A novel RNase 3/ECP peptide for *Pseudomonas aeruginosa* biofilm eradication. Combining antimicrobial, lipopolysaccharide binding and cell agglutinating activities.

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Supplemental Materials

Supplemental Materials and Methods

LUV (large unilamellar vesicle) liposome preparation

LUVs containing DOPC, DOPG or DOPC/DOPG (3:2 molar ratio) of a defined size (approximately 100 nm) were prepared as described previously (1). LUVs were obtained from a vacuum-drying lipid chloroform solution by extrusion through 100nm polycarbonate membranes. The lipid suspension was frozen and thawed ten times before extrusion. A 1 mM stock solution of liposome suspension in 10 mM phosphate buffer and 100 mM NaCl (pH 7.4) was prepared.

Liposome leakage assay

The ANTS/DPX liposome leakage fluorescence assay was performed as described previously (1). Briefly, a unique population of LUVs of DOPC/DOPG (3:2 molar ratio) lipids was obtained containing 12.5 mM ANTS, 45 mM DPX, 20 mM NaCl and 10 mM Tris/HCl (pH 7.4). The ANTS/DPX liposome suspension was diluted to a 30 μM concentration and incubated at 25°C in the presence of protein/peptide. Leakage activity was followed by monitoring the increase in fluorescence at 535 nm (2).

Liposome agglutination assay

Liposome agglutination was analyzed by dynamic light scattering (DLS) using a Malvern 4700 photon correlation spectrometer (Malvern Instruments). An argon laser (λ=488 nm) was used to cover the wide size range involved. Hydrodynamic radius measurements were always carried out at a reading scattering angle of 90°. From the intensity measurements recorded, data were processed by the CONTIN software (Malvern), and the hydrodynamic diameter, the polydispersity index and the total number of counts were calculated. The incubation buffer was 10 mM Tris/HCl and 100 mM NaCl (pH 7.4). Measurements were performed at 25°C, a 200 μM final liposome concentration, and a 5 μM protein/peptide concentration.

Supplemental Figures and Tables

Table S1. Sequence information and chemical properties of the assayed antimicrobial peptides.

Peptide	Amino acid sequence	MW (Da)	Net charge at pH 7	Hydropathicity (GRAVY scale)
RN3(1-36)	RPPQFTRAQWFAIQHISLNPPRCTIAMRAINNYRWR-NH2	4411.1	+7	-0.6639
RN3(5-17P22-36)	RPFTRAQWFAIQHISPRTIAMRAINNYRWR-NH2	3758.3	+7	-0.6667
GL-13	GQIINLKASLDLL-NH2	1397.6	+1	+0.8000
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES-NH2	4493.3	+7	-0.7243
WY12	YVLWKRKRFIFI-NH2	1669.0	+5	+0.3000
CA-M	KWKLFKKIGIGAVLKVLTTGLPALIS-NH2	2795.5	+6	+0.8423

Table S2. Antimicrobial and agglutinating activities of RNase 3, RN3(1-36) and RN3(5-17P22-36). The 100% minimum bactericidal concentration (MBC), and the minimum agglutinating concentration (MAC) were calculated as described in Materials and Methods. N.D.: not detected at the assayed concentration range (up to 50 μM). Values are indicated as mean ± SEM.

	MBC (μM)		MAC (μM)	
	E. coli	*S. aureus*	*E. coli*	*S. aureus*
RNase 3	0.20 ± 0.05	0.20± 0.06	*0.20 ± 0.05*	N.D.
RN3(1-36)	1.20 ± 0.03	1.20 ± 0.13	0.45 ± 0.05	N.D.
RN3(5-17P22-36)	0.20 ± 0.06	0.60± 0.05	0.87 ± 0.05	N.D.

Table S3. LPS binding of RNase 3, RN3(1-36) and RN3(5-17P22-36). The effective concentration for 50 % displacement (ED_{50}) and the percentage of maximum displacement were calculated as described in the Materials and Methods. 100% refers to a total displacement whereas 0% is for no displacement of the dye, indicating no binding. Values are indicated as mean ± SEM.

	LPS binding	
	ED_{50} (μM)	$\%_{max}$
RNase 3	1.79 ± 0.32	99.85 ± 9.51
RN3(1-36)	2.91 ± 0.43	90.35 ± 5.03
RN3(5-17P22-36)	2.11 ± 0.12	92.16 ± 2.06

TableS4. Hemolytic (HC_{50}) activities and cytotoxicity on MRC-5 cell line (IC_{50}) by RNase 3 and RN3(5-17P22-36). Values are indicated as mean ± SEM.

