Richard M. Epand *Editor*

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Preface

Host defense peptides have been studied over several decades. Interest in the application of these agents for therapy is growing. This book surveys our current state of knowledge of host defense peptides and considers their potential for clinical application as well as some of the barriers to this development.

Host defense peptides are part of the innate immune system of multicellular eukaryotes. The range of organisms in which host defense peptides have been discovered is large. These peptides have diverse structures as reviewed in the chapter by Monique van Hoek. These peptides have been most extensively studied as antibacterial agents. They have antimicrobial activity not only in the host in which they are produced, but many of these agents have been shown to be effective when administered to other hosts. In this chapter some of the unanswered questions and ongoing areas of development are highlighted in boxes inserted in the text. The activity of many of these agents is not limited to bacteria, but as reviewed by Lohner and Leber, some of these agents are antifungal. The structure and chemical composition of fungi are more similar to mammalian cells than they are to bacteria. Nevertheless, differences exist in fungi that can be exploited for developing antifungal agents. These include the nature of their cell wall, the structure of their membrane sterol, ergosterol that is different from cholesterol, as well as the chemical structure of fungal sphingosine. Host defense peptides can also be immunomodulatory and inflammo-modulatory. It is therefore not surprising that these agents can also have antiviral activity. The antiviral activity of host defense peptides is reviewed by Sousa, Casanova, Stevens, and Barlow. In addition to their stimulation of inflammation and the immune system, these antiviral host defense peptides can also directly affect viral particles and have broad spectrum antiviral activity. The mechanism of action of some of these agents is summarized in a table and the therapeutic potential of the host defense peptides as antiviral agents is discussed. A very different application of host defense peptides is discussed by Gaspar and Castanho, regarding their use in cancer therapy. There is evidence

suggesting this as a possible application of these agents, but it is suggested that further development of the application of host defense peptides in this area will require a more complete understanding of their mechanism of anticancer action. An application of host defense peptides not often discussed is that of plant host defense peptides and their possible application in agriculture. Goyal and Mattoo review this field and show that host defense peptides from plants are structurally diverse and have a variety of mechanisms of action, including damaging the cell membrane as well as having intracellular targets.

One of the properties of host defense peptides is that they indirectly protect against pathogens by mechanisms involving inflammation and immunity. Eicosanoids play an important role in regulating innate immunity and host defense. One source of the interaction is from the influence of eicosanoids and of arachidonic acid in the expression of host defense peptides. In addition, some host defense peptides stimulate the synthesis of eicosanoids, which themselves are immunomodulatory. The relationship between host defense peptides and eicosanoids is outlined in the chapter by Wan, Tang, and Haeggström.

One of the reasons that there is an immediate need to develop novel and potent host defense peptides is that many organisms are developing resistance to traditional antibiotics. It had been initially thought that since antimicrobial peptides have been effective throughout evolution it would be less likely that resistance would develop. In addition, many of these agents act at the level of the membrane of the pathogen, giving less opportunity for the development of altered metabolic or genetic properties of the pathogen. However, over time resistance has developed to virtually every antibiotic. In long term, it might require something like multidrug evolution strategies to reverse antibiotic resistance (Baym et al. 2016). However, until such strategies become developed, drugs to inhibit resistance mechanism may provide an interim solution, as described by Phoenix, Dennison, and Harris. There is also a family of compounds that has been used in conjunction with traditional antibiotics to reverse multidrug resistance in bacteria. These agents are oligomers of acyl-lysines that are described in the review by Mor. These compounds can also be linked to insoluble resins for the removal and detection of bacteria.

While there is a large variety of chemical structures and mechanisms of action of natural host defense peptides, none of them have properties to make them ideally suited for therapeutic application. There have thus been efforts, such as those described by Mor, to design novel agents. In addition to novel compounds like the oligo-acyl-lysines, efforts are being made to utilize the common features of host defense peptides. Wang describes the development of an algorithm to reveal the features that are common among host defense peptides from a wide range of organisms. He suggests that there are two alternative paths that can be used to optimize the properties of the designed peptide. One is by a combinatorial synthesis and drug screening. The other is by structure-based rational drug design. The two methods are not mutually exclusive, but can be used in combination. In the next

article, Deshayes, Lee, Schmidt, Xian, Kasko, and Wong present the dilemma that most antimicrobial peptides are both non-specific and toxic to the host and are of low potency against pathogens, requiring the use of high concentrations. However, the alternative is the use of antibiotics that are highly potent and specific but against which the resistance easily develops. This chapter suggests that hybrid molecules can be designed to combine the best features of non-specific antimicrobial peptides with the high potency of antibiotics. Additionally, the drug can be made resistant to proteolytic degradation by using β-peptide linkages.

The final section discusses the perspectives for a more widespread clinical use of host defense peptides. The role of bacterial infection as a contributor to preterm birth in humans is discussed in the chapter by James and Bajaj-Elliott. Among the host defense mechanisms are the actions of host defense peptides. Mansour, Hancock, and Otto focus their discussion specifically on the treatment of infections by *Staphylococcus aureus*. This is the most abundant bacteria in the microbiome of the skin. This bacteria has developed resistance to almost all known antibiotics. Infections with methicillin-resistant *Staphylococcus aureus* (MRSA) have claimed more lives than HIV/AIDS. Challenges to the development of therapies based on the use of host defense peptides include the low potency and the weak specificity of these agents, thus creating a narrow therapeutic window. Host defense peptides have a short half-life in the body, largely as a result of proteolytic degradation, thus limiting their efficacy. Another difficulty with the clinical use of host defense peptides is that they are immunogenic. Thus, these peptides have a dual role in stimulating the immune system. On the one hand, this property increases their effectiveness against the invading pathogens; on the other hand, the host defense peptide itself can become an immunogen, especially because its low potency requires that it be used at high concentrations. This antigenicity reduces the effectiveness of these peptides and can even lead to cross-reaction with endogenous proteins, resulting in autoimmune diseases. Another limitation for the commercialization of host defense peptides as drugs is the high cost of production of peptide synthesis. This could be ameliorated with the use of shorter peptides or non-peptide drugs. Further improvements could include synergistic cocktails, stimulation of endogenous production of host defense peptides, and using drugs to overcome resistance mechanisms.

There has been much progress in the identification of host defense peptides in a large number of organisms. Attempts have been made in developing host defense peptides for diverse applications including in the field of urology for the use in removing kidney stones; as stimulants of insulin release with potential for type II diabetes therapy; as useful agents in lung infections; as coatings for implanted devices such as catheters and other applications. The mechanism of action of host defense peptides is varied but in general these compounds tend to be non-specific and do not have very high potency. They also stimulate resistance mechanisms.

Nevertheless, currently many efforts are made to overcome these limitations and there is hope that we are at the beginning of a new period in which host defense peptides will be developed for a variety of therapeutic applications.

Richard M. Epand
Raquel F. Epand

Reference

Baym M, Stone LK, Kishony R (2016) Multidrug evolutionary strategies to reverse antibiotic resistance. Science 351:aad3292

Contents

Part I
Biological Targets of Host Defense Peptides

Chapter 1
Diversity in Host Defense Antimicrobial Peptides

Monique L. van Hoek

Abstract Host defense antimicrobial peptides are part of the innate immune system of organisms in multicellular eukaryotes. Following the identification of the first insect antimicrobial peptide, cecropin, in moths in 1980 (Eur J Biochem 106(1):7–16, 1980; Nature 292(5820):246–248, 1981) and the first amphibian peptide magainin in 1987 (Proc Natl Acad Sci USA 84(15):5449–5453, 1987), scientists have been exploring the diversity of animal antimicrobial peptides through examination of their sequences, structures and functions. The sequences of antimicrobial host defense peptides are surprisingly diverse as is the lack of commonalities between animals across phyla. Although peptides are classified into categories such as cathelicidins and defensins, the similarity between the active peptides within these categories can sometimes be difficult to find. This is because these peptides share function, structure, and mechanism within groups but often have very different sequences (Clin Microbiol Rev 19(3):491–511, 2006). That is, they have significant structural conservation often without significant sequence conservation. Sorensen and Borregaard (Comb Chem High Throughput Screen 8(3):273–280, 2005) beautifully described the diversity of host defense and antimicrobial peptides as "nature's attempt at combinatorial chemistry" (Comb Chem High Throughput Screen 8(3):273–280, 2005). In this chapter, we will discuss the diversity of antibacterial peptides from insects and oysters to reptiles and humans. Questions that could be of interest for future research and seem to be currently unanswered are highlighted in boxes throughout the text.

1.1 Cathelicidins

The classic example of extreme diversity in antimicrobial peptides is found in the cathelicidins, a major class of antimicrobial peptides of vertebrates. They are characterized as being processed from a propeptide that includes an N-terminal

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"cathelin domain" and a C-terminal domain that is cleaved to release the functional cathelicidin peptide (Kosciuczuk et al. 2012; Lehrer and Ganz 2002; Tomasinsig and Zanetti 2005). The active antimicrobial cathelicidin peptides are typically amphipathic, cationic peptides that are produced through proteolytic processing due to host-derived (or occasionally pathogen-derived) proteases. These peptides have many activities: they can form pores in bacterial membranes, bind to bacterial lipopolysaccharide, have intracellular bacterial targets and also exert host-directed activities such as immune cell recruitment and keratinocyte chemotaxis (Zanetti 2005; Sorensen and Borregaard 2005). Members of the cathelicidin class of peptides can also exhibit antiviral, antifungal, and antiparasitic activities, but the focus below will be on peptides with antibacterial activity.

Cathelicidin genes have been identified in an ever-increasing number of organisms. The hagfish, which may be 300 million years old, is perhaps the most evolutionarily ancient organisms to have a cathelicidin identified. Cathelicidin genes and active peptides have also been identified in more modern animals such as pandas (Yan et al. 2012) and wallabys (Carman et al. 2009). Some organisms, like primates, have only a single alpha-helical cathelicidin gene, while other organisms such as horse, cattle, sheep, and pigs have multiple cathelicidin genes (Tomasinsig and Zanetti 2005).

Most of the known cathelicidins adopt a helical structure in membranes. However, some members of the cathelicidin family such as protegrin, prophenin, indolicidin, and bactenecin differ from the "helical" cathelicidins in important ways (Tomasinsig and Zanetti 2005). These peptides can contain proline rich peptide sequences or cysteine-stapled beta-hairpin peptide structures (Lee et al. 2008). For example, protegrin's N-terminal domain has a stimulatory effect on cathepsin-L (Zhu 2008), opposite to the effect of the human cathelin domain on cathepsin-L. These unusual cathelicidins represent another facet of the diversity of antimicrobial peptides. Members of this group of cathelicidin peptides have been shown to have antimicrobial activities against important pathogens including *Burkholderia pseudomallei* (Madhongsa et al. 2013; Wang et al. 2004) as well as host-directed activities (Baumann et al. 2014). The remainder of this section will focus primarily on the helical cathelicidin peptides (Fig. 1.1).

Humans and most primates produce on only a single cathelicidin. In humans, cathelicidin peptides are stored in the azurophilic granules of neutrophils as the inactive propeptide, and are processed by enzymes (neutrophil elastase (Cole et al. 2001) or a serine protease (Ponkham et al. 2010)) to generate the mature active peptide (Tongaonkar et al. 2012). In humans and higher vertebrates, the active cathelicidin peptide is almost always encoded on Exon 4 of the cathelicidin encoding gene (Kosciuczuk et al. 2012; Nizet and Gallo 2003; Zanetti et al. 2000; Tomasinsig and Zanetti 2005). Four cathelicidin-like peptides have been identified in the chicken (*Gallus gallus domesticus*) (van Dijk et al. 2011), including fowlicidin-1, -2 and -3 (also known as chCATH-1, chCATH-2/CMAP27, chCATH-3) (van Dijk et al. 2005), and chCATH-B1/chCATH-4 (Xiao Y et al. 2006). In humans, the cathelicidin hCAP18 is processed by proteinase 3 inside the neutrophil granule (Ponkham et al. 2010) or neutrophil elastase in the extracellular

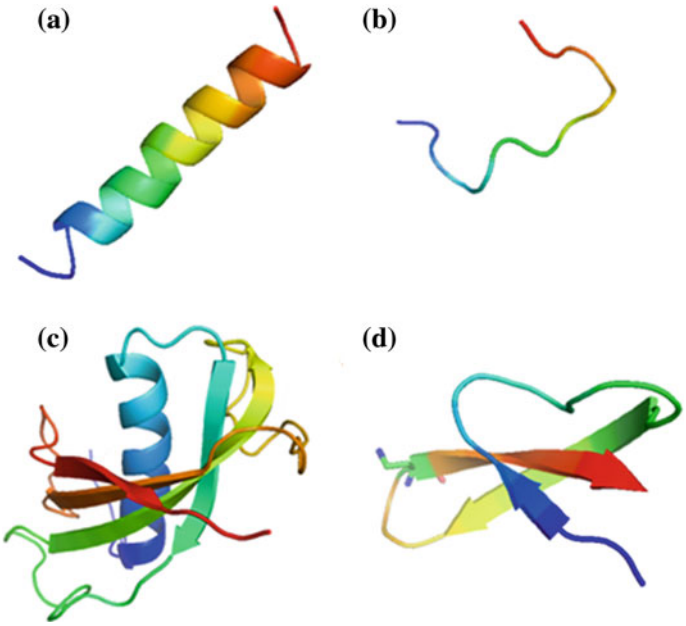

Fig. 1.1 Classes of CAMP secondary structures. Adapted from "Various AMPs" by Ymahn, licensed under CC BY 2.0. **a** α-helical: magainin (Gesell et al. 1997), **b** unstructured or random coil: indolicidin (Rozek et al. 2000), **c** mixed: protegrin-1 (Yang et al. 2003), **d** β-sheet: human α-defensin-1 (Zhang et al. 1992). https://commons.wikimedia.org/wiki/File:Various_AMPs.png#/media/File:Various_AMPs.png

space (Li et al. 2012) to its active form, LL-37. In the case of chicken cathelicidin, the pro-cathelin domain peptide is cleaved by a serine protease to release the mature peptide following stimulation of heterophils with bacterial products (van Dijk et al. 2009).

The human cathelicidin antimicrobial peptide, LL-37, has been studied extensively, and this peptide and smaller fragments have been shown to have both antimicrobial as well as antibiofilm activities against multiple important pathogens, including *Pseudomonas, Burkholderia, Staphylococcus*, and *Mycobacteria* (Blower et al. 2015; Dean et al. 2011a, b; Amer et al. 2010; Rivas-Santiago et al. 2013; Overhage et al. 2008) as well as antifungal (Rapala-Kozik et al. 2015) and antiviral activities (Barlow et al. 2011). The various "domains" of this peptide are the subject of current study (Mishra et al. 2013; Wang et al. 2014; Molhoek et al. 2009; Nagant et al. 2012) to better understand the mechanisms and biological role of these different activities at a molecular level.

Research question: What sequences define the cleavage site for release of the cathelicidin active peptide in nonmammalian species?

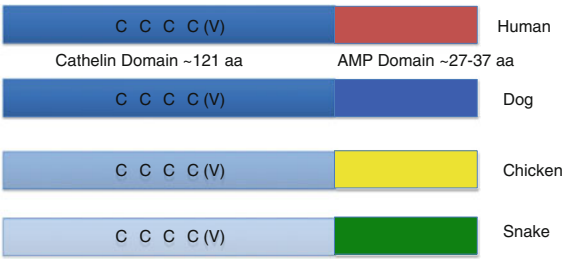

Fig. 1.2 Generalized organization of cathelicidin genes and relative comparison between four species. Cathelicidins are defined and identified by the relatively homologous cathelin domain. Four cysteines are commonly found in the cathelin domain, and a Valine is commonly found just prior to the cleavage site for the active peptide. The active antimicrobial peptide portion is nonconserved, represented by different colored antimicrobial peptide domains. As the sequences diverge from the human CAMP, the conservation of the N-terminal domain decreases, indicated by the decrease in intensity of the shading

The active cathelicidin antimicrobial peptides are different in their sequences between almost all organisms (Fig. 1.2). The features that they most highly share are their net positive charge (cationic), and the active peptide typically is unstructured in aqueous solutions but acquires a helical structure in membranes, as well as having an amphipathic face on the helix, although alternative structures of the active peptide can be found in this class (Tomasinsig and Zanetti 2005). This striking lack of conservation in the cathelicidin antimicrobial peptide sequence plus the relative conservation of the N-terminal domain raises interesting questions about the evolutionary selective pressure that may have been applied to these cathelicidin genes (Zhu and Gao 2009; Cheng et al. 2015). Why has each animal developed a different solution to the ~27–37 amino acid antimicrobial peptide that will be active in innate immunity? That is, instead of converging on a similar and perhaps best peptide, why does each animal appear to discover a novel peptide with sufficient antimicrobial activity against a variety of gram-positive and gram-negative bacteria? Is this an actively selected feature (as it is for the variable domain of antibodies for example)? Or is the selection pressure due to the presence of the cathelin domain (Tomasinsig and Zanetti 2005), which is more conserved, even though it does not have a direct "antimicrobial" activity? Perhaps it has some other important property?

Research question: What is the evolutionary selection pressure to conserve the N-terminal domain of cathelicidins?

Cathelicidin genes share a general structure, including an N-terminal cathelin domain and the C-terminal active peptide. Many papers state that the N-terminal cathelin domain is "highly conserved" (Wang et al. 2008), but this point deserves reexamination as more cathelicidins are identified in nonmammalian species.

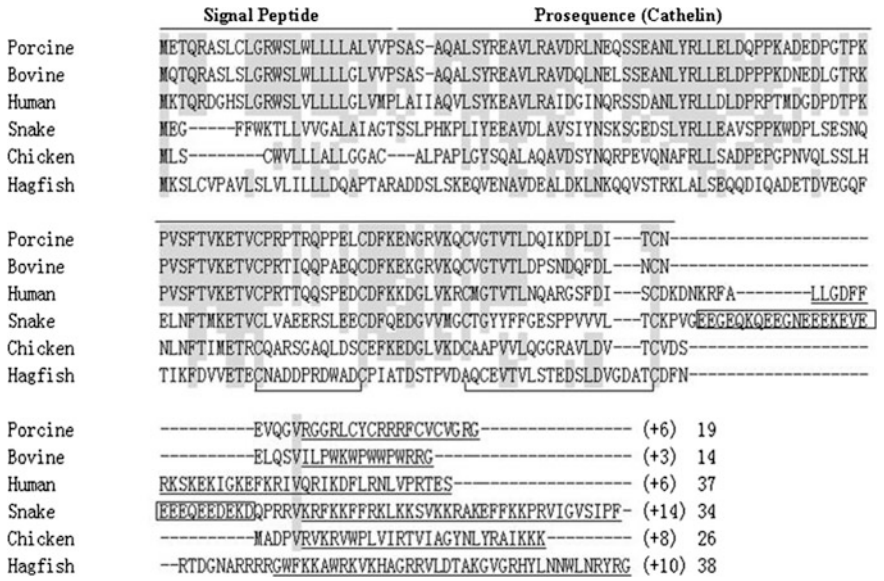

Fig. 1.3 Multiple sequence alignment of snake cathelicidin with other representative cathelicidins. This figure (from Wang et al. 2008, "Snake Cathelicidin from *Bungarus fasciatus* Is a Potent Peptide Antibiotics", Plos One (Wang et al. 2008)) is used following the terms of the Creative Commons Attribution License. The figure legend reads: "Multiple sequence alignment of snake cathelicidin with other representative cathelicidins. Cathelicidin-BF precursor is aligned with porcine, bovine, human, chicken and hagfish cathelicdins. *Dashes* are inserted to optimize the alignment, and conserved residues are *shaded*. Two intramolecular disulfide bonds in the cathelin pro-sequence are shown. Mature cathelicinds are *underlined*, and their net charge (*in parenthesis*) and length are also indicated. The acidic fragment insertion in cathelicidin-BF is *boxed*."

For example, see Fig. 1.3 from Wang et al. (2008), which shows the cathelin domain alignment between pig, cow, and human cathelicidins compared to snake, chicken, and hagfish cathelicidins. Previously reported analyses may have been somewhat limited by the small number of nonmammalian cathelicidin sequences that had been deposited in the databases. In this paper regarding snake cathelicidins and their alignments and phylogenetic analysis (Wang et al. 2008), it is suggested that mammalian N-terminal domains are "highly conserved" with each other, as stated in many other papers. However, examination of the chicken, fish, and snake N-terminal cathelin domains demonstrates significant divergence (Cheng et al. 2015).

Research question: Does the N-terminal domain of hagfish, chicken, or reptile cathelicidins also inhibit cathepsin or cysteine proteases?

The extreme diversity of the known cathelicidin peptides is further illustrated in the phylogenetic tree in Fig. 1.4 from Wang et al. (2008), demonstrating how "distant" the fish cathelicidins are from mammalian cathelicidins (Wang et al. 2008), and overall identifying three main clusters of cathelicidins within the vertebrates. With the continued discovery of more cathelicidins from other phyla, it is likely that there are less well conserved cathelin domains in other eukaryotes.

Cathelicidin peptides have been identified and characterized in the elapid snake family. Highly related cathelicidins were identified in *Bungarus fasciatus* (BF-CATH/Bf-CRAMP, Accession B6D434), *Ophiophagus hannah* (OH-CATH/Oh-CRAMP, Accession B6S2X2) and *Naja atra* (NA-CATH/Na-CRAMP, Accession B6S2X0) (Zhao et al. 2008), and these snake-encoded cathelicidin peptides appear to be generally similar throughout the reptiles (Zhao et al. 2008; Wang et al. 2008), called "cathelicidin–OH like" in the databases. The cathelicidin peptide from the Chinese King cobra, *N. atra*, has been particularly well studied. The full-length NA-CATH peptide was synthesized and was found to be antimicrobial against a wide variety of bacteria (de Latour et al. 2010; Dean et al. 2011a, b; Amer et al. 2010). Smaller fragments of this peptide (ATRA peptides) have been identified and found to be highly effective against pathogenic and multidrug resistant bacteria (de Latour et al. 2010; Dean et al. 2011a, b; Amer et al. 2010), which could be useful for therapeutic applications.

The precise evolutionary relationship of turtles with other reptiles continues to be a matter of current debate. Their genomes appear to encode cathelicidin antimicrobial peptides that are "snake-like" (van Hoek 2014) but encode a defensin-type peptides (gallinacin-like) that may be more similar to "avian" beta-defensin peptides. These differences in the turtle antimicrobial host defense "peptidome" may reflect the arguments currently ongoing in the literature regarding the precise genomic placement of turtles within the reptilian lineage (Wang et al. 2013; Badenhorst et al. 2015).

One of the most interesting examples of the *in vivo* function of host defense antimicrobial peptides is when the lizard loses its tail. The tail regenerates and the wound bed is rarely infected during this process. Cathelicidin-like peptides Ac-CATH-1, Ac-CATH-2a, Ac-CATH-2b, and Ac-CATH-3 have been reported in the genome of the Carolina anole lizard, *Anolis carolinensis* (Dalla Valle et al. 2012). In addition, cathelicidin 1 and 2 antibody reactive peptides have been identified by immunocytochemistry staining within granules of heterophilic and basophilic granulocytes (Alibardi 2014b). Cathelicidin-antibody staining material was identified in the tail stump wound epidermis and associated with bacteria within those wounds (Alibardi 2014b).

It has been relatively difficult to predict new, active cathelicidin peptides from genomic sequences due to the low sequence conservation or homology, and a lack of understanding about the sequence determinants for cleavage of the active peptide (Cheng et al. 2015). A potentially useful approach to predicting the cathelicidin peptide in potential genes is to take a structural approach, accounting for the C-terminal 28–37 amino acid cationic helical peptide located at the end of the gene. For example, the following five genes in the Chinese alligator genome (Table 1.1)

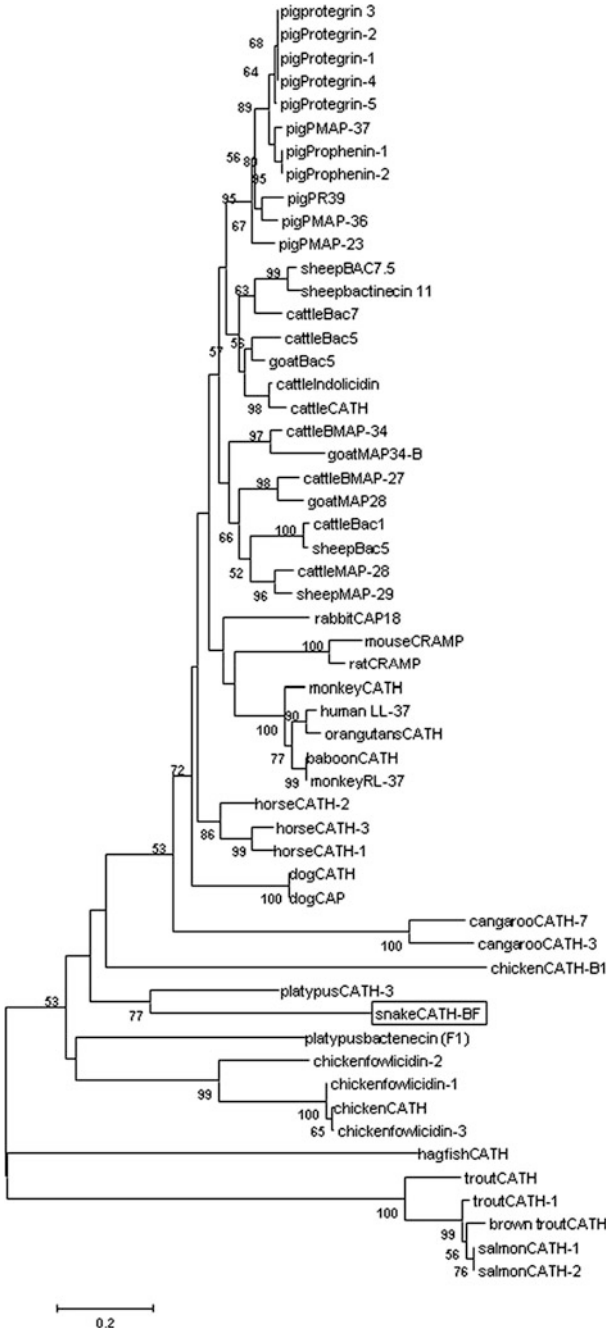

Fig. 1.4 Phylogenetic analysis of cathelicidins. This figure is from Wang et al. (2008), "Snake Cathelicidin from *Bungarus fasciatus* Is a Potent Peptide Antibiotics", Plos One, (Wang et al. 2008) and is used following the terms of the Creative Commons Attribution License. The figure legend reads: "Phylogenetic analysis of cathelicidins"

Table 1.1 Predicted *Alligator sinensis* Cathelicidin-OH antimicrobial peptide-like genes

GeneID (% Identity to OH-CATH)	Cathelicidin-OH antimicrobial peptide-like amino acid sequence. (OH-CATH sequence: KRFKKFFKKLKNSVKKRAKKFFKKPRVIGVSIPF)(Zhao et al. 2008)	Size and net charge of predicted 35 aa C-terminal peptide (APD2 (Wang et al. 2009; Wang and Wang 2004))
102373900 XM_006026411.1 XP_006026473.1 (45 %)	METCLRLLLLLGVATAVATAQAQAQAQTQSQSQAGYEDAVTTAV DIFNQESGLPQAYRLLEAEPQSEWNPSSQAAQPLKFSVKETVCPI AQKGNLKQCDFKENGLVKDCSGLFTAGKKPPVTAVKCEDTSQEP ELVTRRKFWKKVLNGALKIAPFLLG	35 aa (grey), +3, helical. Predicted peptide extends into predicted OH-CATH cathelin domain (ending in V).
102377572 XM_006026425.1 XP_006026487.1 (39 %)	MVSDLASQPVQPLKFSIKETECLVSEKRDISQCPFKDKGLVKDCKG LYAEEKEPPVITAKCEDAGQEPQLVKRVNWAKVGRTALKLLPYIF G	35 aa (grey), +3, helical. Predicted peptide extends into predicted OH-CATH cathelin domain (which is too short here).
102382109 XM_006037224.1 XP_006037286.1 (42 %)	MGRWGWGVLLGLAMLVASASASQHKTLSYEEAVSLAVDFYNQE PGIDHAFRLLRADPQPAWDMTSKPRQELRFVVRETVCPRAQDPP ASECDFKDNGLVRNCTGLFSTERESPTVIITCDTVTPGQHVRVRR SGWWNGHKRRRGSGSRHGQYSSTKYGGRKRPRKRPGSGSWLSH DTPHVAPIAKGHVG	35 aa (grey), +3, not helical.
102373644 XM_006026410.1 XP_006026472.1 (42 %)	MGRWGWGVLLGLATFVAVALGSQHKTLSYEEAVSLAVDFYNQG PGIDHAFRLLRADPQPAWDMTSKPRQELRFVVRETVCPRAQDPP ASECDFKDNGLVRNCTGLFSTERESPTVIITCDTVTPGQHVRVRR SGWWNGHKRRRGSGTRRGRFSHIAHGGRKGHERIA	35 aa (grey), +9, helical. Predicted peptide appends almost exactly to predicted OH-CATH cathelin domain (ending in V).
102379244 XM_006037211.1 XP_006037273.1 (55 %)	MQTCWVILLLPLLGAASTELPTPGTDPPQLTPTYAQALATAVDVY NQGPGVDFAFRLLEAESRDDWDASTDPLRQLEFTLKETECPVGE DQPLDQCDFKDGGAVLDCTGTFSCSEASLMVLVTCQPAEPLPDR VRRGLFKKLRRKIKKGFKKIFKRLPPVGVGVSIPLAGRR	35 aa (grey), +13, helical, Predicted peptide appends almost exactly to predicted OH-CATH cathelin domain (ending in V).

are all annotated as "cathelicidin-OH like" genes, based on the N-terminal cathelin homology. Applying sequence analysis, these genes score relatively high in identity to OH-CATH (39–45 %). However, when examined using this structural C-terminal focussed approach, we can place the following two requirements: (1) requiring the presence of a highly charged, cationic, helical region that extends 28–37 amino acids towards the N-terminus from the C-terminal end (we typically used 35 aa), and (2) not allowing the potential cathelicidin peptide sequence to enter the predicted cathelin domain (which is usually from amino acid 1–121, Conserved domains: pfam00666, smart00043). From this analysis, it can be concluded that only two of these five genes (GeneID 102373644 and 102379244) even have the potential to actually encode a C-terminal cathelicidin-like antimicrobial host defense peptide of the appropriate charge, length, and helicity. If the C-terminal 35 amino acids are then compared to OH-CATH, it becomes obvious that only the last gene on the table (GeneID 102379244) has the potential to be the homolog of OH-CATH, as it is 55 % identical.

The last gene (102379244), most closely aligns with the physical and structural parameters of a potential cathelicidin and also has good homology to the OH-CATH peptide (55 % identical). Most importantly, it meets the physicochemical parameters of a cathelicidin peptide. The second to last gene (102373644) is still potentially of interest, as it matches the physical parameters of a potential cathelicidin, but has a divergent C-terminal sequence that has low similarity to other cathelicidin peptides in the APD2 databases (Wang et al. 2009), thus it would be of interest for further study.

Although cathelicidin genes are often active against a broad-spectrum of gram-negative and gram-positive bacteria, some are antimicrobial only against the pathogens to which the host is normally exposed. In their recent paper, (Sun et al. 2015) demonstrated that the cathelicidin newly identified from the toad *Bufo bufo gargarizans* Cantor had poor antimicrobial activity against common human pathogens such as *Staphylococcus*, *E. coli*, and *Pseudomonas*, but demonstrated strong antimicrobial activity against various aquatic bacteria that are likely part of the toad's natural habitat, including *Aeromonas hydrophila* and *Vibrio* species. When we seek novel antimicrobial peptides for human use, we should perhaps be guided by the evolutionary microbial environment to which different animals are exposed (Bishop et al. 2015; van Hoek 2014).

1.2 Cecropins

Insects express many unique classes of antimicrobial peptides that differ from those found in vertebrates (Yi et al. 2014; Ezzati-Tabrizi et al. 2013; Lehrer and Ganz 1999; Bulet and Stocklin 2005); however, here we will briefly focus on cecropins. Cecropins are an insect-specific group of antimicrobial peptides (AMPs) that were first discovered in the cecropia moth, *Hyalophora cecropia* (Steiner et al. 1981). Cecropin peptides have been found to be antimicrobial for both gram-positive and

gram-negative bacteria (Steiner et al. 1981; Otvos 2000). Cecropins share some "thematic similarities" to cathelicidins, but are considered to be a separate group and are only found in insects. These similarities to cathelicidins include:

i. Cecropin transcripts are proproteins that get processed to the final active AMP.
ii. Cecropins are cationic peptides.
iii. Cecropin peptide shape is disordered in aqueous solutions and assumes an alpha- helix in the context of membranes.
iv. Cecropins are 3–4 Daltons in size.
v. Cecropins are divergent in their active antimicrobial peptide sequence.
vi. Cecropins are widely expressed within the insect phylum.
vii. The cecropin peptides can bind bacterial lipopolysaccharide (LPS).
viii. Cecropin peptides can destabilize bacterial membranes and cause bacterial cell lysis.

However, the active antimicrobial peptides of cecropins can have varied and highly complex structures (Harikrishna et al. 2012), and are significantly different from the relatively simple alpha-helical peptides like LL-37.

Cecropin peptides remain an area of active study and research, and new cecropin peptides are still being discovered (Lee et al. 2013; Hu et al. 2013), such as in the silkworm, *Bombyx mori* (Cheng et al. 2006; Hong et al. 2008).

In addition, hybrid synthetic peptides using part of cecropin peptide fused with part of maganin and LL-37 or other peptides are proving to be highly effective against multiple pathogenic bacteria such as *Bacillus anthracis, Burkholderia cepacia, Francisella tularensis LVS*, and *Yersinia pseudotuberculosis* (Fox et al. 2012).

1.3 Beta-Defensins

Defensins are one of the major classes of antimicrobial peptides in higher vertebrates and are also present in insects. Insect defensins are typically only active against gram-positive bacteria (Otvos 2000), while vertebrate defensins have a broader range of action. Vertebrate defensins are organized into three main subclasses: α, β-, and θ-defensins. These $\sim$4 kDa cationic peptides are characterized by having six cysteines arranged in three disulfide bonds, with the pairing of the bonds being highly characteristic for each type of defensin. Defensins have predominantly β-sheet characteristic with some α-helices plus intramolecular disulfide bonds (Fig. 1.4). Defensins are encoded in the genome, and processed from a pro-defensin molecule by various proteases (Wilson et al. 2009). Defensins are known to be critical components of innate immunity (Zhao and Lu 2014). The expression of these defensin peptides are often induced following bacterial or viral infection as part of the innate immune response (Pierson et al. 2013; Han et al. 2008) except for hBD1 which appears to be constitutively expressed in humans.

θ-Defensins are not known outside of primates, and are not expressed in humans but have been shown to be very active antiviral peptides (Zhao and Lu 2014).

