# Structural and Functional Characterization for Fingerprinting Biosimilars

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## Abstract

A battery of analytical methods have been developed to probe biochemical, biophysical and biological properties of several biosimilar molecules. Orthogonal methodologies, using a range of state-of-the-art techniques, have been employed to demonstrate analytical similarity. Structure and function relationships are essential to a risk assessment for ranking and prioritizing quality attributes. Quantitative measurement for attributes impacting biological activity, immunogenicity, PK/PD and safety (toxicity) has been investigated. Structural and functional data relationships including minor glycan structures and ADCC bioactivity, aggregation and proliferation potency, and methionine residue oxidation and FcRn binding potency are presented. Furthermore, the structural quality attributes and product degradation pathways are examined in forced degradation studies.

## Introduction

Correlation of structure and function is crucial to establishing critical quality attributes. Several aspects ranging from the primary amino acid sequence to post-translational modifications (PTMs) have been known to affect various biological functions of biotherapeutics. Deamidation and oxidation on a CDR impacts antibody activity and further impacts safety or efficacy. Glycation and glycosylation impacts PK. Glycosylation and fucosylation play a major role in ADCC. When a particular structural element or binding interaction is involved in exerting the mechanism of action, it is considered as a critical quality attribute and must be closely monitored through process development and process changes to be within desired specifications. For a Biosimilar product, in addition to such monitoring, the attributes must closely match that of the reference product. Fingerprinting can be thought of as balancing the various structural differences in order to achieve similar biological activity. Orthogonal testing by several rigorous methods is necessary to establish overall functional similarity between a Biosimilar and its originator.

## Case Study 1: Fingerprint ADCC Similarity-Part A

### Introduction

Antibody-Dependent Cell Cytotoxicity (ADCC) is an effector function mediated by the simultaneous binding of an antibody to antigen expressed on target cell membrane and to the Fc receptor FcgRIIIa, expressed on immune effector cells and leads to target cell death. Glycosylation of a conserved Asp residue in the Fc domain is essential to FcgRIIIa binding, while fucosylation of the glycan interferes with this binding, and decreases ADCC. The afucosylated species consist of four variants G0, G1, G2 and the high mannose species. While several publications show a positive correlation between ADCC and total % afucosylated species, correlation with individual afucosylated glycoforms has not been studied.

ADCC is hypothesized to be a secondary MoA of the originator molecule in this case study. ONS-biosimilar showed a higher ADCC activity compared to the originator. In-depth analysis of biosimilar product obtained different clones and different processes was performed and statistical analysis was applied to obtain a correlation between structure and function and guide further development of the Biosimilar.

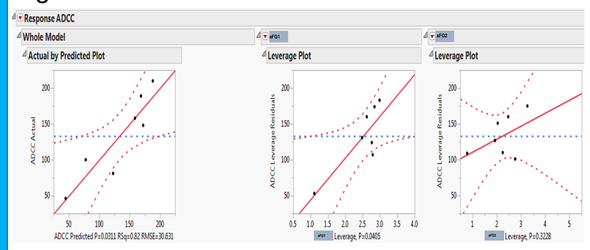
### Methods

- *ADCC activity* was measured in an assay using target cells expressing membrane bound antigen and freshly isolated PBMC as effector cells
- *Glycan analysis* was performed using an LC-MS method
- *Statistical analysis* was performed using SAS JMP software

### Results

- A tight correlation between ADCC activity and two of the afucosylated forms (aFG1, and aFG2) was observed
- From the statistical analysis of the data, an equation was derived that predicts required % aFG1 and aFG2 in order to achieve target ADCC potency values

Fig. 1. Correlation between ADCC and %aFG-1




ADCC = -53.7 + 58.3*aFG1+18.2*aFG2

### Conclusions

- The afucosylated glycoform aFG1 contributes the majority of ADCC functionality with some contribution from aFG2
- Since aFG2 is known to increase immunogenicity, process development efforts focused on reducing aFG1, without affecting aFG2

## Case Study 1: Fingerprint ADCC Similarity-Part B

### Introduction

As concluded in Part A of Case Study 1, process improvements focused on reducing % aFG1. To further confirm the %aFG1 and ADCC correlation, and to identify the % aFG1 that yields optimum ADCC, samples with varying levels of %aFG1 were generated. Previous data indicated that process impurities can have variable impact on different analytical methods. Hence, a two-step purification was performed and for each sample, material obtained from both steps were analyzed for glycoform quantitation, FcgRIIIa binding and ADCC. In addition, since CDC is a secondary MoA for this drug, CDC activity was also measured to ensure that it was not impacted.

