FDA Public Meeting

BsUFA III Regulatory Science Program Interim Public Meeting

Event Materials for FDA Public Meeting

September 18, 2025 / 9:00 am – 3:00 pm EST

This document contains the Scientific Posters from research awardees of the BsUFA III Regulatory Science Pilot Program who are participating in the Poster Session.

Note: These scientific posters represent the views of the awardees and not necessarily those of the FDA.

Scientific Posters under Research Priority B: Explore how modernization of analytical technologies could better and/or more efficiently detect relevant quality attributes	
Diane McCarthy, PhD Senior Scientific Director Global Biologics U.S Pharmacopeia (USP)	**Poster: Comparison of Multi-Attribute Method (MAM) to Conventional Methods**
Tongzhong Ju, MD, PhD Senior Pharmaceutical Scientist Office of Pharmaceutical Quality Research (OPQR) Office of Pharmaceutical Quality (OPQ)| CDER | FDA	**Poster Title: Establishment of a Fesible Method to Quantify Glycofroms of Human IgG1 mAb Drugs and their biosimilars in Culture Media as a Component of Process Analytic Technology**
Tongzhong Ju, MD, PhD Senior Pharmaceutical Scientist Office of Pharmaceutical Quality Research (OPQR) Office of Pharmaceutical Quality (OPQ)| CDER | FDA	**Poster Title: A Chemoenzymatic Method for Simultaneous profiling of N and O-glycans in one-pot**
Scientific Posters under Research Priority C: Define best-practices for assessing and reporting quality attributes	
Reza Nejadnik, PhD Associate Professor University of Iowa College of Pharmacy	**Poster Title: Formulation and Characterization of Biosimilars: Scratching the Surface**
Anna Schwendeman, PhD Larry and Ann Hsu Professor of Pharmaceutical Sciences University of Michigan, Ann Arbor	**Poster Title: Multimodal Mass Spectrometric Characterization of Structural Microheterogeneity in Rituximab Reference and Biosimilars**
Scientific Posters under Research Priority D: Develop alternatives to the comparative clinical immunogenicity assessment(s)	
Anne De Groot, MD Chairman of the board and CMO	**Poster Title: ISPRI-HCP Can Predict the Immunogenicity Risk of Protein Impurities,**

EpiVax, Inc.	**Improving Biosimilar Product Development and Interchangeability Assessment**
Daniela Verthelyi, MD, PhD Supervisory SBRBPAS Expert Division Director Office of Pharmaceutical Quality Research (OPQR) IV Office of Pharmaceutical Quality (OPQ)| CDER | FDA	**Poster Title: Develop acceptance parameters and standards for the Innate Immune Response Modulating Impurities (IIRMI) assays in the Biosimilar space**
Kristina Howard, DVM, PhD Research Veterinary Medical Officer Division of Applied Regulatory Science (DARS) Office of Clinical Pharmacology (OCP) Office of Translational Sciences (OTS) | CDER | FDA	**Poster Title: Neo-Thy immune-humanized mice can produce anti-drug antibodies to support immunogenicity assessment for biological drug products**
Kristina Howard, DVM, PhD Research Veterinary Medical Officer Division of Applied Regulatory Science (DARS) Office of Clinical Pharmacology (OCP) Office of Translational Sciences (OTS) | CDER | FDA	**Poster Title: Data mining study: In vitro immunogenicity assay submissions in biosimilar drug applications**
Scientific Posters under Research Priority E: Define approaches that will increase feasibility of biosimilar development (e.g., PD biomarkers, modeling and simulation)	
Kristina Howard, DVM, PhD Research Veterinary Medical Officer Division of Applied Regulatory Science (DARS) Office of Clinical Pharmacology (OCP) Office of Translational Sciences (OTS) | CDER | FDA	**Advancing Pegylated Drug Evaluation: A Novel Approach to Pharmacokinetic Assessment of Pegfilgrastim**
Yow-Ming Wang, PhD Associate Director for Biosimilars Office of Clinical Pharmacology (OCP) Office of Translational Sciences (OTS) CDER|FDA	**Poster Title: Evidence-based Approach to The Design of Clinical Pharmacology Studies Supporting Biosimilars Development and Approval – Aim 3&4: Failed Studies (2 posters)**

Have a question for the Q&A Session?

Submit your question via email to BsUFARegSciProgram@fda.hhs.gov or scan the QR code and include *Interim Public Meeting* in the subject line.

Comparison of Multi-Attribute Method (MAM) to Conventional Methods

Tim Guo#, Sheila Mugabe#, Vishal Amancha*, Nithin Singam*, Gantasala Subramanya Sameer Kumar*, Amy Liu#, Zhiwen Yang#, Shubrata Khedkar*, Subbalakshmi V*, Li Jing#, Niomi Peckham#, Jarkko Huuskonen#, Dipankar Das*, and Diane McCarthy#

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Abstract

The mass spectrometry-based Multi-Attribute Method (MAM) has gained traction due to its potential to streamline lab work and decrease development time. The capability and sensitivity of MAM to provide more detailed information on primary structure, post-translational modifications, and relative quantitation of Product Quality Attributes (PQAs) at low levels, along with its potential to replace multiple conventional methods, has led to adoption of MAM throughout multiple stages of development. While replacing multiple QC tests provides an opportunity to streamline lab work and decrease development time and post-approval costs, several challenges remain. This project addresses one of the key areas of consideration for implementation of MAM in QC as outlined in a 2019 publication from FDA staff [1]: the performance of MAM vs. conventional methods. This study compared performance of MAM versus conventional QC methods for characterization of product quality attributes (PQAs) using adalimumab and etanercept as examples of monoclonal antibody and fusion protein therapeutics. Analysis focused on glycosylation, charge variants, size variants and other molecular modifications. Samples were also tested for bioactivity, binding affinity, and structure. While some limitations were encountered for sialylated glycans, overall, the MAM method not only provided comparable quantitation of product variants but was able to provide superior specificity and detect modifications that were not detected using conventional methods. The knowledge base and methods from this study can lower the barrier to adoption of MAM and enable wider use of MAM by biosimilar manufacturers, ultimately increasing efficiency of biosimilar development.

Introduction

LC-MS peptide-based MAM has the ability to simultaneously determine multiple attributes with greater specificity than conventional methods, and the potential to replace multiple conventional methods, thereby improving efficiency of development (Table 1). The MAM workflow consists of a characterization phase, which leverages MS/MS to identify molecular modifications and a monitoring phase, which provides relative quantitation of previously identified modifications.

Table 1. Common PQAs Measured by MAM *vs.* Conventional Methods*

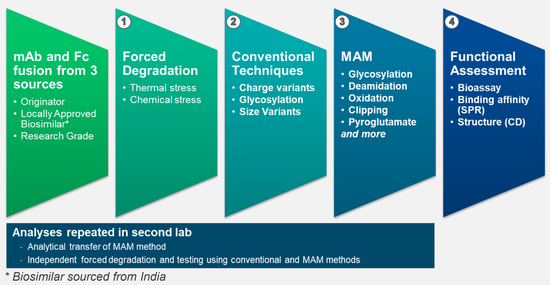
Product Quality Attribute		MAM	Conventional Method				
		Peptide Mapping LC-MS	SEC	IEX/cIEF/icIEF	rCE-SDS	nrCE-SDS	Glycan by HILIC
Identity		+	–	+/–	–	–	–
Soluble aggregates		–	+	–	–	+/–	–
Fragments or clips		+	+/–	–	+	+	–
Amino acid mutation or misincorporation		+	–	–	–	–	–
Cys-related modifications#	Unpaired Cys	+	–	+/–	–	–	–
	Disulfide isoform	+	–	–	–	–	–
	Thioether	+	–	–	+/–	–	–
Glycosylation	N-Glycan	+	–	+/–	–	–	+
	Non-glycosylated	+	–	–	+	–	–
	O-Glycan (Ser, Thr)	+	–	+/–	–	–	–
Isomerization (Asp)		+	–	+/–	–	–	–
Oxidation (Met, Trp)		+	–	–	–	–	–
Hydroxylysine		+	–	–	–	–	–
Charge variants	Deamidation (Asn, Gln)	+	–	+	–	–	–
	Glycation	+	–	+	–	–	–
N-Terminal modifications	Signal peptide	+	–	+/–	–	–	–
	N-Terminal pyroglutamate	+	–	+	–	–	–
C-Terminal modifications	Lys deletion	+	–	+	–	–	–
	Amidation	+	–	+	–	–	–

Key
"+" application can be used;
"-" application not commonly used;
"+/-" application may be used

*adapted from USP General Chapter *<1060> Mass Spectrometry Based Multi-Attribute Method for Therapeutic Proteins*

The disulfide isoforms and unpaired cysteines would need to be measured from a peptide map generated from a nonreduced protein sample, as they involve structural features that do not persist or cannot be distinguished upon reduction.

This study evaluated the performance of MAM vs. conventional methods using adalimumab and etanercept as examples of a monoclonal antibody and a fusion protein, respectively. While adalimumab is a fairly typical monoclonal antibody with a single N-glycosylation site on each heavy chain, etanercept has 3 N-linked glycosylation sites and 13 potential O-linked glycosylation sites, with highly sialylated glycans. In order to generate a range of modifications for method comparison, adalimumab and etanercept were acquired from three different sources, including the originator, a biosimilar from India, and a research-grade product, and subjected to forced degradation (Aim 1). Samples were then assessed using conventional (Aim 2) and MAM methods (Aim 3), as well as functional and structural tests (Aim 4) (Figure 1).



* *Biosimilar sourced from India*

Figure 1. Study Design

Methods

Forced degradation included thermal stress at 40 °C for up to 6 weeks, as well as chemical stress (oxidation for adalimumab and alkaline stress for etanercept). Samples were screened by SEC and icIEF to select stressed samples for further analysis. The MAM method was also transferred to a second lab, where the forced degradation study and analysis under Aims 2 and 3 were repeated. Functional and structural analyses were also performed in Lab 2 (Aim 4). Methods are summarized in Table 2, along with the Standards used to establish System Suitability.

Table 2. Methods and System Suitability Standards

Method	Source	Standards for Assessing System Performance
SEC-HPLC	*<129> Analytical Procedures for Recombinant Therapeutic Monoclonal Antibodies*	Monoclonal IgG System Suitability RS
IEX	in-house protocol	USP mAb 001 RS
rCE-SDS, nrCE-SDS	*<129> Analytical Procedures for Recombinant Therapeutic Monoclonal Antibodies*	Monoclonal IgG System Suitability RS
N-Glycan profiling	In-house method using LC-FLR analysis of Rapi-Fluor labeled released glycans	USP mAb 001 RS
Sialic acid quantitation	*<210> Monosaccharide Analysis*	N-acetyleuraminic acid RS N-glycolyneuraminic acid RS
MAM	In-house protocols – following best practices from <1060> *Mass Spectrometry-Based Multi-Attribute Method for Therapeutic Proteins*	USP mAb 001 RS
Bioassay	In-house method – following best practices from *<1032> Design and development of biological assays, <1033> Validation of biological assays* and *<1034> Analysis of biological assays*	Zero time point control Also evaluated vs. IS
SPR binding assay	In-house method – following best practices from *<1032> Design and development of biological assays, <1033> Validation of biological assays, <1034> Analysis of biological assays* and *<1105> Immunological Test Methods—Surface Plasmon Resonance*	Zero time point control
Circular Dichroism	In-house method	Lysozyme

The assessment of PQAs by MAM followed the principles outlined in USP general chapter <1060> *Mass Spectrometry Based Multi-Attribute Method for Therapeutic Proteins*, which provides information on best practices to ensure the quality and consistency of the MAM assay. The tryptic digestion method was optimized to minimize sample preparation-induced artifacts, and a fit-for-purpose qualification was performed. Qualification parameters included specificity, precision, linearity, and sample stability (freeze thaw and autosampler). MAM system readiness was established using a panel of 7 peptides from USP mAb001 RS, which were evaluated at the beginning and end of each run for TIC signal intensity, mass accuracy, retention time, integrated peak area, and artifactual oxidation (one peptide).

A total of 68 modifications were monitored for adalimumab and 59 modifications were monitored for etanercept (Table 3).

Table 3. Modifications Detected by MAM

Modification	Modified Amino Acid and Location	
	Adalimumab	Etanercept
Amidation	-	P465
Deamidation	Q3, N77, Q315, N319, N329, N388, N393, Q3(LC)	Q4, Q26, Q29, Q82, Q92/N93, N306, N345, N381, N404/N409/N410
DP Clipping	D274	D290
Glycation	-	K337, K346
Isomerization	-	D300, D421
Lysine Clipping	K450	K467
N-glycosylation	N301 *(multiple glycan structures)*	N149 (low recovery), N171 (low recovery), N317 *(multiple glycan structure)*
O-glycosylation	-	T8, S186/S199/T200, T243/S259 *(multiple glycan structures)*
Oxidation	M34, W47, W53, M83, M256, W385, M432, M4(LC)	M30, M187, M272, M378, W401, W437, M448
Pyroglutamate	E1	-
Succinimide	N319, N388, N394, Q442	Q82, N306, N335, N381, N404/N409/N410, Q438, N454

Results

Since both the conventional methods and MAM can generally distinguish individual glycan structures (at released glycan and glycopeptide levels, respectively), relative quantities can be compared directly (Figure 2). Results showed that:

- Adalimumab N-glycan profiles detected by MAM showed good correlation with conventional methods (Figure 2A) and allowed resolution of glycans that could not be resolved using conventional methods (M5 and A1G1F).
- When plotting only lower abundance glycans from adalimumab, the relative percentages were slightly lower in MAM but strong correlation was still observed (R^2 >0.98).
- Both conventional and MAM glycan analysis results revealed some limitations for etanercept (Figure 2B).
  - Conventional methods for released N-glycans could not distinguish between the glycan populations on each of the 3 N-linked sites. Analysis of O-glycans with conventional methods was not performed due to low robustness of the method.
  - MAM was able to detect both N- and O-glycans on a site-specific basis. However, MAM yielded low recovery of highly sialylated glycans.

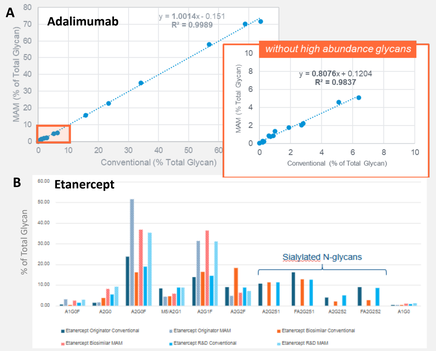


Figure 2: Comparison of N-Glycan Analysis using MAM vs. Conventional Methods

Conventional methods and MAM measure charge variants differently, so results cannot be compared directly. The ion exchange chromatography methods used for conventional methods separate variants based on net surface charge, charge distribution, and protein conformation, whereas MAM detects variants at the individual amino acid level. Therefore, to compare methods, individual modifications detected by MAM were categorized as either acidic or basic and combined to compare to conventional methods (Figure 3).

- Trends observed with stress were consistent for both MAM and conventional methods, but MAM provided superior specificity.
- MAM identified specific peptides and modifications that contribute to the increase in acidic species. MAM also detected basic variants not resolved in the conventional methods.

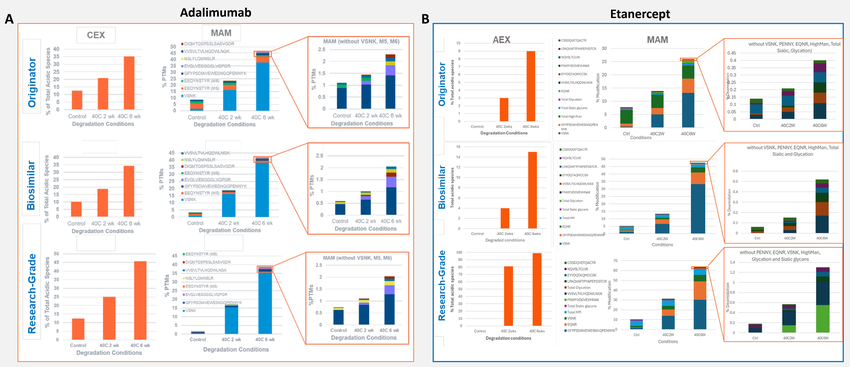


Figure 3: Comparison of Charge Variants using MAM vs. Conventional Methods

Conclusions

- Strong correlation was observed for glycans between conventional and MAM results for adalimumab and MAM was able to resolve some glycans that coeluted in conventional methods. Both MAM and conventional methods exhibited limitations for analysis of etanercept. While conventional methods cannot distinguish the location of each glycan, MAM also had limitations due to low recovery of highly sialylated glycans.
- Charge variants could not be correlated directly due to differences in specificity and measurement, so comparisons focused on trends. Both methods showed similar trends with stress, but the MAM method provided superior specificity by detecting individual sites of modifications and providing relative quantitation at each site. MAM was also able to detect other modifications that are not readily detected using conventional methods.
- Because stress conditions induce a variety of simultaneous molecular changes, it is not possible to correlate changes in function or structure to individual variants. For example, aggregation, oxidation and deamidation all increased with thermal stress. However, MAM results are consistent with several previous literature reports that identified specific amino acids and modifications that impacted activity, including modifications that impact Fc binding for adalimumab, and functional activity of etanercept (data not shown).
- Overall, the MAM method not only provided comparable quantitation of product variants but was also able to provide superior specificity and detect modifications that were not detected using conventional methods. Some limitations were observed for MAM analysis of highly sialylated glycans. For fusion proteins and other highly sialylated biotherapeutics, additional or orthogonal methods may be needed. However, since most mAbs contain trace levels of sialylated glycans, the MAM method should be suitable for analysis of most mAb products.

