Final report on the project: "Overcoming multi-drug resistance in bacteria: the role of antimicrobial peptides"

In brief, the present work had as main objective to investigate the possible use of antimicrobial peptides in combination with conventional antibiotics to restore antibiotic susceptibility of antibiotic-resistant strains and/or prevent emergence of antibiotic resistance during antibiotic therapy. To achieve this goal, we studied 1) the relationship between bacterial resistance to antibiotics and to antimicrobial peptides, 2) how frequent bacteria develops resistance to antimicrobial peptides, 3) how frequent are collateral sensitivity and synergism combinations between antimicrobial peptides and antibiotics, and 4) how can this information be used to develop new peptide-based antimicrobials.

The results were published as part of three peer-reviewed publications and are summarized here:

1) Lazar, **Martins** et al, Nature Microbiology **3**, 718–731, 2018 (first authorship shared with Viktória Lázár)

To study whether antibiotic resistance in *E. coli* leads to cross-resistance or collateral sensitivity towards antimicrobial peptides, we measured the changes in the susceptibilities of 60 antibiotic-resistant strains to a set of 24 peptides. Peptides were chosen based on the following criteria: diverse sources (synthetic/natural), different putative mechanisms of action, structural diversity, and clinical relevance.

We observed that antibiotic-resistant bacteria show a high frequency of collateral sensitivity to antimicrobial peptides (AMPs), whereas cross-resistance is relatively rare (Figure 1), and we identified the main antibiotic-resistance mechanisms behind these interactions:

- Multidrug resistance mutations in MarR increase the negative surface charge of the bacterial cell and thus cause collateral sensitivity to membrane-interacting peptides.
- Aminoglycoside resistance induces cross-resistance to AMPs most likely through mutations in *sbmA* - involved in the uptake of proline-rich peptides.
- Mutations in the EnvZ/OmpR two-component regulatory system, and in the outer membrane porin C (*ompC*) cause collateral sensitivity to pore-forming peptides.

