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Review

Function and therapeutic potential of host defence peptides¶

JOSEPH B. MCPHEE and ROBERT E. W. HANCOCK*

Department of Microbiology and Immunology and Centre for Microbial Diseases and Immunity Research, Lower Mall Research Station, University of British Columbia, 2259 Lower Mall, BC, Canada

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Abstract: Cationic host defence (antimicrobial) peptides are an important component of the innate immune systems of a wide variety of plants, animals, and bacteria. Although most of these compounds have direct antimicrobial activities under specific conditions, a greater appreciation for the diversity of functions of these molecules is beginning to develop in the field. In addition to their directly antimicrobial activities, they also have a broad spectrum of activity on the host immune system, with both pro-inflammatory and anti-inflammatory effects being invoked. Increasingly sophisticated approaches to understand the role of host defence peptides in modulating innate immunity are already serving to guide the development of novel therapeutics.

Keywords: antimicrobial peptide; clinical trials; peptide design; innate immunity

INTRODUCTION

The discovery of antibiotics in the 1930s led to one of the most revolutionary periods in the history of medicine. Alexander Fleming's discovery of penicillin, and the subsequent commercialization of this and other classes of compounds is estimated to have increased average life expectancy by up to 10 years [1]. This represented the largest single increase in life expectancy since the introduction of public hygiene protocols at the end of the 19th century. In recent years, however, society is beginning to see a gradual claw-back of these impressive early gains due in part to increasing rates of antibiotic resistance. This is further illustrated by the fact that until the introduction of linezolid in 2000, there had not been a novel class of antibiotics introduced to the market since the 1960s. This disheartening observation is further exacerbated by the alarming abandonment of antimicrobial drug discovery by a number of major pharmaceutical companies. In spite of these disheartening observations, a number of large pharma and biotech companies are still developing antimicrobial therapeutics, one class of which includes variants of natural cationic host defence peptides, also termed cationic antimicrobial peptides. We prefer the former term as several of these peptides have been shown to have both antimicrobial and immunomodulatory activities, both of which can be utilized for treatment and prophylaxis of infectious diseases. In this review, we will focus on advances in understanding structure–activity relationships for both actions of this diverse class of compounds and highlight the promising advances made in recent years to bring the potential of this class of compounds into the clinic.

The term cationic antimicrobial peptide is classically applied to a peptide with direct antibiotic activity, which is less than 50 amino acids in length, with an overall charge ranging from +2 to >+10 due to the presence of lysine and/or arginine residues. The peptide will generally adopt an amphipathic or amphiphilic structure either in solution, or more typically, upon interaction with membranes. This attribute is considered crucial for activity because, in most cases, cationic peptides must interact with a biological membrane in order to exert their activity. Most studies with cationic peptides have tended to focus exclusively on their potential as direct antimicrobials. This antimicrobial activity is an important one for many peptides that have been studied, but especially for the natural peptides, there is an important caveat. To merit the descriptor antimicrobial, the peptide(s) should exhibit significant antimicrobial activity under physiological conditions and at a local concentration consistent with that observed *in vivo*. Although the concentration at particular *in vivo* sites, especially living tissues, is often difficult to measure accurately, this definition serves to discriminate between peptides for which antimicrobial activity is the most important function and those for which immunomodulatory activity is more important. These activities are not mutually exclusive, and a single peptide can, in principle, express these different activities at separate tissue sites; however, a number of recent studies have

* Correspondence to: R. E. W. Hancock, Department of Microbiology and Immunology and Centre for Microbial Diseases and Immunity Research, Lower Mall Research Station, University of British Columbia, 2259 Lower Mall, Room 232, Vancouver, BC, Canada, V6T 1Z4;
e-mail: bob@cmdr.ubc.ca
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demonstrated that these activities are separable [2,3]. Both antimicrobial and immunomodulatory cationic peptides are widespread throughout the living kingdom, being found in virtually every species examined. Although depending upon the host species and site of infection examined, the relative contribution of each type of activity to defence may vary.

CLASSES OF CATIONIC PEPTIDES

In spite of the structural diversity displayed by cationic peptides, they are generally capable of folding into an amphipathic or amphiphilic structure, either in free solution or upon interaction with biological membranes. Many cationic peptides can be roughly classified on the basis of their major structural class. It should be stressed that although these categories are useful for describing the structural folds that a given peptide possesses, there is little, if any, relationship between the class of peptide and any description of its biological activity. Table 1 displays some of the physical properties of peptides described in this review.

The most widespread and the best-characterized class of cationic peptides is the amphipathic α-helical class of peptides. These are peptides that can, upon interaction with membranes, adopt an α-helical conformation, in which one face of the helix contains a large proportion of polar amino acids, while the opposite face contains a majority of hydrophobic residues. Members of the α-helical class of compounds include the frog skin secretion peptide, magainin, the honeybee venom, mellitin, and the dipteran insect defence peptides, the cecropins. These three peptides are highly antimicrobial, but also exhibit selective toxicity toward mammalian cells [4–6]. Thousands of synthetic α-helical peptides have also been synthesized and studied for the relative contributions of charge, hydrophobicity, hydrophobic moment, and helicity to their antimicrobial and hemolytic activities [7–10].

