Teaching old proteases new tricks: Proteolytic degradation of Aβ and IL-13

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Why are we considering therapeutic protease approaches?

- Catalytic mechanism
- Irreversible inactivation

- Stoichiometric binding
- Reversible binding

Expected benefits
- Lower dose
- Greater potency
- Avoidance of “antigen sink”
Why is the scope of therapeutic proteases limited?

- There are a few FDA-approved protease drugs

<table>
<thead>
<tr>
<th>Usage</th>
<th>Protease</th>
<th>Indications</th>
<th>Source of protein</th>
<th>Target protein or pathway</th>
<th>Type of protease</th>
<th>Year approved by FDA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombolysis</td>
<td>Urokinase (u-PA)</td>
<td>Thrombus, catheter clearing</td>
<td>Extracted from urine or from primary kidney cell culture</td>
<td>Converts plasminogen into plasmin</td>
<td>Serine</td>
<td>1978</td>
</tr>
<tr>
<td></td>
<td>T-PA (alteplase, Activase®)</td>
<td>AMI, stroke, catheter clearing</td>
<td>Recombinant in CHO cells</td>
<td>Plasminogen activator</td>
<td>Serine</td>
<td>1987 (AMI) 1996 (stroke) 2002 (catheter clearing)</td>
</tr>
<tr>
<td></td>
<td>Retepase (Retevase)</td>
<td>AMI</td>
<td>Recombinant in E. coli</td>
<td>Plasminogen activator</td>
<td>Serine</td>
<td>1996</td>
</tr>
<tr>
<td></td>
<td>TNK-IPA (tenecteplase, Metalyse®)</td>
<td>Myocardial infarction</td>
<td>Recombinant in CHO cells</td>
<td>Plasminogen activator</td>
<td>Serine</td>
<td>2000</td>
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<tr>
<td>Procoagulant</td>
<td>FIX</td>
<td>Haemophilia B</td>
<td>Human plasma</td>
<td>FX activator</td>
<td>Serine</td>
<td>1990</td>
</tr>
<tr>
<td></td>
<td>FIX (BeneFIX®)</td>
<td>Haemophilia B</td>
<td>Recombinant in CHO cells</td>
<td>FX activator</td>
<td>Serine</td>
<td>1997</td>
</tr>
<tr>
<td></td>
<td>FVIIa (NovoSeven®)</td>
<td>Haemophilia A and B</td>
<td>Recombinant in CHO cells</td>
<td>FX and FIX activator</td>
<td>Serine</td>
<td>1999</td>
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<tr>
<td></td>
<td>Topical thrombin in bandages</td>
<td>Bleeding</td>
<td>Bovine</td>
<td>Fibrinogen activator</td>
<td>Serine</td>
<td>2006</td>
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<tr>
<td></td>
<td>Thrombin (Recothrom®)</td>
<td>Bleeding</td>
<td>Recombinant in CHO cells</td>
<td>Fibrinogen activator</td>
<td>Serine</td>
<td>2008</td>
</tr>
<tr>
<td>Sepsis</td>
<td>Activated protein C, (drotrecogin alfa, Xigris®)</td>
<td>Sepsis, septic shock</td>
<td>Recombinant in human cell line</td>
<td>Plasminogen activator</td>
<td>Serine</td>
<td>2001</td>
</tr>
<tr>
<td>Neuromuscular</td>
<td>Botulinum toxin A (Botox®)</td>
<td>Various muscle spasms</td>
<td>Bacterial (C. botulinum)</td>
<td>Syntxin and SNAP-25 deactivator</td>
<td>Zinc</td>
<td>1989</td>
</tr>
<tr>
<td></td>
<td>Botulinum toxin B (Myobloc)</td>
<td>Cervical dystonia</td>
<td>Bacterial (C. botulinum)</td>
<td>SNAP25 deactivator</td>
<td>Zinc</td>
<td>2000</td>
</tr>
<tr>
<td>Digestion</td>
<td>Zenpep® (pancrelipase)</td>
<td>Exocrine Pancreatic Insufficiency</td>
<td>Porcine pancreatic extract</td>
<td>Aids digestion of protein</td>
<td>Serine</td>
<td>2009</td>
</tr>
</tbody>
</table>

Craik CS 2011 Biochem J. 435 16

- Specificity engineering difficult: How do you find a novel protease against a molecular target of choice?
Neprilysin

- Neprilysin, or neutral endopeptidase (NEP), is a Zn-dependent metalloprotease that inactivates a broad range of peptide hormones including Aβ.
- It is a type II membrane protein expressed over a wide range of tissues and the active domain (80kD) is arranged as a shell around the catalytically active core.

