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# Review

# Plasticins: membrane-damaging peptides with 'chameleon-like' properties

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**Abstract.** Plasticins belong to the dermaseptin superfamily of gene-encoded, membrane-active host defense peptides produced by the skin of hylid frogs. The plasticins, which are rich in Gly and Leu residues arranged in regular 5-mer motifs GXXXG (where X is any amino acid residue), have very similar amino acid sequences, hydrophobicities, and amphipathicities but differ markedly in their net charge, conformational plasticity, and activity spectra. The intrinsic flexibility and structural malleability of plasticins modulate their ability to bind to and disrupt the membranes of prokaryotic and eukaryotic cells, and/or to reach intracellular targets, therefore triggering functional versatility. This family of closely related but functionally divergent peptides constitutes a good model to address the relationships between structural polymorphism, membrane-interacting properties, and the biological activity of antimicrobial, cell-penetrating, and viral fusion peptides. Plasticins could thus serve as templates to design potent multifunctional drugs that could act simultaneously against bacterial pathogens and viruses.

**Keywords.** Frog skin secretion, antimicrobial peptide, dermaseptin-related peptide, plasticin, membrane-active peptide, structural malleability, membrane-damaging property.

## Introduction

Gene-encoded peptide antibiotics are an essential defense component of prokaryotic and eukaryotic organisms, destined to challenge invading pathogens and proliferation of commensals [1]. Small-sized antimicrobial peptides, termed bacteriocins, have been produced by a number of Gram-positive and Gram-negative bacteria for millions of years to contain the proliferation of microorganisms that are closely related or confined within the same ecological niche, thus helping the producing microbe to compete for limited resources [2]. In vertebrates, polycationic, membrane-active antimicrobial peptides (AMPs), 10 – 46 residues long, that are secreted by specialized epithelial cells offer a first line of defense against infectious agents by limiting bacterial invasion and/or colonization of the respiratory tract, the gastrointestinal and genital systems, and the skin [3 – 8]. Some of them act synergistically, increasing the microbicidal activity [9 – 14]. Most interestingly, several AMPs are effective against strains of antibiotic-resistant bacteria [5]. The activity of AMPs principally results from their capacity to bind to the outer leaflet of bacterial bilayers and, once bound, to insert into the membrane, thereby triggering transient wormhole formation and membrane disruption [15 – 20]. However, it should be

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noted that more recent investigations provide evidence that the antibiotic activity is also due to interactions of these peptides with intracellular targets [3].

Plasticins are members of the dermaseptin superfamily of gene-encoded, membrane-active host defense peptides produced by the skin of hylid frogs. These peptides are genetically related, with a remarkable identity in signal sequences and acidic propieces of their preproforms, but have clearly diverged to yield several families of microbicidal cationic peptides that are structurally distinct. These families include: (a) the dermaseptins *sensu stricto* from *Phyllomedusa sauvagei* and *P. bicolor*, amphipathic alpha-helical peptides all of which have a conserved tryptophan residue at position 3 and a positive net charge attributable to the presence of Lys residues that punctuate an alternating hydrophobic and hydrophilic sequence; (b) the dermaseptin S9, from *P. sauvagei*, a β-sheeted non-amphipathic peptide that has a hydrophobic core sequence flanked at both termini by several positively charged residues, and (c) the plasticins, which are rich in Gly and Leu residues arranged in regular 5-mer motifs GXXXG (where X is any amino acid residue).

This review summarizes the structural and functional properties of this singular family, which displays considerable structural polymorphism leading to functional versatility. We illustrate how this peptide family constitutes a good model to address the relationships between structural polymorphism, membrane-interacting property, and biological activity of antimicrobial, cell-penetrating, and viral fusion peptides [9, 21, 22].

## Biogenesis of plasticins: orthologous peptides with dissimilar structural and functional properties

Hylid frogs synthesize in their skin various peptides belonging to the dermaseptin superfamily that protect them against noxious microorganisms. The dermal serous glands of these frogs produce huge amounts of biologically active peptide stored in secretory granules [23, 24] (Fig. 1) that are very similar to mammalian neuropeptides and hormones [25–30]. They also contain a rich arsenal of broad-spectrum, cytolytic antimicrobial peptides [8, 31] that are released onto the skin surface by a holocrine mechanism to provide an effective and fast-acting defense. These peptides are derived from precursors that all show a highly conserved N-terminal prepro-sequence and markedly varied carboxy-terminal domains (Fig. 2A) corresponding to very different antimicrobial peptides with very various lengths, charges, and antimicrobial spectra [32] (Fig. 2B). The vast number of different peptides encoded by this gene family reflects an unprecedented degree of gene diversification, similar to that of the gene families that mediate interactions between organisms, such as immunoglobulins [33, 34] or venom-derived toxins [35, 36]. These peptides have all been given individual names and grouped to form distinct families on the basis of their structural characteristics. So, the plasticins, dermaseptin-related peptides (DRPs) from *P. bicolor*, *Pachymedusa dacnicolor, Agalychnis annae*, and *A. callidryas*, are rich in Gly and Leu residues with regular, repeated 5-mer motifs GXXXG. Among the plasticins, DRP-PBN2 and DRP-PD 3-6 are two orthologous peptides from *P. bicolor* and *P. dacnicolor*, respectively, that differ by 10 amino acids (56 % amino acid identity). They also differ in their net charge, DRP-PBN2 containing two positively charged lysine residues. These lysine residues are replaced by asparagine and glycine in DRP-PD 3-6. An additional aspartate residue in position 5 of DRP-PD 3-6 generates a peptide with a net charge of 0 (Fig. 2C). Plasticins share characteristics with other frog skin antimicrobial peptides such as temporins [37], bombinins [38] and aureins [39]: they are relatively hydrophobic and poorly charged with a maximum of +3 net charge for DRP-PBN2a. Phylogenetic analysis has shown that DRP-PBN2 from *P. bicolor* develops very different properties from neutral ANC, the common ancestor of DRP-PD 3-6 from *P. dacnicolor*, DRP-AA 2–5 from *A. annae,* and DRP-AC1 and 2 from *A. callidryas* (Fig. 2D) that mainly arose from two substitutions (Asn8/Lys and Gly12/Lys) between the two peptide sequences. A combination of phylogenetic reconstructions, analysis of the mutation rates, and geophysical models for the sequence of fragmentation of Gondwana suggests that positive selection operated at a molecular level in the early stage of frog species divergence to produce a novel antimicrobial function from an otherwise inactive, neutral ancestral peptide (ANC), and that lysine residues were selected [9, 32, 40].

