Monitoring Charge Heterogeneity of Antibody-Maytansinoid Conjugates (AMC) with iCIEF

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CE Pharm 2008
Outline

I. Introduction of AMC and its charge heterogeneity
II. Analytical methods employed for monitoring charge heterogeneity
III. Use of iCIEF on MAb and AMC analysis
IV. Demonstration of using iCIEF for quantitation of unconjugated antibody (uMAb) in AMC
Antibody Maytansinoid conjugate (AMC)

- Combines high specificity of mAb with the cell killing ability of small molecule cytotoxic agent
  - AMC approach to be discussed is with ImmunoGen’s TAP technology
  - 7 TAP compounds in clinic
- mAb
  - Monoclonal antibodies recognizing tumor-specific antigens
  - IgG1 or IgG4 utilized thus far
- Linker
  - Heterobifunctional linker attached to mAb via Lysines
  - Cytotoxic agent attached to linker by “reducible” or “non-reducible” bonds
- Maytansinoid (MAY) cytotoxic agent (e.g. DM1, DM4)
  - Members of Ansamitocin family
  - Highly potent (IC50: 10 – 100 pM)
  - Inhibits tubulin polymerization
How do TAP compounds work?

- TAP compounds are designed to bind to targets on the surface of cancer cells.
- They are brought into the cell by natural processes.
- Once inside the cell, the cytotoxic agent is freed and is able to kill the cancer cell.
Basic structure of AMC

- Average of 3 – 4 molecules of MAY per MAb molecule
- Choice of linker depends on the antibody and target (among other factors) and impacts, stability, clinical safety, and efficacy
- Neither the linker nor Maytansinoid possess any charge

SPP-linked AMC (reducible)
SPDB-linked AMC (reducible)
SMCC-linked AMC (non-reducible)
Source of Charge Heterogeneity in AMC

- Unconjugated antibody itself contains charge heterogeneity (e.g. C-terminal Lys, N-terminal pyro-Glu, deamidation, etc.)
- Lysine is a basic amino acid, so the pI of the antibody decreases each time a lysine residue is modified by a linker molecule
- Resulting AMC is a mixture of molecules where each antibody has different numbers of DM4 covalently conjugated to it (D0, D1, D2, etc.)
- Charge heterogeneity in any AMC sample is complicated by all the above factors
- It is important to monitoring charge heterogeneity in AMC, because:
  - Understand and characterize AMCs
  - Lot-to-lot consistency
AMC: IMGN242

- IMGN242 is a treatment for tumors that express the CanAg antigen (e.g. gastric, pancreatic)
- Currently in Phase II clinical trials
- Antibody: huC242 (MW = 147 kDa)
- Cytotoxic agent: DM4
- Linker: SPDB
- Average DM4-to-Ab ratio = 3.7
Explored Analytical Methods for Monitoring Charge Heterogeneity in AMC

<table>
<thead>
<tr>
<th>Method</th>
<th>Drawbacks</th>
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<tbody>
<tr>
<td>Weak cation exchange (WCX) chromatography</td>
<td>Very poor resolution of different species in AMCs</td>
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<tr>
<td></td>
<td>Quantitation cannot be carried out</td>
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<tr>
<td>Gel Isoelectric Focusing (IEF)</td>
<td>Quantitation is difficult to carry out</td>
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<td></td>
<td>Poor reproducibility</td>
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<td></td>
<td>Labor-intensive</td>
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<tr>
<td>Capillary Isoelectric Focusing (CIEF)</td>
<td>Time-consuming (focusing followed by mobilization)</td>
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<td></td>
<td>Resolution lost during mobilization</td>
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</table>
AMC Charge Profile by WCX

Charge heterogeneity of AMC cannot be monitored by this method

- Antibody

- AMC

Unconjugated MAb
AMC Charge Profile by CIEF
Charge heterogeneity of AMC also cannot be monitored by this method

**CIEF Profile of IMGN242**

- Absorbance @ 280 nm
- Migration Time (min)
Imaged Capillary Isoelectric Focusing (iCIEF)

- Instrument: iCE280 (Convergent Biosciences, Canada)
- Focusing is captured in real time, so there is no need for mobilization after focusing
  - Higher throughput than CIEF methods
  - High pI reproducibility
  - High resolution
- Electropherograms can be exported and integrated for quantitation
Process of CIEF

1. Sample and carrier ampholytes (CA) are injected into the capillary.

2. The CA forms a pI gradient across the capillary. Species will start migrating according to its pI.

3. Eventually, species will focus at the pH where its charge is neutral.

In iCIEF, absorbance at 280 nm within the capillary are taken every 30 seconds during focusing.
Test Samples

- Hemoglobin standards
  - System suitability
- mAb: huC242
  - Assay evaluation
- AMC: IMGN242
  - Charge heterogeneity profile
  - Quantification of unconjugated antibody (umAb)
- All samples are analyzed using iCE280
- All electropherograms were exported to Empower (Waters Corporation, Milford, MA) for integration
Focussing of hemoglobin is indicative of the system’s performance.

Sample composition: 8% pH 3-10 Pharmalytes, 2 internal pI Markers (4.22, 9.46) in 0.35% Methyl Cellulose (MC)

The four subunits must be well-resolved under the specified condition.