The beta-defensin peptides are the most conserved of the antimicrobial host defense peptides among the vertebrates, likely due to the large number of characteristic cysteines. Beta-defensins are defined as peptides that contain a highly conserved pattern of 6 cysteines with a clearly defined pattern of intramolecular bonding: Cys1–Cys5, Cys2–Cys4, and Cys3–Cys6 (Wu et al. 2003) (Fig. 1.4). In humans, β-defensins are commonly expressed in epithelial cells, and are widely expressed in the body (Garcia et al. 2001). Other than the highly conserved cysteines, however, there is significant diversity in the intervening sequences of beta-defensin peptides (Semple et al. 2006; Cheng et al. 2015).

The first detailed report of an in vivo role for β-defensin peptide expression was in the anole lizard, *Anolis carolinensis*. Lizards can lose their tails during predator escape, and the tail can then regenerate. In this process, a wound is formed, which does not typically get infected. β-Defensin peptides are found to be expressed both within the azurophilic granulocytes in the wound-bed as well as in the associated epithelium (Alibardi 2013b, 2014a), and are observed in phagosomes containing degraded bacteria. While there is a distinct lack of inflammation in the wound, which is associated with regeneration (Alibardi et al. 2012), there is a high level of expression of AcBD15 and AcBD27 (two of the most highly expressed β-defensins in that tissue) (Alibardi 2013a). Thus, there is a potential role of these defensins in infection prevention, wound healing and regeneration of the anole lizard tail.

Eggs contain lots of readily available biological material and are interesting places to discover antimicrobial peptides. Recently, it was found that the β-defensin-like peptide pelovaterin, identified in the eggshell of the Chinese softshelled turtle, has antimicrobial activity, and that these peptides may also play an additional role in the formation of the eggshell through aggregation (Lakshminarayanan et al. 2008). This may be similar to the role of the gallin, an ovodefensin which is a beta-defensin-like peptide in avian eggs (Lakshminarayanan et al. 2008; Herve-Grepinet et al. 2010; Mine et al. 2003).

1.4 Diverse Requirements for Folding

The beta-defensin peptides also demonstrate diverse requirements of folding for activity. Human beta-defensin 2 (hBD-2) for example must be folded correctly with the cysteines in the correct bonding pattern to exhibit its full antimicrobial activity (Wu et al. 2003). For hBD3, it has recently been demonstrated that the disulfide bonds are dispensable for antimicrobial activity but required for chemotactic activity (Wu et al. 2003). Furthermore, small fragments of hBD3 that are designed to be linear also have high antimicrobial activity (Papanastasiou et al. 2009). This finding has important implications for the potential mechanism of hBD3 action on

the host and on pathogens, and demonstrates a separation of these two activities though differences in the structure of the peptides.

Interestingly, almost the opposite is true for hBD1, the constitutively expressed AMP. Schroeder et al. (2011a, b) demonstrated that by reducing all the disulfide bonds within hBD1, the antimicrobial activity actually is significantly increased (Schroeder et al. 2011a, b) compared to the fully oxidized form, which is generally inactive for antimicrobial activity. This finding, combined with an evaluation of the redox potential in the lung and other epithelial surfaces, has led to a new appreciation for the potential role of hBD1 in host defense.

1.5 Alpha Defensins

Alpha defensins are highly expressed in many but not all higher organisms (Das et al. 2010) and are peptides that contain a highly conserved pattern of 6 cysteines with a clearly defined pattern of intramolecular bonding: Cys1–Cys6, Cys2–Cys4, and Cys3–Cys5 (Wu et al. 2003). For example, human neutrophil peptide HNP-1, also called α-defensin 1, is highly expressed in neutrophils and other leukocytes in humans. HNP-1 is important in the ability of white blood cells to deal with bacterial pathogens (Lehrer and Lu 2012). Alpha defensins are different from beta-defensins in their bonding pattern of the six characteristic cysteines, and their more limited tissue expression pattern.

Although considered by phylogenetic analysis to be in the same phyla as avians (Sauropsida), reptiles are still generally thought of by most of us as being quite separate from birds (Alfoldi et al. 2011). Within the reptiles, crocodilians are considered to be the most closely related to the avian branch. Interestingly, this evolutionary connection is reflected in the antimicrobial profile ("peptidome") in that neither avians nor reptilians encode α-defensin antimicrobial peptides (Xiao et al. 2004), which are a critical part of mammalian innate immunity (Wilson et al. 2009; Zhao and Lu 2014; Lehrer and Lu 2012; Tongaonkar et al. 2012). Reptile neutrophil-like cells have granules that contain both cathelicidin-like and β-defensin peptides, but unlike mammals, no α-defensin peptides. By doing an in-depth analysis of multiple alpha- and beta-defensin genes, Xiao et al. (2004) conclude that mammalian alpha-defensin genes may have arisen from early beta-defensin genes through a process of gene duplication and evolution (Xiao et al. 2004).

Research question: Do chickens and reptiles use beta-defensins in heterophils in the same way as humans use alpha defensins in neutrophils in response to infection?

1.6 Big-Defensins

Big-defensins (Schmitt et al. 2012; Schulenburg et al. 2007), first identified in horseshoe crabs in 1995 (Saito et al. 1995), are peptides involved in innate immunity in invertebrates, such as horseshoe crabs, oysters and mussels. big-defensins are an unusual antimicrobial peptide when compared to mammalian or vertebrate defensins and further reflect the diversity of antimicrobial peptides found in nature (Schmitt et al. 2012). These 79 amino acid long peptides have two distinct domains: a highly hydrophobic, cationic and likely helical N-terminal domain and a cationic C-terminal domain with 6 cysteines that closely resembles mammalian beta-defensins (Saito et al. 1995; Kouno et al. 2008) (Fig. 1.5). Each domain separately can exert antimicrobial activity. The N-terminal region was shown to be antimicrobial against gram-positive bacteria, the C-terminal region was shown to be antimicrobial against gram-negative bacteria, and there appears to be some combined synergy of the two domains together with regards to LPS binding (Rosa et al. 2011; Saito et al. 1995; Kouno et al. 2009). Many of the big-defensin genes are inducible by bacterial products, and their expression is not observed in uninfected oysters (Rosa et al. 2011).

These "big-defensins" are evolutionarily related to vertebrate defensins in their C-terminal domain in terms of structure, but still surprisingly distant from human

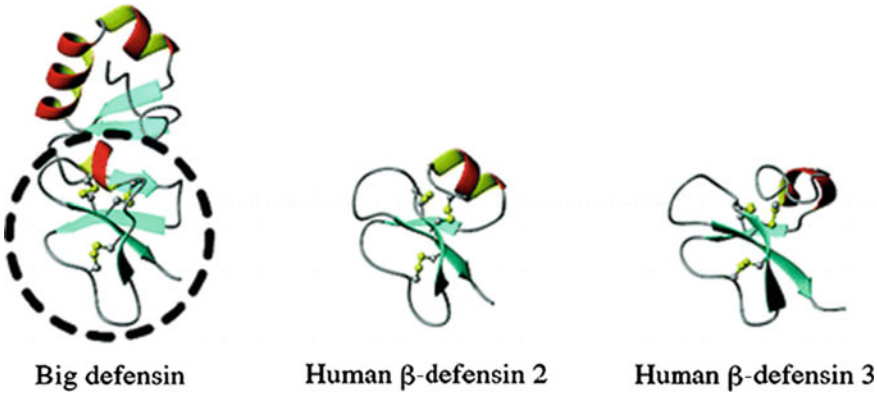

Fig. 1.5 Structure of big-defensins compared to beta-defensins. The N-terminal region of the big-defensin "forms a hydrophobic globular domain, and the C-terminal domain adopts a beta-defensin like shape with the typical beta-defensin Cys1–5, 2–4, 3–6 bonding pattern." Figure is reprinted with permission (Kouno et al. 2008). Copyright 2008 American Chemical Society

beta-defensins by sequence (Rosa et al. 2015). There are at least 17 different big-defensins known or predicted in different invertebrates (Schmitt et al. 2012). Oysters, such as *Crassostrea gigas* (Rosa et al. 2015; Gonzalez et al. 2007; Gueguen et al. 2006), have big-defensins that are active only against gram-positive bacteria. Oyster big-defensins were shown to exert their antibacterial activity by interfering with *Staphylococcus aureus* Lipid II and thus interfering with the bacteria's peptidoglycan synthesis (Schmitt et al. 2010), revealing the diversity of bacterial targets for antimicrobial peptides. Horseshoe crabs express a big-defensin tachyplesin, which can bind LPS and has activity against both gram-positive and gram-negative organisms under MIC conditions (Saito et al. 1995; Kushibiki et al. 2014).

1.7 Hepcidins

Hepcidin peptides are liver-expressed peptides containing eight cysteines and are involved in ferroportin and iron binding through the N-terminus (Rodriguez et al. 2014; Park et al. 2001). Hepcidins appear to have very little direct antimicrobial activity against pathogens, and exert their activity primarily though regulating free iron. The host defense hepcidin peptides have a very conserved pattern of 8 cysteines (thus containing four disulfide bonds) with highly characteristic bridging pattern (C7–C22; C10–C13; C11–C19; C14–C22) (Jordan et al. 2009; Clark et al. 2011). However, the intervening sequences are highly divergent, leading to overall relatively low sequence similarity between distant vertebrates (Table 1.2). Hepcidin-like sequences were identified in amphibians and reptiles, including the painted turtle and the anole lizard (Hilton and Lambert 2008). In contrast to the beta-defensins and cathelicidins, the chicken genome does not appear to encode a gene for hepcidin peptides (van Hoek 2014), while reptile genomes do, highlighting an interesting difference between these otherwise highly related organisms. Chickens do have the ferroportin gene (Tako et al. 2010), so it might be expected that there is a hepcidin partner of some kind, despite the fact that it can not be identified by searching the genome.

Research question: Do chickens regulate iron homeostasis with a ferrpotin/hepcidin system with an as yet unidentified hepcidin or do they regulate iron homeostasis via some other mechanism?

Table 1.2 Comparison of predicted hepcidin sequences across multiple phyla

Animal	Identification number (APD2 or Gene ID number)	Sequence
Human	AP00193	DTHFPICIFCCGCCHRSKCGMCCKT
Buffalo	AP01682	DTHFPICIFCCGCCHRSKCGMCCKT
Cow	512301	DTHFPICIFCCGCCRKGTCGMCCRT
Sheep	100500730	DTHFPICIFCCGCCRKGTCGICCKT
Panda bear	301771027	DTHFPICLFCCGCCNKSKCGICCKT
Mouse	14211542	DTNFPICIFCCKCCNNSQCGICCKT
Lizard (*Anolis carolinensis*)		NSHISICTYCCNCCKNKGCSFCCRT
Painted Turtle	530604487	NSHFPICTYCCKCCRNQGCGFCCRT
Western clawed frog	161611805	QSHLSICVHCCNCCKYKGCGKCCLT
Carp (fish)	AP02445	QSHLSLCRYCCNCCRNKGCGYCCKF
Salmon	929066985	QSHLSLCRWCCNCCHNKGCGFCCKF
Chickens	None found	None found

The highly conserved cysteines are shown in black, and some of the different amino acids are highlighted in other colors, roughly corresponding to different phyla

1.8 Protein Fragments with Antimicrobial Activity

Over the past decade, there have been increasingly reports of peptides that are cleavage products of other proteins that have no known role in antimicrobial response, in which the cleaved products are then shown to have direct antimicrobial activity. One example of this is the release of histone-derived peptides including buforin and others, which have antimicrobial activity against many different bacteria (Pavia et al. 2012; Bustillo et al. 2014). Similarly, reports of antimicrobial activity of fragments of lactoferrin show that these fragments have antimicrobial activity (Bolscher et al. 2006).

Another very interesting example is the release of a c-terminal fragment of the interferon-inducible CXC chemokines, which have been shown to be antimicrobial (Crawford et al. 2009). Hughes et al. have demonstrated that the fragment of CXCL10 is antimicrobial against *Bacillus anthracis* vegetative cells by targeting the cell division protein FtsX (Crawford et al. 2011). This example suggests a possible novel host defense role for these protein fragments that is separate from the previously understood role of CXCL10 in the host response to bacterial infection (Crawford et al. 2010).

Using a custom-made particle for harvesting native peptides, and de novo-assisted peptide sequencing, my collaborators and I have identified antimicrobial peptide fragments from alligator plasma, including several that we call "Apo" peptides, which are C-terminal, alpha-helical cleavage products of alligator Apolipoprotein C (Bishop et al. 2015; Juba et al. 2015). These apo-peptides were highly active in EC50 assays against a range of both gram-negative and gram-positive bacteria (Bishop et al. 2015). The role of these fragments in the innate immunity of the alligator is not yet known, nor whether their production is regulated in the face of infection.

Research Question: Is the generation of apo-peptide fragments altered in the presence of a bacterial infection? Is the expression of the parental peptide regulated by exposure to bacterial products?

1.9 Conclusions

Antimicrobial peptides are known throughout most of the animal kingdom, from invertebrates to vertebrates. Although canonical "classes" of antimicrobial peptides are identified, there is surprising diversity of peptides even within common classes. The evolutionary reasons for this diversity are unclear. Is there evolutionary pressure to have a diverse arsenal of peptides in different animals, or are the different peptides "equivalent" solutions to the problem of innate immune host defense?

Host defense antimicrobial peptides have been classified into families, but especially within the cathelicidin family, they dominantly share physicochemical properties within the families, such as size, net charge, physical structure and location within the encoding gene. Taking a structural approach (rather than a sequence-based approach such as genomics or proteomics) to understand the relatedness of host defense antimicrobial peptides can be helpful, especially with respect to identifying new cathelicidin peptides, for example.

With the advent of high-throughput sequencing, genomes of previously unstudied animals are being sequenced and their transcriptomes are being determined. As more peptides are identified and characterized, some of the remaining issues identified here will be clarified, such as whether cathelicidin prosequences from organisms distant to mammals will still retain a "cathelin" domain, either in sequence or in function. The comparison of reptiles to mammals provides useful examples of the diversity of antimicrobial peptides found within higher organisms. The known cathelicidin active peptides from reptiles are very different in sequence than cathelicidin peptides from any other phylum, and the same is true for almost every active cathelicidin peptide from any animal known. The beta-defensins are more conserved between phyla, but mostly due to the sequence constraints of the 6-cysteine pattern. The alpha defensins are also more conserved, but interestingly are not expressed in the avian or non-avian reptiles. Even the hepcidin peptides predicted in reptiles are highly divergent from the mammalian hepcidin in the amino acid sequence despite the high constraint of the 8 conserved cysteines out of 25 amino acids. Interesting peptides also include the cecropins and the big-defensins.

Overall, antimicrobial peptides employ a diverse range of novel and effective mechanisms to kill bacteria. Some of these mechanisms may surely be harnessed and enhanced for use by humans in the battle against infection. There are many interesting research questions to pursue within the area of host defense antimicrobial peptides, as the field continues working to potentially develop these peptides as potential therapeutics for clinical use. In itself, the diversity of antimicrobial peptide sequences suggests that these peptides may be good platforms to develop further into potential therapies as there are a wide variety of mechanisms employed by each of these peptides and each peptide may present another of nature's solutions to the problem of how to kill bacteria.

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References

Alfoldi J, Di Palma F, Grabherr M, Williams C, Kong L, Mauceli E, Russell P, Lowe CB, Glor RE, Jaffe JD, Ray DA, Boissinot S, Shedlock AM, Botka C, Castoe TA, Colbourne JK, Fujita MK, Moreno RG, ten Hallers BF, Haussler D, Heger A, Heiman D, Janes DE, Johnson J, de Jong PJ, Koriabine MY, Lara M, Novick PA, Organ CL, Peach SE, Poe S, Pollock DD, de

Queiroz K, Sanger T, Searle S, Smith JD, Smith Z, Swofford R, Turner-Maier J, Wade J, Young S, Zadissa A, Edwards SV, Glenn TC, Schneider CJ, Losos JB, Lander ES, Breen M, Ponting CP, Lindblad-Toh K (2011) The genome of the green anole lizard and a comparative analysis with birds and mammals. Nature 477(7366):587–591. doi:10.1038/nature10390

Alibardi L (2013a) Granulocytes of reptilian sauropsids contain beta-defensin-like peptides: a comparative ultrastructural survey. J Morphol 274(8):877–886. doi:10.1002/jmor.20143

Alibardi L (2013b) Ultrastructural immunolocalization of beta-defensin-27 in granulocytes of the dermis and wound epidermis of lizard suggests they contribute to the anti-microbial skin barrier. Anat Cell Biol 46(4):246–253. doi:10.5115/acb.2013.46.4.246

Alibardi L (2014a) Histochemical, biochemical and cell biological aspects of tail regeneration in lizard, an amniote model for studies on tissue regeneration. Prog Histochem Cytochem 48 (4):143–244. doi:10.1016/j.proghi.2013.12.001

Alibardi L (2014b) Ultrastructural immunolocalization of chatelicidin-like peptides in granulocytes of normal and regenerating lizard tissues. Acta Histochem 116(2):363–371. doi:10.1016/j.acthis.2013.08.014

Alibardi L, Celeghin A, Dalla Valle L (2012) Wounding in lizards results in the release of beta-defensins at the wound site and formation of an antimicrobial barrier. Dev Comp Immunol 36(3):557–565. doi:10.1016/j.dci.2011.09.012

Amer LS, Bishop BM, van Hoek ML (2010) Antimicrobial and antibiofilm activity of cathelicidins and short, synthetic peptides against Francisella. Biochem Biophys Res Commun 396 (2):246–251. doi:10.1016/j.bbrc.2010.04.073

Badenhorst D, Hillier LW, Literman R, Montiel EE, Radhakrishnan S, Shen Y, Minx P, Janes DE, Warren WC, Edwards SV, Valenzuela N (2015) Physical mapping and refinement of the painted turtle genome (chrysemys picta) inform amniote genome evolution and challenge turtle-bird chromosomal conservation. Genome Biol Evol 7(7):2038–2050. doi:10.1093/gbe/evv119

Barlow PG, Svoboda P, Mackellar A, Nash AA, York IA, Pohl J, Davidson DJ, Donis RO (2011) Antiviral activity and increased host defense against influenza infection elicited by the human cathelicidin LL-37. PLoS ONE 6(10):e25333. doi:10.1371/journal.pone.0025333

Baumann A, Demoulins T, Python S, Summerfield A (2014) Porcine cathelicidins efficiently complex and deliver nucleic acids to plasmacytoid dendritic cells and can thereby mediate bacteria-induced IFN-alpha responses. J Immunol 193(1):364–371. doi:10.4049/jimmunol.1303219

Bishop BM, Juba ML, Devine MC, Barksdale SM, Rodriguez CA, Chung MC, Russo PS, Vliet KA, Schnur JM, van Hoek ML (2015) Bioprospecting the American alligator (Alligator mississippiensis) host defense peptidome. PLoS ONE 10(2):e0117394. doi:10.1371/journal.pone.0117394

Blower RJ, Barksdale SM, van Hoek ML (2015) snake cathelicidin NA-CATH and smaller helical antimicrobial peptides are effective against burkholderia thailandensis. PLoS Negl Trop Dis 9 (7):e0003862. doi:10.1371/journal.pntd.0003862

Bolscher JG, van der Kraan MI, Nazmi K, Kalay H, Grun CH, Van't Hof W, Veerman EC, Nieuw Amerongen AV (2006) A one-enzyme strategy to release an antimicrobial peptide from the LFampin-domain of bovine lactoferrin. Peptides 27(1):1–9. doi:10.1016/j.peptides.2005.06.012

Bulet P, Stocklin R (2005) Insect antimicrobial peptides: structures, properties and gene regulation. Protein Pept Lett 12(1):3–11

Bustillo ME, Fischer AL, LaBouyer MA, Klaips JA, Webb AC, Elmore DE (2014) Modular analysis of hipposin, a histone-derived antimicrobial peptide consisting of membrane translocating and membrane permeabilizing fragments. Biochim Biophys Acta 1838 (9):2228–2233. doi:10.1016/j.bbamem.2014.04.010

Carman RL, Old JM, Baker M, Jacques NA, Deane EM (2009) Identification and expression of a novel marsupial cathelicidin from the tammar wallaby (Macropus eugenii). Vet Immunol Immunopathol 127(3–4):269–276. doi:10.1016/j.vetimm.2008.10.319

Cheng T, Zhao P, Liu C, Xu P, Gao Z, Xia Q, Xiang Z (2006) Structures, regulatory regions, and inductive expression patterns of antimicrobial peptide genes in the silkworm Bombyx mori. Genomics 87(3):356–365. doi:10.1016/j.ygeno.2005.11.018

Cheng Y, Prickett MD, Gutowska W, Kuo R, Belov K, Burt DW (2015) Evolution of the avian beta-defensin and cathelicidin genes. BMC Evol Biol 15:188. doi:10.1186/s12862-015-0465-3

Clark RJ, Tan CC, Preza GC, Nemeth E, Ganz T, Craik DJ (2011) Understanding the structure/activity relationships of the iron regulatory peptide hepcidin. Chem Biol 18 (3):336–343. doi:10.1016/j.chembiol.2010.12.009

Cole AM, Shi J, Ceccarelli A, Kim YH, Park A, Ganz T (2001) Inhibition of neutrophil elastase prevents cathelicidin activation and impairs clearance of bacteria from wounds. Blood 97 (1):297–304

Crawford MA, Zhu Y, Green CS, Burdick MD, Sanz P, Alem F, O'Brien AD, Mehrad B, Strieter RM, Hughes MA (2009) Antimicrobial effects of interferon-inducible CXC chemokines against Bacillus anthracis spores and bacilli. Infect Immun 77(4):1664–1678. doi:10.1128/IAI.01208-08

Crawford MA, Burdick MD, Glomski IJ, Boyer AE, Barr JR, Mehrad B, Strieter RM, Hughes MA (2010) Interferon-inducible CXC chemokines directly contribute to host defense against inhalational anthrax in a murine model of infection. PLoS Pathog 6(11):e1001199. doi:10.1371/journal.ppat.1001199

Crawford MA, Lowe DE, Fisher DJ, Stibitz S, Plaut RD, Beaber JW, Zemansky J, Mehrad B, Glomski IJ, Strieter RM, Hughes MA (2011) Identification of the bacterial protein FtsX as a unique target of chemokine-mediated antimicrobial activity against Bacillus anthracis. Proc Natl Acad Sci USA 108(41):17159–17164. doi:10.1073/pnas.1108495108

Dalla Valle L, Benato F, Maistro S, Quinzani S, Alibardi L (2012) Bioinformatic and molecular characterization of beta-defensins-like peptides isolated from the green lizard Anolis carolinensis. Dev Comp Immunol 36(1):222–229. doi:10.1016/j.dci.2011.05.004

Das S, Nikolaidis N, Goto H, McCallister C, Li J, Hirano M, Cooper MD (2010) Comparative genomics and evolution of the alpha-defensin multigene family in primates. Mol Biol Evol 27 (10):2333–2343. doi:10.1093/molbev/msq118

de Latour FA, Amer LS, Papanstasiou EA, Bishop BM, van Hoek ML (2010) Antimicrobial activity of the Naja atra cathelicidin and related small peptides. Biochem Biophys Res Commun 396(4):825–830. doi:10.1016/j.bbrc.2010.04.158

Dean SN, Bishop BM, van Hoek ML (2011a) Natural and synthetic cathelicidin peptides with anti-microbial and anti-biofilm activity against Staphylococcus aureus. BMC Microbiol 11:114. doi:10.1186/1471-2180-11-114

Dean SN, Bishop BM, van Hoek ML (2011b) Susceptibility of pseudomonas aeruginosa biofilm to alpha-helical peptides: D-enantiomer of LL-37. Front Microbiol 2:128. doi:10.3389/fmicb.2011.00128

Ezzati-Tabrizi R, Farrokhi N, Talaei-Hassanloui R, Alavi SM, Hosseininaveh V (2013) Insect inducible antimicrobial peptides and their applications. Curr Protein Pept Sci 14(8):698–710

Fox MA, Thwaite JE, Ulaeto DO, Atkins TP, Atkins HS (2012) Design and characterization of novel hybrid antimicrobial peptides based on cecropin A, LL-37 and magainin II. Peptides 33 (2):197–205. doi:10.1016/j.peptides.2012.01.013

Garcia JR, Jaumann F, Schulz S, Krause A, Rodriguez-Jimenez J, Forssmann U, Adermann K, Kluver E, Vogelmeier C, Becker D, Hedrich R, Forssmann WG, Bals R (2001) Identification of a novel, multifunctional beta-defensin (human beta-defensin 3) with specific antimicrobial activity. Its interaction with plasma membranes of Xenopus oocytes and the induction of macrophage chemoattraction. Cell Tissue Res 306(2):257–264. doi:10.1007/s004410100433

Gesell J, Zasloff M, Opella SJ (1997) Two-dimensional 1H NMR experiments show that the 23-residue magainin antibiotic peptide is an alpha-helix in dodecylphosphocholine micelles, sodium dodecylsulfate micelles, and trifluoroethanol/water solution. J Biomol NMR 9 (2):127–135

Gonzalez M, Gueguen Y, Desserre G, de Lorgeril J, Romestand B, Bachere E (2007) Molecular characterization of two isoforms of defensin from hemocytes of the oyster Crassostrea gigas. Dev Comp Immunol 31(4):332–339. doi:10.1016/j.dci.2006.07.006

Gueguen Y, Herpin A, Aumelas A, Garnier J, Fievet J, Escoubas JM, Bulet P, Gonzalez M, Lelong C, Favrel P, Bachere E (2006) Characterization of a defensin from the oyster Crassostrea gigas. Recombinant production, folding, solution structure, antimicrobial activities, and gene expression. J Biol Chem 281(1):313–323. doi:10.1074/jbc.M510850200

Han S, Bishop BM, van Hoek ML (2008) Antimicrobial activity of human beta-defensins and induction by Francisella. Biochem Biophys Res Commun 371(4):670–674. doi:10.1016/j.bbrc.2008.04.092

Harikrishna N, Rao MS, Murty US (2012) Immune peptides modelling of Culex pipiens sp by in silico methods. J Vector Borne Dis 49(1):19–22

Herve-Grepinet V, Rehault-Godbert S, Labas V, Magallon T, Derache C, Lavergne M, Gautron J, Lalmanach AC, Nys Y (2010) Purification and characterization of avian beta-defensin 11, an antimicrobial peptide of the hen egg. Antimicrob Agents Chemother 54(10):4401–4409. doi:10.1128/AAC.00204-10

Hilton KB, Lambert LA (2008) Molecular evolution and characterization of hepcidin gene products in vertebrates. Gene 415(1–2):40–48. doi:10.1016/j.gene.2008.02.016

Hong SM, Kusakabe T, Lee JM, Tatsuke T, Kawaguchi Y, Kang MW, Kang SW, Kim KA, Nho SK (2008) Structure and expression analysis of the cecropin-E gene from the silkworm, Bombyx mori. Biosci Biotechnol Biochem 72(8):1992–1998. doi:10.1271/bbb.80082

Hu H, Wang C, Guo X, Li W, Wang Y, He Q (2013) Broad activity against porcine bacterial pathogens displayed by two insect antimicrobial peptides moricin and cecropin B. Mol Cells 35 (2):106–114. doi:10.1007/s10059-013-2132-0

Hultmark D, Steiner H, Rasmuson T, Boman HG (1980) Insect immunity. Purification and properties of three inducible bactericidal proteins from hemolymph of immunized pupae of Hyalophora cecropia. Eur J Biochem 106(1):7–16

Jenssen H, Hamill P, Hancock RE (2006) Peptide antimicrobial agents. Clin Microbiol Rev 19 (3):491–511. doi:10.1128/CMR.00056-05

Jordan JB, Poppe L, Haniu M, Arvedson T, Syed R, Li V, Kohno H, Kim H, Schnier PD, Harvey TS, Miranda LP, Cheetham J, Sasu BJ (2009) Hepcidin revisited, disulfide connectivity, dynamics, and structure. J Biol Chem 284(36):24155–24167. doi:10.1074/jbc.M109.017764

Juba ML, Russo PS, Devine M, Barksdale S, Rodriguez C, Vliet KA, Schnur JM, van Hoek ML, Bishop BM (2015) Large scale discovery and De Novo-assisted sequencing of cationic antimicrobial peptides (CAMPs) by microparticle capture and electron-transfer dissociation (ETD) mass spectrometry. J Proteome Res 14(10):4282–4295. doi:10.1021/acs.jproteome.5b00447

Kosciuczuk EM, Lisowski P, Jarczak J, Strzalkowska N, Jozwik A, Horbanczuk J, Krzyzewski J, Zwierzchowski L, Bagnicka E (2012) Cathelicidins: family of antimicrobial peptides. Rev Mol Biol Rep 39(12):10957–10970. doi:10.1007/s11033-012-1997-x

Kouno T, Fujitani N, Mizuguchi M, Osaki T, Nishimura S, Kawabata S, Aizawa T, Demura M, Nitta K, Kawano K (2008) A novel beta-defensin structure: a potential strategy of big defensin for overcoming resistance by Gram-positive bacteria. Biochemistry 47(40):10611–10619. doi:10.1021/bi800957n

Kouno T, Mizuguchi M, Aizawa T, Shinoda H, Demura M, Kawabata S, Kawano K (2009) A novel beta-defensin structure: big defensin changes its N-terminal structure to associate with the target membrane. Biochemistry 48(32):7629–7635. doi:10.1021/bi900756y

Kushibiki T, Kamiya M, Aizawa T, Kumaki Y, Kikukawa T, Mizuguchi M, Demura M, Kawabata S, Kawano S (2014) Interaction between tachyplesin I, an antimicrobial peptide derived from horseshoe crab, and lipopolysaccharide. Biochim Biophys Acta 1844 (3):527–534. doi:10.1016/j.bbapap.2013.12.017

Lakshminarayanan R, Vivekanandan S, Samy RP, Banerjee Y, Chi-Jin EO, Teo KW, Jois SD, Kini RM, Valiyaveettil S (2008) Structure, self-assembly, and dual role of a beta-defensin-like peptide from the Chinese soft-shelled turtle eggshell matrix. J Am Chem Soc 130(14):4660–4668. doi:10.1021/ja075659k

Lee E, Jeong KW, Lee J, Shin A, Kim JK, Lee J, Lee DG, Kim Y (2013) Structure-activity relationships of cecropin-like peptides and their interactions with phospholipid membrane. BMB Rep 46(5):282–287

Lee JY, Yang ST, Lee SK, Jung HH, Shin SY, Hahm KS, Kim JI (2008) Salt-resistant homodimeric bactenecin, a cathelicidin-derived antimicrobial peptide. FEBS J 275 (15):3911–3920. doi:10.1111/j.1742-4658.2008.06536.x

Lehrer RI, Ganz T (1999) Antimicrobial peptides in mammalian and insect host defence. Curr Opin Immunol 11(1):23–27

Lehrer RI, Ganz T (2002) Cathelicidins: a family of endogenous antimicrobial peptides. Curr Opin Hematol 9(1):18–22

Lehrer RI, Lu W (2012) Alpha-defensins in human innate immunity. Immunol Rev 245 (1):84–112. doi:10.1111/j.1600-065X.2011.01082.x

Li SA, Lee WH, Zhang Y (2012) Efficacy of OH-CATH30 and its analogs against drug-resistant bacteria in vitro and in mouse models. Antimicrob Agents Chemother 56(6):3309–3317. doi:10.1128/AAC.06304-11

Madhongsa K, Pasan S, Phophetleb O, Nasompag S, Thammasirirak S, Daduang S, Taweechaisupapong S, Lomize AL, Patramanon R (2013) Antimicrobial action of the cyclic peptide bactenecin on Burkholderia pseudomallei correlates with efficient membrane permeabilization. PLoS Negl Trop Dis 7(6):e2267. doi:10.1371/journal.pntd.0002267

Mine Y, Oberle C, Kassaify Z (2003) Eggshell matrix proteins as defense mechanism of avian eggs. J Agric Food Chem 51(1):249–253. doi:10.1021/jf020597x

Mishra B, Epand RF, Epand RM, Wang G (2013) Structural location determines functional roles of the basic amino acids of KR-12, the smallest antimicrobial peptide from human cathelicidin LL-37. RSC Adv (42). doi:10.1039/C3RA42599A

Molhoek EM, den Hertog AL, de Vries AM, Nazmi K, Veerman EC, Hartgers FC, Yazdanbakhsh M, Bikker FJ, van der Kleij D (2009) Structure-function relationship of the human antimicrobial peptide LL-37 and LL-37 fragments in the modulation of TLR responses. Biol Chem 390(4):295–303. doi:10.1515/BC.2009.037

Nagant C, Pitts B, Nazmi K, Vandenbranden M, Bolscher JG, Stewart PS, Dehaye JP (2012) Identification of peptides derived from the human antimicrobial peptide LL-37 active against biofilms formed by Pseudomonas aeruginosa using a library of truncated fragments. Antimicrob Agents Chemother 56(11):5698–5708. doi:10.1128/AAC.00918-12

Nizet V, Gallo RL (2003) Cathelicidins and innate defense against invasive bacterial infection. Scand J Infect Dis 35(9):670–676. doi:10.1080/00365540310015629

Otvos L Jr (2000) Antibacterial peptides isolated from insects. J Pept Sci 6(10):497–511. doi:10.1002/1099-1387(200010)6:10<497:AID-PSC277>3.0.CO;2-W

Overhage J, Campisano A, Bains M, Torfs EC, Rehm BH, Hancock RE (2008) Human host defense peptide LL-37 prevents bacterial biofilm formation. Infect Immun 76(9):4176–4182. doi:10.1128/IAI.00318-08

Papanastasiou EA, Hua Q, Sandouk A, Son UH, Christenson AJ, Van Hoek ML, Bishop BM (2009) Role of acetylation and charge in antimicrobial peptides based on human beta-defensin-3. APMIS 117(7):492–499. doi:10.1111/j.1600-0463.2009.02460.x

Park CH, Valore EV, Waring AJ, Ganz T (2001) Hepcidin, a urinary antimicrobial peptide synthesized in the liver. J Biol Chem 276(11):7806–7810. doi:10.1074/jbc.M008922200

Pavia KE, Spinella SA, Elmore DE (2012) Novel histone-derived antimicrobial peptides use different antimicrobial mechanisms. Biochim Biophys Acta 1818(3):869–876. doi:10.1016/j.bbamem.2011.12.023

Pierson T, Learmonth-Pierson S, Pinto D, van Hoek ML (2013) Cigarette smoke extract induces differential expression levels of beta-defensin peptides in human alveolar epithelial cells. Tob Induc Dis 11(1):10. doi:10.1186/1617-9625-11-10

Ponkham P, Daduang S, Kitimasak W, Krittanai C, Chokchaichamnankit D, Srisomsap C, Svasti J, Kawamura S, Araki T, Thammasirirak S (2010) Complete amino acid sequence of three reptile lysozymes. Comp Biochem Physiol C: Toxicol Pharmacol 151(1):75–83. doi:10.1016/j.cbpc.2009.08.010

