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Enhancing Antibiotic Efficacy through Antimicrobial Peptide-Mediated Modulation: The Sub-MIC Approach

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A. Introduction and goals

Antimicrobial resistance (AMR) poses a significant public health risk by making infections harder to treat. Two primary mechanisms contribute to antibiotic resistance in bacteria: intrinsic mechanisms and adaptive resistance. To combat AMR, innovative approaches are being developed, including new antibiotics, antibiotic adjuvants, monoclonal antibodies, phage therapy, and agents altering membrane permeability.

One promising strategy is targeting the transmembrane electrochemical gradient in bacterial cells. While membrane depolarisation by antibiotics is well-studied, the impact of hyperpolarisation on antibiotic susceptibility is less understood. Recent studies show that some inorganic compounds and antimicrobial peptides (AMPs) can kill bacteria by hyperpolarizing the membrane. AMPs, which primarily target bacterial membranes, often synergise with antibiotics, sensitise antibiotic-resistant bacteria, and limit resistance development.

PGLa, an AMP from frog skin, may be a promising candidate for combination therapy. It disrupts bacterial membranes through hydrophobic interactions and can permeabilise membranes at low concentrations without forming pores. PGLa derivatives with improved properties might disrupt bacterial ion homeostasis and membrane potential at sub-inhibitory concentrations, and work as adjuvants to enhance the sensitivity of antibiotic-resistant bacteria to conventional antibacterial agents. Furthermore, by introducing β-amino acids into the structure we aimed to reduce hydrophobic stabilisation of the helix-membrane interaction in PGLa molecules, which would promote selective ion transport through the intact membrane and improve resistance to human proteases. Thus, we designed novel peptidomimetic foldamers to enhance antibiotic activity against resistant bacteria using sub-MIC. We also investigated the mechanism of these peptides relating to membrane potential modulation and ion transport activity.

Again, we wanted to investigate if membrane potential modulation underlies the effects of AMPs at sub-MIC. AMPs are typically studied for their lytic mechanisms above the MIC, but their action at sub-MIC levels is less understood. Studying a diverse set of AMPs under identical conditions may identify a generalised mechanism at sub-MIC levels. Thus, we examined 17 diverse cationic peptides, evaluating their effects on membrane potential and ion transport under consistent conditions.

B. Methods

Circular Dichroism (CD) Measurements

The CD spectra of all peptides were analysed with and without LUVs using a Jasco J-1100 CD spectrometer with a 1 mm quartz cuvette, a scanning rate of 100 nm/min, and 5 accumulations from 260 to 190 nm. A 100 μM peptide stock solution was made in 10 mM Na-phosphate buffer (pH 7.2), and LUVs were prepared similarly but without NaCl.

Preparation of large unilamellar vesicles (LUVs)

20 mM LUVs were prepared by mixing a 7:3 molar ratio of DOPC and DOPG from chloroform stocks. The mixture was purged with nitrogen to form a thin lipid film, dried with hot air, and hydrated with 20 mM HEPES buffer (pH 7.2) containing 100 mM NaCl/NaH2PO4 for 1 hour, then kept overnight at 4 °C. After 12 freeze-thaw cycles, the vesicles were extruded through a 200 nm extruder and unencapsulated NaCl/ NaH_2PO_4 was removed by dialysis with 20 mM HEPES buffer (pH 7.2) containing 100 mM KCl/ $NaNO_3$/ NaH_2PO_4.

NMR measurements

A Bruker Ascend 500 spectrometer with a 5 mm BBO Prodigy Probe recorded the NMR spectra for the ion transport study at 310 K. For ^{23}Na NMR, 0.5 μL of shift reagent, 1M $Dy(PPP)_2^{7-}$ was added to 500 μL of 20 mM LUVs with NaCl inside and KCl outside. For ^{35}Cl NMR, 2 μL of 1M $Co(NO_3)_2$ was added to 500 μL of LUVs with NaCl inside and $NaNO_3$ outside. For ^{31}P NMR, 500 μL of 20 mM LUVs with NaH_2PO_4 inside and NaCl outside was used.

Membrane polarisation in LUV model

Fluorescence measurements were conducted using an Optima Fluostar plate reader with LUVs containing NaCl inside and NaCl or KCl or NaH2PO4 or NaNO3 outside. Oxonol VI dye (0.45 μM) was added to 0.2 mM LUVs, and the excitation/emission wavelengths were set to 580/640 nm with a 10 nm slit width. Aliquots of 290 μL were placed in 96-well plates, followed by 10 μL of peptide stock or buffer for background. Measurements at 37°C normalised fluorescence intensity as $(F - F_0)/F_0$.

C. Result and discussion

1. A structure-based strategy was employed to modify PGLa, resulting in the synthesis of novel α/β-foldamer adjuvants (PGLb1, PGLb2, and PGLb3) by targeting different sites that interact with the bacterial membrane, aimed at improving antibiotic efficacy against resistant bacteria and enhancing resistance to human proteases (Figure 1).

