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Membre en qualité de Correspondant à titre étranger (Russie)
Derniers développements de l'ingénierie des protéines pharmaceutiques avec en particulier la polysialylation comme alternative à la PEGylation

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3. OAO PharmSyntez, S-Petersburg, 194358, Russia

4. Xenetic Biosciences Inc. Lexington, MA, USA

5. Institut de Biologie Structurale, DYNAMOP, 38000 Grenoble, France
The ways of recombinant protein drugs clearance from patients

- Capture by cells and lysosome degradation
- Clearance by kidney
- Proteolytic degradation from bloodstream
Site-directed mutagenesis allow to form inactive drug complexes, which are slow dissociate in point of injection give the drug to bloodstream. Commercial drug Lantus (Aventis Pharma Deutschland GmbH)
Strategies of enhancement of therapeutic effects. How we may do long-living therapeutics?

“Antibody-protection technology”. Antibody-protected therapeutic is destroyed only upon interaction with corresponding receptor.
A. Modification of the gene to arrange new sites of glycosylation - Darbepoetin, Tenecteplase.
B. Chemical modification. Covalent conjugation of recombinant protein by polymeric molecules. Pegylation – Pegintron, Pegasys, Neulasta, Mircera, Oncaspar etc.
Chemical Polysialylation VS PEGylation

Oxydized colominic acids (CA0)
is a mix of homopolymers of sialic acid
with average molecular weight of 27 kDa

<table>
<thead>
<tr>
<th></th>
<th>Oxydized colominic acids (CA0)</th>
<th>Polyethyleneglycol</th>
</tr>
</thead>
<tbody>
<tr>
<td>FDA approved</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>In vitro modification</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>Biodegradable</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>Source</td>
<td>E.coli</td>
<td>Chemical syntesis</td>
</tr>
</tbody>
</table>
N-terminal conjugate of human oxyntomodulin and polysialic acid
Recombinant gut hormone oxyntomodulin (OXM) is known as a satiety signal in human subjects and has a therapeutic potential as an appetite control agent. The only form of this hormone that has a use prospective is the prolonged one, since intact OXM has a very short half-disappearance time. Conjugation of OXM and the natural hydrophilic polymer polysialic acid (PSA) may significantly improve it’s half-life.
Synthesis and purification of PSA-OXM conjugate

• Process outline is the same to insulin conjugation
• Productive conjugation occurs only at mildly acidic pH around 6.0
• Purification by reverse phase + anion exchange
• Conjugation yield 50%, purification yield 60%
• Product purity by RP-HPLC over 95%
RP-HPLC analysis of the PSA-OXM

Analytical chromatograms of different OXM species: OXM – blue curves, de-polysialylated PSA-OXM conjugate – black curves, sample of crude PSA-OXM synthesis reaction – violet curves, sample of combined reverse phase purified fractions (after the first purification stage) RXN-RP – cyan curves, and purified PSA-OXM – green curves. Panel “A” represents runs with 0.1% v/v TFA, panel “B” - runs with 0.05% w/v TEA.
Peptide map of the PSA-OXM

Analytical chromatograms of Asp-N proteolytic reactions. Green line represent OXM peptide proteolytic digestion fragments, blue line represent deglycosylated PSA-OXM proteolytic reaction and the black line represents unmodified PSA-OXM conjugate’s Asp-N digestion fragments. Number of collected fraction and its peptidic content (determined later by MALDI MS) is signed over each fraction peak.
MALDI-TOF determination of the modified fragment

Comparative mass-spectra of Asp-N protease digests of OXM (green), deglycosylated PSA-OXM (dG, red) and PSA-OXM (blue). Molecular mass is in Da.
Effects on cumulative food intake after an overnight fasting in C57Bl6 mice, after ip administration of vehicle or OXM (at 200 or 3000 nMol/kg) or PSA-OXM (at 3000 or 14700 nMol/kg). Results are means±s.e.m. (n=10-12) * P<=0.05; ** P<=0.01 compared with saline group using two-way statistical analysis of variance followed by pos-hoc Dunnet’s test.
Phase I study: «Single center randomized in parallel groups clinical study of pharmacokinetics, pharmacodynamics, safety and tolerance of Oxyntolong on healthy volunteers.

