ADC Analyte Diversity and Appropriate PK Assays
Part I: Background & Bioanalytical Strategy

European Bioanalysis Forum – ADC Training Day
Bringing ADC into Practice
Defining the Bioanalytical Strategy

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BioAnalytical Sciences, Genentech
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Overview

- Antibody drug conjugate (ADC) background
- Molecular complexity & the need for multiple PK analytes
- Importance of understanding catabolism in vivo
- Appropriate quantitative assays for pharmacokinetics
- Immunogenicity overview
- Summary and acknowledgments
What Are Antibody Drug Conjugates (ADCs)?

- Targeted and selective cancer treatment with reduced side effects

S. Kaur, EBF ADC Training, Analyte Diversity Part I, Lisbon, 06.20.17
The Long Road to ADCs in the Clinic

Success required overcoming challenges across multiple fields

• High immunogenicity of murine/chimeric monoclonal antibodies
• Unstable linkers and high systemic toxicity from released cytotoxin
• Cytotoxins with insufficient potency
• Poor internalization and insufficient delivery of cytotoxin
• Limited expression of the target antigen

“Magic bullet” Concept
Paul Ehrlich 1900

1980s Early ADCs

2000 Mylotarg® Approval (withdrawn 2010)

2011 Adcetris® Approval
2013 Kadcyla® Approval

Many Clinical Trials Ongoing
ADC Success Required Advances in Technology Across Multiple Fields

- Humanized monoclonal antibody production
- Stable chemical linker chemistries
- Cytotoxins with appropriate potency and mechanism of action
- Genomic profiling to identify unique tumor antigens
- Novel hybrid large molecule/small molecule bioanalytical technologies
FDA NEWS RELEASE: Aug. 19, 2011 (Seattle Genetics)
FDA approves Adcetris® to treat two types of lymphoma
“The U.S. Food and Drug Administration today approved Adcetris® (brentuximab vedotin) to treat Hodgkin lymphoma (HL) and a rare lymphoma known as systemic anaplastic large cell lymphoma (ALCL)”
http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncementsucm268781.htm

FDA NEWS RELEASE: Feb. 22, 2013 (Genentech/Roche)
FDA approves new treatment for late-stage breast cancer
The U.S. Food and Drug Administration today approved Kadcyla® (ado-trastuzumab emtansine), a new therapy for patients with HER2-positive, late-stage (metastatic) breast cancer
http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncementsucm340704.htm

Footnote: Mylotarg® (gemtuzumab ozogamicin), accelerated approval 2000 for acute myeloid leukemia (AML), withdrawn 2010 after results from subsequent trial raised safety concerns and failure to demonstrate clinical benefit
ADC Components

### Antibody
- Reasonable kinetics
- High specificity (i.e., limited antigen expression in normal tissues)
- High affinity
- Acceptable manufacturing obstacles
  - Humanized/chimera
  - IgG class
  - Ab fragments

### Linker
- Stability in systemic circulation
- “Release” of biologically active drug
  - Acid labile hydrazones (cleavable)
  - Disulfides (cleavable)
  - Dipeptides (cleavable)
  - Thioethers (uncleavable)

### Payload
- Amenable to conjugation
- Highly potent agent
  - 0.0003% - 0.08% of an injected Ab dose/gram of tumor
- Target must be inside the cell
- Molecular structure small (immunogenicity)
- Solubility aqueous buffers
- Stable in plasma (Ab half-life)

ADCs are based on a limited number of toxic payloads targeting:
- Tubulin filaments (maytansinoids, auristatins)
- DNA (calicheamicin, CC-1065 analogs)
- RNA (amanitin)

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Example of Transcript Profiling to Identify Suitable Tumor Targets for ADCs

Target expressed in lung, ovarian, and thyroid cancers
Example of In Vitro Cellular Internalization Data for an ADC