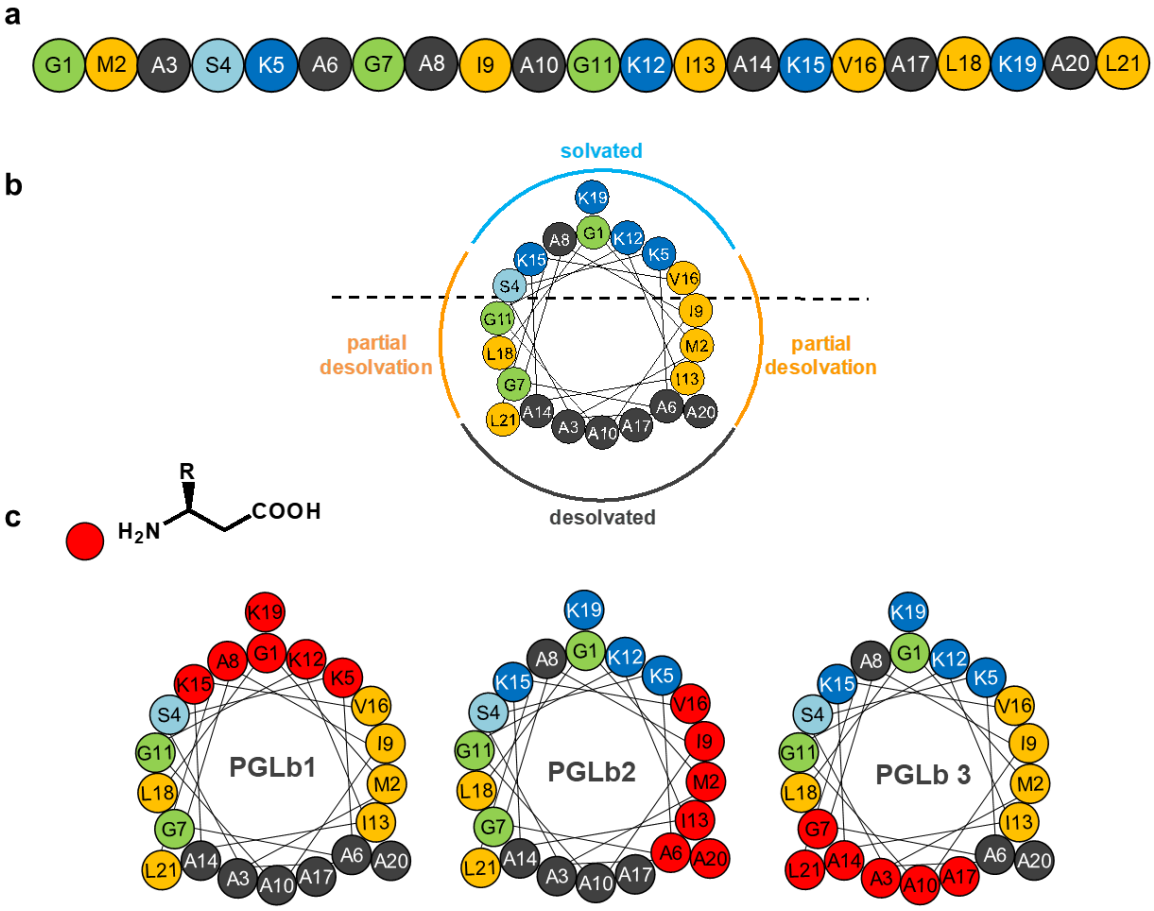

Figure 1: PGLa and the designed foldamers analogues. (a) The sequence of PGLa. (b) different solvation states of PGLa helix. Dash line represents the membrane outer layer (c) Modification positions (homologous α → β^3 amino acid replacements marked by red colour): PGLb1, PGLb2, and PGLb3.

2. Despite their weak intrinsic antibacterial activity, PGLb1 and PGLb2 demonstrated strong synergistic effects with Nalidixic acid across multiple *E. coli* isolates, in contrast to the primarily additive effect observed with PGLa (Figure 2).

