Review

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Bacterial membrane lipids in the action of antimicrobial agents‡

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Many antimicrobial agents that target bacteria are cationic and can interact with the anionic lipid components that are exposed on the bacterial membrane. Bacteria vary widely in the nature of the major lipid components that are in the cell membrane. Those bacteria with both anionic as well as zwitterionic or neutral lipids can be induced to form domains in the presence of antimicrobial peptides possessing several cationic charges. This segregation of anionic and zwitterionic lipids into domains can result in the arrest of cell growth or in cell death. Such agents are generally more toxic to Gram-negative bacteria, than to Gram-positive ones. These findings emphasize the importance of the lipid composition of bacterial membranes in determining the susceptibility of the organism to the action of certain antimicrobial agents. **Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.**

Keywords: antimicrobial peptide; membrane domain; bacterial membrane

Introduction

There has been considerable effort in recent years to develop alternative therapies to traditional antibiotics for the treatment of infectious diseases. The need for new drugs is made more acute by the increased emergence of antibiotic-resistant strains of bacteria.

One group of agents that has attracted interest are cationic antimicrobial peptides. These comprise both natural defence peptides as well as designed peptides and represent a diverse group of compounds in several respects. Despite this diversity, a common feature of most of these peptides is that they are polycationic. The potency of these peptides is not very high, generally in the micromolar range. This relatively low potency is in accord with the fact that they are not designed to interact with a specific target. As a consequence, there is no single mechanism of action. There are at least three recognized major types of antimicrobial action:

1. *Interaction with the bacterial membrane*. This can include breaching the membrane barrier or affecting specific membrane functions such as inhibiting a membrane-bound enzyme.
2. *Interaction with an intracellular target*. Several antimicrobial peptides are known to interact with DNA, leading to a cytotoxic action [1–3]. This is not surprising as these peptides carry multiple positive charges and the nucleic acid is polyanionic.
3. The peptides can stimulate the innate immunity system by promoting the release of natural defence peptides as well as stimulating phagocytic cells.

Although the mechanism of action of antimicrobial peptides is diverse, complex and is not highly specific, some of these peptides can exhibit broad range toxicity, i.e. they are effective against most bacterial species, while other peptides can have a more limited range, acting only against defined bacterial species.

With regard to antimicrobial agents that target the bacterial cell membranes, there are several mechanisms that have been proposed. The mechanism by which many antimicrobial agents induce membrane damage has been suggested to be by formation of pores [4] lined by both lipids and peptides [5] or by a more general 'carpet mechanism' [6], but other specific mechanisms have also been suggested [7–11]. In addition to having a mechanism to damage bacterial membranes, in order to be effective therapeutic agents these peptides must show selectivity for bacterial membranes and not damage the membranes of eukaryotic cells. In general, there is not a single mechanism by which any of these agents function, but rather a combination of mechanisms.

In this review we will focus on one of the reasons for the difference in activity of certain antimicrobial agents against different bacterial species. This will also introduce a novel mechanism of toxicity. We will discuss how the clustering of anionic lipids by cationic antimicrobial agents will lead to alterations in the properties of cell membranes and describe why this effect is more important for some bacterial species than for others.

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Abbreviations used: *AFM, atomic force microscopy; CL, cardiolipin; DiSC3, 3,3′, diethylthiodicarbocyanine iodide; DSC, differential scanning calorimetry; LPS, lipopolysaccharide; MAS/NMR, magic angle spinning NMR; MIC, minimal inhibitory concentration; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; POPE, 1-palmitoyl-2-oleoylphosphatidylethanolamine; TMCL, tetramyristoylcardiolipin; TOCL, tetraoleoylcariolipin.*

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Biography

Raquel F. Epand received her Ph.D. in the Chemistry Department at Cornell University. She is currently a researcher in the Department of Biochemistry and Biomedical Sciences at McMaster University and has published over 150 papers in peer reviewed journals. Her research interests are in the area of peptide and protein interactions with model and biological membranes.

