Capillary electrophoresis leading the way to an enhanced understanding of conjugational heterogeneity in an ADC

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Researcher Analytical Development for New Chemical Entities
Byondis At a Glance

Clinical stage biopharmaceutical R&D company

Dutch owned, based in Nijmegen, the Netherlands

Creating precision medicines

mAbs and ADCs

Experienced and highly educated staff
### Byondis Pipeline

<table>
<thead>
<tr>
<th>Target</th>
<th>Modality</th>
<th>Compound</th>
<th>Clinical Indication</th>
<th>Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>HER2</td>
<td>Biosimilar</td>
<td>Trastuzumab</td>
<td>Breast and gastric cancer</td>
<td>Submitted/Marketed</td>
</tr>
<tr>
<td>HER2</td>
<td>ADC</td>
<td>SYD985</td>
<td>Breast Cancer</td>
<td>Phase II</td>
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<tr>
<td>c-MET</td>
<td>ADC</td>
<td>BYON3521</td>
<td>Endometrial cancer</td>
<td>FIH 2022</td>
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<tr>
<td>SIRPα</td>
<td>mAb</td>
<td>BYON4228</td>
<td>2nd line HER2-expressing Uterine Serous Carcinoma (USC)</td>
<td>FIH 2023</td>
</tr>
<tr>
<td>CD123</td>
<td>ADC</td>
<td>BYON4413</td>
<td></td>
<td>FIH 2023</td>
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</tbody>
</table>

**Research & Platform Development**

- **Worldwide license agreement with Amgen/Allergan (marketed since 2018 as Kanjinti®)**

- **3rd line HER2-positive metastatic breast cancer (MBC)**

- **2nd line HER2-expressing Uterine Serous Carcinoma (USC)**

**Preclinical**

- SYD985: Breast Cancer

**Clinical Indications**

- Breast and gastric cancer
- Breast Cancer
- Endometrial cancer
- 2nd line HER2-expressing Uterine Serous Carcinoma (USC)
Antibody-Drug Conjugates (ADCs)

Uptake of ADC by internalization and intercellular release of payload

Proteolytic cleavage and subsequent release of payload in tumor microenvironment

Diffusion of active payload to neighboring tumor cells (bystander effect)
Randomly Cysteine-conjugated ADC

mAb \[ \text{TCEP - SS reduction} \] Partially reduced mAb \[ \text{Linker-drug conjugation} \]
Conjugational Heterogeneity in an ADC

Drug

Fab position f
Hinge position h
Drug-to-Antibody Ratio (DAR) by HIC-HPLC

10-May-23
Improvement of ‘Second Generation’ ADC Technologies

- Protein Concentration
- Purity
- Drug load
- Aggregation

Quality Attributes

Process Parameters

- Ratio TCEP
- pH
- Temperature
- Reduction time

Research → Process Development → Early Clinical Supply (Ph I/II) → Process Scale up (Ph II/III) → Process Qualification (Ph III/Commercial)
Quality Target Product Profile (QTPP)

Safety and efficacy

Patient

Process

Product

CMAs, CPPs

QTPP, CQAs
Critical Quality Attributes (CQA) of an ADC

QTPP → Critical Quality Attributes → CMC development strategy

- Protein Concentration
- Purity
- Drug load and distribution (DAR)

Product and process characterization
Understanding and controlling CQAs:
- Safety, efficacy and cytotoxicity
- Selected process technology
- Process capability
- Specifications

Quality attributes to ensure the safety and efficacy as promised
Impact mAb Reduction on Product Quality and Consistency

Molar Ratio mAb/Reducing agent
Temperature
Time
pH

Partially reduced mAb

TCEP - SS reduction

byondis
Quantification of the Impact by Reduction Time on DAR-related Attributes

ADCs were prepared in two consecutive experiments using the same conditions, except for the reduction time of the mAb:

• The first experiment comprising ADCs with reduction times of **0.5, 1, 4, and 24 hours**
• And the second experiment comprising ADCs with reduction times of **1, 2, 3, and 4 hours**.

• Disulfide bond reduction was ended at each time point by proceeding to the conjugation step and attaching linker-drug molecules to the available cysteines.

