4. Membrane interaction of polymyxin B and synthetic analogues studied in biomimetic systems: Implications for antibacterial action

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Abstract. Membrane-active antimicrobial peptides, such as polymyxin B (PxB), are currently in the spotlight as potential candidates to overcome bacterial resistance. We have designed synthetic analogs of PxB in order to determine the structural requirements for membrane action. Since the mechanism of action of PxB involves interaction with both the outer membrane and the cytoplasmic membrane of Gram negative bacteria, we have used an approach based on mimicking the outer layers of these membranes using monolayers, Langmuir-Blodgett films and unilamelar vesicles, and applying a battery of biophysical methods in order to dissect the different events of membrane interaction. Collectively, results indicate that the PxB analogues act in the bacterial membrane by the same mechanism than PxB, and that cationic amphipathicity determines peptide activity.

Introduction

The global emergence and spread of multidrug-resistant bacteria (defined as resistant to three or more antibacterial drug classes) is an important clinical

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problem. Some factors that have contributed to the development of bacterial resistance include inappropriate use of antibiotics, such as the overuse of powerful, broad-spectrum antibiotics, the presence of antibiotics in the food/livestock industry and the inclusion of antimicrobials in household products [1, 2]. The World Health Organization recognizes antimicrobial resistance as one of the three greatest threats to human health [3]. However, the antimicrobial pipeline remains unacceptably lean, in fact, the ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* species) have outpaced the drug discovery process [4, 5]. The number of antimicrobial agents approved by the United States Food and Drug Administration (FDA) and the European Medicines Agency (EMA) has sharply decreased over the last 25 years (Fig. 1). This has left few treatment options for the most stubborn forms of MRSA (methicillin-resistant *Staphylococcus aureus*). The picture is even bleaker for infections caused by Gram-negative bacteria, which are occasionally resistant to all the antibiotics now in the market. All these facts have made it necessary to launch different initiatives and efforts towards discovering new, safe and effective antimicrobials in the next decade [6]. All this work is beginning to crystallize, and in the last decade 20 new antibiotics have been launched and approximately 40 compounds are currently in active clinical development [7].

In this context, there is now renewed interest in the search for drugs that have more than one target on the bacterial cell, rather than a specific chiral receptor or enzyme, with a focus on modes of therapy with mechanisms less likely to promote resistance. Antimicrobial peptides (AMPs) are a class of antibiotics that has attracted great interest in the last few years because they rarely spur the development of resistant organisms. They are a diverse group of molecules that share a few common features, the most important is that their main target is the lipid bilayer itself, and in consequence to develop genetic resistance is very costly for bacteria.
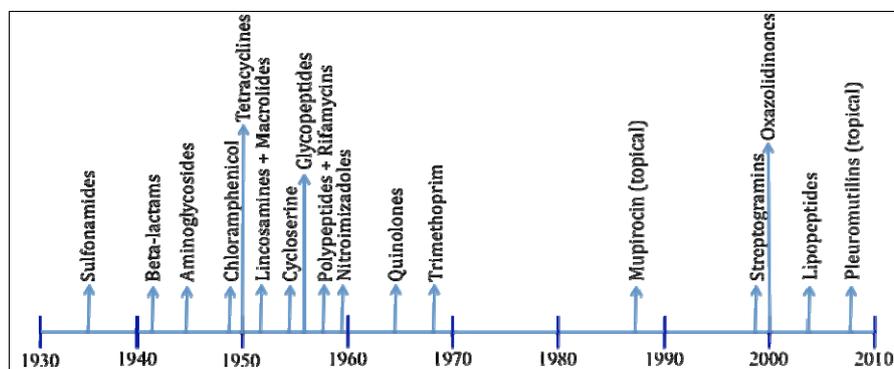

Figure 1. Between 1935 and 1968, 14 classes of antibiotics (with novel mechanisms of action) were introduced for human use; since then only 5 new classes have been described.

1. Antimicrobial peptides (AMPs)

Antimicrobial peptides are natural weapons produced by a variety of organisms, from single-celled microbes to vertebrates [8]. They are very diverse in their sequences, length (generally short) and structures (some of them are linear while others are cyclic due to disulfide bridges, with a variety of secondary structures) but they all adopt an amphipathic conformation with opposing hydrophobic and polar charged faces that allows them to interact and disrupt selectively negatively charged microbial membranes. They can be classified as follows [9]:

- Anionic peptides, such as dermcidin from humans and daptomycin.
- Linear cationic α-helical peptides, as for example magainin 2 and cecropins.
- Anionic and cationic peptides enriched for specific amino acids, like tryptophan-rich indolicin from cattle and Dab-rich polymyxins.
- Peptides that contain cysteine and form disulphide bonds, such as defensins and protegrins.
- Peptide fragments of larger proteins, as for example lactoferricin from lactoferrin.

