Original Article

Comparative biosimilar quality studies between a rituximab product and MabThera

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ABSTRACT

In this work a rituximab follow-on biologic, Zytux is tested for its biosimilarity to the reference originator product, MabThera. The test involved an analytical assessment exercise done head-to-head on samples of Zytux and MabThera withdrawn randomly from the Iraqi governmental hospitals. This analytical assessment encompassed methods for comparing the quality aspects of the product such as protein (rituximab) content, purity, integrity, primary structure, charge variants, higher-order structure, and *in vitro* biological activity. Rituximab concentration and pH of Zytux and MabThera were comparable and lay within the product specification. Both products showed comparable integrity and purity profiles when tested by SDS-PAGE analysis. Rituximab from both products showed a similar primary structure profile using HPLC-based peptide mapping. The results also showed comparable charge variant profiles obtained by cation exchange chromatography analysis, comparable secondary and tertiary structures probed by circular dichroism (CD) scan and CD-melt, respectively, and finally comparable biological activity of both products using complement-dependent cytotoxicity (CDC) assay. Overall results obtained in this study suggest a high degree of similarity. Therefore, Based on these studies one can conclude that Zytux is a likely biosimilar to MabThera, even though more studies are needed as part of the "totality of evidence" required to ensure the biosimilarity of rituximab follow-on biologic to the reference product.

Keywords: Biosimilars, Rituximab, MabThera, Zytux, Monoclonal antibody, Analytical

Introduction

A biosimilar product is a biological medicinal product that is highly similar to the originator stand-alone biological product developed earlier. Unlike small-molecule drugs which are supposed to be identical in generic medicinal products, it is difficult to make exact copies of a biological product [1]. The difficulty in making identical copies comes from the complex nature of these products and the way they have been made [2, 3]. Biological products are usually protein in nature with a large molecular weight. The large size of proteins leads to the formation of higher-order structures, namely secondary and tertiary structures. These higher-order structures are maintained through a set of weaker interactions than the covalent bonds holding the primary structure together such as hydrogen bonds, ionic bonds, and Van der Waals forces. Thus, proteins are considered as delicate molecules compared to small molecules and need special conditions in production and handling [4]. Biological medicinal products including biosimilars are also produced in living cells such as mammalian cells, yeast, and bacteria. Therefore, any change in the production processes can lead to significant changes in the protein properties produced in these cells due to the high sensitivity of the protein-producing machinery of these cells to variation in production conditions such as temperatures, agitation rates, dissolved oxygen concentration, and type and amount of nutrients [5].

Despite the above-mentioned difficulties, there has been an urgent need to facilitate and encourage the replication of some of the patent-expired originator biological products to create competitors to these products and reduce the cost of biological therapy for patients who need them in their treatment protocols

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[6]. Therefore, stringent regulatory authorities such as the European Medicines Agency (EMA) and the US FDA have set the stage for the introduction of biosimilars through the introduction of abbreviated approval pathways [2, 7, 8]. The Iraqi National Regulatory Authority (NRA), for example, follows EMA guidelines for the registration of biosimilars [9]. These pathways are different from the new drug application pathways, mainly in the size of clinical studies required to prove the efficacy and safety of the product. A biosimilar testing requires an extensive analytical characterization to prove its similarity to the originator product but less of the expensive clinical studies.

The analytical biosimilarity exercise involves a set of head-to-head characterization methods using the proposed biosimilar product and the originator product under the same experimental conditions [10, 11]. These characterizations involve methods for characterizing the protein amino acid sequence, which should be the same as the originator product. Other methods are looking for the similarity in the protein higher-order structure which has to be the same to ensure proper product stability and proper interaction with the target receptor. Comparing the protein purity, integrity, and posttranslational profiles are other crucial critical quality attributes to ensure that a biosimilar product can have the same clinical efficacy as the originator product [12]. These comparisons serve the basis of waiving any unnecessary clinical studies and allow the "extrapolation of indications" principle to take place and faster approval of biosimilars [13, 14].

Rituximab is a chimeric anti-CD20 monoclonal antibody (mAb) that has been used in the treatment of several types of cancers such as non-Hodgkin's lymphoma and chronic lymphocytic leukemia. Rituximab is also used in the treatment of rheumatoid arthritis, pemphigus vulgaris, as well as granulomatosis with polyangiitis, and microscopic polyangiitis. Rituximab was first developed and sold under the name MabThera (or Rituxan®, USA). However, MabThera went off patent in 2013 (Europe) and pharmaceutical companies started developing rituximab follow-on biologics (i.e. biosimilar) such as Rixathon, Truxima, Ruxience, and Zytux [15, 16].

