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Antimicrobial Peptide prodrugs and mimetics

Éanna Forde[1, 2], André Schütte[3], Andrea Molero-Bondia[1], Louise Sweeney[4], Emer Reeves[5], Catherine Greene[5], Hilary Humphreys[2, 6], Ronan Mac Loughlin[4], Marcus Mall[3], Deirdre Fitzgerald-Hughes[2], Marc Devocelle[1,*]

[1] Department of Pharmaceutical & Medicinal Chemistry, Royal College of Surgeons in Ireland;
[2] Department of Clinical Microbiology, Royal College of Surgeons in Ireland;
[3] Department of Translational Pulmonology, University of Heidelberg;
[4] Aerogen Ltd, Galway;
[5] Department of Medicine, Royal College of Surgeons in Ireland;
[6] Department of Microbiology, Beaumont Hospital, Dublin

* Corresponding author: mdevocelle@rcsi.ie

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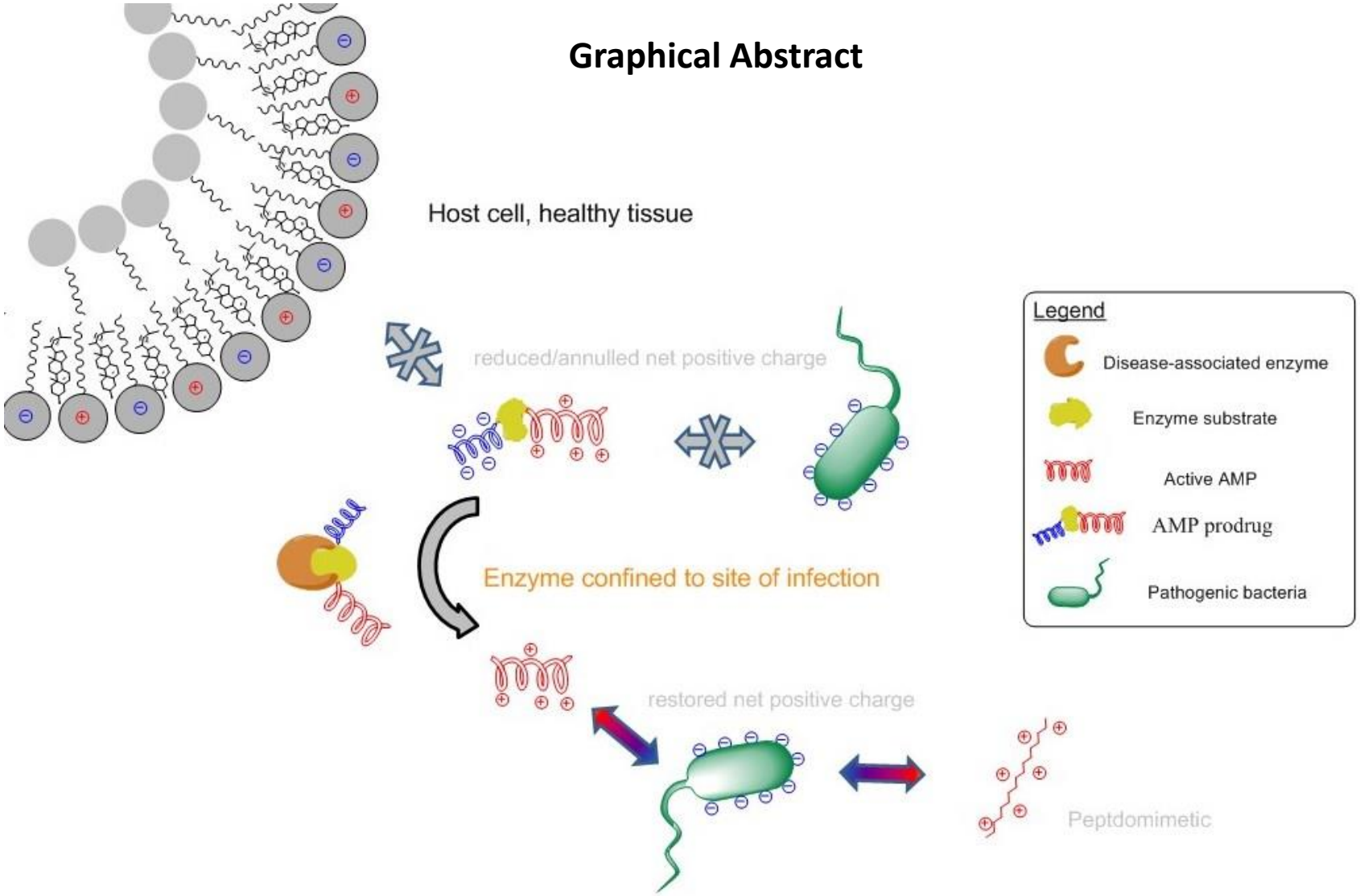
Antimicrobial Peptide prodrugs and mimetics

Graphical Abstract



Abstract: Antimicrobial Peptides (AMPs) represent one of the most durable and effective defence of multicellular organisms against bacterial infections. These cationic and amphipathic peptides represent promising leads for the development of antibiotics against resistant bacteria. However, their clinical applications have been limited by an inadequate margin of safety.

A prodrug approach can overcome a toxicity barrier in drug delivery. Prodrugs of AMPs can be generated by transiently reducing their net positive charges by attaching a negative promoiety through a linker which can be degraded by an enzyme (bacterial or human) confined to sites of infection. For example, neutrophil elastase (NE), a human protease involved in chronic airway inflammation and infections associated with cystic fibrosis (CF), can restore the cationic property of AMPs modified with oligo-glutamate promoieties. Their bactericidal activities against the CF pathogen *Pseudomonas aeruginosa* are restored by NE in CF bronchoalveolar lavage fluids. The potential of this prodrug approach in reducing the safety barrier in the clinical use of AMPs was evaluated *in vivo*, in a murine model of lung delivery.

