The Basics of Analytical Comparability of Biosimilar Monoclonal Antibody for Regulatory Reviewers

IPRP Biosimilars Working Group

Disclaimer :
This document reflects the views of subject matter experts participating in the IPRP Biosimilars (BWG) Working Group and should not be construed to represent the official views of any given regulatory authority participating in the IPRP.

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I. Disclaimer

Disclaimer

☐ This material is a compilation of publicly available information on the current approach for analytical comparability of biosimilars, especially monoclonal antibodies.

☐ This material does not include any specific recommendations of the IPRP BWG and the views and opinions expressed in this material are those of the individuals who serve in his/her personal capacity and do not necessarily reflect the official policy or position of any agency or organization.

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☐ This material is intended to help regulatory reviewers before he or she begins to review quality of biosimilars, who has certain level of understanding for biotherapeutics and review experiences.

☐ This material could be used as initiation step for training of biosimilarity as a complementary tool and interactive course such as hands-on training.

II. Concepts of the Biosimilar

1. Definition of Biosimilar

☐ WHO - Similar biotherapeutic product (SBP) is a biotherapeutic product which is similar in terms of quality, safety and efficacy to an already licensed reference biotherapeutic product. (*ref: WHO, Guidelines on Evaluation of Similar Biotherapeutic Products (SBPs), 2009*)

☐ EMA - A biosimilar is a biological medicinal product that contains a version of the active substance of an already authorised original biological medicinal product (reference medicinal product) A biosimilar demonstrates similarity to the reference medicinal product in terms of quality characteristics, biological activity, safety and efficacy based on a comprehensive comparability exercise.
(*ref: EMA, Guideline on similar biological medicinal products, 2014*)

☐ US FDA – The biological product is highly similar to the reference product notwithstanding minor differences in clinically inactive components, and there are no clinically meaningful differences between the biological product and the reference product in terms of the safety, purity, and potency of the product.
(*ref: Section 7002(b)(3) of the Affordable Care Act, adding section 351(i)(2) of the PHS Act*)

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2. Definition of Similarity/Biosimilarity

☐ Similar does not equal to Same

- ✓ **Highly similar** to the reference product in all clinically relevant quality attributes, i.e. product attributes that may impact clinical performance. (*ref: WHO, Guidelines on Evaluation of Similar Biotherapeutic Products (SBPs), 2009*)
- ✓ highly similar to the reference product notwithstanding **minor differences** in clinically inactive components, and that there are **no clinically meaningful differences** between the biological product and the reference product in terms of the safety, purity, and potency of the product. (*ref: Section 7002(b)(3) of the Affordable Care Act, adding section 351(i)(2) of the PHS Act*)

☐ Biotherapeutics are **almost impossible to be produced as the same molecule** of reference products

☐ Why?

a) Biotherapeutics are very complex and heterogeneous molecules

b) Sensitive to differences in cell lines, manufacturing processes and formulation

☐ **A comprehansive comparability exercises are needed** to demonstrate biosimilarity between reference products and biosimilars!!

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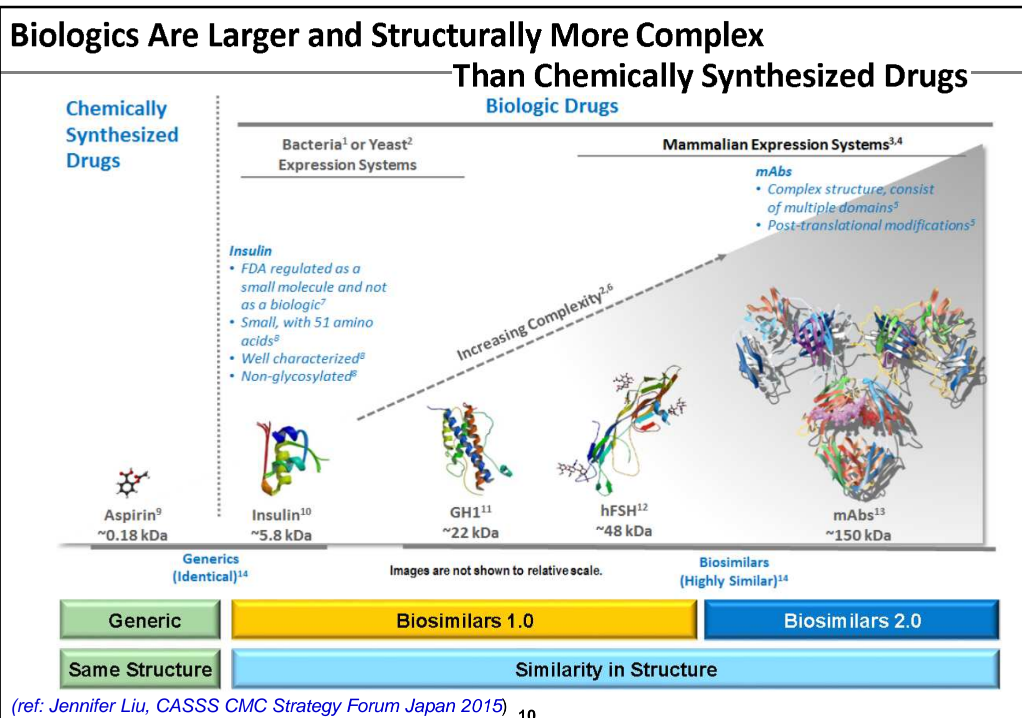

3. Development Process of Biosimilar

☐ Increased knowledge of the relationship between biochemical, physico-chemical and biological properties of the product and clinical outcomes facilitates development of a biosimilar.

☐ General considerations

a) Biosimilar **shall utilize the potential mechanism(s) of action** for the reference product.

b) Has the **same route of administration and dosage form** as the reference product.

c) **Differences from the reference product as regards strength, pharmaceutical form, formulation, excipients or presentation require justification.** If needed, additional data should be provided. Any difference should not compromise safety.

(ref: EMA, Guideline on similar biological medicinal products, 2014)

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3. Development Process of Biosimilar

☐ Development of Biosimilar

a) Understanding of reference product and defining critical quality attributes (CQAs) and additional attributes to monitor

- publically available information (literatures etc.), extensive characterization data of reference products
- Known (all possible) mechanism of actions, biological functions, purity, safety, and immunogenicity profiles etc.

b) Establishment of target quality product profile for Biosimilar

- Most of CQAs may be already established early in development

c) Manufacturing development to match the reference product profile

d) Demonstration of Analytical Comparability

e) Non-clinical studies and Clinical studies

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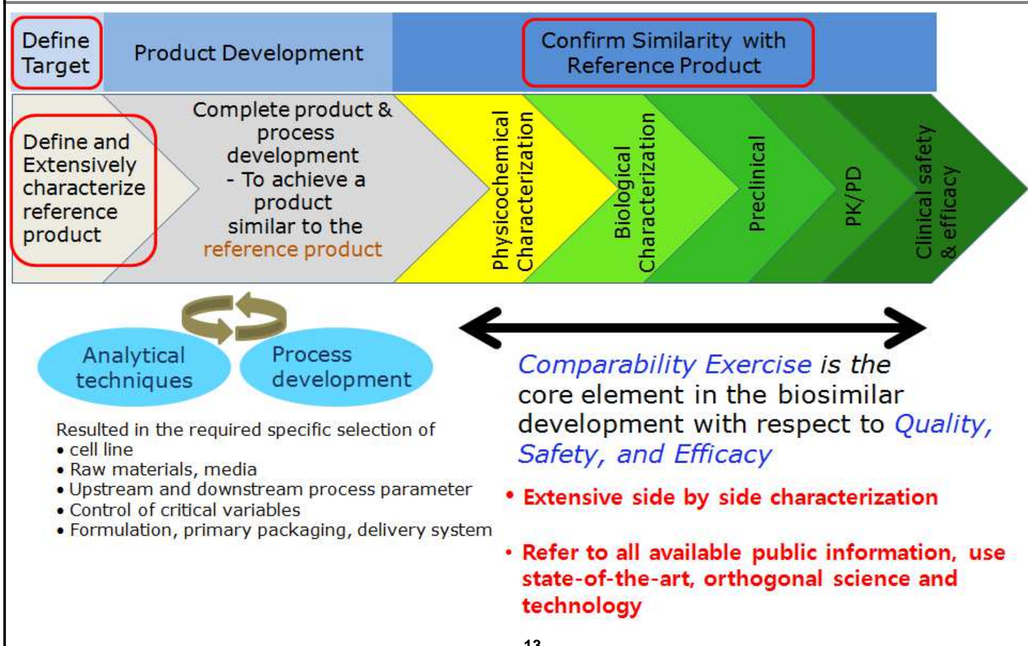
3. Development Process of Biosimilar



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4. Demonstration of Similarity/Biosimilarity

☐ **Stepwise approach**

a) Demonstration of similarity of a biosimilar and a reference product in terms of quality is a prerequisite for reducing the nonclinical and clinical data set required for licensure.

b) Move onto the next level to address a residual uncertainty if any.

☐ **Totality of evidence**

a) The decision to license a biosimilar product should be based on comprehensive evaluation of the whole data package for each of Quality, Non-Clinical and Clinical parameters to demonstrate similarity to a Reference Product.

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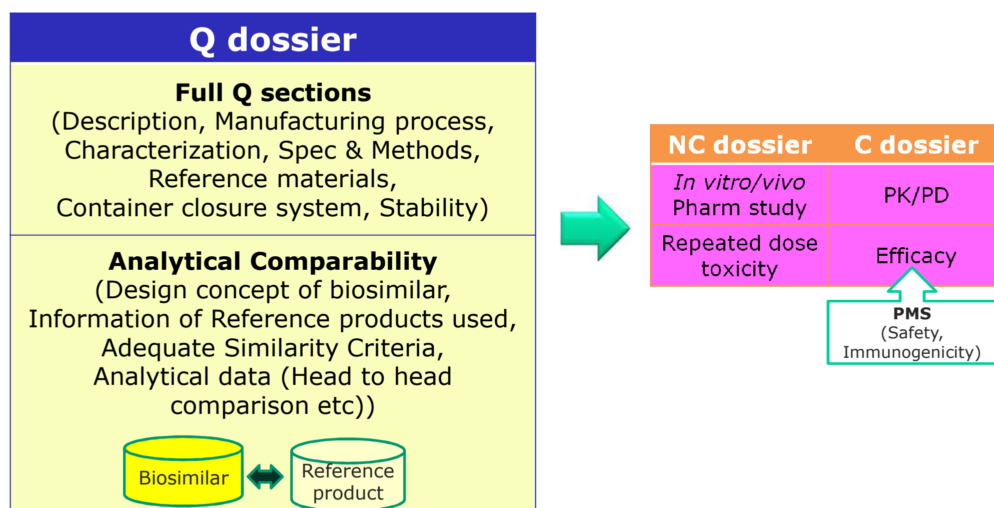
4. Demonstration of Similarity/Biosimilarity



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5. Reference Product

☐ must be a medicinal product **approved** within the regulated territory, on the basis of a **complete dossier**. (*ref: EMA, Guideline on similar biological medicinal product, 2014*)

☐ **A single reference product should be used** as the comparator throughout the comparability programme for quality, safety and efficacy studies during the development of a biosimilar in order to allow the generation of coherent data and conclusions. (*ref: EMA, Guideline on similar biological medicinal product, 2014*)

☐ **Shifts in quality profile of Reference Product**

a) Such events could occur during the development of a biosimilar and may result in a development according to a QTPP which is no longer fully representative of the reference product available on the market.

b) The ranges identified before and after the observed shift in quality profile could normally be used to support the biosimilar comparability exercise at the quality level, as either range is representative of the reference medicinal product.

(ref: EMA, Guideline on similar biological medicinal products containing biotechnology-derived proteins as active substance: quality issues, 2014)

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5. Reference Product

☐ The majority of the regulations necessarily require demonstration of comparability to a local reference product approved in their jurisdiction.

a) Possibility of geographic divergence of reference products in quality attributes

- Variations from different supply chain (e.g. Difference of manufacturing sites)
- Variations after separation of license holders & independent change
- Variations from sequential application of a manufacturing process change

☐ The Use of a Foreign Reference Product

a) To facilitate global development, most NRAs accept the use of non-local reference products by demonstrating the equivalence of the local and foreign reference products **(Bridging study)**.

(see also Appendix II, 'A. Understanding Reference product')

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III. Analytical Comparability Assessment

6. Role of quality analysis

☐ **Quality analytics are an essential tool for establishing similarity.**

a) Analytics are typically more sensitive than traditional clinical endpoints in this respect.

b) Clinical studies play a role in supporting biosimilarity.

(ref: Supporting biosimilarity and extrapolation, GABI Journal, vol 4 (4), 2015)

☐ **Robust characterization is essential.**

a) The more comprehensive and robust the characterization data,

⇨ the stronger the justification for selective and targeted approach to animal and human testing

⇨ the stronger the justification for differences

6. Role of quality analysis

Criteria	Less data	More data
Thorough understanding of reference product	Allows for good justification for similarity window	
Expression cell line and formulation	Same	Different
Amino acid sequence	Identical	May not be a biosimilar
Structure	Highly similar	Different
Post translational modification	Highly similar	Different
Kinetics, Binding	Equivalent	Not equivalent - May not be a biosimilar
Process & Product related impurity	Highly similar	Different - non-clinical data may be needed (toxicity)
Forced degradation	Highly similar	Different
Comprehensive understanding	Expected	

(*ref: Ramanan S (Amgen) AHC Biotherapeutics Workshop 2015*)

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7. Structure vs Function/Immunogenicity of a mAb

☐ Biotherapeutics, especially monoclonal antibodies(mAb) are very large, complex and heterogeneous molecules.

☐ **Structure vs Function**

a) Fab function : biological activity via binding to specific target

b) Fc function : binding to FcγR/C1q etc ⇨ CDC, ADCC, ADCP etc.

binding to FcRn ⇨ protecting IgG from lysosomal degradation, PK profile

☐ **Structure vs Immunogenicity**

a) Process-related impurities (Host cell protein, endotoxin etc.)

b) Product-related substances/impurities

: Non-human oligosaccharides (glycosylation profile), Aggregates etc.

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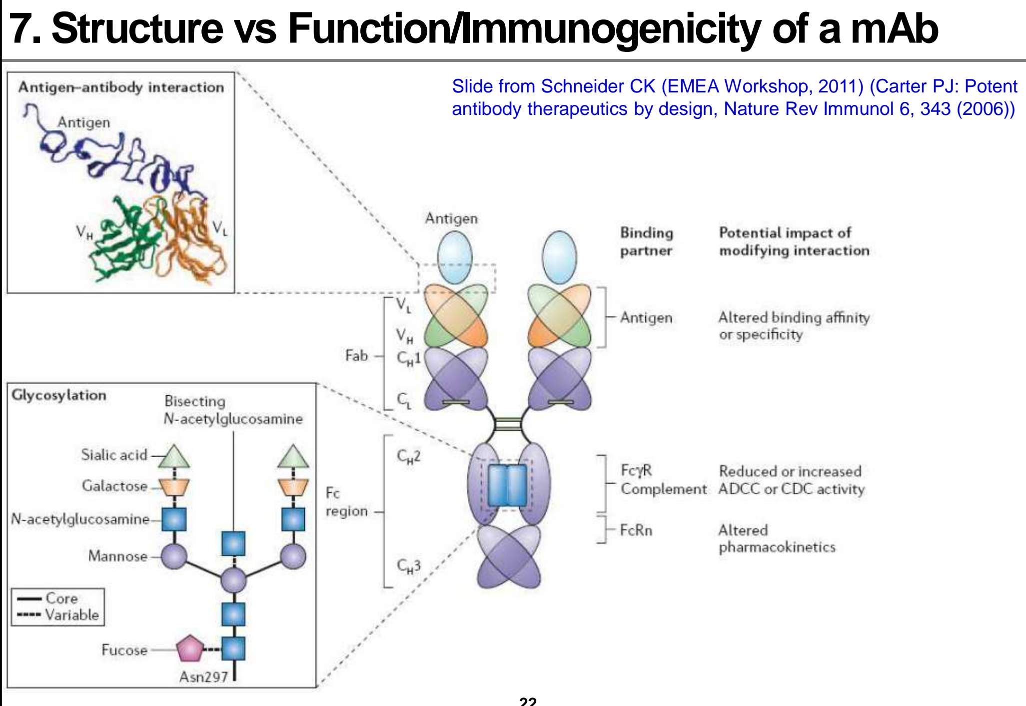

7. Structure vs Function/Immunogenicity of a mAb
Slide from Schneider CK (EMEA Workshop, 2011) (Carter PJ: Potent antibody therapeutics by design, Nature Rev Immunol 6, 343 (2006))
Antigen-antibody interaction
Antigen
VH
VL
Glycosylation
Bisecting N-acetylglucosamine
Sialic acid
Galactose
N-acetylglucosamine
Mannose
Core
Variable
Fucose
Asn297
Antigen
Fab
VL
VH
CH1
CL
Fc region
CH2
CH3
Binding partner
Potential impact of modifying interaction
Antigen
Altered binding affinity or specificity
FcγR
Complement
Reduced or increased ADCC or CDC activity
FcRn
Altered pharmacokinetics
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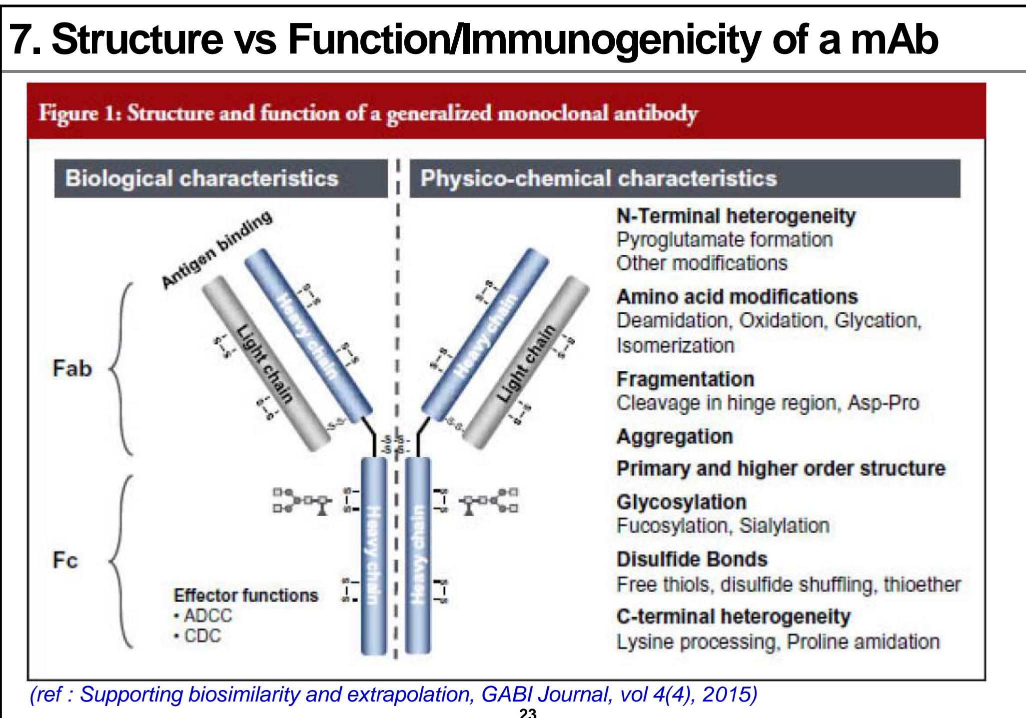

7. Structure vs Function/Immunogenicity of a mAb
Figure 1: Structure and function of a generalized monoclonal antibody
Biological characteristics
Physico-chemical characteristics
Antigen binding
Fab
Fc
Light chain
Heavy chain
Effector functions
• ADCC
• CDC
N-Terminal heterogeneity
Pyroglutamate formation
Other modifications
Amino acid modifications
Deamidation, Oxidation, Glycation, Isomerization
Fragmentation
Cleavage in hinge region, Asp-Pro
Aggregation
Primary and higher order structure
Glycosylation
Fucosylation, Sialylation
Disulfide Bonds
Free thiols, disulfide shuffling, thioether
C-terminal heterogeneity
Lysine processing, Proline amidation
(ref : Supporting biosimilarity and extrapolation, GABI Journal, vol 4(4), 2015)
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7. Structure vs Function/Immunogenicity of a mAb

☐ **Process-related impurities**

(*ref: EMA, Guideline on similar biological medicinal products, 2014*)

a) Process-related impurities may differ between the originator and biosimilar products, although these should be minimised. It is preferable to rely on purification processes to remove impurities rather than to establish a non-clinical testing program for their qualification. Differences that may confer a safety advantage (e.g. lower levels of impurities) should be explained but are unlikely to preclude biosimilarity.

b) Process-related impurities (e.g. host cell proteins, host cell DNA, reagents, downstream impurities, etc.) are expected to differ qualitatively from one process to another. Therefore, **the qualitative comparison of these parameters may not be relevant in the biosimilar comparability exercise**. Nevertheless, **state-of-the-art analytical technologies** following existing guidelines and compendial requirements **should be applied, and the potential risks related to these identified impurities (e.g. immunogenicity) will have to be appropriately documented and justified**.

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7. Structure vs Function/Immunogenicity of a mAb

☐ **Heterogeneity in recombinant mAbs**

a) Monoclonal antibodies commonly display several sources of heterogeneity. (e.g. C- terminal lysine processing, N-terminal pyroglutamate, deamidation, oxidation, isomerisation, fragmentation, disulfide bond mismatch, N-linked oligosaccharide, glycation), which lead to a complex purity/impurity profile comprising several molecular entities or variants. (*ref: EMA, Guideline on Development, Production, Characterization and Specifications for Monoclonal Antibodies and Related Products, 2009*)

b) All of these product-related variants may alter the biological properties of the expressed recombinant protein. Therefore, **identification and determination of the relative levels of these protein variants should be included in the comparative analytical characterization studies.** (*ref: US FDA, Guidance, Quality Considerations in Demonstrating Biosimilarity of a Therapeutic Protein Product to a Reference Product, 2015*)

c) **Also should evaluate the impact on potency, immunogenicity and PK/PD etc.**

ex) C-terminal Lysine : variability of truncation level

⇨ variability of charge profile (i.e., charge heterogeneity)

⇨ but doesn't seem to impact potency or safety profile

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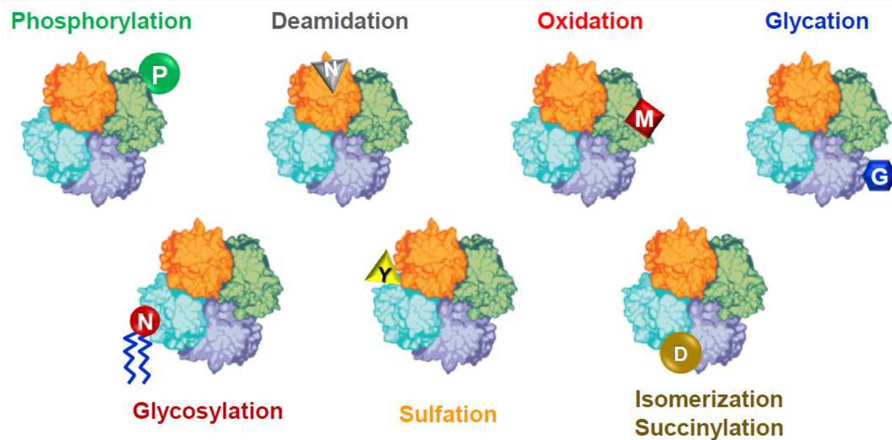
7. Structure vs Function/Immunogenicity of a mAb

☐ **Post-Translational Modifications (PTMs)**

a) Various PTM can contribute to the structural and functional diversity

b) **impacted by the cell line and production process**



1. Mann M, et al. *Nature Rev Biotech*. 2003;21:255-261; 2. Medzihradszky KF, et al. *Mol Cell Proteomics*. 2004;5:429-440; 3. Karve TM, et al. *J Amino Acids*. 2011;2011:1-13.

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7. Structure vs Function/Immunogenicity of a mAb

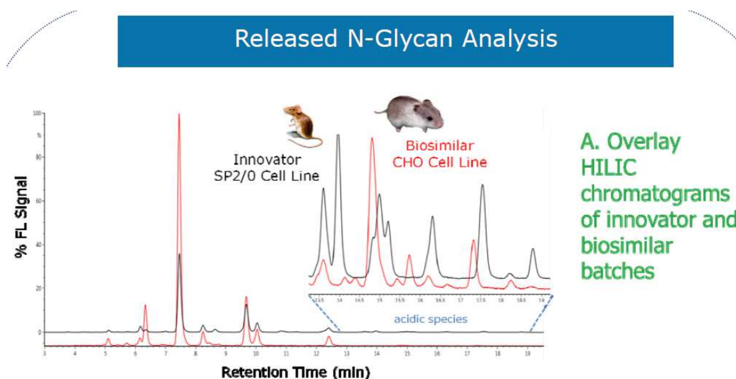
☐ **Glycosylation : a complex heterogeneous PTM of proteins**

Depending on the expression host, the glycosylation composition and patterns or glycoforms in a mAb or Fc-fusions can be significantly different, which can have **significant impacts on the PK and/or PD of monoclonal antibodies, resulting in potentially altered efficacy and safety profiles.** (*ref: Liu L, Antibody Glycosylation and Its Impact on the Pharmacokinetics and Pharmacodynamics of Monoclonal Antibodies and Fc-Fusion Proteins, 2015*)

Released N-Glycan Analysis

Deploying an integrated LC/UV/MS system for assessing structural comparability of innovator and biosimilar Infliximab

(Henry Shion et al, Waters Corporation, 2014 WCBP Symposium poster)



A. Overlay HILIC chromatograms of innovator and biosimilar batches

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7. Structure vs Function/Immunogenicity of a mAb

☐ Summary of (potential) key Impacts of Glycosylation on the PK and PD of mAb and Fc-fusion proteins

Glycan	Impacts
Mannose	• Increases the clearance of mAb • Enhances FcrRIIIa binding/ADCC of mAb • Reduces C1q binding/CDC of mAb
Fucose	• Interferes with binding to FcrRIIIa • Defucosylation enhances FcrRIIIa binding/ADCC activity
Galactose	• Exposed galactose may increase the clearance of mAb • Enhances CDC of mAb
GlcNAc	• Bisecting GlcNAc enhance FcrRIIIa binding/ADCC • Increases the clearance of Fc-fusion proteins
Sialic acid NANA	• Critical for reducing the clearance of Fc-fusion proteins • Anti-inflammatory activity
Sialic acid NGNA	• Interferes with FcrRIIIa binding and reduce ADCC activity of mAb • May be immunogenic in humans
Galα1-3Galβ1-GlcNAc-R	• Immunogenic in humans and may induce anaphylaxes

(ref: Liu L, Antibody Glycosylation and Its Impact on the Pharmacokinetics and Pharmacodynamics of Monoclonal Antibodies and Fc-Fusion Proteins, 2015)

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8. Quality Attributes (QAs)

☐ The quality target product profile (QTPP) of a biosimilar should be based on data collected on the chosen reference medicinal product, including publicly available information and data obtained from extensive characterisation of the reference medicinal product. (*ref: EMA, Guideline on similar biological medicinal products containing biotechnology-derived proteins as active substance: quality issues, 2014*)

☐ Physicochemical and functional characterization studies should be sufficient to establish relevant quality attributes including those that define a product's identity, quantity, safety, purity, and potency. (*ref: US FDA, Guidance, Quality considerations in Demonstrating Biosimilarity of a Therapeutic Protein Product to a Reference Product, 2015*)

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8. Quality Attributes (QAs)

☐ The Identification of the **potential correlations between QAs** (or orthogonal methods) are important to evaluate clinical relevance.
ex) %Afucosylation ⇨ FcrRIIIa binding ⇨ ADCC ⇨ Clinical relevance

☐ Some QAs should consider the age of the different batches of reference product.
ex) Size and charge variants : can be changed with the passage of time at the recommended storage condition ⇨ may analyze the data by plotting against the estimated material age at the time of testing.

☐ **Acceptable differences and Impacted quality attributes**
a) Expression system : may result in undesired consequences, such as atypical glycosylation pattern, higher variability or a different impurity profile, as compared with those of the reference medicinal product.
b) Formulation : purity/impurity level, stability profile etc.
c) Container/closure system : compatibility profile, stability profile etc.

(see also Appendix II, 'B. Differences of Producing cell lines' and 'C. Differences of Formulation')

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8. Quality attributes (QAs)

☐ Process-related impurities (e.g. host cell protein, DNA)
a) specific to the individual process
b) It is preferable to rely on purification processes to remove impurities. Differences that may confer a safety advantage (e.g. lower levels of impurities) should be explained. *(ref: EMA, Guideline on similar biological medicinal products, 2014)*

☐ Particular attention should be given to quality attributes that might have an impact on immunogenicity or potency, or that have not been identified in the reference medicinal product. *(ref: EMA, Guideline on similar biological medicinal products containing biotechnology-derived proteins as active substance: quality issues, 2014)*

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8. Quality Attributes (QAs)

□ Quality Attributes of mAb (Example)

a) Primary structure (amino acid sequence, N/C-terminal sequence, Molecular weight, peptide mapping profile, Disulfide bonds structure, etc.)

b) Higher order structure (secondary, tertiary and quaternary structure)

c) Additional Post-translational modifications (Oxidation, Deamidation, Glycation etc.)

d) Charge variants (pI value, qualitative and quantitative profile of acidic/main/basic species)

e) Size variants (qualitative and quantitative profile of High/Low molecular weight species, aggregates, sub-visible particles etc)

f) Glycosylation profile (Glycosylation profile, site-specific profile, site-occupancy etc.)

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8. Quality Attributes (QAs)

□ Quality attributes of mAb (Examples)

g) Strength/Content (Protein concentration/amount, Volume in container)

h) Potency (target binding, mechanism of action exploration)

i) Process-related Impurities (host-cell protein, host-cell DNA etc.)

j) Formulation (pH, excipient content etc.)

k) Degradation/Stability profiles

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9. Assessment of Critical Quality Attributes

☐ **Identification of CQAs** : Considering the impact on **clinical performance** and **degree of uncertainty** in each quality attribute

a) used to guide the product and process development.

b) should be considered to determine similarity in quality and impact on extrapolation of indications.

c) should be considered to design the control strategy of the quality and manufacturing process.