• Prior to analysis, the ADC samples were purified to remove non-conjugated mAb and process-related impurities and finally formulated to drug substance at 10 mg/mL.
## DAR Analysis by HIC-HPLC

### Analytical Variation

<table>
<thead>
<tr>
<th>Batch</th>
<th>Reduction time (h)</th>
<th>DAR0 (%)</th>
<th>DAR1 (%)</th>
<th>DAR2 (%)</th>
<th>DAR4 (%)</th>
<th>DAR6 (%)</th>
<th>Average DAR</th>
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<tr>
<td>N/A</td>
<td></td>
<td>0.1</td>
<td>0.3</td>
<td>0.9</td>
<td>0.3</td>
<td>0.7</td>
<td>0.03</td>
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<td>ADC.e01/t0.5 h</td>
<td>00:31</td>
<td>0.3</td>
<td>0.8</td>
<td>71.8</td>
<td>22.9</td>
<td>4.1</td>
<td>2.6</td>
</tr>
<tr>
<td>ADC.e01/t1 h</td>
<td>01:07</td>
<td>0.2</td>
<td>0.5</td>
<td>71.6</td>
<td>23.5</td>
<td>4.2</td>
<td>2.6</td>
</tr>
<tr>
<td>ADC.e02/t1 h</td>
<td>01:03</td>
<td>0.4</td>
<td>0.4</td>
<td>71.6</td>
<td>24.1</td>
<td>3.6</td>
<td>2.6</td>
</tr>
<tr>
<td>ADC.e02/t2 h</td>
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<td>0.3</td>
<td>0.5</td>
<td>72.1</td>
<td>23.7</td>
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<td>2.6</td>
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<tr>
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<td>0.3</td>
<td>0.6</td>
<td>72.1</td>
<td>23.7</td>
<td>3.3</td>
<td>2.6</td>
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<tr>
<td>ADC.e02/t4 h</td>
<td>04:06</td>
<td>0.3</td>
<td>0.5</td>
<td>71.9</td>
<td>24.0</td>
<td>3.3</td>
<td>2.6</td>
</tr>
<tr>
<td>ADC.e01/t4 h</td>
<td>04:13</td>
<td>0.2</td>
<td>0.5</td>
<td>72.0</td>
<td>23.7</td>
<td>3.6</td>
<td>2.6</td>
</tr>
<tr>
<td>ADC.e01/t24 h</td>
<td>24:11</td>
<td>0.3</td>
<td>0.7</td>
<td>72.6</td>
<td>23.3</td>
<td>3.2</td>
<td>2.6</td>
</tr>
</tbody>
</table>
Conjugational Heterogeneity of Randomly Cysteine-conjugated ADCs

Drug Distribution (isomeric DAR species) by CE-SDS

10 kDa marker

Light chain (LC)

Heavy chain (HC)

Heavy-heavy chain (HH)

Heavy-light chain (HL)

Heavy-heavy-light chain (HHL)

Intact (HHLL)
Positional Isomers Characterization by CE-SDS

<table>
<thead>
<tr>
<th>Batch</th>
<th>Reduction time (h)</th>
<th>LC (%)</th>
<th>HC (%)</th>
<th>HL (%)</th>
<th>HH (%)</th>
<th>HHL (%)</th>
<th>HHLL (%)</th>
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</thead>
<tbody>
<tr>
<td>Analytical Variation</td>
<td>N/A</td>
<td>0.7</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.6</td>
<td>0.2</td>
</tr>
<tr>
<td>ADC.e01/t0.5 h</td>
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<td>20.3</td>
<td>1.6</td>
<td>9.1</td>
<td>11.0</td>
<td>50.7</td>
<td>6.3</td>
</tr>
<tr>
<td>ADC.e01/t1 h</td>
<td>01:07</td>
<td>19.9</td>
<td>1.8</td>
<td>11.1</td>
<td>10.4</td>
<td>49.7</td>
<td>6.2</td>
</tr>
<tr>
<td>ADC.e02/t1 h</td>
<td>01:03</td>
<td>20.2</td>
<td>1.7</td>
<td>10.7</td>
<td>10.5</td>
<td>49.8</td>
<td>6.1</td>
</tr>
<tr>
<td>ADC.e02/t2 h</td>
<td>02:05</td>
<td>19.4</td>
<td>1.8</td>
<td>12.6</td>
<td>9.2</td>
<td>49.5</td>
<td>6.4</td>
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<tr>
<td>ADC.e02/t3 h</td>
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<td>18.8</td>
<td>1.9</td>
<td>14.1</td>
<td>8.3</td>
<td>49.3</td>
<td>6.6</td>
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<tr>
<td>ADC.e02/t4 h</td>
<td>04:06</td>
<td>18.4</td>
<td>2.0</td>
<td>15.4</td>
<td>7.7</td>
<td>48.9</td>
<td>6.6</td>
</tr>
<tr>
<td>ADC.e02/t5 h</td>
<td>04:13</td>
<td>18.0</td>
<td>2.1</td>
<td>15.9</td>
<td>7.4</td>
<td>48.9</td>
<td>6.8</td>
</tr>
<tr>
<td>ADC.e01/t24 h</td>
<td>24:11</td>
<td>16.7</td>
<td>2.2</td>
<td>19.2</td>
<td>4.9</td>
<td>49.0</td>
<td>7.0</td>
</tr>
</tbody>
</table>