- Single SC administration
- 12 healthy subjects
- 3 subsequent dose cohorts (0.25; 0.75; 1.5 mg/kg)
- Randomized (3:1 in each cohort), placebo-controlled
Oxyntolong (PSA- Oxyntomodulin)/OxyL01/01 Study

AUC (PSA-OXL) Insulin- 435 mkME
AUC (Placebo) Insulin- 282 mkME
Conclusions:

- OXM peptide can be conjugated to PSA with the high yield at the N-terminus.
- The conjugate is completely insensitive to DDP-IV – dipeptidyl aminopeptidase, rapidly inactivating free OXM in vivo.
- PSA-OXM posseses significantly decreased specific activity, but in high dose reduces food consumption in mice for 8 h. Anorexigenic effect of free OXM in this animal model is limited to 2 h at maximal tolerable concentration.
- Single SC administration of PSA-OXM to humans increases insulin secretion.
Polysialylated Erythropoietin: Next Generation of Erythropoiesis Stimulating Agent

Enhancing lives and improving drugs
PolyXen: Better performance for peptide and protein drugs

- Uses polysialic acid (PSA), a natural polymer, to extend the active life of bioactives

- PEGylation has provided a clinical road map

- Key benefits over established PEGylation:
  - Biodegradability and better efficacy
  - Avoidance of:
    - toxicity, injection site-reactions, immunogenicity, anti-genicity,
    - and high viscosity

- Other benefits
  - Fewer doses and improved stability
  - Reduced immunogenicity and anti-genicity
  - Ability to improve the clinical profile (PK/PD/half life) of most protein drugs
  - Applicable to over 200 marketed drugs and 2000+ candidates in development
**PSA-EPO for anaemia treatment**

- A long acting EPO with a gentler mode of action (likely to be less toxic)
- Data so far supports potential use of the product on a once monthly basis
  - Current market - US$9bn for EPO products (EPO and Aranesp) need to be given 1 to 3 times a week
- Excellent freedom to operate and patent coverage
- Convenient to patients and health professionals
- Ongoing explorations for a wide range of anemia indications

Half-life and dosing schedule of various erythropoiesis stimulating agents in man

<table>
<thead>
<tr>
<th>Agent</th>
<th>Population</th>
<th>Route</th>
<th>Half-life (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>i.v.</td>
<td></td>
</tr>
<tr>
<td>Epoetin alpha</td>
<td>Healthy volunteers</td>
<td>s.c.</td>
<td>19.4 ± 10.7</td>
</tr>
<tr>
<td>Epoetin beta</td>
<td>Healthy volunteers</td>
<td>i.v.</td>
<td>8.8 ± 2.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>s.c.</td>
<td>24.2 ± 11.2</td>
</tr>
<tr>
<td>Darbepoetin alpha</td>
<td>PD patients</td>
<td>i.v.</td>
<td>25.3 ± 2.2</td>
</tr>
<tr>
<td>Aranesp®</td>
<td></td>
<td>s.c.</td>
<td>48.8 ± 5.2</td>
</tr>
<tr>
<td>C.E.R.A</td>
<td>Healthy volunteers</td>
<td>i.v.</td>
<td>133 ± 9.8</td>
</tr>
<tr>
<td>Mircera®</td>
<td></td>
<td>s.c.</td>
<td>137 ± 21.9</td>
</tr>
<tr>
<td></td>
<td>PD patients</td>
<td>i.v.</td>
<td>134 ± 19</td>
</tr>
<tr>
<td></td>
<td></td>
<td>s.c.</td>
<td>139 ± 20</td>
</tr>
<tr>
<td>Omontys®</td>
<td>Healthy volunteers</td>
<td>i.v.</td>
<td>23.5</td>
</tr>
<tr>
<td></td>
<td>PD patients</td>
<td>i.v.</td>
<td>41</td>
</tr>
<tr>
<td>ErepoXen®</td>
<td>Healthy volunteers</td>
<td>s.c.</td>
<td>121</td>
</tr>
<tr>
<td>(15 KDa PSA-EPO;</td>
<td>PD patients</td>
<td></td>
<td>&gt;121</td>
</tr>
<tr>
<td>N-terminal derivatisation; Xenetic)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*PK profile required to meet desired hemoglobin levels in patients.*
The conformation of PSA-EPO is same as EPO

