Proceedings of the 36th European Peptide Symposium
Michal Lebl (Editor)
European Peptide Society, 2022 *https://doi.org/10.17952/36EPS.2022.105*

Novel Polymyxins with Reduced Toxicity and Modulated Spectrum of Activity

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Introduction

Antibacterial resistance to almost all available antibiotics is increasing worldwide, and Gram-negative multidrug-resistant bacteria are particularly worrisome. Moreover, the extensive use of antibiotics in the context of the COVID-19 pandemic has accelerated the threat of running out of them. Here, we report a series of polymyxin E (colistin) synthetic analogues with a disulfide bond-containing scaffold and modifications in the hydrophobic domains (Figure 1). By means of biophysical techniques, we explore the interaction of these compounds with models that mimic the bacterial inner and outer membranes, resulting in a selective effect on anionic membranes.

Results and Discussion

Design and synthesis

In previous work, we demonstrated that the introduction of a disulfide bond within the macrocycle peptide structure reduces the toxicity compared to that displayed by natural polymyxins [1,2]. In the present work, we have modulated the hydrophobicity of these molecules by changing the length of the fatty acyl chain and the amino acid at position 6. All analogues have been synthesized by Fmoc/tBu solid-phase peptide synthesis (SPPS).

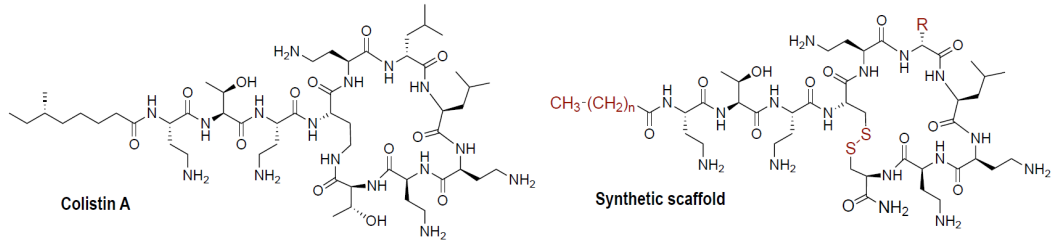

Fig. 1. Structure of natural colistin A (left) and general structure of the synthetic analogues (right).

In vitro activity and hemolysis assays

The activity against bacteria has been assessed in terms of the minimum inhibitory concentration. Two Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) and one Gram-positive (*Staphylococcus aureus*) have been evaluated. Hemolytic activity was assessed using fresh rabbit erythrocytes and determined by measuring the absorbance at 540 nm of released hemoglobin in the supernatant after peptide exposure. As it can be shown in Table 1, a minimum length of seven carbons in the fatty acyl chain is needed for activity. Analogues with longer acyl chains induce higher degree of hemolysis (Figure 2). On the other hand, the sPE-10 analogue showed selectivity towards *P. aeruginosa* and very low hemolytic activity.

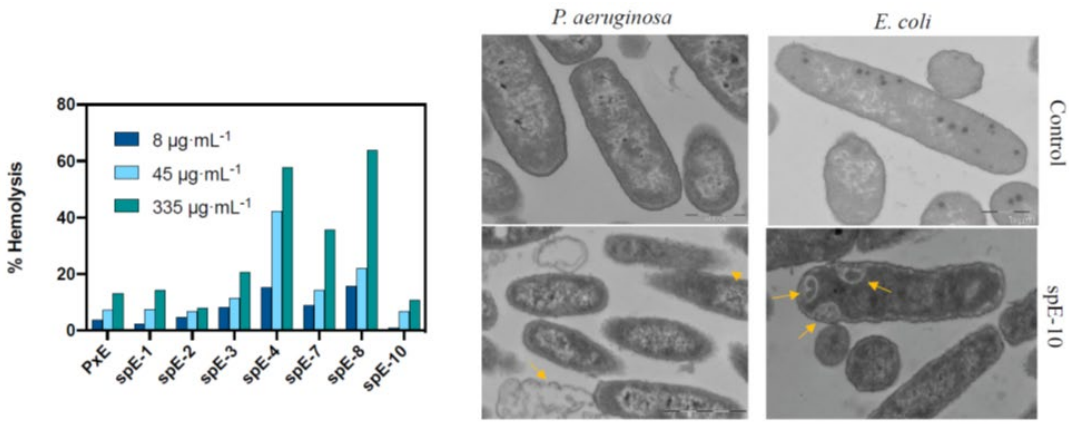

Fig. 2. Hemolysis induced by synthetic analogues(left). TEM images for control samples and bacteria treated with analogue spE-10 at its MIC value (right). Yellow arrows indicate rupture of the cell membrane and cytoplasmic clear zones.