Target-Negative Controls

Target = Red, Lysosome = Green, Lysosome & ADC = Yellow

Target
Color Overlay
Lysosomes
Membrane
### ADC Linkers and Properties

- Serum stability
- Cleavage mechanism

<table>
<thead>
<tr>
<th>MOLECULE</th>
<th>LINKER</th>
<th>STRUCTURE</th>
<th>CLEAVAGE MECHANISM</th>
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<tbody>
<tr>
<td>Adcetris, vc-MMAE</td>
<td>Peptide (VC)</td>
<td><img src="image" alt="Peptide" /></td>
<td>Protease (cathepsinB)</td>
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<tr>
<td>Doronina et al. Nature Biotech 2003, 778</td>
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<td>Mylotarg, BR96-dox</td>
<td>Hydrazide</td>
<td><img src="image" alt="Hydrazide" /></td>
<td>Acid</td>
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<td>Hamann et al. Bioconj Chem 2002, 47</td>
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<td>Mylotarg, maytansines</td>
<td>Disulfide</td>
<td><img src="image" alt="Disulfide" /></td>
<td>Glutathione</td>
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<tr>
<td>Kadcyla/MCC-DM1, MC-MMAF</td>
<td>“Non-cleavable”</td>
<td><img src="image" alt="“Non-cleavable”" /></td>
<td>Ab degradation leads to aa-L-D</td>
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<tr>
<td>Erickson et al. Cancer Res 2006, 4426</td>
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## Examples of ADC Cytotoxic Drugs

### Tubulin Inhibitors

**Maytansine**
- Potent anti-mitotic macrolide with cytotoxic activity in broad range of tumor types
- Semi-synthetic maytansine analogs, DM1 and DM4
- Inhibits mitosis by interfering with microtubule polymerization and assembly

**Auristatin**
- Highly potent fully synthetic analog of natural product, dolastatin-10
- MMAE (membrane permeable)
- MMAF (membrane impermeable)
- Inhibits tubulin polymerization

### DNA Alkylating Agents

**Duocarmycin**
- Highly active DNA alkylating agent, picomolar activity
- Binds to DNA minor groove, resulting in double stranded DNA breakage and cell death

**Calicheamicin**
- Potent naturally occurring hydrophobic antibiotic cytotoxin
- Approximately 1000-fold more active than doxorubicin against xenograft tumors
- Binds to the minor groove in DNA, resulting in double stranded DNA breakage and cell death
Therapeutic Index (TI) = toxic dose/therapeutic dose

- measure of relative safety of the drug for a particular treatment
Overview

- Antibody drug conjugate (ADC) background
- Molecular complexity & the need for new analytics
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- Appropriate quantitative assays for pharmacokinetics
- Summary and acknowledgments
ADCs have Complex Structures: Large/Small Molecule Features & Heterogeneity

2nd generation ADCs

Kadcyla™

Adcetris™

3rd generation ADCs

Components: Antibody, conjugation site, linker and payload

Panowski et al. mAbs (2014)

S. Kaur, EBF ADC Training, Analyte Diversity Part I, Lisbon, 06.20.17
Potential for Additional Heterogeneity In Vivo: Active Large Catabolites & Small Catabolites Possible

Kaur, Xu, Saad, Dere, Triguero, Bioanalysis (2013)
Saad et al., Bioanalysis 7(13), 1583-1604 (2015)
Bioanalytical Experience from a Rich ADC Pipeline

Bioanalytical strategies based on experience from a diverse ADC platform and a rich pipeline

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**Late Stage Research**

- Molecule Selection
  - Early ADCs

**Preclinical Development**

- IND Enabling Studies
  - Pre-IND ADCs

**Clinical Development**

- Phase I
- Phase II
- Phase III

**Filing/Approval**

- IND
- Multiple ADCs in Development

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*KADCYLA® (ado-trastuzumab emtansine) approved in 2013 for HER2+ mBC*
ADCs are Complex: Many Analyte Choices….
Pharmacokinetics (PK) of Mixtures is a New Paradigm

1. Total Antibody

2. Conjugate
   Conjugated Antibody or Antibody-Conjugated Drug

3. Unconjugated Drug

Three key analytes measured for PK

Multi-disciplinary bioanalytical team to enable innovation:

- ADC structural characterization *in vivo* by affinity capture LC-MS
- Three key pharmacokinetic assays
  1) Total antibody by LBA or immunoaffinity LC-MS/MS
  2) Antibody-conjugated drug (conjugate) by immunoaffinity LC-MS/MS
  3) Unconjugated drug by LC-MS/MS
- Catabolite assays in circulation & tissues, as needed
- Immunogenicity assays