Multiple studies have shown that AMPs have very good activities (in the micromolar range), and broad spectrum of activity against a range of bacteria, fungi, enveloped viruses and parasites [10] and represent a promising novel class of antibiotics. Traditionally, the search of novel AMPs involved the identification of active peptides from natural sources followed by the design of synthetic peptide analogs, but nowadays design is accompanied by structure-function studies, or *de novo* design that includes high-throughput combinatorial library screening, structure-based modeling, predictive algorithms and the introduction of non-coded modifications to conventional peptide chemistry [11]. Some AMPs have even been discovered through sequence searches within large proteins (such as lactoferricin or hemoglobin). Some examples of AMPs introduced or in advanced stages of development can be found in Table 1.

2. Mechanism of action of AMPs

Although the mode of action of antimicrobial peptides involves disrupting the integrity of the bacterial membrane in different ways, new evidence points to intracellular targets, including DNA and protein synthesis, protein folding, enzymatic activity and cell wall synthesis. In any

Table 1. Representative antimicrobial peptides in different stages of the drug-discovery process (M = Marketed).

Name	Description	Activity	Phase	Observations
Polymyxin B (PxB)	10-amino acid lipopeptide	Antibacterial Gram-	M	First discovered in the 1940s, but their use was discontinued due to nephrotoxicity and neurotoxicity
Colistin (Polymyxin E)	10-amino acid lipopeptide	Antibacterial Gram-	M	Discovered in the 1950s, differs in 1 amino acid from PxB, less active than PxB but also less toxic and so preferentially used.
Nisin	34-amino acid polycyclic peptide	Antibacterial Gram+	M	In 2001, approved as antimicrobial food additive
Daptomycin	13-amino acid lipopeptide	Antibacterial Gram+	M	Originally developed by Lilly in the early 1980s but abandoned after early clinical trials. Cubist Pharmaceuticals launched daptomycin in 2003
Telavancin	lipoglycopeptide	Antibacterial Gram+	M	Approved by the FDA in 2009 for complicated skin and skin structure infections
Pexiganan (MSI-78)	22-amino acid analogue of magainin 2	Broad-spectrum antibacterial	III	No advantage was demonstrated over existing therapies
Iseganan (IB-367)	synthetic protegrin 1	Broad-spectrum antibacterial and antifungal	III	No advantage was demonstrated over existing therapies
Omiganan (MBI-226)	synthetic analogue of indolicidin	Broad-spectrum antibacterial and antifungal	III	Failed in phase III of catheter-related infections but is actually under Phase III trials for acne and rosacea
PAC-113	12-amino acid peptide based on histatin	Candidiasis (*C. albicans*)	II	Phase I and Phase II completed with promising results, no Phase III trials proposed yet
XOMA 629	9-amino acid peptide derived from bactericidal/permeability-increasing protein (BPI)	Impetigo	II	First tested for acne without demonstrating benefit over existing therapy, actually in Phase II trials for treatment of impetigo
HB1345	synthetic lipohexapeptide	Acne (*Propionibacterium acnes*)	Pre-clinical	Promising due to short length

case, membrane interactions are important even for intracellular-targeting peptides, because they must translocate the lipid membrane. Diverse studies have indicated that the interaction of AMPs with the bacterial membranes is specifically based on their structural properties. It is thus their sequence, size, cationic nature, hydrophobicity and amphipathicity that govern their interaction with target cells [12, 13].

