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An N-capping asparagine–lysine–proline (NKP) motif contributes to a hybrid flexible/stable multifunctional peptide scaffold

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INTRODUCTION

Structural diversity drives multiple biological activities and mechanisms of action in linear peptides. Here we describe an unusual N-capping asparagine-lysine-proline (NKP) motif that confers a hybrid multifunctional scaffold to a computationally designed peptide (PaDBS1R7). This peptide was characterized for its antibacterial, antibiofilm, anticancer, anti-infective and wound healing properties.

MATERIAL AND METHODS

Structural studies
- Peptide design (flexibility inducer residues)
- Circular dichroism (CD)
- Nuclear magnetic resonance (NMR)
- Molecular dynamics (MD) simulations

Biological studies
- Antibacterial, antibiofilm, anti-infective
- Anticancer
- NO and cytokines modulation
- Wound healing

Mechanisms of action
- CD with different mimetic vesicles
- Leakage assays
- MD simulations

RESULTS AND DISCUSSION

PaDBS1R7 has a shorter α-helix segment than other computationally designed peptides of similar sequence but with key residue substitutions (Fig. 1A – J). Although this motif acts as an α-helix breaker in PaDBS1R7, the Asn5 presents exclusive N-capping effects, forming a belt to establish hydrogen bonds for an amphipathic α-helix stabilization. The combination of these different structural profiles was described as a coil/N-cap/α-helix scaffold, which was also observed in diverse computational peptide mutants. Biological studies revealed that all peptides displayed antibacterial activities (Fig. 3 A – J). However, only PaDBS1R7 displayed anticancer properties, eradicated *Pseudomonas aeruginosa* biofilms, decreased bacterial counts by 100-1,000-fold *in vivo* (Fig. 4C), reduced lipopolysaccharide-induced macrophages stress (Fig. 4D), and stimulated fibroblast migration for wound healing (Fig 5A – H). This study extends our understanding of an N-capping NKP motif to engineering hybrid multifunctional peptide drug candidates with potent anti-infective and immunomodulatory properties.

