Characterization of Filgrastim using Intact and Top-down MS

Mass Spec 2015 conference

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Sandoz Biopharmaceuticals
Outline

- Approval of biosimilar Zarxio – the totality of evidence
- Characterization of product variants using intact and top-down MS
- Comparison of filgrastim products with regard to product variants
- Intact and top-down MS of PEGylated filgrastim
Zarxio™ is the first biosimilar approved in the US

- 2004: EU draft general guidelines adopted
- 2008: Zarxio™ approved in EU
- 2009: Japan regulatory guidelines
- 2010: US regulatory pathway established
- 2012: US Draft Guidance
- 2014: EU revised overarching guideline
- 2015: Zarxio™ approved in US

Zarxio™ /Zarzio™ is marketed in more than 60 countries (as Zarzio outside the US) generating 7.7 million patient-days of exposure experience until 31 Jul 2014, Date of PSUR: 29 Aug 2014
The FDA’s totality of evidence approach

The *residual uncertainty* defines the extent of the clinical program.

- **Physico-chemical characterization**
- **Functional characterization**
- **Clinical Non-clinical**

Filgrastim
~19 kDa

Mab
~150 kDa
Analytical characterization was the foundation for the approval of Zarxio™ as the first US Biosimilar

In case of Zarxio™ the analytical similarity data did not raise residual uncertainties*

*January 7 2015 Oncology Drugs Advisory Committee Meeting

Neupogen is a registered trademark of Amgen Inc.
MS as key technology for the characterization of product-related variants

Filgrastim
18.8kDa
PDB: 1RHG

<table>
<thead>
<tr>
<th>Quality attribute</th>
<th>Criticality</th>
<th>Characterized by</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidation</td>
<td>High</td>
<td>MS</td>
<td>Present in DS</td>
</tr>
<tr>
<td>N-term acetylation</td>
<td>High</td>
<td>MS</td>
<td>Present only in Intermediates</td>
</tr>
<tr>
<td>N-term phosphogluconooylation</td>
<td>High</td>
<td>MS</td>
<td>Present only in Intermediates</td>
</tr>
<tr>
<td>N-term truncations</td>
<td>Low</td>
<td>MS</td>
<td>Present in DS</td>
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</tbody>
</table>
Top-down MS of filgrastim

25% sequence coverage using HCD at 35eV
Methionine oxidation site-assignment

Met1

1  M T P L G P A S S L P Q S F L L K C L E Q V R K I Q C D G A A L Q E
35  K L C A T Y K L C H P E E L V L L G H S L G I P W A S C R S Q
69  A L Q L A G C L S Q L H S G L F L Y Q G L Q A L E G I S P E L G P
103  T L D T L Q L D V A D F A T T I W Q Q M E E L G M A P A L Q R T Q G
137  A M P A F A S A F Q R R A G V G L V A S H L M E E L G M A P A L Q R T Q G
171  H L A Q P

Met122

Met127

Met138

Diagnostic fragment ions used for methionine oxidation site-assignment

y50  y47  y78  b97

native filgrastim
[M+H]^+
Methionine oxidation site-assignment

0.1% oxidation at Met122 and Met127

Neupogen® (25 weeks after end of shelf-life)

Neupogen® (9 weeks before end of shelf-life)

Filgrastim Sandoz development sample

Filgrastim Sandoz development sample

0.1% oxidation at Met122 and Met127

doubly oxidized filgrastim [M+H]+
Characterization of an acetylated variant

**IB solubilization**

**Refold**

**Purification Step A**

**Purification Step B**

**Purification Step C**

**Zarxio™ DS**

RPC of a fraction from Step A

N-term acetylation
Characterization of a phosphoglucunoylated variant

IB solubilization

Refold

Purification Step A

Purification Step B

Purification Step C

Zarxio™ DS

RPC of a fraction from Step B

+258Da modification

modified b-series (+258Da) starting at b33

native y-series up to y79
Characterization of a phosphogluconooylated variant

IB solubilization

Refold

Purification Step A

Purification Step B

Purification Step C

Zarxio™ DS

variant isolated

N-term phosphogluconooylation

6-phosphogluconolactone
Semi-quantification of product variants in DSP intermediates by MS

