Ion Channel Modulating Nanobodies: two \textit{in vitro} to \textit{in vivo} case studies

April 2015
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Ablynx

Corporate snapshot

CORPORATE
- Drug discovery and development company in Ghent, Belgium
- >300 employees

TECHNOLOGY
- Pioneer in next generation biological drugs – Nanobodies®
- >500 granted and pending patents
- >30 programmes – six at the clinical development stage
- Three clinical proof-of-concepts (POC)
- 2 wholly-owned products in later stage clinical development (Phase III & Phase II)
- >10 new clinical programmes anticipated over the next 3 years

PRODUCTS
- AbbVie, Boehringer Ingelheim, Eddingpharm, Merck & Co, Merck Serono and Novartis

PARTNERS

FINANCIALS
- €206M in cash at December 31st 2014
What are Nanobodies?
Nanobodies

Derived from heavy-chain only antibodies

- *Camelid* heavy-chain only antibodies are stable and fully functional
- Nanobodies represent the next generation of antibody-derived biologics

**Ablynx’s Nanobody**
- small
- robust
- sequence homology comparable to humanised/human mAbs
- easily linked together
- nano- to picomolar affinities
- intractable targets
- multiple administration routes
- manufacturing in microbial cells
Ablynx’s platform
Rapid generation of high quality biologics

Immunise llamas with antigen or use synthetic library

Wide range of highly diverse Nanobodies with 0.1-10nM affinities

Formatted* Nanobodies ready for in vivo testing

Cloning and production in microbial systems

~12-18 months

*Glycine-serine linkers from C-terminus to N-terminus
## Nanobody platform

### Competitive advantages

<table>
<thead>
<tr>
<th>Mix and match</th>
<th>Alternative delivery routes</th>
<th>Customised half-life extension</th>
<th>Challenging and intractable targets</th>
<th>Cell killing</th>
<th>Cell- / tissue-homing</th>
<th>Manufacturing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Targeting different pathways at once with a single Nanobody construct, e.g. multiple checkpoint inhibitors</td>
<td>Inhalation</td>
<td>Weeks/days/hours</td>
<td>Nanobodies against ion channels and GPCRs</td>
<td>Oral-to-topical</td>
<td>Cell specificity</td>
<td>High-yield, high-concentration, low-viscosity, microbial production</td>
</tr>
<tr>
<td></td>
<td>Needle-free</td>
<td>Fc</td>
<td>Nanobodies can reach conserved cryptic epitopes</td>
<td>Ocular</td>
<td>Immune cell recruitment</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Albumin binding Nanobody</td>
<td></td>
<td></td>
<td>Tissue-specific targeting</td>
<td></td>
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<td></td>
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</tbody>
</table>
Ion channel targeting Nanobodies

Ion channels represent a major class of validated drug targets

- Ion channels represent a major class of cell function regulatory proteins
- More than 50% of drugs target only four key gene families:
  - class I GPCRs
  - nuclear receptors
  - ligand-gated ion channels
  - voltage-gated ion channels
  \(~14\%

