoflow liquid chromatography separation system Agilent Polaris_HR-Chip-3C18 (Agilent Technologies, Santa Clara, CA, USA) with a gradient mode of acetonitrile (from 1% to 45% in 80 min) in water solution of formic acid/trifluoroacetic acid (0.08/0.01% vol) followed by identification using quadrupole time-of-flight mass spectrometer 6550 QTOF (Agilent Technologies). The data was processed using Peaks AB v 2.0 (Bioinformatics Solutions, Ontario, Canada) and BiopharmaFinder v.3.2 (Thermo Scientific).

2.4. RP HPLC tryptic peptide mapping

The test samples were denaturated with 8 M urea, reduced with DTT followed by alkylation with iodoacetic acid. Excess reagents were removed and samples were re-buffered into ammonium hydrogen carbonate solution by means of ultrafiltration using Amicon Ultra-15 centrifugal filter unit with Ultracel-10 membrane (Merck Millipore) prior to specific proteolysis with trypsin at 37 °C for 4 h. Resulting peptides were separated using HPLC system Alliance e2695\2489 (Waters) equipped with a C18 reversed phase column AdvanceBio PeptidePlus 2.1 * 150 mm, 2.7 μm (Agilent Technologies). Elution was achieved by a gradient of acetonitrile (from 0% to 45% in 120 min) in 20 mM ammonium acetate solution, pH 4.0. The eluted peptides were detected at 210 nm.

2.5. Intact mass measurement by infusion ESI-TOF-MS and middle-up analysis by HPLC- ESI-TOF-MS

Intact protein samples after purification by dialysis by means of Slide-A-Lyzer Mini devices (Thermo Scientific) to remove polymers and low-molecular-weight impurities were analyzed using Nexera X2 HPLC system (Shimadzu Corp., Kyoto, Japan) coupled with quadrupole time-of-flight mass spectrometer 6550 QTOF 6550 QTOF (Agilent Technologies). Experimental data were processed using UniDec v. 4.1.2. software (Agilent Technologies). The calculation of the average theoretical molecular weight of the major form of eculizumab (147,874 Da) was carried out taking into account the main modifications: *G0F* glycans, all C-terminal lysine residues are cleaved, pyroglutamates at the N-termini of heavy chains, as well as 17 disulfide bonds.

The determination of the molecular weights of the light and heavy chains of eculizumab was carried out after samples denaturation with guanidine hydrochloride followed by the reducing of disulfide bonds with dithiothreitol. The resulting fragments were separated by reversed phase HPLC-MS mode using a BioResolve RP mAb Polyphenyl column (Waters) in gradient of acetonitrile (from 15% to 55% in 12 min) in 0.15% difluoroacetic acid. Molecular weight measurements for $F(ab')_2$ and Fc/2 fragments were carried out after specific proteolysis by IdeS enzyme (Promega Corporation) between amino acid residues V**AG**PSV (highlighted in bold) located in heavy-chain positions 237–238. Subsequent chemical cleavage of disulfide bonds using tris(2-carboxyethyl) phosphine (TCEP) divided the $F(ab')_2$ subunit into two fragments: LC (light chain) and Fd subunit. The resulting fragments were separated by reversed phase HPLC-MS mode using the same conditions as for separating LC and HC. The obtained mass spectra were processed using MassHunter Qualitative Analysis B.09.00 software (Agilent Technologies) and deconvoluted using UniDec v. 4.1.2. software (Agilent Technologies).

2.6. Disulfide bonds mapping (RP-HPLC ESI-MS/MS)

Native protein samples were treated with iodoacetic acid to alkylate the unpaired cysteine residues followed by specific proteolysis with trypsin at 70 °C for 1.5 h. Reduced protein samples were denaturated and treated with DTT before alkylation. The analysis of all samples was done using 6550 QTOF mass spectrometer (Agilent Technologies) with high resolution tandem data dependent acquisition mode.

2.7. Ellman's assay

For free-Cys determination the spectroscopic method developed by Ellman was used [14]. Free-Cys in a protein are reacted with difluoroacetic acid, 5,5-dithio-bis(2-nitrobenzoic acid) forming stable yellow-colored 2-nitro-5-thiobenzoic acid (TNB), which can be quantified by measuring the absorbance at 412 nm and applying a molar extinction coefficient of 13,600 M^{-1} cm^{-1}.

2.8. Near UV-circular dichroism (Near UV-CD)

Near-UV Circular Dichroism **(**CD) spectra were measured at 20 °C after removing polysorbate 80 from test samples (by dialysis against SP buffer - 20 mM sodium phosphate, 50 mM sodium chloride, pH 6.8, in dialysis bags with 3.5 kDa cut-off limit) on spectropolarimeter J-810 (JASCO Corp, Tokyo, Japan) using cuvettes with a path length of 1.0 cm. The protein concentration ranged from 3.9 to 4.3 μM. The spectra were recorded with wavelength range from 250 to 350 nm (step size 1.0 nm, bandwidth 2 nm, response time 1 s, averaging time of data 5 s), and smoothed by 5-point averaging after subtracting the absorption spectrum of the buffer solution. The subtraction / smoothing of the spectra and the calculation of the molar ellipticity of the samples were performed using the Spectra Analysis v.1.53.04 (JASCO Corpe and OriginPro 9.0 (OriginLab Corp., Northampton, MA, USA) software, respectively.

2.9. Far UV-circular dichroism (Far UV-CD)

Far-UV CD spectra were measured at 20 °C after removing polysorbate 80 from test samples (by dialysis against SP buffer - 20 mM sodium phosphate, 50 mM sodium chloride, pH 6.8, in dialysis bags with 3.5 kDa cut-off limit) on spectropolarimeter J-810 (JASCO Corp) using cuvettes with a path length of 1.0 cm. The protein concentration ranged from 0.67 to 0.69 μM. The spectra were recorded with wavelength range from 200 to 250 nm (step size 1.0 nm, bandwidth 2 nm, response time 2 s, averaging time of data 5 s), and smoothed by 5-point averaging after subtracting the absorption spectrum of the buffer solution. The subtraction / smoothing of the spectra and the calculation of the molar ellipticity of the samples were performed using the Spectra Analysis v.1.53.04 (JASCO Corp) and OriginPro 9.0 (OriginLab Corp.), respectively.

