Antimicrobial peptides and induced membrane curvature: Geometry, coordination chemistry, and molecular engineering

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ABSTRACT

Short cationic, amphipathic antimicrobial peptides are multi-functional molecules that have roles in host defense as direct microbicides and modulators of the immune response. While a general mechanism of microbicidal activity involves the selective disruption and permeabilization of cell membranes, the relationships between peptide sequence and membrane activity are still under investigation. Here, we review the diverse functions that AMPs collectively have in host defense, and show that these functions can be multiplexed with a membrane mechanism of activity derived from the generation of negative Gaussian membrane curvature. As AMPs preferentially generate this curvature in model bacterial cell membranes, the selective generation of negative Gaussian curvature provides AMPs with a broad mechanism to target microbial membranes. The amino acid constraints placed on AMPs by the geometric requirement to induce negative Gaussian curvature are consistent with known AMP sequences. This 'saddle-splay curvature selection rule' is not strongly restrictive so AMPs have significant compositional freedom to multiplex membrane activity with other useful functions. The observation that certain proteins involved in cellular processes which require negative Gaussian curvature contain domains with similar motifs as AMPs, suggests this rule may be applicable to other curvature-generating proteins. Since our saddle-splay curvature design rule is based upon both a mechanism of activity and the existing motifs of natural AMPs, we believe it will assist the development of synthetic antimicrobials.

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1. Introduction

Cationic antimicrobial peptides (AMP) are present in virtually every multicellular organism, and comprise an important part of the innate host defense [1–6]. Collectively, AMPs display broad spectrum antimicrobial activity and have a rapid mechanism of action [1,2,5]. Unlike many so-called conventional antibiotics used clinically to treat bacterial infections like β-lactams, quinolones, macrolides, tetracyclines which have core structural features that are primarily responsible for their antibacterial activities, AMP sequences are highly diverse and do not have a common core structure. Instead, most AMPs share a fundamental motif: they are cationic and amphipathic [1]. From in vitro studies, the general

mechanism of AMP activity is believed to involve the selective disruption and permeabilization of microbial membranes. It is now understood that their microbicidal activities often extend to acting as metabolic inhibitors by binding to intracellular targets [2]. Furthermore, a large number of studies have established that the role of mammalian AMPs in host defense is not restricted to acting as direct microbicides, and that many AMPs can strongly modulate the immune response through interactions with components of the innate and adaptive immune systems [7]. Based on these results the emerging picture is that AMPs are multi-functional tools which participate in many facets of the immune response.

The establishment of robust structure-activity relationships between the amino acid sequences of AMPs and their microbicidal activities has remained elusive. It is generally accepted that the baseline membrane activity shared by most AMPs is derived from their common fundamental motif. However, the potency and selectivity of membrane-active peptides can widely vary with the types and amounts of cationic and hydrophobic residues, as well as their specific arrangements in the peptide. The multi-functionality of AMPs can further complicate relating peptide sequence to membrane activity, since certain parts of the sequence may be more important for encoding other functions and less vital for membrane disruption. For example, in addition to broad spectrum

antimicrobial activity, the 37 amino acid sequence of human cathelicidin LL37 imparts the peptide with diverse biological activities [8,9]. LL37 has been shown to inhibit biofilm formation, bind and neutralize LPS. LL37 also has immunomodulatory abilities: it is a chemo-attractant for both innate and adaptive immune cells, it can induce other immune mediators and help regulate the inflammatory response. In addition to the above, LL37 can promote angiogenesis and wound closure [8,9]. Differentiating the peptide characteristics responsible for membrane activity from those responsible for other biological activities like immunomodulation is not straightforward, especially since some characteristics may be important for more than one function. In principle, if one could relate sequence characteristics of AMPs to their membrane mechanisms of activity, such structure-activity relationships could be used as 'design rules' to guide the synthesis of new membrane-active antimicrobials in a deterministic way.

Here we review peptide-membrane interactions in the context of AMPs, with an emphasis on our recent work on peptide-induced membrane curvature and its causative effect on destabilization of the barrier function in bacterial membranes. We argue that non-specific electrostatic and hydrophobic interactions between AMPs and membranes will lead to local membrane curvature deformations, and the specific types of membrane curvature generated will depend on the physical chemistry of AMPs and cell membranes. Among the taxonomy of curvature deformations certain types are expected to enable membrane destabilization. Specifically, negative Gaussian membrane curvature (equivalently saddle-splay curvature) should be especially disruptive since it is topologically necessary for a variety of known membrane destabilizing processes. Our previous in vitro studies demonstrated that prototypical AMPs generated saddle-splay curvature in model membranes with physicochemical properties characteristic of bacteria membranes but not in membranes with properties that resembled eukaryotic cell plasma membranes [10,11]. The selective generation of saddle-splay curvature in bacterial cell membranes provides AMPs with a broad mechanism to specifically target the membranes of microbes. Furthermore, the geometric requirement to induce saddle-splay curvature places specific constraints on the relative amounts of arginine, lysine, and hydrophobic amino acids used by AMPs. Interestingly, this 'saddle-splay curvature design rule' is consistent with the naturally occurring, cationic AMPs in the AMP database (http://aps.unmc.edu/AP/main.php) [12]. The sequence constraints imposed by saddle-splay curvature generation are not strongly restrictive, which provides AMPs significant freedom to adopt other useful functions like multiplexing membrane activity with inhibiting metabolic processes by binding to intracellular targets, or by acting as immunomodulatory agents. Since our saddle-splay curvature design rule is based upon both a mechanism of activity and the existing motifs of natural AMPs, we believe it will assist the development of synthetic antimicrobial agents by providing guidelines for their construction.

2. Structural features

To date well over 1000 AMPs have been discovered. Despite the enormous diversity of AMP sequences, most share a few broad features. AMPs are short (<50 amino acids), have net positive charge (+2 to +9), and contain a significant proportion of hydrophobic amino acids (>30%) [1–6]. Generally their cationic and hydrophobic residues spatially partition into discrete clusters to form an amphipathic secondary structure. AMPs are often classified by these folded structures. One subgroup consists of linear peptides such as magainins [13] from frogs, and LL-37 [8] from humans. These peptides adopt alpha helical structures when they partition into a membrane interface which segregates their polar and hydrophobic residues on opposite faces of the helix. Another subgroup of AMPs includes peptides that consist of beta sheet structures stabilized by disulfide bonds from cysteine residues, such as protegrins [14] from pigs, and defensins [15–17]. Mammalian defensins include α-defensins such as cryptdins [18] from mouse, β-defensins like the human β-defensins [16] and θ-defensins [19] which are found only in old world monkeys. A third subgroup is categorized by extended linear peptides with sequences dominated by a few amino acids, like the tryptophan-rich indolicidin [20] from cattle, and the arginine and proline-rich PR-39 [21] from pigs. Lastly there are peptides derived from proteolysis of proteins such as buforin II [22] from histone 2A which is expressed in frogs, and lactoferricin B [23] from gastric pepsin digestion of bovine lactoferrin.

Based on the hypothesis that the antimicrobial activities of many AMPs depend more on their fundamental cationic and amphipathic properties than on their precise amino acid sequences and secondary structures, a variety of synthetic antimicrobials have been designed using different chemistries. One method is to modify existing AMP sequences to maximize antimicrobial activity while also minimizing hemolytic activity to optimize therapeutic potential. Examples of synthetic AMPs which have been developed by modifying natural versions include MS-78 (pexiganan) [24], a variant of the AMP magainin from frogs, iseganan [25], a variant of protegrin from pigs, and omiganan [26], a cattle indolicidin variant. By using the positively charged amphipathic features as templates, synthetic AMP analogs have been constructed. These analogs include model antimicrobials based upon mimicking the peptide primary structure (peptidomimetics), and ones that use non-peptidic compounds [27,28]. Peptidomimetic antimicrobials have been made using β-peptides [29–31] and peptoids [32]. These peptides folded into amphipathic structures and demonstrated potent in vitro antibacterial activity. Furthermore, unlike the α-peptide backbone of natural AMPs, both β-peptides and peptoids offer the additional advantage of being resistant to proteases. Non-peptidic compounds include facially amphipathic acrylamide polymers [33], phenylene ethynylene molecules [34], polymethacrylate derivatives [35], and polynorborene derivatives [36], which all displayed potent selective activity against Gram-positive and Gram-negative bacteria. (These are described in the review by Henderson and Lee, elsewhere in this issue.) Since these synthetic antimicrobials are also cationic and amphipathic, it is believed their mechanisms of activity are similar to AMPs.

