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Selectivity of antimicrobial peptides: a complex interplay of multiple equilibria

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Abstract

Antimicrobial peptides (AMPs) attack bacterial membranes selectively, killing microbes at concentrations that cause no toxicity to the host cells. This selectivity is not due to interaction with specific receptors, but is determined by the different lipid composition of the membranes of the two cell types, and by the peculiar physico-chemical properties of AMPs, particularly their cationic and amphipathic character. However, the available data, including recent studies of peptide-cell association, indicate that this picture is excessively simplistic, because selectivity is modulated by a complex interplay of several interconnected phenomena. For instance, conformational transitions and self-assembly equilibria modulate the effective peptide hydrophobicity, the electrostatic and hydrophobic contributions to the membrane binding driving force are non-additive, and kinetic processes can play an important role in selective bacterial killing in the presence of host cells. All these phenomena, and their bearing on the final activity and toxicity of AMPs, must be considered in the definition of design principles to optimize peptide selectivity.

Keywords: antimicrobial peptides, host defense peptides, selectivity, toxicity, peptide/membrane association, aggregation, hydrophobicity, amphipathicity.

1. Introduction

The scientific and medical interest for antimicrobial peptides (AMPs), short peptides produced by most organisms as part of their innate immune defenses, derives from their wide-spectrum bactericidal properties and their possible application to fight drug-resistant bacteria. However, in view of clinical applications, the absence of significant toxicity is almost as important as a good activity. In this respect, one of the appealing properties of many AMPs is their cell selectivity, *i.e.* the ability to kill bacterial cells at concentrations significantly lower than those causing damage to cells of the host organism, at least in *in vitro* tests. Still, potential toxicity is commonly listed as one of the challenges limiting the clinical application of AMPs as systemic drugs (Hancock 2006; Eckert 2011; Yeung 2011; Seo 2012; Carneiro 2015; Pachón-Ibáñez 2017), and therefore, several research efforts are devoted to understand and further improve AMP selectivity.

This chapter discusses AMP selectivity, the origin and the structural determinants of this property, the design strategies available to improve it, and the results of recent studies on the quantitative determination of peptide-cell association. Overall, the available data indicate that selectivity is the results of a complex interplay of several interconnected phenomena, including peptide association to target and host cells, peptide conformational equilibria and AMP aggregation. Any modification to the peptide sequence and structure necessarily affects all of these processes, which must therefore be fully understood and considered in the rational design of new peptide or peptidomimetic molecules with improved selectivity properties. Our attempt is to give a critical overview of the available evidences, in order to provide a rationale for future efforts in this area. To this end, we have strived to derive, whenever possible, generalizations of the findings reported in the literature, but we have to stress from the beginning that the presence of exceptions to every rule is the norm, in such a diverse set as AMPs, also as a consequence of the complications mentioned above.

The different aspects of AMP selectivity have last been reviewed by Matsuzaki in 2009 (Matsuzaki 2009). Selectivity or toxicity have often been considered in general review articles on AMPs (Alba 2012; Teixeira 2012; Oddo 2017; Hollmann 2018). Some reviews have summarized our current knowledge on the structural determinants of AMP activity and selectivity (Takahashi 2010; Huang 2010; Strömstedt 2010; Tossi 2011; Ruiz 2014; Ebenhan 2014 a). Finally, for a recent discussion on how the interaction of AMPs with target and host cells determines their selectivity, see (Savini 2018).

As illustrated in other chapters of this book, AMPs have multiple functions, including anticancer, antifungal and antiviral activities. For the sake of brevity and simplicity, in this chapter we will essentially limit ourselves to discuss selectivity for bacterial versus host cells. Selectivity of anticancer peptides has been reviewed by Phoenix and coworkers (Phoenix 2012; Harris 2013).

2. AMPs are selective for microbial cells

AMPs have been isolated from natural sources based on their antimicrobial activity. The minimum inhibitory concentration (MIC, *i.e.* the lowest concentration of antimicrobial agent that inhibits the visible growth of a microorganism) (Wiegand 2008) or the minimum bactericidal concentration (MBC, *i.e.* the minimal drug dosage killing at least 99.9% of the bacterial cells) (Lorian 2005) for AMPs are usually in the low μM range (Giacometti 1998). AMPs are typically bactericidal, and therefore the MIC and MBC values are usually similar (Giacometti 1998).

The active concentration of a bioactive, therapeutically useful molecule must be much lower than the concentration causing toxic effects to the host cells. This property is quantified by the therapeutic index (TI), *i.e.* the ratio of the active concentration to the toxic concentration (see the legend to Table 1 for a detailed definition of these parameters). In the case of AMPs, whose main mechanism of bactericidal action is membranolytic (as discussed in Section 3) toxicity is most commonly assessed by measuring the lysis of

erythrocytes (Figure 1). Table 1 summarizes some TI values of natural and artificial AMPs, which are typically in the range 10-1000. However, it should be considered that unfortunately a strong variability is present in the literature regarding the definition of the toxic concentration, because different thresholds of lysed red blood cells (RBCs) are utilized to define the minimum hemolytic concentration (MHC), ranging from barely detectable to full hemolysis (see references cited in Table 1) (Bacalum 2015). In addition, MIC values depend on the specific strains tested in the assay.

When toxicity is assayed on other human cells, the results are generally not very different from those obtained using hemolysis (Table 2), but combining the two toxicity tests obviously provides a clearer picture of the selectivity of AMPs (Bacalum 2015).

AMP activity and toxicity are usually measured in separate assays performed under rather different conditions (for instance regarding cell density, see Section 9) (Matsuzaki 2009). We have argued that experiments on bacterial and human cells in co-culture would provide a more stringent test of peptide selectivity (Savini 2017, 2018). However, this approach has been employed only in a few cases. These studies, discussed in detail in Section 9.3 demonstrated that AMPs are selective even when acting on bacteria co-cultured with mammalian cells (Figure 2 a).

Several evidences indicate that AMPs are selective also *in vivo*. For instance, a large body of studies starting in 1999 (Welling) has shown that radiolabeled (Lupetti 2003; Brouwer 2008; Akhtar 2012; Ebenhan 2014 a) or fluorescent (Akram 2015) AMPs can be used to image infections *in vivo*, and can even discriminate between infection and inflammation, thanks to their specific binding to bacterial cells (Welling 2000) (Figure 2 b and c). Among the peptides used for this purpose, there are defensins, cathelicidins, lactoferricins, histatins, artificial peptoids, and particularly sequences derived from ubiquicidin (Lupetti 2003; Brouwer 2008; Akhtar 2012; Ebenhan 2014 a, b; Dutta 2017; Lei 2018). Several imaging studies employed ubiquicidin 29-41 (Meléndez-Alafort 2004; Akhtar 2005; Vallejo 2008; Gandomkar 2009; de Murphy 2010; Assadi 2011; Ostovar 2013; Saeed 2013; Kahrom 2014; Ebenhan 2018; Bhatt 2018), which has moderate activity and selectivity in the standard assays (MIC 40 μ M, TI>5) (Brouwer 2006; Lupetti 2008), but accumulates at the site of infection. For instance, one study reported overall values of sensitivity, specificity, and accuracy for infection detection of 100%, 80%, and 94% (Akhtar 2005). Analogs of the ubiquicidin peptide have been used also for targeted delivery of traditional antibiotics to the infection site (Chen 2015).

Although *in vivo* studies of the activity of AMPs abound, similar investigations characterizing their toxicity are more sparse (Mahlapuu 2016). Some TIs derived from animal studies are summarized in Table 3, and the range of values is similar to that obtained *in vitro*.

The selectivity of AMPs for bacterial cells is demonstrated also by the fact that these peptides have been exploited in sensing elements that can detect infection (Mannoor 2010; Shriver-Lake 2012; Silva 2014; Hoyos-Nogués 2018), even in whole blood (Shi 2017) or other complex biological samples (Qiao 2017).

Overall, the results collected in the literature support an interesting selectivity of AMPs for target versus host cells. The origin of this property is necessarily related to the mechanism of action of AMPs.

3. Cellular membranes are the main target of AMPs

Selectivity is not surprising when a biomolecule associates to a specific receptor or protein (Le Joncour 2018). However, this is not the case for most AMPs. In general, natural AMPs and their enantiomers comprising all D amino acids have a comparable antimicrobial activity (Figure 3), while any interaction of a peptide with a protein, due to the chirality of both systems, would be favored for one enantiomer over the other. As is often the case for AMPs, exception to this rule have been reported (Otvos 2000; Bulet 2005; de la Fuente-Núñez 2015), showing that the mechanism of action of a minority of AMPs could be receptor mediated. On the

other hand, microbiological assays of membrane permeability, and microscopic imaging of bacteria treated with AMPs clearly show that cell membranes are damaged, and that, as a consequence, transmembrane gradients are dissipated (Tiozzo 1998; Arcidiacono 2009; Hartmann 2010; Agrawal 2018) (Figure 4). Usually, membrane perturbation and bacterial killing are correlated, further supporting membrane disruption as the main bactericidal mechanism. However, also in this case exceptions exist, indicating that a subclass of AMPs might act through different killing mechanisms (He 2014; Friedrich 2000). Finally, it is worth mentioning that AMPs usually are able to perturb the permeability of artificial membranes, comprising only phospholipids (Figure 4 c) (Orioni 2009; Bocchinfuso 2011; Braun 2017; Savini 2018;). This observation demonstrates that the membrane-perturbing activity is purely the result of physico-chemical interaction between the peptide and the lipid bilayer, and not the consequence of some biological process.

Overall, literature data clearly demonstrate that membrane perturbation is the main mechanism of direct bacterial killing for most AMPs. Even for those AMPs that act through a different antibacterial mechanism (Nicolas 2009; Otvos 2017), the cell envelope is the first cell component that the peptides encounter, and they have to cross the extracellular membrane and the cell wall (when present) to reach the plasma membrane and eventually the cell interior.

Incidentally, the fact that AMPs target microbial membranes determines their broad spectrum activity, the fact that they are bactericidal, rather than bacteriostatic, and also the higher difficulty for bacteria in developing resistance against them (compared to resistance against conventional antibiotics acting on a protein target) (Perron 2006; Otvos 2017).

4. Bacterial and host cells have different membrane structure and composition

If membranes are the target, then it is conceivable that selectivity arises from a difference in membrane composition of the various cell types. Indeed, bacterial and eukaryotic cells have very different cell envelopes (Wang 2017). Bacteria can be divided in Gram-positive and Gram-negative, depending on whether they are colored by the Gram stain or not. This assay reflects differences in the composition of the cell envelope. In both cases, the plasma membrane is surrounded by a cell wall. However, in Gram positive bacteria, this is formed by a thick peptidoglycan and lipoteichoic acid layer (40-80 nm). By contrast, in Gram negatives, a thin peptidoglycan layer (8 nm thick) is contained in a second (outer) membrane, with asymmetric composition: phospholipids are the main components of the inner leaflet, while the outer layer is mainly formed by lipopolysaccharides (LPS). On the other hand, eukaryotic cells only have the plasma membrane, with asymmetric lipid composition in the two leaflets of the bilayer (Figure 5).

In addition to the different structures of the cell envelope, important differences are present in the lipid composition of the cellular membranes. Tables 4 and 5 summarize the lipid content of bacterial and RBC membranes.

Bacterial membranes contain a significant fraction of negatively charged lipids: in Gram-negatives both membranes contain phosphatidylglycerol (PG, ~20% overall) and cardiolipin (CL, ~5% overall); in Gram positive bacteria, the content of anionic lipids is much higher, again with PG and CL being the most important components (Malanovic 2016). However, the membranes of these cells can also contain the positively charged L-lysyl-PG (LPG). In both cases, the main zwitterionic component is phosphatidylethanolamine (PE) and no sterols are present. Of course, the values for the composition reported in Table 4 are only approximate, since they change with the specific strain and growth conditions. In addition, lipid composition is not homogeneous over the cell surface (Renner 2011; Oliver 2014).

Human cells contain cholesterol and have no anionic phospholipids in the outer leaflet of their cell membrane. Some negatively charged glycolipids, such as gangliosides, are present on the cell surface (Miyazaki 2012),

but they are minor components in most cell membranes (with the exception of nerve cells) (Storch 1985). These properties are exemplified by RBCs, which are commonly used to test toxicity and selectivity (Table 5). For eukaryotes, the main zwitterionic components are phosphatidylcholine (PC), sphingomyelin (SM) and PE.

Overall, we can translate these differences in lipid composition in distinct physico-chemical properties. Bacterial membranes contain more anionic lipids in the outer surface of their bilayers than eukaryotic cells. This difference combines with the additional negative charges conferred to bacterial cells by teichoic and teichuronic acids and LPS. Furthermore, the transmembrane potential of bacterial cells is more inside-negative than that of normal mammalian cells (Yeaman 2003). For all these reasons, bacteria have stronger electrostatic interactions with positively charged molecules than eukaryotic cells. Another difference is that bacterial membranes are more disordered and less well packed than those of eukaryotes, due to the lack of cholesterol. In addition, they contain larger amounts of “non bilayer” lipids, with negative or positive values for the “intrinsic curvature”, such as PE, CL and PA, or LPG, respectively (McMahon 2015; Malanovic 2016). This property depends on the relative sizes of the phospholipid head-groups and acyl chains. Lipids where the cross-sectional area occupied by head-groups and tails is similar (e.g. PC, PG, PS) are said to have a cylindrical shape, and pack well in locally flat bilayer structures (zero intrinsic curvature). By contrast, lipids where the head-group is smaller than the tails (e.g. PE or PA) favor concave shapes of the monolayer (negative curvature). The opposite is true for lipids with comparatively larger polar heads (e.g. LPG) which have a positive curvature (Koller 2014).