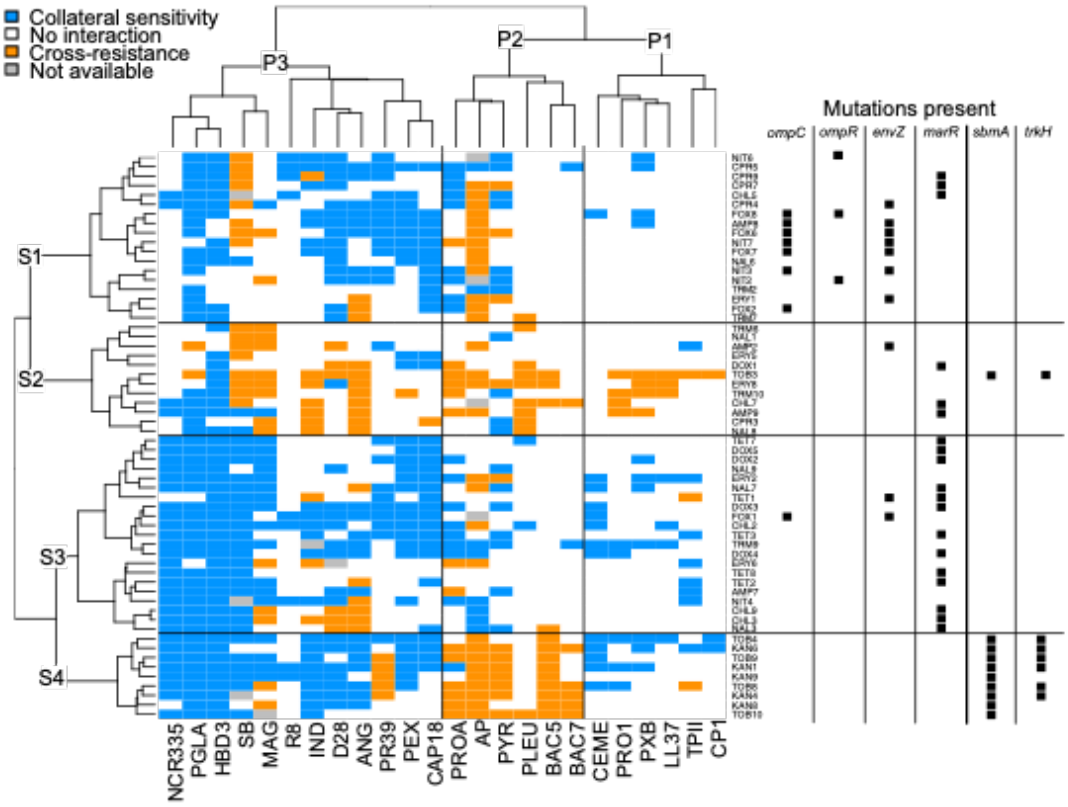

Figure 1. Susceptibility profiles of 60 laboratory-evolved antibiotic-resistant *E. coli* strains
Hierarchical clustering of 60 antibiotic-resistant strains (rows) and a set of 24 antimicrobial peptides (columns) based on the cross-resistance and collateral sensitivity interactions between them. Hierarchical clustering was performed separately on rows and columns, using Ward's method. Black squares on the right side of each antibiotic-resistant strain denote previously identified mutations in antibiotic-resistance genes that were significantly enriched in one or more strain clusters ($p<0.05$, two-sided Fisher's exact test). S1 strains were enriched in *envZ*, *ompR* and *ompC* mutations, whereas S3 strains were enriched in *marR* mutations ($P<0.05$ for all cases, two-sided Fisher's exact test). While S3 strains show widespread collateral sensitivity to antimicrobial peptides, especially to P1 and P3 peptides, aminoglycoside-resistant strains (S4) show extensive cross-resistance to proline-rich peptides (P2) ($p<0.0001$, two-sided Fisher's exact test).

We also observed that:

- Susceptibility of clinical-derived *E. coli* strains towards AMPs revealed that collateral sensitivity is at least partly conserved across multiple genetic backgrounds.
- Gene expression changes in LPS biosynthesis contribute to collateral sensitivity.
- Collateral sensitivity of the marR mutant to peptides occurs via modulation of the LPS phosphorylation pathway, through upregulation of the waaY. We propose that the consequent altered outer membrane composition facilitates the interaction of antimicrobial peptides with the cell membrane and thereby enhances their killing efficiency
- Antibiotic resistant strains show high prevalence of collateral sensitivity to the peptide PGLA.

- A strong synergism between PGLA and antibiotics is prevalent in antibiotic-resistant strains carrying mutations in *marR*, *envZ* or *ompF* genes, known to influence membrane permeability.
- Subinhibitory doses of PGLA caused up to 30-fold increase in susceptibility in laboratory-evolved antibiotic-resistant bacteria and this result is conserved across multiple genetic backgrounds and species.