☐ **Potential clinical impact of quality attributes**

a) efficacy

b) pharmacokinetics

c) immunogenicity (which remains the main reason of clinical studies)

d) safety/toxicity : pharmacological toxicity (biological activities) & off-target toxicity (rare with biologicals since they are highly specific to their target)

☐ Degree of Uncertainty : the level of attribute present, the possibility of deviation occurs, and the assay sensitivity

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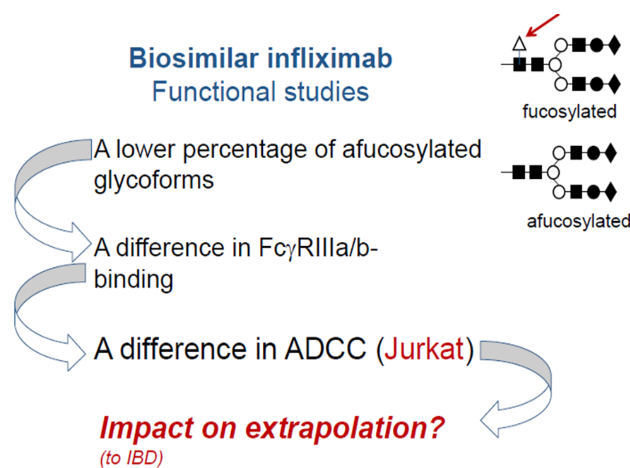
9. Assessment of Critical Quality Attributes

☐ Efficacy : Example - Afucosylation and ADCC



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9. Assessment of Critical Quality Attributes

☐ QAs that affect the immunogenicity *(ref: Supporting biosimilarity and extrapolation, GABI, Vol 4, 2015)*

Table 1: Immunogenicity: critical attributes are well known

Attribute (example)	Comment/analytical methods (examples)
Amino acid sequence	Must be identical/orthogonal peptide maps with high resolution MS and MS/MS sequencing
Aggregates	Critical factor/SEC, FFF, MALLS, DLS, AUC, imaging methods, particle characterization
Folding, disulphide bridges, free cysteines	CD spectroscopy, H-D-Exchange, FT-IR, X-ray, 1D and 2 D NMR, peptide mapping
Degradation	Degradation products that do not occur in the body potentially immunogenic/RP-HPLC, CEX, Papain-HIC, Papain-IEX, peptide map, MS
Hostcell proteins	Adjuvant effect or complex formation/ELISA, mass spectrometry
Leachables/extractables	Adjuvant effect or effect on folding/aggregation; HPLC with highly sensitive detectors, mass spectrometry
Glycosylation: Galactose-α1,3-Galactose	Reported for cetuximab patients pre-sensitized by tick bites only/ NP-HPLC of 2AB-labeled glycans coupled to ESI-MS, exoglycosidase digestion, MALDI TOF/TOF, CGE, peptide map
Glycosylation:N-glycolyl-neuraminic acid (NGNA)	NP-HPLC, WAX, HPAEC, RP-HPLC after DMB-labelling, mass spectrometry

Immunogenicity evaluation remains the main reason for clinical studies

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9. Assessment of Critical Quality Attributes

☐ Quality attributes that affect the Pharmacokinetics (ADME)

(*ref: Supporting biosimilarity and extrapolation, GABI Journal, vol 4 (4), 2015*)

Table 2: Pharmacokinetics: attributes critical to absorption, distribution, metabolism and excretion are well known

Attribute (example)	Comment/analytical methods (examples)
Amino acid sequence	Must be identical/orthogonal peptide maps with high resolution MS and MS/MS sequencing
Folding, disulphide bridges, free cysteines	Misfolding leads to faster clearance/CD spectroscopy, H-D-Exchange, FT-IR, X-ray, 1D and 2D NMR, peptide mapping
Oxidation (methionine)	Can decrease binding to FcRn and thus lead to increased exposure/ RP-HPLC, Papain-HIC, peptide map, mass spectrometry
Degradation	Degraded product is cleared quickly/RP-HPLC, CEX, Papain-HIC, Papain-IEX, peptide map, MS
Glycosylation: Sialylation	Reduced clearance via liver asialo-glycoprotein receptors, increased proteolytic stability; no major impact for mAbs/NP-HPLC, WAX, HPAEC, RP-HPLC after DMB-labelling, mass spectrometry

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9. Assessment of Critical Quality Attributes

☐ Example (ref: https://wwwparexelcom/files/3114/3385/6985/Quality_Data_for_Demonstrating_Biosimilarity_Articlepdf)

ATTRIBUTE	KEY SIMILARITY CONSIDERATIONS
Product-related	Percent protein content, pH, osmolarity, qualitative and quantitative composition of key excipients
Structure of active substance	Primary structure must match reference product, differences in higher order structure (folding) in theory may impact pharmacokinetics, efficacy, immunogenicity, and safety; and any differences will need to be justified robustly as having low or no impact
Isoforms of active substance	Differences in isoform distribution such as side chain variants, glycosylation and N or C terminal truncation or modification must be robustly justified; differences are assumed to be significant until proven otherwise
Impurities	Process-related impurities (host cell proteins, trace solvents and leachables) may not impact biologic activity, but may adversely affect the immunogenicity profile; product-related impurities – depending on % content, activity level, and potential for undesirable activity/immunogenicity – have a variable impact, from very low to very high
Biological activity	Potency, receptor binding and, for mAbs, both target (antigen) and Fc binding

■ = always very high criticality
■ = at least high criticality
■ = variable criticality

9. Assessment of Critical Quality Attributes

☐ Example : Zarxio™ (ref: FDA Advisory Committee Briefing Document, 2015)

Table 5 - Criticality of quality attributes and their impact on clinical parameters

Quality Attribute	Criticality	Relevant for	Methods Used
Amino acid sequence	Very High	Efficacy, Safety, Immunogenicity	Edman, peptide mapping, MS
Potency	Very High	Efficacy Safety	Bioassay
Target binding	Very High	Efficacy Safety	Surface plasmon resonance
Protein concentration	Very High	Efficacy	Content determination
Subvisible particles	High	Immunogenicity	Light obscuration
Oxidized variants	High	Efficacy	Reversed phase chromatography
Higher order structure	High	Efficacy Immunogenicity	CD and NMR spectroscopy
High-molecular weight variants/aggregates	High	Immunogenicity	Size exclusion chromatography
Truncated variants	Low	None	Reversed phase chromatography coupled with MS
Norleucine	Very Low	None	Reversed phase chromatography
Deamidation	Very Low	None	Cation exchange chromatography

10. Selection & Suitability of the Analytical Methods

☐ Assays should provide results that are meaningful (Relevance) and Reliable.

☐ **Selection of Methods**

a) based on the nature of the mAb and knowledge regarding the structure and heterogeneity of the reference product and biosimilar product, including those characteristics critical to product performance

- capable of elucidating and comparing the Quality Attributes
- evaluate the all (potential) MOAs, Structure/function relationships and clinical relevance
- evaluate the Degradation/Stability profiles
- evaluate Lot-to-lot variations

b) State-of-the-art technologies should be used.

c) Orthogonal methods should be used.

- The methods used should separate and analyse different variants of product based upon different underlying chemical, physical and biological properties of protein molecules.*(ref: WHO, Guidelines on Evaluation of Similar Biotherapeutic Products(SBPs), 2009)*

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10. Selection & Suitability of the Analytical Methods

☐ **Suitability of methods**

a) Analytical method capability impact the assessment of similarity.

(ref: WHO, Guidelines on Evaluation of Similar Biotherapeutic Products (SBPs), 2009)

⇨ should be able to discern potential structural and functional differences wherever possible.

⇨ Knowledge of the analytical limitations of each technique used to characterize the product (e.g. limits of sensitivity, Resolving power) should be applied when determining similarity.

b) Adequately qualified for intended use

⇨ Sensitivity, sufficient Resolution and acceptable Intermediate Precision etc.

⇨ Sample manipulation prior to analysis or analysis conditions can affect the results. (example: Concentrating sample can affect the properties of the protein leading to homodimerization)

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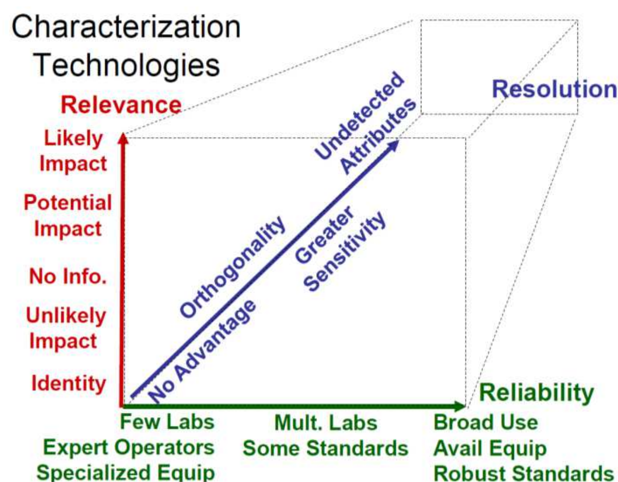
10. Selection & Suitability of the Analytical Methods



(ref: Kozlowski, S (CDER), BiomanufacturingTechnology Summit, Rockville, MD, June 13, 2014)

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11. Quality Attributes vs Analytical Methods

□ Characterization studies

Attributes	Potential effect	Examples of Analytical methods
1. Primary Structure		
Amino acid sequence	- Basic characterization of all effect - Should be identical to reference product	R Peptide mapping with UV and MS detection, MS/MS sequencing(HPLC-ESI-MS)
Terminal variants (C-terminal Lysine, N-terminal pyroglutamate)	- Heterogeneity - C-terminal Lys : Generally no impact - N-terminal pyroglutamate : No impact on biological function but may have influence on pharmacokinetics - Impact on Mw and charge profiles	Peptide mapping with MS and MS/MS Sequencing
Molecular Weight	- Heterogeneity due to PTMs and terminal mode	Peptide mapping with MS and MS/MS (Intact, Reduced and Deglycosylated)
Disulfide bond	-Disulfide bond is key contributor for conformation of structure	R/NR RP-HPLC–ESI–MS peptide mapping Ellman's assay(free thiol)

(ref: Schiestle M, 2015 AHC Biotherapeutics Workshop with modification by KIM JA)

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11. Quality Attributes vs Analytical Methods

Attributes	Potential effect	Examples of Analytical methods
2. Post-translational modification		
Deamidation Isomerization Oxidation Glycation	- May impact on biological functions or immunogenicity (Deamidation, Oxidation) - May be immunogenic (IsoAsp etc) - May impact on stability profile - Impact on charge profile, glycan profile…	Ion exchange chromatography (CEX, IEX) Boronate affinity chromatography HI-HPLC Peptide mapping with MS and MS/MS(HPLC-ESI-MS)
3. Higher order Structure		
Higher order structure	-Folding linked to conformation of structure -Impact on target binding, biological function	Far/near-UV CD, FT-IR, hydrogen deuterium exchange (HDX)-MS, DSC 1D/2D NMR, X-ray crystallography

(*ref: Schiestle M, 2015 AHC Biotherapeutics Workshop with modification by KIM JA*)

11. Quality Attributes vs Analytical Methods

Attributes	Potential effect	Examples of Analytical methods
4. Glycosylation		
Fucosylation Mannose X	- Afucosylated variants lead to higher ADCC in some cases - Mannose X variants lead to higher ADCC in some cases	Exoglycosidase digestion 2AB labelled-NP HPLC/UPLC and MS HILIC ESI-MS MALDI TOF-MS CE-SDS Peptide mapping (UPLC and MS) *N-linked Glycan : PNGaseF etc
High Mannose	- May increase serum clearance and impact on PK area under the curve (AUC) - Potentially immunogenic	
Galactosylation	- Higher galatosylation lead to higher CDC in some cases	
Galactose-α-1,3-galactose	- Potentially immunogenic (especially in Fab region : Type I hypersensitivity)	
Sialylation	- Impact on PK profile in some cases - Higher sialylation leads to lower ADCC - Sialylation in some Fc fusion protein (-cept) may impact on biological activity - N-glyxolylneuraminic acid(NGNA) form is potentially immunogenic	NP-HPLC Weak Anion Exchange Chromatography DMB labelled RP-HPLC and MS

(*ref: Schiestle M, 2015 AHC Biotherapeutics Workshop with modification by KIM JA*)

11. Quality Attributes vs Analytical Methods

Attributes	Potential effect	Examples of Analytical methods
5. Variants		
Size variants	- Aggregate form (and/or HMWS) may have less biological activity and also may be immunogenic - Fragments/Cleavage may have less biological activity - May impact on Stability profile	R/NR SDS-PAGE, CE-SDS SEC FFF MALLS DLS AUC Particle characterization (HIAC, MFI)
Charge variants	- Arise from PTM or incomplete processing of C-terminal Lys - Generally no impact on biological activity but some charge variants in critical region may influence on biological activity	Ion-exchange chromatorgraphy (CEX, IEX), Gel & Capillary electrophoresis (IEF, icIEF) *Using Carboxypeptidase B
Hydrophobicity	- Influenced from aggregation	RPC, HIC
6. Process impurity		
Host cell proteins	- Adjuvant effect or complex formation - May be immunogenic (may have an adverse impact upon Safety)	ELISA, 2-D electrophoresis, LC-MS
Host cell DNA	- May have an adverse impact upon Safety	Q-PCR

(ref: Schiestle M, 2015 AHC Biotherapeutics Workshop with modification by KIM JA)

11. Quality Attributes vs Analytical Methods

Attributes	Structural element	Examples of Analytical methods
7. Biological Function		
Binding to target	Fab	ELISA, SPR, FRET Cell-based binding assay
Programmed cell death, Neutralization assay	Fab	Cell-based apoptosis assay Reporter gene assay
Fc-effector function	Fc : FcγR binding	SPR, FRET, Alphascreen Cell-based binding assay
	Fc : C1q binding	SPR, ELISA
	Fab & Fc ; ADCC, CDC	Cell-based ADCC assay, Cell-based CDC assay
PK	Fc : FcRn binding	SPR, Alphascreen

(see also Appendix II, 'F. ADCC : Physiological system & Exaggerated system', 'G. CDC' and 'H. Allotype of Fc gamma Receptors')

(ref: Schiestle M, 2015 AHC Biotherapeutics Workshop with modification by KIM JA)

11. Quality Attributes vs Analytical Methods

Attributes	Structural element	Examples of Analytical methods
8. General properties		
Protein Content	- according to pharmaceutical design (strength)	UV 280, HPLC
Extinction coefficients	- An intrinsic property of the product - Not expected to have lot-to-lot variation	Amino acid analysis
Volume, appearance etc	According to pharmaceutical design (strength etc)	Volume in container

(*ref: Schiestle M, 2015 AHC Biotherapeutics Workshop with modification by KIM JA*)

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11. Quality Attributes vs Analytical Methods

☐ Forced Degradation Studies

Attributes	Potential effect	Examples of Analytical methods
High Temperature	- Denaturation, Aggregation, Fragmentation	CQA and/or Stability-indicating items
Light (Photostability)	- Denaturation, Aggregation, Fragmentation	CQA and/or Stability-indicating items
Low pH	- Denaturation, Aggregation, Fragmentation	CQA and/or Stability-indicating items
High pH	- Deamidation (Usually Lysine residues), etc.	CQA and/or Stability-indicating items, Peptide mapping with MS and MS/MS Sequencing (ID of deamidated residues)
H2O2	- Oxidation (Usually Methionine residues) - May influence on pharmacokinetics (Dependant on the region of oxidated sites)	CQA and/or Stability-indicating items, Peptide mapping with MS and MS/MS Sequencing (ID of oxidated residues)
Addition of metal ion catalysts (Fe2+ or Cu2+ etc.)	- May be relevant in formulations and manufacturing process, etc. - May lead to oxidation	CQA and/or Stability-indicating items

(*ref: Schiestle M, 2015 AHC Biotherapeutics Workshop with modification by KIM JA*)

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12. Analytical Comparability Assessment

□ Considerations for analytical comparability program

a) Cumulative knowledge of reference products on the market helps to understand range and variability of the innovator manufacturing process.

b) Comprehensive Analytical Comparability Studies

- **Extensive Characterization studies and Forced-degradation studies**

c) The rationale for the analytical similarity assessment should be clearly described.

- known quality attributes and performance characteristics of the RP (*US FDA, Guidance, Quality Considerations in Demonstrating Biosimilarity of a Therapeutic Protein Product to a Reference Product, 2015*)

d) Age of sample at the time of testing should be factored when comparing stability-indicating attributes.

e) **Analytical differences** should be characterized through orthogonal methods, and **should have no clinically meaningful impact on safety and efficacy** of biosimilars.

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12. Analytical Comparability Assessment

□ Requirements of the batch to be analyzed

a) Similarity assessments should be performed for **to-be-commercial batches of biosimilar**.

b) **predominately analyzed in Drug Product lots**, but certain parameters can be analyzed in Drug Substance lots (DS lots should be representative for DP lots appropriately).

- quality attributes specific to drug product : protein concentration, volume, sub-visible particles and stability/degradation products
- quality attributes specific to drug substance : glycosylation profile, ADCC, CDC etc.

c) Elements of the to-be-commercial include:

- Representative scale
- Same unit operations and same critical raw materials for non-clinical, clinical and commercial batches

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12. Analytical Comparability Assessment

□ **Extensive Characterization Studies**
(Structural, physicochemical, biological characterization studies)

a) Compared with Reference product
- **Side-by-side** characterization : minimize the interference of the interpretation of results
  ⇨ especially important for analytical methods which do not have high 'intermediate precision' or for assays where an internal standards should be tested simultaneously etc.
- **Independent data comparisons** from multiple assays **in a collective manner**
  ⇨ especially important for methods that have higher 'intermediate precision'

b) Use state-of-the-art/orthogonal techniques

c) **Evaluate the All (potential) MOAs**

(see also Appendix II, 'D. Example of Analytical Comparability Assessment')

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12. Analytical Comparability Assessment

□ **Forced Degradation Studies**

a) Under defined stressed conditions, **degradation/stability profile should be similar**.
(i.e. similar degradation pathway, no new degradants…)

b) It is important to set the various and appropriate degradation conditions and select analytical methods to monitor the CQA affected.

c) Consider ages of Biosimilar and Reference product.

(see also Appendix II, 'D. Example of Analytical Comparability Assessment')

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12. Analytical Comparability Assessment

☐ **Acceptance Similarity Criteria**

a) The acceptance similarity criteria and justifications should be provided.

b) **Quantitative ranges** should be based primarily on the measured quality attribute ranges of the reference product and **should not be wider than the range of the variability** of the representative reference product batches, **unless otherwise justified** .

- taking into account the number of reference medicinal product lots tested, the quality attribute investigated, the age of the batches at the time of testing and the test method used.(*ref: EMA, Guideline on similar biological medicinal products Guideline containing biotechnology-derived proteins as active substance: quality issues, 2014*)

c) Number of batches depends on assay and batch variability.

d) A **descriptive statistical approach** to establish ranges for quality attributes could be used, if **appropriately justified**. *(ref: EMA, Guideline on similar biological medicinal products Guideline containing biotechnology-derived proteins as active substance: quality issues, 2014*)

(see also Appendix II, 'E. Acceptance Similarity Criteria and Statistical Approaches')

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12. Analytical Comparability Assessment

☐ Possible statistical solutions

a) Pros and Cons

- Advantage: Provide a consistent decision-rule for all biosimilar submissions
- Disadvantage/challenge: Statistical equivalence test for analytical biosimilarity assessment is challenging due to limited sample sizes and lack of scientific knowledge of the equivalence margins.

b) Statistical approach used should be justified.

c) Example

- 2 or 3 standard deviation (mean±2SD or 3SD), Tolerance Interval, Equivalence testing
- 3-tiered approach *(US FDA's current thinking; ref: Tsong Y, DIA/FDA statistics Forum 2015 etc.)*

(see also Appendix II, 'E. Acceptance Similarity Criteria and Statistical Approaches')

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12. Analytical Comparability Assessment

☐ Quality attribute values which are outside or between the range(s) determined for a quality attribute of the reference medicinal product should be appropriately justified with regard to their potential impact on safety and efficacy.

- It should also be noted that there is no regulatory requirement for re-demonstration of biosimilarity once the Marketing Authorisation is granted.

(ref: EMA, Guideline on similar biological medicinal products containing biotechnology-derived proteins as active substance: quality issues, 2014)

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13. Assessment of Residual Uncertainty

☐ Not-similar results ('Not-identical', 'Different', 'No equivalent' etc.)

a) Need more data to demonstrate no effect on safety, purity, and potency.

b) Justifications of Differences

- additional studies (orthogonal methods, additional batches), relevant literatures etc.

c) The more comprehensive and robust data will reduce the degree of uncertainty.

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13. Assessment of Residual Uncertainty

☐ **Factors considered in the assessment of residual uncertainty** may include:

(*ref: Lemery SJ et al..; Biosimilars: Here and Now, Am Soc Clin Oncol Educ Book, 2016*)

a) which specific attributes were tested, understanding that is its necessary to assess for differences in any critical quality attributes;

b) the number of attributes tested (in a theoretical example, a more extensive characterization with a fingerprint-like analysis could reduce uncertainty);

c) the number of lots tested for both the proposed biosimilar product and the reference product; and

d) what differences, if any, were observed between products and what impact the differences could have on safety and efficacy

14. Analytical comparability and Potential impact on Extrapolation

☐ For extrapolation, the structural elements relevant to **immunogenicity** and to the **mechanism(s) of action** in the different indications are especially important .

(*ref: Supporting biosimilarity and extrapolation, GABI Journal, vol 4 (4), 2015*)

☐ Potential clinical impact of quality attributes

a) efficacy

b) pharmacokinetics

c) immunogenicity (which remains the main reason of clinical studies)

d) safety/toxicity : pharmacological toxicity (biological activities) & off-target toxicity (rare with biologicals since they are highly specific to their target)

☐ Extrapolation of data is already an established scientific and regulatory principle that has been exercised for many years, for example, in the case of major changes in the manufacturing process of originator biologicals.

(*ref: Weise M et al.., Biosimilars: the science of extrapolation, Blood 124, 3191-3196, 2014*)

☐ For more details of principles of the extrapolation of indications, refer to the Reflection Paper on Extrapolation of Indications in Authorization of Biosimilar Products.

(*ref: Reflection Paper of IRPF BWG, 2017*)

15. Summary

☐ **The similar-but-not-identical paradigm**

a) Microheterogeneity is not specific to biosimilars; it is a 'normal' feature of any biologicals. (*ref: Schneider CK, Biosimilars in rheumatology: the wind of change, Ann Rheum Dis 72 (3), 315-318, 2013*)

b) The resulting biosimilar and the reference product can technically not be entirely identical, because biosimilar developers have to establish their own independent manufacturing process . *(ref: Weise M, Biosimilars: the science of extrapolation, Blood 124, 3191-3196, 2014)*

☐ **Foundation vs supporting data to demonstrate Similarity**

a) Comparative analytical data provide the foundation for a biosimilar development program and can influence decisions about the type and amount of animal and clinical data needed to support a demonstration of biosimilarity.

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15. Summary

☐ **Understanding CQA**

a) Biosimilar should be highly similar to the reference product in all clinically relevant quality attributes, ie product attributes that may impact clinical performance. *(ref: WHO, Guidelines on Evaluation of Similar Biotherapeutic Products (SBPs), 2009)*

b) That means all critical quality attributes (i.e. those important for the function of the molecule) must be comparable. *(ref: EMA, Guideline on similar biological medicinal products containing biotechnology-derived proteins as active substance: quality issues, 2014)*

☐ **Analytical comparability assessment using state-of-the-art analytical tools**

a) Thorough characterization of both reference product and biosimilar should be carried out using appropriate, state-of-the-art biochemical, biophysical and biological analytical techniques. (*ref: WHO, Guidelines on Evaluation of Similar Biotherapeutic Products (SBPs), 2009*)

b) Meaningful assessment depends on the capabilities of available state-of-the-art analytical assays. *(ref: US FDA, Guidance, Quality Considerations in Demonstrating Biosimilarity of a Therapeutic Protein Product to a Reference Product, 2015)*

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15. Summary

☐ **Potential impact on extrapolation**

a) Thus, a biosimilar with highly similar structure, chemical, physical and biological attributes would be expected to produce the same pharmacology and thus highly similar safety and efficacy as the reference in every clinical indication.
(*ref: Gerrard TL etc., Biosimilars: extrapolation of clinical use to other indications, GABI Journal, 4(3), 2015*)

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IV. Appendix 1

- Additional Information

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IV. Appendix 1 : Additional Information

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A. Understanding Reference Product

☐ A single reference product should be used as the comparator throughout the comparability programme for quality, safety and efficacy studies. *(ref: EMA, Guideline on similar biological medicinal products, 2014)*

☐ The majority of the regulations necessarily require demonstration of similarity to a local reference product approved on that market.

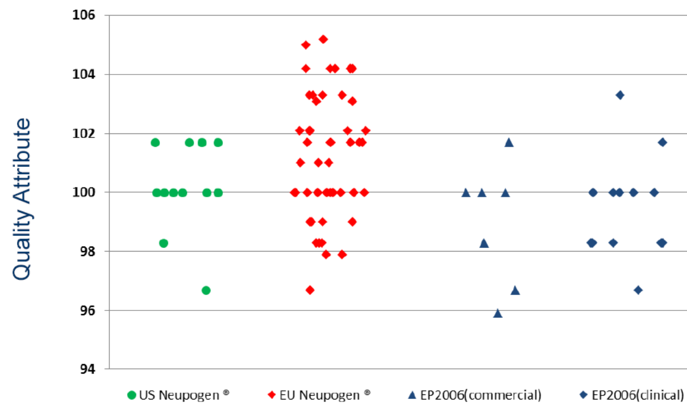
a) Possibility of geographic divergence in originator product quality attributes

- Geographical separation of the supply chain
  (e.g. Difference of manufacturing sites)
- License separation, and then independent development
- Sequential application of a manufacturing process change

A. Understanding Reference Product

✓ Regional variation?

- It may affect the adequacy of the quality range of selected Reference products and QTPP of biosimilar



Reference: Sandoz and FDA presentations for the January 7, 2015 Meeting of the Oncologic Advisory Committee

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A. Understanding Reference Product

☐ Shifts in quality profile of Reference Product

a) Such events could occur during the development of a biosimilar medicinal product and may result in a development according to a QTPP which is no longer fully representative of the reference medicinal product available on the market.
(*ref: EMA, Guideline on similar biological medicinal product—Quality issues, 2014*)

b) The ranges identified before and after the observed shift in quality profile could normally be used to support the biosimilar comparability exercise at the quality level, as either range is representative of the reference medicinal product.
(*ref: EMA, Guideline on similar biological medicinal product—Quality issues, 2014*)

c) Data from pre- and post- change batches should be clearly highlighted and separated in the dossier.

< Batch-to-Batch variation and Shift in quality profile to Reference Product !! >

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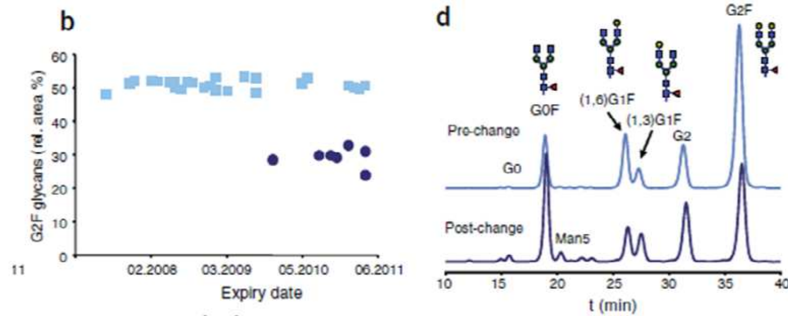
A. Understanding Reference Product

Example 1) Enbrel : Change in glycosylation profile

- Post change: N-glycan G2F decreased by almost 2 fold



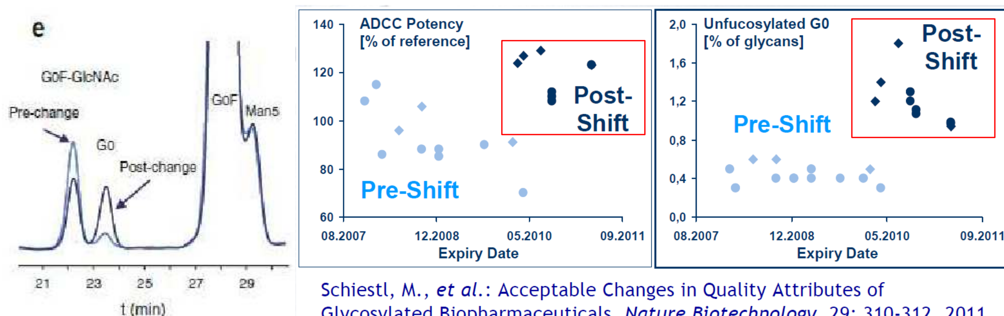
Comparison of the different pre- and post-change batches of Enbrel
b) Relative amount of the G2F glycan of the pre-change(n=25) and the post-Change (n=9) batches d) Exemplary glycan mapping chromatograms

Schiestl M et. al, : Acceptable Changes in Quality Attributes of Glycosylated Biopharmaceuticals, Nature Biotechnology 29, 310-312 (2011)

A. Understanding Reference Product

Example 2) Rituximab : Change in glycosylation profile and biological activity

- Post-change : Abundance of unfucosylated product increased by 3-fold ADCC response increased



Schiestl, M., *et al.*: Acceptable Changes in Quality Attributes of Glycosylated Biopharmaceuticals. *Nature Biotechnology*, 29: 310-312, 2011

A. Understanding Reference Product

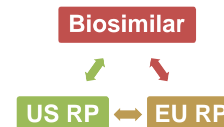
☐ The Use of a Foreign Reference Product

a) To facilitate global development, it is possible to use foreign reference product by demonstrating the equivalence of the local and foreign reference products.

b) Conditions for choice of reference product

⇨ Approval, sourcing, bridging study conditions by countries to countries

c) As a scientific matter, the type of bridging data needed will always include data from analytical studies (e.g., structural and functional data) that compare all three products (the proposed biosimilar, the EEA-authorised reference product and the non EEA-authorised comparator), and may also include data from clinical PK and/or PD bridging studies for all three products. (*EMA, Guideline on similar biological medicinal products, 2014*)



A. Understanding Reference Product

☐ The Use of a Foreign Reference Product

d) Issues that a sponsor may need to address to use a non-US-licensed comparator product in a biosimilar development program include, but are not limited to, the scientific bridge between the non-US-licensed comparator product and the US-licensed reference product, including comparative physicochemical characterization, biological assays/functional assays, degradation profiles under stressed conditions, and comparative clinical PK and, when appropriate, PD data, to address the impact of any differences in formulation or primary packaging on product performance. *(ref: US FDA, Guidance, Quality Considerations in Demonstrating Biosimilarity of a Therapeutic Protein Product to a Reference Product, 2015)*

A. Understanding Reference Product

✓ Example of CT-P13 (RemsimaⓇ/InflectraⓇ)

a) MFDS (Republic of Korea) had recommended :

- Demonstrate the Comprehensive analytical similarity between CT-P13, US-licensed Remicade and EU-approved Remicade.