The differences in lipid composition and in physical properties between bacterial and human cell membranes are considered to be the origin of AMP selectivity. Similar considerations on membrane composition (particularly regarding the content of anionic lipids and sterols) have been proposed to explain the selectivity for cancer cells (Hoskin 2008; Schweizer 2009; Phoenix 2012; Gaspar 2013), fungi (van der Weerden 2013; Rautenbach 2016), protozoa (Rivas 2009) and enveloped viruses (Aloia 1993; Findlay 2013), since in all cases the lipid distribution is different from that of a normal eukaryotic cell.

5. Lipid composition determines the affinity of AMPs for lipid bilayers

The hypothesis of a selectivity based on differences in lipid composition has been tested by studying the interaction of AMPs with model membranes mimicking the composition of the natural bilayers. With liposomes, it is possible to vary the lipid composition at will, and to measure both peptide-membrane association, and peptide-induced membrane permeability (Bocchinfuso 2011; Savini 2018). The role of various membrane properties in AMP selectivity is summarized in the following Sections.

5.1 Membrane charge

In model membranes, the presence of anionic lipids increases peptide association to the bilayer, and, as a consequence, peptide-induced leakage. (Matsuzaki 1989, 1995; Gazit 1995; Abraham 2005; Sood 2008 a; Russell 2010; Bobone 2013; Golbek 2017; Maturana 2017). On the other hand, the positively charged lipid lysyl-PG, present in Gram+ bacteria, inhibits AMP activity (Nishi 2004; Andra 2011). These findings are a straightforward consequence of electrostatic interaction of the membranes with the positively charged AMPs (see Section 6). Regarding the anionic gangliosides present in the outer leaflet of eukaryotic membranes, Matsuzaki and coworkers (Miyazaki 2012) demonstrated that, although their acidic moieties favor the association of AMPs to model membranes, this interaction does not lead to strong membrane perturbation, since the peptides remain trapped in the sugar region.

5.2 Cholesterol content

Several studies also reported an AMP-inhibitory effect of cholesterol. For instance, the presence of cholesterol inhibits the membrane-perturbing activity of magainin, pardaxin, LL-37, temporin L, human defensin HNP1 and other AMPs (Matsuzaki 1995; Tytler 1995; Hallock 2002; Sood 2008 a, b; Gonçalves 2012; Verly 2008; Wu 2010; McHenry 2012). The membrane-ordering effects of cholesterol in fluid bilayers are well established: insertion of the rigid ring structure of the sterol limits the possibility for trans-gauche isomerization for adjacent phospholipid tails, leading to an increase in bilayer order, packing, thickness and rigidity (Henriksen 2006; Mouritsen 2004). All these effects could contribute to reduce peptide binding and membrane perturbation (McIntosh 2002). However, the relevance of cholesterol for AMP selectivity has been recently questioned. While all the investigations listed above were performed on simple lipid mixtures, a comprehensive study by Ramamoorthy and coworkers on more realistic lipid compositions showed that cholesterol's protective effect against AMPs does not occur in lipid systems containing raft domains and presenting phase separation (McHenry 2012; Brender 2012). There are examples where the activity of AMPs is not affected by the presence of cholesterol even in simple lipid mixtures (Bobone 2013). On the other hand, Matsuzaki (1995) demonstrated an AMP-inhibiting effect of cholesterol in real cells, by artificially varying the cholesterol content of RBCs.

It is worth mentioning that the inhibitory effect is specific of cholesterol, while ergosterol, present in fungal membranes does not appear to inhibit peptide binding and activity to the same extent, in agreement with the specific activity of antifungal peptides (Sood 2008 b; Gonçalves 2012) and with the comparatively smaller effects of ergosterol on membrane order (Henriksen 2006).

5.3 Intrinsic curvature

The situation is less clear regarding the effect of the presence of negative curvature lipids (PE) in bacterial membranes. PE has been shown to inhibit pore formation by magainin, melittin, alamethicin, PMAP-23, mastoparan X (Matsuzaki 1998; Allende 2005; Lee 2005; Bobone 2012). On the other hand, the activity of some AMPs is favored by the presence of PE (Schröder-Borm 2003; Epand 2006; Leite 2015). Inhibition of pore formation by PE can be understood by considering that the peptides act by inserting in the head-groups region of the membrane, thus imposing a positive curvature strain, which is released after a threshold of membrane-bound peptide concentration is reached, through the formation of membrane defects or pores. The presence of lipids with negative intrinsic curvature would counteract this mechanism (Matsuzaki 1998, Lee 2005). Similar considerations, on the other hand, suggest that PE can favor membrane binding of AMPs, by reducing the intrinsic curvature strain needed for peptide insertion in the polar region of the bilayer; an increased binding to PE containing membranes has been reported for some AMPs (Schröder-Borm 2003; Phoenix 2015). However, reasoning only in terms of intrinsic curvature might be misleading. For instance, the peculiar lipid-lipid interactions made possible by the structure of PE could inhibit peptide insertion: this phospholipid contains a primary amine (lacking in PC), which allows it to form strong hydrogen bonds with phosphate or CO groups in other lipids (Lewis 2005). This H-bond network is responsible for the melting temperatures of PE lipids being higher than those of their corresponding PC analogues (Lewis 2005). This difference might lead to an increase in the energy needed to insert a peptide in the bilayer, or to open a pore, in the presence of PE. Finally, an additional mechanism invoked to explain different activities on vesicles lacking or containing PE involves peptide-induced formation of lipid domains (Epand 2006). Overall, these considerations can explain why different final effects are observed on the membrane-perturbing activity of AMPs, depending on which of the various phenomena predominates in each specific case. In any case, it is difficult to ascribe a well defined role in AMP selectivity to the PE content.

6. Thermodynamics of peptide-membrane association

In principle, selectivity for different membrane compositions could result from two effects. AMPs could have a higher affinity for bacterial membranes than for human bilayers, or they could be more effective in perturbing the former, once inserted (Wimley 2011). The data on model membranes presented above clearly indicate that differential binding is an important aspect of AMP selectivity.

AMPs are usually short (about 10–50 residues in length) and their sequences and structures have no common features, except for the cationic charge (most AMPs fall in the range of +2 to +4 e), and amphipathic character, with an overall content of about 50% hydrophobic residues (Wang 2017). The role of these properties is easily rationalized: charge imparts selectivity toward bacterial versus eukaryotic membranes and apolar residues provide a hydrophobic driving force for binding and insertion into membranes, leading to perturbation of bilayer integrity. Indeed, peptides interacting only electrostatically usually do not cause significant membrane leakage, because their depth of insertion is too shallow (Wimley 2010 a).

6.1 Hydrophobic and electrostatic driving forces are non-additive

Different treatments are used in the literature to describe peptide-membrane interactions, and comprehensive reviews are available on this topic (White 1999; Wieprecht 2002; Simon 2002; Santos 2003; Seelig 2004; Wimley 2010 a). Here we will just briefly mention that, since peptide/membrane association does not have a specific stoichiometry, it is not correctly described by a binding equilibrium, and it is better treated as a partition equilibrium between the water and the membrane phase (White 1999; Wieprecht 2002; Santos 2003; Wimley 2010 a). In this view, the main effect of Coulombic interactions can be described as an increase in local peptide concentration in the vicinity of the bilayer, according to Gouy Chapman theory (Beschiaschvili 1990; Wieprecht 2002; Seelig 2004). However, the thermodynamic contributions of electrostatic and hydrophobic effects to the driving force of peptide water/membrane partition are not simply additive (Ladhokin 2001). This finding is due mainly to the different depths of polar and aliphatic moieties of phospholipids in the bilayer: charged groups are located on the surface of the membrane, well separated from the hydrocarbon core, and the physico-chemical properties of the bilayer vary steeply in the head-group region (Wimley 2010 a). As a consequence, the depth of insertion of a peptide in the membrane is determined by the interplay between hydrophobic effect and Coulombic forces (Wimley 2010 a). Highly charged, hydrophilic molecules sit on the membrane surface, strongly hydrophobic peptides insert into the hydrocarbon core, while cationic, amphipathic peptides are located at an intermediate position, which depends on their specific properties (Bocchinfuso 2009; Farrotti 2015). In turn, the depth of insertion in the bilayer modulates the intensity of electrostatic and hydrophobic contributions: strongly simplifying, one could say that the position of a peptide in the membrane determines the average distance between the peptide and the charged lipid moieties, and the degree of insertion of the peptide in the water-free hydrocarbon core. An increase in peptide hydrophobicity ultimately reduces the effect of electrostatic interactions; on the other hand, augmenting the Coulombic forces diminishes the hydrophobic contribution to the binding free energy (Ladhokin 2001).

6.2 Multiple interconnected equilibria modulate peptide activity and selectivity

Several artificial peptides were designed having the required characteristics of cationic charge and amphipathic character. However, in many cases such peptides turned out to be highly toxic (Dathe 1996; Cornut 1994; Bobone 2013). These findings demonstrated that a cationic charge is not sufficient for specificity and provided a first indication of the complexities of peptide-membrane interaction discussed above. Peptides in solution can assume different conformations and aggregation states, once membrane-

bound they can change conformation, orientation, insertion depth and aggregation state (Figure 6). All these phenomena are regulated by interconnected equilibria and therefore they contribute in determining the final membrane-perturbing activity (Stella 2004; Mazzuca 2005; Gatto 2006; Bobone 2013). Every modification in peptide properties can affect all these processes (Gatto 2006). In our opinion, this is the reason why the rational design of peptides with improved selectivity has met with limited success, and it has progressed through a trial and error process. Even so, several useful principles for the optimization of AMP selectivity have been defined.

7. Selectivity of AMPs is determined by their physico-chemical properties

Helical peptides are the most abundant and best-characterized class of AMPs. Investigations on the structural determinants of AMP selectivity have mostly focused on this type of peptides. They are usually disordered in solution, but attain a helical conformation when membrane bound, with a spatially amphipathic distribution of the side chains, where most of the hydrophobic residues face towards the membrane center, and the polar and charged residues are oriented towards the water phase. From a physico-chemical point of view, they can be characterized by several parameters, such as charge, hydrophobicity or amphipathicity. In addition to the considerations discussed in the previous Section, on the multiple processes involved in peptide/membrane interactions, investigations on the role of each of these parameters are complicated by the fact that varying the sequence by a single amino acidic substitution usually causes a variation in multiple physico-chemical properties of the peptides (Wieprecht 1997 a; Dathe 2001). For instance, inserting an additional cationic residue does not vary only the peptide charge, but also its hydrophobicity and amphipathicity. However, some systematic studies have been performed where the authors tried to compare peptide sequences where multiple substitutions were inserted to cause the significant variation of one parameter only, while the others were kept as constant as possible (Dathe 1997; Wieprecht 1997 a, b, c; Dathe 1999; Dathe 2001; Giangaspero 2001; Dathe 2002; Zelezetsky 2005, 2006 a).

7.1 Cationic charges favor selectivity

Based on the results on the importance of anionic lipids for the membrane activity of AMPs, it is not surprising that a positive correlation between peptide positive charge and antimicrobial activity and selectivity has often been described (Bessalle 1992; Matsuzaki 1997; Dathe 2001; Giangaspero 2001; Zelezetsky 2006 a; Bobone 2011). However, several studies reported that increasing cationicity above a certain level (+5, +8 or +9 depending on the specific case) is not beneficial and might even cause a decrease in activity or selectivity (Dathe 2001; Giangaspero 2001; Zelezetsky 2006 a, Jiang 2008). This last finding might be due an overly shallow insertion of the peptide in the bilayer (Wimley 2010 a) and to the non-additivity of electrostatic and hydrophobic effects, discussed in Section 6.

One of the ways to increase the total positive charge of the peptide is C-terminal amidation, which is frequent in natural sequences and has the additional advantage of reducing susceptibility to proteolytic degradation (Huang 2010; Mura 2016). However, this approach to increase peptide selectivity is not generally valid, possibly because it also affects the stability of helical conformations in solution (Dennison 2009) (see Section 8).