The ancestral peptide ANC and peptide analogs with altered net charges, DRP-PBN2a, [$K^{8,12}$]-ANC, [$K^{8,12}$]-DRP-PD 3-6, and [$K^{8,12}$, $F^{18}$]-DRP-PD 3-6 (Fig. 2C), constitute a good model to address the relationships between structural polymorphism, membrane-interacting property and biological activity [9, 21, 22].

Figure 3 illustrates the diversity of physico-chemical properties of the plasticin family in comparison with two members of the dermaseptin superfamily, dermaseptin B2 and dermaseptin S9. The three representative plasticins have identical overall hydrophobicities (0.39) and amphipathicities, corresponding to a hydro-

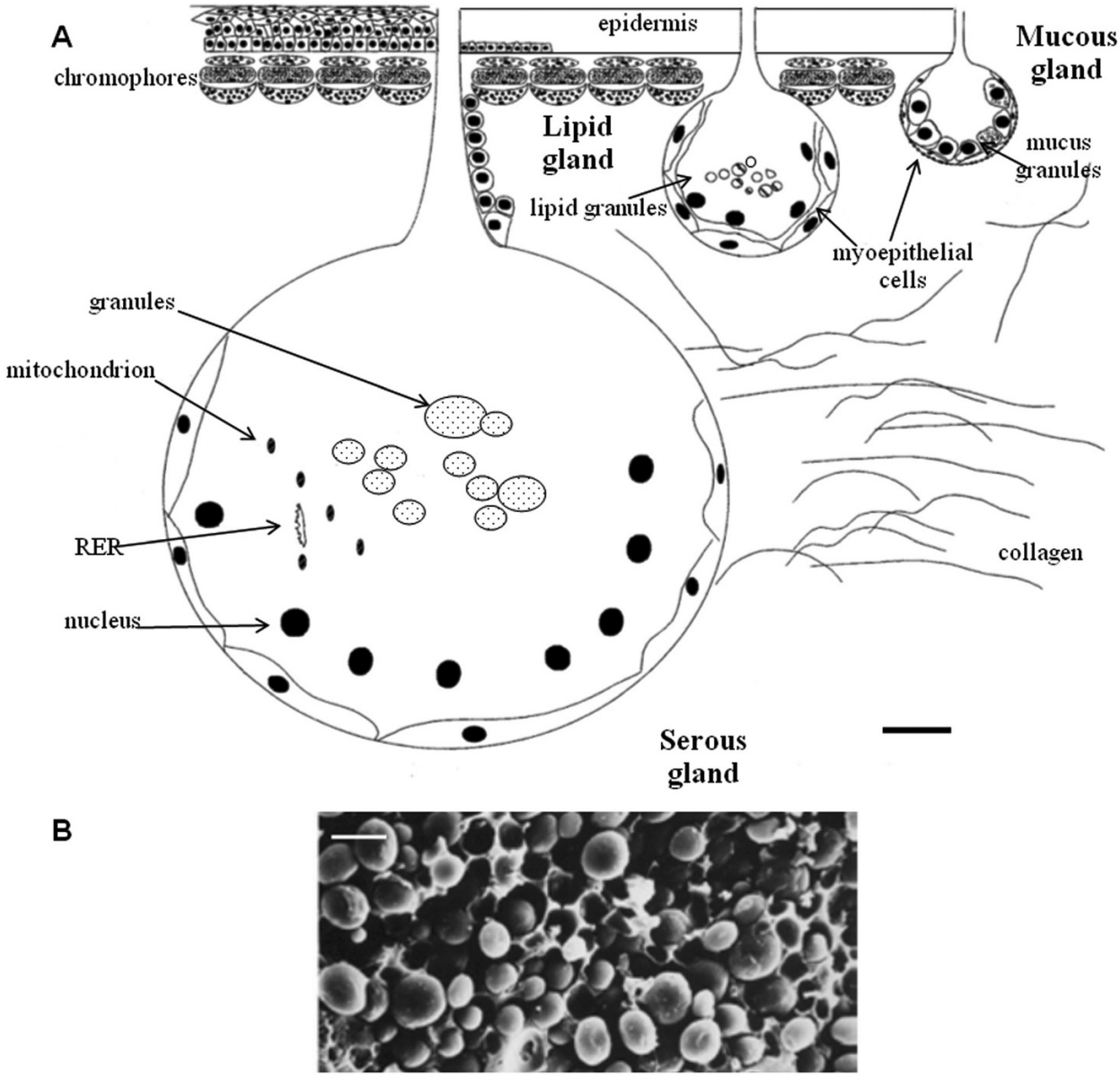


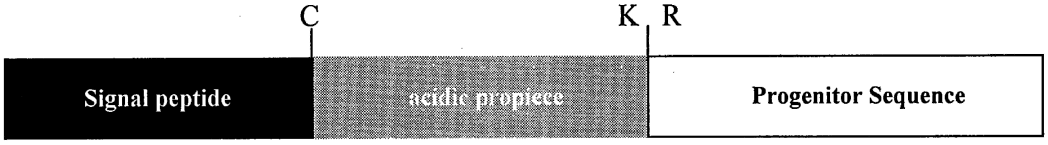

**Figure 1.** (*A*) Diagram of the skin of *Phyllomedusa bicolor* showing the three types of gland (mucous, lipid, and serous) (bar, 100 μm). (*B*) Secretory granules of serous glands containing bioactive peptides as revealed by a scanning electron micrograph of freeze-fractured skin of an adult frog (bar, 25 μm) [23].

phobic sector that subtends a radial angle of 180° on a helical wheel projection, in contrast to dermaseptin B2 [41, 42] presenting a radial angle of 120°. Interestingly, dermaseptin S9 does not resemble any of the antimicrobial peptides identified to date, and in sharp contrast to known helical antimicrobial peptides, dermaseptin S9 has a highly hydrophobic core sequence comprising 10 bulky aliphatic and aromatic residues, which is flanked on either side by polar/cationic residues [43]. It is also noticeable that positive charge distribution differs for plasticins and the two selected dermaseptins modeled as ideal α-helices. Dermaseptins B2 and S9 appear uniformly positively charged and thus may have a larger impact on their surrondings, while the positive charges of plasticins are presented as Nterminal lobes.

