In vitro effect of the acidic pH on the susceptibility of the antimicrobial peptide Esc(1-21) against respiratory pathogens in cystic fibrosis lung environment

Giacomo Cappella[1], Maria Rosa Loffredo[1], Bruno Casciaro[1], Floriana Cappiello[1], Arianna Turchini[1], Maria Luisa Mangoni[1]

[1]*Department of Biochemical Sciences, Laboratory Affiliated to Istituto Pasteur Italia - Fondazione Cenci Bolognetti, Sapienza University of Rome, Rome, Italy*

https://doi.org/10.17952/37EPS.2024.P2100

Background

Esculentin(1-21) [Esc(1-21)] is a short-sized cationic antimicrobial peptide (AMP) corresponding to the N-terminal region of the frog-skin AMP esculentin-1a. It is endowed with a fast rate of killing activity, especially against Gram-negative bacteria, with a membrane perturbing mechanism of action.

Esculentin-1a

GIFSKLAGKKIKNLLISGLKNVGKEVGMDVVRTGIDIAGCKIKGEC

Esc(1-21) **GIFSKLAGKKIKNLLISGLKG-NH_2** **+6** **Basic residues**

- **Short size**
- **A large spectrum of activity (Gram$^-$, Gram$^+$, fungi)**
- **A potent activity against planktonic and biofilm forms of *Pseudomonas aeruginosa* (Gram$^-$).**

Rana esculenta

Antimicrobial activity of Esc(1-21) against reference strains and clinical isolates of *P. aeruginosa*

[PEPTIDE] CAUSING 99,9% KILLING OF PLAKTONIC CELLS	[PEPTIDE] CAUSING 95% KILLING OF BIOFILM CELLS
1 µM	12.5-25 µM

Premise and aim of the work

The number of antibiotic-resistant microbial infections is dramatically increasing, while the discovery of new antibiotics is significantly declining. Furthermore, the activity of antibiotics is negatively influenced by the ability of bacteria to form sessile communities, called biofilms, and by the microenvironment of the infection, characterized by an acidic pH, especially in the lungs of patients suffering from cystic fibrosis (CF). AMPs represent interesting alternatives to conventional antibiotics, and with expanding properties. Here, we explored the effects of an acidic pH on the antimicrobial and antibiofilm activities of the AMP Esc(1-21).

1. Antimicrobial Activity of AMPs and Antibiotics at Different pH Values, against Reference Gram-Negative Bacterial Strains

The capability of Esc(1-21) and various AMPs in inhibiting microbial growth of three reference Gram-negative bacterial strains was studied compared to conventional antibiotics, in 10% TSB (Tryptic soy Broth) pH 7.5.

Compound	MICs (µg/mL)		
	A. baumannii ATCC 19606	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853
Esc(1-21)	4.3	4.3	8.7
Bombinin H2	15.3	61.3	245
Temporin L	3.25	6.5	6.5
LL-37	9.0	9.0	4.5
Colistin	0.5	0.5	0.25
Tobramycin	2.0	1.0	0.125
Ciprofloxacin	0.25	0.25	0.125

Table 1. The data are the modal values from three independent experiments. Table taken from ref. **[1]**

The MIC values were then determined in media with increasing acidity.

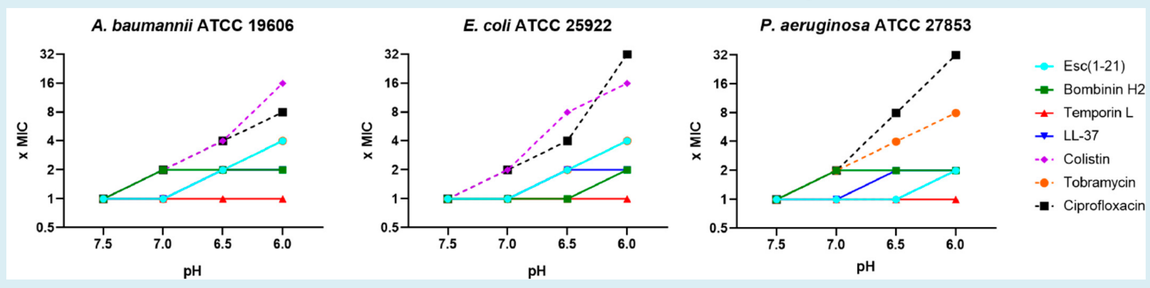


Figure 1. Variation in the MIC (**expressed as fold-change in MIC value**) of AMPs and antibiotics against the same strains as above, in 10% TSB at different pH values. The plotted data represent the modal values of three independent experiments. Graph taken from ref **[1]**

Subsequently, the activity of Esc(1-21) was evaluated against a panel of different Gram-negative strains, including reference and multidrug-resistant clinical isolates. Tobramycin was also included for comparison.