Rapala-Kozik M, Bochenska O, Zawrotniak M, Wolak N, Trebacz G, Gogol M, Ostrowska D, Aoki W, Ueda M, Kozik A (2015) Inactivation of the antifungal and immunomodulatory properties of human cathelicidin LL-37 by aspartic proteases produced by the pathogenic yeast Candida albicans. Infect Immun 83(6):2518–2530. doi:10.1128/IAI.00023-15

Rivas-Santiago B, Rivas Santiago CE, Castaneda-Delgado JE, Leon-Contreras JC, Hancock RE, Hernandez-Pando R (2013) Activity of LL-37, CRAMP and antimicrobial peptide-derived compounds E2, E6 and CP26 against Mycobacterium tuberculosis. Int J Antimicrob Agents 41(2):143–148. doi:10.1016/j.ijantimicag.2012.09.015

Rodriguez R, Jung CL, Gabayan V, Deng JC, Ganz T, Nemeth E, Bulut Y (2014) Hepcidin induction by pathogens and pathogen-derived molecules is strongly dependent on interleukin-6. Infect Immun 82(2):745–752. doi:10.1128/IAI.00983-13

Rosa RD, Santini A, Fievet J, Bulet P, Destoumieux-Garzon D, Bachere E (2011) Big defensins, a diverse family of antimicrobial peptides that follows different patterns of expression in hemocytes of the oyster Crassostrea gigas. PLoS ONE 6(9):e25594. doi:10.1371/journal.pone.0025594

Rosa RD, Alonso P, Santini A, Vergnes A, Bachere E (2015) High polymorphism in big defensin gene expression reveals presence-absence gene variability (PAV) in the oyster Crassostrea gigas. Dev Comp Immunol 49(2):231–238. doi:10.1016/j.dci.2014.12.002

Rozek A, Friedrich CL, Hancock RE (2000) Structure of the bovine antimicrobial peptide indolicidin bound to dodecylphosphocholine and sodium dodecyl sulfate micelles. Biochemistry 39(51):15765–15774

Saito T, Kawabata S, Shigenaga T, Takayenoki Y, Cho J, Nakajima H, Hirata M, Iwanaga S (1995) A novel big defensin identified in horseshoe crab hemocytes: isolation, amino acid sequence, and antibacterial activity. J Biochem 117(5):1131–1137

Schmitt P, Wilmes M, Pugniere M, Aumelas A, Bachere E, Sahl HG, Schneider T, Destoumieux-Garzon D (2010) Insight into invertebrate defensin mechanism of action: oyster defensins inhibit peptidoglycan biosynthesis by binding to lipid II. J Biol Chem 285(38):29208–29216. doi:10.1074/jbc.M110.143388

Schmitt P, Rosa RD, Duperthuy M, de Lorgeril J, Bachere E, Destoumieux-Garzon D (2012) The Antimicrobial Defense of the Pacific Oyster, Crassostrea gigas. How Diversity may Compensate for Scarcity in the Regulation of Resident/Pathogenic Microflora. Front Microbiol 3:160. doi:10.3389/fmicb.2012.00160

Schroeder BO, Stange EF, Wehkamp J (2011a) Waking the wimp: redox-modulation activates human beta-defensin 1. Gut Microbes 2(4):262–266. doi:10.4161/gmic.2.4.17692

Schroeder BO, Wu Z, Nuding S, Groscurth S, Marcinowski M, Beisner J, Buchner J, Schaller M, Stange EF, Wehkamp J (2011b) Reduction of disulphide bonds unmasks potent antimicrobial activity of human beta-defensin 1. Nature 469(7330):419–423. doi:10.1038/nature09674

Schulenburg H, Boehnisch C, Michiels NK (2007) How do invertebrates generate a highly specific innate immune response? Mol Immunol 44(13):3338–3344. doi:10.1016/j.molimm.2007.02.019

Semple CA, Gautier P, Taylor K, Dorin JR (2006) The changing of the guard: molecular diversity and rapid evolution of beta-defensins. Mol Divers 10(4):575–584. doi:10.1007/s11030-006-9031-7

Sorensen OE, Borregaard N (2005) Cathelicidins–nature's attempt at combinatorial chemistry. Comb Chem High Throughput Screen 8(3):273–280

Steiner H, Hultmark D, Engstrom A, Bennich H, Boman HG (1981) Sequence and specificity of two antibacterial proteins involved in insect immunity. Nature 292(5820):246–248

Sun T, Zhan B, Gao Y (2015) A novel cathelicidin from Bufo bufo gargarizans Cantor showed specific activity to its habitat bacteria. Gene 571(2):172–177. doi:10.1016/j.gene.2015.06.034

Tako E, Rutzke MA, Glahn RP (2010) Using the domestic chicken (Gallus gallus) as an in vivo model for iron bioavailability. Poult Sci 89(3):514–521. doi:10.3382/ps.2009-00326

Tomasinsig L, Zanetti M (2005) The cathelicidins–structure, function and evolution. Curr Protein Pept Sci 6(1):23–34

Tongaonkar P, Golji AE, Tran P, Ouellette AJ, Selsted ME (2012) High fidelity processing and activation of the human alpha-defensin HNP1 precursor by neutrophil elastase and proteinase 3. PLoS ONE 7(3):e32469. doi:10.1371/journal.pone.0032469

van Dijk A, Veldhuizen EJ, van Asten AJ, Haagsman HP (2005) CMAP27, a novel chicken cathelicidin-like antimicrobial protein. Vet Immunol Immunopathol 106(3-4):321–327. doi:10.1016/j.vetimm.2005.03.003

van Dijk A, Tersteeg-Zijderveld MH, Tjeerdsma-van Bokhoven JL, Jansman AJ, Veldhuizen EJ, Haagsman HP (2009) Chicken heterophils are recruited to the site of Salmonella infection and release antibacterial mature Cathelicidin-2 upon stimulation with LPS. Mol Immunol 46 (7):1517–1526. doi:10.1016/j.molimm.2008.12.015

van Dijk A, Molhoek EM, Bikker FJ, Yu PL, Veldhuizen EJ, Haagsman HP (2011) Avian cathelicidins: Paradigms for the development of anti-infectives. Vet Microbiol 153:27–36

van Hoek ML (2014) Antimicrobial peptides in reptiles. Pharmaceuticals (Basel) 7(6):723–753. doi:10.3390/ph7060723

Wang Z, Wang G (2004) APD: the Antimicrobial Peptide Database. Nucleic Acids Res 32 (Database issue):D590–592. doi:10.1093/nar/gkh025

Wang Y, Walter G, Herting E, Agerberth B, Johansson J (2004) Antibacterial activities of the cathelicidins prophenin (residues 62 to 79) and LL-37 in the presence of a lung surfactant preparation. Antimicrob Agents Chemother 48(6):2097–2100. doi:10.1128/AAC.48.6.2097-2100.2004

Wang Y, Hong J, Liu X, Yang H, Liu R, Wu J, Wang A, Lin D, Lai R (2008) Snake cathelicidin from Bungarus fasciatus is a potent peptide antibiotics. PLoS ONE 3(9):e3217. doi:10.1371/journal.pone.0003217

Wang G, Li X, Wang Z (2009) APD2: the updated antimicrobial peptide database and its application in peptide design. Nucleic Acids Res 37 (Database issue):D933–937. doi:10.1093/nar/gkn823

Wang Z, Pascual-Anaya J, Zadissa A, Li W, Niimura Y, Huang Z, Li C, White S, Xiong Z, Fang D, Wang B, Ming Y, Chen Y, Zheng Y, Kuraku S, Pignatelli M, Herrero J, Beal K, Nozawa M, Li Q, Wang J, Zhang H, Yu L, Shigenobu S, Wang J, Liu J, Flicek P, Searle S, Wang J, Kuratani S, Yin Y, Aken B, Zhang G, Irie N (2013) The draft genomes of soft-shell turtle and green sea turtle yield insights into the development and evolution of the turtle-specific body plan. Nat Genet 45(6):701–706. doi:10.1038/ng.2615

Wang G, Mishra B, Epand RF, Epand RM (2014) High-quality 3D structures shine light on antibacterial, anti-biofilm and antiviral activities of human cathelicidin LL-37 and its fragments. Biochim Biophys Acta 1838(9):2160–2172. doi:10.1016/j.bbamem.2014.01.016

Wilson CL, Schmidt AP, Pirila E, Valore EV, Ferri N, Sorsa T, Ganz T, Parks WC (2009) Differential processing of {alpha}- and {beta}-defensin precursors by matrix metalloproteinase-7 (MMP-7). J Biol Chem 284(13):8301–8311. doi:10.1074/jbc.M809744200

Wu Z, Hoover DM, Yang D, Boulegue C, Santamaria F, Oppenheim JJ, Lubkowski J, Lu W (2003) Engineering disulfide bridges to dissect antimicrobial and chemotactic activities of human beta-defensin 3. Proc Natl Acad Sci USA 100(15):8880–8885. doi:10.1073/pnas.1533186100

Xiao Y, Hughes AL, Ando J, Matsuda Y, Cheng JF, Skinner-Noble D, Zhang G (2004) A genome-wide screen identifies a single beta-defensin gene cluster in the chicken: implications for the origin and evolution of mammalian defensins. BMC Genom 5(1):56. doi:10.1186/1471-2164-5-56

Xiao Y, Cai Y, Bommineni YR, Fernando SC, Prakash O, Gilliland SE, Zhang G (2006) Identification and functional characterization of three chicken cathelicidins with potent antimicrobial activity. J Biol Chem 281(5):2858–2867. doi:10.1074/jbc.M507180200

Yan X, Zhong J, Liu H, Liu C, Zhang K, Lai R (2012) The cathelicidin-like peptide derived from panda genome is a potential antimicrobial peptide. Gene 492(2):368–374. doi:10.1016/j.gene.2011.11.009

Yang Y, Sanchez JF, Strub MP, Brutscher B, Aumelas A (2003) NMR structure of the cathelin-like domain of the protegrin-3 precursor. Biochemistry 42(16):4669–4680. doi:10.1021/bi027133c

Yi HY, Chowdhury M, Huang YD, Yu XQ (2014) Insect antimicrobial peptides and their applications. Appl Microbiol Biotechnol 98(13):5807–5822. doi:10.1007/s00253-014-5792-6

Zanetti M (2005) The role of cathelicidins in the innate host defenses of mammals. Curr Issues Mol Biol 7(2):179–196

Zanetti M, Gennaro R, Scocchi M, Skerlavaj B (2000) Structure and biology of cathelicidins. Adv Exp Med Biol 479:203–218. doi:10.1007/0-306-46831-X_17

Zasloff M (1987) Magainins, a class of antimicrobial peptides from Xenopus skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor. Proc Natl Acad Sci USA 84(15):5449–5453

Zhang XL, Selsted ME, Pardi A (1992) NMR studies of defensin antimicrobial peptides. 1. Resonance assignment and secondary structure determination of rabbit NP-2 and human HNP-1. Biochemistry 31(46):11348–11356

Zhao H, Gan TX, Liu XD, Jin Y, Lee WH, Shen JH, Zhang Y (2008) Identification and characterization of novel reptile cathelicidins from elapid snakes. Peptides 29(10):1685–1691. doi:10.1016/j.peptides.2008.06.008

Zhao L, Lu W (2014) Defensins in innate immunity. Curr Opin Hematol 21(1):37–42. doi:10.1097/MOH.0000000000000005

Zhu S (2008) Positive selection targeting the cathelin-like domain of the antimicrobial cathelicidin family. Cell Mol Life Sci 65(7–8):1285–1294. doi:10.1007/s00018-008-8070-x

Zhu S, Gao B (2009) A fossil antibacterial peptide gives clues to structural diversity of cathelicidin-derived host defense peptides. FASEB J 23(1):13–20. doi:10.1096/fj.08-114579

Chapter 2
Antifungal Host Defense Peptides

Karl Lohner and Regina Leber

Abstract Fungi infect billions of people every year, yet their contribution to the global burden of disease is largely unrecognized and the repertoire of antifungal agents is rather limited. Thus, treatment of life-threatening invasive fungal infections is still based on drugs discovered several decades ago. In addition, recent data on resistance emergence of fungi emphasize the urgent need for novel antifungal treatments. One alternative strategy is based on host defense peptides. Among the large number of antimicrobial peptides, a group of peptides show primarily antifungal activity by interfering with enzymes of cell wall biosynthesis or specific membrane lipids such as ergosterol. Both are promising targets for antifungal peptides, as they are absent in mammalian cells and hence low toxicity of peptides can be expected. However, most of the antimicrobial peptides exhibit a broad spectrum activity including antifungal activity. These peptides act on the cell membrane level and although their structures vary largely, they share a positive net charge, which facilitates electrostatic interactions with negatively charged lipids of the target cell, and an amphipathic structure, which facilitates incorporation into the cell membrane and in turn membrane disruption. Thereby, membrane lipids differing between mammals and fungi play a central role concerning specificity and efficacy of these peptides. Hence, understanding their molecular mechanism(s) of action will aid in the design of novel antifungal agents. Finally, some of these peptides were shown to act synergistically with conventional drugs, which would further widen the armory to treat especially life-threatening invasive fungal infections.

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2.1 Introduction

Of the 1.5 million fungal species, around 300 are reported to be pathogenic in humans (Taylor et al. 2001). Superficial and mucosal fungal infections are extremely common, but life-threatening invasive fungal infections have increased in importance (Polvi et al. 2015; Warnock 2007). In a very recent review about yeast pathogens, *Cryptococcus neoformans* was described as the leading cause of deaths due to fungal infections, with a global burden of nearly 1 million cases annually, and more than 620,000 deaths worldwide (Polvi et al. 2015; Park et al. 2009). Further, cryptococcal meningitis contribute up to 20 % of AIDS-related mortality in low-income and middle-income countries every year (Loyse et al. 2013). *Candida albicans,* another important fungal pathogen, causes more than 400,000 deaths per year due to invasive candidiasis (Horn et al. 2009). Risk factors for invasive candidiasis include surgery (especially abdominal surgery), burns, long-term stay in an intensive care unit, and previous administration of broad spectrum antibiotics and immunosuppressive agents (Kontoyiannis et al. 2003; Zaoutis et al. 2005; Sydnor and Perl 2011; Pfaller and Diekema 2004; Spampinato and Leonardi 2013). The Centers for Disease Control and Prevention, Atlanta, reported that roughly one third of patients, who suffer from bloodstream infections caused by drug-resistant *Candida* spp., die during their hospitalization in the US (http://www.cdc.gov/drugresistance/threat-report-2013/pdf/ar-threats-2013-508.pdf). Finally, patients with impaired immune function are also often infected by *Aspergillus* species (Polvi et al. 2015; Warnock 2007).

Despite the profound impact of fungal pathogens on human health worldwide, treatment can be hampered by toxicity, poor tolerability, or a narrow activity spectrum of antifungal drugs. Nevertheless, invasive fungal infections remain understudied and underdiagnosed as compared to other infectious diseases (Brown et al. 2012). Further, the repertoire of antifungal agents is rather limited and therefore treatment of life-threatening invasive fungal infections is still mainly based on drugs discovered several decades ago (Butts and Krysan 2012). Polyenes, azoles, allylamines, and echinocandins represent the most common classes of antifungals currently used in the clinics. These agents demonstrate high levels of antifungal activity, although resistance is reported for all classes including echinocandins, which represent the first and so far only class of licensed antifungal peptides (Polvi et al. 2015; Drgona et al. 2014; Spampinato and Leonardi 2013; Chen and Sorrell 2007; Perlin 2015). The fungal-derived echinocandins are cyclic hexapeptides with N-linked acyl lipid side chains, which inhibit cell wall biosynthesis at the level of (1,3)-β-D-glucan synthase (Boucher et al. 2004). Whereas native echinocandins were hemolytic and had poor solubility in water, chemical modifications resulted in molecules with improved properties (Luca and Walsh 2000; Denning 2002). The first licensed echinocandin product was caspofungin acetate (Cancidas®; Merck) (Denning 2002). Currently, also micafungin (Mycymine®; Astella Pharma) and anidulafungin (Ecalta®, Pfizer) are available for treatment of invasive fungal infections. The inhibitory spectrum of these

Table 2.1 Secondary structure and physico-chemical properties of antifungal peptides comprised of ≤50 amino acids[a]

Net charge[b]	Secondary structure	Hydrophobic residues (%)									Number of peptides
		≤10	11–20	21–30	31–40	41–50	51–60	61–70	71–80	81–90	**855**
Positive	α-helix	3	1	8	31	38	31	13	3	0	128
	β-sheet	0	0	3	16	9	13	1	0	0	42
	α + β (combined)	0	0	3	19	6	1	0	0	0	29
	Neither α nor β	0	0	0	1	1	2	0	0	0	4
	Disulfide	0	0	11	65	22	15	2	0	0	115
	Rich in unusual aa	8	1	1	3	1	0	0	0	0	14
	Unknown	0	2	22	68	159	116	91	4	1	463
		11	**4**	**48**	**203**	**236**	**178**	**107**	**7**	**1**	**795**
Zero	α-helix	0	0	0	0	0	0	0	0	0	0
	β-sheet	0	0	0	0	0	0	0	0	0	0
	α + β (combined)	0	0	0	0	0	0	0	0	0	0
	Neither α nor β	0	0	0	0	0	0	0	0	0	0
	Disulfide	0	0	0	2	0	0	0	0	0	2
	Rich in unusual aa	4	2	0	0	0	0	0	0	0	6
	Unknown	1	1	3	9	6	4	3	1	0	28
		5	**3**	**3**	**11**	**6**	**4**	**3**	**1**	**0**	**36**
Negative	α-helix	0	0	0	1	2	0	1	0	0	4
	β-sheet	0	0	0	1	0	0	0	0	0	1
	α + β (combined)	0	0	0	1	0	0	0	0	0	1
	Neither α nor β	0	0	0	0	0	0	0	0	0	0
	Disulfide	0	0	1	0	0	0	0	0	0	1
	Rich in unusual aa	1	0	0	0	0	0	0	0	0	1
	Unknown	1	3	1	5	2	1	0	3	0	16
		2	**3**	**2**	**8**	**4**	**1**	**1**	**3**	**0**	**24**

[a]Data from AP Database (Wang and Wang 2004; Wang et al. 2009)
[b]At physiological pH

synthetically modified lipopeptides, however, does not include the leading fungal pathogen *Cryptococcus neoformans* since this pathogen has little or no (1,3)-β-D-glucan synthase enzyme (Denning 1997; Hector 1993).

Currently, there is considerable interest in antifungal properties of antimicrobial peptides (AMPs) and research on this topic has strongly expanded during the past decade. Antimicrobial peptides are produced by diverse life forms including mammals, plants, amphibians, insects, fungi, and bacteria. More than 2500 natural or synthetic AMPs are listed in the Antimicrobial Peptide Database (APD, http://aps.unmc.edu/AP) of which around 900 have antifungal activity (Wang and Wang 2004; Wang et al. 2009) including some proteins such as ribonucleases and proteases. The vast majority of AMPs with antifungal activity is positively charged and for almost two third of these peptides no secondary structure is determined so far (Table 2.1). About 15 % of the peptides exhibit either an α-helix or structures stabilized by disulfide bonds, while peptides that adopt a β-sheet make up to only ~5 %. A similar amount of peptides have a combined α-helical/β-sheet structure and a very minor fraction (2 %) is rich in unusual amino acids. It is of interest to note that peptides containing unusual amino acids have a very low content of hydrophobic residues (mostly ≤10 %), while the percentage of hydrophobic residues of the majority of peptides with antifungal activity is around 50 % (Table 2.1). Excellent reviews about antifungal peptides originating from insects and plants have very recently been published (Faruck et al. 2015; Lacerda et al. 2014; Vriens et al. 2014; Silva et al. 2014; Nawrot et al. 2014). Furthermore, Matejuk et al. described peptide-based strategies for antifungal therapies against emerging infections emphasizing that these peptides may have specific targets showing selective toxicity or may be multifunctional in their mode of action (Matejuk et al. 2010). The number of peptides exhibiting primarily antifungal activity such as echinocandins is much lower than peptides exhibiting a broad antimicrobial activity supposedly resulting in lysis of the cytoplasmic membrane. This review will focus on the different fungal targets of peptides that have shown selective toxicity against fungal pathogens in vitro or in vivo. Further, we will briefly discuss mechanisms of membrane lysis and describe co-applications of standard drugs and antifungal peptides.

2.2 Targets for Antifungal Therapy

In terms of numbers of classes of agents that can be used to treat life-threatening mycoses, the targets of antifungal agents are heavily focused, directly or indirectly, on the cell envelope (wall and plasma membrane), and particularly on the fungal membrane sterol, ergosterol, and its biosynthesis (Odds et al. 2003) (Table 2.2, Fig. 2.1). From the 1950s until the discovery of azoles, polyene antifungal agents such as amphotericin B, which are known to cause significant nephrotoxicity, represented the standard of therapy for systemic fungal infections (Ghannoum and Rice 1999). Amphotericin B (AmpB) has been proposed to interact with plasma

Table 2.2 Proposed targets of selected antifungal peptides

Target		Peptide	Sequence/Net charge	Reference(s)
Cell wall	Glucan synthase inhibitors	Echinocandines	Cyclic hexapeptides	Boucher et al. (2004)
	Chitin synthase inhibitor	Nikkomycin, polyoxin	Peptidyl nucleoside antibiotics	Nix et al. (2009)
	Chitin binding	Hevein-like SmAMP3	VGPGGECGGRFGGCAGGQCCSRFGFCGSGPKYCAH net charge: +2	Rogozhin et al. (2015) (APD ID: AP02585)[a]
	Chitin binding	CTB1	Cyclic thiopeptide	Mizuhara et al. (2011)
	Chitin binding	Penaeidin-4d	HSSGYTRPLRKPSRPIFIRPIGCDVCYGIPSSTARLCCFRYGDCCHL net charge: +5	(Cuthbertson et al. 2002; Destoumieux et al. 2000) (APD ID: AP00420)
Plasma membrane[b]	Ergosterols facilitating pore formation of SE	Syringomycin E (SE)	cyclic lipodepsi-nonapeptide	(Lucca et al. 1999; Feigin et al. 1997)
	High ergosterol affinity, (mitochondria?)	Human neutrophil peptide-1 (HNP-1)	ACYCRIPACIAGERRYGTCIYQGRLWAFCC net charge: +3	(Selsted et al. 1985; Gonçalves et al. 2012a; Lehrer et al. 1988) (APD ID: AP00176)
	Interaction with sphingolipids, nucleus (cell cycle control)	Psd1	KTCEHLADTYRGVCFTNASCDDHCKNKAHLISGTCHNWKCFCTQNC net charge: +1	(Almeida et al. 2000; Medeiros et al. 2010; Lobo et al. 2007) (APD ID: AP00483)
	Interaction with sphingolipids	DmAMP1	ELCEKASKTWSGNCGNTGHCDNQCKSWEGAAHGACHVRNGKHMCFCYFNC net charge: +1	(François et al. 2002; Thevissen et al. 2000) (APD ID: AP00918)
	Interaction with sphingolipids	LTX109	Synthetic peptidomimetic (arginine-tertbutyl tryptophan-arginine-phenylethan) net charge: +2	(Isaksson et al. 2011; Bojsen et al. 2013)
	Interaction with sphingolipids	RsAFP2	QKLCQRPSGTWSGVCGNNNACKNQCIRLEKARHGSCNYVFPAHKCICYFPC net charge: +6	(Terras et al. 1993; Thevissen et al. 2004) (APD ID: AP00287)
	Interaction with sphingolipids	Heliomycin	DKLIGSCVWGAVNYTSDCNGECKRRGYKGGHCGSFANVNCWCET net charge: +1	(Lamberty et al. 2001; Thevissen et al. 2004) (APD ID: AP00031)

(continued)

Table 2.2 (continued)

Target		Peptide	Sequence/Net charge	Reference(s)
	Interaction with sphingolipids	Sugarcane defensin (Sd5)	HTPTPTPICKSRSHEYKGRCIQDMDCNAACVKESESYT GGFCNGRPPFKQCFCTKPCKRERAAATLRWPGL net charge: +6	(De-Paula et al. 2008) (APD ID: AP02018)
	Interaction with sphingolipids	Drosomycin	DCLSGRYKGPCAVWDNETCRRVCKEEGRSSGHCSPSLKCWCEGC net charge: +1	(Fehlbaum et al. 1994; Gao and Zhu 2008) (APD ID: AP00672)
	Interaction with sphingolipids, blocks mammalian L-type Ca^{2+} channel	MsDef1	RTCENLADKYRGPCFSGCDTHCTTKENAVSGRCRDDFRCWCTKRC net charge: +3	(Ramamoorthy et al. 2007; Spelbrink et al. 2004) (APD ID: AP00978)
Intracellular	Mitochondria	Human histatin 5 (Hst5)	DSHAKRHHGYKRKFHEKHHSHRGY net charge: +5	(Oppenheim et al. 1988; Helmerhorst et al. 1999) (APD ID: AP00505)
	Nucleus	Auristatin PHE (synthetic product of marine dolastatin 10)	Pentapetide (dovaline-valine-dolaisoleunine-dolaproine-phenylalanine-methyl-ester)	Woyke et al. (2001, 2002)
	Intracellular (DNA damage)	Dermaseptin S3 (DsS3 (1-16))	ALWKNMLKGIGKLAGKAALGAVKKLVGAES net charge: +5	(Mor et al. 1994; Morton et al. 2007) (APD ID: AP00159)
	intracellular (DNA damage)	Magainin 2	GIGKFLHSAKKFGKAFVGEIMNS net charge: +3	(Zasloff 1987; Morton et al. 2007) (APD ID: AP00144)
	Acts on vacuolar proton pump, seems to act via Ca^{2+} influx through L-type Ca^{2+} channels	PA1b	ASCNGVCSPFEMPPCGTSACRCIPVGLVIGYCRNPSG net charge: +1	(Chouabe et al. 2011; Gressent et al. 2011)

[a]ID number of peptides in the AP Database (Wang and Wang 2004; Wang et al. 2009)
[b]Membrane-active peptides with broad spectrum activity are not listed. Examples can be deduced from the AP Database and from Table 2.2 (Matejuk et al. 2010)

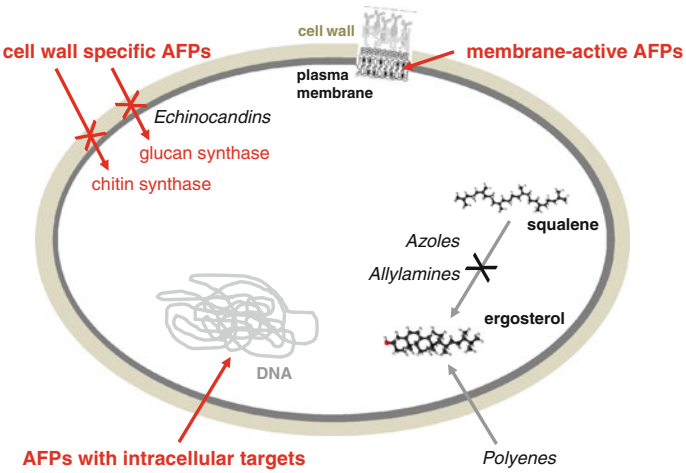

Fig. 2.1 Potential targets of antifungal peptides and conventional antifungal drugs. Latter interfere mainly with the biosynthesis of ergosterol and its physiological function, while the former predominantly interfere with cell wall biosynthesis and cell membrane integrity

membrane ergosterol resulting in the formation of ion channel aggregates that are inserted into lipid bilayers and thereby permeabilize and kill yeast (Kruijff and Demel 1974; Holz 1974). Anderson et al., however, reported that AmpB exists primarily in the form of large, extra-membranous aggregates that kill yeast by extracting ergosterol from lipid bilayers (Anderson et al. 2014; Lohner 2014). The clinical efficacy and safety of azoles, in particular fluconazole, has led to their extensive use. The primary target of azoles is a heme protein, which catalyzes cytochrome P-450-dependent 14-α-demethylation of lanosterol (Hitchcock et al. 1990). Accumulation of zymosterol and squalene was observed, when *C. albicans* cells were treated with voriconazole (Sanati et al. 1997). Mammalian cholesterol synthesis is also blocked by azoles at the stage of 14-α-demethylation, however, the dose required to effect the same degree of inhibition is much higher than that required for fungi (Hitchcock et al. 1990; van den Bossche et al. 1978; Ghannoum and Rice 1999). Allylamines, such as terbinafine and naftifine, have primarily fungicidal action against many fungi as a result of its specific inhibition of squalene epoxidase (Ryder 1992). Treated fungi accumulate squalene, while becoming deficient in ergosterol, which leads to inhibition of growth. Terbinafine has no effect on cholesterol biosynthesis in vivo (Ryder 1992). Regarding antifungal proteins and peptides, potential targets of fungal cells including several intracellular targets were described earlier (Theis and Stahl 2004; Matejuk et al. 2010). Novel antifungal drugs need to act on targets that are absent or different in mammalian cells.

2.3 Cell Wall-Specific Antifungal Peptides

For pathogenic fungi, the cell wall (Fig. 2.2) is critical for invading the host and resisting against host defense mechanisms (Latgé and Beauvais 2014). It provides the cell with sufficient mechanical strength to withstand changes in osmotic pressure imposed by the environment. The fungal cell wall is a complex structure composed typically of chitin, 1,3-β- and 1,6-β-glucan, mannan and proteins, although cell wall composition frequently varies markedly between species of fungi (Adams 2004). Enzymes catalyzing the synthesis of cell wall components are promising targets for antifungal peptides as they are absent in mammalian cells and hence low toxicity of peptides can be expected. Disruptions of cell wall structure have a profound effect on the growth and morphology of the fungal cell, often rendering it susceptible to lysis and death (Bowman and Free 2006).

2.3.1 Inhibitors of Glucan Synthase

Glucan is the major structural polysaccharide of the fungal cell wall, constituting approximately 50–60 % of the wall by dry weight (Fleet 1985; Kapteyn et al. 1999). The 1,3-β-glucan serves as the main structural constituent to which other cell wall components are covalently attached. As a result, the synthesis of 1,3-β-glucan is required for proper cell wall formation and the normal development of fungi (Bowman and Free 2006).

Echinocandins (caspofungin, micafungin, and anidulafungin) are now the preferred first line therapy for patients with invasive candidiasis (Spampinato and Leonardi 2013). These semi-synthetic lipopeptides are non-competitive inhibitors of (1,3)-β-D-glucan synthase, an enzyme complex that forms glucan polymers in fungal cell walls (Denning 1997). This leads to the formation of fungal cell walls

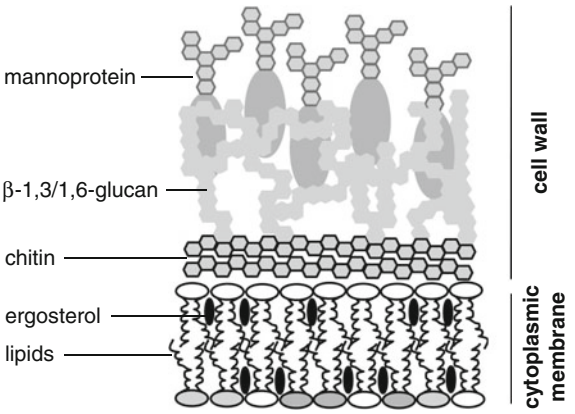

Fig. 2.2 Schematic representation of the fungal cell envelope highlighting the most important components of the cell wall and cytoplasmic membrane. Membrane proteins were omitted for clarity

with impaired structural integrity, which in turn results in cell vulnerability to osmotic lysis (Grover 2010). Their low toxicity may reflect the fact that their target, (1,3)-β-D-glucan synthase, is not found in humans (Perlin 2015). Echinocandin drugs are potentially fungicidal against most clinically important *Candida* spp. but, are considered fungistatic against *Aspergillus* (Barchiesi et al. 2005; Ernst et al. 1999; Bowman et al. 2002; 2006; Pfaller et al. 2003). Although these types of drugs were licensed first in 2001, reports on *Candida* spp. isolates resistant to echinocandins are increasingly reported (Perlin 2015). Resistance is attributed to point mutations in the *FKS1* gene, which encodes the major subunit of the glucan synthase complex (Perlin 2007).

2.3.2 *Inhibitors of Chitin Synthase*

Chitin, a long linear homo-polymer of β-1,4-linked N-acetylglucosamine, is a structurally important component of the fungal cell wall. Chitin accounts for only 1–2 % of the yeast cell wall by dry weight (Klis 1994; Klis et al. 2002), whereas the cell walls of filamentous fungi, such as *Neurospora* and *Aspergillus*, are reported to contain 10–20 % chitin (Nobel et al. 2000; Bartnicki-Garcia 1968; Bowman et al. 2006). Disruption of chitin synthesis leads to disordered cell walls and the fungal cell becomes malformed and osmotically unstable (Bago et al. 1996; Specht et al. 1996).

Nikkomycins are a group of peptidyl nucleoside antibiotics produced by *Streptomyces ansochromogenes* (Chen et al. 2000) and *Streptomyces tendae* (Brillinger 1979). Acting as competitive inhibitors of chitin synthase, nikkomycins inhibit the growth of filamentous fungi and yeasts (Dähn et al. 1976; Feng et al. 2014). Compared to conventional antifungal agents, including fluconazole and amphotericin B, nikkomycin Z resulted in greater killing of *Coccidioides* spp. and was able to sterilize lung lesions in seven of eight mice dosed with 50 mg/kg/day for 6 days, while the conventional agents tested did not sterilize lung lesions in any case (Hector et al. 1990). Nikkomycin Z has been used in Phase I clinical trials for the treatment of coccidioidomycosis (Nix et al. 2009). However, the peptidyl nucleoside was degraded in rat, mouse and rabbit plasma much faster than in pH 7.5 buffer (Tokumura and Horie 1997). Recently, two novel nikkomycin analogs (nikkomycin Px and Pz) were generated by mutasynthesis showing similar antifungal activities to those of natural nikkomycins, but with improved stabilities under different pHs and temperatures (Feng et al. 2014). Polyoxins, which were isolated from the culture broth of *Streptomyces cacaoi*, are closely related to nikkomycins and also act as specific inhibitors of chitin synthase (Hector 1993; Isono et al. 1969). Polyoxins, which contained hydrophobic amino acids, retained strong chitin synthase inhibitory activity and were resistant to cellular hydrolysis of *C. albicans* (Smith et al. 1986).

2.3.3 Chitin Binding Peptides

Members of the family of hevein-like antimicrobial peptides carry a conserved chitin binding site. The hevein-like peptides belong to a unique class of plant antimicrobials that show resemblance to hevein, the antimicrobial peptide (AMP) from latex of *Hevea brasiliensis* (van Parijs et al. 1991; Rogozhin et al. 2015). Their antifungal activity is supposed to be associated with their chitin binding activity. Binding to chitin is believed to interfere with hyphal growth resulting in abnormal branching, retardation of elongation and swelling. Hevein-like peptides are rarely found outside the plant kingdom. Novel hevein-like peptide precursors were identified by similarity search methods, including one from a fungal source (Porto et al. 2012). SmAMP3, a new member of the hevein-like family peptides was isolated recently from leaves of a weed species *S. media* (Rogozhin et al. 2015). It is basic and cysteine-rich, with six cysteines linked to form three disulfide bridges. SmAMP3 demonstrated significant inhibition of spore germination of fungi with highest activity against *B. cinerea* (Rogozhin et al. 2015).

