Dual “Hybrid” and Regular LC-MS/MS Assay for the Quantitation of Unconjugated and Conjugated Calicheamicin in Support of Mylotarg (gemtuzumab ozogamicin) Pediatric Study

Day 2 – Thursday 17 Nov 2016,
LARGE MOLECULE LC-MS Session,
16:15 – 16:35

Leo Kirkovsky
Pfizer San Diego, California, USA
Clinical Assay Group (CAG) @ Clinical Pharmacology
Outline

• ADC analytes and analytical methods
• Calicheamicin as a payload for ADCs
• LCMS method for unconjugated Calicheamicin
• “Hybrid” LCMS methods for conjugated Calicheamicin in ADCs
• Bioanalysis for Mylotarg pediatric study
  • MyeChild 01 pediatric clinical trials and selection of PK assays
  • Challenges with unconjugated Calicheamicin assay validation
  • Dual-Analyte assay for Mylotarg PK for clinical studies
  • Lessons Learned and Remaining Questions to Answer
• Acknowledgements
Cleavable and Non-Cleavable Linkers

Assumptions and terminology:

- Cleavable linkers have a reactive moiety ('soft spot') that can be cleaved by lysosomal (and other) enzymes or body chemicals (e.g., acids)
- Non-cleavable linkers designed to be stable against certain enzymes
- Conjugated payload - non toxic
- Un-conjugated (‘free’) payload – toxic
- Linkers – designed to be non toxic
- Number of payload molecules per ADC molecule: Drug Antibody Ratio (DAR)
## Potential Analytes for ADC With Maximum DAR=3
(Hypothetical Example)

<table>
<thead>
<tr>
<th>Analyte name</th>
<th>Analyte Structure</th>
<th>Analytical methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dosed drug and reference standard</td>
<td><img src="image" alt="Structure" /></td>
<td>Multiple methods</td>
</tr>
<tr>
<td>Total mAb</td>
<td><img src="image" alt="Structure" /></td>
<td>ELISA, LCMS</td>
</tr>
<tr>
<td>ADC (DAR &gt;0)</td>
<td><img src="image" alt="Structure" /></td>
<td>ELISA</td>
</tr>
<tr>
<td>Conjugated payload</td>
<td><img src="image" alt="Structure" /></td>
<td>LCMS</td>
</tr>
<tr>
<td>Total payload</td>
<td><img src="image" alt="Structure" /></td>
<td>LCMS</td>
</tr>
<tr>
<td>Unconjugated payload</td>
<td><img src="image" alt="Structure" /></td>
<td>LCMS</td>
</tr>
<tr>
<td>Naked mAb</td>
<td><img src="image" alt="Structure" /></td>
<td>ELISA, LCMS</td>
</tr>
<tr>
<td>Linker &amp; payload metabolites</td>
<td><img src="image" alt="Structure" /></td>
<td>LCMS</td>
</tr>
</tbody>
</table>
What Components of ADC May Exist In Vivo?

ELISA

ELISA, LCMS

LCMS, ELISA

LCMS, ELISA

ELISA

ELISA, LCMS

LCMS, ELISA

Payload metabolites LCMS

Proteins & peptide secondary conjugates
What Components of ADC May Exist In Vivo?

- Proteins & peptide secondary conjugates
- Payload metabolites

Methods:
- ELISA
- LCMS
Calicheamicin as a Payload for ADCs
History of Calicheamicin

- Calicheamicin was isolated originally in the mid-1980s from the chalky soil, or "calichi pits", located in Kerrville, Texas

Adrienne Mayor, Stanford University's Departments of Classics and History of Science
- An extraordinarily toxic bacterium harbored by the "infernal" Styx River might have been the fabled poison rumored to have killed Alexander the Great more than 2,000 years ago.
- Calicheamicin, a secondary metabolite of *Micromonospora echinospora*, is what gave the Styx river (now called Mavroneri, Greece) its toxic reputation.