### Methods

- *ADCC activity* was measured using a Promega reporter gene assay using cells expressing membrane bound antigen as target cells
- *FcgRIIIa binding* was measured using an SPR-based method
- *CDC* activity was measured in a cell-based assay using cells expressing membrane bound antigen and human serum complement
- *Glycan* method was further optimized from that used in Part A, to improve the recovery of glycoforms

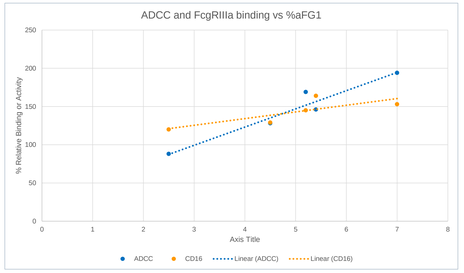
### Results

- Correlation between %aFG1 and ADCC and FcgRIIIa binding is confirmed
- %aFG1 does not impact CDC activity
- Process impurities have variable impact on different methods

Table1. Process Development Sample Analysis

| Sample | aFG1 | ADCC (Rel. Potency by EC50) | FcgRIIIa binding | CDC |
|---|---|---|---|---|
| Originator- lot 1 (reference std) | | 100 | 100 | 100 |
| ONS- Sample 1-Step 1 | 2.5 | 109 | 120 | 120 |
| ONS- Sample 1-Step 2 | | 88 | 126 | 112 |
| ONS- Sample 2-Step 1 | 4.5 | 139 | 148 | 125 |
| ONS- Sample 2-Step 2 | | 128 | 129 | 113 |
| ONS- Sample 3-Step 1 | 7.0 | 178 | 170 | 117 |
| ONS- Sample 3-Step 2 | | 194 | 153 | 111 |
| ONS- Process A-BDS | 5.5 | 146 | 164 | 119 |
| ONS- Sample 6 | 0.9 | 113 | 94 | 123 |
| Originator lot 2 | | 99 | 98 | 122 |

Fig. 2.Correlation between ADCC, FcgRIIIa and %aFG1




### Conclusions

- ADCC fingerprinting can be achieved by establishing a structure function relationship between activity and individual glycoforms and optimizing the abundance of the relevant afucosylated glycoforms.

## Case Study 2: FcRn Binding

### Introduction

Oxidation of methionine residues can occur due to exposure to certain types of stress. Methionine 258 residue in the Fc region, is important for FcRn binding and oxidation at this site negatively impacts FcRn binding. FcRn, or the neonatal Fc receptor, plays a role in protecting serum IgG and increasing its half-life and thus, FcRn binding is closely related to PK.
In this case study, originator molecule and ONS-Biosimilar product were subjected to stress resulting in methionine oxidation and the impact on various functions was investigated.

### Methods

- *Oxidative stress* was induced either by treating samples with 1%TBHP for different durations, or by exposing to 5X light treatment
- *FcRn binding* was measured using an SPR based method
- *Potency* was measured in a cell based assay

Table 2.A. Analysis of TBHP treated Samples

| Sample | Hrs of treatment with 1%TBHP | | | PTM | |
|---|---|---|---|---|---|
| | | % Potency | % FcRn | HC M34 | HC M258 |
| Originator | 0 | 100 | 100 | 0.7 | 2.5 |
| | 2 | --- | 73 | 0.7 | 25.4 |
| | 4 | --- | 68 | 0.6 | 36.5 |
| | 8 | --- | 53 | 0.6 | 51.9 |
| | 24 | --- | ND | 0.7 | 79.9 |
| | 48 | 96 | ND | 1 | 90.5 |
| ONS- Biosimilar BDS | 0 | 101 | 105 | 0.8 | 3.4 |
| | 2 | --- | 79 | 0.6 | 24.7 |
| | 4 | --- | 65 | 0.6 | 38 |
| | 8 | --- | 43 | 0.6 | 51.6 |
| | 24 | --- | ND | 0.9 | 77.7 |
| | 48 | 92 | ND | 1.1 | 90.1 |

Table 2.B. Analysis of 5X light exposed Samples

| Sample | Treatment condition (5 X ICH light and dark) | % Potency | %HC M34 |
|---|---|---|---|
| Originator | Dark | 100 | 0.6 |
| | 5X | 35 | 2.1 |
| ONS- Biosimilar BDS | Dark | 101 | 0.8 |
| | 5X | 30 | 2.2 |

### Results and Conclusions

- Impact of methionine oxidation on specific functional activities depends on the location of the affected methionine residues
- Oxidation of HC M258 impacts FcRn binding but not potency, whereas oxidation of the HC M34 results in decreased potency

## Discussion and Conclusions

1.Monoclonal antibodies are multi-functional molecules and structural changes can impact the various functions differently. Moreover certain structural attributes can show opposing effects on function.
2.Fingerprinting functional activities, or overall functional similarity can be achieved by establishing the correlation between specific structural attributes and the corresponding functional activities.

Comparability for Biologics, Biotherapeutics Analytical Summit, March 2015