Production of an anti-CD20 immunocytokine for the treatment of Non-Hodgkin lymphoma in *N. benthamiana*

Marusic C., Novelli F., Salzano A.M., Scaloni A., Benvenuto E., Pioli C. and Donini Marcello
• **NHLs** rank fifths in cancer incidence and mortality worldwide

• NHLs are of B-cell origin and more than 90% express leukocyte antigen CD20

• CD 20 is an integral membrane phosphoprotein (33-37 kDa) expressed on all stages of B cell development except the first and last. It is present in B-cell precursors, mature B-cells but not on early pro-B cells and plasma cells.

• Classical standard therapies are chemotherapy or radiotherapy

• Anti CD-20 C2B8 (Rituxan, Roche) is currently used for regression of B-cell lymphoma also in combination therapies

• Its mechanism of action is not well understood but it mediates (ADCC, CDC and apoptosis).
mAb ‘Blockbusters’

<table>
<thead>
<tr>
<th>mAb (target)</th>
<th>Companies</th>
<th>Global 2011 sales (US$ billions)*</th>
<th>Expiry of constraining product patent*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infliximab (TNF)</td>
<td>Johnson &amp; Johnson; Merck &amp; Co.; Mitsubishi Tanabe</td>
<td>$9.0</td>
<td>Sep 2018     Aug 2014</td>
</tr>
<tr>
<td>Adalimumab (TNF)</td>
<td>Abbott; Eisai</td>
<td>$8.2</td>
<td>Mar 2019     Apr 2018</td>
</tr>
<tr>
<td>Etanercept† (TNF)</td>
<td>Amgen; Pfizer; Takeda</td>
<td>$7.9</td>
<td>Nov 2023     Feb 2015</td>
</tr>
<tr>
<td>Rituximab (CD20)</td>
<td>Roche</td>
<td>$6.8</td>
<td>Dec 2015     Nov 2013</td>
</tr>
<tr>
<td>Bevacizumab (VEGF)</td>
<td>Roche</td>
<td>$6.0</td>
<td>Jul 2019     Apr 2018</td>
</tr>
<tr>
<td>Trastuzumab (HER2)</td>
<td>Roche</td>
<td>$5.9</td>
<td>Nov 2023     Jul 2014</td>
</tr>
<tr>
<td>Ranibizumab (VEGF)</td>
<td>Novartis; Roche</td>
<td>$3.8</td>
<td>Jul 2019     Apr 2018(^{\dagger})</td>
</tr>
<tr>
<td>Cetuximab (EGFR)</td>
<td>Bristol-Myers Squibb; Merck Serono</td>
<td>$1.9</td>
<td>Jun 2015     Sep 2014</td>
</tr>
<tr>
<td>Natalizumab (α4 integrin)</td>
<td>Biogen Idec</td>
<td>$1.5</td>
<td>Mar 2015     Aug 2015(^{\dagger})</td>
</tr>
<tr>
<td>Omalizumab (IgE)</td>
<td>Novartis; Roche</td>
<td>$1.2</td>
<td>Jan 2020     Aug 2017</td>
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</tbody>
</table>

*Estimated patent expiry dates and 2011 sales data provided by Thomson Reuters. †The main patent on etanercept had been expected to expire in October 2012, but Amgen recently secured an additional 17 years of protection owing to a filing loophole. \(^{\dagger}\)Supplementary protection certificates have been filed for these products in some European Union member states, potentially adding up to an additional 5 years of protection. EGFR, epidermal growth factor receptor; IgE, immunoglobulin E; mAb, monoclonal antibody; TNF, tumour necrosis factor; VEGF, vascular endothelial growth factor.

Rituximab

• The effectiveness of Rituximab (C2B8) in the treatment of B-cell lymphoma is variable.

• Necessity to induce multiple mechanisms such as: CDC (complement dependent cytotoxicity) ADCC antibody dependent cell cytotoxicity and apoptotic signaling.