2.10. Fourier-transfrom IR spectroscopy (FTIR)

Fourier-transfrom IR spectra after removing polysorbate 80 from test samples (by dialysis against SP buffer - 20 mM sodium phosphate, 50 mM sodium chloride, pH 6.8, in dialysis bags with 3.5 kDa cut-off limit) were measured at 20 °C on FTIR spectrometer Nicolet 6700 FT-IR (Thermo Scientific). The optical path length of a CaF_2 cell was calculated for each measurement proceeding from the test sample optical density at 3404 cm^{-1} and using the water absorption value of 0.533 AU at an 1-μm optical path length adjusted for the sample protein concentration. The protein concentration ranged from 39.1 to 69.3 mg/mL. Each protein spectrum after subtraction the absorption spectrum of the buffer solution was adjusted for the spectral contribution of water and CO_2 vapors with further testing in the wave number range of 1725–1481 cm^{-1} with a resolution of 1 cm^{-1}. The obtained values of the content of secondary structure elements were averaged.

2.11. Intrinsic fluorescence

The intrinsic fluorescence of eculizumab samples was compared by means of Cary Eclipse fluorescence spectrometer (Agilent Technologies) with fluorescence excitation wavelength 280 nm. The spectral width of the exciting and analyzing monochromators was 5 nm and 2.5 nm, respectively. Measurements of the intrinsic fluorescence spectra of protein solutions (0.46–0.62 μM) after removing polysorbate 80 by dialysis were carried out at 20 °C in a 1 cm optical quartz cell in the wavelength range from 290 to 420 nm with a step of 2 nm and averaging time of data at each point equal to 2 s. The spectral contribution of buffer solution was subtracted from the fluorescence spectra of protein samples, the obtained difference spectra were corrected for the spectral sensitivity of the device and described by a log-normal function in the wavelength range of 300–420 nm using the LogNormal software (IBI RAS, Pushchino, Russian Federation).

2.12. Differential scanning calorimetry (DSC)

The thermal stability of was evaluated using DASM-4 M scanning microcalorimeter (Biopribor, Pushchino, Russian Federation) after removing polysorbate 80 from test samples by dialysis against SP buffer - 20 mM sodium phosphate, 50 mM sodium chloride, pH 6.8, in dialysis bags with 3.5 kDa cut-off limit). *Cp* profiles were measured from 20 °C to 90 °C at a scan rate of 0.86 K/min. To determine the thermal transition temperatures, the DSC v.1.2 (IBI RAS) and OriginPro 9.0 (OriginLab Corp.) software were used.

2.13. Hydrophilic liquid interaction chromatography (HILIC)

GlycoPrep® Rapid N-Glycan Preparation with InstantAB™ kit (Agilent Technologies) was used to N-glycan sample preparation. The test samples were enzymatically processed with PNGase F, followed by labeling of the released glycans with high-performance fluorescent dye InstantAB [15]. The fluorescently labeled glycans were separated by HILIC using HPLC system Alliance e2695\2475 (Waters with AdvanceBio Glycan Map column (Agilent Technologies) using 60 mM ammonium formate mobile phase pH 4.5 with a decreasing acetonitrile gradient (from 70% to 60% in 80 min) at $\lambda_{ex} = 278$ nm, $\lambda_{em} = 344$ nm. Data was acquired and processed by Empower™ 3 (Waters) software.

2.14. Cation-exchange high-performance liquid chromatography (CEX-HPLC)

Analysis of eculizumab charge isoform distribution was performed using weak CEX-HPLC using HPLC system Alliance e2695\2489 (Waters) with ProPac WCX-10 BioLC 4 × 250 mm ID column (Thermo Scientific) at 40 °C. Runs were performed with growing sodium chloride content (from 20 mM to 100 mM in 30 min) in 20 mM MES, 1 mM ethylenediaminetetraacetic acid mobile phase at pH 5.5 with detection at a wavelength of 280 nm. Data was acquired and processed by Empower™ 3 (Waters) software. Pretreatment with CpB was carried out at (37 ± 2)°C for 30 min, then the samples were analyzed under the same conditions as intact.

2.15. Isoelectric point

Isoelectric point and the content of charge-related protein isoforms of eculizumab has been determined using Isoelectric Focusing (IEF) in native condition (10% D-Sorbitol) in IEF CleanGel with pH gradient of 3–10. Coomassie Blue R-250 was used to stain the gel. Pretreatment of CpB samples was carried out at (37 ± 2)°C overnight, then the samples were analyzed under the same conditions as intact. The visualization of the gels and the calculation of the results were carried out using the ImageQuant TL 8.1 (GE Healthcare) software.

2.16. Size-exclusion high-performance liquid chromatography (SEC-HPLC)

SEC-HPLC was performed using HPLC system Alliance e2695\2489 (Waters) with a TSKgel G3000SWXL 5 μm 7.8 × 300 mm column (Tosoh Bioscience, Tokyo, Japan) at room temperature with 0.01 M sodium phosphate dibasic, 0.15 M sodium chloride mobile phase at pH 7.0, at flow rate 1.0 mL/min with detection at a wavelength of 214 nm. Data was acquired and processed by Empower™ 3 (Waters) software.

2.17. Hydrophobic interaction chromatography (HIC-HPLC)

HIC-HPLC analysis was carried out using HPLC system Alliance e2695\2489 (Waters) with MabPac HIC-10 1000 A 5 μm, 4.6 × 100 mm column (Thermo Scientific) at 40 °C. The runs were performed with flow rate 0.8 mL/min of mobile phase, containing 100 mM sodium phosphate dibasic at pH 7.0 with decreasing gradient of ammonium sulfate (from 0.7 M to 0 M in 30 min) with detection at 280 nm. Data was acquired and processed by Empower™ 3 (Waters) software.

2.18. Non-reduced capillary electrophoresis

Capillary electrophoresis was performed with polyacrylamide PA 800 plus SDS MW gel using PA800 plus system (Sciex, Danaher Corp., Washington, D.C., USA). Test samples after denaturation with sodium dodecyl sulfate (SDS) were injected into a fused-silica and separated inside the porous matrix under applied external electric field with detection at a wavelength of 220 nm.

2.19. C5-binding specific activity by ELISA

To characterize C5-binding capacity of Elizaria® DP and Eculizumab RP was used an enzyme-linked immunosorbent assay (ELISA). An interaction of serially diluted samples with an Purified Human Complement Protein C5 forms “C5-eculizumab” complex followed by adding a horseradish peroxidase-labeled antibody specific to human IgG4 Fc-fragment to detect this complex. Visualization of “C5-eculizumab-antibody” complex is achieved by adding a chromogenic peroxidase substrate that breaks down forming a stained product. Optical density at 405/492 nm of the solution in each well is proportionate to the quantity of the C5-bound eculizumab. Eculizumab concentration/response curve is approximated using 4-parameter logistic fit using GraphPad Prism 6.0 software (GraphPad Software, Inc., San Diego, CA, USA). The parameter EC_{50} describes the product concentration corresponding to a 50% response (optical density) level and is inversely related to the product functional activity. Specific C5-binding activity (BU/mg, units of binding per mg of protein) is then evaluated for test product, taking account of its EC_{50} value, as well as reference product EC_{50} value and known C5-binding activity.

