Phylogica Phylomer platform & Functional penetrating peptides - a new type of cell penetrating peptide for efficient drug delivery into mammalian cells

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Research manager
What are Phylomers?

- Phylomers® are peptides derived from evolutionary diverse eukaryotic and archaebacterial genomes and more recently from viral genomes.
- Enriched for natural secondary structures which have evolved for high affinity and biological activity.
- Provide a rich source of structural motifs for screening against a wide range of targets or potential for cell penetration.

**Diagram:**

- Bacteria
  - Cyanobacteria
  - Chlorobi
  - Spirochetes
  - Thermotogales
  - Purple bacteria
- Archaea
  - Archaeoglobi
  - Methanococci
  - Thermococci
- Crenarchaeota
  - Thermoprotei
  - Pyrodictium
  - Deinococcus
- Eukarya
  - Aquilox
  - Halobacteria
  - Thermoplasma
  - Methanobacteria

**Viruses**

- Adenovirus
- Adeno-associated virus
- Epstein-Barr virus
- Measles
- Newcastle disease
- Parainfluenza
- Respiratory syncytial virus
- Rotavirus
- Reovirus
- Papillomavirus
- Adeno-associated virus
- SV40
- Measles
- New Castle Disease
- Parainfluenza
- Respiratory syncytial virus
- Rotavirus
- Reovirus
- Papillomavirus

**Categories:**

1. Aeropyrum pernix
2. Archaeoglobus fulgidus
3. Bacillus subtilis
4. Bordetella pertussis
5. Borrelia burgdorferi
6. Campylobacter jejuni
7. Chlorobium tepidum
8. Clostridium acetobutylicum
9. Deinococcus radiodurans
10. Escherichia coli
11. Haemophilus influenzae
12. Halobacterium salinarum
13. Helicobacter pylori
14. Listeria innocua
15. Methanococcus jannaschii
16. Neisseria meningitides
17. Pseudomonas aeruginosa
18. Pyrococcus horikoshii
19. Salmonella enterica
20. Shigella flexneri
21. Staphylococcus aureus
22. Streptomyces avermitilis
23. Sulfolobus solfataricus
24. Thermoplasma volcanium
25. Thermotoga maritima
Phyloemer platform for discovery of structure-rich peptides

** PHYLOMER Peptide therapeutics **

- Effective at endosomal escape
- Carry *functional* cargoes into a range of cell types
- “Functional Penetrating Peptides” or Phyloemer FPPs

** Antimicrobial targets **

** Extracellular targets **

** Intracellular targets **

- Transcription factors e.g. MYC in cancers
- Phyloemer Myc inhibitor in lead optimisation stage
FPP Delivery Platform Features

1. Endosomal escape trap, identification of new Phylomer FPPs

2. \textit{In vitro} validation and maturation

3. Evidence of \textit{in vivo} efficacy
The Problem: drug cargoes are trapped in the endosomes

- Conventional CPPs are often only active at concentrations of > 10 uM (limiting feasible clinical application, toxicity and high costs)
- Search for more efficient CPPs with high level of endosomal escape
Based on the specific biotinylation of the 15 aa avi-tag by *E. coli* biotin ligase (BirA)

- Mammalian target cells have to be stably transformed to express BirA in the cytoplasm
Phylogica’s Endosomal Escape Trap

Phage is taken up by endosomes

Avitag  Phylomer

Biotin ligase

T7 Phage Library
Phylogica’s Endosomal Escape Trap
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- Phage are amplified and BirA Trap cell incubations are repeated 2-3 times
- MiSeq NGS sequencing
- Validation as recombinant fusion proteins
FPP Delivery Platform Features

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Split-GFP complementation assay validates endosomal escape of FPP fusions
Split-GFP complementation assay validates endosomal escape of FPP fusions

- Only cargo that escaped the endosome can fluoresce in this assay

International patent no: PCT/AU2014/050094
Split GFP assay shows superior FPP uptake & cytoplasmic delivery vs other CPPs in live cells

- All CPP/FPP_S11 fusions shown here were capable of complementing GFP fluorescence *in vitro*
- Phylomer FPP caused higher cytoplasmic delivery than conventional CPPs – especially at lower concentrations
**β-lactamase: new, medium through-put CPP-validation assay**

**β-lactamase (BLA)**

- Bacterial enzyme, cleaves beta-lactam antibiotics (penicillin, cephalosporins)
- CCF2-AM: cell-permeable, self loading substrate for BLA with a FRET-pair of fluorophores
  - *Uncleaved* CCF2: excitation 408 nm: green fluorescence
  - *Cleaved* CCF2: excitation 408 nm: blue fluorescence

**Cell assay:**
- CHO-K1 cells + protein incubated at 37°C for 1 h
- Trypsin treatment
- Loading with 1 uM CCF2-AM

**Mammalian cells loaded with CCF2-AM**

**Cell entry of FPP_BLA**
- BLA activity in the cell
- Blue fluorescence
- Microscopy or Flow Cytometry
BLA assay can detect cell entry of cargo concentrations at 0.5 uM