7.2 Hydrophobicity is necessary for activity, but correlates with toxicity: the two thresholds

The other main parameter influencing peptide affinity for membranes is hydrophobicity. Several studies concur to support the view that two hydrophobicity thresholds exist (Dathe 1997; Kondejewski 1999, 2002;

Stark 2002; Chen 2007; Glukhov 2008; Mojsoska 2015; Uggerhøj 2015). A first threshold hydrophobicity value must be reached to obtain peptides with significant membrane binding and insertion, and thus endowed with antimicrobial activity. However, if hydrophobicity surpasses a second, higher threshold, toxicity is observed, because binding to neutral membranes becomes significant. The difference between these two thresholds is due to the electrostatic contributions to peptide binding to bacterial membranes. Therefore, an optimal range of hydrophobicity values exists, in which peptides exhibit antimicrobial activity, but no significant toxicity. Above a third, even higher threshold, activity decreases, because of peptide aggregation and lack of solubility (Gatto 2006; Chen 2007; Chu-Kung 2010; Wimley 2010 a). It is difficult to provide quantitative values for these thresholds, since different hydrophobicity scales are used in the literature. Just as an example, Deber and coworkers identified values of 0.4 and approximately 2 in the Liu-Deber scale for the activity and toxicity thresholds, respectively, for the hydrophobicity of the core segment of a series of model peptides (Glukhov 2008).

It is interesting to note that hydrophobicity affects binding to neutral membranes more than to charged bilayers, and hemolysis more than bactericidal activity (Wieprecht 1997 a; Dathe 2002). The rationale underlying this finding is not immediately obvious, since the hydrophobic driving force is present for both membrane types, and therefore any variation in hydrophobicity should affect both antimicrobial activity and toxicity to the same extent. The experimental observations can be explained based on the non-additivity of electrostatic and hydrophobic effects (Section 6).

In the case of highly hydrophilic peptides, modifications that increase hydrophobicity can enhance the antimicrobial activity (first threshold), without inducing strong toxicity (second threshold). Malmsten and coworkers have reported addition of hydrophobic oligopeptide stretches to the N or C-terminus of the sequence as a way to improve peptide activity and selectivity (Pasupuleti 2009; Schmidtchen 2009, 2011, 2014). Comparison of different hydrophobic modifications indicated that tagging by oligo-Trp sequences at the C-terminus is the most effective one, leading to a substantial increase in activity, without significant enhancement of toxicity. Trp residues have peculiar properties, since they are known to have an affinity for membrane interfaces, thanks to their ability to interact both with hydrophobic moieties and with charged groups (through cation-aromatic interactions) (Yau 1998). It has been speculated that the specificity enhancing effect of Trp might be linked to the difficulty of inserting such a bulky residue in the tightly packed, cholesterol-containing membranes of eukaryotes (Pasupuleti 2009; Schmidtchen 2009, 2011, 2014). However, preferential interaction of Trp with cholesterol has also been hypothesized (de Kruijff 1990), although it is disputed (Holt 2008), and in some cases introduction of Trp residues has been linked to enhanced peptide toxicity (Oddo 2017; Matsuzaki 1997). Another common approach to increase the hydrophobicity of highly hydrophilic peptides is lipidation (Gatto 2006). Shai's group demonstrated that highly polar peptides, originally devoid of antimicrobial activity, can become antimicrobial, but not toxic, after this modification (Avrahami 2004; Malina 2005; Makovitzki 2006, 2008). However, in other cases, lipidation led to strong toxicity (Chu-Kung 2004; Laverty 2010), or even to loss of activity, when it compromised peptide solubility (Toniolo 1996; Gatto 2006; Chu-Kung 2010). These findings highlight the fine tuning of AMP hydrophobicity needed for optimal activity and selectivity properties.

7.3 Excessive amphipathicity causes toxicity

The total quantities of charged and hydrophobic residues provide only a very rough measure of peptide properties, since also their position in the sequence and structure are obviously important. Amphipathicity measures the degree of asymmetry in the distribution of polar and hydrophobic residues. This property can be quantified by the hydrophobic moment. This quantity is usually defined assuming an ideal helical structure, and summing the vectors indicating the position of each residue with respect to the helix axis, multiplied by their respective hydrophobicity values (in analogy with the definition of an electric dipole). To

compare sequences of different lengths, the mean hydrophobic moment can be obtained by normalizing for the number of amino acids (Eisenberg 1982; Phoenix 2002). Peptide amphipathicity is a very important parameter for determining the free energy of membrane binding (Fernández-Vidal 2007). As early as 1981, De Grado demonstrated that amphipathicity is sufficient to induce lytic activity in a helical peptide (De Grado 1981). The specific value of the hydrophobic moment becomes particularly important for selectivity in an intermediate range of hydrophobicity values, when the hydrophilic or hydrophobic components of the peptide do not predominate in determining its behavior (Dathe 1999; Dathe 2002). Similarly to what has been reported for hydrophobicity, an increased hydrophobic moment affects the activity on neutral membranes more than that on charged bilayers (Wieprecht 1997 b; Dathe 2002). Increasing amphipathicity above a critical threshold results in strong interaction with neutral membranes, leading to toxicity (Wieprecht 1997 b; Dathe 1999; Fernández-Vidal 2007; Kindrachuk 2010). As discussed above for hydrophobicity, also in this case it is difficult to provide a quantitative, generally valid value for this threshold.

Another measure of the distribution of polar and hydrophobic residues is the angle subtended by the polar face of the amphipathic helix, again assuming an ideal conformation and looking along the helix axis (Uematsu 2000). The available data on the role of this property in selectivity are limited, but a comprehensive study by Dathe and coworkers (Dathe 2002) provided some indications. As discussed above, hydrophobicity and hydrophobic moment mostly affect the affinity for neutral membranes, and thus the toxic activity. By contrast, in model membranes the polar angle affects AMP ability to perturb the bilayer, after membrane binding: in charged bilayers peptide-induced membrane leakage decreases with increasing polar angle, while it is essentially unaffected in neutral membranes (Dathe 2002). However, the effects of the polar angle in cellular assays of activity and toxicity are more limited (Dathe 2002).

8. Conformational and aggregation equilibria play an important role in membrane selectivity: the concept of effective hydrophobicity

All the considerations reported in the previous Section are based on hydrophobicity values determined from the peptide amino acidic composition, and on amphipathicity calculated assuming an ideal helical conformation. In addition, a monomeric peptide state is always considered. However, as discussed in Section 6, peptides in solution and in the membrane attain specific ensembles of conformations, which can deviate significantly from an ideal alpha helix. In addition, amphipathic peptides have a strong tendency to aggregate (Figure 6). Conformational equilibria and self-assembly affect the degree to which the hydrophobic moieties of AMPs are exposed to the water phase, and therefore modulate the hydrophobic driving force for membrane binding (Bobone 2013). Similarly, water-membrane partition is affected by the peptide conformation, orientation and depth of insertion in the bilayer. Based on these considerations, in our opinion AMP selectivity is not determined by the “ideal” peptide hydrophobicity or amphipathicity, but by what we call “effective” hydrophobicity and amphipathicity, *i.e.* the value these parameters assume in the actual conformation and aggregation state attained by the peptide in solution and in the bilayer (Bobone 2013; Uggerhøj 2015).

If peptide conformation and aggregation influence peptide hydrophobicity, the opposite is also true: high hydrophobicity and amphipathicity values favor a stable secondary structure by allowing the formation of intramolecular interactions between apolar residues (Fernández-Vidal 2007). Peptide structure is influenced by self-assembly processes, too (Sal-Man 2002). Therefore, in order to fully understand the determinants of peptide selectivity, conformational and self-assembly equilibria should be considered.

8.1 Helicity correlates with toxicity

A correlation between peptide helicity and toxicity has been reported in many studies (Tossi 2000; Giangaspero 2001; Zelezetski 2005; Chen 2005; Khandelia 2006; Zhang 2011; Mangoni 2011; Chapuis 2012; Bobone 2013; Cherry 2014). In addition, helix destabilizing Gly or Pro residues are often present close to the center of the sequence of natural, selective AMPs that attain a helical conformation in membranes (Tossi 2000, Bobone 2013). These amino acids are important for peptide selectivity, since their deletion, substitution or insertion significantly affects toxicity, through the perturbation of the secondary structure (Thennarasu 1996; Zhang 1999; Shin 2001; Yang 2002; Lee 2004; Song 2004; Yang 2006 a, b; Lee 2007; Carotenuto 2008; Bobone 2013; Wang 2015). An increase in selectivity with a reduction in helical structure has been reported for other helix-breaking strategies, such as the insertion of D-amino acids (Shai 1996; Oren 1997; Shai 2001; Papo 2002; Chen 2005; Zhu 2007 c; Kaminski 2011; Nan 2012; Huang 2014), or peptoid residues (N-substituted glycines, which lack a H-bonding proton on the N backbone atom and comprise a flexible main-chain methylene group) (Song 2005; Zhu 2007 a, b; Kim 2010). Incidentally, these non proteinogenic residues have the added advantage of reducing peptide susceptibility to proteolysis (Papo 2002; Kim 2010).

The correlation between helicity and toxicity was tentatively explained by proposing that a stable helical conformation enhances the peptide propensity to aggregate (Kindrachuk 2010; Vermeer 2012): in helical amphipathic peptides, the hydrophobic face of the helix is totally exposed to the aqueous phase, and therefore aggregation is hydrophobically favored. Aggregation, in turn, would inhibit crossing of the LPS layer and cell wall, and thus access to the plasma membrane of bacteria (see below). However, helicity normally affects toxicity, rather than antibacterial activity. In addition, in the peptides we investigated, aggregation was significant only at concentrations higher than the membrane-perturbing values, and therefore it was not relevant for activity (Bobone 2013). Probably a higher tendency to aggregate and an enhanced toxicity are just two independent consequences of the hydrophobicity induced by a stable helical structure, but the lack of selectivity in helical peptides is not caused by peptide aggregation (see also below). Based on a systematic study in which a central proline residue was moved along the sequence or deleted, we obtained data supporting an alternative explanation (Bobone 2013). In a perfectly helical, amphipathic structure, the apolar residues are completely exposed on the hydrophobic face of the helix. Even though short peptides are often unstructured in water, helical conformations can be at least partially populated, also thanks to the stabilization due to the interaction between hydrophobic side chains aligned along the helix (a motif often called “leucine zipper” (Asthana 2004)). White and coworkers reported a strong correlation between the amphiphilicity of a peptide sequence in an ideal helical conformation and both the degree of helicity in solution and the affinity for neutral membranes (Fernández-Vidal 2007). Destabilization of the helical conformation allows the peptide to fold onto itself, hiding the apolar side chains from the water phase, and reducing the effective hydrophobicity of the peptide, and thus the driving force for binding neutral membranes (Bobone 2013; Büttner 1992). Destabilization of the helix also increases the entropic cost of membrane binding, since association to the bilayer is normally followed by peptide structuring (Zelezetsky 2005). Interestingly, once membrane bound, the helix-destabilizing modifications do not preclude the attainment of an amphipathic helical conformation (Bobone 2013; Orioni 2009; Oren 2000). Therefore, variations in the free energy of membrane binding are determined essentially by changes in the solution conformation. Unstructured conformations would inhibit binding to neutral membranes, but would affect only marginally the affinity for charged bilayers, leading to enhanced selectivity. Related to this interpretation is the concept of position-dependent hydrophobicity: hydrophobic residues in unstructured regions of the peptide contribute to the effective hydrophobicity and to toxicity less than those in helical segments (Tachi 2002).

The effective, conformation-dependent hydrophobicity can be calculated from peptide structures (Gaillard 1994), but it can also be determined experimentally. Reversed-phase chromatography retention times have

resulted to be an accurate measure of the effective hydrophobicity of peptides (Krause 1995; Zhou 1990; Kim 2005). Interestingly, strong correlation between RP-HPLC retention times and the hemolytic activity of AMPs has been reported (Blondelle 1991; Kondejewski 1999; Tachi 2002).

8.2 Imperfect amphipathicity optimizes selectivity

Another idea related to effective hydrophobicity is imperfect amphipathicity. Insertion of a polar/charged residue in the hydrophobic face of an amphipathic helix has proven to be a reliable method to increase AMP selectivity (Asthana 2004; Chen 2005; Ahmad 2006; Hawrani 2008; Ahmad 2009 a, b; Pandey 2010; Pandey 2011; Jiang 2011, 2014; Son 2013; Dalzini 2016; Zhang 2016; Jiang 2018). Hodges and coworkers even termed these misplaced polar residues “specificity determinants” (Jiang 2011, 2014; Jiang 2018), even though exceptions to the selectivity-improving effect of this approach have been reported (Wang 2018). Interestingly, an imperfectly amphipathic structure is a common property of many natural, selective AMPs (Wimley 2010 b; Orioni 2009). Again, this approach reduces the hydrophobic driving force for binding neutral membranes. On the other hand, antimicrobial activity is usually not affected significantly by these changes: in charged bilayers, binding takes place all the same, thanks to the electrostatic attraction; once membrane-bound the peptide is able to attain a (possibly distorted) helical conformation, as demonstrated by spectroscopic and simulative studies (Hawrani 2008; Orioni 2009). In the bilayer, imperfect amphipathicity will contribute to membrane disruption, by driving some polar head-groups in the hydrophobic core of the membrane, as we observed for PMAP-23 (Orioni 2009) (Figure 7). This type of membrane activity has been termed “interfacial activity” by Wimley (2010 b).