In parallel, a novel class of peptidomimetics with antimicrobial activities similar to AMPs, against Gram-positive bacteria, has been developed. Their spectrum of activity is currently extended to Gram-negative organisms.

Keywords: Antimicrobial Peptides; Prodrugs; Peptidomimetics; Antibiotics for Cystic Fibrosis.

Introduction – Antibiotic resistance

'A post-antibiotic era—in which common infections and minor injuries can kill—far from being an apocalyptic fantasy, is instead a very real possibility for the 21st century ' [1]

- **Antibiotic resistance in common bacteria is ready to become a global public health crisis, arising from a situation described as 'a perfect storm'. [2]**

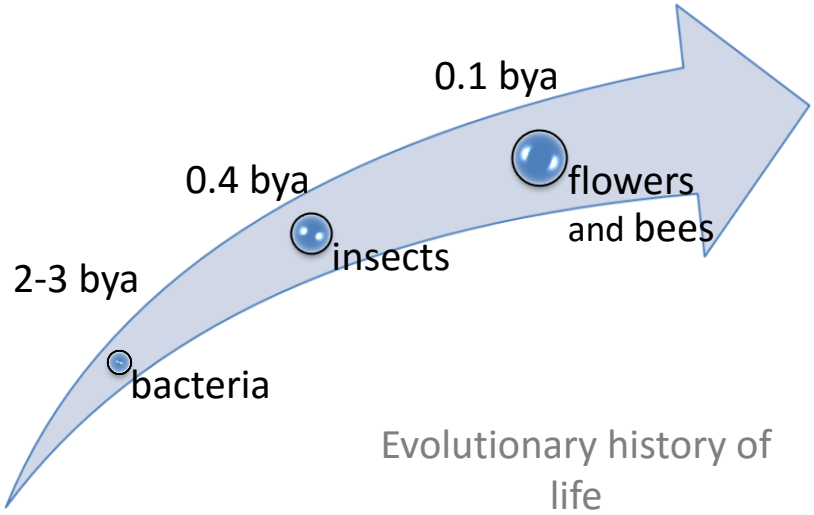
- **It cumulates a shortage of treatment options for an increasing number of widespread infections, a lack of new antibiotics in development and the unbalanced rates of anti-infective drug development (on average 12 years) and antibiotic emergence (*e.g.* adaptation rates of 12 days against ciprofloxacin [3]).**

- **Novel strategies , including therapeutic, which can potentially delay the emergence of antibiotic resistance, are therefore desirable.**

Introduction – Antimicrobial Peptides (AMPs)

- **Also called Host Defence Peptides (HDPs), they are multifunctional molecular effectors of innate immunity, the first line of defence against infection in multicellular organisms. [4]**

- **Some living organisms (*e.g.* plants, insects) totally rely on these peptides to fight infections and have used them for million of years, without facing significant resistance mechanisms from bacteria.**



Evolutionary history of life

- **Slow emergence of resistance attributed to the polypharmacology of these peptides and their use in combinations.**

Introduction – AMPs as antibiotic candidates

- Substantiated by their direct antimicrobial activity, but also immuno-enhancing potential and anti-inflammatory activity.
- Note that AMPs are also currently investigated as novel drug candidates for anticancer therapy.

- Advantages of AMPs:

✓Low propensity to select resistant mutants

✓Synergistic activity with classical chemotherapies

✓Active against both dividing and non-dividing cells

- Limitations of AMPs:

✘Unknown systemic toxicity

✘ Rapid metabolic degradation

Introduction – Prodrug approach to address AMPs' clinical shortcomings

- **Prodrugs are inactive precursors of pharmaceutical agents that are activated *in vivo*.**

- **Targeted delivery of an active parent drug can be achieved by a prodrug strategy, if the activation is mediated by a chemical and/or biochemical reaction confined to a specific body site: it can therefore address a toxicity issue in drug development.**

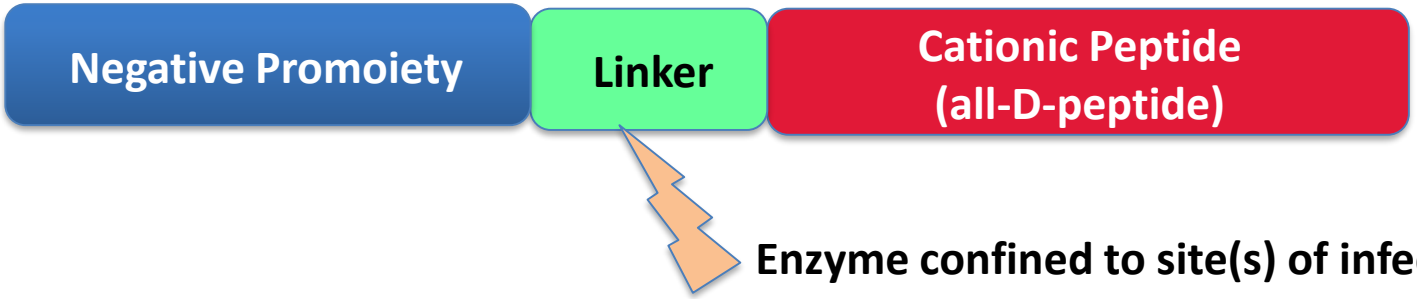
- **AMPs are amphipathic peptides; one of the main activity determinants is a net positive charge.**

- **An AMP prodrug can be generated by reversible reduction of this net charge.**

Introduction – AMPs prodrugs

- AMP Prodrug: reduced/ annulled net positive charge

- Active AMP sequence assembled from D-amino acids, to prevent proteolytic degradation



