Video Article

In Vitro Methods for Comparing Target Binding and CDC Induction Between Therapeutic Antibodies: Applications in Biosimilarity Analysis

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Abstract

Therapeutic monoclonal antibodies (mAbs) are relevant to the treatment of different pathologies, including cancers. The development of biosimilar mAbs by pharmaceutical companies is a market opportunity, but it is also a strategy to increase drug accessibility and reduce therapy-associated costs. The protocols detailed here describe the evaluation of target binding and CDC induction by rituximab in Daudi cells. These two functions require different structural regions of the antibody and are relevant to the clinical effect induced by rituximab. The protocols allow the side-to-side comparison of a reference rituximab and a marketed rituximab biosimilar. The evaluated products showed differences both in target binding and CDC induction, suggesting that there are underlying physicochemical differences and highlighting the need to analyze the impact of those differences in the clinical setting. The methods reported here constitute simple and inexpensive *in vitro* models for the evaluation of the activity of rituximab biosimilars. Thus, they can be useful during biosimilar development, as well as for quality control in biosimilar production. Furthermore, the presented methods can be extrapolated to other therapeutic mAbs.

Video Link

The video component of this article can be found at https://www.jove.com/video/55542/

Introduction

Therapeutic antibodies are recombinant monoclonal antibodies (mAbs) developed for the treatment of different pathologies, including cancers, autoimmune and chronic diseases, neurologic disorders, and others[1]. Currently, the FDA has granted approval to more than 40 therapeutic mAbs, and more are expected to reach the market in the following years.

Rituximab is a high-affinity chimeric monoclonal IgG1 antibody approved for the treatment of $CD20^+$ B-cell non-Hodgkin's lymphoma (NHL), $CD20^+$ follicular NHL, chronic lymphocytic leukemia, and rheumatoid arthritis[2,3]. The recognition of CD20, which is overexpressed in B cells, by rituximab induces apoptosis; complement activation; and antibody-dependent cell mediated cytotoxicity (ADCC)[3]. The patents of this drug expired in Europe and in the U.S. in 2013 and 2016, respectively. Thus, pharmaceutical companies worldwide are developing rituximab biosimilars. As in any other drug for human consumption, biosimilars require approval from regulatory agencies. International guidelines indicate that for mAbs, biosimilarity should be demonstrated by comparing the physicochemical characteristics, pharmacokinetics, efficacy, and safety of the new and reference products[4].

Accordingly, the methodologies used in such comparisons must assess the structural and functional characteristics of the mAbs, especially those with clinical relevance. To that end, *in vitro* assays show several advantages over *in vivo* experiments (reviewed in Chapman *et al.*)[5]: i) *in vitro* studies are more sensitive to differences between the proposed biosimilar and the reference product; ii) *in vivo* studies must be performed in relevant species, which for many mAbs are non-human primates; and iii) since the mechanism of action, the preclinical toxicology, and the clinical effects of the reference product are well known, *in vivo* studies with biosimilars may not provide additional useful information. Accordingly, the European Union's Guidance for biosimilars allows candidates to enter clinical trials based on robust *in vitro* data alone[6].

Here, we present two fast, economic, and simple assays that evaluate the biological activity of rituximab using $CD20^+$ cultured cells. These assays can be included as part of the comparability exercise for rituximab biosimilar candidates.

Protocol

1. Evaluation of Target Binding by Flow Cytometry

1. **Preparation of biological materials and reagents**
   1. Make 500 mL of RPMI culture medium supplemented with 10% heat-inactivated fetal bovine serum (H-IFBS).
   2. Culture Daudi Burkitt's Lymphoma (Daudi) cells and Daudi GFP$^+$ cells using RPMI and 75-cm^2 culture flasks. Maintain the cultures at 37 °C in a 5% CO_2 humidified atmosphere until they reach 6 - 9 x 10^5 cells/mL.
   3. Make 50 mL of staining buffer by diluting 1/100 H-IFBS in PBS; this buffer is stable at 2 - 8 °C for at least one month.
   4. Prepare the test solutions for the reference and biosimilar mAbs. Make ten 1:2 serial dilutions (500 µL each) in staining buffer, starting from 5 µg/mL.
   5. Use staining buffer to dilute human IgG (isotype control) to 5 µg/mL and PE-Cy5 mouse anti-human IgG (secondary antibody) to the concentration suggested by the manufacturer.
   6. Prepare 4% paraformaldehyde in PBS (fixation buffer).