Cyclothiazomycin B1 (CTB1) is an antifungal cyclic thiopeptide isolated from the culture broth of *Streptomyces* sp. HA 125-40. CTB1 inhibited the growth of several filamentous fungi including plant pathogens along with swelling of hyphae and spores, which indicates serious effects on cell wall rigidity. CTB1 does not inhibit chitin synthase activity, but it induces cell wall fragility by binding to chitin (Mizuhara et al. 2011). Also the antifungal activity of penaeidins, a family of antimicrobial peptides characterized in the shrimp *Penaeus vannamei*, can be related to their chitin binding ability (Destoumieux et al. 2000).

2.4 Membrane-Active Antifungal Peptides

As mentioned in the introduction and described above host defense peptides with primarily antifungal activity are much less abundant than peptides with broad antimicrobial activity. This is most likely due to evolution creating molecules that can protect the host from a variety of invaders. Therefore, the predominant fraction of these peptides shows a broad spectrum activity against bacteria, fungi and even viruses (Cole and Ganz 2000). Within this plethora of peptides, which predominantly act on the plasma membrane level, there are some, which interact with specific membrane lipid components such as ergosterol and sphingolipids, described in Sects. 2.4.2 and 2.4.3. However, most of them are supposed to induce lysis of the cell membrane. The molecular mechanism(s) of membrane rupture mutually depends on the nature of the peptide and membrane lipid composition (Lohner and Blondelle 2005; Lohner 2009). Thus, in terms of antifungal drug design it is crucial that antifungal peptides can discriminate between target and host membrane (Lohner 2001).

2.4.1 Broad Spectrum Antimicrobial Peptides

Mammalian and fungal membranes are composed of proteins and three main lipids: phospholipids, sphingolipids, and sterols (Zinser et al. 1993; Löffler et al. 2000; van Meer and de Kroon 2011). The phospholipid classes of eukaryotic plasma membranes are asymmetrically distributed as they actively sequester phosphatidylcholine (PC) and sphingomyelin (SM) within the outer monolayer of the membrane (van Meer et al. 2008; Devaux and Morris 2004). PC accounts for >50 % of the phospholipids in most eukaryotic membranes. It self-organizes spontaneously as a planar bilayer, in which PC has a nearly cylindrical molecular geometry (Fig. 2.3). Most PC molecules have one *cis*-unsaturated fatty acyl chain, which renders the membrane fluid at room temperature (van Meer et al. 2008; van Meer and de Kroon 2011). Sphingolipids usually contain a long to very long saturated fatty acid (C16–C32) with an amide linkage to the sphingoid base. They generally adopt a solid gel phase, but are fluidized by sterols, which supposedly preferentially interact with them in the membrane (van Meer and de Kroon 2011). Phosphatidylethanolamine (PE) as well as the negatively charged lipids phosphatidylserine (PS) and phosphatidylinositol (PI) are found almost exclusively in the inner leaflet of the bilayer

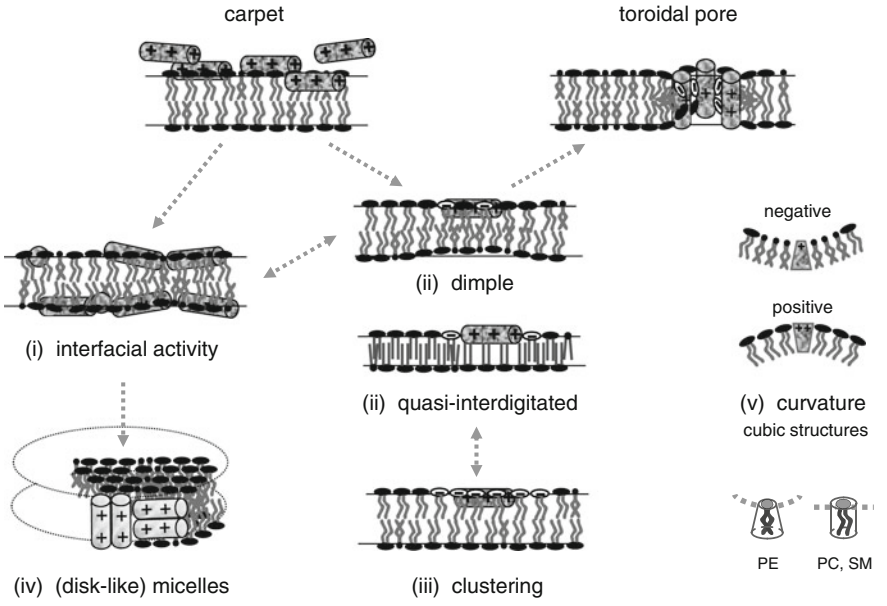

Fig. 2.3 Schematic representation of various modes of action of membrane-active peptides. *Arrows* indicate some possible mutual reactions, e.g., after peptide binding to and accumulation at the membrane surface followed by insertion into the membrane interface (*carpet model*) various molecular mechanisms may occur, which strongly depend on the nature of both peptide and lipids. At high peptide concentrations micellization may occur. In the *right hand lower corner* the molecular shape of representative lipids are indicated

(van Meer et al. 2008; Devaux and Morris 2004). The non-bilayer propensity of PE is essential for the functional embedding of membrane proteins and for processes such as membrane fusion and fission (Kruijff 1997; Lohner 1996). PE assumes a conical molecular geometry because of the relatively small size of its polar headgroup (Fig. 2.3) (Seddon and Templer 1995). Membrane asymmetry is known to affect various bilayer properties, including membrane potential, surface charge, permeability, shape as well as stability (Devaux 1991; Cheng et al. 2009; Marquardt et al. 2015). Owing to this asymmetric phospholipid distribution mainly uncharged, zwitterionic phospholipids are exposed to the outside of the cell membrane of eukaryotes (Lohner 2001).

Studies on the surface potential and the translocation of anionic phospholipids in *Saccharomyces carlsbergensis* unveiled that about 5 % of anionic phospholipids are in the exofacial side of the plasma membrane (Cerbón and Calderón 1994). When cations were added to the culture medium this value increased slightly but significantly to 7 %. On the other hand, most of the membrane-active antimicrobial peptides exhibit a positive net charge under physiological conditions, which facilitates electrostatic interactions with negatively charged lipids of the target cell, while their amphipathic structure facilitates incorporation into membrane layers (Tossi et al. 2000; Lohner and Blondelle 2005). Therefore, owing to the comparatively low content of anionic lipids at the surface of fungi as compared to bacteria membrane-destabilization of antifungal peptides was suggested not to be facilitated by strong electrostatic interactions but rather by cell leakage due to pore formation, which is supposed to appear far below micromolar concentrations (Matsuzaki et al. 1995; Matsuzaki 1998). In the toroidal pore model (Fig. 2.3) peptides together with lipids form transmembrane pores, with the hydrophilic residues facing the lumen of the pore (Matsuzaki et al. 1996; Huang 2006). However, similar amounts of anionic lipids, i.e., PS, were found to be exposed on the outer membrane leaflet of cancer cells (Riedl et al. 2011a) shown to be sufficient to render them as target for cationic antimicrobial peptides without affecting significantly membranes of normal cells (Riedl et al. 2011b; Riedl et al. 2015; Hoskin and Ramamoorthy 2008). Thus, membrane permeabilization of fungal membranes may also occur by other modes of action than pore formation. In this respect, the most frequently discussed mechanism is the carpet model (Fig. 2.3), where AMPs accumulate at the cell membrane being aligned parallel to the bilayer surface and insert into the membrane above a certain threshold concentration resulting in membrane permeabilization and eventually disruption (Shai 2002). At the molecular level different processes may apply that can lead to loss of membrane integrity briefly listed here and schematically shown in Fig. 2.3:

(i) interfacial activity model, defined as the propensity of amphipathic peptides to partition into the membrane interface in a way to disrupt the normally strict segregation of polar and non-polar groups of the lipids (Wimley 2010),
(ii) free volume model, interfacial alignment parallel to the membrane plane creating “voids” in the hydrophobic core of the membrane, which leads to a quasi-interdigitated structure in the gel phase and membrane thinning/dimple formation in the fluid phase (Lohner 2009; Sevcsik et al. 2007; Huang 2000),

(iii) phase separation, creating domains with different physico-chemical properties between lipid bulk and peptide-enriched domains (Arouri et al. 2009; Epand et al. 2010; Epand and Epand 2009, 2011; Lohner 2009),
(iv) disruption of the membrane similar to detergents occurring particularly at high peptide concentration (Bechinger and Lohner 2006),
(v) modifying membrane curvature strain (Koller and Lohner 2014; Lohner and Blondelle 2005).

These models may be considered as special cases within the complex interaction of amphipathic peptides and membrane lipids, which besides of their nature also depend on a number of factors including environmental factors such as pH, ionic strength or temperature. Taking this into consideration and the fact that both molecules are highly dynamic, the SMART (soft membranes adapt and respond) model was introduced to account for the full range of possibilities (Bechinger 2015). Notably, the fast killing rate within minutes (Boman 2003) as well as the nature of the target (lipids of the plasma membrane) makes the occurrence of resistance less likely, since substantial modification of the lipid composition would affect fungal cell viability.

Although mammalian and fungal plasma membranes are similar in structure and composition, differences may arise when fungal species that infect humans switch from yeast cells to mycelium, which is considered to be an important factor in pathogenesis and in turn may facilitate the design of novel antifungal peptides. The primary function of hypha formation is to invade the substrate they are adhered to (Brand 2012). The levels of total lipids, sterols and phospholipids were found to be different in the mycelial form (log phase) of *Candida albicans* and in its yeast form (Mishra and Prasad 1990; Goyal and Khuller 1994). The contents of PC, PS, and PI in the mycelial form are higher than in the yeast form, whereas the opposite is true for PE (Mishra and Prasad 1990; Goyal and Khuller 1994). Analyses of the fatty acid composition showed that mycelial apolar and polar lipid fractions contained higher levels of polyunsaturated fatty acids (C18:2 and C18:3) as well as C16:0, C16:1 and C18:0, but lower levels of oleic acid (C18:1) than the corresponding yeast fractions (Ghannoum et al. 1986). The differences in the fatty acid pattern resulted in alterations in the thermotropic phase behavior and thus physico-chemical properties of *C. albicans* membrane lipids corresponding to its morphological form (Goyal and Khuller 1994; Ghannoum et al. 1986). The fatty acid pattern of mycelial lipids from *A. niger* were also different from its yeast form lipids (Chattopadhyay et al. 1985). An unusual lipid species, pyrophosphatidic acid (pyro-PA), was identified in *Cryptococcus neoformans* (Itoh and Kaneko 1977). Pyro-PA may have a potential role in signaling and stress response in *C. neoformans* and it is important for the mammalian immune response (Shea et al. 2006; Balboa et al. 1999). Unlike other fungi, membranes of clinical isolates of the pathogenic yeast *Cryptococcus neoformans* contain obtusifoliol as major sterol, followed by ergosterol (Ghannoum et al. 1994). Obtusifoliol is an important intermediate in the synthesis of sterols and has been observed in several fungal species following treatment with azoles (Vanden Bossche et al. 1990; Ghannoum et al. 1994). As with the total sterol

content, there was considerable variation in the types and quantities of sterols present in isolates from individual patients (Ghannoum et al. 1994). In contrast to *C. neoformans*, *C. albicans* does not show significant strain-to-strain variation in sterol patterns. Moreover, ergosterol is the predominant sterol in *C. albicans* (Ghannoum et al. 1994). In this respect, it is highly interesting to note that minor structural differences of sterols as deduced from NMR experiments can account for differential binding of amphotericin B to ergosterol (strong), cholesterol (weak) and lanosterol (no binding) (Anderson et al. 2014). It was suggested that this has also important implications for the design of novel antifungal compounds that distinguish between ergosterol of fungal and cholesterol of mammalian cell membranes thereby reducing unwanted side effects (Lohner 2014).

2.4.2 *Antifungal Peptides and Ergosterol*

Fungal membranes differ from those of higher eukaryotes concerning sterols, which regulate membrane fluidity. Ergosterol is the major sterol in the membranes of lower eukaryotes like yeast and fungi, whereas cholesterol predominates in the plasma membrane of mammalian cells (Henriksen et al. 2006). Antifungal substances like polyenes, azoles, and allylamines act on ergosterol or its synthesis (Ryder 1992; Sabatelli et al. 2006; Anderson et al. 2014). Cholesterol and ergosterol are similar molecules, but there are slight structural differences: ergosterol has two additional double bonds as well as a methyl group on the side chain (Hsueh et al. 2007). These small differences in sterol structure, however, result in stronger conformational ordering of lipid acyl chains in case of cholesterol and weaker effects on membrane packing for ergosterol (Hsueh et al. 2007; Urbina et al. 1995).

The small cyclic lipodepsipeptide syringomycin E from *Pseudomonas syringae* is a potent antifungal peptide (Segre et al. 1989; Lucca et al. 1999). Syringomycin E acts on the fungal plasma membrane and alters several of its functions, including ion transport, protein phosphorylation, and H^+-ATPase activity (Zhang and Takemoto 1986; Suzuki et al. 1992; Reidl et al. 1989; Feigin et al. 1997). The antifungal activity of syringomycin E is dependent on the presence of sterols in the plasma membrane of the fungal cells (Takemoto et al. 1993). Furthermore, the pore-forming activity of syringomycin E can be modulated by the type of sterol. The energy barrier for the channel formation in membrane bilayers was highest in presence of cholesterol, while ergosterol was promoting pore-forming activity of this lipopeptide (Feigin et al. 1997; Blasko et al. 1998). Although syringomycins are fungicidal against important human pathogenic yeasts, they caused lysis of sheep erythrocytes (Sorensen et al. 1996).

Psd1, a defensin isolated from seeds of the pea *Pisum sativum* with a compact cysteine-stabilized α/β motif, showed high partitioning into ergosterol-containing membranes (as fungal membranes), whereas partitioning of Psd1 into cholesterol-containing membranes was undetectable (Gonçalves et al. 2012b). This suggests low toxicity of Psd1 to mammalian (cholesterol-rich) membranes. The

cationic Psd1 has also increased affinity for membranes containing glycosylceramide, which is the most common fungal glycosphingolipid (Gonçalves et al. 2012b; Vriens et al. 2014; Wilmes et al. 2011). Upon interaction with their target membrane, plant defensins are either internalized by the fungal cell and interact with internal targets, or they stay at the cell surface and induce cell death through induction of a signaling cascade (Vriens et al. 2014).

The human neutrophil peptide 1(HNP1) showed low interaction with glucosylceramide rich membranes, but high sterol selectivity for ergosterol-containing membranes in vitro (Gonçalves et al. 2012a). The histidine-rich glycoprotein (HRG) as well as the epithelium-produced growth factor midkine preferentially lysed ergosterol-containing liposomes over cholesterol-containing ones, indicating a specificity for fungal versus mammalian membranes (Rydengård et al. 2008; Nordin et al. 2012). Although these peptides show selectivity for fungal membranes in vitro, their therapeutic application would be accompanied by dose-limited toxicities towards human cells.

2.4.3 *Antifungal Peptides and Sphingolipids*

Sphingolipids are potentially specific targets for antifungal molecules due to structural differences between fungal and mammalian sphingolipids such as 9-methyl group branching of the sphingoid base and different degrees of unsaturation in fungal sphingolipids (Thevissen et al. 2005). Sphingolipids and their biosynthesis have been investigated intensively for the yeast *S. cerevisiae*. The three types of sphingolipids (IPC, MIPC, and M(IP)2C) are located primarily in the plasma membrane (Patton and Lester 1991; Hechtberger et al. 1994). Disruption of the biosynthetic pathway for the sphingolipid mannosyl di-(inositol phosphoryl) ceramide (M(IP)2C) in *S. cerevisiae* resulted in resistance to the plant defensin DmAMP1 and the synthetic amphipathic peptide mimetic LTX109 indicating that M(IP)2C is essential for their antifungal action (Thevissen et al. 2000; Bojsen et al. 2013). DmAMP1 was shown to bind to purified M(IP)2C and this binding was enhanced in the presence of ergosterol (Thevissen et al. 2003).

Another plant defensin, RsAFP2, as well as the insect defensin-like heliomicin, selectively binds to glucosylceramide from fungi like *P. pastoris* and *C. albicans*, but not to glucosylceramide from human source (Thevissen et al. 2004). *S. cerevisiae* that do not contain this sphingolipid is resistant to RsAFP2-induced permeabilization and growth inhibition. In contrast to DmAMP1, the interaction of RsAFP2 with glucosylceramide was not increased in the presence of ergosterol (Thevissen et al. 2004).

Other plant and insect defensins interacting specifically with sphingolipids are Psd1 isolated from pea seeds (Medeiros et al. 2010; Wilmes et al. 2011); Sd5 isolated from *Saccharum officinarum* (De-Paula et al. 2008); MsDef1 from Medicago sativa (Ramamoorthy et al. 2007) and Drosomycin, an inducible insect defensin isolated from Drosophila (Gao and Zhu 2008).

2.5 Intracellular Targets

Some antifungal peptides enter the fungal cell and interact with intracellular targets after crossing the plasma membrane. Nevertheless, membrane lipids play a role concerning specificity and efficacy of these antifungal peptides.

Histatin 5 (Hst5), a human basic salivary peptide with strong fungicidal properties in vitro, becomes internalized and targets to energized mitochondria (Helmerhorst et al. 1999). The killing of *C. albicans* by Hst5 is accomplished by an increase in membrane potential and permeability and the subsequent release of intracellular ATP (Koshlukova et al. 1999, 2000; Bobek and Situ 2003). However, non-respiring yeast cells were protected against histatin 5 killing activity (Helmerhorst et al. 1999). The importance of metabolic activity in the susceptibility of *C. albicans* cells to basic proteins, like protamine or HNP-1, was already reported by Olson et al. (1977) and Lehrer et al. (1988). Interestingly, the amino acid sequence of histatin 5 resembles the mitochondrial targeting sequence characteristic for mitochondrial proteins that target proteins from cytosol to mitochondria (Nicolay et al. 1994; Helmerhorst et al. 1999). Perturbation of mitochondrial membranes by antifungal peptides may be facilitated by the divalent negative phospholipid cardiolipin, which is highly enriched in the inner mitochondrial membrane (Daum 1985).

Antifungal peptides may also cause inhibition of nuclear migration and nuclear division as shown for the penta-peptide auristatin PHE (Woyke et al. 2002), which has fungicidal activity against *C. neoformans.* This peptide caused complete disruption of both spindle and cytoplasmic microtubules in *C. neoformans*. As a consequence cell cycle arrest was leading to uninucleate, large-budded cells. The nucleus itself is the intracellular target of the plant defensin PsD1 (Lobo et al. 2007). PsD1 was shown to interact with the cell cycle control protein cyclin F from *N. crassa* cells and thereby impaired the progression of the cell cycle (Lobo et al. 2007).

Dermaseptin S3(1-16) and magainin 2 are two unrelated, amphibian-derived cationic peptides that interacted with DNA in vitro. Both peptides also interfered with DNA integrity of *S. cerevisiae* in vivo (Morton et al. 2007). This implies that both peptides are able to pass through the cytoplasmic membrane of yeast cells and damage DNA.

PA1b (pea albumin 1 subunit b) is a plant peptide of 37 amino acids purified from *Pisum sativum* and acts as an insecticide. The toxicity of PA1b is due to a specific and direct interaction with the V0 complex of the vacuolar proton pump (Chouabe et al. 2011). PA1b adopts a typical knottin fold with a triple-stranded antiparallel ß-sheet and three buried interlocked disulfide bonds (Jouvensal et al. 2003). Antifungal activity has been reported for the knottin-type peptides Mj-AMP1 and PAFP-S (Cammue et al. 1992; Gao et al. 2001; van der Weerden et al. 2013).

2.6 Synergism with Conventional Antifungal Drugs

An attractive therapeutic option might be a combination of antifungal peptides with conventional antifungal drugs like amphotericin B and azoles. In fact, a substantial cooperative effect of lactoferrin with amphotericin B, fluconazole, and 5-fluorocytosine was observed against *Candida* species (Kuipers et al. 1999). The combination of lactoferrin and fluconazole appeared to be the most successful combination. Wakabayashi et al. reported on cooperative effects of lactoferrin with clomitrazole agents against *Candida* growth (Wakabayashi et al. 1996). Lactoferrin is an innate host defense protein, which exerts a candidacidal effect in a cation concentration-dependent manner (Viejo-Díaz et al. 2004). Peptide 2, a short and potent lactoferrin derivative, suppressed the growth of *Candida* cells additively by a combination of peptide 2 with amphotericin B or miconazole (Ueta et al. 2001). Furthermore, in pilot experiments the effect on the minimal inhibitory concentration of amphotericin B, fluconazole, and 5-fluorocytosine upon addition of sub-inhibitory concentrations of the frog skin antimicrobial peptide, PGLa, as well as of Hst5 and designed analogs was tested (van't Hof et al. 2000). Thereby, addition of the peptides to amphotericin B resulted in a synergistic effect against several *Aspergillus*, *Candida* and *Cryptococcus* strains, while no enhanced activity was found in combination with fluconazole or 5-fluorocytosine. Tanida et al. also reported that Hst5 and the human neutrophil peptide, HNP1, acted synergistically with amphotericin B and itraconazole to suppress *Candida* colony formation (Tanida et al. 2006). The synergism between HNP1 and itraconazole was weak compared to combinations with other peptides. Inhibition of sterol synthesis by itraconazole might reduce membrane affinity of HNP1 as this peptide was shown to have high sterol selectivity for ergosterol-containing membranes in vitro (Gonçalves et al. 2012a). A number of studies concerning synergism between antifungal peptides of the echinocandin family and amphotericin B or azoles were performed. Disturbing the integrity of fungal cell walls by echinocandins may facilitate access of polyenes and triazoles to the cell membrane. Synergy between cilofungin and amphotericin B, a polyene derivative, was first reported for a murine model of candidiasis in 1991 (Hanson et al. 1991). Anidulafungin increased the antimycotic efficacy of amphotericin B and fluconazole against *Candida spp.* (Rosato et al. 2012) and pneumocandin L-743,872 enhanced the efficacy of fluconazole and amphotericin B in vitro against *C. neoformans* (Franzot and Casadevall 1997). Caspofungin and amphotericin B were synergistic or synergistic to additive for a number of clinical isolates of *Aspergillus* and *Fusarium* spp. (Arikan et al. 2002). A successful combined antifungal treatment of a life-threatening systemic fungal infection by *Aspergillus flavus* was reported by Krivan et al. (2006). The infection which developed in a central venous catheter tunnel progressed rapidly in spite of conventional and subsequent liposomal amphotericin B therapy. However, the deep fungal infection resolved after 30 days of dual therapy with liposomal amphotericin B and caspofungin. Therapy with co-administration of two or three antifungals has been applied by clinicians in

difficult-to-treat infection. However, there is still no support from randomized, controlled clinical trials (Hatipoglu and Hatipoglu 2013). Nevertheless, in summary these studies indicate that the growth inhibitory activity of conventional antifungal drugs can be enhanced by sub-inhibitory concentrations of antimicrobial peptides without affecting the cytotoxic activity against mammalian cells, suggesting that combination therapy can be a promising strategy for treatment of fungal infections.

2.7 Concluding Remarks

A global rise in incidences of invasive fungal infections has been reported, although true mortality rates are unknown because of a lack of good epidemiological data. This development has been largely related to modern medical interventions and immunosuppressive diseases (Brown et al. 2012). For example, in her annual report of 2011 the UK Chief Medical Officer Dame Sally C. Davies summed up: "Thus we are now seeing the paradoxical emergence of new infectious disease threats, and the re-emergence of infections that had previously been thought to be a problem of the past, as a direct consequence of the success of modern medicine. Examples include the increased risk of infection in general, but also of unusual infections such as invasive fungal disease, in patients being treated for non-infectious diseases, such as patients on immunosuppressive treatments for cancer or inflammatory disease." Further, demographic changes resulting in an ever elderly population favors such disease pattern and demand to manage also infectious complications common in patients undergoing dialysis for renal failure, and surgery, especially organ transplantation. Unfortunately, clinically available drugs have had only modest success in reducing the high mortality rates of invasive fungal infections such as candidiasis and cryptococcosis, their treatment relying on a limited number of antifungal drugs. In terms of such life-threatening systemic infections amphotericin B, which was brought onto the market in the 1950s, still remains the first line treatment and is considered as the gold standard despite its low therapeutic index, which may cause severe side effects. Furthermore, recent data indicate the emergence of drug-resistant fungi within hospitals and possibly the larger environment (Mesa-Arango et al. 2012). Therefore, as a consequence of the current situation Brown et al. proposed to tackle human fungal infections by (i) raising the general awareness of the problem, (ii) developing rapid, simple, and cheap diagnostics as well as (iii) safer and more effective antifungal drugs (Brown et al. 2012).

In this contribution, we focused on one alternative strategy for the development of novel specific antifungal drugs, which is based on host defense peptides. Among these peptides a minor group shows primarily antifungal activity, while the majority of peptides exhibit broad antimicrobial activity. Both classes have targets, which are absent in mammalian cells and therefore will have strongly reduced or no side effects. Peptides belonging to the former group of peptides bind to (i) enzymes, which are essential for the biosynthesis of the cell wall, (ii) ergosterol and (iii) sphingolipids, both being essential for plasma membrane function. The latter

group of peptides interacts with the cytoplasmic membrane inducing membrane permeabilization and cell lysis. Biophysical studies on membrane mimetic systems demonstrated that these membrane-active peptides have no specific receptor and thus they should be less prone to resistance development. The molecular mechanism(s) of killing depends on both the physico-chemical properties of the peptides and the membrane lipid composition. A detailed mechanistic understanding of antifungal activity will be important to understand the molecular basis for selective targeting of fungal cells. This in turn is essential for the rational development of novel antifungal agents that lead to more specific and hence safer therapeutics. Finally, these peptides may also be used synergistically in combination with conventional antifungal drugs, which would further widen the armory to treat especially life-threatening invasive fungal infections.

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References

Adams DJ (2004) Fungal cell wall chitinases and glucanases. Microbiology (Reading, Engl) 150 (Pt 7):2029–2035. doi:10.1099/mic.0.26980-0

Almeida MS, Cabral KM, Zingali RB, Kurtenbach E (2000) Characterization of two novel defense peptides from pea (*Pisum sativum*) seeds. Arch Biochem Biophys 378(2):278–286. doi:10.1006/abbi.2000.1824

Anderson TM, Clay MC, Cioffi AG, Diaz KA, Hisao GS, Tuttle MD, Nieuwkoop AJ, Comellas G, Maryum N, Wang S, Uno BE, Wildeman EL, Gonen T, Rienstra CM, Burke MD (2014) Amphotericin forms an extramembranous and fungicidal sterol sponge. Nat Chem Biol 10 (5):400–406. doi:10.1038/nchembio.1496

Arikan S, Lozano-Chiu M, Paetznick V, Rex JH (2002) In vitro synergy of caspofungin and amphotericin B against *Aspergillus* and *Fusarium* spp. Antimicrob Agents Chemother 46 (1):245–247

Arouri A, Dathe M, Blume A (2009) Peptide induced demixing in PG/PE lipid mixtures: a mechanism for the specificity of antimicrobial peptides towards bacterial membranes? Biochim Biophys Acta 1788(3):650–659. doi:10.1016/j.bbamem.2008.11.022

Bago B, Chamberland H, Goulet A, Vierheilig H, Lafontaine J, Piché Y (1996) Effect of Nikkomycin Z, a chitin-synthase inhibitor, on hyphal growth and cell wall structure of two arbuscular-mycorrhizal fungi. Protoplasma 192(1–2):80–92. doi:10.1007/BF01273247

Balboa MA, Balsinde J, Dillon DA, Carman GM, Dennis EA (1999) Proinflammatory macrophage-activating properties of the novel phospholipid diacylglycerol pyrophosphate. J Biol Chem 274(1):522–526

Barchiesi F, Spreghini E, Tomassetti S, Arzeni D, Giannini D, Scalise G (2005) Comparison of the fungicidal activities of caspofungin and amphotericin B against *Candida glabrata*. Antimicrob Agents Chemother 49(12):4989–4992. doi:10.1128/AAC.49.12.4989-4992.2005

Bartnicki-Garcia S (1968) Cell wall chemistry, morphogenesis, and taxonomy of fungi. Annu Rev Microbiol 22:87–108. doi:10.1146/annurev.mi.22.100168.000511

Bechinger B (2015) The SMART model: soft membranes adapt and respond, also transiently, in the presence of antimicrobial peptides. J Pept Sci 21(5):346–355. doi:10.1002/psc.2729

Bechinger B, Lohner K (2006) Detergent-like actions of linear amphipathic cationic antimicrobial peptides. Biochim Biophys Acta 1758(9):1529–1539. doi:10.1016/j.bbamem.2006.07.001

Blasko K, Schagina LV, Agner G, Kaulin YA, Takemoto JY (1998) Membrane sterol composition modulates the pore forming activity of syringomycin E in human red blood cells. Biochim Biophys Acta 1373(1):163–169

Bobek LA, Situ H (2003) MUC7 20-Mer: investigation of antimicrobial activity, secondary structure, and possible mechanism of antifungal action. Antimicrob Agents Chemother 47 (2):643–652. doi:10.1128/AAC.47.2.643-652.2003

Bojsen R, Torbensen R, Larsen CE, Folkesson A, Regenberg B (2013) The synthetic amphipathic peptidomimetic LTX109 is a potent fungicide that disturbs plasma membrane integrity in a sphingolipid dependent manner. PLoS ONE 8(7):e69483. doi:10.1371/journal.pone.0069483

Boman HG (2003) Antibacterial peptides: basic facts and emerging concepts. J Intern Med 254 (3):197–215

Boucher HW, Groll AH, Chiou CC, Walsh TJ (2004) Newer systemic antifungal agents. Drugs 64 (18):1997–2020. doi:10.2165/00003495-200464180-00001

Bowman SM, Free SJ (2006) The structure and synthesis of the fungal cell wall. BioEssays 28 (8):799–808. doi:10.1002/bies.20441

Bowman JC, Hicks PS, Kurtz MB, Rosen H, Schmatz DM, Liberator PA, Douglas CM (2002) The antifungal echinocandin caspofungin acetate kills growing cells of *Aspergillus fumigatus* in vitro. Antimicrob Agents Chemother 46(9):3001–3012

Bowman JC, Abruzzo GK, Flattery AM, Gill CJ, Hickey EJ, Hsu MJ, Kahn JN, Liberator PA, Misura AS, Pelak BA, Wang TC, Douglas CM (2006) Efficacy of caspofungin against *Aspergillus flavus*, *Aspergillus terreus*, and *Aspergillus nidulans*. Antimicrob Agents Chemother 50(12):4202–4205. doi:10.1128/AAC.00485-06

Brand A (2012) Hyphal growth in human fungal pathogens and its role in virulence. Int J Microbiol 2012:517529. doi:10.1155/2012/517529

Brillinger GU (1979) Metabolic products of microorganisms. 181. Chitin synthase from fungi, a test model for substances with insecticidal properties. Arch Microbiol 121(1):71–74

Brown GD, Denning DW, Levitz SM (2012) Tackling human fungal infections. Science 336 (6082):647. doi:10.1126/science.1222236

Butts A, Krysan DJ (2012) Antifungal drug discovery: something old and something new. PLoS Pathog 8(9):e1002870. doi:10.1371/journal.ppat.1002870

Cammue BP, de Bolle MF, Terras FR, Proost P, van Damme J, Rees SB, Vanderleyden J, Broekaert WF (1992) Isolation and characterization of a novel class of plant antimicrobial peptides form *Mirabilis jalapa* L. seeds. J Biol Chem 267(4):2228–2233

Cerbón J, Calderón V (1994) Surface potential regulation of phospholipid composition and in-out translocation in yeast. Eur J Biochem 219(1–2):195–200

Chattopadhyay P, Banerjee SK, Sen K, Chakrabarti P (1985) Lipid profiles of *Aspergillus niger* and its unsaturated fatty acid auxotroph, UFA2. Can J Microbiol 31(4):352–355

Chen SCA, Sorrell TC (2007) Antifungal agents. Med J Aust 187(7):404–409

Chen W, Zeng H, Tan H (2000) Cloning, sequencing, and function of *sanF*: a gene involved in nikkomycin biosynthesis of *Streptomyces ansochromogenes*. Curr Microbiol 41(5):312–316

Cheng H, Megha London E (2009) Preparation and properties of asymmetric vesicles that mimic cell membranes: effect upon lipid raft formation and transmembrane helix orientation. J Biol Chem 284(10):6079–6092. doi:10.1074/jbc.M806077200

Chouabe C, Eyraud V, Da Silva P, Rahioui I, Royer C, Soulage C, Bonvallet R, Huss M, Gressent F (2011) New mode of action for a knottin protein bioinsecticide: pea albumin 1 subunit b (PA1b) is the first peptidic inhibitor of V-ATPase. J Biol Chem 286(42):36291–36296. doi:10.1074/jbc.M111.281055

Cole AM, Ganz T (2000) Human antimicrobial peptides: analysis and application. BioTech 29 (4):822–826, 828, 830–831

Cuthbertson BJ, Shepard EF, Chapman RW, Gross PS (2002) Diversity of the penaeidin antimicrobial peptides in two shrimp species. Immunogenetics 54(6):442–445. doi:10.1007/s00251-002-0487-z

Dähn U, Hagenmaier H, Höhne H, König WA, Wolf G, Zähner H (1976) Stoffwechselprodukte von mikroorganismen. 154. Mitteilung. Nikkomycin, ein neuer hemmstoff der chitinsynthese bei pilzen. Arch Microbiol 107(2):143–160

Daum G (1985) Lipids of mitochondria. Biochim Biophys Acta 822(1):1–42

de Kruijff B (1997) Biomembranes. Lipids beyond the bilayer. Nature 386(6621):129–130. doi:10.1038/386129a0

de Kruijff B, Demel RA (1974) Polyene antibiotic-sterol interactions in membranes of *Acholeplasma laidlawii* cells and lecithin liposomes. III. Molecular structure of the polyene antibiotic-cholesterol complexes. Biochimica et Biophysica Acta (BBA) Biomembranes 339 (1):57–70. doi:10.1016/0005-2736(74)90332-0

de Luca AJ, Walsh TJ (2000) Antifungal peptides: origin, activity, and therapeutic potential. Rev Iberoam Micol 17(4):116–120

de Lucca AJ, Jacks TJ, Takemoto J, Vinyard B, Peter J, Navarro E, Walsh TJ (1999) Fungal lethality, binding, and cytotoxicity of syringomycin-E. Antimicrob Agents Chemother 43 (2):371–373

de Medeiros LN, Angeli R, Sarzedas CG, Barreto-Bergter E, Valente AP, Kurtenbach E, Almeida FCL (2010) Backbone dynamics of the antifungal Psd1 pea defensin and its correlation with membrane interaction by NMR spectroscopy. Biochim Biophys Acta 1798 (2):105–113. doi:10.1016/j.bbamem.2009.07.013

de Nobel H, van Den Ende H, Klis FM (2000) Cell wall maintenance in fungi. Trends Microbiol 8 (8):344–345