Antoinette Hayes, toxicologist at Pfizer
- Calicheamicin is produced by extremely toxic, gram-positive soil bacterium and has only recently come to the attention of modern science. It was discovered in the 1980s in caliche, crusty deposits of calcium carbonate that form on limestone and is common in Greece.

http://en.wikipedia.org/wiki/Calicheamicin
Calicheamicin as a Payload for ADCs

**N-Ac-γ-Calicheamicin DMH**

- A derivative of γ-Calicheamicin, a cytotoxic enediyne class of natural products synthesized by *Micromonospora echinospora ssp. calichenis*
- Binds DNA in minor groove, inhibits formation of transcription complex, and causes relatively sequence-specific (T'CCT) double-strand breaks leading to apoptosis
- Significantly more potent than traditional cytotoxic chemotherapeutics

Patrick van Berkel, 2016 World ADC San Diego
Cytotoxic Mechanism of Action of $\gamma$-Calicheamicin

N-Ac-$\gamma$-Calicheamicin DMH
Considered reactive and toxic

N-Ac-$\varepsilon$-Calicheamicin
Considered inactive and non-toxic

Hydrogen abstraction from solvent/matrix

Bergman cyclization

Double-strand DNA Breaks

Hydrogen abstraction from phosphodiester backbone of DNA

Michael addition

-S-S- Reduction

GSH
GST
LCMS method for unconjugated Calicheamicin
Clinical LCMS Assay for N-Ac-γ-Calicheamicin DMH

**Analyte**

N-Acetyl-γ-Calicheamicin DMH  
LogD @ pH 7.4 = 3.74 (Pfizer RGate)

Chemical Formula: C61H84IN5O23S3 / Exact Mass: 1477.38  
1477.38 (100.0%), 1478.38 (70.2%), 1478.37 (1.8%)  
1479.38 (29.6%), 1479.37 (13.6%)  
1480.38 (13.5%), 1480.39 (5.0%)  
1481.38 (4.1%), 1481.39 (2.0%)  
1482.38 (1.2%)

Example: ULOQ = 10 mg/mL, SIL-IS (M+5) = 1 ng/mL  
ULOQ contribution to IS = 10*0.06=0.6 ng/mL (60% of IS signal)

**Analog Internal Standard**

N-Propionyl-γ-Calicheamicin DMH  
Chemical Formula: C62H86IN5O23S3 / Exact Mass: 1491.39

- Mass difference with analyte = 14 Da
- Log D @ pH 7.4 = 4.25 (Pfizer RGate)
- Prepared by acylation of γ-Calicheamicin by propionic anhydride.
- Impurity of acetic anhydride in propionic anhydride may lead to contamination of IS with analyte
  - Example; LLOQ = 0.05 ng/mL; IS = 1 ng/mL (with 1% of N-Ac-g-Calicheamicin DMH);  
  - IS contribution to analyte LLOQ = 1*0.1 = 0.01 ng/mL or 20% of LLOQ
- Required <1% of analyte impurity in IS
- [IS] = 3.2 ng/mL - no contribution to analyte demonstrated
Clinical LCMS Assay for N-Ac-γ-Calicheamicin DMH—Chromatography and Calibration Curve

LCMS trace at LLOQ (0.05 ng/mL)

Analyte transition: 1478.5/200.2

IS transition: 1492.9/214.2

LCMS trace at ULOQ (10 ng/mL)

Analyte transition: 1478.5/200.2

IS transition: 1492.9/214.2

LCMS trace in matrix bank with IS

Analyte transition: 1478.5/200.2

IS transition: 1492.9/214.2

A representative linear (1/x^2) calibration curve

Analyte area / IS area

LCMS trace at LLOQ (0.05 ng/mL)

Analyte transition: 1478.5/200.2

IS transition: 1492.9/214.2

A representative linear (1/x^2) calibration curve

Analyte area / IS area

Analyte peak count ~ 40 (~ 10% of LLOQ peak count)

20Jun12_115600-05. rdb (CL-184538): "Li near" Regression (1/\(x^2\) weighting): \(y = 0.374x + 0.000927\) (\(r = 0.9982\))

Analyte Conc. / IS Conc.