2. **Target binding**
   1. Collect the Daudi and Daudi GFP$^+$ cell suspensions from the 75-cm^2 culture flasks and transfer them to a 15-mL centrifuge tube. Centrifuge at 400 x g for 5 min.
   2. Wash the cells by adding 5 mL of PBS and centrifuging the cell suspension at 400 x g for 5 min.
   3. Resuspend the cells in PBS and perform a cell count and viability analysis with trypan blue. Use cultures with cell viability levels ≥ 95% for the analysis.
   4. Dilute the cell suspension to 4 x 10^6 cells/mL with cold staining buffer.
   5. In 1.5-mL microcentrifuge tubes, add 50 µL of the cell suspension to 100 µL of the different test concentrations of the reference or biosimilar mAbs. Include replicates for each experimental condition.
   6. Prepare additional tubes for the isotype control (human IgG1 instead of rituximab) and negative control (secondary antibody without primary antibody).
   7. Incubate at 4 °C for 20 - 30 min.
   8. Wash the cells by adding 1 mL of PBS and centrifuging the cell suspension at 400 x g for 5 min at 10 °C. Discard the supernatant.
   9. Suspend the cells in 100 µL of the secondary antibody and incubate for 20 - 30 min at 4 °C, protected from light.
   10. Wash the cells twice with PBS and suspend them in 200 µL of fixation buffer.
   11. Analyze the cells on a flow cytometer.
       NOTE: The signal remains stable for several days if the samples are stored at 4 °C and protected from light.

3. **Data acquisition**
   1. Open two dot-plots on a worksheet of the flow cytometer operating software. Set the FSC-A *versus* FSC-H in the first and the FSC-A versus SSA-A in the second. Open a histogram for the PE-Cy5 channel.
   2. In the FSC-A versus FSC-H plot, make a gate (R1) selecting singlet events (**Figure 1A**).
   3. Set the R1 population in the FSC-A versus SSA-A dot-plot and then make a new gate (R2) selecting target cells (**Figure 1B**). Set the R2 population in the PE-Cy5 intensity histogram to view the frequency distribution of the cells.
   4. Adjust the lower fluorescence intensity (FI) limit for the PE-Cy5 channel using the negative and isotype control (**Figure 1C**).
   5. Acquire 10,000 events within R2 from the sample with the higher concentration of the reference product. FI of this sample should be the highest expected (**Figure 1C**).
   6. Acquire the rest of the samples.
   7. For each sample, get the median fluorescence intensity (MFI) in the PE-Cy5 channel.
   8. For samples with the reference or biosimilar mAb, calculate the difference between sample MFI and that of the isotype control (ΔMFI).

2. Assessment of CDC

1. **Preparation of biological materials and reagents**
   1. Prepare cell culture medium and culture Daudi and Daudi GFP$^+$ cells as described above (steps 1.1.1 - 1.1.2).
      NOTE: Additionally, the CDC assay requires serum-free RPMI.
   2. Dilute normal human serum complement (NHSC) 1:2 with serum-free RPMI. Prepare 2.5 mL.
   3. Prepare 1 mL of heat-inactivated (30 min/56 °C) NHSC diluted 1:2 with RPMI.
   4. Prepare sets of tests solutions for the reference and biosimilar mAbs in serum-free RPMI. Make ten dilutions (200 µL each) from 1 to 0.025 µg/mL.

2. **CDC assay**
   1. Collect the Daudi and Daudi GFP$^+$ cells from the cultures and quantify the cell viability (see steps 1.2.1 - 1.2.3).
   2. Prepare a cell suspension with 4 x 10^5 cells/mL in serum-free RPMI.
   3. Add 50 µL of cell suspension to 50 µL of each reference or biosimilar mAb test concentration in 96-well conical (V)-bottom microplates. Include replicates for each experimental condition.
   4. Prepare additional wells for the negative control (*i.e.,* without mAb), basal death control (*i.e.,* heat-inactivated NHSC in the presence of mAb), and staining positive control (*i.e.,* cells exposed to 50 µL of 70% EtOH).
   5. Incubate the cells for 20 - 30 min at 37 °C in a 5% CO_2 humidified atmosphere.
   6. Add 50 µL of NHSC (diluted 1:2) to each well and incubate the opsonized cells for 2.5 h at 37 °C in a 5% CO_2 humidified atmosphere. Use heat-inactivated NHSC in the basal death control wells.

7. Centrifuge at 400 x g for 5 min at 10 °C. Discard the supernatant.
8. Wash the cells by adding 150 µL of PBS and centrifuging the cell suspension for 5 min at 400 x g and 10 °C. Discard the supernatant.
9. Stain the samples with 7-aminoactinomycin (7-AAD), as previously described[7,8].
10. Analyze the cells on a flow cytometer on the same day.

