Design of Host Defence Peptides for Antimicrobial and Immunity Enhancing Activities

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Abstract: Host defense peptides are a vital component of the innate immune systems of humans, other mammals, amphibians, and arthropods. The related cationic antimicrobial peptides are also produced by many species of bacteria and function as part of the antimicrobial arsenal to help the producing organism reduce competition for resources from sensitive species. The antimicrobial activities of many of these peptides have been extensively characterized and the structural requirements for these activities are also becoming increasingly clear. In addition to their known antimicrobial role, many host defense peptides are also involved in a plethora of immune functions in the host. In this review, we examine the role of structure in determining antimicrobial activity of certain prototypical cationic peptides and ways that bacteria have evolved to usurp these activities. We also review recent literature on what structural components are related to these immunomodulatory effects. It must be stressed however that these studies, and the area of peptide research, are still in their infancy.

INTRODUCTION

In the past 15 years, research on cationic antimicrobial peptides has exploded into a promising new field of study and a potential source of new therapeutics. Cationic antimicrobial peptides are short (usually less than 50 amino acids) proteins possessing excess positively charged lysine and/or arginine or histidine residues (the latter being charged at acidic pH only) and a large percentage (around 50% or so) of hydrophobic amino acids. They are produced by virtually every species of life examined, and a single species may produce dozens of chemically distinct types of peptides. Although structurally diverse, upon interaction with bacterial membranes cationic peptides generally adopt a strongly amphipathic or amphiphilic three-dimensional structure. This ability to adopt an amphipathic structure is what allows cationic peptides to insert into biological membranes, and can explain many of their biological properties. However, despite the vast amount of research that has been carried out on these compounds, the sheer diversity within the class of compounds makes it difficult to draw general conclusions about the precise mechanisms of killing.

This review will focus primarily on recent advances in the understanding of how the structure of various cationic peptides affects their antimicrobial activities against Gram-negative and Gram-positive bacteria. Thus, although cationic peptides are extremely diverse from the perspective of secondary structure, some general principles for their design can be applied. We also review the mechanisms by which a number of these bacteria (particularly Gram-negative bacteria) have evolved resistance to these compounds, and what this may mean in terms of peptide design. Although certain cationic peptides have been shown to have potent activity against fungi and enveloped viruses, these activities are beyond the scope of the current review.

In addition to their known antimicrobial role, mammalian cationic peptides are also an extremely important player in mediating innate immune responses to infection. Very little is currently known about how structural aspects of these molecules lead to their effects on the innate immune system, but some intriguing observations indicate that we are beginning to understand how to address these types of problems. Although the great diversity of structures and functions of the many cationic peptides suggest that this class of molecules will be extremely valuable as new therapeutics, this development has been hampered by a number of technological problems that need to be overcome before they can achieve their full commercial potential. A number of these issues will be described, and approaches for circumventing them will be reviewed.

DISCOVERY OF CATIONIC ANTIMICROBIAL PEPTIDES

Although the first cationic antimicrobial peptide, nisin, was discovered in 1928 in cultures of lactic acid bacteria, little was known about how it actually killed other bacteria until the late 1960s and early 1970s, when the cytoplasmic membrane was shown to be the likely target. Polymyxin B, discovered in 1947 as a product of the soil-dwelling bacterium, *Bacillus polymyxa*, is active against Gram-negative bacteria. As with nisin, little was known about how it interacted with bacteria, until work revealed that resistance seemed to occur by alteration of the outer membrane. Both of these agents have become important commercial products as antimicrobials. As more antimicrobial peptides were discovered and studied, it became clear that the outer membrane of Gram-negative bacteria was not the only site of action for this class of compounds. Recently, there have also been hints that there may be intracellular targets for certain

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1386-2073/05 $50.00+.00

cationic peptides, since DNA and RNA synthesis are affected before any killing occurs with these peptides [1,2].

Cationic antimicrobial peptides have now been isolated from and characterized in most animals including insects and other invertebrates, amphibians, birds and mammals. In insects and other invertebrates, these peptides serve as the host's primary defense against bacterial infection. Several recent reviews describe the diversity of peptides isolated from insects and their role in the insect immune system [3-5]. Similarly, the isolation of several structurally diverse families of peptides from the skin of several species of frogs rapidly led to potential commercial applications, although none of these has been successful to date [6]. As in invertebrates, the cationic peptides of the amphibians are primarily involved in protection of the host against infection. However, the antimicrobial role of cationic peptides in host defense is not the only function of these compounds (and in many cases may not be the primary function) [7]. Indeed, in recent years it has become clear that in addition to the antimicrobial effects exerted by cationic peptides, they are also extremely important in the regulation of immunity in mammals, including humans. Thus we have tended in recent years to adopt the moniker "host defence peptides" for these molecules.

PEPTIDE FAMILIES

Cationic peptides typically exhibit very little sequence similarity. Despite this, they are often grouped according to the major structural conformation found in their membrane-associated state. Even this may be a somewhat controversial assertion, as a given peptide may sample multiple conformations when in a non-membrane mimetic environment. For this reason, peptide structural data is often determined when the peptides are interacting with model membranes or in membrane-mimetic environments. It must be stressed however, that the structure of a particular peptide does not define the mode of action or actions for that particular peptide towards a given bacterial cell.

AMPHIPATHIC α-HELICAL CATIONIC PEPTIDES

The most abundant class of peptides is the amphipathic α-helical class, which upon interaction with target membranes, folds into an amphipathic α-helix with one face of the helix containing the majority of hydrophobic amino acids, and the opposite face containing the majority of polar or charged amino acids. This class includes some of the best-characterized antimicrobial peptides such as alamethicin [8], the lantibiotic food preservative nisin [9], and magainins from the skin of many *Xenopus* species [10]. This class also includes the human cathelicidin LL-37 [11] (hCAP-18) which although a relatively weak antimicrobial agent, plays an extremely important role in immune system signaling [12-14]. Although structurally conserved, the mode of action of this class of peptides appears quite diverse.

β-STRAND CATIONIC PEPTIDES

The second large class of peptides includes the β-stranded peptides, also isolated from diverse sources. These peptides are stabilized by two or more disulfide bonds or by cyclization. They include the relatively short and highly antimicrobial β-hairpin tachyplesins [14,15] and polyphemusins [16] from the Asian and American horseshoe crabs, and protegrins from pig neutrophils [17] which each contain two disulfide bonds stabilizing a two-stranded β-hairpin. Gramicidin S , an already commercialized cyclic β-stranded decapeptide antibiotic produced by *Bacillus brevis*, has been extensively characterized and indeed has spawned many derivatives [18,19].

There are also a vast number of 3 to 4 disulphide bond-stabilized peptides, the most prominent of which are the defensins which are a major component of innate immunity in plants, insects and mammals. However, due to their structural complexity and generally muted antimicrobial activities under physiological conditions (i.e. high salt) [20,21] they have not been a major target for antimicrobial design (due largely to cost of synthesis), and these will not be discussed in detail here.

OTHER STRUCTURAL CLASSES

A number of other cationic antimicrobial peptides are characterized by their composition, containing high proportions of amino acids such as tryptophan, histidine, or proline. Most of these appear to adopt extended structures upon interaction with membranes such that the structure is stabilized by hydrogen bonding and Van der Waals forces with lipids, rather than intra-peptide interactions. The peptides indolicidin [22] of bovine neutrophils and the synthetic peptide tritrpticin [23] are relatively small (13 residues each) and contain a large proportion of tryptophan residues. In SDS micelles, they both form a boat-like structure that is unique among peptides examined to date. Other unusual peptides include PR-39 from porcine neutrophils, which contains an amazing proportion of proline residues (50 %) and affects a large number of physiological responses in its host including maintenance of tissue oxygenation during sepsis [24], recruitment of neutrophils [25], and wound healing [26]. The histatin family of peptides [27,28] of humans and other primates contains ~27% histidine residues and is involved in protection of the buccal mucosa from pathogenic yeast [29]. The mode of action of many of these types of peptides are not well characterized, although PR-39 and indolicidin appear not to cause bacterial membrane disruption at their effective concentrations [2,30].

Other peptides are cyclic due to ring closure or a single disulphide bond. The bovine neutrophil peptide bactenecin (also called dodecapeptide) fits into this class. the topical antibiotic polymyxin B from *Bacillus polymyxa* contains a lipid tail appended to a cyclic peptide that contains a type II β-turn similar to that found in polyphemusin and tachyplesin [31].