	HC_{50} (µM)	IC_{50} (µM)
RNase 3	217,15 ± 42,17	200,79 ± 40,24
RN3(5-17P22-36)	178,33 ± 81,72	57,59 ± 13,21

TableS5. Minimum inhibitory concentration (MIC) of RNase 3 and RN3(5-17P22-36) for *Pseudomonas aeruginosa* PAO1 and three clinical isolates (Hospital Valle Hebron, Spain). Assays were performed in triplicate. Values are indicated as mean ± SEM.

	MIC (μM)	
	RNase 3	RN3(5-17P22-36)
P. aeruginosa PAO1	0.83 ± 0.4	0.52 ± 0.2
P. aeruginosa isolate 1	1.0 ± 0.4	2.0 ± 0.3
P. aeruginosa isolate 2	1.0 ± 0.4	2.0 ± 0.3
P. aeruginosa isolate 3	4.5 ± 1.5	6.0 ± 1.0

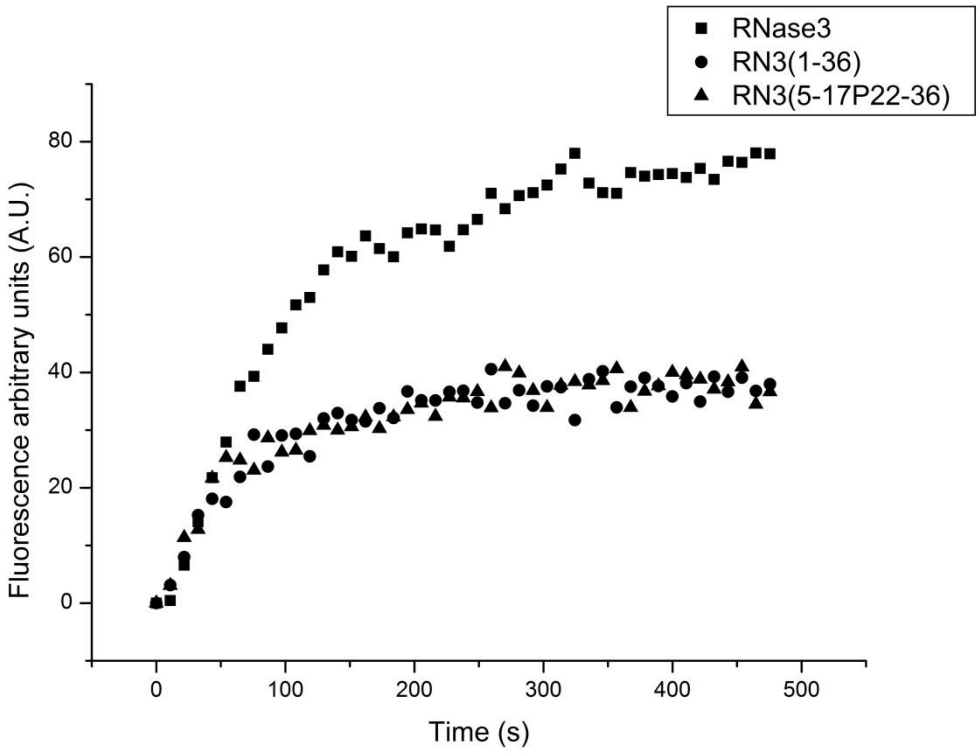


Figure S1. Membrane disruption was determined by ANTS/DPX leakage assay after incubation of 5 μM of RNase 3, RN3(1-36) and RN3(5-17P22-36) with LUVs of DOPC/DOPG (3:2 molar ratio) as a function of time.