Mice, rats, and humans all possess α-helical members of the cathelidicin family of peptides. In the mouse, this peptide is called cathelin-related antimicrobial peptide (CRAMP), while the rat homologue is named rCRAMP. Humans also contain a homologue of this peptide called hCAP18/LL-37. Although LL-37 has been shown to be antimicrobial *in vitro*, it is severely antagonized by the presence of physiological salt concentrations, especially of divalent cations, and it is thus likely that its antimicrobial role has been overstated in the past [11].

The second major class of cationic peptides includes the β-sheet class of peptides. This is a large family of peptides that is defined by the presence of two or more β-strands, stabilized by the presence of one or more disulphide bonds. This large class of peptides includes several sub-classes of antimicrobial and host defence peptides, especially the α-defensin and β-defensin classes of mammalian peptides. Recently, another class of defensins has been discovered in rhesus macaque neutrophils, the θ-defensins, which are cyclic [12]. Humans possess a homologous pseudo-gene to the θ-defensin gene, but a premature stop codon prevents it from being expressed [13]. The α-defensins contain from 29 to 35 residues and three disulphides that stabilize the three-stranded β-sheet. These disulphides are formed between C1→C6, C2→C4, and C3→C5. This linkage pattern differentiates them from the β-defensins, which contain 34–47 residues and are linked C1→C5, C2→C4, and C3→C6 [14].

A particularly potent group of β-stranded cationic peptides have a hairpin structure (antiparallel β-strands) interconnected by a type II β-turn [15,16]. They are stabilized by the presence of one or two disulphide bonds between the β-strands. The prototypes of the double disulphide stabilized peptides are the tachyplesins and polyphemusins which are the major components of the innate immune system of Asian and American horseshoe crabs, respectively [17,18]. These peptides show an exceptionally potent salt-insensitive antimicrobial activity against Gram-negative and Gram-positive bacteria, as well as antifungal activity [19]. There is also a burgeoning interest in this class of peptides because homologues of polyphemusin II have been shown to be specific inhibitors for CXCR4, the receptor required for HIV internalization [20]. Possibly related to these peptides is a small class of cyclic peptides containing a single disulphide bond with a β-turn, in between the interconnected cysteine residues, e.g. bactenecin or dodecapeptide from cattle neutrophils [15,16].

A class of extended cationic peptides is not really defined structurally, but rather for its lack of typical secondary structure. These peptides usually contain a high proportion of amino acids that promote the adoption of novel folds. Examples of this class include the bovine neutrophil peptide indolicidin and the porcine peptide fragment, tritrpticin. These peptides have tryptophan residues at 3 (tritrpticin) or 5 (indolicidin) of their 13 amino acid positions. Indolicidin and tritrpticin exhibit moderate broad-range activity against both Gram-positive and Gram-negative bacteria. The tertiary structure of both these peptides has been solved through 2D-NMR [21,22]. They are characterized by the formation of a boat-like extended structure upon interaction with diphosphotidylcholine (DPC) micelles. The tryptophan-rich regions in the middle of the peptides interact with one layer of the membrane, orienting such that they straddle the aqueous/membrane interface [23]. The *N*- and *C*-termini, containing the cationic lysine and arginine residues are oriented toward the aqueous environment. Histatins are a class of peptides produced by human salivary glands. They contain a very high

proportion of histidine residues (18–29%), are highly cationic (+7 to +8), and exhibit antimicrobial activity against fungi [24]). Other members of this class include a series of proline-rich peptides. These can be found as the proteolytic peptide fragments of larger peptides like Bac-5 and Bac-7 from bovine neutrophils. These peptides have the ability to translocate model membrane systems, and also have antimicrobial activity. Several studies have shown that these two activities are separable [25]. Peptides rich in proline are also found in many species of insects [4]. Another group of glycine-rich peptides, have also been identified in the skin secretion of a number of amphibian species, and in a large number of insect species [4,26]. Members of the glycine-rich peptide family may also have some structural similarities to other classes of peptides such as melittin, cecropin, or attacin. A number of insect-derived antimicrobial peptides belonging to proline-rich and glycine-rich families may also be modified by the addition of glycosyl groups [4,27].

TARGETS OF CATIONIC ANTIMICROBIAL PEPTIDES

For many years, all cationic antimicrobial peptides were assumed to act through membrane permeabilization. Although this may be true at very high concentrations (well above the minimal inhibitory concentration (MIC)), it is now well established that this is not the case for many peptides at their lowest effective concentrations. It is also clear that even for those peptides that are membrane active, a remarkable heterogeneity may exist in the mode of interaction of a particular peptide for a particular membrane system. For peptides that appear to have non-membrane targets, although the mechanism of action is often not highly specific, it appears to involve multiple targets and thus can be highly effective, as evidenced by the very low rates at which cationic antimicrobial peptide resistance emerges [28,29].