PEPTIDES AS ANTIMICROBIAL AGENTS

There is a tendency in the field to label peptides as "potent" when activities have only been tested in dilute media such as 10 mM phosphate buffer or highly diluted growth medium. Indeed when tested in either 100 mM NaCl (the concentration of NaCl in the blood) or even more importantly 2 mM Mg^{2+} or Ca^{2+} (which is present in every

fluid of the human body, and has an effect equivalent to 200 mM NaCl) these antimicrobial activities are often revealed to be rather weak [32]. There are however some peptides that maintain their activity in a physiologically relevant environment. Cationic peptides selected for commercial development can have potent activity against bacterial cells, but generally have reduced toxicity towards eukaryotic cells. Such peptides achieve this selectivity by utilizing the intrinsic differences between eukaryotic and prokaryotic membrane structure. Eukaryotic membranes typically have 45%-55% phosphatidylcholine (PC) and 15-25% phosphatidylethanolamine (PE) lipids on their surfaces, lipids which have a net charge of zero at pH 7. Mammalian membranes also contain 10-20% cholesterol. In contrast, the outer membrane of Gram-negative bacteria contains a highly negatively charged polyanionic lipopolysaccharide (LPS) on the surface of its outer membrane, while the cytoplasmic membrane of all bacteria contains up to 30% negatively charged lipids like phosphatidylglycerol (PG), and cardiolipin (CL). These would tend to attract the binding of peptides. There is also a large difference in the electrochemical gradient across the bacterial cytoplasmic membrane (~ -130 to -150 mV) whereas most eukaryotic cells possess a transmembrane potential ($\Delta\Psi$) of around –15 mV. This greater bacterial $\Delta\Psi$ is oriented in such a fashion that it may electrophorese these peptides into cells and thus be a major factor in determining cationic peptide susceptibility.

Although the majority of work on cationic peptides has studied the membrane active effects, it has now become clear that many peptides are capable of acting on intracellular targets, although it should be stressed that even for these peptides interaction with (and translocation across) the membrane is also required [33].

STRUCTURE-ACTIVITY RELATIONSHIPS OF α-HELICAL PEPTIDES

The α-helical cationic peptides are an ideal model system for determining the role of structure on activity. The ideal α-helix can be described by a relatively small number of parameters. One parameter is the helicity of the molecule. This is simply a measure of the likelihood that a given peptide will assume an α-helical conformation in a given environment. The charge on a particular peptide also plays a role in determining the antimicrobial activity of that peptide. Another important parameter is the hydrophobicity of the molecule, which simply describes the ability of the peptide to preferentially partition from an aqueous environment into a hydrophobic one. Two properties related to hydrophobicity include the hydrophobic moment, which is a measure of the separation of polar and hydrophobic faces on the helix, and the proportion of the peptide that represents the hydrophilic face (often called the subtended angle and derived by model building). In this way, a peptide that is more amphipathic will have a larger hydrophobic moment, while a peptide with a very large angle covering the hydrophilic face might interact quite strongly with the head groups of the lipid via this face of the molecule, but the smaller hydrophobic region will not penetrate very deeply into the membrane bilayer. A diagram of these properties is given in Fig. (**1**). Although a number of studies have been carried out in which all of the above characteristics have been systematically altered, in reality it is not possible to completely isolate one variable from all of the others. Despite this, general trends can be deciphered.

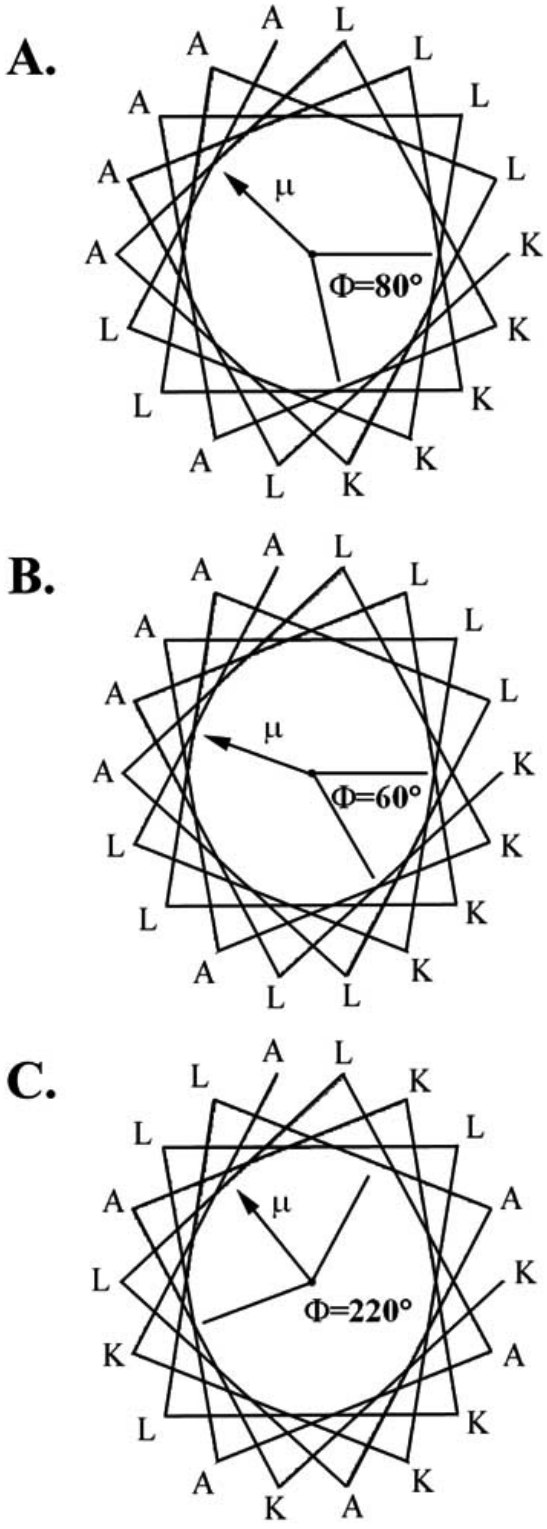

Fig. (1). Helical wheel representation of three hypothetical cationic α-helical peptides with varying hydrophobic moment, subtended angle, and hydrophobicity.

Hydrophobicity, hydrophobic moment, and the angle subtended by the hydrophobic face are a perfect example of

this phenomenon. Generally, a cationic antimicrobial peptide must be hydrophobic enough to partition into the bacterial membrane, but not so hydrophobic that it can enter any membrane or that solubility becomes an issue. Thus, most cationic peptides tend to fall into a window of hydrophobicity somewhere between these two extremes. High hydrophobicity seems to be correlated with the ability to cause hemolysis [34]. As an example of the potential effects of changing even a single residue, Juvvadi *et al.* showed that they could affect the antimicrobial activity of cecropin-melittin hybrid peptides by altering the hydrophobicity of the residue at position 8 [35]. When an I8→L8 substitution was made, the peptide maintained antimicrobial activity, while a hydrophilic I8→S8 substitution reduced activity. Likewise, when the hydrophobicity of synthetic KLAL peptides or magainin 2 analogues were varied, both antimicrobial and hemolytic activities were affected, but as hydrophobicity dropped below a certain threshold, the therapeutic index (ratio of bacterial cell killing activity to the eukaryotic cell killing activity) became much lower, and although activity was improved, selectivity was lost [36-38].

In practice, many cationic peptides are capable of interacting with both microbial and with eukaryotic membranes. To assure a high therapeutic index it is necessary to maximize those interactions that improve antimicrobial activity, while minimizing those that result in hemolysis. Generally this means creating a peptide that has a strong binding preference for negatively charged membranes, readily folds into a stable structure within the membrane and is not too hydrophobic.

The hydrophobic moment (μ) of a peptide gives a directional component to the overall hydrophobicity of a given peptide. Studies on this property are fairly complicated because of the difficulty of altering this variable without affecting others. Nonetheless, several studies have successfully shown that this is a very important variable for determining activity. A series of magainin 2 analogues were synthesized with altered μ values [37]. This study indicated that within the small window of hydrophobic moments examined, the antimicrobial activity and the hemolytic activity of the peptides could be altered, but that peptides that possessed better antibacterial activity were also endowed with greatly increased hemolytic activity.

In many cases, improved antimicrobial activity comes as a result of the charge of the peptide. This is because the initial interaction between the cationic peptide and the target membrane are primarily driven by charge-charge interactions. A number of studies have shown that increasing the positive charge of a particular peptide can increase its antimicrobial activity [39-41], however we and others have observed that adding positively charged residues beyond a certain number (usually 5-6) may have little effect.