Biography

Richard M. Epand received his A.B. from the Johns Hopkins University and his Ph.D. in Biochemistry from Columbia University. He then gained research experience in the laboratories of Harold Scheraga at Cornell University and with Professor Luis Leloir at the Instituto de Investigaciones Bioquimicas in Buenos Aires. Dr. Epand took up his first faculty position at the University of Guelph in Ontario in the Department of Chemistry as Assistant Professor. From there he joined McMaster University where he spent most of his professional career in the Department of Biochemistry and Biomedical Sciences.

Dr. Epand received the Avanti Award in Lipids from the Biophysical Society and has been elected a Fellow of that society. He has also been granted a Senior Investigator Award from the Canadian Institutes of Health Research. Dr. Epand is currently the Executive Editor of the Biomembranes section of *Biochimica Biophysica Acta*. He has over 450 manuscripts published in peer reviewed journals as well as having edited several books. The research interests of Dr. Epand's laboratory have focused on the properties of membranes and on the interaction between membrane proteins and the surrounding lipids with a goal of understanding how these interactions modulate biological function. Among his current research activities are studies of viral fusion, domains in membranes, proteins that bridge mitochondrial membranes, membrane properties of diacylglycerol kinases and the mechanism of antimicrobial agents.

Experimental Findings Demonstrating the Clustering of Anionic Lipids by Certain Antimicrobial Agents

A simple test to determine if a cationic antimicrobial agent will cluster anionic lipids is to study lipid phase transition behaviour using DSC. A mixture of an anionic and a zwitterionic lipid is chosen that exhibits miscibility. The lipids used in this work are defined in Table 1 together with their charge at neutral pH and phase transition properties. Structures of the peptides used are given in Table 2. The DSC curve for such a mixture will show a single broad phase transition that will be largely reversible in heating and cooling scans. If a cationic agent preferentially interacts with the anionic lipid component, then the mixture will exhibit a DSC curve in which, at least in part, the anionic and zwitterionic components will give separate transitions (Figure 1A). The two transitions will not be equivalent to a combination of the transitions of each of the separate components, as the segregation of the two lipids would not be complete. Furthermore, the transition properties of the anionic lipid component will be more perturbed as a consequence of interacting with the cationic agent. In contrast, the zwitterionic lipid component will be enriched in a region of the membrane and hence have phase transition properties closer to those of the pure zwitterionic lipid component. This in general will result in the phase transition of this domain being sharper (i.e. having a higher cooperativity) and having a temperature closer to, but not exceeding that of the pure zwitterionic component. These changes should be observed on cooling and heating DSC scans, and be reversible. The phenomenon should be present regardless of which of the two lipids is the higher melting component (Figure 1A and B). Note that when the major lipid component POPE is the higher melting component (Figure 1A), addition of the peptide shifts the phase transition to higher temperatures as POPE becomes separated from the mixture. In contrast, when the zwitterionic POPE is the lower melting component (Figure 1B), addition of the peptide results in a shift of the phase transition to lower melting temperatures. Also, Figure 1B shows that the properties of the lipid mixture in the presence of peptide are largely independent of kinetic factors, particularly with regard to the cooling scans, that are completely scan independent. An additional criterion is that the cationic agent should not greatly affect the phase transition behaviour of the pure form of the zwitterionic lipid component. This method of assessing anionic lipid clustering requires that the sample be taken into the gel state, so that the phase transition can be observed. Nevertheless, by approaching the phase transition temperature from higher to lower temperatures should largely reflect the organization of the lipid in the liquid crystalline phase. It is advantageous, however, to test the conclusions about lipid segregation using other independent criteria. Several such criteria have been used for these systems.