Promoiety should be non-toxic

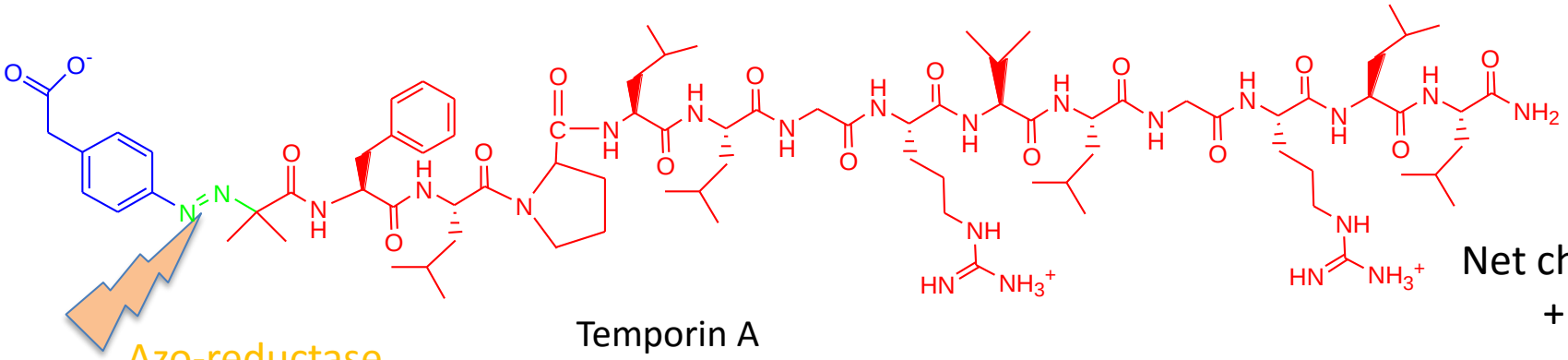
Active AMP: restored net positive charge

Targeted delivery relies essentially on the linker

Results and discussion – 1st example of AMP Prodrug candidate

- **Azo-reductase dependent co-drug: mutual prodrug of an AMP and an anti-inflammatory agent (4-aminophenyl acetic acid, 4-APAA, see slide 10) [5].**
- **Azo bond is metabolically stable and can only be cleaved by azo-reductases.**
- **These enzymes are only secreted by anaerobic bacteria and essentially confined to the colon.**
- **These co-drugs can target the colonic bacteria *Clostridium difficile*; among colonic bacteria, this organism secretes the highest quantities of reductases, endowed with the highest reduction rates.**
- **4-APAA is a potent inhibitor of *C. difficile* toxin A-induced colonic inflammation.**

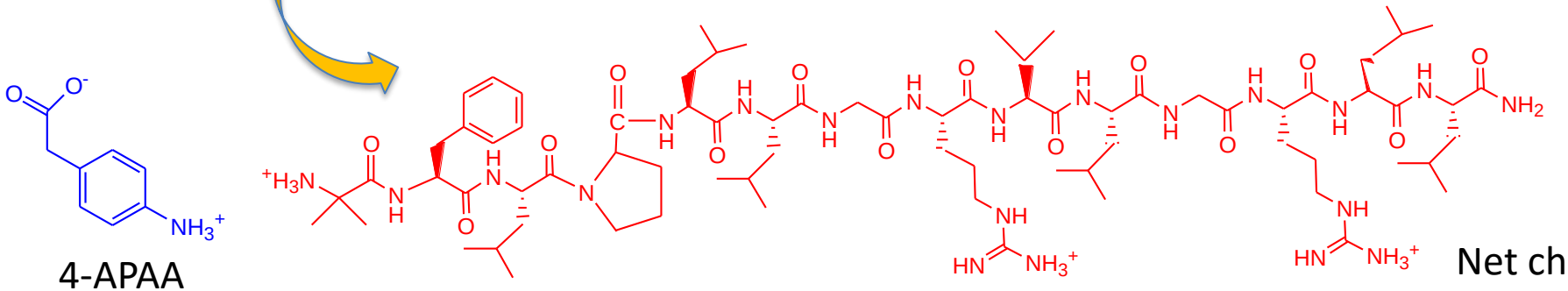
Azo-reductase dependent co-drug



Azo-reductase

Temporin A
(Wade D, Kuusela P, *FEBS Letters,* 2000)

Net charge: +1



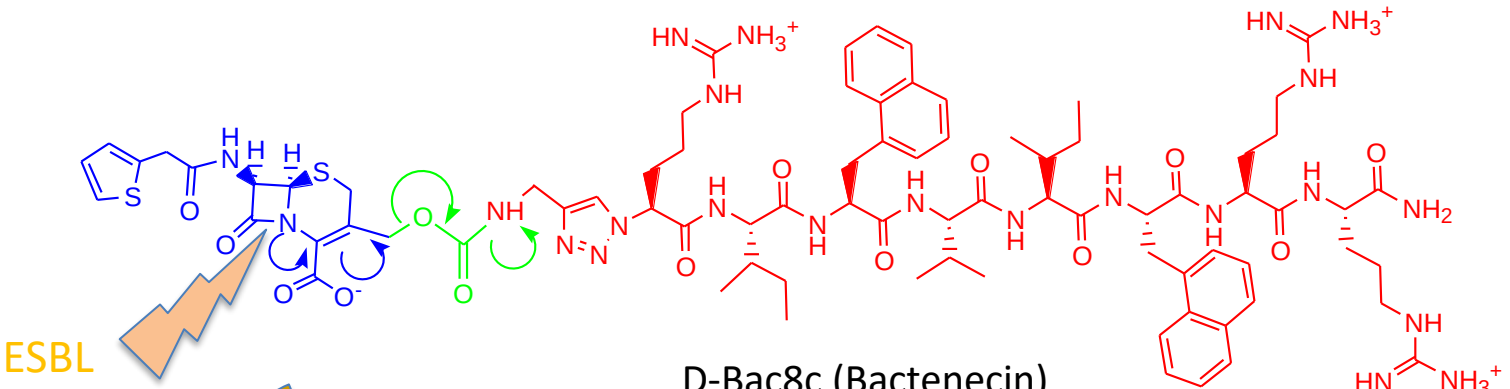
4-APAA
(NSAID)