⇒ CELLTRION : submission of a 3-way analytical bridging data

b) US FDA provided the following recommendations :

- Demonstrate PK similarity between CT-P13, US-licensed Remicade and EU-approved Remicade based on the following PK variables (AUCinf, Cmax and AUClast).
- Assess safety and immunogenicity in the setting of patients who undergo a single transition from EU-approved Remicade to CT-P13 to provide a descriptive comparison with patients who continue on EU-approved Remicade.

⇒ CELLTRION : submission of a 3-way analytical bridging data and a 3-way clinical PK Study

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B. Differences of Producing cell lines

* i.e. Difference of Host Cell Line and Expression System

☐ Allows for the Use of different expression system that provide similar quality attributes and have equal or better safety and efficacy profile.

☐ Can result in the various type and degree of PTM, which may impact on Potency and Immunogenicity.

a) Glycosylation patterns can vary significantly between different host cell types.

b) Especially non-human glycan types can generate immunogenic reactions.

✓ Two critical differences have been identified between humans and most other mammals: humans have lost the ability to biosynthesize both the terminal Gal•1-3Galb1-(3)4GlcNAc (alpha-Gal) epitope, and a major mammalian sialic acid, *N-glycolylneuraminic acid (Neu5Gc)*, structures that are widely present on non-human mammalian cells (Padler-Karavani and Varki, 2011)

✓ Normal humans have antibodies directed against these structures

c) Affect the types and levels of process/product-related substances and impurities.

☐ Therefore choice of expression system for biosimilar needs careful consideration including the impact on clinical effects of reference product.

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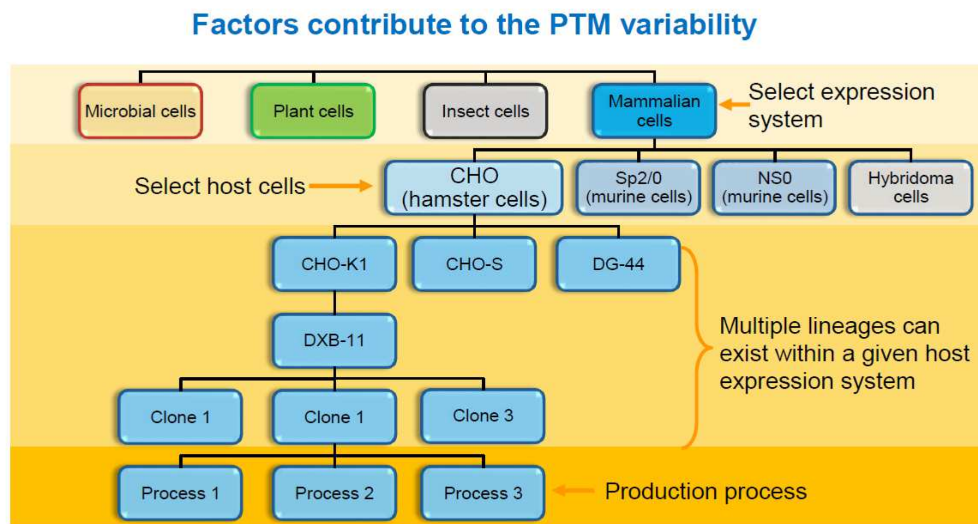
B. Differences of Producing cell lines



(Ref : Ramanan S, AHC Biotherapeutics Workshop 2015)

B. Differences of Producing cell lines

✓ Example (1) : Cetuximab (Erbitux®)

- Chimeric Mab expressed in Sp2/0 myeloma cells
- Murine cell lines express both Neu5Gc and alpha-Gal, similar to CHO and other mammalian cell lines, but at considerably higher levels. (*Muchmore EA et al, 1989*)
- Thus, therapeutic glycoproteins produced in murine cell line are more likely to be immunogenic.
- Both Neu5Gc and alpha-Gal have been described as part of an additional N-glycan in the Fab fragment of the Mab. (*Qian J et al, 2007*)
- The alpha-Gal epitope on Cetuximab has been shown to induce anaphylaxis in patients triggered by pre-existing anti-Gal IgE antibodies. (*Chung CH et al, 2008*)

B. Differences of Producing cell lines

✓ Example (2) : Reference product from Sp2/0 ⇨ Biosimilar from CHO

- Closely related systems but, CHO has even better safety track record.
- CHO cell and Sp2/0 can show the differences in C-terminal lysine variants and glycosylation pattern, but these differences have been reported that do not impact significantly on efficacy, safety and pharmacokinetics.
- C-terminal lysine : lower levels in the CHO *(Dick LW et al, 2008)*
  - ⇨The removal of the carboxy-terminal lysine from the heavy chains is routinely observed upon the characterization of monoclonal antibodies and is caused by intracellular enzymes.
  - ⇨From a regulatory aspect, this 'lysine clipping' is not regarded as critical under the condition that a potency assay is available that proofs the quality of the mAb. *(Bernhard A et al, 2007)*
- Murine cell lines express both Neu5Gc & alpha-Gal, similar to CHO and other mammalian cell lines, but at considerably higher levels. *(Muchmore EA et al, 1989)*
  - ⇨ Thus, therapeutic glycoproteins produced in murine cell line are more likely to be immunogenic.
- Murine cell lines show higher sialylation compared to CHO cells. *(Byrne B et al, 2007; Yoo EM et al, 2002)*
  - ⇨ May or may not impact on PK.

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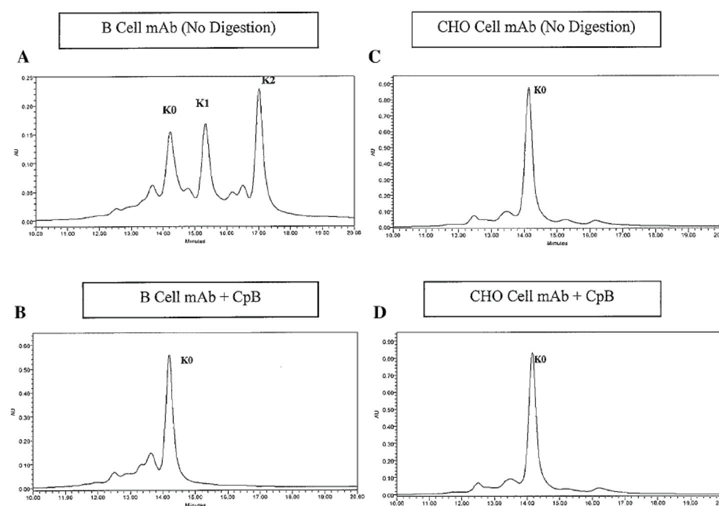
B. Differences of Producing cell lines

C-Terminal Lysine Variants in Fully Human Monoclonal Antibodies: Investigation of Test Methods and Possible Causes

(Dick LW et al, 2008)

*B cell : murine hybridoma cell line



Ion exchange chromatography of (A) B cell-produced antibody (B) B cell-produced antibody digested With CpB (C) CHO cell-produced antibody and (D) CHO cell-produced antibody digested with CpB

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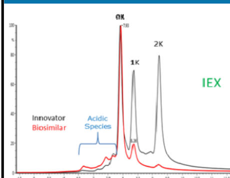
B. Differences of Producing cell lines

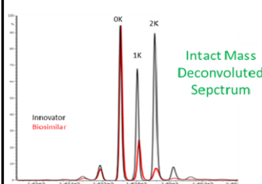
Deploying an integrated LC/UV/MS system for assessing structural comparability of innovator and biosimilar Infliximab

(Shion H et al, Waters Corporation, 2014 WCBP poster)

IEX Detects Charge Heterogeneity

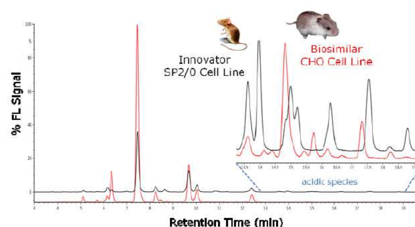


(A) Cation exchange chromatography comparison between an innovator batch and a biosimilar batch of IgG1 (Infliximab)

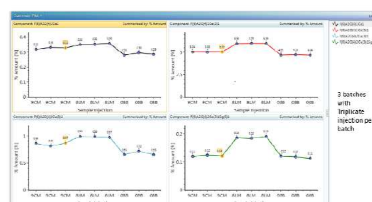


(B) Deconvoluted intact mass spectra comparison of deglycosylated innovator and biosimilar proteins. Lysine variants are labeled (0K, 1K, 2K)

Released N-Glycan Analysis



A. Overlay HILIC chromatograms of innovator and biosimilar batches



B. Alpha-Gal containing glycans in innovator batches

A. Overlay HILIC chromatogram with fluorescence detection (top) of released, 2-AB labeled N-glycans profiles that show significant differences due to the cell line selections. B. Four glycans from the innovator's batch have alpha-gal. The relative % of these glycans are illustrated in line chart for three batches (and with triplicate injections). The alpha-gal and NeuGc (not shown) were not observed in the Biosimilar candidate batches.

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C. Differences of Formulation

☐ Biosimilar product should be a pharmaceutically acceptable product and achieve the similarity to the Reference Product.

a) The formulation of the biosimilar does not need to be identical to that of the reference product, but Need to match Stability Profile.
- no new degradation species
- similar trend and levels of degradation species under the same conditions

b) Analyze the stability-indicating data and impurity data by considering the effects of similarity assessment.

✓ Regardless of the formulation selected, the suitability of the proposed formulation with regards to stability, compatibility (i.e. interaction with excipients, diluents and packaging materials), integrity, activity and strength of the active substance should be demonstrated. If a different formulation and/or container/closure system to the reference medicinal product is selected (including any material that is in contact with the medicinal product), its potential impact on the efficacy and safety of the biosimilar should be appropriately justified. *(ref: EMA, Guideline containing biotechnology-derived proteins as active substance: quality issues, 2014)*

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C. Differences of Formulation

□ Formulation differences can have huge effects on the stability profile and which types of new impurities form on stability.

a) Many types of non-enzymatic reactions occur spontaneously and generally the rates are affected by pH and temperature (deamidation, oxidation, glycation etc.).

b) Impurities to be created are often formulation dependant and can be affected by concentration or choice of excipients/surfactants (aggregate, fragment, unfolded proteins etc.).

□ To consider of the effects from the formulation differences, identify the types of tests or data that should be focused on to confirm similarity in stability.

a) The differences of purity/impurity measurements may be observed between Reference products and Biosimilar products.

b) Consider the appropriate conditions for comparative forced-degradation studies. (High Temperature, Light, Low/High pH, Oxidation by H_2O_2 and/or metal ions etc.)

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D. Example of Analytical Similarity Assessment

(Methods and Acceptance criteria)

(1) Primary Structure and Molecular Weight

Properties	Attribute	Test Method	No of Lots (RP/Similar)	Similarity Acceptance Criteria	Result	Conclusion	Method suitability
Primary Structure	Amino acid Composition	Hydrolysis and HPLC	3/3	within the variability of method	Similar	Acceptable	Cal (Side-by-side)
	Amino acid sequence	Peptide mapping by HPLC	3/6	Identical profile to the RBP	Identical	Acceptable	Cal (Side-by-side)
	Amino acid sequence	Amino acid sequencing by LC-ESI-MS/MS	3/6	Identical to the RBP	Identical	Acceptable	Cal (Side-by-side)
	N/C-terminal sequence	N/C-terminal sequencing by peptide mapping (LC-MS), Edman degradation	3/6	Identical to the RBP	Identical	Acceptable	Cal (Side-by-side)
	Molecular mass	SDS-PAGE, MALDI-TOF, ESI-QTOF-MS	3/6	within ± X%(ppm) of the predicted MW Or Identical to the RBP	Similar	Acceptable	Cal (Side-by-side)

* This example is virtual.
* Abbreviations in method suitability,
- Val : validation, Qual : Qualification, Cal : calibration

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D. Example of Analytical Similarity Assessment

(Methods and Acceptance criteria)

(2) Post-translational Modifications

Properties	Attribute	Test Method	No of Lots (RP/Similar)	Similarity Acceptance Criteria	Result	Conclusion	Method suitability
PTMs	N/C-terminal heterogeneity	LC-MS	10/6	within the variability of method	Not Similar -Difference of the level of C-term Lys - Only detected the specific N-term variant in Biosimilar	Orthogonal discussion (charge profile, functional testing etc), Additional batches, Literatures ⇒ No clinical impact ⇒ Acceptable	Cal (Side-by-side)
	Oxidation	LC-MS	10/6	Identical profile to the RBP	Not Similar -Difference of the level at some sites	Orthogonal discussion (Forced degradation studies, functional testing etc), Additional batches, Literatures ⇒ No clinical impact ⇒ Acceptable	Qual/Cal (Side-by-side)
	Deamidation	LC-MS	10/6	Identical to the RBP	Not Similar -Difference of the level at some sites	Orthogonal discussion (Forced degradation studies, functional testing etc), Additional batches, Literatures ⇒ No clinical impact ⇒ Acceptable	Qual/Cal (Side-by-side)
	Glycation	LC-MS	10/6	Identical to the RBP	Identical	Acceptable	Cal (Side-by-side)

D. Example of Analytical Similarity Assessment

(Methods and Acceptance criteria)

(3) Higher Order Structure

Properties	Attribute	Test Method	No of Lots (RP/Similar)	Similarity Acceptance Criteria	Result	Conclusion	Method suitability
Higher Order Structure	Secondary structure	UV absorption	3/3	Similar profile	Similar	Acceptable	Cal/SST (Side-by-side)
	Secondary/ Tertiary Structure	Far/Near-UV CD	3/3	Similar profile	Similar	Acceptable	Cal/SST (Side-by-side)
	Secondary structure	FT-IR	3/3	Similar profile	Similar	Acceptable	Cal/SST (Side-by-side)
	Secondary structure	DSC	3/3	Similar profile	Similar	Acceptable	Cal/SST (Side-by-side)
	Secondary structure	HDX	3/3	Similar profile	Similar	Acceptable	Cal/SST (Side-by-side)
	Disulfide linkage Structure	Peptide mapping/ LC-MS	3/6	Identical to the RBP	Identical	Acceptable	Cal (Side-by-side)
	Free thiol	Thiol assay kit	3/6	Similar to the RBP	Not similar	But, Very low level in Biosimilar and Reference product) (< 1 mol/mol) Acceptable	Qual/Cal (Side-by-side)

D. Example of Analytical Similarity Assessment

(Methods and Acceptance criteria)

(4) Glycosylation

Properties	Attribute	Test Method	No of Lots (RP/Similar)	Similarity Acceptance Criteria	Result	Conclusion	Method suitability
Glycosyl-ation	N-linked glycosylation site	LC-MS	3/6	Identical to the expected site	Identical	Acceptable	Cal (Side-by-side)
	N-glycan structure	HPLC-MS, UPLC-MS	30/10	T-sided TI, Or Mean±3SD	Identified, similar	Acceptable	Cal (Side-by-side)
	N-glycan profile (%Afucosylated/ %G0F/%G1F/%G2F/ %High Man)	HPLC (2-AB), HILIC	30/10	T-sided TI, Or Mean±3SD	NOT similar -different of relative areas of some glycan species	Orthogonal discussion (charge profile, functional testing etc), Additional batches, Literatures ⇒ No clinical impact ⇒ Acceptable	Val/Qual
	Sialic acid	HPLC (DMB), LC-MS	30/10	T-sided TI, Or Mean±3SD	NOT similar	Orthogonal discussion (charge profile, functional testing etc), Additional batches, Literatures ⇒ No clinical impact ⇒ Acceptable	Val/Qual

D. Example of Analytical Similarity Assessment

(Methods and Acceptance criteria)

(5) Charge Heterogeneity

Properties	Attribute	Test Method	No of Lots (RP/Similar)	Similarity Acceptance Criteria	Result	Conclusion	Method suitability
Charge Hetero-geneity	Charge isoforms	IEF	30/10	Similar pI range, Similar band pattern	NOT similar -difference of band pattern (basic or acidic shift etc)	Orthogonal discussion (peak ID, functional testing etc), Additional batches, Literatures ⇒ No clinical impact ⇒ Acceptable	Val
	Charge Profile (%acidic/ %main/ %basic)	IEX, icIEF (with CpB)	30/10	T-sided TI, Or Mean±3SD	NOT similar -difference of relative amount of charge variants	Orthogonal discussion (peak ID, functional testing etc), Additional batches, Literatures ⇒ No clinical impact ⇒ Acceptable	Val
	Charge Profile (%acidic/ %main/ %basic)	IEX, icIEF (without CpB)	30/10	T-sided TI, Or Mean±3SD	NOT similar -difference of relative amount of charge variants	Orthogonal discussion (peak ID, functional testing etc), Additional batches, Literatures ⇒ No clinical impact ⇒ Acceptable	Val

D. Example of Analytical Similarity Assessment

(Methods and Acceptance criteria)

(6) Size Heterogeneity

Properties	Attribute	Test Method	No of Lots (RP/Similar)	Similarity Acceptance Criteria	Result	Conclusion	Method suitability
Size Hetero-geneity	Size distribution (%main/%HMWS/%LMWS)	HPLC(SEC)	30/10	1 Similar profile 2 T-sided TI, Or Mean±3SD	NOT similar -difference of relative amount of minor isoforms	Orthogonal discussion (peak ID, functional testing, stability profile etc), Additional batches, Literatures ⇒ No clinical impact ⇒ Acceptable	Val
	Size distribution (%main/%HMWS/%LMWS)	CE-SDS(R/NR), SDS-PAGE(R/NR)	30/10	1 Similar profile 2 T-sided TI, Or Mean±3SD	NOT similar -difference of relative amount of minor isoforms	Orthogonal discussion (peak ID, functional testing, stability profile etc), Additional batches, Literatures ⇒ No clinical impact ⇒ Acceptable	Val
	HMWS profile	SV-AUC	6/6	1. Similar profile 2. Report results	similar (All dimer)	Acceptable	Qual/SST (Side-by-side)
	HMWS profile	SEC-MALS	6/6	1. Similar profile 2. Report results	similar (All dimer)	Acceptable	Qual/SST (Side-by-side)

D. Example of Analytical Similarity Assessment

(Methods and Acceptance criteria)

(7) Additional Physico-chemical characteristics

Properties	Attribute	Test Method	No of Lots (RP/Similar)	Similarity Acceptance Criteria	Result	Conclusion	Method suitability
Biophysical analysis	Determination of extinction coefficient	Amino acid analysis	6/6	N/A (Similar estimated values)	Similar	Acceptable	Qual/SST (Side-by-side)
	Sub-visible particles	MFI	30/10	N/A	Similar	Acceptable	Val/SST (Side-by-side)
	Protein concentration	UV/VIS at A280	30/10	T-sided TI, Or Mean±3SD	Similar	Acceptable	Val/SST

Analytical Methods for Characterizing & Quantifying Aggregates and Particles

Courtesy of John Carpenter, Univ Colorado

Biopharma Excellence

D. Example of Analytical Similarity Assessment

(Methods and Acceptance criteria)

(8) Biological activities : Fab-related

Properties	Attribute	Test Method	No of Lots (RP/Similar)	Similarity Acceptance Criteria	Result	Conclusion	Method suitability
Biological activity (Fab -related)	Target binding (soluble target)	SPR, ELISA, FRET, Alpha screen	30/10	T-sided TI, Or Mean±3SD	Similar	Acceptable	Qual/Cal (Side-by-side)
	Target binding (membrane-bound)	FACS, Cell based ELISA	30/10	T-sided TI, Or Mean±3SD	Similar	Acceptable	Qual/Cal (Side-by-side)
	Potency assay	Neutralization assay etc.	30/10	T-sided TI, Or Mean±3SD	Similar	Acceptable	Qual/Cal (Side-by-side)

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D. Example of Analytical Similarity Assessment

(Methods and Acceptance criteria)

(9) Biological activities : Fc-related

Properties	Attribute	Test Method	No of Lots (RP/Similar)	Similarity Acceptance Criteria	Result	Conclusion	Method suitability
Biological Activity (Fc-Related)	FcrR binding	ELISA, SPR, Alpha screen	30/10	T-sided TI, Or Mean±3SD	Similar	Acceptable	Qualification (Side-by-side)
	ADCC	ADCC assay -PBMC assay -modified NK cell assay -Reporter gene assay	30/10	T-sided TI, Or Mean±3SD	Similar	Acceptable	Qual/Cal (Side-by-side)
	C1q binding	ELISA	30/10	T-sided TI, Or Mean±3SD	Similar	Acceptable	Qual/Cal (Side-by-side)
	CDC	CDC assay	30/10	T-sided TI, Or Mean±3SD	Similar	Acceptable	Qual/Cal (Side-by-side)
	FcRn binding	ELISA, SPR	30/10	T-sided TI, Or Mean±3SD	Similar	Acceptable	Qual/Cal (Side-by-side)

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D. Example of Analytical Similarity Assessment

(Methods and Acceptance criteria)

(10) Evaluation of the Comparability of Stability Profiles
(including Forced Degradation Studies)

Attribute	Test Method	No of Lots (RP/Similar)	Similarity Acceptance Criteria	Result	Conclusion
Stability Profile	real-time/real-temperature (5±3℃)	3:3	No new degradants, Similar stability profile	Similar	Acceptable
	accelerated (25±2℃/60±5%RH)	3:3	No new degradants, Similar stability profile	Similar	Acceptable
	stress conditions (40±2℃/75±5%RH)	3:3	No new degradants, Similar stability profile	Similar	Acceptable
Forced Degradation Studies	Photo-stability	1:1	No new degradants, Similar degration profile	Similar	Acceptable
	Low pH	1:1	No new degradants, Similar degradation profile	Similar	Acceptable
	High pH	1:1	No new degradants, Similar degradation profile	Similar	Acceptable
	Oxidation (H2O2 etc)	1:1	No new degradants, Similar degradation profile	Similar	Acceptable

E. Acceptance Criteria and Statistical approaches

☐ **Qualitative comparison** by side-by-side manner

a) amino acid sequence, S-S linkage, peptide mapping profile, IEF profile etc.

☐ **Quantitative ranges** should be established for the biosimilar comparability exercise, where possible. *(ref: EMA, Guideline containing biotechnology-derived proteins as active substance: quality issues, 2014*)

a) should be based on data from testing of a sufficient number of Reference product batches.

b) using statistical approach (The statistical approach used should be justified.)

- Advantage: Provide a consistent decision-rule for all biosimilar submissions.
- Disadvantage/challenge: Statistical equivalence test for analytical biosimilarity assessment is challenging due to limited sample sizes and lack of scientific knowledge of the equivalence margins.

☐ The Number of biosimilar batches for Comparability

a) The higher the number, the better the analysis.

b) Be considered to achieve appropriate confidence interval.

E. Acceptance Similarity Criteria and Statistical approaches

☐ Examples of Quantitative Range

1. Min-Max : range defined by the minimum value & maximum value of the reference product lot measurements

2. Mean±X Standard Deviation (±2SD or ±3SD etc.)

a) based on the Reference lots
b) Usually easy to apply and be consistent with quality control principle.
c) Should consider the method variability.
d) If data are normally distributed then simply a number of coverage intervals may be expressed as follows. *(NIST/SEMATECHe-Handbook)*
- ±1SD interval around the mean has coverage of 67% of total data
- ±2SD interval around the mean has coverage about 95% of total data
- ±3SD interval around the mean has coverage of about 99.7% of total data
- These percent coverages are true only when population mean and SD are known

e) If sample size is big enough, ±3SD and TI with 99.7 % coverage are close to each other.

E. Acceptance Similarity Criteria and Statistical approaches

3. Tolerance Interval

a) An interval that contains a certain proportion of the data population with a specified degree of confidence.
- At least a certain proportion (p) of the population falls with a given level of confidence (1–α).

b) If the data normally distributed, the two-sided TI can be determined by using the following equation. (*Howe, 1969*):
- Mean±k·s

$$k = Z_{(1-p)/2}\sqrt{\frac{N-1}{\chi^2_{(\gamma,N-1)}}}\sqrt{1+\frac{1}{N}}$$

s : standard deviation
k : multiplier to adjust the width of the interval defined
N : sample size that was used to estimate the mean and SD)
$\chi^2_{(\gamma,N-1)}$: the critical value of the chi-square distribution with degrees of freedom N-1 that is exceeded with probability *r*
$Z_{(1-p)/2}$: the critical value of the normal distribution which is exceeded with probability (1-p)/2

c) The width of the TI is dependent upon the sample size, confidence level, and coverage level.
d) Considering the uncertainty associated with such a small data set, TI with an appropriate confidence level would be the recommended statistical method.
e) example : Two-sided/One-sided TI with 95 % confidence level/95 % population (95/95 TI), N=35
- Two-sided (*Howe, 1969*) : k = ± 2490
- One- sided (*Hatrella, 1963*) : k = ± 2157

E. Acceptance Similarity Criteria and Statistical approaches

4. Equivalence Testing

a) If Inferential statistics is used, testing for equivalence should generally be applied.

b) Equivalence margin is determined prior to experimentation using confidence level and power.

c) Equivalence margin interrelates strongly to sample sizes, allowable difference, significance level and power.

d) Assess if the mean difference (and confidence interval on the mean difference) is within acceptable margin.

- Confidence interval is within the similarity limit => equivalent
- Two-sided test : upper limit and lower limit
- One-sided test could be acceptable for certain QAs (eg impurities)

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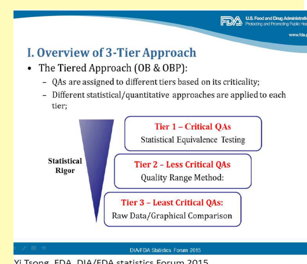
E. Acceptance Similarity Criteria and Statistical approaches

✓ **US FDA's current thinking considering statistical approach to support the decision of similarity** *(see also Section III Inflectra Case)*

1. Risk based approach for quality review (e.g. tier approach)
   a) 1st step : Evaluate the criticality of quality attributes,
      - considering impact on clinical performance and degree of uncertainty in impact
   b) 2nd step : Assign quality attributes to different tiers based on their criticality
      - Risk ranking should take into account probability and severity impact (on efficacy, safety & immunogenicity) as well as the uncertainty associated with the evidence for the impact.

2. 3-tiered approach
   a) Tier 1 (Critical QAs) : equivalence test
      - Analytically similar
        if 90% confidence interval of the true mean difference is within equivalence margins ($\delta 1$, $\delta 2$)
      - increasing sample size : minimum of 6 lots
        (10 or more required to achieve appropriate power levels)
   b) Tier 2 (Less Critical QAs) : Quality range +/-X SD
   c) Tier 3 (Least Critical Qas) : Data/Graphical comparison



Yi Tsong, FDA, DIA/FDA statistics Forum 2015

3. Inflectra and Zarxio : Equivalence margin = 1.5SD ($\delta = 1.5\sigma R$)

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F. ADCC : Physiological system & Exaggerated system

☐ Principles of ADCC assay

a) NK cells recognize their target cells via FcγRIIIa (CD16) that bind to antibody bound to the surface of the target cells.

b) Binding of NK cells to their target cells induces the release of preformed cytotoxic mediators by granule exocytosis.

c) The lysis of the target cells is extracellular, requires direct cell-to-cell contact, and does not involve complement.

☐ Factors impacting Sensitivity and its Relevance to physiological conditions

a) Target cells : expression of different levels of target ligand

b) Effector cells : PBMCs from healthy donor/patient,
Isolated primary NK cells from healthy donor/patient

c) Different E/T ratios

d) Presence or absence of autologous serum

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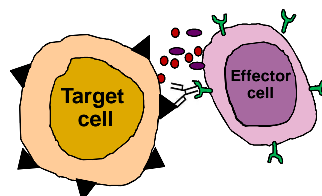
F. ADCC : Physiological system & Exaggerated system

☐ Systems

a) Classical methods (primary cell-based assays)
- Effector cells : PBMCs from healthy donor/patient,
Isolated primary NK cells from healthy donor/patient
- End-point methods : 51Cr measurement, LDH release assay, FACS
- Drawbacks : Requirement of fresh blood from donors, highly variable as a result of donor differences and the requirement for cell culture and expansion

b) ADCC-Reporter gene assay
- Effector cells : Engineered Jurkat cell (overexpression of human FcγRIIIa) and NFAT-luciferase reporter gene
- End-point methods : Luciferase expression (ie equivalent to classic LDH release assay)



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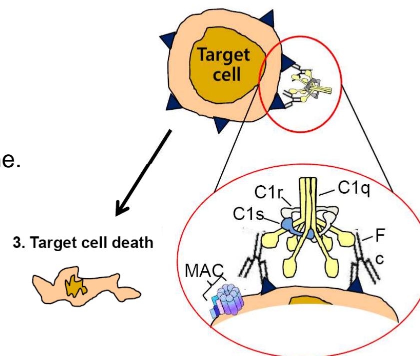
G. CDC

☐ Principles

a) The therapeutic antibody is diluted in the complement matrix and added to a target cell line.

b) Antibody bound to the target cell surface fixes complement resulting in the assembly of the membrane attack complex and finally in the perforation of target cell membrane.

c) Cells are lysed.



☐ Methods

a) FACS

- 7-Amino-actinomycin D (7-AAD) intercalates into double-stranded nucleic acids; it is excluded by viable cells but can penetrate cell membranes of dying or dead cells;
- a flow cytometer can be used to measure the dose-dependent complement-derived cytotoxicity

b) ATP measurement (Cell Titer-Glo®Luminescent Cell Viability Assay)

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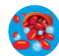
H. Allotype of Fc gamma Receptors

✓ In the clinic the FcgRIII 158V/F polymorphism interfere with the ability to generate ADCC responses in vitro during trastuzumab/rituximab treatment
(Not all studies show a significant correlation between FcrR variants and clinical responses)

Mellor *et al. Journal of Hematology & Oncology* 2013, **6**:1
http://www.jhoonline.org/content/6/1/1



JOURNAL OF HEMATOLOGY & ONCOLOGY

REVIEW **Open Access**

A critical review of the role of Fc gamma receptor polymorphisms in the response to monoclonal antibodies in cancer

James D Mellor[1,2,3,8*], Michael P Brown[4], Helen R Irving[2], John R Zalcberg[5,6] and Alexander Dobrovic[3,6,7]

Abstract

Antibody-dependent cellular cytotoxicity (ADCC) is a major mechanism of action of therapeutic monoclonal antibodies (mAbs) such as cetuximab, rituximab and trastuzumab. Fc gamma receptors (FcgR) on human white blood cells are an integral part of the ADCC pathway. Differential response to therapeutic mAbs has been reported to correlate with specific polymorphisms in two of these genes: *FCGR2A* (H131R) and *FCGR3A* (V158F). These polymorphisms are associated with differential affinity of the receptors for mAbs. This review critically examines the current evidence for genotyping the corresponding single nucleotide polymorphisms (SNPs) to predict response to mAbs in patients with cancer.