## GXXXG motifs and self-association of plasticins

The mode of antimicrobial action of most of the preprodermaseptin-derived peptides from hylid frogs is believed to be the permeation/disruption of the lipid plasma membrane of the target cells, either through a 'carpet,' 'toroidal pore,' or 'leakyslit' mechanism [18, 44]. Once bound, the hydrophobic

**A/**




**B/**

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DRP-PBN2     MAFLKKSLFLVLFLALVPLSICEE-KKSEEENEEKQEDD-QSEEKRGLVTSLIKGAGKLLGGLFGSVTGGQS
DRP-AA-2-5   MAFLKKSLFLVLFLAIVPLSICEEEKREEENEEK-QEDDDQS-KRGLVSGLLNTAGGLLGDLLGSLGSLSGgES
DRP-PD-3-6   MAFLKKSLFLVLFLALVPLSICEAEKREEENEEK-QEDDDESEKKRGVVTDLLNTAGGLLGNLVGSLSGgER
DRP-AC1      MAFLKKSLLLVLFLGLVSLSICEEEKRENEDEEK-QEDDDQSENKRGLLSGILNTAGGLLGNLIGSLSNgES
DRP-AC2      MAFLKKSLLLVLFLALVPLSICEEEKREEEDEEK-QEDDDQSENKRGLLSGILNSAGGLLGNLIGSLSNgES
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**C/**

| DRP-plasticins | Sequence | Net charge |
|---|---|---|
| DRP-AA 2-5* | GLVSGLLNTAGGLLGDLLGSLGSLSGGES | 0 |
| DRP-AC1** | GLLSGILNTAGGLLGNLIGSLSNGES | 0 |
| DRP-AC2** | GLLSGILNSAGGLLGNLIGSLSNGES | 0 |
| **DRP-PBN2**** | GLVTSLIKGAGKLLGGLFGSVTGGQS | +2 |
| **DRP-PBN2a***** | GLVTSLIKGAGKLLGGLFGSVTGa | +3 |
| **DRP-PD 3-6**** | GVVTDLLNTAGGLLGNLVGSLSGa | 0 |
| **[$K^{8,12}$]-DRP-PD 3-6***** | GVVTDLLKTAGKLLGNLVGSLSGa | +2 |
| **[$K^{8,12}$,$F^{18}$]-DRP-PD3-6***** | GVVTDLLKTAGKLLGNLFGSLSGa | +2 |
| **ANC***** | GLVTGLLNTAGGLLGDLFGSLTGa | 0 |
| **[$K^{8,12}$]-ANC***** | GLVTGLLKTAGKLLGDLFGSLTGa | +2 |

* [101] ; ** [32] ; *** [9]

**D/**

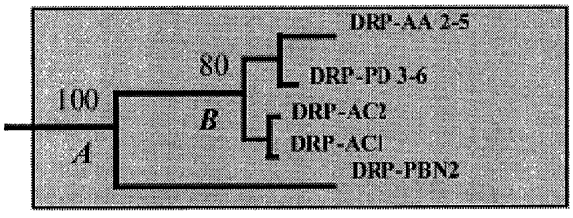


**Figure 2.** Conserved preproregions and hypervariable antimicrobial domains of preprodermaseptins. (*A*) Diagram of preprodermaseptin. (*B*) Alignment of the predicted amino acid sequences of the plasticin preproregions. Gaps (–) have been introduced to maximize sequence similarities. Identical (black background) amino acid residues are highlighted. DRP, dermaseptin-related peptide (appended with PB, AA, AC, or PD to indicate that the sequences were identified from *Phyllomedusa bicolor, Agalychnis annae, A. callidryas,* and *Pachymedusa dacnicolor*, respectively). (*C*) Plasticin sequences including peptide orthologs and analogs. The most studied peptides are emphasized in bold case. ANC is inferred to peptide ancestor. The GXXXG motifs are underlined; a, amidated. (*D*) Plasticin phylogram.

face of the amphipathic peptide would then permit the peptide to enter the membrane interior, thereby triggering local fusion of the membrane leaflets, pore formation, cracks, and membrane disruption. Therefore, it is likely that the selectivity of antimicrobial peptides may additionally depend on properties other than their lipid-binding potency, because peptides need to cross the cell wall before reaching the cytoplasmic membrane, a process that should depend on the structure and oligomeric state of the peptide [5]. It has also been proposed that some peptides which disrupt membranes via a carpet mechanism approach the membrane in an oligomeric state and have a highly α-helical arrangement before coming in contact with the membrane [45]. The amino acid sequences of plasticins resemble those of transmembrane protein segments, and encompass three GXXXG motifs that are known to mediate

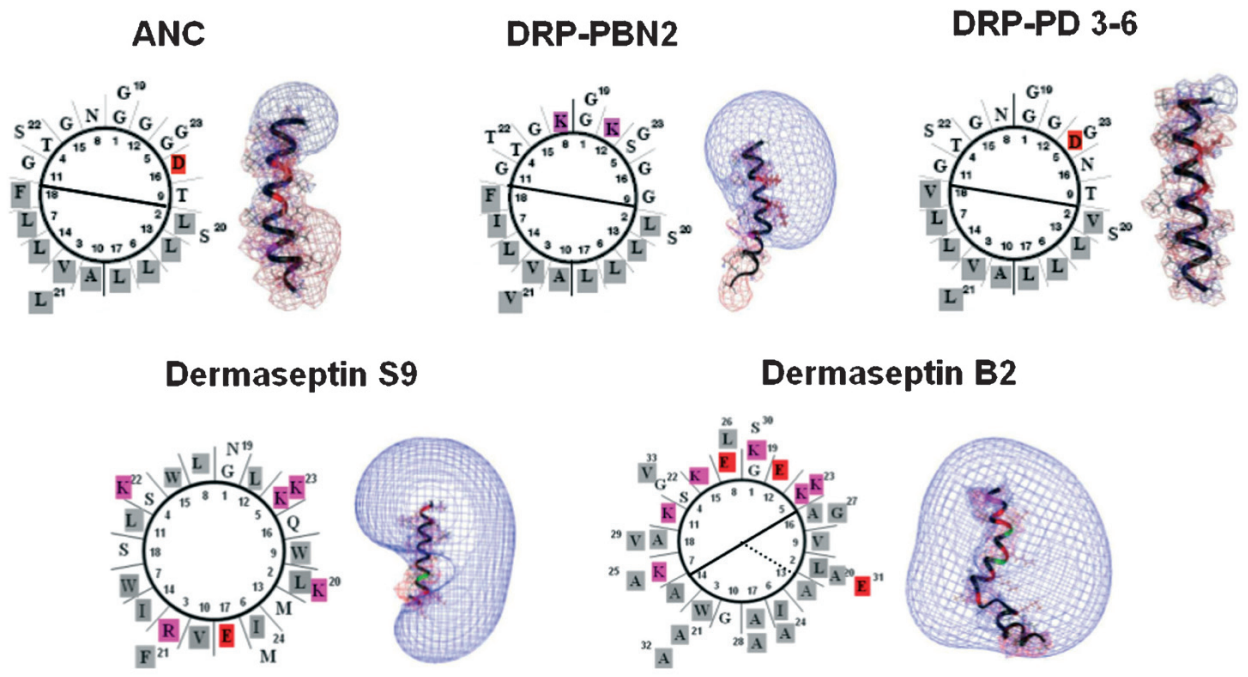


**Figure 3.** Helical wheel projections showing the distribution of hydrophilic and hydrophobic amino acids, and electrostatic diagrams of the three main plasticins in comparison with two representative dermaseptins with diverging physico-chemical properties, the recently cloned dermaseptin S9 [43] and the well-known dermaseptin B2. Hydrophobic (gray background), positively charged (pink background), and negatively charged (red background) side chains are highlighted. The blue and red meshes represent the 1 kT/e positive and 1 kT/e negative electrostatic fields of the peptides calculated from the finite difference solution of the Poisson-Boltzmann equation.

interactions between transmembrane or fusion peptide helices [46, 47].