Finally, it is worth mentioning that we recently observed an effect of imperfect amphipathicity on toxicity also in the case peptidomimetic antimicrobial molecules (Konai 2018). Two small amphipathic, cationic molecules, were characterized by the same compositional hydrophobicity, but had very different selectivity. By combining molecular dynamics simulations and RP-HPLC retention times, we demonstrated that this was due to imperfect amphipathicity and lower effective hydrophobicity of the selective analogue compared to the toxic compound.

8.3 Effects of peptide aggregation in the aqueous phase on activity and toxicity are system-dependent

AMPs, due to their amphipathic nature, are susceptible to aggregation in water (Tian 2015). Some peptides oligomerize through the interaction of the apolar sides of their amphipathic helices (Oren 1999; Asthana 2004; Raimondo 2005; Ahmad 2006;), while others form micellar structures (Liu 2009; Wang 2010; Joshi 2014; Lin 2015; Haney 2017; Lei 2018), fibrils (Tu 2007; Chen 2010; Chen 2013; Shankar 2013; Chairatana 2014; Ravi 2015), or even hydrogels (Veiga 2012; McCloskey 2014; Haney 2017). Often the aggregates disassemble into monomers once membrane-bound (Ghosh 1997). The critical concentration for self-assembly can vary significantly from one specific case to the other, also depending on the experimental conditions, and particularly on salt concentration.

The results of experimental and theoretical studies on the effects of aggregation on peptide activity and selectivity are extremely contradictory. Both a negative (Feder 2000; Kustanovich 2002; Chen 2006, 2007; Daschbach 2012; Lin 2015; Farrotti 2017; Haney 2017; Bagheri 2018; Zou 2018) and positive (Sal-Man 2002; Avrahami 2002; Liu 2009; Chen 2010; Joshi 2014; Ravi 2015; Lei 2018) correlation between aggregation and activity have been reported, as well as lack of activity changes following aggregation (Chen 2013). Similarly, some studies found that toxicity was not significantly affected by aggregation (Joshi 2014; Lei 2018;), while others reported an increase (due to differential effects on membrane binding) (Zelezetsky 2006 b; Chen 2013; Lin 2015), or a decrease (Kustanovich 2002; Chen 2006; Chen 2007) in selectivity upon self-assembly. Shankar

et al. (2013) suggested that toxicity of self-assembled lipopeptides depends on the specific structure of the fibrillar aggregates.

The reported discrepancies are most likely due to the fact that peptide aggregation is usually controlled by varying the peptide properties, or by modulating electrostatic interactions by changing the ionic strength of the solution. It is therefore difficult to discriminate between the direct effects of these changes (*e.g.* an increase in hydrophobicity) and the consequence of the variations they induce in aggregation. One approach to solve this problem is covalent linking of the monomers (Sal-Man 2002; Dempsey 2003), but it does not exactly mimic self-assembly driven by hydrophobic interactions.

Thermodynamic considerations on the interconnected equilibria involved in AMP activity indicate a possible positive role of aggregation in enhancing peptide selectivity. Aggregation, which is hydrophobically driven, reduces the effective peptide hydrophobicity by hiding the apolar moieties in the molecule from the aqueous phase. As a consequence, the hydrophobic driving force for membrane binding is reduced in the aggregates (Stella 2004; Mazzuca 2005; Gatto 2006; Chen 2007; Chu-Kung 2010; Farrotti 2017). Considering the various hydrophobicity thresholds discussed above, aggregation could therefore lead to a reduced toxicity. At the same time, preassembly of AMPs causes a local release of a high concentration at a single site in the membrane, and this could cause higher activity (Ravi 2015). In addition, computational studies suggested that self assembly could lead to membrane selectivity also by affecting the kinetics of membrane binding (Lin 2015): binding to host mammalian membranes will be slow and inefficient as long as the lipopeptides are micellized in solution, while binding to the bacterial surface will still be efficient, thanks to electrostatic interactions and to the higher fluidity of the membrane. On the other hand, in cellular assays the large size of the aggregates, compared to monomers, could impair selectivity: preassembled AMPs might be unable to cross the LPS layer or the cell wall, and thus to reach the plasma membrane of bacteria. At the same time, they would still be able to interact with the “naked” membrane of host cells (Oren 2000; Kustanovich 2002; Sal-Man 2002; Mangoni 2009).

Discussing aggregation, it is important to note that this phenomenon reduces susceptibility to proteolytic degradation and affects the pharmacokinetics and pharmacodynamics *in vivo* (Raimondo 2005; Tu 2007; Chen 2013; Lei 2018). It is also worth mentioning that human α -defensin 6 (HD6) has negligible direct killing activity, but prevents infections by self-assembling into a network of fibrils, that capture pathogens and thus contrast microbial invasion (Chairatana 2014).

9. AMP binding to cells

As discussed in the previous Sections, AMP selectivity is usually interpreted essentially on the basis of the different affinities observed in liposome studies for bilayers mimicking the membranes of bacteria or eukaryotes. However, quite surprisingly, peptide affinities towards the two types of cells are largely uncharacterized. In addition, if peptide activity is modulated by a cell-binding equilibrium, it should depend on the density of cells, but antimicrobial activity and toxicity assays are usually carried out using standardized, fixed cell densities, which are not necessarily representative of the cell concentrations present in a typical infection site (Savini 2018). Finally, bactericidal and hemolytic activities are routinely determined in separate assays, but when the two cell populations are present at the same time, they compete for peptide association. All these aspects have received limited attention, until quite recently. Biophysical studies on model membranes allow the determination of both membrane binding and bilayer perturbing activity, while microbiological studies usually report activities only in terms of total peptide concentration. We recently reviewed the few studies that are trying to apply to cellular experiments the same quantitative approaches normally used with model systems (Savini 2018). Here, only the aspects relevant to AMP selectivity are summarized.

9.1 AMPs have a higher affinity for bacterial than for eukaryotic cells

Only a handful of studies reported data on AMP binding to bacterial and eukaryotic cells. As soon as 1988, Bruce Merrifield and his group (Steiner 1988) measured binding of cecropin A and some of its analogs to *Escherichia coli*, *B. megaterium*, *B. thuringiensis* and *P. aeruginosa* cells and to erythrocytes. While binding to the bacteria was significant (between 70 and 80% for the natural peptide, under the conditions studied) no detectable association was observed for RBCs, at a cell density corresponding to a membrane area similar to that present in the experiments with bacteria. Welling (2000) measured the binding of defensin 1-3, ubiquicidin and human lactoferrin to bacteria and activated murine peritoneal leucocytes. In the presence of the same cell density (2×10^7 cell/mL) the peptides bound 5-500 times more efficiently to bacteria than to mammalian cells, even though the latter are much bigger. Similarly, Ferro-Flores (2003) reported that, in the presence of 2×10^7 cell/mL, an ubiquicidin analogue was 35% bound in the case of bacteria (*S. aureus*), while less than 4% in the case of human tumor cell lines LS174T and ACHN (which, again, are significantly bigger than bacteria). Comparable results have been reported for two ubiquicidin analogues (approximately 45-100% binding to *S. aureus*, while only 10% to leukocytes, in the presence of 2×10^5 cell/mL), although in this case selectivity was surprisingly observed also for an anionic peptide used as negative control (Ebenhan 2014 b). Wimley and coworkers (Starr 2016) measured the binding of the artificial AMP ARVA to *E. coli*, *S. aureus* and RBCs. In all cases, association to bacteria was more favorable than to RBCs. After accounting for the differences in cell size, the authors estimated that the affinity for bacterial membranes was more than 2 orders of magnitude higher than for erythrocytes. Finally, an analog of LL37 was reported to bind *E. coli*, *S. aureus* and *M. smegmatis*, but not to hepatic cells, under conditions of comparable cell numbers (Dutta 2017). Overall, these data indicate that the differential affinity routinely observed with model bilayers is present also for the membranes of real cells.

9.2 Activity and toxicity are cell-density dependent

Another aspect that has remained essentially uncharacterized until very recently is whether the activities of AMPs depend on the density of cells present in the assays. Based on a partition equilibrium, the fraction of membrane-bound peptide obviously depends on the concentration of cells in the sample. Therefore, it is to be expected that MIC/MBC/MHC values depend on the concentration of cells used in the assays. In broth dilution assays of antimicrobial activity the recommended value for the initial cell density (inoculum) is 5×10^5 cell/mL (Patel 2012), which was selected for minimizing false positive and false negative results in the clinical practice (Wiegand 2008). However, bacterial cell-densities in clinically relevant infections range from 1 to 10^9 cell/mL. Similarly, hemolytic activity assays are normally performed with 5×10^8 cell/mL, which is 1/10 of the cell density in whole blood (Savini 2018 and references therein). Matsuzaki (2009) pointed out that the cell densities in the two assays are very different, also considering that the membrane area of an erythrocyte is approximately 10 times bigger than that of a typical bacterium. Therefore, he wondered if TI values such as those reported in Table 1 are an experimental artifact due simply to the fact that more peptide is probably needed to kill a higher number of bigger cells.

In the case of traditional antibiotics, it is well known that the MIC often depends on the size of the bacterial inoculum ("inoculum effect"). By contrast, in the case of AMPs, this possible dependence has been investigated only in very few studies (Savini 2018). In the 1990's, Levison (1993) reported that the bactericidal activity of magainins against *P. aeruginosa* was inoculum dependent above 3×10^5 cell/mL, but it did not vary if the inoculum was reduced below this value. Similarly, Jones (1994) observed an inoculum effect for lactoferricin B against *E. coli*, with a plateau at inoculum densities below 10^6 cell/mL. Ulrich and coworkers measured MIC values for Gramicidin S and PGLa at two cell density values and observed a cell-density

dependence (Hartman 2010). More recently, we measured the MBC values for a fluorescent analogue of PMAP-23 in the presence of different *E. coli* cell densities (Savini 2017). Also in our case, the MBC increased with inoculum size, but reached a plateau at densities of 5×10^6 cell/mL and below. A similar trend was reported by Poon and coworkers for pexiganan (Jepson 2016). Finally, a recent study reported an inoculum effect also for LL-37 (Snoussi 2018).

Overall, these studies show that AMP activity is strongly dependent on the density of cells in the assay, with a linear (Savini 2017) or sublinear (Jepson 2016) trend. If a threshold concentration must be reached in the membrane to form pores (Melo 2009), it is obvious that more membrane-bound peptide molecules are needed to kill a higher number of cells (Savini 2017, 2018). Therefore, under conditions of relatively high cell densities, where the peptide is completely bound to the cells, a strong cell-density dependence of the activity is expected. Data by Jepson *et al.* (2016) and Snoussi *et al.* (2018) indicate that this effect might be due also to peptide sequestration by strong binding to killed bacterial cells. We tentatively explained the plateau in the low cell-density regime as due to the cell binding equilibrium. At low cell densities, some of the peptide remains free in solution, and this fraction increases with decreasing concentrations of cells. As a consequence, the two effects (when there are less cells to kill also a lower fraction of peptide is cell-bound) cancel each other, leading to a plateau in the total peptide concentration needed in the sample to kill the bacteria (Savini 2017, 2018).

Interestingly, we observed a cell-density dependence also for the hemolytic activity, with a plateau at densities below 10^7 cell/mL (Savini 2017).

9.3 Competition for cell binding in co-culture experiments might be regulated by kinetic phenomena

Since both antimicrobial activity and toxicity seem to depend on the density of cells, the effective selectivity also depends on the value of this parameter used in the two assays (MIC and MHC). The quantity of peptide that binds to a type of cell or to the other is determined by the respective affinities, but also by the concentration of cells of each type. In the end, the peptide is active and/or toxic if the respective threshold of bound peptide needed for membrane perturbation is reached. In principle, under conditions where the host cells are in large excess (as in the case of systemic treatment of an infection), lack of toxicity is expected even if the affinities for the two cell types are similar (Matsuzaki 2009, Savini 2017).

A limited number of studies has tested peptide activity and toxicity in assays where both bacteria and mammalian cells were present. Mor and coworkers showed that AMPs bound to RBCs are able to transfer to microbial cells, exerting their activity (a phenomenon they termed “affinity driven molecular transfer”) (Feder 2001). Derivatives of lentivirus lytic peptides killed *P. aeruginosa* bacteria interacting with cultured human airway epithelial cells, at peptide concentrations that only moderately affected the cell monolayer (Phadke 2003). Fluorescence microscopy images showed that in a co-culture of *S. aureus* and human cells (endothelial cells or neutrophils) AMPs concentrate on the bacterial cells (Matsuzaki 2009; Akram 2015) (Figure 2 a). Chen (2013) showed that the artificial AMP CL-1 is able to kill selectively *S. aureus* in co-culture with human cells and Malmsten and coworkers reported activity without hemoliticity in bacteria-supplemented blood for an engineered AMP (Schmidtchen 2011). A striking evidence of selectivity is provided by the fact that some AMPs (*e.g.* LL-37) are able to kill *S. aureus* bacteria internalized into mammalian cells (Noore 2012). Selectivity in co-culture has been reported also for AMP-inspired systems, such as peptidomimetics, cationic peptidopolysaccharides and peptide hydrogels (Salick 2007; Li 2012; Konai 2018). Some co-culture data have been reported also for antifungal, antiprotozoan and anticancer activities. For instance, an analogue of the antifungal peptide PAF26 concentrated on fungal cells in co-culture with human lung epithelial cells (Mendive-Tapia 2016); an artificial anticancer peptide concentrated in cancer cells, co-cultured with primary cells (Chen 2014). Some AMPs (*e.g.* dermaseptins or NK-lysin) are able to kill protozoan parasites such as *P.*

falciparum or *T. cruzi* inside human cells, disrupting the plasma membrane of the intracellular parasites without harming that of the host (Ghosh 1997; Krugliak 2000; Jacobs 2003; Gelhaus 2008).