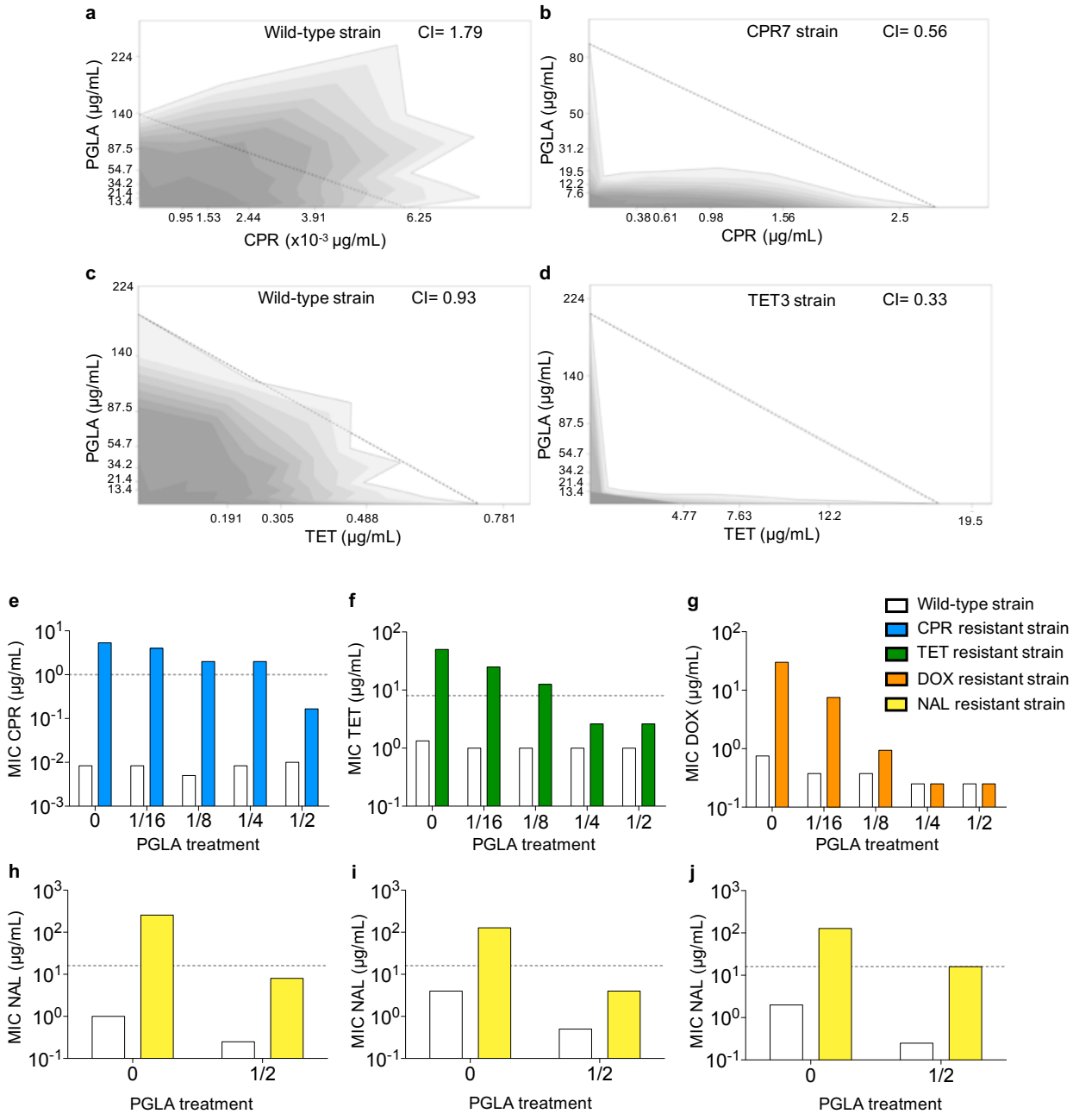

Figure 2 - Interaction of PGLA and antibiotics, when applied in combination

Antibiotic-PGLA interactions were determined in *E. coli* K12 BW25133 wild-type and corresponding antibiotic-resistant strains. Figures **a-d** show the combination effect of PGLA and ciprofloxacin (CPR) or tetracycline (TET) on the wild-type strain (**a** and **c**), ciprofloxacin-resistant strain (CPR7) (**b**) and tetracycline-resistant strain (TET3) (**d**). While the combination shows strong antagonism (**a**) or no interaction (**c**) in the wild-type strain, the interaction shifted to strong synergism in the resistant strain (**b** and **d**). Dashed line represents no