Analyte area / IS area
“Hybrid” LCMS method for Conjugated Calicheamicin in ADCs
• Separate unconjugated payload from ADC
  • LLE removal of unconjugated Calicheamicin
  • Tried other methods (Protein A) but did not work

• Cleave conjugated payload from ADC
  • In-situ reductive cyclization leading to N-Ac-ε-Calicheamicin
  • Tried hydrolysis but did not work

• Measure cleaved payload
  • LLE followed by LCMS quantitation of N-Ac-ε-Calicheamicin

• Report results as either:
  • Antibody-conjugated payload
  • Total ADC based on fixed initial DAR value (but DAR changes with time)
Schematic of “Hybrid” LCMS Methods for Conjugated Calicheamicin Quantitation

- Serum sample
  - Ether extraction
  - Forced cleavage and cyclization by DTT
- Washed serum sample
- Cyclized payload in serum sample
  - Ether extraction
  - Extracted payload

**Legend:**
- γ-Calicheamicin DMH (active toxin)
- ε-Calicheamicin (inactive toxin)
Clinical LCMS Assay for Conjugated Calicheamicin:
Cleavage from ADC with Formation of N-Ac-ε-Calicheamicin

N-Ac-ε-Calicheamicin
Clinical LCMS Assay for ADC: Calibration Standards and Internal Standards

Calibration standard - ADC

Measured Analyte - N-Ac-ε-Calicheamicin
Exact Mass: 1333.34

Added IS - N-Propionyl-γ-Calicheamicin DMH
Exact Mass: 1491.39

Measured IS - N-Propionyl-ε-Calicheamicin
Exact Mass: 1347.36

This is a surrogate & analog IS
Clinical LCMS Assay for InO – Calibration Curve and Chromatography

Representative linear calibration curve in human serum

Assay Range:
1.00 to 500 ng/mL of InO
Bioanalysis for Mylotarg pediatric study
Mylotarg (gemtuzumab ozogamicin, GO) ADC –
History and Current Status

- Wyeth’s application for Mylotarg was approved by the FDA in 2000 for treating acute myeloid leukemia (AML)

- CD33 mAb / conventional Lys conjugation of Calicheamicin

- Pfizer voluntarily withdrawn Mylotarg from the US market in 2010

- Mylotarg is still available in Japan

- Currently Mylotarg is been evaluated in a pediatric study with fractionated dose administration
• MyeChild 01 is a randomized open-label multicenter dose-escalating trial to evaluate toxicity, safety, and activity of a first-line fractionated GO regimen in combination with intensified induction regimens in at least 550 pediatric patients, aged 1 month to less than 18 years, with newly-diagnosed, de novo or secondary AML.

• The study is sponsored by the University of Birmingham, United Kingdom and conducted by a group of academic institutions working in a collaborative agreement with Pfizer Inc.

• In this study, 3 mg/m$^2$ GO will be administered IV over a 2-hour period on Study Days 4, 7 and 10, similar to the previous fractionated regimen used in Study ALFA-0701.