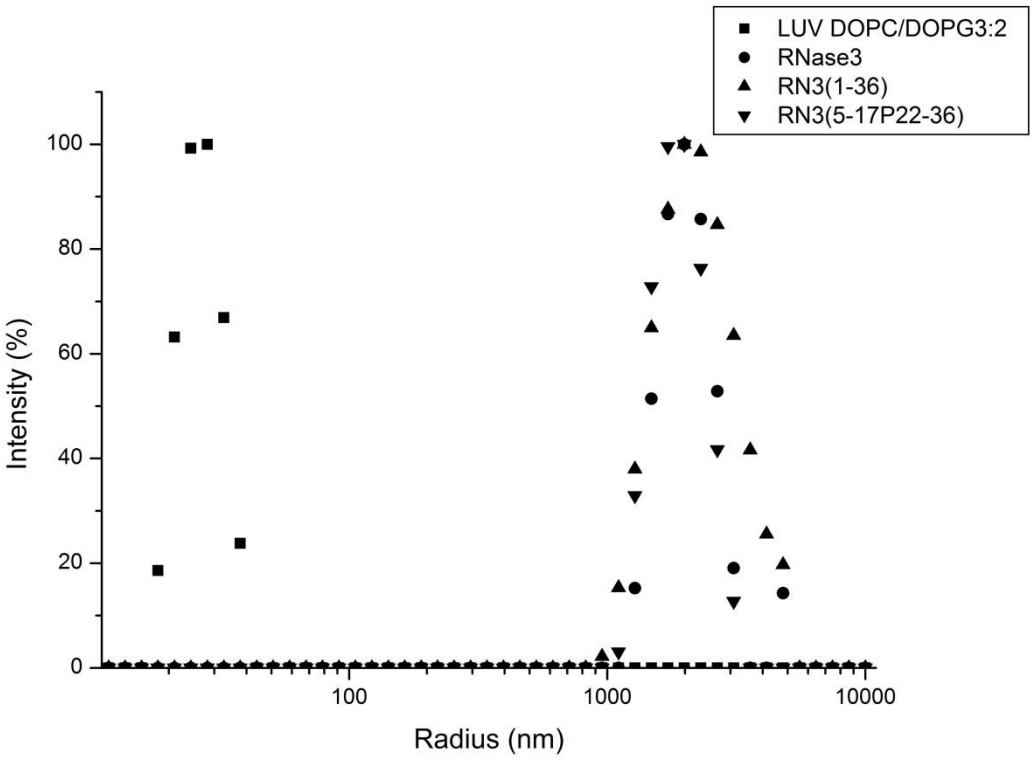
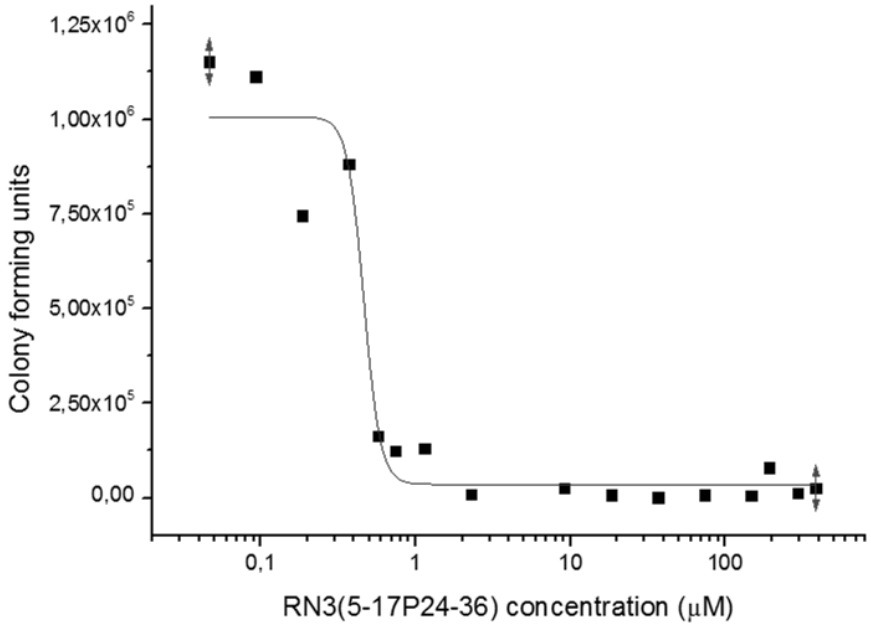


Figure S2. Membrane aggregation was assessed by DLS. An aliquot of 5 µM of RNase 3, RN3(1-36) and RN3(5-17P22-36) were incubated with 200 µM LUVs of DOPC/DOPG (3:2 molar ratio) for 1h at room temperature. Liposome aggregation is depicted as a function of time.