Diagram showing the separation of positional isomers with peaks for light chain (LC), heavy-light (HL), heavy-heavy-light (HHL), and heavy-heavy-heavy-light (HHLL).
Dynamics of Conjugational Heterogeneity by Reduction Time

A

B

Drug
Fab position f
Hinge position h

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Exchange of Positional Isomers as Function of Reduction Time

LS = Large at scale manufacturing
Characterization of Conjugated Cysteines by nrPEM LC-MS/MS

![Graph showing relative peak area (%)]

- ADC Large Scale 3
- ADC.e02/t1 h
- ADC.e02/t2 h
- ADC.e02/t3 h
- ADC.e02/t4 h

- Conjugated Fab interchain (%)
- Conjugated Hinge interchain (%)

10-May-23
### Residual TCEP Analysis by LC-MS

<table>
<thead>
<tr>
<th>Batch</th>
<th>Reduction time (h)</th>
<th>TCEP (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduction buffer</td>
<td>N/A</td>
<td>141.5</td>
</tr>
<tr>
<td>ADC.e01/t0 h</td>
<td>00:00</td>
<td>1.5</td>
</tr>
<tr>
<td>ADC.e01/t0.5 h</td>
<td>00:31</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>ADC.e01/t1 h</td>
<td>01:07</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>ADC.e01/t4 h</td>
<td>04:13</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>ADC.e01/t24 h</td>
<td>24:11</td>
<td>&lt;0.2</td>
</tr>
</tbody>
</table>

Molar Ratio mAb/Reducing agent

TCEP - SS reduction

![TCEP reaction](image-url)
Peak Annotation of HIC Chromatogram
The nrCE-SDS method employed in this characterization study was validated to enable quantification of each individual ADC constituent, as well as the overall purity.

### Intended purpose: Drug distribution and purity

<table>
<thead>
<tr>
<th>Performance characteristics</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Accuracy</td>
<td>99 – 102 %</td>
</tr>
<tr>
<td>Precision</td>
<td>&lt; 2.0 %</td>
</tr>
<tr>
<td>Linearity</td>
<td>&gt; 0.99</td>
</tr>
<tr>
<td>Specificity</td>
<td>No interfering compounds and stability indicating.</td>
</tr>
<tr>
<td>Reportable range</td>
<td>50 – 150 % (relative to nominal concentration)</td>
</tr>
<tr>
<td>LOQ</td>
<td>0.05 % Area</td>
</tr>
</tbody>
</table>
The conjugational heterogeneity of cysteine-conjugated ADCs is a complex interplay between DAR species and positional isomers.

Characterization of process parameters, like mAb reduction, leads to better prediction of the reduced interchain disulfides, thereby controlling the composition of DAR species.

The reduction time influences the dynamics in the population of positional isomers:
- Shorter reduction times lead to predominance of Fab conjugated cysteines
- Prolonged reduction times shifts the population to more hinge conjugated cysteines.

This unexpected observation, revealed by nrCE-SDS, emphasizes the importance of CE for the characterization of randomly cysteine-conjugated ADCs.

nrCE-SDS was found to be an excellent complement to HIC and alternative for RP-HPLC as a fast and easy substitute assay for monitoring purity, the conjugated positions, and the consistency therein.
Acknowledgements

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