The first rituximab follow-on biologic that was used in Iraq before the introduction of the Iraqi biosimilars registration guidelines was Zytux [9, 17, 18]. This product was procured for the Iraqi Ministry of Health (MoH) to be used in replacement of MabThera (which is also available in the Iraqi hospitals) to reduce the cost of biological products and reallocating the money saved to procure other essential medicines. Therefore, this work aimed to assess the similarity of Zytux, which was already supplied to the Iraqi hospitals, to the originator product, MabThera. The assessment included a set of analytical studies as part of the "totality of evidence" exercise of demonstrating biosimilarity of follow-on biologic to its originator counterpart.

Materials and Methods

Random Zytux (AryoGen Pharmed) and MabThera (Roche) unexpired samples (500 mg-vials of each product) were obtained from Iraqi hospitals, and the following analyses were performed as part of assessing similarity between the two products using the available resources to draw as much similarity as possible between the two products.

Rituximab bioinformatics data analyses

The amino acid sequence for the chimeric monoclonal antibody rituximab was obtained from DrugBank for both the light and the heavy chains [19]. The sequence was analyzed using the well-known protein bioinformatics website ExPASy-ProtParam tool (https://web.expasy.org/protparam/) to calculate several rituximab parameters such molecular weight (a non-glycosylated variant of the drug), theoretical isoelectric point (pI), amino acid composition, atomic composition, and extinction coefficient. This information helps in comparing Zytux to the reference product, MabThera, and gives the reader an introduction to the protein.

Determination of rituximab concentration

Rituximab concentration was determined for both Zytux and MabThera by measuring the absorbance at 280 nm. Samples were prepared by diluting the drug with the appropriate volume of phosphate buffer saline (PBS) to reach a rituximab nominal concentration of 0.5 mg/ml. An extinction coefficient of 235,380 $M^{-1}cm^{-1}$ (assuming all pairs of Cys residues form cystines) was used in the calculations to translate the absorbance into molar concentration then into mg/ml concentration.

Determination of pH

Determination of pH was carried out by placing a small volume of the rituximab solution into a clear small beaker and the pH of the solution was measured by the potentiometric method using a calibrated pH apparatus according to the method reported in European pharmacopeia, EP (2.2.3). The pH meter was calibrated in three different pHs using calibration solutions of pH 4, 7, and 10, respectively. The measurements were taken at the same temperature which was room temperature.

SDS-PAGE analyses

The electrophoretic mobility patterns of Zytux and MabThera were compared by SDS-PAGE analysis, as previously described [20]. In this analysis, a 12% acrylamide gel was used, and the samples were analyzed under both reducing and non-reducing conditions. Samples were prepared by mixing about 4 µg of the sample with the appropriate volume of the sample loading buffer, either non-reducing buffer or reducing buffer (containing 2-mercaptoethanol). The samples were placed into a Thermo Block preheated to 90°C for 7-10 minutes and then loaded into the gel wells. A molecular weight marker was also loaded on the gel to determine the approximate molecular weight of the proteins. The gel was then run for 60 minutes at 180 volts using a Bio-Rad power supply (PowerPac™ Basic Power). Coomassie blue R-250 (Bio-Rad) solution was used to develop the gel and visualize the protein bands after destaining. The gel was pictured and saved on the computer using a phone camera.

Cation exchange chromatography (CEX) of intact rituximab

A 300 µL sample of 1 mg/mL rituximab was prepared using mobile phase A (10 mM phosphate buffer pH 7.5) as diluent. Then, 5 µL of 5 mg/mL carboxypeptidase B from the porcine pancreas (Sigma, C9548) solution was added and the mixture was incubated for 2 hours at 37 °C to eliminate any charge heterogeneity coming from the C-terminal lysine clipping [21]. Samples of 80 µL of the digestion mixture were injected into the TSKgel CM-STAT ion-exchange column (7um, 4.6 x 100 mm) using an HPLC system, a flow rate of 0.8 mL, gradient elution with buffer B which is the same as A plus 100 mM NaCl. The protein was monitored using a UV
absorbance detector at 280 nm.