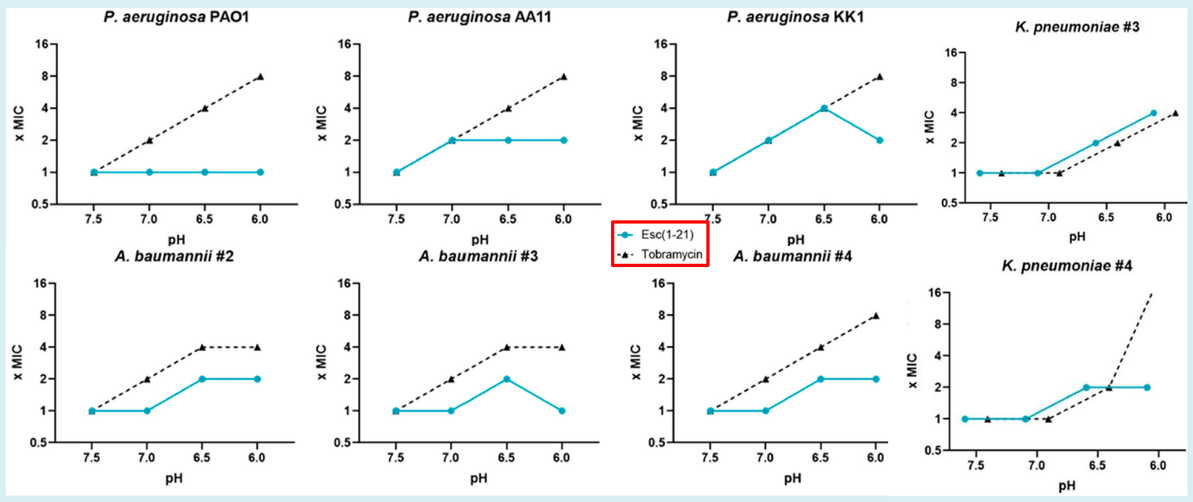


Figure 2. Variation in the MIC values (**expressed as fold-change in MIC value**) of Esc(1-21) and tobramycin at different pH values against several Gram-negative strains, in 10% TSB at different pH values. The data are the modal values from three independent experiments. Graph taken from ref **[1]**

Overall, these experiments underline the capability of Esc(1-21) to better withstand acidic conditions, retaining its antimicrobial activity more effectively than conventional antibiotics.

2. Antibiofilm Activity of Esc(1-21) Compared to Tobramycin against Preformed Biofilm in Luria–Bertani Broth (LB)

Gram-negative bacteria easily colonize biological surfaces by creating matrix-enclosed aggregates, called biofilms, in which the pH can vary within the biofilm microenvironment. Therefore, it is crucial to investigate the activity of antibiotic compounds against the sessile form of bacteria at different pH levels.