It is interesting to note however, that the ability of a peptide to form a structure compatible with the membrane may also impact on activity and selectivity. A series of peptides based upon an idealized amphipathic α-helical peptide, KLALKLALKALKAAKLA-NH_2, and variants containing double substitutions of D-amino acids along the helix were constructed and the antimicrobial and hemolytic activity was examined [36]. As the substitutions approached the middle region of the helix, the helicity of the peptide was markedly reduced changing the hydrophobic moment. Interestingly, this loss of helicity caused a reduction in the hemolytic activity of the peptide, while the antimicrobial activity was affected to a lesser degree. This is likely because the initial interaction with bacterial cells appears to be predominantly due to charge-charge interactions, which would be unaffected by changes in helicity, but insertion into the membrane requires folding into an amphipathic conformation which would affect insertion into both negatively charged bacterial membranes and the less charged erythrocyte membranes. Indeed the strength of interaction of these variant KLAL peptides with anionic or zwitterionic phospholipids depended on both the tendency of the peptide being examined to form an α-helix and the nature of the lipids in the model system.

STRUCTURE-ACTIVITY RELATIONSHIPS OF β-SHEET PEPTIDES

Gramicidin S (GS) is a cyclic decapeptide consisting of the sequence VOL_DFPVOL_DFP, where O is the cationic amino acid ornithine, and the prefix $_D$, denotes an amino acid present as the *d*-enantiomer of that amino acid. The structure of GS includes two 3-residue antiparallel β-strands connected by two type II' β turns and stabilized by four H-bonds. The structure of GS was determined by NMR in 1995 and demonstrates high amphipathicity, with the positively charged ornithine residues located on the same side of the molecule (Fig. (**2**)) [42]. The opposite face contains the two leucine residues and two valine residues. The proline residues are located in the turn region of the peptide, while the two phenylalanine residues are oriented toward the same face as the ornithine residues, at approximately a 45 degree angle relative to the plane of the β-sheet [42]. Unlike many α-helical peptides, the structure of GS is quite stable in both aqueous and membrane mimetic environments. This amphipathic structure allows strong interaction of GS with many biological membranes. GS is highly antimicrobial against a wide-variety of bacteria and fungi [43]. However, the therapeutic index for GS is quite low due to the high haemolytic activity of this compound [44,45].

Many variants of GS have been synthesized and characterized. These include GS10, GS12 and GS14, cyclic peptides that contain 10, 12, and 14 residues, respectively [46-48]. These peptides are also altered such that the positively charged residues are lysine rather than ornithine. These gramicidin variants have similar structures, differing mainly in the size of the β-sheet and in the number of H-bonds stabilizing the β-sheet, with GS12 and GS14 having five or six H-bonds respectively, rather than the normal four. In an attempt to improve the therapeutic index of these peptides, diasteriomeric (D-amino acid) variants of these peptides have been synthesized in which single amino acid residues were systematically altered to their diastereomer. All of the variants synthesized were characterized by CD-spectroscopy, by hydrophobic partitioning, and by antimicrobial and haemolytic activity. Since all of the peptides within a given series (GS10, GS12, or GS14) have identical intrinsic hydrophobicity, any changes in observed

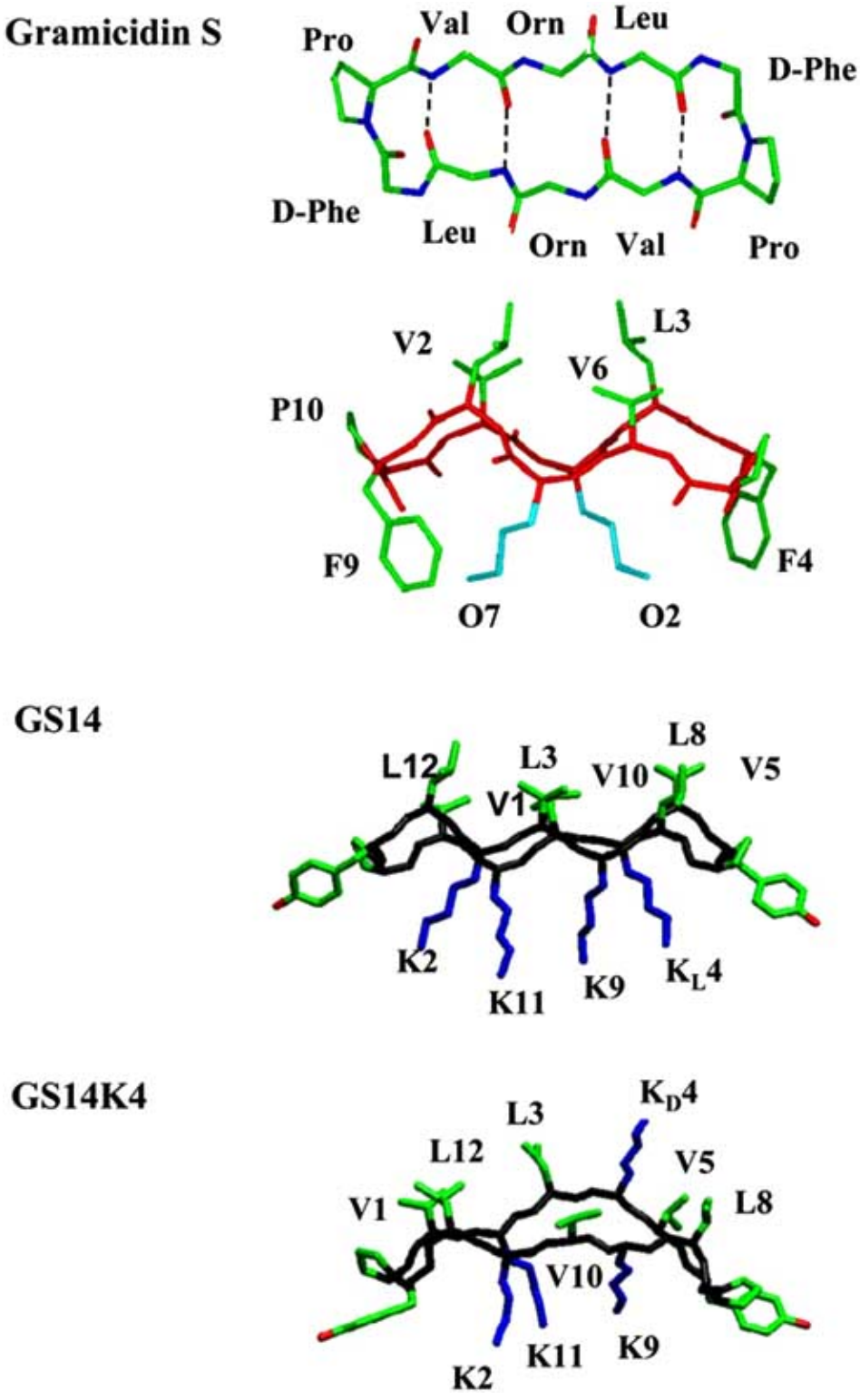

Fig. (2). Structure of gramicidin S showing A) the β-sheet structure of the peptide and B) the orientation of the charged ornithine residues with respect to the hydrophobic valine and leucine residues. The phenylalanine residues are also located in an orientation suitable for interaction with the interface between the hydrophobic acyl chains of the lipids and the polar head groups.

hydrophobicity/ amphipathicity must be due to differences in the structure of the peptide.

It was found that despite the relatively conservative changes in sequence, the amphipathicity of the peptides was drastically decreased, with most variants exhibiting reduced retention times when assessed by reverse phase HPLC, as compared to the parent compound [46-48]. This reduction correlated very well both with reduced LPS binding ability and with lowered hemolytic activity. Interestingly however,

despite the requirement for some amphipathic character to get antimicrobial activity, when the amphipathicity was very high, there was a very low therapeutic index, because of both the lack of antimicrobial activity (possibly due to an inability to dissociate from surface LPS or teichoic acid) and because of a very high hemolytic activity. Consistent with this observation the most amphipathic molecules, (GS10, GS12, and GS14) while inactive against Gram-negative and Gram-positive bacteria, were highly active against *Acholeplasma laidlawii* B, a Gram-positive bacterium lacking a cell wall [49].