Keywords: FCGR2A, FCGR3A, trastuzumab, rituximab, cetuximab, ADCC

In a pre-clinical study, trastuzumab-mediated ADCC of autologous peripheral blood mononuclear cells (PBMNCs) was measured by a chromium-51 release assay using a *HER2*-positive human breast cancer cell line (MDA-MB-361) as a target. The ADCC analysis showed that PBMNCs of *FCGR2A* H/H and/or *FCGR3A* V/V genotypes caused significantly higher trastuzumab mediated cytotoxicity than PBMNCs of other genotypes [2].

A retrospective, non-randomised study of trastuzumab in 54 patients with HER2-positive metastatic breast cancer found a significant difference in the objective response rate depending on the *FCGR2A* and *FCGR3A* genotypes [2]. Patients were treated with trastuzumab

The largest study to date examining the effects of FcgR polymorphisms on the response to trastuzumab is that of Hurvitz et al. [23]. The patients were part of the Breast Cancer International Research Group (BCIRG)-006 study of patients receiving adjuvant trastuzumab with chemotherapy for HER2-positive early stage breast cancer. BCIRG-006 was a randomized clinical trial in which two trastuzumab containing experimental arms (both using the same dose of trastuzumab – 8 mg/kg loading does followed by 6 mg/kg every 3 weeks for a total of 12 months) were compared to a non-trastuzumab control arm. Germline DNA from 1218 patients and 1189 patients was genotyped for the H131R and V158F SNPs, respectively. There was no statistically significant difference in disease free survival (DFS) based on FcgR genotypes (*FCGR2A* H/H vs H/R vs R/R, log rank test, $p = 0.81$, and *FCGR3A* V/V vs V/F vs F/F, log rank test, $p = 0.33$). *Interestingly*, in the trastuzumab arms, there was no statistically significant difference in DFS by *FCGR2A* ($p = 0.76$) or *FCGR3A* ($p = 0.98$) genotype. Furthermore, when a sub-

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H. Allotype of Fc gamma Receptors

FcγRIIIa-158V/F Polymorphism Influences the Binding of IgG by Natural Killer Cell FcγRIIIa, Independently of the FcγRIIIa-48L/R/H Phenotype

By Harry R. Koene, Marion Kleijer, Johan Algra, Dirk Roos, Albert E.G.Kr. von dem Borne, and Masja de Haas

We analyzed a genetic polymorphism of Fcγ receptor IIIa (CD16) that is present on position 158 (Phe or Val) in the membrane-proximal, IgG-binding domain. With a polymerase chain reaction–based allele-specific restriction analysis assay we genotyped 87 donors and found gene frequencies of 0.57 and 0.43 for *FcγRIIIA-158F* and *−158V,* respectively. A clear linkage was observed between the *FcγRIIIA-158F* and *−48L* genotypes on the one hand and the *FcγRIIIA-158V* and *−48H* or *−48R* genotypes on the other hand (χ^2 test; $P <$ .001). To determine the functional consequences of this FcγRIIIa-158V/F polymorphism, we performed IgG binding experiments with natural killer (NK) cells from genotyped donors. All donors were also typed for the recently described triallelic FcγRIIIa-48L/R/H polymorphism. NK cells were treated with lactic acid to remove cell-associated IgG. FcγRIIIa^{NK}-158F bound significantly less IgG1, IgG3, and IgG4 than did FcγRIIIa^{NK}-158V, irrespective of the FcγRIIIa-48 phenotype. Moreover, freshly isolated NK cells from FcγRIIIa-158VV individuals carried significantly more cytophilic IgG than did NK cells from FcγRIIIa-158FF individuals. In addition, CD16 monoclonal antibody (MoAb) MEM154 bound more strongly to FcγRIIIa-158V, compared with -158F, again independently of the FcγRIIIa-48 phenotype. The binding of MoAb B73.1 was not influenced by the FcγRIIIa-158V/F polymorphism, but proved to depend solely on the amino acid present at position 48 of FcγRIIIa. In conclusion, the previously reported differences in IgG binding among the three FcγRIIIa-48L/R/H isoforms are a consequence of the linked, biallelic FcγRIIIa-158V/F polymorphism at amino-acid position 158.

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H. Allotype of Fc gamma Receptors

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ORIGINAL PAPER

FCGR3A-158 polymorphism influences the biological response to infliximab in Crohn's disease through affecting the ADCC activity

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Abstract An association between *FCGR3A*-158 V/F polymorphism and biological responses to infliximab has been reported in Crohn's disease (CD) in Western countries. However, little is known about the mechanism by which gene polymorphism affects the responses to infliximab. The aims of this study were to confirm the association in Japanese CD patients and to reveal the effect of gene polymorphism on biological responses to infliximab. Japanese CD patients were examined retrospectively at weeks 8 and 30. Clinical and biological responses were assessed by the Crohn's disease activity index and C-reactive protein levels, respectively. The infliximab-binding affinity of natural killer (NK) cells from *FCGR3A*-158 V/V, V/F and F/F donors was examined. Infliximab-mediated antibody-dependent cell-mediated cytotoxicity (ADCC) activities were also determined using transmembrane TNF-α-expressing Jurkat T cells as target cells and peripheral blood mononuclear cells (PBMCs) from V/V, V/F and F/F donors as effector cells. Biological responses at week 8 were statistically higher in V/V patients, whereas no significant differences were observed in either clinical responses at weeks 8 and 30 or biological responses at week 30 among the three genotypes. NK cells and PBMCs from V/V patients also showed higher infliximab-binding affinity and infliximab-mediated ADCC activity, respectively. Our results suggest that *FCGR3A*-158 polymorphism is a predicting factor of biological responses to infliximab in the early phases. *FCGR3A*-158 polymorphism was also found to affect the infliximab-binding affinity of NK cells and infliximab-mediated ADCC activity in vitro, suggesting that an effect on ADCC activity influences biological responses to infliximab in CD patients.

Keywords Infliximab · Crohn's disease · *FCGR3A* · Polymorphism · ADCC

Polymorphism in IgG Fc receptor gene *FCGR3A* and response to infliximab in Crohn's disease: a subanalysis of the ACCENT I study

Edouard J. Louis[a], Hervé E. Watier[b], Stefan Schreiber[d,e], Jochen Hampe[d,*], François Taillard[f], Allan Olson[g], Nicole Thorne[g], Hongyan Zhang[g] and Jean-Frédéric Colombel[c]

Recently, it has been shown that *FCGR3A*-158 gene polymorphism is associated with biological and possibly clinical response to infliximab in Crohn's disease. We further assessed this association in a subset of 344 patients from the large and well-defined cohort of 573 patients with Crohn's disease from the ACCENT I study. No association could be observed between *FCGR3A*-158 gene polymorphism and the clinical response to infliximab, which was primarily defined as a decrease of ≥ 70 points in the Crohn's disease activity index or clinical remission (Crohn's disease activity index <150). We did, however, confirm a trend towards a greater decrease in C-reactive protein after infliximab in V/V homozygotes as compared with V/F heterozygotes and F/F homozygotes (−79.4, −76.5, and −64.3%, respectively, at week 6; $P = 0.085$; one-tailed $P = 0.043$). This finding has no immediate clinical impact but may enhance the understanding of the complex mechanisms of action of anti-tumor necrosis factor agents in Crohn's disease. *Pharmacogenetics and Genomics* 16:911–914 © 2006 Lippincott Williams & Wilkins.

Pharmacogenetics and Genomics 2006, **16**:911–914

Keywords: C-reactive protein, Crohn's disease, IgG Fc receptor, infliximab, polymorphism, tumor necrosis factor

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Conflicts of interest: S. Schreiber is a speaker for Essex Pharma GmbH, and a consultant for Centocor and Schering-Plough. E. Louis is a senior research associate at the FNRS, Belgium. F. Taillard, A. Olson, N. Thorne, and Z. Hogyan are employees of Centocor, Inc.

Received 11 May 2006 **Accepted** 6 July 2006

I. Relevant Guidelines

☐ World Health Organization (WHO)

- Guidelines on evaluation of similar biotherapeutic products (2009)
  (http://www.who.int/biologicals/publications/trs/areas/biological_therapeutics/TRS_977_Annex_2.pdf?ua=1)
- Guidelines on the quality, safety, and efficacy of biotherapeutic protein products prepared by recombinant DNA technology (2013)
  (http://www.who.int/biologicals/biotherapeutics/TRS_987_Annex4.pdf?ua=1)
- Guidelines on evaluation of monoclonal antibodies as similar biotherapeutic products (SBPs) (2016)
  (http://who.int/biologicals/expert_committee/mAb_SBP_GL-ECBS_review_adoption-2016.10.26-11.7post_ECBS-Clean_Version.pdf?ua=1)

☐ European Medicines Agency (EMA)

- Guideline on Similar Biological Medicinal Products (2014)
  (http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2014/10/WC500176768.pdf)
- Guideline on similar biological medicinal products containing biotechnology-derived proteins as active substance: quality issues (2014)
  (http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2014/06/WC500167838.pdf)

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I. Relevant Guidelines

☐ Food and Drug Administration (US FDA)

- Scientific Considerations in Demonstrating Biosimilarity to a Reference Product (2015)
  (http://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm291128.pdf)
- Quality Considerations in Demonstrating Biosimilarity of a Therapeutic Protein Product to a Reference Product (2015)
  (http://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm291134.pdf)

☐ Health Canada (HC)

- Information and Submission Requirements for Subsequent Entry Biologics (2010)
  : Under revision

☐ Pharmaceuticals and Medical Devices Agency (PMDA)

- Guideline for the Quality, Safety and Efficacy Assurance of follow-on biologics (2013)
  (https://www.pmda.go.jp/files/000153851.pdf)

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I. Relevant Guidelines

☐ Ministry of Food and Drug Safety (MFDS, Republic of Korea)

- Guidelines on the Evaluation of Biosimilar Products (2014)

(http://www.mfds.go.kr/eng/eng/download.do?boardCode=17840&boardSeq=70199&fileSeq=1)

☐ Swissmedic

- Authorization of similar biological products(biosimilars) (2014)

(https://www.swissmedic.ch/ZL101_00_002e_VV)

J. List of Abbreviations

ADCC	Antibody Dependent Cell mediated Cytotoxicity
ADCP	Antibody Dependent Cell mediated Phagocytosys
BWG	Biosimilars Working Group
C dossier	Clinical dossier
CASSS Forum	Californian Separation Science Society Forum
CDC	Complement dependent Cytotoxicity
CMC	Chemistry, Manufacturing and Controls
CQAs	Critial Quality Attributes
DS	Drug Substance
EEA	European Economic Area
IBD	Inflammatory Bowel Disease
IPRF	International Pharmaceutical Regulators Forum
MOA	Mode of Action
NC dossier	Non-clinical dossier
NRA	National Regulatory Authority
PD	Pharmacodynamics
PK	Pharmacokinetics
PMS	Post Marketing Surveilance
PTM	Post Tnaslational modification
Q dossier	Quality dossier
QTPP	Quality Target Product Profile
RP	Reference Product
SD	Standard Deviation
WCBP symposium	Well Characterized Biotechnology Pharmaceuticlas symposium

V. Appendix 2

- Case Study : Remsima/Inflectra (Celltrion Inc.)

- ✓ **Focused on introducing the content of the public information, FDA Review report of the CT-P13 (Inflectra/Remsima), an approved biosimilar to Remicade® (infliximab) by FDA.**
- ✓ **Any additional review comments are not included.**
- ✓ **It is just a case and it can not be concluded that this case is a standard data set for a monoclonal antibodies as a biosimilar.**

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1. What is Remsima/Inflectra?

1.1. What is Remsima/Inflectra (CT-P13) ?

☐ Remsima/Inflectra (code name CT-P13) is a biosimilar to Remicade

☐ Date of Authorization : EU (10/09/2013), US (2016)

☐ Developer/Manufacturer : Celltrion, Inc.

☐ CT-P13 is currently licensed in 67 countries, including countries in the EU, Canada, Japan, and South Korea

1. 2. Physicochemical & Functional characteristics of CT-P13

☐ Active substance : inflximab (chimeric mAb against TNF-alpha, IgG1)

☐ Dosage form : Liquid, lyophilized powder, stored in 2~8℃
<must be same by regulatory requirement>

☐ Route of administration : IV infusion
<must be same by regulatory requirement>

☐ Indications : Same to US-licensed Remicade

- Mechanisms of Action : binding and neutralization of soluble and transmembrane Tumor-Necrosis Factor Alpha (sTNFα and tmTNFα) <must be same by regulatory requirement>

☐ Strength : 100 mg/vial <Difference is acceptable, But Same>

☐ Expression system: Sp2/0 <Difference is acceptable, But Same>

☐ Formulation : includes the same inactive ingredients as US-licensed Remicade
<Difference is acceptable, But Same>

☐ Container/Closure system : Type I Glass Vial <Difference is acceptable, But Same>

1 .2. Physicochemical & Functional characteristics of CT-P13

Table 1. CT-P13 vs Remicade: Summary of Strength, Formulation, Presentation and Container Closure System

Component	Function	Reconstituted CT-P13	Reconstituted US Remicade	Reconstituted EU Remicade
Infliximab	Active pharmaceutical ingredient	10 mg/mL	10 mg/mL	10 mg/mL
Sucrose	Stabilizing agent	50 mg/mL	50 mg/mL	50 mg/mL
Monobasic sodium phosphate monohydrate	Buffering agent	0.22 mg/mL	0.22 mg/mL	0.22 mg/mL
Dibasic sodium phosphate dihydrate	Buffering agent	0.61 mg/mL	0.61 mg/mL	0.61 mg/mL
Polysorbate 80	Surfactant	0.05 mg/mL	0.05 mg/mL	0.05 mg/mL
Container closure	Container closure	20 mL Type I glass vial, butyl rubber stopper	20 mL Type I glass vial, butyl rubber stopper	20 mL Type I glass vial, butyl rubber stopper

2. The Strategy of Analytical Comparability Assessments

2.1. Summary

□ Executive summary : Extensive analytical data intended to support,

a) a demonstration that CT-P13 and US-licensed Remicade are highly similar,

b) a justification of the relevance of comparative data generated using the EU-approved Remicade to support a demonstration of the biosimilarity of CT-P13 to US-licensed Remicade,

c) a demonstration that CT-P13 can be manufactured in a well-controlled and consistent manner, leading to a product that is sufficient to meet required quality standards

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2.1. Summary

□ The results of these comparisons,

a) 3 products met the pre-specified criteria :

- analytical similarity, statistical criteria for the critical potency bioassay(TNF-α neutralization), TNF-α binding strength

  ⇨ a pair-wise analytical comparison of CT-P13 to US Remicade is consistent with the conclusion that CT-P13 is highly similar to the reference product (US Remicade)

b) Adequate analytical bridge between EU Remicade, US Remicade, and CT-P13

- to justify the relevance of the comparative data generated using EU Remicade to support a demonstration of the biosimilarity of CT-P13 to US-Remicade

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2.2. Assessment Program

(Determining analytical similarity of CT-P13 to US-licensed Remicade)

□ Analytical Similarity assessments

a) Comparative Structural and Physicochemical Similarity assessment

b) Functional and Biological Similarity assessment

□ Residual uncertainties arising from physicochemical and structural studies

a) Little or no impact on biological activities and the absence of clinical impact is supported by clinical studies

□ Consideration of Analytical bridge between CT-P13, US Remicade, and EU Remicade

a) CT-P13 has already been licensed from EMA

b) Development of CT-P13 was conducted entirely outside of the US

c) Directed towards meeting the product approval requirements of non-US regulatory agencies

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2.2. Assessment Program

(Determining analytical similarity of CT-P13 to US-licensed Remicade)

□ Analytical Similarity Data package : 2 sets, each side-by-side testing

a) 2-way analytic similarity assessment (CT-P13 and EU Remicade)

b) 3-way analytic similarity assessment of physicochemical similarity

- Data from side-by-side testing of the 3 products using the same method but conducted at different times were combined for statistical analysis

□ Numbers of analyzed lots (All lots were within the expiry date at the time of testing)

a) 3~26 lots of CT-P13, 3~30 lots of EU Remicade, 3~36 lots of US Remicade were assessed in 3-way biosimilarity studies

b) considered to reflect a range of expiration dates and product ages

- CT-P13 lots : manufactured between Feb 2012 and May 2015 (included testing after 9~21 months storage)
- US Remicade : Expiration dates were between Feb 2015 and Feb 2018 (included testing after 2~29 months storage)
- EU Remicade : Expiration dates were between Mar 2013 and Feb 2018 (included testing after 4~36 months storage)

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2.3. Analytical Techniques

Table 2. Quality Attributes and Methods Used to Evaluate Analytical Similarity of CT-P13, US-licensed Remicade, and EU-approved Remicade

Quality Attribute	Methods
Primary structure	• Peptide mapping with ultraviolet (UV) and mass spectrometry (MS) detection • Amino Acid Analysis • Post-translational modification (MS/MS) • Intact Mass Reduced (LC-MS) • Peptide mapping coupled with tandem mass spectrometry (MS/MS)
Protein content	• UV280
Higher order structure	• Far and Near UV circular dichroism • FTIR • Free thiols • Antibody Array • Liquid chromatography coupled with mass spectrometry (LC-MS)(disulfide bond characterization) • Differential scanning calorimetry

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2.3. Analytical Techniques

High molecular weight species/aggregates	• Size exclusion chromatography (HPLC) • Size exclusion chromatography (SEC-MALS) • CE-SDS (reduced and non-reduced) • Analytical Ultracentrifugation
Charge	• IEF • IEC-HPLC
Glycosylation	• Oligosaccharide profiling • N-linked Glycan analysis • Sialic Acid analysis • Monosaccharide Analysis

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2.3. Analytical Techniques

Potency	• *In vitro* TNF-α neutralization assay
Binding assay – TNF	• ELISA • Cell based binding affinity
Binding assay – Fc	• NK cell binding affinity via Fc receptors (in presence of 50% serum or 1% BSA) • FcγRIIIa V and F type binding affinity (SPR) • FcγRIIIb binding affinity (SPR) • FcγRIIa binding affinity (SPR) • FcγRIIb binding affinity (SPR) • FcγRI binding affinity (ELISA) • FcRn binding affinity (SPR) • C1q binding assay (ELISA) • C1q binding assay (ELISA)
Bioassay/ mechanism of action exploration	• ADCC (PBMC as effectors) • ADCC (NK cells as effectors) • ADCC (LPS-stimulated monocytes as targets) • CDC • Induction of apoptosis by reverse signaling • Inhibition of pro-inflammatory cytokine release by reverse signaling (Caco-2 cells) • Wound healing (closure %) • Inhibition of T Cell proliferation (MLR) • Induction of regulatory macrophages

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<Consideration 1> Mechanisms of Action of Infliximab

✓ Infliximab is an IgG1 kappa monoclonal antibody, with a high avidity for TNF-α, both soluble and membrane-bound forms

✓ Mechanisms of Action (MoA)
- (primarily) sTNF-α binding via the variable region complementary determining region (CDR)
  ⇨ neutralizing and sequestering excess sTNF-α produced in local inflammatory sites
- (another potential) binding and cross-linking mTNF on inflammatory cells or induction of regulatory macrophages
  ⇨ apoptosis by reverse signaling
- (some potential) effector function of Fragment crystallizable region (Fc) part of the antibody
  ⇨ ADCC or CDC of lysis of mTNF+ inflammatory T-cells or other cells associated with particular disease states

✓ The relative importance of merely sequestering sTNF vs eliciting other effecter functions on mTNF+ cells may vary between disease states
- high affinity binding and neutralization of sTNFα is important across all Remicade indications
- Binding to tmTNFα may especially contribute to MoA in treating CD and UC

✓ Thus, all potential activities of infliximab were investigated as part of biosimilarity studies

✓ (Another Clinical Relevance) Binding to FcRn influences PK

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<Consideration 1> Mechanisms of Action of Infliximab

Table 3. Known and Potential (Likely or Plausible) Mechanisms of Action of US-licensed Remicade in the Licensed Conditions of Use

MOA of Remicade	RA	AS	PsA	PsO	CD, Pediatric CD	UC, Pediatric UC
Mechanisms involving the Fab (antigen binding) region:						
Blocking TNFR1 and TNFR2 activity via binding and neutralization of s/tmTNF	Known	Known	Known	Known	Likely	Likely
Reverse (outside-to-inside) signaling via binding to tmTNF:	-	-	-	-	Likely	Likely
Apoptosis of lamina propria activated T cells	-	-	-	-	Likely	Likely
Suppression of cytokine secretion	-	-	-	-	Likely	Likely
Mechanisms involving the Fc (constant) region:						
Induction of CDC on tmTNF-expressing target cells (via C1q binding)	-	-	-	-	Plausible	Plausible
Induction of ADCC on tmTNF-expressing target cells (via FcγRIIIa binding expressed on effector cells)	-	-	-	-	Plausible	Plausible
Induction of regulatory macrophages in mucosal healing	-	-	-	-	Plausible	Plausible
ADCC: antibody-dependent cellular cytotoxicity; AS: ankylosing spondylitis; CD: Crohn's Disease; CDC: complement-dependent cytotoxicity; MOA: mechanism of action; PsA: psoriatic arthritis; PsO: plaque psoriasis; RA: rheumatoid arthritis; UC: ulcerative colitis; sTNF: soluble TNF; tmTNF: transmembrane TNF						

Source: FDA summary of existing literature on the topic of mechanisms of action of US-licensed Remicade[8,9]

2.4. Similarity Acceptance Criteria and Statistical analysis

* In accordance with FDA recommendations,

(1) Physicochemical biosimilarity Studies

☐ Criticality of Quality Attributes

a) Factor 1 : Evaluation of the clinical relevance and possible impact on activity, PK/PD, safety, immunogenicity, and efficacy in the identification of Quality Target Product Profile (QTPP) and Critical Quality Attributes (CQA) based on literature data

b) Factor 2 : the level of attribute present

c) Factor 3 : assay sensitivity

2.4. Similarity Acceptance Criteria and Statistical analysis

Table 4. Factors Included in Criticality Ranking for Physicochemical Test Data

Factor	Physicochemical Test Scoring System
Factor 1 Clinical impact	**10**: Clinical impact was considered to be high if the attribute is known to impact biological activity, safety, or immunogenicity **5** : Clinical impact was considered to be medium if the attribute has the potential to impact biological activity, safety, or immunogenicity **1**: Clinical impact was considered to be low if the attribute does not impact biological activity, safety, or immunogenicity
Factor 2 Level of attribute in infliximab	**5**: > 30% high content **3**: 10 - 30% medium content **1**: < 10% low content
Factor 3 Sensitivity of assay to detect difference	**5**: Validated/qualified in house with %CV ≤ 10% **3**: Validated/qualified in house with %CV > 10% **1**: CRO method or in-house method not fully qualified

CV: Coefficient of variation, CRO: Contract research organization

2.4. Similarity Acceptance Criteria and Statistical analysis

☐ Example of statistical analysis : A tiered approach based on a criticality risk ranking (ref: S. Chow, On assessment of analytical similarity in biosimilar studies, Drug Designing 3 (3) 2014)

a) Tier 1: Equivalence test with the null hypothesis

- The Equivalence Margin for the CI of mean difference was defined as ± 1.5SD based on reference product variability ($\delta = 1.5\sigma R$)
- Defining the EM as ±1.5σ assures 85% power of accepting the equivalence hypothesis, if the true mean difference is 1/8 times the σR with 10 biosimilar product lots and 10 comparator product lots used for testing and assuming a Type I error rate of 5% (CI of 90%) for the equivalence testing procedure
- Results are shown as 'within EM(Equivalence Margin)' or 'not within EM'
- Applied : extinction coefficient, protein concentration, Micro-flow Imaging, and HIAC

2.4. Similarity Acceptance Criteria and Statistical analysis

□ Statistical analysis : A tiered approach based on a criticality risk ranking

b) Tier 2: Quality range (QR) approach ('mean ± xσ' of reference product)

- σR : variation or reference product,
  x : multiplicity of the unit reference product variation
- QR limits : mean±3SD (Based on FDA criteria, high similarity was considered to have been demonstrated if 90% of data points were within the QR of US Remicade lots *(Tsong et al, 2015)*)
- Results are shown as the % of lots within the QR of US Remicade
- Most of Physicochemical tests were generally assigned to Tier 2

c) Tier 3: Presentation of raw/graphical data (Visual comparison)

- Inappropriate statistical analysis : no variability in the RP, qualitative testing etc.
- Results are shown as 'high' or 'not high'

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2.4. Similarity Acceptance Criteria and Statistical analysis

(2) Biological and Functional Assays

□ Criticality of Quality Attributes

a) based on: relationship to MoA or PK (Factor 1) and assay sensitivity (Factor 2)

- greater weight than physicochemical tests in criticality ranking by increasing the scoring for Factor 1 since the biological activity assays directly measure biological activities related to mechanisms of action, PK, and efficacy
- Biological assays with criticality scores of 100 and above : Tier 1

b) High score : *in vitro* TNFα Neutralization, TNFα Binding Affinity, Cytokine Suppression in Caco-2 cells, FcRn binding etc and FcRn binding affinity

- related to neutralizing the activity of sTNFα ⇨ relevant to all indications
- FcRn ⇨ PK

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2.4. Similarity Acceptance Criteria and Statistical analysis

c) Moderate score : Cell Based Binding Affinity, Inhibition of Cytokine Release by Reverse Signaling etc.

- related to binding to tmTNFα and inhibition of cytokine release through reverse signaling into the tmTNFα binding cells ⇨ particularly relevant to CD & UC

d) Tier 3 (qualitative tests) : ADCC using LPS stimulated monocytes as target cells because no measureable activity was obtained, Wound healing by induced regulatory macrophages because this was a qualitative assay

2.4. Similarity Acceptance Criteria and Statistical analysis

Table 5 Factors Included in Critically Ranking for Biological Assay Data

Factor	Biological Assay Scoring System
Factor 1 Relationship to MoA or PK	**100**: The assay measures a biological activity key to MoA or PK in all indications **50**: The assay measures a biological activity which may contribute to MoA or PK in some indications **10**: The assay measures a biological activity which is not important to MoA or PK in any indication
Factor 2 Sensitivity of Assay to Detect Difference	**5**: In-house fully validated/qualified method with %CV < 15% **3**: In-house fully validated/qualified method with %CV ≥ 15% **1**: Not fully qualified due to inherent variability

CV: Coefficient of variation, MoA: Mechanism of action

2.4. Similarity Acceptance Criteria and Statistical analysis

□ Statistical analysis : A tiered approach based on a criticality risk ranking

a) Tier 1: Equivalence test with the null hypothesis

- Biological assays with criticality scores of 100 and above
- EM of $\delta = 1.5\sigma R$ of US Remicade data
- Required sample size : 10 lots (based on the variability of 7 reference product lots in the test method with greatest variability, CDC and Suppression of Cytokine Release by Reverse Signaling)
- Combining data at multiple concentrations for statistical analysis to provides increased power (justified based on all concentrations being within the linear portion of the dose- response curve and the use of relative values (compared to internal reference standard) in these assays)
- Results are shown as 'within EM' or 'not within EM' for CI of mean difference based on EM

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2.4. Similarity Acceptance Criteria and Statistical analysis

b) Tier 2: Quality range (QR) approach ('mean ± xσ' of reference product)

- Assays with criticality scores below 100
- Generally mean±3SD
- Where assay variability (%RSD) was greater than 20%, the QR was decreased to mean±2SD (corresponding to 95% coverage of reference product values) to ensure that any differences between the products were not masked by assay variability
- Results are shown as the % of lots within the QR of US Remicade
- Considered to high similarity where ≥ 90% of the lots were within the QR of US Remicade

c) Tier 3: Visual comparison

- ADCC using LPS stimulated monocytes as target cells, wound healing by induced regulatory macrophages (no measurable activity)
- Results are shown as 'high' or 'not high' or the assay result is reported

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2.5. Evaluation data and Results of the Analytical similarity

Attribute	Clinical Relevance	Potential Impact[1]	Test	Biosimilarity CT-P13 vs US (Highly Similar)	Analytic Bridge EU vs US (Highly Similar)
Content	Efficacy	High	Protein Concentration (UV_{280})	Yes	Yes
Purity/Impurity	Efficacy & Immunogenicity	High	SEC-HPLC	No[2]	Yes
		High-Low	SEC-MALS	No[2]	Yes
		High-Moderate	Analytical Ultracentrifugation (AUC)	Yes	Yes
		High-Moderate	Micro-flow Imaging (MFI)	Yes	Yes
		High-Moderate	Light Obscuration (HIAC)	Yes	Yes
	Efficacy	Moderate	Non-reduced CE-SDS	No[2]	Yes
		Moderate	Reduced CE-SDS	Yes	Yes
Charge Variants	Efficacy	Moderate	IEF	Yes	Yes
		Mid-Low	IEC-HPLC	Yes No: Peak 1, Peak 4[2]	Yes No: Peak 3, Peak 5, Peak 6[2]
Glycosylation	Immunogenicity	Mid-Low	Optimized HPAEC-PAD	Yes No: G0[2]	Yes
		Mid-Low	NP-UPLC Glycan Analysis	Yes No: G0, G1F-GN, G1, G1F, Unknown 1, G1F'+NGNA, G2F+NGNA, G2F+2NGNA[2]	-
		Mid-Low	N-linked Glycan Analysis	Yes No: G0, G1F1NGNA, G2F1NGNA[2]	Yes No: G0, G1F1NGNA[2]
		Mid-Low	Sialic Acid Analysis	Yes	Yes
		Low	Monosaccharide Analysis	Yes	Yes
		Low	Glycation	No[2]	Yes
Excipients	Efficacy, Safety, Immunogenicity	Moderate	pH Polysorbate 80 Sucrose	Yes	Yes

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< 1. Physicochemical studies >

Table 6 Conclusions of Statistical Analysis of the 3-way Physicochemical Similarity Study

Attribute	Clinical Relevance	Potential Impact[1]	Tier	Test	Measurement	Biosimilarity CT-P13 vs US Remicade (equivalence/ % within QR/visual similarity)	Analytic Bridge EU vs US Remicade (equivalence/ % within QR/ visual similarity)
Primary Structure	Efficacy, Safety, Immuno-genicity	High	3	Peptide Mapping (HPLC)	Visual comparison	High	High
		High-Low	2	Peptide Mapping (LC-MS)	% Deamidation HC Asn57 Asn318 Asn364 Asn387 Asn41 % Oxidation HC Met255 % C-terminal lysine variant HC Lys450	 100 100 100 100 100 100 100	 100 100 100 100 100 100 88
		Low	3	Intact Mass (Reduced) (LC-MS)	Mass (Da) HC K0: 4 masses HC K1: 3 masses LC: 1 mass	 High High High	 High High High
		Low	3	Amino Acid Analysis	Robust amino acids	High	High
			1 & 2		Extinction coefficient	Within EM 100	- -
			2		Tyrosine	100	-
		Low	3	N-terminal Sequencing	HC:EVKLEESGGGLVQP GGSMK LC:DILLTQSPAILSVSPG ER	High High	High High
		Low	3	C-terminal Sequencing	HC:SLSLSPGK / SLSLSPG LC:SFNRGEC	High High	High High

