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Review

Temporins and their synergism against Gram-negative bacteria and in lipopolysaccharide detoxification

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ABSTRACT

Ribosomally synthesized antimicrobial peptides (AMPs) represent an essential component of the ancient and non-specific innate immune system in all forms of life, with the primary role of killing infectious microorganisms. Amphibian skin is one of the richest storehouses for them. Each frog species produces its own set of peptides with up to 10 isoforms, as in the case of the species *Rana temporaria*. Nowadays, human health is facing two major threats: (i) the increasing emergence of resistant pathogens to one or more available drugs, and (ii) the onset of septic shock, which is associated with the release of lipopolysaccharide (LPS) from the cell walls of Gram-negative bacteria, particularly upon antibiotic treatment. AMPs are considered as potential new anti-infective compounds with a novel mode of action, because many of them can kill bacteria and, at the same time, neutralize the toxic effects of LPS. Recent studies have suggested that the production of large number of structurally similar AMPs within the same animal is a strategy used by nature to increase the spectrum of antimicrobial activities, by using combinations of the peptide's isoforms. The biological rationale for their coexistence within the same organism is discussed. In addition, the distinctive and attractive synergistic effects of temporins in both antimicrobial and anti-endotoxin activities are reviewed, along with their plausible underlying molecular mechanism.

Contents

Abbreviations: AMP, antimicrobial peptide; CD, circular dichroism; DPC, dodecylphosphocholine; FITC, fluorescein-isothiocyanate; IC_{50}, peptide concentration causing 50% inhibition of microbial growth; IM, inner membrane; LPS, lipopolysaccharide; MIC, minimal inhibitory concentration; NMR, nuclear magnetic resonance; OM, outer membrane; rho-temp, rhodamine-labeled temporin; SDS, sodium dodecyl sulfate

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1. Amphibian antimicrobial peptides — discovery and function

The amphibian skin is one of the richest sources of biologically active peptides displaying pharmacological as well as antimicrobial properties [1–4]. Antimicrobial peptides (AMPs) represent the major component, with sizes ranging from 10 to 46 amino acid residues and constitute the

Table 1
Primary structure of some temporins.

Temporin	Frog species	Sequence	Ref.
ALa	*Amolops loloensis*	FLPIVGKLLSGLSGLL–NH_2	[87]
1ARa	*Lithobates areolatus*	FLPIVGRLISGLL–NH_2	[88]
1AUa	*Rana aurora*	FLPIIGQLLSGLL–NH_2	[89]
1BYa	*R. boylii*	FLPIIAKVLSGLL–NH_2	[90]
1Ca	*L. clamitans*	FLPFLAKILTGVL–NH_2	[91]
1Cb		FLPLFASLIGKLL–NH_2	[91]
1Ec	*Pelophylax lessonae*	FLPVIAGLLSKLF–NH_2	[92]
1DRa	*R. draytoni*	HFLGTLVNLAKKIL–NH_2	[31]
1DRb		NFLGTLVNLAKKIL–NH_2	[31]
1Ga	*L. grylio*	SILPTIVSFLSKVF–NH_2	[93]
1Gb		SILPTIVSFLSKFL–NH_2	[93]
GH	*Hylarana güntheri*	FLPLLFGAISHLL–NH_2	[94]
1HKa	*L. heckscheri*	SIFPAIVSFLSKFL–NH_2	[95]
1Ja	*R. japonica*	ILPLVGNLLNDLL–NH_2	[96]
1La	*R. luteiventris*	VLPLISMALGKLL–NH_2	[19]
1Lb		NFLGTLINLAKKIM–NH_2	[19]
1Lc		FLPILINLIHKGLL–NH_2	[19]
1M	*R. muscosa*	FLPIVGKLLSGLL–NH_2	[8]
1Oa	*R. ornativentris*	FLPLLASLFSRLL–NH_2	[97]
1Od		FLPLLASLFSGLF–NH_2	[97]
1OLa	*L. okaloosae*	FLPFLKSILGKIL–NH_2	[95]
1OLb		FLPFFASLLGKLL–NH_2	[95]
1P	*L. pipiens*	FLPIVGKLLSGLL–NH_2	[19]
1PLa	*L. palustris*	FLPLVGKILSGLI–NH_2	[98]
1PRa	*R. pirica*	ILPILGNLLNGLL–NH_2	[99]
1SHa	*Pelophylax saharicus*	FLSGIVGMLGKLF–NH_2	[32]
1SHb		FLPIVTNLLSGLL–NH_2	[32]
1SPa	*L. septentionalis*	FLSAITSILGKFF–NH_2	[100]
1SPb		FLSAITSLLGKLL–NH_2	[100]
1TGa	*R. tagoi*	FLPILGKLLSGIL–NH_2	[101]
1Ta	*R. temporaria*	FLPLIGRVLSGIL–NH_2	[20]
1Tb		LLPIVGNLLKSLL–NH_2	[20]
1Td		LLPIVGNLLNSLL–NH_2	[20]
1Tg		FFPVIGRILNGIL–NH_2	[20]
1Th		LSP–––NLLKSLL–NH_2	[20]
1Tl		FVQWFSKFLGRIL–NH_2	[20]
1Va	*L. virgatipes*	FLSSIGKILGNLL–NH_2	[102]
1Vb	*Odorrana versabilis*	FLPLVGKILSGLI–NH_2	[103]

Basic and acidic residues are indicated by red and blue letters, respectively. Gaps (-) are inserted to maximize identities. The species names of frogs and peptides' abbreviations reflect the new nomenclature proposed by J.M. Conlon [25].The sequence of temporins ALa, 1ARa, 1AUa, 1BYa, 1Ca/Cb, 1Ec, 1Ga/Gb, GH, 1HKa/1OLa/1OLb, 1Ja, 1Oa/Od, 1PLa, 1PRa, 1SPa/SPb, 1TGa, 1Va, 1Vb are reported in references [87], [88], [89], [90], [91], [92], [93], [94], [95], [96], [97], [98], [99], [100], [101], [102], [103], respectively.

effector molecules of innate immunity [5]. These AMPs are produced by dermal glands and released into the skin secretion, in a holocrine manner, upon stress or injury, as a result of contraction of myocytes surrounding the glands [3]. All together, frog skin represents a good model system to investigate the *in vivo* role of AMPs in vertebrates [6], and various reports have highlighted their functional importance in host protection against pathogenic microorganisms [7,8]. All frog species are endowed with their own unique set of AMPs, constituting families of 2–100 closely related members [9] (see also the review of J.M. Conlon, the same issue), but very little is known about the biological significance of multiple forms of a prototypic peptide sequence within the same organism. The principal classes of structurally similar amphibian AMPs encompass bombinins and bombinins H from the European toads *Bombina variegata* and *Bombina orientalis* [10–13] (see also a chapter in this issue); magainins from the African clawed frog *Xenopus laevis* [14], dermaseptins, originally isolated from the South American arboreal frog *Phyllomedusa sauvagii* [15,16], and those from the *Rana* genus (e.g., brevinins, ranalexins, ranatuerins, esculentins and temporins) [17–21]. Recent studies have demonstrated that several amphibian AMPs synergize with each other in the antimicrobial activity [7,22,23]. This review summarizes relevant details in these studies and shows how nature provided each animal with a specific and fast-acting defence system that also allows synergism between several peptide combinations. More specifically, this review focuses on the unique synergistic effects of some temporins (1Ta, 1Tb and 1Tl) in inhibiting both Gram-negative bacterial growth and the toxic effect of lipopolysaccharide (LPS). Besides their scientific importance, studies along this line should open additional avenues for the design of new anti-infective AMPs.