Impact

- This study yielded an extensive, public dataset* that provides insight into advantages and limitations of MAM vs. conventional methods. The study also yielded a comparative dataset that can support regulatory decision making, including MAM vs. conventional methods as well as lab to lab variability and comparisons of MAM vs. functional and structural assessments.
- The MAM methods used in this study provide a starting point for biosimilar manufacturers to help facilitate adoption. The strategies and criteria used to evaluate MAM system readiness can also facilitate more consistent implementation of MAM in analytical comparability and QC testing.
- Overall, results of this study create a knowledge base that can help create a common understanding of the performance of MAM vs. conventional methods and set industry and regulatory expectations for implementation of MAM to assess analytical comparability and PQAs of biosimilars. Methods and system readiness criteria developed in this study can also lower the barrier to adoption of MAM and enable wider use of MAM by biosimilar manufacturers.

* *Publications under preparation.*

References:
1. Rogstad, S., Yan, H., Wang, X., Powers, D., Brorson, K., Damdinsuren, B., & Lee, S. (2019). Multi-Attribute Method for Quality Control of Therapeutic Proteins. Analytical Chemistry, 91(22), 14170–14177

Establishment of A Feasible Method to Quantify Major Glycoforms of Human IgG1 mAb Drugs in the Production Media as a Component of Process Analytical Technology

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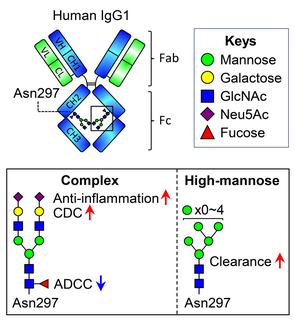
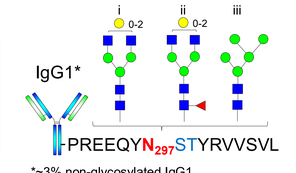
Abstract

Monoclonal antibody (mAb) drugs including their biosimilars constitute nearly a half of total protein drugs. Fully-human and humanized IgG1 mAb drugs produced from CHO cells often contain three major glycoforms due to heterogeneous N-glycosylation at Asn_{297} residue in their Fc domain: **aFucosylated**, **Fucosylated**, and **High-mannose**. N-glycosylation is a critical quality attribute (CQA) for many IgG1 mAb drugs as it impacts on their effector functions/efficacies, such as antibody-dependent cell-mediated cytotoxicity (ADCC) or complement dependent cytotoxicity (CDC), and PK/PD, and thus needs to be controlled. A small fraction of IgG1 mAb is not N-glycosylated (**non-glycosylated**), which lacks the effector activity. Mass spectrometry, fluorescence-tagging coupled with (U)HPLC are the current tools for the quantification of those glycoforms in the purified IgG1 mAbs. However, these methods are not practical biosimilar production cell line development and advanced manufacturing or continuous manufacturing of the mAb drugs. To address the unmet need, we are developing a feasible, quick, and high-throughput method to quantify relative amount (%) of three major glycoforms of IgG1 mAb drugs with a biolayer interferometry (BLI) format. We have generated and characterized the mouse monoclonal antibodies, mAb-NG and mAb-G that specifically recognize the non-glycosylated (NG) and differently glycosylated (G) human IgG1 mAbs, respectively. The mAb-G requires both sugar and Fc peptide for the binding with high affinity at 1~2 nM range. We first tested on an ELISA-based assay using those mouse mAbs, resulting in the consistent measurement of % glycoforms of therapeutic IgG1 drugs, as compared to MALDI-TOF/MS analysis. Applying in a BLI platform, the % glycoforms of IgG1 mAbs and their biosimilars were consistently measured. The optimization and validation of the BLI method with different products and their biosimilars as well as with production media is ongoing. Once validated, this approach could facilitate product cell line development and provide a mechanism of real time feedback during manufacturing that could help address chemistry, manufacturing, and controls (CMC) considerations for biosimilar manufacturers. This technology as a component can be also potentially incorporated into the process analytical technology (PAT) for advanced manufacturing of human IgG1 mAb drugs.

Introduction





Three major glycoforms of mAb drugs produced in CHO cells:

i, aFucosylated
ii, Fucosylated | Complex type
iii, High-mannose type

- **Glycosylation is of regulatory interest as it is a CQA.**
- **High-throughput quantification of % glycoforms of IgG1 mAb biosimilars in unpurified or production media is challenging.**

Methods & Results

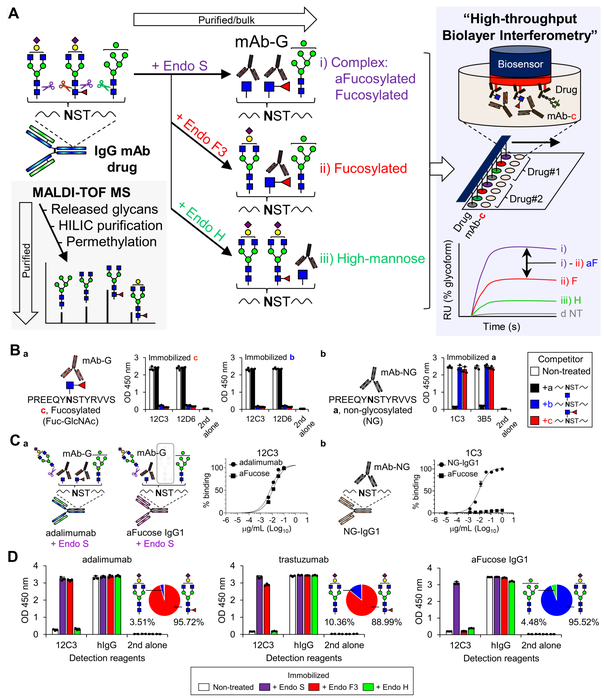


Figure 1. Measurement of glycoforms on human IgG1 mAb drugs. (A) A schematic illustration of the strategy to quantify % glycoforms of human IgG1 drugs treated with different endoglycosidases. Endo S cleaves all complex types (aFucosylated and Fucosylated, **i**), Endo F3 only cleaves Fucosylated (**ii**), and Endo H cleaves all High-mannose types (**iii**), to create those unique glyco-epitopes, (±Fuc)-GlcNAc-IgG1 Fc that are recognized by a specific mouse monoclonal antibody (mAb-G). A biolayer interferometry (BLI) offers high-throughput measurement of the mAb-G toward those glycosidases-treated IgG1. Binding unit (RU) represents the degrees of N-glycosylation (% glycoforms) of IgG1 drugs. **(B)** Generation of mouse monoclonal antibodies using Fuc-GlcNAc-Fc glycopeptides (glycopeptide-**c**, **a**), or non-glycosylated Fc (peptide-**a**, **b**) to generate specific antibodies (mAb-G and mAb-NG, respectively). Obtained hybridomas of mAb-G (12C3 and 12D6), or mAb-NG (1C3 and 3B5) were analyzed by ELISA with or without competitors as indicated. Dot-bar graphs with error bars (±SEM, triplicates) show one representative data of at least two experiments (n=2). **(C)** Binding profiles of mAb-G (12C3, **a**) to Endo S-treated adalimumab, (±Fuc)-GlcNAc-IgG1, and aFucose IgG1 that lacks fucose, or mAb-NG (1C3, **b**) to fully-non-glycosylated IgG1 (NG-IgG1) and intact aFucose IgG1 in a dose escalation. Graphs with error bars (±SEM, triplicates) were shown one representative. **(D)** Quantification of % glycoforms of IgG1 drugs (adalimumab, trastuzumab, and aFucose IgG1) by ELISA format using mAb-G (12C3). Dot-bar graphs with error bars (±SEM, triplicates) show one representative data of three independent experiments (n=3). The pie charts show the average of % glycoforms of human IgG1 drugs. See the summary in **Table 1**.

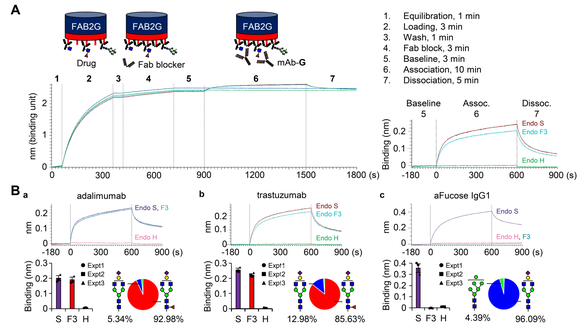


Figure 2. Establishment of a high-throughput biolayer interferometry (BLI) platform to measure % glycoforms of therapeutic mAb drugs. (A) A schematic procedure to measure % glycoforms of therapeutic mAb drugs (10 µg/mL, trastuzumab as a representative): The procedure takes 7-Steps, and the binding unit (nm) was adjusted by the baseline (step 5) set as 0, then calculated % glycoforms based on the mAb-G binding (12C3, 200 nM). (**B**) Measurement of % glycoforms of human IgG1 mAbs with BLI: Intact IgG1 mAbs (adalimumab, a; trastuzumab, b; aFucose IgG1, c) were individually treated with endoglycosidases, and applied into 96-well plate, then the binding units were measured with mAb-G (12C3, 200 nM) following the procedure (Detection, Steps 5-7 were shown) (upper panel). Dot-bar graphs with error bars (±SEM) show the binding units (n=3), corresponding to the pie charts showing the average of % glycoforms on IgG1 mAbs (lower panel) (summarized in **Table 1**).

Table 1. Comparison of analytical methods to measure % glycoforms on therapeutic mAb drugs

	% adalimumab			% trastuzumab			% aFucose IgG1		
	Fucosylated	aFucosylated	High-mannose	Fucosylated	aFucosylated	High-mannose	Fucosylated	aFucosylated	High-mannose
MS	92.24 ± 0.35	5.58 ± 0.23	2.18 ± 0.19	88.98 ± 1.94	10.41 ± 1.80	0.61 ± 0.14	n.d.*	95.73 ± 0.43	4.27 ± 0.35
ELISA	95.72 ± 0.31	3.51 ± 0.27	0.77 ± 0.45	88.99 ± 1.59	10.36 ± 1.89	0.65 ± 0.49	-0.21 ± 0.98	95.52 ± 0.74	4.48 ± 0.74
BLI	92.98 ± 2.41	5.34 ± 2.18	1.68 ± 0.30	85.63 ± 1.73	12.98 ± 2.17	1.39 ± 0.44	-0.48 ± 0.60	96.09 ± 0.86	4.39 ± 0.52

* Not detected

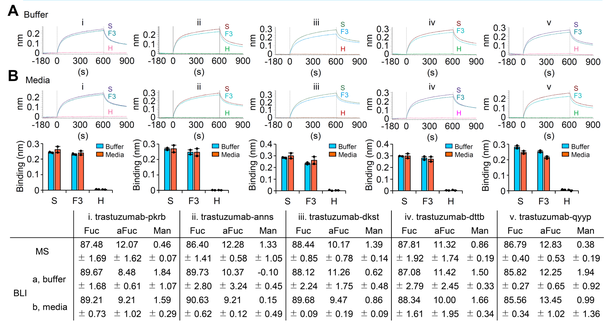


Figure 3. Measurement of % glycoforms of mAb biosimilars in buffer or media. Five purified biosimilars of trastuzumab (i, trastuzumab-pkrb; ii, -anns; iii, -dkst; iv, -dttb; v, -qyyp) were diluted in buffer (**A**), or media (**B**), and treated with endoglycosidases, and measured on BLI as in **Figure 2**. Dot-bar graphs with error bars (±SEM) show the binding units (n=2) in comparison. The average of % glycoforms of biosimilars are summarized in the table as compared to the data from MALDI-TOF/MS analysis.

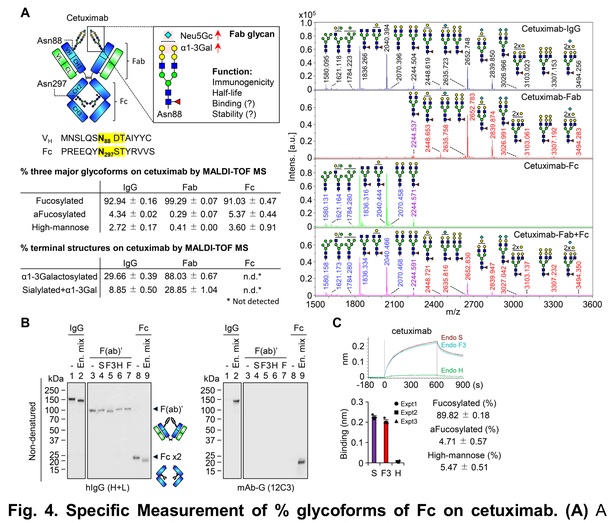


Fig. 4. Specific Measurement of % glycoforms of Fc on cetuximab. (A) A schematic structure of cetuximab: human IgG1 mAb drug cetuximab carries N-glycans on both Fab and Fc domains. Glycans at N88 of variable region of heavy chain (VH) in the Fab are structurally different from that at N299 in the Fc. The N-glycans from intact IgG1, and the Fab and Fc which were separated by protein A purification after IdeZ digestion, were released by PNGase F at denatured conditions, permethylated and analyzed on MALDI-TOF MS, respectively. The % major glycoforms were summarized in the table (n=3). (**B**) Western blotting analyses: Fab domain of cetuximab was treated with Endo S, Endo F3, Endo H, or PNGase F in a native condition, and analyzed by immunoblotting with anti-human IgG (H+L) and mAb-G, respectively. Full length (IgG) and Fc domain of cetuximab were treated with Endo S+F3+H mix (En. mix) as positive controls. (**C**) Measurement of the % glycoforms of cetuximab: intact cetuximab was treated with three different endoglycosidases, and the % glycoforms were measured on BLI following the same procedure in **Fig. 2.** A dot-bar graph (±SEM) summarized the binding units (n=3), and the average of % glycoforms of cetuximab was calculated.

Summary

1. Generated mouse monoclonal antibodies, mAb-G or mAb-NG that specifically recognize glycosylated or non-glycosylated human IgG1.
2. Characterized specificity and affinity of the mAb-G or mAb-NG by ELISA, WB, and BLI.
3. Developed ELISA (1.5 days) and BLI (~2 hrs) methods to measure the % glycoforms of IgG1 mAb drugs and biosimilars either purified or in conditioned media, confirmed by MALDI-TOF/MS (~4 days).

Regulatory Impacts

This technology can: 1) facilitate the human IgG1 mAb biosimilar development; 2) be potentially incorporated into the process analytical technology (PAT) for advanced manufacturing of human IgG1 mAb drugs.

Acknowledgements: This work was supported by the BsUFA-III Regulatory Research Pilot Program, OPQ's Center of Excellence (CoE) for Manufacturing Science and Innovation (MS&I), and CDER Domestic Manufacturing initiatives, at the United States Food and Drug Administration (US FDA).

A chemoenzymatic method for simultaneous profiling N- and O-glycans on glycoproteins using one-pot format

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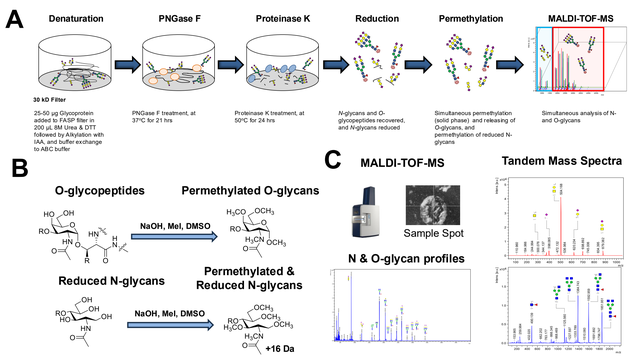
Abstract

Glycosylation is generally characterized and controlled as a critical quality attribute for therapeutic glycoproteins, including biosimilars, because glycans can impact protein drug product efficacy, half-life, stability, and safety. Analytical procedures to characterize N-glycans are relatively well established, but the characterization of O-glycans is challenging due to complex workflows and a lack of enzymatic tools. Here, we establish a simplified chemoenzymatic method to simultaneously profile N- and O-glycans from the same sample using a one-pot format by mass spectrometry (MS). N-glycans were first released by PNGase F, followed by O-glycopeptide generation by proteinase K, selective N-glycan reduction, and O-glycan release by β-elimination during permethylation of both N- and O-glycans. Glycan structural assignments and determination of the N- to O-glycan ratio were obtained from the one-pot mass spectra. The streamlined, one-pot method is a reliable approach that will facilitate advanced characterizations for biosimilars to assess their similarity to reference products regarding glycosylation. The method also ensures lot-to-lot consistency in glycosylation of glycoprotein drugs, including biosimilars.