3. Mechanisms of activity

A general mechanism of AMP activity is selective disruption of cell membranes leading to membrane permeabilization, depolarization, dissipation of electrochemical gradients, leakage, and eventual cell death [1,37]. Support for this comes from studies showing that AMPs composed of D amino acids showed identical antibacterial and hemolytic activities to their all-L natural versions, indicating specific interactions with chiral membrane receptors and enzymes are not responsible for activity [38–41]. Therefore, membrane disruption is thought to be a consequence of non-specific interactions between the AMP and the cell membrane. Evidence that AMPs can directly target lipids, the principal components in cell membranes, comes from an extensive number of in vitro studies using distinct techniques including X-ray, NMR, AFM, Raman, circular dichroism, differential scanning calorimetry, and fluorescence experiments [2,42] (and references therein). Furthermore, numerous leakage assays on liposomes have demonstrated that AMPs can permeabilize lipid bilayers. These studies have established that AMPs strongly interact with and alter the intrinsic structure of lipid bilayers.

AMP selectivity is thought to be a consequence of compositional differences between bacterial cell membranes and the membranes of the host cells [1,2,5,43–46]. Microbial cell surfaces are decorated with polyanionic molecules like lipopolysaccharides in Gram-negative bacteria, and lipoteichoic acids in Gram-positive bacteria [5]. Additionally, the outer leaflet of bacterial plasma membranes contains large amounts of anionic lipids such as those with phosphatidylglycerol (PG) and diphosphatidylglycerol (dPG, also called "cardiolipin" which is abbreviated as CL) head groups. For example, the membranes of the Gram positive bacteria *Staphylococcus aureus*, and *Streptococcus pneumoniae* are composed primarily of PG and CL lipids. Phosphatidylethanolamine (PE) is the principle zwitterionic phospholipid found in Gram negative bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium* and in the Gram positive genus *Bacillus*.[47] The lipid compositions of animal cell membranes are differ from bacteria plasma membranes in at least three ways [1,48,49]: First, they are more heavily populated by lipids with neutral zwitterionic head groups such as phosphatidylcholine (PC) and sphingomyelin (SM). Second, their lipid compositions are asymmetrically distributed between the inner and outer bilayer leaflets. For example, the primary lipids in outer leaflet of human erythrocytes are PC and SM, while PE and the anionic lipids phosphatidylserine (PS), and phosphatidylinositol (PI) are segregated on the cytosolic side of the cell. Third, sterols such as cholesterol constitute a major component (for cholesterol ~30% by mole) of animal plasma membranes. Numerous in vitro studies on both natural and synthetic cationic membrane-active antimicrobials have shown that the presence of anionic lipids increases membrane disruption and permeabilization [43]. Recently we have shown that AMPs can act against membranes with specific distributions of lipids, rather than target specific lipid molecules themselves. Anionic membranes that are also enriched with negative intrinsic curvature lipids such as PE and CL are more susceptible to destabilization compared with those containing zero intrinsic curvature lipids like PC [10,50,51]. Moreover, it was found that the existence of anionic lipids and the existence of negative intrinsic curvature lipids are both necessary conditions for activity, but neither is a sufficient condition. Finally, the presence of cholesterol in eukaryotic and model cell membranes can inhibit membrane insertion and disruption by AMPs [52–55], which has been attributed to the rigidifying effects of cholesterol on lipid bilayers [56,57]. These results indicate that AMPs target general differences in the physical chemistry between bacterial plasma membranes and host cell membranes, due to differences in the amounts of multiple lipid species.

From in vitro studies, amphipathic AMPs are inferred to disrupt membranes through a combination of non-specific electrostatic and hydrophobic interactions [1,2,42–45]. In this mechanism the cationic and hydrophobic moieties play complementary roles. Interaction between the anionic membrane and the cationic AMP leads to strong effective electrostatic attraction and binding of the AMP to the membrane. Upon adsorption to the membrane the hydrophobic portions of the AMP insert into the non-polar region of the bilayer leading to membrane destabilization and loss of barrier function [1,2,42–45]. Based on studies of AMPs with model membranes composed of single or mixed lipids three models have been previously proposed to describe the insertion of AMPs into the bilayer: the toroidal pore, carpet, and barrel-stave models [2,43].

In the "toroidal-pore model", insertion of peptides into the membrane eventually induces a self-connection between the two monolayer leaflets, creating a pore that is lined with both lipid head groups and AMPs. Under specific experimental conditions toroidal pores have been observed for protegrin-1 [58], melittin [59], magainin-2 [58,60], with X-ray and neutron scattering techniques. Similar results were found for protegrin-1 [61], and the synthetic analog of magainin-2, MSI-78 [62] using NMR spectrometry. In the "carpet model" the peptides bind and cover the membrane surface until a threshold concentration is reached. The adsorbed peptides then disintegrate the membrane in a detergent-like manner into micelles [43]. This model has been used to explain the membrane activities of the dermaseptins [63,64] and cecropins [65,66], since they exhibit maximal permeation after coating the surfaces of liposomes. In the "barrel stave model" the peptides aggregate in the bilayer and then span it to form a transmembrane pore. The hydrophobic portions of the peptides face the non-polar interior of the bilayer and the hydrophilic portions are exposed to solution. Results from oriented circular dichroism [67], neutron scattering [59,68], and X-ray scattering experiments [68] suggest that alamethicin induces this type of membrane pore.

These three models do not exhaust the types of membrane disruptions that have been observed for membrane active antimicrobials, however. There are many ways in which AMPs can act against bacterial membranes. For example, SEM micrographs of *P. aeruginosa* after a few minutes of exposure to the sheep cathelicidin SMAP29 showed the presence of large blebs on the bacterial cell surface [69,70]. Electron microscopy on cross sections of *E. coli* after treatment with the antimicrobial polymyxin B showed significant surface vesicularization [71], thin, finger-like membrane protrusions that break down into small vesicles. And electron micrographs show treatment of *E. coli* cells with protegrin-1 produces numerous microvilli in the bacterial membrane [72]. It has been shown that the pore-forming peptide melittin can induce budding in vesicles by promoting lipid phase separation [73]. In particular, this last study illustrates that the specific form of membrane disruption depends on both the peptide and on the physiochemical properties of the membrane. Overall, this diversity of outcomes suggests that in addition to pore formation AMPs can destabilize membranes and compromise their barrier function through multiple processes including blebbing, budding, and vesicularization. Furthermore, the resultant membrane destabilization mechanism is due to interplay between the unique properties of both the AMP and membrane. Therefore, it is often the case that a specific type of membrane destabilization mechanism cannot be described as the membrane mechanism of activity for an AMP.