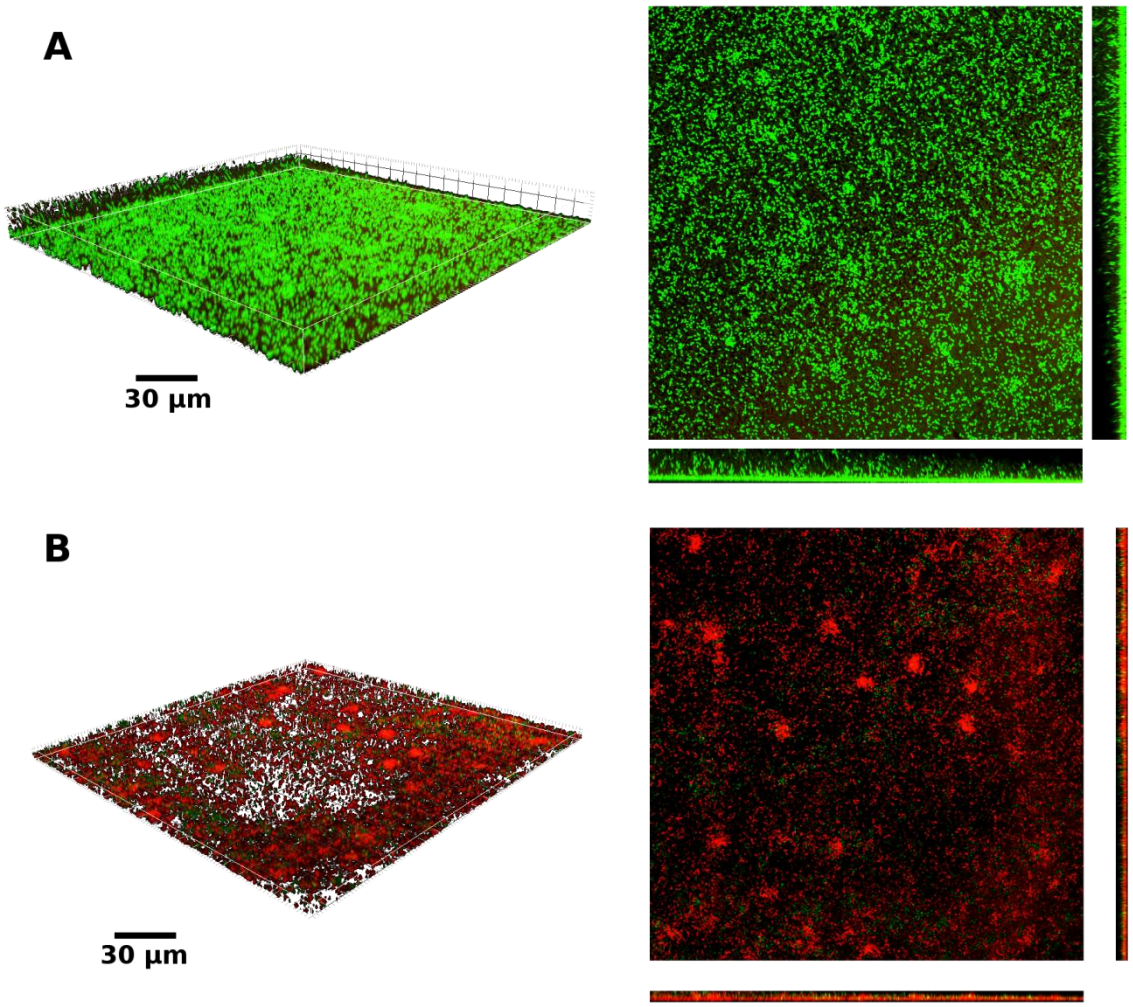
A



B

	Biofilm ED_{50} (μM)	**Supernatant ED_{50} (μM)**	**Biofilm MBEC (μM)**	**Supernatant MBEC (μM)**
RNase 3	0.55 ± 0,02	1.31 ± 0.37	≤ 5	≤ 20
RN3(5-17P22-36)	0.46 ± 0,03	1.57 ± 0.43	≤ 5	≤ 20

Figure S3. Effect on pre-established *P. aeruginosa* biofilms by RNase 3 and RN3 peptide determined by CFU counting. For each protein/peptide concentration the remaining CFUs in the treated biofilm and supernatant fractions were calculated by plating LB-Agar Petri dishes after 4h of incubation as described in methodology. A) Plotting of CFUs against the RN3(5-17P22-36) peptide. B) Calculated 50% effective dose (ED_{50}) and MBEC values for biofilm and supernatant fractions. Values are indicated as mean ± SEM.



C

	Cell mortality (%)	Biofilm depth (µm)
Control	-	12.28 ± 5.75
RNase 3	75,36 ± 7,39	5.50 ± 0.85
RN3(5-17P22-36)	90,03 ± 9,01	5.77 ± 2.53

Figure S4. Analysis by confocal microscopy of pre-established *P. aeruginosa* biofilms treated with 10 µM of protein/peptide for 4h at 37ºC. A and B) 3D reconstruction and projections using the IMARIS software® of control (A) and treated biofilm with the RN3(5-17P22-36) peptide (B). C) Bacterial cell mortality quantification using the Live/Dead staining and biofilm depth estimation as described in the methodology. Values are indicated as mean ± SEM.

REFERENCES

1. **Torrent M, Cuyas E, Carreras E, Navarro S, Lopez O, de la Maza A, Nogues MV, Reshetnyak YK, Boix E.** 2007. Topography studies on the membrane interaction mechanism of the eosinophil cationic protein. Biochemistry **46:**720-733.
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