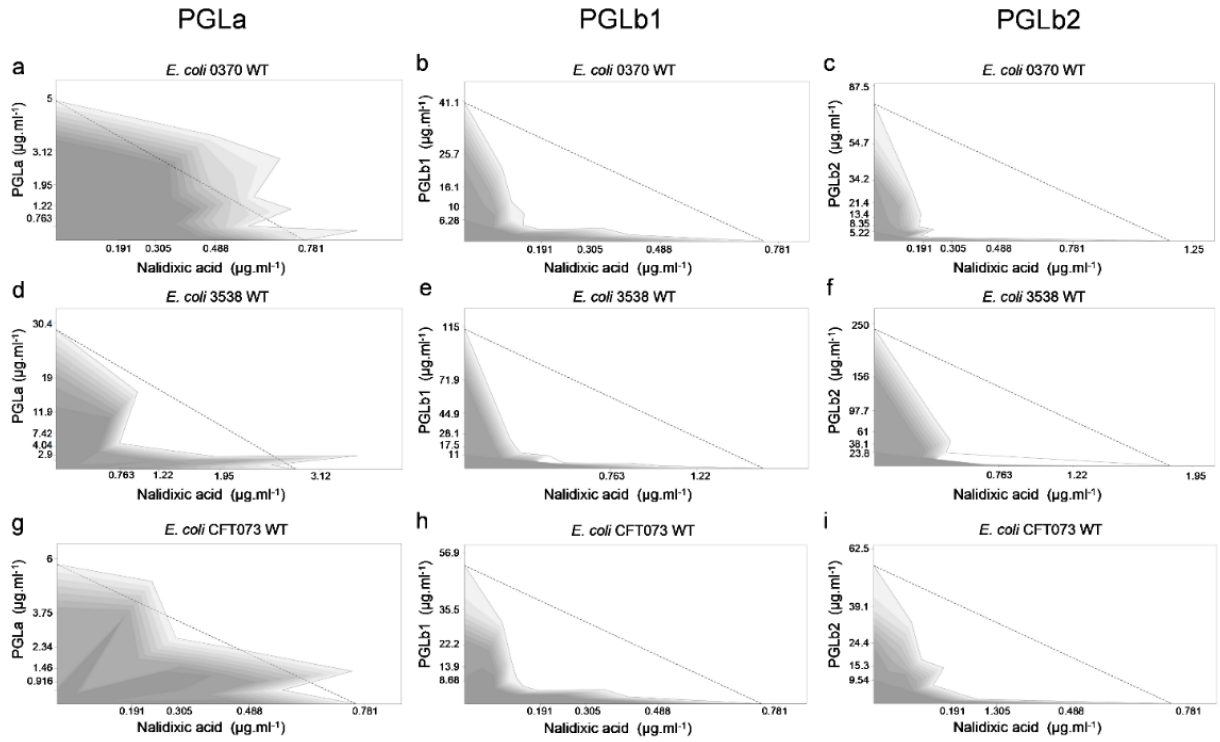

Figure 2: Interactions between Peptides and Nalidixic Acid. (a-c) wild-type *E. coli* 0370, (d-f) *E. coli* 3538 and (g-i) *E. coli* CFT073 strains. Grey shade represents the growth rate of the bacteria with darker shades indicating higher growth rates. The dashed line represents no interaction.

3. In clinical isolates of nalidixic acid-resistant *E. coli, K. pneumoniae*, and *S. flexneri*, PGLb1 and PGLb2 significantly reduced antibiotic resistance at sub-MIC. These *in vitro* findings were validated *in vivo*, with PGLb1 notably enhancing the survival rate of *G. mellonella* larvae infected with nalidixic acid-resistant *E. coli* (Figure 3).