While membrane permeabilization is a general feature of AMP activity, there is abundant evidence that certain AMPs utilize other mechanisms of microbial killing, as a number of AMPs have been shown to bind to intracellular targets and consequently inhibit metabolic processes in bacteria. For example, some AMPs have been shown to directly bind DNA. Buforin II was found to penetrate *E. coli* without lysing the cell membrane and inhibit cellular functions by binding to DNA and RNA [22]. Tachyplesin I from horseshoe crab penetrates bacterial membranes without lysing them and directly interacts with the minor groove of the DNA duplex [74]. Bovine indolicidin can kill bacteria without lysing the cell membrane by binding DNA [75]. Indolicidin is also an inhibitor of nucleic acid and protein synthesis, and can induce filamentation in *E. coli* as a result of preferential inhibition of DNA synthesis [76]. Other AMPs have been shown to inhibit macromolecular synthesis. PR39 from pigs halts synthesis of nucleic acids and proteins which leads to their degradation and bacterial cell death [77]. In addition to permeabilizing both the outer and inner membranes of E. coli, human α-defensins HNP-1 and -2 cease DNA, RNA, and protein synthesis [78]. And at their minimum inhibitory concentrations pleurocidin from flounder and pleurocidin-derived AMPs can translocate into bacterial cells and exhibit their antimicrobial activities by inhibiting macromolecular synthesis [79]. It is important to note however that even though membrane permeabilization does not account for full antimicrobial activity of certain AMPs, it is still a necessary condition for their activity because the peptide must cross the cytoplasmic membrane in order to

reach its internal target. Therefore, membrane interaction remains an important activity component of non-lytic AMPs, even if their membrane interactions differ from those of their lytic counterparts.

In addition to their direct antimicrobial abilities many AMPs play other indirect roles in the host immune response. Mammalian defensins and cathelicidins have been implicated as modifiers of the innate immunity and in bridging the innate and adaptive immune systems. HNP-1 and -2 are chemotactic in vitro for human monocytes [80] and T-cells [81], as well as naive T-cells and immature dendritic cells [82]. Human β-defensins also act as chemokines, as human β-defensin-2 promoted an adaptive immune response by recruiting immature dendritic cells and memory T-cells through interaction with human chemokine receptor 6 (CCR6) [83], and hBD-3 & -4 are chemotactic for monocytes [84,85]. Similarly, the cathelicidins LL37 [86], indolicidin [87], PR39 [88], and CRAMP from mouse [89] have all been shown to chemoattract various types of leukocytes. Neutrophil α-defensins from human, rat, guinea pig, and rabbit all induced histamine secretion from mast cells [90], as did hBD-2 and LL37 [91], indicating they can directly trigger inflammatory response. And HNPs [92,93] and LL37 [94] stimulate release of cytokines such as interleukin 8 (IL-8) from epithelial cells and keratinoctyes, respectively. These results demonstrate that mammalian AMPs have a broad range of immune activation functions.

In contrast to the above, certain AMPs have been associated with the prevention of deleterious effects to the host from inflammatory response. For example, LL37 partially protected mice against lethal endotoxic shock induced by high concentration lipopolysaccharide (LPS) [95]. Both indolicidin and LL37 inhibited lipopolysaccharide (LPS)-induced tumor necrosis factor alpha (TNF-α) release in human macrophage/monocyte-like THP-1 cells indicating they have anti-endotoxin ability [87]. Like other AMPs, both of these cathelicidins can directly bind to LPS [96], which may prevent proinflammatory cytokine secretion since the neutralized LPS is unavailable to pattern recognition receptors. However, similar studies showed LL37 and bovine cathelicidin BMAP-27 suppress proinflammatory transcriptional responses in human monocytes to LPS [97], suggesting these peptides have multiple endotoxin-neutralizing activities. Finally, LL37 can induce angiogenesis [98] and promote keratinocyte migration [99,100], which suggests the peptide can assist in tissue repair and wound healing. HNPs have also been shown to assist cell division in epithelial cells and fibroblasts [101], induce epithelial cell proliferation [102], and enhance wound closure [103]. These results provide evidence that mammalian host defense peptides can have growth-promoting properties which help to counteract the destructive effects of infection and inflammation.

From the above summary, it can be seen that there is abundant evidence that AMPs are multifunctional molecules that have a range of roles in host defense. They are generally microbicidal, but many also display specialized immune modulation functions. It is known that collectively, AMPs share the fundamental structural motif of amphiphilicity. A number of natural questions emerge. How does this motif connect to the specific activity of AMPs against bacterial membranes but not eukaryotic membranes, in the form of membrane permeation or translocation? Why do not all amphiphiles have these functions? It is not generally known whether there are additional systematic patterns in AMP sequences, and how these patterns relate to peptide-lipid interactions that enable selective membrane destabilization. Identification of such patterns can in principle lead to deterministic molecular engineering of AMPs. Finally, how do relatively short simple amino acid sequences such as those found in AMPs evolve such a broad range of functions? We now sketch some answers based on our recent work.

Generic electrostatic and hydrophobic interactions between a cationic, amphiphilic AMP and a cell membrane lead to strong binding between the peptide and membrane and subsequent partial insertion of the peptide into the bilayer. We argue that the physical chemistry of AMPs and cell membranes leads to a whole taxonomy of local membrane distortions, specific combinations of which are topologically active and can lead to membrane destabilization. From lyotropic systems, examples of these curvatures include positive mean curvature and positive Gaussian curvature seen in micelles, negative mean curvature seen in inverted hexagonal phases and negative Gaussian (saddle-splay) curvature seen in cubic phases. Furthermore, saddle-splay curvature membrane deformations are expected to be especially disruptive since this type of curvature is geometrically necessary [104] for pore formation and a broad range of known AMP-induced membrane destabilizing processes. Comparison of the types of membrane curvature produced by arginine, lysine, and hydrophobicity suggests that certain amino acid combinations can induce saddle-splay curvature. We show that the sequences from this 'saddle-splay curvature design rule' are consistent with trends in the compositions of natural AMPs as well as cell-penetrating peptides, a cognate class of known molecular pore formers. The relation of these AMP sequences to synthetic membrane active antimicrobials are discussed. Finally we explore the implications of this design rule. The generation of saddle-splay membrane curvature under-determines the sequences of AMPs, a fact that allows the multiplexing of additional functions in the greater context of host immunity and beyond.

4. Background

4.1. Formalisms of membrane curvature

As a first approximation, biological membranes can be thought of as a bilayer consisting of two leaflets that are in a fluid state. Since hydrophobic interactions prevent lipid diffusion out of the membrane there is a well-defined average matter density distribution along the bilayer normal. Combined X-ray and neutron diffraction measurements have determined the time-averaged transmembrane probability distribution curves of water and lipid components (e.g. phosphates, methylenes) for a lipid bilayer in a liquid state [105,106]. These results reveal there is significant out-of-plane thermal motion, based on the widths of the probability densities. Therefore, the water–membrane interface is not a single plane but instead has an associated thickness, which is typically defined by the distribution of the water hydrating the lipid headgroups (∼15A for DOPC) [107]. Furthermore, a liquid state lipid bilayer is a highly heterogeneous region where large changes in polarity occur over sub-nanometer length scales [108]. The thickness of the membrane-water interface, and the sharp decrease in polarity across it make a fluid state membrane amenable to the partitioning of an amphipathic AMP from solution into the membrane.