Based on association equilibria, when the two cell types are present at the same time, competition for peptide binding should take place: the antimicrobial activity and/or the toxicity of the peptides should be inhibited by sequestration of a fraction of the peptide molecules due to binding to the other cell population. Very surprisingly, we observed that this is not the case (Savini 2017). We measured both bacterial killing and hemolysis for analogues of PMAP-23 and esculentin, in a mixed population of *E. coli* and erythrocytes, and compared these results with the traditional assays performed on the two cell types separately (Figure 8). The activities on bacteria and on RBCs were essentially unaffected by the presence of the other cell population, in contradiction with the predictions based on binding equilibria. Data from Wimley's lab provide further support to the conclusion that out of equilibrium, kinetic phenomena are at play when two cell populations are present at the same time. They showed that the results of such experiments depend on the order of addition of the different components: like in our case, no change in the MIC was observed when AMPs were added to a mixture of bacteria and RBCs, or to bacteria alone. However, the antimicrobial activity was significantly inhibited when the peptide was incubated with RBCs first, and then both were added to the bacterial culture (Starr 2016) (Figure 8). Actually, while equilibrium processes can be invoked before the cell membranes are perturbed, it is easy to realize that this approach is too simplistic in the case of bilayer disruption, which allows access to multiple additional binding targets (*e.g.* inside the cell) (Snoussi 2018).

Overall, quantitative measurements of peptide interactions with cells confirmed that AMPs have a higher affinity for bacterial than for host cells. Experiments performed with varying cell densities indicated that both activity and toxicity depend on this parameter (even though a plateau is observed at low cell densities) and that therefore the measured selectivity depends on the specific conditions of the experiments. Finally, experiments with mixed bacterial and eukaryotic cells showed that, contrary to expectations based on equilibrium considerations, competition for peptide binding does not lead to a loss in activity. This finding definitely warrants further co-culture studies.

10. Concluding remarks

A large body of studies has been devoted to characterize, understand and improve the selectivity of AMPs. These data support the view that selectivity arises due to the different lipid composition of bacterial and host cell membranes. AMPs are able to discriminate between the two types of bilayers thanks to their physico-chemical properties. We can summarize here the main guidelines for optimization of peptide selectivity:

- Increasing the cationic charge, by C-terminal amidation, substitution of anionic residues, or insertion of cationic amino acids in the polar side of the peptide structure, leads to a better selectivity. However, an excessive increase in positive charge, above a threshold that depends on each specific case, might be ineffective or even detrimental.
- Reducing hydrophobicity, amphipathicity and helicity is an effective strategy. These properties are necessary for activity, since they are responsible for the hydrophobic driving force for membrane binding and for insertion in the bilayer. However, several studies have demonstrated that they affect toxicity more than activity. This finding is probably a consequence of the non-additivity of electrostatic and hydrophobic effects. Therefore, an intermediate range of hydrophobicity and amphipathicity values optimizes selectivity.
- The real determinants of selectivity are the effective, conformation dependent, hydrophobicity and amphipathicity values (rather than the parameters determined based on an idealized conformation). They can be optimized by introducing:
 - polar residues in the hydrophobic side of the peptide helix (imperfect amphipathicity).

- helix-breaking residues, such as Pro, Gly, D amino acids, or peptoids.
- Aggregation in the aqueous phase modulates selectivity, too. Often, self-assembly is beneficial for selectivity.

In this chapter, we tried to generalize and simplify as much as possible the results collected over many years of studies. However, the readers that had the patience to follow us until here must have realized that the literature on AMP selectivity is full of exceptions and contradictions. Peptide association to bacterial and host cells is modulated (in a non-additive way) by electrostatic and hydrophobic interactions. The effective peptide hydrophobicity is determined by peptide conformation and aggregation state. Therefore, AMP selectivity is finely regulated by interconnected binding, aggregation and conformational equilibria. Any variation in peptide property will affect all of these phenomena. Therefore, in order to predict, or at least to understand, the effect of peptide modifications on the final selectivity, all the possible processes involved in peptide behavior in the aqueous and membrane phases must be considered.

We still do not fully understand what happens when AMPs act on bacteria and human cells together. Recent data indicate that in this case even the complex scenario outlined above is overly simplified, since kinetic phenomena probably have to be taken into account. Further studies in this area are definitely warranted and hold the promise to provide a better understanding of AMP selectivity.

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TABLES

Name	Sequence	TI	Calculated as	Reference
NATURAL PEPTIDES				
Temporin-L	FVQWFSKFLGRIL-NH ₂	1	HC ₅₀ /MIC (Gram + &-)	Mangoni 2011
Magainin-1	GIGKFLHSAGKFGKAFVGEIMKS	2.5-4	HC ₅ /MIC (<i>E. coli</i>)	Bacalum 2015
PMAP-36	GRFRRLRKKTRKRLKKIGKVLKWIPPVIGSIPLGCG-NH ₂	3	HC ₅ /MIC (Gram + &- and <i>C. albicans</i>)	Lyu 2016
Mastoparan X	INWKGIAAMAKKLL-NH ₂	3.4	HC ₅ /MIC (<i>E. coli</i>)	Henriksen 2014
Arenicin-1	RWCYAYVRVRGVLVRYRRCW	5	HC ₅₀ /MIC (Gram + &-)	Panteleev 2015
Polistes Mastoparan	VDWKKIGQHILSVL-NH ₂	6	HC ₅ /MIC (<i>E. coli</i>)	Bacalum 2015
Magainin 2B	GIGKFLHAAKKFAKAFVAEIMNS	9	HC ₅₀ /IC ₅₀ (<i>B. anthracis</i>)	Dawson 2011 a
Dermaseptin 1	ALWKTMLKKLGTMALHAGKAALGAAADTISQGTQ	0.83-7	HC ₅ /MIC (<i>E. coli</i>)	Bacalum 2015
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	12	HC ₅ /MIC (<i>E. coli</i> , <i>S. aureus</i>)	Luo 2017
Indolicidin	ILPWKWPWWPWRR-NH ₂	13 4-11	HC ₅₀ /MIC (Gram + &-) HC ₅ /MIC (<i>E. coli</i>)	Nan 2009 Bacalum 2015
Gramicidin-S	VOLFPVOLFP	1-18 2	HC ₅₀ /MIC (Gram + &-) HC ₁₀₀ /MIC (Gram + &-)	Swierstra 2016 Kondejewski 1999
XT-7	GLLGPLLKIAAKVGSNLL	18	HC ₅₀ /MIC (<i>E. coli</i>)	Kamech 2012
PGLa	GMASKAGAIAGKIAKVALKAL-NH ₂	24	HC ₁₀ /MIC (Gram + &-)	Strandberg. 2007
Tachyplesin I	KWCFRVCYRGICYRRCR-NH ₂	2-24	HC ₅ /MIC (<i>E. coli</i>)	Bacalum 2015
SMAP-29	RGLRRLGRKIAHGVKKYGPVLRIRIAG	26	HC ₅₀ /IC ₅₀ (<i>B. anthracis</i>)	Dawson 2011 b
Ascaphin-8	GFKDLLKGAALKVKTVLF	37	HC ₅₀ /MIC (<i>E. coli</i>)	Kamech 2012
Papillosin	GFWKKVGSAAWGGVKAAGAAVGGGLNALAKHIQ	34	HC ₅₀ /IC ₅₀ (<i>B. anthracis</i>)	Dawson 2011 b
PMAP-23	RIIDLLWRVRPQKPKFVTWVVR	23 >57	HC ₅ /MIC (Gram+ & -) HC ₀ /MIC (Gram + &-)	Velduhizen 2017 Kang 1999
Ascaphin-1	GIRDVLKGAAKAFVKTVAGHIAN-NH ₂	>60	HC ₅₀ /MIC (<i>E. coli</i>)	Juretic 2009
Magainin 2	GIGKFLHSAKKFGKAFVGEIMNS	3-82	HC ₅ /MIC (<i>E. coli</i>)	Bacalum 2015
Cecropin B	KWKVFKKIEKMGRNIRNGIVKAGPAIAVLGEAKAL-NH ₂	20-130	HC ₅ /MIC (<i>E. coli</i>)	Bacalum 2015
Cecropin A	KWKLFKKIEKVGGQNIKAGPAVAVVGQATQIAK-NH ₂	177-579	HC ₅ /MIC (<i>E. coli</i>)	Bacalum 2015

MODIFIED ANALOGUES OF NATURAL PEPTIDES				
Mastoparan-X Ala1	ANWKGIAAMAKLL-NH ₂	7	HC ₅ /MIC (<i>E. coli</i>)	Henriksen 2014
PMAP-36 T115	TRKRLKKIGKVLKWI-NH ₂	29	HC ₅ /MIC (Gram + &- and <i>C. albicans</i>)	Lyu 2016
Gramicidin GS14K4	VKLkV _y PLKVKLyP	31	HC ₁₀₀ /MIC (Gram + &-)	Kondejewski 1999
D-Piscidin1 19K	ffhhifrgkvhvgtihrlvtg-NH ₂	33	HC ₅₀ /MIC (<i>A. baumannii</i>)	Jiang 2014
Arenicin-1 V8R	RWCYAYRRVRGVLVRYRRCW	80	HC ₅₀ /MIC (Gram + &-)	Panteleev 2015
[K2, K16] XT-7	GKLGPLLKIAAKVGSKLL	>130	HC ₅₀ /MIC (<i>E. coli</i>)	Kamech 2012
Indolicidin-A7	ILKWKWKWWKWRR-NH ₂	190	HC ₅₀ /MIC (Gram + &-)	Nan 2009
D-Dermaseptin S4 L7K,A14K	alwmtlkkvklkakalnavlvgana-NH ₂	219	HC ₅₀ /MIC (<i>A. baumannii</i>)	Jiang 2014
[I2, K19] ascaphin-8	GIKDLLGAALKALVKTVLK	>480	HC ₅₀ /MIC (<i>E. coli</i>)	Kamech 2012
Gramicidin V3/A3	AKLkAyPLKAKLyP	520	HC ₁₀₀ /MIC (<i>C. xerosis</i>)	Kondejewski 2002
DESIGNED PEPTIDES				
V13K	Ac-KWK _S FLKTFKS _{AKK} TVLHTALKAISS-NH ₂	163	HC _{>0} /MIC (Gram + &-)	Chen 2005
P5	KWKLLKKPLLKLLKKL-NH ₂	>150	HC ₌₀ /MIC (Gram + &-)	Park 2003
Pep-1-K	KKTWWKTWWTKWSQPKK _{RR} KV	174	HC _{>0} /MIC (Gram + &-)	Zhu 2009
PK-12-KKP	KKPWWKPWWPKWKK	200	HC _{>0} /MIC (Gram + &-)	Zhu 2009
D16	Ac-klksllk _{tl} skakkk _{kl} tl _l kalsk-NH ₂	890 3355	HC ₅₀ /MIC (<i>P. aeruginosa</i>) HC ₅₀ /MIC (<i>A. baumannii</i>)	Jiang 2011

Table 1.

Therapeutic index (TI) of natural and artificial AMPs.

The TI is defined as the ratio of hemolytic to inhibitory peptide concentration. HC_x is defined as the peptide concentration causing the x% of hemolysis. HC_{>0} is the minimal peptide concentration that produces detectable hemolysis; HC₌₀ is the highest peptide concentration that causes no detectable release of hemoglobin. HC₁₀₀ is the minimal concentration causing total lysis. MIC is defined as the minimum concentration that inhibits bacterial growth; when calculated on more than one strain, the geometric mean of the values is reported. Data presented as a range result from different MIC values reported in the literature for the same peptide. -NH₂ in the peptide sequence indicates amidation at the C-terminus, while Ac- indicates acylation at the N-terminus. O is pyrrolisine. Amino acids are colored in blue, red, orange and green based on their cationic, anionic, polar and hydrophobic character, respectively. Lowercase letters indicate D amino acids.