According to the Shai-Matsuzaki-Huang (SMH) model [14-16], unstructured peptides adopt a three-dimensional structure when interacting with the bacterial membrane and fold into amphiphilic molecules, with positively charged sides directly interacting with the anionic lipid head-groups of the bacterial membrane. The lipids are then displaced by the peptides, causing a thinning of the outer leaflet of the bacterial membrane. Right after this, the peptides aggregate and form a channel that allows for peptide translocation into the interior of the target cell (Fig. 2). At this point, there are three different models that have been proposed to explain the method of pore formation; the barrel-stove model, the torodial pore (wormhole) model and the micellisation model [17, 18]. There does not appear to be one model that fits all peptides, with various factors contributing to the process of pore formation. The actual antimicrobial activity in order to
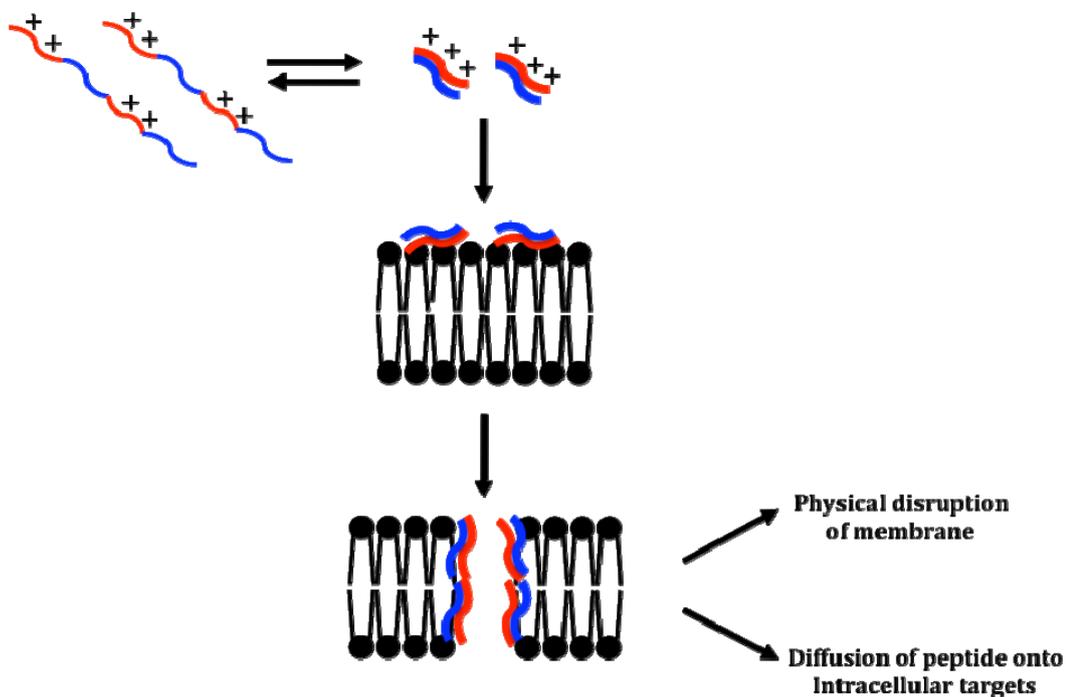

Figure 2. The SMH model for the mechanism of action of an AMP. The various steps include: carpeting of the outer leaflet, insertion of the peptide into the bilayer and finally collapse of the membrane or in some cases diffusion of the peptide to target the intracellular target.

kill bacteria resides in part in the disruption of the bacterial cell membrane, which can occur in a number of ways. These include membrane depolarization, permeabilization or creation of pores which could cause cellular contents to leak out, degradation of cell walls, and alteration of the lipid composition in the membrane bilayer which could lead to the disturbance of membrane functions [8, 19].

A variety of techniques have been used to assess the mechanisms of antimicrobial peptide activity. Each method provides a slightly different view of peptide activity and no single technique is capable of adequately determining the mechanism of action of AMPs. Such techniques include:

- Biophysical studies with model membranes: To provide insights into mechanisms of activity and therefore determine the type of damage induced by the peptides, we can study the interaction of AMPs with phospholipids in model membranes (liposomes or monolayers), of single or mixed lipids in order to mimic the different composition of bacterial membranes. The use of fluorescent dyes can also be useful, such as encapsulated dyes to detect the formation of pores or labeled lipids to determine the effect on the bilayer [13].
- Microscopy: The use of microscopy techniques to visualize the effects of AMPs on microbial cells has helped identify general target sites. Scanning and transmission electron microscopy have been used to demonstrate the ultrastructural damaging effects of antimicrobial peptides [20]. This analysis has shown that different peptides have different effects on microbial cells, indicating that they might have different target sites or mechanisms of activity.
- Circular dichroism (CD) or oriented circular dichroism (OCD): The orientation and secondary structure of an AMP bound to a lipid bilayer can be measured by CD in a controlled humidity environment with light incident normal to the sample surface [21].
- Flow cytometry (FC): Flow cytometry in conjunction with different dyes can be used to determine the effects of the peptides on bacteria, such as propidium iodide (PI) to detect membrane permeability or bis-(1,3-dibutylbarbituric acid) trimethine oxonol [DiBAC$_4$(3)] to detect depolarization of the membrane [22].
- Solid-state NMR spectroscopy (ssNMR): Solid-state NMR measures the secondary structure, orientation and penetration of AMPs into lipid bilayers in the biologically relevant liquid-crystalline state [23]. These data help define the interactions of AMPs with bacterial membranes and the effects of peptide and membrane composition on activity.