An amphipathic molecule partitioned into the membrane interface will deform the membrane. These membrane curvature deformations can be described using ideas from geometry, which we briefly review here. Imagine a curved two dimensional surface. At each point on the surface, there are an infinite number of ways to draw different curves with different curvatures ($C = 1/R$) through that point, so we must find a consistent way to characterize two-dimensional curvature. It turns out there are two special directions in which the directional curvatures are extremal (maximum and minimum). Moreover, these two directions are always orthogonal to one another. The curvatures along these two principal directions are called principal curvatures ($c_1 = 1/R_{max}$ and $c_2 = 1/R_{min}$). Once
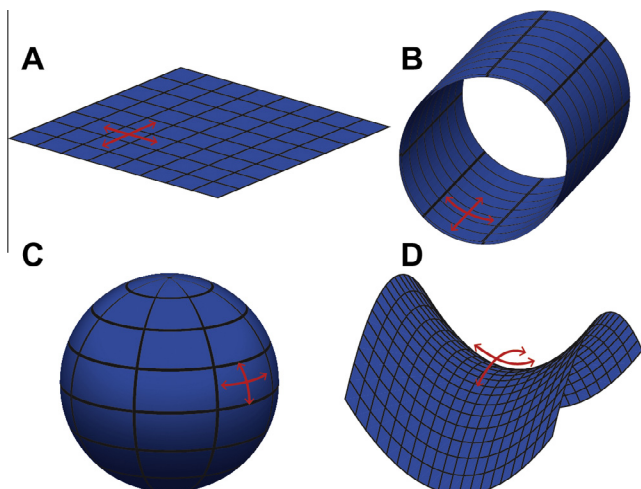
Fig. 1. The common types of curvature seen in lyotropic systems. (A) A plane has $c_1 = c_2 = 0$, so it has zero mean curvature, $H = \frac{1}{2}(c_1 + c_2) = 0$, and zero Gaussian curvature, $K = c_1 c_2 = 0$ everywhere. (B) Cylindrical shapes such as membranes arranged into the inverted hexagonal phase have $c_1 < 0$, $c_2 = 0$, so $H < 0$ and $K = 0$. (C) Spherical shapes like spherical micelles have $c_1 > 0$, $c_2 > 0$, so every point on the surface has positive Gaussian curvature, $K > 0$. (D) Saddle-shaped surfaces have $c_1 < 0$, $c_2 > 0$, so they have negative Gaussian curvature, $K < 0$. A minimal surface is the special case where $c_1 = -c_2$ everywhere.

these principal curvatures are known, we can calculate every directional curvature using a formula first derived by Euler [109]:

$$c(\alpha) = c_1 \cos^2 \alpha + c_2 \sin^2 \alpha$$

where α is the angle between the chosen direction and the principal direction of c_1. These principle curvatures can be combined into two different general expressions to describe deformations of a surface. Their average or mean curvature is defined as $H = \frac{1}{2}(c_1 + c_2)$, while their product is known as the Gaussian curvature, $K = c_1 c_2$. For example, in the context of membranes, lamellar phases (L_α) are locally flat everywhere and have zero mean curvature and zero Gaussian curvature (Fig. 1A). The inverted hexagonal phase (H_{II}) composed of a 2D hexagonal arrangement of cylindrical solution channels wrapped by lipid monolayers has $c_1 < 0$ in the direction

of wrapping and $c_2 = 0$ along the cylinder axis, so H_{II} phases have negative mean curvature and zero Gaussian curvature (Fig. 1B). Spherical objects like spherical micelles have both non-zero mean curvature and positive Gaussian curvature, since c_1 and c_2 have the same sign (Fig. 1C). If c_1 and c_2 have opposite sign, then the surface has negative Gaussian curvature and will locally have a saddle-shape (Fig. 1D). Surfaces with $c_1 = -c_2$ everywhere are known as minimal surfaces and have zero mean curvature and negative Gaussian curvature (Fig. 2C). Therefore, the interactions between amphipathic molecules such as AMPs and the membrane interface can be described by the resulting mean and Gaussian curvatures they impart to the membrane.

The elastic energy costs of deforming the membrane away from its unperturbed state is generally described using the approach from Helfrich [110]. The curvature elastic energy per unit area of bending a membrane is:

$$f = 2\kappa(H - c_0)^2 + \kappa_G K$$

Here c_0 is the intrinsic curvature. The bending modulus, κ, is a constant that describes the stiffness of the membrane, while the Gaussian curvature modulus, κ_G, is a constant related to the resistance of the membrane to topological transitions, such as the formation of holes. In other words, the first term in the equation measures the energetic penalty of bending the membrane from its intrinsic curvature, while the second term measures the energetic penalty of Gaussian curvature distortions which bend and stretch the membrane and are prerequisite to topological transitions [111,112].

Bilayer intrinsic curvature arises from asymmetries across monolayer leaflets. If two monolayers with equivalent non-zero intrinsic curvature combine to form a membrane, the bilayer intrinsic curvature of the membrane will be zero to avoid producing energetically costly voids. The physicochemical properties of lipids and the interactions between them determine the monolayer intrinsic curvature. In lipid membranes, monolayer intrinsic curvature arises because the lipid–lipid interactions in the monolayer will depend on the position in the monolayer [113]. For example, at the hydrophilic surface the interactions between the lipid headgroups will generally produce different in-plane forces than the interactions between the lipid tails at the hydrophobic surface. If the sum of the in-plane forces is balanced for an area which is smaller at the headgroups than at the tail ends, then the
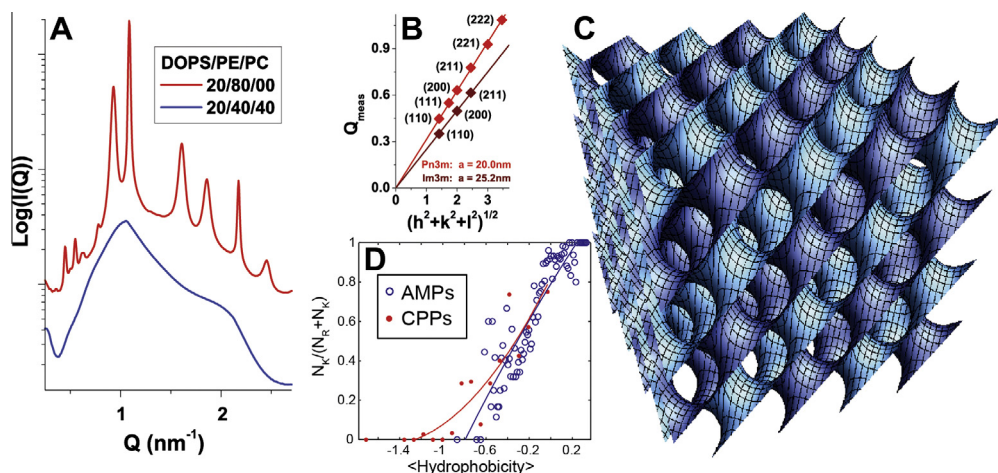

Fig. 2. SAXS measurements of the membrane curvatures generated by prototypical AMPs leads to the saddle-splay curvature selection rule. (A) The membrane curvatures generated by mouse defensin Crp4 depend on lipid composition. In model membranes with lower PE content, PS/PE/PC = 20/40/40, Crp4 induced lamellar phase with spacing d = 6.0 nm. In membranes enriched with $c_0 < 0$ lipids, DOPS/DOPE = 20/80, a Pn3m cubic phase with lattice parameter a = 20.0 nm is observed. Peptide to lipid molar ratios are P/L = 1/45, & 1/90 for the 40% PE and 80% PE membranes, respectively. (B) Indexing of the Pn3m cubic phase peaks, and the less intense coexisting Im3m cubic peaks from the Crp4-DOPS/DOPE = 20/80 sample from A. (C) Illustration of the minimal surface of the Pn3m cubic. Opposite sides of the surface are different colors. Every point on this surface is (locally) saddle-shaped. (D) Saddle-splay curvature selection rule plots for antimicrobial peptides (AMPs) and cell-penetrating peptides (CPPs). At a given Lys/Arg ratio, the CPPs tend to be less hydrophobic than the AMPs.

minimum-energy configuration is a monolayer which bends back toward the headgroups. In this situation the monolayer is said to have negative intrinsic curvature. Lipids which tend to promote this configuration are called negative intrinsic curvature lipids. They are typically illustrated with small headgroups and splayed tails, or geometrically as having a traffic cone shape. At physiological conditions the physical and chemical properties of PE and CL often render them negative intrinsic curvature lipids. In contrast, the properties of PC, PG, and PS lipids typically lead to in-plane forces at their headgroup and tail regions which are balanced for equivalent areas [114]. These lipids typically self-assemble to form flat membranes, and they are often geometrically represented as having cylindrical shape with zero intrinsic curvature.