Circular dichroism
PSA-EPO has higher thermal stability

Temperature (°C)
Dose dependent haemoglobin response: Human data (Phase I)
PSA-EPO clinical trials

cGMP facility for manufacturing of PSA-EPO is established

- Positive Phase-I (SC) trial
  - Conducted by development partner Serum Institute of India (SII)
  - 64 healthy volunteers, including placebos
  - No safety issues and well tolerated in the trial
  - Sustained rise in haemoglobin levels for 28 days
  - Anti-EPO, Anti-PSA & Anti-PSA-EPO IgG antibodies not detected

- Positive Phase-Ila (SC) trial
  - 30 patients
  - No safety issues and well tolerated in the trial
  - Clear reticulocyte response
  - Anti-EPO, Anti-PSA & Anti-PSA-EPO IgG antibodies not detected

- Positive Phase-I/II (IV) trial
  - 40 patients (On going)

- Promising long term (6 months) as well as developmental animal toxicity data

- Data support potential use of the product on a once monthly basis
ErepoXEN (Epolong, PSA EPO)/EVA 12 (PSA-EPO-05) study

PSA-EPO-05 study (Russia)

Open-label, comparative, multicenter, randomized study to evaluate the efficacy, safety and tolerance of Epolong® for the correction of anemia and maintenance of the hemoglobin levels in EPO-naïve patients with chronic kidney disease who are not on dialysis.

---

* in the maintenance phase
EPO = erythropoietin
Q2W = every other week
Q4W = every 4 weeks
Treatment response:

**RBC Aranesp/Epolong**

**Hb Response Aranesp/Epolong**
• The first subject (group III, Epolong (1 per 4 weeks, double dose) reached the maintenance phase
• Epolong is safe at dose 3,5 mcg/kg
• More than 15 g/L Hb increase during 4 weeks
• Long-acting features

Epolong, 1 per 4 weeks

<table>
<thead>
<tr>
<th>Week 17</th>
<th>Week 19</th>
<th>Week 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb, g/L</td>
<td>Hb, g/L</td>
<td>Hb, g/L</td>
</tr>
</tbody>
</table>

3,5 mcg/kg
Summary

• The work till date have provided ample evidence that polysialylation is a next generation approach for improving the PK and PD of therapeutics.

• PSA therapeutics not only retain much of their activity, they also exhibit prolongation of pharmacological function.

• In contrast to PEG, PSA is biodegradable and non-immunogenic.

• Clinical studies in progress with PSA-EPO and support the notion that polysialylation is a credible alternative to PEGylation

• Polysialylation is likely to become the mainstay of optimal drug delivery in due course.
Pulmoxen (PSA-DNAse)

- Superiority to Pulmozyme confirmed by UNC CF Center ex vivo studies

- Phase I clinical trial in healthy volunteers completed:

A phase I, single center open-label nonrandomized clinical trial to evaluate the safety and tolerability of PulmoXEN in two subsequent groups with multiple inhalations on healthy volunteers.

Safety results:
3 patients got productive cough (smokers). Mild (I degree in accordance with CTCAE 4.0), IMP related.

Conclusion:
- Daily inhalation (up to 7 doses) of Pulmoxen is safe for the doses of 2500 and 5000 IU.