2. Temporins

2.1. Biosynthesis and structural characterization

Temporin-like peptides were initially identified in methanol extracts of the skins of the Asian frog *Rana erythraea* and the European hybrid frog *Rana esculenta* [24] (reclassified as *Pelophylax lessonae*/*ridibundus* [25]). They were described as *Vespa*-like peptides, because of their sequence similarity to chemotactic peptides isolated from the venom of wasps of genus *Vespa* [26]. Afterwards, in 1996, 10 structurally related peptides, endowed with antimicrobial properties, were discovered by Simmaco et al. [20]. They were isolated from skin secretions of mild-electrically stimulated specimens of the European red frog *Rana temporaria* and were properly designated as temporins, from A to L [(now re-named temporins 1Ta–1Tl, according to the new nomenclature proposed by J.M. Conlon [25], see Table 1)]. New members were then identified in skin secretions of other ranid frogs of both North American and Eurasian origin (Table 1), enlarging temporin family to more than 100 different isoforms [9]. Although some of them contain up to 17 amino acids, temporins are among the smallest AMPs (10–14 residues long) found in nature to-date and with a low net positive charge at neutral pH (0 to +3), due to the presence of only 1 or 2 basic residues in their sequence. This low cationic character is unique for them and is an exception compared to known AMPs from other sources. Temporins are synthesized as precursors (Fig. 1) bearing a strictly conserved N-terminal domain and an acidic propiece ending with the pair Lys-Arg [20]. These structural features are similar to those present in the precursors of other AMPs from the *Rana* genus [18,27] and the *Phyllomedusinae* subfamily [28]. A single copy of the mature peptide is located at the C-terminus of the acidic prosequence, and a Gly residue, which flanks the carboxyl-terminus of the mature peptide, serves as its

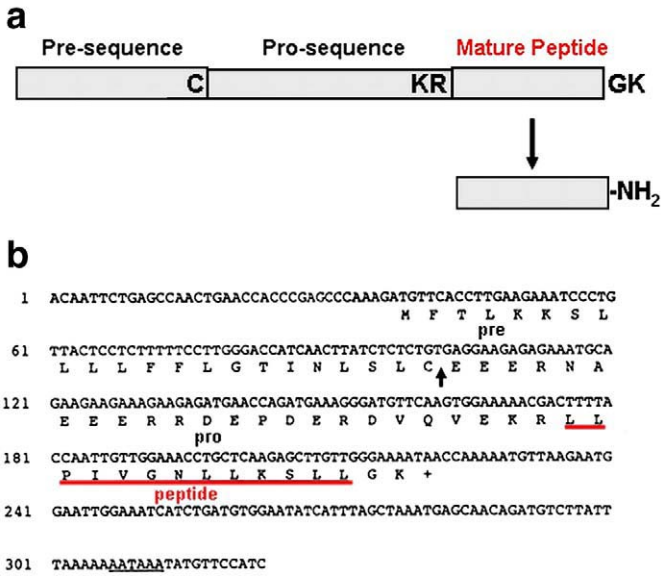

Fig. 1. (a) Schematic representation of the precursors encoding for temporins. C and KR indicate the highly conserved amino acid residues ending the pre- and pro-peptide sequence, respectively. The G residue flanking the C-terminus of the mature peptide serves as its amide donor. (b) Nucleotide sequence and deduced primary structure of the pre–pro-peptide of temporin-1Tb [20]. The sequence of the mature peptide is underlined in red; in black the polyadenylation site at the 3′-end. Vertical arrow denotes the probable site of cleavage by the signal peptidase.

amide donor. In fact, unlike the majority of Ranidae AMPs that contain a C-terminal heptapeptide ring, stabilized by a disulphide bridge, temporins are amidated at their carboxyl end (Table 1), as a result of a post-translational enzymatic reaction, where the penultimate Gly residue in the pro-peptide (Fig. 1) acts as a substrate for peptidyl-glycine α-amidating monooxygenase [29].

2.2. Target cell selectivity

Functional studies have pointed out that despite their small size and low net positive charge (+2 for most isoforms), temporins act efficiently and rapidly against Gram-positive bacteria, including clinical isolates that are resistant to conventional antibiotics (see the review of J.M. Conlon, the same issue). Their Minimal Inhibitory Concentration (MIC) ranges from 2.5 to 20 μM [9,30]. Interestingly, other isoforms with similar sequences, namely, temporin-1Tl and temporin-1Dra from *R. temporaria* and *Rana draytonii*, respectively, are highly active also against Gram-negative bacteria, fungi, and erythrocytes [31,32]. Furthermore, protozoa and viruses have been reported to be targets of this family of AMPs. Indeed, temporins-1Ta and 1Tb from *R. temporaria* and temporin-1Sa from the North African ranid *Pelophylax saharica* [33,34] exert potent antiparasitic activity, *in vitro*, on promastigotes (insect stage) and amastigotes (mammalian stage) of *Leishmania* genus. In particular: (i) the isoforms Ta and Tb induce complete inhibition of viability of *Leishmania donovani* and *L. pifanoi* (causing the visceral and cutaneous leishmaniasis, respectively), with severe damage to the plasma membrane [35]; (ii) temporin-1Sa displays an antiparasitic effect (IC_{50} ~ 20 μM) on promastigotes and axenic amastigotes of *Leishmania infantum* [33]. Temporin-1Ta also reduces the infectivity of channel catfish virus and frog virus 3 [36].

Noteworthy to recall that in addition to antimicrobial and antiviral properties, a significant number of amphibian AMPs were shown to have selective cytotoxic activity against neoplastic cells [37]. In addition to temporin-1Tl, which is active on human erythroleukaemic and human cutaneous T lymphoma cells, other small-sized AMPs from frog skin have been found to display a lytic effect on tumor cells. Among these, the aurein 1.2 (13 residues long) from the Australian bell frog *Litoria raniformis* [38]; the citropin 1.1 (16 residues long) from the tree frog *Litoria citropa*, [39]; some members of the gaegurin family [40]; magainins, from the African toad *X. laevis* and their derivatives [41].