Denning DW (1997) Echinocandins and pneumocandins—a new antifungal class with a novel mode of action. J Antimicrob Chemother 40(5):611–614

Denning DW (2002) Echinocandins: a new class of antifungal. J Antimicrob Chemother 49 (6):889–891

De-Paula VS, Razzera G, Medeiros L, Miyamoto CA, Almeida MS, Kurtenbach E, Almeida FCL, Valente AP (2008) Evolutionary relationship between defensins in the Poaceae family strengthened by the characterization of new sugarcane defensins. Plant Mol Biol 68(4–5):321–335. doi:10.1007/s11103-008-9372-y

Destoumieux D, Muñoz M, Cosseau C, Rodriguez J, Bulet P, Comps M, Bachère E (2000) Penaeidins, antimicrobial peptides with chitin-binding activity, are produced and stored in shrimp granulocytes and released after microbial challenge. J Cell Sci 113(Pt 3):461–469

Devaux PF (1991) Static and dynamic lipid asymmetry in cell membranes. Biochemistry 30 (5):1163–1173

Devaux PF, Morris R (2004) Transmembrane asymmetry and lateral domains in biological membranes. Traffic 5(4):241–246. doi:10.1111/j.1600-0854.2004.0170.x

Drgona L, Khachatryan A, Stephens J, Charbonneau C, Kantecki M, Haider S, Barnes R (2014) Clinical and economic burden of invasive fungal diseases in Europe: focus on pre-emptive and empirical treatment of *Aspergillus* and *Candida* species. Eur J Clin Microbiol Infect Dis 33 (1):7–21. doi:10.1007/s10096-013-1944-3

Epand RM, Epand RF (2009) Lipid domains in bacterial membranes and the action of antimicrobial agents. Biochim Biophys Acta 1788(1):289–294. doi:10.1016/j.bbamem.2008.08.023

Epand RM, Epand RF (2011) Bacterial membrane lipids in the action of antimicrobial agents. J Pept Sci 17(5):298–305. doi:10.1002/psc.1319

Epand RF, Maloy WL, Ramamoorthy A, Epand RM (2010) Probing the "charge cluster mechanism" in amphipathic helical cationic antimicrobial peptides. Biochemistry 49 (19):4076–4084. doi:10.1021/bi100378m

Ernst EJ, Klepser ME, Ernst ME, Messer SA, Pfaller MA (1999) In vitro pharmacodynamic properties of MK-0991 determined by time-kill methods. Diagn Microbiol Infect Dis 33(2):75–80

Faruck MO, Yusof F, Chowdhury S (2015) An overview of antifungal peptides derived from insect. Peptides. doi:10.1016/j.peptides.2015.06.001

Fehlbaum P, Bulet P, Michaut L, Lagueux M, Broekaert WF, Hetru C, Hoffmann JA (1994) Insect immunity. Septic injury of *Drosophila* induces the synthesis of a potent antifungal peptide with sequence homology to plant antifungal peptides. J Biol Chem 269(52):33159–33163

Feigin AM, Schagina LV, Takemoto JY, Teeter JH, Brand JG (1997) The effect of sterols on the sensitivity of membranes to the channel-forming antifungal antibiotic, syringomycin E. Biochim Biophys Acta 1324(1):102–110

Feng C, Ling H, Du D, Zhang J, Niu G, Tan H (2014) Novel nikkomycin analogues generated by mutasynthesis in *Streptomyces ansochromogenes*. Microb Cell Fact 13(1):59. doi:10.1186/1475-2859-13-59

Fleet GH (1985) Composition and structure of yeast cell walls. Curr Top Med Mycol 1:24–56

François Isabelle E J A, Bolle De, Miguel FC, Dwyer G, Goderis Inge J W M, Woutors PFJ, Verhaert PD, Proost P, Schaaper WMM, Cammue BPA, Broekaert WF (2002) Transgenic expression in Arabidopsis of a polyprotein construct leading to production of two different antimicrobial proteins. Plant Physiol 128(4):1346–1358. doi:10.1104/pp.010794

Franzot SP, Casadevall A (1997) Pneumocandin L-743,872 enhances the activities of amphotericin B and fluconazole against *Cryptococcus neoformans* in vitro. Antimicrob Agents Chemother 41(2):331–336

Gao B, Zhu S (2008) Differential potency of drosomycin to *Neurospora crassa* and its mutant: implications for evolutionary relationship between defensins from insects and plants. Insect Mol Biol 17(4):405–411. doi:10.1111/j.1365-2583.2008.00810.x

Gao GH, Liu W, Dai JX, Wang JF, Hu Z, Zhang Y, Wang DC (2001) Solution structure of PAFP-S: a new knottin-type antifungal peptide from the seeds of *Phytolacca americana*. Biochemistry 40(37):10973–10978

Ghannoum MA, Rice LB (1999) Antifungal agents: mode of action, mechanisms of resistance, and correlation of these mechanisms with bacterial resistance. Clin Microbiol Rev 12(4):501–517

Ghannoum MA, Janini G, Khamis L, Radwan SS (1986) Dimorphism-associated variations in the lipid composition of *Candida albicans*. J Gen Microbiol 132(8):2367–2375. doi:10.1099/00221287-132-8-2367

Ghannoum MA, Spellberg BJ, Ibrahim AS, Ritchie JA, Currie B, Spitzer ED, Edwards JE, Casadevall A (1994) Sterol composition of *Cryptococcus neoformans* in the presence and absence of fluconazole. Antimicrob Agents Chemother 38(9):2029–2033

Gonçalves S, Abade J, Teixeira A, Santos NC (2012a) Lipid composition is a determinant for human defensin HNP1 selectivity. Biopolymers 98(4):313–321

Gonçalves S, Teixeira A, Abade J, de Medeiros LN, Kurtenbach E, Santos NC (2012b) Evaluation of the membrane lipid selectivity of the pea defensin Psd1. Biochim Biophys Acta 1818(5):1420–1426. doi:10.1016/j.bbamem.2012.02.012

Goyal S, Khuller GK (1994) Structural and functional role of lipids in yeast and mycelial forms of *Candida albicans*. Lipids 29(11):793–797

Gressent F, Da Silva P, Eyraud V, Karaki L, Royer C (2011) Pea Albumin 1 subunit b (PA1b), a promising bioinsecticide of plant origin. Toxins (Basel) 3(12):1502–1517. doi:10.3390/toxins3121502

Grover ND (2010) Echinocandins: a ray of hope in antifungal drug therapy. Indian J Pharmacol 42(1):9–11. doi:10.4103/0253-7613.62396

Hanson LH, Perlman AM, Clemons KV, Stevens DA (1991) Synergy between cilofungin and amphotericin B in a murine model of candidiasis. Antimicrob Agents Chemother 35(7):1334–1337

Hatipoglu N, Hatipoglu H (2013) Combination antifungal therapy for invasive fungal infections in children and adults. Expert Rev Anti Infect Ther 11(5):523–535. doi:10.1586/eri.13.29

Hechtberger P, Zinser E, Saf R, Hummel K, Paltauf F, Daum G (1994) Characterization, quantification and subcellular localization of inositol-containing sphingolipids of the yeast. *Saccharomyces cerevisiae*. Eur J Biochem 225(2):641–649

Hector RF (1993) Compounds active against cell walls of medically important fungi. Clin Microbiol Rev 6(1):1–21

Hector RF, Zimmer BL, Pappagianis D (1990) Evaluation of nikkomycins X and Z in murine models of coccidioidomycosis, histoplasmosis, and blastomycosis. Antimicrob Agents Chemother 34(4):587–593

Helmerhorst EJ, Breeuwer P, van't Hof W, Walgreen-Weterings E, Oomen LC, Veerman EC, Amerongen AV, Abee T (1999) The cellular target of histatin 5 on *Candida albicans* is the energized mitochondrion. J Biol Chem 274(11):7286–7291

Henriksen J, Rowat AC, Brief E, Hsueh YW, Thewalt JL, Zuckermann MJ, Ipsen JH (2006) Universal behavior of membranes with sterols. Biophys J 90(5):1639–1649. doi:10.1529/biophysj.105.067652

Hitchcock CA, Dickinson K, Brown SB, Evans EG, Adams DJ (1990) Interaction of azole antifungal antibiotics with cytochrome P-450-dependent 14 alpha-sterol demethylase purified from *Candida albicans*. Biochem J 266(2):475–480

Holz RW (1974) The effects of the polyene antibiotics nystatin and amphotericin B on thin lipid membranes. Ann N Y Acad Sci 235:469–479

Horn DL, Neofytos D, Anaissie EJ, Fishman JA, Steinbach WJ, Olyaei AJ, Marr KA, Pfaller MA, Chang C, Webster KM (2009) Epidemiology and outcomes of candidemia in 2019 patients: data from the prospective antifungal therapy alliance registry. Clin Infect Dis 48(12):1695–1703. doi:10.1086/599039

Hoskin DW, Ramamoorthy A (2008) Studies on anticancer activities of antimicrobial peptides. Biochim Biophys Acta 1778(2):357–375. doi:10.1016/j.bbamem.2007.11.008

Hsueh Y, Chen M, Patty PJ, Code C, Cheng J, Frisken BJ, Zuckermann M, Thewalt J (2007) Ergosterol in POPC membranes: physical properties and comparison with structurally similar sterols. Biophys J 92(5):1606–1615. doi:10.1529/biophysj.106.097345

Huang HW (2000) Action of antimicrobial peptides: two-state model. Biochemistry 39(29):8347–8352

Huang HW (2006) Molecular mechanism of antimicrobial peptides: the origin of cooperativity. Biochim Biophys Acta 1758(9):1292–1302. doi:10.1016/j.bbamem.2006.02.001

Isaksson J, Brandsdal BO, Engqvist M, Flaten GE, Svendsen JSM, Stensen W (2011) A synthetic antimicrobial peptidomimetic (LTX 109): stereochemical impact on membrane disruption. J Med Chem 54(16):5786–5795. doi:10.1021/jm200450h

Isono K, Asahi K, Suzuki S (1969) Studies on polyoxins, antifungal antibiotics. 13. The structure of polyoxins. J Am Chem Soc 91(26):7490–7505

Itoh T, Kaneko H (1977) The in vivo incorporation of [32P]-labeled orthophosphate into pyrophosphatidic acid and other phospholipids of *Cryptococcus neoformans* through cell growth. Lipids 12(10):809–813

Jouvensal L, Quillien L, Ferrasson E, Rahbé Y, Guéguen J, Vovelle F (2003) PA1b, an insecticidal protein extracted from pea seeds (*Pisum sativum*): 1H-2-D NMR study and molecular modeling. Biochemistry 42(41):11915–11923. doi:10.1021/bi0348031

Kapteyn JC, van Den Ende H, Klis FM (1999) The contribution of cell wall proteins to the organization of the yeast cell wall. Biochim Biophys Acta 1426(2):373–383

Klis FM (1994) Review: cell wall assembly in yeast. Yeast 10(7):851–869. doi:10.1002/yea.320100702

Klis FM, Mol P, Hellingwerf K, Brul S (2002) Dynamics of cell wall structure in *Saccharomyces cerevisiae*. FEMS Microbiol Rev 26(3):239–256

Koller D, Lohner K (2014) The role of spontaneous lipid curvature in the interaction of interfacially active peptides with membranes. Biochim Biophys Acta 1838(9):2250–2259. doi:10.1016/j.bbamem.2014.05.013

Kontoyiannis DP, Mantadakis E, Samonis G (2003) Systemic mycoses in the immunocompromised host: an update in antifungal therapy. J Hosp Infect 53(4):243–258. doi:10.1053/jhin.2002.1278

Koshlukova SE, Lloyd TL, Araujo MW, Edgerton M (1999) Salivary histatin 5 induces non-lytic release of ATP from *Candida albicans* leading to cell death. J Biol Chem 274(27):18872–18879

Koshlukova SE, Araujo MW, Baev D, Edgerton M (2000) Released ATP is an extracellular cytotoxic mediator in salivary histatin 5-induced killing of *Candida albicans*. Infect Immun 68 (12):6848–6856

Kriván G, Sinkó J, Nagy IZ, Goda V, Reményi P, Bátai A, Lueff S, Kapás B, Réti M, Tremmel A, Masszi T (2006) Successful combined antifungal salvage therapy with liposomal amphotericin B and caspofungin for invasive *Aspergillus flavus* infection in a child following allogeneic bone marrow transplantation. Acta Biomed 77(Suppl 2):17–21

Kuipers ME, de Vries HG, Eikelboom MC, Meijer DK, Swart PJ (1999) Synergistic fungistatic effects of lactoferrin in combination with antifungal drugs against clinical *Candida* isolates. Antimicrob Agents Chemother 43(11):2635–2641

Lacerda AF, Vasconcelos EAR, Pelegrini PB, de Sa Grossi, Maria F (2014) Antifungal defensins and their role in plant defense. Front Microbiol 5:116. doi:10.3389/fmicb.2014.00116

Lamberty M, Caille A, Landon C, Tassin-Moindrot S, Hetru C, Bulet P, Vovelle F (2001) Solution structures of the antifungal heliomicin and a selected variant with both antibacterial and antifungal activities. Biochemistry 40(40):11995–12003

Latgé J, Beauvais A (2014) Functional duality of the cell wall. Curr Opin Microbiol 20:111–117. doi:10.1016/j.mib.2014.05.009

Lehrer RI, Ganz T, Szklarek D, Selsted ME (1988) Modulation of the in vitro candidacidal activity of human neutrophil defensins by target cell metabolism and divalent cations. J Clin Invest 81 (6):1829–1835. doi:10.1172/JCI113527

Lobo DS, Pereira IB, Fragel-Madeira L, Medeiros LN, Cabral LM, Faria J, Bellio M, Campos RC, Linden R, Kurtenbach E (2007) Antifungal *Pisum sativum* defensin 1 interacts with *Neurospora crassa* cyclin F related to the cell cycle. Biochemistry 46(4):987–996. doi:10.1021/bi061441j

Löffler J, Einsele H, Hebart H, Schumacher U, Hrastnik C, Daum G (2000) Phospholipid and sterol analysis of plasma membranes of azole-resistant *Candida albicans* strains. FEMS Microbiol Lett 185(1):59–63

Lohner K (1996) Is the high propensity of ethanolamine plasmalogens to form non-lamellar lipid structures manifested in the properties of biomembranes? Chem Phys Lipids 81(2):167–184

Lohner K (2001) The role of membrane lipid composition in cell targeting of antimicrobial peptides. In: Lohner K (ed) Development of novel antimicrobial agents: emerging strategies. Horizon Scientific Press, Wymondham, Norfolk, U.K., pp 149–165

Lohner K (2009) New strategies for novel antibiotics: peptides targeting bacterial cell membranes. gpb 28(2):105–116. doi: 10.4149/gpb_2009_02_105

Lohner K (2014) Antimicrobial mechanisms: a sponge against fungal infections. Nat Chem Biol 10(6):411–412. doi:10.1038/nchembio.1518

Lohner K, Blondelle SE (2005) Molecular mechanisms of membrane perturbation by antimicrobial peptides and the use of biophysical studies in the design of novel peptide antibiotics. Comb Chem High Throughput Screen 8(3):241–256

Loyse A, Thangaraj H, Easterbrook P, Ford N, Roy M, Chiller T, Govender N, Harrison TS, Bicanic T (2013) Cryptococcal meningitis: improving access to essential antifungal medicines in resource-poor countries. Lancet Infect Dis 13(7):629–637. doi:10.1016/S1473-3099(13)70078-1

Marquardt D, Geier B, Pabst G (2015) Asymmetric lipid membranes: towards more realistic model systems. Membranes 5(2):180–196. doi:10.3390/membranes5020180

Matejuk A, Leng Q, Begum MD, Woodle MC, Scaria P, Chou S, Mixson AJ (2010) Peptide-based antifungal therapies against emerging infections. Drugs Future 35(3):197

Matsuzaki K (1998) Magainins as paradigm for the mode of action of pore forming polypeptides. Biochim Biophys Acta 1376(3):391–400

Matsuzaki K, Murase O, Fujii N, Miyajima K (1995) Translocation of a channel-forming antimicrobial peptide, magainin 2, across lipid bilayers by forming a pore. Biochemistry 34 (19):6521–6526

Matsuzaki K, Murase O, Fujii N, Miyajima K (1996) An antimicrobial peptide, magainin 2, induced rapid flip-flop of phospholipids coupled with pore formation and peptide translocation. Biochemistry 35(35):11361–11368. doi:10.1021/bi960016v

Mesa-Arango AC, Scorzoni L, Zaragoza O (2012) It only takes one to do many jobs: Amphotericin B as antifungal and immunomodulatory drug. Front Microbiol 3:286. doi:10.3389/fmicb.2012.00286

Mishra P, Prasad R (1990) An overview of lipids of *Candida albicans*. Prog Lipid Res 29(2):65–85

Mizuhara N, Kuroda M, Ogita A, Tanaka T, Usuki Y, Fujita K (2011) Antifungal thiopeptide cyclothiazomycin B1 exhibits growth inhibition accompanying morphological changes via binding to fungal cell wall chitin. Bioorg Med Chem 19(18):5300–5310. doi:10.1016/j.bmc.2011.08.010

Mor A, Hani K, Nicolas P (1994) The vertebrate peptide antibiotics dermaseptins have overlapping structural features but target specific microorganisms. J Biol Chem 269 (50):31635–31641

Morton CO, Hayes A, Wilson M, Rash BM, Oliver SG, Coote P (2007) Global phenotype screening and transcript analysis outlines the inhibitory mode(s) of action of two amphibian-derived, alpha-helical, cationic peptides on *Saccharomyces cerevisiae*. Antimicrob Agents Chemother 51(11):3948–3959. doi:10.1128/AAC.01007-07

Nawrot R, Barylski J, Nowicki G, Broniarczyk J, Buchwald W, Goździcka-Józefiak A (2014) Plant antimicrobial peptides. Folia Microbiol (Praha) 59(3):181–196. doi:10.1007/s12223-013-0280-4

Nicolay K, Laterveer FD, van Heerde WL (1994) Effects of amphipathic peptides, including presequences, on the functional integrity of rat liver mitochondrial membranes. J Bioenerg Biomembr 26(3):327–334

Nix DE, Swezey RR, Hector R, Galgiani JN (2009) Pharmacokinetics of nikkomycin Z after single rising oral doses. Antimicrob Agents Chemother 53(6):2517–2521. doi:10.1128/AAC.01609-08

Nordin SL, Sonesson A, Malmsten M, Mörgelin M, Egesten A (2012) The epithelium-produced growth factor midkine has fungicidal properties. J Antimicrob Chemother 67(8):1927–1936. doi:10.1093/jac/dks136

Odds FC, Brown AJ, Gow NA (2003) Antifungal agents: mechanisms of action. Trends Microbiol 11(6):272–279. doi:10.1016/S0966-842X(03)00117-3

Olson VL, Hansing RL, McClary DO (1977) The role of metabolic energy in the lethal action of basic proteins on *Candida albicans*. Can J Microbiol 23(2):166–174

Oppenheim FG, Xu T, McMillian FM, Levitz SM, Diamond RD, Offner GD, Troxler RF (1988) Histatins, a novel family of histidine-rich proteins in human parotid secretion. Isolation, characterization, primary structure, and fungistatic effects on *Candida albicans*. J Biol Chem 263(16):7472–7477

Park BJ, Wannemuehler KA, Marston BJ, Govender N, Pappas PG, Chiller TM (2009) Estimation of the current global burden of cryptococcal meningitis among persons living with HIV/AIDS. AIDS 23(4):525–530. doi:10.1097/QAD.0b013e328322ffac

Patton JL, Lester RL (1991) The phosphoinositol sphingolipids of *Saccharomyces cerevisiae* are highly localized in the plasma membrane. J Bacteriol 173(10):3101–3108

Perlin DS (2007) Resistance to echinocandin-class antifungal drugs. Drug Resist Updat 10(3):121–130. doi:10.1016/j.drup.2007.04.002

Perlin DS (2015) Mechanisms of echinocandin antifungal drug resistance. Ann N Y Acad Sci. doi:10.1111/nyas.12831

Pfaller MA, Diekema DJ (2004) Rare and emerging opportunistic fungal pathogens: concern for resistance beyond *Candida albicans* and *Aspergillus fumigatus*. J Clin Microbiol 42(10):4419–4431. doi:10.1128/JCM.42.10.4419-4431.2004

Pfaller MA, Messer SA, Boyken L, Rice C, Tendolkar S, Hollis RJ, Diekema DJ (2003) Caspofungin activity against clinical isolates of fluconazole-resistant *Candida*. J Clin Microbiol 41(12):5729–5731

Polvi EJ, Li X, O'Meara TR, Leach MD, Cowen LE (2015) Opportunistic yeast pathogens: reservoirs, virulence mechanisms, and therapeutic strategies. Cell Mol Life Sci 72(12):2261–2287. doi:10.1007/s00018-015-1860-z

Porto WF, Souza VA, Nolasco DO, Franco OL (2012) In silico identification of novel hevein-like peptide precursors. Peptides 38(1):127–136. doi:10.1016/j.peptides.2012.07.025

Ramamoorthy V, Cahoon EB, Li J, Thokala M, Minto RE, Shah DM (2007) Glucosylceramide synthase is essential for alfalfa defensin-mediated growth inhibition but not for pathogenicity of *Fusarium graminearum*. Mol Microbiol 66(3):771–786. doi:10.1111/j.1365-2958.2007.05955.x

Reidl HH, Grover TA, Takemoto JY (1989) 31P-NMR evidence for cytoplasmic acidification and phosphate extrusion in syringomycin-treated cells of *Rhodotorula pilimanae*. Biochim Biophys Acta 1010(3):325–329

Riedl S, Rinner B, Asslaber M, Schaider H, Walzer S, Novak A, Lohner K, Zweytick D (2011a) In search of a novel target—phosphatidylserine exposed by non-apoptotic tumor cells and metastases of malignancies with poor treatment efficacy. Biochim Biophys Acta 1808(11):2638–2645. doi:10.1016/j.bbamem.2011.07.026

Riedl S, Zweytick D, Lohner K (2011b) Membrane-active host defense peptides—challenges and perspectives for the development of novel anticancer drugs. Chem Phys Lipids 164(8):766–781. doi:10.1016/j.chemphyslip.2011.09.004

Riedl S, Leber R, Rinner B, Schaider H, Lohner K, Zweytick D (2015) Human lactoferricin derived di-peptides deploying loop structures induce apoptosis specifically in cancer cells through targeting membranous phosphatidylserine. Biochim Biophys Acta 1848(11 Pt A):2918–2931. doi: 10.1016/j.bbamem.2015.07.018

Rogozhin EA, Slezina MP, Slavokhotova AA, Istomina EA, Korostyleva TV, Smirnov AN, Grishin EV, Egorov TA, Odintsova TI (2015) A novel antifungal peptide from leaves of the weed *Stellaria media* L. Biochimie 116:125–132. doi:10.1016/j.biochi.2015.07.014

Rosato A, Piarulli M, Schiavone BIP, Montagna MT, Caggiano G, Muraglia M, Carone A, Franchini C, Corbo F (2012) In vitro synergy testing of anidulafungin with fluconazole, tioconazole, 5-flucytosine and amphotericin B against some *Candida* spp. Med Chem 8(4):690–698

Rydengård V, Shannon O, Lundqvist K, Kacprzyk L, Chalupka A, Olsson A, Mörgelin M, Jahnen-Dechent W, Malmsten M, Schmidtchen A (2008) Histidine-rich glycoprotein protects from systemic *Candida* infection. PLoS Pathog 4(8):e1000116. doi:10.1371/journal.ppat.1000116

Ryder NS (1992) Terbinafine: mode of action and properties of the squalene epoxidase inhibition. Br J Dermatol 126(Suppl 39):2–7

Sabatelli F, Patel R, Mann PA, Mendrick CA, Norris CC, Hare R, Loebenberg D, Black TA, McNicholas PM (2006) In vitro activities of posaconazole, fluconazole, itraconazole, voriconazole, and amphotericin B against a large collection of clinically important molds and yeasts. Antimicrob Agents Chemother 50(6):2009–2015. doi:10.1128/AAC.00163-06

Sanati H, Belanger P, Fratti R, Ghannoum M (1997) A new triazole, voriconazole (UK-109,496), blocks sterol biosynthesis in *Candida albicans* and *Candida krusei*. Antimicrob Agents Chemother 41(11):2492–2496

Seddon JM, Templer RH (1995) Polymorphism of lipid-water systems. In: Lipowsky R, Sackmann E (eds) Handbook of biological physics: structure and dynamics of membranes. Elsevier SPC, Amsterdam, pp 97–160

Segre A, Bachmann RC, Ballio A, Bossa F, Grgurina I, Iacobellis NS, Marino G, Pucci P, Simmaco M, Takemoto JY (1989) The structure of syringomycins A1, E and G. FEBS Lett 255(1):27–31

Selsted ME, Harwig SS, Ganz T, Schilling JW, Lehrer RI (1985) Primary structures of three human neutrophil defensins. J Clin Invest 76(4):1436–1439. doi:10.1172/JCI112121

Sevcsik E, Pabst G, Jilek A, Lohner K (2007) How lipids influence the mode of action of membrane-active peptides. Biochim Biophys Acta 1768(10):2586–2595. doi:10.1016/j.bbamem.2007.06.015

Shai Y (2002) Mode of action of membrane active antimicrobial peptides. Biopolymers 66 (4):236–248. doi:10.1002/bip.10260

Shea JM, Henry JL, Del Poeta M (2006) Lipid metabolism in *Cryptococcus neoformans*. FEMS Yeast Res 6(4):469–479. doi:10.1111/j.1567-1364.2006.00080.x

Silva PM, Gonçalves S, Santos NC (2014) Defensins: antifungal lessons from eukaryotes. Front Microbiol 5:97. doi:10.3389/fmicb.2014.00097

Smith HA, Shenbagamurthi P, Naider F, Kundu B, Becker JM (1986) Hydrophobic polyoxins are resistant to intracellular degradation in *Candida albicans*. Antimicrob Agents Chemother 29 (1):33–39

Sorensen KN, Kim KH, Takemoto JY (1996) In vitro antifungal and fungicidal activities and erythrocyte toxicities of cyclic lipodepsinonapeptides produced by *Pseudomonas syringae* pv. *syringae*. Antimicrob Agents Chemother 40(12):2710–2713

Spampinato C, Leonardi D (2013) *Candida* infections, causes, targets, and resistance mechanisms: traditional and alternative antifungal agents. Biomed Res Int 2013:204237. doi:10.1155/2013/204237

Specht CA, Liu Y, Robbins PW, Bulawa CE, Iartchouk N, Winter KR, Riggle PJ, Rhodes JC, Dodge CL, Culp DW, Borgia PT (1996) The *chsD* and *chsE* genes of *Aspergillus nidulans* and their roles in chitin synthesis. Fungal Genet Biol 20(2):153–167. doi:10.1006/fgbi.1996.0030

Spelbrink RG, Dilmac N, Allen A, Smith TJ, Shah DM, Hockerman GH (2004) Differential antifungal and calcium channel-blocking activity among structurally related plant defensins. Plant Physiol 135(4):2055–2067. doi:10.1104/pp.104.040873

Suzuki YS, Wang Y, Takemoto JY (1992) Syringomycin-stimulated phosphorylation of the plasma membrane H-ATPase from red beet storage tissue. Plant Physiol 99(4):1314–1320

Sydnor ERM, Perl TM (2011) Hospital epidemiology and infection control in acute-care settings. Clin Microbiol Rev 24(1):141–173. doi:10.1128/CMR.00027-10

Takemoto JY, Yu Y, Stock SD, Miyakawa T (1993) Yeast genes involved in growth inhibition by *Pseudomonas syringae* pv. *syringae* syringomycin family lipodepsipeptides. FEMS Microbiol Lett 114(3):339–342

Tanida T, Okamoto T, Ueta E, Yamamoto T, Osaki T (2006) Antimicrobial peptides enhance the candidacidal activity of antifungal drugs by promoting the efflux of ATP from *Candida* cells. J Antimicrob Chemother 57(1):94–103. doi:10.1093/jac/dki402

Taylor LH, Latham SM, Woolhouse ME (2001) Risk factors for human disease emergence. Philos Trans R Soc Lond B Biol Sci 356(1411):983–989. doi:10.1098/rstb.2001.0888

Terras FR, Torrekens S, van Leuven F, Osborn RW, Vanderleyden J, Cammue BP, Broekaert WF (1993) A new family of basic cysteine-rich plant antifungal proteins from *Brassicaceae* species. FEBS Lett 316(3):233–240

Theis T, Stahl U (2004) Antifungal proteins: targets, mechanisms and prospective applications. Cell Mol Life Sci 61(4):437–455. doi:10.1007/s00018-003-3231-4

Thevissen K, Cammue BP, Lemaire K, Winderickx J, Dickson RC, Lester RL, Ferket KK, van Even F, Parret AH, Broekaert WF (2000) A gene encoding a sphingolipid biosynthesis enzyme determines the sensitivity of *Saccharomyces cerevisiae* to an antifungal plant defensin from dahlia (*Dahlia merckii*). Proc Natl Acad Sci U S A 97(17):9531–9536. doi:10.1073/pnas.160077797

Thevissen K, François IEJA, Takemoto JY, Ferket KKA, Meert EMK, Cammue BPA (2003) DmAMP1, an antifungal plant defensin from dahlia (*Dahlia merckii*), interacts with sphingolipids from *Saccharomyces cerevisiae*. FEMS Microbiol Lett 226(1):169–173

Thevissen K, Warnecke DC, François IEJA, Leipelt M, Heinz E, Ott C, Zähringer U, Thomma BPHJ, Ferket KKA, Cammue BPA (2004) Defensins from insects and plants interact with fungal glucosylceramides. J Biol Chem 279(6):3900–3905. doi:10.1074/jbc.M311165200

Thevissen K, Francois IEJA, Aerts AM, Cammue BPA (2005) Fungal sphingolipids as targets for the development of selective antifungal therapeutics. Curr Drug Targets 6(8):923–928

Tokumura T, Horie T (1997) Kinetics of nikkomycin Z degradation in aqueous solution and in plasma. Biol Pharm Bull 20(5):577–580

Tossi A, Sandri L, Giangaspero A (2000) Amphipathic, alpha-helical antimicrobial peptides. Biopolymers 55(1):4–30. doi:10.1002/1097-0282(2000)55:1<4:AID-BIP30>3.0.CO;2-M

Ueta E, Tanida T, Osaki T (2001) A novel bovine lactoferrin peptide, FKCRRWQWRM, suppresses *Candida* cell growth and activates neutrophils. J Pept Res 57(3):240–249

Urbina JA, Pekerar S, Le HB, Patterson J, Montez B, Oldfield E (1995) Molecular order and dynamics of phosphatidylcholine bilayer membranes in the presence of cholesterol, ergosterol and lanosterol: a comparative study using 2H-, 13C- and 31P-NMR spectroscopy. Biochim Biophys Acta 1238(2):163–176

van den Bossche H, Willemsens G, Cools W, Lauwers WF, Le Jeune L (1978) Biochemical effects of miconazole on fungi. II. Inhibition of ergosterol biosynthesis in *Candida albicans*. Chem Biol Interact 21(1):59–78

van der Weerden NL, Bleackley MR, Anderson MA (2013) Properties and mechanisms of action of naturally occurring antifungal peptides. Cell Mol Life Sci 70(19):3545–3570. doi:10.1007/s00018-013-1260-1

van Meer G, de Kroon Anton I P M (2011) Lipid map of the mammalian cell. J Cell Sci 124(Pt 1):5–8. doi:10.1242/jcs.071233

van Meer G, Voelker DR, Feigenson GW (2008) Membrane lipids: where they are and how they behave. Nat Rev Mol Cell Biol 9(2):112–124. doi:10.1038/nrm2330

van Parijs J, Broekaert WF, Goldstein IJ, Peumans WJ (1991) Hevein: an antifungal protein from rubber-tree (*Hevea brasiliensis*) latex. Planta 183(2):258–264. doi:10.1007/BF00197797

van't Hof W, Reijnders IM, Helmerhorst EJ, Walgreen-Weterings E, Simoons-Smit IM, Veerman EC, Amerongen AV (2000) Synergistic effects of low doses of histatin 5 and its analogues on amphotericin B anti-mycotic activity. Antonie Van Leeuwenhoek 78(2):163–169

Vanden Bossche H, Marichal P, Willemsens G, Bellens D, Gorrens J, Roels I, Coene MC, Le Jeune L, Janssen PA (1990) Saperconazole: a selective inhibitor of the cytochrome P-450-dependent ergosterol synthesis in *Candida albicans*. *Aspergillus fumigatus* and *Trichophyton mentagrophytes*. Mycoses 33(7–8):335–352

Viejo-Díaz M, Andrés MT, Fierro JF (2004) Effects of human lactoferrin on the cytoplasmic membrane of *Candida albicans* cells related with its candidacidal activity. FEMS Immunol Med Microbiol 42(2):181–185. doi:10.1016/j.femsim.2004.04.005

Vriens K, Cammue BPA, Thevissen K (2014) Antifungal plant defensins: mechanisms of action and production. Molecules 19(8):12280–12303. doi:10.3390/molecules190812280

Wakabayashi H, Abe S, Okutomi T, Tansho S, Kawase K, Yamaguchi H (1996) Cooperative anti-*Candida* effects of lactoferrin or its peptides in combination with azole antifungal agents. Microbiol Immunol 40(11):821–825

Wang Z, Wang G (2004) APD: the antimicrobial peptide database. Nucleic Acids Res 32(Database issue):D590–2. doi:10.1093/nar/gkh025

Wang G, Li X, Wang Z (2009) APD2: the updated antimicrobial peptide database and its application in peptide design. Nucleic Acids Res 37(Database issue):D933–7. doi:10.1093/nar/gkn823

Warnock DW (2007) Trends in the epidemiology of invasive fungal infections. Nippon Ishinkin Gakkai Zasshi 48(1):1–12. doi:10.3314/jjmm.48.1

Wilmes M, Cammue BPA, Sahl H, Thevissen K (2011) Antibiotic activities of host defense peptides: more to it than lipid bilayer perturbation. Nat Prod Rep 28(8):1350–1358. doi:10.1039/c1np00022e

Wimley WC (2010) Describing the mechanism of antimicrobial peptide action with the interfacial activity model. ACS Chem Biol 5(10):905–917. doi:10.1021/cb1001558

Woyke T, Pettit GR, Winkelmann G, Pettit RK (2001) In vitro activities and postantifungal effects of the potent dolastatin 10 derivative auristatin PHE. Antimicrob Agents Chemother 45(12):3580–3584. doi:10.1128/AAC.45.12.3580-3584.2001

Woyke T, Roberson RW, Pettit GR, Winkelmann G, Pettit RK (2002) Effect of Auristatin PHE on microtubule integrity and nuclear localization in *Cryptococcus neoformans*. Antimicrob Agents Chemother 46(12):3802–3808. doi:10.1128/AAC.46.12.3802-3808.2002

Zaoutis TE, Argon J, Chu J, Berlin JA, Walsh TJ, Feudtner C (2005) The epidemiology and attributable outcomes of Candidemia in adults and children hospitalized in the United States: a propensity analysis. Clin Infect Dis 41(9):1232–1239. doi:10.1086/496922

Zasloff M (1987) Magainins, a class of antimicrobial peptides from *Xenopus* skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor. Proc Natl Acad Sci U S A 84(15):5449–5453

Zhang L, Takemoto JY (1986) Mechanism of action of *Pseudomonas syringae* phytotoxin, syringomycin. Interaction with the plasma membrane of wild-type and respiratory-deficient strains of *Saccharomyces cerevisiae*. Biochim Biophys Acta 861(1):201–204

Zinser E, Paltauf F, Daum G (1993) Sterol composition of yeast organelle membranes and subcellular distribution of enzymes involved in sterol metabolism. J Bacteriol 175(10):2853–2858

Chapter 3
Antiviral Host Defence Peptides

Filipa Henderson Sousa, Victor Casanova, Craig Stevens and Peter G. Barlow

Abstract The ongoing global mortality and morbidity associated with viral pathogens highlights the need for the continued development of effective, novel antiviral molecules. The antiviral activity of cationic host defence peptides is of significant interest as novel therapeutics for treating viral infection and predominantly due to their broad spectrum antiviral activity. These peptides also display powerful immunomodulatory activity and are key mediators of inflammation. Therefore, they offer a significant opportunity to inform the development of novel therapeutics for treating viral infections by either directly targeting the pathogen or by enhancing the innate immune response. In this chapter, we review the antiviral activity of cathelicidins and defensins, and examine the potential for these peptides to be used as novel antiviral agents.

