STUDIES ON THE MODE OF ACTION OF CATIONIC β-HAIRPIN ANTIBIOTICS

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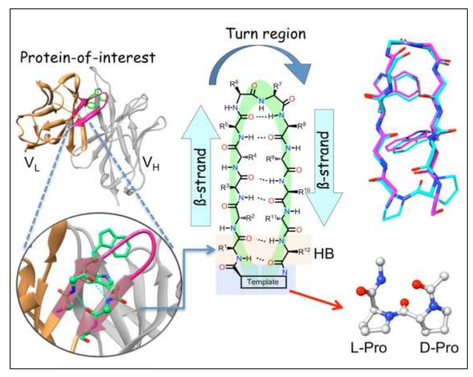
With increasing **bacterial resistance** to existing antimicrobial drugs, the need for new antibiotics active against drug-resistant microorganisms is becoming a pressing issue. Conformationally restrained **peptidomimetics (PEMs)**[1] based on naturally occurring antimicrobial peptides (AMPs) of the **innate immune system** are gaining importance in the discovery of new biologically active molecules[2]. Our research focuses on utilizing **β-hairpins**, a recurrent motif that participates in many important biological processes. Several AMPs from the innate immune system have been identified as having this structural motif, making the synthesis and use of stable, **structurally constrained** peptides that mimic these secondary structures of great interest. Our PEMs contain 12 residues linked to a β-hairpin stabilizing **D-Pro-L-Pro template**, which can be synthesized by **Solid Phase Peptide Synthesis (SPPS)**. The systematical and methodical **study of the mode of action** of these potent new antimicrobial drugs is of first interest. The identification of new mechanisms for bacterial killing and new targets that could be useful in the fight against resistant bacteria and could prove to be the key to winning the war against these agressive pathogens.



Earlier efforts to discover new cyclic cationic AMPs that adopt stable β-hairpin structures led to the discovery of L27-11, which has a potent and selective antimicrobial activity against Pseudomonas sp. by targeting the outer membrane protein LptD, inhibiting its key role in outer membrane biogenesis. Driven by this discovery, the synthesis and screening of libraries of β-hairpin peptidomimetics, led to the discovery of **JB-95**, a novel AMP showing a potent activity against E. coli sp. Combining several techniques to methodically analyse the potential targets, we were able to determine a likely mechanism of action where the compound selectively disrupt the **outer membrane** in **Escherichia coli.**

ANTIMICROBIAL RESISTANCE

Facts*:

illnesses

2,049,442

deaths

23,000

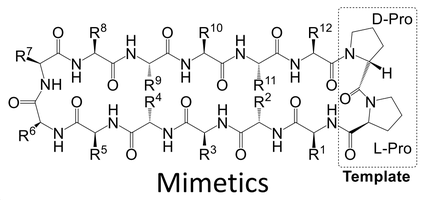
annual cost to the healthcare system

20,000,000,000 $

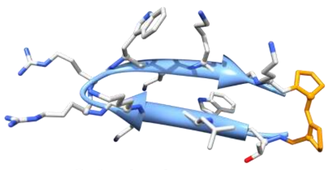
*CDC estimations for 2015 in the US

β-HAIRPIN PEMs: A SOURCE OF ANTIBIOTICS

Protegrin I
- Isolated from porcine leukocytes
- Broad spectrum antibiotic
- Lytic mechanism of action

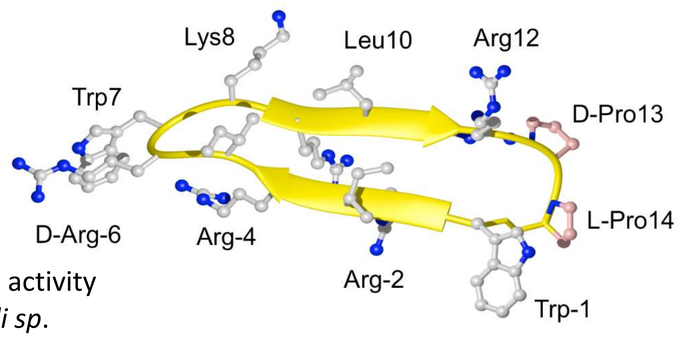


Mimetics
12-residues + template



β-hairpin PEM L27-11[3]
- 12 residues + template
- Shows potent antimicrobial activity against Gram-negative *Pseudomonas* sp.