2.20. C5-component binding by surface plasmon resonance (SPR)

The dose-dependent specific C5 protein binding of Elizaria® DP and Eculizumab RP was investigated by SPR by Biacore 8 K (GE Healthcare) using CM5 sensor chip series S (GE Healthcare) with covalently immobilized antibodies recognizing Fc part of IgG. The rate of formation and degradation of antigen / antibody complexes was observed in real time, followed by calculation of the Ka (association constant) and Kd (dissociation constant). Experimental sensograms were adopted with mathematical model corresponding to 1:1 Langmuir interactions.

2.21. Specific anti-haemolytic activity

Determination of the specific antihemolytic activity of eculizumab is performed in an in vitro test using human complement-containing serum and chicken red blood cells (RBC) coated with polyclonal antibodies

against RBC. Human serum complement induces hemolysis of antibody-sensitized chicken erythrocytes in the absence of eculizumab. Eculizumab binds the C5 complement component and prevents hemolysis. The tested samples in serial dilutions supplemented with 5% normal human complement-containing serum and chicken erythrocytes, sensitized with rabbit polyclonal antibodies against chicken RBC. Following incubation for 40 min at 37 °C optical density at 415 nm was measured. The results were processed with GraphPad Prizm 6.0 software (GraphPad Software, Inc.) using a 4-parametric log function of dependence of the three-replication averaged optical density against eculizumab content.

2.22. C1q-binding

Determination of interaction of eculizumab with human complement component C1q was carried out by enzyme-linked immunosorbent assay. Purified human complement C1q protein binds to eculizumab immobilized on the surface of the ELISA plate wells. After incubation and washing, the formed complex is detected using anti-C1q-specific sheep IgG antibodies conjugated with horseradish peroxidase as described in details [16]. The results were processed with GraphPad Prizm 6.0 software (GraphPad Software, Inc.) using a 4-parametric log function.

2.23. FcRn binding

The dose-dependent specific eculizumab binding with neonatal Fc-receptor (FcRn) was characterized by SPR by Biacore 8 K (GE Healthcare) using CM5 sensor chip series S (GE Healthcare) with covalently immobilized antibodies recognizing hexa-histidine sequence of protein molecule. When various concentrations of the test samples are added at a pH of 6.0 to the FcRn immobilized on the sensor surface, the "FcRn-eculizumab" complex is formed in a dose-dependent manner. Observation of the association and dissociation of the complex is carried out in real time followed by calculation of the Ka and Kd. Experimental sensograms were adopted with mathematical model corresponding to 1:1 Langmuir interactions.

2.24. Fcγ-associated receptors binding

For comparative characteristics of Elizaria® DP and Eculizumab RP, a method of surface plasmon resonance on Biacore 8 K (GE Healthcare) for recording of formation and dissociation of a complex at pH 7.4 was applied. The antibodies specific to hexahistidine sequence were covalently immobilized on all 8 channels of the CM5 sensor (GE Healthcare) in advance. Then, human recombinant Fc-receptors having six C-terminal amino acid residues of histidine were specifically immobilized on the surface of the working flow cells of the certain channel of the activated sensor. Following the receptor immobilization, the rates of formation and degradation of the receptor-antibody complexes were observed in real time by pulsing the increasing concentrations of eculizumab over the immobilized Fc receptor in so-called single cycle mode of analysis. The equilibrium dissociation constant was calculated (if possible) as the ratio of the dissociation rate constant to the association rate constant (for CD64) or in steady state affinity analysis as a concentration of eculizumab essential to achieve a half of the maximal amount of comlex to be formed (CD16, CD32).

2.25. Statistics

In accordance with the FDA recommendations [10], the analytical similarity assessment was performed with an adequate number of meaningful lots and attributes/assays. A three-level differentiation of statistical generalization was used [17,18]. Equivalence testing (Tier 1) is applied for attributes with the highest potential clinical impact and assays that explain clinically relevant mechanism of action of eculizumab. Two one-sided tests on the mean difference of developing and the reference products are used with an interval (−1.5xSD +1.5xSD) that can support 78–84% confidence interval is recommended for number of lots n = 6–8 [17]. Most of the attributes with medium risk rank are reported as mean ± SD where standard deviation could be calculated (Quality ranges approach, Tier 2). If necessary, a statistical comparison between the results of two separate analytical sets (Set 1 and Set 2, were used QR_{pooled} limits, based on Pooled Standard Deviation (SD_{pooled}) calculated from Set 1 and Set 2 SDs (an example of calculation is given in the Appendix 1 in the electronic supplementary material [ESM]). Evaluation through visual comparison (Tier 3) were applied for attributes with the lowest risk ranking.

3. Results and discussion

Extensive physicochemical and structural testing of Elizaria® DP and Eculizumab RP was performed with state-of-the-art technologies, using EU-sourced Eculizumab RP (Soliris®, Alexion Pharmaceuticals) produced over several years and at different stages of the life cycle (12 eculizumab EU lots, in total). This retrospective approach allows us to estimate the range of changes in COAs of the original drug in terms of lot-to-lot variability. Due to the orphan status of eculizumab and the limited number of Eculizumab RP batches available on the market at a particular point in time, head-to-head comparative studies were conducted repeatedly, by the same methods. Quality parameters changing over time, such as, for example, the amount of impurities and biological activities, were assessed using Elizaria® DP and Eculizumab RP with close production dates. Compared Elizaria® DP batches were produced using both pilot and industrial processes, including the batches involved in preclinical and clinical studies, as well as process validation lots (19 Elizaria® DP batches, in total). All Elizaria® DP and Eculizumab RP lots were stored under the recommended storage condition and tested prior to the specified expiration date.

The similarity test plan for Elizaria® DP and Eculizumab RP listing all evaluated analytical techniques and attributes is shown in Table 1. Orthogonal techniques were used whenever possible to comprehensively analyze the structural characteristics and biological functions of eculizumab. Table 2 presents a summary of the resulting assessments of the Quality Attributes (COAs).

3.1. Primary structure

The primary structures of eculizumab in the Elizaria® DP and the Eculizumab RP (Soliris®) were compared using a wide range of methods, which are listed in Table 1.