The connection between molecular pore formers like AMPs and monolayer intrinsic curvature can be understood by relating the bilayer intrinsic curvature, c_0, bending modulus, κ, and Gaussian curvature modulus, κ_G, to their monolayer parameters (denoted by m). For symmetric bilayers the expressions are [115]:

$$c_0 = 0$$

$$\kappa = 2\kappa^m$$

$$\kappa_G = 2\kappa_G^m - 4\kappa^m c_0^m d$$

The first expression restates that the bilayer is symmetric, and the second expression explains that the bilayer bending modulus is twice the monolayer bending modulus. The third expression is less straightforward. The bilayer Gaussian modulus is not simply twice the monolayer Gaussian modulus, κ_G^m, it also depends on the monolayer bending modulus, κ^m, the monolayer intrinsic curvature, c_0^m, and d, the distance from the middle of the bilayer to the monolayer pivot plane. The Gauss-Bonnet theorem shows the formation of a toroidal pore in a membrane changes the Gaussian curvature, K, in the membrane by an amount $\Delta \int K \cdot dA = -4\pi$. It follows that the energy change from pore formation is $\Delta E = \kappa_G \int K \cdot dA = -4\pi\kappa_G$; pore formation is energetically favorable when $\kappa_G > 0$ [114,115]. Typically the negative value of the monolayer Gaussian bending modulus ($\kappa_G^m < 0$, from the lateral stress profile of the monolayer) [116] ensures that κ_G remains negative, which exacts an energetic penalty for pore formation. Physically both κ^m and d must be positive, so the sign of the second term in the expression for the bilayer Gaussian modulus depends on the monolayer intrinsic curvature, $\kappa_G \propto -c_0^m$. (Similar expressions have been derived for block copolymers [117]). Therefore, enriching membranes with negative intrinsic curvature lipids decreases the energetic barrier to pore formation [114], and membranes with elevated concentrations of these lipids should be more susceptible to membrane disruption by pore-forming peptides like AMPs. This has been experimentally observed that bacterial membranes with elevated concentrations of PE lipids are more susceptible to natural and synthetic antimicrobials [10,118].

4.2. Membrane interface partitioning

The initial attraction of a cationic AMP to an anionic membrane is driven by the entropy gain from releasing territorially bound counterions [119]. While the initial attraction is electrostatic, partitioning into the membrane often requires peptide folding. For example, at the membrane interface α-helical peptides undergo secondary structural transitions. Circular dichroism experiments on the AMPs magainin-2 [120] and melittin [121] have shown that while they are unstructured in solution, both peptides adopt α-helical conformations when partitioned into membranes. Studies of the free energies of transfer of peptides from water to bilayer and water to octanol have shown that a helical structure transition is a consequence of the high energetic cost of partitioning peptide

bonds into a membrane interface. Formation of secondary structures such as α-helices allows peptide bonds to form hydrogen bonds which reduce the energetic cost of transfer (free energy reduction of roughly −0.5 kcal/mol per residue) [122]. In the case of α-helical AMPs, the stabilized helix structure renders them facially amphipathic. Helical wheel projections of many α-helical AMP sequences show their hydrophobic amino acids segregate along one face, while their polar and charged residues occupy the opposite face [123]. Amphipathicity is also observed in β-sheet AMPs stabilized by disulfide bonds, as their secondary structures often display discrete cationic and hydrophobic patches [1,3,109,117]. The effect of a steep decrease in polarity across the membrane interface is to promote AMP amphipathicity and to align the peptide with the interface gradient. The end result is a membrane interface-partitioned AMP oriented such that its hydrophobic residues are directed toward the non-polar region of the bilayer while its cationic residues remain close to the charged and zwitterionic lipid headgroups.

4.3. AMP-membrane interactions generate membrane curvature deformations

Electrostatic and hydrophobic interactions between an amphipathic AMP and the membrane interface generate complementary curvature deformations. Entropic gain from counterion release is the driving force for the effective electrostatic attraction between peptide and membrane. Since counterion release will be maximized when the cationic moieties of a polymer are closely associated with the anionic and polar headgroups of the membrane, there is a tendency for the membrane to wrap around the polymer to maximize contact between the charged portions of the polymer and membrane [124]. The membrane will curve toward the solution. Therefore, electrostatic interactions between a cationic AMP and an anionic membrane will generally result in negative membrane curvature generation. Additionally, embedding an AMP into the membrane interface requires displacing lipids in order to accommodate the peptide. The extent of the displacement will be related to AMP hydrophobicity. The hydrophobic force pushes hydrophobic portions of the embedded peptide into the non-polar regions of the membrane, producing an increase of hydrophobic volume in the perturbed monolayer in a manner dependent on the hydrophobic properties of the AMP [125–127]. A gain in hydrophobic volume has the effect of bending the membrane toward its interior. Therefore, hydrophobic interactions between an amphipathic AMP and a membrane will generally result in positive membrane curvature generation.

The ability of a cationic and amphipathic peptide to generate both negative and positive membrane curvature at the same nanoscopic location is central to its ability to destabilize membranes. A membrane deformation that consists of both these curvature types in different directions will locally have the shape of a saddle. The membrane will bend upwards ($c_1 < 0$) towards the peptide in one direction and downwards ($c_2 > 0$) in the other direction (equivalently, 'wrapping' the membrane in one direction and 'pinching' the membrane in another). When the principle curvatures in orthogonal directions have opposite sign, $K < 0$, the surface exhibits negative Gaussian (equivalently "saddle-splay") curvature (Fig. 1D). Saddle-splay curvature is a topological requirement for toroidal pore formation [104,114,115,128]. For example, objects like spheres which do not contain holes have c_1 and c_2 the same sign and therefore have positive Gaussian curvature everywhere on their surfaces (Fig. 1C). However, for 'holey' objects such as a torus the surface bends toward itself as the interior of the pore is traced, whereas moving out of the pore the surface bends away from itself. In addition to being required for pore formation, saddle-splay curvature is also necessary for membrane protrusions,

such as blebs and buds. If a protrusion remains connected to the bulk membrane, the Gauss–Bonnet Theorem shows this configuration has the same total Gaussian curvature as the unperturbed flat membrane, i.e. 0. The top of the protrusion which has positive Gaussian curvature will be exactly cancelled by the negative Gaussian curvature at the base of the protrusion [129]. Therefore, saddle-splay membrane curvature is required for a variety of known membrane destabilization processes like pore formation, as well as in membrane blebbing, budding, and vesicularization events, all of which lead to the loss of barrier function of cell membranes.

5. SAXS studies on peptide–membrane interactions

5.1. Antimicrobial peptides

In previous work [10,11] we have investigated mammalian defensins, an important class of membrane disruptive AMPs with, in vitro, broad spectrum and selective microbicidal activities against a variety of pathogens including Gram positive and Gram negative bacteria, fungi, protozoa, and enveloped viruses. Their common characteristics include molecular weights of 2–5 kDa, β-strand secondary structures stabilized by conserved tri-disulfide bonds from six cysteine residues, amphipathicity, and high cationic net charge [15–17]. They can be broadly grouped into three types: θ-defensins from old world monkeys, α-defensins found in mammals, and β-defensins found in mammals, birds, and reptiles [15–17]. Collectively, both θ-defensins and α-defensins utilize the cationic amino acid arginine over lysine, while β-defensins have more intermediate arginine to lysine ratios [16,130]. The preference for arginine in β-sheet defensins is different than in the case for α-helical AMPs such as magainins which generally use much more lysine than arginine. Since many aspects of defensin biology are known, they are an ideal prototypical family of AMPs to investigate their antibacterial abilities from selective membrane disruption.