3. Mechanism of action of polymyxins

Emergence of nosocomial bacterial pathogens with acquired resistance to almost all available antibiotics, namely "superbugs", is a growing medical concern. The major challenge is regarding the treatment of multidrug-resistant Gram-negative bacteria, such as *Pseudomonas aeruginosa* or *Acinetobacter baumannii*, since no new antibiotic is even in the drug development pipeline with the sole exception of tigecycline. Consequently, there has been a resurgence of old antibiotics, such as polymyxins discovered in the 1940s [24], as drugs of last resort for the treatment of infections caused by MDR Gram-negative pathogens, despite their toxicity (nephro- and neurotoxicity) and the lack of clinical efficacy data [25, 26].

Polymyxin B (PxB) and polymyxin E (colistin) are two secondary metabolite nonribosomal cyclic lipopeptides produced by the soil bacterium *Bacillus polymyxa,* and are the two polymyxins used clinically. Both are specific and highly potent against Gram negative bacteria. They share a common primary sequence, with five positives charges due to L-α,γ-diaminobutyric acid (Dab), the only difference being at position 6, which is occupied by D-Phe in PxB and D-Leu in colistin (Fig. 3).

The mechanism of action of polymyxins, and in particular of PxB, has been studied in detail [27]. The initial target is anionic lipopolysaccharide (LPS) in the outer membrane (OM) of Gram negative bacteria, resulting in OM permeabilizing action. The high affinity for LPS binding can be attributed to the polycationic character of PxB together with its amphipathicity, and the polar and hydrophobic domains folding in two distinct faces when binding to the bacterial membrane. LPS binding results in
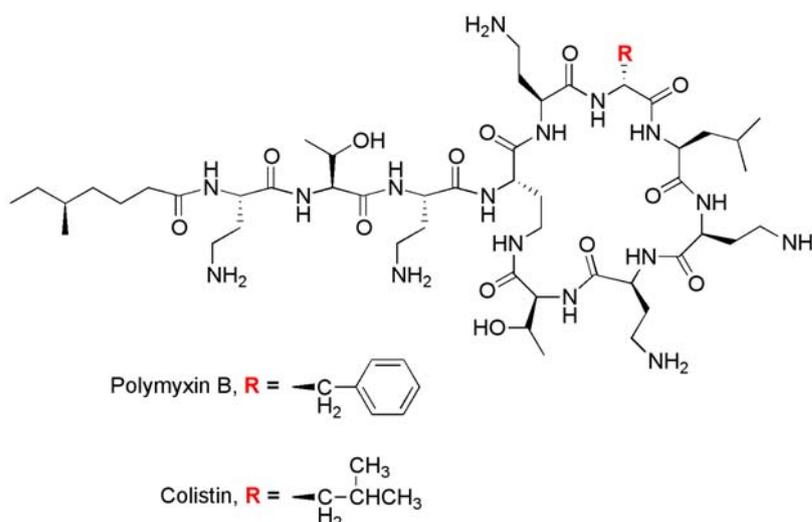

Figure 3. Structures of polymyxin B and colistin.

divalent cation displacement, and leads to PxB self-promoted uptake into the periplasmic space [28]. This process is necessary but not sufficient for bacterial killing, and subsequent interaction with the cytoplasmic membrane is necessary for antibiotic activity [28, 29]. The next step remains unclear at the molecular level, but biophysical studies with model membranes have given insight into the mechanism of bacterial killing by PxB. One possibility involves the insertion of PxB into the cytoplasmic membrane and the disruption of its integrity via membrane thinning or pore formation. However, several reports clearly show that permeabilization or depolarization of the membrane take place at concentrations of polymyxin well above the minimal inhibitory concentration (MIC), and thus other mechanisms of bactericidal action should be considered [12].

A more consistent mechanism of action is based on the formation of periplasmic membrane contacts between outer and inner membranes. According to this model, once in the periplasmic space PxB forms contacts between the two enclosed phospholipid interfaces and promotes a fast exchange of certain phospholipids. The resulting changes in the membrane lipid composition trigger an osmotic imbalance that leads to bacterial stasis and cell death. This phenomena has been proposed as the mechanism of antibacterial action of PxB and several other antibiotic peptides, including cecropins [30-32], and has major implications in bacterial resistance, since it would not be susceptible to generate stable genetic resistance. In fact, acquisition of resistance by a sensitive microbial strain against antimicrobial peptides is surprisingly improbable [8].