PI Side opinion – “smokers were detected in healthy volunteer cohort, cough was caused by drug effects”.

- Setting up Phase II-III in Russia (Registration Study)
- Setting up IND enabling preclinical safety in US
Insulin

- Polypeptide hormone, 7700 Da.
- Produced by β-cells in pancreas.
- Discovered in 1921 by the group of John McLeod.
- Successfully used for human therapy in 1922, manufactured at large scale in 1923.
- Structure determined by Frederick Sanger in 1955.
- First recombinant protein drug (Eli Lilly, Genentech).

John McLeod
Nobel Prize 1923

Frederick Sanger
Nobel Prize 1958
Diabetes mellitus

**Classification:** type I (autoimmune) and type II (acquired insulin resistance).

**Background:** type I is genetically predisposed, type II mainly due to obesity, unhealthy diet, sedentary lifestyle.

**Epidemiology:** 7-8% of population.

**Typical complications:** Retinopathy, nephropathy, cardiovascular, diabetic ulcer.

**Morbidity:** 3 200 000 deaths/year worldwide.

**Therapy:** Oral insulin secretagogues, insulin.
PSA – Insulin

Polysialylated insulin: synthesis and activity

Sanjay Jain\textsuperscript{a,b}, Dale H. Hreczuk-Hirsch\textsuperscript{a}, Agamemnon Epenetos\textsuperscript{a}, Peter\textsuperscript{b}

\textsuperscript{a}Lipoxen Technologies Ltd, \textsuperscript{b}The School of Pharmacy, University of London

SDS-PAGE of intact insulin, insulin polysialylated with 22- or 39-kDa CA and molecular weight markers. Lane 1, molecular weight markers; lane 2, intact insulin; lane 3, insulin incubated for 48 h in the presence of non-oxidized CA (process control); lanes 4 and 5, reaction mixtures of insulin and CA (22 or 39 kDa, respectively).

Blood glucose levels in T/O mice after subcutaneous injection of intact insulin (\textbullet{}), polysialylated insulin using 22-kDa (\textblacksquare{}) and 39-kDa (\textblacktriangle{}) CA (upper panel), and of equivalent doses of 22-kDa (\textblacksquare{}) and 39-kDa (\textblacktriangle{}) CA alone (lower panel). Values with intact insulin in the lower panel (also shown in the upper panel) are plotted for comparison. When appropriate, animals received 0.3 units of insulin based on protein content. Values are mean ± S.D. (\textit{n} = 5).
Initial production process

Conjugation:
- pH 6.0
- Sodium acetate, 10 mM
- Insulin - 1 mg/ml
- 15 kDa CAO - 10 mg/ml
- +4 °C
- Reaction time – 24 h

Insulin conversion (SEC): 55%

Purification:
1. Hydrophobic chromatography, step elution
2. Anion exchange chromatography, step elution
3. Ultrafiltration, diafiltration
4. Fill and finish

Purification yield (insulin): 65%

Pharmaceutical composition prototype
- Protein - 15 mg/ml
- Sodium phosphate - 10 mM, pH 7.4
- m-cresol – 2.7 mg/ml
Initial production process, purity

Reverse phase chromatography at pH 6.2

SDS-PAGE, non-reducing
Revised production process

Conjugation:
• pH 6.2
• Sodium phosphate 100 mM
• Insulin - 4 mg/ml
• 15 kDa CAO - 24 mg/ml
• +37 °C
• Reaction time – 3 h

Insulin conversion (RP-HPLC): 50-60%
Target product (RP-HPLC): 20-25%

Reverse phase chromatography at pH 6.2
Revised production process

Purification:
1. Reverse phase chromatography, SOURCE RPC 15 resin, 4 mg/ml load, triethylammonium acetate pH 6.2, ethanol gradient elution
2. Anion exchange chromatography, SOURCE Q 15 resin, 4 mg/ml load, sodium phosphate pH 7.4, NaCl gradient elution
3. Ultrafiltration and formulation, 5 kDa PES cross-flow cassette
4. Fill and finish