**Table 1. Current Ion Channel Drugs**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Commercial Name</th>
<th>Target Channel</th>
<th>Disease Indication</th>
</tr>
</thead>
<tbody>
<tr>
<td>Verapamil</td>
<td>Verelan</td>
<td>L-type CaV</td>
<td>Angina, hypertension, arrhythmia</td>
</tr>
<tr>
<td>Diltiazem</td>
<td>Cardizem</td>
<td>L-type CaV</td>
<td>Angina, hypertension, arrhythmia</td>
</tr>
<tr>
<td>Amiodipine</td>
<td>Norvasc</td>
<td>L-type CaV</td>
<td>Angina, hypertension, arrhythmia</td>
</tr>
<tr>
<td>Nimodipine</td>
<td>Nimotop</td>
<td>L-type CaV</td>
<td>Angina, hypertension, arrhythmia</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>Procardia</td>
<td>L-type CaV</td>
<td>Angina, hypertension, arrhythmia</td>
</tr>
<tr>
<td>Sotalol</td>
<td>Betapace</td>
<td>HERG</td>
<td>Arrhythmia</td>
</tr>
<tr>
<td>Amiodarone</td>
<td>Cordorone</td>
<td>HERG</td>
<td>Arrhythmia</td>
</tr>
<tr>
<td>Flecaïnide</td>
<td>Tambocor</td>
<td>NaV1.5</td>
<td>Arrhythmia</td>
</tr>
<tr>
<td>Gabapentin</td>
<td>Neurontin</td>
<td>CaV a2d</td>
<td>Pain</td>
</tr>
<tr>
<td>Ziconotide</td>
<td>Prialt</td>
<td>CaV2.2</td>
<td>Severe chronic pain</td>
</tr>
<tr>
<td>Lidocaine</td>
<td>Lidocaine</td>
<td>NaV</td>
<td>Local anesthesia</td>
</tr>
<tr>
<td>Phenytoin</td>
<td>Dilantin</td>
<td>Brain NaV</td>
<td>Epilepsy</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>Tegetrol</td>
<td>Brain NaV</td>
<td>Epilepsy</td>
</tr>
<tr>
<td>Lamotrigine</td>
<td>Lamictal</td>
<td>Brain NaV</td>
<td>Epilepsy, bipolar disorder</td>
</tr>
<tr>
<td>Topiramate</td>
<td>Topamax</td>
<td>Brain NaV</td>
<td>Epilepsy</td>
</tr>
<tr>
<td>Flupirtine</td>
<td>Flupirtine</td>
<td>KCNQ2/3</td>
<td>Epilepsy</td>
</tr>
<tr>
<td>Benzodiazepine</td>
<td>Diazepam</td>
<td>GABA</td>
<td>Depression</td>
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<tr>
<td>Gilbenclimide</td>
<td>Gilmepride</td>
<td>K\textsubscript{ATP}</td>
<td>Diabetes</td>
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<tr>
<td>Lubiprostone</td>
<td>Amitzia</td>
<td>CLC2</td>
<td>Constipation</td>
</tr>
</tbody>
</table>

Discovery Medicine 2010 9 (46) 253
Nature Reviews Drug Discovery 2006 5 993
Ion channel targeting Nanobodies

Established ion channel targeting approaches are suboptimal

- Established compound technologies are addressing this target space inadequately
  - Paucity of initial leads
  - Lack of selectivity (SME, many toxins)
  - Lack of developability (toxins)

- Nanobodies represent a unique solution
  - Selective (vs. small molecules)
  - Developable (vs. toxins)
  - Target cryptic epitopes/clefts (vs. mAbs)
  - Target multiple epitopes at once (vs. other biologics)

Ablynx’s five ion channel programmes

- Kv1.3: highly potent and selective Nanobodies with *in vivo* PoC
- P2X7: agonist and antagonist Nanobodies with *in vivo* PoC
- Ion-gated: functional Nanobodies discovered
- Ligand-gated: binders discovered
- Voltage-gated: lead discovery ongoing
Kv1.3 (KCNA3)

Channel family, function and structure

- Kv family ion channel (Kvα1.x - Shaker-related: Kv1.1- Kv1.8)
- Structural representative for the 6TM class of ion channels
- Active form is a homo-tetramer; hetero-tetramerization with other Kv1 possible, several beta-subunits possible (e.g. integrins)
- Voltage gated
- Highly selective for K⁺

Overall topology:
Kv1.3 (KCNA3)

Kv1.3 is critical for T-cell activation

- Kv1.3 channels provide the counterbalancing K\(^+\) efflux for Ca\(^{2+}\) entry into T\(_{EM}\) cells to facilitate sustained Ca\(^{2+}\) influx.
- Activated T\(_{EM}\) cell population is critically dependent on Kv1.3 for K\(^+\) flux.
- Wide applicability to autoimmune and inflammatory diseases
  - Multiple Sclerosis, Psoriasis, Type 1 Diabetes, ...
Kv1.3 (KCNA3)

Epitopes for inhibitory Nanobodies

- Sequence conservation within Kv1 family
  - ECL3 – highest conservation (~80%); moderate-high risk of lack of specificity
  - ECL1, ECL2 – (20-60%); low risk of lack of specificity
- Sequence analysis vs. rat Kv1.3
  - ECL1-ECL3 – (≥85%); good chance of cross-reactivity
- Expected chance for inhibition if binding to loop: ECL3>ECL2>>ECL1

