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LPS is a Key Molecule in the Synergistic Effect of Temporins on Gram-Negative Bacteria

Maria Luisa Mangoni[1]*, Ludovica Marcellini H.G. [1], Donatella Barra[1], Maurizio Simmaco[1] and Yechiel Shai[2]

[1] Dipartimento di Scienze Biochimiche and Unità di Diagnostica Molecolare Avanzata, II Facoltà di Medicina e Chirurgia, Università La Sapienza, Azienda Ospedaliera S. Andrea, 00189 Roma, Italy; [2] Department of Biological Chemistry, The Weizmann Institute of Science, Rehovot, 76100, Israel
E-mail: marialuisa.mangoni@uniroma1.it

Introduction

The growing emergence of multidrug-resistant microbes has led to an urgent need for alternative approaches to treat infections. Naturally occurring antimicrobial peptides (AMPs), produced by almost all forms of life, are attractive candidates for therapeutic development. They represent key components of the immune system, a fast-acting weapon against invading pathogens, before the adaptive immune response is activated [1,2]. There is compelling evidence that, unlike conventional antibiotics, most gene-encoded AMPs interact with the bacterial membrane and increase its permeability as part of their killing mechanism. However, before reaching it, they need to cross the cell wall that, in Gram-negative bacteria, is surrounded by the lipopolysaccharide (LPS)-outer membrane, which forms a very efficient barrier against a variety of hydrophilic and hydrophobic molecules [3,4]. In Amphibia, temporins are the largest family of AMPs (more than 50 members) and are among the shortest ones with an alpha-helical structure in a membrane mimicking environment. Interestingly, up to ten isoforms have been found within the same specimen [5]. However, the biological significance of the coexistence of so many isoforms in a single living organism is not clear. We addressed this question using temporins A, B and L (Table 1) isolated from *Rana temporaria* skin secretion [6].

Table 1. Primary structure of temporins

Peptide	Sequence[a]	Net charge
TEMPORIN A	FLPLIGRVLSGIL-NH_2	+2
TEMPORIN B	LLPIVGNLLKSLL-NH_2	+2
TEMPORIN L	FVQWFSKFLGRIL-NH_2	+3

[a], basic residues are indicated in red

Results and Discussion

Recently, we have demonstrated that both temporins A and B, which are only weakly active on Gram-negative bacteria, can synergize when combined with temporin L, to overcome bacterial resistance imposed by the LPS protective layer [7]. We have shown that this effect is highly dependent on the type of LPS. More specifically, we compared the ability of temporins A, B, L and their combinations to inhibit the growth of *E. coli* O111:B4 and *E. coli* O26:B6, the latter endowed with a shorter LPS-carbohydrate chain.

The antimicrobial activity of temporins, tested by the checkerboard titration method, was also assayed against three different cell-wall defective mutant strains of *E. coli* D21, which have lost increasing amounts of sugar residues in the LPS backbone (*E. coli* D21e7; D21f1 and D21f2) [8]. Interestingly, there is a clear synergism for temporins A+L and B+L against *E. coli* O111:B4, whereas an indifferent interaction is found toward *E. coli* O26:B6 and the other bacterial strains (Table 2). This suggests that the loss of a synergistic effect between temporins on Gram-negative bacteria is parallel to the shortening of the LPS-polysaccharide chain length of the target microorganism.

Table 2. Antimicrobial activity of temporins on different E. coli strains

Bacterial strains	MIC (μM)			FIC index[a]		
	A	B	L	A+L	B+L	A+B
E. coli O111:B4	100	50	10	0.48	0.50	0.56
E. coli O26:B6	50	50	10	0.56	0.61	0.56
E. coli D21 e7	25	25	2,5	0.56	0.58	0.56
E. coli D21 f1	12.5	25	2.5	0.56	0.57	0.57
E. coli D21 f2	6	12	2.5	0.66	0.9	0.56

[a], FIC index: ≤ 0.5, synergism; 0.51- 4, no interaction; > 4, antagonism

To verify whether the behaviour of A+L and B+L on the two bacterial strains reflected a different organization of the peptides when in contact with different types of LPS, we analyzed the effect of purified LPS from *E. coli* O111:B4 and O26:B6 on temporins which were labelled, at their N-terminus, with rhodamine (rho). Notably, when rhodamine-labelled monomers are self-associated, the result is self-quenching of the emission fluorescence, whereas the proteolytic cleavage of the oligomer leads to

an increase of the fluorescent signal. A marked quenching of fluorescence, in a dose-dependent manner, was detected immediately after addition of each type of LPS to rho-temporin A and B, indicating oligomerization of the two peptides upon contact with LPS. In contrast, an increase of fluorescence was manifested in the case of rho-temporin L, suggesting a partial disaggregation of this temporin, regardless the type of LPS used.

To get insight into the reason for the loss of synergism of A+L and B+L on *E. coli* O26:B6, we studied the effect of temporin L on the oligomeric state of A and B, when in contact with the two types of LPS. For that purpose, we recorded the fluorescence of the labelled peptides, when used alone or in combination with equimolar concentrations of each unlabeled temporin, before and after the addition of LPS.

The results of these experiments have pointed out that temporin L inhibits the self-association of temporins A and B induced by LPS O111:B4 (as revealed by the slight fluorescence quenching). In contrast, this effect could not be detected in the presence of LPS O26:B6.

Overall this study reveals two important findings:

(i) temporins A and B are not active on Gram-negative bacteria, because of their oligomerization when in contact with the outer membrane. Thus, their larger size should interfere with the peptide translocation through the cell wall into the target cytoplasmic membrane;

(ii) the synergistic activity between temporins on Gram-negative bacteria is related to the ability of temporin L to assist temporins A and B to traverse the LPS layer by preventing their oligomerization. However, this effect is highly dependent on the LPS type (Fig. 1).

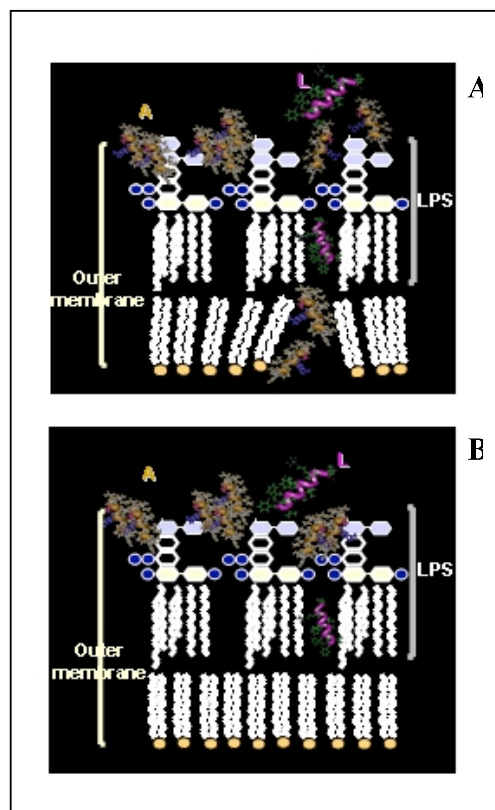

Fig. 1. Schematic representation of a possible mechanism for the synergism (panel A) or non-synergism (panel B) between temporins A and L, at the level of LPS O111:B4 (panel A) and O26:B6 (panel B) layer of Gram-negative bacteria.

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