interaction calculated based on the Loewe additivity model. Growth rate is represented in the combination space by the shade of the grey color with darker shades denoting higher growth rates Figures **e-j** show the effect of subinhibitory concentrations of PGLA on antibiotic activity. Ciprofloxacin-resistant CPR7 (**e**), tetracycline-resistant TET3 (**f**) and doxycycline-resistant DOX3 (**g**) strains, derived from *E. coli* K12 BW25133 were treated with subinhibitory concentrations of PGLA, while measuring the MIC for the given antibiotic to which they were adapted. The concentrations of PGLA used were 1/16, 1/8, 1/4 and 1/2 of its MIC against the wild-type strain. The minimal inhibitory concentration of nalidixic acid (NAL) was measured in *E. coli* clinical isolates 0370 (**h**), 3539 (**i**) and CFT073 (**j**), and their corresponding nalidixic acid-resistant strains in the presence of 1/2 of the MIC for PGLA. None of the PGLA concentrations, when applied alone, affected the growth of the wild-type or the resistant strains (the only exception being the 40% growth rate reduction of the tetracycline (TET) resistant strain in response to ½ MIC PGLA). Dashed lines represent the clinical breakpoints for the antibiotics in *E. coli* (not available for doxycycline (DOX)). Data in this figure is representative of at least 2 biological replicates.

- PGLA could select against de novo evolution of resistance against tetracycline and ciprofloxacin.