Russo 2005 FEBS Lett 579 6027

Neprilysin levels in brain
Engineering Neprilysin to an Aβ selective protease

- 10 µl culture growth of random neprilysin variants expressed in *S. cerevisiae*
- Peptide substrates (Aβ and various other peptide targets against which binding should be reduced) are coupled with a fluorophore and biotin for streptavidin binding
- Assays performed in femtolitre (fl) of culture supernatant
- Read out is sensitive to size of the fluorophore labelled part and therefore measures peptide cleavage
  - Red fluorophore label for Aβ(1-40)-peptide
  - Blue fluorophore label for off-target peptides ie. several off-targets can be pooled in one assay

- Simultaneous measurement of Aβ and off-target activity in the same well allows ratiometric selection

```
Abeta   Bt  1. NEP   low anisotropy
\textcolor{red}{\Delta}  \\
off-target   Bt  2. +SA  high anisotropy
\textcolor{blue}{\Delta}  \\
```

Webster 2014 PLoS One 9 e104001
Single and double mutation variants of neprilysin with increased proteolytic activity against Aβ
Improved neprilysin variants
G399V and G714K variant is both more active against Aβ and less promiscuous.
Neprilysin variant shows minimal structural alteration

• Indirect local changes:
  – Reduced size of substrate binding site
  – Reducing plasticity

• Pharmacology:
  – Potent Aβ reduction in plasma of mouse and monkeys
  – No reduction of central Aβ
  – *Henderson 2014 Brain 13 553*
How do we discover proteases against novel targets? The matrix

- Our approach:
  - Screening of broad panel of human proteases vs validated therapeutic targets
  - Identify specific and neutralising cleavage
As expected, natural proteases show substrate promiscuity

![Table showing substrate cleavage results for various proteases and proteins](image)

- **Not cleaved**
- **Site-specific cleavage**
- **Non-specific cleavage**
- **Low activity (≤50% 24h)**
- **Low activity and non-specific**
- **Not tested**

*Urbach 2015 Chem Biol 22:1442*
Three activities looked particularly promising

1: substrate
2: substrate + protease (stopped: + inhibitor)
3: substrate+protease+inhibitor
4: protease + inhibitor

Urbach 2015 Chem Biol 22:1442
Hydrolysis of IL-13 by MMP catalytic domains

![Image of Figure 3](image)

**Table 2**

<table>
<thead>
<tr>
<th>MMP</th>
<th>Cleavage of IL-13 in 24 h (^a) (%)</th>
<th>Activity Half Life (^b) (h)</th>
<th>Stability group (^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-1</td>
<td>≥ 50</td>
<td>210</td>
<td>High</td>
</tr>
<tr>
<td>MMP-2</td>
<td>0</td>
<td>2</td>
<td>Low</td>
</tr>
<tr>
<td>MMP-3mut</td>
<td>≤ 50</td>
<td>230</td>
<td>High</td>
</tr>
<tr>
<td>MMP-7</td>
<td>≥ 50</td>
<td>49</td>
<td>Medium</td>
</tr>
<tr>
<td>MMP-8</td>
<td>≥ 50</td>
<td>3</td>
<td>Low</td>
</tr>
<tr>
<td>MMP-9</td>
<td>0</td>
<td>27</td>
<td>Medium</td>
</tr>
<tr>
<td>MMP-10 mut</td>
<td>≤ 50</td>
<td>65</td>
<td>Medium</td>
</tr>
<tr>
<td>MMP-12</td>
<td>≥ 50</td>
<td>3.5</td>
<td>Low</td>
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<tr>
<td>MMP-12 mut</td>
<td>≥ 50</td>
<td>47</td>
<td>Medium</td>
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<tr>
<td>MMP-13</td>
<td>≥ 50</td>
<td>4.6</td>
<td>Low</td>
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<tr>
<td>MMP-20</td>
<td>≥ 50</td>
<td>165</td>
<td>High</td>
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</table>

\(^a\) CLEavage assay performed in the presence of 10 nM IL-13 and MMPs for 24 h at 37°C. 
\(^b\) Assayed by measuring fluorescence at 365 nm, excitation at 280 nm. 
\(^c\) Determined by evaluating Cleavage assay with IL-13. 

*Urbach 2015 Chem Biol 22:1442*
Hydrolysis of IL-13 in BAL

![Graph showing the hydrolysis of IL-13 in different MMP concentrations.](Image)

- **MMP conc. 1μM**

Urbach 2015 Chem Biol 22:1442
The concept of captivases

- Captivases: binding and catalysis are uncoupled, yet active simultaneously
- Maximises therapeutic benefit of both functionalities
  - Specificity of an antibody
  - Mechanism of a protease

Gordon 2015 in preparation
Natural proteases employ remote binding sites for specificity

- Exosites—surface sites on proteinases physically separated from the active site residues (e.g. blood coagulation factors)
- Distinct binding domains separate from catalytic domain
- Modular systems are more evolvable—both in nature and in the lab
Captivase mechanism: activity towards IL-13 is greatly enhanced

<table>
<thead>
<tr>
<th></th>
<th>Anti-IL-13 scFv</th>
<th>Soluble IL-13Ra2</th>
<th>Captivase</th>
<th>Inactive Captivase</th>
<th>MMP-8</th>
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<tbody>
<tr>
<td>M</td>
<td>+</td>
<td></td>
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<tr>
<td></td>
<td>+</td>
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<tr>
<td>Anti-IL-13 scFv</td>
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<tr>
<td>Active MMP-8</td>
<td>+</td>
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<td>5 min</td>
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<td>2 h</td>
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<td>24 h</td>
<td>+</td>
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<tr>
<td>96 h</td>
<td>+</td>
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</tbody>
</table>

Gordon 2015 in preparation
Conclusions

- Proteolytic degradation of a therapeutic target is an attractive approach for drug discovery
- Protease promiscuity can be readily addressed through engineering
- Promiscuity makes it possible to find novel specificities
- Captivase might be a generic and modular approach to reduce $K_M$ and the impact of promiscuity
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