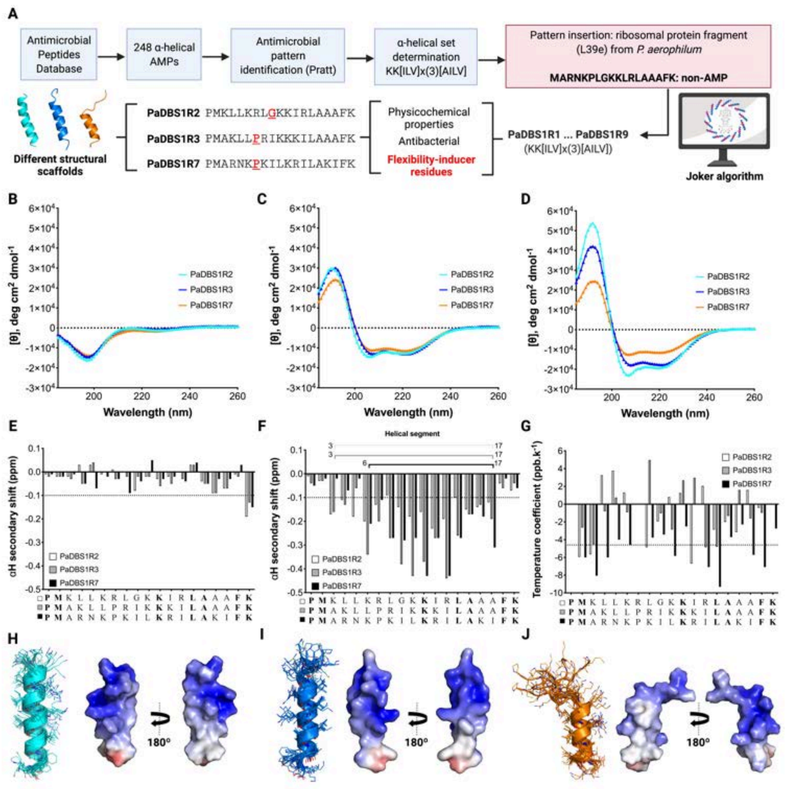


Fig. 1. Computer-aided design of PaDBS1 peptides and selection of PaDBS1R2, R3 and R7 for structural analysis. (A) For the computer-aided design of PaDBS1 peptides, 248 α-helical AMP sequences were retrieved from the Antimicrobial Peptide Database (APD). CD spectra were obtained in ultrapure water (B), 30% TFE in water (v/v) (C) and 50 mmol L^{-1} SDS (D). αH secondary chemical shifts in 90% H_2O (v/v) and 10% D_2O (v/v) (E), 60% H_2O (v/v) 30% TFE-d3 (v/v) and 10% D2O (v/v) (F) and amide proton (NH) temperature coefficients (G), of PaDBS1R2, R3 and R7 are also shown. Values more negative than -0.1 ppm (dashed lines in (E) and (F)) were interpreted as α-helix formation. Hydrogen bonds were considered for amide temperature coefficient values more positive than -4.6 ppb K_{-1} (dashed line in (G)). Conserved residues between all the peptides listed are in bold type. The superposition of the ten lowest energy NMR structures for PaDBS1R2 (PDB: 6CSK) (H), R3 (PDB: 6CSZ) (I) and R7 (PDB: 6CTI) (J) are also shown (H-J).

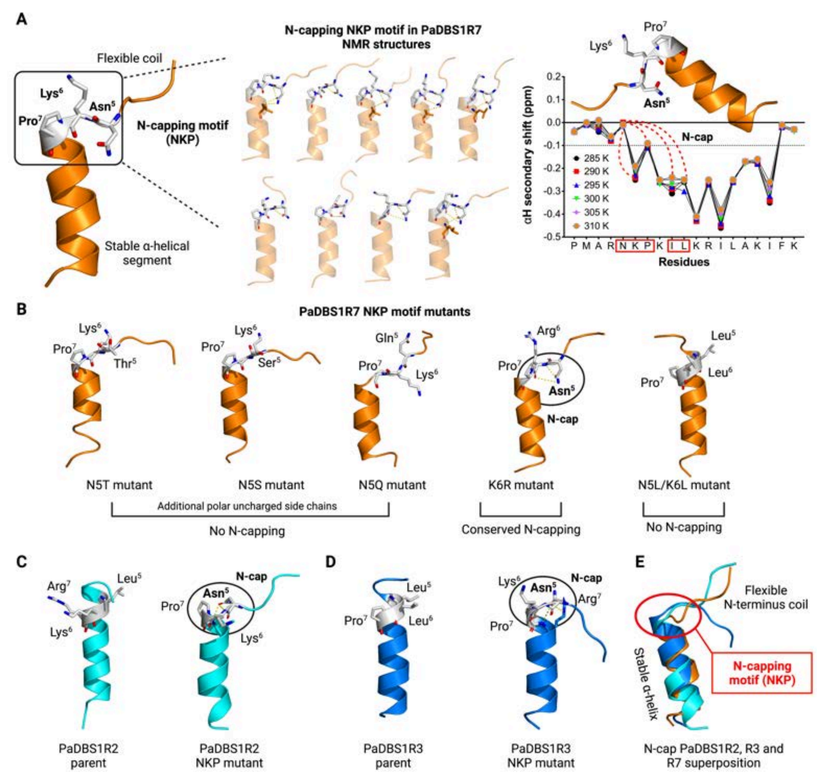


Fig. 2. An NKP motif in PaDBS1R7 triggers the coil/N-cap/α-helix structural scaffold. (A) The lowest energy NMR structure for PaDBS1R7 presents an NKP motif that influences on the peptide's scaffold by interrupting the α-helical segment at Pro7. This coil/N-cap/α-helix structural scaffold was observed for all ten lowest energy PaDBS1R7 NMR structures. The αH secondary shifts at different temperatures are also shown, revealing that the coil/N-cap/α-helix scaffold is conserved from 285 to 310 K, with primary N-capping involving Asn5 side chain atoms and Lys6, Pro7, Ile9 and Leu10 backbone atoms (red dashes). (B) Computational PaDBS1R7 mutants at position 5 replacing asparagine by other polar uncharged residues were also generated, leading to N-cap effect loss (C – E).