In a related study, the therapeutic index of a highly amphipathic molecule (GS14) was altered by incorporating a charged amino acid (lysine) into the hydrophobic face of the molecule [50], leading to reduced hydrophobicity as assessed by reversed phase HPLC. This also greatly reduced the hemolytic activity and increased the antimicrobial activity against both Gram-negative and Gram-positive bacteria. In aqueous environments, circular dichroism (CD) spectroscopy indicated that there was a large reduction in β-sheet content for one of the analogues (GS14K4) that appeared to be restored when placed into a membrane-mimetic environment containing TFE. The structure of these modified variants was determined by NMR in 30% TFE environments, and they showed a highly ordered β-sheet structure, with the added lysine facing into the normally hydrophobic region of the peptide [50]. Thus, while amphipathicity seems to be an important component of the antimicrobial activity of a particular cationic peptide, it is possible that if the molecule is too amphipathic it will remain bound at the polar/apolar interface of the membrane, without destabilizing the membrane.

NMR studies on a 19F-labelled gramicidin S variant indicated that when bound to membranes, the molecule is initially oriented in a way such that the hydrophobic face of the molecule is inserted into the outer leaflet of a PC bilayer, while the hydrophilic side chains are oriented toward the external side of the bilayer [51]. It was demonstrated that when the membrane was in the liquid crystalline phase, the peptide was quite dynamic, wobbling around the bilayer normal, while if the membrane was in the gel phase, the peptide was virtually immobile. Presumably, this membrane interaction is relevant to the mode of action of GS. However, it is not yet entirely clear how this membrane insertion leads to membrane disruption, although several lines of evidence have suggested that the insertion of GS into model membranes results in the formation of non-lamellar structures. Indeed, differential scanning calorimetry (DSC) spectra of GS:PC (1:25) mixtures exhibited non-isotropic peaks, with the appearance of a high-temperature shoulder at the transition temperature [52]. These observed patterns are consistent with the peptide partitioning at the lipid head group at the polar/apolar membrane interface. The effect on lipid packing/disruption would be a secondary effect of this GS-lipid interaction [52,53].

The polyphemusins and tachyplesins are related classes of peptides containing a single two-stranded β-pleated sheet that is stabilized by two disulfide bonds. Structures for polyphemusin, tachyplesin, and several analogues have been determined by nuclear magnetic resonance (NMR) spectroscopy [54,55]. They exhibit extremely high affinity for LPS from Gram-negative bacteria and they also have very low MIC values for both Gram-negative and Gram-positive bacteria [56-58].

Several synthetic variants of polyphemusin have also been designed to be more amphipathic [56]. Interestingly, all of these variants (PV5, PV7, and PV8) showed very similar affinity for LPS, and all showed improved ability to protect mice from lethal endotoxemia, as compared to polyphemusin I. The variants also all displayed reduced antimicrobial activity and decreased haemolytic activity [56]. This suggests that although LPS binding is an important interaction with respect to a particular bacterium, it cannot be the sole determinant of the antimicrobial activity of a given peptide. The variant polyphemusins also showed reduced ability to insert into lipid bilayers, as demonstrated by smaller pressure increases in (PC/PG/CL) lipid monolayers. This suggested that although able to interact with membranes to a certain degree, the depth of insertion into the monolayer might be lower than for polyphemusin I [56], possibly consistent with the variant peptides being more stable at the polar/apolar membrane interface and less likely to insert into the bilayer. The variant polyphemusins also showed drastically different killing kinetics toward *E. coli* UB1005, with polyphemusin I causing complete killing within 5 minutes, while the variant peptides required one hour to reach the same level of killing. Indeed, these same correlations between improved amphipathicity and decreased activity were also noted for gramicidin S analogues GS14K4 and GS14K3L4 as described above [50].

Recently, high resolution NMR structures have been determined for both polyphemusin I and for PV5 [54]. These structures show that both peptides are very structurally conserved with respect to the previously determined structure for tachyplesin [55]. Interestingly the structures of all of these peptides suggest that although the β-turn region is highly constrained in all NMR structures, there is a great deal of flexibility in the β-stranded region, especially adjacent to the disulphide bond closest to the ends of the peptide. This structural flexibility may play a role in the interaction of these peptides with LPS and with lipid bilayers. Indeed, the structure of tachyplesin interacting with PC micelles has also been determined, and shows a prominent bend in the β-sheet structure [55]. Similar studies using CD spectroscopy to characterize the structural changes of tachyplesin upon phosphatidylglycerol (PG) small unilamellar vesicles (SUVs) or LPS/PG SUVs have also shown that the β-strand and β-turn structures are stabilized upon interaction with either of these systems [59]. This conformational change may allow the peptide to change its amphipathic characteristics upon membrane interaction, perhaps leading to membrane perturbations as a consequence.

STRUCTURE ACTIVITY RELATIONSHIPS IN OTHER PEPTIDES

A third well-studied group of peptides includes the polymyxins and synthetic variants. Polymyxin B is highly active against Gram-negative but not Gram-positive bacteria. It consists of a seven-membered cyclic peptide moiety with a three amino acid residues tail to which is attached an nine-carbon fatty acid that is required for strong antimicrobial

activity. In the absence of the lipid tail, polymyxin B nonapeptide does not cause killing but can sensitize bacterial cells to the action of other antibiotics that are normally excluded by the outer membrane. As with gramicidin S and analogues, binding to the membrane is not sufficient for killing, but rather deep insertion into the membrane seems to be required. However whether the actual target is the cytoplasmic membrane, has been disputed [60]. The observation that a lipid tail is required for polymyxin B activity has been extrapolated to permit improvement of the activity of a number of different antimicrobial peptides by covalently linking fatty acyl groups to them. In this way, the antimicrobial activity of magainin analogues was expanded to include fungi [61]; that of cecropin-magainin hybrids was broadened to be leishmanicidal [62]; and the antibacterial activity of human lactoferricin was increased by two orders of magnitude [63].

MECHANISM OF ACTION – GRAM-NEGATIVE OUTER MEMBRANE

Two membranes surround Gram-negative bacteria. The inner cytoplasmic membrane has a typical bilayer structure composed of phospholipids, with a number of integral and peripheral membrane proteins. The outer membrane is asymmetric, with the inner leaflet composed of phospholipids and the outer leaflet being the polyanionic glycolipid LPS [64,65]. The negative charges on LPS, due to a high content of phosphates and acidic sugars, are bridged by divalent cations that serve to partially neutralize the negative charge and stabilize the outer membrane [66]. These divalent cations bind with moderate affinity to the LPS, and these sites serve as the part of the outer membrane at which self-promoted uptake of polycations, like the cationic antimicrobial peptides, occurs.

Polymyxin B has long been known to increase the permeability of the outer membrane and to sensitize Gram-negative cells to antibiotics that are normally unable to cross the outer membrane [67]. Also the ability of polymyxin B to bind to and neutralize endotoxin was first described in the 1960s [68-70]. In addition, mutant strains resistant to polymyxin B bound less polymyxin B with lower affinity than wild-type cells [67,71,72]. Based on this background and the isolation of a mutant that mimicked cells grown on low Mg^{2+} and was cross resistant to polymyxin B, gentamicin and EDTA (now recognized to be altered in signaling through PhoPQ) [73], the self-promoted uptake hypothesis was proposed [65]. This hypothesis proposes that polycationic molecules bind to the divalent cation binding sites on LPS at the surface of the outer membrane by displacing native divalent cations such as Mg^{2+} or Ca^{2+} and disrupting the stabilization of LPS by divalent cation cross-bridging, leading to localized disruption of the bilayer. The disrupting polycation is then taken up through the destabilized membrane, hence the name for the process, self-promoted uptake [65]. This hypothesis was subsequently extended to embrace cationic antimicrobial peptides, e.g. by demonstrating that cationic peptides bind to LPS, perturb the outer membrane permeability barrier and cause disruption as revealed by the formation of membrane blebs at MIC concentrations. Binding to LPS is required for lethality and indeed explains the preferential activity of many cationic peptides against Gram-negative bacteria, but are not thought to be the direct cause of lethality since cells can remain viable even when the outer membrane is completely removed, as is the case with spheroplasts. Importantly in the area of peptide design, binding to a divalent cation binding site explains the above-mentioned observation that divalent cations, such as Mg^{2+} and Ca^{2+} are far more antagonistic to peptide activity, than are monovalent cations like Na^{+} or K^{+}.