• The MyeChild 01 Study is comprised of 2 phases: a sequential-group dose-finding phase to identify the safe and optimum number of doses of GO in combination with AraC plus mitoxantrone or liposomal DNR in induction, followed by a randomization phase to compare 1 dose with 2 or 3 doses of GO in order to identify the optimum number of fractionated doses of GO when given with induction chemotherapy. The PK of GO following this fractionated 3 mg/m$^2$ regimen will be evaluated in a subset of approximately 50 patients during the dose-finding phase.
Challenges with Legacy Mylotarg PK Methods To Use in Support of MyeChild 01 Study

- Original Wyeth’s application methods:
  - Unconjugated (“free”) Calicheamicin by ELISA
  - Total Calicheamicin by ELISA (as a measure of ADC + Free Calicheamicin)
  - Total mAb by ELISA
- Unconjugated (“free”) Calicheamicin by ELISA
  - Legacy ELISA reagents were no longer available – no cross-comparison with legacy data even if newly developed reagents recreated
  - Sensitivity (LLOQ) 0.250 ng/mL (not enough for current study)
  - ELISA is not selective for γ-Calicheamicin vs ε-Calicheamicin:
    - Legacy ELISA results represented a sum of both forms perhaps with e-form more present
- Total Calicheamicin by ELISA
  - Legacy ELISA reagents were no longer available – no cross-comparison with legacy data even if newly developed reagents recreated
  - Sensitivity (LLOQ) 1-10 ng/mL (1 ng/mL may be sufficient for current study)
  - Total Calicheamicin assay measures both “free” and “conjugated” payload
    - May be acceptable for initial part of PK but not clear for terminal phase
- Limited blood sample size
  - Pediatric subjects
  - Measuring other analytes besides PK (e.g., immunogenicity)
- Lower doses
  - Fractionated doses
LCMS Methods for Calicheamicin-Based ADCs

- **Unconjugated Calicheamicin by LCMS**
  - API 6500
  - Human serum – may have analyte stability challenges
  - Sample aliquot = 50 uL
  - LLOQ = 0.050 ng/mL

- **Conjugated Calicheamicin methods by LCMS**
  - API 4000
  - Sample aliquot = 150 uL
  - InO: LLOQ = 0.070 ng/mL of ε-Calicheamicin (DAR = 6.8)
  - EFNA4: LLOQ = 0.100 ng/mL of ε-Calicheamicin
LCMS Methods for Mylotarg

• Unconjugated Calicheamicin by LCMS
  • API 6500
  • Human serum – may have analyte stability challenges
  • Sample aliquot = 50 uL
  • LLOQ = 0.050 ng/mL

• Conjugated Calicheamicin methods by LCMS
  • API4000
  • Sample aliquot = 150 uL
  • lnO: LLOQ = 0.070 ng/mL of ε-Calicheamicin (DAR = 6.8)
  • EFNA4: LLOQ = 0.100 ng/mL of ε-Calicheamicin

• Conjugated Calicheamicin methods for GO in a dual-analyte LCMS method
  • API6500
  • Sample aliquot = 75 uL (vs 200 uL with 2 separate methods)
  • Unconjugated Calicheamicin: LLOQ = 0.020 ng/mL
  • Mylotarg: LLOQ = 0.025 ng/mL of ε-Calicheamicin
Mylotarg - Principles of a Dual-Analyte Method
Mylotarg Dual-Analyte Method: Challenges with Unconjugated Calicheamicin Assay

• **Validation**
  - Initial acceptance criteria as for conventional small molecules (A&P 20%/15%)
  - Initial validation showed higher data variability (A&P ~ 20%+)

• **To continue validation or relax A&P?**
  - Is relaxing criteria in the middle of validation a deviation?
  - The FDA Bioanalytical guidance – following literally or conceptually?

• **What impact on assay implementation?**
  - Delay with measuring PK
  - Original A&P might lead to higher assay failure and non-reportable results
  - Is slightly less accurate data better than no data?
  - Most of unconjugated Calicheamicin data for similar ADCs were BQL
  - The use of unconjugated Calicheamicin assay results:
    - PK profile, when feasible
    - Link to any AEs

• **Is relaxing criteria justifiable by actual assay performance?**
Mylotarg Dual-Analyte Method:
γ-Calicheamicin Assay A&P Acceptance Criteria

• γ-Calicheamicin is not a conventional “small molecule”
  • Natural product with a molecular weight of ~ 1368 Da