Following L27-11, other β-hairpin PEMs with potent antimicrobial activity were discovered.

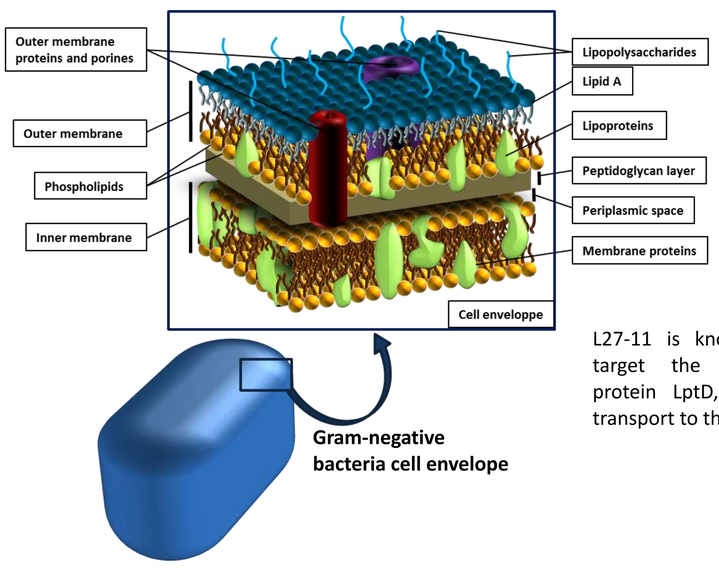


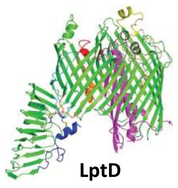
β-hairpin PEM JB-95[4]
- 12 residues + template
- Shows potent antimicrobial activity against Gram-negative *E. coli sp*.

PEM	Strains			% Hemolysis at 100 µg/mL
	E. coli ATCC 25922	*P. aeruginosa* PAO1	*S. aureus* ATCC 29213	
Protegrin I	0.25 - 0.5	nd	2	41
L27-11	>64	0.01	>64	
JB-95	0.25 - 0.5	32	2	1.6
999	0.25 - 0.5	1	16	2.1

Minimal inhibitory concentration (MIC) in µg/ml
(nd indicates not determined).

MECHANISM OF ACTIONS OF PEMs?





LptD

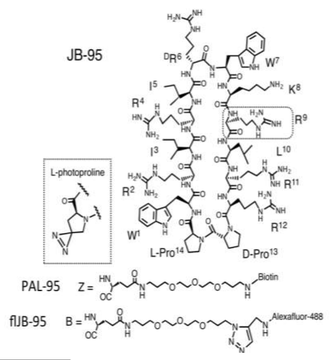
L27-11 is known to specifically target the outer membrane protein LptD, required for LPS transport to the cell surface[3].

- Because of their cationic, amphipatic nature, β-hairpins AMPs are known to bind efficiently to bacterial membranes
- Lots of β-hairpins AMPs cause membrane disruption due to electrostatic interactions but many other modes of action have been reported
- The specific interation of AMPs with bacterial membranes is of great interest to fight bacterial resistances and develop drugs with low toxicity