Table 1 provides some examples of antimicrobial peptides that contain GXXXG or YXXXY (where Y is either A or S) motifs. It is noticeable that these peptides principally belong to the bacteriocin family, or originate from frog skins (bombinins [38], PGLa [19]). Deber and coworkers [48] have examined peptides containing AXXXA sequence motifs and showed that their antimicrobial activity was enhanced due to dimerization, or higher oligomerization states. In particular, the obliquely tilted T-state represents an antiparallel dimer of PGLa that is formed in the membrane at increasing concentrations possibly via the central GXXXG motif as assessed by a solid-state nuclear magnetic resonance (NMR) study [49]. Stepwise site-directed mutagenesis into the corresponding peptides (Ent-alpha and Ent-beta) of the two-peptide bacteriocin enterocin 1071 (Ent) revealed the importance of GXXXG in the beta peptide that may be involved in a specific interaction with the cognate alpha peptide for antimicrobial potencies and specificities [50]. Recent studies also strongly suggest that covalent cross-linking of AMPs might be useful to strengthen the activity of otherwise monomeric peptides and increases resistance against degradation by bacterial proteases [51]. Disulfide dimerization of pore-forming, amphipathic helical peptides was also demonstrated to be a general approach to generate AMPs having activity at very low concentrations [52]. Matsuzaki and coworkers [53] have emphasized that dimerization is a prerequisite for cell disruption and that monomer-dimer equilibrium in the case of magainin 2 is an obligatory step for the formation of pentameric pores.

The impact of GXXXG motifs on plasticin self-association was assessed by SDS-PAGE and measurement of translational diffusion coefficients by pulse field gradient (PFG)-NMR experiments [P. Joanne, O. Lequin, F. Bruston, P. Nicolas and C. El Amri, unpublished data]. The GXXXG motifs were not shown to promote oligomerization of plasticins to the same extent as the dimeric transmembrane segment of glycophorin A [46]. Other factors may be involved, such as the sequence context or intrinsic conformational flexibility. Figure 4 represents a generic view of structural interconversions and plasticin self-association in the presence of anionic membrane for the two most potent plasticins, DRP-PBN2a and [$K^{8,12}$, $F^{18}$]-DRP-PD 3-6. Fluctuations in their antimicrobial potencies (see below) appear to be related to shifts within the autoassociation stage altering the sequentiality of peptide oligomerization.

**Table 1.** Examples of antimicrobial peptides containing small residue-XXX-small residue (GXXXG, SXXXG, SXXXS, GXXXS, AXXXA, SXXXA, GXXXA, AXXXG) motifs.

| Peptide | Sequence | Status | Reference |
|---|---|---|---|
| Cytolisin $CylL_s$ | TTPACFTI**G**LGV**G**ALFSAKFC | *Enterococcus faecalis* | 102 |
| Lactococcin Gβ | KKWGWLAWVDPAYEFIK**G**FGK**GA**IKE**G**NKDKWKNI | design peptide from *Lactobacillus* | 50 |
| Designed peptide | KKKKKK**A**AXA**A**W**A**AXA**A**-$NH_2$, where X = Phe/Trp | design peptide | 48 |
| PGLa | GMASKA**G**AIA**G**KIAKVALKAL-$NH_2$ | *Xenopus laevis* | 19 |
| Plantaricin E | FNRG**G**YNF**G**KSVRHVVDAI**G**SVA**G**ILKSIR | bacteriocin-like peptides from *Lactobacillus plantarum* C11 | 103 |
| Plantaricin F | VFHAYSARGVRNNYKS**A**VGP**A**DWVI**S**AVR**G**IHG | | |
| Plantaricin J | GAWKNFWSSLRK**G**FYD**G**EAGRAIRR | | |
| Plantaricin K | RRSRKNGI**G**YAI**G**YAF**GA**VER**A**VLG**G**SRDYNK | | |
| Bombinin BLP-3 | **G**IGA**A**IIS**A**GK**S**ALK**G**LAK**G**LAEHF-$NH_2$ | *Bombina orientalis* | 104 |
| Bombinin H2 | IL**G**PVL**G**LVG**S**ALG**G**LLKKI-$NH_2$ | H-like bombinin from *Bombina* | 105 |
| Bombinin H4 | $II_{allo}$**G**PVL**G**LVG**S**ALG**G**LLKKI-$NH_2$ | | |
| Bombinin H6 | IL**G**PIL**G**LVG**S**NAL**G**GLL-$NH_2$ | | |
| Bombinin H7 | $IL_D$**G**PIL**G**LVG**S**NAL**G**GLL-$NH_2$ | | |
| Temporin A | FLPLI**G**RVL**S**GIL-$NH_2$ | *Rana temporaria* | 13 |