NMR, using the solid-state method of MAS/NMR, is a powerful method for studying biological membranes, allowing for high resolution spectra of high molecular weight slowly tumbling membrane samples that would give broad lines in liquid state NMR. One of the convenient isotopes for membrane studies is ^{31}P. The isotope has close to 100% natural abundance and for most phospholipids there is only one phosphate per lipid molecule, avoiding the necessity of resonance assignments. In addition, the NMR signal from ^{31}P is detected with high sensitivity, comparable to that of protons. The main application of ^{31}P NMR has been to assign lipid phases on the basis of the shape and width of the

Table 1. Properties of selected lipids at neutral pH

Abbreviation	Acyl chains	Overall charge	Phase transition[a]
POPE	16 : 0/18 : 1	~0	25
TOCL	$(18:1)_4$	−2[b]	>0
TMCL	$(14:0)_4$	−2[b]	~40[c]

[a] Gel to liquid crystalline phase transition temperature (°C).
[b] Charge at neutral pH is between 1 and 2 [12].
[c] TMCL exhibits gel state polymorphism [13].

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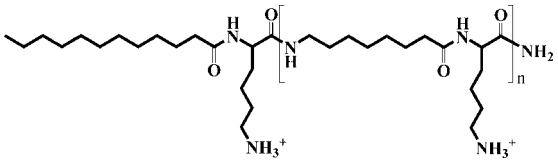
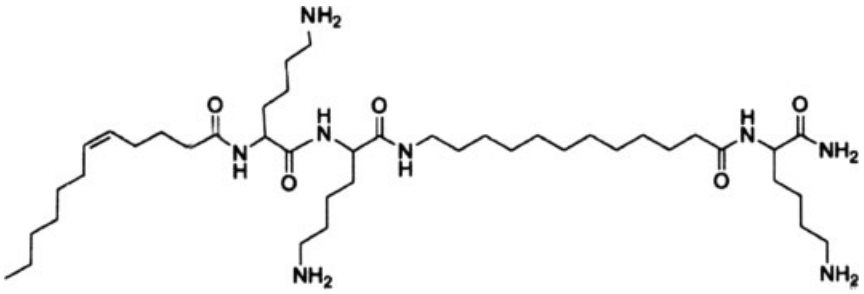
Table 2. Structure of antimicrobial peptides referred to

Agent	Structure
MSI-78	GIGKFLKKAKKFGKAFVKILKK-NH_2
MSI-103	KIAGKIAKIAGKIAKIAGKIA-NH_2
MSI-469	Octyl-KIAGKIAKIAGKIAKIAGKIA-NH_2
MSI-843	Octyl-OOLLOOLOOL-NH_2
MSI-1254	Octyl-XXLLXXLXXL-NH_2
KR-12	KRIVQRIKDFLR-NH_2
GF-17	GFKRIVQRIKDFLRNLV-NH_2
GF-17 D3	GFKRiVQRiKDFIRNLV-NH_2
PR-9	PFWRIRIRR-NH_2
RR-9	RRPFWIIRR-NH_2
PI-9	PRFRWRIRI-NH_2
Cateslytin	RSMRLSFRARGYGFR
$C_{12}K\text{-}7\alpha_8$	 n = 7
$C_{12(\omega7)}K\text{-}\beta_{12}$	

O, ornithine; X, 2,4 diaminobutyric acid (Dab) (lower case letters correspond to D-amino acids).

Table 3. ^{31}P MAS/NMR POPE : TOCL (3 : 1) with the OAK $C_{12}K\text{-}7\alpha_8$ Linewidth (Hz)

	TOCL peak			POPE peak		
Temperature (°C)	TOCL	POPE/TOCL	POPE/TOCL + 10 mol% OAK	POPE	POPE/TOCL	POPE/TOCL + 10 mol% OAK
20	47	36	47	138[a]	34	38
25	–	35	53	42	34	41
30	41	34	52	42	35	44

[a] The linewidth is particularly large because this is the only case that corresponds to gel state lipid.

static NMR powder pattern, although there have also been other applications. We have recently employed ^{31}P MAS/NMR for the study of domain formation in membranes induced by cationic antimicrobial agents [14,15]. The two principal features of the ^{31}P MAS/NMR spectra used to assess anionic lipid clustering are the chemical shift and the linewidth.