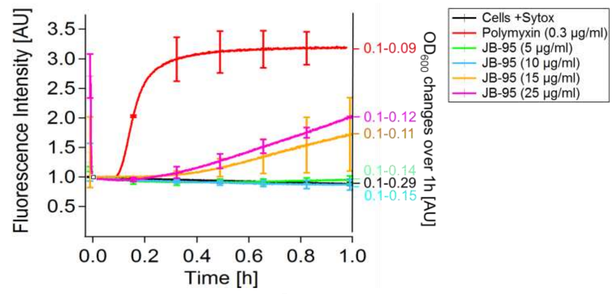
JB-95 A PROMISING ANTIMICROBIAL COMPOUND

Drugs	Strains			
	E. coli ATCC 25922	*E. coli* 2138/ 2151	*E. coli* 2143/ 2154	*E. coli* 3459/ 2150
JB-95	0.25	0.25	0.25	0.25
ceftriaxone	0.06	0.06	0.12	>64
ampicillin	2	>64	>64	>64
rifampicin	8	8	>64	8
erythromycin	>64	>64	64	>64

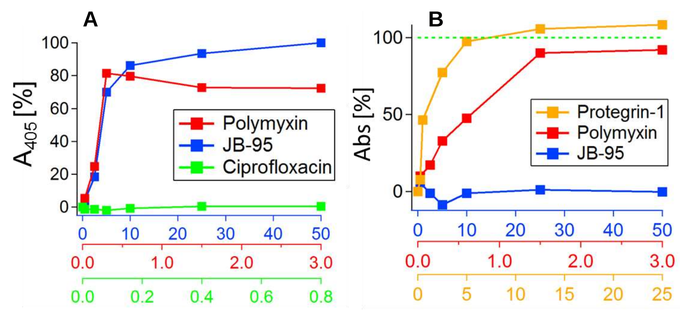
Minimal inhibitory concentrations (MIC) in µg/ml.



1. Cell envelope permeabilisation (Sytox Green uptake assay)

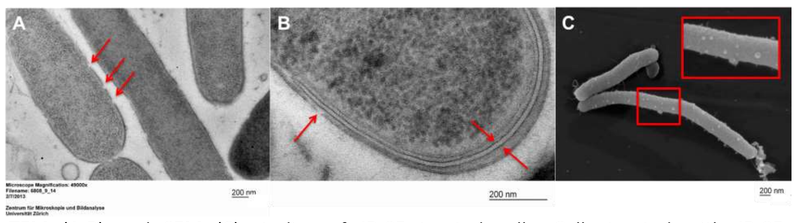


2. Effect on outer membrane (OM) or inner membrane (IM)

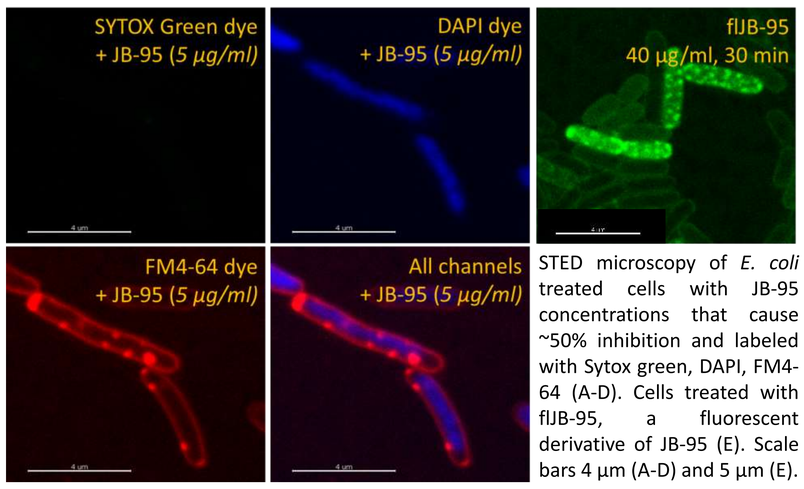


Dose response curves. β-lactamase assay (A) , β-galactosidase assay (B).