Peptide mapping analyses

Rituximab (deglycosylated using PNGase F) samples of 10 mg/mL concentration were denatured by adding denaturing buffer (6 M guanidine HCl, 1 mM EDTA, and 250 mM Tris HCl, pH 7.5). Dithiothreitol (DTT) solution was added to the samples, and the samples were incubated at 37°C for 30 min. Then, iodoacetamide (IAA) solution was added to the samples, and the samples were incubated at room temperature for 30 min. To prepare the mixture for trypsin digestion, digestion buffer (2 M urea in 100 mM Tris HCl, pH 7.8) was added. Digestion continued for 18 hours at room temperature. Digestion was quenched by adding trifluoroacetic acid solution, and the samples were injected into a C18 column (Grace Vydac, 4.6 × 150 mm, 5µm, 300 A° column) using an HPLC system, where the detector was set at 280 nm wavelength.

Circular Dichroism (CD) analyses

Zytux and MabThera samples were prepared for CD analyses by purifying rituximab from the formulation buffer using a protein A affinity spin column (NAb Spin Kit, Peirce), according to the manufacturer's directions. Protein fractions resulting from the spin column were desalted through a 10 kDa MWCO filter (Sartorius). Samples were diluted to 200 µg/mL with 10 mM potassium phosphate buffer pH 7.6 for CD analysis. Far UV-CD spectra (from 260-200 nm) were recorded using a Jasco 810 spectrometer at 20 °C, using a 1mm path length cuvette and a scan speed of 12 nm/min. Three replica scans were collected and the average was recorded. Final spectra represent buffer subtracted data.

The samples were also subjected to thermal melt analysis (CD-melt) which was carried out at 220 nm over a temperature ramp between 20 - 95°C. Melting temperatures were calculated from the inflection point at which the fraction of unfolded protein was 0.5 [22].

Biological activity

The biological activity of Zytux was compared to Mabthera using a complement-dependent cytotoxicity (CDC) *in vitro* assay. Serial dilution of Zytux and MabThera were prepared, and then 50 µL of each concentration was transferred into a 96-well assay microplate. A cell suspension of CD20-positive WIL2-S cells with a density of 0.7- 0.8×10^6 cells/ml was prepared. Then 50 µl of cell suspension was added to each well of the 96-well assay microplate. After that, 50 µl of diluted complement was added to wells except for the cell control wells. The plate was stained with Alamar blue (Bio-Rad) and incubated for 16-24 hours. The plate was read using a multi-detection microplate reader at the excitation wavelength of 540 nm and emission wavelength of 590 nm with a cut-off at 590 nm. Finally, the biological activity of Zytux and its relative potency to reference product MabThera were measured by a parallel line assay using PLA 3.0 software (Stegmann Systems)

Results and Discussion

Rituximab properties using bioinformatics data analysis

The calculated rituximab parameters such as molecular weight (non-glycosylated variant of the drug), theoretical isoelectric point (pI), amino acid composition, and extinction coefficient **(Figure 1)** can help in comparing Zytux to the reference product, MabThera. It is important to note that the data obtained from this bioinformatics exercise are for the non-glycosylated variant of rituximab. But they represent a good value to start with then add glycosylation, which is widely known to be a biantennary of complex-type glycans [23]. The isoelectric point (pI) can be also shifted from the theoretical values because the website ExPASy-ProtParam did not take into account the charges that might exist on the glycan part of rituximab, especially the negative charge of sialic acid. Some of the calculated properties such as the rituximab extinction coefficient and molecular weight are utilized in this work while other calculated parameters can be used for further rituximab biosimilars characterization, such as the use of pI in capillary isoelectric focusing (cIEF) for the determination of charge variants.

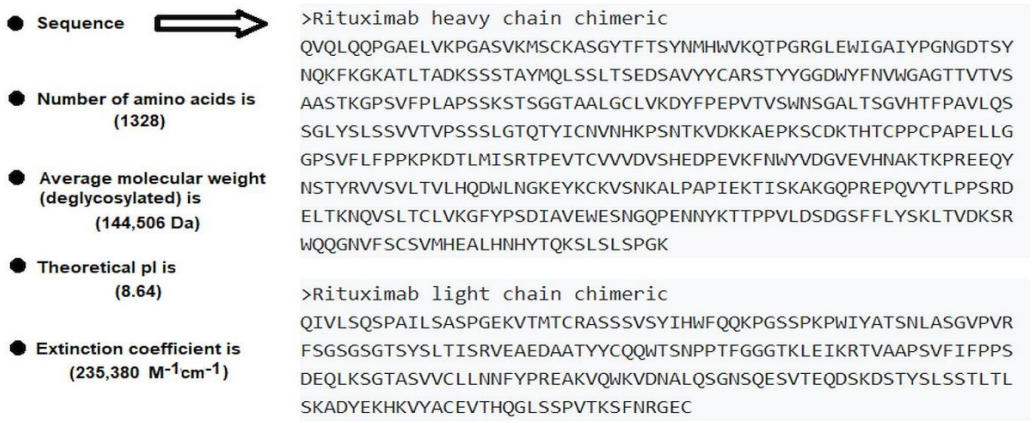


Figure 1. Primary structure of rituximab along with several of its theoretical properties calculated based on the available bioinformatics database