• γ-Calicheamicin is reactive
  • Can be metabolized chemically and enzymatically in biological matrices
  • Light sensitive

• Co-existing ADC may release γ-Calicheamicin
  • Mylotarg uses a cleavable linker for Calicheamicin conjugation

• Method uses an analog Internal Standard (IS) rather than a SILIS
  • N-propionyl version of γ-Calicheamicin DMH is used as an analog IS
  • Calicheamicin structure does not allow for introducing sufficient number of stable label isotopes

• A&P acceptance criteria for method validation have been relaxed by 5% (25%/20% vs 20%/15%)
  • All subsequent validation runs were within this new range
Unconjugated Calicheamicin in clinical PK samples

- Out of ~1000 analyzed samples, only ~7% are quantifiable
- The rest of the samples are below LLOQ (0.050 ng/mL)
- Only one sample > 3xLLOQ

ISR

- To not conduct ISR at all due to poor statistical significance?
- To conduct ISR with one or perhaps few more future samples but not use ISR for data acceptance?
- To use the samples below 3xLLOQ?
- Any other thoughts?
Unconjugated Calicheamicin in EFNA4 ADC Clinical Trials: ISR

- **Unconjugated Calicheamicin in clinical PK samples**
  - Out of ~ 1000 analyzed samples, only ~7% are quantifiable
  - The rest of the samples are below LLOQ (0.050 ng/mL)
  - Only one sample > 3xLLOQ

- **ISR**
  1. To not conduct at all because there are no formally eligible samples?
  2. To re-analyze existing low number of eligible samples (>3xLLOQ) but do not use ISR data acceptance?
  3. To conduct ISR with the samples < 3xLLOQ but do not use ISR data acceptance?
  4. To spike measurable samples and re-analyze them for ISR?

**Measurable unconjugated calicheamicin concentrations (ng/mL) in quantifiable samples**
FDA Experience with Unconjugated Payload Data Use

- Question: Should unconjugated clinical payload assays be treated as ‘fit-for-purpose’ (similar to biomarker assays)?
  - Monitor and quantify different forms of payload with ‘qualified’ assays
  - Once a ‘useful’ analyte / assay identified, fully validate or at least ‘qualify’?

**Adcetris Payload PK/PD**

Supplemental Studies—MMAE increased (but not dramatically)

LFTs, AEs were not predicted by payload PK/PD

**ADCs—What Moieties Should We Measure?**

Did we measure all of the correct moieties?

- Increases in MMAE ... but they do not explain all of the AEs
  - Some deaths occurred at low MMAE concentrations?
- Should we be monitoring something else?
  - Is there a deep tissue reservoir for some moiety?
- Are we not monitoring patients treated with ADCs long enough?

Brian Booth  (FDA), 2015 WRIB
Mylotarg Assay: Unconjugated Calicheamicin Curve, Chromatography and IS Performance

Calibration curve (linear)

Analyte and IS chromatograms at LLOQ

Analyte and IS chromatograms at ULOQ

Analyte and IS chromatograms in matrix blank

Area

Injections

IS performance

S:N = 32:1
Mylotarg Assay: Conjugated Calicheamicin Curve, Chromatography and IS Performance

Calibration curve (quadratic)

IS performance

Analyte and IS chromatograms at LLOQ

S:N = 252:1

Diastereomers

Analyte and IS chromatograms at ULOQ

Diastereomers

Analyte and IS chromatograms in matrix blank

Diastereomers
### Mylotarg Assay Performance During Validation (1)