3. Bacterial cytological profiling and fluorescent AMP derivative imaging

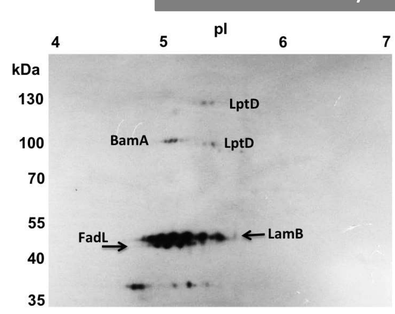


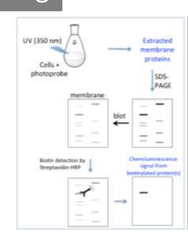
TEM (A,B) and SEM (C) studies of JB-95 treated cells. Cells treated with JB-95 concentrations that cause ~50% inhibition (5 µg/ml)



STED microscopy of *E. coli* treated cells with JB-95 concentrations that cause ~50% inhibition and labeled with Sytox green, DAPI, FM4-64 (A-D). Cells treated with fIJB-95, a fluorescent derivative of JB-95 (E). Scale bars 4 µm (A-D) and 5 µm (E).

4. Photoaffinity labeling



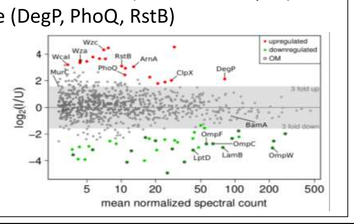


PAL-95 labeled OM proteins and biotin detection.
2D-SDS-PAGE (10%)

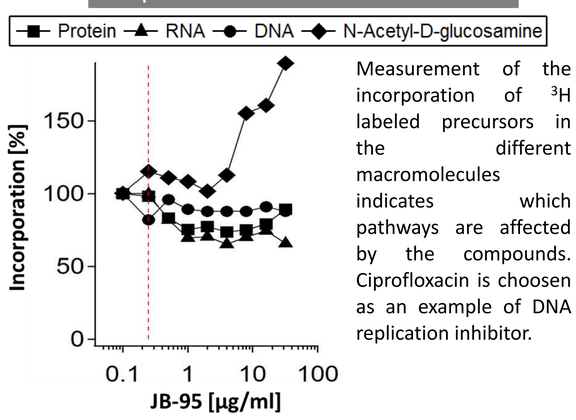
5. Proteomic studies

Upregulated proteins
→ Membrane maintenance proteins
- → capsule biosynthesis and export (Wzc, Wza, Gmd)
- → LipidA synthesis and modification (ArnA, ArnC, EptA)
- → OM stress response (DegP, PhoQ, RstB)

Downregulated proteins
→ β-barrel OM proteins
- → LptD
- → Porins
- → Transporters
- → NOT BamA



6. Incorporation of radiolabeled precursors



Measurement of the incorporation of ^3H labeled precursors in the different macromolecules indicates which pathways are affected by the compounds. Ciprofloxacin is choosen as an example of DNA replication inhibitor.

JB-95 disrupts specifically the OM (but not IM) by targeting clusters of OM proteins[4].

CONCLUSIONS

Cationic β-hairpin PEMs have shown their potential as antimicrobial drugs. The study of their mode of action is of great interest to identify new potential targets in order to fight resistant bacteria

In the case of JB-95, killing of *E. coli* bacteria seems to happen via a specific OM permeabilisation at key points that could be clusters of β-barrel proteins in the OM.

→IM appears to be unaffected
→OM targeting is of great interest to specifically kill Gram-negative bacteria
→Such a mechanism of action has not been, to our knowledge, reported for any synthetic or naturally occurring antibiotic

REFERENCES

1) Robinson, J. A. (2013), *J. Peptide Sci.* 19(3), 127–140.
2) Obrecht, D., et al (2012), *Drug Discovery Today: Technologies.* 9(1), e63-e69.
3) Srinivas, N., et al (2010), *Science.* 327, 1010-1013.
4) Urfer, M., et al. (2016), *J. Biol. Chem.* 291(4), 1921–1932.