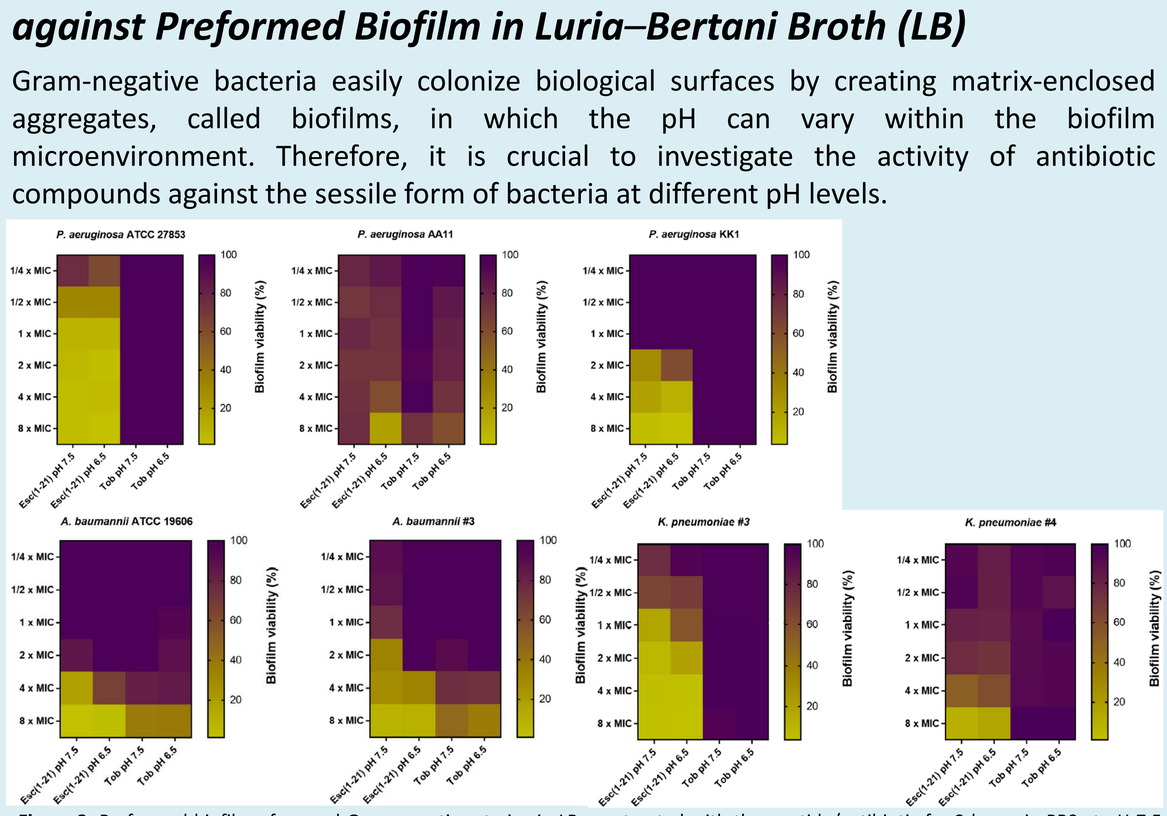
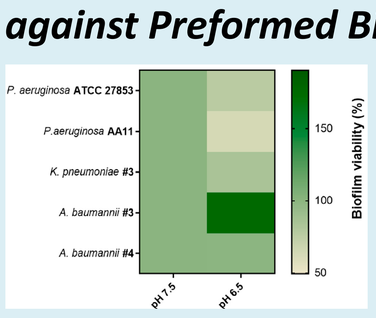


Figure 3. Preformed biofilm of several Gram-negative strains in LB was treated with the peptide/antibiotic for 2 hours in PBS at pH 7.5 and 6.5. The colors represent the percentage of biofilm viability from 0% (yellow) to 100% (purple). Graph taken from ref **[1]**

Notably, the activity of the peptide remained almost unchanged in the trend. These results highlight the potential of AMPs as novel antimicrobial agents, considering the enhanced resistance to antibiotics of the bacteria embedded in biofilms.

3. Antibiofilm Activity of Esc(1-21) Compared to Tobramycin against Preformed Biofilm Grown in m63 Medium



The acidic pH is also known to influence biofilm formation. For this reason, we also investigated the capability of different strains to form biofilm at two different pH conditions, pH 7.5 and 6.5. To ensure a constant pH during biofilm formation, m63 medium was used.

Figure 4. Percentage of biofilm viability of different Gram-negative strains in m63 at pH 6.5 with respect to pH 7.5 (set as 100%). The colors represent the percentage of biofilm viability from 0% (light green) to 100% (dark green). The statistical analysis was conducted with the *t* test for all strains between the two different pH values: all differences were found to be significant ($p < 0.05$), with the sole exception of *A. baumannii* #4 ($p = 0.765$). Graph taken from ref **[1]**

Then, Esc(1-21) was tested against these preformed biofilms for two hours in PBS at the corresponding pH, while tobramycin was used as the antibiotic control.