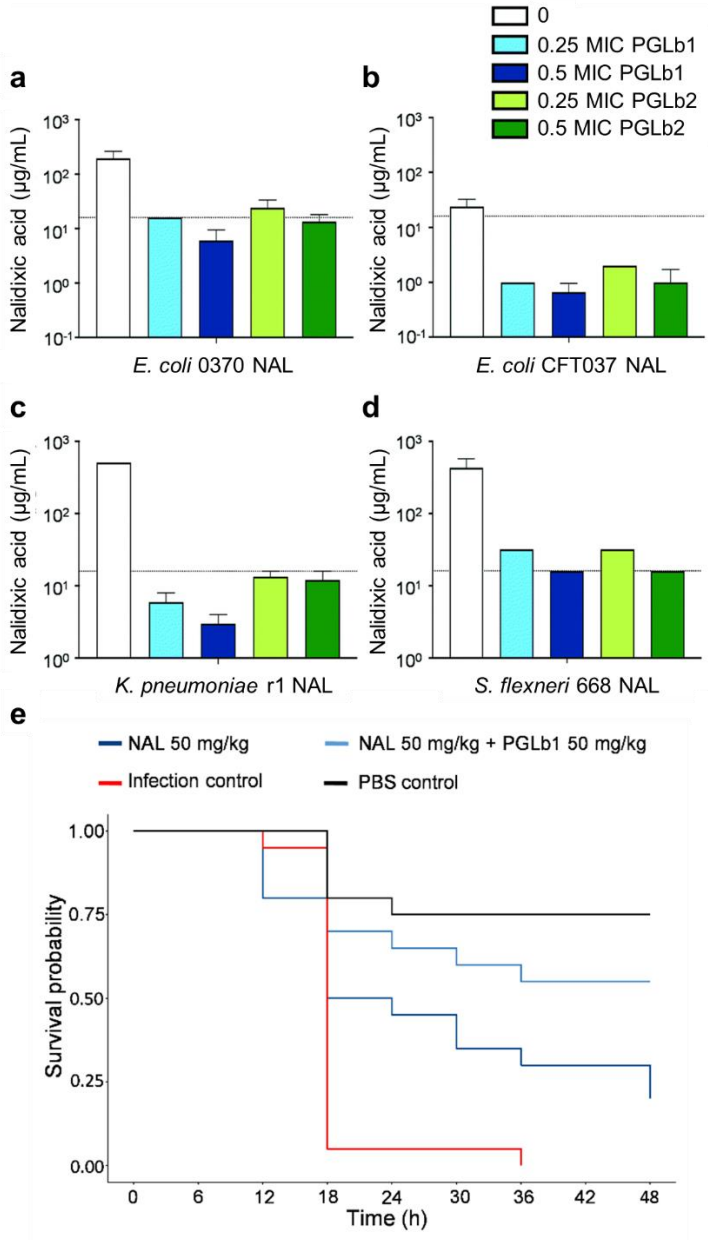

Figure 3: Interaction between foldamers and nalidixic acid. The MIC of nalidixic acid (NAL) was assessed in NAL resistant (a) *E. coli* clinical isolates 0370 and (b) CFT073, (c) *Klebsiella pneumoniae* r1, (d) *Shigella flexneri* 668 in the presence of 0.5 MIC and 0.25 MIC of the peptides. Dashed line represents resistance breakpoint for NAL (i.e., 16mg.l^{-1}) and (d) Combination therapy of PGLb1 and nalidixic acid *in vivo*.

4. Flow cytometric analysis of *E. coli* revealed that PGLb1 and PGLb2 induce substantial and prolonged hyperpolarisation of the bacterial membrane, in contrast to the mild and transient effect observed with PGLa, providing insights into the mechanism behind the sub-MIC effect (Figure 4).

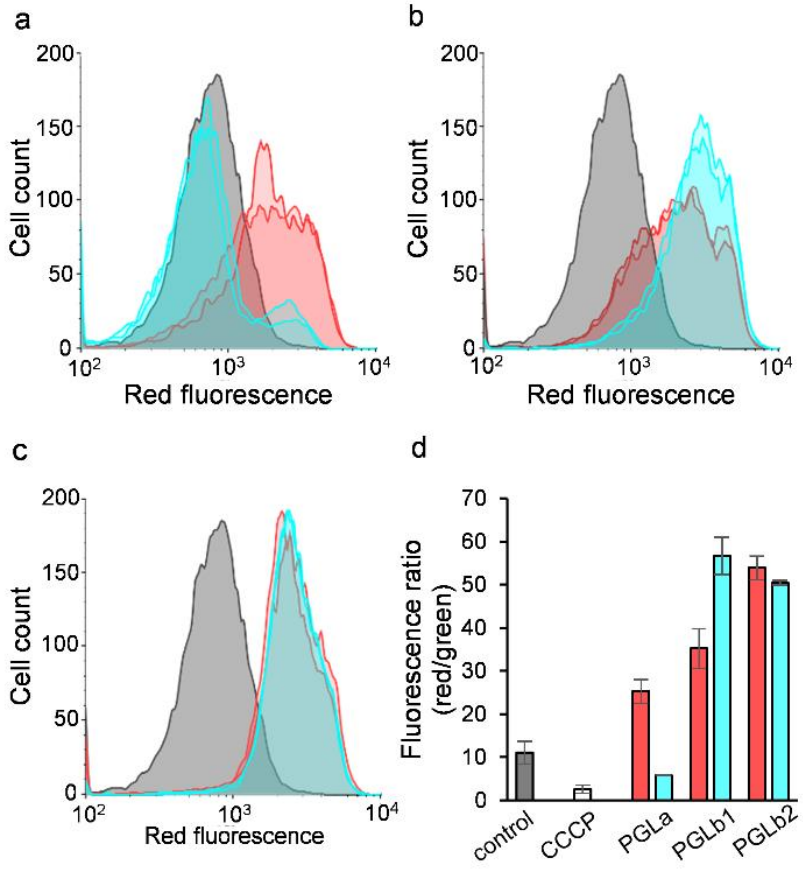

Figure 4: Flow cytometric membrane polarisation assay (BacLight) in *E. coli*. Red channel histograms for (a) PGLa, (b) PGLb1, and (c) PGLb2 are displayed, respectively, after 15 minutes (red) and 30 minutes (blue) of incubation. The control cells are plotted in grey. (d) The ratio of red and green fluorescence represented. CCCP used as depolarising ionophore. Red and blue bars represent measurements after 15 and 30 minutes of incubation, respectively, with a decreased ratio indicating depolarisation and an increased ratio indicating hyperpolarisation.

5. Studies using a LUV model demonstrated that PGLb1 and PGLb2 elicit the diffusion potential through time-dependent selective transport of cations (K^+ over Na^+) and anions (Cl^- over NO_3^-), providing deeper insights into the mechanisms underlying membrane hyperpolarisation (Figure 5).