Membrane Targets

Cationic antimicrobial peptides generally take advantage of the intrinsic differences between eukaryotic and prokaryotic membranes (Figure 1). The major differences are the much higher concentration of negatively charged lipids on the surface monolayer of bacterial cytoplasmic membrane, the high electrical potential gradient across these membranes, and the high proportion of unusual lipids like cholesterol in eukaryotic membranes. Bacterial membrane lipids typically consist of approximately 30% anionic phosphatidylglycerol and cardiolipin and 70% phosphatidylcholine. This excess of anionic lipids contrasts with the outer monolayer of the membrane of higher eukaryotes, which

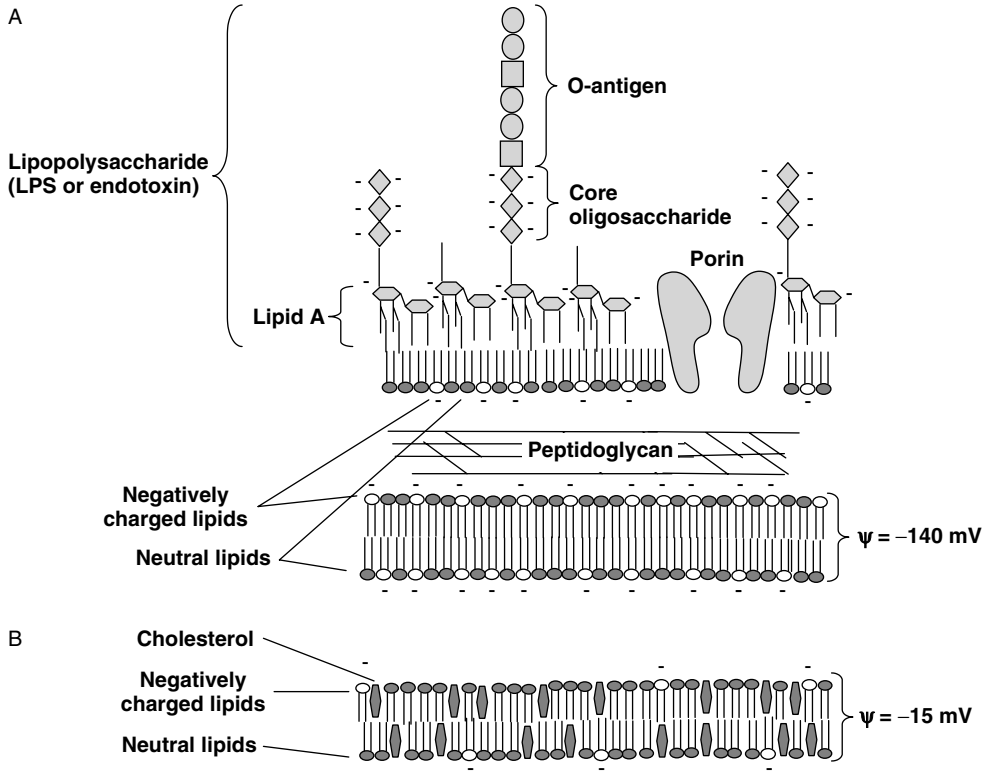

Figure 1 (A). Major characteristics of the Gram-negative bacterial cell envelope. Note the high proportion of negatively charged lipids as well as the anionic character of the LPS and the large transmembrane potential. (B). Major lipid components of a typical eukaryotic membrane. Note the lower proportion of anionic lipids as well as the high percentage of cholesterol.

Table 1 Primary Sequence and Structural Class of Cationic Peptides Described in this Review