3. **Data acquisition**
   1. Open two dot-plots on a worksheet of the flow cytometer operating software. Set those plots as in steps 1.3.1 - 1.3.3 (**Figure 2A-B**). Create a third plot that is a dot-plot for GFP versus 7-AAD on the R2 population.
   2. Define the adequate FI limits using the Daudi cells, Daudi GFP^+ cells, and death positive control (**Figure 2C**).
   3. For each sample, measure the percentage of $7\text{-}AAD^+$ target cells. Acquire at least 5,000 events from R2.
   4. Calculate the specific mAb-induced cytotoxicity by subtracting the percentage of $7\text{-}AAD^+$ in the basal death control from the percentage found in samples with different concentrations of mAbs (**Figure 2D**).

3. Biosimilarity Analysis

1. Enter the concentration and response values into a graphing software.
2. Generate graphs and calculate non-linear regressions with the following considerations: i) use the log-transformation of the mAb concentration as "X"; ii) use the variable slope mathematical model (Y = minimum response + (maximal response - minimum response)/ 1+10^((LogEC_{50}-X)*Hill slope)); and iii) constrain the bottom values to zero, since the basal response has been subtracted.
   NOTE: Curves with a symmetrical sigmoidal shape are expected.
3. Compare both non-linear fits with a global fit using an F-test (many graphing software programs include this feature).
   NOTE: Such tests establish as the null hypothesis that the maximal response, logEC_{50}, and the Hill slope are the same for the two datasets, which matches the biological question intended to be addressed.

Representative Results

Using the protocols described above, target binding and the CDC induction of reference rituximab were compared in parallel with those of a biosimilar rituximab produced and commercially available in Asia.

In Daudi cells, both mAbs bound CD20 in a concentration-dependent manner (**Figure 1D**). Non-linear regressions of binding data displayed an r^2 of 0.978 and 0.848 for reference and biosimilar rituximab, respectively (**Figure 1E**). Statistical analysis of the concentration-response curves showed that they, and therefore the pharmacodynamic parameters calculated from them, are significantly different between mAbs ($P < 0.0001$). The maximal response for the biosimilar was 2.16-fold lower than that of the reference product. These results suggest that the two evaluated mAbs have different capacities to bind CD20 expressed on the membrane of leukemic cells.

CDC induction was also compared to the two mAbs. Reference and biosimilar products stimulated CDC in Daudi cells in a concentration-dependent manner (**Figure 2E**). Importantly, the concentrations at which the mAbs induced CDC were different than those required for target binding. Non-linear regressions of the CDC data showed $r^2 > 0.980$ for both products. The statistical comparison of the concentration-response curves indicated that they are significantly different ($P < 0.01$), making the biosimilar less potent. These data indicate that the capacity to induce CDC is different for the analyzed mAbs.