ECL1: 41 aa  ECL2: 14 aa  ECL3: 41 aa
Kv1.3 (KCNA3)

Sea Anemone Potassium Channel Toxin (ShK)

- ShK variant (Ph 1b) and PAP-1 (SME, Ph 1) most advanced compounds
- Efficacy demonstrated in various animal models
  - Multiple Sclerosis
  - Type 1 diabetes
- Docking of ShK on homology model of the Kv1.3 channel based on the crystal structure of the bacterial potassium channel KcsA (pore-forming helices: S5/S6)
Kv1.3 (KCNA3)

High affinity binding and further improvement through formatting

- Monovalent Nanobodies identified with low nanomolar affinity
  - multiple Nanobodies derived from two B-cell lineages
- Nanobodies are cross-reactive with preclinical development relevant species
  - cynomolgus monkey
  - rat
- Bivalent constructs show improved affinity and rat cross-reactivity
Kv1.3 (KCNA3)

Kv1.3 Nanobodies recognize novel epitope

- Nanobodies require 1st extracellular loop ECL1 for binding

![Graph showing concentration vs. MCF](image)
**Kv1.3 (KCNA3)**

**Exquisite selectivity for Kv1.3 over nearest family members**

- Selectivity was evaluated over closest related Kv1 family members and hERG
- Lack of measurable off-target current block in ephys demonstrates selectivity over highly related ion channels

<table>
<thead>
<tr>
<th>Channel</th>
<th>anti-Kv1.3</th>
<th>ShK-186</th>
<th>Biological role</th>
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</thead>
<tbody>
<tr>
<td>Kv1.2</td>
<td>ongoing</td>
<td>700</td>
<td>Neuronal</td>
</tr>
<tr>
<td>Kv1.4</td>
<td>ongoing</td>
<td>n.t.</td>
<td>Neuronal</td>
</tr>
<tr>
<td>Kv1.5</td>
<td>&gt;10,000</td>
<td>&gt;1,500</td>
<td>Cardiac</td>
</tr>
<tr>
<td>Kv1.6</td>
<td>&gt;10,000</td>
<td>250</td>
<td>Neuronal</td>
</tr>
<tr>
<td>hERG</td>
<td>&gt;10,000</td>
<td>&gt;1,500</td>
<td>Cardiac</td>
</tr>
</tbody>
</table>

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**Graphs**

- **Kv1.3**
- **Kv1.5**
- **Kv1.6**
- **hERG**
Kv1.3 (KCNA3)

Fast onset of blocking and increase in duration as bivalent

- Avidity enables long target residence time
  - Monovalent format washes out rapidly, bivalent format does not (within 30 min recording time)
Kv1.3 (KCNA3)

Formatting allows the combination of functional profiles

- Combination of Nanobodies with different signatures leads to mixed profiles
Kv1.3 (KCNA3)

High affinity binding and further improvement through formatting

- Functional activity comparable to benchmark toxin
  - Freshly isolated CD45RA-CCR7- T cell subset
  - Stimulated with plate-coated αCD3 only
  - 72h incubation at 37 °C
  - Read out: IFNγ release

![Graphs showing IFNγ release](image)
## Kv1.3 (KCNA3)

**in vivo PoC study design**

### Study groups | Dosing | Timing
--- | --- | ---
Vehicle | topical dose | 1hr and 6hr post challenge
Dexamethasone | topical dose | 12h and 1h pre-challenge
ShK | 10 µg/kg, s.c. | 12h and 1h pre-challenge
bivalent, non-HLE | equimolar, s.c. | 12h and 1h pre-challenge
trivalent, HLE | equimolar, s.c. | 12h and 1h pre-challenge
trivalent, HLE | equimolar, s.c. | 1h pre-challenge

**Study groups and dosing:**
- **Vehicle:** Topical dose 1hr and 6hr post challenge
- **Dexamethasone:** Topical dose 12h and 1h pre-challenge
- **ShK:** 10 µg/kg, s.c. 12h and 1h pre-challenge
- **Bivalent, non-HLE:** Equimolar, s.c. 12h and 1h pre-challenge
- **Trivalent, HLE:** Equimolar, s.c. 12h and 1h pre-challenge
- **Trivalent, HLE:** Equimolar, s.c. 1h pre-challenge