Attribute	Clinical Relevance	Potential Impact[1]	Tier	Test	Measurement	Biosimilarity CT-P13 vs US Remicade (equivalence/ % within QR/visual similarity)	Analytic Bridge EU vs US Remicade (equivalence/ % within QR/ visual similarity)
Higher Order Structure	Efficacy & Immuno-genicity	Low	3	Fourier Transform Infrared Spectroscopy (FTIR)	Amide I Amide II A B C	High High High High High	High High High High High
		Low	3	Differential Scanning Calorimetry (DSC)	Transition 1: 67 - 68°C Transition 2: 74 - 75°C Transition 3: 83 - 84°C	High High High	High High High
		Low	3	Circular Dichroism (CD)	Visual comparison	High	High
		Moderate	2	Free Thiol Analysis	Average free SH/IgG (mol/mol)	100	100
		Moderate	3	Disulfide Bond	Visual comparison: 8 peaks matched	High	High
		High	3	Antibody Array	Visual comparison of ELISA signal of 34 pAbs	High	High

Attribute	Clinical Relevance	Potential Impact[1]	Tier	Test	Measurement	Biosimilarity CT-P13 vs US Remicade (equivalence/ % within QR/visual similarity)	Analytic Bridge EU vs US Remicade (equivalence/ % within QR/ visual similarity)
Purity/ Impurity	Efficacy & Immuno-genicity	High	2	SEC-HPLC	% Monomer	0	100
		High-Low	2	SEC-MALS	% Monomer % HMW MW Monomer MW HMW	0 0 71 86	100 100 100 100
		High-Moderate	2	Analytical Ultra-centrifugation (AUC)	% Monomer % Higher Species	100 100	100 100
		High-Moderate	1	Micro-Flow Imaging (MFI)	Sub-visible particles 1 ≤, < 100 (μm) 2 ≤, < 100 (μm) 5 ≤, < 100 (μm) 10 ≤, < 100 (μm)	 Within EM Within EM Within EM Within EM	 Within EM Within EM Within EM Within EM
		High-Moderate	1	Light Obscuration (HIAC)	Sub-visible particles 2 ≤ (μm) 5 ≤ (μm) 10 ≤ (μm)	 Within EM Within EM Within EM	 Within EM Within EM Within EM

Attribute	Clinical Relevance	Potential Impact[1]	Tier	Test	Measurement	Biosimilarity CT-P13 vs US Remicade (equivalence/ % within QR/visual similarity)	Analytic Bridge EU vs US Remicade (equivalence/ % within QR/ visual similarity)
	Efficacy	Moderate	2	Non-reduced CE-SDS	% Intact IgG	0	97
		Moderate	2	Reduced CE-SDS	% Sum H+L chains	96	94
					% Non-glycosylated HC	96	94
		Moderate	3	IEF	8 bands identified	High	High
Content	Efficacy	High	1 & 2	Protein Concentration (UV_{280})	Reconstituted product	Within EM 100	- 92

Attribute	Clinical Relevance	Potential Impact[1]	Tier	Test	Measurement	Biosimilarity CT-P13 vs US Remicade (equivalence/ % within QR/visual similarity)	Analytic Bridge EU vs US Remicade (equivalence/ % within QR/ visual similarity)
Charge Variants	Efficacy	Mid-Low	2	IEC-HPLC	% Peak 1 % Peak 2 % Peak 3 % Peak 4 % Peak 5 % Peak 6	40 100 100 0 100 100	90 100 70 90 80 70
Glycosyl-ation	Immuno-genicity	Mid-Low	2	Oligo-saccharide Profiling Using HPAEC-PAD	% G0F % Man5 % G0 % G1F % G2F % SA1 % SA2	100 100 9 100 100 100 100	100 100 100 97 97 100 100
		Mid-Low	2	Sialic Acid Analysis	Molar ratio sialic acid/protein (mol/mol)	100	100
		Low	2	Monosaccharide Analysis	Molar ratio monosaccharide/ protein (mol/mol) Fuc GlcN Gal Man	 100 100 100 100	 100 100 100 100
		Low	2	Glycation (LC-ES-MS)	% Glycated LC % Glycated HC	0 0	100 100

Attribute	Clinical Relevance	Potential Impact[1]	Tier	Test	Measurement	Biosimilarity CT-P13 vs US Remicade (equivalence/ % within QR/visual similarity)	Analytic Bridge EU vs US Remicade (equivalence/ % within QR/ visual similarity)
Glycosyl-ation		Mid-Low	2	NP-UPLC Glycan Analysis	G0F-GN	100	100
					G0	0	100
					G0F	100	100
					Man5	100	100
					G1F-GN	0	100
					G1	87	96
					G1F	0	100
					G1F'	100	100
					Unknown 1	4	100
					G2	100	100
					G2F	100	100
					G1-GN+NGNA	100	100
					G1F-GN+NGNA	100	100
					G1F+NGNA	100	100
					G1F'+NGNA	4	100
					G2+NGNA	100	100
					G2F+NGNA	87	100
					Unknown 2	100	100
					Unknown 3	96	100
					G2F+2NGNA	39	100

Attribute	Clinical Relevance	Potential Impact[1]	Tier	Test	Measurement	Biosimilarity CT-P13 vs US Remicade (equivalence/ % within QR/visual similarity)	Analytic Bridge EU vs US Remicade (equivalence/ % within QR/ visual similarity)
Glycosyl-ation		Mid-Low	2	N-linked Glycan Analysis	% Man5	100	100
					% G0F-GlcNAc	100	100
					% G0	0	87
					% G0F	100	100
					% G1F	100	100
					% G2F	100	100
					% G1F1NGNA	0	87
					% G2F1NGNA	0	100
Excipients	Efficacy, Safety, Immunogenicity	Moderate	2	pH	pH	100	100
				HPLC	Polysorbate 80 (%w/v)	100	100
				HPAEC-PAD	Sucrose (%w/v)	92	100

Attribute	Clinical Relevance	Potential Impact[1]	Tier	Test	Measurement	Biosimilarity CT-P13 vs US Remicade (equivalence/ % within QR/visual similarity)	Analytic Bridge EU vs US Remicade (equivalence/ % within QR/ visual similarity)
Purity/ Impurity	Efficacy & Immuno-genicity	High	2	SEC-HPLC	% Monomer	0	100
		High-Low	2	SEC-MALS	% Monomer % HMW MW Monomer MW HMW	0 0 71 86	100 100 100 100
		High-Moderate	2	Analytical Ultra-centrifugation (AUC)	% Monomer % Higher Species	100 100	100 100
		High-Moderate	1	Micro-Flow Imaging (MFI)	Sub-visible particles 1 ≤, < 100 (μm) 2 ≤, < 100 (μm) 5 ≤, < 100 (μm) 10 ≤, < 100 (μm)	Within EM Within EM Within EM Within EM	Within EM Within EM Within EM Within EM
		High-Moderate	1	Light Obscuration (HIAC)	Sub-visible particles 2 ≤ (μm) 5 ≤ (μm) 10 ≤ (μm)	Within EM Within EM Within EM	Within EM Within EM Within EM

< 2. Biological and Functional Testing >

Table 7 Results of Statistical Analysis of the 3-way Biological Activity Similarity Studies – Tier1

Activity	Clinical Relevance	Potential Impact[1]	Assay	Measurement	Biosimilarity US vs CT-P13[2]	Analytic Bridge US vs EU[2]
Binding to sTNFα	MoA & Efficacy- all indications	High	*In Vitro* TNFα Neutralization	% Relative activity by cell viability	Within EM	Within EM
		High	TNFα Binding Affinity (ELISA)	% Relative binding (EC_{50})	Within EM	Within EM
	MoA & Efficacy- CD & UC	High	Caco-2 (Cytokine Suppression)	% Relative activity Combined conc. At 10 μg/mL At 2 μg/mL At 0.4 μg/mL	Within EM Within EM Within EM Within EM	Within EM Within EM Within EM Not within EM[3]
Binding to tmTNFα	MoA & Efficacy- CD & UC	Moderate	Cell Based Binding Affinity	% Relative binding (EC_{50})	Within EM	Within EM
		Moderate	Inhibition of Cytokine Release by Reverse Signaling	% Relative activity Combined conc. At 5.3 μg/mL At 2.4 μg/mL At 1.1 μg/mL	Within EM Within EM Within EM Within EM	Within EM Within EM Not within EM[4] Within EM
FcRn Binding	PK- all indications	High	FcRn Binding Affinity (SPR)	% Relative binding affinity by KD	Within EM	Within EM

< 2. Biological and Functional Testing >

* foot note

3. The CI for mean difference for EU Remicade at the lowest concentration 04 μg/mL (-0.47, 6.67) was slightly outside the EM of US Remicade (-6.61, 6.61). The data are presented in Figure 27 (page 195).

4. A single lot of EU Remicade had a low relative activity at 24 μg/mL of 65%. The data are presented in Figure 30 (page 197).

Table 8. Results of Statistical Analysis of the 3-way Biological Activity Similarity Studies – Tier 2 & 3

Activity	Clinical Relevance	Potential Impact[1]	Tier	Assay	Measurement	Biosimilarity US vs CT-P13[2] (% within QR/visual comparison)	Analytic Bridge US vs EU[2] (% within QR/visual comparison)
Binding to tmTNFα	MoA & Efficacy- CD & UC	Low	2	Induction of Apoptosis by Reverse Signaling (FACS)	% Relative apoptotic cells Combined conc. At 1.0 μg/mL At 0.6 μg/mL At 0.3 μg/mL At 0.2 μg/mL	100 90 100 100 100	100 100 100 100 100
Binding to tmTNFα-Fc	MoA & Efficacy- CD & UC	Low	2	Induction of Regulatory Macrophages[4,5]	% Induced Regulatory Macrophage from Total PBMC At 2.5 μg/mL At 0.625 μg/mL At 0.156 μg/mL	100 100 100	67 [3] 100 [3] 100 [3]
		Low	2	Suppression of T cell Proliferation by Regulatory Macrophages[4]	% Relative Proliferation Combined conc. At 125 ng/mL At 63 ng/mL At 31 ng/mL	87 100 80 60	100 [3] 100 [3] 100 [3] 67 [3]
		Low	3	Would Healing by Regulatory Macrophages	Estimate of % Closure	Highly similar	Highly similar

C1q Binding & CDC Activity	Immune system mediator - classical complement pathway	Low	2	C1q Binding Affinity (ELISA)	% Relative binding affinity	100	100
		Low	2	CDC	% Relative binding by EC_{50}	92	91
Fc Binding	Immune system mediator	Low	2	FcγRIIIa V Type Binding Affinity (SPR)	% Relative binding affinity KD	85	100
		Low	2	FcγRIIIa F Type Binding Affinity (SPR)	% Relative binding affinity KD	61	100
		Low	2	FcγRIIIb Binding Affinity (SPR)	% Relative binding affinity KD	90	100
		Low	2	FcγRIIa Binding Affinity (SPR)	% Relative binding affinity KD	100	100
		Low	2	FcγRIIb Binding Affinity (SPR)	% Relative binding affinity KD	100	100
		Low	2	FcγRI Binding Affinity (ELISA)	% Relative binding affinity EC_{50}	100	100
	Immune system	Low	2	*Ex Vivo* Binding to NK Cells in 1% BSA[3]	% Relative binding Combined conc. At 50 μg/mL At 10 μg/mL At 2 μg/mL	33 0 0 33	100 100 67 100
		Low	2	*Ex Vivo* Binding to NK Cells in 50% Serum[3]	% Relative binding Combined conc. At 50 μg/mL At 10 μg/mL At 2 μg/mL	89 100 100 67	89 100 33 100

tmTNFα & Fc Binding	MoA & Efficacy- CD & UC	Low	2	ADCC using PBMC (Healthy Donor)	% Relative activity	100	100
		Low	2	ADCC using NK Cells (Healthy Donor)	% Relative activity Combined conc. At 8 ng/mL At 4 ng/mL At 2 ng/mL	96 100 96 96	99 100 97 97
		Low	3	ADCC using LPS-stimulated Monocytes and NK Cells (Healthy Donor)	At 8 concentrations (0.000013 μg/mL to 1 μg/mL)	Highly similar (no activity)	Highly similar (no activity)
		Low	3	ADCC using IBD patient-derived LPMC and NK Cells	At 10 μg/mL and 50 μg/mL	Highly similar (low-no activity)	-

* foot note
2. The QR (unless otherwise indicated) : mean±3SD
3. Only 3 lots of CT-P13, US and EU Remicade were included due to availability of cells.
4. The QR : mean±2SD for these research assays due to inherent assay variability
   - Only 3 lots of EU Remicade were included in this assay
5. Results for Induction of Regulatory Macrophages are absolute values and were not compared to internal reference standard.

Table 9. Conclusions of Functional Assays Related to Mechanism of Action in IBD and Results of Statistical Analysis

Assay	Tier	Target	Cells	Biosimilarity CT-P13 vs US (within EM/ % within QR/visual comparison)	Analytic Bridge EU vs US (within EM/ % within QR/ visual comparison)
Caco-2 (Cytokine Suppression)[1]	1	sTNFα	Caco-2 (Human epithelial colorectal adenocarcinoma cells)	Within EM	Within EM[2]
Cell Based Binding Affinity	1	tmTNFα	tmTNFα Jurkat Cells	Within EM	Within EM
Inhibition of Cytokine Release by Reverse Signaling[1]	1	tmTNFα	LPS-stimulated PBMC	Within EM	Within EM[3]
Induction of Apoptosis by Reverse Signaling[1]	2	tmTNFα	tmTNFα Jurkat Cells	100[4]	100
Induction of Regulatory Macrophages	2	tmTNFα-macrophage	Mixed lymphocytes	100	100[5,6]
Suppression of T- cell Proliferation by Regulatory Macrophages[1]	2	tmTNFα-macrophage	Mixed lymphocytes	87[7]	100[5,8]
Wound Healing by Regulatory Macrophages	3	tmTNFα-macrophage	HCT 116 colon epithelial cells & induced regulatory macrophages	Highly similar	Highly similar
ADCC	2	tmTNFα of Jurkat cell-FcγRIIIa of NK cell	tmTNFα-overexpressing Jurkat cell & NK cells	96	99
	2	tmTNFα of Jurkat cell-FcγRIIIa of PBMC	tmTNFα-overexpressing Jurkat cell & PBMC	100	100
	3	tmTNFα of monocytes-FcγRIIIa of NK cell	LPS-stimulated monocytes & NK cells	Highly similar (no activity)	Highly similar (no activity)
	3	tmTNFα of LPMC-FcγRIIIa of NK cell	IBD patient-derived LPMC & NK cells	Highly similar (low-no activity)	Highly similar (low-no activity)

* foot note
1. Conclusions of statistical analysis of combined concentration data are shown
2. EU Remicade outside of EM at 0.4 μg/mL
3. EU Remicade outside of EM at .24 μg/mL due to a single lot with low relative activity
4. 90% of CT-P13 lots within QR at 1 μg/mL
5. Reduced number of EU Remicade lots tested
6. 67% EU Remicade within QR at 25 μg/mL concentration
7. 100% CT-P13 within QR at 125 ng/mL; 80% CT-P13 within QR at 63 ng/mL; 60% CT-P13 within QR at 31 ng/mL
8. 67% EU Remicade within QR at 31 ng/mL concentration

2.6. Assessment of Uncertainties

☐ Uncertainties were thoroughly investigated using a step-wise approach involving:

a) In vitro studies to characterize differences
b) Biological assays to investigate impact
c) Data from forced degradation studies used to identify thresholds
d) *Ex vivo* studies performed to determine the impact on human cells
e) Clinical studies to address any remaining uncertainty

Table 10 Conclusions of Statistical Analysis of the 3-way Physicochemical Similarity Study

Attribute	Clinical Relevance	Potential Impact[1]	Test	Biosimilarity CT-P13 vs US (Highly Similar)	Analytic Bridge EU vs US (Highly Similar)
Primary Structure	Efficacy, Safety, Immunogenicity	High	Peptide Mapping (HPLC)	Yes	Yes
		High-Low	Peptide Mapping (LC-MS)	Yes	Yes No: HC Lys450[4]
		Low	Intact Mass (Reduced) (LC-MS)	Yes	Yes
		Low	Amino Acid Analysis	Yes	Yes
			Extinction Coefficient ($L \cdot g^{-1} \cdot cm^{-1}$)	Yes	-
		Low	N-terminal Sequencing	Yes	Yes
		Low	C-terminal Sequencing	Yes	Yes
Higher Order Structure	Efficacy & Immunogenicity	Low	Fourier Transform Infrared Spectroscopy (FTIR)	Yes	Yes
		Low	Differential Scanning Calorimetry (DSC)	Yes	Yes
		Low	Circular Dichroism (CD)	Yes	Yes
		Moderate	Free Thiol Analysis	Yes	Yes
		Moderate	Disulfide Bond	Yes	Yes
		High	Antibody Array	Yes	Yes

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Table 11. Residual Uncertainties Identified in Physicochemical and Structural Analyses, Potential Impact, and Studies to Address Uncertainty

Physicochemical Uncertainty	Level (Mean Value) CT-P13	Level (Mean Value) US Remicade	Level (Mean Value) EU Remicade	Potential Impact	Studies to Address Uncertainty	Conclusions of Studies to Address Uncertainty
Intact IgG (H2L2) (CE-SDS NR)	95.1%	98.2%	98.3%	Biologic Activity	Functional assays to compare biological activity	Theoretically translates to 1.5% difference in TNFα binding; No impact on biological activities
Charge Variants (C-terminal lysine) (IEC-HPLC,%)	Peak 1: 6.6 ± 0.9 Peak 2: 12.1 ± 0.4 Peak 3: 39.8 ± 0.8 Peak 4: 24.9 ± 0.6 Peak 5: 3.5 ± 0.3 Peak 6: 13.1 ± 0.8	Peak 1: 3.8 ± 0.8 Peak 2: 9.6 ± 1.4 Peak 3: 40.7 ± 2.8 Peak 4: 19.7 ± 0.8 Peak 5: 4.9 ± 0.7 Peak 6: 21.6 ± 3.4	Peak 1: 2.8 ± 0.8 Peak 2: 7.9 ± 1.5 Peak 3: 37.0 ± 6.5 Peak 4: 20.6 ± 0.8 Peak 5: 5.6 ± 1.8 Peak 6: 26.1 ± 6.5	Biologic Activity	*In vitro* and *in vivo* tests Functional assays to compare biological activity	CT-P13 has higher levels without C-terminal lysine (Peak 1 and Peak 2) and with a single C-terminal lysine (Peak 4), and lower levels of infliximab with 2 C-terminal lysine residues (Peak 5 & Peak 6). C-terminal lysines are removed from the molecule rapidly in serum and *in vivo*. Addressed in clinical studies
High Molecular Weight Forms (SEC-HPLC) (SEC-MALS)	0.8% 0.5%	0.2% 0.2%	0.2% 0.2%	Immunogenicity	Assessment of immunogenicity in clinical studies	Addressed in clinical studies

Physicochemical Uncertainty	Level (Mean Value)			Potential Impact	Studies to Address Uncertainty	Conclusions of Studies to Address Uncertainty
	CT-P13	US Remicade	EU Remicade			
Glycation (LC-ES-MS, %)	LC: 2.4 ± 0.1 HC: 4.0 ± 0.2	LC: 0.8 ± 0.1 HC: 0.8 ± 0.1	LC: 0.7 ± 0.0 HC: 0.8 ± 0.1	Biologic Activity	Functional assays to compare biological activity	CT-P13 has higher levels of glycation (non-enzymatic addition of glucose to lysine residues) in LC and HC. The sites of glycation are the same. None of the sites of glycation are within the TNFα binding region. No impact on biological activities was detected. No impact on immunogenicity.
G0 Content (HPAEC-PAD) (N-linked glycan) (NP-UPLC)	0.72% 1.1% 0.7%	1.74% 2.2% 1.4%	1.67% 2.4% 1.3%	Biologic Activity	Functional assays to compare biological activity	Impact on FcγRIIIa binding affinity, translating into lower binding to NK cells *ex vivo* in the absence of serum. No impact on NK cell binding in the presence of serum. High similarity in all 3 ADCC assays. Slightly lower (6% cytotoxicity at 8 ng/ml) mean ADCC activity using NK cells of V/F FcγRIIIa allotype unlikely to be clinically significant as this was not observed in the other ADCC assays and little or no ADCC activity was detected using IBD patient cells.

3. Detailed Results

- ✓ Summary of key data from Section 4 and 5 of FDA Briefing Document prepared by Celltrion

3.1. Primary Structure and Molecular Weight

*Unless otherwise indicated, the data described in this section are from the 3-way similarity analysis

Proper-ties	Attribute	Test Method	Similarity Acceptance Criteria(Tier)/Measurement	(CT-P13 vs US RBP)	(EU vs US RBP)	Results and Conclusion	Method Suitability
Primary structure	Peptide Mapping	Peptide mapping (HPLC)	Tier 3 Visual comparison	High	High	Highly similar profile and retention times to RBP (Acceptable) No missing or additional significant peptides	
	Amino acid sequence	Peptide mapping (LC-ES-MS/MS)		Identical		Matched the expected peptides Sequence coverage 100% (Acceptable)	
	N/C-terminal sequence	LC-ES-MS/MS	Tier 3 (identical to the RPB)	High	High	identical in 3 products and match the expected sequence (C-term : with and without a terminal lysine residue in all 3 products)	
	Molecular mass	LC-ES-MS (intact, reduction)	Tier 3 HC K0: 4 masses HC K1: 3 masses LC: 1 mass	All High	All High	closely match with the expected mass, and Highly similar to RBP	
	Amino acid Composition	Amino acid Analysis	Tier 3 (Robust amino acids)	High	High	Similar in 3 products	

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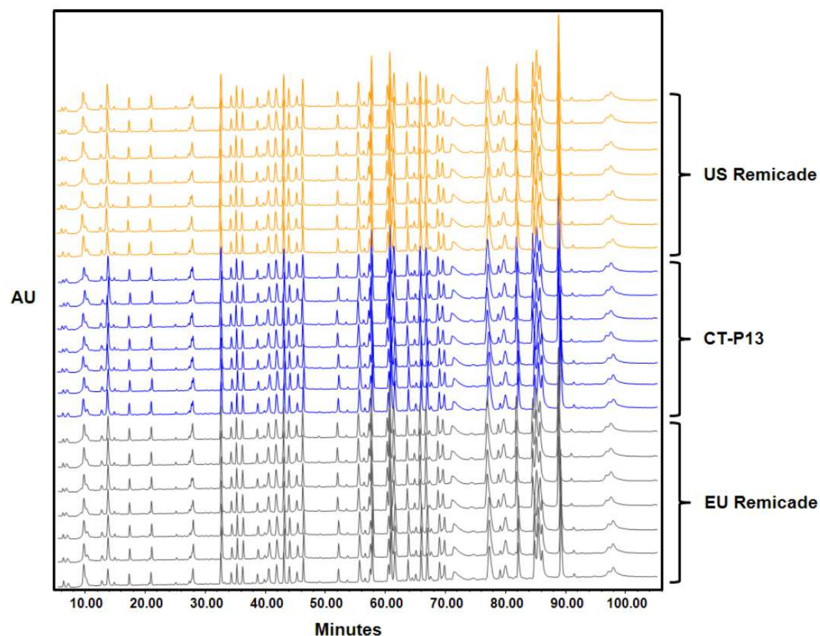
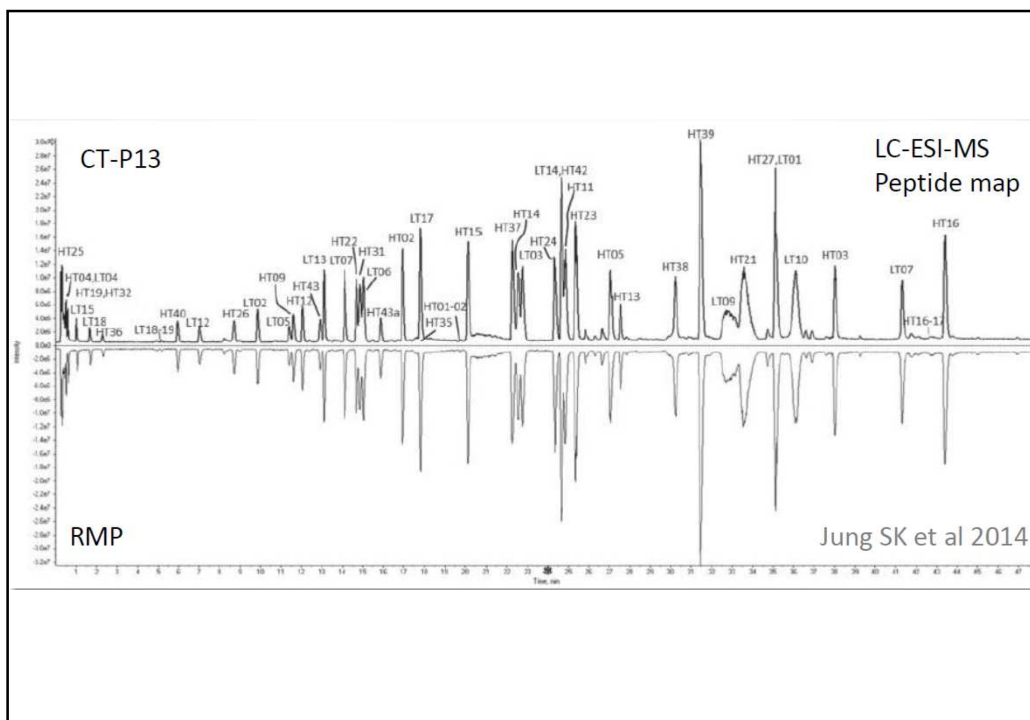
Figure 1. Representative Overlaid Chromatograms of Peptide Mapping for US Remicade, CT-P13 and EU Remicade from the 3-Way Similarity Study





3.2. Post-translational Modifications (except Glycosylation profile)

Properties	Attribute	Test Method	Similarity Acceptance Criteria(Tier)/Measurement	(CT-P13 vs US RBP)	(EU vs US RBP)	Results and Conclusion	Method Suitability
Post-Translational Modification	% Oxidation HC	LC-MS	Tier 2 : ± 3SD Met255(HC)	100	100	Similar levels in the 3 products	
	Deamidation	LC-MS	Tier 2 : Asn57, Asn318,Asn364, Asn387, Asn41(HC)	All sites, 100	All sites, 100	Highly similar levels in the 3 products	
	% C-terminal Lys variant HC	LC-MS	Tier 2 : Lys450(HC)	100	88	Slightly higher levels of K0 and K1 than US/EU RBP	
	Glycation	LC-ES-MS	Tier 2 : % Glycated LC % Glycated HC	0 0	100 100	• Identical the glycation site profile • Higher levels than US/EU RBP	

[Justification of Differences]

A. C-term Lys variants : No clinically meaningful

B. Glycation

- The glycation site profile was the same for the 3 products, confirming structural similarity
- Although CT-P13 contained higher levels of glycated forms, the level of glycation of CT-P13 remained low : 24% (LC) & 40 % (HC)
- None of the glycation sites of CT-P13 or Remicade were located in the TNFα binding region
- According to literatures, none of the glycation sites reside near the FcγRIIIa binding region (next Figure)
- No differences in biological activities by data from similarity studies of biological assays including TNFα binding and neutralization assays
- Data from samples of CT-P13 and US Remicade with artificially created levels of glycation showed that glycation has no impact on FcγRIIIa binding affinity of CT-P13 or US Remicade
- No impact on immunogenicity is expected as Abs are glycated on incubation with serum and in vivo (Goetze et al, 2012)

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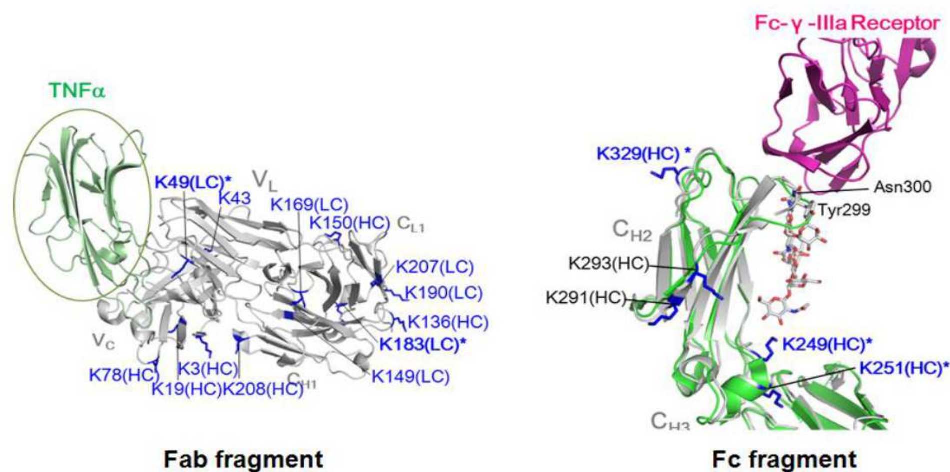
Figure 2. Locations of Identified Glycation Sites in Fab and Fc Regions of CT-P13 with TNFα Binding and FcγRIIIa Receptor Binding Sites



Glycated residues are shown in blue and highly glycated residues are shown in blue with an asterisk.
Left Hand Side: TNFα shown in green; Fab region of CT-P13 shown in grey
Right Hand Side: FcγRIIIa shown in pink; Fc complex shown in green; Fc region of CT-P13 is overlaid in grey.