Concentration and pH of the solution

The concentration measurement carried out on the diluted product using UV-spectrophotometer and a calculated extinction coefficient of 253,380 $M^{-1}cm^{-1}$, showed a concentration of 0.51 mg/ml ($Abs._{280nm}$= 0.83) and 0.53 mg/mL ($Abs._{280nm}$= 0.87) for Zytux and MabThera, respectively. These concentrations translate back into a vial (500 mg vial) content of 510 mg and 530 mg of Zytux and MabThera, respectively assuming the same nominal filling volume of 500 mL for both products. Both concentrations lie within the acceptable range of total protein specification indicating the same amount of active drug substances can be delivered to the patient using the same volume of either product [24]. The concentration similarity among the two products can also indicate a uniform content for both products and an accurate formulation for the biosimilar products, Zytux.

Furthermore, pH measurements which were carried out on the undiluted trastuzumab solution of Zytux and MabThera, gave a pH of 6.5 And 6.7 for Zytux and MabThera, respectively which indicates quite a similar pH for both products where both of the values lie within the consensus finished product pH specification range of 6.2-6.8 [25]. Maintaining the pH of the finished rituximab product within a specific range is usually achieved by using certain formulation buffers such as phosphate and citrate buffers. These buffers help prevent many posttranslational modifications such as acid and base-catalyzed deamidation and oxidation which are known to affect protein integrity, stability, and biological activity [26]. It is also important to mention that keeping the pH of the drug product within the release specification pH (6.2-6.8) can keep the protein positively charged since this pH is below the theoretical pI (8.64) by more than 1 pH unit. Hence, the positive charge on rituximab can reduce its aggregation which is beneficial because aggregation can cause a loss of activity and possible immunogenicity [27].

SDS-PAGE analysis

The results from SDS-PAGE analyses are shown in **Figure 2** which illustrates a similar electrophoretic mobility pattern for Zytux and MabThera under both non-reducing conditions and reducing conditions. Under non-reducing conditions, IgG-isotype mAbs show a single band usually around 150 kDa. This band is slightly broader than usual due to the presence of N-glycans in the C_H2 domain of the heavy chain of these mAbs imparting them some sort of microheterogeneity. However, under reducing conditions the single band should separate into two bands, one around 50 kDa and the second around 25 kDa due to the reduction of the disulfide bonds which are usually holding the heavy and light chains of the IgG homodimer together [28]. There is also a very faint band underneath the heavy chain major band which usually corresponds to the non-glycosylated heavy chain. This faint band also indicates a high percentage of glycosylation site occupancy at the C_H2 domain of the heavy chain to support the rituximab structural stability and mediate the effector functions [29]. Also, the analyses show no additional bands below or above the expected bands from an IgG mAb sample which indicates the absence of proteolytic and aggregation species, respectively. The SDS-PAGE analyses gave a clear picture of the purity and integrity expected from biosimilar as well as originator mAb therapeutic products.

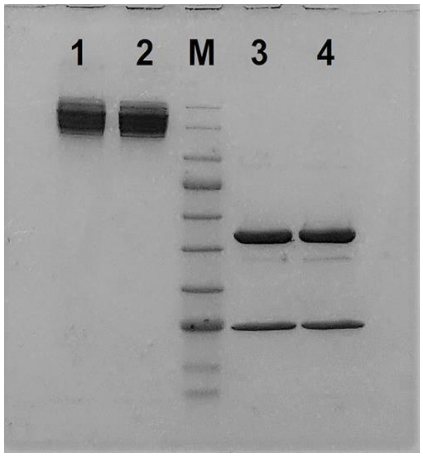
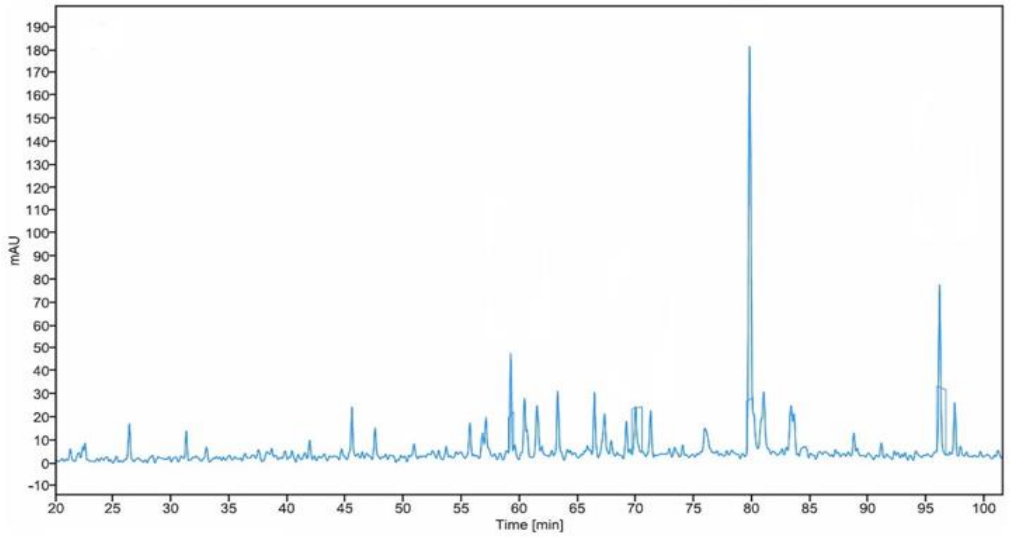