By means of HPLC-QTOF MS the complete amino acid sequence of eculizumab in Eculizumab RP and Elizaria® DP was confirmed with 100% coverage (see Fig. S1 in ESM). *De novo* sequencing of peptide fragments has not identified any residual signal terminal sequences. Peptide mapping by RP-HPLC after deglycosilation and enzymatic hydrolysis with trypsin and subsequent mass-spectrometric identification of the peptides produced demonstrated the overlap of eculizumab chromatographic profiles for Eculizumab RP and Elizaria® DP (Fig. 1). More than 50 chromatographic peaks have been identified that matched 100% of eculizumab amino acid sequence (see Fig.S2 and Table S1 in ESM).

Analysis of post-translational modifications (PTMs) showed a comparable level of oxidation of methionine residues in positions M4, M48, M81, M253, M359 and M429, not exceeding 5% (Table 2). The largest difference in PTMs level was registered for deamidation at sites 316 and 385 of heavy chain: despite the close range of scatter of the proportions, the content of the modified form in Eculizumab RP is 1.2–3 times higher than that in the Elizaria® DP. In all other sites the low percentage of absolute content of deamidated forms reduced its potential effect to eculizumab efficiency and safety. There is also a slight difference in the levels of clipped C-terminal lysine, not exceeding 2%, the low absolute difference between average percentage of this modification and its low

Table 2
Analytical similarity assessment data for selected attributes for Elizaria® DP and Eculizumab RP.

COAs	Elizaria® DP range*	Eculizumab RP range*
Intact Whole Protein, Da	*6 lots*	*6 lots*
Glycosylation G0F/G0F	147874 ± 0	147873 ± 1
Proteolytic Subunits, Da	*6 lots*	*6 lots*
$F(ab')_2$	97479.0 ± 0.6	97478.3 ± 0.3
Fc/2	25216.5 ± 0.2	25216.7 ± 0.1
Reduced Light chain, Da	*6 lots*	*6 lots*
	23420.5 ± 0.1	23420.5 ± 0.1
Reduced Heavy chain, Da	*6 lots*	*6 lots*
	51562.0 ± 0.2	51562.0 ± 0.2
Post Translational Modification, %	*6 lots*	*6 lots*
Deamidation Light chain, N53	0.2–0.4	0.1–0.9
Deamidation Light chain, N137	0.7–26.1	2.2–24.8
Deamidation Heavy chain, N180	0–3.1	0–3.6
Deamidation Heavy chain N316	0–34.2	3.2–45.5
Deamidation Heavy chain, N363	0–1.1	0–0.8
Deamidation Heavy chain N385	0.2–32.9	12.3–34.2
Oxidation Light chain, M4	0.1–1.9	0–2.2
Oxidation Heavy chain, M81	0.5–4.1	0.5–4.6
Oxidation Heavy chain, M359	0.5–2.7	0.5–3.7
Pyroglutamate formation Heavy chain, Q1	100	100
N-glycosylation Heavy chain, N298	84.6–88.9	84.2–90.6
C-terminal lysine clipping Heavy chain, 448		
Glycan profile, %	*6 lots*	*6 lots*
Galactosylated	17.9–21.0	15.0–17.0
Afucosylated	4.5–6.6	6.3–7.6
High-mannose	1.3–3.1	4.0–5.1
Sialylated	0.2–0.3	1.7–2.2
RP-HPLC peptide mapping profile similarity	*6 lots*	*6 lots*
	Yes	Yes
Near UV-CD spectral similarity	*8 lots*	*6 lots*
	Yes	Yes
Far UV-CD spectral similarity, %	*8 lots*	*6 lots*
α-helices	2.7–3.1	2.8–3.0
β-sheets	41.1–41.7	40.9–42.1
Turns	22.7–23.0	22.8–23.1
Random coil	31.8–32.6	31.6–32.6
FTIR spectral similarity, %	*8 lots*	*6 lots*
α-helices	3.8–5.3	4.3–5.2
β-sheets	39.8–43.2	40.0–42.5
Turns	11.1–11.5	11.1–11.6
Random coil	35.8–39.9	35.5–40.7
Intrinsic Fluorescence spectral similarity	*8 lots*	*6 lots*
	Yes	Yes
Differential scanning calorimetry, °C	*8 lots*	*6 lots*
Tm_1	68.6–69.1	68.6–69.0
Tm_2	69.5–69.7	69.4–69.7
Tm_3	78.9–79.3	79.1–79.4
Free thiols content, units per mol of eculizumab	*3 lots*	*3 lots*
	0.1–0.8	0.3–0.9
Impurities (SEC-HPLC), %	*19 lots*	*12 lots*
LMW	0.0–0.3	0.0–0.3
Eculizumab monomer	99.0–99.8	98.6–99.5
HMW	0.2–0.8	0.4–1.1
Charge isoforms (IEX-HPLC), %	*6 lots*	*6 lots*
Lys 0	49.2–62.7	52.1–55.4
Lys 1	17.2–31.1	17.1–20.5
Lys 2	7.3–13.3	5.8–7.1
Acidic	2.9–13.0	6.6–23.7
Charge isoforms (IEF), %	*3 lots*	*3 lots*
Isoform 1 (pI ~ 6.23)	7.1–8.5	4.1–4.9
Isoform 2 (pI ~ 6.05)	20.7–21.9	15.8–18.4
Isoform 3 (pI ~ 5.90)	44.1–46.1	39.4–43.2
Isoform 4 (pI ~ 5.75)	18.3–19.5	23.9–26.3
Isoform 5 (pI ~ 5.65)	5.6–6.2	9.7–14.3

pI isoelectric point, *Tm* thermal transition temperature
* Mean±SD

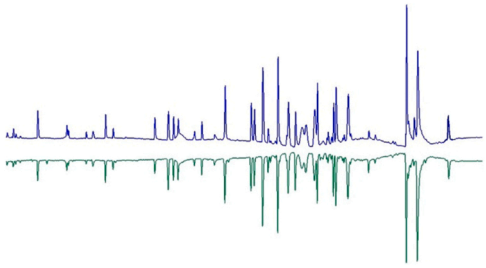

Fig. 1. Peptide mapping by RP-HPLC, Eculizumab RP; Elizaria® DP.

functional significance suggests that it doesn't affect the therapeutical properties of product, because in clinical use after intravenous injection, the lysine is rapidly cleaved by endogenous carboxypeptidases [19]. Other modifications including pyroglutamate formation at N-terminal Q448 and glycosylation at heavy chain N298 of a high degree of similarity of post-translational modification profiles of Elizaria® DP and Eculizumab RP, no significant differences have been identified. No O-glycosylation sites have been detected.