3.1 Introduction

The ongoing mortality and disease associated with circulating viral infections of humans and animals, together with the ongoing threat of emerging viral strains underscores the requirement for the development of novel antiviral molecules. While vaccination against common viral pathogens is effective and desirable, direct antiviral therapeutics play a key role in treating diseases caused by viral pathogens that have no vaccine, that lack a global vaccination programme, or those with vaccines of limited efficacy. In addition, as the resistance of viral pathogens to common antiviral drugs increases, new classes of antiviral molecules could provide a strategy for treatment of both existing and emerging infections for decades to come.

The antiviral activity of Cationic Host Defence Peptides (CHDP; also known as antimicrobial peptides) is of increasing interest for informing the development of novel antiviral therapeutics. Due to their broad spectrum activity, CHDP play a key

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role in the innate immune response to both bacterial and viral pathogens. In addition, CHDP have substantial immunomodulatory activities that can contribute to the rapid clearance of infections. Therefore, a better understanding of the antiviral and immunomodulatory activities of CHDP in the context of viral infection will be of great importance in the race to develop new treatments that are broadly effective against viral pathogens.

CHDP are small, evolutionarily conserved peptides with a positive charge that have broad spectrum activity against a range of pathogens, both in vitro and in vivo. In humans, there are two major families of CHDP; the cathelicidins and the defensins, however, more families exist in mammals, birds, fish, reptiles, arthropods and plants, each showing antimicrobial potential. While the antiviral activity of many peptides has been established in a significant number of studies, a comprehensive understanding of the mechanism(s) of action involved remains elusive. Of particular interest are the immunomodulatory and inflammomodulatory activities of CHDP, from gaining a more fundamental understanding of innate responses to infection in addition to informing development of novel therapeutics. CHDP have been shown to have the capacity to modulate cell death pathways in infected cells, assist in the recruitment of immune cells to sites of infection, promote angiogenesis, alter immune cell differentiation and to mediate production of pro- and anti-inflammatory cytokines.

In this chapter, we review the antiviral activities of both cathelicidins and defensins, and also highlight key CHDP from other species that demonstrate antiviral potential, either through direct antiviral activity or by modulation of the immune response to the infection.

3.2 The Antiviral Activity of Cathelicidins

Cathelicidins range from 12–88 amino acids in length and are characterised by the presence of an N-terminal signal sequence which directs the newly synthesised protein towards the secretory pathway, a conserved cathelin-like domain which has a high sequence homology with the porcine cysteine protease inhibitor, cathelin and a variable C-terminal antimicrobial domain which becomes the mature functional peptide upon proteolytic cleavage.

Cathelicidins were first identified in bovine neutrophils and are widely distributed in mammals including humans, rhesus monkeys, rats, mice, guinea pigs, rabbits, sheep, cows, horses and dogs and also in non-mammalian species including chicken, rainbow trout and hagfish (Zanetti et al. 1993). In humans, only one cathelicidin has been described—the cationic antimicrobial peptide of 18kDa (hCAP18), which can be found at high concentrations in the specific granules of neutrophils and can be expressed by epithelial cells of skin and mucosa of the respiratory, urogenital and gastrointestinal tracts (De et al. 2000). hCAP18 is cleaved extracellularly by proteinase-3 to generate its active form LL-37, a linear 37 aminoacids peptide with two leucine residues at the N-terminal and an amphipathic

α-helical structure (Sorensen et al. 2001). The peptide is known to be expressed by macrophages, eosinophils, lymphocytes, mast cells and NK, T- and B-cells (Agerberth et al. 2000).

Mice express the cathelicidin mCRAMP (murine cathelin-related antimicrobial peptide), which has high sequence identity with hCAP18 and the porcine cathelicidin PR-39. mCRAMP shows similar expression and function patterns to its human ortholog (Gallo et al. 1997) that is stored in neutrophil granules and is expressed by epithelial cells and leukocytes. mCRAMP knock-out mice have a high susceptibility to infections when compared to wild type mice (Huang et al. 2007; Iimura et al. 2005; Kovach et al. 2012).

In contrast to humans and mice, which only express one cathelicidin, pigs express a variety of cathelicidins which differ in activity and structure. The porcine cathelicidins include five different protegrins (PGs), three α-helical peptides (PMAP-23, -36, -37), two prophenins (PF-1, -2) and the PR-39 peptide (Zhang et al. 2000). PGs are produced and stored by porcine neutrophils as inactive propeptides but are proteolytically cleaved into their active forms by neutrophil elastase in the extracellular environment. Their expression is enhanced by bacterial LPS, IL-6, retinoic acid and salmonella infections (Wu et al. 2000) and PG-1 has been shown to have the broadest antimicrobial activity spectrum (Yasin et al. 1996a, b). Cathelicidins have also been characterized in many other species, such as sheep, monkeys, horses and cows. In sheep, eight cathelin-associated peptides have been identified and SMAP-29 (sheep myeloid antimicrobial peptide 29) is one of the most potent cationic host defence peptide known in terms of antimicrobial activity, having a wide spectrum of activity against bacteria, fungi and virus (Tomasinsig and Zanetti 2005).

Cathelicidins play key roles in host defence via direct antimicrobial activity (Putsep et al. 2002), by acting as critical immunomodulatory molecules and in the control of inflammation. Recently, a number of studies conducted in humans, mice and other in vitro models have highlighted the potent antiviral activity of cathelicidins. Viruses that are most susceptible to cathelicidins include enveloped DNA and RNA viruses, and while all of the antiviral mechanisms remain to be elucidated, there does appear to be a direct effect on the viral envelope. However, non-enveloped viruses such as adenovirus can also be inactivated by cathelicidins (Gordon et al. 2005; Barlow et al. 2014). Therefore, the antiviral activity of these peptides likely comprises complex array of mechanisms that cannot all be explained by a direct effect on the virus particles.

3.3 RNA Viruses

3.3.1 Influenza Virus (IAV)

Influenza virus (IAV) is an enveloped virus from the *Orthomyxoviridae* family. Viral influenza has caused the death of more people in short periods of time than any other infectious disease (Taubenberger and Morens 2006). There is a well-established

global vaccination programme in place for preventing influenza infection on an annual basis, but this is limited to protecting against the prevalent circulating strains, and therefore emerging new strains can potentially lead to a global pandemic outbreak. Antiviral treatments are available, although the emerging resistance of circulating strains to neurominidase inhibitors, one of the current front-line treatment, is of serious concern (Nitsch-Osuch and Brydak 2014; Hurt 2014).

Studies have demonstrated the antiviral potential of several cathelicidins against influenza virus. A study by Barlow et al. 2011 demonstrated the antiviral properties of human and murine cathelicidins in vivo and in vitro (Barlow et al. 2011). mCRAMP and LL-37, but not porcine cathelicidin PG-1, showed antiviral properties when pre-incubated with IAV in vitro. Tripathi et al. (2013) further demonstrated that pre-incubation of LL-37 peptide with IAV is necessary for optimal inhibition of IAV, although a host cell pre-treatment or a delayed treatment with exogenous LL-37 also inhibits IAV replication to an extent (Tripathi et al. 2013). This reveals that LL-37 peptide has the ability to interact with epithelial cells although the most potent activity of the peptide involves direct interactions with the virus particles.

In in vivo studies, murine models receiving LL-37 or mCRAMP treatment showed a significant increase in survival compared to saline treated mice. Mice treated with PG-1 showed no significant alterations compared to the control group (Barlow et al. 2011). This demonstrates that antiviral activities of cathelicidins are species-specific, and it was proposed that LL-37 protects against IAV infection through modulation of inflammatory response in the lungs. Mice infected with IAV exhibited a pronounced up-regulation of numerous pro-inflammatory cytokines, although this was attenuated in LL-37-treated mice, suggesting that LL-37 modulates the inflammatory response by inhibiting excessive inflammation (Barlow et al. 2011).

In order to determine the effects of LL-37 on the cellular uptake and replication of IAV, qPCR was used to quantify viral infection and replication. The results showed that LL-37 did not reduce the number of virus particles associated with cells after 45 min of infection, although at 24 h post-infection, LL-37 caused a significant reduction in the amount of virus present in cells and in the cell culture supernatant (Tripathi et al. 2013). Electron microscope images revealed that LL-37 had induced disruption of viral membranes, which may be one possible mechanism of antiviral activity.

Interestingly, pandemic IAV strains have shown to be more resistant to innate inhibitors of seasonal IAV strains, such as human and murine cathelicidins. A recent study compared the antiviral activities of LL-37 and derived fragments from LL-37 against seasonal and pandemic strains of influenza virus and revealed that the central fragment of LL-37 showed greater activity against the pandemic IAV strain than LL-37 in vitro (Tripathi et al. 2015). This finding suggests the possibility that synthetic derivatives of LL-37 with more potent antiviral activity could be used as a potential therapeutic for this infection.

3.3.1.1 Human Immunodeficiency Virus (HIV)

HIV is an enveloped *lentivirus* of family *Retroviridae* that causes HIV infection and acquired immune deficiency syndrome (AIDS). An estimate of 34.2 million people worldwide live with HIV and around 2.5 million new infections and 1.7 million deaths were detected in 2011 only (Piot and Quinn 2013). The hallmark of this infection is the gradual loss of $CD4^+$ T-cells which leads to an acquired immune deficiency syndrome or AIDS (Simon et al. 2006). The production of up to 10^{10} viral particles per day together with low fidelity of reverse transcription and recombination generate viral quasi-species in chronically infected subjects makes lifelong treatment with a combination of highly active antiretroviral drugs (HAART) the only option capable of keeping the infection controlled.

Indolicidin, a cathelicidin isolated from large granules of bovine neutrophils, was the first to show anti-HIV activity (Robinson et al. 1998). It is thought that the potent antimicrobial properties of indolicidin are related to their ability to disrupt pathogen membranes. Experimentally, indolicidin directly inactivated the HIV-1 virus particles, an observation that was attributed to a membrane-mediated antiviral mechanism.

A in vitro study by Bergman et al. (2007) showed that LL-37 could inhibit HIV-1 replication in peripheral blood mononuclear cells (PBMC), including primary $CD4^+$ T-cells. This was shown to be independent of formyl peptide receptor-like-1 (FPRL-1) signalling, a receptor which is thought to be partially responsible for mediating some of the chemotactic and immunomodulatory effects of LL-37 (Bergman et al. 2007; Barlow et al. 2006). Another study examined the anti-HIV effects of LL-37 and its derived fragments, together with BMAP-18, a fragment derived from bovine cathelicidin BMAP-27, in vitro (Wang et al. 2008). The peptide sequence order, aromatic residues and helical structures were examined and were shown to play an important role in HIV inhibition. Again, a central fragment of LL-37 (known as GI-20), and BMAP-18, were the most active against HIV-1 compared to LL-37 and BMAP-27. This study essentially provides new antimicrobial templates that could be used to develop novel anti-HIV therapies.

A more recent study examined the effects of LL-37 and its derived fragments on HIV-1 reverse transcriptase, HIV-1 integrase and HIV-1 protease (Wong et al. 2011). It was shown that all peptides tested lacked the ability to inhibit translocation of HIV-1 integrase, an enzyme essential for HIV replication. The most potent inhibitory effects of the peptides were seen on HIV-1 reverse transcriptase, and the central peptide, LL13-37, was revealed to have the strongest inhibitory activity.

hCAP-18 has been shown to be strongly expressed in the epithelium of the epididymis, which suggests an important role of hCAP-18 in the antimicrobial protection of the reproductive male system (Malm et al. 2000). It has also been shown that vaginal fluid of healthy women has intrinsic anti-HIV-1 properties and these were conferred by cationic polypeptides. A depletion of cationic polypeptides caused a reduction of the intrinsic anti-HIV-1 activity (Venkataraman et al. 2005) and, in addition to this, cervicovaginal secretions (CVS) of Kenyan women in HIV-serodiscordant relationships contained HIV neutralizing activity and CVS

which revealed no intrinsic anti-HIV activity could be enhanced by the addition of recombinant LL-37 (Levinson et al. 2012). Further studies have revealed that high concentrations of LL-37 were found in cervicovaginal secretions (CVS) collected from Kenyan sex workers with bacterial transmitted infections, which are associated with increased HIV acquisition (Levinson et al. 2009). This finding does not seem to correlate with previous studies, where CVS contain HIV neutralizing activity conferred by cationic polypeptides. Therefore, there is a pressing need to determine the in vivo significance of LL-37 peptide in HIV infections.

3.3.1.2 Junin Virus (JV)

Junin virus (JV) is an enveloped virus of the *Arenaviridae* family, which causes Argentine haemorrhagic fever (AHF). A study by Albiol Matanic and Castilla (2004) assessed the antiviral effects of indolicidin against junin virus, although it was determined that this cathelicidin was not able to induce substantial rates of viral inactivation (Albiol Matanic and Castilla 2004). However, the relatively low virucidal action of indolicidin against junin virus that was observed was thought to be due to direct inactivation of the virus particles, similar to the antiviral mechanisms proposed for the action of cathelicidins against HIV-1 (Robinson et al. 1998).

3.3.1.3 Dengue Virus (DENV)

Dengue virus (DENV) is an enveloped member of the *Flaviviridae* family which causes dengue fever—a mosquito-borne tropical disease. An important target for antiviral therapies against dengue virus is NS2B/NS3 serine protease, as disruption of NS2B/NS3 serine protease functions inhibit virus replication. A study by Tambunan and Alamudi (2010) demonstrated that cationic cyclic peptides have a high potential to inhibit NS2B/NS3 serine protease activities of dengue virus (Tambunan and Alamudi 2010). A more recent study proposed that protegrin-1 (PG-1), a cationic cyclic peptide which was originally isolated from porcine blood cells, is able to inhibit NS2B/NS3 serine protease activity, thus translating to reduced viral replication in vitro (Rothan et al. 2012).

3.3.1.4 Human Respiratory Syncytial Virus (RSV)

RSV is an enveloped virus and a member of the *Paramyxoviridae* family, and has been shown to be responsible for significant numbers of respiratory tract infections. It is the major cause of viral bronchiolitis in young children (Nair et al. 2010). A recent study demonstrated that human cathelicidin displays concentration-dependent antiviral activity against RSV in vitro at physiologically relevant concentrations of 25 μg/ml (Currie et al. 2013). LL-37 inhibited RSV replication and decreased the spread of infection, and these effects were highest when the peptide

was pre-incubated with RSV or added to cells simultaneously. This study demonstrated that LL-37 mediates direct effects against the virus particles, similar to the action of LL-37 against influenza A virus (Tripathi et al. 2013). It was also demonstrated that a delayed LL-37 exposure, taking place 2 h after infection, resulted in the loss of antiviral effects which revealed the inability of the peptide to rescue infected cells. However, pre-treating cells with LL-37 prior to infection resulted in a reduction of infectivity, suggesting that the peptide is retained by the epithelial cells exerting a protective antiviral state. These results indicate that LL-37 can protect epithelial cells from viral infection through a mechanism distinct from direct antiviral activity. In addition, LL-37 was also shown to actively protect RSV-infected epithelial cells from cell death.

3.3.2 DNA Viruses

3.3.2.1 Vaccinia Virus (VV)

Vaccinia virus (VV) is an enveloped poxvirus, of the family *Poxviridae*, which is the active constituent of the vaccine that eradicated smallpox. It has been demonstrated that individuals with atopic dermatitis (AD) have a predisposition to develop eczema *vaccinatum* in response to the vaccine, and that, in these individuals, the expression of hCAP18 is reduced (Howell et al. 2006). It has been shown that in normal skin biopsies, the expression of LL-37 was induced by vaccinia virus, but this was not observed in not in AD skin. Furthermore, a study by Howell et al. (2004) demonstrated that both LL-37 and the murine cathelicidin, mCRAMP, have antiviral activity against vaccinia virus, and the antiviral mechanism by which LL-37 exerts its effect involves the removal of the outer membrane of vaccinia virus, thus causing envelope damage (Howell et al. 2004; Dean et al. 2010).

3.3.2.2 Herpes Simplex Virus (HSV)

Herpes simplex virus 1 and 2 (HSV-1 and -2) are enveloped viruses of the family *Herpesviridae*, that are widely found in humans and are particularly infectious. While they can be suppressed by some antiviral drugs, they are not normally susceptible to complete eradication from a host. A study by Yasin et al. (2000) screened 20 host defence peptides to test their antiviral activity against HSV type 1 and 2 (Yasin et al. 2000). LL-37 was shown to have very little capacity to induce viral inactivation. However, the bovine cathelicidin, indolicidin, displayed potent antiviral activity against both HSV types.

Other peptides which were investigated for anti-HSV activity include BMAP-27 and -28 (acronym of "bovine myeloid antimicrobial peptides"), which are cathelicidins found in bovine neutrophils. BMAP-27 and -28, and their synthetic 1–18 fragments, were analysed for their in vitro antiviral activity against HSV-1. Only

BMAP-28 was shown to provide some protection in vitro against HSV-1 whereas all other peptides were ineffective at non-cytotoxic concentrations (Benincasa et al. 2003). Another study examined the underlying mechanism for the potent antiviral activity of indocilidin observed against HSV type -1 and -2 in vitro, and suggested that the mechanism underlining the antiviral activity was related to the ability of the peptide to disrupt the viral envelope, thus inactivating the virus particles (Albiol Matanic and Castilla 2004). However, contrary to previous reports, Gordon et al. 2005 reported a potent antiviral activity of LL-37 against HSV-1 in vitro (Gordon et al. 2005).

It is known that a subgroup of patients with atopic dermatitis (AD) will develop eczema herpeticum (ADEH) due to a disseminated infection with HSV (Wollenberg et al. 2003). Patients with AD have decreased expression of host defence peptides, and it has been suggested that a deficiency of LL-37 may cause patients with AD to be more susceptible to ADEH (Ong et al. 2002). A study by Howell et al. (2006) showed that LL-37 exhibited direct antiviral activity against HSV-2 in vitro. In addition, a particularly physiologically relevant model was employed whereby human keratinocytes cells were pre-incubated with HSV for a period of 6 h before treatment with LL-37 for 18 h to assess whether intracellular viral replication could be inhibited with physiologic concentrations of LL-37 (Howell et al. 2006). This study showed that the peptide was able to significantly reduce the levels of HSV gene expression in infected keratinocytes. In vivo studies using mice deficient in mCRAMP revealed higher rates of HSV replication compared to the wild type mice, indicating an important role for host defence peptides in controlling HSV in skin infection.

Interestingly, a recent study tested and compared two different approaches to fight HSV-1 corneal infection. A sustained release of LL-37 delivered through nanoparticles incorporated within corneal implants was compared with a cell-based delivery of LL-37 cDNA transfected into HCECs (human corneal epithelial cells). LL-37 released from implants showed an ability to inhibit HSV-1 activity, but did not clear HSV-1 from infected cells. HCEC producing LL-37 also showed direct anti-HSV-1 activity, although none of these approaches were able to completely eliminate the virus infection (Lee et al. 2014).

3.3.2.3 Adenovirus (Ad)

Adenovirus is non-enveloped virus that is part of the *Adenoviridae* family. Adenoviruses are a major cause of conjunctivitis and keratoconjunctivitis, but can also cause upper and lower respiratory and gut infections. Although usually self-limiting, human adenovirus (HAdV) infections are quite contagious and put immunocompromised individuals at serious risk of severe and recurrent pulmonary infections, with mortality rates that reach up to 55 % (Lion 2014). Treatment for an infection is largely supportive therapy rather than direct antiviral therapeutics.

A study by Gordon et al. (2005) investigated the antiviral activity of LL-37 against different adenovirus serotypes (Ad19, Ad8, Ad5 and Ad3) (Gordon et al. 2005). LL-37 demonstrated a significant reduction of Ad19 titer in vitro (2 log

Table 3.1 Antiviral activities of cathelicidins

Cathelicidin	Structure	Source	Virus	Genome	Mechanism	References
LL-37	α-helix	Human	IAV	RNA	Viral envelope damage; cellular target; modulation of inflammatory response in vivo;	Tripathi et al. (2013), Barlow et al. (2011)
			HIV	RNA	Inhibition of HIV-1 reverse transcriptase	Wong et al. (2011)
			RSV	RNA	Direct effects on virus particles; cellular target;	Currie et al. (2013)
			VV	DNA	Viral envelope damage	Dean et al. (2010)
			HSV	DNA	Viral envelope damage; virus inactivation;	Howell et al. (2006)
			Ad	DNA	Unknown	Gordon et al. (2005)
mCRAMP	α-helix	Mouse	IAV	DNA	Virus inactivation; modulation of inflammatory response in vivo;	Barlow et al. (2011)
			VV	DNA	Unknown	Howell et al. (2004)
			HSV	DNA	Unknown	Howell et al. (2006)
Indocilidin	Extended	Bovine	HIV	RNA	Viral envelope damage	Robinson et al. (1998)
			JV	RNA	Viral inactivation	Albiol Matanic and Castilla (2004)
			HSV	DNA	Viral envelope damage	Albiol Matanic and Castilla (2004)
BMAP-18 (fragment derived from BMAP-27)	α-helix	Bovine	HIV	RNA	Viral inhibition	Wang et al. (2008)
BMAP-28	α-helix	Bovine	HSV	DNA	Viral inhibition	Benincasa et al. (2003)
Protegrin-1	β-sheet	Porcine	DENV	RNA	Inhibit NS2B/NS3 serine protease activity; viral inhibition	Rothan et al. (2012a, b)

reduction over 4 h). This study also provided some insight on a potential antiviral mechanism of cathelicidins, based on the comparison of HSV and Ad19 time-kill assays. The data demonstrated a rapid killing of HSV-1 which suggested a disruption of the viral lipid membrane as a possible mechanism. Several other studies have also proposed similar mechanisms for other cathelicidins (Robinson et al. 1998; Albiol Matanic and Castilla 2004; Dean et al. 2010). Interestingly, for the Ad19 strain, LL-37 produced a much slower progressive reduction in virus titers. As adenovirus lacks a viral envelope, this suggests that the direct antiviral mechanism of cathelicidins does not involve membrane disruption. Alternative mechanisms have been proposed such as disruption of the adenovirus particles (detergent-effect) and/or blockage of viral entry into the cell.

3.3.3 Summary—The Antiviral Activity of Cathelicidins

In summary, a review of the current literature shows that cathelicidins have antiviral properties against a broad spectrum of viruses; the underlying mechanisms likely involve a direct effect on viral particles as well as the capacity to modulate host immune responses that may contribute in the clearance of infection. A better understanding of how cathelicidins interact with virus particles directly, in addition to their effects on infected host cells, remains to be established. However, it is clear that cathelicidins are ideal targetable components of the innate immune system that can be used to inform the development of novel therapeutics with broad activity targeting a number of viruses (Table 3.1).

3.4 The Antiviral Activity of Defensins

Defensins are small, cysteine-rich cationic peptides that act as important effectors of the innate immune system (Ding et al. 2009; Ganz 2003). Human defensins are classified according to their structure, α and β-defensins differing in their disulphide bond pairing, and θ-defensins, being present in Old World monkeys, displaying a circular structure (Lehrer 2004). Of note, despite RNA transcripts for θ-defensins are found in human bone marrow cells, a premature stop codon prevents protein translation (Nguyen et al. 2010).

Neutrophil α-defensins (HNPs 1–4) are mainly synthesized as prepropeptides in promyelocytes in the bone marrow, and the mature peptide is stored in the granules of neutrophils. These peptides are found in lower concentrations in NK-cells, B-cells, γδ T-cells, monocytes, macrophages and immature dendritic cells (Rehaume and Hancock 2008). In contrast, human alpha defensins 5 and 6 (HD5 and HD6) are constitutively expressed and secreted as a propeptide in Paneth cells, salivary glands and also in genital mucosa (Ouellette 2006). Human β defensins (HBDs) 1-3 are mainly expressed in epithelial cells, but can also be found in

immune cells, mainly monocyte/macrophages and dendritic cells, whereas 3 θ-defensins (RTD1-3) have been found in rhesus macaque leukocytes (Ganz 2003; Tang et al. 1999). Retrocyclin, an artificial peptide based on the human θ defensins pseudogene has been also shown to possess antimicrobial capacity (Tran et al. 2008; Yasin et al. 2004).

Defensins have broad antimicrobial activities, including the capacity to inhibit viral infections. Despite structural and physical similarities, such as overall size and positive charge, each defensin has variable antiviral activity. The differences in activity are likely due to the mechanism of action exhibited by each peptide towards a particular DNA or RNA virus.

3.4.1 DNA Viruses

3.4.1.1 Herpes Simplex Virus

One of the first viruses which was shown to be inhibited by α-defensins was HSV-1, which was inactivated after incubation with HNP-1, -2 and -3, an effect that was abrogated by serum addition (Daher et al. 1986). Further work has shown that one β defensin (hBD3) and all six α-defensins inhibited HSV-2 infection (Hazrati et al. 2006). Interestingly, while HNP1-3 and HD5 interacted with the viral glycoprotein Gb2 present on the viral envelope, HNP-4 and HD6 bound to heparan sulfate and heparin, cellular receptors used for HSV-2 to gain entry to cells. All of these interactions have been shown to result in diminished viral entry. In contrast, the same study demonstrated that β-defensin 1 and 2 (hBD1 and hBD2) which lack anti-HSV-2 effects, do not bind to neither Gb2 nor heparan sulfate.

Similar antiviral effects against HSV are displayed by θ defensins, as both rhesus θ defensins 3 (RTD3) and retrocyclins 1 and 2 (RC1 and RC2) have been demonstrated to inhibit HSV viral entry to cells (Yasin et al. 2004). Of these peptides, retrocyclin 2 was shown to interact with the viral glycoprotein Gb2, thus interfering with viral entry without causing significant cytotoxicity to target cells. Defensins often display an antiviral activity that spans different steps on the target virus cycle. In this regard, HNP-1 and HD5 are able to block HSV viral gene expression even when added after the virus infection, indicating that defensins are also able to block post-entry events in the HSV cycle.

3.4.1.2 Human Papilloma Virus (HPV)

HNP-1-3 and HD5 but not hBD1, hBD2 or HD6 have been shown to exhibit antiviral activity against HPV, which is the primary cause of cervical cancer in sexually transmitted infections (Buck et al. 2006). HNP-1-3 and HD5 block HPV infection by impairing virion escape from endocytic vesicles. A recent study also suggested that HD5 blocks viral entry by interacting with viral particles and

blocking L2 cleavage, a necessary step for successful viral entry and post-entry events (Wiens and Smith 2015). Detailed studies on HD5 and HPV interactions suggest that hydrophobic residues, in particular, Arg-28, are very important for the antiviral activity of HD5 against HPV and other non-enveloped viruses (Gounder et al. 2012; Tenge et al. 2014).

3.4.1.3 Vaccinia Virus (VV)

Several defensins have been shown to have varying potential to neutralize VV infection. Incubating VV for 24 h with hBD3 was shown to reduce the number of viral plaques formed on BSC-1 green monkey kidney cells. In addition, hBD3 reduced the expression of viral DNA-dependent RNA polymerase (Howell et al. 2007) during viral infection. However, HNP-1, hBD1 and hBD2 showed no activity against this enveloped virus (Howell et al. 2004).

3.4.1.4 JC Polyomavirus (JCPyV) and BK Virus

JC polyomavirus (also known as John Cunningham virus) is a member of the family *polyoviridae*, and chronically infects between 70–90 % of the human population. This pathogen only tends to cause clinical symptoms in immunocompromised individuals, where infection spreads to the central nervous system (Shackelton et al. 2006; Wollebo et al. 2015). In experimental studies, a panel of α and β-defensins were tested for their capacity to neutralize JCPyV infection, resulting in HD5 and hBD3 being able to block the infection when incubated at 100 μg/ml with the virus for 1 h before infecting SVG-A human foetal glial cells (Zins et al. 2014). Interestingly, hBD3 showed significant cytotoxicity on cells at this concentration, whereas HD5 neutralized JCPyV in a dose-dependent manner (Zins et al. 2014; Dugan et al. 2008). Of note, HNP1-3 and hBD1, 2 and 4 showed no anti JCPyV activity. Further experiments also showed that HD5 was able to interact with JCPyVvirions, stabilizing the viral capsid and thus preventing the viral genome release (Zins et al. 2014), a novel mechanism of action for a peptide that is generally thought to employ detergent-like modes of antiviral activity.

BK virus shares 75 % of its genome with JCPyV, and was first isolated from the urine of a renal transplant individual (Gardner et al. 1971). Around 80 % of healthy individuals in England have been shown to display antibodies against the virus, generally in the absence of symptoms (Gardner 1973). If symptoms are noted, these can consist of fever and nonspecific upper respiratory infection which might lead to kidney manifestations such as cystitis or nephritis in immunocompromised individuals or those receiving transplants (Reploeg et al. 2001).

HNP1 and HD5 were shown to reduce viral V-protein expression in VERO cells when incubated directly with the virus at 20 or 50 μg/ml for 1 h, while hBD2 only was effective at 50 μg/ml. In contrast, hBD1 showed no inhibitory effect (Dugan et al. 2008). When the direct antiviral effect of defensins was studied further, HD5

was shown to interact directly with BK virus particles, inducing aggregation, and thus reducing viral attachment to cell membranes. Of note, similar antiviral effects of HD5 were also observed against other related polyomaviruses such as simian virus-40 (SV40). It was also shown that both HNP-1 and HD5 were also able to block adenovirus escape from endosomes.

3.4.1.5 Adenovirus (Ad)

It has been shown that hBD-1 and HD-5, when expressed on eukaryotic cell lines, showed potential to protect those cells against adenoviral infection, in particular from Av1CF2 (Gropp et al. 1999). Different HAdV species show variable susceptibility to defensin actions. In this regard, HNP-1 inhibited HAdV A, B and C infection, while increased HAdV D, E and F infectivity (Smith et al. 2010). HD5 showed similar actions, blocking HAdV A, B, C and E while increasing D and F infectivity. Interestingly, and similar to other non-enveloped viruses, HD5 interacts with adenoviral capsids preventing viral uncoating and avoiding its release from endocytic vesicles (Nguyen et al. 2010; Smith et al. 2010; Smith and Nemerow 2008). Further studies confirmed that Arg-28 residues of HD5 are critical for the antiviral effects seen against adenovirus (Gounder et al. 2012). Much less is known about other defensins effects on adenovirus. However, HNP-1 was shown to be effective at a concentration of 50 μg/ml in blocking adenovirus-type 5 infection of 293 cells, whereas hBD-2 showed a reduced effect (Bastian and Schäfer 2001).

3.4.1.6 Cytomegalovirus (CMV)

CMV is an enveloped virus that belongs to the *Herpesviridae* family, and is the most common congenital infection, affecting up to 0.2–2.2 % of all live births (Huygens et al. 2014). Foetal or perinatal infections can have devastating neurological consequences for the baby. However, post-natal CMV infections are usually asymptomatic, establishing lifelong infections without severe consequences on immune competent individuals.

In the context of CHDP activity against this virus, super physiological concentrations (100 and 200 μg/ml) of HNP-1 peptide were shown to directly inhibit CMV viral particles reducing the PFU/ml by 0.29 and 0.81 $\log_{10}$, respectively (Daher et al. 1986). However, this virus notably showed less susceptibility to HNP-1 compared to HSV-1, which was also assessed in the same study.

3.4.1.7 Baculovirus

Baculovirus naturally infects insect larvae hosts, usually *Lepidoptera* (Butterflies and moths), with no known diseases caused by this virus in organisms others than arthropods (Airenne et al. 2013). Due to its efficient reproduction cycle and ability

to carry large DNA inserts, baculovirus are extensively used as tools for gene delivery and recombinant production of proteins in insect cells. Baculovirus gp64 protein allows viral fusion in an acidified endosome, an event seen upon infection of *Spodoptera frugiperda* insect cells, resulting in gp64 expression and the formation of cell syncytia (Leikina et al. 2005). However, it has been shown that RC-2 peptide is capable of blocking baculovirus fusion with host cells, inhibiting the virus-mediated syncytium formation.

3.4.2 RNA Viruses

3.4.2.1 Human Immunodeficiency Virus (HIV)

It has been shown that a variety of defensin peptides can have an impact on HIV infection. HNP-1 is clearly deleterious for viral dissemination, having a direct effect against a low multiplicity of infection (MOI) of viral particles in the absence of serum, whereas in the presence of serum HNP-1 acts on host cells (Chang et al. 2005). One of the suggested mechanisms for such cellular effects is the HNP-1 mediated inhibition of the PKC signalling pathway, which is required for viral uncoating (Fields et al. 1988). Importantly, this HNP-1 effect did not affect the expression of viral receptors CD4, CXCR4 or CCR5 (Chang et al. 2003). Interestingly, as a part of its direct effects on HIV viral particles, HNP1-3 peptides were shown to interact with gp120, a viral glycoprotein, thus impairing viral attachment to cell membranes. This effect was further potentiated by HNP-1-3 interactions with cellular CD4. Both antiviral effects were reduced, but not completely abolished by the presence of serum (Demirkhanyan et al. 2012; Furci et al. 2006; Wang et al. 2004). A closely related peptide, HNP-4 was also able to bind gp120 and CD4 (Wu et al. 2005). A more indirect but also effective anti-HIV effect of HNP-1-3 peptides is their capacity to increase the secretion of the C-C chemokines MIP-1α, MIP-1β and RANTES, which can bind to CCR5 to act as antagonists for viral R5 strains that use CCR5 as a co-receptor (Guo et al. 2004).