<table>
<thead>
<tr>
<th></th>
<th>Unconjugated Calicheamicin</th>
<th>Conjugated Calicheamicin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Matrix</strong></td>
<td>Human K2EDTA plasma</td>
<td></td>
</tr>
<tr>
<td><strong>Sample volume</strong></td>
<td>75 uL</td>
<td></td>
</tr>
<tr>
<td><strong>Mass Spectrometer</strong></td>
<td>API 6500</td>
<td></td>
</tr>
<tr>
<td><strong>Extraction type</strong></td>
<td>LLE</td>
<td>LLE/LLE</td>
</tr>
<tr>
<td><strong>MRM for analyte</strong></td>
<td>1500.25 &gt; 1321.30</td>
<td>1356.3 &gt; 1196.20</td>
</tr>
<tr>
<td><strong>MRM for IS</strong></td>
<td>1514.30 &gt; 1335.30</td>
<td>1370.3 &gt; 1210.20</td>
</tr>
<tr>
<td><strong>Calibration Range</strong></td>
<td>0.020 – 2.00 ng/mL</td>
<td>0.025 – 25.0 ng/mL</td>
</tr>
<tr>
<td><strong>Calibration Range for ADC</strong></td>
<td>n/a</td>
<td>1.04 – 1040 ng/mL</td>
</tr>
<tr>
<td><strong>QC concentrations (ng/mL)</strong></td>
<td>0.020, 0.040, 0.200, 1.500</td>
<td>0.025, 0.075, 0.500, 3.00, 18.0</td>
</tr>
<tr>
<td><strong>Regression / Weighting</strong></td>
<td>Linear, 1/concentration²</td>
<td>Quadratic, 1/concentration²</td>
</tr>
<tr>
<td><strong>QC Intra-assay Statistics (%)</strong></td>
<td><strong>A (Accuracy)</strong>/<strong>P (Precision)</strong></td>
<td><strong>A (Accuracy)</strong>/<strong>P (Precision)</strong></td>
</tr>
<tr>
<td></td>
<td>P: from 2.19% to 15.4%</td>
<td>P: from 0.998% to 12.2%</td>
</tr>
<tr>
<td></td>
<td>A: from -21.4% (LLOQ)/-12.7% to 5.92%</td>
<td>A: from -5.78% to 5.87%</td>
</tr>
<tr>
<td><strong>QC Inter-assay Statistics (%)</strong></td>
<td><strong>A (Accuracy)</strong>/<strong>P (Precision)</strong></td>
<td><strong>A (Accuracy)</strong>/<strong>P (Precision)</strong></td>
</tr>
<tr>
<td></td>
<td>P: from 3.08% to 15.1% (LLOQ)</td>
<td>P: from 4.30% to 9.43%</td>
</tr>
<tr>
<td></td>
<td>A: from -10.6% to 3.29%</td>
<td>A: from -3.36% to 1.33%</td>
</tr>
<tr>
<td><strong>Analyte recovery</strong></td>
<td>56%</td>
<td>47%</td>
</tr>
<tr>
<td><strong>IS recovery</strong></td>
<td>69%</td>
<td>~66% (due to pre-existing ε-form in ε-IS material?)</td>
</tr>
<tr>
<td><strong>Dilution linearity</strong></td>
<td>5 fold down from 0.200 ng/mL</td>
<td>5 fold down from 0.500 ng/mL 10 fold down from 100.0 ng/mL</td>
</tr>
</tbody>
</table>
Mylotarg Assay Performance During Validation (2)

<table>
<thead>
<tr>
<th>Blood Stability*</th>
<th>Unconjugated Calicheamicin</th>
<th>Conjugated Calicheamicin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single analyte, RT</td>
<td>at least 1 hour**</td>
<td>at least 1 hour**</td>
</tr>
<tr>
<td>Wet-ice</td>
<td>2 hours**</td>
<td>2 hours**</td>
</tr>
<tr>
<td>Both analytes, RT</td>
<td>at least 1 hour**</td>
<td>at least 1 hour**</td>
</tr>
<tr>
<td>Wet-ice</td>
<td>2 hours</td>
<td>2 hours</td>
</tr>
<tr>
<td>Plasma Stability*</td>
<td>at least 1 hour**</td>
<td>at least 2 hours</td>
</tr>
<tr>
<td>Single analyte, RT</td>
<td>at least 4 hours**</td>
<td>2 hours</td>
</tr>
<tr>
<td>Wet-ice</td>
<td></td>
<td>8 hours</td>
</tr>
<tr>
<td>Both analytes, RT</td>
<td>at least 4 hours**</td>
<td>6 hours</td>
</tr>
</tbody>
</table>

