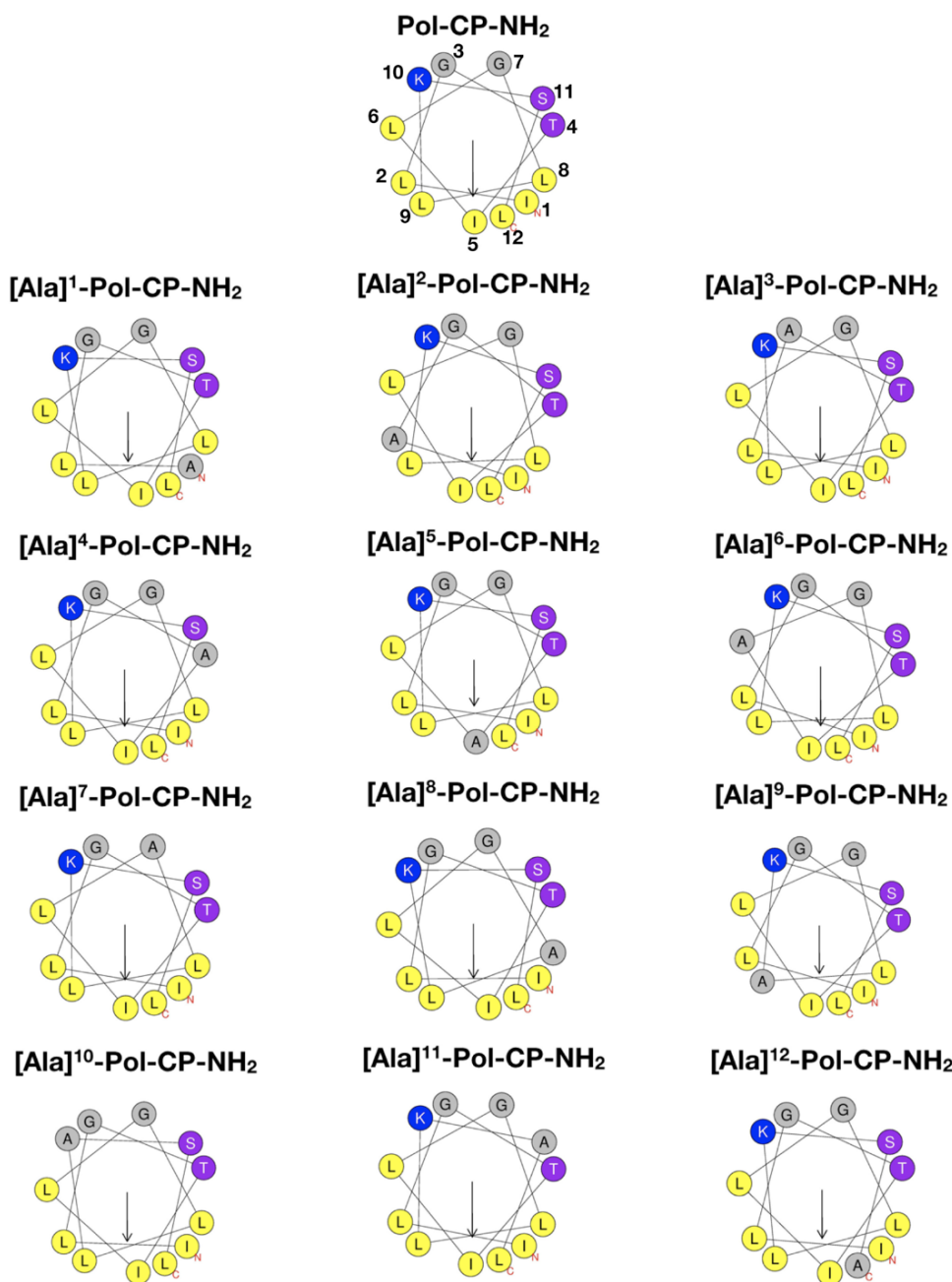