Name	Sequence	TI (cells)	Calculated as	TI (RBCs)	Calculated as	Reference
NATURAL PEPTIDES						
Magainin2-NH ₂	GIGKFLHSAKKFGKAFVGEIMNS-NH ₂	6	LC ₉₉ (3T3)/MIC (Gram+ & -)	>130	HC ₁₀₀ /MIC (Gram+ & -)	Javadpour 1996
Polistes Mastoparan	VDWKKIGQHLSVL-NH ₂	7	LC ₅₀ (PBMC)/MIC (<i>E. coli</i>)	6	HC ₅ /MIC (<i>E. coli</i>)	Bacalum 2015
Dermaseptin 1	ALWKTMLKKLGTMLHAGKAALGAAADTISQGTQ	1-7	LC ₅₀ (PBMC)/MIC (<i>E. coli</i>)	1-10	HC ₅ /MIC (<i>E. coli</i>)	Bacalum 2015
Lasioglossin III	VNWKKILGKIIVVK-NH ₂	>8	LC ₅₀ (HUVEC)/MIC (Gram+ & -)	>105	HC ₅₀ /MIC (Gram+ & -)	Slaninová 2012,
Magainin I	GIGKFLHSAGKFGKAFVGEIMKS	7-12	LC ₅₀ (PBMC)/MIC (<i>E. coli</i>)	2.5-4	HC ₅ /MIC (<i>E. coli</i>)	Bacalum 2015
Indolicidin	ILPWKPWWPWRR-NH ₂	6-17	LC ₅₀ (PBMC)/MIC (<i>E. coli</i>)	4-11	HC ₅ /MIC (<i>E. coli</i>)	Bacalum 2015
BMAP-28	GGLRSLGRKILRAWKKYGPIIPIIRIG	17	LC ₉₅ (HN)/MIC (Gram+ & -)	55	HC ₉₀ /MIC (Gram+ & -)	Skerlavaj 1996
BMAP-27	GRFKRFRKKFKLSPVILLHLG	18	LC ₈₀ (HN)/MIC (Gram+ & -)	59	HC ₃₀ /MIC (Gram+ & -)	Skerlavaj 1996
PMAP-23	RIIDLWVRPQPKFVTWVVR	23	LC ₃₀ (IPEC-J2)/MIC (Gram+ & -)	23 >57	HC ₅ /MIC (Gram+ & -) HC ₌₀ /MIC (Gram + & -)	Velduhizen 2017, Kang 1999
Cecropin B- NH ₂	KWKVFKKIEKMGRNIRNGIVKAGPAIAVLGEAKAL-NH ₂	29	LC ₉₉ (3T3)/MIC (Gram+ & -)	>86	HC ₁₀₀ /MIC (Gram+ & -)	Javadpour 1996
Tachyplesin I	KWCFRVCYRGICVRRR-NH ₂	3-30	LC ₅₀ (PBMC)/MIC (<i>E. coli</i>)	2-24	HC ₅ /MIC (<i>E. coli</i>)	Bacalum 2015
Magainin2	GIGKFLHSAKKFGKAFVGEIMNS	3-82	LC ₅₀ (PBMC)/MIC (<i>E. coli</i>)	3-82	HC ₅ /MIC (<i>E. coli</i>)	Bacalum 2015
Cecropin A	KWKLFKKIEKVGQNIIRDGIKAGPAVAVVGOATQIAK-NH ₂	27-180	LC ₅₀ (PBMC)/MIC (<i>E. coli</i>)	20- 130	HC ₅ /MIC (<i>E. coli</i>)	Bacalum 2015
MODIFIED ANALOGUES OF NATURAL PEPTIDES						
A(A1R A8R I17K)	RIGSILGRLAKGLPTLKSNIK-NH ₂	1.5-3	LC ₅₀ (L929)/MIC (Gram+ & -)	10-16	HC ₁₀ /MIC (Gram+ & -)	Zhang 2016
DESIGNED PEPTIDES						
D-LAK120 AP13	kklaalakkwplakklaalakk-NH ₂	4	LC ₅₀ (RAW 264.7)/MIC (Gram -)	67	HC ₅₀ /MIC (Gram-)	Vermeer 2012
WK12	KWWKWWKWWKK	>10	LC ₂₀ (PBMC)/MIC (Gram-)	>10	HC ₁₀ /MIC (Gram-)	Deslouches 2016
WR12	RWWRRWRRWRR	>20	LC ₂₀ (PBMC)/MIC (Gram-)	>20	HC ₁₀ /MIC (Gram-)	Deslouches 2016
(KLAKLA) ₂	KLAKKLAKLAKLA	>45	LC ₉₉ (3T3)/MIC (Gram+ & -)	>125	HC ₁₀₀ /MIC (Gram+ & -)	Javadpour 1996
(KLAKLAK) ₂	KLAKLAKKLAKLAK	>86	LC ₉₉ (3T3)/MIC (Gram+ & -)	>125	HC ₁₀₀ /MIC (Gram+ & -)	Javadpour 1996

Table 2.

Comparison of TI values determined with RBCs or with other eukaryotic cells.

LC: lethal peptide concentration. LC_x is defined as the peptide concentration killing x% of the cells. For HC and MIC definitions, and for the color code used for the sequences, please see Table 1. When data are presented as a range, they result from different MIC values reported in the literature for the same peptide. HBRC: human red blood cells. PBMC: peripheral blood mononuclear cells (3·10⁶ /mL). 3T3 cells: murine fibroblast cell line (2·10⁵/mL). HUVEC cells: Human umbilical vein endothelial cells (2·10⁴/mL); HN: human neutrophils (4·10⁶/mL). IPEC-J2: porcine intestinal epithelial cells (1.5·10⁵/mL). L929: mouse fibroblast cells (1.5·10⁴/mL). RAW 264.7: murine macrophage cells (3·10⁵/mL). The density of RBCs used in the hemolytic activity assays (when specified in the original reference) ranged from 10⁶ to 10⁹ cells/mL. Lowercase letters indicate D amino acids.

Name	Sequence	TI	Calculated as	Bacterial strain	Reference
NATURAL PEPTIDES					
P3	VNFKLLSHSLVTLASHL	3	LD ₅₀ /ED ₆₀	<i>E.coli</i>	Zhang 2015
OH-CATH30	KFFKLLKNSVKKRAKKFFKKPRVIGVSIPF	12	LD ₅₀ /ED ₈₀	<i>E.coli</i>	Li 2012
BMAP-27	GRFKRFRKKFKKLSPVILLHLG	47-55 190-220	LD ₅₀ /ED ₁₀₀ LD ₅₀ /ED ₁₀₀	<i>P.aeruginosa</i> <i>E.coli</i>	Benincasa 2003
BMAP-28	GGLRSLGRKILRAWKKYGPIIVPIIRIG	24-27 47-55	LD ₅₀ /ED ₁₀₀ LD ₅₀ /ED ₁₀₀	<i>E.coli</i> <i>S.aureus</i>	Benincasa 2003
MODIFIED ANALOGUES OF NATURAL PEPTIDES					
HD5-myr	ATCYCRTGRCATRESLSGVCEISGRLYRLCCR-myr	>3	LD ₀ /ED ₉₀	<i>E. coli</i>	Lei 2018
RN7-IN8	FLGGLIKWPWWPWRR-NH ₂	4	LD ₁₀₀ /ED ₅₀	<i>S. pneumoniae</i>	Jindal 2017
JH3	RRFKLLSHSLVTLASHL	4.5	LD ₅₀ /ED ₉₀	<i>E.coli</i>	Zhang 2015
TP3	FIHHIIGGLFSVGKHIHSLIHGH	7 7 >24	MTD/ED ₆₀ MTD/ED ₇₀ MTD/ED ₁₀₀	<i>A.baumannii</i> <i>K pneumoniae</i> MRSA	Pan 2015 Pan 2015 Huang 2015 a
D-OH-CATH30	kffkklknsvkkrakffkprvigsipf	8	LD ₅₀ /ED ₁₀₀	<i>E.coli</i>	Li 2012
OH-CM6	KFFKLLKKA VKKGFKKFAKV	10	LD ₅₀ /ED ₇₀	<i>E.coli</i>	Li 2012
TP4	FIHHIIGGLFSAGKAIHRLIRRRRR	20 20 >24	MTD/ED ₉₀ MTD/ED ₉₀ MTD/ED ₁₀₀	<i>A.baumannii</i> <i>K pneumoniae</i> MRSA	Pan 2015 Pan 2015 Huang 2015 b
DESIGNED PEPTIDES					
A3-APO	(Chex-RPEKPRPYLPRPRPPRPVR) ₂ -Dab-NH ₂	2.5	LD ₅₀ /ED ₁₀₀	<i>E.coli</i>	Szabo 2010
Onc72	VDKPPYLPRPRPPROIYNO-NH ₂	>20	LD ₅₀ /ED ₅₀	<i>E.coli</i>	Knappe 2012
(LLKK) ₂ C	LLKKLLKKC	28	LD ₅₀ /ED ₅₀	<i>A baumannii</i>	Huang 2012
C(LLKK) ₂ C	CLLKKLLKKC	34	LD ₅₀ /ED ₅₀	<i>A baumannii</i>	Huang 2012

Table 3.

TI values obtained from in vivo studies.

TI was calculated as the ratio of the toxic or lethal dose to the effective dose of peptides in mice. LD_x is the minimum dose that was lethal for at least x % of animals; LD₀ is the highest peptide concentration that caused no deaths; MTD is minimum dose that caused toxicity (narrowing of the eyes was taken as symptom), MLD is the minimum lethal dose, ED_x is the effective dose, *i.e.* the minimum dose causing the survival of at least x% of the animals. Bacteria and peptides were injected intraperitoneally, except for (Huang 2015 a, b) and (Pan 2015), where toxicity tests were performed by intramuscular injection. -myr indicates myristoylation at the C-terminus. Chex =1-amino-cyclohexane carboxylic acid; Dab=2,4-diamino-butyric acid; O=pyrrolysine. MRSA is methicillin resistant *S. aureus*. For the color code used for the sequences, please see Table 1. Lowercase letters indicate D amino acids

PHOSPHOLIPID COMPOSITION OF BACTERIAL MEMBRANES					
	PE	PG	CL	PA	L-lysyl PG
Total charge	0	-1	-2 (-1)	-1	+1
Intrinsic curvature	-	0	-	-	+
<i>E. coli</i> (both membranes) (Ames 1968)	69	19	6.5		
(Raetz 1978)	75-85	10-20	5-15		
(Morein 1996)	79	17	4		
(Rowlett 2017)	78	12	6	<3	
<i>E. coli</i> (cell membrane) (Morein 1996)	75	19	6		
<i>S. typhimurium</i> (both membranes) (Osborn 1972)	86	13	1		
(Ames 1968)	78	18	3		
<i>S. typhimurium</i> (cell membrane) (Osborn 1972)	76	21	3		
<i>B. subtilis</i> (Op den Kamp 1969)*	30	36	12		22
(Bishop 1967)**	34	49	11		
<i>S. aureus</i> (Hayami 1979): (strain Newman)		49	13	0.6	34
(strain Tazaki)		47	10	1	38

Table 4

Phospholipid composition of the membranes of Gram-negative (name highlighted in red) and Gram-positive (name highlighted in blue) bacteria.

PE: phosphatidylethanolamine; PG: phosphatidylglycerol; CL: cardiolipin; PA: phosphatidic acid; L-lysyl PG: L-Lysyl phosphatidylglycerol. Phospholipid composition data are expressed as molar percentages. In the case of (Hayami 1979), data were calculated converting the % of phosphorus to molar % by considering two P atoms per CL molecule, and one for the other lipids. In the case of (Osborn 1972), data were calculated converting the % of [2-³H] glycerol to molar %, by considering three ³H atoms per CL molecule, two per PG and one for the other lipids. * indicates a growth condition without glucose and sulfate. ** indicates that 6% of lipo amino acids were also recovered. Data on total charge at physiologic pH and intrinsic curvature were taken from (Marsh 1990; McMahon 2015; Malanovic 2016; Boyd 2017).

PHOSPHOLIPID COMPOSITION OF HUMAN RBC MEMBRANES						
	PC	PE	SM	PI	PS	PA
Total charge	0	0	0	-1	-1	-1 (-2)
Intrinsic curvature	0	-	0	+	0	-
<i>Both leaflets</i> (Dodge 1967)	29.2±1.5	27.5±1.5	25.4±1.4	0.6±0.5	14.8±1.7	1.1±0.5
(Broekhuijse 1969)	28.3±2.1	26.7±1.0	25.8±1.7	1.9±0.6	12.7±1.3	
(White 1973)	34.7	28.0	20.1		14.3	
(Verkeleij 1973)	28	26	24		13	
(Van Meer 1981)	29.5	25.9	25.3		12.2	
<i>Outer leaflet</i> (Verkeleij 1973)	42	10	40		0	
(Virtanen 1998)	44.8	11.1	42.1			
<i>Inner leaflet</i> (Verkeleij 1973)	14	42	8		26	
(Virtanen 1998)	14.0	43.9	9.1	1.2	29.6	2.2
<i>Asymmetry of distribution</i> <i>(%in the outer leaflet)</i> (Verkleij 1973)	76	20	82		0	
(Zwaal 1973)	62		83			
(Gordesky 1973)		15			0	
(Zwaal 1975)	75					
(Gordesky 1975)		33			0	
(Van Meer 1981)	78	20	80			
(Bütikofer 1990)				24		
(Gascard 1991)				20		
CHOLESTEROL CONTENT AS CHOLESTEROL/PHOSPHOLIPID MOLAR RATIO						
(Cooper 1975)	0.95					
(Ballas 1980)	0.75					
(Chabanel 1983)	0.80					

Table 5

Phospholipid and cholesterol content of human erythrocytes membrane.