Kaur et al., *Bioanalysis* 5 (2) 201-26 (2013)
Gorovits et al., *Bioanalysis* 5 (9) 997-1006 (2013)
Overview

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- ADC complexity & drug development bioanalysis
- Understanding biotransformations in vivo
- Appropriate quantitative assays for pharmacokinetics
- Summary and acknowledgments

S. Kaur, EBF ADC Training, Analyte Diversity Part I, Lisbon, 06.20.17
Quantitative LBA Assays for ADCs: More Complex than LBA for mAb Therapeutics

ADC Standard curve may not represent analytes in vivo
Understanding Biotransformations of ADCs *In Vivo*: Essential Information for Designing Appropriate PK Assays


- S. Kaur, K. Xu and O. Saad, “Analysis of antibody drug conjugates by bead based affinity capture and mass spectrometry” United States patent S 8541178, issued 24 Sept 2013

- S. Kaur, K. Xu and O. Saad, European patent 2277044, issued 17 June 2015
Profound Impact of Antibody Conjugation Site on the ADC Stability Identified by Affinity Capture LC-MS

In Vitro Human Plasma Stability by Affinity Capture LC-MS

B. Shen and K. Xu et al., Nature Biotechnology, 30, 184-189, (2012)

Linker Deconjugation

Retro-Michael Rxn

Linker Stabilization

Retro-Michael Rxn

Catabolites identified maleimide hydrolysis as key mechanism
Overview

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Biotransformation Data Important for PK Assays
Plasma Stability Study using HIC shows New ADC DARs Formed

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Graph A:
- DAR0, DAR2, DAR4, DAR6, DAR8
- 0 hour is Similar to Assay Standard

Graph B:
- DAR1, DAR3, DAR5
- 96 hour Differs from Assay Standard
Characterize Ligand Binding Assays with Individual DARs Identified in Plasma to Evaluate Assay performance

- Used individual DAR controls in plasma to test with ELISA ligand binding reagents
- No single anti-Drug mAb reagent in conjugate Ab assay appropriate for all DARs

<table>
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<tr>
<th>Assay</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>6</th>
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<tbody>
<tr>
<td>Total Antibody ELISA (% recovery)</td>
<td>88</td>
<td>78</td>
<td>86</td>
<td>92</td>
<td>92</td>
</tr>
<tr>
<td>Conjugated-Antibody ELISA (% recovery)</td>
<td>NA</td>
<td>11</td>
<td>102</td>
<td>99</td>
<td>64</td>
</tr>
</tbody>
</table>

1 Based on expected nominal concentration of individual DARs spiked into serum
Develop Appropriate PK Assays:
Immunoaffinity LC-MS/MS & LBA Conjugate Assay Formats

- Detection reagent (Biotin antigen/SA-HRP or Biotin anti-CDR/SA-HRP)
- ADC (carrying 1 or more drugs)
- Capture reagent (anti-drug mAb)
- Linker cleavage/ADC digestion step
- LC-MS/MS quantification of released drug
- ADC and Endogenous IgG
- Capture by Protein A (resin)
Example ADC Pharmacokinetics (2.4 and 1.4 mg/kg):
Antibody-Conjugated Drug, Total Antibody, Unconjugated Drug

At a dose of 2.4 mg/kg (Q3W MTD) and 1.4 mg/kg (Q1W MAD):
acDrug clearance was ~26 and ~30 mL/day/kg;
half-life of acDrug was ~5 and ~1.8 days.

Jian Xu, Rong Zhang, Ola M. Saad, Joyce F. Liu, Kathleen N Moore, Howard A Burris 3rd, Eric W. Humke, Kirsten Achilles Poon, Sandhya Girish Poster, ASCPT, 2015
Comparable Antibody Quantification Data by Hybrid Binding LC-MS/MS vs. ELISA

ADC Total Antibody ELISA
- Detection reagent (anti-human mAb or anti-CDR mAb)
- ADC (conjugated & unconjugated)
- Capture Reagent (Antigen, anti-CDR mAb, or anti-human mAb)

Hybrid Binding LC-MS/MS
- Trypsin
- Protein A, anti-human or anti-CDR Capture Bead
- Magnet
- Signature peptide(s)