Background

- Protein drugs and their biosimilars, such as Fc-fusion proteins are often modified by complex glycans with heterogeneity
- Glycosylation can impact safety, stability and efficacy of protein drugs, therefore in-depth characterization of N-and O-glycans is critical
- Analysis of protein glycosylation poses major challenges
- No generic method for release of all glycans is available
- Poor ionization of neutral carbohydrates requires derivatization such as permethylation or labeling to enhance detectability of glycans during mass spectrometric analysis

Methodology



Workflow of the one-pot method for simultaneously analysis of N-, and O-glycans of glycoproteins: A. Isolation of N-glycans and O-glycopeptides in tandem by FASP filter. **B**. Differentiation of permethylated N and O-glycans by unique reducing ends. **C**. Mass Spectrometry analysis of N and O-glycans in one pot by MALDI-TOF/MS.

Results and Discussion

1

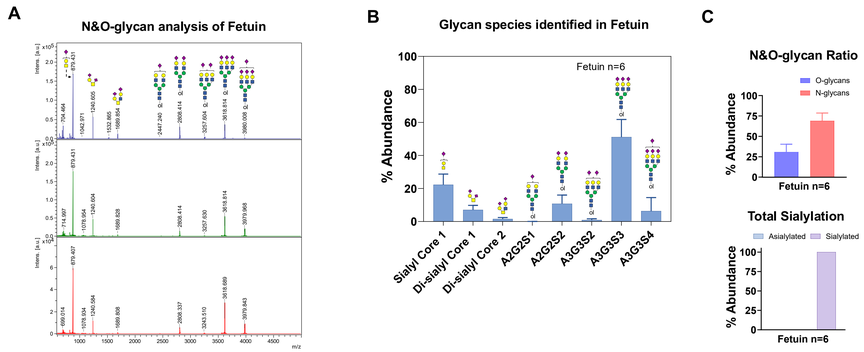


Figure 1 Legend:
(A) One-pot N- and O-glycan profiling of a model glycoprotein fetuin.
(B) Summary of glycans identified in six reactions of fetuin plotted by % abundance (mean ± SD).
(C) N- to O-glycan ratio of fetuin (top right) and abundance of the total sialylated N- and O-glycan species of fetuin (bottom right). Data are represented as mean ± SD.

2

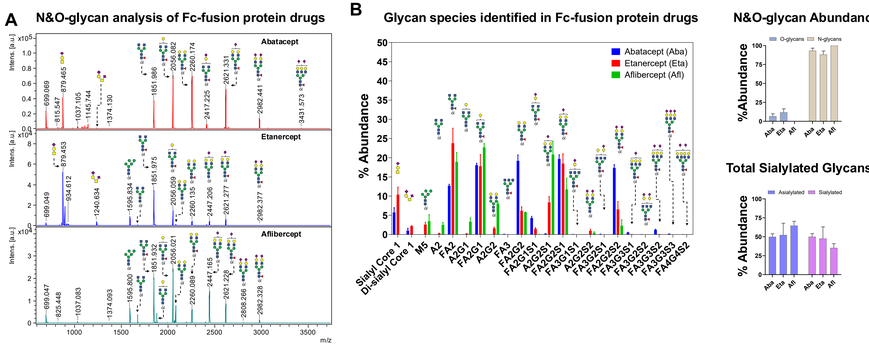


Figure 2 Legend:
(A) MALDI-TOF/MS spectra of N- and O-glycans of Fc-fusion proteins: abatacept, etanercept, and aflibercept (n = 3).
(B) Summary of unique glycan abundance found in abatacept, etanercept, and aflibercept followed by the N- and O-glycan ratio of each glycoprotein and total % sialylation of N- and O-glycans combined.

3

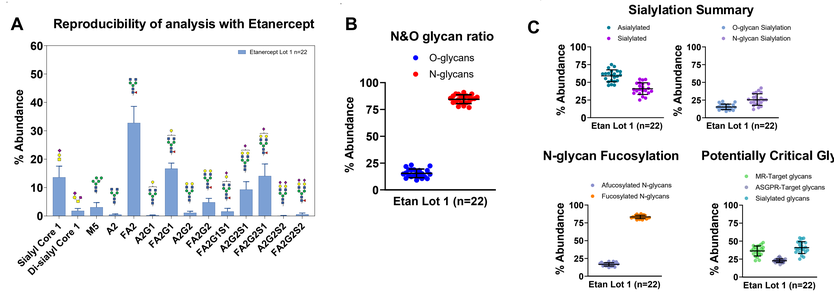


Figure 3 Legend:
(**A**) Summary of the reproducibility of one-pot method using the data from 22 reactions of etanercept lot 1 with the abundance of glycan species identified in etanercept. (**B**) N- and O-glycan ratio. (**C**) Summary of abundance of total sialylated glycan species; abundance of sialylated N- and O-glycan species is shown in the upper panel. Other glycan determinants include N-glycan fucosylation, total sialylation, and abundance of mannose receptor (MR) and asialoglycoprotein receptor (ASGPR) target glycans, summarized in the lower panel. Data are represented as mean ± SD.

Summary

- We developed a reproducible method for simultaneously profiling N- and O-glycans from purified proteins and cellular proteins from cell lysates in One-Pot.
- Relative abundances of unique N-and O-glycan species were determined consistently from three independent preparations of Fetuin and Fc-Fusion Proteins
- N- and O-glycans were differentiated by tandem mass spectra based on unique mass shifts (+16 Da) associated by reduced N-glycan core fragments.

Regulatory Impacts

1. For industry:

1) The available one-pot method will promote the development of biosimilar programs by facilitating the production cell line development to identify and develop the cell clones whose products have similar glycosylation profiles: N- and O-glycan ratio, levels of sialylation and PK-relevant glycan determinants.
2) The one-pot method can be utilized for characterization and/or release testing of the glycosylation of therapeutic proteins/biosimilars to ensure the similarity and lot-to-lot consistency in glycosylation.
3) Advancement of the method will increase its applicability and usability in characterization and release testing of therapeutic proteins and biosimilars.

2. For regulatory agency:

1) Structure-function assessments and comparisons related to O- and N-glycans of biosimilar products will be easier for Assessors to perform and correlate with other quality and safety attributes.
2) The data from the one-pot glycomic method can greatly assist the quality Assessors in assessing: a) the quality attributes of glycosylation in glycoprotein products: efficacy, PK or PD and safety; b) the lot-to-lot comparability in glycosylation of the products/biosimilars; and c) glycosylation similarity of a biosimilar to their reference product.

Acknowledgements

This work is supported by the FDA BsUFA III Regulatory Science Pilot Program, CDER Domestic Manufacturing Initiatives, OPQ Centers of Excellence. This work was also supported by an appointment to the Research Participation Program at the U.S. Food and Drug Administration administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the U.S. Department of Energy and the U.S. Food and Drug Administration.

Reference:

Ortega-Rodriguez, *et al.*, (2024) A chemoenzymatic method for simultaneous profiling N- and O-glycans on glycoproteins using a one-pot format. *Cell Reports Methods*, 4(8):100834. https://doi: 10.1016/j.crmeth.2024.100834

Formulation and Characterization of Biosimilars: Scratching the Surface

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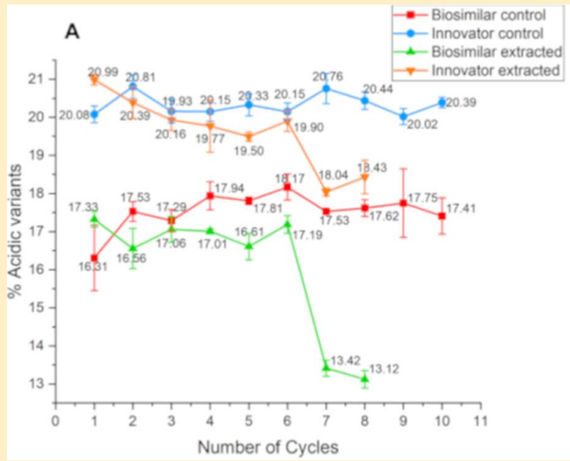
Background

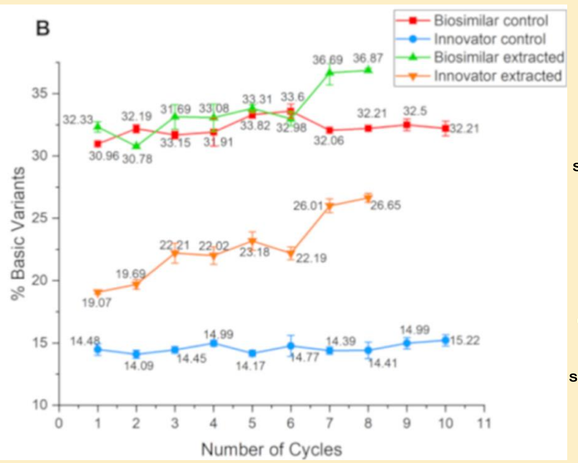
There is a great interest in development of biosimilars and the number of FDA-regulated biosimilar products is increasing at a fast pace. There are, however, different formulations and characterization platforms used in biosimilar development even for a single API that make the scene complex and raise questions regarding the best and potentially problematic practices.

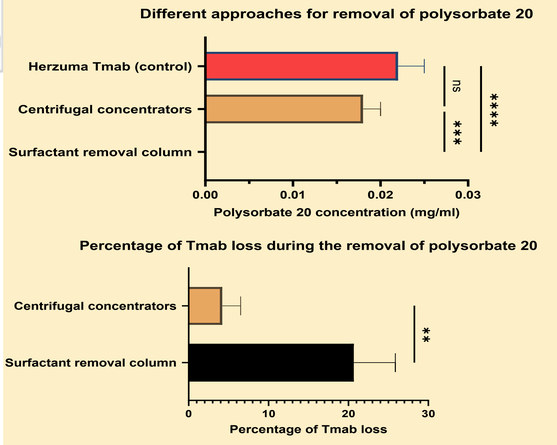
This study aimed to shed light on some of these questions by addressing shortcomings in characterization methods and by looking into formulation components of biosimilars using trastuzumab (Tmab) as a model.

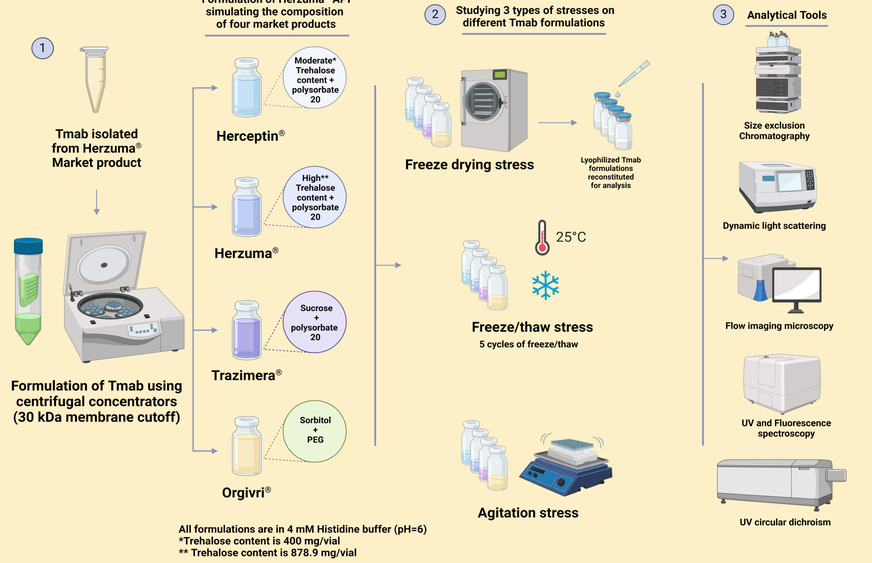
Biosimilar Brand Name	Manufacturer	Formulation	Reference product and its formulation
Ogivri®	Mylan/Biocon	Sorbitol - PEG	Herceptin® Moderate trehalose content – PS 20
Herzuma®	Celltrion	High trehalose content – PS 20	
Trazimera®	Pfizer	Sucrose – PS 20	
Ontruzant®	Samsung Bioepis	Moderate trehalose content – PS 20	
Kanjinti®	Amgen	Moderate trehalose content – PS 20	
Hercessi®	Henlius Biotech	Low trehalose content – PS 20	
PS: Polysorbate, High: 879 mg/vial, Moderate: Around 400 mg/vial, Low: 150 mg/vial			

Removal of excipients may be needed for various reasons including but not limited to assessment of similarity at the API level or interference of certain excipients with specific analytical methods. The effects of the removal process, however, is non-trivial.









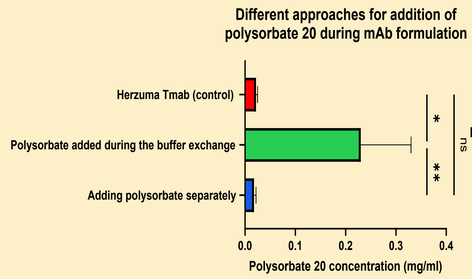
Selection of a specific formulation may be because of scientific reasons, or patents filed on formulation and/or components, or simply because of platform processes and company preferences.

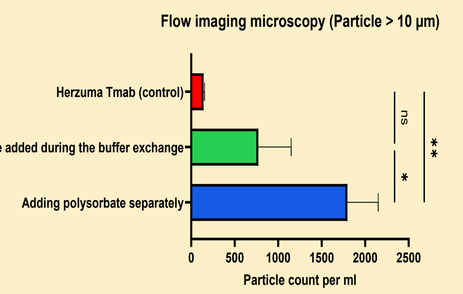
Pfizer approach

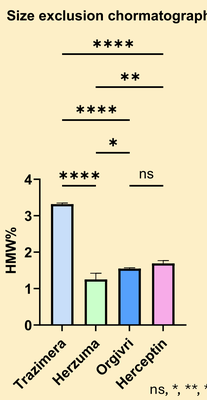
Biosimilar	Active ingredient	Dosage form	Excipients in biosimilar	Excipient in reference
Zirabev®	Bevacizumab-bvzr	lyophilized	Sucrose PS 80	Trehalose PS 80
Trazimera®	Trastuzumab-qyyp	lyophilized	Sucrose PS 20	Trehalose PS 20
Inflectra®	Infliximab-dyyb	lyophilized	Sucrose PS 80	Sucrose PS 80
Abrilada®	Adalimumab-afzb	Prefilled syringe	Sucrose PS 80	Mannitol PS 80
Ruxience®	Rituximab-pvvr	lyophilized	Sucrose PS 80	No sugar PS 80
Ixifi®	Infliximab-qbtx	lyophilized	Sucrose PS 80	Sucrose PS 80
Nyvepria®	Pegfilgrastim-apgf	Prefilled syringe	Sorbitol PS 20	Sorbitol PS 20
Nivestym®	Filgrastim-aafi	Prefilled syringe	Sorbitol PS 80	Sorbitol PS 80

Amgen approach

Biosimilar	Active ingredient	Dosage form	Excipients in biosimilar	Excipient in reference
Kanjinti®	Trastuzumab-anns	lyophilized	Exactly the same as in the reference product formulation	Trehalose PS 20
Avsola®	Infliximab-axxq	lyophilized		Sucrose PS 80
Amjevita®	Adalimumab-atto	Prefilled syringe		Sucrose PS 80
Mvasi®	Bevacizumab-awwb	Liquid		Trehalose PS 20
Riabni®	Rituximab-pvvr	Liquid		No sugar PS 80
Wezlana®	Ustekinumab-auub	Liquid		Sucrose PS 80

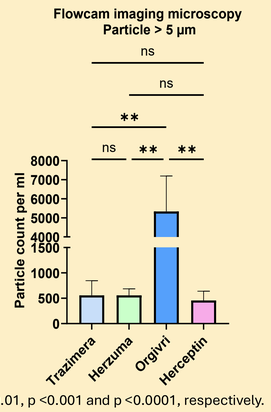






Upon lyophilization, Trazimera® formulation (sucrose) shows the highest HMW% while Herzuma® formulation (Original formulation) shows the Least HMW%

Upon agitation stress, Ogivri® formulation (sorbitol and PEG) shows the highest particle count, while all other formulations show minimal particle counts



ns, *, **, ***, and **** refer to statistically non-significant, p<0.05, p <0.01, p <0.001 and p <0.0001, respectively.

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Acknowledgement

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Disclaimer: The views and opinions presented here represent those of the speakers and should not be considered to represent advice or guidance on behalf of the U.S. FDA.