The molecular mechanism governing cell selectivity of temporins, as well as of many other AMPs, is not clear. However, recent studies have suggested that cell specificity is dictated by both physico-chemical characteristics of a peptide (sequence, charge distribution, oligomeric state, amphipathicity and helicity), and by the type of the target cell surface and its metabolism. Cell specificity can also be controlled by combinations of AMPs, as will be outlined below, for temporins-1Ta, Tb and Tl.

3. Temporins-1Ta, Tb and Tl

The isoform temporins-1Ta, 1Tb and 1Tl from *R. temporaria* skin secretion are among the most studied temporin peptides for their mechanism of action on both intact bacteria and artificial systems.

3.1. Mode of action on intact bacteria

Regardless of the precise mode of action, the effect of AMPs in general depends upon their interaction with the microbial membrane [42]. The first step in this interaction is the electrostatic attraction between the cationic peptide and the negatively charged components of the cell envelope. These include the phosphate groups within the LPS in the outer membrane (OM) of Gram-negative bacteria, or the lipoteichoic acid (LTA) on the surface of Gram-positive bacteria. The inner leaflet of the OM in Gram-negative bacteria is composed of phospholipids resembling those of the cytoplasmic membrane, whereas the outer leaflet contains mostly LPS, which encompasses three parts: (i) a lipid A region consisting of a disaccharide of phosphorylated glucosamines attached to six or seven saturated fatty acids chains, some of which are hydroxylated; ii) a hydrophilic O-antigenic domain, made of repeating saccharide units, varying among different bacterial species, which protrude into the surrounding medium [43,44]; and (iii) an oligosaccharide core, which connects the two portions.

Initially, AMPs traverse the LPS layer in a self-promoted uptake process driven by hydrophobic interactions [42], and subsequently reach the cytoplasmic inner membrane (IM). With regard to temporins, most studies have revealed that the killing process is rapid and concomitant with the permeation of the IM [9,35]. Furthermore, differing from those AMPs that interfere with intracellular functions without destabilizing the bacterial membrane, temporin-1Tl, for example, perturbs the membrane even at concentrations that are significantly lower than the MIC [45]. However, in contrast with many natural and *de novo* designed AMPs, temporin-1Tl does not lyse bacteria, but rather causes them a ghost-like shape with a deep roughening of their surface and a collapse of the cellular structure (Fig. 2).

3.2. Conformation and orientation within model membranes

Most of the temporins, like other AMPs derived from frog skin, adopt mainly a random coil conformation in aqueous solution. However, there are exceptions such as the isoform Tl, which adopts an α-helical

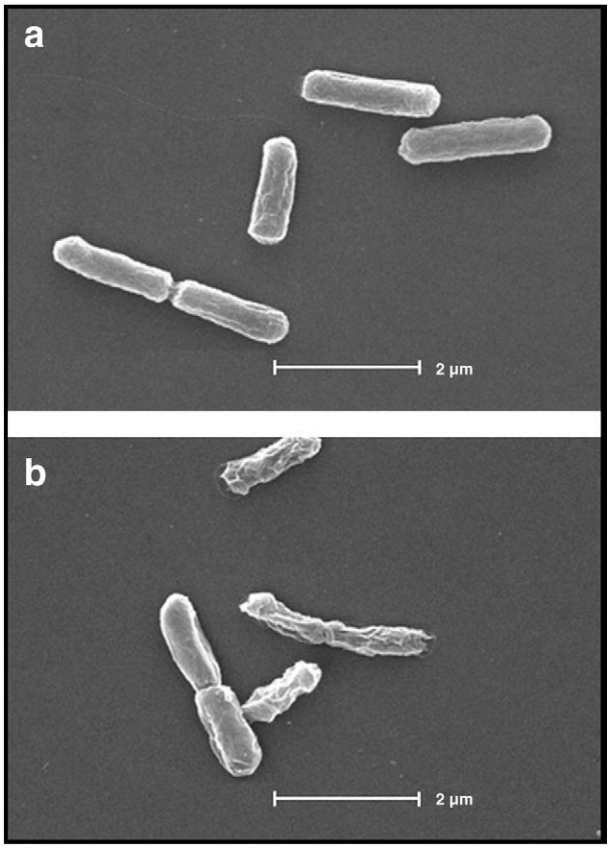

Fig. 2. Scanning electron microscopy of temporin-1Tl-treated *E. coli* D21. (a) Control bacteria; (b) bacteria after peptide treatment for 15 min at a lethal concentration (50 μM with 1×10^7 cells). Each figure has been magnified × 15,000. A prominent collapse of the cell structure and a deep roughening of the cell surface are visible after microbial exposure to the peptide.

structure under this condition, probably due to its oligomerization. As the majority of AMPs, temporins form an amphipathic structure in a biological membrane or in a membrane-mimetic environment. In this context, temporins-1Ta and Tl have been analyzed by spectroscopic techniques (CD and NMR) and molecular dynamics simulation, in the presence of sodium dodecyl sulfate (SDS) and dodecylphosphocholine (DPC) micelles, mimicking the negatively charged and zwitterionic membranes, respectively. In SDS, the peptides are located at the micelle-water interface, whereas in DPC, they prefer a location perpendicular to the micellar surface, with the N-terminus embedded in the hydrophobic core. However, there are discrepancies between the two peptides: temporin Tl has a higher propensity, compared with Ta, to form α-helical structures in both membrane-mimetic systems, as well as a higher propensity to penetrate the lipid vesicles.

The loss of α-helical structure along the entire sequence of temporin-1Ta in DPC (Fig. 3a), correlates with the peptide's inability to deeply insert into the hydrophobic core of this type of vesicles, which can be the reason for the weak lytic effect of Ta on red blood cells. Such a result is in contrast with the strong haemolytic activity of Tl and its profound penetration into DPC micelles, where the α-helicity of the peptide extends from the N- to the C-terminal amino acid (Fig. 3a). In SDS micelles, both temporins prefer a distribution at the lipid-water interface, parallel to the micellar surface (as also found for the temporins from *P. saharica* [34]). In addition, they have a lower tendency to form an α-helical structure: in Ta, it encompasses the central residues 6–9, and in Tl, residues 3–11 (Fig. 3b). Studies on their topological orientation suggest the formation of a "dynamic peptide-lipid supramolecular complex". Based on this, the peptide could bind the negatively charged lipids and form transient pores that increase in size as the peptide-to-lipid molar ratio also increases [46]. When the peptide-to-lipid ratio is very high, this results in detergent-type micellization of the membrane, as described by the "carpet" model [47].