• Currently combination therapy of Rituximab and IL2, stimulator of T cells and NK cells, have shown promising results in clinical studies

• The aim of this project is to produce in plant a tumor-targeting, engineered IL-2 immunocytokine variant of the C2B8 antibody
### Immunocytokines in cancer therapy

#### Table 1: Overview of the immunocytokines in clinical development

<table>
<thead>
<tr>
<th>Immunocytokine</th>
<th>Company</th>
<th>Format</th>
<th>Illustration</th>
<th>Antigen</th>
<th>Indication</th>
<th>Stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>FL6-IL2</td>
<td>Philogen</td>
<td>Diabody</td>
<td></td>
<td>A1 domain</td>
<td>Breast cancer, lung cancer</td>
<td>Phases 1b/1I</td>
</tr>
<tr>
<td>(Teleukin)</td>
<td></td>
<td></td>
<td></td>
<td>of Tenascin C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hu14.18-IL2</td>
<td>Merck KGaA</td>
<td>IgG</td>
<td></td>
<td>GD2</td>
<td>Melanoma, neuroblastoma</td>
<td>Phases II</td>
</tr>
<tr>
<td>(EMD273063)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L19-IL2</td>
<td>Philogen</td>
<td>Diabody</td>
<td></td>
<td>EDB</td>
<td>Melanoma, pancreas, RCC</td>
<td>Phases 1b</td>
</tr>
<tr>
<td>(Darleukin)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NHS-ILZLT</td>
<td>Merck KGaA</td>
<td>IgG</td>
<td></td>
<td>DNA</td>
<td>Solid tumors, NHLymphoma, NSCL carcinoma</td>
<td>Phases 1/II</td>
</tr>
<tr>
<td>(EMD 521873, Selectikine)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BC1-IL2</td>
<td>Antisoma/Novartis</td>
<td>IgG</td>
<td></td>
<td>Domain VII of Fibronectin</td>
<td>Melanoma</td>
<td>Phases 1/II</td>
</tr>
<tr>
<td>(AS1409)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NHS-IL2</td>
<td>Merck KGaA</td>
<td>IgG</td>
<td></td>
<td>DNA/histone</td>
<td>Various solid tumors</td>
<td>Phases 1</td>
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<tr>
<td>(hTNT3-IL12, MSB0010360)</td>
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<td></td>
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<tr>
<td>L19-TNF</td>
<td>Philogen</td>
<td>scFv</td>
<td></td>
<td>EDB</td>
<td>Melanoma</td>
<td>Phases 1/II</td>
</tr>
<tr>
<td>(Fibromun)</td>
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</tbody>
</table>


- IL-2 based immunocytokines have proved better efficacy than combination of IL2 and immunoglobulin
Examples of Immunocytokines used in active clinical trials

Advantages of immunocytokines:

- Far better antitumour activity than the combination of antibody and cytokine separately.
- Concentration of payload at tumour site, reduction of side effects.
- Long term resistance to subsequent tumour challenge
- Bivalent recombinant antibodies such as scFv-Fc fusions display long residence time at the site of disease and rapid blood clearance.

Hypothetical mechanism of immunocytokines antitumour action
C2B8 (Rituximab)-based plant immunocytokine
Production of recombinant antibodies using vacuum-agroinfiltration

Agrobacterium tumefaciens

[GOI]

N. benthamiana plant

Vacuum

Plant Cell

Hydroponic cultivation of *N. benthamiana* and infiltration facility at ENEA
Western blot analysis of plant extracts (anti-γ)

2B8-Fc-hIL2

A

B

+ : P19 AMCV

2B8-Fc
Western blot analysis of plant extracts

2B8-Fc-hIL2

Anti-hIL2
Protein-A purification and antibody characterization

All bands were digested and subjected to extensive peptide mapping experiments by combined MALDI-TOF-MS and nLC-ESI-LIT-MS/MS analysis.