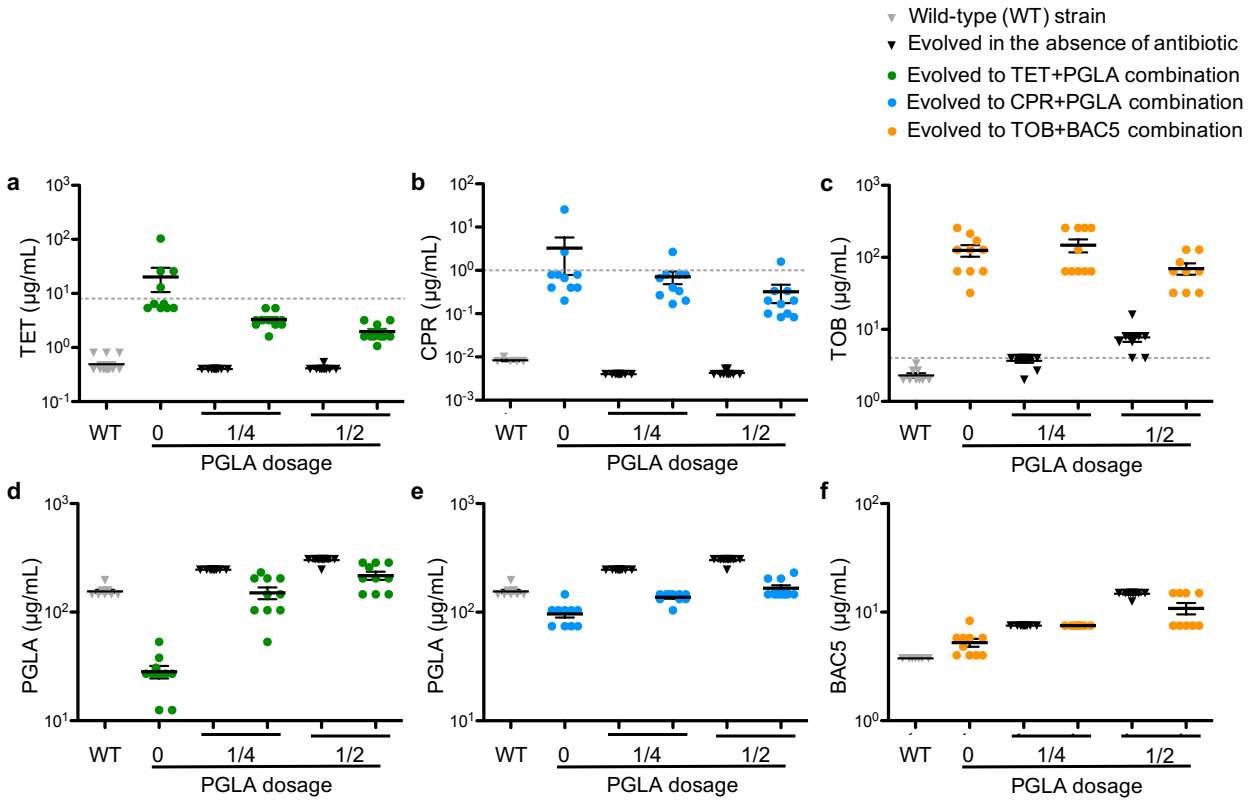

Figure 3 - Minimum inhibitory concentrations (MICs) of laboratory-evolved lines adapted to antibiotics in the absence and the presence of subinhibitory dosage of antimicrobial peptides
MIC was measured following a laboratory evolution of the wild-type *E. coli* strain to tetracycline (TET, green), ciprofloxacin (CPR, blue) and tobramycin (TOB, orange) in the absence or in the presence of ¼ or ½ of the MIC of the antimicrobial peptides PGLA (**a**, **b**, **d**, **e**) or BAC5 (**c**, **f**) against the wild-type strain. MICs of the wild-type and both PGLA and BAC5 evolved lines (in the absence of antibiotic) are represented by grey and white colored bars, respectively. Each data point represents the MIC value of one of each ten parallel-evolved lines. Error bars represent the mean ± standard error of the mean for each experimental condition. Dashed

lines represent clinical breakpoints for TET, CPR or TOB in *E. coli*. Both the CPR-PGLA and the TET-PGLA combinations, which are representatives of collateral sensitive interactions (Figure 1), significantly slowed down the evolution of resistance towards the given antibiotic when administered together. Reassuringly, the control combination (BAC5-TOB), representing a cross-resistance interaction, did not reduce the rate of TOB resistance evolution (P=0.0834, 1-way ANOVA).

2. Spohn et al, Nature Communications, **10**:4538, 2019

Following the above results, it became increasingly important to understand whether the use of antimicrobial peptides (although promising against antibiotic resistant bacteria) could lead to the evolution of bacterial resistance against AMPs themselves. Furthermore, possible cross-resistance between AMPs is of main importance due to concerns that therapy using AMPs could diminish the response of our own immune system that uses its own peptides in response against infections.

With the aim of answering these questions, we systematically studied the evolution of resistance of *E. coli* K12 to a chemically diverse set of AMPs overlapping with that used before. We observed that:

- Laboratory evolution of resistance to AMPs was less efficient and more heterogenous than to antibiotics. To note that evolution of resistance was particularly difficult against some peptides, including PGLA.
- Using two examples (TPII and PXB), we observed that similar tendency is observed when laboratory evolution was studied using four sensitive reference strains representative of four ESKAPE pathogens: *E. coli* ATCC 25922, *Salmonella enterica* serovar Typhimurium LT2, *Klebsiella pneumoniae* ATCC 10031 and *Acinetobacter baumannii* ATCC 17978.
- In sharp contrast to the high frequency of cross-resistance interactions between antibiotics, cross-resistance interactions among AMPs were rare.

Whole-genome sequencing revealed that the most relevant mutated proteins are involved in lipopolysaccharide (LPS) modification and transport (waa-pathway), phospholipid trafficking (mla-pathway), and a two-components sensor system (BasR-BasS). When inserted individually into wild-type *E. coli* K12, we observed that those mutations generally conferred resistance to the original peptide towards which they emerged, but no cross-resistance was observed.

3. Bhaumik, Hetényi, Olajos, **Martins**, et al. Mol. Syst. Des. Eng., **7**, 21, 2022

(first authorship shared with Bhaumik, Hetényi, Olajos who were responsible for the chemistry part of the work, i.e. synthesis and chemical characterization of the foldamers)

Inspired by the molecular scaffold of the antimicrobial peptide PGLa, we have developed in collaboration with the group of Tamás Martinek antimicrobial foldamers with a computer-guided design strategy, to obtain compounds that would be active as adjuvants to antibiotics. PGLa has the propensity to fold into an amphiphilic helix upon interaction with bacterial membranes. Using molecular dynamics (MD) simulations, we modelled its sequential membrane-induced folding to identify the key amino acid residues involved in

this process. Two foldamers, PGLb1 and PGLb2, were synthetized based on the *in silico* modelling.