We used small-angle X-ray scattering (SAXS) to characterize the structures and phase behavior of the peptide-lipid complexes generated by representative defensins from each of the three types [10]. The membrane curvature deformations induced by defensins were probed by modifying the biophysical properties of the membrane by changing the membrane lipid composition. In particular we assayed the effects of negative intrinsic curvature lipids on phase behavior, as bacteria plasma membranes are known to have higher concentrations of negative intrinsic curvature lipids (PE, CL) compared to eukaryotic cell membranes. In general, the defensins exhibited a tendency to generate saddle-splay curvature in membranes enriched with DOPE, a prototypical negative intrinsic curvature lipid. Fig. 2A shows representative SAXS spectra for mouse α-defensin Cryptdin-4 (Crp4) and DOPS/DOPE/DOPC lipid membranes. In membranes with equal concentrations of DOPE ($c_0 < 0$) and DOPC ($c_0 \approx 0$), DOPS/DOPE/DOPC = 20/40/40, correlation peaks are observed at integral Q values, indicating Crp4 reorganizes membranes with similar amounts of PE and PC lipids into multilamellar phases, flat stacks of membranes with Crp4 intercalated between adjacent bilayers. More correlation peaks appear when the membrane PE content is raised to amounts characteristic of the inner membranes of Gram negative bacteria such as *E. Coli*. The peaks have characteristic ratios, $\sqrt{2}:\sqrt{3}:\sqrt{4}:\sqrt{6}:\sqrt{9}:\sqrt{12}$, indicating the presence of the Pn3m 'double-diamond' cubic phase (Fig. 2B). The Pn3m is a bicontinuous cubic phase where the lipid membrane separates space into two equivalent, non-intersecting regions (Fig. 2C). The middle of the bilayer traces out a surface with principal axes that are equal and opposite, $c_1 = -c_2$, everywhere on the surface [131]. Such

surfaces are called minimal surfaces and they have zero mean curvature and negative Gaussian curvature at every point. Therefore, the α-defensin Crp4 induces membrane destabilizing saddle-splay curvature in model bacterial cell membranes, but not in model eukaryotic cell membranes. Similar phase behavior was observed for representative θ- and β-defensins. Subsequent experiments showed that other β-sheet and α-helical AMPs (Table 1) induce saddle-splay curvature in model bacterial membranes but not in eukaryotic membranes, suggesting that the selective generation of saddle-splay curvature is a general feature of AMPs.

5.2. Cell penetrating peptides

Generation of saddle-splay curvature by arginine-rich defensins is similar to the behavior observed for a cognate class of known pore forming peptides, the cell-penetrating peptides [132–134]. Cell-penetrating peptides are short cationic peptides which have the ability to translocate across the plasma membranes of eukaryotic cells [135,136]. While controversy still surrounds their uptake mechanism, the emerging body of evidence suggests cell-penetrating peptides use multiple mechanisms to enter cells [60,137–139]. They can directly cross membranes [140–142]. They can also employ the cell's endocytotic machinery for translocation, as studies have shown uptake via clathrin-mediated endocytosis [143], caveolin-dependent endocytosis [144], and macropinocytosis [145,146]. The most widely studied cell-penetrating peptide, the TAT peptide from the HIV transactivator protein is an 11 amino acid sequence peptide which has a net charge of +8 from 6 arginines and 2 lysines [147–149]. We have previously shown that the TAT peptide induced the Pn3m cubic phase in membranes [132]; like defensins, the TAT peptide can generate saddle-splay curvature. As this curvature is geometrically necessary for pore formation and endocytosis, saddle-splay curvature deformations may lower the free energy barriers of the translocation mechanisms used by the TAT peptide and thereby enable its entry into cells. Arginine has an essential role in the translocation abilities of the TAT peptide. Polyarginine sequences with sufficient length display equivalent or better uptake compared with the TAT peptide [150,151]. However, homopeptides of lysine, histidine, and ornithine with the same charge as the TAT peptide cannot enter cells [150]. The improved uptake of poly-Arg compared to poly-Lys has been attributed to the ability of the guanidinium group of arginine to form stable bidentate hydrogen bonds with anionic membrane components such as the phosphate groups of phospholipids, compared with the monodentate hydrogen bonding abilities of the amine group of lysine [150,152,153]. Previous explanations for the differential uptake profiles of Arg vs Lys emphasized the importance of bidentate vs monodentate H-bonding. However, these descriptions did not provide a molecular understanding for how the interactions of guanidinium groups with anionic membrane components lead to translocation while the interactions of amine groups with these components do not.

5.3. Multivalent Arg/Lys-lipid interactions and membrane curvature

To understand these differences at the molecular level we used quantum mechanical (QM) calculations on a minimal model to gain insight into the interactions of Arg and Lys with phosphate groups of the lipid bilayer [154]. Unexpectedly, single guanidinium groups and amine groups coordinated 2 phosphates with the same complexation energy. The observed diphosphate-guanidinium complex, stabilized by two (bidentate) H-bonds between the guanidinium group and each phosphate, was not more energetically favorable than the diphosphate-amine complex, formed by single (monodentate) H-bonds between the amine group and each phosphate. Instead, the differences between the two cationic

Table 1
List of natural and modified AMPs that selectively induce negative Gaussian curvature in model bacterial cell membranes.

AMP	Sequence
Cryptdin-4	GLLCYCRKGHCKRGERVRGTCGIRFLYCCPRR
(R/K) Cryptdin-4	GLLCYCKKGHCKKGEKVKGTCGIKFLYCCPKK
TAT-Cryptdin-4	YGRKKRRQRRRGLLCYCRKGHCKRGERVRGTCGIRFLYCCPRR
R$_9$-Cryptdin-4	RRRRRRRRRGLLCYCRKGHCKRGERVRGTCGIRFLYCCPRR
HBD-2	DPVTCLKSGAICHPVFCPRRYKQIGTCGLPGTKCCKKP
HBD-3	GIINTLQKYYCRVRGGRCAVLSCLPKEEQIGKCSTRGRKCCRRKK
RTD-1	GFCRCLCRRGVCRCICTR
BTD-7	GFCRCFCRRGVCRCVCTR
Protegrin-1	RGGRLCYCRRRFCVCVGR-NH$_2$
RMAD-4	RRTCRCRFGRCFRRESYSGSCNINGRIFSLCCR
(R/K) RMAD-4	KKTCKCKFGKCFKKESYSGSCNINGKIFSLCCK
(R$_{1,2,5,33}$/K) RMAD-4	KKTCKCRFGRCFRRESYSGSCNINGRIFSLCCK
Magainin-2	GIGKFLHSAKKFGKAFVGEIMNS-NH$_2$
(F$_{12}$/W, N2$_2$/C) Magainin-2 Dimer	(GIGKFLHSAKKWGKAFVGEIMCS-NH$_2$)$_2$
(F$_{12}$/W, N2$_2$/C, R/K) Magainin-2 Dimer	(GIGRFLHSARRWGRAFVGEIMCS-NH$_2$)$_2$
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ-NH$_2$
LL37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES
pLL37	LLGNFFRKSKQKIGKQFKRIVQRIKNFLRNLVPRTQS
Granulysin-peptide 31-50	TRVSRTGRSRWRDVSRNFMR
Granulysin-peptide 31-50 (V$_{44}$/W)	TRVSRTGRSRWRDWSRNFMR