## Antimicrobial activity of cationic plasticins and their interaction with anionic membrane models

Only cationic plasticins display potent antimicrobial activities against a broad spectrum of microorganisms. The activity scale is as follows: DRP-PBN2a > DRP-PBN2 > [$K^{8,12}$, $F^{18}$]-DRP-PD 3-6 > [$K^{8,12}$]-ANC > [$K^{8,12}$]-PD 3-6 [22]. However, a detailed analysis shows that discrepancies appear within Gram-negative and/or Gram-positive strains for some of these peptides. Some Gram-negative strains were resistant to [$K^{8,12}$]-PD 3-6 and [$K^{8,12}$, $F^{18}$]-DRP-PD 3-6, whereas [$K^{8,12}$]-ANC was more potent than [$K^{8,12}$, $F^{18}$]-DRP-PD 3-6 against Gram-positive strains, suggesting involvement of the lipopolysaccharide (LPS) environment [54]. This was confirmed by membrane leakage experiments using $\beta$-galactosidase and O-nitrophenyl β-D galactopyranoside (ONPG) [9]. Plasticins also vary substantially in their ability to depolarize the membrane, as observed by the use of the DiS-C3(5) fluorescent probe at various concentrations. No absolute correlation between the ability to permeabilize the cytoplasmic membrane and antibacterial activity was observed (Table 2A). For example, DRP-PBN2a exhibits good bactericidal activity against *Escherichia coli* but presents a reduced ability (50%) to dissipate the bacterial membrane potential [22]. Moreover, this plasticin is found to be bactericidal, while [$K^{8,12}$, $F^{18}$]-DRP-PD 3-6, which is bacteriostatic, has the greatest ability to depolarize the membrane. At concentrations threefold the minimal inhibitory concentration (MIC), [$K^{8,12}$]-ANC has no effect on the membrane potential but is found to be bacteriostatic. These various behaviors for closely related peptides are in part thought to be due to LPS, the major molecular component of the outer membrane of Gram-negative bacteria that protects them from the surroundings [54, 55]. As previously suggested by Wu et al. [56], although the primary targets of plasticins are plasma membranes, inducing irreversible depolarization, secondary modes of action might be at work, involving the bacterial cell wall and membrane, such as activation of bacterial autolytic enzymes [57], endogenous phospholipases in host cells [58], or interactions of the AMPs with internal targets once the membrane is breached [3].

Cell membranes of many bacterial pathogens are predominantly composed of phosphatidylglycerol (PG), cardiolipid (CL), or phosphatidylserine (PS) and are highly electronegative; these properties can be mimicked *in vitro* by anionic 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylglycerol (DMPG) bilayers or vesicles [59]. We now consider relations between anionic membrane disruptive properties of cationic plasticins and antimicrobial potency (Table 2A). It is becoming increasingly clear that perturbation by antimicrobial peptides of the membrane near the hydrophobic-hydrophilic interface leads to a thinning of the lipid bilayer that precedes loss of the permeability

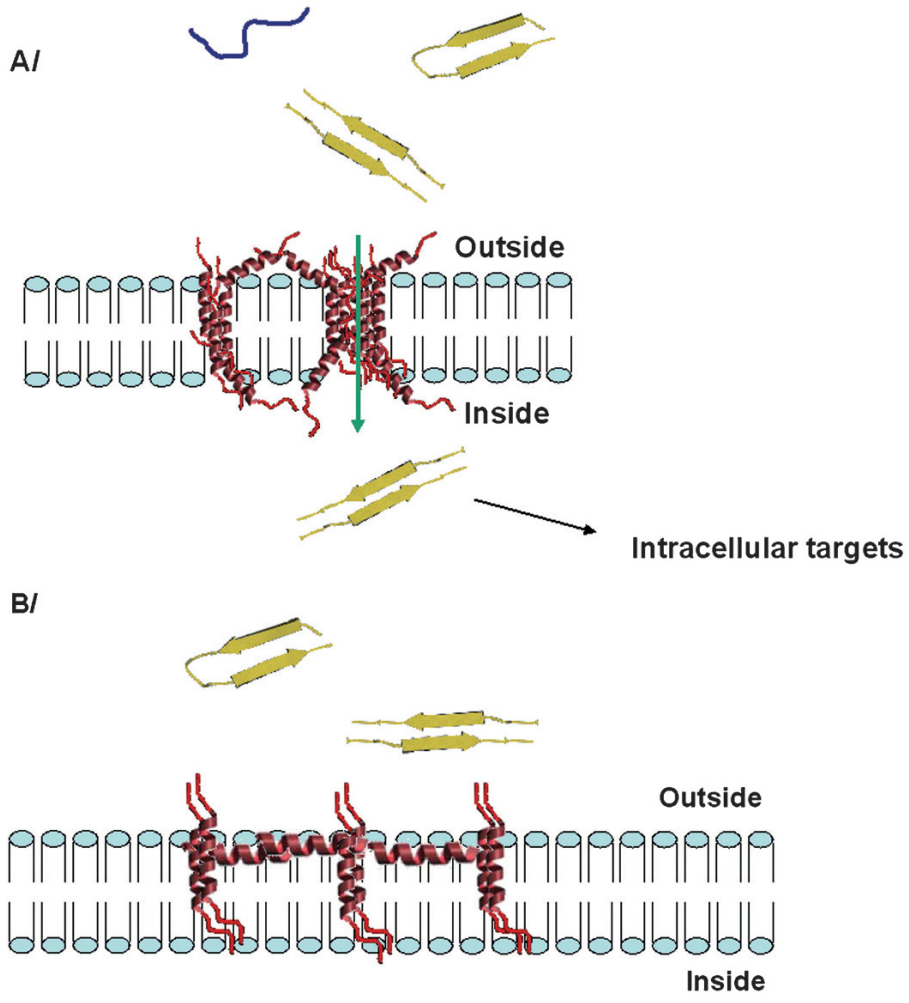


**Figure 4.** Cartoon illustrating and summarizing structural interconversions at the membrane interface, self-association properties, and the various modes of interaction between the two most active cationic plasticins and anionic membrane, highlighting divergence in their antimicrobial mechanism of action. Beta structures are colored in yellow and α helices in red. (*A*) DRP-PBN2a; (*B*) [$K^{8,12}$, $F^{18}$]- DRP-PD 3-6.

barrier [44]. The degree of perturbation is thus in part linked to peptide shape [60].

Like the dermaseptins S [12, 14] and B [18, 41], the dermatoxins [61], the phylloxins [62], and the phylloseptins [63], cationic plasticins are mainly helical when bound to anionic mimetic membranes but can also adopt alternative structural fittings including β-hairpin-like structures at this interface [21, 22] (Table 2 and Figure 4). This is in contrast to the well-known antimicrobials, magainin and cecropin, that also adopt helical structures at an air-water interface, whereas melittin only adopts a helical structure in the presence of lipids [64]. This behavior has also been related to the different charge distributions on the peptide amino acid sequences [65]. The affinity of antimicrobial peptides for anionic phospholipid bilayers is a critical factor influencing their selectivity and potency [18, 66–68]. Cationic plasticins form peptide multilayers when adsorbed onto the DMPG bilayer and bind to anionic membranes in a two-step process. In a first step, the cationic peptide binds to the membrane surface because of the hydrophobic effect and several non-specific interactions. In a second step, the subsequent formation and adjustment of the peptide helix result in tightly bound peptide-lipid complexes (PL*) that are primarily stabilized by the aliphatic and aromatic residues. Plasticins encompassing a phenylalanine residue form denser PL* complexes with a DMPG bilayer than does [$K^{8,12}$]-DRP-PD 3-6. The presence of an aromatic residue clearly prolongs the contact time between the peptide and the membrane [22], allowing further structural rearrangements [69]. In addition to being conformationally different from other small membrane-damaging cationic linear peptides such as magainins [19, 70, 71], cecropins [44, 72], and dermaseptins [15, 73], as seen above, plasticins also differ in their membrane-binding modes. For example, dermaseptin B2 binding to DMPG bilayers is characterized by a first binding step governed by non-specific long-range electrostatic interactions between the peptide lysine residues and the anionic headgroups of the phospholipids [41, 42]. Cationic plasticins interact with DMPG by decreasing the associated water content of the phospholipid heads,