MECHANISM OF ACTION – CYTOPLASMIC MEMBRANE

All cationic peptides must interact with the cytoplasmic membrane (in both Gram negative and Gram positive bacteria) to lead to lethality. Indeed if high enough concentrations of cationic amphipathic peptides are used these usually cause membrane disruption. Without re-iterating arguments already made [2, 25], it is quite clear that at the minimal lethal concentration not all peptides kill through membrane disruption and many studies of mechanism of action suffer from the fact that no meaningful studies are performed in intact cells and the basis for concluding a membrane-lytic mechanism of action relates to studies done with model membrane systems and high ratios of cationic peptides to lipids. Indeed from a broad range of studies performed, it is clear there is considerable heterogeneity in the mode of interaction of individual cationic peptides with model membranes [60].

Four major models have been suggested for how peptides interact with the cytoplasmic membrane. These are the barrel-stave model, the carpet model, the toroidal pore model and the membrane aggregate model (with the latter two being somewhat related). These are described in many reviews [e.g. 74-76]. We favor the latter model, as it explains most experimental observations and also why some (or possibly many) peptides translocate across the membrane. However all of these models could be correct under specific circumstances, depending upon the peptide being investigated and the composition of the membrane used in the study [25].

NON-MEMBRANE TARGETS FOR PEPTIDES

Several peptides that have been examined in detail are clearly able to translocate across the cytoplasmic membrane. It was shown that exposure to various cationic peptides like the indolicidin analogues CP11CN and CP10A and a bactenecin derivative Bac2A caused septation defects and nuclear condensation in *Staphylococcus epidermidis*, without obvious membrane lysis (emptying out of cytoplasmic contents) [1,32]. Loss of macromolecular synthesis at around the minimal inhibitory concentration was observed for a number of peptides including fish pleurocidin, bovine indolicidin and indolicidin analogues [1,2,77]. Immunogold labeling of both lactoferricin B and magainin 2 also demonstrated that cationic peptides can enter into the cytoplasm of bacterial cells, suggesting the presence of non-membrane targets for these systems [78]. The frog peptide buforin can also kill cells without major impact on the membrane [79].

Most of the studies of cationic peptide activity have focused on the interaction of cationic peptides with bacterial membranes, but specific interactions with a number of other bacterial components have also been reported. The cationic peptide pyrrhocoricin, originally isolated from the European sap-sucking bug, has been shown to specifically bind to and inhibit the ATPase activity of *E. coli* DnaK, leading to an increase in misfolded protein and ultimately the death of the cell [80,81]. A number of structurally unrelated cationic peptides were recently been shown to inhibit several aminoglycoside modification enzymes [82]. The site of interaction was modeled with the cationic peptide binding within a large negatively charged cleft in the enzyme. Structure-activity relationships with one of these cationic peptides indicated, however, that both ionic and hydrophobic interaction were important for the interaction to take place [83].

BACTERIAL RESISTANCE TO CATIONIC ANTIMICROBIAL PEPTIDES

Growth of many bacteria including, *Salmonella* sp., *E. coli*, *Pseudomonas* sp., *Yersinia*, and others in media containing low concentrations of divalent cations, especially Mg^{2+} or Ca^{2+} results in the activation of a two-component regulatory system, PhoP-PhoQ and greatly increased resistance to cationic antimicrobial peptides [84-86]. A second two-component system PmrA-PmrB also regulates resistance to cationic peptides in a number of bacterial species including *Salmonella* sp., *Pseudomonas* sp., and *E. coli* [87,88]. In *Salmonella* sp. or *E. coli* this system regulates of number of genes involved in resistance to cationic peptides. One gene regulated by PhoP is the *pagP* gene; the PagP protein is localized in the outer membrane and catalyzes the transfer of palmitate from a phospholipid to the to N-linked myristoyl residue of the proximal sugar residue [89]. The addition of this extra lipid increases the hydrophobicity of the Lipid A moiety, presumably making it more difficult to destabilize by interaction with cationic peptides. Activation of the PhoP-PhoQ system also leads to the activation of a second two-component system, PmrA-PmrB. The PmrA-PmrB system directly activates two operons involved in Lipid A modification. Upregulation of the *ugd* gene and the *pmrHFIJKLM*.operon result in the production of a Lipid A molecule containing 4-amino-4-deoxyarabinose at the 1 and/or 4' phosphates. A detailed pathway for the synthesis of this compound has been proposed by Raetz and coworkers and the model is being tested in detail [90-94]. In addition to these changes, lipid A purified from an *E. coli* mutant that is resistant to polymyxin B has been shown to also contain phosphatidyl ethanolamine at the 1 and/or 4' positions [92,95]. The genes involved in the addition of phosphatidyl ethanolamine to lipid A have not yet been identified. The addition of phosphatidyl ethanolamine and/or 4-amino-4-deoxyarabinose serves to decrease the charge of the LPS molecules and alleviates the requirement for divalent cation stabilization. This also reduces the affinity of cationic peptides for the outer membrane, thereby making the cells containing these modifications more resistant to peptide induced lysis.

Recently, it has become increasingly clear that certain pathogens are capable of detecting the presence of cationic peptides and mounting a defense response leading to enhanced resistance. *P. aeruginosa* isolates from patients with cystic fibrosis (CF) can have a Lipid A molecule that contains an extra acyl group [96]. This modification, reminiscent of the above mentioned changes, would presumably make the outer membrane more stable against outer membrane disorganizing agents. Such isolates also show evidence of possessing N_4-aminoarabinose modified Lipid A changes that are characteristic of growth in media containing low concentrations of divalent cations [96]. This is in spite of the fact that the concentrations of these in surface airway fluid is in the millimolar range, which normally represses such LPS modifications. We have recently shown that when *Pseudomonas* is grown in media containing high concentrations of Mg^{2+} and sub-inhibitory concentrations of cationic peptides, conditions that mimic the CF lung, genes involved in cationic peptide resistance are induced [88]. This pre-exposure to sub-MIC concentrations is protective in wild-type *Pseudomonas*, but not protective in strains lacking the LPS modification genes responsible for the addition of N_4-aminoarabinose to lipid A (McPhee and Hancock, unpublished results). Although we previously published that the *pmrAB* operon was upregulated in the presence of e.g. bovine indolicidin, we recently showed that induction of the LPS modification operon by peptides appears to be independent of the PhoPQ and PmrAB signaling systems. Interestingly, the ability of peptides to induce these operons is not uniform. In this way, we have found that peptides that tend to be the best antimicrobials are also the worst at causing *P. aeruginosa* to respond to them and vice versa. This observation suggests that there are design principles that would allow the development of highly active peptides that do not result in adaptive resistance.

An analogous response has been seen in *Salmonella* species, but there are several important differences. Like *Pseudomonas*, *Salmonella* detects the presence of cationic peptides [97]. Unlike *Pseudomonas* however, the response of *Salmonella* appears to depend upon the *phoP* gene, since strains lacking *phoP* are incapable of adaptive resistance to cationic peptides. Interestingly, in *Salmonella* the presence of cationic peptides also results in RpoS-dependent protection against reactive oxygen species. These results indicate that the ability to respond to cationic peptides has evolved to specialized protection. Thus in *Salmonella*, we see resistance to cationic peptides and reactive oxygen species, both of which are typically encountered by the bacterium in the *Salmonella*-containing vacuole of host macrophages.

In addition to the LPS modifications described above, other mechanisms for cationic peptide resistance at the outer membrane level are known. *Salmonella* sp. have been shown to produce an outer membrane protease, PgtE, a member of the OmpT family of serine proteases that specifically cleaves certain α-helical cationic peptides and rendering cells more resistant to killing by these peptides [98]. OmpT has also been shown to prevent killing of *E. coli* by protamine [99]. Interestingly, a *Yersinia pestis* homologue of this protein, Pla, is known to be expressed at body temperature and is a virulence factor involved in the mobility of the bacterium from a subcutaneous wound to distal sites [100]. Furthermore, studies that looked at the susceptibility of both

Gram-positive and Gram-negative bacteria in the presence of protease inhibitors suggested that a number of intracellular proteases like DegP, also contributed to the intrinsic resistance of a number of bacteria to cationic peptides [101].

Yersinia pestis species have also been shown to have altered resistance to cationic peptides due to modification of its outer membrane. However, unlike in the species described above, these changes are not due to Lipid A modification, but rather due to the presence of a terminal galactose or heptose in the core oligosaccharide [102]. PhoP mutants of this strain are altered in that the terminal sugar of the *phoP*- strain consists of only a terminal heptose. These mutant strains are approximately 8-fold more sensitive to cecropin P1 and more than 100-fold more sensitive to polymyxin B.