A possible explanation for the different orientations of temporins in DPC versus SDS may relate to the balance between the net positive charge and the hydrophobicity of a particular peptide. Analogs with high hydrophobicity can bind electrostatically to the negatively charged head groups of SDS and would be kept on the surface. However, in zwitterionic DPC they will have sufficient hydrophobicity that will allow them to penetrate into the hydrophobic core of the micelles. In contrast, peptides with low hydrophobicity, will bind electrostatically to the surface of SDS, and will not have sufficient hydrophobicity to penetrate into the DPC micelles.

Overall, our data led to the assignment of different molecular mechanisms underlying the antimicrobial and haemolytic activities of these temporins [46]: a "carpet" mechanism and the formation of a "barrel-stave" pore in negatively charged and zwitterionic membranes, respectively (Fig. 3). Interestingly, a general "carpet-like" mode of interaction of AMPs when bound to negatively charged membranes has been reported. According to this mechanism, the peptides are located first on the surface of phospholipid bilayers until a local threshold concentration has been reached, which further lead to the formation of local defects in them [47]. The list of these AMPs includes cathelicidin LL-37 [48]; the granulysin [49]; the tachyplesin [50] and polyphemusin [51].

Note that temporins are too short (only 13 amino acids) to span the entire membrane and to act via the "barrel-stave" mechanism in zwitterionic membranes. This implies that they might form dimers head-to-tail or via other orientations; however, this hypothesis needs to be further investigated.

Although temporins differ from most cationic AMPs by having only 1–2 positively charged amino acids, they are able to disrupt anionic membranes to a similar extent of that of most highly positively charged AMPs, the reason for which is yet not known.

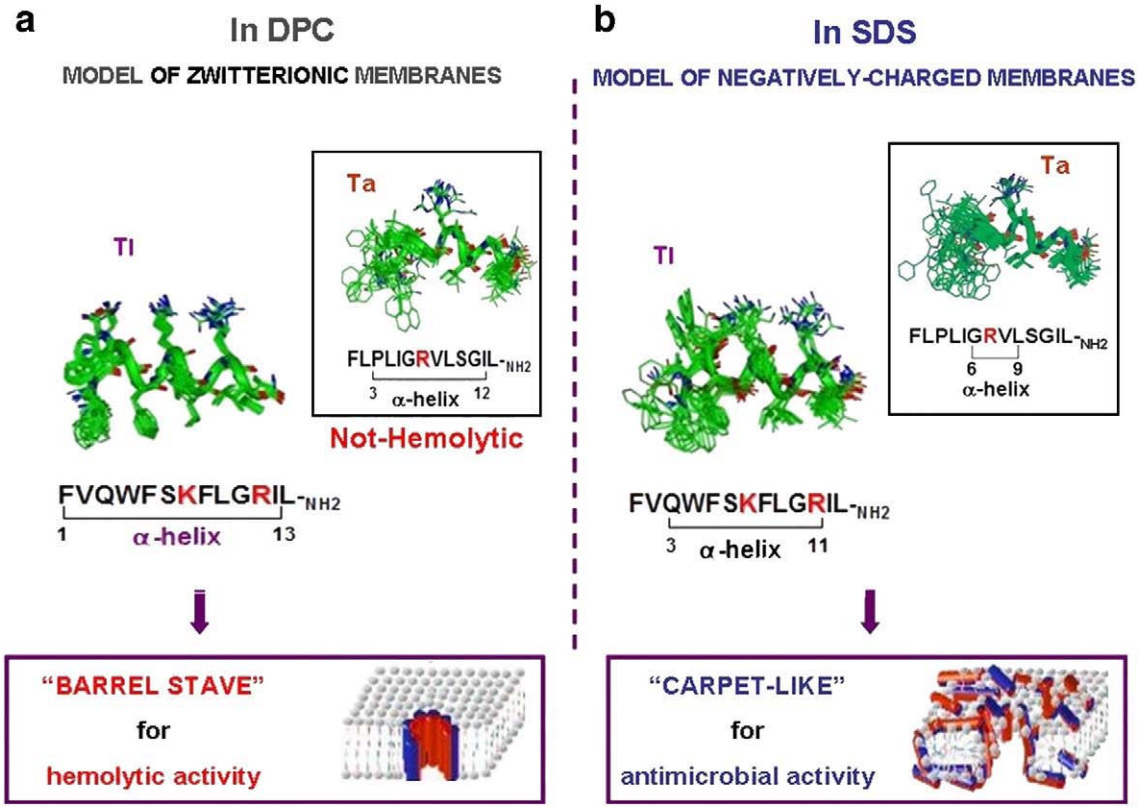

Fig. 3. Structure of temporins-1Ta and Tl in DPC (a) and SDS (b) micelles, as determined by NMR studies [46]. The backbone of the lowest energy structure in each peptide is shown as a ribbon. Heavy atoms are shown with different colours (carbon, green; nitrogen, blue; oxygen, red). The molecular mechanisms proposed for the haemolytic and antibacterial activities of the temporins are schematized inside boxes. The red and blue colours of the peptide (cylinder) indicate its hydrophilic and hydrophobic regions, respectively.

4. Synergism between frog's AMPs: general aspects

The finding of a synergism between AMPs is not surprising, considering the simultaneous presence of several isoforms within the same animal. The occurrence of synergism was first observed in the dermaseptin family, where the antimicrobial action of combined dermaseptins was higher than the sum of individual peptides [7]. As indicated in the previous paragraphs, Gram-negative bacteria are resistant to temporins-1Ta and Tb; however, a combination of each of them with a sub-inhibitory concentration of temporin-1Tl overcomes resistance [52]. Surprisingly, this synergistic effect is more pronounced against *Aeromonas hydrophila*, which is an opportunistic pathogen living in healthy frogs, but can cause high mortality in amphibian populations, leading to diseases such as the natural outbreaks of "red leg" [8]. Note that this bacterium is not susceptible to several AMPs from frog skins, e.g., magainin I, magainin II, PGLa, ranalexin, and dermaseptin [8] and to several conventional antibiotics [53]. In addition, in humans, this microbe is responsible for a variety of infections (e.g., gastroenteritis), especially in immunocompromised individuals [54,55]. These examples indicate that the production of a large number of structurally similar AMPs, within a single frog species, can protect the animal from a wider range of microorganisms. Because of the continuous growth of resistant clinical isolates to currently used antibiotics, studies aimed at the development of strategies for the production of new non-single-peptide-based antimicrobials are also in progress. Nowadays, a number of AMPs reached the phase of clinical evaluation and potential marketing as novel anti-infective compounds [56]. Furthermore, the requirement of a lower concentration for each component of the therapeutic formulation would reduce the problems associated with its production-cost and side effects on patients.