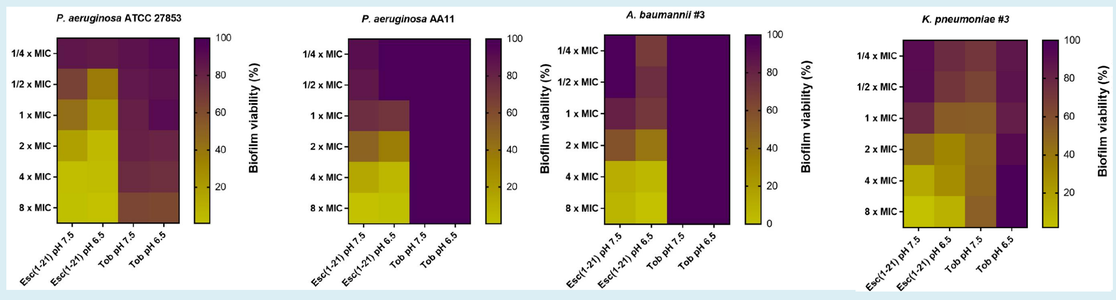


Figure 5. The effect of Esc(1-21) and tobramycin (Tob), against preformed biofilms of several Gram-negative bacterial strains in m63 medium at two different pHs, i.e., 7.5 and 6.5. Data were collected from three independent experiments. Graph taken from ref **[1]**

The activity of Esc(1-21) was enhanced against the biofilms of all the tested strains at pH 6.5. On the contrary, tobramycin had a significantly weaker activity at the two different pHs.

4. Activity of Esc(1-21) at pH 7.5 and 6.5 against Model Membranes of Gram-Negative Bacteria

Esc(1-21)'s primary mechanism of action is membrane disruption, leading to cell death. To assess the impact of pH variations, Esc(1-21) was tested at pH 7.5 and 6.5 on large unilamellar vesicles (LUVs), mimicking Gram-negative bacterial membranes and loaded with the fluorescent probe carboxyfluorescein (Cyf).

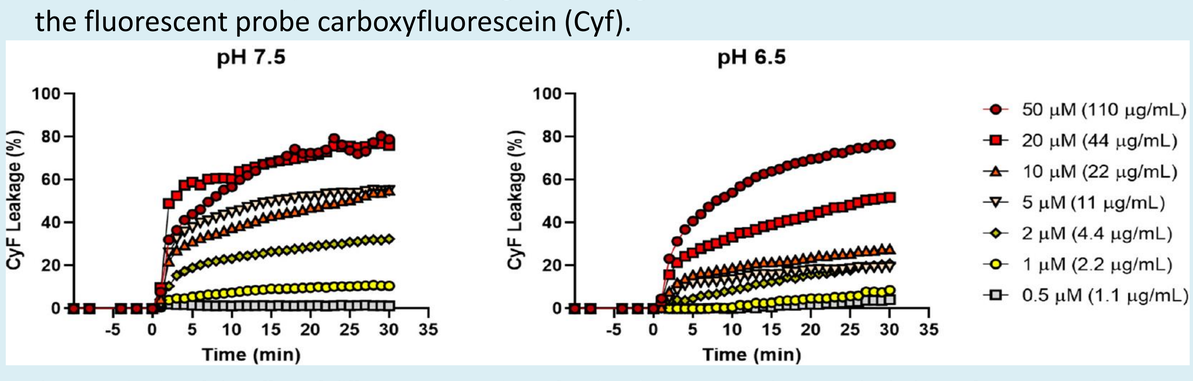


Figure 6. Kinetics of the effect of different concentrations of Esc(1-21) at pH 7.5 and 6.5, on the leakage of CyF encapsulated into POPE/POPG (ratio 7:3 mol-mol) LUVs. LUVs were used at a final lipid concentration of 100 µM. Data points are the mean of three different experiments. Graph taken from ref **[1]**

After peptide addition (time 0), a fast and dose-dependent increase in the fluorescence signal was recorded, according to the membrane-perturbing mechanism of action of Esc(1-21).
At pH 7.5, the percentage of CyF leakage was >60% at 50 and 25 µM, while 20–50% leakage was recorded for the lowest concentrations. At pH 6.5, the peptide retained its membrane-perturbing activity, albeit to a lower intensity, with a CyF leakage of about 60% at 25 µM.