Peptide	Source	Structural class	Sequence
Cecropin A	Silk moth (*Hyalophora cecropia*)	α-helical	KWWLFKKIEKVGQNIRDGIIKAGPAVVGQ ATQIAK
Magainin II	Clawed frog (*Xenopus laevi*)	α-helical	GIGKYLHSAKKFGKAWVGEIMNS
Buforin II	Asian toad (*Bufo bufo* gargarizans)	α-helical	TRSSRAGLQFPVGRVHRLLRK
CRAMP	Mouse (*Mus musculus*)	α-helical	ISRLAGLLRKGGEKIGEKLKKIGQKIKNFF QKLVPQPE
LL-37	Human (*Homo sapiens*)	α-helical	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRN LVPRTES
Melittin	Honey bee (*Apis mellifera*)	α-helical	GIGAVLKVLTTGLPALISWIKRKRQQ
hBD-1	Human (*Homo sapiens*)	β-sheet β-defensin	DHYNC$_1$VSSGGQC$_2$LYSAC$_3$PIFTKIQGTC$_2$ YRGKAKC$_1$C$_3$K
HNP-1	Human (*Homo sapiens*)	β-sheet α-defensin	AC$_1$YC$_2$RIPAC$_3$IAGERRYGTC$_2$IYQGRLWA FC$_3$C$_1$
RTD-1	Rhesus macaque (*Macaca mulatta*)	β-sheet θ-defensin	Cyclic RC$_1$IC$_3$TRGFC$_1$RC$_2$LC$_3$RRGVC$_2$
Polyphemusin I	American horseshoe (*Limulus polyphemus*)	β-sheet	RRWC$_1$FRVC$_2$YRGFC$_2$YRKC$_1$R
Indolicidin	Cow (*Bos taurus*)	Extended (W-rich)	ILPWKWPWWPWRR
Tritrpticin	Pig (*Sus scrofa*)	Extended (W-rich)	VRRFPWWWPFLRR
Histatin I	Human (*Homo sapiens*)	Extended (H-rich)	DSHEERHHGRHGHHKYGRKFHEKHHSH RGYRSNYLYDN
Bactenecin 5	Cow (*Bos taurus*)	Extended (P-rich)	RFRPPIRRPPIRPPFYPPFRPPIRPPIFPPIRPP FRPPLGPFP
Pyrrocoricin	Sap-sucking bug (*Pyrrhocorus apterus*)	Extended (P-rich)	VDKGSYLPRPTPPRPIYNRN
Drosocin	Fruit fly (*Drosophila melanogaster*)	Extended (P-rich)	KPRPYSPRPTSHPRPIR
Apidaecin	Carder bee (*Bombus pascuorum*)	Extended (P-rich)	GNRPVYIPPPRPPHPRL

Table 2 List of Cationic Antimicrobial Peptide Drugs, Company Responsible for Commercialization, Testing Status, and the Disease it is Designed to Treat

Compound	Company	Testing status	Disease drug designed to treat
Pexiganan	Magainin Pharmaceuticals	Phase III completed	Infection of diabetic foot ulcers
Iseganan	IntraBiotics Corporation	Phase III halted prematurely	Ventilator-associated pneumonia
rBPI21	Xoma Ltd.	Phase III completed	Severe bacterial meningitis
Omiganan	Migenix (formerly Micrologix)	Phase III completed	Infection at site of in-dwelling catheter insertion
IMXC001	Inimex Pharmaceuticals	Preclinical	Sepsis

typically contains predominantly uncharged lipids at neutral pH (Figure 1B). This increases the electrostatic interactions between cationic peptides and the bacterial cytoplasmic membrane. In addition to this significant structural difference, the transmembrane electrical potential of bacteria is much higher than that of eukaryotic cells (~-15 mV), being approximately −140 mV (i.e. tending to 'electrophorese' the cationic peptides from the external surface of the cytoplasmic membrane into the membrane and/or cytoplasm).

The structural differences between Gram-negative bacterial outer membrane and eukaryotic surface membranes are even more unusual (Figure 1A). The outer membrane is an asymmetric bilayer, with a lipid composition of the inner phospholipid monolayer similar to that of the cytoplasmic membrane. The outer leaflet consists of a matrix of lipopolysaccharide (LPS) and an amphiphilic polyanionic glycolipid consisting of three main components, Lipid A, core oligosaccharide, and O-antigen. The Lipid A moiety is the most responsible for the barrier functions of the outer membrane and consists of a diglucosamine sugar unit that is phosphorylated at its C1 and C 4′ positions. The phosphate groups are bridged by divalent Ca^{2+} or Mg^{2+} cations, which serve to stabilize the LPS by preventing the charge–charge repulsion that would normally occur. Lipid A is N- or O-linked to between four and seven acyl chains that form the outer leaflet of the outer membrane. The core oligosaccharide is linked

to the Lipid A moiety through a unique sugar, 2-keto-3-deoxyoctulosonic acid (KDO). The core oligosaccharide may also be extensively modified by the addition of phosphates, pyrophosphates, ethanolamine and amino acids. The presence of phosphates and pyrophosphates in the core oligosaccharide, the O-antigen and the Lipid A region all contribute to the high negative charge on the surface of the Gram-negative outer membrane. Peptides interact with the divalent cation binding sites on LPS and cause localized disruption of the outer membrane, leading to what has become known as self-promoted uptake of the peptide across the outer membrane.

There are several physical parameters that mainly determine whether or not a given peptide will tend to interact preferentially with prokaryotic or eukaryotic membranes. Although many of these principles have been determined using wholly synthetic peptide variants, they can be applied to improving the activity of natural peptide variants. The first variable is charge, with a strong relationship existing between overall charge of a particular peptide and the strength of the initial electrostatic interaction, thus influencing antimicrobial activity [7,30]. Another important variable is hydrophobicity, in that, as hydrophobicity increases, a corresponding increase in activity is generally seen. However, once hydrophobicity exceeds a certain level, selectivity between prokaryotic and eukaryotic membranes is lost, with a concomitant increase in cytotoxicity [8]. For a more detailed discussion on structure–activity relationships in several classes of antimicrobial peptides, readers are referred to a number of recent reviews [31–33].