Transmission electron microscopy

The effect of analogue spE-10 on *E. coli* and *P. aeruginosa* has been studied by transmission electron microscopy (TEM). As shown in Figure 2, the treatment of *P. aeruginosa* with spE-10 causes significant rupture of the cell membranes and cytoplasmic clear zones. In the case of *E. coli*, this analogue similarly induces the formation of intracellular membranous structures and cytoplasmic clear zones.

Table 1. Sequences and antimicrobial activities of the peptide analogues determined as the minimal inhibitory concentration (MIC) calculated for different bacteria strains.

	Sequence	*MIC (μg.mL^{-1})*		
		EC[1]	*PA*	*SA*
spE-1	hexanoyl-Dab-Thr-Dab-Cys-Dab-*Leu*-Leu-Dab-Dab-*Cys*	>32	>32	>32
spE-2	octanoyl-Dab-Thr-Dab-Cys-Dab-*Leu*-Leu-Dab-Dab-*Cys*	16	4	>32
spE-3	decanoyl-Dab-Thr-Dab-Cys-Dab-*Leu*-Leu-Dab-Dab-*Cys*	8	2	8
spE-4	dodecanoyl-Dab-Thr-Dab-Cys-Dab-*Leu*-Leu-Dab-Dab-*Cys*	8	1	8
spE-7	decanoyl-Dab-Thr-Dab-Cys-Dab-*Nle*-Nle-Dab-Dab-*Cys*	2	2	8
spE-8	dodecanoyl-Dab-Thr-Dab-Cys-Dab-*Nle*-Nle-Dab-Dab-*Cys*	4	4	4
spE-10	heptanoyl-Dab-Thr-Dab-Cys-Dab-*Aoc*-Nle-Dab-Dab-*Cys*	8	1	>32
Polymyxin B	R1-Dab-Thr-Dab-Dab-Dab-*Phe*-Leu-Dab-Dab-Thr	1	0.5	>32
Colistin	R1-Dab-Thr-Dab-Dab-Dab-*Leu*-Leu-Dab-Dab-Thr	1	0.5	>32

D-amino acids are denoted in italics, and underlined residues denote bond formation. R1: natural mixture of branched C7-C9 fatty acyl moieties

Biophysical studies

The binding affinity of colistin and the synthetic analogues with model lipid membranes has been studied by using monolayers at the air-water interface. Different lipid compositions including LPS, POPE:POPG (6:4), POPG and POPC have been tested. According to the results obtained, the synthetic analogues as well as colistin show a high binding affinity for LPS, which mimics the outer membrane of Gram-negative bacteria. In contrast, the change in surface pressure for zwitterionic POPC membrane is low, indicating a low affinity of the analogues and colistin to bind the eukaryotic membrane model.

Colistin is able to induce the aggregation of vesicles forming clusters, what facilitates the selective exchange of phospholipids. The change in light scattering of the synthetic analogues has been evaluated with liposomes of POPE:POPG and, like colistin, the lipopeptides show an increase of the scattered light, indicating vesicle aggregation (Figure 3). In order to study if the analogues induce mixing, a FRET experiment using vesicles containing 0.6% NBD-PE (donor vesicles) and vesicles containing 0.6% Rh-PE (acceptor vesicles) was conducted. All synthetic analogues induced the lipid mixing between anionic vesicles of POPE:POPG (6:4), similarly to colistin.

Table 2. Increase in surface pressure ($\Delta\pi$/mN·m^{-1}) upon penetration of the antimicrobial lipopeptides into monolayers.

*Composition**	***Increase in surface pressure ($\Delta\pi$/mN·m^{-1})***							
	spE-1	*spE-2*	*spE-3*	*spE-4*	*spE-7*	*spE-8*	*spE-10*	*PxE*
LPS	10.6	9.5	10.0	11.4	14.2	12.5	14.8	11.1
POPE:POPG	4.1	6.4	7.9	9.1	6.8	9.8	8.2	7.9
POPG	2.8	4.3	5.7	5.8	8.2	7.4	0.8	5.3
POPC	0.3	0.6	1.3	2.1	1.4	1.3	1.8	0.8