Figure 2. A comparison of SDS-PAGE analysis of Zytux and MabThera. Lane 1 and 2 represent Zytux and MabThera, respectively under non-reducing conditions. Lanes 3 and 4 represent Zytux and MabThera, respectively under reducing conditions (2-mercaptoethanol added). M lane represents a marker with the size of the following components in kDa (from top to bottom): 180, 135, 100, 75, 63, 48, 35, 25, 17, 11

Primary structure comparison

Peptide mapping is an essential tool for identifying the protein and comparing the protein amino acid sequence (primary structure) to a reference standard, as it serves as a fingerprinting tool for any known protein. In this analysis, the protein-long polypeptide chain was cut into smaller peptides using certain peptidases such as trypsin used here. Trypsin usually cleaves the peptide bond between the carboxyl group of the amino acid lysine or arginine and the amine group of any adjacent amino acid except proline. The resulting smaller peptides have different but distinct retention times on a C18 column, for example, using an HPLC instrument coupled to a UV detector. Comparing the resultant peaks belonging to these peptides can serve as a powerful tool to compare biosimilars to their originators or to compare different lots of the same product. Results from this test showed that both Zytux and MabThera exhibited similar peptide mapping profiles **(Figure 3)** which indicate that both products share the same primary structure to a large extent [30]. It is also worth mentioning that peptide mapping should be better done on the deglycosylated protein samples to excludes any differences in the protein primary structure which can come from the different retention times and intensities of glycopeptides, which are usually heterogeneous.



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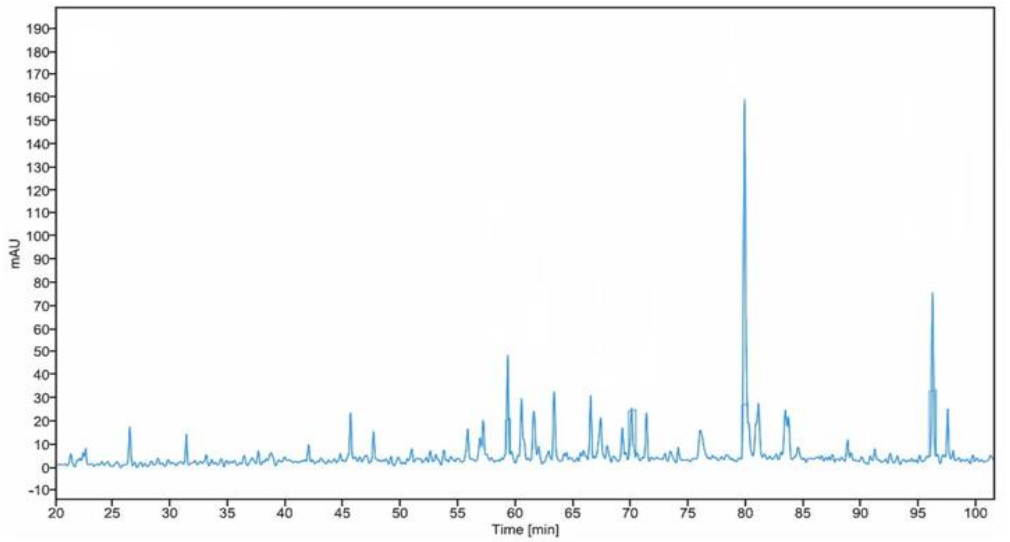