To be considered as membrane active, a peptide should exhibit significant membrane disruptive activity at the MIC. In theory this may involve complete membrane dissolution (although in our experience this is rare), the formation of small holes which may lead to the loss of the transmembrane electrochemical gradient, or the leakage of intracellular contents leading to cell death (i.e. lysis which is similarly rare). The actual mechanism by which membrane disruption occurs is a matter of much debate and is outside of the scope of this review. However, a number of excellent reviews have been written on the subject recently and the reader is referred to them for detailed discussions [31–34]. Our own suggestion is that peptides first associate with the outer monolayer of the cytoplasmic membrane and insert into a position parallel to the bilayer at the interface of the hydrophilic and hydrophobic portions of the membrane. At a given critical concentration, peptide molecules reorient themselves to form transmembrane aggregate containing lipid and peptide molecules, and create conductance pathways that permit the leakage of protons, other ions, and possibly larger molecules [35,36].

Intracellular Targets

An expanding body of evidence suggests that many cationic peptides have intracellular targets. In fact, it has been known for some time that cationic peptides are capable of translocating across the cytoplasmic membrane without causing significant leakage of intracellular contents or loss of transmembrane potential [37]. Additionally, it was demonstrated that a human neutrophil peptide (HNP-1) and a human platelet microbicidal protein 1 (tPMP-1) exert their bactericidal effect by interfering with intracellular processes [38]. Consistent with this, pretreating *Staphylococcus aureus* cells with either a DNA gyrase inhibitor (novobiocin) or with protein synthesis inhibitors (azithromycin, quinupristin, or dalfopristin) significantly reduced the killing effects caused by exposure to tPMP-1 or HNP-1, while antagonism was demonstrated between tetracycline and tPMP-1 [38]. Other groups also observed antagonism between cationic peptides like buforin II, magainin II, and cecropin P1 and the protein synthesis inhibitor chloramphenicol, toward the opportunistic pathogen *Stenotrophomonas maltophilia*, although the authors did not deem the antagonism to be significant [39].

Similarly, several proline-rich insect antimicrobial peptides including pyrrhocoricin, apidaecin, and drosocin inhibited the activity of the cytoplasmic heat shock chaperone DnaK [40]. This activity was shown to involve the inhibition of ATPase activity by pyrrhocoricin and required specific residues in the pyrrhocoricin peptide [41,42]. This inhibition of DnaK activity led to the accumulation of misfolded proteins within the cytoplasm, resulting in cell death. Similarly, the overexpression of GroE and DnaK in a sensitive background strain decreased the growth inhibitory effect of magainin 2, buforin II, and poly-L-lysine [43]. These results thus support the concept that several cationic peptides have intracellular targets.

Work from our lab has shown that, at concentrations of twice the MIC where little killing was observed, indolicidin and the indolicidin variants CP11CN and CP10A caused strong inhibition of RNA and protein synthesis in *S. aureus*, with more modest effects on DNA synthesis [44]. Exposure to twice the MIC of the cecropin–mellitin hybrid protein CP29 also led to a decrease in macromolecular synthesis, but with preferential effects on DNA and protein synthesis, and lesser effects on transcription [45]. Exposure to the bactenecin analogue Bac2A showed drastic losses of all three types of macromolecular synthesis at both twice and ten times the MIC. Generally, in these experiments, there was little killing observed at twice the MIC while at ten times the MIC, the decrease in macromolecular synthesis was accompanied by a 1–4 log order reduction in cell number, depending on the peptide examined. These data are thus consistent with the hypothesis that peptides can enter bacterial cells, as opposed to merely interacting with and destroying

bacterial membranes. Electron microscopic studies of the action of these peptides were similarly consistent with this conclusion [46]. Similar trends were observed when a Gram-negative bacterium *Escherichia coli* was exposed to certain derivatives of the winter flounder peptide, pleurocidin [47], in that changes in macromolecular synthesis occurred in the absence of major changes in cell viability or membrane potential. It is important to note, however, that at a concentration equal to 10 times the MIC, pleurocidin derivatives could completely collapse the membrane potential, indicating that at high enough concentrations, damage to the membrane could ensue.

Strong, but indirect, evidence that cationic peptides exert profound intracellular effects on bacteria comes from the evidence that bacteria undergo a specific adaptive response to sub-MIC concentrations of cationic peptides. The first instance of this came from work in *E. coli*, where exposure to the insect defence peptides cecropin A or cecropin B resulted in the specific upregulation of the *osmY* and *micF* genes [48,49]. These genes are involved in the *E. coli* hyperosmotic response and their upregulation is consistent with a mechanism of action that involves similar stresses. Transcriptional profiling of the *E. coli* response to cecropin A also indicated that this peptide could cause major changes in gene expression at sub-inhibitory concentrations [50]. Of note is the fact that these peptides were previously suggested to act on membranes, and yet clearly have effects on cells at concentrations below their MICs.