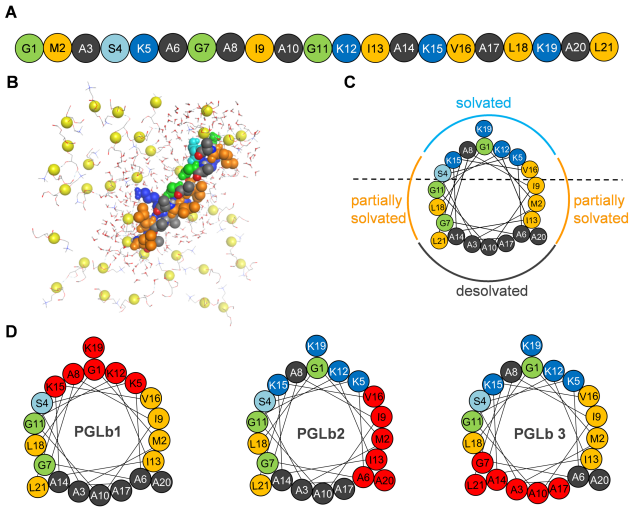

Figure 4. Structures of PGLa and the designed foldamers.

The sequence of PGLa (a) PGLa in parallel orientation with the membrane surface. Yellow spheres indicate the phosphorus atoms of the lipid, surrounding waters are drawn with ball and stick model. (b) Helix wheel representation of PGLa and the different solvation states of residues along the helix surface. (c) Positions of modifications (homologous α → β3 amino acid replacements) are marked with red circles for the three designed foldamers: PGLb1, PGLb2, and PGLb3 (d).

In vitro studies showed that PGLb1 and PGLb2:

a) are weak antibacterial compounds.
b) induce strong synergism both in antibiotic-sensitive and resistant strains.

Table 1 - Combination index (CI) values of peptide-antibiotic combinations on *E. coli* clinical isolates and the respective antibiotic-resistant strains. CI was estimated in *E. coli* 0370, *E. coli* 3538 and *E. coli* CFT073 strains and their corresponding nalidixic acid- and ampicillin-resistant derivatives. The cut-off values were CI≥1.14 for antagonism (orange); CI≤0.86 for synergism (blue); and 0.86<CI<1.14 for no interaction (grey).

Combination Index (CI)					
Strain	resistant to:	Antibiotic	Peptide		
			PGLa	PGLb1	PGLb2
E. coli 0370	Wild type	Nalidixic acid	1.26	0.47	0.31
		Ampicillin	1.30	0.74	0.63
E. coli 0370	Nalidixic acid	Nalidixic acid	1.09	0.91	0.36
	Ampicillin	Ampicillin	0.96	0.66	0.45
E. coli 3538	Wild type	Nalidixic acid	0.75	0.45	0.44
		Ampicillin	0.94	0.96	0.85
E. coli 3538	Nalidixic acid	Nalidixic acid	1.01	0.55	0.46
	Ampicillin	Ampicillin	0.84	0.94	0.60
E. coli CFT073	Wild type	Nalidixic acid	1.08	0.48	0.44
		Ampicillin	1.10	0.85	0.74
E. coli CFT073	Nalidixic acid	Nalidixic acid	0.85	0.81	0.36
	Ampicillin	Ampicillin	0.91	0.61	0.62

c) reduce the level of antibiotic resistance of *E. coli* 0370, *Klebsiella pneumoniae* r1 and *S. flexneri* 668 clinical isolates adapted to nalidixic acid.