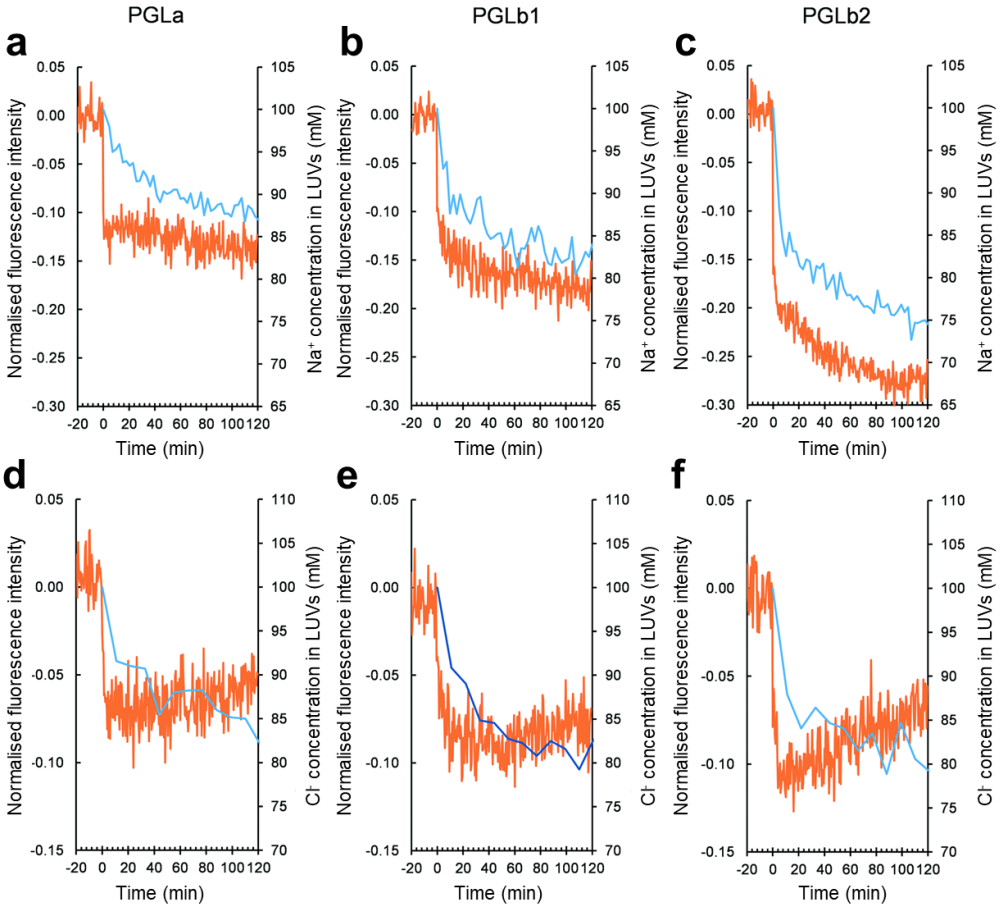

Figure 5: Membrane polarisation and ion transport assessments. Membrane potential (red curve) and inside Na^+ concentration (blue curve) changes are shown in the Na^+/K^+ exchange (100 mM NaCl inside and 100 mM KCl outside) upon adding (a) PGLa, (b) PGLb1 and (c) PGLb2. Membrane potential (red curve) and inside Cl^- concentration (blue curve) changes are shown in the Cl^-/NO_3^- exchange (100 mM NaCl inside and 100 mM $NaNO_3$ outside) upon adding (d) PGLa, (e) PGLb1 and (f) PGLb2.

6. An expanded study of 17 AMPs revealed that those with higher structure inducibility, as confirmed by CD measurements, caused membrane hyperpolarisation in *E. coli* at subinhibitory doses, indicating a common mechanism of action involving ion transport (Figure 6).