**Table 2.** Recapitulative data for cationic plasticins in the membrane models 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylglycerol (DMPG) and 1,2-dimyristol-*sn*-glycero-3-phosphatidylcholine (DMPC), related to structure, adsorption density, lipid perturbations, and biological activities [22].

| | | DRP-PBN2 | DRP-PBN2a | [$K^{8,12}$]-ANC | [$K^{8,12}$]-DRP-PD 3-6 | [$K^{8,12}$, $F^{18}$]-DRP-PD 3-6 |
|---|---|---|---|---|---|---|
| **DMPG and bacteria** | | | | | | |
| Structure on DMPG vesicles | α-helix (%) | 75 [1] | 65 | 54 | 68 | 63 |
| | β-structure (%) | 12 [1] | 0 | 17 | 4 | 18 |
| Adsorption density on DMPG bilayer | ng · $mm^{-2}$ [a] | nd | 2.8 | 3.3 | 3.0 | 3.5 |
| | PL* (ng · $mm^{-2}$) [b] | nd | 1.2 | 1.2 | 1.0 | 1.2 |
| DMPG vesicle perturbation | Membrane interface (%) [c] | nd | 23 | 17 | 17 | 23 |
| | Bilayer core (%) [d] | nd | 5 | 6 | 3 | 3 |
| Antimicrobial activity on bacteria strains | Effect [e] | B | B | b | b | b |
| | MIC (μM) [f] | 6.25 | 6.25 | 25.00 | 12.50 | 12.50 |
| | Membrane potential [g] | ++ [1] | ++ | + | nd | ++++ |
| **DMPC and eukaryotes** | | | | | | |
| Structure on DMPC vesicles | α-helix (%) | | 47 | 38 | 51 | 60 |
| | β-structure (%) | | 27 | 36 | 35 | 30 |
| Adsorption density on DMPC bilayer | ng · $mm^{-2}$ [a] | nd | 1.2 | 1.7 | 4.2 | 3.7 |
| | PL* (ng · $mm^{-2}$) [b] | nd | 0.3 | 0.5 | 0.2 | 0.3 |
| DMPC vesicle perturbation | Membrane interface (%) [c] | nd | 13 | 7 | 3 | 7 |
| | Bilayer core (%) [d] | nd | 4 | 4 | 3 | 2 |
| Cytotoxicity | Cytotoxicity to HeLa cells [h] | nd | ++ | +++ | nd | ++ |
| | Erythrocyte hemolysis at 50 μM (%) [i] | 30 | 35 [1] | 22 | 35 | 25 |

[1] Unpublished data.
[a] Adsorption density obtained by surface plasmon resonance (SPR).
[b] PL*, represents the complex between the peptide and the lipid corresponding to the penetration of the peptide into the hydrophobic core of the bilayer as quantified after 10 min desorption by SPR.
[c] 1727/1742 ratio increase (%) corresponding to membrane interface (νCO) determined by Fourier transform infrared spectroscopies.
[d] Percentage of $\nu_{AS}CH$ modification compared to pure phospholipids, first established by FTIR spectroscopies.
[e] B, bactericidal; b, bacteriostatic.
[f] Minimal inhibitory concentrations (MIC) for *Escherichia coli* B.
[g] Disruption of the electric potential of bacterial membranes measured by DiS-C3(5) fluorescent probe.
[h] Cytotoxicity to HeLa cells: qualitative evaluation by video microscopy.
[i] Erythrocyte haemolysis.

replacing water molecules by peptides themselves (Table 2) [22]. This strongly suggests that they may act via other ways than the single breakdown of cytoplasmic membrane permeability. The interaction of the peptide with alkyl chains of DMPG phospholipids results in noticeable disordering of the acyl chain region of the fluid bilayer, probably due to gel to liquid-crystalline phase transition, or as a consequence of peptide insertion [74, 75]. Moreover, the aspartate residue at position 16 ([$K^{8,12}$]-DRP-PD 3-6 and [$K^{8,12}$, $F^{18}$]-DRP-PD 3-6) or at position 5 (ANC) promotes or weakens the sequestration time of the peptide by lipids, inducing greater rotational mobility of the alkyl chain, highlighting preferential interactions with anionic phospholipids. DRP-PBN2a does not bear a D residue, explaining its intermediate status. The abilities of plasticins to act on lipid membranes were also related to their potential to cross membranes, thus targeting the cytoplasm or nucleus. Consequently, DRP-PBN2, giving the highest PL* complex density in a DMPG bilayer (Table 2), is believed to have the strongest capacity to cross membranes. Differential scanning microcalorimetry (DSC) experiments performed on selected plasticins support these various membrane disruptive modes [P. Joanne, C. Lacombe, S. Castano, B. Desbat, P. Nicolas, C. El Amri, unpublished data].

## Cytotoxic activity of cationic plasticins and their interaction with zwitterionic membrane models

Eukaryotic cell killing by host defense peptides can be achieved via different mechanisms: (i) induction of necrosis resulting from the disruption of the cytoplasmic membrane, and (ii) induction of apoptosis triggered by the binding of the peptides to the mitochondrial membrane [54]. All cationic plasticins induce erythrocyte hemolysis [9] (Table 2). Incubation of HeLa cells with 100 μM DRP-PBN2 or [$K^{8,12}$]-ANC

induces cell necrosis after 3 h, whereas after 24 h, these plasticins at a concentration of 10 μM provoke damage in the plasma membrane and changes in cell morphology; [$K^{8,12}$]-ANC, which is twice as densely packed as DRP-PBN2a, caused greater changes [22]. Both peptides cause local membrane fusion, the formation of large polynucleated cells, and they also alter the nuclei, influencing condensation and fragmentation as attested by the presence of apoptotic bodies. No strong correlation exists between bilayer core perturbations and cytotoxicity, but there is a tight correlation between PL* complex density and cytotoxicity (Table 2). This behavior has also been noted by analysis of a series of dermaseptin S4 derivatives, revealing that the potency of highly cytolytic peptides such as K(4)K(20)-S4 is not due to the highest membrane adhesion affinity but to the highest propensity for the inserted state. Similarly, truncated derivatives of 16, 13, and 10 residues showed a progressive reduction in cytotoxicity that best correlated with a progressive reduction in insertion affinity [76]. The outer membrane of cancer cells contains only a small amount of negatively charged PS (3–9% of the total membrane phospholipids), being only slightly more negative than that of normal eukaryotic cells [77, 78]. Because of this, some cationic AMPs are more toxic to cancer cells than to normal cells. Furthermore, the presence of a higher negative potential within cancer cells compared to non-cancer cells may contribute to the selective lytic activity of AMPs [78]. In addition, membrane fluidity is typically increased in cancer cells relative to their healthy counterparts, which may facilitate cancer cell membrane destabilization by membrane-bound cationic AMPs [79].