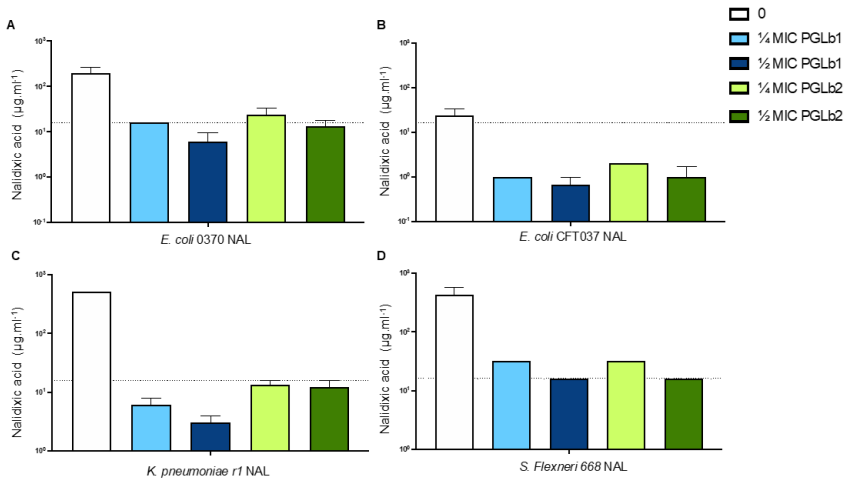

Figure 5. Impact of sub-inhibitory concentrations of foldamers on antibiotic activity against nalidixic acid-resistant bacteria.

The MIC of nalidixic acid (NAL) was assessed in *E. coli* clinical isolates 0370 (a) and CFT073 (b), *K. pneumoniae* r1 (c) and *S. flexneri* 668 (d) strains, all of which are resistant to NAL, in the presence of 1/2 × MIC and 1/4 × MIC of the peptide against each strain. When applied alone, neither of these peptide concentrations interfered with the growth of any of the strains. Dashed line represents resistance breakpoint for NAL (i.e. 16 mg.l^{-1}) suggested by the CLSI (Clinical Laboratory Standards Institute). Data are based on at least two biological replicates.

d) decrease the level of resistance against moxifloxacin in moxifloxacin-resistant strains of *Enterobacter cloacae*, *Acinetobacter baumannii*, and *K. pneumoniae.*

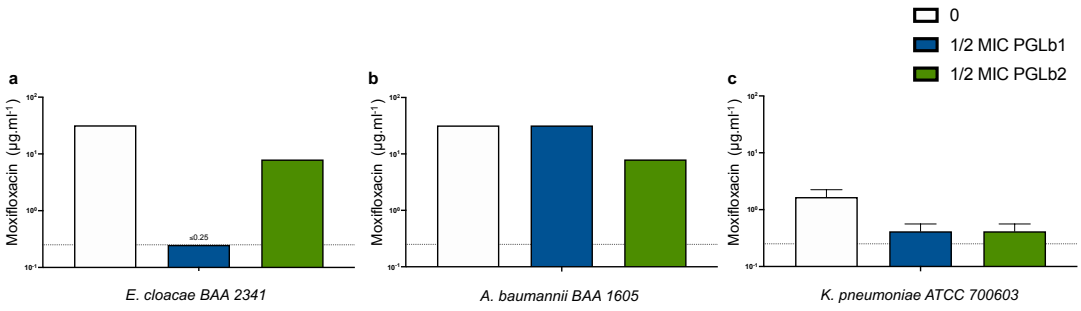

Figure 6. Impact of sub-inhibitory concentrations of PGLb1 and PGLb2 on antibiotic activity against moxifloxacin-resistant pathogenic strains.