A link has also been established between pre-exposure to sub-MIC cationic peptides and resistance. In both *Pseudomonas aeruginosa* and *Salmonella enterica* sv. Typhimurium (S. Typhimurium), exposure to cationic peptides at concentrations below the MIC led to a marked induction of specific cationic peptide resistance determinants [51,52]. These include the *pmrHFIJKLM* genes of *P. aeruginosa*, and homologous genes in *Salmonella.* This operon is involved in modifying the surface of the bacteria to be less charged, and thus lowers the strength of the initial ionic interaction of cationic peptides with the bacterial cell envelope. In *Salmonella*, peptide-directed upregulation depends on the PhoPQ two-component regulatory system, while in *P. aeruginosa*, it is independent of both PhoPQ and a second two-component regulation system PmrAB. Intriguingly, the concentration of cationic antimicrobial peptide in *P. aeruginosa* that leads to an increase in resistance can be as little as 1% of the MIC, indicating that in this species at least, and possibly many others, bacteria have evolved a system to respond to the presence of certain cationic antimicrobial peptides to reduce their activity.

Owing to the large number of effects that cationic peptides can exert on bacterial cells, we initially suggested a 'multi-hit' mechanism of action [32]. This model predicts that due to their highly charged nature, cationic antimicrobial peptides that are capable of translocating across the cytoplasmic membrane will be able to tightly bind to and inhibit the activity of a number of anionic molecules, including enzymes and nucleic acids. Consistent with this concept, a recent publication demonstrated direct inhibition of aminoglycoside modifying enzymes. These enzymes, which are noted for having an open anionic aminoglycoside binding surface, can be specifically inhibited by several cationic peptides belonging to a variety of different structural classes [53]. This data, along with the observation of macromolecular biosynthesis inhibition as outlined above, provides some support for the multi-hit hypothesis. This multi-hit hypothesis also helps to explain why it is so difficult for bacteria to evolve high-level resistance to cationic antimicrobial peptides.

PEPTIDE DESIGN

There are more than 700 peptides known from Nature [54] and more than 50 individual structures solved [32]. This provides tremendous impetus to the design of novel and improved antimicrobial peptides. To date, most peptide development has involved a structure-assisted design in which a modest number of changes are made to the parent molecules to optimize certain key features. These features usually include amphiphilicity (separation of charged/polar and hydrophobic residues into separate regions in the final peptide structure), number of charged and hydrophobic residues, flexibility, and compatibility with a particular secondary structure. Many of these design principles, especially with the α-helical structural class have come from the study of the highly simplified Lys-Leu-rich peptides. These are peptides that contain only – two to three different amino acid residues. They have been used to elucidate the role of helicity, hydrophobic moment, overall hydrophobicity, and the stereospecificity to antimicrobial activity [7,9,55]. Overall, this approach has been reasonably successful in optimizing activities and molecules with activities equivalent to the very best antibiotics against particularly recalcitrant (multiply antibiotic resistant) bacteria. However, such an approach limits the number of organisms that can be screened. For this reason, we are interested in developing approaches that permit random screening of peptides. To date, there are several promising approaches. The first, developed by Blondelle and Houghten [56], involved the construction of combinatorial libraries of peptides, followed by a mixed peptide deconvolution strategy. Unfortunately, such a strategy is somewhat limited to smaller peptides, although this permitted the authors to screen a hexamer library and identify a six amino acid peptide with antimicrobial activity for *S. aureus*. A second

strategy would involve recombinant procedures in which a recombinantly produced peptide was randomly mutated by introducing mixed nucleotides at various positions of synthetic genes encoding the peptide in question. Unfortunately, the screening procedures with these recombinantly produced peptides are difficult. A third method that we are currently developing is to robotically synthesize multiple peptides on cellulose supports [57].

CATIONIC HOST DEFENCE PEPTIDES AS EFFECTORS OF INNATE IMMUNITY

The case for a primary role for natural cationic peptides in host defences is becoming increasingly convincing [14,58–62]. While this involves in part the direct antimicrobial action of these peptides, we have argued that the concentrations found at many body sites (e.g. the mucosa) are inconsistent with a primary role in direct antimicrobial (killing) action, although it seems certain that very potent peptides (e.g. pig protegrin) and those found in high concentrations at specific body sites (e.g. α-defensins in phagocytic granules or in intestinal crypts at mg/ml concentrations) are able to directly kill microbes. Recent data have indicated that peptides have an important (immunomodulatory) role in the orchestration of innate immunity. Indeed, many of these functions, including an ability to mediate protection against endotoxic shock [63], the promotion of wound-healing [64,65], the stimulation of chemokine synthesis [66] and mast cell chemotaxis [67], the promotion of angiogenesis [68], and an adjuvant activity in the adaptive immune response [69] have been demonstrated in animal models in the complete absence of infectious organisms. It is further worth noting that the role of these host defence peptides is quite novel; unlike the natural innate immune response, there is a mixture of anti-inflammatory and pro-inflammatory responses, such that the peptides can resolve infections even while suppressing the production of pro-inflammatory cytokines [58].