Net charge: +3

Results and discussion – 2nd example of AMP Prodrug candidate

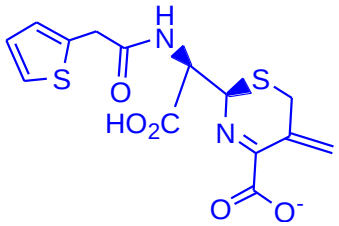
- **Extended Spectrum β-Lactamases (ESBLs) dependent prodrug (see slide 12) [6].**
- **ESBLs are enzymes of resistance against β-lactam antibiotics, the cornerstone of the antibiotic arsenal.**
- **ESBLs are produced by (Multi-Drug) Resistant Gram-negative bacteria, organisms against which therapeutic options (existing and in development) are currently severely limited.**
- **ESBL-producing, in particular Metallo β-Lactamases-producing Enterobacteriaceae, produce enzymes with the highest catalytic efficiencies and broadest spectrum of substrates and are therefore ideal targets of these prodrugs.**
- **In these prodrugs, the promoiety is a cephalosporin which releases the active peptide upon degradation by a β-lactamase.**

ESBLs-activated AMP prodrugs

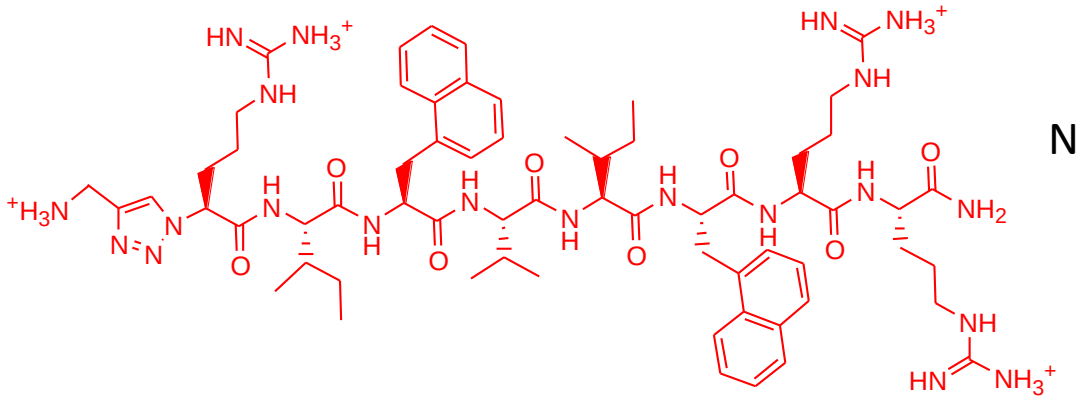


D-Bac8c (Bactenecin)
(Hancock R, *Nat. Biotechnol*. 2005)

Net charge:
+2



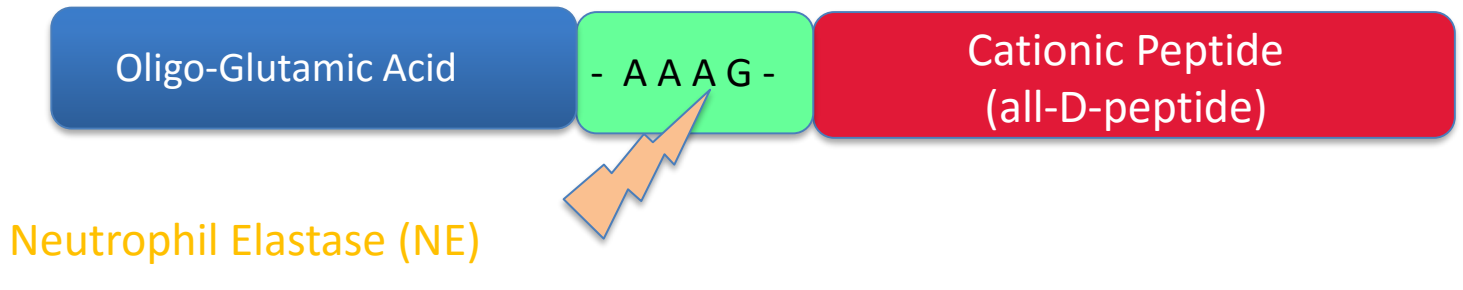
Degraded cephalosporin
(inactive as antibiotic)



Net charge:
+4

Results and discussion – 3rd example of AMP Prodrug candidate

- **Neutrophil Elastase (NE)-dependent AMP prodrugs [7].**
- **Chronic infections in Cystic Fibrosis patients are localised to the endobronchial space.**
- **As a result, neutrophil-dominated immune response releases large quantities of NE into the endobronchial space**
- **Prodrugs can be designed by using an oligo-glutamic acid promoiety [8] and a substrate of NE (A-A-A-G peptide sequence) as a linker.**



NE-dependent AMP Prodrug candidates

- Bac8c: bactenecin optimized sequence [9]

  (r-l-w-v-l-w-r-r-NH_2) 8-mer, net charge +4

- Bac8c prodrug: 16-mer, net charge -1

  Ac-E-E-E-E-A-A-A-G-r-l-w-v-l-w-r-r-NH_2

- HB43: AMP for Cystic Fibrosis [10]

  (f-a-k-l-l-a-k-l-a-k-k-l-l-NH_2) 13-mer, net charge +5

  HB43 prodrug: 21-mer, net charge 0

- Ac-E-E-E-E-A-A-A-G-f-a-k-l-l-a-k-l-a-k-k-l-l-NH_2

- P18: cecropin A-magainin 2 hybrid sequence [11]