Biophysical studies using model membranes have demonstrated that at the concentrations around the MIC, PxB induces the apposition of anionic vesicles and the formation of functional vesicle-vesicle contacts that support a fast and selective exchange of phospholipids exclusively between the outer monolayers of the vesicles in contact and maintaining intact the inner monolayers and the aqueous contents, as schematized in Fig. 4 [33-35].
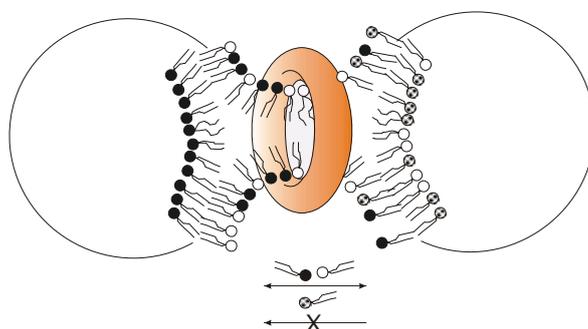

Figure 4. Model showing the formation of membrane contacts mediated by PxB. Key points are: *(i)* contacts involve only the outer monolayers of the apposed vesicles; *(ii)* bilayer structure is maintained; *(iii)* lipid transfer is selective and depends on the lipid headgroup composition; *(iv)* PxB is accessible from the aqueous phase; *(v)* contacts are stable (from [33-35]).

A battery of physicochemical and biophysical methods has been applied to dissect and study the different events of the interaction of polymyxin with the lipid membrane. Overall, these methods amplificate the effect caused by very few molecules of peptide bound to the membrane. Unilamelar vesicles and monolayers at the air/water interface have been prepared with lipopolysaccharide (LPS from *Salmonella minnesota*) to reproduce the outer membrane of Gram-negative bacteria. To mimic the cytoplasmic membrane, mixtures of 1-palmitoyl-2-oleoylglycero-*sn*-3-phosphoglycerol (POPG) and 1-palmitoyl-2-oleoylglycero-*sn*-3-phosphoethanolamine (POPE), or lipid extracts of *Escherichia coli,* have been used.

Lipid monolayers are valuable simplified models of biomembranes, and have been extensively used in the study of the interfacial properties of membrane active peptides. Monolayers are bidimensional model membranes with a planar geometry where the lipid molecules have a defined orientation, and are very suitable for thermodynamic analysis [36]. Transferred to a solid support, lipid or lipid-peptide monolayers can be investigated by atomic force microscopy (AFM), a powerful technique to study the structure and properties of the lipid surface at a nanometric scale [37].

Thermodynamic analysis of mixed monolayers formed by PxB and *E. coli* lipids shows non-ideal mixing consistent with lipid-PxB domain formation [38]. At very low mole fractions of PxB at the interface, below 0.04 mol fraction, monolayers had associated small positive values of excess Gibbs energy, thus suggesting immiscibility and formation of PxB clusters in the dominant lipid phase. AFM observation of transferred mixed and pure monolayers at the membrane equivalence pressure reveals that at the low concentrations of PxB required for contact formation, $X_{PxB}<0.04$, the antibiotic interacts with the bacterial lipids in a very unique way, inducing the formation of well-defined microdomains that are clearly distinguishable from the structures formed at lytic concentrations of PxB (Fig. 5).

Interaction with LPS monolayers was also determined by the Langmuir trough technique, maintaining constant either the surface pressure or the area. In addition, morphological changes in the monolayer upon peptide insertion have been observed by Brewster angle microscopy (BAM). BAM images of monolayers of PxB-containing LPS or its anchoring part, lipid A, show that PxB induces important changes in the film morphology with the formation of more condensed phases at lower surface pressures [39].