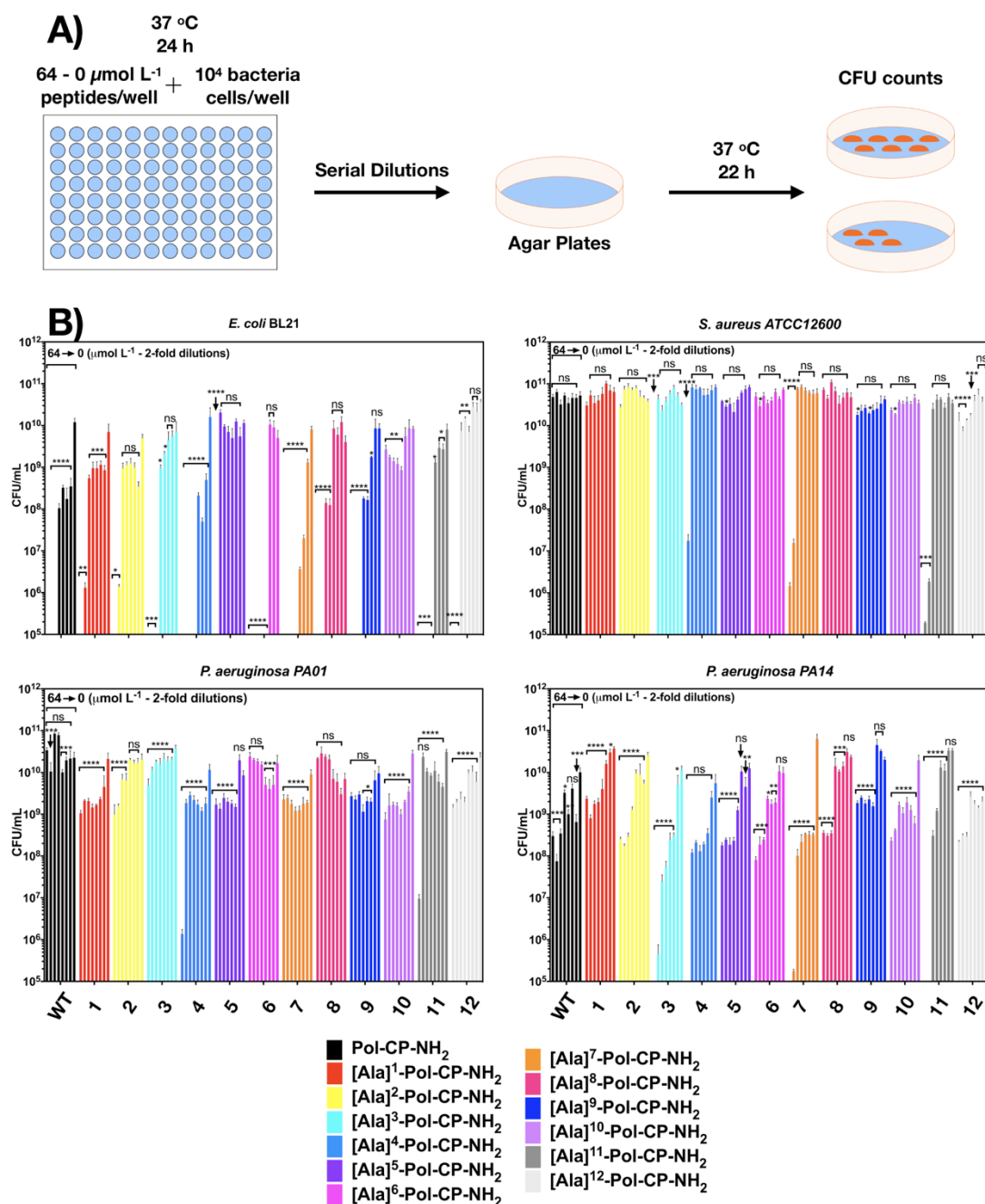