  (k-w-k-l-f-k-k-i-p-k-f-l-h-l-a-k-k-f-NH_2) 18-mer, net charge +8.5

- P18 prodrug: 26-mer, net charge +3.5

  Ac-E-E-E-E-A-A-A-G-k-w-k-l-f-k-k-i-p-k-f-l-h-l-a-k-k-f-NH_2

- (Residual AAG- or AG- amino acids from NE-sensitive linker on activated AMPs) [12]

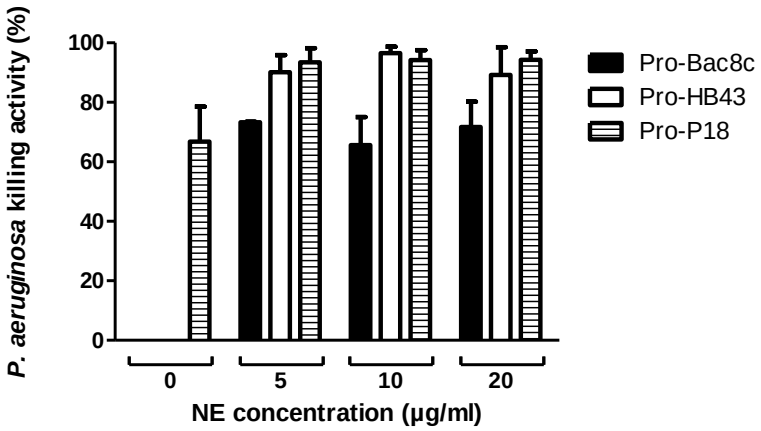
Results and discussion - Susceptibility testing

MICs *vs. P. aeruginosa* strains (µg/ml) Active peptides Prodrug peptides

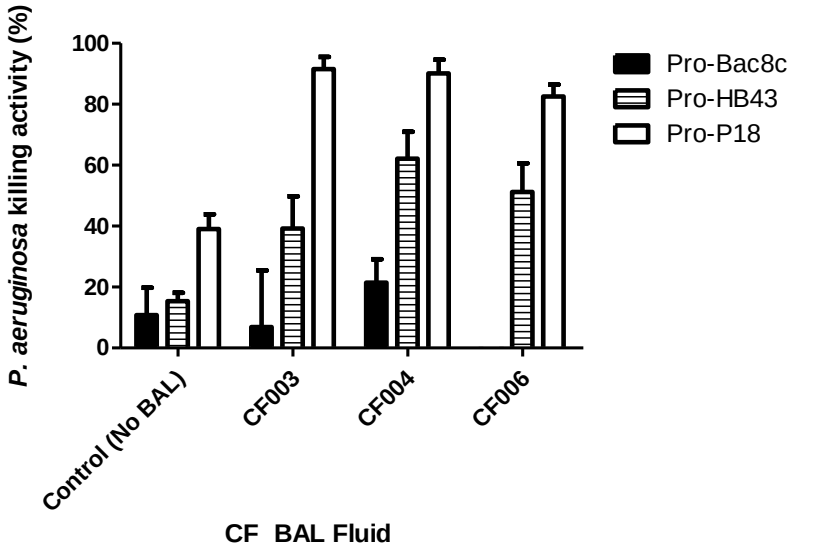
Peptide	PAO1	PABH01	PABH02	PABH03	PABH04
AAG-Bac8c	4	8	8	16	8
Bac8c prodrug	> 64	> 64	> 64	> 64	> 64
AAG-P18	2	2	4	4	2
P18 prodrug	> 64	64	64	> 64	64
AG-HB43 (TFA salt)	8	4	8	4	4
AG-HB43 (hydrochloride)	8	4	8	4	4
HB43 prodrug	> 64	> 64	> 64	> 64	> 64

- **Prodrug modification can mask antimicrobial activity**

Results and discussion - Activation assays



- Assays performed with 6.25 µg/ml prodrug peptides and purified NE against *P. aeruginosa* (PAO1)

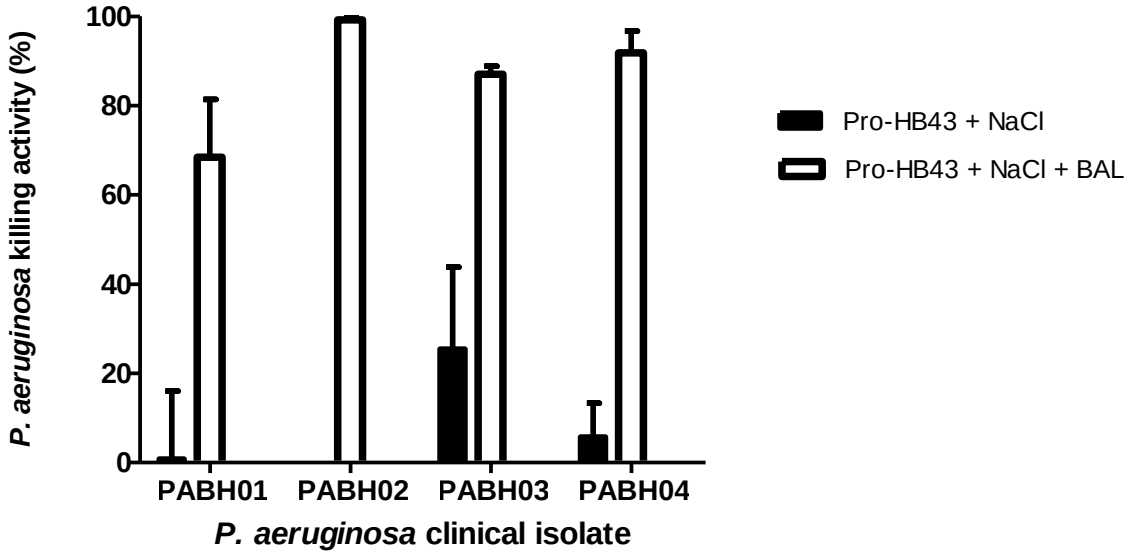


- Assays performed with

25 mg/ml prodrug peptides, 25% (v/v) CF Broncho Alveolar Lavage fluids and 300 mM NaCl (PAO1)