4. Polymyxin B synthetic analogues

In view of the excellent antimicrobial activity of the naturally occurring polymyxins and relatively low prevalence of resistance, it is not surprising that there has been substantial effort into discovery of polymyxin analogues with improved microbiological, pharmacological, and toxicological profiles [27].
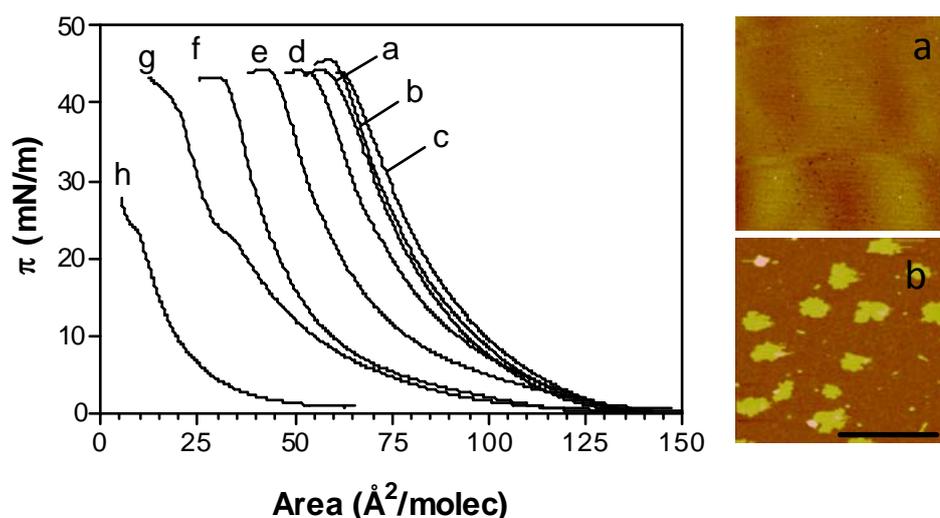
Figure 5. Variation of the surface pressure with the mean molecular areas of mixed ECL-PxB monolayers at various peptide mol fractions: (a) 0; (b) 0.005; (c) 0.01; (d) 0.17; (e) 0.36; (f) 0.56; (g) 0.77; and (h) 1. AFM images of the ECL-PxB L-B monolayers at 32 mN/m are shown at the right side. Bar represents 500 nm [38].

We have designed and synthesized a series of PxB-analogues that mimic the primary and secondary structure of PxB [39-43]. Peptide synthesis was performed manually following standard Fmoc/^{t}Bu procedures, using DIPCDI/HOBt activation on a benzhydrylamine resin. Homogeneity of peptide crudes was assessed by analytical HPLC, and peptides were subsequently purified by preparative HPLC. Cyclization of peptides through disulphide bonds was carried out by air oxidation. Final purity was greater than 95%. Peptides were characterized by amino acid analysis with a Beckman 6300 analyser and by MALDI-TOF with a Bruker Model Biflex III.

The first analogue obtained by this method (sp-B, structure in Fig. 6), closely mimics the primary and secondary structure of PxB. However, to ensure the economic viability of the potential new drug, the simplicity of the synthetic process had to be considered. For this purpose, the complex bond between Dab^4 and Thr^{10} of natural PxB was substituted by cyclization via an intramolecular disulphide bridge resulting from oxidation of two cysteine residues inserted in the primary amino acid sequence, as had previously been reported [44], and linear nonanoic acid was introduced at the N-terminus.

Penetration of sp-B into monolayers of LPS gives information on the affinity of the lipopeptide for the main component of the Gram negative outer membrane [39]. Experiments conducted at constant pressure or at constant area showed that sp-B readily inserted into LPS monolayers, with even higher levels of affinity than the parent compound.
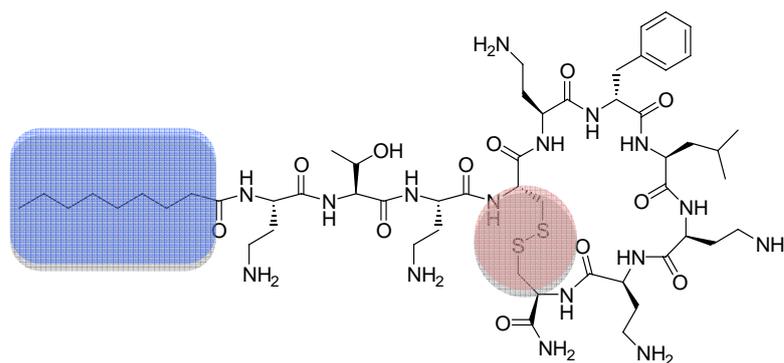
Figure 6. Sequence of PxB synthetic analogue sp-B. The two principal changes introduced to simplify the synthesis are highlighted.