The chemical shift or position of the peak in the NMR spectrum is slightly different for different phospholipids with differing chemical structures. In mixtures of two miscible lipids the change in environment of the phosphate group of each of the lipids will result in a change of chemical shift compared with that of the pure lipid components. The signals from each of the two lipids will become less separated but will generally still be resolvable, even if the lipids are completely mixed. Upon addition of a cationic antimicrobial agent to a lipid mixture composed of a zwitterionic and an anionic lipid, the resonance peak for the zwitterionic component will shift toward that of the pure form, whereas that of the anionic lipid will shift further from that of the position of the pure anionic component. These opposite shifts for the two lipids can be understood as a consequence of the zwitterionic lipid being enriched in a domain that is depleted of anionic lipid and therefore acquiring a chemical shift close to that of the pure zwitterionic component. At the same time, the anionic lipid is also segregating into a domain in which it is enriched compared with the initial mixture. However, unlike the zwitterionic lipid, the anionic component is interacting with the positive charges of the added agent and hence its chemical shift will be further removed from that of a pure anionic lipid.

The linewidth of each of the two overlapping peaks can be determined using computer programs for curve deconvolution.

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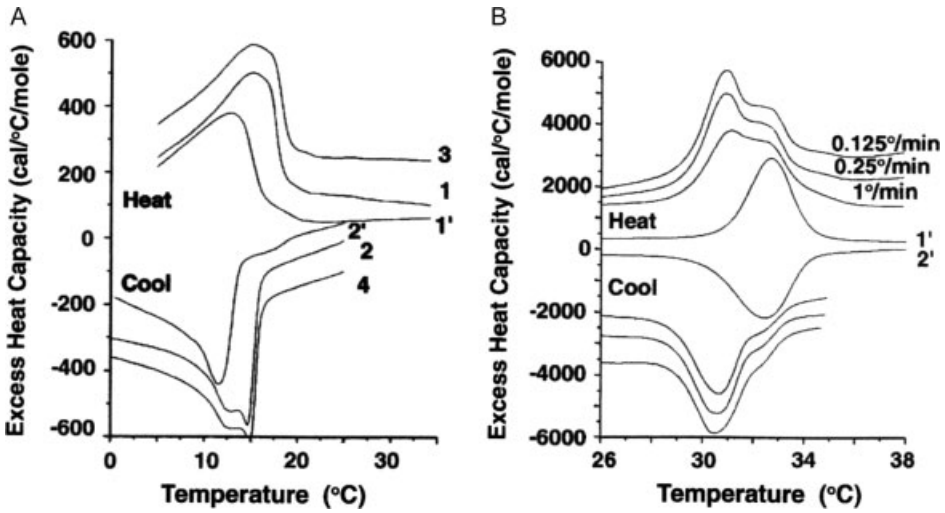

Figure 1. DSC of peptide RR9 added to hydrate a film of (A) POPE : TOCL 75 : 25 in which the highest melting component is the zwitterionic lipid POPE. Scan rate of 1°/min. Curves 1′ and 2′ are the heating and subsequent cooling scans, respectively, of the pure lipid mixture in the absence of peptide. Curves 1 and 2 are sequential heating and cooling scans, respectively, of the lipid mixture in the presence of the peptide RR9; this heating and cooling cycle was repeated again and shown in curves 3 and 4. (B) POPE : TMCL 60 : 40 in which the highest melting component is the anionic lipid TMCL. Curves 1′ and 2′ are the heating and cooling scans, respectively of the pure lipid mixture in the absence of peptide. Other scans are in the presence of peptide and measured at different scan rates, as indicated. Lipid to peptide ratio was 20. Phase separation occurs regardless of whether the anionic component is the higher or the lower melting component of the mixture.

The linewidth is influenced by the motional properties of the lipid. For example, gel state lipid has broader lines than lipid in the liquid crystalline phase. Similarly, anionic lipid clustered into a domain in which the motion of the headgroup is restricted because of interaction with cationic agents will also have broader lines. Such a phenomenon has been observed (Table 3) [14,15].