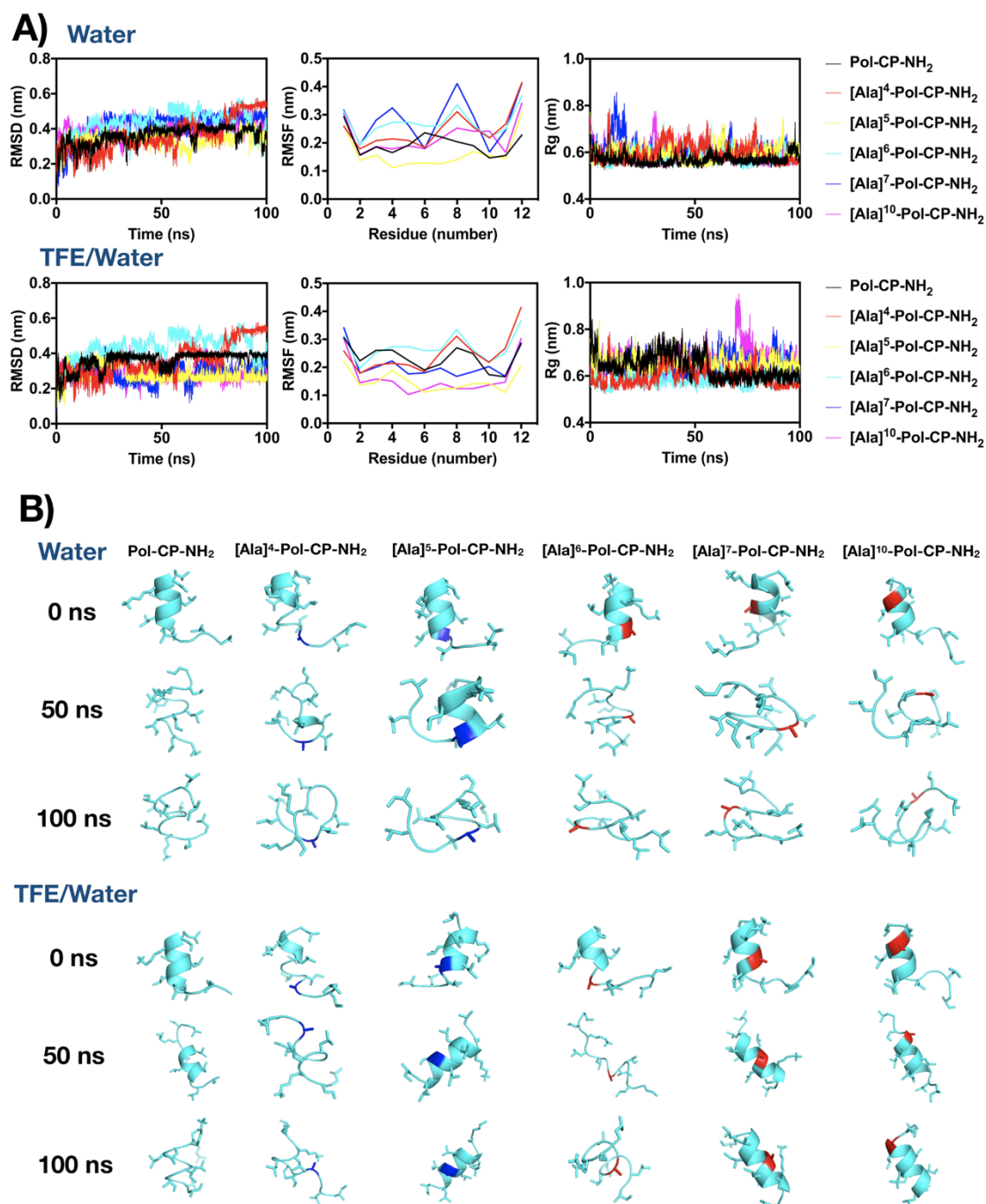
Supplementary Information: Structure-Function-Guided Exploration of the Antimicrobial Peptide Polybia-CP Identifies Activity Determinants and Generates Synthetic Therapeutic Candidates