Multimodal Mass Spectrometric Characterization of Structural Microheterogeneity in Rituximab Reference and Biosimilars

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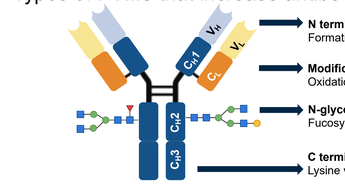
OVERVIEW

- Development of monoclonal antibody biosimilars necessitates replicating the sequence, efficacy, and safety features of their respective innovators.
- IgG1 biopharmaceuticals possess the inherent structural variability due to post-translational modifications (PTMs) such as N-glycosylation, N-terminal pyro-Glu formation, deamidation, oxidation, and C-terminal Lys clipping.
- These structural heterogeneities may negatively impact efficacy and safety by altering their native conformation, stability, target protein binding, Fc domain receptor interactions, and/or pharmacokinetics.
- Mass spectrometry (MS) offers a sensitive and robust method for analyzing the structural attributions of proteins.
- We conducted the comprehensive protein analysis between rituximab innovators and its biosimilars by employing various analytical techniques within the MS workflow.
- Additionally, we assessed ADCC activity to see how PTMs variations impact rituximab's mechanisms of action (MOAs)

INTRODUCTION

Therapeutic Monoclonal Antibody

- **Rituximab**, a chimeric anti-CD20 IgG1
  - Innovator: Rituxan® (Genentech)
  - Truxima® (Celltrion, Nov 2019)
  - Ruxience™ (Pfizer, Jan 2020)
  - Riabni (Amgen, Jan 2021)
- Types of **PTMs** that increase antibody microheterogeneity

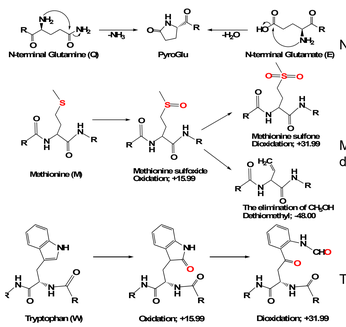


N terminal heterogeneity
Formation of Pyroglutamate

Modifications of amino acids
Oxidation, Isomerization, Deamidation, etc

N-glycosylation
Fucosylation, Sialylation, Galactosylation

C terminal heterogeneity
Lysine variants (clipping)



METHODS

- Intact MS: LC-MS (Q-Exactive MS)
  - Intact workflow – fully glycosylated and de-glycosylated (by PNGase F) samples, intact mass analysis

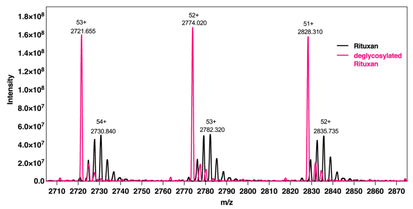


Figure 1. Intact MS analysis under native and deglycosylated conditions. (A) Deconvoluted mass spectrometry of intact untreated (black) and PNGase F-treated (pink) Rituxan

- Released Glycan Analysis: LC-FLR-MS (Xevo G2-XS QToF)
  - Released Glycan (N-linked) workflow – Instant PC labeled, chromatogram analysis

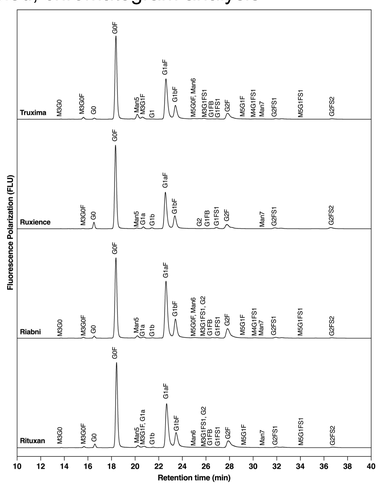


Figure 2. N-glycan profiles of rituximab products by fluorescence LC analysis. LC plots of released glycan which labeled with instant PC. Glycans were detected using fluorescence and identified using the Protein Metrics released glycan workflow. (Lot N=3)

- Peptide Mapping: LC-MS/MS (Orbitrap)
  - PTM workflow – Trypsin and Lys-C cleavage, peptide-level analysis

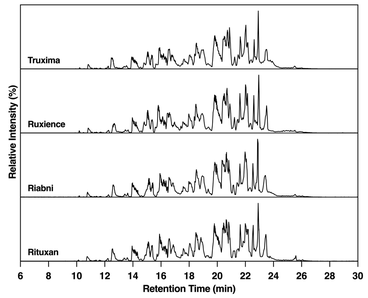
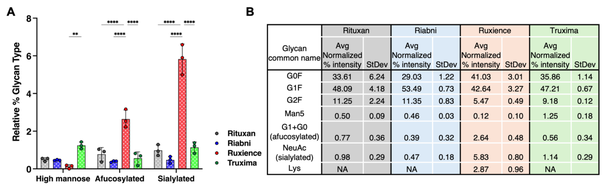


Figure 3. Total ion chromatogram (TIC) of tandem MS analysis of rituximab products obtained in peptide mapping experiments.

RESULTS



B

Glycan common name	Rituxan Avg Normalized % intensity	Rituxan StDev	Riabni Avg Normalized % intensity	Riabni StDev	Ruxience Avg Normalized % intensity	Ruxience StDev	Truxima Avg Normalized % intensity	Truxima StDev
G0F	33.61	6.24	29.03	1.22	41.03	3.01	35.86	1.14
G1F	48.09	4.18	53.49	0.73	42.64	3.27	47.21	0.67
G2F	11.25	2.24	11.35	0.83	5.47	0.49	9.18	0.12
Man5	0.50	0.09	0.46	0.03	0.12	0.10	1.25	0.18
G1+G0 (afucosylated)	0.77	0.36	0.39	0.32	2.64	0.48	0.56	0.34
NeuAc (sialylated)	0.98	0.29	0.47	0.18	5.83	0.80	1.14	0.29
Lys	NA		NA		2.87	0.96	NA	

Figure 4. Relative percentage contribution of each glycan type by intact MS. (A) Relative percentage contribution of each glycan type in rituximab products, identified by intact MS. Detailed values are provided in Table (B). Lot N = 3; Statistical analyses were performed using Tukey's multiple comparison test; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$.

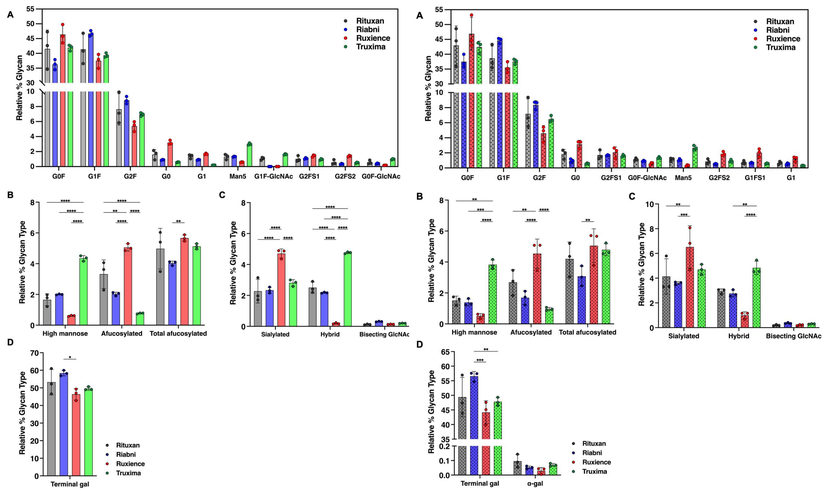


Figure 5. Relative percentage contribution of each glycan type by released glycan analysis (Instant-PC). (A) The top 10 glycans in Rituxan identified by LC-FLR-MS, categorized by glycan type: (B) percentage of mannosylated and afucosylated glycans, (C) percentage of sialylated, hybrid, and bisecting GlcNAc glycans, and (D) percentage of terminal galactosylated glycans. Glycans were identified using Protein Metrics. (Lot N = 3; error bars represent standard deviation. Statistical analyses were performed using Tukey's multiple comparison test; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$.)

Figure 6. Relative percentage contribution of each glycan type by peptide mapping analysis (LC-MS/MS). (A) The top 10 glycans in Rituxan identified by LC-MS/MS, categorized by glycan type: (B) percentage of mannosylated and afucosylated glycans, (C) percentage of sialylated, hybrid, and bisecting GlcNAc glycans, and (D) percentage of terminal galactosylated glycans. (Lot N = 3; error bars represent standard deviation. Statistical analyses were performed using Tukey's multiple comparison test; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$)

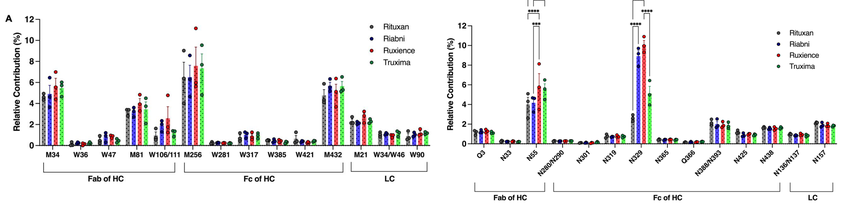


Figure 7. LC-MS/MS analysis of tryptically digested rituximab products. (A) Total contribution of oxidation, dioxidation, and dethiomethylation at Trp (W) or Met (M) residues, relative to the XIC AUC sum of all identified peptides. (B) Total contribution of succinimidation and deamidation at Asn (N) or Gln (Q) residues, relative to the XIC AUC sum of all identified peptides. HC: Heavy Chain; LC: Light Chain. (Lot N = 3; error bars represent standard deviation. Statistical analyses were performed using Tukey's multiple comparison test; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$.)

	Variable position protein	Sequences	Rituxan (%)	Riabni (%)	Ruxience (%)	Truxima (%)
Heavy Chain	1 Gln→pyroGlu -17.0265	-.qVQLQQPGAELVK.P	1.15± 0.27	1.23± 0.06	1.03± 0.10	1.03± 0.06
		-.qVQLQQPGAELVKPGASVK.M	98.44± 0.26	98.64± 0.07	98.23± 0.13	98.63± 0.04
	451 Lys-loss -128.0950	K.SLSLSPGk.-	99.03± 0.47	98.90± 0.46	91.50± 4.85	99.37± 0.06
Light Chain	1 Gln→pyroGlu -17.0265	-.qIVLSQSPAILSASPGEK.V	86.90± 0.85	90.80± 0.81	74.13± 4.92	84.27± 0.74

Table 1. Summary of identified tryptic peptides, including pyroGlu formation and Lys truncation in rituximab samples (Lot N = 3). Data are presented as mean ± standard deviation.

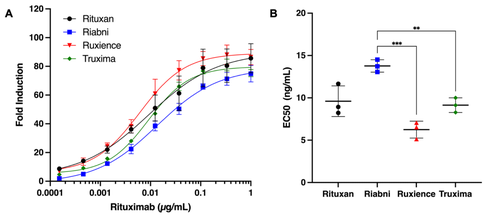


Figure 8. Comparison of the ADCC activity of rituximab products. Results of ADCC Reporter bioassay of rituximab products are shown as (A) a fold induction vs. mAb concentration curve and (B) EC50 values derived from the curve in (A). Fold of induction was calculated by dividing RLU (induced-background) by the RLU (no antibody control - background). (N = 3; error bars represent standard deviation. Statistical analyses were performed using Tukey's multiple comparison test; **p < 0.01, ***p < 0.001, ****p < 0.0001.)

CONCLUSION

Using rituximab innovator and biosimilar pairs, we investigated PTMs through various analytical techniques and mass spectrometry methods. We compared the results obtained from these different analyses and found that, although the precise numerical values slightly differed, the trends in PTMs were consistent across analyses. These findings highlight our ability to comprehensively analyze PTMs using methodologies that each offer unique strengths in terms of sensitivity and robustness.

Despite the balanced glycan effects on ADCC, the total afucosylated glycan abundances (sum of high mannose and afucosylated) are highly correlated with ADCC in vitro

Upon completing this research, we expect to lay the groundwork for developing an orthogonal method for assessing microheterogeneity in the development and regulatory approval of biosimilars.

ACKNOWLEDGEMENTS

I thank to the Schwendeman lab for their continuous support in completing experiments, analyzing data, and compiling. I also would like to thank Michael Ford for running samples on the mass spectrometer, St John Skilton for his help in analyzing the MS data using ProteinMetrics Byos software.

This study was supported by FDA BsUFA grant **1 U01FD007763-01-Schwendeman.**

ISPRI-HCP Can Predict the Immunogenicity Risk of Protein Impurities, Improving Biosimilar Product Development and Interchangeability Assessment

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EpiVax, Inc., Providence, Rhode Island, USA

Introduction

- Chinese Hamster ovary (CHO) cells dominate biotherapeutic protein production due to proper post-translational modifications, high productivity, and established safety.
- CHO-derived proteins persist as residual impurities called host cell proteins (HCPs) in final drug products.
- HCPs are critical impurities that can compromise drug efficacy, stability, and safety by triggering immune responses or degrading therapeutic proteins.
- Recent studies use LC-MS/MS to identify and characterize common HCPs in biopharmaceutical products.
- This research has created detailed lists of problematic CHO-derived HCPs affecting product quality and patient safety.
- In silico HCP prediction enables proactive immunogenicity assessment before expensive clinical trials. Improved models may make it possible to use “in silico only” analysis of new HCP for potential clinical immunogenicity.

Purpose of this Study

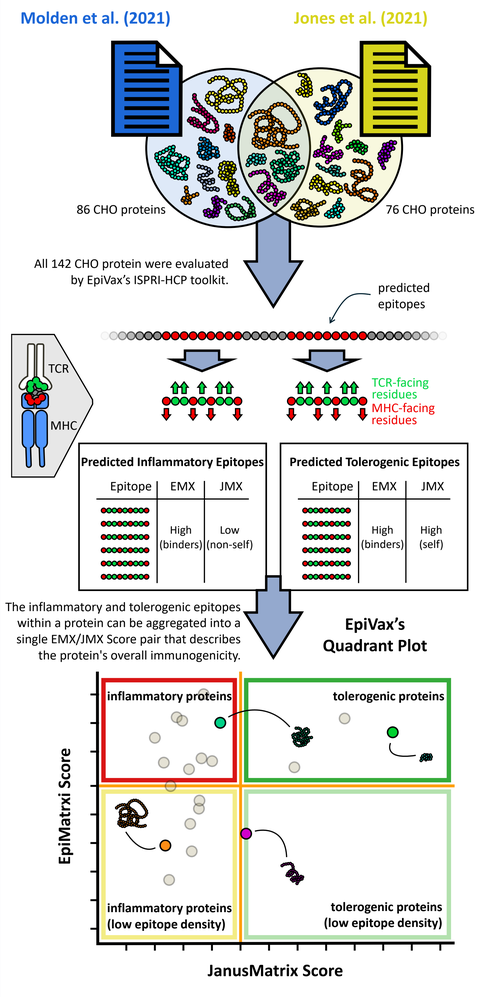
- The study aims to develop and validate improved in silico methods for assessing CHO host cell protein immunogenic potential in biopharmaceutical products.
- Different antigen formats will be evaluated to determine the most efficient immunogenicity screening approach.
- A comprehensive T cell immunogenicity dataset will be generated for 87 common CHO HCP impurities.
- This dataset addresses the assessment of commonly found HCP impurities and informs in silico models designed to assess immunogenicity risk.

Methods

- 142 frequently seen CHO host cell proteins, some of which are categorized as high-risk, were assessed using EpiVax’s ISPRI-HCP toolkit, which first predicts MHC class II T cell epitopes, followed by an assessment of epitope density and an evaluation of the TCR-binding residues into ‘foreign’ and ‘self’. Resulting protein scores are visualized using quadrant plots to identify HCPs of concern.
- In addition, 30 out of the 142 CHO HCPs were evaluated in vitro using a T cell proliferation assay.
- Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors and labeled with Carboxyfluorescein succinimidyl ester (CFSE).
- Labeled PBMCs were cultured for seven days stimulated with either HCP peptides or appropriate control conditions.
- Proliferating cells were identified by flow cytometry, gating on CD3+ CD4+ CFSE- cells following the seven-day incubation.
- Data gathered over the course of these studies is being used to improve the existing HCP immunogenicity prediction platform.

Conclusions

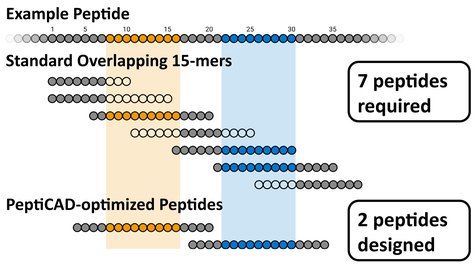
The present study systematically evaluated the ISPRI-HCP computational toolkit for predicting the immunogenicity of CHO host cell protein. This was achieved through correlation with experimental T cell proliferation assays. The majority of CHO proteins demonstrated foreign-like characteristics with low JanusMatrix scores, consistent with their potential to trigger immune responses. While a positive correlation between in silico predictions and CD4+ T cell responses was observed, discordance in a subset of proteins indicates opportunities for model refinement.



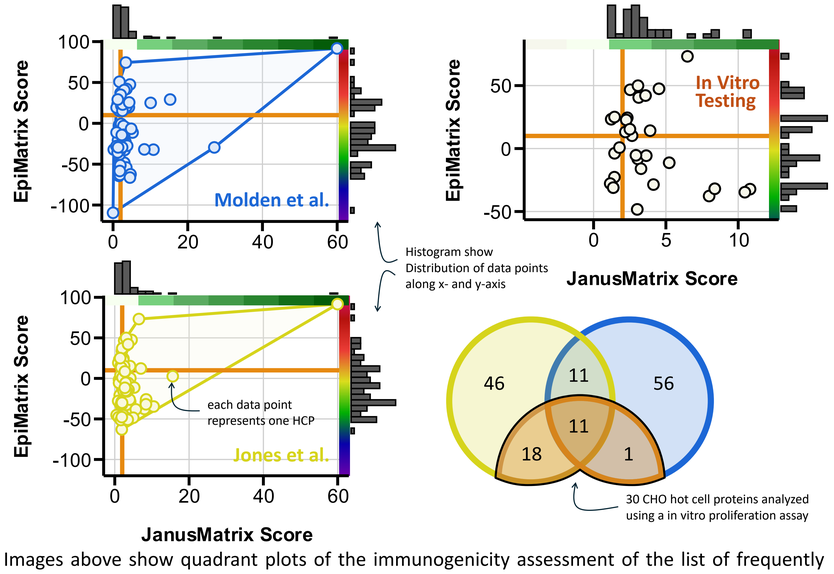
For protein immunogenicity evaluation, amino acid sequences are parsed into overlapping 9-mer frames using single-amino acid shifts. Each 9-mer is evaluated for (1) MHC binding based on amino acid residues predicted to bind MHC pockets, and (2) TCR-facing residues responsible for T cell binding and recognition. Combined evaluations determine whether input sequences will (1) trigger inflammatory immune responses, (2) trigger tolerogenic immune responses, or (3) remain immunologically silent.