^{2}H NMR has also been used to evaluate the extent of lipid phase segregation [16]. ^{2}H NMR has often been applied in the study of membrane properties to evaluate the order parameter that depends on the extent of molecular motion. An advantage of ^{2}H NMR is that it requires labelling of the lipid with ^{2}H and hence can selectively introduce this NMR-active probe to only a portion of the sample. In addition, compared with fluorescence or spin-labelled probes, this probe is rather innocuous; being only a different isotope of hydrogen it will not perturb the system. Fully deuterated fatty acids are commercially available and relatively inexpensive. They can be used to synthesize specifically deuterated lipids. The NMR spectrum from such lipids is characterized by each position in the acyl chain giving rise to a pair of peaks, each pair being separated by nuclear–quadrupole interactions. The extent of this peak splitting i.e., the distance between the pair of peaks in the NMR spectrum can be used to calculate the order parameter. The spectrum has to be deconvoluted to calculate the individual order parameters for each position on a perdeuterated acyl chain [17]. These experiments have contributed to our understanding of the gradient of motion in membrane bilayers with the methyl terminus of the acyl chain being the most mobile and therefore having the lowest order parameter. In a mixture of anionic and zwitterionic lipids, each of the lipid components can be separately monitored, according to which of the components is labelled with ^{2}H. The method has been applied to the cationic antimicrobial agent, cateslytin [16]. It was found that there were two populations of the zwitterionic lipid that were in slow exchange on the NMR time scale. One population had order parameters similar to those of the anionic lipid component, while another had order parameters similar to the pure zwitterionic lipid. It was suggested that cateslytin promoted the formation of domains, one more ordered and enriched with anionic lipids, while the other domain contained mostly the zwitterionic lipid [16].

A related strategy, also using deuterium-labelled lipids, employs infrared spectroscopy to separately monitor protonated and deuterated lipid components. In a recent example, the temperature dependence of the antisymmetric CH_2 stretching band was used to assess the phase transition characteristics of different lipids in a mixture to provide evidence for lipid phase separation induced by small cationic antimicrobial peptides [18].

Direct imaging of domains can also provide strong evidence for the formation of domains. To do this, however, the domains must be sufficient large so that they can be resolved by the methods employed. There has been much discussion about the limitation of light microscopy in this respect, but methods are being developed to detect domains smaller than the wavelength of light even with this method. A more general solution is to use electron microscopy. It has been suggested that membrane domain formation can be detected by freeze fracture electron microscopy [19–21]. This method has also been applied successfully to show similar morphological rearrangements in membranes of a mixture of anionic and zwitterionic lipids when small cationic antimicrobial peptides are added [22].

Another high-resolution imaging method is AFM. This method can provide information about the topology of a membrane surface. The method is particularly sensitive to height differences in different parts of the specimen. More ordered domains will be thicker and can be resolved over a background of a second more disordered domain. The method is particularly powerful when combined with spectroscopic tools, so that the topological information can be compared with information about the chemical or physical properties of the domains. Such a study has been carried out using the peptide PFWRIRIRR-amide to induce clustering of anionic lipids [23]. The time dependence of the formation of domains and the distribution of sizes of these domains was demonstrated by AFM. Measuring

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Table 4. Lipid composition and minimal inhibitory concentrations (MICs) of the OAK $C_{12}K$-7α_8 against various species of bacteria

Species	% CL	% PG	% PE	MIC (μM)
Gram-negative bacteria (with PE)				
Escherichia coli	5	15	80	1.6–3.1
Enterobacter cloacae	3	21	74	1.6
Yersinia kristensenii	20	20	60	1.6
Proteus mirabilis	5	10	80	6.2
Klebsiella pneumoniae	6	5	82	3.1
Pseudomonas aeruginosa	11	21	60	6.2
Gram-positive bacteria (no PE)				
Staphylococcus aureus	42	58	0	50
Streptococcus pneumonia	50	50	0	50
Gram-positive bacteria (with PE)				
Bacillus cereus	17	40	43	12
Bacillus polymyxa	8	3	60	6.2

