Bioactivity Assays: 
Putting the Puzzle Together

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Outline

• General Remarks
• MoA reflecting bioassays
  • Proliferation Assays: G-CSF, EPO
  • MoA assays for mAbs
    • Bevacizumab
    • Adalimumab, Infliximab
    • Rituximab
      • ADCC, CDC, Apoptosis, ADCP
• Beyond lot release and stability
  • Forced degradation
  • Biocomparability
• Summary
Bioassays - Past, Present and Future

History:
• GMP-compliant relative bioactivity determination for later phase lot release and stability testing, often with proliferation readout

Paradigm change:
• Reflection of mechanism of action (MoA) required
• Bioassays needed in earlier phase
• Bioassays used for biocomparability, accelerated stress condition studies, confirmation of successful product up scaling
• Often more than one bioassay needed in the beginning if drug follows more than one mechanism of action \textit{in vivo}
• Rising acceptance of surrogate approaches to replace tedious primary cell based assays
Bioassays in the Life Cycle of a Biotherapeutic

- Method development
- Method transfer
- Method optimization
- ICH-compliant method validation
- Lot release testing for drug substance and drug product
- Stability testing
- Biocomparability testing
- Accelerated stress condition testing
- Short term dilution stability
- Confirmation of successful production up scaling
Functional assays for mAb therapeutics: Prerequisites

- Confirmation of MoA
- Suitable cell line with constant cell surface receptor expression
- Appropriate choice of the source of primary material
- Appropriate choice of reference material
- Robust and precise assay setup
- Suitable for statistical evaluation
- Suitable for validation
- Stability indicating properties
- Determination of range
- Determination of LOD/LOQ
**G-CSF and EPO Bioassay**

*In vitro* assay to determine the ability of G-CSF or EPO to stimulate cell proliferation

- **G-CSF**
  - M-NFS-60 cells
  - Incubation for 44-48 hours
  - CellTiter 96® AQueous One Solution
  - M-NFS-60 cells
  - Quantitation of cell proliferation and cell viability

- **EPO**
  - UT-7 cells
  - Incubation for 3 days
  - CellTiter 96® AQueous One Solution
  - UT-7 cells

*Statistical evaluation and determination of relative potency*
- Red: Reference standard
- Blue: Test item

Reference:
- European Pharmacopoeia, “Filgrastim Concentrated Solution”
Bevacizumab

- Recombinant humanized mAb that blocks angiogenesis by inhibiting vascular endothelial growth factor A (VEGF-A)
- Used in the treatment of cancer, often in combination with chemotherapy
- Off-label use for treatment of age-related macular degeneration (AMD) and diabetic retinopathy
- First commercially available angiogenesis inhibitor
- VEGF neutralizing assay is MoA reflecting in vitro bioactivity assay
Bevacizumab proliferation assay
primary HUVEC cells are replaced by an engineered HEK293 reporter cell line expressing NFAT-luc2P/KDR
Bevacizumab Reporter Bioassay
limited dilution linearity

<table>
<thead>
<tr>
<th>nominal potency [%]</th>
<th>determined relative potency [%]</th>
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<tbody>
<tr>
<td>50</td>
<td>53.6</td>
</tr>
<tr>
<td>70</td>
<td>70.8</td>
</tr>
<tr>
<td>100</td>
<td>98.3</td>
</tr>
<tr>
<td>130</td>
<td>137.1</td>
</tr>
<tr>
<td>150</td>
<td>157.3</td>
</tr>
</tbody>
</table>

Coefficient of Correlation $[R]$: 0.997

Coefficient of Determination $[R^2]$: 0.995
Bevacizumab Reporter Bioassay

Repeatibility
TNF Signaling Pathway via TNF Receptor Type 1
Proliferation Assay for TNF\(\alpha\) mAbs

- mAb
- Cytokine
- Overnight incubation
- CellTiter 96® AQueous One Solution
- Quantitation of cell proliferation and cell viability
- Statistical evaluation and determination of relative potency

Graph: Reference standard and test item
Additional Bioassays for TNFα mAbs

**Caspase 3/7 Apoptosis Assay**

- **U937 cells**
- **Response duration (2.5 hr)**
- **Caspase-Glo® 3/7 Reagent**

**Reporter Assay**

- **Frozen, Thaw-and-Use**
- **NFκB-RE luc2 HEK293 Cells**
- **Response induction (5 hr)**
- **Luciferase Detection Reagent**
Comparison of Bioassays for TNFα mAbs