PC: Phosphatidylcholine; PE: phosphatidylethanolamine; SM: sphingomyelin; PI: phosphatidylinositol; PS: phosphatidylserine; PA: phosphatidic acid. Phospholipid composition data are expressed as molar percentages. Data from Verkeleij (1973) were derived from a figure in the cited reference. Data on total charge at physiologic pH and intrinsic curvature were taken from (Marsh 1990; McMahon 2015).

FIGURES

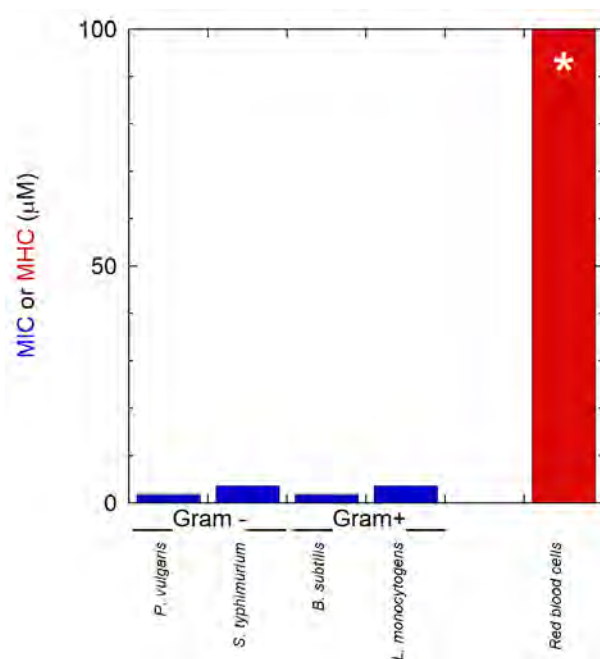


Fig. 1

Selective cytotoxicity in in vitro assays.

Minimal inhibitory concentrations against different Gram- and Gram+ bacterial strains, and minimal hemolytic concentration for the designed artificial AMP P5. The asterisk indicates that no hemolysis was observed for P5 in the peptide concentration range investigated (up to 100 μ M). Adapted, with permission, from research originally published in (Bobone 2013) (published by the European Peptide Society and John Wiley & Sons, Ltd.).

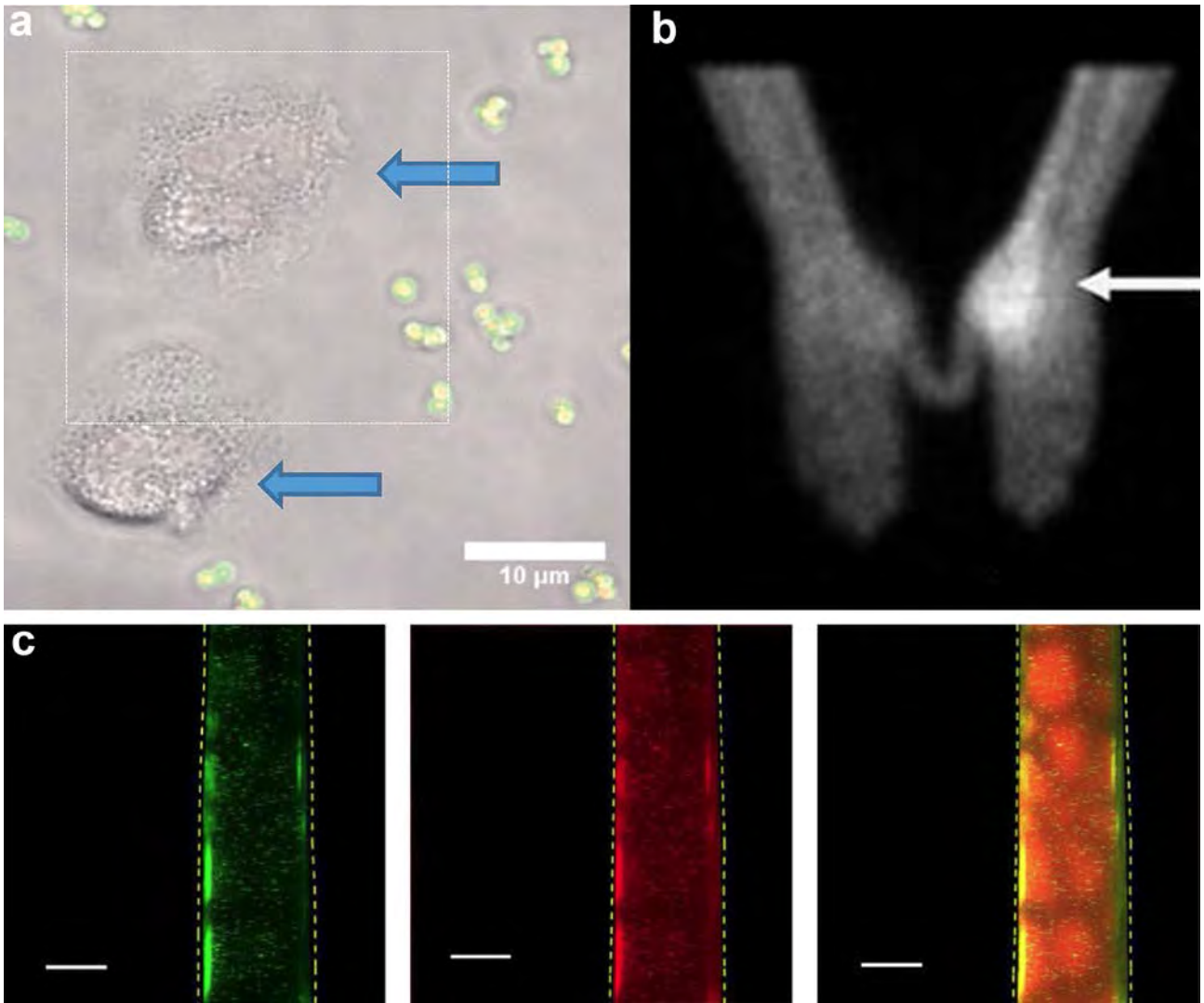


Fig. 2

Selective targeting of bacterial cells by AMPs in vitro and in vivo.

a) optical and fluorescence microscopy image of a labeled ubiquicidin analogue (visible by the green fluorescence) selectively binding to *S. aureus* bacteria, in co-culture with isolated human neutrophils (blue arrows). Adapted, with permission, from research originally published in (Akram 2015) (published by The Royal Society of Chemistry).

b) Positron emission tomography image of a patient with an infection in the left hand (indicated by the arrow), traced with a radiolabeled ubiquitin analogue. No significant peptide uptake in the contralateral hand was noted. The image was obtained 30 min after tracer administration. Reproduced, with permission, from research originally published in (Akhtar 2012).

c) Visualization of *in vivo* targeting of human α -Defensin 5 (HD5) toward *E. coli* cells. The mesenteric vein was imaged intravitaly in mice by two-photon laser scanning microscopy, 30 minutes after injection of *E. coli* cells expressing a green fluorescent protein (visualized in the left panel), and treatment with HD5 labeled with a red fluorescent probe (imaged in the center panel). Colocalization is demonstrated by the overlapped images (right panel). Scale bars: 50 μ m. Adapted, with permission, from (Lei 2018). Copyright (2018) American Chemical Society.

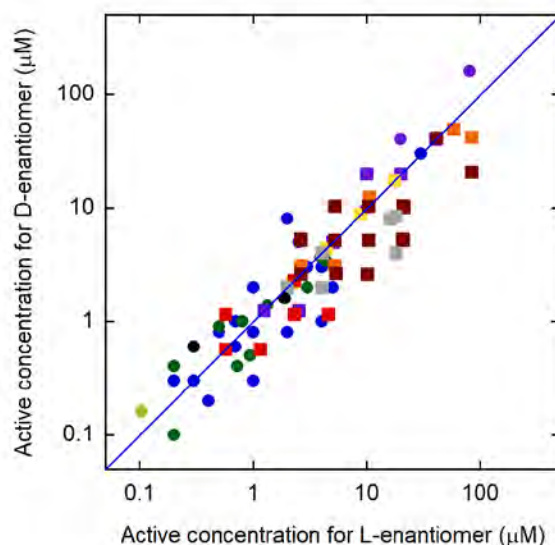


Fig. 3

Activity of natural AMPs and their enantiomeric analogues.

Comparison of the antibacterial activities (circles for MBC, squares for MIC) of enantiomeric peptides. Data refer to: magainin-2: (Bessalle 1990) (violet), cecropin A (Wade 1990) (blue), melittin (Juvvadi 1996) (dark green), LL-37 (Dean 2011) (light green), KSLK (Hong 1999) (yellow), temporin A (Wade 2000) (orange; data refer to IC_{50} values), camel 48 (Oh 2000) (red), V681 and analogues (Chen 2006) (dark red, data refer to LC_{50} values), lactoferricin B analogues (Wakabayashi 1999) (silver), cecropin B (Bland 2001) (black). The blue line is the diagonal of the plot (corresponding to identical activity for D and L enantiomers) and not a fit. A version of this figure with a more limited set of data has been published previously (Savini 2018).

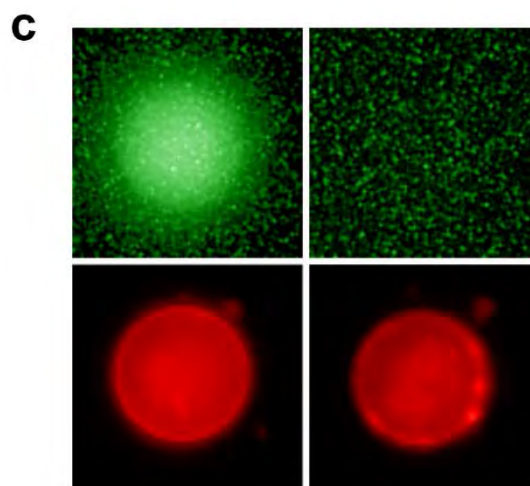
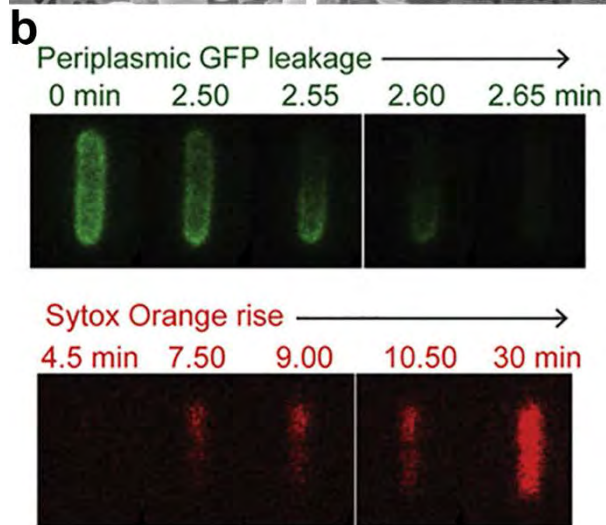
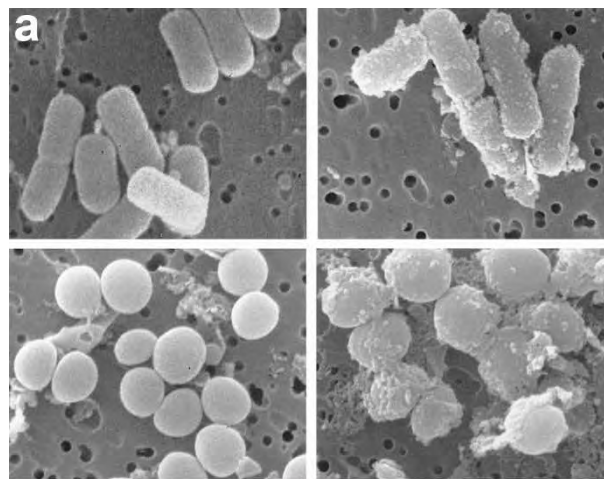


Fig. 4

Fluorescence and electron microscopy images of the effects of AMPs on bacterial and artificial membranes.

a) scanning electron micrographs of *E. coli* (top) and *S. aureus* (bottom) before (A and C) and after (B and D) treatment with the synthetic AMP PGYa (30 min, 10 μ M). The images show a considerable roughening of the bacterial membranes and formation of blebs on the cell surface, in contrast to the smooth surfaces of untreated bacteria, providing a strong indication that the membrane is being considerably altered by the peptide. Adapted, with permission, from research originally published in (Tiozzo 1998) © Elsevier.

b) fluorescence microscopy images of an *E. coli* cell attacked by the AMP cecropin A (0.5 μ M). The leakage of periplasmic green fluorescent protein (GFP), shown by the green fluorescence, indicates perturbation of the outer membrane, while uptake of the DNA stain Sytox Orange (red fluorescence) demonstrates pore formation in the plasma membrane. Adapted, with permission, from research originally published in (Agrawal 2018) © Elsevier.

c) fluorescence microscopy images of perturbation of a giant unilamellar vesicle by the AMP PMAP-23. The top panels report the green fluorescence emission from carboxyfluorescein molecules entrapped inside the GUV, which were completely released after peptide addition (right). By contrast, the vesicle was still present after peptide addition, as indicated by the red fluorescence of rhodamine labeled phospholipids located in the GUV bilayer (bottom panels). Taken together, these images demonstrate pore formation by the AMP. The vesicle diameter is about 20 μ m. Adapted, with permission, from research originally published in (Orioni 2009) © Elsevier.