The quantity of disulfide bonds and unpaired cysteines and their locations for Elizaria® DP and Eculizumab RP were determined by HPLC-MS/MS analysis. The visual comparison of base peak current (BPC) chromatograms of native and reduced samples (Fig. S3 in EMS) show multiple variations in elution profiles that could be associated with the disulfide-containing peptides. The location of all identified disulfide bonds is typical for the IgG2 molecule; the only exception is the absence of the disulfide bond between cysteine residues in 23 and 88 sites of light chain, that has been identified for both Elizaria® DP and Eculizumab RP. The detailed disulfide bonds locations in hinge region were not determined due to high heterogeneity of their variants in both molecules that is typically for the IgG2 antibodies [20]. However, in the hinge region of all Elizaria® DP and Eculizumab RP samples we always saw three disulfide bonds and one pair of free cysteine residues. Analysis of non-reduced, alkylated tryptic peptides by RP-HPLC ESI-MS/MS confirmed that 30 (15 pairs) out of a total of 36 Cys residues form disulfide bonds within the heavy chain, within the light chain, and between the heavy and light chains in full eculizumab molecules from both Elizaria® DP and Eculizumab RP samples. In addition, 6 unpaired cysteines were found in location LC 23, LC 88 and HC 228 (hinge region) (Fig. S4 in EMS). Free-cysteine residues due to incomplete disulfide bond formation in mAbs IgG2 can lead to aggregation [21,22] and negatively affect antibody stability [23]. Quantitation of free-Cys by Ellman's method demonstrated comparability of Elizaria® DP and Eculizumab RP samples (Table 2), thus, the same behavior of the compared drugs can be expected despite the absence of guidelines from regulators on acceptable levels of free-cysteine residues.

The previously obtained results [24] of molecular mass measurement and glycoform content estimation for intact protein in the test samples of Elizaria® DP and Eculizumab RP after deconvolution are presented in Table 2, Fig S5 and Fig.S6 in ESM. Comparison of raw mass spectra show that Elizaria® DP and Eculizumab RP forms an ion clusters with charge distribution between approximately 35 + and 65 +, that is typical for denaturated IgG molecules; the most intense signal was registered from the 40 + and 50 + charge state range. The measured average molecular mass for the major *G0F/G0F* glycoform was in good agreement with the estimated value 148,878 Da, while the measurement error did not exceed 5 ppm (1 Da). In general, for Eculizumab RP eight proteoforms were detected and seven for Elizaria® DP, of which 2 proteoforms differed in the glycan profile, and the other two differed in the content of uncleaved C-terminal lysine (see Fig.S6 in ESM). The content of major glycoforms *G0F/G0F*, *G1F/G0F*, and *G1F/G1F* of eculizumab in Elizaria® DP and Eculizumab RP was similar with more variable content of minor glycoforms (see Fig S6 in EMS). For Eculizumab RP, minor

glycoforms *G0F-GN/G0F-GN*, *G0F/G0F-GN*, *G2F/G2F+Hexose* have been found that are absent in Elizaria® DP. The presence of glycoform *G0F/G0* is characteristic only for Elizaria® DP. These differences are due to the use of the production CHO cell line instead of the NS0 line and do not reduce the parameters of the efficacy and safety of Elizaria®, because eculizumab lacks glycosylation-related activities and did not elicit effector function through FcγRs or C1q to mediate ADCC or CDC [25].

For reduced Eculizumab RP and Elizaria® DP has been found that the measured molecular weights of the light (LC) and heavy (HC) chains of eculizumab are in line with prediction at 23,421 and 51,562 Da, respectively (Table 2), with the error not exceeding 19.3 ppm (1.3 Da). It was also confirmed the similarity of molecular weight distributions for $F(ab')_2$ and Fc/2 fragments, what are in line with predictions at 97,480 and 25,217 Da, respectively (Table 2), with the error not exceeding 15.2 ppm (1.0 Da). Additional treatment of F(ab')2 and Fc/2 fragments with TCEP yielded three well chromatographically separated subunits, Fc/2, LC and Fd (Fig. S7 in EMS). Annotated mass spectra of the obtained subunits are shown in Fig. S8 in EMS. The molecular weights of all subunits were equal to the calculated ones, the maximum difference from the expected value did not exceed 0.6 Da (40 ppm). Due to the Middle Up analysis, it was possible to establish that there is no reliable difference in glycoforms *G1F* and *G2F* contents, shown earlier for intact protein (see Table S2 in EMS); oligosaccharides *G0F-GN* and *G0* are unique to Eculizumab RP and Elizaria® DP, respectively.

3.2. Higher-order structure

Integral parameters of the secondary structure of Elizaria® DP and Eculizumab RP were analyzed using two orthogonal analysis: CD in the far-UV spectral regions and FTIR spectroscopy in the medium infra-red (IR) spectral region. The obtained Far UV-CD spectra show a high degree of similarity with an absorption minimum at 217 nm and a maximum of approx. 203 nm (Fig. S9A in EMS), which is typical for proteins with a predominance of the β-sheets. Fig. S9B in EMS shows the overlap of Elizaria® DP and Eculizumab RP FTIR spectra in the wave number range of 1725–1481 cm^{-1}, which additionally confirms the similarity of the secondary structure. Both of Elizaria® DP and Eculizumab RP are characterized by prevalence of β-sheets and negligible contribution of α-helices. The resulting estimated content of the secondary structure elements in the samples tested is outlined in Table 2 and are in perfect agreement between the two orthogonal methods. Slight differences in the content of secondary structure elements were within the experimental error.

Tertiary structure analysis of Elizaria® DP and Eculizumab RP by means of Near UV CD, demonstrate overlap of the obtained spectra (Fig. S10A in EMS), which indicates the similarity of the local environmental symmetry of aromatic amino acid residues and confirms the comparability of the tertiary structure of proteins. Investigation of the state of the local environment of tryptophan and tyrosine residues in the eculizumab tertiary structure was carried out by protein's intrinsic fluorescence method. The shape of emission spectra for Elizaria® DP and Eculizumab RP (Fig. S10B in EMS) indicates the predominance of the spectral contribution of tryptophan residues. Maximum of the fluorescence spectra at 335 nm indicates a high availability of tryptophan residues to the solvent, which provide a predominant spectral contribution. The overlay of the obtained spectra and the similarity of the values of log-normal components (after spectra deconvolution by log-normal function) confirm that Elizaria® DP and Eculizumab RP have a highly similar tertiary structure.