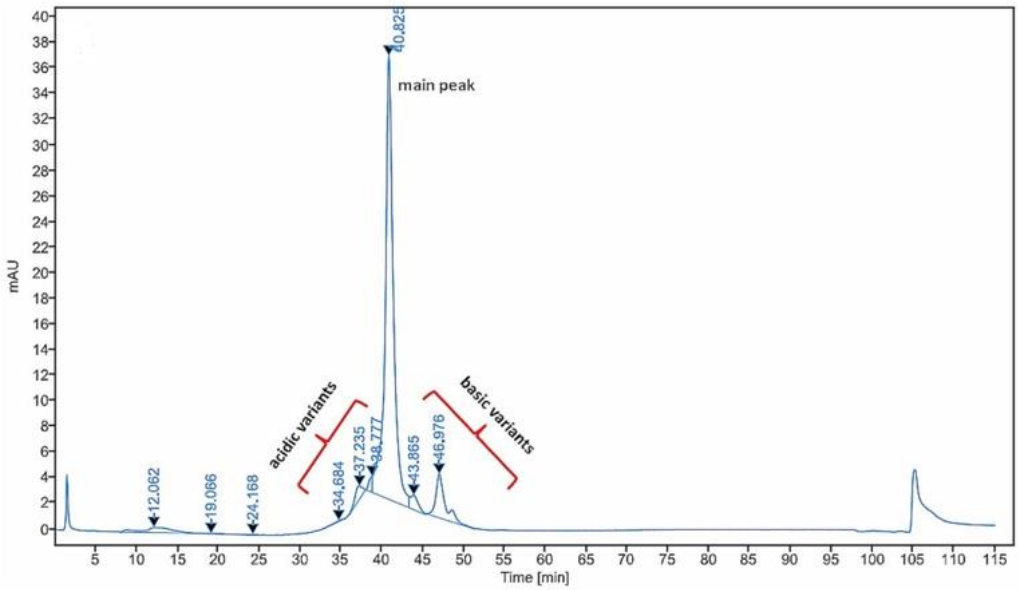
b)

Figure 3. Peptide mapping for primary structure analysis of Zytux and MabThera using trypsin digestion on a C18 column and HPLC UV detector. Panel a is for Zytux and panel b is for the reference product, MabThera

Charge variants by cation exchange chromatography

Charge variants, specifically the acidic and basic isoforms, of Zytux and MabThera were measured using cation exchange chromatography (CEX) and compared to each other. The chromatograms from the CEX are shown in **Figure 4**. Adding up the areas under the peak of the acidic isoforms (all the peaks adjacent and left to the main peak) resulted in 3.5% and 3.7% acidic isoforms, while adding up the areas under the peak of the basic isoforms (all the peaks adjacent and right to the main peak) resulted in 12.4% and 4.8% basic isoforms for Zytux and

MabThera, respectively. These results indicate that the charge variants profiles for the products are comparable and acceptable since none of the isoforms exceeded 20% of the main peak which should constitute more than 60% [24, 30].



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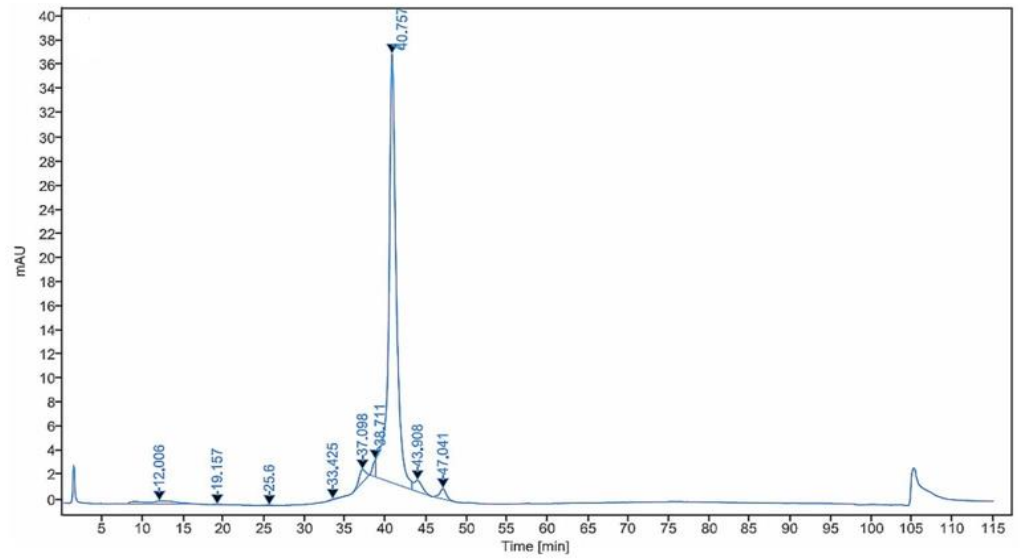


b)