peptides are a result of placing their side chains close to one another. The planar Y-shape of the guanidinium group along with its bidentate H-bonding ability allows it to rigidly arrange the two phosphates along the planar shape of the group. A consequence of this coordination is that by 'face to face' stacking their guanidinium groups nearby Arg side chains can still maintain diphosphate coordination at spacings characteristic of poly-Arg. Conversely, the I-shape of the amine group along with its monodentate H-bonding abilities cannot organize phosphates in such a structured manner, which results in a significant energetic penalty when amine groups are placed at separations characteristic of poly-Lys. In poly-Arg, the stacking arrangement allows each membrane-associated guanidinium to remain H-bonded with two phosphates and thereby "cross-link" the lipid headgroups into a composite with a large headgroup area, effectively producing a series of stacked positive intrinsic curvature amphiphiles along the peptide chain. This organization of bulky lipid headgroups will generate a stress on the membrane from lipid steric crowding interactions, and produce positive curvature strain along the peptide as the membrane bends toward its interior. By generating positive membrane curvature along the peptide from excluded volume interactions in conjunction with negative membrane curvature generation perpendicular to the peptide via electrostatically-based wrapping effects, poly-Arg is able to generate saddle-splay membrane curvature. In support of this model we find that increasing the spacing between side chains in guanidinium homopolymers decreases the amount of saddle-splay curvature they can generate, since increasing the average distance between guanidinium groups relieves the stress from molecular crowding interactions that lead to positive curvature generation [134,154]. Furthermore, a critical number of arginines are needed in poly-Arg before the peptide can generate saddle-splay curvature; tetra-arginine (R$_4$) produced inverted hexagonal phases with negative mean curvature only, and a minimum number of 5 residues are necessary before oligo-arginine can generate cubic phases [134]. Finally, the QM calculations show that it is energetically costly for amine groups in poly-Lys to maintain diphosphate coordinations, consistent with our empirical observations that Lys$_8$ generates inverted hexagonal phases. Since poly-Lys is unable to duplicate the crowding interactions of poly-Arg, it generates pure negative mean curvature from membrane wrapping.

Recently the Cui and Yethiraj groups have investigated the interactions of arginine/lysine homopolymers and lipid membranes with MD studies (see the review by Cui, et al. 2013).

Using a coarse-grained BMW-MARTINI model they simulated the phase behaviors of poly-Arg and poly-Lys with DOPS/DOPE = 20/80 membranes. In exact agreement with our SAXS studies, poly-Arg induced the negative Gaussian curvature-rich Pn3m cubic phase while poly-Lys induced an inverted hexagonal phase with negative mean curvature. In atomistic simulations they observed that the main difference between Arg and Lys is the ability of guanidinium to simultaneously coordinate the glycerols of lipids in addition to lipid headgroups. The results of their MD simulations in conjunction with our QM calculations demonstrate how the multivalent nature of guanidinium allows it to strongly interact with multiple lipid components and thereby generate negative Gaussian curvature. In this emerging picture molecular scale differences in how guanidinium groups and amine groups interact with anionic membrane components like lipid headgroups lead to distinct types of curvature generation by arginine and lysine, which influence their abilities to compromise the barrier function of cell membranes.

5.4. Design rule for saddle-splay curvature generation

The fundamental structural motif of AMPs, cationicity and hydrophobicity, can be recast in terms of membrane curvature generation. Since a general mechanism of action for AMPs is membrane disruption, the topological requirement to generate saddle-splay curvature constrains their arginine, lysine, and hydrophobic content. There will be a compositional trade-off between the relative amounts of arginine and lysine plus hydrophobicity used in an AMP. Since arginine can generate saddle-splay curvature (both positive and negative curvature) while lysine generates negative curvature only and hydrophobicity generates positive curvature only, this implies the curvature generating abilities of arginine can be supplemented using a combination of lysine and hydrophobicity. This saddle-splay curvature selection rule was tested by analyzing the sequences of 1080 cationic AMPs in the antimicrobial peptide database [10,12]. AMP hydrophobicity was determined by using three distinct, widely used hydrophobicity scales: the Kyte-Doolittle scale [155], the Eisenberg Consensus scale [156], and the Wimley-White biological scale [157]. Consistent with the saddle-splay curvature selection rule (see Fig. 2D for plot with Eisenberg Consensus scale), all three scales show a strong positive trend between the average hydrophobicity and lysine content in AMPs, indicating that, on average, more hydrophobic peptides use lysine over arginine [10]. Similar trends are

observed for cell-penetrating peptides (Fig. 2D) [134]. The good correspondence between the trends in AMP cationicity and hydrophobicity with those predicted by the selection rule supports our model that the selective generation of saddle-splay membrane curvature is a general mechanism of AMP activity.

5.5. Implications of design rule

The saddle-splay curvature selection rule is informative about the average amounts of arginine and lysine plus hydrophobicity that are needed to equip a peptide with membrane disruption ability. In this sense the saddle-splay curvature selection rule places constraints on the sequences of AMPs. Since many different versions of cationic, amphipathic molecules satisfy the criteria for membrane activity, the saddle-splay curvature selection rule under-determines AMP sequence. The sequence degeneracy for membrane activity is consistent with observations that, collectively, AMP sequences are labile and highly diverse [1]. It also implies that AMPs have a certain degree of compositional freedom to multiplex membrane activity with other useful functions such as inhibiting essential metabolic processes in bacteria, and modulating the host immune response. As summarized above, a variety of AMPs kill microbes by binding to intracellular targets. Most AMPs are polycationic, so they cannot enter bacterial cells by passive diffusion through membranes. Instead, they reach the cell interior by compromising the barrier function of bacterial membranes via permeabilization processes which require saddle-splay curvature. Therefore, the capability to generate saddle-splay curvature remains necessary condition for non-lytic AMPs which act as metabolic inhibitors by binding intracellular targets, even if it is not a sufficient condition for full activity. We expect non-lytic AMPs will follow the saddle-splay curvature selection rule, just like their lytic counterparts. Conversely, acquiring the ability to bind an internal target is contingent on whether the sequence requirements for the additional function are compatible with the compositional requirements set by the saddle-splay curvature selection rule. For example, the AMPs indolicidin [75], buforin [22], and tachyplesin [74] have all been shown to bind DNA. The high amount of cationic charge necessary for DNA binding is readily compatible with membrane activity and saddle-splay curvature generation. Finally, if membrane activity remains important to the microbicidal mechanisms of action for AMPs that also have immunomodulatory functions, they should retain the ability to generate saddle-splay curvature. In summary, the lack of strong compositional constraints imposed by the saddle-splay curvature selection rule allows membrane-active AMPs to multiplex other useful functions with baseline membrane activity.

The saddle-splay curvature selection rule does not account for the effects of peptide sequence, interactions between amino acids, and secondary structure on AMP membrane activity. Many AMPs can fold into or have built in secondary structures that render them amphipathic, and a number of studies have shown attenuated or abolished activity from sequence mutations, substitutions, and scramblings which destroy the partitioning of cationic and hydrophobic residues [148] (and references therein). Surprisingly, synthetic mimics of antimicrobial peptides (SMAMPs) [35,158–160] devised as random copolymers consisting of cationic and hydrophobic side chains have demonstrated selective antimicrobial activity comparable to AMPs, even though they possess heterogeneous sequences, lengths, and secondary structures. To investigate the homogeneous/heterogeneous dichotomy between AMPs and SMAMPs we examined the abilities of methacrylate-based random copolymers to generate membrane curvature, and used a bioinformatics approach similar to the one used for AMPs to quantify their average charge and hydrophobicity [161]. Three different polymers were studied: inactive, selectively active against bacteria and non-hemolytic, non-specifically active against both bacteria and red blood cells. The ability of the polymers to generate saddle-splay curvature was similar to natural AMPs and it tracked with their activity profiles, as the non-specifically active polymer induced saddle-splay curvature over a wider range of lipid compositions. However, the SMAMPs utilized significantly higher amounts of both average polymer hydrophobicity and cationic charge than AMPs. This is attributed to the randomness of the SMAMPs, as opposed to the organized clustering of cationic and hydrophobic residues in AMPs. Peptides with charge and hydrophobic distributions that are better arranged for maximal counterion release and hydrophobic insertion will require less cationic charge and hydrophobicity than random polymers which have suboptimal arrangements. The 'inefficient' arrangement in SMAMPs can be compensated by increasing the average amount of both curvature-generating ingredients for the random copolymers, while the 'efficient' AMPs can produce similar saddle-splay curvature deformations with fewer charged and hydrophobic residues. These results provide possible explanations for why amphipathicity can be important for natural AMPs, yet random copolymers can act as membrane-active antimicrobials.