Investigation of thermal stability of Elizaria® DP and Eculizumab RP by means of DSC demonstrated the visual similarity of specific heat profiles obtained (Fig. S11 in EMS), quantitative values of the measured transition temperature (Tm) and the specific enthalpy (Δhcal) presented in Table 2. For the eculizumab, transitions have been assigned to the loss of native structure of the CH2 antibody domain (Tm_1 about 68.8 °C), CH3 antibody domain (Tm_2 about 69.5 °C), and Fab (Tm_3 about 79.1 °C), where the latter transition may also involve early about stages of aggregation. The obtained DSC data confirm comparable thermal and conformational stability of Elizaria® DP and Eculizumab RP.

3.3. Glycosylation profile

In addition to mass spectrometric techniques, N-glycosylation profiles comparison was carried out using HILIC and data are shown in Fig. 2A. The shape of the obtained chromatograms is characteristic for standard IgG molecules and highly similar; the quantitative content of glycan groups (galactosylated, afucosylated, high-mannose and sialylated) is presented in Table 2. The main contribution to the glycoprofile of Elizaria® DP and Eculizumab RP is made by Fc-complex-type glycans containing 0, 1 and 2 terminal galactose residues (*G0*, *G1* and *G2*, respectively). It was found that there is a certain difference in glycans patterns mainly due to different producing cell lines (NS0 for Eculizumab RP, CHO - for Elizaria® DP). Nevertheless, as for unique species found in both products their total content is no more than 4% thus assuming a major (≥96%) part of observed glycans to be identical for Elizaria® DP and Eculizumab RP.

Eculizumab RP has a higher level of high mannose glycans (4.0–5.1% vs 1.3–3.1 in Elizaria® DP), which potentially can lead to decrease mAb thermal stability or on propensity towards aggregation [26], also possible changes in PK profiles (increased serum clearance) provided by mannose receptor pathway [27], however for substantial change in PK properties an overall high-mannose glycans abundance has to be as more as 20% or even bigger up to 80–100% [28], therefore, the difference in high-mannose content can reasonably be considered insignificant.

For monoclonal antibodies, it has been determined that complex afucosylated glycans initiate a greater affinity when interacting with the human FcγRIIIA receptor and enhance ADCC (antibody dependent cell mediated cytotoxicity); and galactosylated glycans stimulate complement-dependent cytotoxicity (CDC), another effector function of IgG [29]. Thus, slightly different contents of galactosylated and afucosylated glycans in Elizaria® DP compared to Eculizumab RP are not significant in context of reduced eculizumab ADCC and CDC potencies [30], also does not affect in vivo clearance [31].

The expected difference in sialylation levels was also detected, due to the difference in the producer's cell lines. Eculizumab RP, produced by NS0 cells, is characterized by the presence of the predominant sialylated glycans containing 5-glycolylneuraminic acid (NGNA). Besides allergenic NGNA for Eculizumab RP, an immunogenic α-Gal epitope was detected (0.6–1.3%), which is naturally absent in Elizaria® DP (Fig. 2B). In Elizaria® DP these oligosaccharides are absent, which is typical for products from CHO cells and provides enhanced safety characteristics of the drug [32]. The results obtained are consistent with those for ABP 959 – CHO-derived Amgen's eculizumab biosimilar: low levels of α-Gal in eculizumab RP and its absence in ABP 959 [4]. A generally low (<2.5%) sialylated glycans content together with overall reduced effector functions of IgG2 allows to expect no influence on any activity characteristics. Since PK of eculizumab is to be provided via FcRn-mediated interaction one may suppose a negligible sialic acids effect here too.

These given conclusions were confirmed by direct pre-clinical and clinical studies of Elizaria® DP thus suggesting physiologicaly insignificant difference with Eculizumab RP due to glycosylation pattern diversity.

3.4. Charge isoforms distribution

Charge isoforms distribution of eculizumab was examined by two orthogonal methods such as cation-exchange chromatography (CEX-HPLC) and isoelectric focusing (IEF).

As follows from the presented CEX-HPLC profiles (Fig. 3 A), Elizaria® DP and Eculizumab RP are characterized by the presence of three major charged isoforms differing by the number of C-terminal lysines

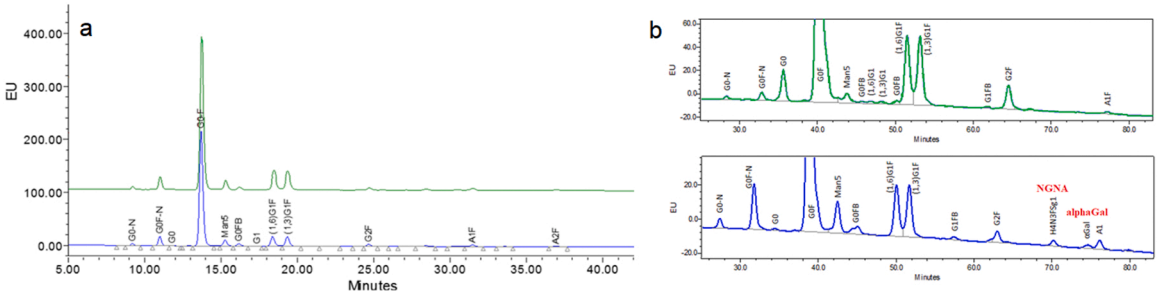

Fig. 2. Glycan profiling by HILIC. a. General view, b – Up-scaled fragment. Eculizumab RP; Elizaria® DP.

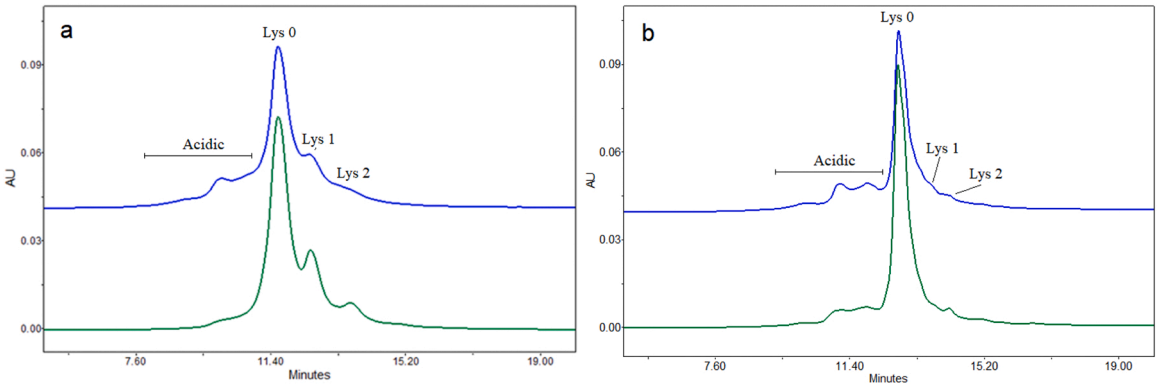

Fig. 3. CEX-HPLC profiles of Elizaria® DP and Eculizumab RP before (a) and after (b) CpB treatment, Eculizumab RP; Elizaria® DP.