In stark contrast to HNP1-3, HD5 and HD6 seem to increase viral infectivity without any blockage of post-entry viral events (Klotman et al. 2008). Further assessment of this unexpected effect suggested that HD5 and HD6 actually enhance viral attachment to the cells (Rapista et al. 2011). Another study, however, showed HD5 to inhibit HIV infection by blocking gp120–CD4 interaction (Furci et al. 2012). Further work is needed to clarify HD5 effects on the HIV infection process, particularly when considering sexually transmitted infections may increase HD5 and HD6 secretions in the genital mucosa prior to a possible HIV infection (Klotman et al. 2008).

Given the importance of HIV interaction with mucosal surfaces, β-defensin impact on HIV infection has also been investigated. HIV was shown to upregulate hBD2 and hBD3 release by human oral epithelial cells, and incubation of viral particles with these peptides protected cells from infection (Quiñones-Mateu et al.

2003). Interestingly, hBD1 was neither upregulated nor protective in this context. However, hBD2 and hBD3 did appear to act both directly on viral particles, and also on the host cell, by downregulating CXCR4 expression. Accordingly, these peptides were found to be more effective against X4 viral isolates. A later study found a similar anti-HIV activity of hBD2 and hBD3 to both X4 and R5 strains, indicating that defensin treatment inhibited early products of reverse transcription (Sun et al. 2005).

Given the interest in the broad antiviral effects of defensins, rhesus macaque θ-defensins (RTD) and synthetic retrocyclins (RC) have been also tested for their anti-HIV activity. RC-1 and RC-2 were shown to bind glycosylated gp120 and CD4, thus explaining the previously observed protective activity (Cole et al. 2002; Wang et al. 2003). Interestingly, RTD peptides also showed anti-HIV activity (Wang et al. 2004). Further work demonstrated that RC-1 was able to block HIV-1 fusion with target cells (Gallo et al. 2006). Finally, rhesus macaque α-defensins 4 (RMAD 4) also showed anti-HIV activity at 150 μg/ml by blocking viral entry (Tanabe et al. 2004). Interestingly, the same study showed that cryptidin-3 (a mouse derived α-defensin) actually increased HIV-1 replication. In this regard, guinea pig, rabbit and rat α-defensins have also been tested for anti-HIV activity, demonstrating an ability to inhibit infection of T-cell lines (Nakashima et al. 1993).

3.4.2.2 Hepatitis C (HCV)

Hepatitis C infection primarily affects the liver, being a major cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma (Maheshwari et al. 2008). HCV infection is estimated to affect 170 million people worldwide, is frequently asymptomatic, and spontaneous clearance is seen in a third of infected individuals. Chronic infection (with viral replication detected for more than 6 months) leads to hepatic complications. Little is known about the capacity of defensins to impact viral hepatitis. However, it has been shown that HCV core protein have been shown to activate the transcription of alpha-defensin genes, indicating that they may play a role in the innate immune response to the virus (Aceti et al. 2006).

3.4.2.3 Influenza A Virus (IAV)

Early studies have reported HNP-1-3 activity against the A/WSN (H1N1) strain of IAV (Daher et al. 1986). More detailed work has shown that HNP-1 affects influenza replication by blocking the PKC signalling pathway on host cells, needed for viral endosomal trafficking (Salvatore et al. 2007). It was shown that HNP-1 did not have a direct impact on viral particles, but did increase viral clearance by neutrophils (Tecle et al. 2007). A similar effect was also observed with HNP-2, HD5 and to a lesser extent with hBD2 but not with HNP-3 or hBD1. Another β-defensin, hBD3, was also shown to block IAV infection by blocking hemagglutinin-mediated fusion and immobilizing membrane proteins (Leikina et al.

2005). Interestingly, RC2 also showed similar effects to hBD3. Of note, murine β-defensin 2 and 3 (mBD2 and mBD3) protected mice from infection with the A/PR/8 (H1N1) IAV strain, and in similarity to their human analogues, their activity was attributed to impaired viral entry (Gong et al. 2010; Jiang et al. 2012).

3.4.2.4 Respiratory Syncytial Virus (RSV)

Defensins are expressed at high concentrations in the inflamed lung, and are usually upregulated during viral infections of the airway epithelia. In this context, it has been demonstrated that RSV infection increased hBD2 secretion by alveolar A549 cells, which was subsequently able to block further viral entry by directly disrupting the viral envelope (Kota et al. 2008). Interestingly RSV did not induce the expression of hBD1, as this is constitutive, but hBD1 lacked direct anti-RSV activity. Although alveolar macrophages are permissive to RSV infection, they do not tend to influence the later development of disease (Pribul et al. 2008). However, this interaction can result in increased secretion of pro-inflammatory cytokines that can impact on β-defensin expression in the lungs (Becker et al. 1950).

3.4.2.5 Rhinovirus (HRV)

Rhinovirus is a major cause of common cold and it is associated with asthma exacerbations (Jartti and Korppi 2011). It has been demonstrated that RV16 infection of primary bronchial epithelial cells results in the induction of hBD2 and hBD3 mRNA, whereas hBD1 remains unaltered (Duits et al. 2003). It was also demonstrated that the same serotype also upregulated hBD2 mRNA and protein in A549 alveolar epithelial cells. Interestingly this effect was conserved in other major group serotype HRV-14 or in 2 minor group serotypes such as HRV-2 and HRV-1A.

Further work has investigated the inoculation of human subjects divided into non-smokers, smokers and COPD groups with RV-16 (Proud et al. 1950). Interestingly, a larger proportion of smokers (78.6 %) were successfully inoculated with the virus, compared to those with COPD (66.7 %) or non-smokers (58 %). RV16 was shown to increase α-defensin secretion in the sputum 9 days post-infection compared to baseline, but this was only observed in the COPD group (Mallia et al. 2012). RV-16 infection also resulted in increased neutrophil elastase, which may subsequently degrade secreted host defence peptides.

3.4.2.6 Echovirus and Reovirus

The Enteric Cytopathic Human Orphan (ECHO) virus, belongs to the *Picornaviridae* family. Enteroviruses are mainly found in the gastrointestinal tract, and can cause opportunistic infections mainly in children by indirect faecal–oral

transmission, causing febrile illness (Sherris Medical Microbiology 2004). HNP-1 peptide was tested for its antiviral activity against this non-enveloped RNA virus without showing any direct antiviral effect at concentrations of peptide known to inhibit HSV-1 (Daher et al. 1986).

In similarity to Echovirus, HNP-1 showed no direct activity against this dsRNA, non-enveloped virus, which generally causes a mild and limited upper respiratory and gastrointestinal tract infection which can spread across individuals, and also across species (Daher et al. 1986). Interestingly, the use of reovirus is being contemplated in cancer therapy due to its oncolytic activity in melanoma lines and xenografts (Galanis et al. 2012).

3.4.2.7 Vesicular Stomatitis Indiana Virus (VSIV)

VSIV belongs to the *Rhabdoviridae* family and is an arthropod-borne virus primarily affecting rodents, cattle, swine and horses. Infected livestock or sand flies can be a source of infection for humans, which develops as mild flu-like symptoms (Kuzmin et al. 2009). This enveloped RNA virus showed an intermediate susceptibility to direct inactivation by HNP-1 peptide, reducing its PFU/ml by 0.74 and 0.84 $\log_{10}$ with 50 or 100 μg/ml of peptide, respectively (Daher et al. 1986).

3.4.2.8 SARS Coronavirus (SARS-CoV)

Severe acute respiratory syndrome (SARS) affected over 8000 individuals in 2002–2003, causing almost 800 deaths, underscoring the importance of testing antiviral agents (Peiris et al. 2003). It has been shown that intranasal injections of ~125 μg RTD-1 peptide prior to virus inoculation protected BALB/c mice against a mouse-adapted strain of SARS-CoV (Wohlford-Lenane et al. 2009). Of note, untreated animals exhibited around 75 % mortality rate, whereas treated ones showed a 100 % survival. RTD-1 treatment altered lung cytokine responses to the virus, suggesting immunomodulatory effects where at least in part, behind the protective action of RTD-1. Interestingly *DEFA-3* AND *DEFA-4* genes (coding for HNP-3 and -4) were upregulated in blood samples of patients suffering acute SARS coronavirus infection (Lee et al. 2005).

3.4.2.9 Dengue Virus (DENV)

Recombinant RC-1 peptide has shown to reduce DENV-2 viral replication in VERO cells when incubated directly with the viral particles, but also showed a moderate effect when pre-treating or treating cells post-viral entry (Rothan et al. 2012). This study suggested that RC-1 impacts DENV-2 replication by inhibiting the activity of viral NS2B-NS3 serine protease. Interestingly further studies showed

that human skin fibroblasts release HD5 and hBD2 upon infection with DENV-2 (Bustos-Arriaga et al. 2011).

3.4.2.10 Sindbis Virus (SINV)

SINV, a member of the *Togaviridae* family, is transmitted by a mosquito vector, and causes sindbis fever which results in arthralgia, malaise and rash. It has been shown that both RC2 and hBD3 peptide were able to block the virus fusion with target cells (Leikina et al. 2005) (Table 3.2).

3.5 Other CHDP with Antiviral Activity

In addition to the well-characterized activity of cathelicidins and defensins against a wide variety of viral pathogens, a number of CHDP from other non-human organisms have been the focus of a number of studies attempting to understand whether these peptides, or synthetic derivatives, could be used to inform the design of novel therapeutics against viral infections specific to humans.

A substantial diversity in CHDP exists across a number of other species that possess activity against human pathogens. The Antimicrobial Peptide Database (http://aps.unmc.edu/AP) now contains in excess of 2,500 peptides with demonstrable antimicrobial activity isolated from six kingdoms, and includes peptides from bacteria, fungi, plant, amphibians, fish, reptiles and birds. Interestingly, approximately 170 of these peptides (which include cathelicidins and defensins) demonstrate antiviral activity against a broad range of viral pathogens.

3.5.1 Cecropin

A family of CHDP with particularly potent activity against human viral pathogens is the cecropins. These peptides, identified initially in the Cecropia moth (*Hyalophoracecropia*) form part of the immune response to infection in a number of insect orders including Diptera and Lepidoptera, and are typically 30–39 amino acids in size (Boman 1991). Cecropin and cecropin-like peptides are a highly conserved, predominantly alpha helical group of peptides that have broad spectrum antibacterial and antifungal activity (Bulet and Stocklin 2005).

A recent study revealed that the mature form of an induced cecropin-like peptide found in the salivary glands of the female mosquito, *Aedesaegypti*, had substantial antiviral activity against Dengue virus (Luplertlop et al. 2011). The virus utilizes *A.aegypti* as a vector for transmission to humans and induces an innate immune response, characterized by the expression of a cecropin-like peptide, produced by up-regulation of the AAEL000598 gene. The peptide is subsequently cleaved from

Table 3.2 Antiviral activities of defensins

Defensin	Structure	Source	Virus	Genome	Antiviral mechanism	REF
HNP-1-3 (including HNP-1)	α-defensin	Human	HSV-2	dsDNA	Interacts with viral gb2; prevents binding to target cell; also blocks post-entry events	Yasin et al. (2004), Hazrati et al. (2006)
			HPV	dsDNA	Blocks virus escape from endocytic vesicles	Buck et al. (2006)
			VV	dsDNA	No antiviral effect	Howell et al. (2004)
			JCPyV	dsDNA	No antiviral effect	Zins et al. (2014)
			BK virus	dsDNA	Inhibits infection; mechanism unknown	Dugan et al. (2008)
			HAdV A, B1,B2,C	dsDNA	Inhibits infection; direct interaction with viral particles preventing uncoating	Smith et al. (2010), Bastian and Schäfer (2001)
			CMV	dsDNA	Modest direct antiviral effect	(Daher et al. 1986)
			HIV-1	ssRNA	Direct effect on virion; blocks viral gp120–cellular CD4 interaction; blocks host PKC signalling, affecting reverser transcription and integration; increased secretion of C–C chemokines	Chang et al. (2003), Demirkhanyan et al. (2012), Furci (2006), Wang et al. (2004), Guo (2004)
			VSIV	(−)ssRNA	Direct effect on virus, inhibits infection. mechanism unknown	Daher et al. (1986)
			HCV	(+)ssRNA	HCV core protein activates HNP transcription (antiviral activity untested)	Aceti et al. (2006)
			Echovirus	(+)ssRNA	Lack of direct effect on virion	Daher et al. (1986)
			Reovirus (type 3)	dsRNA	Lack of direct effect on virion	Daher et al. (1986)
			IAV	(−)ssRNA	Inhibition of PKC pathway in host cells; blocks viral replication and protein synthesis; increased uptake and clearance by neutrophils	Salvatore et al. (2007), Tecle et al. (2007)

(continued)

Table 3.2 (continued)

Defensin	Structure	Source	Virus	Genome	Antiviral mechanism	REF
HNP-4	α-defensin	Human	HSV-2	dsDNA	Interacts with heparan sulfate on host cells; prevents viral entry	Hazrati et al. (2006)
			JCPyV	dsDNA	No antiviral effect	Zins et al. (2014)
			HIV-1	ssRNA	Lectin independent antiviral effect; weak interaction with gp120 and CD4	Wu et al. (2005)
RMAD3	Rhesus Macaque α-defensin 3	Rhesus	HIV-1	ssRNA	Moderate direct antiviral effect	Tanabe et al. (2004)
RMAD4	Rhesus Macaque α-defensin 4	Rhesus	HIV-1	ssRNA	Blocks viral entry	Tanabe et al. (2004)
HD5	α-defensin	Human	HSV-2	dsDNA	Interacts with viral gb2; prevents binding to target cell; blocks post-entry events	Hazrati et al. (2006)
			HPV	dsDNA	Blocks virus escape from endocytic vesicles; blocks virus unfolding (host mediated capsid L2 cleavage)	Yasin et al. (2004), Hazrati et al. (2006), Buck et al. (2006), Gounder et al. (2012)
			JCPyV	dsDNA	Binds to viral particles inhibiting genome release	Zins et al. (2014), Dugan et al. (2008)
			BK virus	dsDNA	Causes aggregation of viral particles, reducing viral attachment to cells	Zins et al. (2014), Dugan et al. (2008)
			HAdV A,B1,B2,C, E	dsDNA	Inhibits infection by direct interaction with viral particles preventing uncoating	Gounder et al. (2012), Smith et al. (2010)
			HIV-1	ssRNA	Enhances HIV-1 infection by increasing viral attachment; effects independent on CD4 or viral co-receptors in target cells	Klotman et al. (2008), Rapista et al. (2011)

(continued)

Table 3.2 (continued)

Defensin	Structure	Source	Virus	Genome	Antiviral mechanism	REF
			IAV	(−)ssRNA	Increased neutrophil uptake and viral clearance	Tecle et al. (2007)
HD6	α-defensin	Human	HSV-2	dsDNA	Interacts with heparansulfate on host cells; prevents viral entry	Hazrati et al. (2006)
			HPV	dsDNA	No antiviral effect	Buck et al. (2006)
			HIV-1	ssRNA	Enhances HIV-1 infection by increasing viral attachment; effects independent on CD4 or viral co-receptors In target cells	Klotman et al. (2008), Rapista et al. (2011)
NP-1	α-defensin	Rabbit	HSV-1	dsDNA	Direct effect on viral particles; prevents viral entry and VP16 translocation to the nucleus	Sinha et al. (2003)
			HSV-2	dsDNA	Direct effect on viral particles; prevents viral entry and VP16 translocation to the nucleus	Sinha et al. (2003)
			HIV-1	ssRNA	Inhibits HIV infection in transformed cell lines	Nakashima et al. (1993)
α-defensins	α-defensin	Rat	HIV-1	ssRNA	Inhibits HIV infection in transformed cell lines	Nakashima et al. (1993)
α-defensins	α-defensin	Guinea pig	HIV-1	ssRNA	Inhibits HIV infection in transformed cell lines	Nakashima et al. (1993)
Crp 3	α-defensin	Mouse	HIV-1	ssRNA	Moderate increase in HIV infectivity	Tanabe et al. (2004)
Crp 4	α-defensin	Mouse	HIV-1	ssRNA	Moderate antiviral activity	Tanabe et al. (2004)
hBD1	β-defensin	Human	HSV-2	dsDNA	No antiviral effect	Hazrati et al. (2006)
			HPV	dsDNA	No antiviral effect	Buck et al. (2006)
			VV	dsDNA	No antiviral effect	Howell et al. (2004)

(continued)

Table 3.2 (continued)

Defensin	Structure	Source	Virus	Genome	Antiviral mechanism	REF
			JCPyV	dsDNA	No antiviral effect	Zins et al. (2014)
			BK virus	dsDNA	No antiviral effect	Dugan et al. (2008)
			HAdV (Av1CF2)	dsDNA	Inhibits infection; mechanism unknown	Gropp et al. (1999)
			HIV-1	ssRNA	No antiviral effect; not upregulated by virus	Quiñones-Mateu (2003)
			IAV	(−)ssRNA	Increased virus uptake by neutrophils	Tecle et al. (2007)
			RSV	(−)ssRNA	No antiviral effect	Kota et al. (2008)
			HRV	(+)ssRNA	Not induced by virus	Duits et al. (2003)
hBD2	β-defensin	Human	HSV-2	dsDNA	No antiviral effect	Hazrati et al. (2006)
			HPV	dsDNA	No antiviral effect	Buck et al. (2006)
			VV	dsDNA	No antiviral effect	Howell et al. (2004)
			JCPyV	dsDNA	No antiviral effect	Zins et al. (2014)
			BK virus	dsDNA	Moderate inhibitory effect; mechanism unknown	Dugan et al. (2008)
			HAdV (5)	dsDNA	Moderate inhibitory effect; mechanism unknown	Bastian and Schäfer (2001)
			HIV-1	ssRNA	Inhibits both X4 and R5 infection; downregulates CXC4; inhibits early products of reverse transcription	Quiñones-Mateu et al. (2003), Sun et al. (2005)
			IAV	(−)ssRNA	Increased uptake by neutrophils	Tecle et al. (2007)
			RSV	(−)ssRNA	Increased secretion; direct effect on viral particles; disruption of the viral envelope	Kota et al. (2008)
			HRV	(+)ssRNA	Induction of expression by virus	Duits et al. (2003)

(continued)

Table 3.2 (continued)

Defensin	Structure	Source	Virus	Genome	Antiviral mechanism	REF
mBD2	β-defensin	Mouse	IAV (PR8)	(−)ssRNA	Blocks viral entry to target cells	Gong et al. (2010)
			DENV-2	(+)ssRNA	Induction of expression	Bustos-Arriaga et al. (2011)
hBD3	β-defensin	Human	HSV-2	dsDNA	Interacts with heparansulfate on host cells and also viral gb2; prevents viral binding	Hazrati et al. (2006)
			VV	dsDNA	Reduced viral DNA-dependent RNA pol expression and plaque formation	Howell et al. (2004)
			JCPyV	dsDNA	Inhibits infection; cytotoxicity towards host cells	Zins et al. (2014)
			HIV-1	ssRNA	Inhibits both X4 and R5 infection; downregulates CXC4; inhibits early products of reverse transcription	Quiñones-Mateu (2003), Sun et al. (2005)
			IAV	(−)ssRNA	Blockshemagglutinin-mediated viral fusion to host cells	Leikina et al. (2005)
			SINV	(+)ssRNA	Blocks viral fusion with target cells	Leikina et al. (2005)
mBD3	β-defensin	Mouse	IAV (PR8)	(−)ssRNA	Blocks viral entry to target cells	Jiang et al. (2012)
RC1	Inferred from human θ defensin genes	Human	HSV-2	dsDNA	Blocks viral attachment; reduced nuclear translocation of VP16	Yasin et al. (2004)
		Human	HSV-1	dsDNA	Direct effect on viral particles; mechanism unknown	Yasin et al. (2004)
		Human	HIV-1	ssRNA	Blocks viral fusion; binds to viral gp41 and glycosylated gp120 and cellular CD4	Cole et al. (2002), Gallo et al. (2006), Wang et al. (2004)
		Human	HIV-2	ssRNA	Modest inhibition of viral fusion	Gallo et al. (2006)

(continued)

Table 3.2 (continued)

Defensin	Structure	Source	Virus	Genome	Antiviral mechanism	REF
		Human	SIV	ssRNA	Modest inhibition of viral fusion	Gallo et al. (2006)
		Human	DENV-2	(+)ssRNA	Reduces replication by inhibiting viral NS2B-NS3 serine protease	Rothan et al. (2012)
RC2	Inferred from human θ defensin genes	Human	HSV-2	dsDNA	Blocks viral attachment; reduced nuclear translocation of VP16	Yasin et al. (2004)
			HSV-1	dsDNA	Direct effect on viral particles; mechanism unknown	Yasin et al. (2004)
			Baculovirus	dsDNA	Blocks viral fusion with target cells, avoids syncytium formation	Leikina et al. (2005)
			HIV-1	ssRNA	Blocks viral fusion; binds to viral glycosylated gp120 and cellular CD4	Wang et al. (2004), Cole et al. (2002)
			IAV	(–)ssRNA	Prevents hemagglutinin-mediated viral fusion with target cells	Leikina et al. (2005)
			SINV	(+)ssRNA	Prevents viral fusion with target cells	Leikina et al. (2005)
RTD1	Rhesus θ defensin	Rhesus	HSV-2	dsDNA	No antiviral effect	Yasin et al. (2004)
			HSV-1	dsDNA	No antiviral effect	Yasin et al. (2004)
			HIV-1 (various isolates)	ssRNA	Moderate direct antiviral effect; mechanism unknown	Wang et al. (2004)
			SARScoV	(+)ssRNA	Immunomodulatory effect; reduction in lung cytokine secretion	Wohlford-Lenane et al. (2009)

(continued)

Table 3.2 (continued)

Defensin	Structure	Source	Virus	Genome	Antiviral mechanism	REF
RTD2	Rhesus θ defensin	Rhesus	HSV-2	dsDNA	Moderate antiviral effect; mechanism unknown	Yasin et al. (2004)
			HSV-1	dsDNA	No antiviral effect	Yasin et al. (2004)
			HIV-1 (various isolates)	ssRNA	Moderate direct antiviral effect; mechanism unknown	Wang et al. (2004)
RTD3	Rhesus θ defensin	Rhesus	HSV-2	dsDNA	Direct effect on viral particles; cytotoxicity towards host cell	Yasin et al. (2004)
			HSV-1	dsDNA	Direct effect on viral particles; mechanism unknown	Yasin et al. (2004)
			HIV-1 (various isolates)	ssRNA	Moderate direct antiviral effect; mechanism unknown	Wang et al. (2004)

an immature form into a mature active form. Interestingly the immature (MK) form of the cecropin-like peptide was more active against all four strains of Dengue virus tested (Dengue-1, -2, -3 and -4) than the mature (GK) cleaved form. The authors also established that both the GK and MK forms of the peptide were active against Chikungunya virus, a pathogen also transmitted by the *A. aegypti* mosquito that can cause fever and severe long lasting joint pain. Another study revealed that scorpine, a hybrid peptide with structural similarities to cecropins and defensins derived from the venom of the scorpion *Pandinus imperator*, also exhibited antiviral activity against dengue virus by inhibiting the replication of Dengue-2 virus (Carballar-Lejarazu et al. 2008).

Cecropin A is reported to inhibit HIV production by infected T-cells and fibroblasts in a dose-dependent manner (Wachinger et al. 1998). The mechanism responsible for this inhibition was found to be reduced viral gene expression and synthesis of viral products, indicating that Cecropin has an antiviral role beyond that of direct interaction with the HIV virion.

Cecropins have also been shown to have antiviral activity at early and late points in the viral infection and replication cycle. For example, both cecropin P1 and cecropin D have been shown to have antiviral activity at several stages in infection with porcine reproductive and respiratory syndrome virus (PRRSV), an infection which can have substantial financial impact in the pig industry. Cecropin P1 is a peptide initially thought to be produced in the porcine intestine (Lee et al. 1989), but was later revealed to have been produced by the nematode, *Ascarissuum*, which survives in the porcine gut (Andersson et al. 2003). This peptide was shown to block attachment and replication of the virus in kidney and alveolar macrophage cell lines, and also reduced the number of infectious viral particles produced after infection in vitro (Guo et al. 2014). The peptide was also shown to have immunomodulatory properties by preventing the onset of PRRSV-induced apoptosis. Similarly, cecropin D was recently shown to inhibit PRRSV attachment and replication, and also attenuated apoptosis induced by the virus (Liu et al. 2015).

The broad spectrum antiviral activity of cecropins is further evidenced by a study demonstrating that cecropin A could inhibit replication of Junin virus, the cause of Argentine hemorrhagic fever (Albiol Matanic and Castilla 2004). The peptide inhibited Junin virus-related protein production in host cells, but the authors suggested that the antiviral effects were predominantly limited to later stages in the virus replication cycle, since the peptide did not appear to alter viral infectivity. Interestingly, the same study also demonstrated that cecropin A did not exhibit any inhibitory activity against herpes simplex virus types 1 and 2, suggesting that any antiviral activity exhibited by these peptides is pathogen specific. It should be noted that all HSV-1 and HSV-2 contain a DNA genome, in contrast to HIV-1, Junin, PRRSV and Dengue, which are RNA viruses, and thus the activity of cecropins against other DNA viruses remains to be understood.

3.5.2 *Dermaseptin*

There have been a substantial number (~1000) Host Defence Peptides identified from amphibian skin, of which Dermaseptins are a superfamily. These host defence peptides have a cationic charge due to an abundance of lysine residues, and tend to be between 27–34 amino acids in size (Amiche et al. 1994). They have been found in several species of frogs, including the Hylidae (tree frogs) and the Ranidae (true frogs) (Nicolas and El Amri 2009). Dermaseptins have been demonstrated to have antimicrobial activity against a wide range of bacterial pathogens involved in human diseases including *Pseudomonas*, *Salmonella*, *Staphylococcus*, *Escherichia* and *Enterobacter* species. Interestingly, the peptide Dermaseptin S1, a 34-residue peptide isolated from the frog genus *Phyllomedusa*, was shown to have immunomodulatory activity via an ability to stimulate the production of reactive oxygen species and myeloperoxidase by primary human neutrophils (Ammar et al. 1998). Dermaseptin S2 has also been touted as a potential therapeutic molecule in the treatment of cancer in a recent study demonstrating antitumor and angiostatic activity in a range of tumour cell types, likely due to the induction of necrosis (van Zoggel et al. 2012).

In terms of their antiviral activity, dermaseptins have been shown to be highly effective against a broad range of viral pathogens, with activity that affects several steps in the infection and replication process. Dermaseptin-1 was initially identified to have antiviral activity against two viruses that are known to infect ectothermic animals; Frog virus 3 from the family *Iridoviridae*, and Channel catfish virus, a member of the *Herpesviridae* family (Chinchar et al. 2004). This study provided indication that dermaseptin peptides possessed antiviral activity and subsequent studies were extended to examine the activity against human viral pathogens.

Dermaseptins S1-S5 shown to have direct and varying antiviral activity against Herpes Simplex Virus-1 in vitro (Belaid et al. 2002). This study identified Dermaseptin S4 as having the most potent antiviral activity, but only at very early stages in the viral infection process as experiments suggested that it exerted an effect when exposed to the virus prior to infection or during viral attachment. A later study further evaluated the activity of Dermaseptin S1 and derivatives against Herpes Simplex Virus, demonstrating that the antiviral activity of the parent peptide could be increased by alteration of the original sequence (Savoia et al. 2010). Interestingly, the same study also showed that some of the derivatives also exhibited activity against Papillomavirus Psv-16 in vitro, but with very low cytotoxicity towards the host cells. A recent study by Bergaoui et al. also demonstrated that Dermaseptin S4 and synthetic derivatives exhibited activity against Herpes Simplex Virus-2 with reduced cytotoxicity, although in vivo efficacy remains to be determined (Bergaoui et al. 2013).

Other studies have revealed a possible role for Dermaseptins in the treatment of HIV-1 infection. Dermaseptins have been shown to partially inhibit HIV virus infection of T-cells although cytotoxicity was observed at higher concentrations (VanCompernolle et al. 2005). Another study by Lorin et al. identified Dermaseptin

S4 as having potential inhibitory activity against HIV-1 by inhibiting viral infection of human primary T-lymphocytes (Lorin et al. 2005). The authors attributed this activity to a direct disruption of the virion and, while the parent peptide elicited cytotoxicity at higher concentrations, were able to reduce host cell cytotoxicity by reducing the positive charge of the native peptide through amino acid deletion or substitution. Notably, the modified peptides were also able to reduce HIV-1 binding to endometrial cells together with inhibition of capture and transmission of virus from dendritic cells to $CD4^+$ T-cells. Dermaseptin S9 was also identified as having weak activity against HIV-1, but a mutant S9 peptide, where three lysine residues were replaced with arginines, exhibited potent inhibitory activity against HIV-1 although the mechanism underlying this observation remains unclear (Wang et al. 2010).

3.5.3 Magainin

Magainins are cationic host defence peptides that were originally identified in the skin and granular secretions of *Xenopuslaevis* (the African clawed frog) (Zasloff 1987; Giovannini et al. 1987), but have also been identified as inducible peptides in other species in the *Xenopus* genus; *X. borealis, X. clivii, X. muelleri, X. petersii, X. amieti* and *X. andrei* (Conlon et al. 2012). These peptides are 23–34 amino acids in length and the native peptides, together with synthetic derivatives, have been demonstrated to have broad antimicrobial activity against a range of bacterial pathogens (Zairi et al. 2009; Chen et al. 1988). There is, however, a limited amount of information on the activity of magainins against viral pathogens. One study has assessed the activity of a number of synthetic magainin derivatives against Herpes Simplex-1 virus, establishing that several peptide derivatives have the capacity to reduce viral plaque formation in in vitro assays (Egal et al. 1999). Further evidence of antiviral activity of magainins was also revealed in a study by Chinchar et al. (2004) which showed magainin II, but not magainin I, was able to reduce the infectivity of channel catfish virus (CCV) (Chinchar et al. 2004). However, both magainins exhibited less activity against frog virus 3, indicating that the activity was likely virus specific.

3.5.4 Melittin

Melittin is an alpha helical peptide that is 26 amino acids in length and was initially identified in bee venom (Habermann and Jentsch 1967) and later characterized as an amphipathic peptide with broad ranging antimicrobial activity (Terwilliger and Eisenberg 1982; Wade et al. 1992). The native melittin peptide and synthetic analogues have been investigated in a number of studies as potential novel antimicrobial therapeutics, but their well-characterized antiviral activity against a number of pathogens is of particular interest.

One of the first studies to assess the antiviral activity of melittin was published Wachinger et al. (1992) which identified that melittin and six derivatives reduced HIV-1 replication in host cells (Wachinger et al. 1992). A subsequent study revealed that the mechanism underlying this inhibition was that melittin was capable of specifically reducing HIV-associated gene expression in the host cell while not affecting the overall gene expression profile (Wachinger et al. 1998). This suggests an important immunomodulatory role for this peptide, rather than direct antiviral activity, as potentially contributing to the treatment of HIV infection.

Several studies have also characterized the influence of melittin, and synthetic derivatives upon the establishment and progression of Herpes Simplex Virus infection. Baghian et al. (1997) synthesized a number of derivatives based upon the original melittin structure to assess their antiviral activity against HSV-1 virus. One such peptide was called Hecate, which has an altered amino sequence that changes the distribution of charged residues within the peptide without altering the overall amphipathic α-helical structure (Baghian et al. 1997). The authors determined that Hecate prevented HSV-1 plaque formation and prevented virus spread in in vitro models while some synthetic melittin analogues were unable to do so, suggesting that sequence played an important role in the antiviral activity of the peptide. A later study screened a number of known CHDP against HSV-1 and HSV-2 and established that many of the α-helical peptides screened (including magainins, cecropins and cathelicidin) did not display activity against the viruses (Yasin et al. 2000). However, melittin did have substantial activity against both HSV-1 and HSV-2, although the mechanism underlying this observation was not fully described.

More recently, it has been demonstrated that the native melittin peptide showed antiviral activity towards Junin virus (Albiol Matanic and Castilla 2004), and that synthetic melittin analogues showed activity towards Tobacco Mosaic Virus (Marcos et al. 1995). Interestingly, a recent study by Falco et al. (2013) used melittin-loaded liposomes to specifically target fish viral hemorrhagic septicemia rhabdovirus (VHSV) (Falco et al. 2013). The authors coated the immunoliposomes with antibodies targeting the surface G glycoprotein of VHSV, and showed a reduction in infectivity of greater than 95 %. This approach provides an exciting avenue for the targeted delivery of antiviral host defence peptides whilst minimizing cytotoxic damage to host cells.

3.5.5 Tachyplesin and Polyphemusin

Tachyplesin is a 17 amino acid cationic β-sheet peptide that was originally identified in the hemocytes of *Tachypleustridentatus* (Horseshoe crab) and was demonstrated to have broad spectrum antimicrobial activity (Nakamura et al. 1988). Polyphemusin I is a 18 amino acid peptide identified in the hemocytes of the American horseshoe crab, *Limulus polyphemus*. Both peptides are thought to play a key role in the innate immune system of the crab, and have been shown to have potent LPS binding and neutralization activity (Nakamura et al. 1988; Powers et al. 2006).

The antiviral activity of Tachyplesin against HIV was investigated by Morimoto et al. (1991) who showed that the peptide could reduce virus-mediated cytopathic effects by more than 70 % in in vitro models (Morimoto et al. 1991). The peptide also reduced the infectivity of the virus, an effect that was shown to be independent on the reverse transcriptase activity of HIV. Interestingly, subsequent studies by Nakashima et al. (1992), Murakami et al. (1997) and Xu et al. (1999) characterized the activities of the isopeptideT22 ($Tyr^{5,12}$, Lys^{7}-polyphemusin II), and synthetic analogues against HIV, and determined that the antiviral activity was due to ability of the peptide to bind to CXCR4 (Xu et al. 1999; Murakami et al. 1997; Nakashima et al. 1992). CXCR4 is a chemokine receptor used by T-cell tropic strains of HIV to infect host cells. Thus, the host cell mediated mechanism of action of these peptides against HIV contrasts to the direct antiviral activity exhibited by other CHDP.

Tachplesin peptides have also been demonstrated to have activity against other viruses that affect humans. It was shown that Tachyplesin peptides were able to inactivate Vesicular stomatitis virus (VSV; also known as vesicular stomatitis Indiana virus), a zoonotic member of the family *Rhabdoviridae* that can infect cattle and cause disease in humans (Murakami et al. 1991). The same study also identified that Influenza A (H1N1) was also moderately susceptible, although HSV-1 and -2, adenovirus-1, reovirus-2 and poliovirus-1 were not susceptible to the antiviral activities of the peptide. However, a subsequent study, which examined the antiviral activity of tachyplesin against HSV-1 and HSV-2 using an in vitro cytotoxicity model, did suggest that the peptide offered a moderate degree of protection against both virus strains (Yasin et al. 2000).