The MIC of moxifloxacin (MOX) was measured in *Enterobacter cloacae* BAA2341 (a), *Acinetobacter baumannii* BAA 1605 (b), and *Klebsiella pneumonia* ATCC 700603 (c) in the presence of ½ of the peptide MIC against each strain. None of the peptide concentrations, when applied alone, affected the growth of the strains. Dashed line represents the EUCAST suggested break point for MOX, i.e. if MOX MIC is under or equal to 0.25 mg.l^{-1} the strain is considered susceptible to this antibiotic. Data in this figure is representative of at least 2 biological replicates. Note: MIC of PGLb1 and PGLb2 against these strains is >256mg.l^{-1} and therefore the concentrations 256mg.l^{-1} was considered as ½ MIC.

We determined that the observed antibiotic potentiation was mediated by hyperpolarization of the bacterial membrane caused by the alteration of cellular ion transport. PGLb1 and PGLb2 are selective ionophores that enhance the Goldman–Hodgkin–Katz potential

across the bacterial membrane. Moreover, the introduction of non-natural β-amino acids improved resistance of PGLb1 and PGLb2 to human proteases, which can help overcome the traditional drawback of antimicrobial peptides. Neither PGLb1 nor PGLb2 had a significant haemolytic activity (<5%).

After, we tested how relevant are these results in a *in vivo* model. Using a *Galleria mellonella* infection model we observed that:

- When used alone, PGLb1 had no impact on the survival of *G. mellonella* (P = 0.37).
- When used in combination with nalidixic acid, PGLb1 significantly enhanced the larvae's survival upon bacterial infection (P = 0.02).

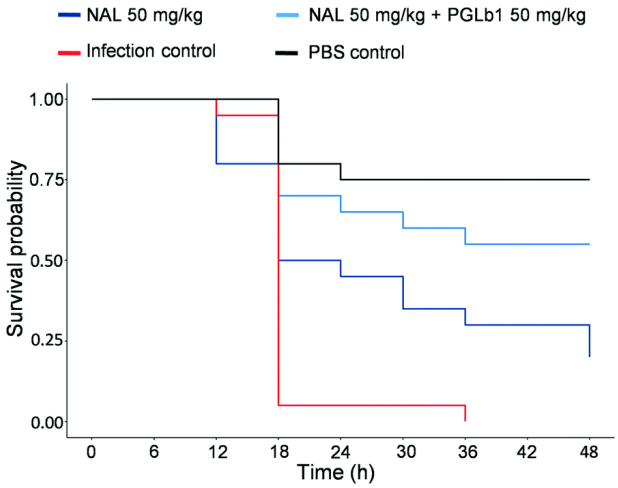

Figure 7. Co-administration of PGLb1 and nalidixic acid in vivo. Cell death kinetics of *E. coli* CFT073 resistant to nalidixic acid, over 48 hours. The phosphate buffered saline (PBS) control group received one injection of sterile phosphate buffered saline, while the other groups were infected with approximately 2 × 10^7 *E. coli* cells. The infection control group received no further treatment, while the other two groups were treated with 50 mg.kg^{-1} nalidixic acid or 50 mg.kg^{-1} nalidixic acid + 50 mg.kg^{-1} PGLb1, respectively. Animals treated with nalidixic acid and PGLb1 in combination showed a significantly higher survival rate than those treated with nalidixic acid alone (P = 0.02) experiments were performed in two biological replicates, with 10 animals per treatment group, hence each curve represents 20 animals.

These findings indicate that manipulating bacterial membrane electrophysiology could be a valuable tool to overcome antimicrobial resistance, while the development of beta-amino acid-containing antimicrobial peptide mimetics can improve *in vivo* stability of AMPs.

This work was presented at four international conferences:

- 26th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID) in Amsterdam, Netherlands from 9 to 12 April 2016
- International Meeting on Antimicrobial Peptides, Copenhagen, 25-27 August 2017
- ASM-ESCMID conference on Drug Development meet the challenge of antimicrobial resistance, Boston, USA, 6-8 September 2017.
- 2022 New Antibacterial Discovery and Development, Gordon Research Seminar and Conference, Renaissance Tuscany Il Ciocco in Lucca, Italy from 23-29 July 2022.