(Lys 2 - C-terminal at both heavy chains, Lys 1 - at one heavy chain, Lys 0 - missing from both heavy chains). Also, in both compared drugs acidic isoforms with similar retention times are found. Elizaria® DP is characterized by a wide scatter in the content of individual Lys-isoforms, moreover, their total content (ΣLys) is, on average, 10% higher than that of Eculizumab RP (Table 2). Also the difference between two products is observed in case of acidic forms, the content of which in Elizaria® DP is 5–10% lower than in Eculizumab RP. Additionally, charge isoforms contents were estimated after successive treatment with CpB, which leads to the removal of C-terminal lysine residues and allows more accurate assess of the content of alkaline charge isoforms in a molecule. The CEX-HPLC profiles obtained after CpB treatment (Fig. 3B) show bigger overlapping, while maintaining a difference in the content of acidic isoforms of 10%.

Isoelectric focusing data confirm the results obtained with the CEX-HPLC. For Eculizumab RP and Elizaria® DP the presence of 5 main bands with pI from 5.50 to 6.50 was detected (Fig. 4). The isoform distribution is indicative of a higher prevalence of basic isoforms (band 1 and 2) in Elizaria® DP vs Eculizumab RP (Table 2). Preliminary treatment of the samples with CpB leads to a decrease in content of the most alkaline isoform (line 1 with pI 6.2 and line 2 with pI 6.0), and reasonable similarity is observed for three major isoforms (bands 3, 4, 5 after CpB treatment). Nevertheless, a certain difference is still obvious and only partial similarity could be concluded about isoforms distribution based on obtained results.

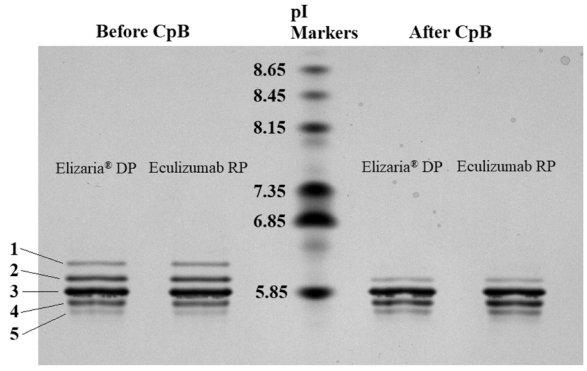

Fig. 4. Isoelectric focusing of Elizaria® DP and Eculizumab RP before (on the left) and after (on the right) CpB treatment.

Increased content of acidic isoforms in Eculizumab RP can be associated with the deamidation of the labile heavy chain asparagine residues (Asn316 and Asn385) mentioned in Section 3.1, as well as sialylation difference (Section 3.3). Revealed small discrepancies cannot be the reason for the difference in efficacy and safety, because the deamidation/sialylation sites capable of acidic isoforms forming are not located in the CDR-regions of the antibody responsible for functional C5-binding. Moreover, for fractionated acidic, basic and the main species no differences were observed during animal pharmacokinetic (PK) and pharmacodynamic (PD) studies [33].

3.5. *Product-related substances and impurities*

Molecular weight distribution of eculizumab was estimated using size-exclusion chromatography (SEC) and capillary gel electrophoresis (CE-SDS) analysis.

SEC-HPLC profiles of Elizaria® DP and Eculizumab RP demonstrate a high degree of similarity, while the retention time (RT) of the eculizumab monomer, high molecular weight (HMW) and low molecular weight (LMW) peaks are the same for the compared drugs, with no new or missing peaks (Fig. 5). The content of HMW, which is mainly a dimer of eculizumab, does not exceed 1.5%, and LMW content is no more 0.5% for both drugs. Both Elizaria® DP and Eculizumab RP contain trace amounts of the eculizumab trimer, which is slightly higher in Eculizumab RP and does not increase during storage of both compared products (data not shown). It is believed that the oligomeric fraction of proteins leads to activity loss and an increased immunological response [34], however the slightly lower content of HMW species for Elizaria® DP

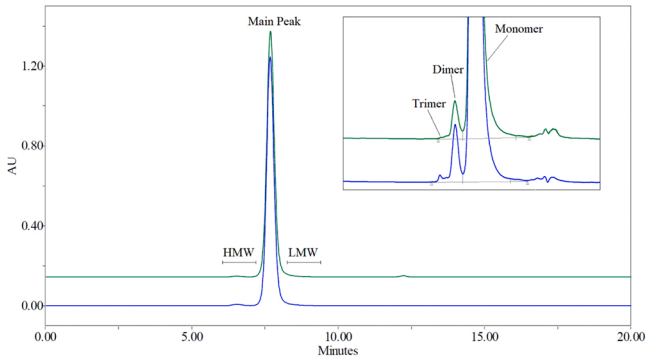

Fig. 5. SEC-HPLC profiles of Elizaria® DP and Eculizumab RP. Up-scaled fragment in frame, Eculizumab RP; Elizaria® DP.

unlikely makes any significant contribution to the potential change in clinical safety.

CE-SDS analysis under non-reducing conditions after samples denaturation with SDS show a presence of several impurities peak with migration time (MT) 12.7, 20.5 and 21.8 min in addition to the eculizumab monomer (MT 22.6 min) with visual similarity of profiles of Elizaria® DP and Eculizumab RP with no new or missing peaks (Fig. S12 in ESM). The tested samples are characterized by a similar degree of purity, constituting 97–98% of eculizumab monomer. The content of truncated forms that can lead to a change in clinical efficacy due to a decrease in the binding activity of monoclonal antibodies is comparable for both drugs.

Analysis of the content of product-related substances and impurities showed no difference in the distribution of hydrophobic variants, determined using HIC-HPLC, between the compared products (Fig. S13 in ESM).

3.6. Specific activity and binding properties

Comparative studies of the biological and functional activities of Elizaria® DP and Eculizumab RP were carried out using a set of analytical methods, including anti-hemolytic assay (anti-HA) measuring the hemoglobin release from chicken red blood cells (RBC), C5-binding specific activity determination by ELISA, C5-component binding evaluation by SPR, FcRn-binding and Fcγ-associated receptors binding assessment by SPR and C1q-binding determination by ELISA.