There is now a growing body of evidence for an impressive variety of immunomodulatory activities of cationic peptides other than direct killing. Such activities would be expected to impact on the quality and effectiveness of innate immune responses and inflammation (see [11,59–61,70] for reviews) but have been only studied sketchily for any given peptide and have not been demonstrated *in vivo*. These activities include (a) direct chemokine activity in attracting neutrophils, monocytes, mast cells, and T helper cells and the ability to induce the production and release of neutrophil and monocyte chemokines from host cells, leading to recruitment of cells of innate immunity to the infection site, (b) promotion of mast cell degranulation leading to histamine release and consequent vasodilation (increase in the permeability of blood vessel to various cells and proteins), (c) the promotion of polarized dendritic cell differentiation, leading to alterations in the function of these cells, (d) the promotion of nonopsonic phagocytosis, (d) the inhibition of fibrin clot lysis by tissue plasminogen activator, which would tend to reduce bacterial spreading, (e) tissue and wound repair through promotion of fibroblast chemotaxis and growth, and (f) promotion of angiogenesis in endothelial cells, and so on. These responses are pro-inflammatory but represent only a moderate subset of typical pro-inflammatory responses to infectious agents and their signalling molecules. It is well known that bacterial molecules like LPS and lipoteichotic acid (LTA) stimulate innate immunity in part by interacting with Toll-like receptors [71,72]. Therefore, it is noteworthy that cationic host defence peptides actually suppress the LPS/LTA-stimulated production of pro-inflammatory cytokines like TNFα and IL6 [59,73]. Consistent with the many responses invoked by cationic peptides *in vitro* and *in vivo*, it has been demonstrated that they induce the expression of hundreds of genes in cells of innate immunity, including monocytes/macrophages and epithelial cells ([66] and R. E. W. Hancock, unpublished results). They do this in part by binding to and trafficking into such cells [74], and stimulating the activation (phosphorylation of the MAP kinases Erk 1/2 and P38) [54,75].

To confirm that these observations have therapeutic potential, we constructed peptides with no direct antimicrobial activity. Despite this, these peptides were able to protect mice against infections with both Gram-positive (*S. aureus*) and Gram-negative (S. Typhimurium) bacteria [11], presumably by boosting innate immunity.

COMMERCIAL DEVELOPMENT AND CLINICAL TRIALS

Although several large pharmaceutical companies have stopped development of new antimicrobials, a number of cationic antimicrobial peptides developed by smaller biotechnology companies have moved into clinical trials, with mixed results. To date, these trials have involved peptides that are primarily antimicrobial in nature, although this may soon change. Table 2 displays a list of cationic peptides that have been designed for therapeutic use and the outcome of clinical trials for these peptides.

Pexiganan (MSI-78) was developed by Magainin Pharmaceuticals (now Genaera of Plymouth Meeting, PA) as a topical antimicrobial for the treatment of infected diabetic foot ulcers. It is a variant of the amphibian peptide magainin 2 with a slightly modified C-terminus to improve its spectrum of activity and to increase stability. It was intended for use in patients

with mild to moderate infections as an alternative to systemic antibiotics, which suffer from limited access to the infected ulcer and a certain degree of toxicity. In Phase III clinical trials, treatment with pexiganan resulted in wound-healing and clinical outcome that was equivalent to those in control subjects treated with ofloxacin. The drug possessed a good safety profile with few reports of toxicity [76]. In spite of these positive results, the FDA rejected the new drug application (NDA) in 1999 as it did not offer greater benefit than the current standard of care.

IntraBiotics Corp. of Mountain View, California developed Iseganan, an analogue of protegrin for treatment of several conditions. These included oral mucositis, an inflammation of the oral mucosa that often follows chemotherapy, lung infections in individuals with cystic fibrosis (CF), and lung infections causing ventilator-acquired pneumonia (VAP). In 2002, results of phase III trials of iseganan for the treatment of oral mucositis showed no difference in clinical outcome between patients receiving iseganan and control patients receiving a placebo [77]. VAP is a common complication of long-term mechanical ventilation, affecting ~15– 30% of individuals who require ventilation for more than 48 h. In 2003, iseganan was granted FDA fast-track status, allowing the rapid enrolment of patients in trials for a treatment that would fulfill a previously unmet need. In 2004, only nine months after the first patients were enrolled, the trial was stopped prematurely due to higher rates of VAP and mortality in patients receiving iseganan. Pre-clinical studies showed that the drug was effective in the rat model of CF *P. aeruginosa* infections. Although the drug also passed phase I safety trials for treatment of CF-associated lung infections, it is no longer in development as an anti-infective agent.