5. Cytotoxic Activity of Esc(1-21) at Different pH against Mammalian Cell Lines

We previously reported the safety profile of Esc(1-21) against several mammalian cell lines through the tetrazolium bromide reduction assay (MTT).
Here, the effect of Esc(1-21) on the viability of different mammalian cell lines, i.e., HaCaT and A549 cells, was studied in acidic conditions by the CCK-8 assay.

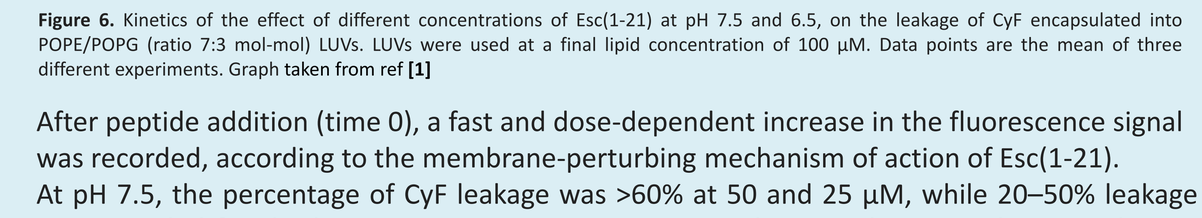


Figure 7. Viability of HaCaT (**A**) and A549 (**B**) cells after 24 h treatment with different concentrations of Esc(1-21) in DMEMg at pH 7.5 and 6.5. Cells not treated with peptides were used as controls. All data are the means of three replicates. Graph taken from ref **[1]**

Esc(1-21) did not markedly reduce the percentage of viable cells at concentrations up to 140 µg/mL; only a slight reduction in cell viability (~20%) was recorded at the highest concentration tested, at pH 6.5 against A549 cells.

Conclusion

The antimicrobial/antibiofilm activity of the AMP Esc(1-21) was explored when used at a pH lower than 7.5. It was found that when tested at pH 6.5, the peptide is practically insensitive to pH changes, highlighting the great potential of Esc(1-21) in the designing and developing of new antimicrobials to fight Gram-negative biofilm bacterial infections.

References

[1] Loffredo, M.R.; Cappiello, F.; Cappella, G.; Capuozzo, E.; Torrini, L.; Diaco, F.; Di, Y.P.; Mangoni, M.L.; Casciaro, B. The pH-Insensitive Antimicrobial and Antibiofilm Activities of the Frog Skin Derived Peptide Esc(1-21): Promising Features for Novel Anti-Infective Drugs. *Antibiotics* **2024**, *13*, 701
[2] Luca, V.; Stringaro, A.; Colone, M.; Pini, A.; Mangoni, M.L. Esculentin(1-21), an amphibian skin membrane-active peptide with potent activity on both planktonic and biofilm cells of the bacterial pathogen Pseudomonas aeruginosa. Cell. Mol. Life Sci. **2013**,70, 2773–2786.
[3] Batoni, G.; Maisetta, G.; Esin, S. Antimicrobial peptides and their interaction with biofilms of medically relevant bacteria. Biochim.Biophys. Acta **2016**, 1858, 1044–1060.

Acknowledgements

The authors thank Alessandra Bragonzi (San Raffaele Institute, Milan, Italy) and Burkhard Tummler (Klinische Forschergruppe, OE 6710, Medizinische Hochschule Hannover, Germany) for the *P. aeruginosa* clinical isolates.

Funding

This research was supported by EU funding within the NextGeneration EU-MUR PNRR. Extended Partnership initiative on Emerging Infectious Diseases (Project no. PE00000007, INF-ACT), and also partially supported by Sapienza University (RP122181619F624E), by Fondazione Italiana per la Ricerca sulla Fibrosi Cistica (Project FFC#4/2022) Delegazione FFC Ricerca di Roma e della Franciacorta e Val Camonica, and National Institutes of Health Grant No. R01 AI176537. M.R.L. is grateful to Pasteur Italia-Fondazione Cenci Bolognetti, as a postdoctoral fellow holder of this Institute.