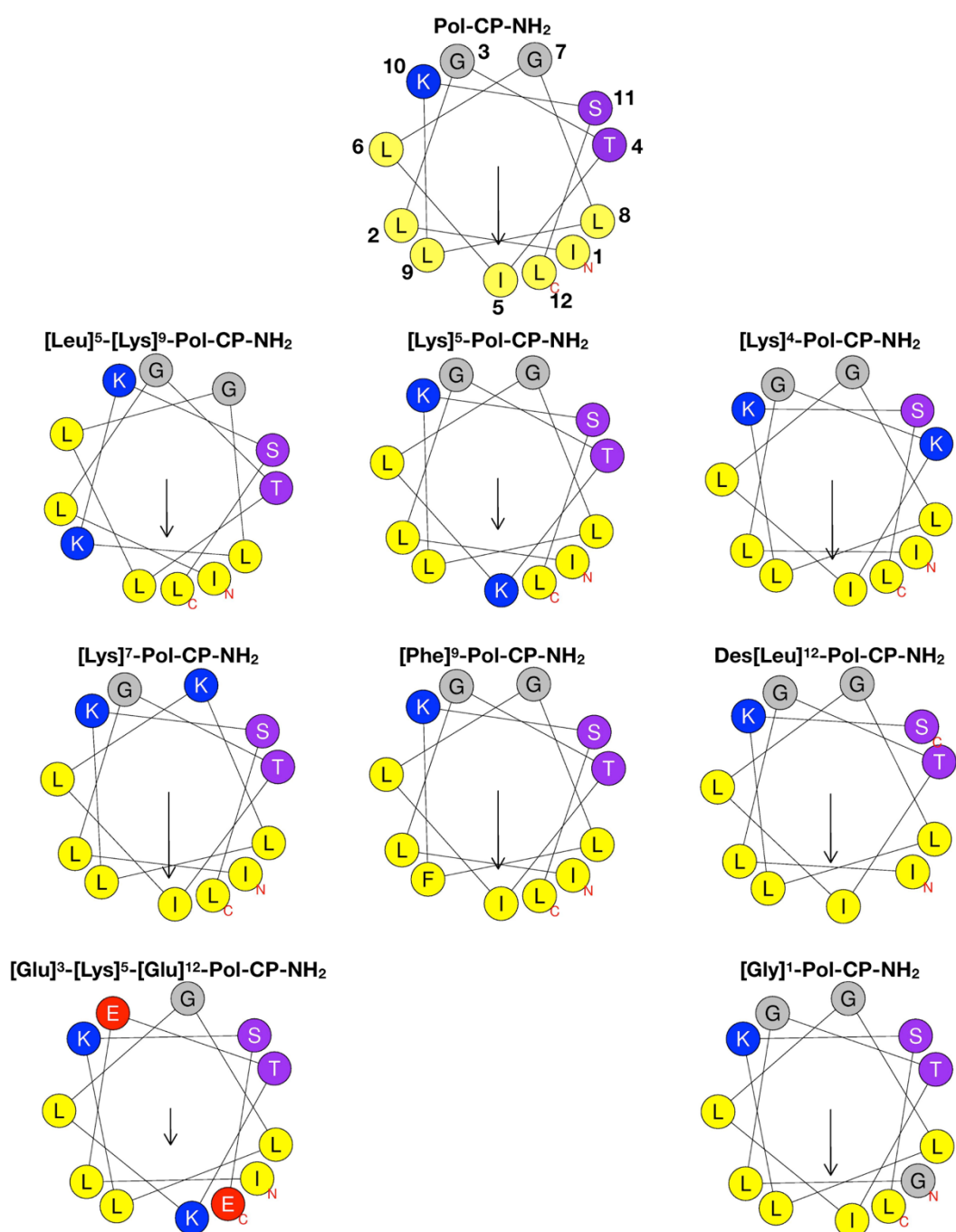
Supplementary Figure 1. Helical wheel representations of the Ala-scan Pol-CP-NH₂ analogs generated using the Heliquest server considering theoretical helical structure and physicochemical properties derived from the amphipathic distribution. Yellow circles indicate hydrophobic/aliphatic residues, blue circles represent positively charged residues, purple circles indicate polar uncharged residues, and gray circles represent residues with hydrophobicity close to zero. The black arrows inside the helical wheel projection of each peptide represent their hydrophobic moment vector, whose magnitude is indicated by the size of the arrows.