MICs are significantly lower when PE is present in the cytoplasmic membrane. CL and PG are anionic lipids, while PE is zwitterionic. Each lipid is presented as the % of total lipids in the bacteria.

the binding of the CL-specific probe, 10-nonyl-acridine orange, specified the location of the anionic lipid, CL. In addition to these differences in bilayer thickness and CL content in different regions of the membrane, application of polarized total internal reflection fluorescence microscopy using the fluorescence probes, 1,1′-dieicosanyl-3,3,3′,3′-tetramethylindocarbocyanine as well as 10-nonyl-acridine orange, differences in the order of different membrane domains were shown [23].

Requirements for Anionic Lipid Clustering by Antimicrobial Agents

The particular interest in the ability of cationic antimicrobial agents to cluster anionic lipids in model membranes comes from the observation that this feature can generally predict the bacterial species specificity of the antimicrobial action.

It is generally appreciated that there are major difference in the membrane organization and composition of membranes from Gram-negative and Gram-positive bacteria. What is sometimes less appreciated is that there are major differences in the phospholipid composition in the cytoplasmic membranes of different bacterial species and some of these differences determine the response of Gram-positive and Gram-negative bacteria to antimicrobial agents. For example, PE is generally more abundant in the membranes of Gram-negative than Gram-positive bacteria [24]. However, there are some exceptions. For example, the Gram-negative bacterial species *Caulobacter crescentus* has little PE content, whereas many species of Gram-positive *Bacillus or Clostridium* have some PE content (Table 4). PE is particularly relevant to the mechanism of anionic lipid clustering. PE is the most prevalent zwitterionic lipid present in bacterial membranes. It was found that bacteriostatic action resulting from anionic lipid clustering correlates with the segregation of anionic lipids from zwitterionic or uncharged lipids (Table 4). Hence, in general agents acting by the lipid clustering mechanism will be more toxic to Gram-negative bacteria than to Gram-positive bacteria. However, the correlation is to the lipid composition of the membrane, not to whether the bacteria are Gram-positive or Gram-negative. Hence, the exceptions of Gram-positive bacteria with high PE will also be sensitive to the lipid clustering mechanism and this provides a good test of the importance of the phenomenon to a particular case (Table 4).

PE is not the only bacterial lipid present that is not anionic. Several bacterial species have a significant content of other zwitterionic or uncharged lipids. Gram-positive bacteria have a thick layer of lipoteichoic acid that makes up the cell wall. The membrane anchor of the lipoteichoic acid is glucosyl diacylglycerol but by being incorporated into the lipoteichoic acid results in glucosyl diacylglycerol not behaving as an uncharged lipid component of the bacterial membrane. However, with the species *S. pyogenes*, a high content of its membrane is glucosyl diacylglycerols, a fraction of which is not incorporated into lipoteichoic acid and acts as an uncharged lipid, making this species susceptible to agents that cluster anionic lipids in the presence of zwitterionic or uncharged lipids [22].

In addition to lipids common to most bacterial species, some bacteria have other lipids including cationic lysyl-PG, aminoacyl-PG, most of which are zwitterionic, as well as zwitterionic lysyl-CL. The most common of these lipid species is lysyl-PG which is a cationic lipid. At higher concentrations such cationic lipids will prevent the binding of cationic antimicrobial agents to the membrane and hence make the bacteria more resistant. This is a known mechanism of acquired resistance of *Staphylococcus aureus* [25,26]. However, in non-resistant strains of *S. aureus* most of the lysyl-PG is found on the cytoplasmic surface of the plasma membrane. Resistance to antimicrobial agents occurs in those strains of *S. aureus* in which the lipid is translocated to the cell exterior by a mechanism facilitated by a membrane protein [27,28]. Although the membrane sidedness of lysyl-PG in *S. aureus* has been determined, in general, there is little information about the sidedness of membrane lipids in bacteria.