Relative abundance of product-related variant [%]

<table>
<thead>
<tr>
<th>Attribute</th>
<th>Refold</th>
<th>Step A</th>
<th>Step B</th>
<th>Zaxio™ DS</th>
<th>Zaxio™ DP</th>
<th>Neupogen® DP</th>
</tr>
</thead>
<tbody>
<tr>
<td>∑ singly oxidized*</td>
<td>4.4</td>
<td>3.2</td>
<td>1.7</td>
<td>1.5</td>
<td>&lt;7.5</td>
<td>&lt;7.8</td>
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<tr>
<td>N-term acetylated</td>
<td>1.2</td>
<td>0.2</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>N-term phosphogluconoylated</td>
<td>2</td>
<td>1.5</td>
<td>0.8</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

* abundance dependent on age of the product
Semi-quantitative assessment of N-terminal truncations by LC-MS

Group I truncated variants

\text{MTPLGPASSLPQSFLKLKCLE}

Group II truncated variants

flexible N-terminal region

Filgrastim 18.8kDa
PDB: 1RHG

native filgrastim
Trace levels of the acetylated filgrastim detectable in some competitor products
Trace levels of tryptophan addition detectable in some competitor products

Neupogen US
Zarxio™/Zarzio™
Filgrastim product C
Filgrastim product F

MS spectra Summed up

18788.6
18902.6
deamidated filgrastim (+1Da)
+114.0 Da
to native filgrastim

TFA adduct

18973.7

1175.9243
z=16

1183.2993
z=16

1176.0509
z=16

1184.1863
z=16

1175.7984
z=16

1184.1847
z=16

1187.6182
z=16

1175.9867
z=16

1183.1733
z=16

1187.4922
z=16

manuscript in preparation

Sandoz
a Novartis company
Trace levels of tryptophan addition detectable in some competitor products

Filgrastim products C, F and G contain ~0.3% additional C-term tryptophan

A leaky UGA stop codon caused by a UGA suppressor tRNA\textsuperscript{Trp} may explain the additional C-term tryptophan (=differences in stop codon usage)

D. Hirsh, L. Gold (1971), J.Mol.Biol
Translation of the UGA triplet in vitro by tryptophan transfer RNA’s

R. H. Buckingham and C. G. Kurland (1977), PNAS
Codon specificity of UGA suppressor tRNA\textsuperscript{Trp} from Escherichia coli

manuscript in preparation
MS of PEGylated Filgrastim

Charge stripping using Triethylamine (TEA) shifting the charge state distribution to a higher m/z.

Huang et al, 2009
Method optimization and transfer to Orbitrap analyzer – direct infusion

Instrument, LTQ Orbitrap XL operated at $R=100,000$ at m/z 400; acquisition time, 10 min;


A direct-infusion- and HPLC-ESI-Orbitrap-MS approach for the characterization of intact PEGylated proteins.
Interfacing to chromatographic separation

A direct-infusion- and HPLC-ESI-Orbitrap-MS approach for the characterization of intact PEGylated proteins.
Characterization of PEG heterogeneity and oxidation site-assignment in pegfilgrastim

A direct-infusion- and HPLC-ESI-Orbitrap-MS approach for the characterization of intact PEGylated proteins.
Summary and conclusion

- Analytical characterization was the foundation for the approval of Zarxio™ as the first US Biosimilar.

- Intact and top-down MS provides enhanced sensitivity and specificity in comparison to RP-HPLC-UV for the identification and monitoring of low-level product variants. However, exact site-assignment is not always possible.

- Orbitrap mass analyzers can be successfully used for the characterization of PEG heterogeneity of a therapeutic protein.

- Owing to its faster scan speed and improved data processing, measurements of PEGylated proteins with the Q Exactive can be efficiently interfaced to online chromatographic separation.
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http://www.uni-salzburg.at/index.php?id=63471&L=1