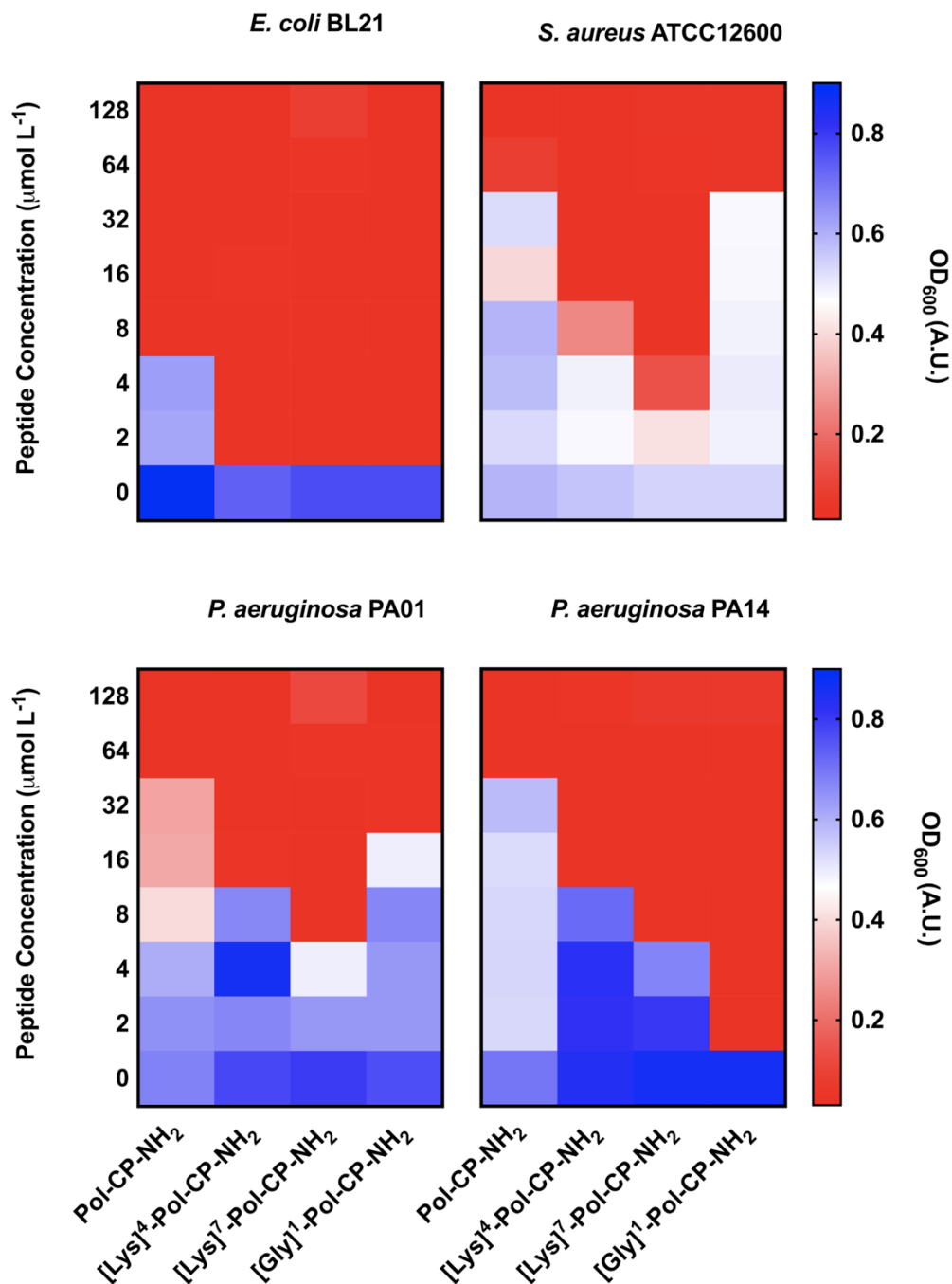
Supplementary Figure 2. (A) Schematic of the in vitro CFU count setup to assess antimicrobial activity of Pol-CP-NH₂ and Ala-scan analogs. Briefly, 10^4 bacterial cells and serially diluted (0 – 64 $\mu\text{mol L}^{-1}$) peptides were added to a 96-well plate and incubated at 37 °C. One day after the exposure, the solution in each well was 10-fold diluted seven times and the serial dilutions were plated in agar plates, which were incubated for 22 h at 37 °C. (B) Next, bacterial colonies were counted. All assays were performed in independent triplicates (statistical significance was determined using two-way ANOVA followed by Dunnett's test, error bars = standard error of the mean, ns = statistically not significant, * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$, **** $p < 0.0001$).