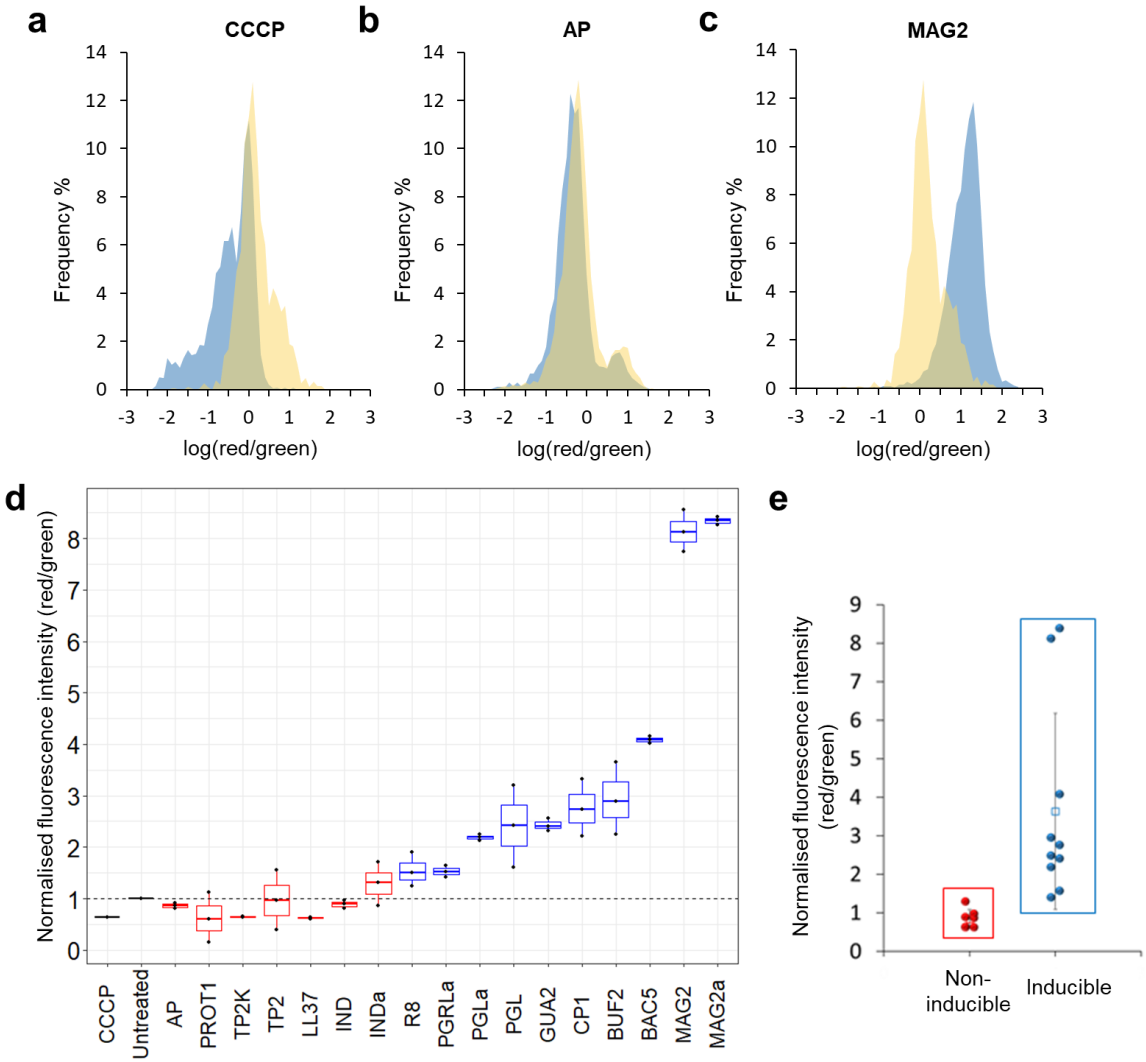

Figure 6: Flow cytometric membrane polarisation assay of AMPs in *E. coli* at 0.5 MIC. Histograms of (a) CCCP representing membrane depolarisation, (b) AP representing no effect on membrane potential, (c) MAG2a representing membrane hyperpolarisation. (d) Boxplot representing membrane polarisation change caused by AMPs. Ratio of red/green fluorescence were calculated using population mean fluorescence intensities for cells treated and normalised with untreated cells. If the ratio is below 1 correspond to depolarisation above 1 is hyperpolarisation. Data are based on 3 biological replicates. Boxplots show the median, first and third quartiles, with whiskers showing the minimum and maximum value. The dashed line represents no change. (c) Membranae polarisation data are shown in relation to the membrane-inducibility of the secondary structure.

7. Membrane polarisation experiments with *S. epidermidis* demonstrated that AMP-induced hyperpolarisation is predominantly limited to Gram-negative bacteria, indicating a significant difference in response between Gram-positive and Gram-negative bacterial membranes (Figure 7).

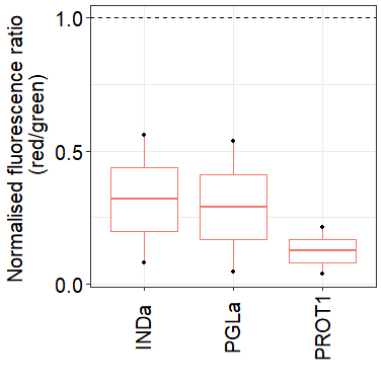

Figure 7: Membrane polarisation assay of AMPs in *S. epidermidis* at 0.5 MIC. The ratio below 1 correspond to depolarisation.

8. Experiments conducted with AMP concentrations ranging from 0.25 to 0.75 MIC in *E. coli* demonstrated increasing membrane hyperpolarisation at lower concentrations, corroborating the diffusion potential mechanism (Figure 3).