Sample	% area content by CEX		
	Acidic variants	Main peak	Basic variants
Zytux	3.5	84.1	12.4
MabThera	3.7	91.5	4.8

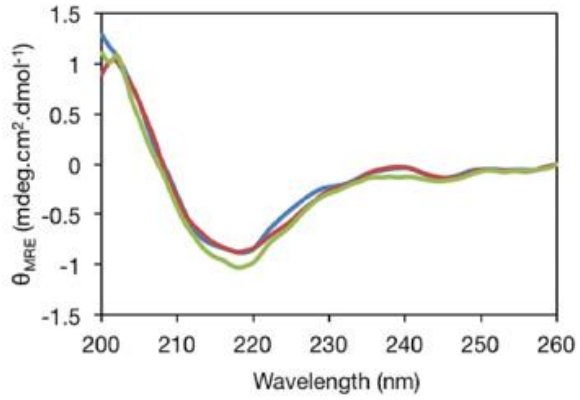
Figure 4. Charge variants comparison between Zytux and MabThera using cation exchange chromatography. Panel A is for Zytux and panel B is for the reference product, MabThera

It is important to clarify that charge variants of monoclonal antibodies can affect many aspects of the antibody, such as its structure and biological activity. These charge variants can result from the protein undergoing different chemical reactions such as deamidation, oxidation, isomerization, phosphorylation, cyclization, and C-terminal lysine clipping, especially in mAbs. Besides affecting biological activities and stability, the percentages of these charge variants are tightly bound to the manufacturing process of the product. Therefore, they are considered as a critical quality attribute and should be controlled carefully [31, 32].

Protein higher-order structure by circular dichroism

Circular dichroism (CD) was used to probe the protein secondary structure and to test its stability by measuring the secondary structure content as a function of increasing

temperature (CD-melt) [22]. Since CD analysis is sensitive to high buffer concentration, rituximab was stripped of its formulation buffer to obtain a better picture for comparing Zytux and MabThera secondary structures. Both products showed comparable far UV-CD spectra with a molar ellipticity (θ) minimum around 218nm-220nm **(Figures 5a and 5b)** which is characteristic of the beta-sheet composition of immunoglobulin G with no alpha helix content [24, 33]. Calculating midpoints of the transition (Tm) from the CD-melt thermograms **(Figures 5c and 5d)** gave values of 78.1 ± 0.3 °C and 77.8 ± 0.1 °C for Zytux and MabThera, respectively.



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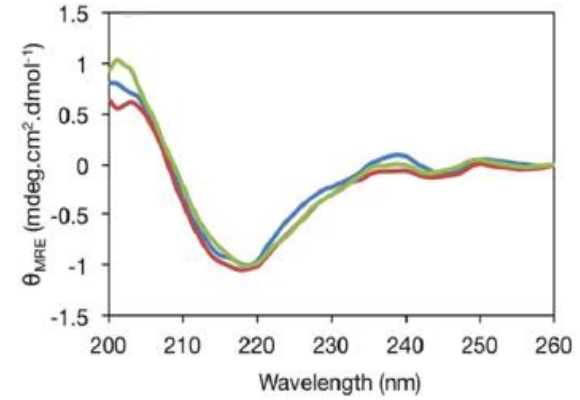

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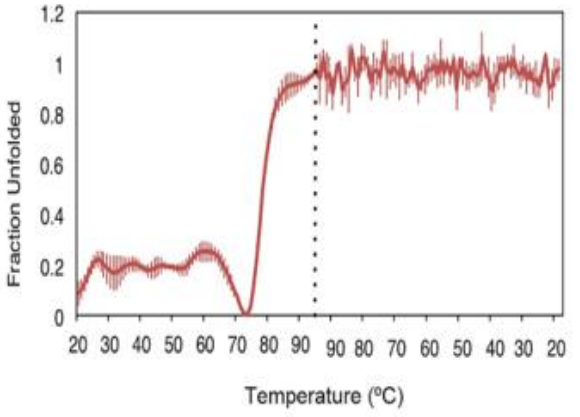