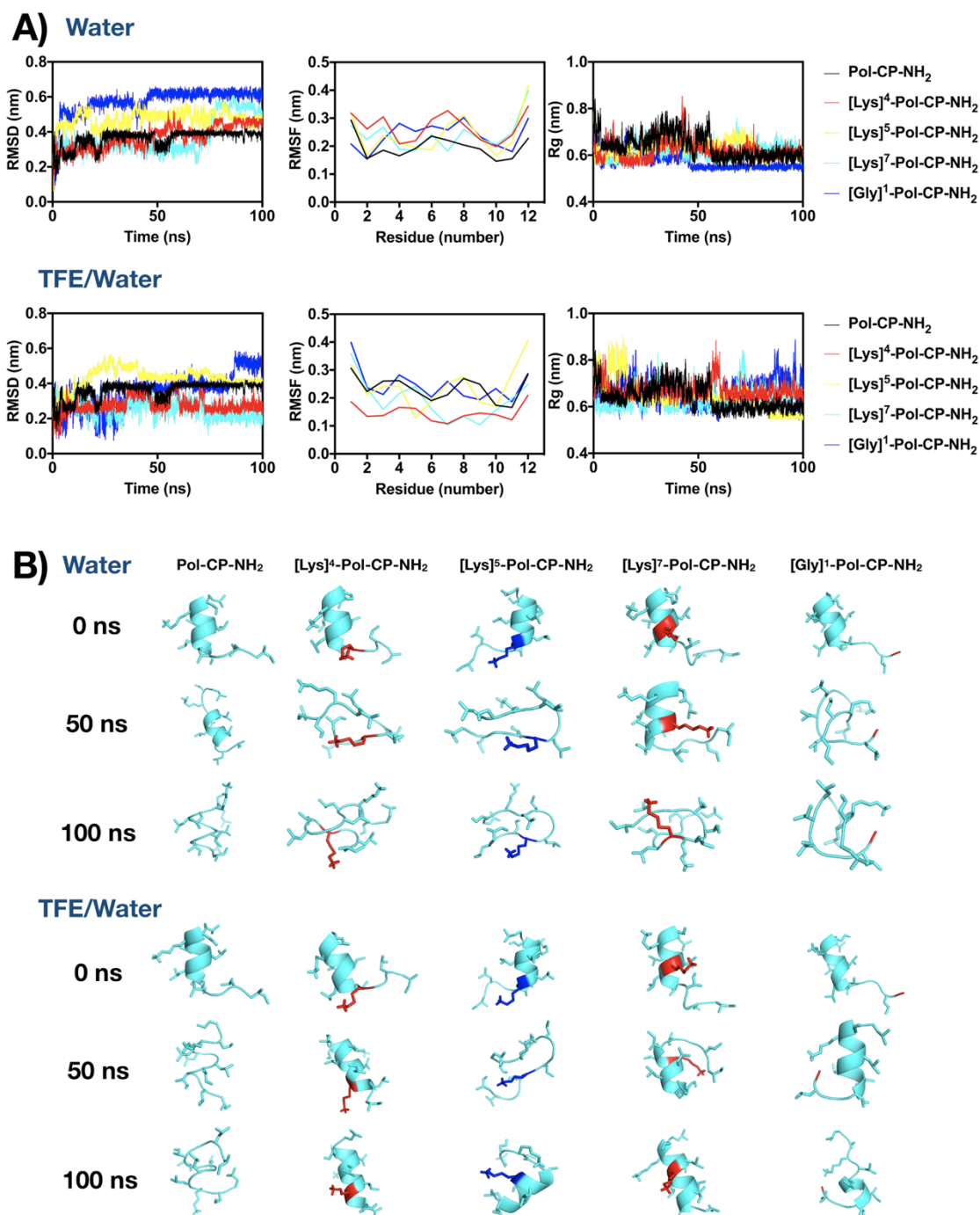
Supplementary Figure 3. (A) Graphical representation of residues movement of Pol-CP-NH₂ and Ala-scan analogs from molecular dynamics simulations in water and TFE/water (3:2, v:v), yielding root mean square deviation, root mean square fluctuation and radius of gyration after 100 ns. **(B)** Three-dimensional theoretical structures snapshots of Pol-CP-NH₂ and Ala-scan derivatives during 100 ns of molecular dynamics simulation. N-terminus of each peptide is always at the bottom.