All 9-mer evaluations are summarized, resulting in a single EpiMatrix and JanusMatrix score describing the overall immunogenicity of the whole protein based on the identified epitopes with immunogenic or tolerogenic potential.

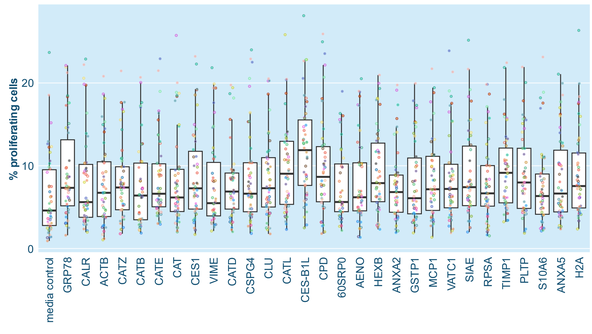
Using the whole protein EpiMatrix and JanusMatrix scores, a plot can be created resulting in a single data point per protein.
Thresholds for EpiMatrix and JanusMatrix scores can be applied which divide the plot into four quadrants, which can be described as ‘highly immunogenic’ or ‘less immunogenic’ with high or low ‘humanness (self)’.



Following epitope identification, EpiVax's PeptiCAD algorithm was used to design peptides. PeptiCAD ensures optimal epitope localization within each peptide sequence with sufficient flanking amino acids. Integrating epitope prediction with PeptiCAD-guided peptide design reduced the number of peptides for each CHO protein.



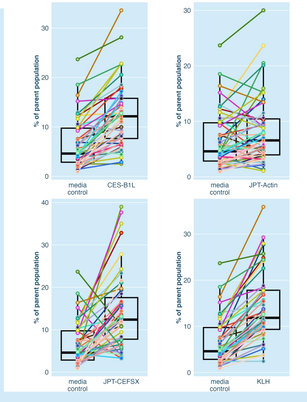
Images above show quadrant plots of the immunogenicity assessment of the list of frequently seen and high-risk HCPs. The majority of CHO proteins showed low JanusMatrix scores, indicating they are likely to be identified as 'foreign' by the immune system.
To evaluate the predictions, 30 out of the 142 CHO HCPs were selected for use in T cell proliferation assays with PeptiCAD-optimized peptides for stimulation. The Venn diagram shows the number of HCPs identified in Molden et al., Jones et al., and the number of proteins investigated during this study using the in vitro assay.



Percentage of proliferating CD4+ T cells following stimulation with HCPs. Each HCP is shown on the x-axis, with its median and interquartile range presented as a boxplots. Individual donor samples are color-coded. Selected HCP vs. media control comparisons are shown below.

Results

This study demonstrates a positive correlation between ISPRI-HCP in silico immunogenicity predictions and experimental CD4+ T cell proliferative responses, validating the computational toolkit's predictive capability for CHO host cell protein immunogenicity assessment. The majority of the 142 evaluated CHO proteins exhibited foreign-like immunogenic characteristics with low JanusMatrix scores, confirming their inherent potential to trigger unwanted immune responses.



However, a subset of CHO HCPs showed discordance between computational predictions and experimental outcomes, revealing critical areas where model refinement can enhance predictive accuracy and clinical relevance.

References

Jones et al. “High-risk Host Cell Proteins (HCPs): A Multi-company Collaborative View.” Biotech and Bioeng 118, no. 8 (2021): 2870–85.
Molden et al. “Host Cell Protein Profiling of Commercial Therapeutic Protein Drugs as a Benchmark for Monoclonal Antibody-Based Therapeutic Protein Development.” mAbs 13, no. 1 (2021): 1955811.

Funding

This research was supported by FDA grant U01FD007760 ("ISPRI-HCP: CHO protein impurity immunogenicity risk prediction for improving biosimilar product development and assessing product interchangeability").

Develop acceptance parameters and standards for the Innate Immune Response Modulating Impurities (IIRMI) assays in the Biosimilar space

Seth Thacker[1], Joseph Balsamo[1], Mark Lowenthal[2], Mirian Mendoza[1], Logan Baker[1], Swetha Tadepalli[1], Svetlana Petrovskaya[1], Karen Phinney[2], Daniela Verthelyi[1]
[1] **CDER. OPQ,** OPQR Div. IV; [2] NIST BMD, BSG.

Introduction:

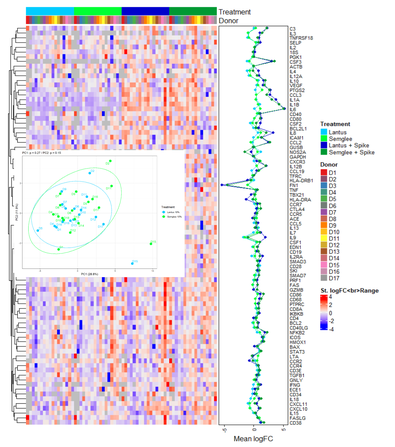
To date, the immunogenicity risk of biosimilars has been addressed primarily by using parallel arm clinical trials, however obtaining clinically informative data often requires large, lengthy, and expensive trials that defy the intent of the abbreviated regulatory path. Mitigating the root causes of residual uncertainty, would facilitate the licensing of biosimilars. A key risk factor that can be mitigated, is potential differences in innate immune response modulating impurities (IIRMIs) including host cell remnants resulting from differences in manufacturing process since IIRMIs can increase local inflammation and act as adjuvants increasing the immunogenicity risk of the drug product. Importantly, IIRMIs activate immune receptors at very low concentrations that frequently cannot be detected using these tests. Therefore, our lab and others have established different in vitro methods to detect and characterize the IIRMI in drug products (Haile et al., 2015; Holley et al., 2021; Jarvi & Balu-Iyer, 2023; Siegel et al., 2024). While these have been useful, in the absence of common reference standards the diversity of assays, testing modalities, and biomarkers have precluded comparisons between products and hindered interpretation of the results. The current studies focus on confirming that these assays can be used for complex proteins as well as the development and characterization of standards that can be used by any sponsor to benchmark their assays to assess (IIRMI). In addition, we examine whether certain product quality attributes such as molecular weight or post-translational modifications modify the sensitivity of the assays. Together, this will inform the expectations of the Agency and provide a roadmap for sponsors to integrate these assays into their immunogenicity risk assessments. Moreover, the use of common standards will allow for metanalysis of the innate immune activation profile across reference products and their licensed biosimilars to establish profiles of innate immune activation that are not associated in clinical trials or commercial use with increased risk of product immunogenicity. The aggregate data will be helpful in establishing a safe margin of in vitro innate immune activation that can aid in assessing the risk for new biosimilar products.

Project Objective:

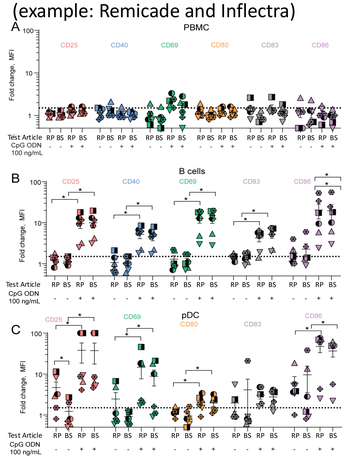
- ❖ Adapt the IIRMI assay for biosimilars :
  - Understanding of impact of product attributes on assay performance
  - Understanding of critical assay attributes
- ❖ Develop suitability standards for the IIRMI
  - Transparent expectation of assay sensitivity for sponsors and reviewers
  - Knowledge gain across methods and products
- ❖ Regulatory relief:
  - Establishment of level of innate immune activation that is not associated with increased immunogenicity risk

Aim 1: Adapt the IIRMI assay for biosimilars:

Gene expression profile of RLD and biosimilar Insulin



Cell-specific surface activation marker profile for RP and biosimilar (BS) mAbs (example: Remicade and Inflectra)



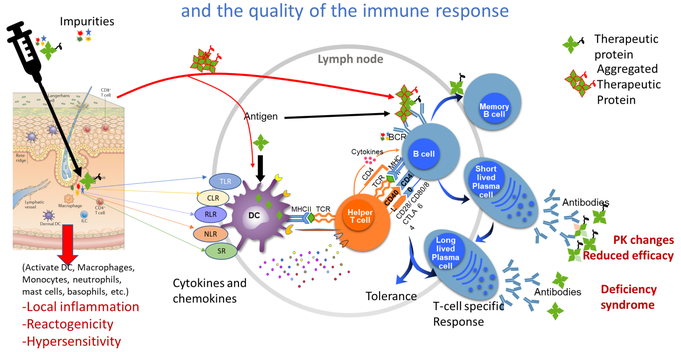
✓In vitro assays can detect differences in innate immune activating impurities in proteins regardless of complexity, although some excipients may mask some impurities. PEGylation does not adversely impact assay sensitivity.

Publications:

1. Her C, Thacker S, Balsamo J, Kelley Baker L, Ireland DD, Pang E, Verthelyi D. Cell-Based Assays to Detect Innate Immune Response Modulating Impurities: Application to Biosimilar Insulin. AAPS J. 2024 Dec 20;27(1):20
2. Balsamo J, Mendoza M, Kelley Baker L, Thacker S G, Verthelyi D. Application of multiplexed spectral flow cytometry for comparative characterization of innate immune response modulating impurities in therapeutics. (In clearance)
3. Mendoza M, Balsamo J, Kelley Baker L, Thacker S G, Verthelyi D. Assessment of Polyethylene Glycol Impact on Innate Immune Activation Assays for Immunogenicity Risk Evaluation of Biosimilar Products. (In preparation)

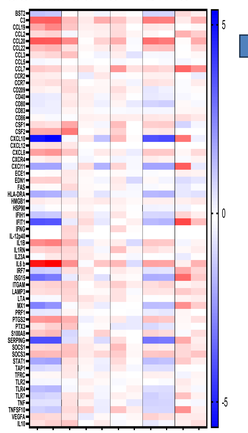
Impurities can increase immunogenicity risk

Residual uncertainty: Immunomodulatory impurities can change the quantity and the quality of the immune response



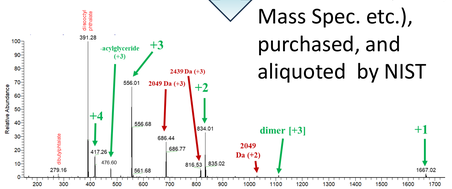
Aim 2: Develop suitability standards for the IIRMI assay and coordinate manufacture, testing and distribution to interested sponsors for testing.

Five suitability standards to control assay sensitivity and specificity were selected based on cell and receptor coverage and consistency of response by cell lines and across donor PBMC.



	Cellular Location	Monocyte	Macrophage	DC	B cell	PMN	pDC
FSL-1 (TLR2/6)	Surface	X	X	X			
MDP (NOD2)	Cytoplasmic	X	X				
D35 (TLR9)	Endosomal						X
CL075 (TLR7,8)	Endosomal			X	X	X	
CU-T12-7 (TLR2/1)	Surface	X	X	X			

Selected controls were characterized (HPLC, Mass Spec. etc.), purchased, and aliquoted by NIST

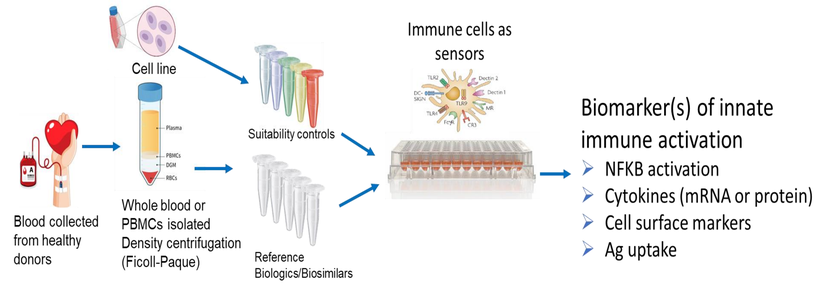


Suitability of controls will be confirmed in multiple assay formats by collaborators in Industry *(in progress).*

Final selection criteria:

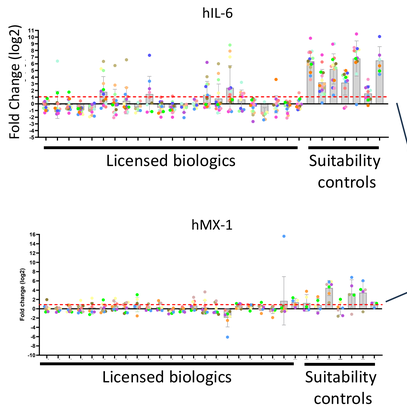
1. PRR range and distribution
2. Demonstrate responsiveness of key innate immune cell types
3. Availability, purity, and consistency of response

Innate immune response assay overview

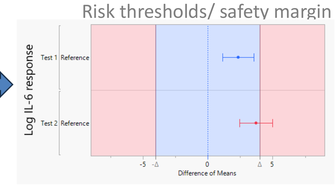


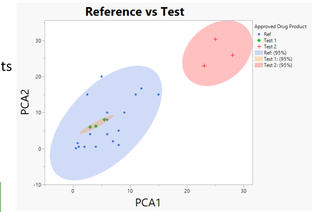
Aim 3: Characterize the IIRMI response profile of range of reference biologics to understand the expected level of innate immune activation relative to suitability controls and identify threshold that could associate with increased immunogenicity risk.

Assessment of approved biologics in PBMC.



Projected results:





Identify range of expression profile of approved Biologic(s) to help define level of innate immune activation unlikely to be associated with adjuvant effect.

Safety margin based on relevant gene/protein/marker expression patterns

Acknowledgments

This work is supported by the FDA Intramural Funding through the BSUFA III Regulatory Science Pilot Program, the FDA Immunogenicity Review Committee, and the Research Participation Program at the U.S. Food and Drug Administration administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the U.S. Department of Energy and the U.S. Food and Drug Administration

Neo-Thy immune-humanized mice can produce anti-drug antibodies to support immunogenicity assessment for biological drug products

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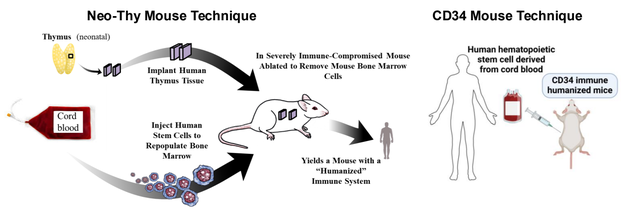
Introduction

Biologically immune-humanized mice represent an advanced animal model that provides a human immune system inside of an immunocompromised mouse strain. These mice have typically been produced in one of three ways: 1) intravenous fusion of human PBMC, 2) transplantation of human hematopoietic stem cells (HSC), referred to as a 'CD34' mouse, or 3) implantation of human thymus under the kidney capsule, along with transplantation of HSC (referred to as Neo/Thy). Having a better understanding of the true capabilities for each of these models would make them more useful to address biosimilar drug development and testing. Development of biosimilars for rare disease biologics as well as for diseases with only one therapeutic available, can be challenging. There are often limited numbers of patients, and reference products may have high immunogenicity occurrence.

The production of mice for the studies, and the studies completed were designed to address two questions: 1) Can Neo/Thy immune-humanized mice make fully mature B-cells responses resulting in production of anti-drug antibodies to drug products? 2) Is there any difference in the ability of Neo/Thy versus CD34 mice, made from the same donor, to produce responses supporting immunogenicity assessment?

To date, there is scant support for any animal model producing robust B-cell responses, including ADAs that are relevant to biosimilar drug products, which is in part, why animal studies have not been used. By confirming the ability of these mice to be able to respond by producing ADAs, it will provide a valuable tool for biosimilars that have limited patient populations, and/or cannot be tested in healthy volunteers.

Study Designs



Production of Immune-humanized mice: Immune humanized mice are an advanced rodent model based on the use of a severely immune-deficient mouse that is transplanted/implanted with hematopoietic stem cells (HSC) from cord blood with/without HLA-matched human thymus tissue. Thymus tissue was obtained either from the organ donor network or from infants undergoing cardiac surgery (due to congenital heart defects). The Neo-Thy mouse develops a human thymus underneath the kidney capsule of the mouse and later receives an HSC transplant. The CD34 mouse only receives HSC transplant. These models were compared to determine, which, if either, could produce antigen specific immune responses to biological drug products to serve as a risk assessment model for immunogenicity.

Study Background: Previous testing in our laboratory of infliximab and two biosimilars (Renflexis, Inflectra) in immune-humanized mice showed no differences between reference and biosimilar products. However, in that study, we had not developed ADA assays to assess the development of ADA to the drug products, and we only evaluated mice produced using Neo/Thy methodology. We previously tested salmon calcitonin and interferon-β in immune-humanized mice and found they also induced immunogenic responses. All of these products are known to induce immunogenicity in patients. These studies tested the effect of a single versus combination of treatments on the ability to induce immunogenicity, as evaluated through ADA development, in immune humanized mice.