**Study timeline:**
- **Day 0:** Sensitization
- **Day 1:** Challenge
- **Day 2:** Read-out
- **Day 3:** PK sampling (Satellite animals)
- **Day 4:** PK sampling (Satellite animals)
- **Day 5:** PK sampling (Satellite animals)
- **Day 6:** PK sampling (Satellite animals)

**DNFB in acetone/olive oil**

**Nanobody/ShK s.c. dose (12h and/or 1h pre-challenge)**
Kv1.3 (KCNA3)

High affinity binding and further improvement through formatting

- Comparable effects of all Nanobody groups and ShK
  - effects are moderate but highly significant (p<0.001 vs. vehicle)
  - both half-life extended (HLE) and non-HLE Kv1.3 Nanobody construct demonstrated efficacy
  - no differences between 1 vs. 2 administrations

Ear thickness readout:

![Ear thickness graph with data for Vehicle, Dexamethasone, ShK, and ShK treatments.](image)
**Kv1.3 (KCNA3)**

**Conclusions**

- Sub nM binding affinity across species in bivalent / biparatopic format
- Greater than 10,000 fold selectivity for Kv1.3 over related ion channels
- Potent gating-dependent channel blocking (↑ inactivation and ↑ accumulation of inactivation), probably through allosteric effect
- Functional Kv1.3 blocking activity on T\textsubscript{EM} cells comparable to reference toxin
- First *in vivo* PoC obtained with Kv1.3 Nanobodies in rat DTH model

- **Flexibility of formatting facilitates improvement in activity and represents an opportunity for heteromultimeric channel complexes**

- **Option to combine different Nanobodies to engineer desired functional profiles**
P2X7

Channel family, function and structure

- Trimeric ligand-operated cation channel
- Widely expressed
  - T cells
  - microglia and macrophages
  - others
- Sensor for high local ATP (extracellular danger signal)
  - after injury, infection, in tumor microenvironment
  - important role in release of pro-inflammatory signals, i.e. IL-1β and IL-18
  - prolonged gating: pore formation → apoptosis
- Role in inflammatory and neurologic disorders
  - RA, IBD, COPD, MS, renal injury and GvHD
  - neuropathic pain, depression
## P2X7

### Pre-clinical validation in inflammatory / neurological diseases

<table>
<thead>
<tr>
<th>Disease</th>
<th>Target validation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Renal disease</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Rheumatoid arthritis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+ A839977, AZ10573295</td>
<td>Honore 2009, Caporali 2008, …</td>
</tr>
<tr>
<td><strong>Respiratory disease</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COPD, asthma</td>
<td>+ A438079, AZ11645373, KN62</td>
<td>Muller 2011, Theatre 2012, …</td>
</tr>
<tr>
<td><strong>Graft versus Host disease</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+ PPDAS, KN62</td>
<td>Wilhelm 2010, …</td>
</tr>
<tr>
<td><strong>Depression</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+ AZ10606120, BBG, JNJ-47965567</td>
<td>Csoelle 2013, Bhattacharya 2013, …</td>
</tr>
<tr>
<td><strong>Pain</strong></td>
<td></td>
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</tr>
</tbody>
</table>

Knock-out and inhibition of P2X7 ameliorate inflammatory, immune and neurological diseases in mouse models.
P2X7

Lead Nanobody characterization

- Three antagonistic P2X7 Nanobodies
  - Dano 1 and 3: human-reactive
  - Dano 2: mouse/rat/human cross-reactive
- Two surrogate mouse-specific P2X7 Nanobodies
  - Dano 4: antagonist
  - Dano 5: agonist

In collaboration with Prof. Koch-Nolte (University Hamburg)
Nanobodies are selective for P2X7

Absence of binding to family members P2X4 (closest relative) and P2X1
Nanobodies can either block or potentiate P2X7 on macrophages

- Nanobodies modulate primary peritoneal macrophages
  - Ca\(^{2+}\) influx
  - IL-1\(\beta\) secretion
**P2X7**

Nanobodies can either block or potentiate P2X7 on T-cells

- ATP-induced gating on T-cells leads to shedding of CD62L
- Bi-valent Nanobodies have significantly increased potency

![Antagonist anti-P2X7 Nanobody](chart1.png)