There are relatively few described mechanisms for bacteria to resist the presence of cationic peptides once the peptides have crossed the outer membrane. In *Neisseria* sp., mutants in the *mtrCDE* system, an active efflux system that is also involved in resistance to detergents like Triton X-100, exhibited ~10-fold increased sensitivity to protegrin 1, a porcine cationic peptide [103]. Other efflux systems have also been implicated in cationic peptide resistance. In *Yersinia* sp., an efflux pump/potassium antiporter system, RosA/RosB has been described that increases the resistance of the bacterium to polymyxin B [104]. In *Vibrio* sp. and *Salmonella* sp. a locus containing the *sapABCD* genes has been proposed to encode an efflux system that increases resistance to cationic antimicrobial peptides [105,106].

As mentioned above, divalent cations can antagonize the action of cationic peptides. In addition polyanions can be antagonistic. *P. aeruginosa*, a major cause of chronic lung infection in cystic fibrosis patients, often converts to a mucoid phenotype characterized by the production of copious amounts of a polyanionic alginate-like exopolysaccharide. *In vitro* experiments have demonstrated that the presence of alginate can raise the MIC to a number of cationic peptides by 8- to 32-fold [32]. Thus it seems likely that the synthesis of alginate in infections could reduce the susceptibility of the organism to cationic peptides.

Resistance to cationic peptides in Gram-positive bacteria also involves covalent modifications of certain cell-wall constituents. Unlike Gram-negative bacteria, Gram-positives contain only a single membrane. However, like Gram-negatives, the envelope of Gram-positive bacteria is negatively charged, due to the presence of teichoic acids and lipoteichoic acids. Teichoic acid consists of repeating polymers of glycerol or ribitol linked by phosphates. The sugar moiety of these cell wall constituents are often modified by the addition of various amino acids. Mutants of *Staphylococcus aureus* that possess decreased alanylation of teichoic acids and lipoteichoic acids have been isolated. The mutation has been mapped to the *dltABCD* operon, which catalyzes the addition of alanine to the sugar moiety of these molecules [107,108]. The loss of this modification makes the teichoic acids even more negatively charged. Another modification involves the addition of lysine to the head group of PG by the MprF protein, decreasing the affinity of cationic antimicrobials for the cytoplasmic membrane [108,109]. Interestingly, homologues of MprF are also found in Gram-negative bacteria, suggesting that modification of cytoplasmic membrane phospholipids may represent a common means of resistance to host defense peptides.

POTENTIAL HURDLES IN PEPTIDE DEVELOPMENT

There are a number of potential barriers to the development of a cationic antimicrobial peptide, although to be fair these hurdles due to toxicity, stability and cost of goods exist for all drugs. The issue of acute toxicity, due to lysis of red blood cells has been discussed above. However there are few to no papers that deal with more subtle toxicities. Anecdotal reports from clinical studies performed with cationic peptides indicate that they tend to not cause any overt effects when applied locally. Even with a relatively hemolytic peptide like protegrin as its parent, the analog peptide IB-367, used as a oral gel in a phase I study of normal human volunteers, was found to give rise to no serious adverse effects, no evidence of allergic or anaphylactoid reactions and no clinically significant changes in vital signs at concentrations that reduced oral microflora by 1000-fold [110]. However subtle toxicities have never been examined although there is a feeling in the field that cationic peptides are toxic when administered systemically. For example it was demonstrated with the cathelicidin variant novispirin G10 that it demonstrated lung toxicity (as revealed by IL-6 induction), in the context of a *Klebsiella* infection but not in uninfected mice [111]. One approach to overcoming toxicity that has been demonstrated with indolicidin is to formulate it in liposomes which permits it to be used in systemic protection [112]. The best studied cationic peptide drugs are the polymyxins. For these drugs the kidney is the primary route of elimination and toxic effects involve the kidney and central nervous system [113]. Conversion of polymyxin E to the methane sulphonate-derivatized pro-drug can reduce but not prevent toxicity.

As natural peptides contain peptide bonds and basic amino acids, they tend to be susceptible to protease digestion. Several approaches have been attempted to increase stability. The natural pro-peptide sequence can be a protease inhibitor [114], pointing the way to potential approaches that conjugate an anti-protease to a protease. In this regard it is helpful that some protease inhibitors are in fact cationic in nature, making it possible that protease inhibition and antimicrobial activity can be intermingled [115]. A second approach is the use of D-amino acids, unnatural amino acids or different peptide backbones [116-118] although the problem with these approaches is that they tend to make an already expensive drug even more expensive. We have also demonstrated that cyclization, that presumably causes steric hindrance of peptide proteolysis, is a reasonable tactic [22].

IMMUNE MODULATING ACTIVITIES OF CATIONIC PEPTIDES

Cationic peptides could potentially be used to treat bacterial, viral or other infections by nature of their direct activity on microbial pathogens as described in detail above. However, peptides can also modulate the host immune

response to indirectly facilitate clearance of microbial infections. The peptides that are effective in modulating the host immune response have a broad range of structural characteristics. For example, peptides of both the α-helical and β-stranded families can indirectly, by inducing chemokine secretion, or directly by acting as a chemokine, recruit inflammatory cells to a site of infection. This suggests that although immunomodulatory peptides have diverse structural characteristics, there is still an overlap in their activities related to the immune response and their contribution to infection clearance. It becomes difficult then to attribute peptide activities to specific structural classes.

The effect of peptides on cell recruitment is an important host defense response. The recruitment of phagocytic cells such as monocytes and dendritic cells brings an antimicrobial arsenal to an infection site to eliminate the pathogen and facilitate pathogen elimination. Cell migration is controlled by a multistep process that includes chemoattraction along a chemoattractant gradient, cell-cell adhesion and, in most cases, transmigration through cell layers [119]. Such chemotactic activities of peptides appear to have *in vivo* relevance since it has been demonstrated that application of the human α-defensin HNP-1 led to reduced *Klebsiella pneumoniae* numbers in murine peritoneal infection model, and this antibacterial activity was accompanied by an enhanced influx of macrophages, granulocytes and lymphocytes into the peritoneal cavity [120]. The activity appeared to be in part mediated by leukocyte accumulation since leukocytopenic mice administrated HNP-1 did not display reduction in bacterial load. A number of the human β-defensin members have similarly been shown to be chemotactic in *in vitro* systems. β-Defensins are β-sheet peptides stabilized by three intramolecular cysteine bonds linking cysteines 1-5, 2-4, and 3-6, whereas the α-defensins have bonds linking cysteines 1-6, 2-4, and 3-5. All defensins tend to have similar tertiary structures with a core of three anti-parallel β-strands, resembling certain chemokines [121]. HBD-1 and HBD-2 are chemotactic for immature dendritic cells and memory T cells through interaction with the receptor CCR6 (also used by the chemokines, MIP-3α, LARC and CCL20) [122]. HBD3 is chemotactic for monocytes although they do not express CCR6 [122-124]. Interestingly, a recent publication demonstrated that disulfide bonding in HBD-3, is required for binding and activation of receptors for chemotaxis, but is not required for its antimicrobial function [125]. In this same publication it was demonstrated that differently folded HBD3 variants have a wide range of chemotactic activities. Peptides that also display chemotactic activity but differ significantly in sequence from the defensins are LL-37 and PR-39. LL-37, a human α-helical peptide of the cathelicidin family, has been suggested to have chemotactic activity for T cells and neutrophils [126]. The proline and arginine rich porcine peptide, PR-39 has calcium-dependent chemotactic activity for neutrophils [25]. A group of synthetic hexapeptides were found to induce intracellular calcium release in a pertussis toxin-sensitive manner and were chemotactic for human monocytes and neutrophils [127]. Although these structurally diverse peptides have been found to be directly chemotactic, certain peptides have also been shown to indirectly enhance cell recruitment. For example, defensins and LL-37 have both been shown to enhance the release of IL-8, a chemoattractant and neutrophil activating cytokine, in airway epithelial cells [13,128]. LL-37 stimulates MAP kinase (Erk 1/2 and P38) signaling leading to transcriptional events that include IL-8 expression [129,130]. The receptors involved in LL-37 interaction with cells appear to be quite complex with different authors finding different receptors according to the biological process stimulated and the cell type examined. Thus peptides have multiple ways of stimulating a range of immune responses.