Study 1:
Objective: Determine if Neo-Thy produced mice could generate ADAs to drug products
- Neo/Thy-humanized mice treated with either saline, KLH, infliximab, interferon-β, or a combination of both biologics
- Infliximab and interferon-β were chosen due to known clinical immunogenicity in patients
- Six groups were tested; each group is composed entirely from a single donor pair of thymus/HSC, essentially serving as 'twins' for the purpose of testing
  - Groups 1, 5, 6: same thymus donor, different stem cell donors (but HLA matched)
  - Groups 2, 3, 4: each had a unique HLA-matched thymus/stem cell donor pairs

Study 2:
Objective: Compare the ability of Neo-Thy versus CD34 mice, produced from the same donor, to make antigen-specific immune responses to biological drug products
- Groups 1, 3, 5, 7: Neo-Thy mice
- Groups 2, 4, 6, 8: CD34 mice
- Treatments: saline, KLH, infliximab, salmon calcitonin, or combination of infliximab & salmon calcitonin

Common to Both Studies:
- Peripheral blood collected at Days 0, 21, 42, and 63
- At study end: lymph nodes, spleen, and bone marrow harvested
- Functional assays performed using freshly isolated cells
  - Flow cytometry (lineage markers + intracellular cytokines)
  - Proliferation
  - Total immunoglobulin production by isotype
  - Anti-drug antibody (ADA) assessment

Acknowledgements

We thank Jose' Austin and Steven Soto for excellent technical support in conducting this research. This project was supported in part by an appointment with the Oak Ridge Institute for Science and Education (ORISE) through an interagency agreement between the U.S. Department of Energy and FDA.

Funding

These projects were supported by funding from the BsUFA Regulatory Science Program.

Disclaimer

This presentation is not an official US Food and Drug Administration guidance or policy statement. No official support or endorsement by the US FDA is intended or should be inferred.

Study 1 Results

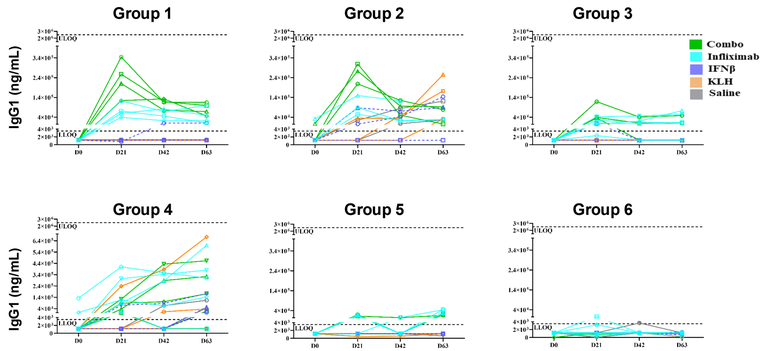


Figure 1. Total IgG1 antibodies in serum. Using a commercial IgG isotyping assay (Millipore-Sigma), IgG1 – IgG4 were measured in serum at Days 0, 21, 42 and 63 (necropsy). Each groups represents one donor, with all mice having a thymus and HSC transplant. Almost all samples show very low to unquantifiable levels of IgG1 and study start which increased over time. Groups 1 and 2 show clear peaks develop at D21 that either decrease or level off over time. Group 4 shows most treated mice with continually increasing IgG1 levels over time through study day 63, suggesting potential ADA formation. Blue dashed line shows LLOQ and black dashed line shows ULOQ.

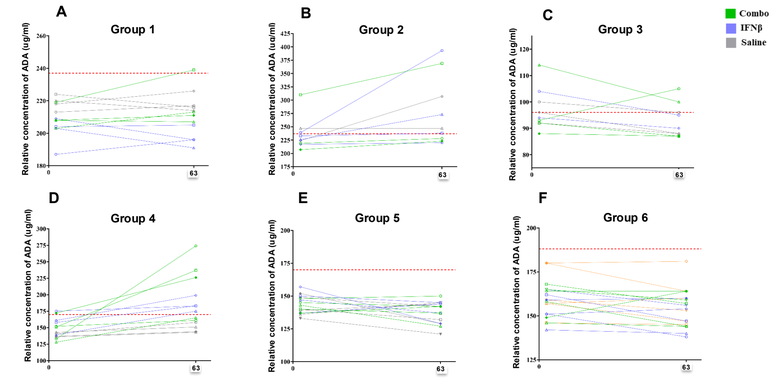


Figure 2. Binding anti-interferon-beta antibodies. Using reference product interferon-beta (Betaseron), an ELISA assay was developed to detect binding ADAs to interferon-beta. Paired samples show day 0 and day 63 results for indicated mice by treatment. Neo-Thy mice in group 2 and4 show binding ADAs in response to treatment. Several mice show initial cross-reactive antibodies to interferon-beta that diminish over time. Red dashed line shows cut point for positive responses.

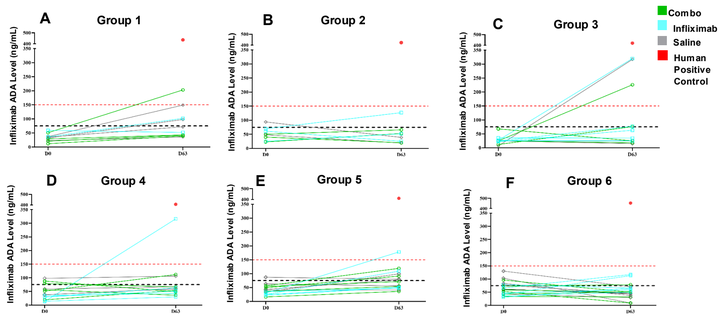


Figure 3. Neutralizing anti-infliximab antibodies. Using reference product infliximab, an ELISA assay was developed to detect neutralizing ADAs to infliximab. Paired samples show day 0 and day 63 results for indicated mice by treatment. Individual Neo-Thy mice in groups 1, 3 and 4 show neutralizing ADAs in response to treatment. Red dot shows values from human positive control serum containing anti-infliximab neutralizing antibodies. Lower black dashed line shows LLOQ and upper red dashed line shows cut point for positive responses.

Study 2 Results

Figure 1. Lineage and activation markers from LN flow cytometry.

Figure 1A compares T cells, B cells, and monocytes, which were comparable between Neo-Thy (n=53) and CD34 (n=52) immune-humanized mouse groups. CD4+CD8+ T cells showed a trend of higher number for Neo-Thy vs CD34, but it was not significant. CD27+IgD- B cells were significantly higher in Neo/Thy vs CD34 mice (p=0.025).

Figure 1B shows results from intracellular cytokine assay for Neo/Thy vs CD34 immune humanized mice. Data showed a significantly greater proportion of IgG+ B cells, and greater proportions of CD25, CD69, IFNγ and IL-2 expressing T cells in Neo/Thy immune humanized mice (n=53) compared to CD34 (n=52). Data displayed as mean ± SEM; multiple unpaired t test. $* p \le 0.05$ and $** p \le 0.0001$.

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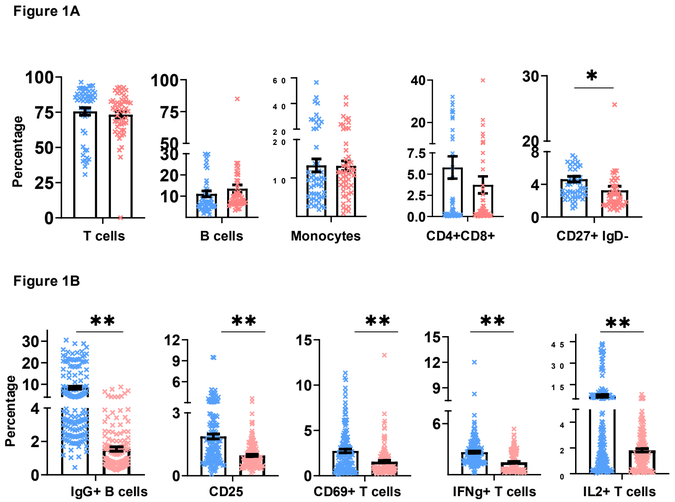

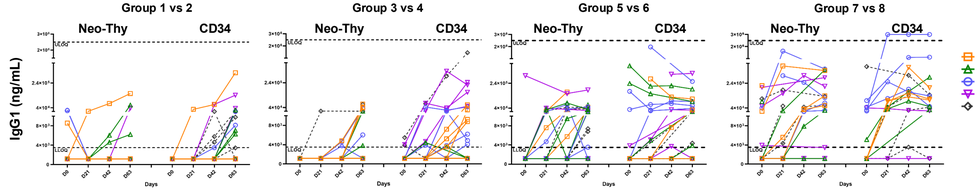

Figure 2. Total IgG1 antibodies in serum. Using a commercial IgG isotyping assay (Millipore-Sigma), IgG1 – IgG4 were measured in serum at Days 0, 21, 42 and 63 (necropsy). Groups from the same donor are shown in pairs, showing mice with a thymus on the left, and those without a thymus on the right. CD34-produced mice for each donor with most treatment groups showed overall higher levels of IgG1 at all time points, including day 0, as compared to those mice with thymus and HSC engraftment. Humanized with a thymus tended to start study with low levels of IgG1, which typically increased over time. Blue dashed line shows LLOQ and black dashed line shows ULOQ.

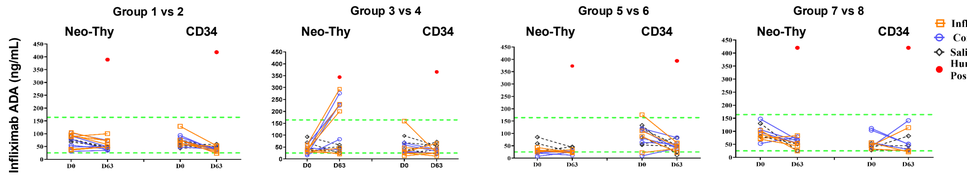

Figure 3. Neutralizing anti-infliximab antibodies. Using reference product infliximab, an ELISA assay was developed to detect neutralizing ADAs to infliximab. Paired samples show day 0 and day 63 results for indicated mice by treatment. Neo-Thy mice in group 3 show neutralizing ADAs in response to treatment, while mice without a thymus from the same donor show no neutralizing ADAs. Red shows values from human positive control serum containing anti-infliximab neutralizing antibodies. Lower green dashed line shows LLOQ and upper green dashed line shows cut point for positive responses.

Conclusions

Study 1

- Neo-Thy immune-humanized mice have a fully functional human immune system and can class switch IgG isotypes
- Immune-humanized mice, with a thymus, can produce anti-IFNβ binding ADAs and anti-infliximab neutralizing anti-drug antibodies

Study 2

- Neo-Thy and CD34 immune-humanized mice have similar cellular composition, but they are less functional in CD34 mice
- CD34 immune-humanized mice produced greater levels of IgG1, but that did not result in the production of neutralizing ADAs
- The presence of a thymus is critical to producing antigen-specific binding and neutralizing ADAs to biological drug products
- Neo-Thy immune-humanized mice can support immunogenicity testing of complex biosimilars for drugs with small patient populations and/or inability to test in healthy volunteers

Data mining study: *In vitro* immunogenicity assay submissions in biosimilar drug applications

U.S. FOOD & DRUG ADMINISTRATION

Jenny Resiliac and Kristina E. Howard

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Abstract

Background: Immunogenicity, the ability of a molecule to stimulate an immune response, is an important safety consideration during drug development. Currently, immunogenicity for biosimilars is assessed in clinical trials by monitoring participants for the development of anti-drug antibodies (ADA). However, there is interest in relying more on nonclinical assessments for derisking immunogenicity questions for biosimilar products. To inform current practices, this project sought to determine if sponsors are submitting in vitro immunogenicity assays as part of their 351(k) biologic license applications (BLAs).

Methods: List of approved BLAs was generated using Purple Book. Then, internal FDA systems were reviewed for individual applications and their associated reference products. Data were then classified by assay groups and cell types. Key search words included: "Immunogenicity", "Assay", "in vitro assays", "ADA", "ELISpot", and "MLR", among others.

Results: 134 biosimilar applications (351(k)) were identified. Of these 91 were either under review or otherwise not approved and therefore not included in our analysis. The remaining 43 approved biosimilars containing immunogenicity data referred to a total of 12 reference products (RP). We identified a range of in vitro assays and found there was diversity in cell types used, assay parameters and protocols. The most common assays identified were binding and neutralizing ADA. Additional in vitro immunogenicity-related assays included cytokine release, mixed lymphocyte reaction (MLR), proliferation, dendritic cell/T-cell proliferation (DC: T-cell assay) and Enzyme-Linked Immunosorbent Spot (ELISpot).

Conclusions: This research has identified that sponsors are conducting in vitro studies to address immunogenicity assessment as part of biosimilar applications. Overall, we found that *in vitro* assays do have predictive capacity but should be carefully selected based on the product's mechanism of action/target, and patient population characteristics (e.g. oncology patients are generally immune suppressed). However, variability in the assays, protocols, and cell types used can make results difficult to interpret and highlights the need for best practices to facilitate data interpretation and usability by review staff as a risk assessment tool.

Study objectives and methodology

Study Objectives:

1) Determine if sponsors were submitting *in vitro* immunogenicity assay data as part of their 351(k) biosimilar application
2) If submitted,
   a) What assay types were included
   b) What types of cells were used in submitted assays
   c) Review protocols for assay conduct
3) Conduct a literature search for published *in vitro* assays; compare results with assays submitted
4) Identify clinical trial results for immunogenicity and determine if *in vitro* assays appear to mirror those results

Methodology:

1) Search FDA internal and external databases to find BLAs
2) Data were cross-referenced across databases and duplicates were removed
3) BLAs under review or those not approved (complete response) were excluded
4) Application details, assay types and parameters including cell types were exported and categorized
5) Assays details were reviewed and summarized
6) Literature search for *in vitro* immunogenicity assays (only for products included in data mining)
7) Literature search clinical trial immunogenicity results (only for products included in data mining)
8) Summarize and compare results

Functional properties of evaluated biosimilars

Blocking: Prevent binding or otherwise block interaction of protein and receptor to inhibit biological activity

Activating: Interact with biological target to stimulate or enhance its activity

Depleting: Engage with cells and promote their elimination (kills target cells) through various immune mechanisms

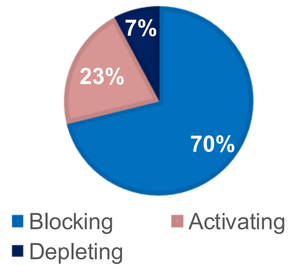


Figure 1. Functional properties of biosimilars evaluated for in vitro immunogenicity. Biosimilars are summarized based on the ability of the product to either activate or block a specific interaction or deplete cells expressing a particular receptor.

Funding

This research was supported by funding from the BsUFA Regulatory Science Program.

Summary of biosimilars evaluated

Product name	Proprietary name	Year of reference approval	Number of approved biosimilars	Date range of biosimilar approvals
Adalimumab	Humira	2008	10	2016 - 2023
Bevacizumab	Avastin	2004	4	2017 - 2022
Denosumab	Prolia/Xgeva	2010	1	2024
Epoetin alpha	Aranesp	2001	1	2018
Etanercept	Enbrel	1998	2	2016 & 2019
Filgrastim	Neupogen	1991	3	2015 - 2022
Infliximab	Remicade	1998	4	2016 - 2023
Pefilgrastim	Neulasta Onpro	2002	6	2018 - 2022
Rituximab	Rituxan	1997	3	2017 - 2020
Tocilizumab	Actemra	2010	1	2023
Trastuzumab	Herceptin	1998	5	2017 - 2020
Ustekinumab	Stelara	2009	3	2016 - 2024

Table 1. Summary of approved biosimilars. A total of 134 biosimilar applications were reviewed in the data mining for *in vitro* immunogenicity assays. Of these 134, those that were either under review, had received a 'complete response', or were pending approval were not included in the evaluation (91 applications). The remaining 43 biosimilar applications, along with their associated RPs (12 products) were reviewed for immunogenicity assays. Approval dates for RP ranged from 1991-2010 while approval for 351 (k) biosimilars dated from 2015-2024.