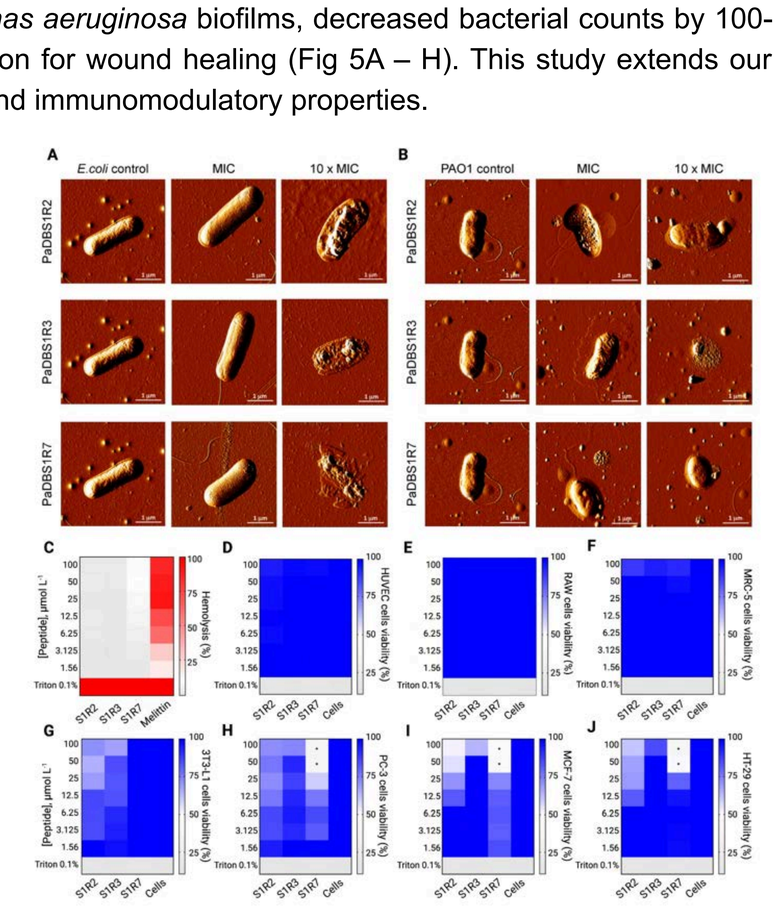


Fig. 3. *Escherichia coli* cells (A) treated with PaDBS1R2, R3 and R7 at their MIC and 10-fold MIC. The same conditions were used for *P. aeruginosa* cells (B) treated with PaDBS1R2, R3 and R7. Total scanning area of 5 × 5 μm; scale bar: 1 μm. The hemolytic activity of PaDBS1R2 (S1R2), R3 (S1R3) and R7 (S1R7) was evaluated using fresh human erythrocytes (C). Cell viability assays were performed using healthy mammalian cells, including HUVEC (D), RAW 267.4 (E), MRC-5 (F) and 3T3-L1 cells (G); and cancer cells, including PC-3 (H), MCF-7 (I) and HT-29 cells (J). All cell lines were treated with PaDBS1R2, R3 and R7 from 1.56 to 100 μmol L^{-1}. *Indicate peptide concentrations at which halfmaximal inhibitory concentrations (IC50) were determined.