An additional consideration is that the cationic antimicrobial agent must access the cytoplasmic membrane of the bacteria in order to promote lipid clustering that will be detrimental to the bacteria. The outer membrane of Gram-negative bacteria can act as a major barrier to reach the cytoplasmic membrane. In the case of the oligo-acyl-lysine (OAK), $C_{12}K$-7α_8, the potency against several species of Gram-negative bacteria was high [15], indicating these agents were permeable to the outer membrane. In contrast, permeability across the outer membrane impacted the relative toxicity of some cationic amphipathic helical antimicrobial peptides against *E. coli*. In some cases, outer membrane permeability was directly tested using *E. coli* ML-35p [29], a strain that was engineered specifically to simultaneously probe penetration across the inner and outer membranes of Gram-negative bacteria [30]. Some antimicrobial peptides, such as the three nonapeptides we had studied, are less toxic than expected against certain strains of Gram-negative bacteria [22] although these peptides are capable of clustering anionic lipids. However, these peptides are very potent against Gram-positive bacteria that have a high concentration of zwitterionic or neutral lipids in their membranes [22]. In accord with a lipid clustering mechanism we find a high ratio of minimal inhibitory concentration values for certain agents against bacteria largely devoid of neutral or zwitterionic lipids *versus* the minimal inhibitory concentration values for the same agents against bacteria with a high content of neutral or zwitterionic lipids in their cytoplasmic membrane (Table 5).

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Table 5. Specificity of antimicrobial agents and relationship to cationic charge

Agent	Charge[a]	Charge per residue	MIC *S. aureus*/MIC *E. coli*
MSI-78	10	0.45	2
MSI-103	7	0.33	15.6
MSI-469	6	0.29	4
MSI-843	6	0.6	4
MSI-1254	6	0.6	1
KR-12	6	0.5	>4
GF-17	6	0.35	0.5
GF-17 D3	6	0.35	>8
PR-9	5	0.56	32[c]
RR-9	5	0.56	64[c]
PI-9	5	0.56	8[c]
Cateslytin	5	0.33	>3
C_{12}K-7α_8	8	8[b]	16
$C_{12(\omega7)}$K-β12	3	3[b]	<0.1

All peptides which function by phase separation exhibit a high number of cationic charges. The ratio of MICs in bacteria where PE is absent *versus* when PE is present is higher than 1, except when there is no phase separation or the peptide is retained in the lipopolysaccharide (LPS) layer.

[a] Estimated for pH 7 using +1/2 for His.

[b] Not a peptide, but an OAK.

[c] Ratio of Gram-positive bacteria without PE (*S. aureus*) to Gram-positive bacteria with PE (*Bacillus megaterium*). See text.

Importance of Anionic Lipid Clustering in the Antimicrobial Action of Cationic Peptides

Current knowledge about the mechanism of action of antimicrobial agents suggests that most of these agents act by a combination of mechanisms, not all of which necessarily even involve the membrane. The strongest argument suggesting that anionic lipid clustering is a major contributing factor to the toxicity of some agents comes from their greater toxicity against bacteria that have both anionic as well as zwitterionic lipids, compared with their lower toxicity against bacteria whose membranes are composed largely of anionic lipids [15,31–33]. Often, this will mean that the toxicity will be more specific to Gram-negative bacteria, as these generally have high concentrations of the zwitterionic lipid, phosphatidyethanolamine in their membranes. If in addition one can show that Gram-positive bacteria with high concentrations of zwitterionic or uncharged lipids are also susceptible (Table 4); this will demonstrate that the correlation to biological potency is with the lipid composition of the membrane, rather as a result of other differences between Gram-positive and Gram-negative bacteria. This biological property combined with a demonstration in model systems that the agent can rearrange lipids to form clusters of anionic lipids, provides good evidence for the important role of lipid clustering in bacterial toxicity.