3.3. Higher Oder Structure

Properties	Attribute	Test Method	Similarity Acceptance Criteria(Tier)/Measurement	(CT-P13 vs US RBP)	(EU vs US RBP)	Results and Conclusion	Method Suitability
Higher Order Structure	Secondary Structure	Fourier Transform Infrared Spectroscopy (FTIR)	Tier 3 : Amide I/Amide II/A/B/C	All High	All High	highly similar spectra	
	Secondary/ Tertiary Structure	Near/Far Circular Dichroism (CD)	Tier 3 : Visual comparison	High	High	highly similar spectra	
	Conformation Stability	Differential Scanning Calorimetry (DSC)	Tier 3 : Transition temperatures 67-68/74-75/83-84 ℃	All High	All High	highly similar thermal unfolding profiles and thermal transition midpoint temperatures	
	Free thiol	Free thiol Analysis	Tier 2 : ± 3SD Average free SH/IgG(mol/mol)	100 (0.14~0.15)	100 (0.14~0.15)	highly similar levels in 3 products	
	Disulfide bond	Peptide mapping /LC-MS	Tier 3 : Visual comparison (8 peaks matched)	High	High	Identical in 3 products	
	Epitope Exposure Analysis	Antibody Array	Tier 3 : Visual comparison (ELISA signal of 34 pAbs)	High	High	Identical in 3 products	

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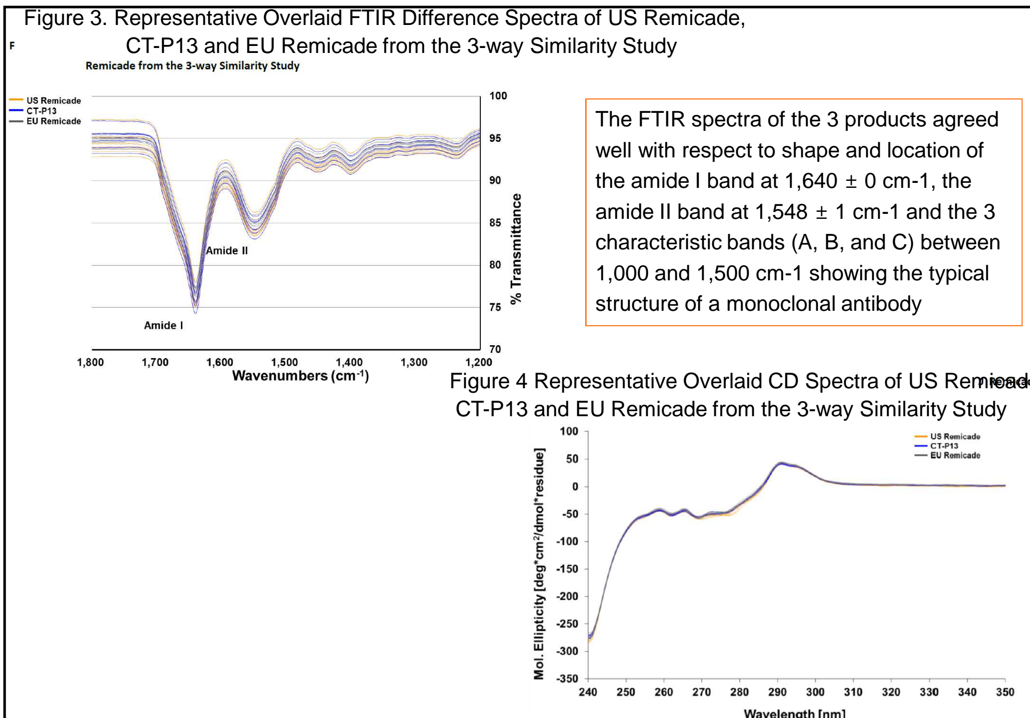
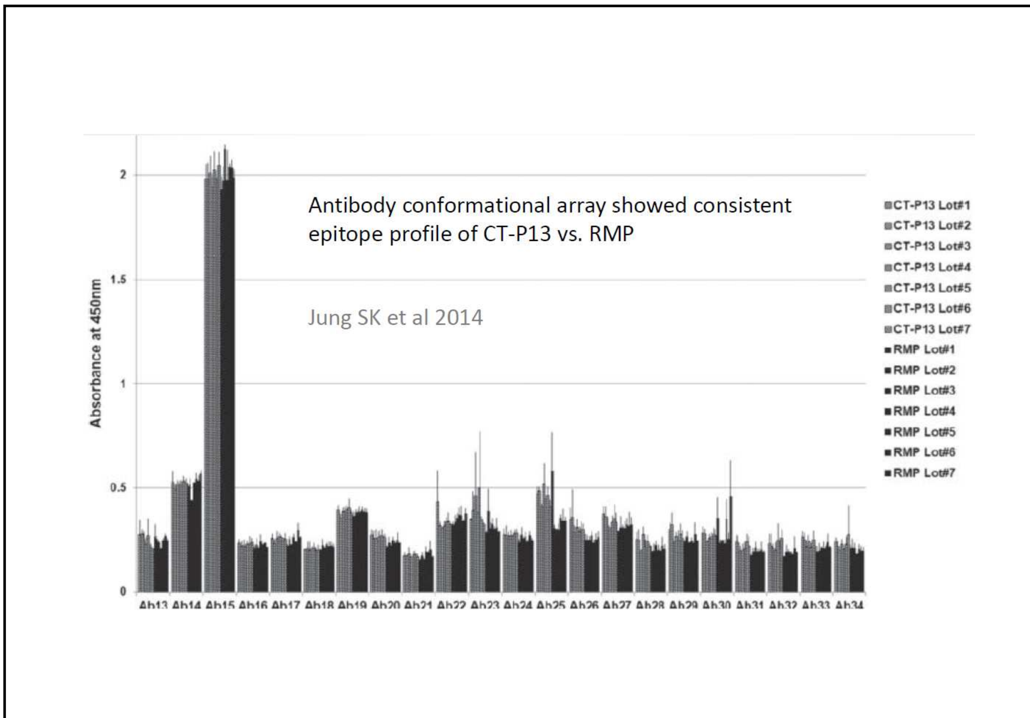


Figure 3. Representative Overlaid FTIR Difference Spectra of US Remicade, CT-P13 and EU Remicade from the 3-way Similarity Study
US Remicade
CT-P13
EU Remicade
Amide I
Amide II
% Transmittance
Wavenumbers (cm-1)
The FTIR spectra of the 3 products agreed well with respect to shape and location of the amide I band at 1,640 ± 0 cm-1, the amide II band at 1,548 ± 1 cm-1 and the 3 characteristic bands (A, B, and C) between 1,000 and 1,500 cm-1 showing the typical structure of a monoclonal antibody
Figure 4 Representative Overlaid CD Spectra of US Remicade, CT-P13 and EU Remicade from the 3-way Similarity Study
Mol. Ellipticity [deg*cm2/dmol*residue]
Wavelength [nm]


Antibody conformational array showed consistent epitope profile of CT-P13 vs. RMP
Jung SK et al 2014
Absorbance at 450nm
CT-P13 Lot#1
CT-P13 Lot#2
CT-P13 Lot#3
CT-P13 Lot#4
CT-P13 Lot#5
CT-P13 Lot#6
CT-P13 Lot#7
RMP Lot#1
RMP Lot#2
RMP Lot#3
RMP Lot#4
RMP Lot#5
RMP Lot#6
RMP Lot#7
Ab13 Ab14 Ab15 Ab16 Ab17 Ab18 Ab19 Ab20 Ab21 Ab22 Ab23 Ab24 Ab25 Ab26 Ab27 Ab28 Ab29 Ab30 Ab31 Ab32 Ab33 Ab34

3.4. Glycosylation Profile

Properties	Attribute	Test Method	Similarity Acceptance criteria	(CT-P13 vs US RBP)	(EU vs US RBP)	Results and Conclusion	Method Suitability
Glycosylation	Site Identification And N-glycan structure Analysis	LC-MS	Tier 2 : % Man5 % G0F-GlcNAc % G0 % G0F % G1F % G2F % G1F1NGNA % G2F1NGNA	 100 100 0 100 100 100 0 0	 100 100 87 100 100 100 87 100	In all 3 products, • only N-glycosylation site of Asn 300 • No O-linked glycans • Major : G0F, G1F • Minor : Man5, G2F, G0 G0F-GlcNAc • Lower levels of G0 - 1.1 ± 0.1% of CT-P13, - 2.2 ± 0.2% of US RBP, - 2.4 ± 0.4% of EU RBP • Lower levels of Man5 (showed high variability of US Remicade lots) - 4.5 ± 0.3% of CT-P13, - 5.1 ± 0.9% of US RBP - 5.0 ± 1.3% of EU RBP	
	N-glycan profiling	HPAEC-PAD	Tier 2 : % G0F % Man5 % G0 % G1F % G2F % SA1 % SA2	 100 100 9 100 100 100 100	 100 100 100 97 97 100 100	• Lower amounts of afucosylated glycans (G0 and Man5) • G0 : 0.72 ± 0.14% of CT-P13, 1.74 ± 0.27% of US RPB, 1.67 ± 0.27% of EU RBP • Man : 4.10 ± 0.55% of CT-P13, 4.31 ± 0.86% of US RBP, 4.18 ± 0.94% of EU RBP	

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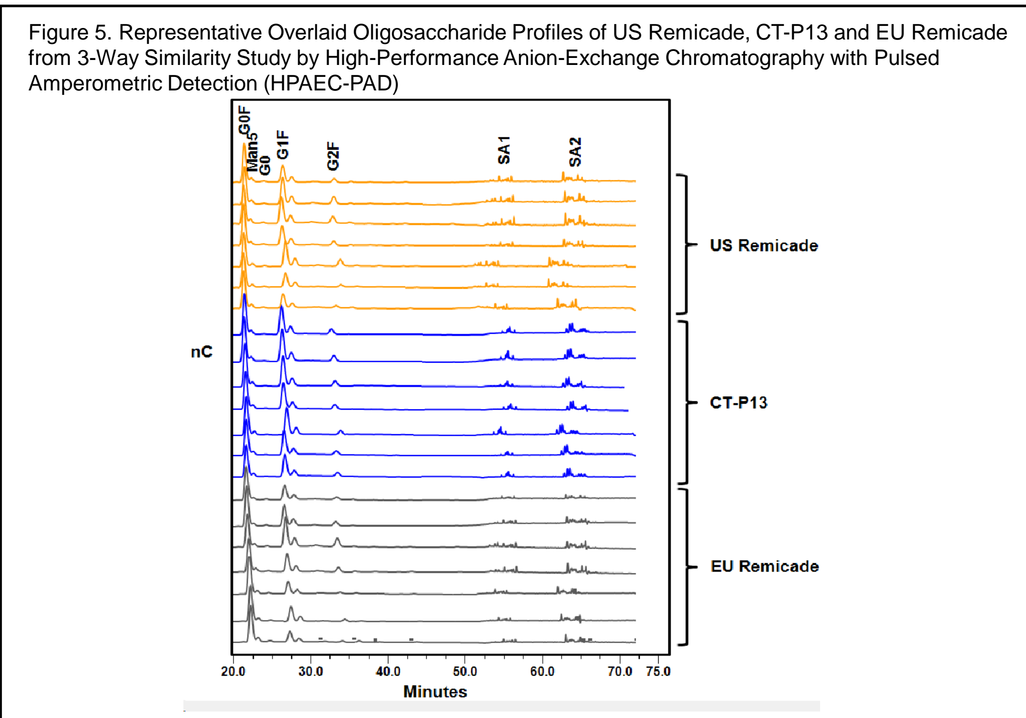
Figure 5. Representative Overlaid Oligosaccharide Profiles of US Remicade, CT-P13 and EU Remicade from 3-Way Similarity Study by High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD)



3.4. Glycosylation Profile(continued)

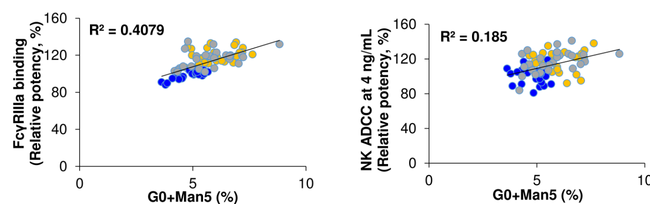
Proper-ties	Attribute	Test Method	Similarity Acceptance criteria	(CT-P13 vs US RBP)	(EU vs US RBP)	Results and Conclusion	Method Suitability
Glycosyl-ation	N-glycan Structure Analysis	NP-UPLC	Tier 2 : G0F-GN G0 G0F Man5 G1F-GN G1 G1F G1F′ Unknown 1 G2 G2F G1-GN+NGNA G1F-GN+NGNA G1F+NGNA G1F′+NGNA G2+NGNA G2F+NGNA Unknown 2 Unknown 3 G2F+2NGNA	 100 0 100 100 0 87 0 100 4 100 100 100 100 100 4 100 87 100 96 39	 100 100 100 100 100 96 100 100 100 100 100 100 100 100 100 100 100 100 100 100	-Slightly lower levels of afucosylated glycans (primarily in G0) -afucosylated glycans : G0, G1, G2, Man5 -G2 content : All lots of CT-P13 were within the QR of US Remicade -G1 : Only 3 lots of CT-P13 had lower levels than the mean ± 3SD range of US Remicade -However, the levels of G1 and G2 are very low; in US Remicade G1 is only 02% of total glycan species and G2 is only 05% of total glycan species -Some differences between CT-P13 and US Remicade in the levels of specific charged glycans such as G1F1NGNA and G2F1NGNA	
	Sialic acid Analysis	HPLC (DMB), LC-MS	Tier 2 : Molar ratio	100	100	-Highly similar for all 3 products (approximately 02 mol sialic acid/mol protein) -NANA levels were too low	
	Monosaccharide Anlaysis	HPLC (DMB), LC-MS	Tier 2 : Molar ratio (Fuc/GlcN/Gal/Man)	All 100	All 100	Highly similar for all 3 products	

[Justification of Differences]

A. Evaluation of the effect of non-glycosylation, agalactosylation, and afucosylation on FcγRIIIa and C1q binding affinity (especially the impact of degree of afucosylation (G0+Man5))

- Non-glycosylation and afucosylation had an impact on FcγRIIIa and C1q binding affinity, but agalactosylation did not.
  (Non-glycosylated form : highly similar level for the 3 products (below 1% in all lots tested.))

Figure 6. Scatter Plots Showing Relationship of % Afucosylated Glycan Species (G0+Man5) With FcγRIIIa Binding Affinity and ADCC Activity



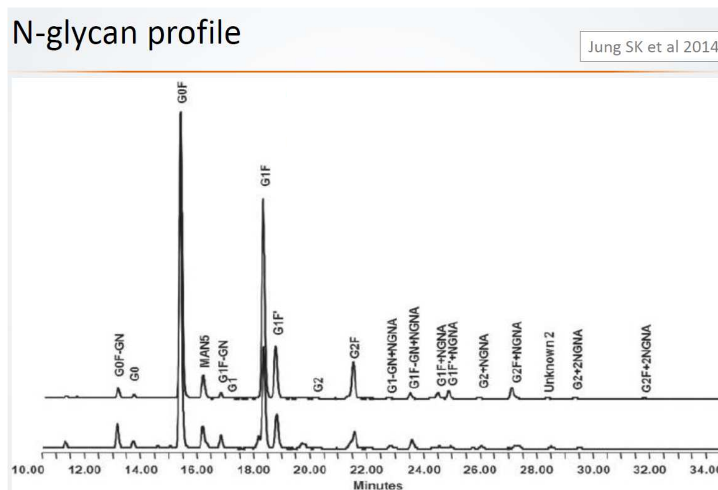
CT-P13 lots are shown in blue; US Remicade lots are shown in yellow; EU Remicade lots are shown in grey

- As there is no association of G0 with immunogenicity and G0 is present on endogenous antibodies, there appears to be no safety impact of the lower level

B. Charged Glycans

- LC-MS & NP-UPLC: some differences in the levels of specific charged glycans such as G1F1NGNA & G2F1NGNA
- Sialic acid (NGNA) Content : The overall NGNA content of the 3 products was highly similar
  • Molar ratios : approximately 0.2 mol sialic acid/mol protein
  • NANA levels were too low to provide robust data for comparison

Figure 7. Comparison of oligosaccharide profiles between CT-P13 and Reference product analysed by normal phase HPLC of AB-labelled N-linked glycans released by PNGase F



3.5. Charge Heterogeneity

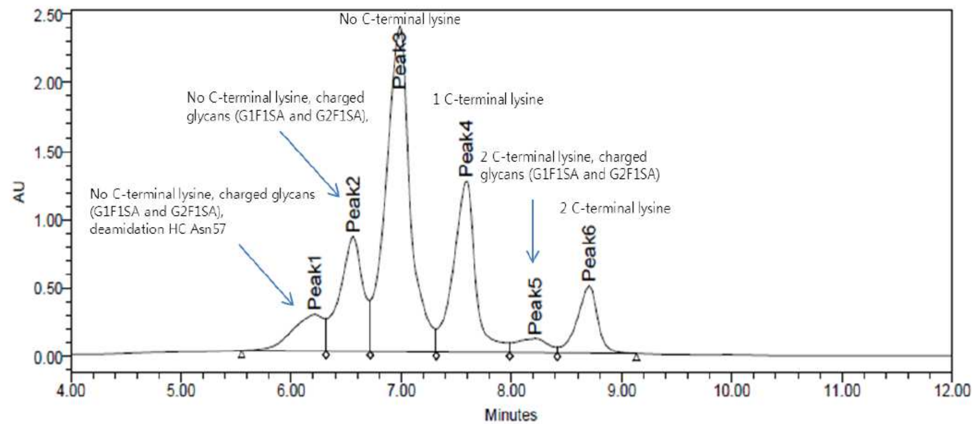
Proper-ties	Attribute	Test Method	Similarity Acceptance criteria	(CT-P13 vs US RBP)	(EU vs US RBP)	Results and Conclusion	Method Suitability
Charge hetero-geneity	Charge isoforms	Iso-Electric Focusing (IEF)	Tier 3 : 8 bands identified	High	High	•Similar band profiles and highly similar pI ranges in 3 products	Acceptable (No clinically meaningful)
	Charge isoforms	IEC-HPLC	Tier 2 : % Peak 1 % Peak 2 % Peak 3 % Peak 4 % Peak 5 % Peak 6	 40 100 100 0 100 100	 90 100 70 90 80 70	• Same charge variant peaks in 3 products • Higher levels of Peak 1 and Peak 4 than US or EU RBP • Mainly due to C-terminal Lys variation -Peak 1/2/3 : K0 variants -Peak 4 : K1 variant -Peak 5/6 : K2 variants	

[Justification of Differences]

A. IEC

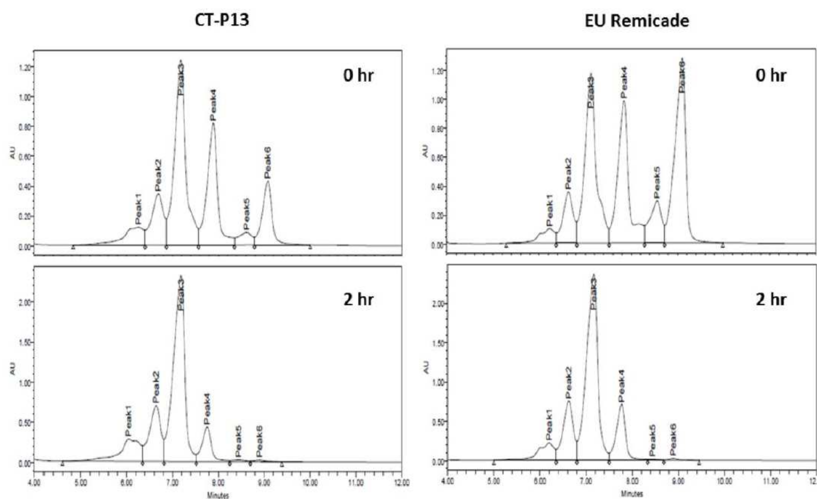
- Identification of IEC-HPLC peaks : confirmed by IEC Analysis with carboxypeptidase treatment
- No clinically meaningful : C-terminal lysine variability had no impact on biological activities in vitro
- Additionally, incubation with IgG-free human serum resulted in rapid clipping of C-terminal lysine residues shown as reductions in Peaks 4, 5, and 6 which contain forms with one or two C-terminal lysine residues (fig 9)
- These results were supported by data obtained from in vitro testing of 12 blood samples taken from patients in the Study CT-P13 12, hereafter referred to as Pilot RA Study, which confirmed rapid C-terminal lysine clipping occurs in blood following infusion

Figure 8. IEC-HPLC Peak Assignment



√ The Results of Peak Identification (fractionation and tryptic peptide mapping)
- The same peaks were detected in 3 products.
- Statistical analysis indicates that CT-P13 contains higher levels of Peak 1 & Peak 4 than US or EU Remicade.
- The difference in IEC-HPLC peaks is attributable to C-terminal lysine variability, with CT-P13 containing higher levels without C-terminal lysine (Peak 1, Peak 2, and Peak 3) and with a single C-terminal lysine (Peak 4), and lower levels of infliximab with 2 C-terminal lysine residues (Peak 5 & Peak 6).

Figure 9. IEC-HPLC Profile of CT-P13 and EU Remicade at 0 and 2 Hours of Serum Incubation



Incubation with IgG-free human serum resulted in rapid clipping of C-terminal lysine residues shown as reductions in Peaks 4, 5, and 6 which contain forms with one or two C-terminal lysine residues.
These results were supported by data obtained from in vitro testing of 12 blood samples taken from patients in the in blood study CT-P13 1.2, hereafter referred to as Pilot RA Study, which confirmed rapid C-terminal lysine clipping occurs following infusion.

3.6. Size Heterogeneity

Properties	Attribute	Test Method	Similarity Acceptance criteria	(CT-P13 vs US RBP)	(EU vs US RBP)	Results and Conclusion	Method Suitability
purity	Size distribution	SEC-HPLC	Tier 2 : % Monomer	0	100	Higher HMW content (mean value 0.8%) and lower monomer content (mean value 99.2%) than US/EU RBP • monomer - 99.2 % of CT-P13, - 99.8% of US RBP - 99.8% of EU RBP	
	Size distribution	R CE-SDS	Tier 2 : % Sum H+L chains, % Non-glycosylated HC	96 96	94 94	• Highly similar to US/EU RBP • %H+L/%NGHC - 99.4 %/0.6 % of CT-P13 - 99.6 %/0.4~0.5 % of US RBP - 99.5 %/0.4~0.5 % of EU RBP	
	Size distribution	NR CE-SDS	Tier 2 : % Intact IgG	0	97	• Slightly lower than US/EU RBP • % IgG - 95.1 % of CT-P13 - 98.2 % of US RBP - 98.3 % of EU RBP • The level of one lot of EU Remicade (97.3%) was also outside the QR (97.38~99.08 %) of US Remicade lots • Fragment : mainly H2L1	

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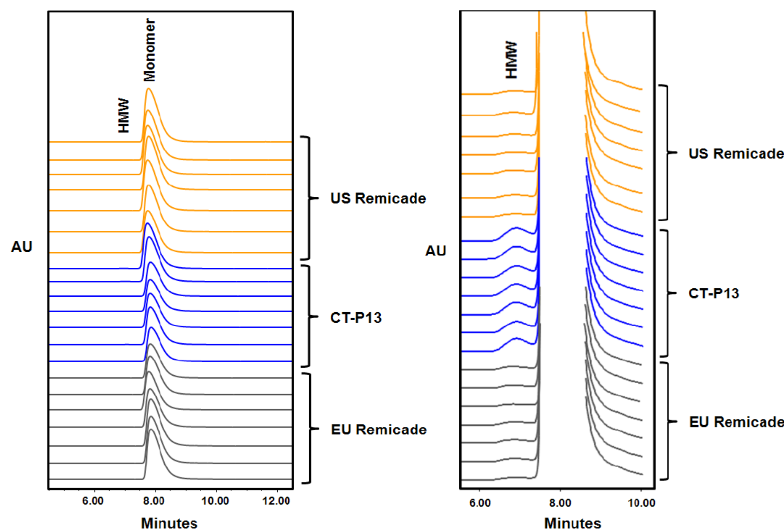
Figure 10. Representative Overlaid SED-HPLC Chromatograms of US Remicade, CT-P13 and EU Remicade from the 3-Way Similarity Study



For all 3 products, a large monomer peak and single peak for HMW species was detected
CT-P13 lots had a higher HMW content (mean value 0.8%) and lower monomer content (mean value 99.2%) than US Remicade lots (99.8% monomer) and EU Remicade lots (99.8% monomer)

3.6. Size Heterogeneity(continued)

Proper-ties	Attribute	Test Method	Similarity Acceptance criteria	(CT-P13 vs US RBP)	(EU vs US RBP)	Results and Conclusion	Method Suitability
purity	HMWS	SV-AUC	Tier 2 : % Monomer % Higher Species	100 100	100 100	highly similar for monomer and HMW content in 3 products •Mainly monomer - 95.6~99.7 % of US Remicade, - 94.2~100 %of EU Remicade, - 95.4~99.8 % of CT-P13 •HMW : dimers~pentamers - 0.3~4.5 % of US Remicade, - 0.0~5.8 %of EU Remicade, - 0.2~4.6 % of CT-P13	
	HMWS	SEC-MALS	Tier 2 : % Monomer % HMW MW Monomer MW HMW	0 0 71 86	100 100 100 100	•Detected mainly monomer & dimer, •Also slightly greater level of HMW forms in CT-P13 - 0.1~0.2 % of US/EU Remicade, - 0.4~0.6 % of CT-P13 •MW monomer - 149~154 kDa of US Remicade, - 150~155 kDa of EU Remicade, - 151~157 kDa of CT-P13, •MW HMW - 286~547 kDa of US Remicade, - 275~537 kDa of EU Remicade, - 244~564 kDa of CT-P13	

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[Justification of Differences]

<u>* higher HMW and lower monomer content : assessed of the clinical relevance in relation to immunogenicity</u>

A. SEC profiles

- Not to be clinically meaningful in repeat-dose studies (RA & AS) : No impact on similarity of PK, immunogenicity, or efficacy (Incidence rates of ADAs, Neutralizing Antibody (NAb) levels and titer values were similar)

B. NR CE-SDS

- Analysis of the TNFα binding affinity and in vitro TNFα neutralization assays of samples with different content of H2L1 : No impact was detected for H2L1 levels up to 5.7%
- Literature reports indicate that monovalent antibody fragments induce apoptosis with equal potency as bivalent molecule (Schaefer, 2011) and have no effect on FcRn binding, providing assurance that tmTNFα signaling activities and PK are unlikely to be affected

✓ **Slightly different between results of SEC-HPLC, SEC-MALS and AUC due to analytic mechanisms**

- SEC-HPLC and SEC-MALS use a chromatography step which may prevent detection of large multimers which do not enter the matrix whereas AUC does not involve a chromatography step.
- However, the limit of detection of AUC has been reported to be higher than that of SEC *(Manning et al, 2014)* .

3.7. Sub-visible particles, Protein content, Absorption Coefficient and Excipients

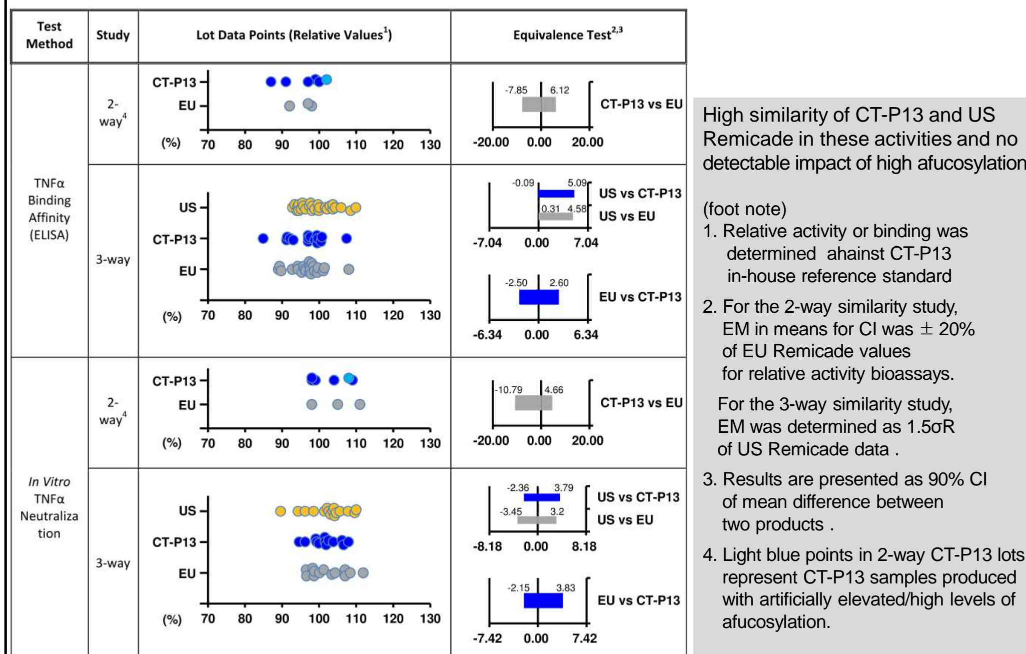
Properties	Attribute	Test Method	Similarity Acceptance criteria	(CT-P13 vs US RBP)	(EU vs US RBP)	Results and Conclusion	Method Suitability
purity	Sub-visible Particulates (1~10 μm size ranges)	Micro-Flow Imaging (MFI)	Tier 1 : 1 ≤, < 100 (μm) 2 ≤, < 100 (μm) 5 ≤, < 100 (μm) 10 ≤, < 100 (μm)	All Within EM	All Within EM	Similar	
	Sub-visible Particulates (1~10 μm size ranges)	Light Obscuration (HIAC)	Tier 1 : 2 ≤ (μm) 5 ≤ (μm) 10 ≤ (μm)	All Within EM	All Within EM	Similar	
Protein content	Protein Content	Protein Concentration (UV280)	Tier 1 & 2 (reconstituted product)	Within EM, 100	- 92	within the EM, statistically within the QR	
	Absorption coefficient	Amino Acid Analysis	Tier 1 & 2	Within EM, 100	- -	Highly similar	
Excipients	pH	pH	Tier 2 :	100	100	Highly similar	
	Polysorbate80 amount	HPLC	Tier 2 : PS80 (w/v%)	100	100	Highly similar	
	Sucrose amount	HPAEC-PAD	Tier 2 : Sucrose (w/v%)	92	100	Highly similar	

* Used the theoretical extinction coefficient of 1.45 which was confirmed by amino acid analysis

3.8. Biological Activities : Fab-related

Properties	Attribute	Test Method	Similarity Acceptance criteria (90% CI of mean diff)	(CT-P13 vs US RBP)	(EU vs US RBP)	Results and Conclusion	Method Suitability
Binding to sTNFα	MoA & Efficacy- all indications	In Vitro TNFα Neutralization	% Relative activity by cell viability	Within EM	Within EM	Similar	
		TNFα Binding Affinity (ELISA)	% Relative binding (EC50)	Within EM	Within EM	Similar	
	MoA & Efficacy- CD & UC	Caco-2 (Cytokine Suppression))	% Relative activity Combined conc At 10 μg/mL At 2 μg/mL At 0.4 μg/mL	Within EM Within EM Within EM Within EM	Within EM Within EM Within EM Not Within EM	Similar	
Binding to tmTNFα	MoA & Efficacy- CD & UC	Cell Based Binding Affinity	% Relative binding (EC50)	Within EM	Within EM	Similar	
		Inhibition of Cytokine Release by Reverse Signaling	% Relative activity Combined conc At 5.3 μg/mL At 2.4 μg/mL At 1.1 μg/mL	Within EM Within EM Within EM Within EM	Within EM Within EM Not Within EM Within EM	Similar	

Figure 11. Results and Statistical Analysis of Binding and Neutralization of sTNFα by CT-P13, ALAG CT-P13, EU Remicade and US Remicade



High similarity of CT-P13 and US Remicade in these activities and no detectable impact of high afucosylation

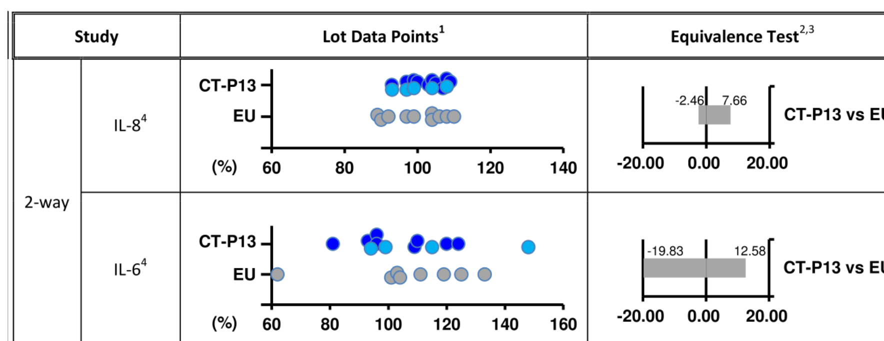
(foot note)

1. Relative activity or binding was determined ahainst CT-P13 in-house reference standard
2. For the 2-way similarity study, EM in means for CI was ± 20% of EU Remicade values for relative activity bioassays.