Importantly, antibiotic therapy against Gram-negative bacterial infections is often accompanied by the release of LPS, also known as endotoxin. The immune system has evolved to recognize LPS as a pathogen-associated molecular pattern (PAMP). LPS is a potent inducer of the innate immune system and is primarily responsible for lethality in sepsis that afflicts about 600,000 individuals in the United States alone, annually [57]. Upon its release, LPS is recognized by mononuclear phagocytes (monocytes and macrophages), which are part of the innate immunity of the host, and it activates them. This enhances their phagocytic activity and significantly raises the secretion level of pro-inflammatory cytokines, e.g. tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), and others [58–64]. Pro-inflammatory cytokine production is essential for initiating the local inflammatory response. However, an excess of LPS, resulting from the invasion of Gram-negative bacteria or as a consequence of intensive antimicrobial chemotherapy [65,66], causes prolonged activation of the immune cells, with an unbalanced systemic secretion of cytokines. This can rapidly give rise to the onset of septic shock syndrome, characterized by endothelial damage, loss of vascular tone, coagulopathy, fever, hypotension and multiple organ failure, which, in extreme cases, leads to death [67,68]. An attractive strategy to combat sepsis is to develop compounds that would sequester LPS, thereby blocking its interactions with the serum and cellular receptors [69,70]. AMPs represent good candidates for this purpose. Indeed, unlike classical antibiotics, several AMPs possess dual functions: they kill bacteria and neutralize the effect(s) of LPS, although the exact mechanism is not yet well understood [52,71–73]. In line with this, a combination between AMPs and drugs, or two different AMPs, may enhance antibacterial/anti-endotoxin activities and hamper the emergence of resistance. The following paragraphs summarize recent studies on the synergistic effects within two pairs of temporins (Ta + Tl and Tb + Tl), in two functions: the growth inhibition of Gram-negative bacteria and the neutralization of LPS-activated macrophages, along with a plausible molecular mechanism.

4.1. Synergism between temporins in antimicrobial activity and the underlying molecular mechanism

Members of the temporins family, namely, 1Ta and 1Tb, were found to display a synergistic effect against Gram-negative bacteria, when each was combined with temporin-1Tl (Fig. 4). Interestingly,

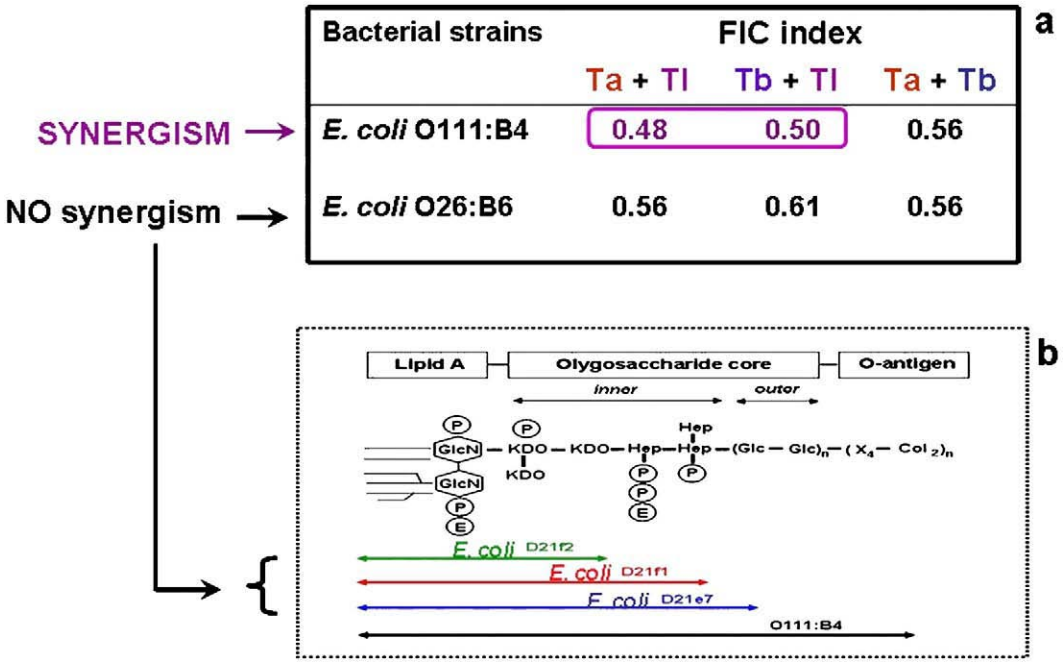

Fig. 4. (a) Fractional inhibitory concentration index (FIC) of temporins Ta + Tl, Tb + Tl and Ta + Tb against the two Gram-negative strains *E. coli* O111:B4 and O26:B6. The fractional inhibitory concentration (FIC) index for combinations of two peptides is calculated according to the following equation: $\sum(FIC_A + FIC_B)/n, = \sum(A/MIC_A + B/MIC_B)/n$, where *A* and *B* are the MICs of drug A and drug B in the combination, MIC_A and MIC_B are the MICs of drug A and drug B alone, FIC_A and FIC_B are the FICs of drug A and drug B, and *n* is the number of wells per plate used to calculate the FIC. The FIC indices are interpreted as follows [86]: FIC ≤ 0.5, synergy; 0.5 < FIC < 1, additivity; 1 ≤ FIC < 4, indifference; FIC ≥ 4, antagonism. (b) Schematic representation of LPS molecules in different *E. coli* strains (D21 e7, D21 f1, D21 f2 and O111:B4). Glc, glucose; Col, colitose; GlcN, N-acetylglucosamine; KDO, ketodeoxyoctonic acid; P, phosphate; E, ethanolamine; X, neutral sugar residue [80].

the synergistic effect was observed with bacterial strains that had long LPS-carbohydrate chains (e.g. *Escherichia coli* O111:B4) (Figs. 4 and 5), but not with *E. coli* O26:B6, which has a shorter LPS-carbohydrate region (Figs. 4 and 5), or with cell-wall defective mutant strains of *E. coli* D21 (e.g., *E. coli* D21 e7; D21f1 and D21f2). The latter have lost increasing amounts of sugar residues in their LPS backbone (Fig. 4). Fluorescence spectroscopy was then used to elucidate the underlying molecular mechanism and to verify whether the different behaviour of Ta + Tl and Tb + Tl on *E. coli* O111:B4 and O26:B6 could reflect different organization of the peptides, when in contact with the two types of LPS. In this assay, rhodamine-labeled temporins (rho-temp) were synthesized and examined for their interaction with purified LPS O111:B4 and O26:B6. When rhodamine-labeled monomers are self-associated, the outcome is self-quenching of the emission fluorescence. However, enzymatic digestion of the rhodamine-labeled peptide should result in the recovery of fluorescence, owing to the dissociation of the oligomers. The addition of LPS to rho-temps Ta and Tb induced a marked and dose-dependent quenching of fluorescence, revealing that both types of LPS triggered the oligomerization of the two peptides [22]. These findings are in line with recent reports revealing that self-assembly of AMPs causes a dramatic reduction in their antimicrobial activity. This is because of the larger size of the oligomers, compared to that of monomers, which prevents the peptide to diffuse through the cell wall [52].