Proliferation assay

Apoptosis assay

Reporter assay

Absorption

Luminescence [RLU]

Adalimumab [ng/ml]

Adalimumab [ng/ml]

Adalimumab [ng/ml]
Bioassays for TNF\(\alpha\) mAbs: Linearity and Range
Bioassays for TNF$_\alpha$ mAbs: Specificity

Proliferation assay

Apoptosis assay

Reporter assay

Absorption

Luminescence [RLU]

Luminescence [RLU]

Conc. mAb [ng/ml]

: test item Rituximab

: reference item Adalimumab
Mechanism of action assays (MoA) for monoclonal antibodies
Rituximab

- Chimeric monoclonal antibody directed against CD20 which is primarily found on the surface of B-cells
- Used in the treatment of many lymphomas, leukemias, transplant rejection and some autoimmune disorders
- Induces the death of target cells via different MoAs
- High effector function potential
- Effector function is part of MoA
Assays for Rituximab

- Binding assay
- Apoptosis assay
- CDC Assay
- ADCC assay with primary effector cells
- ADCC reporter bioassay
- ADCP assay with primary effector cells
- ADCP reporter bioassay
Rituximab Annexin V Apoptosis Assay: Principle

- Annexin V is a calcium-dependent phospholipid binding protein that has a high affinity for phosphatidylserine (PS), a plasma membrane phospholipid.
- One of the earliest features of apoptosis is the translocation of PS from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external environment (loss of membrane asymmetry).
- Annexin V binds to PS exposed on the cell surface.

*Image of diagram showing Annexin V binding to a cell membrane, comparing living and apoptotic cells.*

_Cytometry_ 1998; 31:1-9
Rituximab Annexin V
Apoptosis Assay

Restricted Model

Response vs. dose, Rituximab [ng/ml]

Blank

Rituximab

Counts

Counts
CDC (Complement Dependent Cytotoxicity) Assays: Principle

- The therapeutic antibody is diluted in the complement matrix and added to a target cell line
- 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
- Cells are lysed

Not all therapeutic antibodies are able to induce CDC

*Nat Clin Pract Rheumatol* 3: 86–95
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.

Alternatively, a luminescence base readout is possible using living cell staining (Cell Titer-Glo® Luminescent Cell Viability Assay).
Rituximab CDC Assay (flow-cytometry based): Range

Restricted Model

Measured potency ratio (4PF):

103%  
74%  
136%
Rituximab CDC assay
(luminescence readout: living cells)

4 parameter fit, 95% significance level
dose, Rituximab [ng/ml]

Viability [RLU]

Restricted Model

: test item
: reference item

4 parameter fit, 95% significance level
activity 107%
ADCC Assay

- NK cells recognize their target cells via FcγRIIIA (CD16) that bind to antibody bound to the surface of the target cells
- Binding of NK cells to their target cells induces the release of preformed cytotoxic mediators by granule exocytosis
- The lysis of the target cells is extracellular, requires direct cell-to-cell contact, and does not involve complement
- Typical readout: release of LDH

Source: Satchmo 2000
ADCC Assay with primary effector cells

Quality control of purified NK cells by flow cytometry
Rituximab ADCC assay with primary effector cells

Restricted Model

Comparison of two biosimilars - potency determination using PLA© software

\[
\frac{(\text{experimental release} - \text{mean spontaneous release})}{(\text{mean maximum release} - \text{mean spontaneous release})} \times 100 = \% \text{ specific lysis}
\]

specific lysis [%]

dose, Rituximab [ng/ml]
Rituximab ADCC assay with primary effector cells

Comparison of innovator product and biobetter

Potency determination using PLA software not applicable

Similarity (parallelism) criteria not fulfilled. Absence indicates:

- functional dissimilarity between two preparations
- molecular changes to the test item
- formulation differences between reference and test item
**ADCC Reporter Bioassay**

**Diagram:**

- **FcγRIII Initiated Signaling Events**
  - LCK, ZAP-70, PLCγ, p110, p85, NFATp
  - Rho-family G proteins, PI 3-K
  - Calcium signaling
  - Granule exocytosis, ADCC, transcriptional regulation

**ADCC Cytotoxicity Bioassays**
- Target cells + Antigen + Antibody + FcγRIIIA receptor
  - Primary NK cells
  - Read out: target cell cytotoxicity