In our investigations, 1,2-dimyristol-*sn*-glycero-3-phosphatidylcholine (DMPC) vesicles were used as a model for eukaryotic cell membranes to address the relationships between structure and cytotoxicity of cationic plasticins. Cationic plasticins are mainly helical when bound to zwitterionic DMPC phospholipid vesicles, although the β sheet structure contribution increases (Table 2). They also form peptide multilayers when adsorbed onto the DMPC bilayer and bind to zwitterionic membranes in a two-step process as for anionic membranes. Nevertheless the PL* complex density is weaker, indicating a lower affinity of cationic plasticins for DMPC bilayers compared to DMPG. The binding of peptides at the interface of the zwitterionic membrane results in membrane dehydration and formation of peptide-membrane hydrogen bonds as revealed by Fourier transform infrared (FTIR) spectroscopy [22]. Thus DRP-PBN2a, mainly in α-helix conformation, is adsorbed to the same extent as dermaseptin B2 [41], while [$K^{8,12}$]-ANC undergoes intermolecular associations on a hydrophobic surface. Cytotoxic cationic plasticins, especially DRP-PBN2a and [$K^{8,12}$]-ANC, alter the $\nu_{AS}(CH_2\text{-}CH_3)$ modes, indicating that these peptides interact with the bilayer core (Table 2). Altogether, these biophysical data demonstrate the ability of cationic plasticins to oligomerize in zwitterionic membranes, partially through gain of β-structures. Beta structure contents could thus be used as an indicator of cytotoxic potency in the plasticin family (Table 2). In most cases, invasion of cancerous membrane cells is thought to be mediated by the carpet/toroidal pore model and variants [80]. Sometimes cytotoxicity against tumor lines mirrors antibacterial activity, suggesting common mechanisms of action, but generally this activity could be regarded as a bonus arising from the membrane activity of peptides [81].

## Membrane-active neutral plasticins

Only a few frog skin peptides bearing no net charge have been reported in the literature [9, 82–84] (Table 4). All these peptides were found to be inactive against bacterial strains *in vitro*. The lack of measurable growth-inhibitory activity of neutral peptides toward bacteria is consistent with the proposal that the positive charge on the peptide is important for binding to the negatively charged phospholipids of the bacterial cell membrane [18]. However, it has been shown that neutral frog skin peptides can have a potent activity against microorganisms that the frog may encounter in the wild [1, 85, 86]. Two neutral plasticins have been especially studied: DRP-PD 3-6, which is predominantly β-sheeted in the presence of zwitterionic phospholipid vesicles, and the ancestor, ANC, which is predominantly β-sheeted with both anionic and zwitterionic vesicles (Table 3). The fact that the structure of DRP-PD 3-6 differs greatly in the presence of anionic or zwitterionic vesicles demonstrates that this peptide has versatile conformations at membrane surfaces, i.e., the same amino acid sequence can have several conformations, depending almost exclusively on the lipid molecular environment. Thus, an amphipathic helical structure is not sufficient, in this case, for bacterial membrane disruption. Neutral plasticins, which interact with anionic or zwitterionic phospholipid vesicles, mainly via hydrophobic interactions [22], are weakly adsorbed in the micromolar range of antibacterial activity concentrations (Table 3). The lack of charge did not prevent their adsorption, but could prevent further rearrangements in the bilayer to form PL* complexes with lifetimes compatible with biological activity.

**Table 3.** Recapitulative data for neutral plasticins in the membrane models DMPG and DMPC, related to structure, adsorption density, lipid perturbations, and biological activities [22].

| | | DRP-PD 3-6 | ANC |
|---|---|---|---|
| **DMPG** | | | |
| Structure on DMPG vesicles | α-helix (%) | 64 | 25 |
| | β-structure (%) | 9 | 39 |
| Adsorption density on DMPG bilayer | ng · mm$^{-2}$ [a] | 0.1 | 0.2 |
| | PL* (ng · mm$^{-2}$) [b] | 0 | 0 |
| DMPG vesicle perturbation | Membrane interface (%) [c] | 17 | 17 |
| | Bilayer core (%) [d] | 4 | 5 |
| Antimicrobial activity on bacteria strains | Effect [e] | 350 | $>400$ |
| | MIC (μM) [f] | | |
| **DMPC** | | | |
| Structure on DMPC vesicles | α-helix (%) | 16 | 30 |
| | β-structure (%) | 62 | 50 |
| Adsorption density on DMPC bilayer | ng · mm$^{-2}$ [a] | 0.2 | 0.2 |
| | PL* (ng · mm$^{-2}$) [b] | 0.1 | 0.0 |
| DMPC vesicle perturbation | Membrane interface (%) [c] | 0 | 0 |
| | Bilayer core (%) [d] | 0 | 0 |
| Cytotoxicity | Erythrocyte hemolysis at 50 μM (%) [i] | 42 | 43 |

For details, see footnotes to Table 2.

**Table 4.** Examples of neutral frog skin peptides (the GXXXG motifs are underlined).

| Peptide | Sequence | Frog | Reference |
|---|---|---|---|
| DRP-PD 3-6 | GVVTDLLNTA<u>GGLLGNLVGSLSG</u>-$NH_2$ | *Pachymesusa dacnicolor* | 9 |
| Temporin-1Ja | ILPLVGNLLNDLL-$NH_2$ | *Rana temporaria* (temporin-related peptide) | 83 |
| Bombinin GH-$l_L$ | II<u>GPVLG</u>LVGLVGKPLESLLE-$NH_2$ | Gene-derived bombinins (H-like bombinins) from *Bombina* | 84 |
| Bombinin GH-$l_D$ | II$_D$<u>GPVLG</u>LVGLVGKPLESLLE-$NH_2$ | | |
| Phylloseptin PS14 | FLSIPAAISAVSVSALADHF-$NH_2$ | *Phyllomedusa hypochondrialis azurea* | 82 |

Moreover, DRP-PD 3-6 and ANC are unable to produce bacterial membrane leakage [32]. Nevertheless, they promote perturbations at the peptide-DMPG vesicle interface and in the bilayer alkyl chains at millimolar concentrations, suggesting insertion into bacterial membranes (Table 3). Surprisingly, neutral plasticins induce no perturbation on DMPC vesicles, while they present hemolytic activities *in vitro* (Table 3).