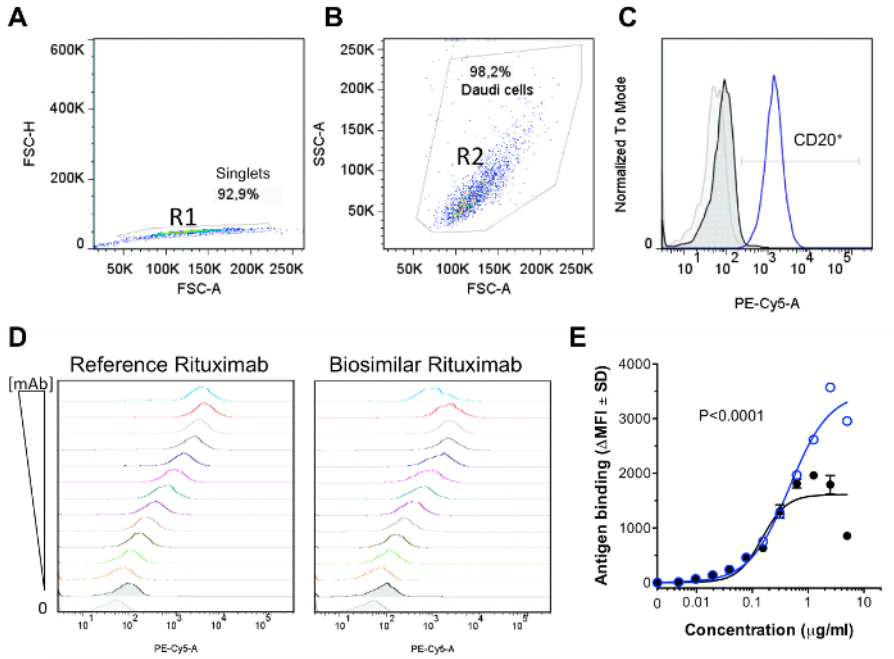

Figure 1. *In Vitro* Target-binding of Anti-CD20 Therapeutic mAbs. Daudi GFP$^+$ cells were exposed to different concentrations of the mAbs (4.8 ng/mL to 5 µg/mL) and then stained with PE-Cy5-conjugated anti-human secondary antibody. Fluorescence intensity (FI) was measured by flow cytometry on single events **(A)**, with size and granularity corresponding to those of the Daudi cells **(B)**. Unstained cells (light grey), isotype controls (dark grey), and 5 µg/mL of the reference rituximab (blue) were employed to set the FI limits **(C)**. Both evaluated mAbs bound Daudi cells in a concentration-dependent manner **(D)**. Responses (ΔMFI; see text) were used to generate concentration-response curves for reference (blue) or biosimilar (black) rituximab **(E)**. Statistical comparison of the non-linear regressions showed differences between the mAbs (P < 0.0001; Fisher exact test). Please click here to view a larger version of this figure.

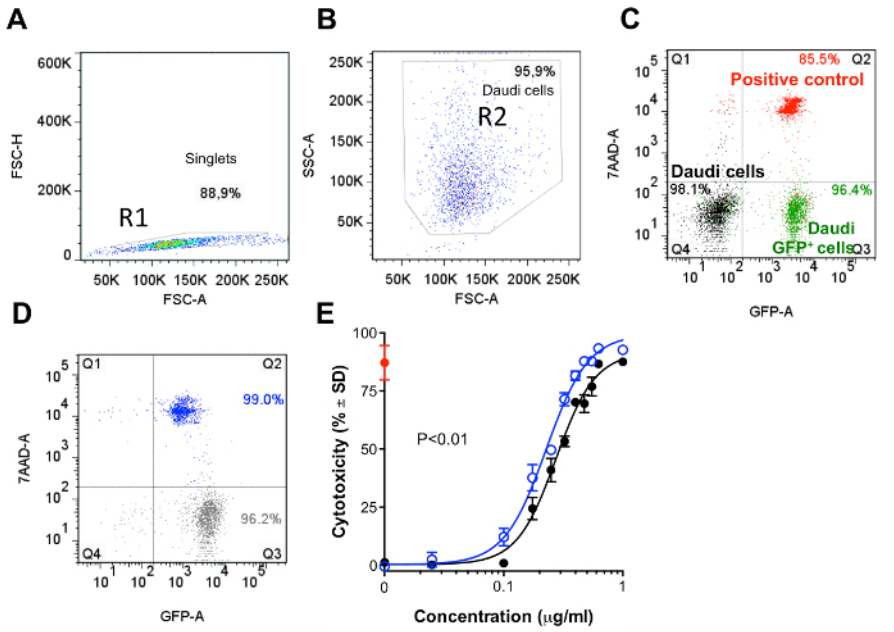

Figure 2. CDC Induction by anti-CD20 Therapeutic mAbs. Daudi GFP$^+$ cells opsonized with different concentrations of mAbs were exposed to the human complement. Cell death was evaluated by 7-AAD staining and the flow cytometric analysis of fluorescence intensity (FI) on single events **(A)**, with size and granularity corresponding to the Daudi cells **(B)**. Unstained GFP- (black) and GFP$^+$ (green) cells and ethanol-killed cells (red) were included as controls **(C)**. Quantification of the 7-AAD$^+$ cells in the basal-death control (grey) and rituximab samples (blue) allowed for the calculation of the mAb-induced cytotoxicity **(D)**. Concentration-response curves obtained for reference (blue) or biosimilar (black) rituximab **(E)**. Statistical comparison of the non-linear regressions showed differences between the responses induced by the two mAbs (P < 0.01; Fisher exact test). Please click here to view a larger version of this figure.