   For the 3-way similarity study, EM was determined as 1.5σR of US Remicade data .
3. Results are presented as 90% CI of mean difference between two products .
4. Light blue points in 2-way CT-P13 lots represent CT-P13 samples produced with artificially elevated/high levels of afucosylation.

Figure 12. Results and Statistical Analysis of sTNFα Blockade Using In Vitro IBD Model Using Caco-2 Cells (Suppression of Cytokines) by CT-P13, ALAG CT-P13, EU Remicade and US Remicade

** ALAG: Artificially elevated levels of afucosylated glycans



✓High similarity of CT-P13 and US Remicade in these activities and no detectable impact of high afucosylation

✓The CI for mean difference for EU Remicade at the lowest concentration 0.4 μg/mL (-0.47, 6.67) was slightly outside the EM of US Remicade (-6.61, 6.61)

✓At both of the higher concentrations, the CI for mean difference for EU Remicade was within the EM of US Remicade and if the data are analyzed using the QR approach, all values for EU Remicade are within QR of US Remicade lots when tested at 0.4 μg/mL

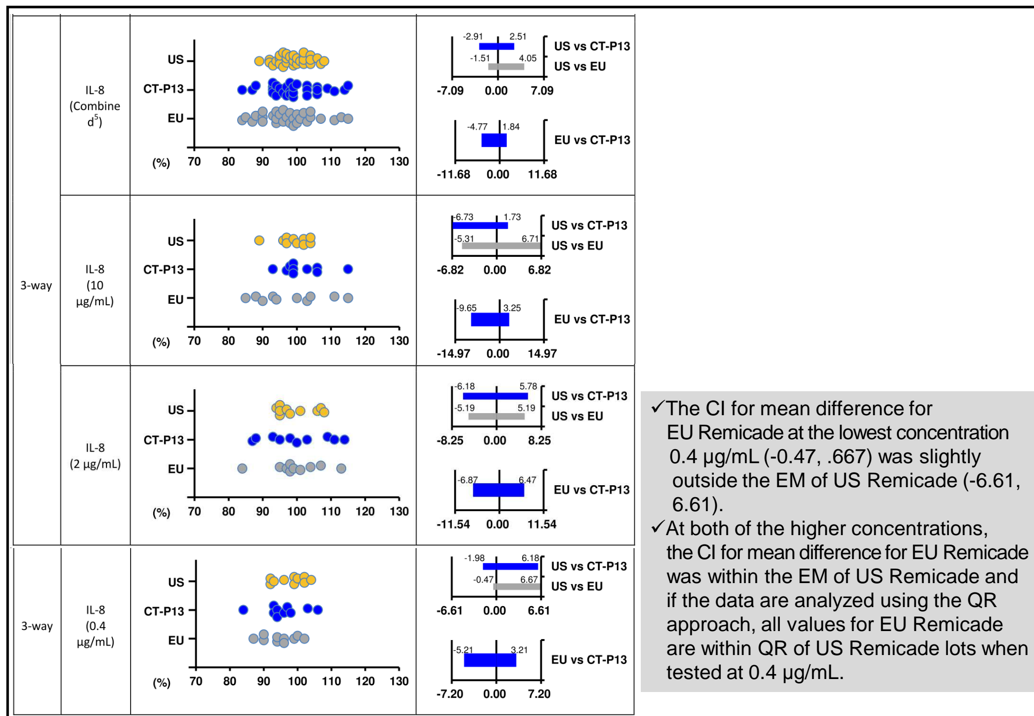
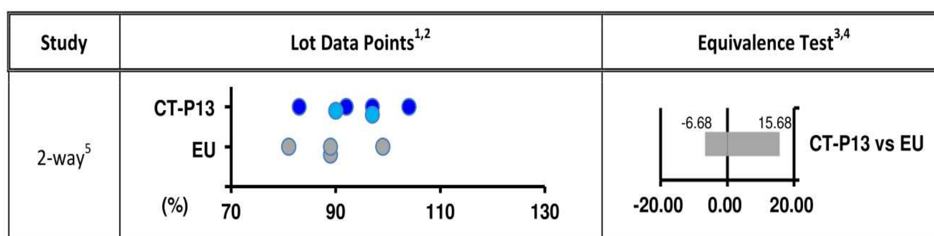


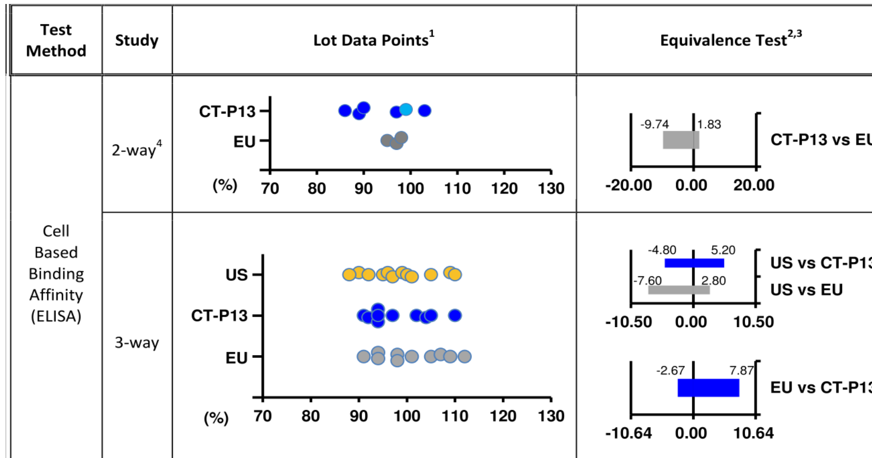
Figure 13. Results and Statistical Analysis of sTNFα Blockade Using In Vitro IBD Model Using Caco-2 Cells (Anti-apoptotic Effect) by CT-P13, ALAG CT-P13 and EU Remicade



- ✓ Study the ability of infliximab to suppress apoptosis through neutralization of sTNFα induced by treatment with cytokines and LPS
- ✓ Afucosylation had no detectable impact on suppression of apoptosis through neutralization of sTNFα
- ✓ Both CT-P13 and EU Remicade can effectively and highly similarly protect intestinal epithelial cells from apoptosis caused by inflammatory cytokines secreted in IBD

< Transmembrane TNFα Binding Activities >

Figure 14. Results and Statistical Analysis of Binding of Transmembrane-Bound TNFα by CT-P13, ALAG CT-P13, EU Remicade and US Remicade (Cell Based Binding Affinity)

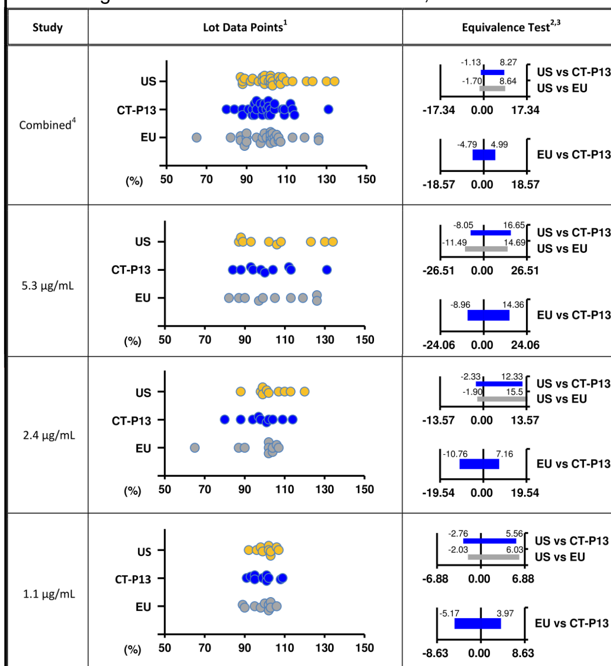


✓The comparative binding affinity of CT-P13, US and EU Remicade to tmTNFα expressed on Jurkat cells was determined using a cell based ELISA at 3 concentrations in 3-way similarity studies, and at 4 concentrations in 2-way similarity studies

✓The comparison of the mean EC50 of the internal reference standard to the mean EC50 of the sample

✓No detectable impact of high afucosylation

Figure 15. Results and Statistical Analysis of Cytokine Release by Reverse Signaling on Binding tmTNFα : Studies of US Remicade, CT-P13 and EU Remicade (3-way Study)



< Cytokine Suppression through Reverse Signaling >

Figure 16. Reverse Signaling: Suppression of TNFα Release from LPS-stimulated PBMC by TNFα inhibitors

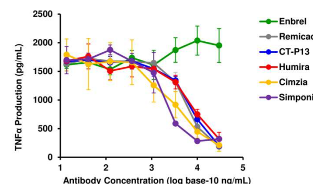
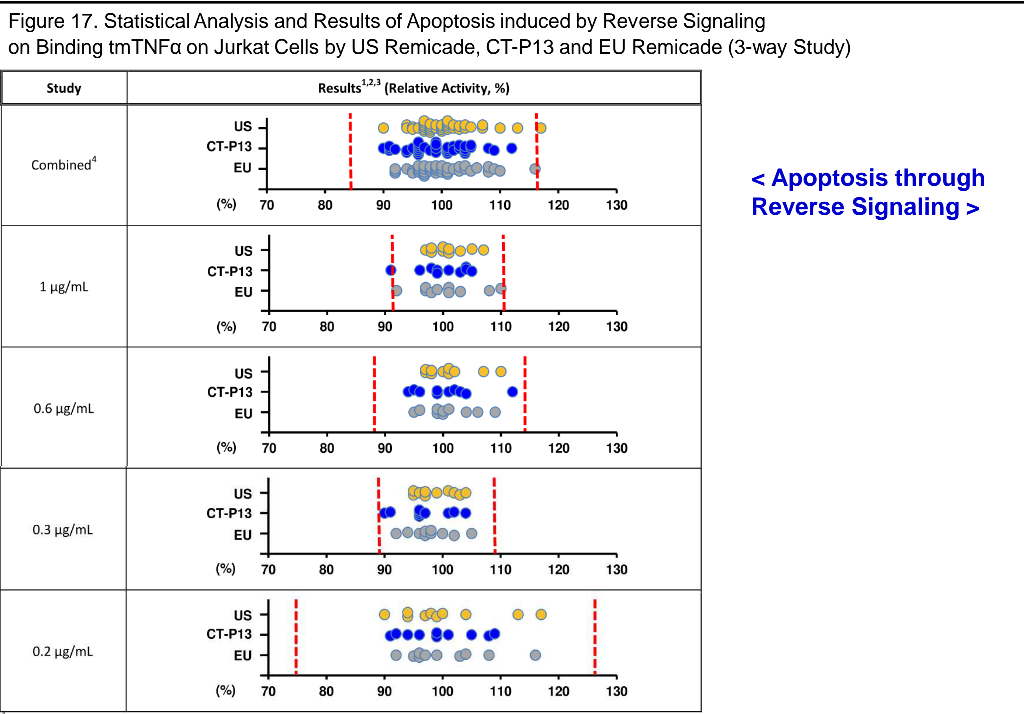


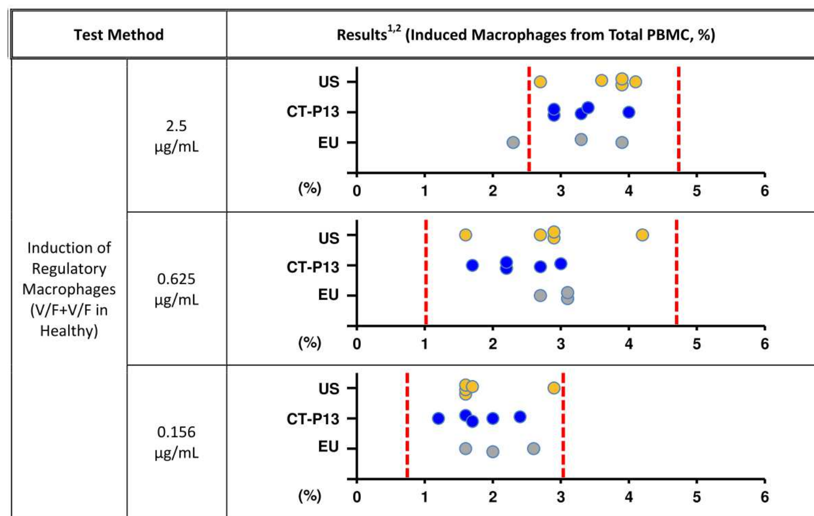
Figure 17. Statistical Analysis and Results of Apoptosis induced by Reverse Signaling on Binding tmTNFα on Jurkat Cells by US Remicade, CT-P13 and EU Remicade (3-way Study)



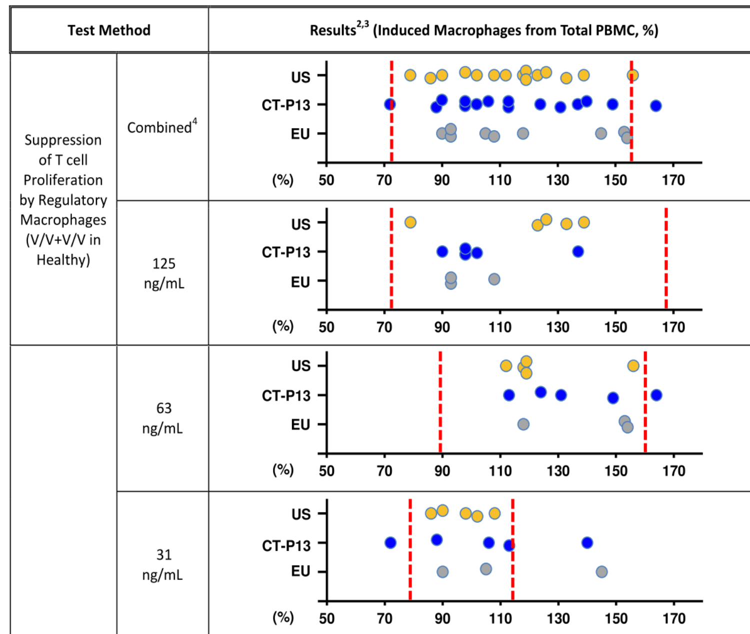
< Apoptosis through Reverse Signaling >

< Induction of Regulatory Macrophages – MLR Assay & Wound Healing >

Figure 18. Quality Range of Statistical Analysis Result for Induction of Regulatory Macrophages By US Remicade, CT-P13 and EU Remicade (3-way Study)

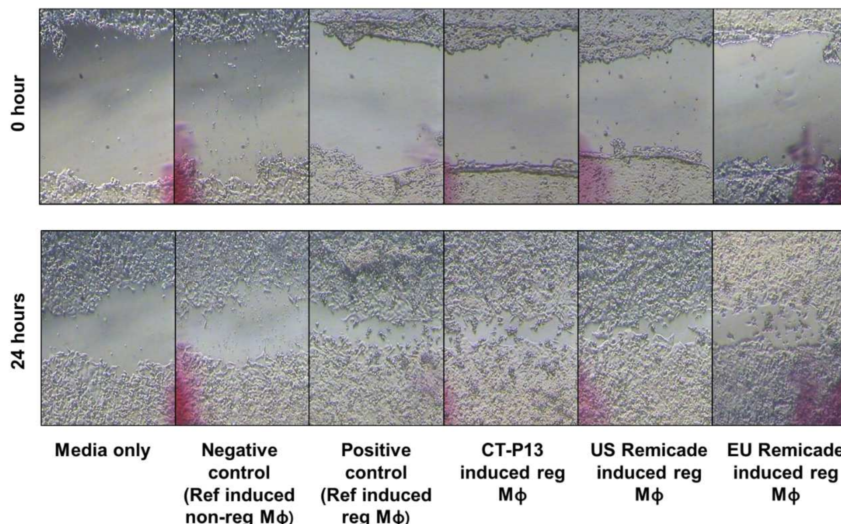


< Induction of Regulatory Macrophages – MLR Assay & Wound Healing >



< Induction of Regulatory Macrophages – MLR Assay & Wound Healing >

Figure 19. Example of Comparative Wound Healing of Colorectal Epithelial Cells by Regulatory Macrophages Induced in a Mixed Lymphocyte Reaction in 3-way Study



Ref: CT-P13 in-house reference standard, reg Mϕ: regulatory macrophages

3.9. Biological Activities : Fc-related

Properties	Attribute	Test Method	Similarity Acceptance Criteria	(CT-P13 vs US RBP)	(EU vs US RBP)	Results and Conclusion	Method Suitability
Binding assay	FcγRI binding affinity	ELISA	Tier 2	100	100	Similar	
	FcγRIIa binding affinity	SPR	Tier 2	100	100	Similar	
	FcγRIIb binding affinity	SPR	Tier 2	100	100	Similar	
	FcγRIIIa V type binding affinity	SPR	Tier 2	85	100	Not similar (Lower)	
	FcγRIIIa F type binding affinity	SPR	Tier 2	61	100	Not similar (Lower)	
	FcγRIIIb type binding affinity	SPR	Tier 2	90	100	Similar, BUT Lower	
	NK cell binding affinity via Fc receptors (in presence of 50% serum or 1% BSA)	Ex Vivo NK Cell Binding, 1% BSA	Tier 2 - % Relative binding combined conc. - At 50 μg/mL - At 10 μg/mL - At 2 μg/mL	33 0 0 33	100 100 67 100	Not similar (Lower)	
		Ex Vivo NK Cell Binding, 50% Serum		89 100 100 67	89 100 33 100	Not similar - Yes: 50 μg/mL, 10 μg/mL - No: 2 μg/Ml	
	FcRn binding affinity	SPR	Tier 2	Within EM	Within EM	Similar	
	C1q binding assay	ELISA	Tier 2	100	100	Similar	

3.9. Biological Activities : Fc-related(continued)

Properties	Attribute	Test Method	Similarity Acceptance criteria	(CT-P13 vs US RBP)	(EU vs US RBP)	Results and Conclusion	Method Suitability
Bioassay/ mechanism of action exploration	CDC		Tier 2	92	91	Similar	
	ADCC	PBMC as effectors	Tier 2	100	100	Similar	
		NK cells as effectors	Tier 2 - % Relative activity - Combined conc - At 8 ng/mL - At 4 ng/mL - At 2 ng/mL	96 100 96 96	99 100 97 97	Similar, BUT Lower	
		LPS-stimulated monocytes as targets (Healthy Donor)	Tier 3 - At 8 concentrations (0.000013 μg/mL to 1 μg/mL)	No activity	No activity	Similar	
		LPS-stimulated monocytes as targets (IBD Patients)	Tier 3 - At 10 μg/mL and 50 μg/mL	No-Low activity	-	Similar	

<FcγRIIIa binding including FcγRIIIa of different allotypes (V/V, V/F, or F/F at position 158)>

Table 12. Binding to FcγIIIa of Different Genotypes

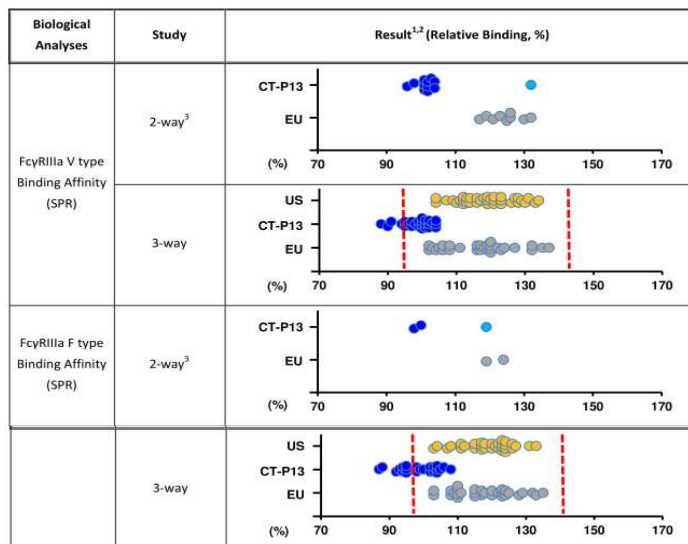
Biological Analysis	Genotype	Product	Absolute Value (KD [μM] or MFI)[3]
FcγRIIIa Binding Affinity (SPR)[1]	V	US Remicade	1.41
		CT-P13	1.79
		EU Remicade	1.40
	F	US Remicade	4.43
		CT-P13	5.33
		EU Remicade	4.52
FcγRIIIa Binding Affinity (*Ex vivo*, 1% BSA)[2]	V	CT-P13	1,475.7
		EU Remicade	1,927.7
	F	CT-P13	1,233.7
		EU Remicade	1,171.0

[1]Absolute values are KD values.
[2]Absolute values are MFI values.
[3]The lower the KD value, the higher the binding affinity.
BSA: Bovine serum albumin , KD: Dissociation equilibrium binding constant, MFI: Mean fluorescence intensity , SPR: Surface plasmon resonance

The difference observed in binding between CT-P13 and both US and EU Remicade is small in comparison to the difference in binding of US Remicade to FcγRIIIa of different genotypes

< FcγRIIIa binding including FcγRIIIa of different allotypes (V/V, V/F, or F/F at position 158) by SPR >

Figure 20. Quality Range Analysis of Fcγ Receptor Binding by CT-P13, ALAG CT-P13, EU Remicade and US Remicade



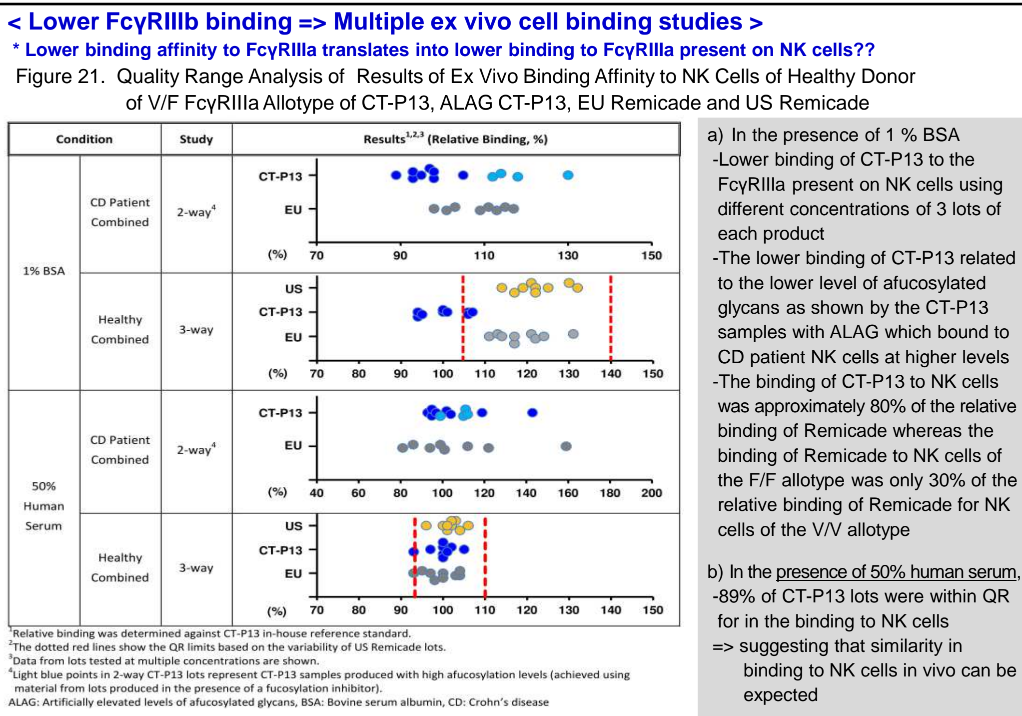
[1]Relative binding was determined against CT-P13 in-house reference standard.
[2]The dotted red lines show the QR limits based on the variability of US Remicade lots.
[3]Light blue points in CT-P13 lots represent CT-P13 samples produced with high afucosylation levels (achieved using material from lots produced in the presence of a fucosylation inhibitor).
ALAG: Artificially elevated levels of afucosylated glycans, SPR: Surface plasmon resonance

- ✓ A slightly lower binding affinity (V&F allotypes) for CT-P13 lots in comparison to EU/US Remicade => reflecting the lower level of afucosylated glycans
- ✓ But, the KD values for US Remicade and CT-P13 fall into a narrow range of roughly, 1.2~2.3 μM for the FcγRIIIa V allotype, 3.9~6.3 μM for the FcγRIIIa F allotype and 9.11~15.6 μM for the FcγRIIIb

< Lower FcγRIIIb binding => Multiple ex vivo cell binding studies >

*** Lower binding affinity to FcγRIIIa translates into lower binding to FcγRIIIa present on NK cells??**

Figure 21. Quality Range Analysis of Results of Ex Vivo Binding Affinity to NK Cells of Healthy Donor of V/F FcγRIIIa Allotype of CT-P13, ALAG CT-P13, EU Remicade and US Remicade



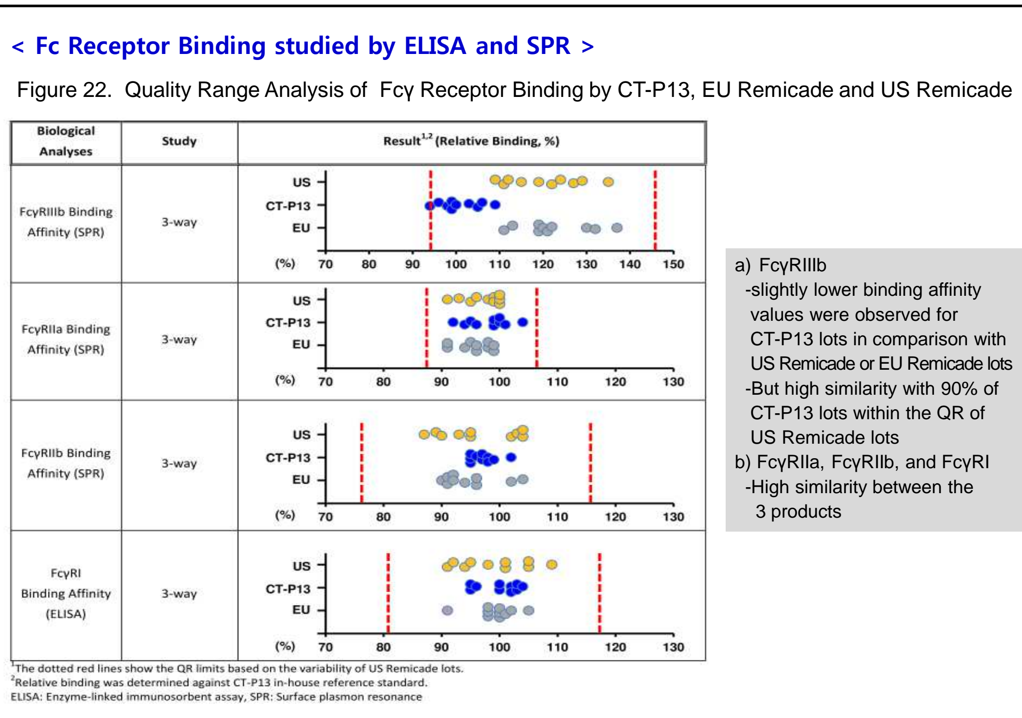
[1]Relative binding was determined against CT-P13 in-house reference standard.
[2]The dotted red lines show the QR limits based on the variability of US Remicade lots.
[3]Data from lots tested at multiple concentrations are shown.
[4]Light blue points in 2-way CT-P13 lots represent CT-P13 samples produced with high afucosylation levels (achieved using material from lots produced in the presence of a fucosylation inhibitor).
ALAG: Artificially elevated levels of afucosylated glycans, BSA: Bovine serum albumin, CD: Crohn's disease

a) In the presence of 1 % BSA
- Lower binding of CT-P13 to the FcγRIIIa present on NK cells using different concentrations of 3 lots of each product
- The lower binding of CT-P13 related to the lower level of afucosylated glycans as shown by the CT-P13 samples with ALAG which bound to CD patient NK cells at higher levels
- The binding of CT-P13 to NK cells was approximately 80% of the relative binding of Remicade whereas the binding of Remicade to NK cells of the F/F allotype was only 30% of the relative binding of Remicade for NK cells of the V/V allotype

b) In the presence of 50% human serum,
- 89% of CT-P13 lots were within QR for in the binding to NK cells
=> suggesting that similarity in binding to NK cells in vivo can be expected

< Fc Receptor Binding studied by ELISA and SPR >

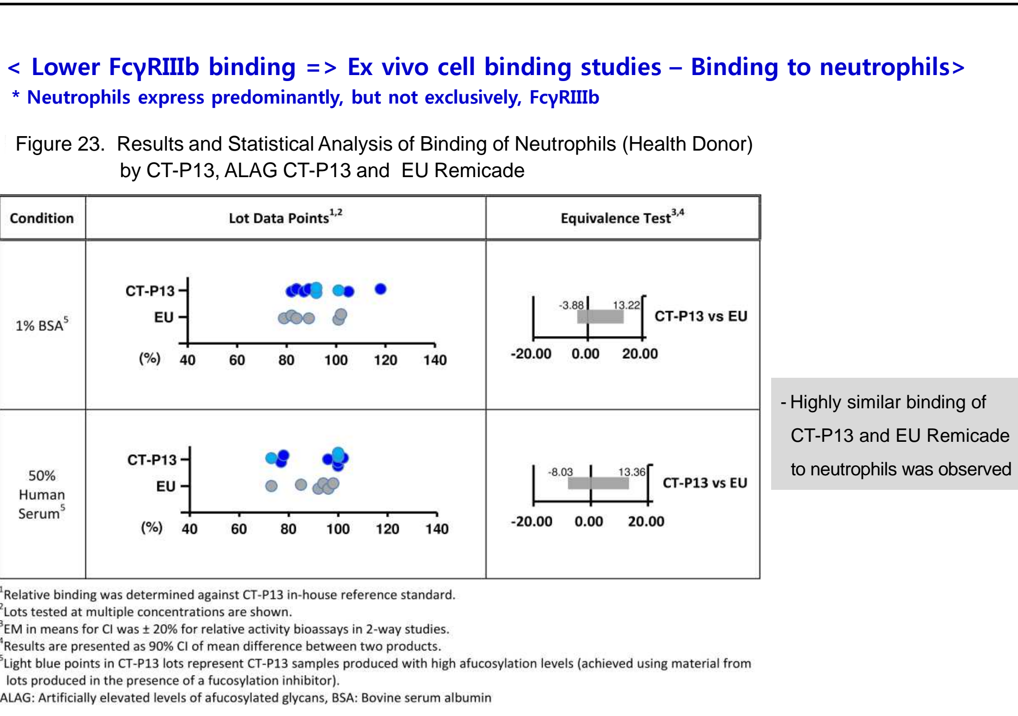
Figure 22. Quality Range Analysis of Fcγ Receptor Binding by CT-P13, EU Remicade and US Remicade