The disparity observed between ANC and DRP-PD 3-6 peptides underlines the essential role played by the position of the D residue in these two plasticins. Recently, Reshetnyak et al. [87] have demonstrated that the protonation state of the aspartate of the C helix of the bacteriorhodopsin-derived peptide pHLIP (pH low insertion peptide) determines the conformation and insertion of the peptide into the membrane bilayer. Protonation induces an increase in peptide hydrophobicity and triggers insertion of peptides into the membrane.

Apart from several transmembrane segments that are designed or derived from membrane proteins, to date only a few biophysical studies are available on the interactions of neutral membrane-active peptides with model membranes. In a recent work, Freitas et al. [88] have dissected out the molecular interactions of the Ebola neutral fusion peptide with raft-like domains, thus demonstrating the feasibility of investigations on neutral membrane-active peptides that are generally limited by their high hydrophobicities.

During bacterial attack, many peptides can be retrieved in frog skin secretions. The resulting cocktail contains simultaneously neutral, anionic, and cationic peptides. We have already shown that inactive neutral peptides enhance the activity of cationic peptides. Thus, neutral peptides can act as primary membrane-disturbing peptides by adopting a transmembrane orientation in synergy with cationic peptides as suggested by [9].

## Structural malleability and functional adaptability of membrane-active plasticins

The intrinsic conformational preferences of plasticins in aqueous solutions have been investigated to probe the extent to which pre-existent peptide conformations may be responsible for variability in bioactive, membrane-bound, conformations and membrane-disturbing properties [21]. DRP-PBN2 and [$K^{8,12}$]-DRP-PD 3-6 display a mixture of unordered and β turn structures in aqueous media, while neutral peptides display high contents of aggregated β structures and turns. [$K^{8,12}$, $F^{18}$]-DRP-PD 3-6 is distinguished by its high content of β hairpin structure together with low amounts of β turns and unordered structure. It is known that the amino acid sequence of a turn can dictate not only the hairpin stability and turn conformation, but also the register of interstrand H bond interactions [89]. The residue at position 8 has been found to play a major role in initiating the folding, while position 12 seems not to be critical. Conformational preferences and stability of plasticins do not exert a profound influence on the antimicrobial potency, and there is no simple correlation between structural flexibility versus rigidity and bioactivity. While cationic plasticins display a pronounced amphipathic character when modeled as a helix (Fig. 3), this property as well as charge repartition are disrupted when peptides are represented in β structures [90]. This was already suggested by Gellman and coworkers [91], who examined the role of structural rigidity in antimicrobial activity of helix-forming oligomers of β amino acids. However, β hairpin foldability and stability may explain subtle differences between the antimicrobial activities and mechanisms of action of the most potent cationic plasticins DRP-PBN2a and [$K^{8,12}$, $F^{18}$]-DRP-PD 3-6. β-Hairpin preformed in solution may act as a conformational lock that prevents the switch to α-helical structure, thus lowering antimicrobial efficiency. In contrast, β hairpin-shaped conformations could also serve as a template for the formation of helical structures at the membrane interface, facilitating a conformational switch during the membrane fusion process as reported for the HIV-1 Gp41 amino-terminal fusion peptide [92, 93].

It is generally assumed that linear amphipathic antimicrobial peptides operate through random coil-to-helix structure transition upon interaction with the microbial membrane [94]. However, a growing number of studies have demonstrated convincingly that structural versatility of the peptides in the vicinity of membranes may lead to alternative mechanisms of action. Clavanin constitutes an interesting example, where conformational flexibility, mainly driven by glycines, is a major determinant for antimicrobial activity [95]. Recently, Wimmer et al. [96] have emphasized the versatility of the interactions of novispirin G-10, an 18-residue, designed cationic peptide derived from the N-terminal part of a sheep antimicrobial peptide, with detergent and lipids. The ability of novispirin to bind to these amphiphiles and to form α-helical structure was found to be sensitive to the electrostatic environment. Several studies have also shown that linearization of AMPs containing β hairpins that are stabilized by disulfide bridges results in unstructured peptides that are still active (e.g., bovine β-defensin 2 C-terminal segment, protegrin, tachyplesin, and gomesin) [97–100]. In the case of tachyplesin I, in particular, with the deletion of all cysteines, it retains antimicrobial and LPS-selective binding but its hemolytic activity is abolished as a consequence of the overall hydrophobicity and amphipathicity disruption [98]. In summary, the antimicrobial potency of linear flexible peptides, adopting various conformational states in equilibrium or not, may be driven by functionally suitable topologies.

## Concluding remarks

Interplays and synergies between antimicrobial effectors together with ongoing studies of natural and synthetic peptides offer great hope for the design of new and specific antimicrobials. Accordingly, the gain of antimicrobial activity during the evolution of DRP-PBN2 may have been driven, at least in part, by environmental pressure to accelerate the adaptation of *P. bicolor* to the particular noxious microbial fauna that this species encounters. Hence, these antimicrobial peptides with such diverse structures and modes of action can be viewed as the successful evolution of a multidrug defense system, which minimizes the chance that microorganisms will develop resistance against individual peptides. Plasticity of interactions could ensure the recognition of a broad spectrum of organisms, a necessity in host defense. Biologically relevant interactions must be specific at the molecular level, and both hydrophobic clustering and electrostatic interactions accompanied by conformational flexibility in a peptide would provide some leeway within this specificity. Studies on plasticins also highlight the various ways a peptide could perturb biomembranes, leading to a biological activity. As such, plasticins can be used as a general model to explore the impact of the structural malleability of membrane-active peptides (antimicrobial, cell-penetrating, and fusion peptides) on their ability to bind to and disrupt membranes of prokaryotic and eukaryotic cells, and/or to reach intracellular targets. Together,

our results have demonstrated that β-hairpin-shaped structures could serve as a conformational template or lock facilitating the conformational switch for the formation of a biologically active helix at the membrane interface during membrane disruptive, fusion, or internalization processes. Our findings are in agreement with structural interconversions reported for viral fusion or cell-penetrating peptides at the membrane interface. We propose that conformational adaptability is one of the major keys for triggering recognition of target cells through membrane micro- or nanodomains, thus allowing a non-overlap of biological activities.

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