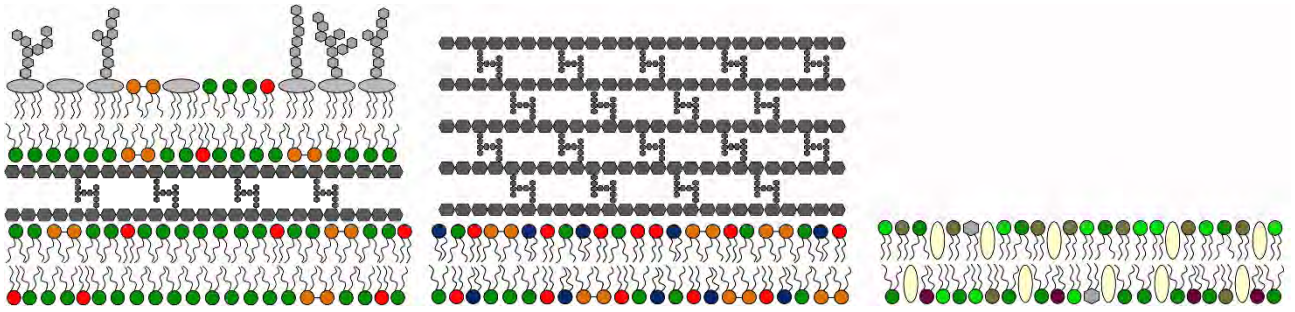


Fig. 5

Schematic depiction of the structure of the cellular envelope in different cell types.

The three panels, from left to right, schematize the structure of the cellular envelope in Gram- and Gram+ bacteria and in human cells, respectively. Proteins, glycolipids and lipoteichoic acids (in Gram+ bacteria have been omitted, for the sake of clarity.

LPS: light grey; peptidoglycan: dark grey; PC: light green; PE: dark green; SM: light brown; PI: light grey; PG: red; PS: dark red; CL: orange; L-lysyl PG: blue; cholesterol: beige.

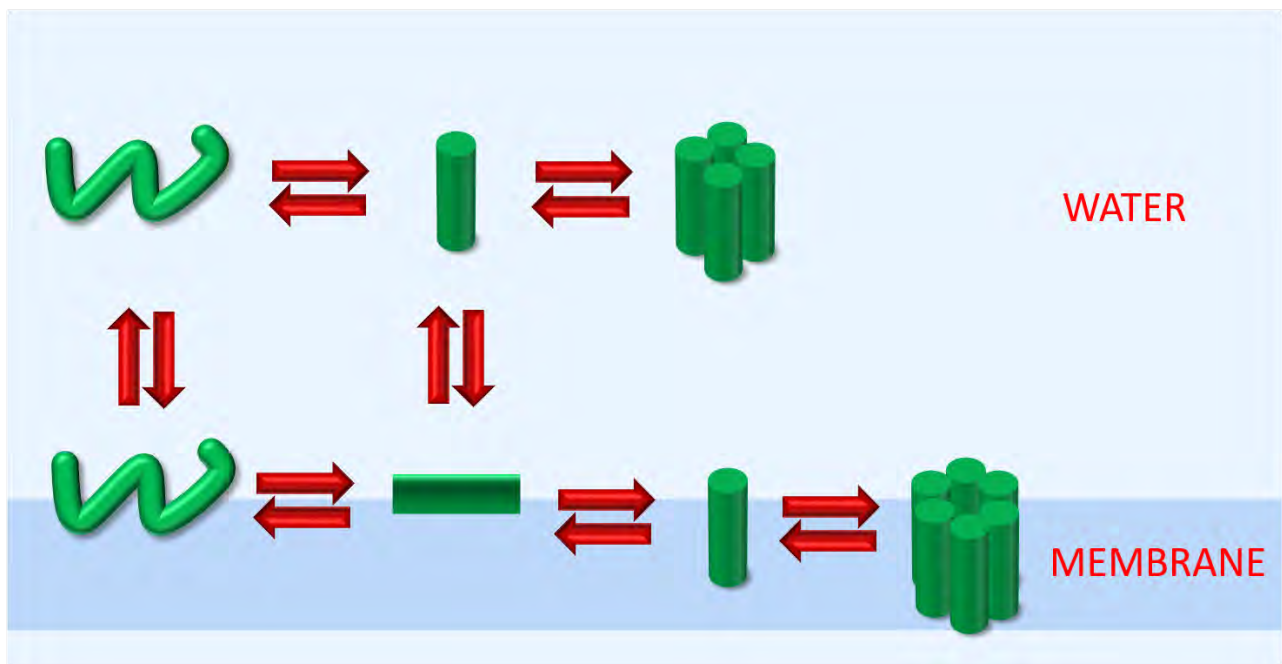


Fig. 6

Schematic depiction of the phenomena involved in peptide/membrane interaction.

In addition to water/membrane partition, conformational, orientational and aggregation equilibria are at play in determining the final peptide activity and selectivity.

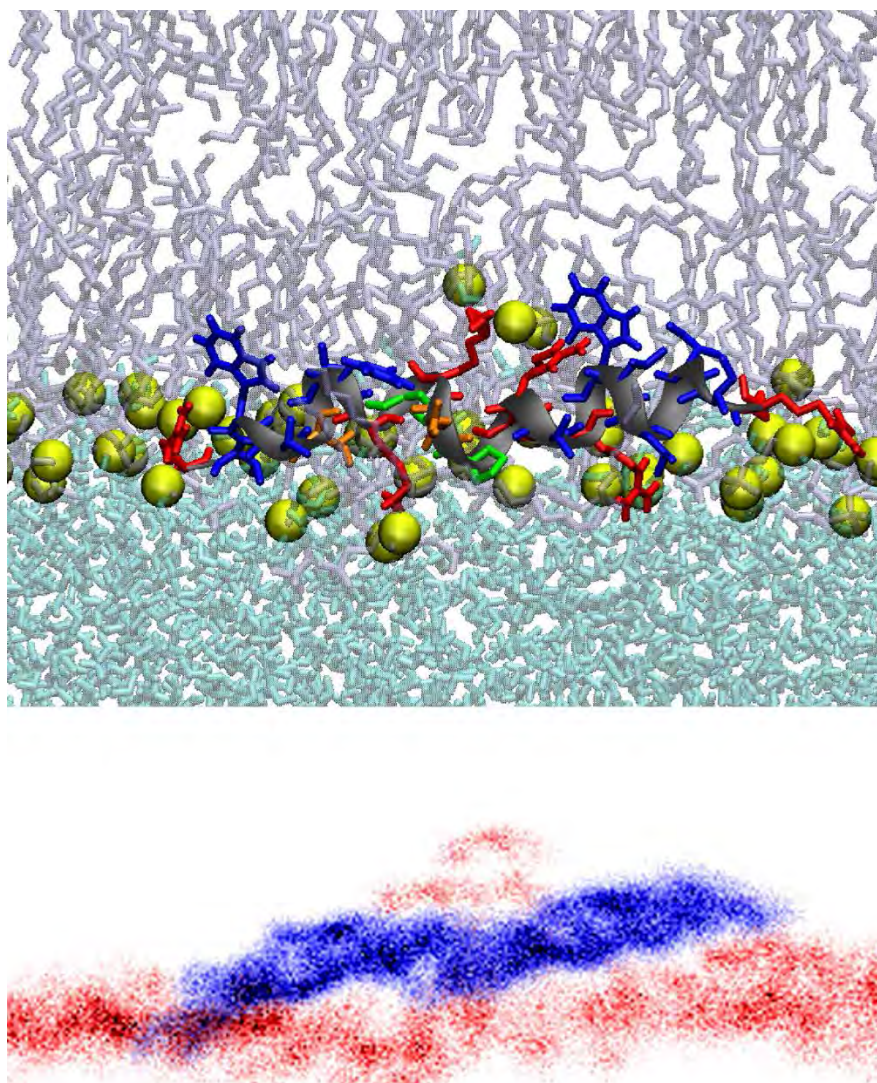


Fig. 7

Interfacial activity of an imperfectly amphipathic AMP

Effects of the imperfectly amphipathic AMP PMAP-23 on the structure of a lipid bilayer, as observed in MD simulations. Two charged residues located on the hydrophobic side of the helix drive three phospholipid headgroups and some water molecules into the hydrophobic core of the membrane.

Water is represented in cyan, phospholipids in gray, and phospholipids' phosphorus atoms as yellow spheres. The peptide backbone is shown in grey, charged side chains in red, polar amino acids in orange, apolar residues in blue, and prolines in green. The lipid composition was POPG/POPC (1:3 mol/mol). The bottom panel reports the density map of the lipid phosphorus atoms (red) and of the peptide backbone atoms (blue). Adapted, with permission, from research originally published in (Orioni 2009) © Elsevier.

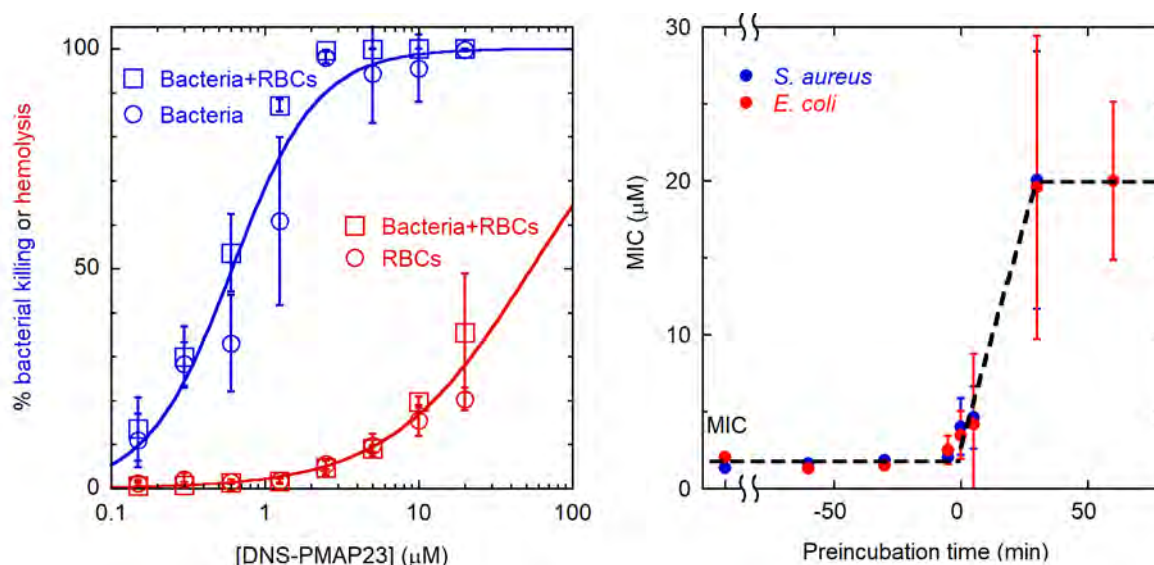


Fig. 8

Antimicrobial and hemolytic activity in assays with both bacterial and erythrocytes

Left panel: bactericidal (blue) and hemolytic (red) activities of the AMP DNS-PMAP23 in the presence of both bacteria and erythrocytes (squares) or of one cell type only (circles). Both activities are only slightly affected by the presence of the other cell population. 4.5×10^7 *E. coli* cells/mL, 4.5×10^8 RBCs/mL. Reproduced with permission from (Savini 2017) <https://pubs.acs.org/doi/abs/10.1021/acscchembio.6b00910> (copyright 2017, American Chemical Society). Further permissions related to the material excerpted should be directed to the ACS.

Right Panel: effect of incubation time on MIC values in assays with both bacteria and erythrocytes. The artificial AMP ARVA-D was tested against *E. coli* (red) and *S. aureus* (blue) under various conditions. "MIC" represents measurements done in the absence of RBCs. All other experiments include 10^9 human RBC/mL. Time zero represents the experiments in which RBC and bacteria were first mixed, followed by peptide addition, *i.e.* no pre-incubation with either cell type. Negative times represent peptide pre-incubation with bacteria before the addition of RBCs. Positive times represent peptide pre-incubation with RBCs, followed by addition of bacteria. Points plotted at 20 μM had MIC values ≥ 20 μM. Significant inhibition of peptide antimicrobial activity due to the presence of RBCs was observed only in the case of pre-incubation with erythrocytes. Proteolytic degradation effects can be ruled out, since ARVA-D is a peptide comprising all D amino acids. Adapted with permission from (Starr 2016) (copyright 2016, American Chemical Society).