In particular, the HbA1c subunit must be resolved as a shoulder or a separate peak.

The difference in pI between HbA1c and HbA is about 0.03
Analysis of huC242 Antibody by iCIEF

- huC242 sample preparation: 0.35% methyl cellulose (MC), 4% pH 8-10.5 Pharmalytes, 7.65 and 9.77 pI markers
- Focusing conditions:
  - Pre-focusing at 500V for 1 min
  - Focusing at 3000V for 10 min
- iCIEF was evaluated for:
  - Reproducibility
  - Linearity
  - Precision
  - Recovery
  - LOD and LOQ
huC242 MAb: Reproducibility and Linearity

<table>
<thead>
<tr>
<th>Summary</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Linearity</strong></td>
<td>$R^2 &gt; 0.99$</td>
</tr>
<tr>
<td></td>
<td>for 0.001-0.1 mg/mL</td>
</tr>
<tr>
<td><strong>Reproducibility of measured pI in linear range</strong></td>
<td>RSD = 0.40%</td>
</tr>
<tr>
<td></td>
<td>from 21 injections at 7 concentrations</td>
</tr>
<tr>
<td><strong>Limit of detection</strong></td>
<td>~ 0.0006 mg/mL</td>
</tr>
<tr>
<td><strong>Limit of quantitation</strong></td>
<td>~ 0.002 mg/mL</td>
</tr>
<tr>
<td><strong>Repeatability of measured pI</strong></td>
<td>RSD = 0.06%</td>
</tr>
<tr>
<td><strong>Day-to-day Precision</strong></td>
<td>&lt; 20% from 0.001 mg/mL</td>
</tr>
<tr>
<td></td>
<td>to 0.1 mg/mL</td>
</tr>
<tr>
<td><strong>Recovery Accuracy</strong></td>
<td>Within 80%-120% for concentrations &gt; 0.004 mg/mL</td>
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Focusing IMGN242 in iCE280

Sample preparation:
- 0.2 mg/mL of IMGN242
- 0.35% MC
- 2% pH 8-10.5 Pharmalytes
- 7.65 and 9.77 pI markers

Focusing condition:
- Pre-focusing at 500V for 1 min
- Focusing at 3000V for 12 min

Focusing is complete when peaks become stationary in the capillary.
Complete focusing is also indicated by current that is close to 0.
Choice of Carrier Ampholyte

- Due to the high pI of IMGN242, choice of carrier ampholyte is limited.
- Narrow range ampholytes are preferred, due to the high degree of resolution desired.
- Carrier ampholytes tested:
  1. Servalyt pH 7-9 and Servalyt pH 9-11 in 1:1 ratio
  2. Pharmalyte pH 8-10.5
- Results:
  - Pharmalyte allows detailed resolution of many species in the AMC
  - Pharmalyte yields a cleaner baseline
Electropherogram of IMGN242 in Empower

- The pI of AMC decreases about 0.1 with each extra DM4 conjugated to it.

<table>
<thead>
<tr>
<th># DM4</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>pI</td>
<td>8.80</td>
<td>8.73</td>
<td>8.63</td>
<td>8.54</td>
<td>8.44</td>
<td>8.33</td>
<td>8.21</td>
<td>8.09</td>
<td>8.00</td>
</tr>
</tbody>
</table>
Comparing iCIEF Profile of IMGN242 with Mass Spectroscopy Profile

- Both methods show similar DM4 distribution profile.
Quantitation of uMAb in IMGN242

<table>
<thead>
<tr>
<th>Injection</th>
<th>Day 1 (#1)</th>
<th>Day 1 (#2)</th>
<th>Day 2 (#1)</th>
<th>Day 2 (#2)</th>
<th>Day 2 (#3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>% uMAb by Peak Area</td>
<td>1.5%</td>
<td>2.1%</td>
<td>2.4%</td>
<td>2.2%</td>
<td>2.2%</td>
</tr>
<tr>
<td>[uMAb] (mg/mL)</td>
<td>0.005</td>
<td>0.007</td>
<td>0.007</td>
<td>0.006</td>
<td>0.007</td>
</tr>
<tr>
<td>% uMAb by Calibration Curve</td>
<td>2.5%</td>
<td>3.7%</td>
<td>3.7%</td>
<td>3.0%</td>
<td>3.3%</td>
</tr>
</tbody>
</table>

- The percentage of umAb was estimated by 2 methods:
  1) Calculating % of total area
  2) Calculating concentration of umAb from the calibration curve, and comparing it with the total concentration of protein in the sample.
- Estimating % of umAb by % of total area is inaccurate, due to the contribution of absorbance at 280nm by DM4.
- The level of umAb detected in the AMC sample is higher than the LOQ of this method.
Conclusions

- Charge heterogeneity in AMCs can be monitored by iCIEF with high resolution.
- The charge profile of IMGN242 from iCIEF is comparable to that obtained from mass spectrometry.
- iCIEF appears to provide a quantitative way to estimate the amount of umAb present in AMC samples.
Acknowledgements

Analytical & Pharmaceutical Sciences
• Alex Lazar, Ph.D. (Senior Scientist)
• Rajesh Krishnamurthy, Ph.D. (Director)

Convergent Biosciences
• Jiaqi Wu, Ph.D.
• Alice Lam

Thank you for your attention!!