6. New contexts: amphipathic helices and membrane curvature

Since the membrane curvature deformations discussed above are the results of non-specific electrostatic and hydrophobic interactions between lipid membranes and cationic, amphipathic molecules, we expect that membrane-associating peptides or proteins which contain domains with similar motifs as AMPs should generate similar types of membrane curvature. Several lines of evidence support this hypothesis. Recent studies have shown that the M2 protein from the influenza A virus can mediate virus budding and scission [162]. M2 is a 97 amino acid protein that self-assembles into a transmembrane homotetrameric proton selective ion channel which is known to play multiple roles during the infectious cycle of the virus [163]. The multi-functional abilities of M2 are encoded by distinct domains which enable one or more functions [163]. Its transmembrane domain is followed by a C-terminal membrane-associated amphipathic α-helix (C-cyto) with similar amino acid composition as AMPs that is capable of inducing budding in vesicles. Reducing the hydrophobicity in the amphipathic helix inhibits membrane scission and virion release [162]. The M2 protein preferentially localizes on the 'necks' of budding virions, the site of maximal negative Gaussian curvature [162]. Using an approach similar to the one for AMPs, we characterized the curvature deformations induced M2 proteins along with variant proteins containing different M2 domains [164]. In general, M2 proteins and variants containing the C-cyto domain induced cubic phases rich in saddle-splay curvature. The structural requirements of scission are more stringent than budding as the neck must constrict to a much smaller size than the virion for 'pinch-off'. Consistent with this restriction we found that M2 proteins are capable of generating saddle-splay curvatures comparable to those on a neck 10 times smaller (under 10 nm) than influenza viruses. Furthermore, the phase behaviors of M2 variants tracked with activity profiles as replacement of the 5 key hydrophobes with alanines generally attenuated saddle-splay curvature generation. While the C-cyto helix is necessary and sufficient to generate saddle-splay curvature, the full length M2 protein and a M2 construct consisting of the transmembrane domain plus the C-cyto helix induced cubic phases over a much wider range of lipid compositions. This suggests that while the C-cyto helix is the primarily responsible for inducing membrane curvature, for proteins like M2 other domains contribute. The M2 protein is a good example of how a curvature generating module

can be integrated into a protein to equip it with multi-functional abilities.

Cationic, amphipathic domains have also been implicated in the mechanisms of action of curvature-generating proteins routinely utilized by eukaryotic cells to produce small vesicle transport carriers for intracellular and extracellular exchange of materials. Emerging evidence suggests that amphipathic helical domains in membrane-cleaving and membrane-sculpting proteins can promote budding and, in certain cases, drive membrane scission [161]. For example, the epsin-1 protein promotes membrane invagination and has been implicated in clathrin-mediated endocytosis. This protein contains a highly conserved lipid binding N-terminal ENTH domain. Upon binding PtdIns(4,5)P$_2$, an amphipathic α-helix is formed in this domain which inserts into the membrane and generates curvature [165]. Both epsin-1 and the ENTH domain can remodel liposomes into tubules [125,165], and vesicularization of liposomes by epsin has been attributed to its ability to destabilize necks and drive membrane scission [166]. Furthermore, certain BAR domains, such as those found in amphiphysins involved in clathrin-mediated endocytosis and endophilins involved in synaptic vesicle endocytosis, contain N-terminal amphipathic helices that are unstructured until they interact with membranes. Upon membrane binding the amphipathic helices in these N-BAR domains fold and insert into the membrane [167–170] and can thereby generate the high membrane curvature necessary for vesicle budding [125,169]. While amphipathic helix insertion can generate high curvatures necessary to constrict membrane necks and drive scission, the rigid, crescent-shaped scaffold formed by dimerized BAR domains acts to stabilize membrane necks by molding them along the scaffold [161]. In this way, the BAR domains in amphiphysin, endophilin, and GRAF proteins acts as antagonists of membrane scission and, instead promote stable neck formation. Interestingly, since proteins like amphiphysins and endophilins contain N-BAR domains consisting of the BAR scaffold and membrane-inserting helices, their ability to promote neck formation or membrane scission depends on specific properties like their scaffold rigidity and the number of amphipathic helices in the protein [161]. Taken together, these results indicate that cells employ proteins equipped with membrane-inserting helices with a similar cationic, amphipathic motif as AMPs to promote the generation of saddle-splay curvature on membrane necks in a controlled manner and thereby tightly regulate intercellular and intracellular transport.

7. Conclusions

Within a more general framework we view AMPs as versatile tools which, collectively, act as direct microbicides by multiplexing interactions with microbial cell membranes and other intracellular targets, and can also act as modulators of the host immune response. Membrane activity is generally conferred by their fundamental structural motif of cationicity (mostly arginine & lysine) and hydrophobicity which allows AMPs to generate strong non-specific electrostatic and hydrophobic interactions with cell membranes. The primary way AMPs compromise the barrier function of membranes is by inducing curvature deformations. Among the possible membrane deformation types we expect generation of saddle-splay membrane curvature to be especially disruptive since it is topologically necessary for a variety of known membrane destabilizing processes included budding, blebbing, vesicularization, as well as pore formation. Saddle-splay membrane curvature generation by an amphipathic AMP is derived from the curvature generating tendencies of its constituent cationic and hydrophobic regions. Induction of negative curvature depends on strong electrostatic interactions between cationic portions of the AMP with anionic membrane components like lipid headgroups. Generation of positive curvature is the result of partial peptide insertion due to the hydrophobic effect and steric crowding interactions from guanidinium-coordinated stacking of lipid headgroups. Since arginine can generate both positive and negative curvature while lysine and hydrophobicity can generate only negative curvature and positive curvature, respectively, a decrease in AMP arginine content can be offset by a corresponding increase in the amount of lysine and hydrophobicity. This is the essence of the saddle-splay curvature selection rule, and it is consistent with natural AMP sequences.

Membrane curvature results from the interplay between an AMP and the membrane. Therefore AMP curvature generation depends on the physical chemistry of both the peptide and membrane. Since bacterial cell membranes contain greater amounts of both anionic lipids (PG and CL) and negative intrinsic curvature lipids (PE and CL) they are more susceptible to membrane destabilizing curvature deformations. Higher membrane charge density implies stronger association occurs between poly-cationic AMPs and bacterial membranes which in turn leads to larger curvature deformations. Greater amounts of negative intrinsic curvature lipids promote membrane destabilizing processes such as pore formation through modification of the bilayer Gaussian modulus. Therefore AMPs target general differences in the physical chemistry between bacterial membranes and eukaryotic membranes.

The saddle-splay curvature selection rule places constraints only on the relative amounts of arginine and lysine plus hydrophobicity needed to make a functional AMP. Therefore, AMPs have significant freedom to adopt other useful characteristics and functions to fight invasive microbes. Since this design rule is based upon both a mechanism of activity and the existing motifs of natural AMPs, we believe it will assist the development of synthetic antimicrobial agents by providing guidelines for their construction. Furthermore since membrane activity does not exhaust the sequence requirements of AMPs we envision other functions might be 'programmed' into synthetic antimicrobials to equip them with multiple mechanisms of action.

Acknowledgements

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