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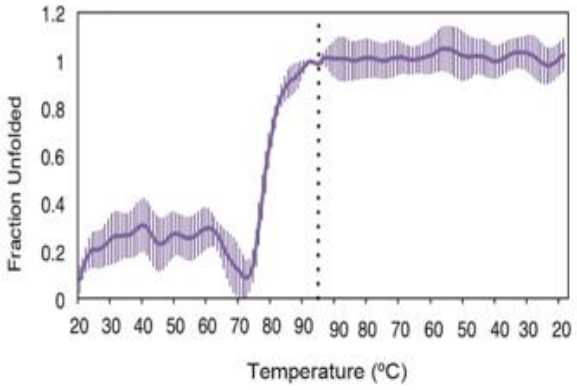


d)

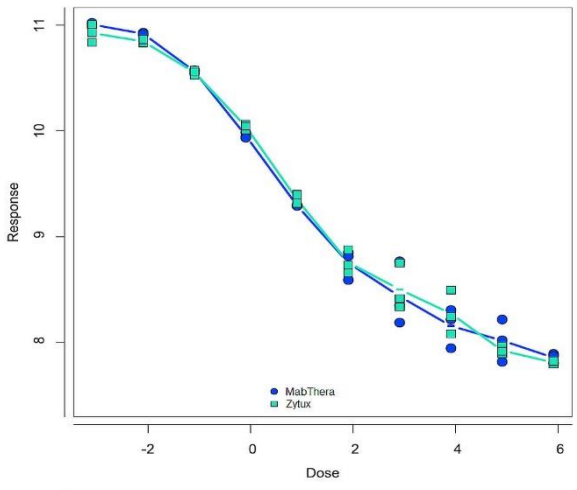
Figure 5. Comparative circular dichroism (CD) analyses of Zytux and MabThera in 10 mM potassium phosphate buffer pH 7.6. Panels (a) and (b) represent CD-scan profiles for Zytux and MabThera, respectively. Panels (c) and (d) represent the thermal stability of Zytux and MabThera by CD as a function of temperature at 220 nm (CD-melt curves), respectively

Thus, Tm is the same for both products indicating comparable higher-order structure composition and stability. Cooling the samples of Zytux and ManThera did not restore the molar ellipticity to their initial values indicating protein irreversible unfolding and loss of higher-order structure upon heating of the samples. Overall results from the CD analyses indicate that rituximab obtained from Zytux and MabThera is folded correctly to give the native secondary structure where this secondary structure is critical for the native protein tertiary structure required for biological activity and stability of mAbs.

Biological activity

The biological activity of a mAb is usually tied to the antibody native structure and the integrity of its Fab, hinge, and Fc regions. The biological activity of Zytux and MabThera was tested using complement-dependent cytotoxicity (CDC) assay. It is well known that the binding of rituximab to its target receptor (CD20) through the Fab region can trigger important effector functions necessary for its mechanism of action such as CDC [34]. The complement system can be activated through the binding of the C1q protein of this system to the Fc region of the rituximab immune complex to result in the formation of the membrane attack complex (MAC) that can induce cell lysis and death.

The *in vitro* CDC assay using CD20 positive WIL2-S cell **(Figure 6)** showed a relative mean potency of Zytux to MabThera of 98%. These results indicate similar CDC activity of Zytux to MabThera since the 90% confidence interval of the ratio of the means lies within the conventional equivalence range of 80% to 125%, and hence similar biological activity.



	Repeat	Potency Ratio	Relative Potency
	1	0.981	
Zytux/ MabThera	2	0.991	98%
	3	0.970	

Figure 6. Complement-dependent cytotoxicity (CDC) assay of Zytux and MabThera using CD20 positive WIL2-S cells. A representative comparative dose-response curves of Zytux (green) and the reference product MabThera (blue). Data points represent the mean ±SD

Conclusion

Biotechnology products, including the anti-CD20 monoclonal antibody rituximab, are complex molecules that are difficult to characterize compared to small-molecule drugs. However, with recent advances in analytical characterization techniques and the introduction of state-of-the-art instruments, the mission can become easier. In this work, it was demonstrated through selected analytical studies that a rituximab product, Zytux, is similar to the originator product MabThera in many physicochemical and biological aspects. The work provided a snapshot of the most important similarity features used in comparing protein therapeutics including rituximab. The features included an analysis of protein purity and integrity through SDS-PAGE, comparing the protein primary structure through peptide mapping analyses, comparing the charge variants profile through CEX, comparting the protein secondary and tertiary structure through CD analyses, and finally comparing the biological activity through a CDC assay. The selected analytical characterizations used herein represent cornerstone evidence that is part of the "totality-of-evidence" required to demonstrate biosimilarity of Zytux to the reference product, MabThera.

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Ethics statement: None

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