Another important feature of peptides in the host immune response is their ability to inhibit inflammatory responses and stimulate angiogenesis. Defensins have been shown to interfere with the activation of neutrophil superoxide-generating NADPH oxidase [131,132]. Reactive oxygen intermediates generated by the phagocyte NADPH oxidase are critically important components of host defense. However they are highly toxic and can cause significant tissue injury during inflammation. It is therefore important that their generation and inactivation are tightly regulated. For example, the implantation of foreign materials into the body results in the production of oxygen free radicals by activated neutrophils [133], which serves to protect the body from infection. Defensins appear to downregulate the ability of the neutrophils to generate superoxide, and consequently modulate host defenses at the site of the foreign implant [134]. PR-39 among other activities has also been shown to inhibit NADPH oxidase activity, which it does by blocking the assembly of this enzyme through interactions with Src homology 3 domains of the p^{47phox} cytosolic oxidase component [135]. This activity of PR-39 is related directly to its primary sequence.

Recent studies have demonstrated that PR-39 also blocks degradation of IκBα and HIF-1α by the proteasome [136]. Yet another study demonstrated that PR-39 is a noncompetitive and reversible inhibitor of proteasome function due to a unique allosteric mechanism allowing for specific inhibition of degradation of selected proteins. It was suggested that PR-39 changes conformational dynamics of the proteasomes by interactions with the non-catalytic subunit R7 in a way that prevents the enzyme from cleaving the substrates of unique structural constraints [137]. In one of several *in vivo* studies, PR-39 was found to alleviate endotoxin-induced liver hypoxia six hours post treatment most likely due to its anti-inflammatory activity and stimulation of angiogenesis [138].

As discussed above, many cationic peptides have the ability to bind to the bacterial component, LPS. This is another feature of the peptides that lends to its role in host defense. During an infection by a pathogen, an innate immune response is usually triggered by binding pathogen-associated molecular patterns or signaling molecules to so-called pattern-recognition receptors, such as Toll-like receptors (TLRs), on the surface of host cells. These signaling molecules are present exclusively on microbes, including bacteria, parasites, viruses, and fungi. For example, LPS present on Gram-negative bacteria, lipoteichoic acid on Gram-positive bacteria, and mannans on yeast cell walls, all trigger a response from the innate immune system. The binding of signaling molecules to TLRs results in rapid marshaling of neutrophils, monocytes,

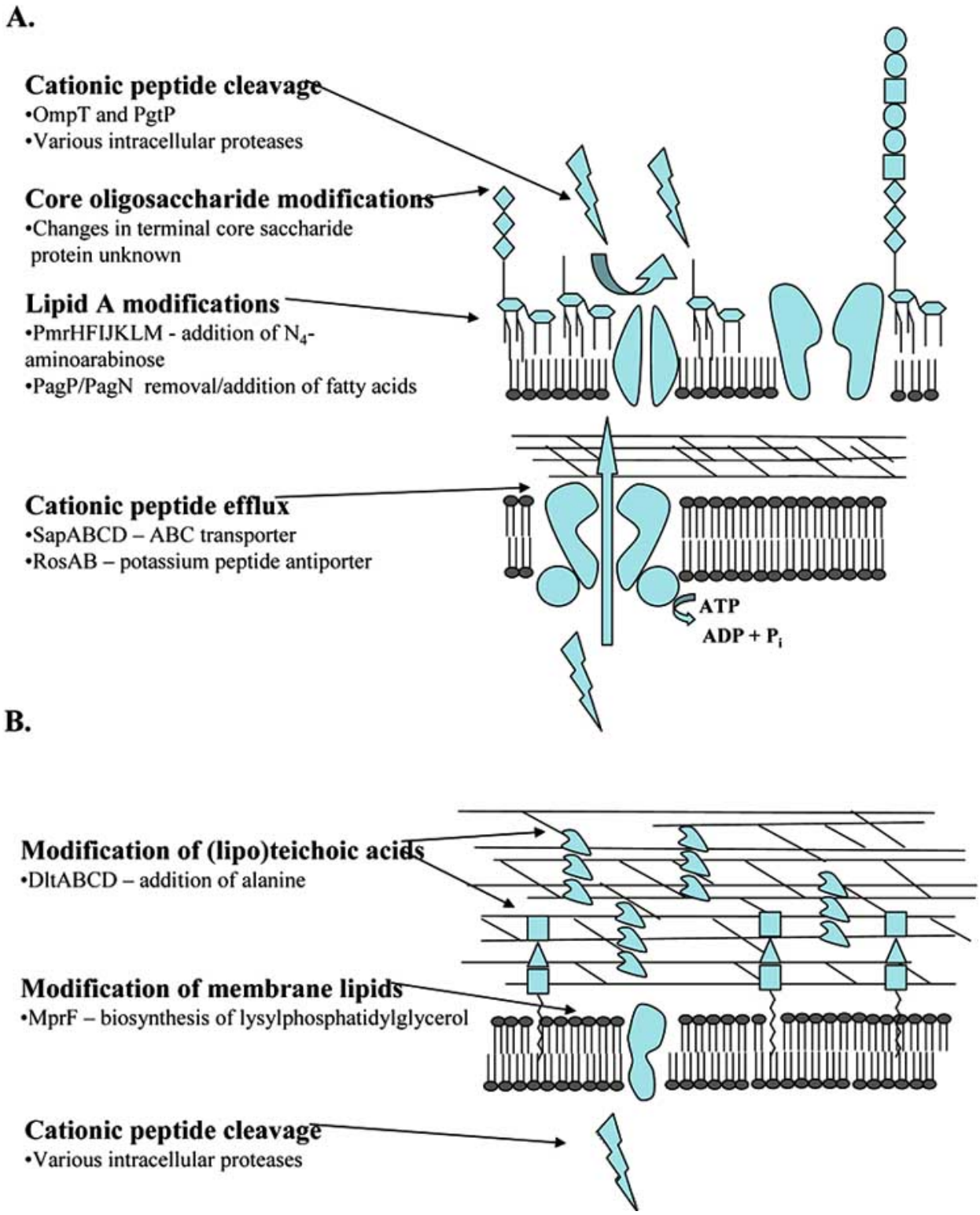

Fig. (3). Diagrams showing modifications of cellular components that give rise to increased cationic peptide resistance. A) Location of cationic peptide resistance determinants in Gram-negative bacteria. B) Location of cationic peptide resistance determinants in Gram-positive bacteria.

macrophages, complement factors, cytokines, antimicrobial peptides, and acute-phase proteins in a complex and highly regulated response against the infection. During an innate immune response, excessive production of certain inflammatory mediators and pro-inflammatory cytokines can lead to a cascade that, if left unchecked, causes tissue damage or sepsis, a potentially lethal condition. An uncontrolled inflammatory response can be triggered by bacterial components released during infection or by the bacteria themselves. Along with the anti-inflammatory