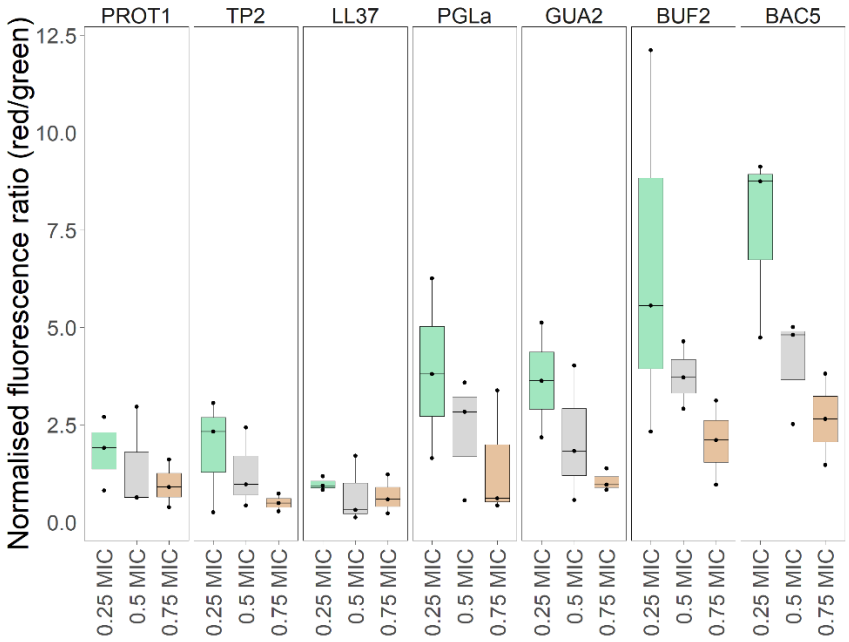

Figure 8: Flow cytometric membrane polarisation assay of AMPs in *E. coli* at different sub-MIC levels.

9. Contrary to literature focusing mainly on cation (especially K^+) transport, our findings indicate that most AMPs can generate membrane potential by transporting both cations and anions, depending on their sequences, thereby maintaining a quasi-steady-state ion gradient (Figure 9).