* Bands with expected N-terminal sequence and c-terminus
→ Bands with incomplete C-terminus

Anti-IL2 ELISA on purified antibodies
Structural analysis of the plant-derived antibodies

2B8-Fc-hIL2

Intact C-terminal sequence: SIISTLT

Most C-terminal sequence: YTLPPSR

N-terminal sequences: 20QIVL (major);

N-terminal sequences: 20QIVL (major);

Most C-terminal sequence: YTLPPSR

2B8-Fc

Intact C-terminal sequence: SLSPGK

Most C-terminal sequence: YTLPPSR

N-terminal sequences: 20QIVL (major);

N-terminal sequences: 20QIVL (major);

Most C-terminal sequence: YTLPPSR

*extensive peptide mapping experiments by combined MALDI-TOF-MS and nLC-ESI-LIT-MS/MS analysis
Gel filtration analysis of purified antibodies

C

mAb H10

2.09

2.54

288-Fc-hIL2

87%

2.10

2.51

2.82

288-Fc

96%

2.12

2.81

Intact scFv-Fcs

Degradation products
**Expression levels of 2B8-Fc-hIL2 and 2B8-Fc in agroinfiltrated *N. benthamiana* leaves.**

<table>
<thead>
<tr>
<th>Construct</th>
<th>Yields* (mg/Kg fresh weight)</th>
<th>Protein A purification yield (mg/Kg fresh weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2B8-Fc-hIL2</td>
<td>28± 6</td>
<td>16</td>
</tr>
<tr>
<td>2B8-Fc</td>
<td>41± 11</td>
<td>28</td>
</tr>
</tbody>
</table>

*Determined by DAS ELISA*
Glycan Analysis

MALDI-TOF-MS spectrum of the peptide (339-347) bearing different complex-type N-linked glycan structures

More than 95% represented by GnGnXF

Homogeneous plant-like Glycosylation at CH2 Asn

MALDI LIFT-TOF/TOF-MS analysis of the glycopeptide (339-347)
CD20 binding on DAUDI cells (Burkitt’s lymphoma)
(Flow cytometry)

2B8-Fc-hIL2 is recognized by an anti-IL2 Ab
CD20 binding on DAUDI cells
(Flow cytometry)

No. of cells

CD20 binding

CD20 binding (MFI)

Anti-γ

Ctrl Ab (µg/ml) 2B8-Fc-hIL2 (µg/ml)

0 5 10 15 20 25 30 35 40

*
Competitive binding of Rituximab and 2B8-Fc-hIL2 to CD20

secondary antibody does not bind 2B8-Fc-hIL2
Human IL2 activity

Proliferation assay on IL2 dependent CTLL-2 cells

2B8-Fc-bound hIL2 stimulates CTLL-2 proliferation

*Concentration refers to the calculated equimolar amount of IL2 between different molecules (taking into account that one antibody molecule is fused to two IL2 molecules)
Antibody-dependent cell-mediated cytotoxicity (ADCC)

- Standard ADCC protocol was used. Daudi cells were cultured with IL-2-activated human PBMC and either Rituximab (RTX), 2B8-Fc-hIL2 or 2B8-Fc for 4 h. For this reason we could not appreciate the contribution of IL2 fusion of 2B8-Fc-hIL2.

- Percentage of cytotoxicity was assessed by LDH release.

Conclusions

• Successful production of a recombinant anti-CD20 scFv-Fc–IL2 immunocytokine. Although some in planta degradation occurs, 90% of protein A purified molecule is constituted by intact assembled antibody.

• The immunocytokine shows similar binding characteristics compared to the IgG₁ rituximab.

• Homogeneous plant-type glycosylation pattern.

• Full biological activity of the fused IL2 was confirmed. The highest yield of plant produced human IL2 (as fusion molecule), is reported.

• ADCC activity of the immunocytokine comparable to Rituximab

Ongoing studies

• Evaluation and comparison of the scFv-Fc–IL2 immunocytokine with ‘modified’ glycosylation pattern in CDC and ADCC.
• Evaluation of immunocytokines in animal models
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