- Good signal:noise ratio for FPP-mediated cell-uptake below 5 uM
- Even cell entry of 0.5 uM FPP_BLA is detectable by BLA-assay
BLA assay can detect cell entry of cargo concentrations at 0.5 uM

Endosomal escape?
• No: full-length BLA assay cannot differentiate endosomal entrapment from escape
• Live cell microscopy suggests that FPP01.1 delivered BLA is mostly into the cytoplasm:

- Good signal:noise ratio for FPP-mediated cell-uptake below 5 uM
- Even cell entry of 0.5 uM FPP_BLA is detectable by BLA-assay

Confocal microscopy (20x mag) of CHO-K1 cells incubated with 4 uM FPP01.1_SpyT/SpyC_BLA for 1 h. Incubation at 20°C instead of 37°C (causing low levels of cell entry)
Development of Split-BLA system to measure endosomal escape

To measure endosomally release only, we are currently developing a split-beta-lactamase assay

- C-terminal part of BLA (CBLA) is stable expressed in the cytoplasm of mammalian cells
- FPP is linked to the N-terminal part of BLA (NBLA)
- Only endosomal escape will lead to beta-lactamase complementation and signal development

First experimental results:

- Strong signal for FPP-mediated cytoplasmic delivery down to 0.5 uM
- Confirmation that FPP01.1 mediates endosomal escape
- Further improvements under way
BLA-assay: Higher test throughput by using SpyCatcher-SpyTag conjugations

SpyCatcher-SpyTag conjugation

- Binding of the 14 aa SpyTag to the 88 aa SpyCatcher autocatalyses an isopeptide bond between the two fragments
- This can be used to covalently link protein/peptide domains which are attached to a SpyCatcher and a SpyTag (efficiency 70-95%)

- Synthetic FPP_SpyTag peptides can be conjugated onto beta-lactamase
- No need for individual cloning, expression and protein purification
BLA-assay allows rapid detection of FPPs with improved cell entry

- Rational design variants of FPP01.1 (all 27 aa) synthesised as SpyT-fusions and conjugated to SpyC-BLA protein
- Conjugation efficiencies: 80-95% - introducing some quantitation error
- Tested on CHO-K1 cells under standard conditions

- Identified several variants of FPP01.1 with up to 3.5-fold improved cell entry
- Parallel Sytox-Red FC analysis did not detect increased toxicity
- Promising maturation variants will be re-tested in the split-GFP or split-BLA assay
FPPs are able to deliver diverse cargo classes

Examples of cargoes delivered with FPP01, FPP01.1 or FFP02

<table>
<thead>
<tr>
<th>Cargo Class</th>
<th>Cargo</th>
<th>Size</th>
<th>IEP</th>
<th>IC50*</th>
<th>MED**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptide</td>
<td>Apoptotic (PAP) PPI inhibitor (DPMIα) Bcl-2 family inhibitory peptides</td>
<td>17 aa ~ 2 kDa 15 aa ~2 kDa 26 aa ~ 3 kDa</td>
<td>10.7</td>
<td>1.7 µM</td>
<td>n/d 1.25 µM</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>8.3</td>
<td>8 µM</td>
<td>1.6 µM</td>
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<td>6.3</td>
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<td>Oligonucleotide</td>
<td>Exon-skipping antisense oligo</td>
<td>24 nu ~ 8 kDa</td>
<td>7.0</td>
<td>n/d</td>
<td>50 nM</td>
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<tr>
<td>Small protein scaffold</td>
<td>Omomyc</td>
<td>11 kDa</td>
<td>9.6</td>
<td>6 µM</td>
<td>n/d</td>
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<tr>
<td>Toxin (protein)</td>
<td>Bouganin</td>
<td>28 kDa</td>
<td>7.8</td>
<td>20 nM</td>
<td>n/d</td>
</tr>
<tr>
<td>Bispecific</td>
<td>Bcl-2 inhibitory peptide + Omomyc scaffold</td>
<td>37 kDa</td>
<td>8.0</td>
<td>190 nM</td>
<td>156 nM</td>
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<td>5.5</td>
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*IC50s tested in various cell lines
**Minimal Effective Dose
n/d (Not determined)
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*IC50s tested in various cell lines  **Minimal Effective Dose  n/d (Not determined)
Delivering Omomyc using Phylogica’s FPP

- Omomyc linked to a Phylomer FPP01.1 (recombinant fusion)
- Omomyc protein alone has low potency due to very poor cell penetration

- Delivery with FPP01.1 significantly increased Omomyc potency
- Most potent direct Myc inhibition described to date