Purification yield (insulin): 60%
Revised production process, purity

Reverse phase chromatography at pH 6.2

<table>
<thead>
<tr>
<th>RT (min)</th>
<th>Peak Type</th>
<th>Area (V*sec)</th>
<th>% Area</th>
<th>Height (V)</th>
<th>% Height</th>
<th>Integration Type</th>
<th>Points Across Peak</th>
<th>Start Time (min)</th>
<th>End Time (min)</th>
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<td>VB</td>
<td>30</td>
<td>18.583</td>
<td>19.100</td>
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SDS-PAGE, non-reducing
# Bioactivity, mice by USSR Pharm. XI

<table>
<thead>
<tr>
<th>No</th>
<th>Sample</th>
<th>Glucose decrease at 40 min, %</th>
<th>Glucose decrease at 120 min, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Standard insulin, 0.08 IU/ml</td>
<td>37</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>CAO-Ins, lot R#132, 1.2 IU/ml</td>
<td>42</td>
<td>52</td>
</tr>
<tr>
<td>4</td>
<td>CAO-Ins, lot R#132, 6 IU/ml</td>
<td>65</td>
<td>54</td>
</tr>
<tr>
<td>5</td>
<td>Control solution</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

IU means 1/27 of 1 mg of any insulin variant (CAO weight not included in calculations).

8 mice in each group, injection volume – 10 µl/g
Quality control, specific tests

1. Identity by RP-HPLC
2. Identity by SDS-PAGE
3. Concentration by RP-HPLC (14.5-15.5 mg/ml)
4. Purity by RP-HPLC (>94%)
5. Dimers by SDS-PAGE (<2%)
6. Free PSA by RP-HPLC (<1%)
7. Free insulin by RP-HPLC (<1%)
8. Residual CN ions (<0.1 ppm)
9. Residual B (<10 ppm)
10. Residual Zn$^{+2}$ (<16 μg/ml)
11. Prolonged action by mice test
Quality control, pharmacopeian tests

1. m-cresol identity by RP-HPLC
2. m-cresol content by RP-HPLC (2.2 – 3.0 mg/ml)
3. Solution transparency
4. pH (6.9 - 7.8)
5. Particles
6. Nominal volume
7. Endotoxins by LAL-test (<20 EU/ml)
8. Sterility
9. Bioactivity by mice test (90-110%)
Bioactivity, mice

Blood glucose at 0 - 330 min. after subcutaneous injection of CAO-Ins or Ins alone (in all cases 0.3 ug/mouse in PBS buffer) in CD-1 female mice at 5-6 weeks Age (weight around 25 g). Data are shown as Means ± SE.
Conclusions

• Conjugate of insulin and polysialic acid can be produced as entirely homogeneous substance with a pharmaceutical quality by a three step purification process.
• Process developed is fully scalable up to tens of grams batches.
• Conjugate produced is active in humans and, according to preliminary data, its activity sustained for more than 13 h after sc injection.
Antidotes toward chemical weapons: promiscuity of catalytic sites.
Poisoning of OPC leads to collapse nervous system

covalent coupling of toxin with acetylcholine esterase leads to increase of acetylcholine in synapse and collapse of nervous system
Question of OPC-poising therapy

- The major target of organophosphorous toxins are cholinesterase-like enzymes;
- Extremely low LD$_{50}$ value
- OPC-associated mortality is 200000 people per year;
- There are real threats of acts of terrorism, for example sarin attack in Tokyo underground at 20 March of 1995
Exposure to OP Poisons Statistics

185 countries in 2012 has joined Chemical Weapons Convention

300,000 cases of domestic lethal poisonings per year according to Red Cross

Undestroyed* reserves:
- 9,450 tonnes - USA
- 12,000 tonnes - Russia

Exposure to domestic pesticides
- 70% - in Sri Lanka
- 62% - in China
- 30% - in India

Out of all cases of domestic poisonings registered in 1998–2000

* based on 2010 reports
Risk group

- Soldier
- Stuff of OP poisons utilization plant
- Farmers
- Users
Butyrylcholinesterase as Bioscavenger

Butyrylcholinesterase (BChE) – a serine hydrolase (EC 3.1.1.8), with different substrate specificity and inhibitor sensitivity.