Bactericidal/permeability increasing (BPI) protein is a component of normal human serum, where it is found as a major component of neutrophil granules. During infection, these neutrophils are attracted to the infection site where degranulation occurs, leading to a high localized concentration of BPI that reduces septicaemia and is proposed to cause killing of Gram-negative bacteria at the site. A recombinant version of a fragment of BPI ($rBPI_{21}$) was developed for treatment of bacterial meningitis by Xoma Ltd. of Berkeley, CA. Clinical trials of $rBPI_{21}$ were carried out on children with severe symptoms of meningitis [78]. The trial was unable to show a statistically significant decrease in mortality compared to a control group. Although the drug did not decrease mortality, it did result in improvement in a number of clinical parameters. An analysis of the data collected in the clinical trial suggested that a multi-endpoint trial may have significantly improved the likelihood of a positive regulatory outcome [79]. Currently, $rBPI_{21}$ has been licensed to Zephyr Sciences of New York City, for development against a number of endotoxin-mediated diseases.

There is also a great deal of interest in treatments that will reduce the number of infections at the site of in-dwelling catheter insertion. In North America, approximately five million in-dwelling catheters are inserted every year, which, in turn, lead to 250 000 to 400 000 infections at the insertion site per year. Migenix (formerly Micrologix) (Vancouver, BC) developed a peptide, based on the bovine peptide indolicidin, for the prevention of infection at sites of in-dwelling catheter insertion. Omiganin (MBI-226) is a topical antibiotic formulation that is applied daily to the peripheral IV insertion site. In phase III clinical trials, the patients receiving the drug experienced a 15% decrease in the rate of infection, which was not considered statistically significant. While the drug did not achieve its primary endpoint of reduced rate of infections, a secondary endpoint of reduced catheter colonization was observed (40% reduction in the group receiving omiganan) as well as a 50% decrease in tunnel infections, and is providing the impetus for a follow up Phase IIIb trial with these endpoints, partnered with Cadence Pharmaceuticals.

Considering these cases, it is worth considering what still needs to be done to move cationic peptides toward commercialization. With the exception of $rBPI_{21}$, the examples listed above all involved topical treatment of difficult infections, and ensuing difficulties in showing efficacy over existing treatment regimens. Topical treatments have the advantage of limiting potential toxicities inherent in systemic drug usage. Unfortunately, the majority of infections that are life threatening, especially those due to multidrug resistant bacteria, require systemic treatment, and no instructive studies of cationic peptide-mediated toxicity have been published. Thus, in moving forward, great emphasis must be placed on studying and understanding any toxicity issues associated with this class of drugs.

An alternative approach would involve utilizing the immunomodulatory activity of cationic peptides as a basis for therapy. Although conventional antibiotic therapy is bedevilled by the emergence of antibiotic resistance, the stimulation of innate immunity, while not inducing or even suppressing harmful pro-inflammatory responses, is an attractive alternative because of the fact that such immunomodulators would act on host cells rather than attack bacteria directly. This approach should prevent resistance from occurring. However, designing drugs that will stimulate innate immunity is significantly more challenging, as there is no convenient *in vitro* susceptibility assay to drive development/refinement of drugs.

We recently reviewed in detail the arguments for utilizing peptides as a potential alternative to antibiotics, by boosting host innate immunity [70]. Inimex Pharmaceuticals (Vancouver, BC) has developed a number of promising leads that are completely

devoid of antimicrobial activity *in vitro* [11]. The peptide IMXC001, now in pre-clinical trials, is a short peptide, based on a natural cationic host defence molecule, which was designed to eliminate antimicrobial activity, while retaining an appropriate immunostimulatory profile. They have a reduced charge compared to the parent peptide and are 11–13 amino acids in length. This peptide does not elicit pro-inflammatory cytokine release (TNF-α), nor is it cytotoxic at concentrations of up to 1 mg/ml. However, in animal models of infection, treatment with IMXC001 significantly reduced bacterial load in the spleen compared with controls [11].

OUTLOOK

When cationic antimicrobial peptides were first brought to clinical trials, there was a great deal of excitement at the prospect of introducing the first novel class of broad-spectrum antimicrobials to the market since the 1960s. Due to a number of complications during clinical development, some of that initial excitement has become muted. We feel that while the results of clinical trials have been somewhat disappointing to date, there is much to remain enthusiastic about. As topical antimicrobials, this class of compounds has started to show some promise and is continuing to be developed for this purpose. Even more exciting, is the increased realization that these compounds have potent immunomodulatory activity. While a number of practical and regulatory hurdles will undoubtedly arise during the development of host defence peptides as immunostimulatory compounds, results from our lab and others suggest that this approach may be an effective treatment for the increasing number of individuals with multidrug resistant bacterial infections.

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