- **Prodrug activation can be mediated by NE concentrations found in CF patients**
  - **Activation in BAL fluids require addition of NaCl (see conclusions**
    - **P18 maintains some activity even as a prodrug**
  - **Bac8c is intolerant to NaCl concentrations required in BAL assays.**

Activation assays – Clinical isolates

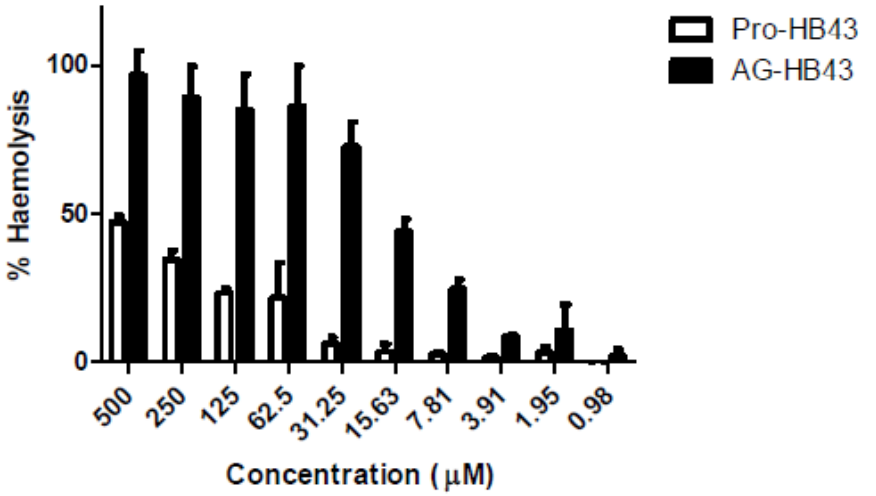


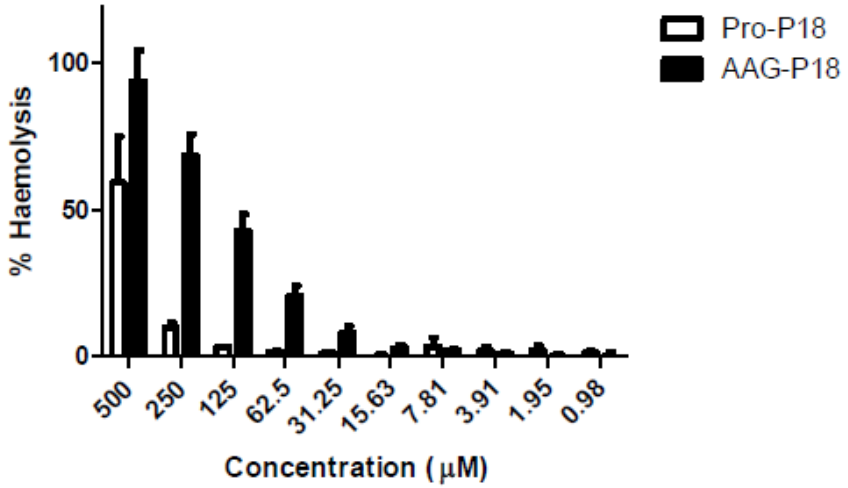
- Assays performed with

25 mg/ml pro-peptide, 25% (v/v) CF BAL fluids and 300 mM NaCl

- **Prodrug activity can be achieved against *P. aeruginosa*, in conditions representative of the CF lung.**

Results and discussion - Toxicity study: haemolytic activities





- Hemolytic activity normalized to 0.1% Triton-X

- **Active AMPs induce haemolysis in a dose dependent manner; prodrug modification prevent haemolysis.**

Toxicity study: cytotoxic activities against CF bronchial epithelial cells

Active peptides Prodrug peptides

Peptide	IC50 (μM) (n =3)
AAG-Bac8c	38.3
Bac8c prodrug	> 300
AG-HB43 (TFA salt)	2.8
AG-HB43 (hydrochloride)	3.7
HB43 prodrug	50.8
AAG-P18	35.5
P18, 4 glutamic acids prodrug	77.3
P18, 5 glutamic acids prodrug	79.4

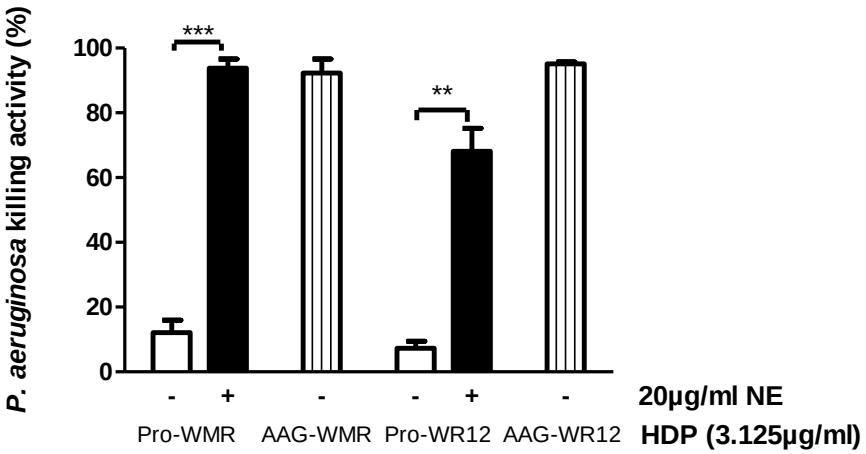
- **Prodrug modification increases therapeutic indices of AMPs.**