Supplementary Figure 4. Helical wheel representations of the second generation of Pol-CP-NH₂ analogs generated using the Heliquet server considering theoretical helical structure and physicochemical properties derived from the amphipathic distribution. Yellow circles indicate hydrophobic/aliphatic residues, blue circles represent positively charged residues, purple circles indicate polar uncharged residues, red circles represent negatively charged residues, and gray circles represent residues with hydrophobicity close to zero. The black arrows inside the helical wheel projection of each peptide represent their hydrophobic moment vector, whose magnitude is indicated by the size of the arrows.



Supplementary Figure 5. In vitro antimicrobial activity of the lead peptides from the second generation of Pol-CP-NH₂ derived agents. Serially diluted (0 – 128 $\mu\text{mol L}^{-1}$) peptides were added to a 96-well plate containing 10^4 bacterial cells in each well and incubated at 37 °C for 24 h. After the exposure, the solution in each well was measured in a microplate reader (600 nm) to check inhibition of bacteria compared to the untreated controls and presented as heat maps of antimicrobial activities ($\mu\text{mol L}^{-1}$) against four bacteria strains: *Escherichia coli* strain BL21, *S. aureus* strain ATCC12600 and *P. aeruginosa* strains PA01 and PA14. Assays were performed in independent triplicates.



Supplementary Figure 6. (A) Graphical representation of residues movement of Pol-CP-NH₂ and second generation of analogs from molecular dynamics simulations in water and TFE/water (3:2, v:v), yielding root mean square deviation, root mean square fluctuation and radius of gyration after 100 ns. **(B)** Three-dimensional theoretical structures snapshots of Pol-CP-NH₂ and derivatives during 100 ns of molecular dynamics simulation. N-terminus of each peptide is always at the bottom.

Supplementary Table 1. Summary of Pol-CP-NH₂ and designed analogs.

Label	Peptide	Sequence	Molecular Weight (Da)	Observed Molecular Weight (Da) ^a	Purity (%) ^b	HPLC Retention Time (min) ^c	HC ₅₀ (μmol L ⁻¹) ^d	MIC Average (μmol L ⁻¹)	SI ^e	Cytotoxicity (μmol L ⁻¹)
WT	Pol-CP-NH ₂	ILGTILGLLKSL-NH ₂	1239.8	1240.9	99	16.5	50.0	16.2	3.1	32.0
1	[Ala] ¹ -Pol-CP-NH ₂	ALGTILGLLKSL-NH ₂	1197.8	1198.7	95	15.8	-	-	-	-
2	[Ala] ² -Pol-CP-NH ₂	IAGTILGLLKSL-NH ₂	1197.8	1198.8	96	15.4	-	-	-	-
3	[Ala] ³ -Pol-CP-NH ₂	ILATILGLLKSL-NH ₂	1253.8	1254.8	96	16.8	-	-	-	>64.0
4	[Ala] ⁴ -Pol-CP-NH ₂	ILGA I ILGLLKSL-NH ₂	1209.8	1210.8	93	16.8	-	-	-	-
5	[Ala] ⁵ -Pol-CP-NH ₂	ILGTALGLLKSL-NH ₂	1197.8	1198.8	94	14.4	-	-	-	>64.0
6	[Ala] ⁶ -Pol-CP-NH ₂	ILGTIAGLLKSL-NH ₂	1197.8	1198.8	93	15.0	-	-	-	-
7	[Ala] ⁷ -Pol-CP-NH ₂	ILGTILALLKSL-NH ₂	1253.8	1254.8	92	16.8	-	-	-	32.0
8	[Ala] ⁸ -Pol-CP-NH ₂	ILGTILGALKSL-NH ₂	1197.8	1198.8	93	15.0	-	-	-	-
9	[Ala] ⁹ -Pol-CP-NH ₂	ILGTILGLAKSL-NH ₂	1197.8	1198.8	90	14.5	-	-	-	-
10	[Ala] ¹⁰ -Pol-CP-NH ₂	ILGTILGLLASL-NH ₂	1182.8	1183.8	92	17.5	-	-	-	-
11	[Ala] ¹¹ -Pol-CP-NH ₂	ILGTILGLLKAL-NH ₂	1223.8	1224.7	95	16.7	-	-	-	64.0
12	[Ala] ¹² -Pol-CP-NH ₂	ILGTILGLLKSA-NH ₂	1197.8	1198.8	92	14.6	-	-	-	-
13	[Leu] ⁵ -[Lys] ⁹ -Pol-CP-NH ₂	ILGTLLGLKKSL-NH ₂	1254.8	1256.0	99	11.5	>100.0	>50.0	-	-
14	[Lys] ⁵ -Pol-CP-NH ₂	ILGTKLGLLKSL-NH ₂	1254.8	1255.9	99	11.5	>100.0	>50.0	-	-
15	[Lys] ⁴ -Pol-CP-NH ₂	ILGKILGLLKSL-NH ₂	1265.8	1266.8	98	15.0	>100.0	3.3	-	32.0
16	[Lys] ⁷ -Pol-CP-NH ₂	ILGTILKLLKSL-NH ₂	1309.8	1310.9	99	16.0	12.5	1.4	9.2	16.0
17	[Phe] ⁹ -Pol-CP-NH ₂	ILGTILGLFKSL-NH ₂	1272.8	1274.0	99	15.7	50.0	20.0	2.5	-
18	Des[Leu] ¹² -Pol-CP-NH ₂	ILGTILGLLKS-NH ₂	1125.8	1126.8	99	13.4	>100.0	>50.0	-	-
19	[Glu] ³ -[Lys] ⁵ -[Glu] ¹² -Pol-CP-NH ₂	ILETKLGLLKSE-NH ₂	1341.8	1341.8	99	9.2	>100.0	>50.0	-	-
20	[Gly] ¹ -Pol-CP-NH ₂	GLGTILGLLKSL-NH ₂	1182.8	1183.8	99	15.5	>100.0	16.7	-	>64.0