Biophysical studies with unilamelar vesicles that model the cytoplasmic membrane reveal that low concentrations of sp-B form functional vesicle-vesicle contacts that are selective for transfer of anionic phospholipids [40, 41]. For example, fluorescence experiments based on the excimer/monomer ratio of pyrene-labeled phospholipids show that only monoanionic phospholipids such as 1-palmitoyl-2-oleoylglycero-*sn*-3-phosphoglycerol (POPG) are transferred, whereas dianionic phospholipids such as 1-palmitoyl-2-oleoylglycero-*sn*-3-phosphate (POPA) are not.

This interaction is equivalent to the previously described for PxB on anionic membranes [35], and therefore sp-B is a valid scaffold to introduce different structural changes with the objective of getting more insight into the molecular basis of the interaction of the peptides with the lipid membrane. In this sense, analogues sp-C and sp-D (sequences in Table 2) were designed and synthesized. The contribution of the hydrophobic domain (D)Phe[6]-Leu[7], highly conserved in the polymyxin family, was assessed with analogue sp-C, where a permutation in the sequence disrupted this hydrophobic domain. The influence of the peptide charges on the interaction has been explored with sp-D, an analogue that contains two residues of L-α,γ-diaminopropionic acid (Dap) in positions 1 and 8. Dap residues have a pK_a approximately of 8.5 and at pH 8.0 will confer the peptide a much lower cationic character compared to PxB (the pK_a of Dab is 9.45).

Table 2. Peptide sequences.

Peptide	Sequence
PxB	R$_1$-Dab-Thr-Dab-*cyclo*[Dab-Dab-*(D)*Phe-Leu-Dab-Dab-Thr]
PxB-np	Thr-Dab-*cyclo*[Dab-Dab-*(D)*Phe-Leu-Dab-Dab-Thr]
sp-B	R$_2$-Dab-Thr-Dab-*cyclo*-[Cys-Dab-*(D)*Phe-Leu-Dab-Dab-Cys]
sp-C	R$_2$-Dab-Thr-Dab-*cyclo*-[Cys-Dab-*(D)*Phe-Dab-Leu-Dab-Cys]
sp-D	R$_2$-Dap-Thr-Dab-*cyclo*-[Cys-Dab-*(D)*Phe-Leu-Dap-Dab-Cys]

[a]R$_1$, (*S*)-6-methyloctanoyl ; [b]R$_2$, nonanoyl; sp-B, sp-C, and sp-D are cyclized by means of a disulfide bond between Cys-4 and Cys-10.

Results in this work are summarized in Fig. 7, and show that insertion into the LPS layer is achieved to different extents by all the lipopeptides assayed, including acyl chain-lacking compound PxB-np, which corresponds to a deacylated PxB decapeptide (Table 2). However, insertion into the anionic phospholipid membrane and adoption of an active form of the peptides, i.e. the ability to induce phospholipid exchange, is more restrictive, and requires the interplay of the two hydrophobic domains of the lipopeptide (the acyl chain and the D-Phe-Leu region), as well as the five positive charges of Dab.

In another study, synthetic fluorescent analogs of sp-B were used to investigate the peptide position and orientation in the intermembrane contacts: sP-Bw, an analog that contains *D*-tryptophan (*D*Trp) instead of the naturally occurring *D*-phenylalanine, and sP-Bpy, incorporating a pyrene group at the N-terminus [41]. The inclusion of *D*Trp is very useful to characterize binding of the peptide to the membrane by fluorescence techniques. Tryptophan fluorescence, anisotropy and quenching measurements performed with sP-Bw indicate that the peptide binds and inserts in anionic vesicles of POPG and POPA adopting different forms. sP-Bw also serves as a donor for intermolecular resonance energy transfer (FRET) experiments with sP-Bpy used as acceptor. FRET experiments are consistent with self-association of sP-B bound to anionic vesicles at the concentrations that induce the vesicle-vesicle contacts (Fig. 8).
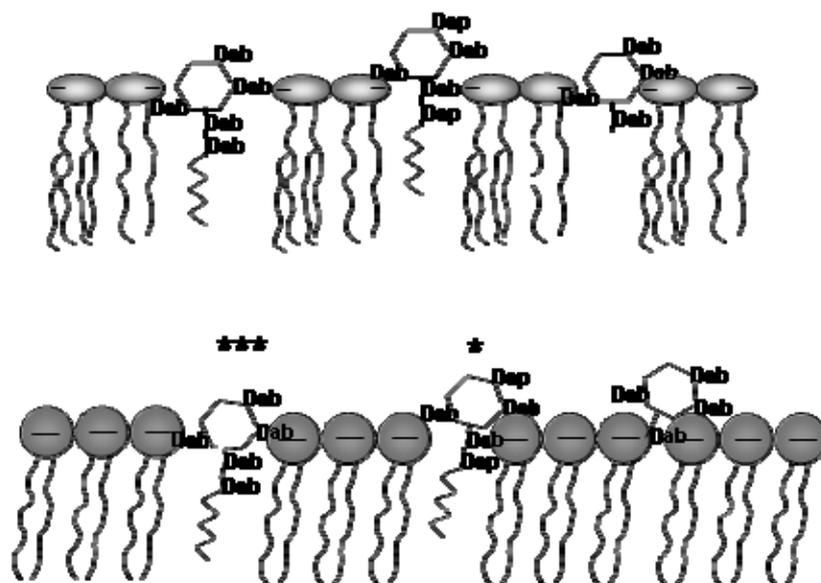