Comparison of ELISA and Hybrid LC-MS/MS

"Plug-and-play" LC-MS/MS approach:
- Generic capture and human Fc peptide for nonclinical studies
- Specific capture for “free vs total”

S. Kaur, EBF ADC Training, Analyte Diversity Part I, Lisbon, 06.20.17
ADC Total Antibody PK Assay:
Generic Framework Peptide Hybrid LC-MS/MS & ELISA Comparable

- **Nonclinical “Plug-and-Play” Hybrid IA-LC-MS/MS**
  - Generic capture & hu Fc peptide-based analyte

- **Clinical Specific Hybrid IA-LC-MS/MS as needed**
  - Specific capture & CDR peptide-based analyte

- **Orthogonal platforms are complementary & help troubleshoot assay performance issues**

**ELISA vs Hybrid LC-MS/MS**

\[ y = 1,1404x + 4,7145 \]
\[ R^2 = 0,9829 \]

**Total Ab ELISA**

- Detection reagent (anti-HulgG, anti-ID)
- ADC (conjugated & unconjugated)
- Target, anti-ID or anti-HulgG

**Hybrid Binding LC-MS/MS**

- Trypsin
- Magnet
- Protein A, anti-HulgG, anti-ID Resin/Bead

- Signature peptide(s) from Fc region/CDR

Kaur et al., Bioanalysis, (2016), 8 (15), 1565–1577
ADC Unconjugated Drug PK Assay: ‘Small’ molecule typically measured by LC-MS/MS

- Typically measured by LC-MS/MS
- Challenges to develop high-sensitivity assays in the presence of ADC
- Additional stability assessments required with ADC present

**LC-MS/MS**

**Competition ELISA**

*Fig. 3* Competition ELISA for quantitation of MMAE. Free MMAE standards (0.025, 0.1, 0.25, 0.5, 1, 2, 3, 4, 5, and 25 nmol/L) mixed with HRP-MMAE (2 ng/mL) compete for binding to anti-MMAE mAb. Unconjugated drug in sample competes with HRP-drug. Detection reagent (HRP-Drug ) and Capture reagent (anti-drug mAb).

*Clin Cancer Res (11), 843-852, (2005)*
**Tiered Approach for ADC Immunogenicity Sample Testing**

1. **Screening Assay**
   - **ADA Positive**
     - **Confirmatory Assay**
       - **Confirmatory Positive**
         - **POSITIVE Sample**
           - Characterize +ve Response
           - Titer Assay
           - ADA domain specificity?
           - NAb activity?
   - **ADA Negative**
     - **Confirmatory Negative**
     - **NEGATIVE Sample**
       - *Presence of ADC in sample may interfere with ADA detection*

**How much characterization and when during clinical development?**

S. Kaur, EBF ADC Training, Analyte Diversity Part I, Lisbon, 06.20.17
Bridging Assay Formats Are Suitable to Screen for ADAs Against ADCs

- Assays should be sensitive and robust in presence of ADC and measure ADAs to antibody, linker-cytotoxic drug, neo-epitopes

**Diagram:**
- Streptavidin Coated Plate
- Incubate sample & reagents overnight
- ADA to Antibody
- ADA to Drug
- Digoxigenin (DIG) ADC Reagent
- Biotinylated ADC Reagent
- Anti-DIG-HRP Detection
- Color
- TMB/H$_2$O$_2$
- HRP

S. Kaur, EBF ADC Training, Analyte Diversity Part I, Lisbon, 06.20.17
**Confirmatory Assay**
Competitive Binding with ADC

**Domain Specificity Characterization**

**Competitive Binding with ADC components**

**Domain Detection Assay using domain-labeled reagents**
(Hoofring et al., 2013)
Summary

• ADCs have complex structures and are dynamically changing mixtures in vivo, requiring measurement of multiple key PK analytes

• Important to understand ADC in vivo biotransformations and analyte structures to best design bioanalytical strategy and ensure assays appropriate

• ADC bioanalysis may use a combination of LBA, LC-MS/MS & Hybrid IA LC-MS/MS methods depending on availability of technologies, reagents, and unique ADC characteristics

• Comprehensive assay characterization critical to understand what is being measured in vivo

• Use caution when comparing/correlating data across assay platforms to ensure same analytes are being measured
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