**New Reporter-Based Bioassay**
- Target cells + Antigen + Antibody + FcγRIIIA (V158) receptor
  - NFAT-RE reporter activation
  - Read out: NFAT-RE reporter activation from effector cells
  - Light emission

[Logo: Charles River]
Rituximab ADCC Reporter Bioassay: Dilution Linearity & Specificity

\[ y = 0.943x + 7.426 \]
\[ R^2 = 0.99 \]

measured relative activity [%]
theoretical relative activity [%]

Mean Graph

- Trastuzumab
- Rituximab

RLU vs. dose [ng/ml]
Rituximab ADCC Reporter Bioassay: Repeatability

Restricted Model

<table>
<thead>
<tr>
<th>Test Item Concentration</th>
<th>RLU</th>
<th>Dose, Rituximab [ng/ml]</th>
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<tbody>
<tr>
<td>50%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>70%</td>
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<tr>
<td>100%</td>
<td></td>
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</tr>
<tr>
<td>130%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>150%</td>
<td></td>
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</tr>
</tbody>
</table>
Rituximab ADCC Reporter Bioassay: Stability (heat stress)
Forced degradation of a mAb therapeutic: ADCC 158 V/V reporter bioassay

Relative Bioactivity [%]

- Untreated
- UV/VIS 1x
- UV/VIS 5x
- UV 1x
- UV 5x
- Freeze/Thaw 15x
- Mechanical Stress 14d
- Heat Stress 40°C, 1d
- Heat Stress 49°C, 1d
- Heat Stress 57°C, 1d
- Heat Stress 57°C, 3d
- Heat Stress 57°C, 7d
- Deamidation 7d
- Oxidation 120 min
- Oxidation 360 min
- Acid Treatment n/e
- Base Treatment 4h
- Disulfide Scrambling 24h

- Photodegradation n/e
- Mechanical Stress 14d n/e
- Oxidation n/e
- Deamidation 7d n/e
Rituximab ADCC Reporter Bioassay: Biosimilars and Biobetters
Comparison of a Biosimilar and a Follow-on Biologic to Rituximab Innovator - Differences between V- and F-variant
ADCP: the neglected MoA

• *In vivo* as well as *in vitro* studies indicate that ADCP is an important and potent effector mechanism for killing of e.g. tumor target cells

• Often it is an additional synergistic pathway to the well characterized ADCC

• ADCP is enhanced *in vivo* by simultaneous treatment with immunomodulatory agents

• Regulatory authorities are requiring data on the impact of ADCP on the antibody mediated cytotoxicity

• Fc$_\gamma$RIIa is a predominant Fc$_\gamma$R in ADCP by macrophages
ADCP Assay with primary effector cells

Labelling of target cells

- addition of therapeutic mAb

Binding

- addition of macrophages

dose-dependent

ADCP

Flow cytometric readout

Isolation of Monocytes from Buffy Coats of healthy donors,
QC by flow cytometry

- Culture with M-CSF

- Culture with IL-10

Macrophages,
QC by flow cytometry
ADCP Assay with primary effector cells

<table>
<thead>
<tr>
<th></th>
<th>w/o Rituximab</th>
<th>0.5 µg/ml Rituximab</th>
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<tbody>
<tr>
<td>t₀</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD14 APC</td>
<td>1.1%</td>
<td>7.2%</td>
</tr>
<tr>
<td>t₃h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD14 APC</td>
<td>1.4%</td>
<td>17.8%</td>
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Graph showing % phagocytosis vs Rituximab concentration.

Another graph showing pHrodo counts at t₀ and t₃h.
ADCP Reporter Assay: Comparison of target cell lines

Primary macrophages as effector cells are replaced by an engineered Jurkat reporter cell line expressing FcγRIIa (H131) and NFAT-RE luc
ADCP Reporter Assay: Specificity and dilution linearity
Partial afucosylation has nearly no impact on the ADCP bioactivity whereas a 3rd generation follow on biologic with another binding epitope was modified towards reduced ADCP activity.
ADCP Reporter Assay: heat stress

8h, 65°C

24h, 65°C
Biocomparability: Rituximab and partially afucosylated Rituximab in different MoA assays
Bioassays - Past, Present and Future

Challenges:

• Extremely short time lines for setup of robust and reliable assays
• Communication as key for success in biosimilar and biobetter testing
• MoA reflecting assays for complex therapeutics that do not follow classical pathways
• Antibody drug conjugates
• Bispecifics
• MoA reflecting assays for cell and gene therapy products
Thank you!

Questions?

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