2nd generation NE-dependent AMP Prodrug candidates

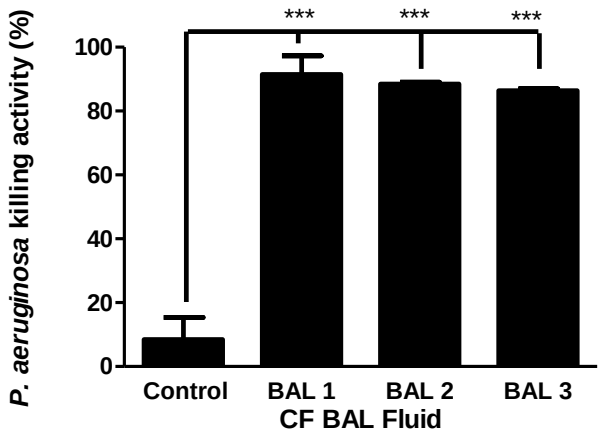
- **From the 1st generation of candidates, HB43 is the best candidate with 18-fold toxicity difference between active and prodrug peptides, while Bac8c is salt intolerant and P18 has residual bactericidal activity and toxicity as a prodrug.**

- **New candidates of similar length and net charge to HB43 :**
  - WR12: engineered cationic AMP [13]
    (r-w-w-r-w-w-r-r-w-w-r-r-NH_2)12-mer, net charge +7
  - WR12 prodrug: 20-mer, net charge +2
    Ac-E-E-E-E-A-A-A-G-r-w-w-r-w-w-r-r-w-w-r-r-NH_2
  - WMR: from hagfish *Myxine glutinosa* [14]
    (w-g-l-r-r-l-l-k-y-g-k-r-s-NH_2) 13-mer, net charge +6
  - WMR prodrug: 21-mer, net charge +1
    Ac-E-E-E-E-A-A-A-G-w-g-l-r-r-l-l-k-y-g-k-r-s-NH_2

Results and discussion - Bactericidal activity



- Assays performed with purified NE against *P. aeruginosa* PAO1

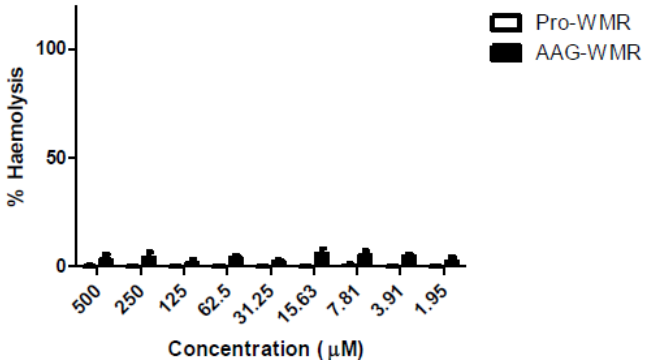


- Assays performed with 25 mg/ml pro-WMR, 25% (v/v) CF BAL fluids and 300 mM NaCl (PAO1)

- **Prodrug activation can be mediated by NE concentrations found in CF patients.**

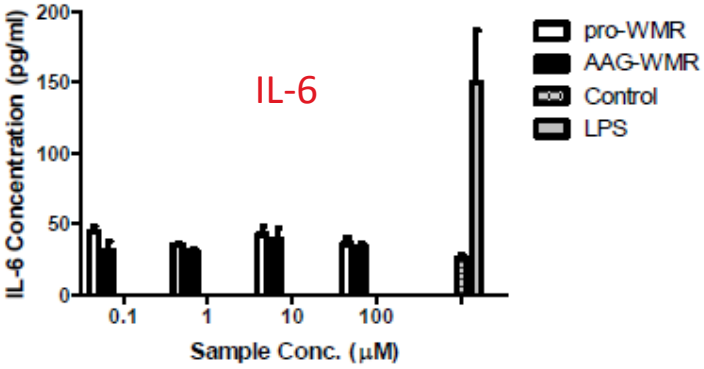
Results and discussion - Toxicity studies

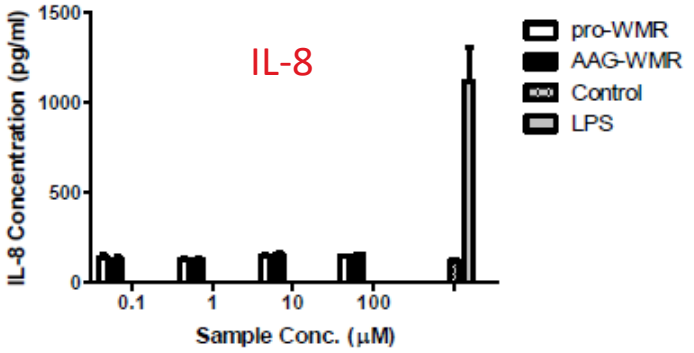
Peptide	CF bronchial epithelial (CFBE) cells	CF tracheal epithelial cells	Neutrophils
AAG-WMR	> 300	> 600	> 300
WMR prodrug	> 300	> 300	> 300



- Hemolytic activity ⇧

- 24 h cytokine release from CFBE cells ⇨





- **Both WMR active and prodrug peptides are non-toxic *in vitro*.**

Nebulisation study, in collaboration with Dr Ronan Mac Loughlin and Ms Louise Sweeney, Aerogen Ltd. Galway

Aerogen®
Pioneering Aerosol Drug Delivery

- Particle sizing of aerosol spray by light-scattering particle size analysis (volumetric median diameter - VMD)
- Droplet diameter of nebulised spray by impaction particle size analysis on 8 stages and end-filter (mass median aerodynamic diameter - MMAD)
- Breathing apparatus: Aerogen Solo nebuliser, with Adapter (Aerogen, Ireland). Salter valved facemask 81070-0 (Salter, USA) and an ASL 5000 active servo lung