Viability of T47D cells 24 h after treatment

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<tr>
<th>Compound</th>
<th>IC₅₀ (µM)</th>
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</thead>
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<tr>
<td>His_Omomyc</td>
<td>&gt;40</td>
</tr>
<tr>
<td>FPP01.1</td>
<td>&gt;40</td>
</tr>
<tr>
<td>FPP01.1_His_Omomyc_V5</td>
<td>5.9</td>
</tr>
<tr>
<td>FPP01.1_His_eGFP_V5</td>
<td>129</td>
</tr>
<tr>
<td>KJ-Pyr9</td>
<td>21.4</td>
</tr>
<tr>
<td>JQ1</td>
<td>12.2</td>
</tr>
<tr>
<td>DMSO</td>
<td>167</td>
</tr>
</tbody>
</table>
### FPP-Omomyc shows potent activity in multiple Myc-driven breast cancer cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Disease</th>
<th>Cell line characteristics</th>
<th>FPP01_Omomyc IC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T47D</td>
<td>Breast cancer</td>
<td>p53 mutₕ(hetero), Myc+++</td>
<td>5.9</td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>Breast cancer</td>
<td>p53 mutₕ(hetero), triple -ve</td>
<td>1.7</td>
</tr>
<tr>
<td>SUM159 *</td>
<td>Breast cancer</td>
<td>Basal, Triple -ve</td>
<td>1.1</td>
</tr>
<tr>
<td>B1.15 (mouse) *</td>
<td>Breast Cancer</td>
<td>Basal, Brca-/-</td>
<td>1.1</td>
</tr>
<tr>
<td>A1.8 (mouse) *</td>
<td>Breast Cancer</td>
<td>Basal, Brca-/-</td>
<td>1.2</td>
</tr>
<tr>
<td>T11 (mouse) *</td>
<td>Breast cancer</td>
<td>Basal, Triple -ve, P53-/-</td>
<td>2.1</td>
</tr>
<tr>
<td>PyMT (Mouse) *</td>
<td>Breast cancer</td>
<td>Luminal/basal</td>
<td>1.0</td>
</tr>
<tr>
<td>MCF10A *</td>
<td>Immortalised tissue</td>
<td>Epithelial breast</td>
<td>&gt;15</td>
</tr>
</tbody>
</table>

- MCF10a cells are not Myc dependent and were not affected by treatment with FPP_Omomyc
- Best in-class potencies for direct Myc inhibition with an unoptimised FPP

* Assoc. Prof. Pilar Blancafort; Cancer Epigenetics Lab, Harry Perkins Institute
FPP Delivery Platform Features

1. Endosomal escape trap, identification of new Phylomer FPPs

2. *In vitro* validation and maturation

3. Evidence of *in vivo* efficacy
Duchenne Muscular Dystrophy (DMD): severe muscle wasting disorder caused by mutations in the gene for dystrophin

- Nonsense mutations; X-linked recessive; affect 1:3,500 male births
- Problems with walking starts at about 4 years, by the age of 12 most boys cannot walk any longer
- So far no cure, current average life expectancy ~26 years
- Treatment by “exon skipping”: 

*D Susan Fletcher, Loren Price and Abbie Adams*
Duchenne Muscular Dystrophy: treatment by exon skipping

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![Diagram showing exon skipping in Duchenne Muscular Dystrophy](image)

- **Pre mRNA**
  - Exon 1
  - 2
  - 21
  - 22
  - 23
  - 24
  - 25
  - 79

- **Normal exon 23**
  - Splicing
  - Spliced mRNA
  - Protein: Fully functional Dystrophin

- **Mutated exon 23**
  - Stop codon mutation
  - Spliced mRNA
  - Protein: Non-functional Dystrophin
**Duchenne Muscular Dystrophy (DMD):** severe muscle wasting disorder caused by mutations in the gene for dystrophin
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![Exon Skipping Diagram]

- **Normal exon 23**
  - Spliced mRNA
    - Fully functional Dystrophin
- **Mutated exon 23**
  - Spliced mRNA
    - Non-functional Dystrophin
- **Exon 23 skipped**
  - Spliced mRNA
    - Functional Dystrophin (shorter but active)
FPP delivery of antisense oligo results in improved phenotype in Duchenne mice

- C57BL/10ScSc<sup>mdx</sup> mice were treated with 5 i.p. injections of FPP-DMD antisense oligo over two weeks

Histology staining: FPP-oligo treatment induced mouse muscle tissue to return to a more-normal phenotype with Dystrophin levels that can lead to functional improvement
Expanding the intracellular landscape for novel biotherapeutics with Phylomers

- Endosome Escape Trap for detection of novel Phylomer FPPs
- Validation of new FPPs by Split GFP and BLA assays
- Using new functional Phylomer FPPs to facilitate efficient delivery of biologic cargoes
- Proof of *in vivo* efficacy of FPPs by showing exon skipping and histological changes in DMD mouse model
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Arne Skerra
Volker Morath

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