Freeze-thaw Stability
after -70°C storage) and thawed on wet ice

<table>
<thead>
<tr>
<th>Extract Stability</th>
<th>108 hours at RT</th>
<th>117 hours at RT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frozen Matrix Storage Stability</td>
<td>75 days at -70°C</td>
<td>75 days at -70°C and at -20°C</td>
</tr>
<tr>
<td>Reinjection Reproducibility</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Impact of hemolysis</td>
<td>acceptable</td>
<td>acceptable</td>
</tr>
<tr>
<td>Impact of lipemia</td>
<td>acceptable</td>
<td>acceptable</td>
</tr>
<tr>
<td>Selectivity in blank plasma</td>
<td>acceptable</td>
<td>acceptable</td>
</tr>
<tr>
<td>Run Length</td>
<td>96 samples</td>
<td>96 samples</td>
</tr>
</tbody>
</table>

Unconjugated and conjugated Calicheamicin assays met the 25%/20% acceptance criteria

- Loss <20% relative to time point zero
- ** - Results generated during development stage
Lessons Learned and Remaining Questions to Answer

- **Single analyte vs Dual analyte assays**
  - Sample size limitations in small species (pediatric subjects or animals)
  - Competition between multiple methods for PK, immunogenicity, and biomarkers (More from Less)

- **PROS and CONS of the dal-analyte assay**
  - **PROS:**
    - More ethical subject treatment (less blood drawn)
    - Potentially faster analysis
    - Sample composition of STD/QC is closer to study samples
    - Same assay can be used in preclinical and clinical studies and even in discovery
  - **CONS:**
    - A loss of one sample leads to a loss of two analytes
    - Regulatory challenges

- **“80:20 rule” for assay development and validations**
  - “Hybrid” conjugated payload assays are robust and may be easier to validate than unconjugated
  - Unconjugated (“free”) payload assay may be more challenging to develop and validate although may have less utility compare to the conjugated payload assays

- **Regulatory requirements for ADC bioanalysis**
  - ADC analytes (and their respective methods) may be treated as small or large molecules
  - No separate bioanalytical guidance exists for ADC bioanalysis
  - **How to fulfill ISR requirements for unconjugated payloads such as Calicheamicin?**
  - **Scientific justification for payload assay validation vs. formal guidance?**
Acknowledgements

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• Jordan Honrine
• Michael Waldron
• William Mylott Jr
Backup Slides
Clinical "Hybrid" LCMS Assay for Calicheamicin-Based ADC: Cross-Validation with ELISA?

- **Fundamental difference between measured analytes**
  - ELISA results vs. LCMS results

- Dynamic ranges of ELISA and LCMS are not fully overlapped
### Hypothetical Example of ADC Quantitation by ELISA vs. LCMS

<table>
<thead>
<tr>
<th>ADC sample composition</th>
<th>Time (relative units)</th>
<th>ADC concentration by ELISA</th>
<th>Payload concentration by LCMS</th>
<th>Equivalent ADC concentration from LCMS payload concentration *</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADC by ELISA</td>
<td>0</td>
<td>3</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>ADC by LCMS</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>1.5</td>
</tr>
<tr>
<td>ADC by ELISA</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0.5</td>
</tr>
<tr>
<td>ADC by LCMS</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Average initial DAR = 2

#### Half-life \( (T_{1/2}) \) based on ELISA vs. LCMS data

- **ADC by ELISA**
- **Payloads by LCMS**