ANTIBODY (TRADE NAME)	TYPE	TARGET	LICENSED INDICATION	TARGET CELLS	REFERENCES
Rituximab (Rituxan)	Chimeric IgG1	CD20	non-Hodgkin's lymphoma (NHL), chronic lymphocytic leukemia (CLL) and rheumatoid arthritis	Daudi, TK, KML-1, Z-138	[17], [18], [19]
Trastuzumab (Herceptin)	Humanized IgG1	HER-2	HER-2 positive breast cancer	Raji	[20]
Infliximab (Remicade)	Chimeric IgG1	TNF-α	Crohn's disease, ulcerative colitis, psoriasis, psoriatic arthritis, ankylosing spondylitis, and rheumatoid arthritis	Jurkat T, Sp2/0-11A5-1 cells transfected with mTNF, SKOV3	[21], [22]
Etanercept (Enbrel)	Fusion protein IgG1	TNF-α	Rheumatoid arthritis, juvenile rheumatoid arthritis, psoriatic arthritis, plaque psoriasis and ankylosing spondylitis	Jurkat T, mononuclear cell-enriched PMC	[21], [22]
Alemtuzumab (Campath)	Humanized IgG1	CD52	B- cell chronic lymphocytic leukemia	Raji, CLL cells	[20], [23]
Adalimumab (Humira)	Human IgG1	TNF-α	Rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis, Crohn´s disease, ulcerative colitis, psoriasis and juvenile idiopathic arthritis	Jurkat T, peripheral blood mononuclear cells (PBMC)	[21], [22]
Cetuximab (Erbitux)	Chimeric IgG1	EGFR	Colorectal cancer and squamous cell carcinoma of the head and neck	A549, H358, Calu-3, H460	[24]
Panitumumab (Vectibix)	Human IgG2	EGFR	Metastatic colorectal carcinoma	A431, MCF7	[25]
Certolizumab Pegol (Cimzia)	Humanized IgG Fab fragment	TNF-α	Crohn's disease, rheumatoid arthritis, psoriatic arthritis	Jurkat T, TNF6.5	[26], [27]
Ofatumumab (Arzerra)	Human IgG1	CD20	CLL	SU-DHL-4, Daudi, Raji	[28]
Golimumab (Simponi)	Human IgG1k	TNF-α	Rheumatoid arthritis, psoriatic arthritis and ankylosing spondylitis	Jurkat T	[26]

Table 1. Monoclonal Antibodies Approved for Therapeutic Use, with Target Cells for the CDC Assay. Please click here to view a larger version of this figure.

Discussion

The patent expiration of a therapeutic mAb is promoting the development of biosimilars. Thus, there is a need for simple methods that can identify differences in clinically relevant activities of these products. $CD20^+$ cultured cells were employed for the evaluation of two key functional characteristics of rituximab: target binding and CDC induction. The former activity requires the recognition of CD20 by the Fab region of the mAb, while the latter depends mainly on the interaction of the Fc region with its complement[9]. Therefore, these assays provide a way to link the structural and functional characteristics of mAbs.

The target binding of therapeutic mAbs is usually evaluated by isothermal titration calorimetry (ITC), surface plasmon resonance (SPR), or biolayer interferometry[10,11,12]. These assays allow affinity calculation, but they require specialized equipment and training. The protocol described here evaluates target binding in a side-to-side comparison to identify differences between products, even without affinity data. The method is simple and employs a relevant cellular context for activity assessment. On the other hand, CDC induction by rituximab can be evaluated by ATP measurement[13], the quantification of released lactate dehydrogenase (LDH)[14] or alamarBlue[15], and MTT assays[16]. The method reported here, using 7-AAD staining, has a low background and can be combined with other stains for multiparametric flow cytometric analysis.

In the representative experiments presented, dose-response curves fitted the four-parameter logistical model, allowing for the calculation of the EC_{50}, Hill slope, and maximal response. Notably, the ranges of concentrations employed to generate such curves were different for each assay, highlighting the importance of analyzing and defining adequate ranges in preliminary experiments. Changes in key reagents, such as fluorochromes and complements, or the use of a cell line with a different target level, may displace the effective range of concentrations.

Statistical analysis identified differences between one batch of a biosimilar rituximab commercially available in Asia and the reference product, both in target binding and in CDC induction. It is important to consider that, even when the manufacturing process of the mAbs is tightly controlled, each attribute of the reference product displays a range. Accordingly, the minimum number of batches that should be tested during the evaluation of a similar biotherapeutic depends on the extent of variability of the reference product and on the assay variability[4].Thus, these protocols must be applied to different batches during the evaluation of comparability.

The presented methods can be extrapolated to other pairs of therapeutic mAbs-targets, as long as the cells expressing the antigen are accessible. **Table 1** lists therapeutic mAbs other than rituximab for which CDC induction is relevant to the clinical efficacy and compiles information on the previously reported cellular models for each mAb.

In conclusion, the two assays described here are simple, fast, and inexpensive, allowing for their execution in most labs. The methods can be used during early steps of biosimilar development or after regulatory approval for batch-to-batch comparison during production.

Disclosures

N. Salinas-Jazmín, E. González-González, and S. M. Pérez-Tapia are employees of UDIBI, which performs biosimilarity studies for several pharmaceutical companies.

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