Figure 7. Cartoon schematic of possible interactions of the peptides with bacterial membranes. (*Top*) Lipopolysaccharide as OM model; (*Bottom*) Anionic phospholipid as IM model. Peptides represent (from left to right): PxB or sp-B; sp-D; PxB-np. Active forms are indicated with asterisks [39].
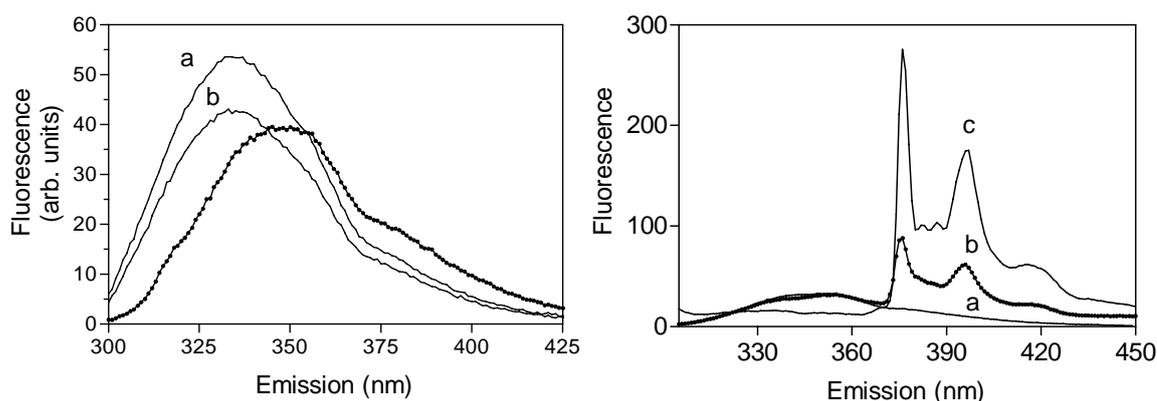
Figure 8. (Left) Tryptophan fluorescence emission spectra of sP-Bw in 10 mM Tris buffer (dotted line) or bound to vesicles of (a) POPG; (b) POPA. (Right) Self-association of sP-B bound to vesicles of POPG detected by intermolecular Trp-pyrene FRET. (a) sP-Bw; (b) mixture of sP-Bw and sP-Bpy; (c) same with POPG vesicles; peptide mol fraction 1% [41].

5. Conclusion

Naturally occurring polymyxins are cationic antimicrobial peptides highly effective against Gram-negative bacteria. The emergence of multiply antibiotic-resistant Gram-negative bacilli, frequently susceptible only to polymyxins, has sparked a renewed interest in these agents, and many efforts are directed towards the generation of new molecules with improved therapeutic index. For this purpose, a detailed knowledge on the mechanism of action of polymyxin and polymyxin derivatives in the membrane is fundamental. In our group we have studied the interaction of polymyxin B (PxB) with lipid vesicles and monolayers prepared with a lipid composition that closely mimics the bacterial inner and outer membranes. Results are consistent with a mechanism of action based on the simultaneous interaction with the two membranes and alteration of the membrane composition by PxB-induced membrane-membrane contacts and lipid exchange. Synthetic PxB-analogues have been specifically designed to get more insight into the mechanism of action at the molecular level. Analogs with intrinsic fluorescence indicate that peptide self-association in the membrane is necessary for intermembrane contact formation, whereas analogs with different modifications in the hydrophobic and polar residues reveal that insertion and membrane activity depends on the lipid composition and is the result of a combination of electrostatic and hydrophobic interactions. Synthetic analogues that act by the same mechanism than the parent peptide are promising molecules in the search for new antibiotics to treat resistant bacteria.

Acknowledgements

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