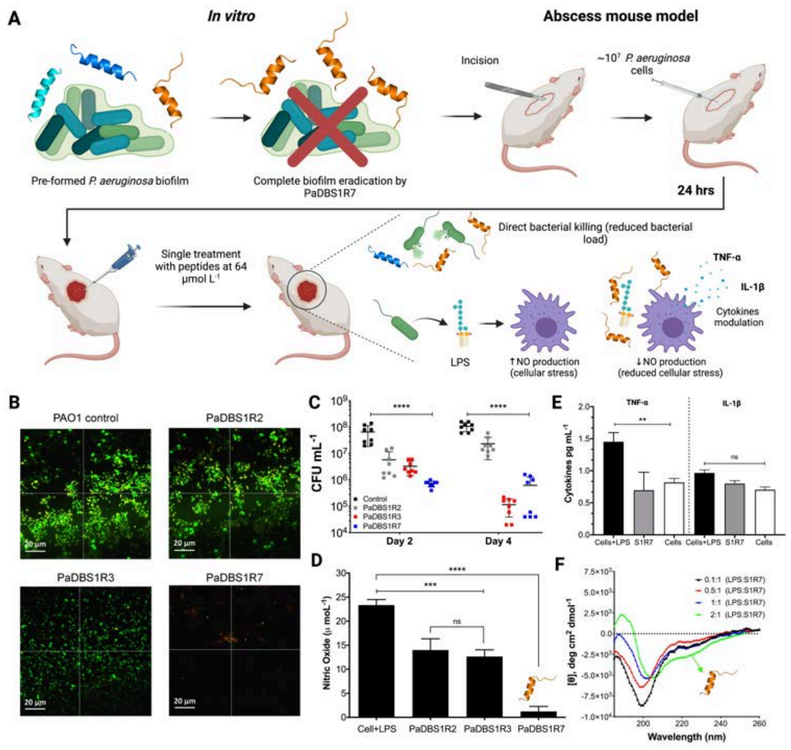


Fig. 4. Flow cell analysis of two-day-old *P. aeruginosa* biofilms (A and B), skin abscess mouse model with *P. aeruginosa* (C), nitric oxide (NO) (D) and cytokines production by macrophages in the presence of LPS (E), and LPS titration using CD experiments (F). Statistical significance was assessed using one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison tests. ** $p < 0.05$; *** $p < 0.001$ **** $p < 0.0001$. The error bars represent standard deviation values. ns: not significant.

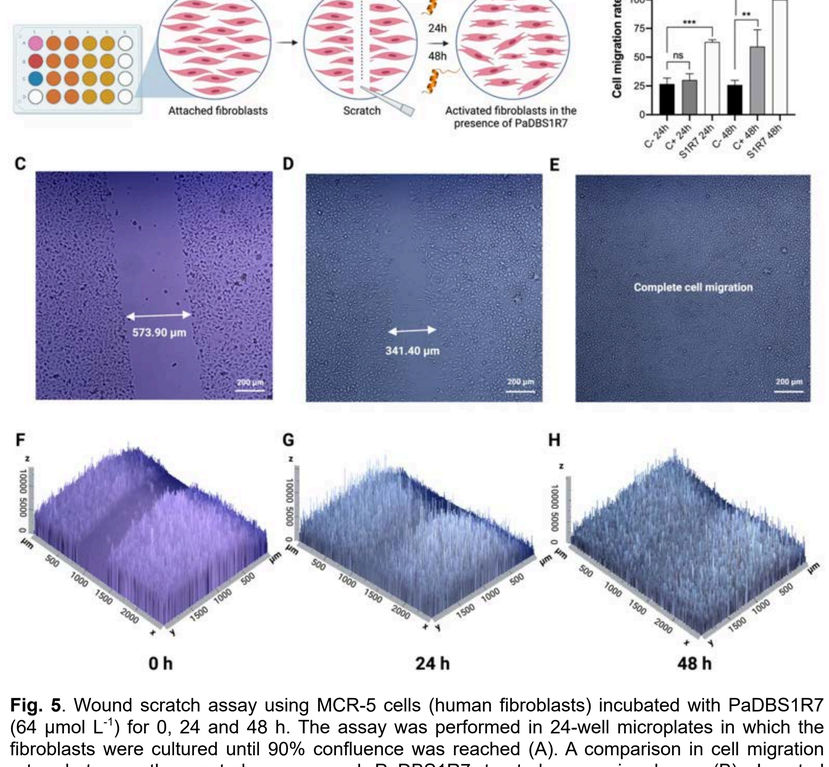


Fig. 5. Wound scratch assay using MCR-5 cells (human fibroblasts) incubated with PaDBS1R7 (64 μmol L^{-1}) for 0, 24 and 48 h. The assay was performed in 24-well microplates in which the fibroblasts were cultured until 90% confluence was reached (A). A comparison in cell migration rates between the control groups and PaDBS1R7 treated group is shown (B). Inverted microscopy images were captured to measure the gap distances in the scratches at 0 (C), 24 (D) and 48 h (E). 2.5D images were also obtained to visualize the gap distances and cell density at the incubation times 0 (F), 24 (G) and 48 h (H).

CONCLUSION

This study extends our understanding of the rules governing AMPs with N-cap motifs. In the peptides studied here, this unusual structural scaffold is triggered by an N-cap NKP motif, which resulted in a multifunctional AMP selective for anionic surfaces and capable of exerting antibacterial, antibiofilm, anticancer and anti-infective (skin infection) effects, also reducing LPS-induced macrophage stress and recruiting fibroblasts to assist wound healing. The biological multifunctionality associated with the N-cap NKP motif makes PaDBS1R7 a highly attractive coil/N-cap/α-helix scaffold for peptide-based drug design.

REFERENCE

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