^aLC/ESI-MS data were obtained on a Model 6130 Infinity mass spectrometer coupled to a Model 1260 HPLC system (Agilent), using a Phenomenex Gemini C18 column (2.0 mm × 150 mm, 3.0 μm particles, 110 Å pores). Solvent A was 0.1% TFA in water, and solvent B was 90% ACN in solvent A. Elution with a 5–95% B gradient was performed over 20 min, 0.2 mL min⁻¹ flow and peptides were detected at 220 nm. Mass measurements were performed in a positive mode with the following conditions: mass range between 100 to 2500 m/z, ion energy of 5.0 V, nitrogen gas flow of 12 L min⁻¹, solvent heater of 250 °C, multiplier of 1.0, capillary of 3.0 kV and cone voltage of 35 V.

^{b,c}HPLC profiles were obtained under the following conditions: Column Supelcosil C18 (4.6 × 150 mm), 60 Å, 5 μm; Solvent System: A (0.1% TFA/H₂O) and B (0.1% TFA in 90% ACN/H₂O); Gradient: 5–95% B in 30 minutes; Flow: 1.0 mL min⁻¹; λ = 220 nm; Injection Volume: 50 μL and Sample Concentration: 1.0 mg mL⁻¹.

^dConcentration needed for 50% hemolysis caused by RBC exposure to peptides.

^eSelectivity Index = HC₅₀/MIC_{average} indicating peptides selectivity when in the presence of human erythrocytes.

Supplementary Table 2. Considerations for each one of the second library analogs designed synthesized in this work to check the importance of different kinds of substitutions and how well can the optimal hotspots describe activity propensities.

Peptide	Design Considerations
ILGTILGLLKSL-NH ₂	Wild-type
ILGTLLGLKKSL-NH ₂	To check if a subtle change in the side chain of the residue at position 5 would alter the peptide biological behavior even with an enhanced net positive charge
ILGTLGLLKSL-NH ₂	To check the importance of Ile presence at position 5 even when substituted by a residue with an additional charge since it decreased when it was substituted by an Ala residue
ILGKILGLLKSL-NH ₂	To check effect of charge introduction at the interface of hydrophobic and hydrophilic faces
ILGTILKLLKSL-NH ₂	Lys is common residue in the 7th position of small wasp venom peptides and Gly substitution by an Ala residue improved activity
ILGTILGLFKSL-NH ₂	To check the effect of increased hydrophobicity
ILGTILGLLKS-NH ₂	To check the effect of increasing hydrophilic/hydrophobic ratio
ILETKLGLLKSE-NH ₂	To check the effect of the introduction of negatively charged residues on the hydrophilic face
GLGTILGLLKSL-NH ₂	Gly is the most common residue in the 1st residue of small cationic amphipathic peptides