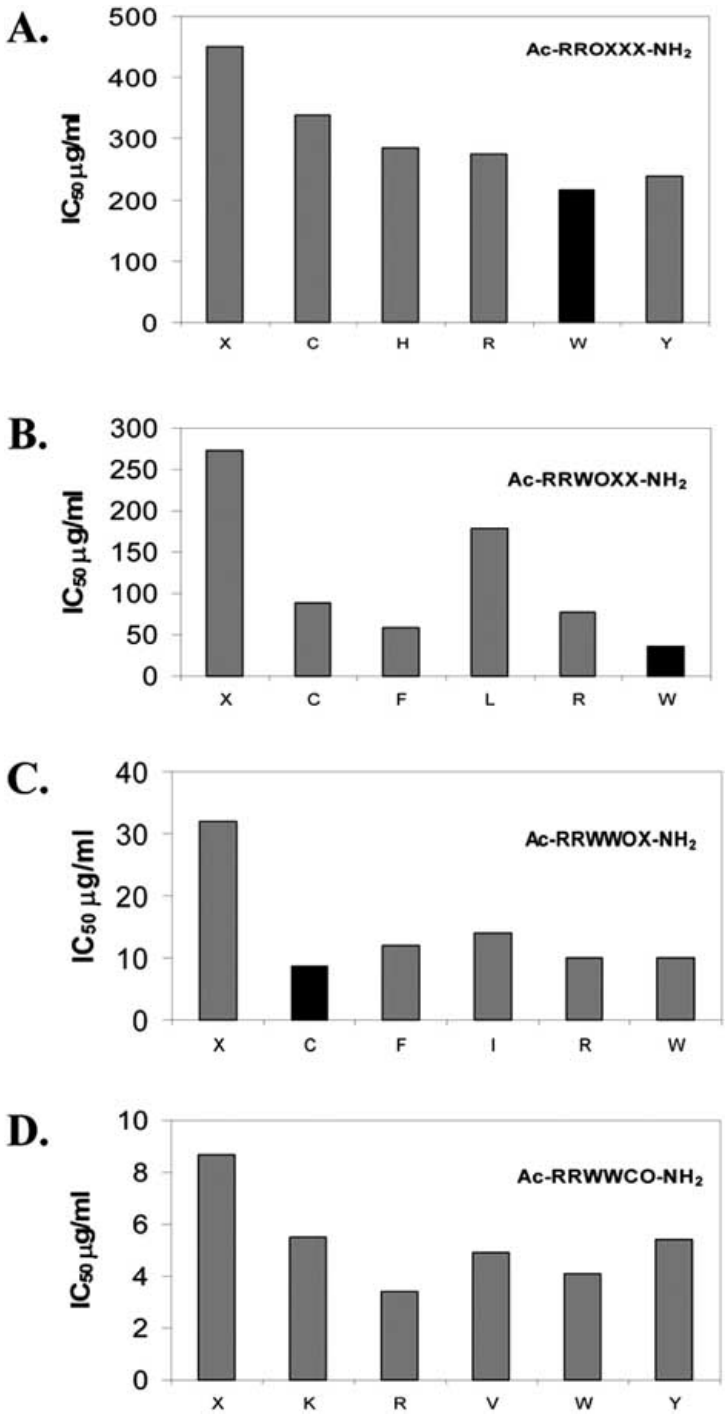

Fig. (4). Example of the development of antimicrobial peptides by using an iterative combinatorial approach [140]. Peptides shown in black serve as the template for the next round of synthesis. A) IC_{50} of a series of peptides (first iteration) consisting of the sequence Ac-WWOXXX-NH2 where O represents the single amino acid shown below each bar and X represents an equimolar mix of all 20 amino acids at that residue. B) Results of second iteration using the most active compound from first round as a template. C) Results of the third iteration. D) Results of the fourth iteration showing that one compound, Ac-RRWWCR-NH_2, has an IC_{50} of 3.4 µg/ml, an improvement of 132-fold over the original mixture of peptides.

activity of the peptides, the ability to bind LPS [139-148] may be an asset. However it is by no means certain that this represents the sole mechanism of suppression of inflammatory responses. Gene array experiments have indicated that human LL-37 [13] and the insect hybrid peptide CEMA [149] causes selective suppression of LPS-upregulated gene expression, whereas simple binding and neutralization of LPS by peptides would be predicted to cause global suppression. Interaction of peptides directly with epithelial cells and monocytes was subsequently shown to lead to up-regulation of the expression of many genes, including anti-inflammatory genes such as IL-10 indicating the potential for indirect suppression of pro-inflammatory responses.

COMBINATORIAL LIBRARY SCREENING FOR CATIONIC PEPTIDE ACTIVITY

The final major barrier to the exploitation of peptides as antimicrobials is cost of goods. Peptides that have been introduced into the clinic to date have been synthesized by solution phase chemistry, and tend to cost up to $200 per gram (a reasonable human daily dose for an antimicrobial). This compares to e.g. gentamicin that costs about $0.80 per gram.

Despite these obstacles, recent advances in peptide synthesis have made feasible the synthesis and screening of large libraries of peptides for a number of different functions. This technology has been particularly useful for determining peptide inhibitors of a particular enzyme, antibody epitope mapping, and generation of antimicrobial peptides. A major advance came in 1991 via the development of an iterative combinatorial process in which a single residue in a peptide is systematically altered while the remaining residues consist of a random mix of amino acids used in the coupling [150]. The most active peptide from each step is used as a template and the next residue is systematically altered. In this way, a single highly active peptide is generated at the end of the process. The process used is outlined in Fig. (**3**). This approach has been adapted to produce reasonably active hexapeptides (IC_{50} = 5-39 μg/ml) [151], and certain others as short as four amino acid residues (IC_{50} = 2-4 μg/ml vs *S. aureus*) when non-natural amino acids are also included in the synthesis [152]. However, one of the major shortcomings of this approach is that due to the large number of peptides within each pool, it is possible and perhaps likely, that a number of highly active peptides within each pool are discarded because they are masked by the low activity of the remaining members of the population.

This approach has been further improved by combining the ability to screen large numbers of antimicrobial compounds with knowledge of the likelihood that a given sequence will adopt a desired conformation. In this way, a known antimicrobial peptide sequence that adopts an α-helical conformation (YKLLKKLLKKLKKLLKKL-NH_2) was examined by substituting positions 4, 7, 11, and 14 on the hydrophobic face of the helix, or by substituting positions 6, 9, 13, and 16 on the hydrophilic face for other amino acids [153]. By using this approach, the original peptide which exhibited a high MIC (30-55 μg/ml) against *S. aureus* and high haemolytic activity (HD_{50} 6.1 μg/ml) was improved both with respect to MIC (2-14 μg/ml) and haemolysis (HD_{50} increased to 24>125 μg/ml) by making a number of substitutions on the hydrophilic face of the peptides, while most changes on the hydrophobic face resulted in peptides with greatly reduced activity.

Another approach that has been used for the production and screening of cationic peptides with improved activity uses recombinantly produced antimicrobial peptide fusions expressed from *E. coli*. The expression vector contains a number of elements including a gene fragment encoding the N-terminal region of RepA, a synthetic cellulose binding domain, a hexa-histidine region, a pre-proregion from human β-defensin 1, a methionine residue, and the gene encoding the antimicrobial peptide [154]. Diversity was generated in specific positions by adding mixtures of phosphoramidites at each of these positions for those residues. Sequencing the genes after they were transformed into the expression host allowed the determination of the peptide sequence of that peptide. In this way the authors were able to generate variant CP2600 peptides exhibiting altered membrane active effects [155].

CONCLUSIONS

It is clear from the literature presented within this review and others that cationic peptides represent exciting and promising new therapeutic potentials. It is perhaps even clearer that a great deal of research remains to be done before this massive potential can be truly harnessed. Studies into structure-activity relationships of certain classes of peptides, especially magainins, the synthetic KLAL class of peptides, gramicidin S, and recently pyrrhocoricin have shone new light into how these peptides exert their antimicrobial effects. Interestingly, a number of studies, particularly those examining pyrrhocoricin and certain α-helical and extended peptides are showing that the mechanism of action of cationic antimicrobial peptides is not necessarily limited to membrane effects. It will be very interesting to see how this field develops over the coming years.

Although many cationic peptides reviewed here (polyphemusins, tachyplesins, magainins) appear to be primarily antimicrobial in nature, for a number of cationic host defense peptides, especially the human classes of α-defensins, β-defensins, and LL-37, the contributions of these peptides to immunomodulatory effects appear to be more important than their direct antimicrobial effects. However despite this observation, the contribution of structure to the immunomodulatory activities of many classes of host defense peptides is still relatively unknown. These problems need to be addressed to permit the rational design of small molecule peptidomimetics, which would have even greater therapeutic potential.

ACKNOWLEDGEMENTS

Financial assistance for our peptide research has come from the Canadian Bacterial Diseases Network and the Functional Pathogenomics of Mucosal Immunity (FPMI) program grant funded by Genome Prairie and Genome BC, with additional assistance from Inimex Pharmaceuticals Inc. JM is the recipient of a Canadian Cystic Fibrosis Foundation Studentship, REWH holds a Canada Research Chair. The authors would like to acknowledge Bob Hodges for providing Fig. (**2**), and Sandeep Tamber and J. P. Powers for helpful discussion and critical reading of the manuscript.

ABBREVIATIONS

SDS = Sodium dodecyl sulfate

PC = Phosphatidylcholine

PE = Phosphatidylethanolamine

PG = Phosphatidylglycerol

CL = Cardiolipin

LPS = Lipopolysaccharide

μ = Hydrophobic moment

$\Delta\Psi$ = Transmembrane potential

GS = Gramicidin S

HPLC = High-performance liquid chromatography

CD = Circular dichroism

DSC = Differential scanning calorimetry

NMR = Nuclear magnetic resonance

SUV = Small unilamellar vesicle

EDTA = Ethylenediaminotetraacetic acid

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Received: 15 March, 2004 **Accepted: 05 July, 2004**