Differing from temporins-1Ta and Tb, an increase in fluorescence was manifested upon addition of LPS to rho-temp Tl, pointing out a partial disaggregation of this peptide, regardless of the type of LPS used. This should make it easier for temporin-1Tl to traverse the LPS leaflet into the target IM [52], and is in agreement with its strong activity on Gram-negative bacteria. Noteworthy, the data from the fluorescence studies fit with the results obtained by circular dichroism in LPS micelles, showing that only temporin-1Tl could adopt an α-helical structure [22]. Since the hydrophobic environment of a membrane induces an α-helix structure to amphipathic peptides, the lack of helicity in Ta and Tb might be due to their binding to those portions of LPS facing the solution, rather than to those in proximity with the inner and more hydrophobic lipid moiety of LPS [22]. This is because both Ta and Tb form oligomers that cannot penetrate easily into the hydrophobic core of LPS.

The next step was to check the effect of unlabeled temporins on the rhodamine-labeled isoforms, upon addition of LPS from both types of bacteria. The presence of unlabeled temporins-1Ta or Tb did not affect the fluorescence quenching of the labeled peptides. In contrast, when rho-temps Ta and Tb were mixed with unlabeled temporin Tl (Fig. 5), a considerable lower quenching of fluorescence was obtained after contact with LPS O111:B4 (Fig. 5, right panels), but not with LPS O26:B6 (Fig. 5, left panels). These results suggest that temporin-1Tl hampers the self-association of Ta and Tb induced by LPS O111:B4 and that this property is lost with LPS with a short sugar chain.

Overall, these studies have revealed two important findings:

(i) Temporins-1Ta and Tb are not active on Gram-negative bacteria, because they oligomerize once bound to bacterial LPS, which makes it hard for them to cross the OM and reach the target cytoplasmic membrane.

(ii) The synergistic activity between temporins Ta + Tl and Tb + Tl towards Gram-negative bacteria takes place on the OM, and is related to the ability of Tl to prevent the self-association of Ta and Tb, and hence to assist them to traverse the cell wall. Such a statement is also supported by the loss of synergism against both *E. coli* spheroplasts, which are devoid of a cell wall and Gram-positive bacteria lacking the OM. Mechanistically, it is

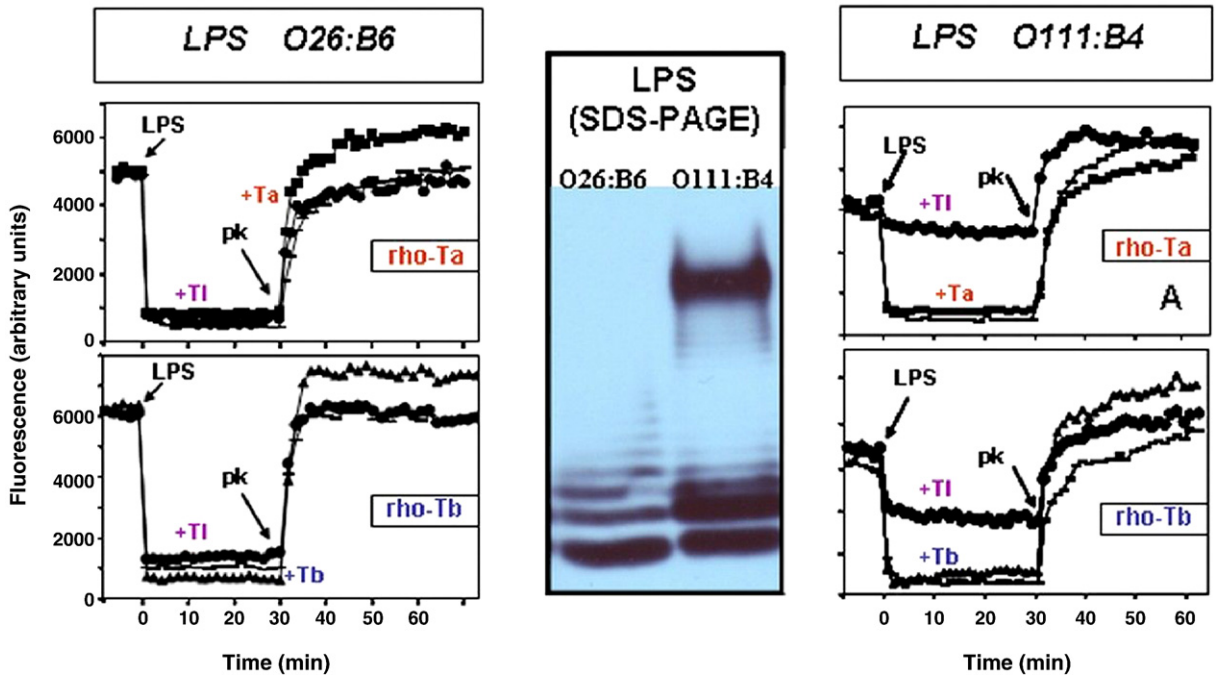

Fig. 5. The effect of unlabeled temporins on the fluorescence of rho-temp Ta (upper panels) and rho-temp Tb (lower panels) in the presence of LPS from *E. coli* O26:B6 (left panels) and O111:B4 (right panels). The labeled peptides were incubated with the same concentration of unlabeled temporin-1Ta (+Ta, ■), temporin-1Tb (+Tb, ▲), and temporin-1Tl (+Tl, ●). Once the fluorescence reached a constant value, LPS was added (first arrow, $t = 0$) and changes in fluorescence were monitored ($\lambda_{exc} = 485$ nm, $\lambda_{ems} = 590$ nm) and plotted as arbitrary units. The rhodamine-labeled peptide-to-LPS molar ratio was 1:4. The second arrow indicates the addition of proteinase-K (pk, 80 μg/ml) to all samples. Control (−) is given by the fluorescent peptide without the addition of unlabeled temporins. The results obtained with unlabeled temporin-1Tb or Ta in the upper or lower panels respectively, are not reported, since they were similar to those shown when unlabeled temporin-1Ta or Tb was used instead, in the respective cases. When rho-temp Ta or Tb were mixed with unlabeled Tl, a less fluorescence quenching than that caused with unlabeled Ta or Tb, was noted upon addition of LPS O111:B4 (right panels), but not with LPS O26:B6 (left panels). The values represent the mean of triplicate samples with S.D. values not exceeding 2% from a single experiment, representative of three different experiments [22,80]. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the two types of LPS, using a gel containing 12% acrylamide and 0.5% SDS is reported in the middle. As indicated by the different molecular weight bands, LPS from *E. coli* O26:B6 has shorter polysaccharide chains with respect to LPS O111:B4.