Elizaria® DP and Eculizumab RP demonstrate similarity of anti-hemolysis, C5- and FcRn-binding by SPR and ELISA, the three biological activities with highest potential clinical impact (Table 3, Fig. 6). By means of SPR, it was determined that of Elizaria® DP and Eculizumab RP have comparable binding kinetics to human neonatal Fc-receptor (FcRn). The assessment of the interactions to human Fcγ-receptors by SPR shows the lack of specific interaction of eculizumab in Elizaria® DP and Eculizumab RP with human CD16 (FcγRIII) and CD64 (FcγRI), which is consistent with the published data [35], and in case of CD32 (FcγRII) shows highly similar binding properties. ELISA also confirmed the comparatively low affinity of eculizumab to C1q for both Elizaria® DP and Eculizumab RP, which is not significant for the functional activity of eculizumab due to the absence of effector functions [2]. These results strongly suggest that Elizaria® DP exhibits highly similar functional properties compared with Eculizumab RP.

Table 3
Biological and functional activities of Elizaria® DP and Eculizumab RP.

Characteristic	Elizaria® range	Eculizumab RP QR
Anti-haemolytic activity	*8 lots*	*6 lots*
RSA, %	96–114	98–106
C5 binding specific activity	*8 lots*	*6 lots*
BU/mg	776 314–1 048 273	835 153–1 076 147
C5 affinity (SPR)	*5 lots*	*3 lots*
Ka, $M^{-1}s^{-1} \times 10^5$	6.11 – 8.01	6.11–6.61
Kd, $s^{-1} \times 10^{-4}$	2.64 – 3.17	2.61–2.92
KD, $M \times 10^{-10}$	4.24 – 4.67	3.94 – 4.78
binding activity (%)	93.0 – 112.6	86.2 – 116.1
FcRn binding (SPR)	*5 lots*	*3 lots*
Ka, $M^{-1}s^{-1} \times 10^6$	1.05 – 1.27	1.01 – 1.1
Kd, $s^{-1} \times 10^{-2}$	4.31 – 5.51	4.19 – 4.46
KD, $M \times 10^{-8}$	3.83 – 4.11	3.80 – 4.40
binding activity (%)	90.4 – 104.1	89.4 – 111.8
CD32(FcγRII) binding (SPR)	*3 lots*	*3 lots*
CD32a KD, $M \times 10^{-5}$	1.66–1.68	1.39–1.62
CD32b/c KD, $M \times 10^{-5}$	5.52 – 5.78	5.59 – 6.29

BU/mg binding unit per mg of protein, Ka (1/Ms) association rate constant, Kd (1/s) dissociation rate constant, KD equilibrium dissociation constant, RSA % relative specific inhibition activity

4. Conclusions

In this study, Elizaria® DP and Eculizumab RP were compared for a total of more than 60 parameters, including general properties such as protein concentration, pH, osmolality, subvisible particle, and so on (data unpublished). Additional investigation of the degradation behavior and trends of eculizumab under the conditions of thermal, oxidative and mechanical stress demonstrated the same stability and impurity profile in the compared Elizaria® DP and Eculizumab RP samples (data unpublished). Taken together, the totality of the analytical data demonstrates that Elizaria® DP and Eculizumab RP are close to identical in physicochemical and structural attributes. The biological activities are highly similar, including critical properties, responsible for the mechanism of action of eculizumab, namely, C5-component binding and hemolysis inhibition. The expected lack of binding with FcγRI (CD64), FcγRIII (CD16) and C1q binding activities was confirmed. Revealed minor differences in a small number of post-translational modifications (deamidation and C-lysine clipping levels) are not considered to be clinically meaningful. The main differences in N-glycosylation profiles, namely the absence of immunogenic α-Gal and NGNA oligosaccharides in Elizaria® DP, which can induce allergic reactions, are due to the different cell lines used for the production of the compared preparations, which is the advantage of Elizaria® DP in terms of product quality and safety profiles. These were confirmed by the comparable results of head-to-head non-clinical and clinical trials.

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CRediT authorship contribution statement

Valentina Gusarova: Conceptualization, Supervision, Methodology, Validation, Investigation, Writing – original draft, Writing – review & editing, Visualization, Formal analysis. **Maxim Degterev**: Methodology, Investigation. **Ivan Lyagoskin**: Supervision, Methodology, Validation, Investigation, Writing – original draft, Formal analysis. **Vladimir Simonov**: Methodology, Validation, Investigation. **Maxim Smolov**: Supervision, Methodology, Formal analysis. **Sergey Taran**: Methodology, Validation, Investigation, Writing – original draft, Formal analysis. **Rahim Shukurov**: Project administration, Conceptualization, Resources.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

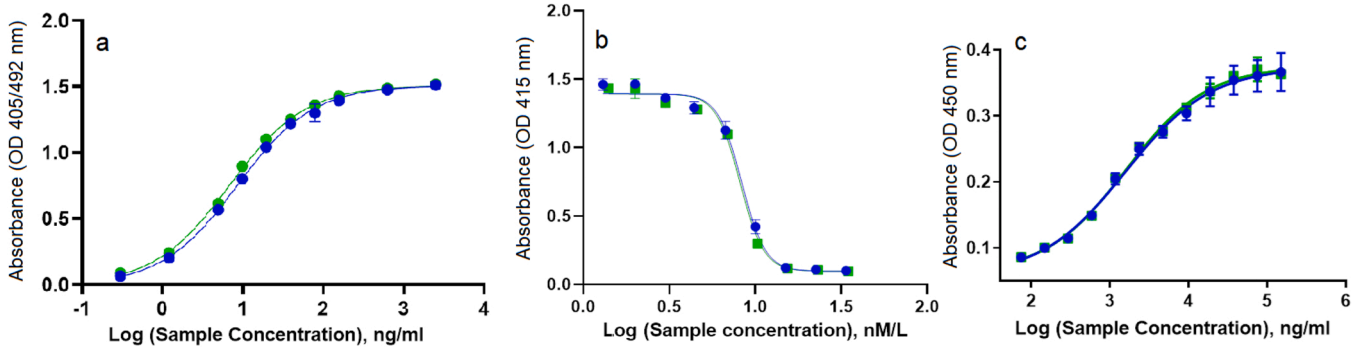

Fig. 6. Biological function of Elizaria® DP and Eculizumab RP. a. C5-binding specific activity; b. Specific anti-hemolytic activity; c. C1q-binding, Eculizumab RP; Elizaria® DP.

Data availability

The authors do not have permission to share data.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpba.2022.115004.

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