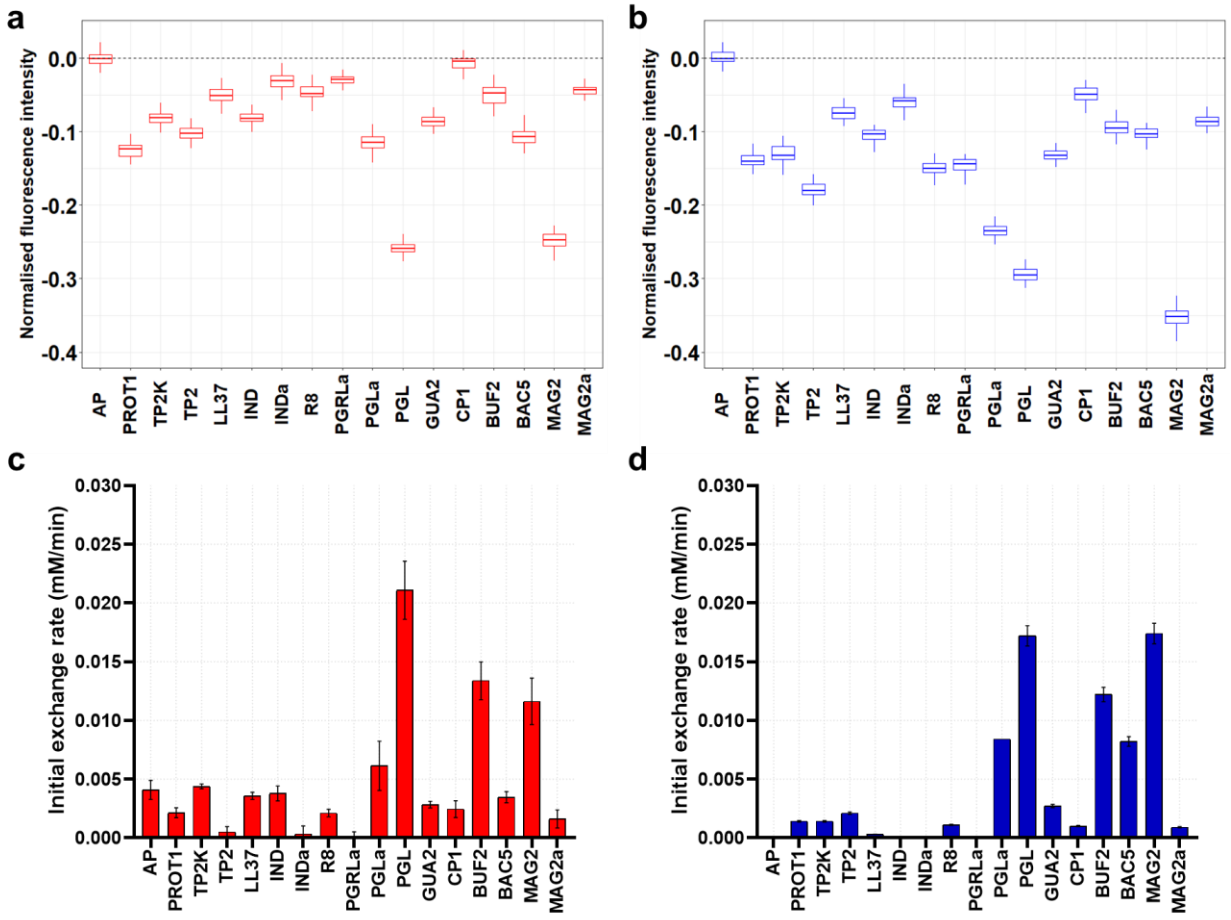

Figure 9: Membrane polarization and Ion transport measurements of AMPs in the LUV model. The negative change indicates a positive inside potential. Boxplots represents data for diffusion potential change for (a) K^+/Na^+ (b) $H_2PO_4^-/ Cl^-$ exchange of all the AMPs. Boxplots show the median, first and third quartiles, with whiskers showing the minimum and maximum value. The dashed line represents no change. Bar plot represents the initial exchange rates observed for AMPs in the (c) Na^+/K^+, (d) $H_2PO_4^- /Cl^-$ gradient model. The ion concentration of Na^+, $H_2PO_4^-$ were determined by ^{23}Na, and ^{31}P NMR assay respectively.

Full publications related to thesis

1. **Bhaumik, K. N**.; Hetényi, A.; Olajos, G.; Martins, A.; Spohn, R.; Németh, L.; Jojart, B.; Szili, P.; Dunai, A.; K. Jangir, P.; Daruka, L.; Földesi, I.; Kata, D.; Pál, C.; A. Martinek, T. Rationally Designed Foldameric Adjuvants Enhance Antibiotic Efficacy via Promoting Membrane Hyperpolarization. *Molecular Systems Design & Engineering* **2022**, *7* (1), 21–33.
2. **Bhaumik, K. N.**; Spohn, R.; Dunai, A.; Daruka, L.; Olajos, G.; Zákány, F.; Hetényi, A.; Pál, C.; A. Martinek, T. Chemically diverse antimicrobial peptides induce hyperpolarization of the *E. coli* membrane. *Communications Biology* **2024,** 7, 1-12.

Other full publication

1. Banerjee, S.; Roy, S.; **Bhaumik, K. N.**; Kshetrapal, P.; Pillai, J. Comparative study of oral lipid nanoparticle formulations (LNFs) for chemical stabilization of antitubercular drugs: physicochemical and cellular evaluation. *Artificial Cells, Nanomedicine, and Biotechnology* **2018**, 46, 540–558.
2. Banerjee, S.; Roy, S.; **Bhaumik, K. N.**; Pillai, J. Mechanisms of the effectiveness of lipid nanoparticle formulations loaded with anti-tubercular drugs combinations toward overcoming drug bioavailability in tuberculosis. *Journal of Drug Targeting* **2020**, 28, 55–69.
3. Hetényi, A.; Szabó, E.; Imre, N.; **Bhaumik, K. N.**; Tököli, A.; Füzesi, T.; Hollandi, R.; Horvath, P.; Czibula, Á.; Monostori, É.; Deli, M. A.; Martinek, T. A. α/β-Peptides as Nanomolar Triggers of Lipid Raft-Mediated Endocytosis through GM1 Ganglioside Recognition. *Pharmaceutics* **2022**, *14* (3), 580.

Scientific lectures related to the thesis

1. **Bhaumik, K. N.**; Hetényi, A.; Olajos, G.; Martins, A.; Spohn, R.; Németh, L.; Jojart, B.; Szili, P.; Dunai, A.; K. Jangir, P.; Daruka, L.; Földesi, I.; Kata, D.; Pál, C.; A. Martinek, T.
   Rationally Designed Foldameric Adjuvants Enhance Antibiotic Efficacy via Promoting Membrane Hyperpolarisation.
   MTA Peptidkémiai Munkabizottság Ülésére (MTA Peptide Chemistry Working Committee Meeting). Balatonszemes, Hungary 2021. October 11-13.
2. **Bhaumik, K. N.**; Hetényi, A.; Olajos, G.; Martins, A.; Spohn, R.; Németh, L.; Jojart, B.; Szili, P.; Dunai, A.; K. Jangir, P.; Daruka, L.; Földesi, I.; Kata, D.; Pál, C.; A. Martinek, T.
   Rationally Designed Foldameric Adjuvants Enhance Antibiotic Efficacy via Promoting Membrane Hyperpolarisation.
   10th Interdisciplinary Doctoral Conference. Pécs, Hungary 2021. November 12-13.
3. **Bhaumik, K. N.**; Hetényi, A.; A. Martinek, T.
   Host defense peptides (HDPs) hyperpolarisation membrane by selective ion transport at sub-MIC concentration. (Poster)
   Peptide Chemistry and Chemical Biology Symposium
   Balatonszemes, Hungary 2022. May 30 – June 1.
4. **Bhaumik, K. N.**; Hetényi, A.; Olajos, G.; Martins, A.; Spohn, R.; Németh, L.; Jojart, B.; Szili, P.; Dunai, A.; K. Jangir, P.; Daruka, L.; Földesi, I.; Kata, D.; Pál, C.; A. Martinek, T.
   Novel foldameric peptide adjuvants counter antimicrobial resistance via promoting membrane hyperpolarisation. (Poster)
   36th European Peptide Symposium and 12th International Peptide Symposium. Barcelona, Spain 2022. August 28 - September 2.