It was found that BChE can effectively bind most of organophosphorus poisons (OP) including warfare agents, such as GA, GB, VX, VR and etc. Thus, butyrylcholinesterase is considered as prime candidate for a bioscavenger for prophylaxis of OP exposure.

Bioscavenger
A protein-based antidote that can inactivate OP compound before it reaches its biological target.

Stoichiometric
Binds OP in ‘one-to-one’ molar ratio

Catalytic
Hydrolyse OP compounds
Current BChE Production Techniques

Outdated human plasma
+ Developed purification protocol
+ Active enzyme
+ No protein modification needed
  - High cost
  - Possibility of contamination with viruses

Transgenic animals
+ Active enzyme
+ High production, up to 5 g/l
  - Protein modifications needed
  - Very expensive

E. coli expression
+ Relatively low cost
  - Inactive enzyme expression

Yeast cells expression
+ Relatively low cost
  - Inactive enzyme expression

CHO cells expression
+ Active enzyme expression
+ FDA approved
+ Scalable for industrial production
  - Low level of expression
  - Additional protein modifications needed

Transgenic plants
+ Active enzyme
  - Protein modification needed
  - Not approved by FDA
A

Polysialylation reaction recovery > 80%
Residual enzyme activity > 90%

B

Ilyushin et al., PNAS 2013
Large-scale production of BCHE-CAO27

A
Stage recovery, %
- Ultraconcentration, cut off 30 kDa:
  - Affinity chromatography on Procainamide Sepharose
  - Anion-exchange chromatography on MonoQ
  - Sialylation
- 98
- 90
- 85
- 80

B
<table>
<thead>
<tr>
<th>Growth media of clone A3h9</th>
<th>Concentrate</th>
<th>Affinity chromatography</th>
<th>Anion-exchange chromatography</th>
<th>Polysialylated BCHE</th>
<th>Purified plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>120 kDa</td>
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</tr>
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<td>70 kDa</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>50 kDa</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>20 kDa</td>
</tr>
</tbody>
</table>

rhBhCE tetramer
Polysialylated hBCHE tetramer
How to characterize polysialylation?

A-I Acid hydrolysis
polysialyc acid

B-I Enzymatic digestion
of polysialic acid

A-II Quantitive analysis
using resorcinol screening

B-II Trypsinolysis
of the conjugate

B-III MS/MS analysis

CAO27 : BChE = 6 : 1

Ilyushin et al., PNAS 2013
Possible Modification Sites Uncovered

Crystal structure of human butyrylcholinesterase
(based on pdb.org 3DKK)
Possible modification sites are marked with arrows

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Position</th>
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<tr>
<td>KPQSLTK</td>
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<tr>
<td>RDNYTKAEEILSRSIVKRWANFAKYGNPNETQNNSTSWPVFKSTEQK</td>
<td>453–499</td>
</tr>
<tr>
<td>DNYTKAEEILSRSIVKRWANFAK</td>
<td>454–476</td>
</tr>
<tr>
<td>LRAQQCRFWTSFFPKVLEMTGNIDEAEWEWK</td>
<td>514–544</td>
</tr>
<tr>
<td>AGFHRWNYYMMDWKNQFNODYTSKK</td>
<td>545–568</td>
</tr>
<tr>
<td>WNNYMMMDWKNQFNODYTSKKESCVGL</td>
<td>550–574</td>
</tr>
</tbody>
</table>