There are exceptions in which lipid clustering can be demonstrated in model systems, but there is no clear correlation of the antimicrobial potency with the lipid composition of the bacterial membrane. One additional feature, referred to above, is the requirement that the agent can pass across the outer membrane of Gram-negative bacteria. There are also cases in which the agent can pass the outer bacterial membrane and can also cluster anionic lipids in model systems, but does not exhibit toxicity specific to certain bacterial species. This does not necessarily mean that the agent is not clustering anionic lipids in the bacteria, but rather that this is not the principal mechanism of toxicity.

An interesting comparison is between the peptide GF-17, a fragment of LL-37 with the sequence FKRIVQRIKDFLRNLV-amide and an analog of this peptide, GF-17D3, in which the three residues in lower case that are underlined are D-amino acids: FKRiVQRiKDFlRNLV-amide. GF-17 forms an amphipathic helix in micelles of SDS or dioctanoyl PG, while GF-17D3 is much less structured, even in a membrane environment [34]. As a result, GF-17 is much more lytic to membranes as shown by its promotion of dye release from liposomes [14]. As a consequence, GF-17D3 exhibits bacterial species specificity as would be expected for an agent that acts primarily through anionic lipid clustering. However, GF-17, that appears to cluster lipids equally as well as GF-17D3, is toxic to both typical Gram-positive and Gram-negative bacteria because it can promote pore formation and therefore has an additional mechanism for killing bacteria whose cytoplasmic membranes are composed largely of anionic lipids.

Mechanism by Which Anionic Lipid Clustering Leads to Bacterial Toxicity

There is a clear phenomenological link between anionic lipid clustering and the bacterial species specificity of a number of antimicrobial agents. However, it is not clear how lipid clustering results in bacterial toxicity. The clustering of anionic lipids to a region of the bacterial membrane would concentrate negative charge in a domain to which cationic peptides would congregate, possibly leading to the formation of a pore. However, antimicrobial agents that cause clustering in general do not cause much leakage of dyes from membrane mimetic liposomes of bacterial cytoplasmic membranes and they are not toxic to *S. aureus*. However, they may breach the membrane barrier to a limited extent, causing slow leakage of contents and/or depolarization of the membrane. Evidence for this comes from studies of C_{12}K-7α_8, one of the best examples of an antimicrobial agent causing clustering. It has been shown with C_{12}K-7α_8 that in the presence of EDTA, added to allow the dye $DiSC_3(5)$ to reach the inner membrane, the cytoplasmic membrane of *E. coli* is depolarized [35] and there is even some slow influx across the cytoplasmic membrane of *E. coli* ML-35 of small organic molecules [36]. This breach of the membrane barrier may be sufficient to cause the bactericidal effect.

In addition to promoting a high concentration of the cationic antimicrobial agent on the membrane's anionic surface, the domain of anionic lipids would be surrounded by an interface with the rest of the membrane that would be under line tension and be less stable. Of course, it is likely that there are always domains in bacterial membranes [37] that have phase boundary defects. However, those that would form in the presence of lipid clustering antimicrobial agents would appear suddenly [23] and the bacteria would not have time to compensate for this rearrangement.

In addition, there can be damage to the bacteria as a result of the redistribution of lipids in the membrane. This may cause the disruption of existing, functional natural domains in the membrane or could decrease the available anionic lipid that may be required for the function of specific cytoplasmic membrane proteins.

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Conclusions

Anionic lipid clustering is a feature of cationic antimicrobial agents that have a sufficiently high density of positive charge and possess a degree of conformational flexibility [33]. Among Gram-positive bacteria, such agents will be most toxic to species that contain significant quantities of neutral or zwitterionic lipids in the membrane. Generally, these agents will be more toxic to Gram-negative than Gram-positive bacteria, as most species of Gram-negative bacteria have high concentrations of the zwitterionic lipid PE, provided that these agents are capable of penetrating the outer membrane. As infections with Gram-negative bacteria are becoming an increasingly important clinical problem [38,39], focus on lipid clustering can specifically contribute to developing new strategies to counter this serious clinical problem.

Acknowledgements

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