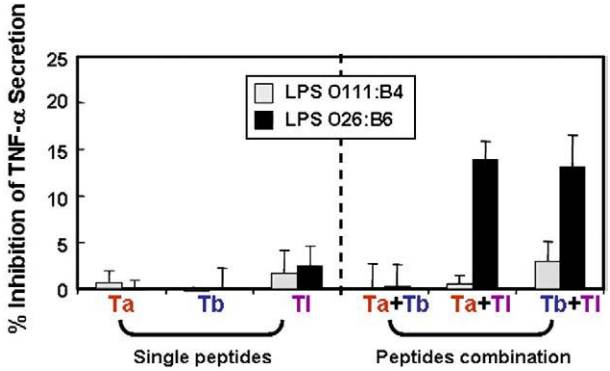

Fig. 6. The effect of temporins on TNF-α secretion by LPS-activated macrophages. RAW 264.7 macrophages were stimulated with LPS (10 ng/ml) derived from *E. coli* O111:B4 or O26:B6 in the presence of 2.5 μM temporins-1Ta, Tb and Tl alone or in combinations of two (Ta + Tb, Ta + Tl or Tb + Tl, 2.5 + 2.5 μM). The percentage of inhibition of TNF-α release was normalized to that of macrophages stimulated with LPS without peptides (0% inhibition). The results are the average of three independent experiments; each experiment was performed in duplicate. Error bars are standard deviations (taken from Ref. [80]).

possible that Tl binds Ta or Tb to form heteroligomers that can diffuse more easily across the LPS leaflet. Alternatively, temporin-1Tl could modify the structure of LPS molecules and, as a consequence, inhibit the LPS-induced self-assembly of Ta and Tb. Noteworthy, it has been found that the synergism of temporins in overcoming bacterial resistance, owing to the LPS barrier, is highly dependent on the length of the polysaccharide region of this molecule. In particular, the disaggregation of the two pairs of temporins, when in contact with the LPS-OM should be hindered by a change in LPS fluidity, which diminishes as the polysaccharide region of LPS reduces its length. This can be interpreted in part as a result of a lower hydration and a lower amount of water molecules in a disordered organization, around LPS with a short carbohydrate region, such as LPS O26:B6. This would increase the rigidity of the structure of this type of LPS to a higher extent than that of LPS O111:B4 (which has longer sugar chains), so as to interfere with the synergistic effect.

To date, only a few peptide combinations synergize in the antimicrobial activity. These include isomers of dermaseptins [7], magainin 2 and PGLa from *X. laevis* [74], the mammalian cathelicidins and defensins [75], and the combination of hepcidin and moronecidin, from bass gill tissue [76]. Nevertheless, with the exception of magainin-2/PGLa, the molecular basis accounting for their synergic activity has not been addressed. Interestingly, the mechanism underlying the synergism between temporins differs from what has been proposed for the pair magainin/PGLa [77]. In the latter case, it is associated with an increasing perturbation of the cytoplasmic membrane, because of increasing pore formation activity by the heterodimer magainin/PGLa, which has a better pore-forming activity than each peptide alone [78,79].

4.2. Synergism of temporins in LPS-detoxification and the underlying molecular mechanism

Temporins-1Ta, Tb, and Tl have been shown to neutralize the toxic effect of LPS derived from various species of *E. coli*, by complexing with it and making it unavailable for interaction with macrophage receptors to stimulate TNF-α secretion (considered to be a primary mediator of endotoxemia). In particular, this process was more pronounced when macrophages were activated with LPS O26:B6 and treated with temporin-1Tl [80]. Most interestingly, the two peptide combinations Ta + Tl and Tb + Tl exhibited a strong synergism in neutralizing LPS O26:B6, but not LPS O111:B4. Indeed, as illustrated in Fig. 6, only when temporins Ta + Tl and Tb + Tl were added to LPS O26:B6-stimulated macrophages, the inhibition of TNF-α release was much greater than the corresponding additive effect of the single peptides. According to what is indicated in the report, this seems to be the first case showing a marked synergism between AMPs from the same specimen in the LPS-detoxification. This event was accompanied by disruption of LPS aggregates to smaller-size particles, as manifested by quasi-elastic light scattering analysis [80]. Conversely, a different distribution in size and polydispersity was noticed when LPS O26:B6 was treated with each peptide alone or with the combination Ta + Tb [80]. This outcome is in agreement with the notion that one of the requirements for temporins to neutralize LPS is linked to its capability to reduce the aggregation state of LPS, probably by forming small complexes between LPS and peptides, similarly to other AMPs [52]. To further examine whether the LPS-detoxification activity of single or mixed temporins reflected their capacity to dissociate LPS micelles, the effect of these peptides on FITC-LPS O111:B4 and FITC-LPS O26:B6 was also investigated. The fluorescence of FITC-LPS aggregates is self-quenched but increases when the aggregates disassemble, because of dequenching [81]. All temporins disaggregated the structure of both types of LPS (Fig. 7), but this effect was higher when Ta + Tl or Tb + Tl were added to FITC-LPS O26:B6 (Fig. 7).

It is important to highlight that, when used alone, temporin-1Tl was more potent in inhibiting LPS-induced activation of macrophages [80], although its ability to dissociate LPS aggregates was weaker than that of Ta and Tb (Fig. 7). This underscores that, besides alteration of the LPS structure, additional processes (e.g., ability to achieve and bind the toxic region of LPS, the lipid A) should be involved and crucial for the endotoxin-neutralization activity of the peptide.

The changes in the LPS organization mentioned above would expose additional peptide binding sites, otherwise hidden within the LPS micelles, to make electrostatic interactions between peptides and LPS molecules more favourable. This interpretation is consistent with the exothermic nature of the binding reactions of temporins Ta + Tl and Tb + Tl to LPS O26:B6 [80]. In comparison, when temporins were added (either alone or in their combinations) to LPS O111:B4, an exothermic binding reaction and an overall compactness of the LPS structure was produced in all cases [80].