**Monolayers mimic the outer membrane of Gram- (LPS), the cytoplasmic membrane of Gram- (POPE/POPG 6:4), or of Gram+ (POPG), or the eukaryotic membrane (POPC)*

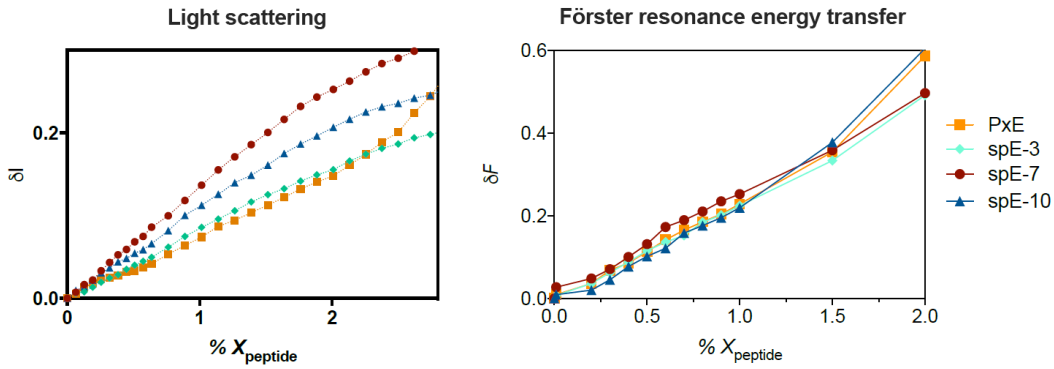

Fig. 3. Change in light scattering (left). On the right, increase in FRET intensity as a function of the mole fraction of lipopeptide added to a (1:1) mixture of vesicles containing 0.6% NBD-PE or Rh-PE.

Finally, we studied the ability of our compounds to induce the leakage of aqueous contents, indicating leaky membrane fusion. In this sense, the increase of fluorescence of ANTS co-encapsulated with the fluorescence quencher DPX was measured. In Figure 4, leakage at three relevant concentrations is shown. As it can be observed, upon peptide addition in membranes of POPE:POPG (6:4), leakage is low in all cases, and at MIC concentrations it remains below 5%, thus indicating a low permeabilization effect in the Gram-negative model.

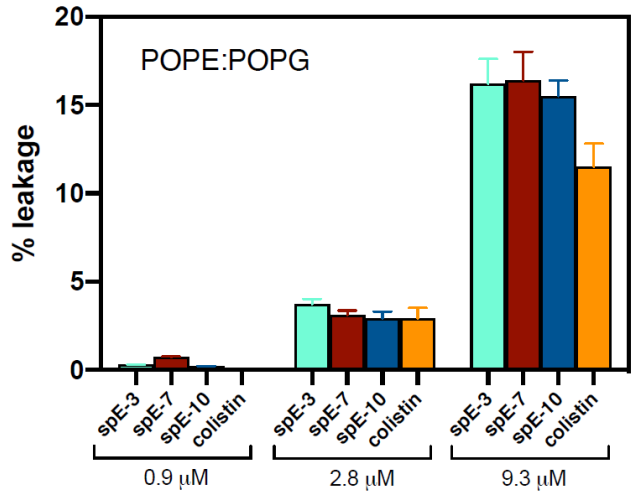

Fig. 4. Leakage from vesicles of POPE/POPG (6:4) at three peptide concentrations. Lipopeptides were added to liposomes co-encapsulating ANTS (12.5 mM) and DPX (45 mM), and leakage was determined as the increase in ANTS fluorescence intensity at 530 nm (excitation 350 nm, lipid concentration 107 μM).

Acknowledgments

We thank the support of the Ministry of Economy and Competitiveness (grant RTI2018-098641-B-I00), Fundació Marató TV3 (ref 201829-10), the University of Barcelona, Fundació Bosch i Gimpera and Agència de Gestió d'Ajuts Universitaris i de Recerca (AGAUR, FI-SDUR grant to JG).

Abbreviations

EC: *Escherichia coli*; PS: *Pseudomonas aeruginosa*; SA: *Staphylococcus aureus*; LPS: Lipopolysaccharide from Salmonella enterica serotype Minnesota Re 595 (Re mutant); POPE: 1-palmitoyl-2-oleoylsn-glycero-3-phosphoethanolamine; POPG: 1-Palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol); POPC: 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine; ANTS: 8-aminonaphthalene-1,3,6-trisulfonic acid; DPX: p-Xylene-bis(N-pyridiniumbromide).

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