Assays identified in biosimilar applications

- 40% were binding ADAs assays using enzyme-linked immunosorbent assay (ELISA), electrochemiluminescence Immunoassay (ECL), radioimmunoassay, or radioimmunoprecipitation
- 30% were neutralizing ADA assays (Nabs)
- 30% were categorized as "Others" included enzyme-linked immunosorbent spot (ELISpot), proliferation, mixed lymphocyte reaction (MLR), apoptosis, and cytokine release

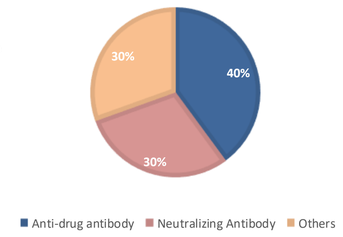


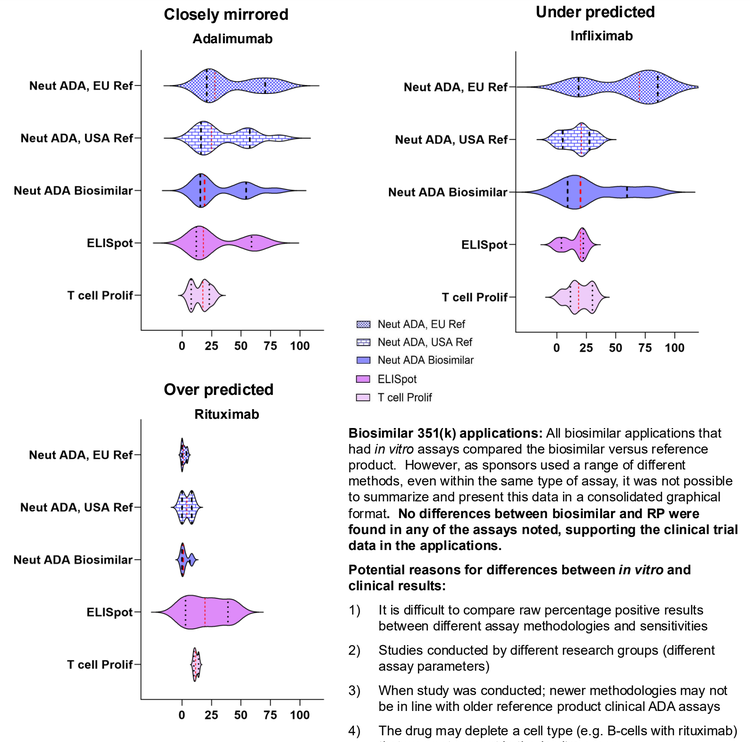
Figure 2. Types of assays identified. All biosimilar applications were reviewed for assays used to assess immunogenicity. Any assay that assessed anti-drug antibodies (binding or neutralizing), or supporting assay was included in the data mining. Overall, 70% of assays found specifically assessed ADAs, while 30% primarily assessed evidence of in vitro immunogenicity responses using adaptive immune system assays.

Cell types used in assays

Abbreviations	Cell identification	Number of times used across applications	Percentage per Total Number of Applications
PBMC	Peripheral blood mononuclear cells	34	53.13%
CHO	Chinese hamster ovary cells	27	42.19%
Jurkat	Immortalized T lymphoblast	16	25.00%
U-937	Pro-monocytic human myeloid leukemia; Histiocytic lymphoma	14	21.88%
HUVEC	Human umbilical vein endothelial cells	13	20.31%

Table 2. Summary of cell types used for assays. A total of 57 cell types were identified, with the top 5 shown in the table. Additional cell lines included natural killer cells-92 (NK-92), human breast cancer cells overexpressing HER2 (BT-474), hypertriploid human breast cancer (SK-BR3), human monocytic cells with recombinant human TNF gene 2aa substitution mutant (MM6 5-48), adherent mouse fibroblast-like (L-929), and human embryonic kidney 293 (HEK 293), among others. The wide variety of cell types made it challenging to summarize results between applications.

Comparison of *in vitro* versus clinical trial results



Biosimilar 351(k) applications: All biosimilar applications that had *in vitro* assays compared the biosimilar versus reference product. However, as sponsors used a range of different methods, even within the same type of assay, it was not possible to summarize and present this data in a consolidated graphical format. **No differences between biosimilar and RP were found in any of the assays noted, supporting the clinical trial data in the applications.**

Potential reasons for differences between *in vitro* and clinical results:

1) It is difficult to compare raw percentage positive results between different assay methodologies and sensitivities
2) Studies conducted by different research groups (different assay parameters)
3) When study was conducted; newer methodologies may not be in line with older reference product clinical ADA assays
4) The drug may deplete a cell type (e.g. B-cells with rituximab) that was not present in the *in vitro* assay

Figure 3. Comparison of in vitro assays in literature to clinical trial results from literature. This figure shows Nabs from clinical trials of reference versus biosimilar drug products with the red dashed line showing median and the two black dashed lines showing the upper and lower quartiles. The ELISpot and T cell proliferation show published data using in vitro assays for the RP. No studies using *in vitro* assays were identified that showed the RP versus biosimilar. The red and black dashed lines show the median and upper/lower quartiles respectively.

Conclusions

- 43 biosimilars, from 12 RPs, were identified and used for data mining
- Sponsors used a greater variety of *in vitro* immunogenicity assays types in the literature than were submitted to the agency
  - Assay types identified were 40% binding ADAs, 30% NAbs, and 30% assessed immunogenicity with assays such as ELISpot, DC:T-cell and proliferation among others
  - *In vitro* assays submitted as part of 351(k) applications showed no differences in adapative immune assay results between the biosimilar and reference product, consistent with clinical trial results for the application
- ELISpot results were most predictive clinical ADAs
  - *In vitro* assays have variable predictive capacity and should be carefully selected based on biological product mechanism of action/target
  - *In vitro* assays show potential for clinical immunogenicity prediction
- Variability in the assays, protocols, and cell types used can make results difficult to interpret
- Results highlight the necessity for development of best practices to facilitate data interpretation and usability by review staff and use of *in vitro* assays as a risk assessment tool

Acknowledgements

This project was supported in part by an appointment to the Research Participation Program at the Office of Generic Drugs, U.S. Food and Drug Administration, administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the U.S. Department of Energy and FDA.

Disclaimer

Advancing Pegylated Drug Evaluation: A Novel Approach to Pharmacokinetic Assessment of Pegfilgrastim

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Abstract

Background: Polyethylene glycol (PEG) a flexible, uncharged and highly hydrophilic polymer, used to prolong the half-life and improve efficacy of therapeutic proteins and nanoparticles. Despite these key advantages of PEG conjugation, biosimilars developed from their respective originator products may fail to meet requirements for FDA approval. This could be broadly attributed to the presence of PEG, which can present challenges in the bioanalytical methods to assess serum concentrations of the PEGylated compound for PK studies

Purpose: With over a dozen PEGylated therapeutics currently on the market, there is a critical need for reliable assays to address variability in pharmacokinetic (PK) measurements for potential biosimilar products. Conventional ELISA assays often lack specificity and show high variability when quantifying PEGylated drugs, creating challenges for biosimilar development. To address these limitations, we focused on pegfilgrastim—a PEGylated G-CSF product with multiple biosimilars as a model drug. Our goal was to develop a cell-based assay (CBA) that targets the PEG backbone, providing a biologically relevant and reproducible alternative to conventional ligand-binding assays for pegfilgrastim detection and PK assessment.

Methodology: A flow cytometry–based cell-based assay (CBA) was developed using AML-193 cells expressing the G-CSF receptor to detect pegfilgrastim in human serum. Pegfilgrastim-spiked samples were incubated with cells, followed by detection with anti-PEG primary antibodies and Alexa Fluor 488–conjugated secondary antibodies. The assay's performance, including linearity and precision, was validated and compared against a commercial ELISA using serum from immune-humanized mice dosed with pegfilgrastim.

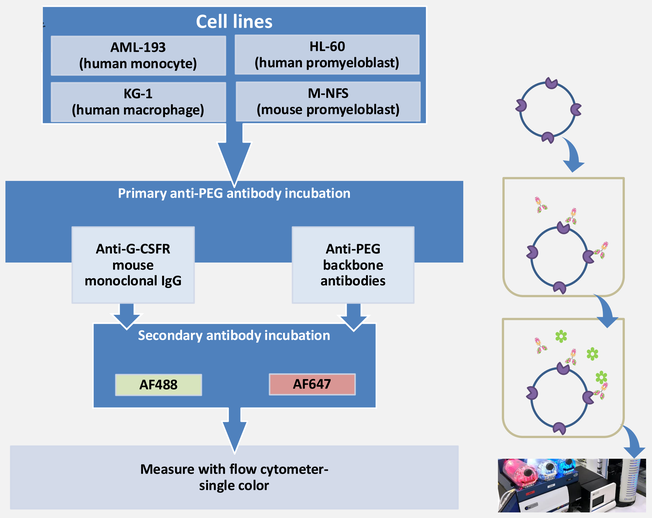
Results: AML-193 cells provided strong and reproducible signal separation, enabling sensitive pegfilgrastim quantification across 1,000–60,000 pg/mL (R^2 = 0.9962). The CBA achieved accurate recovery (±5%) for both reference pegfilgrastim and two FDA-approved biosimilars. When compared with ELISA, the CBA produced similar pharmacokinetic profiles while offering a broader dynamic range and PEG-specific detection.

Methods

Assay Development:
A flow cytometry–based cell-based assay was developed to quantify pegfilgrastim in human serum. Four G-CSF receptor–expressing cell lines were evaluated, and AML-193 cells were selected due to their strong receptor expression and reproducible signal separation. Pegfilgrastim-spiked serum samples were incubated with AML-193 cells, followed by staining with anti-PEG primary antibodies and Alexa Fluor 488–conjugated secondary antibodies. A calibration curve (1,000–60,000 pg/mL) was established to evaluate assay linearity, accuracy, and precision.

Comparison of CBA with G-CSF ELISA:
The CBA was benchmarked against a commercial G-CSF ELISA adapted for pegfilgrastim detection. Immune-humanized mice were dosed with pegfilgrastim, and serum samples were collected from 0 - 72 hours for pharmacokinetic analysis. Both assays were performed on the same samples, and data were analyzed using a four-parameter logistic model in GraphPad Prism, with acceptance criteria of ±20% accuracy for standards and $R^2 \geq 0.99$.

Experimental Design



Funding

This research was supported by funding from the BsUFA Regulatory Science Program.

Identification of Optimal Cell Line

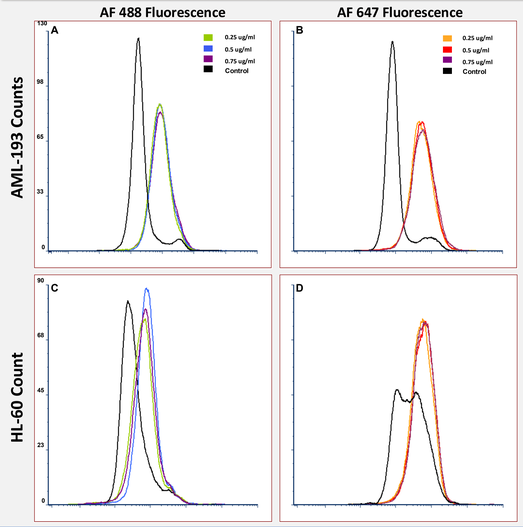


Figure 1 | Flow Cytometry-Based Identification of an Optimal Cell Line for Detection of Pegfilgrastim
Top candidate cell lines, AML-193 (Panel A, B) and HL-60 cells (Panel C, D), were evaluated for their suitability in detecting pegfilgrastim (PFG) using flow cytometry with AF488 (A,B) or AF647 (B,D) fluorophores. Histograms show the fluorescence intensity of cells incubated with pegfilgrastim-spiked serum samples, at a constant fluorophore-labeled antibody concentration. Colored lines represent varied primary antibody concentrations compared to control (Black). AML-193 cells (A, B) demonstrated clear separation between unstained control cells and stained cells for both AF488 (A) and AF647 (B) fluorophore, indicating their suitability for this assay. In contrast, HL-60 cells (C, D) did not achieve optimal peak separation, suggesting limited utility in this context. These results highlight AML-193 as the preferred cell line for accurate and reliable detection of pegfilgrastim.

Secondary Antibody Selection

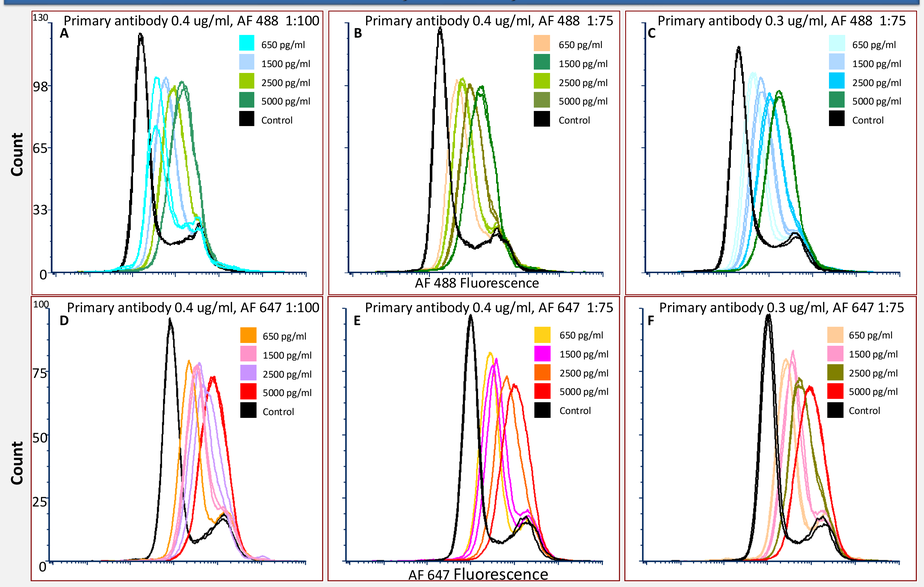


Figure 2 | Evaluation of Secondary Antibodies for Measuring Pegfilgrastim concentrations by Flow Cytometry.
AML-193 cells were tested with two different fluorophore-conjugated secondary antibodies, AF488 (A, B, C) and AF647 (D, E, F), for their ability to detect pegfilgrastim at varying drug concentrations. The color coding corresponds to different pegfilgrastim concentrations as indicated in the legend, illustrating the dose-dependent binding characteristics of pegfilgrastim under different antibody and fluorophore conditions. The AF488-conjugated antibody provided clear and consistent separation between pegfilgrastim-spiked and control samples across all measured pegfilgrastim concentrations, indicating superior sensitivity and specificity. In contrast, the AF647-conjugated antibody failed to achieve optimal peak separation at different drug concentrations and antibodies concentrations, suggesting reduced utility for this assay.

Calibration Curve and Assay Accuracy

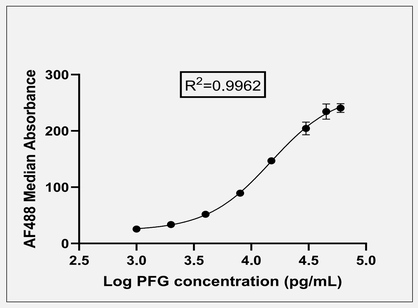


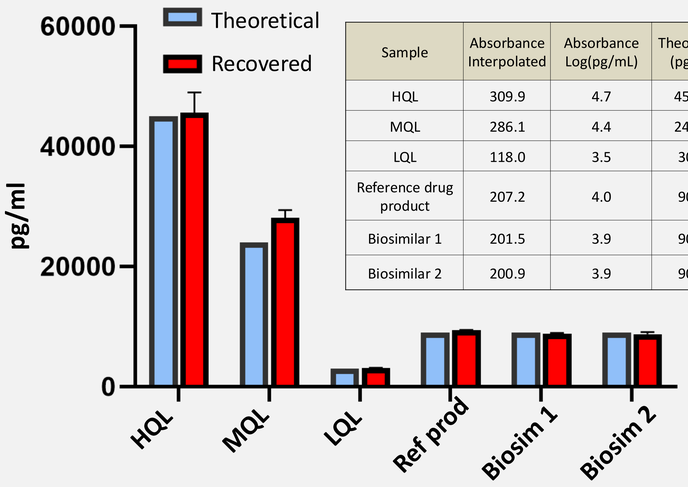
Figure 3 | Final Calibration Curve for Pegfilgrastim Quantification.
The calibration curve was generated by plotting AF488 median fluorescence intensity against the log-transformed pegfilgrastim concentration (pg/mL). The curve range spans from 1,000 to 60,000 pg/mL, showing excellent linearity (R^2 = 0.9962). The accompanying table presents the interpolated values for several test concentrations, comparing measured concentrations with theoretical values. The percentage difference between interpolated and theoretical values is less than 5%, confirming the accuracy and reliability of the assay for pegfilgrastim quantification across a broad dynamic range.

Table 1. Interpolation of Pegfilgrastim Concentrations from Experimental Data

Instrument Reading	Interpolated log (pg/ml)	Interpolated Conc. (pg/ml)	Theoretical Conc. (pg/ml)	Percent Difference (%)
29.5	3.181	1,518	1,500	1.2
42.3	3.489	3,086	3,000	2.9
73.3	3.796	6,246	6,000	4.1
129.5	4.100	12,582	12,000	4.9

Experimental readings were obtained and interpolated to determine the concentration of pegfilgrastim within the curve range of 1,000 to 60,000 pg/ml.

Reliable Quantification of Pegfilgrastim Biosimilars



Sample	Absorbance Interpolated	Absorbance Log(pg/mL)	Theoretical (pg/ml)	Recovered (pg/ml)	% Difference
HQL	309.9	4.7	45000	45521	1.2
MQL	286.1	4.4	24000	28041	16.8
LQL	118.0	3.5	3000	3090	3.0
Reference drug product	207.2	4.0	9000	9375	4.2
Biosimilar 1	201.5	3.9	9000	8757	-2.7
Biosimilar 2	200.9	3.9	9000	8697	-3.4

Figure 4 | Accuracy of CBA Quantification of Pegfilgrastim and Biosimilars.
This figure presents the comparison between theoretical (expected) and measured (recovered) concentrations of pegfilgrastim (Neulasta) and its biosimilars using the cell-based assay-based method. Control samples at high-quantification limit (HQL), medium quantification limit (MQL), and low quantification limit (LQL) were evaluated to assess assay performance across a broad dynamic range. The measured concentrations (red bars) show high concordance with theoretical values (light blue bars), with percentage differences outlined in the accompanying table. These results confirm the assay's capability to accurately quantify pegfilgrastim biosimilars.