Nebulisation Study - Results

Aerogen

Candidate	VMD (μm)	% Fine Particle Fraction <5μm	MMAD (μm)
AAG-WMR	3.8 ± 0.07	66.6	3.14 ± 0.25
WMR prodrug	3.79 ± 0.1	67.1	3.59 ± 0.23

- **Highly respirable (putative respirable range: 1 < MMAD < 5 μm)**
- **Treatment time < 2 minutes.**

Candidate	Inhaled Mass(%)
AAG-WMR	47.58 ± 3.06
WMR prodrug	41.99 ± 3.07

- **Nebulisation data predictive of a high level of dosing in the lung.**

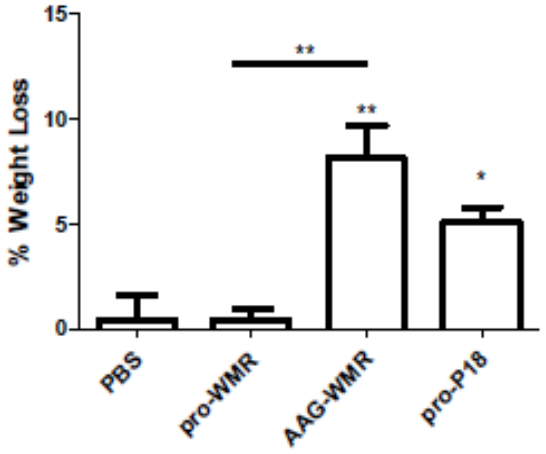
in vivo toxicity study, in collaboration with Prof. Marcus Mall (Translational Lung Research Centre, Heidelberg) [15]

- **β-ENaC-overexpressing mouse model of CF lung disease**

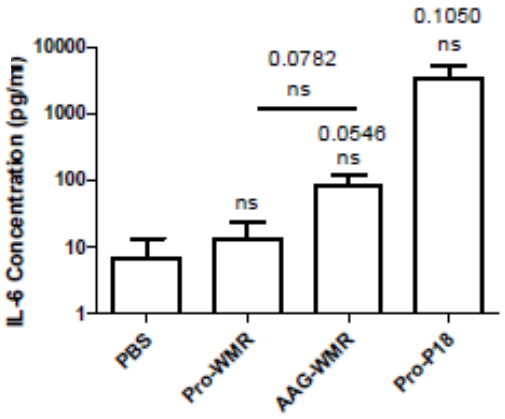
Candidate	Mouse survival
PBS	4 / 4 survived
AAG-WMR	4 / 4 survived
WMR prodrug	4 / 4 survived
AAG-P18	1 / 4 survived
P18 prodrug	4 / 4 survived

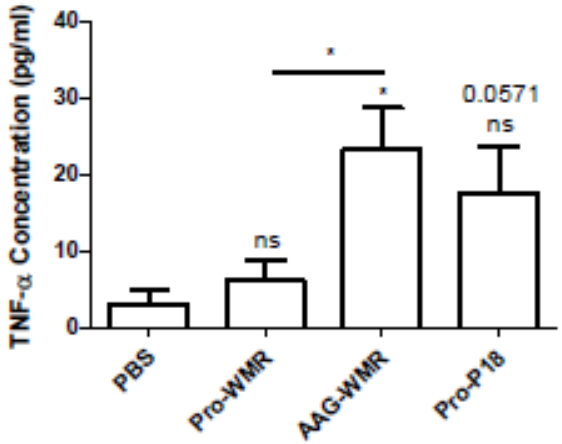
- **Mice intratracheally treated twice with 50 µg of peptide over 24h.**

Results and discussion - *in vivo* toxicity study



- ⇦ % weight loss of wild-type mice
  - ⇩ Cytokine release in CFBE cells





- **Prodrug modification can prevent weight loss.**
- **Increase in cytokine release observed *in vivo* (but not *in vitro*) with the active AMP; no increase in cytokine release both *in vitro* and *in vivo* with the prodrug.**

Conclusions

- **Prodrug modification can mask HDP bactericidal activity and reduce cytotoxic effects *in vivo.***
- **NE-dependent restoration of bactericidal activity under *in vitro* conditions representative of *in vivo* conditions in the CF lung (BAL fluids).**
- **Addition of NaCl required for restoration of bactericidal activity in BAL fluids but inhaled hypertonic saline solutions are used at 1.2 M in CF treatment for the improvement of lung function and could be used to deliver the prodrug.**
- **Nebulisation data predictive of a high level of dosing in the lung.**
- ***in vitro* antimicrobial activities and toxicity of AMPs are not necessarily predictive of *in vivo* efficacy/toxicity.**

Antimicrobial Peptidomimetics

- **Novel method of peptidomimetic generation applied to antimicrobial peptides, to generate antibiotic candidates and/or antimicrobial coatings.**

Peptide / Peptidomimetic Candidate	Length	Ratio	MIC *E. coli*	MIC *S. aureus*
Arg/Nle peptide	10	1/1	-	100
Arg/Nle mimetic	10	2.2/1	> 512	> 512
Arg/Hle mimetic	10	2.2/1	256	256
Arg/Trp peptide	10	1/1	64	8
Arg/Trp mimetic	9-10	1/1.5	128-256	16-32

- **Antimicrobial activities improved over different generations of candidates and approaching activities of the parent peptides. Optimisation, in particular against Gram-negative bacteria, currently in progress.**

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- Prof. Hilary Humphreys (Microbiology, RCSI)
- Prof. Catherine Greene (Respiratory Medicine, RCSI)
- Dr Emer Reeves (Respiratory Medicine, RCSI)
- Dr Ronan Mac Loughlin (Aerogen)
- Ms Louise Sweeney (Aerogen)
- Prof. Marcus Mall (Translational Lung Research Centre Heidelberg)

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