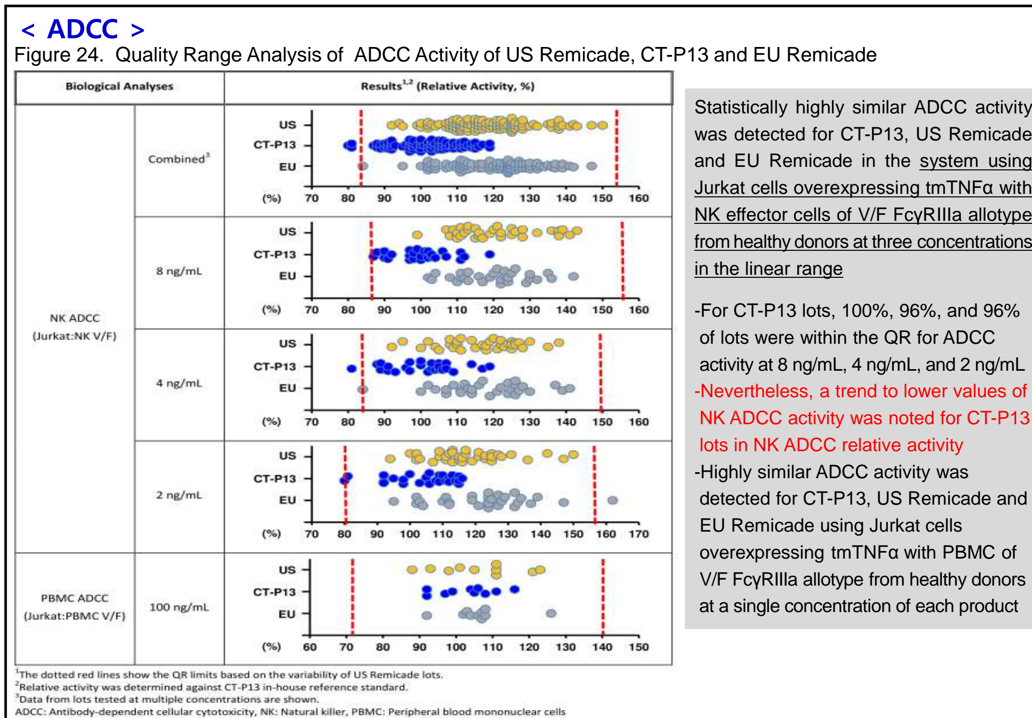
[1]The dotted red lines show the QR limits based on the variability of US Remicade lots.
[2]Relative binding was determined against CT-P13 in-house reference standard.
ELISA: Enzyme-linked immunosorbent assay, SPR: Surface plasmon resonance

a) FcγRIIIb
- slightly lower binding affinity values were observed for CT-P13 lots in comparison with US Remicade or EU Remicade lots
- But high similarity with 90% of CT-P13 lots within the QR of US Remicade lots

b) FcγRIIa, FcγRIIb, and FcγRI
- High similarity between the 3 products



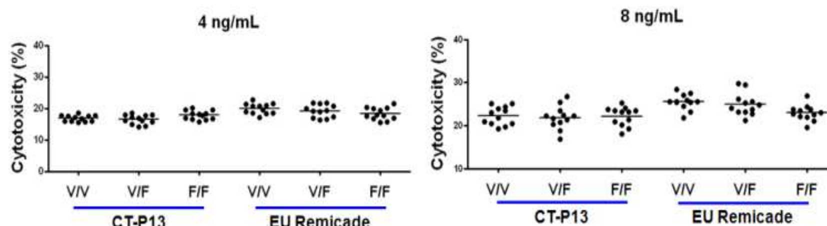
< Lower FcγRIIIb binding => Ex vivo cell binding studies – Binding to neutrophils>
* Neutrophils express predominantly, but not exclusively, FcγRIIIb
Figure 23. Results and Statistical Analysis of Binding of Neutrophils (Health Donor) by CT-P13, ALAG CT-P13 and EU Remicade
Condition
Lot Data Points[1,2]
Equivalence Test[3,4]
1% BSA[5]
CT-P13
EU
(%) 40 60 80 100 120 140
-3.88
13.22
CT-P13 vs EU
-20.00 0.00 20.00
50% Human Serum[5]
-8.03
13.36
- Highly similar binding of CT-P13 and EU Remicade to neutrophils was observed
[1]Relative binding was determined against CT-P13 in-house reference standard.
[2]Lots tested at multiple concentrations are shown.
[3]EM in means for CI was ± 20% for relative activity bioassays in 2-way studies.
[4]Results are presented as 90% CI of mean difference between two products.
[5]Light blue points in CT-P13 lots represent CT-P13 samples produced with high afucosylation levels (achieved using material from lots produced in the presence of a fucosylation inhibitor).
ALAG: Artificially elevated levels of afucosylated glycans, BSA: Bovine serum albumin



< ADCC >
Figure 24. Quality Range Analysis of ADCC Activity of US Remicade, CT-P13 and EU Remicade
Biological Analyses
Results[1,2] (Relative Activity, %)
NK ADCC (Jurkat:NK V/F)
Combined[3]
8 ng/mL
4 ng/mL
2 ng/mL
PBMC ADCC (Jurkat:PBMC V/F)
100 ng/mL
US
CT-P13
EU
(%) 70 80 90 100 110 120 130 140 150 160
(%) 70 80 90 100 110 120 130 140 150 160 170
(%) 60 70 80 90 100 110 120 130 140 150
Statistically highly similar ADCC activity was detected for CT-P13, US Remicade and EU Remicade in the system using Jurkat cells overexpressing tmTNFα with NK effector cells of V/F FcγRIIIa allotype from healthy donors at three concentrations in the linear range
-For CT-P13 lots, 100%, 96%, and 96% of lots were within the QR for ADCC activity at 8 ng/mL, 4 ng/mL, and 2 ng/mL
-Nevertheless, a trend to lower values of NK ADCC activity was noted for CT-P13 lots in NK ADCC relative activity
-Highly similar ADCC activity was detected for CT-P13, US Remicade and EU Remicade using Jurkat cells overexpressing tmTNFα with PBMC of V/F FcγRIIIa allotype from healthy donors at a single concentration of each product
[1]The dotted red lines show the QR limits based on the variability of US Remicade lots.
[2]Relative activity was determined against CT-P13 in-house reference standard.
[3]Data from lots tested at multiple concentrations are shown.
ADCC: Antibody-dependent cellular cytotoxicity, NK: Natural killer, PBMC: Peripheral blood mononuclear cells

< ADCC & FcγRIIIa allotype >

Figure 25. ADCC Activity Using tmTNFα Jurkat Target Cells and NK Effector Cells of Different FcγIIIa Genotypes

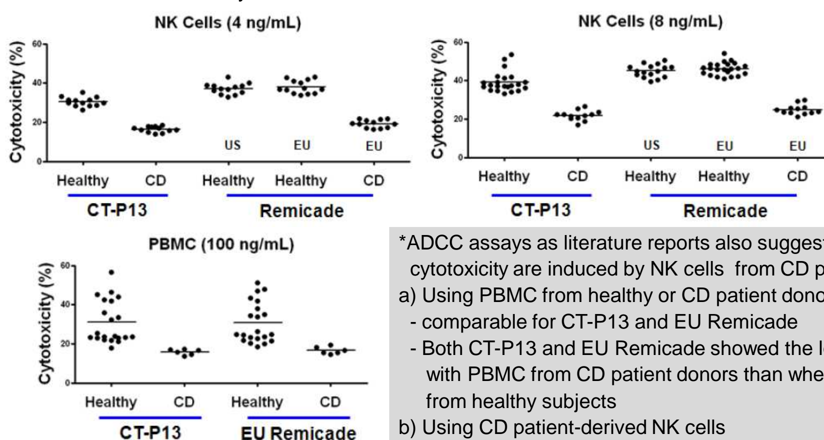


✓ADCC assays with cells from healthy subjects of different FcγRIIIa genotype as the FcγRIIIa allotypes have different binding affinity for the Fc region of IgG1, with NK cells of V/V genotype reported to bind significantly more IgG1 than NK cells of the F/F genotype

- There was significant overlap of the absolute cytotoxicity values obtained using NK cells of the 3 allotypes in NK ADCC assays
- Highly similar ADCC activity was detected for CT-P13 and EU Remicade when using transfected Jurkat target cells and NK effector cells of F/F FcγRIIIa allotype

< ADCC induced by NK cells from CD patients >

Figure 26. ADCC Activity tmTNFα Jurkat Target Cells and NK Cells(V/F) or PBMC (V/F) from Healthy Donors or CD Patients

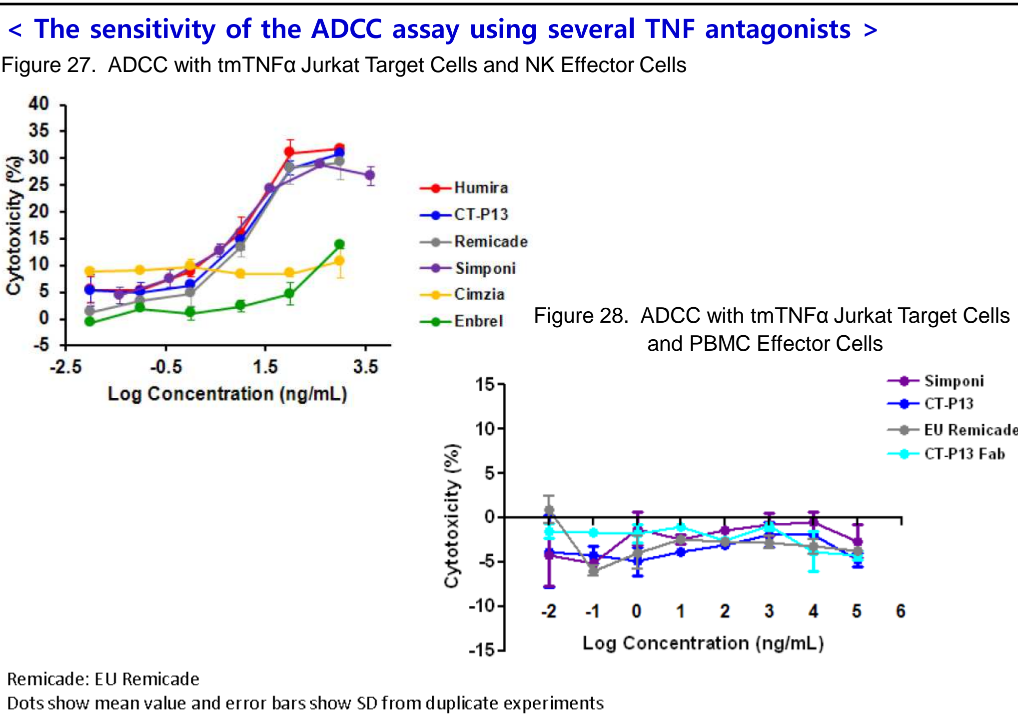


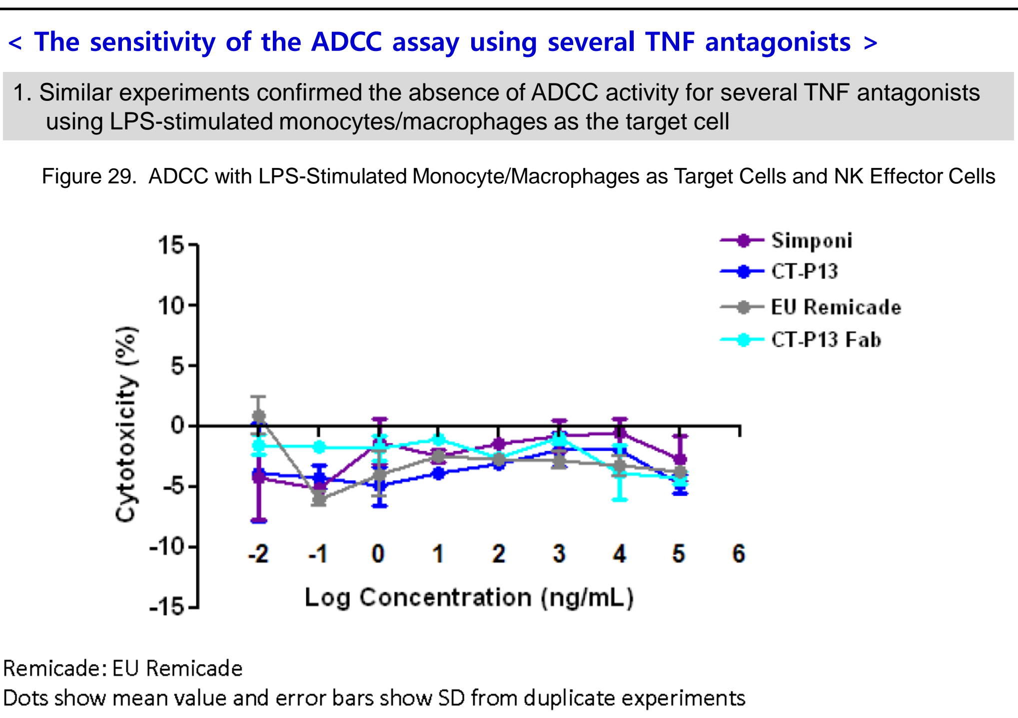
*ADCC assays as literature reports also suggest low levels of cytotoxicity are induced by NK cells from CD patients

a) Using PBMC from healthy or CD patient donors
- comparable for CT-P13 and EU Remicade
- Both CT-P13 and EU Remicade showed the lower cytotoxicity with PBMC from CD patient donors than when using PBMC from healthy subjects

b) Using CD patient-derived NK cells
- lower levels of ADCC activity for both CT-P13 and EU Remicade compared to NK effector cells isolated from healthy donors

⇒ The small difference in mean FcγRIIIa binding affinity, is unlikely to be clinically meaningful in terms of NK ADCC activity in CD patients or in terms of PBMC-mediated ADCC activity in any patient group

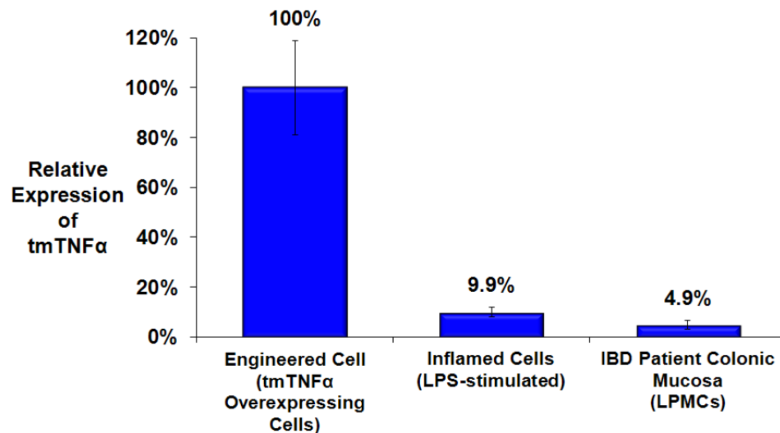

< The sensitivity of the ADCC assay using several TNF antagonists >
Figure 27. ADCC with tmTNFα Jurkat Target Cells and NK Effector Cells
Cytotoxicity (%)
40
35
30
25
20
15
10
5
0
-5
-2.5
-0.5
1.5
3.5
Log Concentration (ng/mL)
Humira
CT-P13
Remicade
Simponi
Cimzia
Enbrel
Figure 28. ADCC with tmTNFα Jurkat Target Cells and PBMC Effector Cells
Cytotoxicity (%)
15
10
5
0
-5
-10
-15
-2
-1
0
1
2
3
4
5
6
Log Concentration (ng/mL)
Simponi
CT-P13
EU Remicade
CT-P13 Fab
Remicade: EU Remicade
Dots show mean value and error bars show SD from duplicate experiments


< The sensitivity of the ADCC assay using several TNF antagonists >
1. Similar experiments confirmed the absence of ADCC activity for several TNF antagonists using LPS-stimulated monocytes/macrophages as the target cell
Figure 29. ADCC with LPS-Stimulated Monocyte/Macrophages as Target Cells and NK Effector Cells
Cytotoxicity (%)
15
10
5
0
-5
-10
-15
-2
-1
0
1
2
3
4
5
6
Log Concentration (ng/mL)
Simponi
CT-P13
EU Remicade
CT-P13 Fab
Remicade: EU Remicade
Dots show mean value and error bars show SD from duplicate experiments

< The sensitivity of the ADCC assay using several TNF antagonists >

2. The expression of tmTNFα on monocytes/macrophages from CD patient LPMC was approximately 50% lower than on monocytes/macrophages from LPS-stimulated PBMC from healthy donors, and tmTNFα was expressed at only 2% (50-fold lower) of the level obtained with tmTNFα Jurkat cells

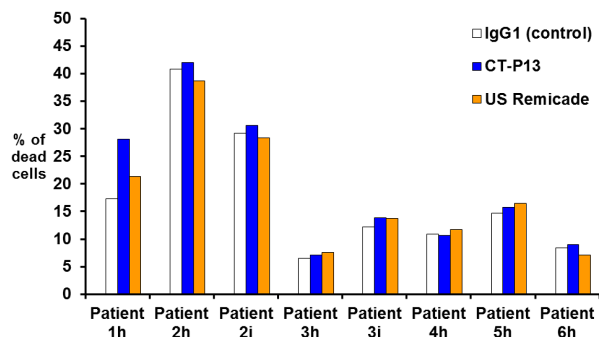
Figure 30. tmTNFα Expression Levels on LPMC from Patient Mucosa, LPS-stimulated Monocytes/Macrophages and tmTNFα Jurkat Cells



LPMC: Lamina propria mononuclear cells, LPS: Lipopolysaccharide , IBD: Inflammatory bowel disease

< The clinical effect of infliximab in IBD >

Figure 31. ADCC Activity of CT-P13 Drug Product and US Remicade Using IBD Patient-derived LPMC as Target Cells



✓ NK cell-mediated ADCC activity of CT-P13 and US Remicade against target cells representative of the inflammatory environment, namely LPMC

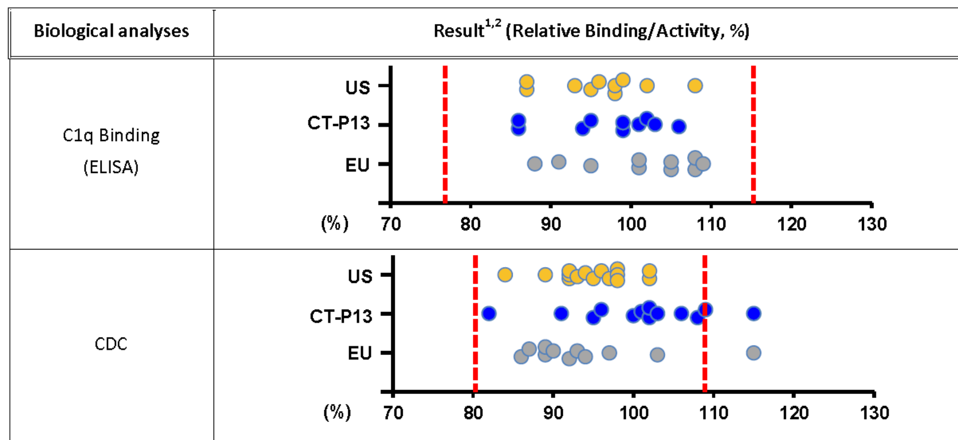
- Infliximab-mediated ADCC of LPMC from the intestine of IBD patients is very low and similar levels of cell death were observed in the presence of a control IgG1
- Robust (non-ADCC-mediated) natural killer cytotoxicity was exerted by these IBD patient-derived NK cells against a cancer cell line
- No difference in ADCC of LPMC was observed between CT-P13 and US Remicade at concentrations (50 μg/mL) equivalent to high-end concentrations achieved in the blood of patients early after infliximab infusion

⇒There is no clinically meaningful difference in ADCC-mediated cell death of IBD patient monocytes conferred by US Remicade and CT-P13

< Complement Binding & CDC Activity >

✓All CT-P13 and EU Remicade lots were within the QR of US Remicade lots for C1q binding
✓CT-P13 and EU Remicade were also highly similar to US Remicade in CDC activity with 92.3% and 90.9% of data points within the QR, respectively

Figure 32. Quality Range Analysis of Binding of C1q and CDC Activity by US Remicade, CT-P13 and EU Remicade (3-way)

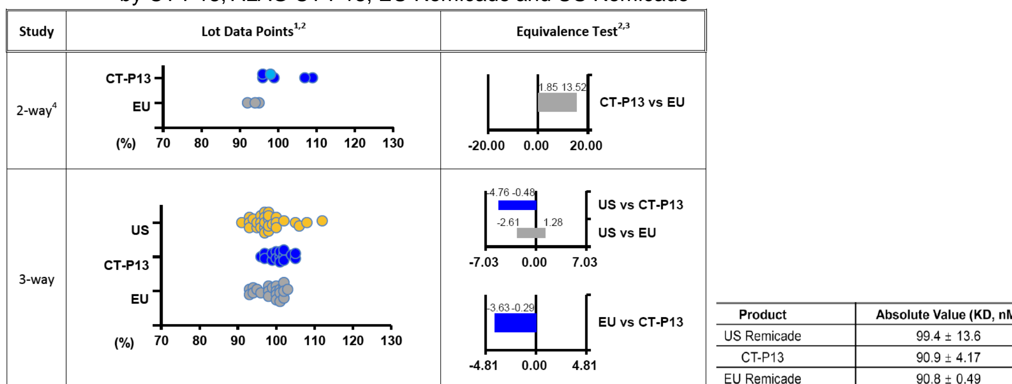


[1]The dotted red lines show the QR limits based on the variability of US Remicade lots.
[2]Relative binding or activity was determined against CT-P13 in-house reference standard.

< FcRn Binding Affinity by SPR >

✓CT-P13 lots were within the EM of US Remicade and of EU Remicade
✓However the 90% CI of mean difference did not include 0
✓The mean and SD values of FcRn binding affinity for CT-P13 was 101±2.7% whereas the mean and SD values for US Remicade were 98±4.7% and for EU Remicade was 99±3.2%

Figure 33 Results and Statistical Analysis of Binding of FcRn by CT-P13, ALAG CT-P13, EU Remicade and US Remicade



Product	Absolute Value (KD, nM)
US Remicade	99.4 ± 13.6
CT-P13	90.9 ± 4.17
EU Remicade	90.8 ± 0.49

[1]Relative binding was determined against CT-P13 in-house reference standard.
[2]For the 2-way similarity study, EM in means for CI ± 20% of EU Remicade values. For the 3-way similarity study, EM was determined as 1.5σ_R of US Remicade data.
[3]Results are presented as 90% CI of mean difference between two products.
[4]Light blue point in 2-way CT-P13 lots represent CT-P13 samples produced with high afucosylation levels (achieved using material from lots produced in the presence of a fucosylation inhibitor).

3.10. Evaluation of the Comparability of Stability Profiles

□ Protocols of the Comparative Stability Studies

a) 2-way data (with EU Remicade)
- Real-time/real-temperature (5±3℃), Accelerated (25±2°C/60±5%RH),
- Stress conditions (40±2°C/75±5%RH)

b) 3-way data (with US Remicade)
- Stress conditions (40±2°C/75±5%RH),
- Forced degradation studies (Low/High pH, Oxidation, High Temp)
  : side-by-side comparison using each 2 batches of CT-P13 and US Remicade

□ Results

a) Real-time/real-temperature conditions : no appreciable changes
b) Accelerated conditions : no significant changes
c) Stress conditions (higher temperature) : trend of decreasing purities (SEC-HPLC, NR CE-SDS)

□ Discussions

a) highly similar stability profiles in all comparative stability studies
b) Under the forced degradation studies : confirmed the comparative degradation profiles

Table 13. Summary of Comparative Stability Studies of CT-P13 and Remicade

Stability	Conditions	Duration	CT-P13 Lots	US Remicade Lots	EU Remicade Lots	Stability Profile
Real-time	5 ± 3°C	36 m (CT-P13) 24 m (Remicade)	✓	-	✓	Comparable
Accelerated	25 ± 2°C, 60 ± 5% RH	6 m	✓	-	✓	Comparable
Stressed	40 ± 2°C, 75 ± 5% RH	3 m	✓	✓	✓	Comparable
In-use (reconstitution)	5 ± 3°C	48 h	✓	-	✓	Comparable
	30 ± 2°C, 65 ± 5% RH					
In-use (infusion)	5 ± 3°C	48 h	✓	-	✓	Comparable
	30 ± 2°C, 65 ± 5% RH					
	30 ± 2°C, 75 ± 5% RH					
	30 ± 2°C, 75 ± 5% RH	48 h	✓	✓	-	Comparable
Forced degradation	Low pH (pH 2.9, 5°C)	4 d, 8 d	✓	✓	-	Comparable
	High pH (pH 11.0, 5°C)	2 d, 4 d				Comparable
	Oxidation (0.005% H_2O_2, 5°C)	12 d, 24 d				Comparable
	High Temp.(45°C)	5 d, 10 d				Comparable

d: days; h: hours; m: months; RH: Relative humidity

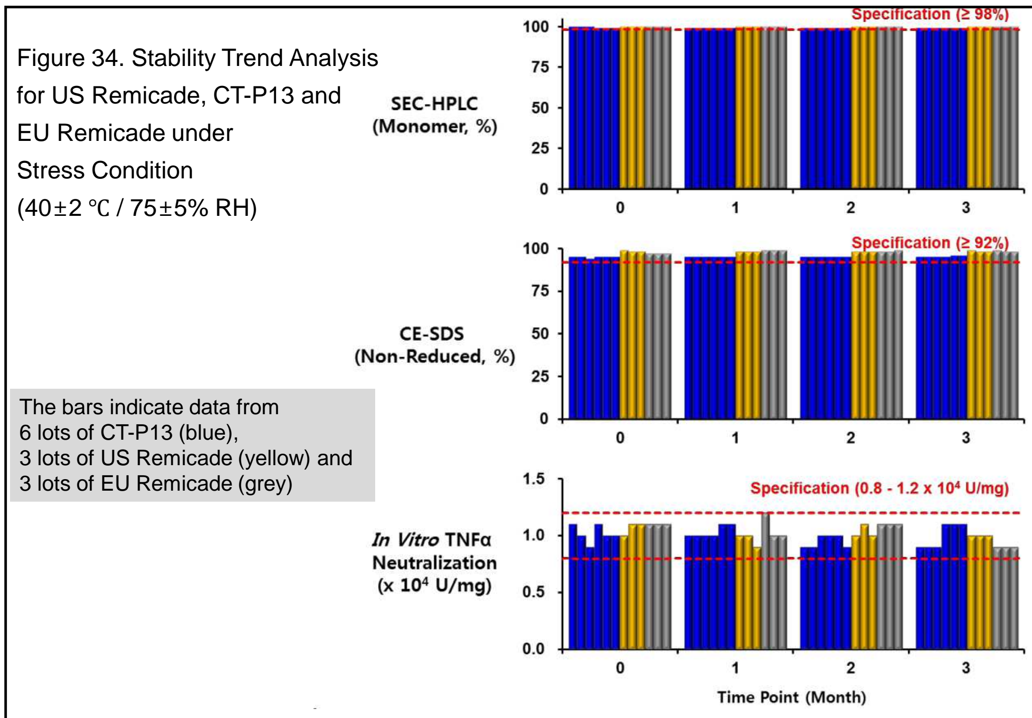


Figure 34. Stability Trend Analysis for US Remicade, CT-P13 and EU Remicade under Stress Condition (40±2 ℃ / 75±5% RH)

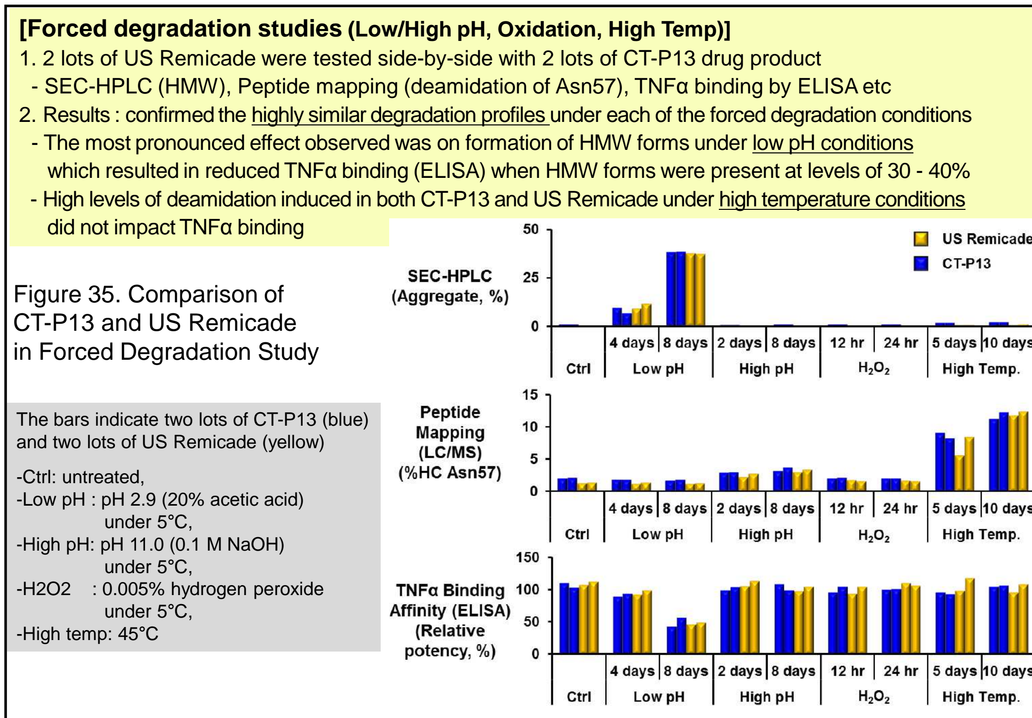


Figure 35. Comparison of CT-P13 and US Remicade in Forced Degradation Study