Ilyushin et al., PNAS 2013
Enzymatic Properties Stay Intact

Enzyme kinetics of butyrylthiocholine hydrolysis

<table>
<thead>
<tr>
<th></th>
<th>$K_M$, μM</th>
<th>$k_{cat}$, min$^{-1}$</th>
<th>$K_{ss}$, mkM</th>
<th>$b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>rhBChE</td>
<td>25±1</td>
<td>49200±800</td>
<td>250±30</td>
<td>2.4±0.2</td>
</tr>
<tr>
<td>rhBChE -CAO27</td>
<td>28±2</td>
<td>50000±600</td>
<td>230±20</td>
<td>2.2±0.2</td>
</tr>
<tr>
<td>hBChE$^+$</td>
<td>23±2</td>
<td>39900±1800</td>
<td>140±20</td>
<td>2.5±0.1</td>
</tr>
</tbody>
</table>

## Polysialylation Increase Blood Stability

### Pharmacokinetics of polysialylated rhBChE in BALB/c mice

<table>
<thead>
<tr>
<th></th>
<th>rhBChE</th>
<th>rhBChE-CAO27</th>
<th>hBChE*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRT, min</td>
<td>220±50</td>
<td>1400±200</td>
<td>2791</td>
</tr>
<tr>
<td>$T_{1/2}^{distr.}$ min</td>
<td>4±1</td>
<td>23±13</td>
<td></td>
</tr>
<tr>
<td>$T_{1/2}^{elimin.}$ min</td>
<td>180±25</td>
<td>1000±135</td>
<td>1683</td>
</tr>
</tbody>
</table>

Toxicity of some warfare agents
LD50 of mice, mkg/kg. Data is shown for i.m. injections

<table>
<thead>
<tr>
<th>Agent</th>
<th>Code</th>
<th>LD50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soman</td>
<td>GD</td>
<td>1400</td>
</tr>
<tr>
<td>Tabun</td>
<td>GA</td>
<td>440</td>
</tr>
<tr>
<td>Sarin</td>
<td>GB</td>
<td>222</td>
</tr>
<tr>
<td>VX</td>
<td></td>
<td>15</td>
</tr>
<tr>
<td>VR</td>
<td></td>
<td>10</td>
</tr>
</tbody>
</table>

Bimolecular inhibition constant

\[ k_i, \text{ M}^{-1} \cdot \text{min}^{-1} \]

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>rhBChE</td>
<td>(5.1\pm0.5)\cdot10^7</td>
</tr>
<tr>
<td>rhBChE -CAO27</td>
<td>(6.1\pm0.6)\cdot10^7</td>
</tr>
<tr>
<td>hBChE</td>
<td>(4.3\pm0.4)\cdot10^7</td>
</tr>
</tbody>
</table>

_Ilyushin et al., PNAS 2013_
**In vivo Studies: Step Two**

- **Open Field test**: Actions observed in treated group compared to control group, %
  - Vertical activity
  - Horizontal activity
- **Treadmill test**: Test passed, %
  - Median and IQR of tested groups
  - IQR control group (Median = 100%)

**i.v. injection of PBS, rhBChE-CAO27 or hBChE, 20 mg/kg**

**30 min**

**i.m. injection of VR dissolved in water**

<table>
<thead>
<tr>
<th></th>
<th>LD$_{50}$ × 10$^3$, mg/kg</th>
<th>Efficacy index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median</td>
<td>95% confidence limits</td>
</tr>
<tr>
<td>rhBChE-CAO27 (n=20)</td>
<td>79</td>
<td>69</td>
</tr>
<tr>
<td>hBChE (n=24)</td>
<td>89</td>
<td>80</td>
</tr>
<tr>
<td>Control (n=61)</td>
<td>19</td>
<td>18</td>
</tr>
</tbody>
</table>
Ex vivo: neurotransmission

If rBChE+CAO27 worked as bioscavenger than neuro-muscular transmission will not be affected.
Merci bien!