**Antagonist P2X7 Nanobody to block inflammatory response (inflammatory / neurological disorders)**

- IC50 0.1 nM

![Potentiating anti-P2X7 Nanobody](chart2.png)

**Potentiating P2X7 Nanobody to activate inflammatory response (Immuono-oncology)**

- EC50 0.1 nM
**P2X7**

**Nanobodies can either block or potentiate P2X7 on T-cells/iNK-T-cells**

- Bivalent P2X7 Nanobodies fused to anti-albumin Nanobody for extended half-life
- Enhancer Dano5 induces shedding of CD27 and cell death of P2X7-positive T-cells *in vivo*
- Blocker Dano4 prevents ligand-induced P2X7 activation *ex vivo*
**P2X7**

**Nanobodies modulate P2X7 dependent pathology *in vivo***

- Antibody-induced nephritis model
  - induction of glomerulonephritis by injection of anti-podocyte serum
  - administration of trivalent half-life extended anti-P2X7 Nanobodies

**Proteinuria (day 15)**

```
<table>
<thead>
<tr>
<th></th>
<th>U_ALB/U_CREA [mg/mg]</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI</td>
<td>0.1</td>
</tr>
<tr>
<td>control</td>
<td>1.0</td>
</tr>
<tr>
<td>agonist</td>
<td>10.0</td>
</tr>
<tr>
<td>antagonist</td>
<td>100.0</td>
</tr>
</tbody>
</table>
```

- * p<0.05 to PI PBS
- # p<0.05 to AP control

**Glomerular damage (day 15)**

- sheep anti-mouse podocyte (AP) or pre-immune serum (PI)
- Urine collection and weight
- IP injection NB: 50 µg
- 25µg
Nanobodies modulate P2X7 dependent pathology *in vivo*

- DNFB-sensitised mouse model for allergic dermatitis
  - C57Bl/6 mice (sensitised on days 0 and 1) were challenged on day 7 by topical application of DNFB to the left ear
  - administration of trivalent half-life extended antagonist anti-P2X7 Nanobody
  - inflammation score (difference in weight between the left and right ears) assessed 24 h after challenge

**Systemic injection of antagonistic anti-P2X7 Nanobody prevents inflammation**
P2X7 Nanobodies modulate Ca\(^{2+}\) influx in human P2X7 transfected HEK cells

- Cells were loaded with the Ca\(^{2+}\) indicator Fluo-4, real time flow cytometry
- Bivalent Nanobodies were pre-incubated or added after addition of ATP
- Antagonistic P2X7 Nanobodies are able to shut down Ca\(^{2+}\) influx after initiation, whereas control mAb is only partially blocking
**P2X7**

**Nanobodies outperform P2X7 benchmark compounds**

- Benchmark compounds have nano- to micromolar potency
  - anti-P2X7 mAb L4
  - small molecule inhibitors (KN-62 and A438079)
- Human reactive anti-P2X7 Nanobodies have pM potencies
  - blockade of IL-1β release in whole human blood
  - prevention of pore formation (→ cell death) of hP2X7 transfected HEK cells
- P2X7 Nanobodies complete block P2X

**Human anti-P2X7 Nanobodies have superior potency and efficacy over existing benchmark compounds in blocking P2X7**
P2X7

Best-in-class opportunity

- Opportunity to access multi-billion dollar market: potential treatment for autoimmune / inflammatory, neurological diseases and oncology
- Best-in-class opportunity: unparalleled combination of developability and high potency / selectivity
  - ease of manufacturing
  - excellent bioavailability
  - absolute specificity for P2X7
  - picomolar potency
  - complete blockade
- *In vivo* PoC in atopic dermatitis and glomerulonephritis mouse models
- *In vitro* PoC for human-reactive Nanobodies
- 2017 IND candidate
Ion channel targeting Nanobodies

Conclusions

• Ion channels represent an important but inefficiently addressed class of drug targets
• Nanobodies have been generated to different types of ion channels
  – highly functional (ephys, functional assays from \textit{in vitro} through to \textit{in vivo})
  – unrivaled selectivity profile
  – proven compatibility with Ablynx’s half-life extension technology
• Formatting flexibility allows freedom to explore ion channel biology in full
  – improvement in potency and crossreactivity
  – opportunity to selectively address heteromultimeric complexes
  – opportunity to dial specific channel activity modulation methods in and out
Questions

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