Pegfilgrastim Detection: CBA vs. ELISA

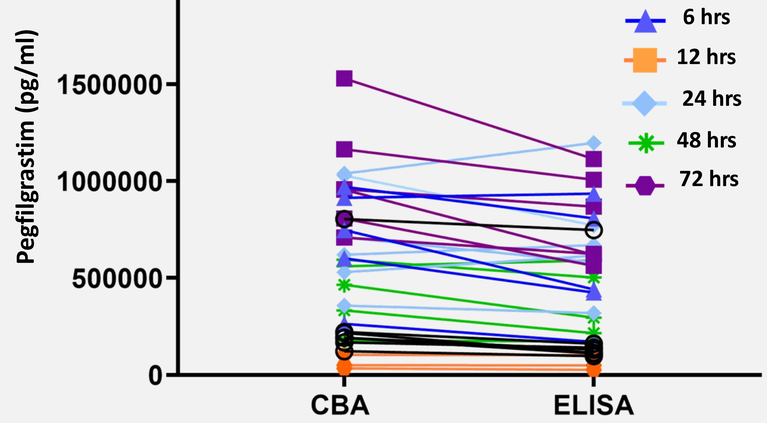


Figure 5 | Comparison of pegfilgrastim plasma levels measured by CBA versus a commercial ELISA.
Humanized mice were administered pegfilgrastim at time zero, and blood samples were collected at multiple time points post-injection (2 to 72 hours). Pegfilgrastim concentrations in plasma were quantified using the newly developed CBA and a commercially available ELISA. Each data point represents the mean of two replicates for an individual mouse sample, and lines connect paired measurements from the same mouse across the two assay platforms. Concentrations are shown in pg/ml. Overall, both assays yielded comparable results across most time points, though minor differences in detection were observed, primarily at later time points.

Conclusions

We developed a robust, flow cytometry–based cell-based assay for quantifying pegfilgrastim that overcomes key limitations of conventional ELISA, including variability and lack of PEG specificity. By targeting the PEG backbone and using G-CSF receptor–expressing AML-193 cells, this assay provides biologically relevant detection with a broad dynamic range and high reproducibility. The CBA successfully quantified both reference pegfilgrastim and FDA-approved biosimilars. The assays performance was comparable to ELISA when applied to *in vivo* samples. This approach offers a scalable platform for pharmacokinetic evaluation and can be adapted to other PEGylated biologics, potentially accelerating biosimilar development and regulatory approval.

Acknowledgements

This project was supported in part by an appointment to the Research Participation Program at the Office of Translational Sciences, U.S. Food and Drug Administration, administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the U.S. Department of Energy and FDA

Disclaimer

Evidence-based Approach to The Design of Clinical Pharmacology Studies Supporting Biosimilars Development and Approval – Aim 3: Failed Studies

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U.S. FOOD & DRUG ADMINISTRATION
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Background

This is an umbrella project with 4 aims. Aim 3 is elaborated in this poster.
When a PK similarity study fails to meet the acceptance criteria, investigating failed PK similarity studies is crucial in biosimilar development as these failures helps sponsors identify issues that could affect clinical performance. A systematic understanding of the underlying reasons for failed PK studies can potentially inform future study designs and help establish more robust development strategies for subsequent biosimilar candidates.

Objectives

- To summarize the clinical pharmacology studies failed to meet PK similarity
- To compare the failed and the follow-up studies for understanding the causes of failed studies

Methods

- Reviewed 128 clinical pharmacology studies assessing PK similarity submitted in 88 biosimilar BLAs received by 12/11/2023.
- Compiled a database containing
  (i) study population,
  (ii) sample size, dose,
  (iii) route of administration,
  (iv) statistical method, and
  (v) study results,
  (vi) summary statistics for primary PK endpoints with relative exposure [geometric mean ratio and corresponding 90% confidence interval].
- Analyzed BLAs with studies that failed to demonstrate similarity

Results

Figure 1. Reference products used in the failed PK similarity studies

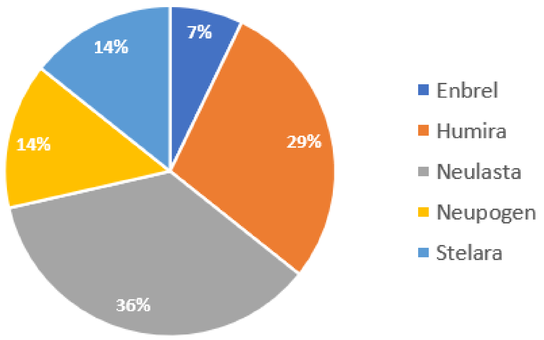

Figure 2. Proportion of PK primary endpoints that failed to meet the acceptance criteria

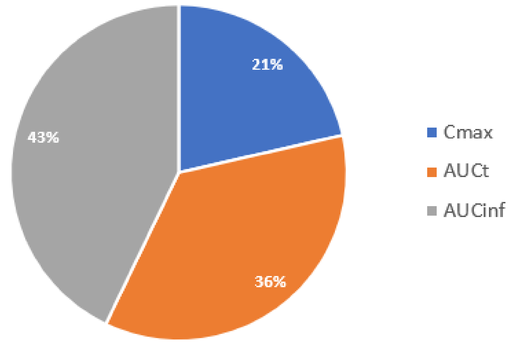

Figure 3. Proportion of comparisons that failed to meet the acceptance criteria

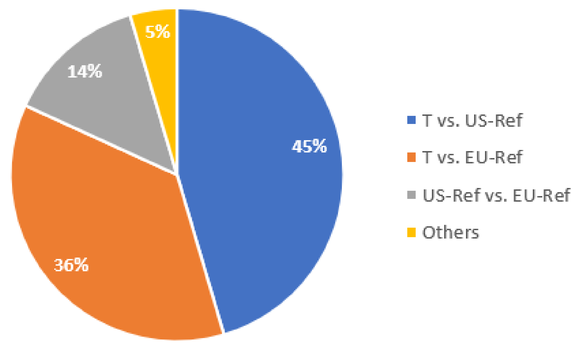

Table 1. The Reasons for not meeting acceptance criteria

(i) a higher-than-expected PK variability
(ii) difference in immunogenicity
(iii) difference in drug content

Table 2. Mitigating factors to meet acceptance criteria

(i) having a larger sample size (Figure 4)
(ii) implementing ANCOVA (Table 3, Study 5)
(iii) adding enrollment /**stratification** criteria (Table 3, Studies 3, 7, 8 & 9)
(iv) utilizing partial reference-replicate design if appropriate (Table 3, Studies 7 & 8)

Figure 4. Sample sizes of failed and follow-up PK similarity studies

Table 3. Covariates used in the failed and follow-up PK similarity studies

	Study design	Failed study	Follow-up study
BLA #1	Crossover	None	None
BLA #2	Parallel	Age	Age, body weight, trial sites
BLA #3	Parallel	body weight category, body weight x treatment	Body weight
BLA #4	Parallel	None	None
BLA #5	Parallel	None	Age, body weight, trial sites
BLA #6	Crossover	None	None
BLA #7	Crossover	None	None
BLA #7	Crossover	None	
BLA #8	Parallel (failed) / Crossover (follow-up)	Body weight, gender	None
BLA #9	Crossover	None	None
BLA #10	Crossover	None	None
BLA #11	Crossover	Group	Group
BLA #12	Parallel	Body weight, trial sites	Body weight, trial sites
BLA #13	Parallel	Body weight	Body weight

Summary

1. Our data showed that clinical pharmacology studies in biosimilar programs have an approximately 11% failure rate (14/128) in meeting PK similarity. Failed studies lead to inefficiency as it requires a subsequent, successful study to achieve approval.
2. A higher-than-expected PK variability is the most cited reason for not meeting PK similarity criteria. Difference in immunogenicity or drug content are also cited.
3. We found an increase in sample size is the primary approach used for addressing failed similarity studies when high PK variability was cited as an explanation for study failure.
4. Other approaches involving modifications of study design and statistical analysis plan could be helpful when pre-specified in the study protocol. For instance, (i) including body weight as a covariate is an approach to improve the precision of statistical comparison in the follow-up studies; (ii) when appropriate, crossover design is more efficient (vs. parallel-group design).

Key Takeaways

1. To improve the efficiency of biosimilar development, identifying potential factors associated with increased risk of study failures is critical.
2. Proactive planning with thoughtful study design and statistical analysis plan (e.g., with consideration of body weight influence on PK) can potentially minimize the risk of study failure.

Limitations

1. There is insufficient information for evaluating root causes of study failure.
2. Immunogenicity is not identified as a potential explanation for study failure due to the heterogeneity of immunogenicity assays among BLAs.
3. While covariate adjustment using ANCOVA may reduce the variability, its use could not address the failed study post hoc. Instead, protocol specification is necessary.
4. Studies that failed to meet similarity criteria may attribute the difference in drug contents and treat it as a covariate. However, drug content is a critical quality attribute and a component of the comparative analytical assessment (CAA) to be controlled through the manufacturing process. Drug content should be part of similarity assessment instead of separating out as a covariate.

Disclaimer: The views expressed on this poster are the authors' and should not be construed as the FDA's policy.

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Evidence-based Approach to The Design of Clinical Pharmacology Studies Supporting Biosimilars Development and Approval – Aim 4: Device bridging

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Background

This poster elaborates on Aim 4 of an umbrella project.

The bridging strategies for a change of injection devices have been decided on a case-by-case basis. A systematic understanding of how the product and device changes impact clinical performance is lacking. Improved understanding could potentially streamline the bridging study for approval of new device presentation of subcutaneous biosimilar products.

Objectives

- To minimize the need for a PK comparability study to support approval of autoinjectors (e.g., bridging from prefilled syringes [PFS] to autoinjectors [AI]), and
- To provide evidence-based recommendation to alternative bridging strategy that can reduce the need of studies involving human subjects

Methods

Scope:

- 351(a) and 351(k) BLAs of monoclonal antibodies (mAbs) and Fc-fusion proteins that have an approved autoinjectors (AI) presentation.
- Approval dates: between October 28, 1996, and June 30, 2024.

Data reviewed and collected:

1. PK comparability studies (design and results) that evaluated PFS and AI presentations included in BLA submissions and those only included in IND submissions; the former is expected to have optimized AI device.
2. Drug product characteristics with the potential to affect devices performance (e.g., viscosity)
3. Device parameters: dosing volume, injection time, injection depth, force parameters
4. Compiled into a database containing

Analyses:

- Investigated factors that may have contributed to differences in PK performance (i.e., not comparable to PFS).

Results

Figure 1: 45 PK comparability studies in 37 BLAs with AI approved

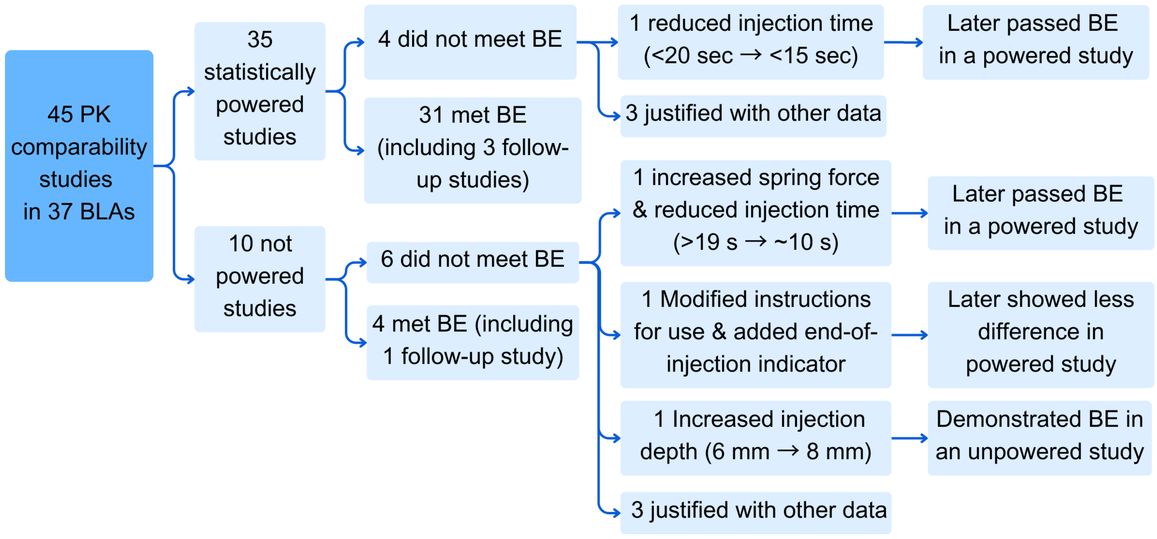

Table 1: Device parameters, product characteristics and PK results in 10 studies with PK-non-BE results from 8 BLAs

BLA	AI	AI platform	Observed values [Their Specifications]				Product Viscosity (cP)	PK results → (Modifications →) Follow-up action
			Dose accuracy*	Inj. depth (mm)	Inj. time (sec)	Activation force (N)		
A	AI-1 mL	a	5.3%	5.84 ± 0.21 [6 ± 2]	4.84 ± 0.27 [≤ 15]	9.40 ± 0.55 [4-18]	14.2	PK-non-BE → modified IFU → new study (PK-BE)
	AI-2 mL	a	9%	7 ± 0.1 [6 ± 2]	7 ± 0.7 [≤ 20]	12 ± 1 [4-23]	9.4	PK-non-BE → Justified by E-R
B	AI	b	NA	NA [NA]	>19 [≤ 20]	NA [NA]	8.8	PK-non-BE → stronger spring → new study
	AI-R	b	13.3%	5.86 ± 0.12 [6 ± 2]	10.30 ± 0.54 [6-19]	11.13 ± 0.32 [≤ 39]	8.8	PK-BE
C	AI	a	NA	NA [4.5-8]	NA [15-20]	NA [4-16]	7.5	PK-non-BE → shorten injection time + add indicators → new study
	AI-R	a	NA	NA [4.5-8]	NA [≤10]	NA [4-16]	7.5	PK-BE
D	AI	b	5.0%	NA [6 ± 2]	≤ 6 [≤15]	9-11 [2-15]	3.8	PK-non-BE → ↑ injection depth (to 8 mm)→ new study
	AI-R	b	5.0%	7.7-8.1 [6.5-9.5]	≤ 6 [≤15]	9-11 [2-15]	3.8	PK-BE
E	AI-1 mL	a	3.1%	NA	NA	NA	8.9	PK-non-BE → USPI says a higher exposure
	AI-2 mL	b	2.7%	6.3 ± 0.18 [6.25±1.75]	10.2 ± 1 [≤12]	9.23 ± 0.23 [2-14]	8.9	PK-non-BE → USPI says a higher exposure
F	AI-1	a	13.2%	6 [6 ± 2]	6.08 ± 1.12 [≤15]	7.3-9.5 [5-15]	5.5	PK-non-BE → Justified by E-R
	AI-2	a	13.2%	6.2-6.4 [6 ± 2]	8.27 ± 0.77 [≤15]	6.2-8.8 [5-15]	14.5	PK-non-BE → Justified by E-R
G	AI	c	3%	7.96 ± 0.28 [7.5 ± 2]	7.5 ± 0.74 [≤15]	3.6-5.5 [2.5-12]	2.75	PK-non-BE → evaluated in Ph 3 OLE
H	AI	b	NA	6.57 ± 0.16 [6.5 ± 2]	3.65 ± 0.68 [≤10]	6.36 ± 0.63 [2-10]	3	PK-non-BE → Justified by E-R

- dosing accuracy expressed as % deviation from target delivery volume.

IFU: Instruction for Use. NA: data not available. R = revised AI

1. Our study identified 10 studies in 8 BLAs not achieving PK comparability between AI and PFS (i.e., PK-non-BE). Notably a higher failure rate in underpowered studies (60%) versus adequately powered studies (11.4%).
2. We investigated AI parameters and product characteristics (namely viscosity) in these 8 BLAs with PK-non-BE results (**Table 1**) one-by-one and did not find any associated with the outcome of PK comparability study. Limited data suggests that injection times > 15 seconds, proper end-of-injection indications and spring force may be important risk factors.
3. After observing PK-non-BE, corrective measures taken included modifications to injection time, spring force, and injection depth. With AI modifications, most subsequent studies achieved or approached PK comparability, suggesting that device parameter adjustments can be effective interventions.
4. Using the same AI device or platform to deliver different products may not consistently achieve comparable PK vs. PFS.
5. Results indicate that successful PK performance likely depends on complex interactions between device parameters, product characteristics, and proper user handling rather than any single factor, highlighting the need for comprehensive approaches to device development and testing.
6. Overall, knowledge gaps exist regarding critical AI device parameters that may influence the PK performance.

Limitations

1. Insufficient understanding of critical device parameters and product characteristics that influence PK performance.
2. Too few cases per parameter category to statistically verify individual parameter influences.
3. Mixed powered and underpowered studies complicated outcome interpretation.
4. Concurrent study design changes (e.g., sample size, injection sites) prevented clear attribution of improvements to device modifications..
5. Attempts to assess relationships between PK comparability outcomes and individual device or product parameters were not fruitful, suggesting that multifactorial interactions may be more important than single-parameter effects.

Communications

1. Publication (2024) PMID: 3816778
2. FDA Student Scientific Research Day poster (2024)
3. OCP Biologics Oversight Board presentations (2023 and 2024)
4. ASCPT poster (2025)
5. Manuscript in preparation

References available upon request.

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