The synergistic effect of temporins in preventing the LPS activation of macrophages relies on the capability of the two pairs of peptides to

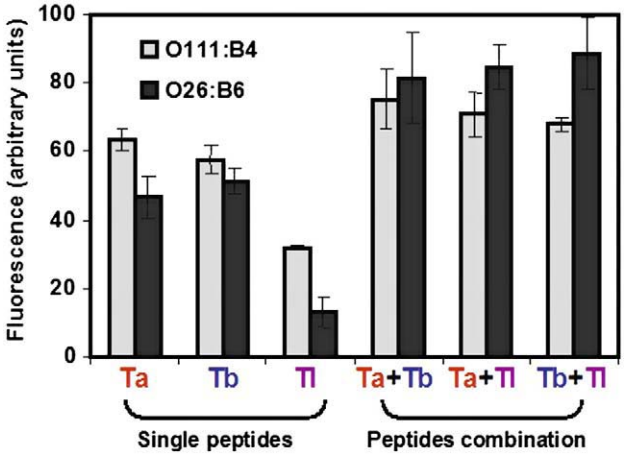

Fig. 7. Effect of temporins on the aggregation state of FITC-labeled LPS from *E. coli* O111:B4 and O26:B6. LPS-FITC (2.5 μg/ml) treated with 1.5 μM temporin-1Ta, Tb or Tl and the combination of temporins Ta + Tb, Ta + Tl and Tb + Tl (1.5 μM each peptide). The change in FITC emission after each treatment was monitored until the fluorescent signal reached a constant value. The increase of fluorescence reflected a change in the LPS-FITC aggregation state. LPS-FITC increases its emission when the distance between its monomers increases, because of dequenching. Standard error of the experimental measurements is also indicated.

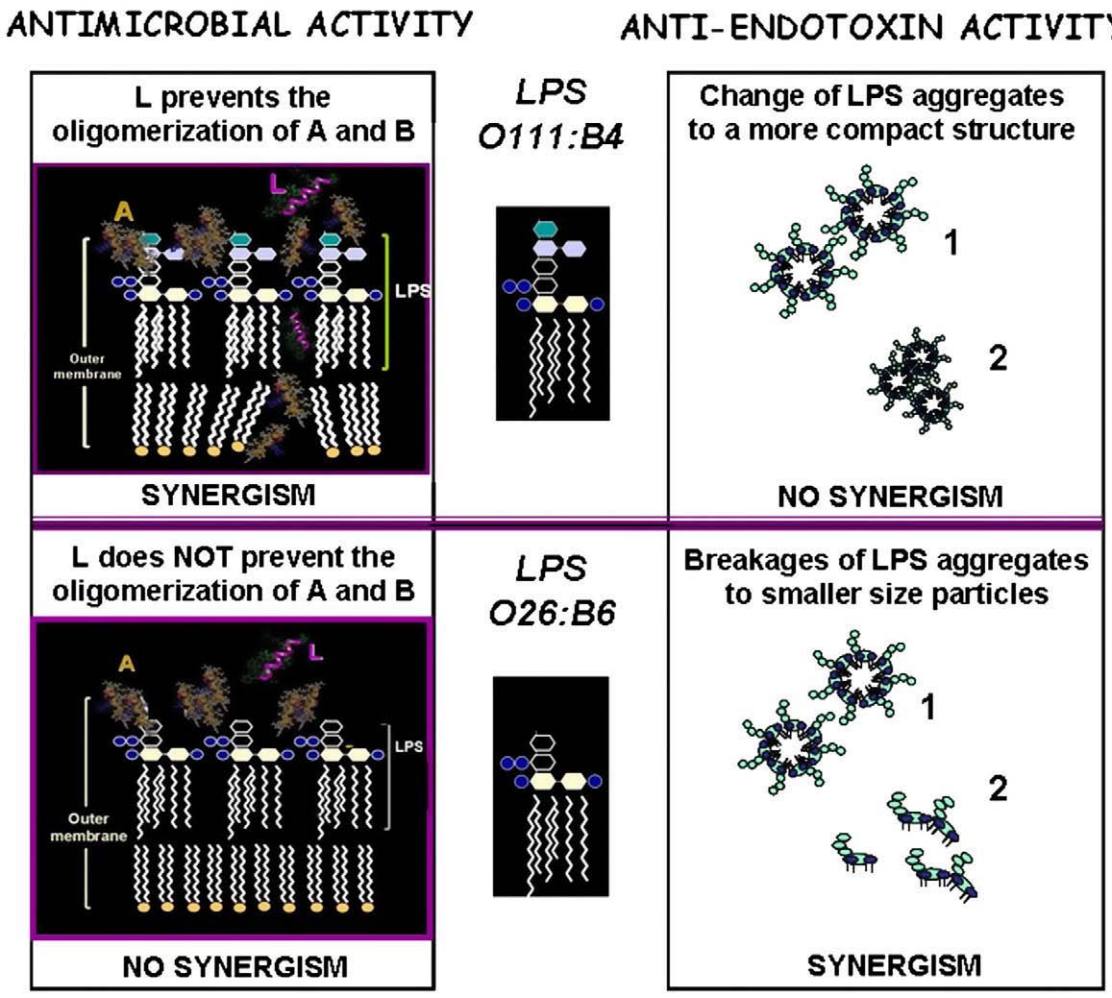

Fig. 8. Left panels: Schematic illustration of the synergistic effect of temporin-1Ta (brown) + Tl (purple) in the antimicrobial activity against *E. coli* O111:B4 and not *E. coli* O26:B6. Right panels: Schematic illustration of the non and synergistic effects of temporins-1Ta + Tl in the anti-endotoxin activity of LPS O111:B4 and O26:B6, respectively.

disrupt the structure of LPS to a greater extent than that induced by each temporin alone.

This finding is favoured by (i) LPS having short carbohydrate side chains, and (ii) peptide molecules folded in an oligomeric state. The second issue matches very well with the knowledge that peptide dimers can alter the structure of LPS more efficiently than the respective single monomers [82].

Overall, the synergism of temporins in inhibiting TNF-α release from LPS-treated macrophages is a specific phenomenon that depends on the type of LPS and peptide combinations, as well as on the type of cytokine secretion pathway (no synergistic effect was detected in the inhibition of other LPS-induced cytokines such as IL-6 [83]). Regarding the corresponding molecular mechanism, there are two remarkable points: (i) the synergistic effect of temporins in the anti-endotoxin activity inversely correlates with the size of the LPS-carbohydrate chains, and therefore occurs with LPS having short length saccharidic portions. (ii) The molecular basis accounting for this synergism differs from that underlying the synergism in the microbial killing which in turn is favoured by LPS with a long polysaccharide region and is linked to the ability of the peptide oligomers to dissociate upon their binding to the bacterial OM (see the scheme of Fig. 8).

5. Concluding remarks

The skin of anurans (frogs and toads) constitutes an important source of biologically active peptides that are used as potential new pharmaceutical agents. One biological rationale for the existence of structurally similar peptides within the same frog species can be attributed to their ability to synergize in both the antimicrobial and anti-endotoxin activities in order to protect the animal from a wider repertoire of infectious microorganisms and/or their septic shock [84,85]. At present, these findings have been described only with peptides derived from amphibian skin, but should be searched also in other families of AMPs. All together, in addition to their contribution to the understanding of the defence mechanism of frogs, such studies should assist in the future development and manufacturing of new natural-like anti-infective and anti-endotoxin drugs, which are urgently needed, because of the emergence of resistant pathogens to conventional antibiotics, as well as because of the high mortality, particularly among hospitalized patients, owing to septic shock.

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