3.6 Conclusion

Collectively, the experimental studies presented here highlight the crucial role that CHDP play in the innate immune response across a wide variety of cell types and species. These peptides possess powerful antiviral activity, and can modulate the cellular immune response to provide a key defence mechanism infection and pathology associated with a myriad of viruses.

While we are beginning to understand some of the underlying mechanisms through which cathelicidins, defensins and other CHDP mediate their antiviral effects; research into the activities of these peptides is still in relative infancy. It is clear, however, that CHDP have huge therapeutic potential as exogenous peptides, and for the development of powerful synthetic analogues that can be directed towards specific viral pathogens.

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References

Aceti A, Mangoni ML, Pasquazzi C, Fiocco D, Marangi M, Miele R et al (2006) Alpha-defensin increase in peripheral blood mononuclear cells from patients with hepatitis C virus chronic infection. J Viral Hepatitis 13:821–827

Agerberth B, Charo J, Werr J, Olsson B, Idali F, Lindbom L et al (2000) The human antimicrobial and chemotactic peptides LL-37 and alpha-defensins are expressed by specific lymphocyte and monocyte populations. Blood 96(9):3086–3093

Airenne KJ, Hu Y-C, Kost TA, Smith RH, Kotin RM, Ono C et al (2013) Baculovirus: an insect-derived vector for diverse gene transfer applications. Mol Ther: J Am Soc Gene Ther 21:739–749

Albiol Matanic VC, Castilla V (2004) Antiviral activity of antimicrobial cationic peptides against Junin virus and herpes simplex virus. Int J Antimicrob Agents 23(4):382–389

Amiche M, Ducancel F, Mor A, Boulain JC, Menez A, Nicolas P (1994) Precursors of vertebrate peptide antibiotics dermaseptin b and adenoregulin have extensive sequence identities with precursors of opioid peptides dermorphin, dermenkephalin, and deltorphins. J Biol Chem 269 (27):17847–17852

Ammar B, Perianin A, Mor A, Sarfati G, Tissot M, Nicolas P et al (1998) Dermaseptin, a peptide antibiotic, stimulates microbicidal activities of polymorphonuclear leukocytes. Biochem Biophys Res Commun 247(3):870–875

Andersson M, Boman A, Boman HG (2003) Ascaris nematodes from pig and human make three antibacterial peptides: isolation of cecropin P1 and two ASABF peptides. Cellular and molecular life sciences: CMLS 60(3):599–606

Baghian A, Jaynes J, Enright F, Kousoulas KG (1997) An amphipathic alpha-helical synthetic peptide analogue of melittin inhibits herpes simplex virus-1 (HSV-1)-induced cell fusion and virus spread. Peptides 18(2):177–183

Barlow PG, Li Y, Wilkinson TS, Bowdish DM, Lau YE, Cosseau C et al (2006) The human cationic host defense peptide LL-37 mediates contrasting effects on apoptotic pathways in different primary cells of the innate immune system. J Leukoc Biol 80(3):509–520

Barlow PG, Svoboda P, Mackellar A, Nash AA, York IA, Pohl J et al (2011) Antiviral activity and increased host defense against influenza infection elicited by the human cathelicidin LL-37. PLoS One 6(10):e25333

Barlow PG, Findlay EG, Currie SM, Davidson DJ (2014) Antiviral potential of cathelicidins. Future Microbiol 9(1):55–73

Bastian A, Schäfer H (2001) Human α-defensin 1 (HNP-1) inhibits adenoviral infection in vitro. Regul Pept 101:157–161

Becker S, Quay J, Soukup J (1991) Cytokine (tumor necrosis factor, IL-6, and IL-8) production by respiratory syncytial virus-infected human alveolar macrophages. Journal of Immunology 147:4307–4312 (Baltimore, Md: 1950)

Belaid A, Aouni M, Khelifa R, Trabelsi A, Jemmali M, Hani K (2002) In vitro antiviral activity of dermaseptins against herpes simplex virus type 1. J Med Virol 66(2):229–234

Benincasa M, Skerlavaj B, Gennaro R, Pellegrini A, Zanetti M (2003) In vitro and in vivo antimicrobial activity of two alpha-helical cathelicidin peptides and of their synthetic analogs. Peptides 24(11):1723–1731

Bergaoui I, Zairi A, Tangy F, Aouni M, Selmi B, Hani K (2013) In vitro antiviral activity of dermaseptin S(4) and derivatives from amphibian skin against herpes simplex virus type 2. J Med Virol 85(2):272–281

Bergman P, Walter-Jallow L, Broliden K, Agerberth B, Soderlund J (2007) The antimicrobial peptide LL-37 inhibits HIV-1 replication. Curr HIV Res 5(4):410–415

Boman HG (1991) Antibacterial peptides: key components needed in immunity. Cell 65(2):205–207

Buck CB, Day PM, Thompson CD, Lubkowski J, Lu W, Lowy DR et al (2006) Human-defensins block papillomavirus infection. Proc Natl Acad Sci 103:1516–1521

Bulet P, Stocklin R (2005) Insect antimicrobial peptides: structures, properties and gene regulation. Protein Pept Lett 12(1):3–11

Bustos-Arriaga J, García-Machorro J, León-Juárez M, García-Cordero J, Santos-Argumedo L, Flores-Romo L et al (2011) Activation of the innate immune response against DENV in normal non-transformed human fibroblasts. PLoS Negl Trop Dis 5:e1420

Carballar-Lejarazu R, Rodriguez MH, de la Cruz Hernandez-Hernandez F, Ramos-Castaneda J, Possani LD, Zurita-Ortega M et al (2008) Recombinant scorpine: a multifunctional antimicrobial peptide with activity against different pathogens. Cellular and molecular life sciences: CMLS 65(19):3081–3092

Chang TL-Y, Francois F, Mosoian A, Klotman ME (2003) CAF-mediated human immunodeficiency virus (HIV) type 1 transcriptional inhibition is distinct from -defensin-1 HIV inhibition. J virol 77:6777–6784

Chang TL, Vargas J, DelPortillo A, Klotman ME (2005) Dual role of α-defensin-1 in anti–HIV-1 innate immunity. J Clin Invest 115:765–773

Chen HC, Brown JH, Morell JL, Huang CM (1988) Synthetic magainin analogues with improved antimicrobial activity. FEBS Lett 236(2):462–466

Chinchar VG, Bryan L, Silphadaung U, Noga E, Wade D, Rollins-Smith L (2004) Inactivation of viruses infecting ectothermic animals by amphibian and piscine antimicrobial peptides. Virology 323(2):268–275

Cole AM, Hong T, Boo LM, Nguyen T, Zhao C, Bristol G et al (2002) Retrocyclin: a primate peptide that protects cells from infection by T- and M-tropic strains of HIV-1. Proc Natl Acad Sci USA 99:1813–1818

Conlon JM, Mechkarska M, King JD (2012) Host-defense peptides in skin secretions of African clawed frogs (Xenopodinae, Pipidae). Gen Comp Endocrinol 176(3):513–518

Currie SM, Findlay EG, McHugh BJ, Mackellar A, Man T, Macmillan D et al (2013) The human cathelicidin LL-37 has antiviral activity against respiratory syncytial virus. PLoS One 8(8): e73659

Daher KA, Selsted ME, Lehrer RI (1986) Direct inactivation of viruses by human granulocyte defensins. J Virol 60(3):1068–1074

De Y, Chen Q, Schmidt AP, Anderson GM, Wang JM, Wooters J et al (2000) LL-37, the neutrophil granule- and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (FPRL1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells. J Exp Med 192(7):1069–1074

Dean RE, O'Brien LM, Thwaite JE, Fox MA, Atkins H, Ulaeto DO (2010) A carpet-based mechanism for direct antimicrobial peptide activity against vaccinia virus membranes. Peptides 31(11):1966–1972

Demirkhanyan LH, Marin M, Padilla-Parra S, Zhan C, Miyauchi K, Jean-Baptiste M et al (2012) Multifaceted mechanisms of HIV-1 entry inhibition by human α-defensin. J Biol Chem 287:28821–28838

Ding J, Chou Y-Y, Chang TL (2009) Defensins in viral infections. J Innate Immun 1:413–420

Dugan AS, Maginnis MS, Jordan JA, Gasparovic ML, Manley K, Page R et al (2008) Human -defensins inhibit BK virus infection by aggregating virions and blocking binding to host cells. J Biol Chem 283:31125–31132

Duits LA, Nibbering PH, van Strijen E, Vos JB, Mannesse-Lazeroms SPG, van Sterkenburg MAJA et al (2003) Rhinovirus increases human beta-defensin-2 and -3 mRNA expression in cultured bronchial epithelial cells. FEMS Immunol Med Microbiol 38:59–64

Egal M, Conrad M, MacDonald DL, Maloy WL, Motley M, Genco CA (1999) Antiviral effects of synthetic membrane-active peptides on herpes simplex virus, type 1. Int J Antimicrob Agents 13(1):57–60

Falco A, Barrajon-Catalan E, Menendez-Gutierrez MP, Coll J, Micol V, Estepa A (2013) Melittin-loaded immunoliposomes against viral surface proteins, a new approach to antiviral therapy. Antiviral Res 97(2):218–221

Fields AP, Bednarik DP, Hess A, Stratford May W (1988) Human immunodeficiency virus induces phosphorylation of its cell surface receptor. Nature 333:278–280

Furci L, Sironi F, Tolazzi M, Vassena L, Lusso P (2006) -defensins block the early steps of HIV-1 infection: interference with the binding of gp120 to CD4. Blood

Furci L, Tolazzi M, Sironi F, Vassena L, Lusso P (2012) Inhibition of HIV-1 infection by human α-defensin-5, a natural antimicrobial peptide expressed in the genital and intestinal mucosae. PLoS One 7:e45208

Galanis E, Markovic SN, Suman VJ, Nuovo GJ, Vile RG, Kottke TJ et al (2012) Phase II trial of intravenous administration of reolysin® (reovirus serotype-3-dearing strain) in patients with metastatic melanoma. Mol Ther 20:1998–2003

Gallo RL, Kim KJ, Bernfield M, Kozak CA, Zanetti M, Merluzzi L et al (1997) Identification of CRAMP, a cathelin-related antimicrobial peptide expressed in the embryonic and adult mouse. J Biol Chem 272(20):13088–13093

Gallo SA, Wang W, Rawat SS, Jung G, Waring AJ, Cole AM et al (2006) Theta-defensins prevent HIV-1 Env-mediated fusion by binding gp41 and blocking 6-helix bundle formation. J Biol Chem 281:18787–18792

Ganz T (2003) Defensins: antimicrobial peptides of innate immunity. Nat Rev Immunol 3:710–720

Gardner SD (1973) Prevalence in England of antibody to human polyomavirus (B.K.). BMJ 1:77–78

Gardner SD, Field AM, Coleman DV, Hulme B (1971) New human papovavirus (B.K.) isolated from urine after renal transplantation. Lancet (London, England) 1:1253–7

Giovannini MG, Poulter L, Gibson BW, Williams DH (1987) Biosynthesis and degradation of peptides derived from Xenopus laevis prohormones. Biochem J 243(1):113–120

Gong T, Jiang Y, Wang Y, Yang D, Li W, Zhang Q et al (2010) Recombinant mouse beta-defensin 2 inhibits infection by influenza A virus by blocking its entry. Arch Virol 155:491–498

Gordon YJ, Huang LC, Romanowski EG, Yates KA, Proske RJ, McDermott AM (2005) Human cathelicidin (LL-37), a multifunctional peptide, is expressed by ocular surface epithelia and has potent antibacterial and antiviral activity. Curr Eye Res 30(5):385–394

Gounder AP, Wiens ME, Wilson SS, Lu W, Smith JG (2012) Critical determinants of human - defensin 5 activity against non-enveloped viruses. J Biol Chem 287:24554–24562

Gropp R, Frye M, Wagner TOF, Bargon J (1999) Epithelial defensins impair adenoviral infection: implication for adenovirus-mediated gene therapy. Hum Gene Ther 10:957–964

Guo C-J, Tan N, Song L, Douglas SD, Ho W-Z (2004) Alpha-defensins inhibit HIV infection of macrophages through upregulation of CC-chemokines. AIDS (London, England) 18:1217–1218

Guo C, Huang Y, Cong P, Liu X, Chen Y, He Z (2014) Cecropin P1 inhibits porcine reproductive and respiratory syndrome virus by blocking attachment. BMC Microbiol 14:273

Habermann E, Jentsch J (1967) Sequence analysis of melittin from tryptic and peptic degradation products. Hoppe Seylers Z Physiol Chem 348(1):37–50

Hazrati E, Galen B, Lu W, Wang W, Ouyang Y, Keller MJ et al (2006) Human - and -defensins block multiple steps in herpes simplex virus infection. J Immunol 177:8658–8666

Howell MD, Jones JF, Kisich KO, Streib JE, Gallo RL, Leung DY (2004) Selective killing of vaccinia virus by LL-37: implications for eczema vaccinatum. J Immunol 172(3):1763–1767

Howell MD, Gallo RL, Boguniewicz M, Jones JF, Wong C, Streib JE et al (2006) Cytokine milieu of atopic dermatitis skin subverts the innate immune response to vaccinia virus. Immunity 24 (3):341–348

Howell MD, Streib JE, Leung DYM (2007) Antiviral activity of human β-defensin 3 against vaccinia virus. J Allergy Clin Immunol 119:1022–1025

Huang LC, Reins RY, Gallo RL, McDermott AM (2007) Cathelicidin-deficient (Cnlp -/-) mice show increased susceptibility to Pseudomonas aeruginosa keratitis. Invest Ophthalmol Vis Sci 48(10):4498–4508

Hurt AC (2014) The epidemiology and spread of drug resistant human influenza viruses. Curr Opin Virol 8:22–29

Huygens A, Dauby N, Vermijlen D, Marchant A (2014) Immunity to cytomegalovirus in early life. Front Immunol 5:552

Iimura M, Gallo RL, Hase K, Miyamoto Y, Eckmann L, Kagnoff MF (2005) Cathelicidin mediates innate intestinal defense against colonization with epithelial adherent bacterial pathogens. J Immunol 174(8):4901–4907

Jartti T, Korppi M (2011) Rhinovirus-induced bronchiolitis and asthma development. Pediatr Allergy Immunol: Eur Soc Pediatr Allergy Immunol 22(4):350–355

Jiang Y, Yang D, Li W, Wang B, Jiang Z, Li M (2012) Antiviral activity of recombinant mouse β-defensin 3 against influenza A virus in vitro and in vivo. Antiviral Chem Chemother 22:255–262

Klotman ME, Rapista A, Teleshova N, Micsenyi A, Jarvis GA, Lu W et al (2008) Neisseria gonorrhoeae-induced human defensins 5 and 6 increase HIV infectivity: role in enhanced transmission. J Immunol 180(9):6176–6185

Kota S, Sabbah A, Chang TH, Harnack R, Xiang Y, Meng X et al (2008) Role of human beta-defensin-2 during tumor necrosis factor-alpha/NF-kappaB-mediated innate antiviral response against human respiratory syncytial virus. J Biol Chem 283:22417–22429

Kovach MA, Ballinger MN, Newstead MW, Zeng X, Bhan U, Yu FS et al (2012) Cathelicidin-related antimicrobial peptide is required for effective lung mucosal immunity in gram-negative bacterial pneumonia. J Immunol 189(1):304–311

Kuzmin IV, Novella IS, Dietzgen RG, Padhi A, Rupprecht CE (2009) The rhabdoviruses: biodiversity, phylogenetics, and evolution. Infect Genet Evol 9:541–553

Lee JY, Boman A, Sun CX, Andersson M, Jornvall H, Mutt V et al (1989) Antibacterial peptides from pig intestine: isolation of a mammalian cecropin. Proc Natl Acad Sci USA 86 (23):9159–9162

Lee Y-S, Chen C-H, Chao A, Chen E-S, Wei M-L, Chen L-K et al (2005) Molecular signature of clinical severity in recovering patients with severe acute respiratory syndrome coronavirus (SARS-CoV). BMC Genom 6:132

Lee CJ, Buznyk O, Kuffova L, Rajendran V, Forrester JV, Phopase J et al (2014) Cathelicidin LL-37 and HSV-1 corneal infection: peptide versus gene therapy. Transl Vis Sci Technol 3 (3):4

Lehrer RI (2004) Primate defensins. Nat Rev Microbiol 2(9):727–738

Leikina E, Delanoe-Ayari H, Melikov K, Cho M-S, Chen A, Waring AJ et al (2005) Carbohydrate-binding molecules inhibit viral fusion and entry by crosslinking membrane glycoproteins. Nat Immunol 6:995–1001

Levinson P, Kaul R, Kimani J, Ngugi E, Moses S, MacDonald KS et al (2009) Levels of innate immune factors in genital fluids: association of alpha defensins and LL-37 with genital infections and increased HIV acquisition. AIDS 23(3):309–317

Levinson P, Choi RY, Cole AL, Hirbod T, Rhedin S, Payne B et al (2012) HIV-neutralizing activity of cationic polypeptides in cervicovaginal secretions of women in HIV-serodiscordant relationships. PLoS One 7(2):e31996

Lion T (2014) Adenovirus infections in immunocompetent and immunocompromised patients. Clin Microbiol Rev 27:441–462

Liu X, Guo C, Huang Y, Zhang X, Chen Y (2015) Inhibition of porcine reproductive and respiratory syndrome virus by Cecropin D in vitro. Infect Genet Evol. 34:7–16

Lorin C, Saidi H, Belaid A, Zairi A, Baleux F, Hocini H et al (2005) The antimicrobial peptide dermaseptin S4 inhibits HIV-1 infectivity in vitro. Virology 334(2):264–275

Luplertlop N, Surasombatpattana P, Patramool S, Dumas E, Wasinpiyamongkol L, Saune L et al (2011) Induction of a peptide with activity against a broad spectrum of pathogens in the Aedes aegypti salivary gland, following Infection with Dengue Virus. PLoS Pathog 7(1):e1001252

Maheshwari A, Ray S, Thuluvath PJ (2008) Acute hepatitis C. The Lancet 372:321–332

Mallia P, Footitt J, Sotero R, Jepson A, Contoli M, Trujillo-Torralbo M-B et al (2012) Rhinovirus infection induces degradation of antimicrobial peptides and secondary bacterial infection in chronic obstructive pulmonary disease. Am J Respir Crit Care Med 186:1117–1124

Malm J, Sorensen O, Persson T, Frohm-Nilsson M, Johansson B, Bjartell A et al (2000) The human cationic antimicrobial protein (hCAP-18) is expressed in the epithelium of human epididymis, is present in seminal plasma at high concentrations, and is attached to spermatozoa. Infect Immun 68(7):4297–4302

Marcos JF, Beachy RN, Houghten RA, Blondelle SE, Perez-Paya E (1995) Inhibition of a plant virus infection by analogs of melittin. Proc Natl Acad Sci USA 92(26):12466–12469

Morimoto M, Mori H, Otake T, Ueba N, Kunita N, Niwa M et al (1991) Inhibitory effect of tachyplesin I on the proliferation of human immunodeficiency virus in vitro. Chemotherapy 37(3):206–211

Murakami T, Niwa M, Tokunaga F, Miyata T, Iwanaga S (1991) Direct virus inactivation of tachyplesin I and its isopeptides from horseshoe crab hemocytes. Chemotherapy 37(5):327–334

Murakami T, Nakajima T, Koyanagi Y, Tachibana K, Fujii N, Tamamura H et al (1997) A small molecule CXCR4 inhibitor that blocks T cell line-tropic HIV-1 infection. J Exp Med 186 (8):1389–1393

Nair H, Nokes DJ, Gessner BD, Dherani M, Madhi SA, Singleton RJ et al (2010) Global burden of acute lower respiratory infections due to respiratory syncytial virus in young children: a systematic review and meta-analysis. Lancet 375(9725):1545–1555

Nakamura T, Furunaka H, Miyata T, Tokunaga F, Muta T, Iwanaga S et al (1988) Tachyplesin, a class of antimicrobial peptide from the hemocytes of the horseshoe crab (Tachypleus tridentatus). Isolation and chemical structure. J Biol Chem 263(32):16709–16713

Nakashima H, Masuda M, Murakami T, Koyanagi Y, Matsumoto A, Fujii N et al (1992) Anti-human immunodeficiency virus activity of a novel synthetic peptide, T22 ([Tyr-5,12, Lys-7]polyphemusin II): a possible inhibitor of virus-cell fusion. Antimicrob Agents Chemother 36(6):1249–1255

Nakashima H, Yamamoto N, Masuda M, Fujii N (1993) Defensins inhibit HIV replication in vitro. AIDS (London, England) 7:1129

Nguyen EK, Nemerow GR, Smith JG (2010) Direct evidence from single-cell analysis that human -defensins block adenovirus uncoating to neutralize infection. J Virol 84:4041–4049

Nicolas P, El Amri C (2009) The dermaseptin superfamily: a gene-based combinatorial library of antimicrobial peptides. Biochim Biophys Acta 1788(8):1537–1550

Nitsch-Osuch A, Brydak LB (2014) Influenza viruses resistant to neuraminidase inhibitors. Acta Biochim Pol 61(3):505–508

Ong PY, Ohtake T, Brandt C, Strickland I, Boguniewicz M, Ganz T et al (2002) Endogenous antimicrobial peptides and skin infections in atopic dermatitis. N Engl J Med 347 (15):1151–1160

Ouellette AJ (2006) Paneth cell alpha-defensin synthesis and function. Curr Top Microbiol Immunol 306:1–25

Peiris JSM, Yuen KY, Osterhaus ADME, Stöhr K (2003) The severe acute respiratory syndrome. N Engl J Med 349:2431–2441

Piot P, Quinn TC (2013) Response to the AIDS pandemic—a global health model. N Engl J Med 368:2210–2218

Powers JP, Martin MM, Goosney DL, Hancock RE (2006) The antimicrobial peptide polyphemusin localizes to the cytoplasm of Escherichia coli following treatment. Antimicrob Agents Chemother 50(4):1522–1524

Pribul PK, Harker J, Wang B, Wang H, Tregoning JS, Schwarze J et al (2008) Alveolar macrophages are a major determinant of early responses to viral lung infection but do not influence subsequent disease development. J Virol 82(9):4441–4448

Proud D, Sanders SP, Wiehler S (2004) Human rhinovirus infection induces airway epithelial cell production of human beta-defensin 2 both in vitro and in vivo. J Immunol 172:4637–4645 (Baltimore, Md: 1950)

Putsep K, Carlsson G, Boman HG, Andersson M (2002) Deficiency of antibacterial peptides in patients with morbus Kostmann: an observation study. Lancet 360(9340):1144–1149

Quiñones-Mateu ME, Lederman MM, Feng Z, Chakraborty B, Weber J, Rangel HR et al (2003) Human epithelial beta-defensins 2 and 3 inhibit HIV-1 replication. AIDS (London, England) 17:F39–48

Rapista A, Ding J, Benito B, Lo Y-T, Neiditch MB, Lu W et al (2011) Human defensins 5 and 6 enhance HIV-1 infectivity through promoting HIV attachment. Retrovirology 8:45

Rehaume LM, Hancock RE (2008) Neutrophil-derived defensins as modulators of innate immune function. Crit Rev Immunol 28(3):185–200

Reploeg MD, Storch Gregory A, Clifford David B (2001) BK virus: a clinical review. Clin Infect Dis 33:191–202

Robinson WE Jr, McDougall B, Tran D, Selsted ME (1998) Anti-HIV-1 activity of indolicidin, an antimicrobial peptide from neutrophils. J Leukoc Biol 63(1):94–100

Rothan HA, Abdulrahman AY, Sasikumer PG, Othman S, Rahman NA, Yusof R (2012a) Protegrin-1 inhibits dengue NS2B-NS3 serine protease and viral replication in MK2 cells. J Biomed Biotechnol 2012:251482

Rothan HA, Han HC, Ramasamy TS, Othman S, Rahman NA, Yusof R (2012b) Inhibition of dengue NS2B-NS3 protease and viral replication in Vero cells by recombinant retrocyclin-1. BMC Infect Dis 12:314

Salvatore M, García-Sastre A, Ruchala P, Lehrer Robert I, Chang T, Klotman Mary E (2007) α-Defensin inhibits influenza virus replication by cell-mediated mechanism(s). J Infect Dis 196:835–843

Savoia D, Donalisio M, Civra A, Salvadori S, Guerrini R (2010) In vitro activity of dermaseptin S1 derivatives against genital pathogens. APMIS. 118(9):674–680

Shackelton LA, Rambaut A, Pybus OG, Holmes EC (2006) JC virus evolution and its association with human populations. J Virol 80:9928–9933

Sherris medical microbiology (2004) An introduction to infectious diseases. 4th ed edn. McGraw-Hill, New York, 979 p

Simon V, Ho DD, Abdool Karim Q (2006) HIV/AIDS epidemiology, pathogenesis, prevention, and treatment. The Lancet 368:489–504

Sinha S, Cheshenko N, Lehrer RI, Herold BC (2003) NP-1, a rabbit alpha-defensin, prevents the entry and intercellular spread of herpes simplex virus type 2. Antimicrob Agents Chemother 47 (2):494–500

Smith JG, Nemerow GR (2008) Mechanism of adenovirus neutralization by Human alpha-defensins. Cell Host Microbe 3:11–19

Smith JG, Silvestry M, Lindert S, Lu W, Nemerow GR, Stewart PL (2010) Insight into the mechanisms of adenovirus capsid disassembly from studies of defensin neutralization. PLoS Pathog 6:e1000959

Sorensen OE, Follin P, Johnsen AH, Calafat J, Tjabringa GS, Hiemstra PS et al (2001) Human cathelicidin, hCAP-18, is processed to the antimicrobial peptide LL-37 by extracellular cleavage with proteinase 3. Blood 97(12):3951–3959

Sun L, Finnegan CM, Kish-Catalone T, Blumenthal R, Garzino-Demo P, La Terra Maggiore GM et al (2005) Human beta-defensins suppress human immunodeficiency virus infection: potential role in mucosal protection. J Virol 79:14318–14329

Tambunan US, Alamudi S (2010) Designing cyclic peptide inhibitor of dengue virus NS3-NS2B protease by using molecular docking approach. Bioinformation 5(6):250–254

Tanabe H, Ouellette AJ, Cocco MJ, Robinson WE (2004) Differential effects on human immunodeficiency virus type 1 replication by -defensins with comparable bactericidal activities. J Virol 78:11622–11631

Tang YQ, Yuan J, Osapay G, Osapay K, Tran D, Miller CJ et al (1999) A cyclic antimicrobial peptide produced in primate leukocytes by the ligation of two truncated alpha-defensins. Science 286(5439):498–502

Taubenberger JK, Morens DM (2006) 1918 Influenza: the mother of all pandemics. Emerg Infect Dis 12(1):15–22

Tecle T, White MR, Gantz D, Crouch EC, Hartshorn KL (2007) Human neutrophil defensins increase neutrophil uptake of influenza a virus and bacteria and modify virus-induced respiratory burst responses. J Immunol 178:8046–8052

Tenge VR, Gounder AP, Wiens ME, Lu W, Smith JG (2014) Delineation of interfaces on human alpha-defensins critical for human adenovirus and human papillomavirus inhibition. PLoS Pathog 10:e1004360

Terwilliger TC, Eisenberg D (1982) The structure of melittin. II. Interpretation of the structure. J Biol Chem 257(11):6016–6022

Tomasinsig L, Zanetti M (2005) The cathelicidins–structure, function and evolution. Curr Protein Pept Sci 6(1):23–34

Tran D, Tran P, Roberts K, Osapay G, Schaal J, Ouellette A et al (2008) Microbicidal properties and cytocidal selectivity of rhesus macaque theta defensins. Antimicrob Agents Chemother 52:944–953

Tripathi S, Tecle T, Verma A, Crouch E, White M, Hartshorn KL (2013) The human cathelicidin LL-37 inhibits influenza A viruses through a mechanism distinct from that of surfactant protein D or defensins. J Gen Virol 94(Pt 1):40–49

Tripathi S, Wang G, White M, Qi L, Taubenberger J, Hartshorn KL (2015) Antiviral activity of the human cathelicidin, LL-37, and derived peptides on seasonal and pandemic influenza A viruses. PLoS One 10(4):e0124706

van Zoggel H, Carpentier G, Dos Santos C, Hamma-Kourbali Y, Courty J, Amiche M et al (2012) Antitumor and angiostatic activities of the antimicrobial peptide dermaseptin B2. PLoS One 7(9):e44351

VanCompernolle SE, Taylor RJ, Oswald-Richter K, Jiang J, Youree BE, Bowie JH et al (2005) Antimicrobial peptides from amphibian skin potently inhibit human immunodeficiency virus infection and transfer of virus from dendritic cells to T cells. J Virol 79(18):11598–11606

Venkataraman N, Cole AL, Svoboda P, Pohl J, Cole AM (2005) Cationic polypeptides are required for anti-HIV-1 activity of human vaginal fluid. J Immunol 175(11):7560–7567

Wachinger M, Saermark T, Erfle V (1992) Influence of amphipathic peptides on the HIV-1 production in persistently infected T lymphoma cells. FEBS Lett 309(3):235–241

Wachinger M, Kleinschmidt A, Winder D, von Pechmann N, Ludvigsen A, Neumann M et al (1998) Antimicrobial peptides melittin and cecropin inhibit replication of human immunodeficiency virus 1 by suppressing viral gene expression. J Gen Virol 79(Pt 4):731–740

Wade D, Andreu D, Mitchell SA, Silveira AM, Boman A, Boman HG et al (1992) Antibacterial peptides designed as analogs or hybrids of cecropins and melittin. Int J Pept Protein Res 40(5):429–436

Wang W, Cole AM, Hong T, Waring AJ, Lehrer RI (2003) Retrocyclin, an antiretroviral -defensin, Is a lectin. J Immunol 170:4708–4716

Wang W, Owen SM, Rudolph DL, Cole AM, Hong T, Waring AJ et al (2004a) Activity of - and -defensins against Primary Isolates of HIV-1. J Immunol 173:515–520

Wang TT, Nestel FP, Bourdeau V, Nagai Y, Wang Q, Liao J et al (2004b) Cutting edge: 1,25-dihydroxyvitamin D3 is a direct inducer of antimicrobial peptide gene expression. J Immunol 173(5):2909–2912

Wang G, Watson KM, Buckheit RW Jr (2008) Anti-human immunodeficiency virus type 1 activities of antimicrobial peptides derived from human and bovine cathelicidins. Antimicrob Agents Chemother 52(9):3438–3440

Wang G, Watson KM, Peterkofsky A, Buckheit RW Jr (2010) Identification of novel human immunodeficiency virus type 1-inhibitory peptides based on the antimicrobial peptide database. Antimicrob Agents Chemother 54(3):1343–1346

Wiens ME, Smith JG (2015) Alpha-defensin HD5 inhibits furin cleavage of human papillomavirus 16 L2 to block infection. J Virol 89:2866–2874

Wohlford-Lenane CL, Meyerholz DK, Perlman S, Zhou H, Tran D, Selsted ME et al (2009) Rhesus theta-defensin prevents death in a mouse model of severe acute respiratory syndrome coronavirus pulmonary disease. J Virol 83:11385–11390

Wollebo HS, White MK, Gordon J, Berger JR, Khalili K (2015) Persistence and pathogenesis of the neurotropic polyomavirus JC. Ann Neurol 77:560–570

Wollenberg A, Wetzel S, Burgdorf WH, Haas J (2003) Viral infections in atopic dermatitis: pathogenic aspects and clinical management. J Allergy Clin Immunol 112(4):667–674

Wong JH, Legowska A, Rolka K, Ng TB, Hui M, Cho CH et al (2011) Effects of cathelicidin and its fragments on three key enzymes of HIV-1. Peptides 32(6):1117–1122

Wu H, Zhang G, Minton JE, Ross CR, Blecha F (2000) Regulation of cathelicidin gene expression: induction by lipopolysaccharide, interleukin-6, retinoic acid, and Salmonella enterica serovar typhimurium infection. Infect Immun 68(10):5552–5558

Wu Z, Cocchi F, Gentles D, Ericksen B, Lubkowski J, DeVico A et al (2005) Human neutrophil α-defensin 4 inhibits HIV-1 infection in vitro. FEBS Lett 579:162–166

Xu Y, Tamamura H, Arakaki R, Nakashima H, Zhang X, Fujii N et al (1999) Marked increase in anti-HIV activity, as well as inhibitory activity against HIV entry mediated by CXCR4, linked to enhancement of the binding ability of tachyplesin analogs to CXCR4. AIDS Res Hum Retroviruses 15(5):419–427

Yasin B, Harwig SS, Lehrer RI, Wagar EA (1996a) Susceptibility of chlamydia trachomatis to protegrins and defensins. Infect Immun 64(3):709–713

Yasin B, Lehrer RI, Harwig SS, Wagar EA (1996b) Protegrins: structural requirements for inactivating elementary bodies of Chlamydia trachomatis. Infect Immun 64(11):4863–4866

Yasin B, Pang M, Turner JS, Cho Y, Dinh NN, Waring AJ et al (2000) Evaluation of the inactivation of infectious Herpes simplex virus by host-defense peptides. Eur J Clin Microbiol Infect Dis 19(3):187–194

Yasin B, Wang W, Pang M, Cheshenko N, Hong T, Waring AJ et al (2004) Defensins protect cells from infection by herpes simplex virus by inhibiting viral adhesion and entry. J Virol 78:5147–5156

Zairi A, Tangy F, Bouassida K, Hani K (2009) Dermaseptins and magainins: antimicrobial peptides from frogs' skin-new sources for a promising spermicides microbicides-a mini review. J Biomed Biotechnol 2009:452567

Zanetti M, Del Sal G, Storici P, Schneider C, Romeo D (1993) The cDNA of the neutrophil antibiotic Bac5 predicts a pro-sequence homologous to a cysteine proteinase inhibitor that is common to other neutrophil antibiotics. J Biol Chem 268(1):522–526

Zasloff M (1987) Magainins, a class of antimicrobial peptides from Xenopus skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor. Proc Natl Acad Sci USA 84(15):5449–5453

Zhang G, Ross CR, Blecha F (2000) Porcine antimicrobial peptides: new prospects for ancient molecules of host defense. Vet Res 31(3):277–296

Zins SR, Nelson CDS, Maginnis MS, Banerjee R, O'Hara BA, Atwood WJ (2014) The Human Alpha Defensin HD5 Neutralizes JC Polyomavirus Infection by Reducing Endoplasmic Reticulum Traffic and Stabilizing the Viral Capsid. J Virol 88:948–960

Chapter 4
Anticancer Peptides: Prospective Innovation in Cancer Therapy

Diana Gaspar and Miguel A.R.B. Castanho

Abstract Current cancer treatments require improvements in selectivity and efficacy. Surgery, radiation, and chemotherapy approaches result in patient's suffering over time due to the development of severe side-effects that simultaneously condition adherence to therapy. Biologically active peptides, in particular antimicrobial peptides (AMPs), are versatile molecules in terms of biological activities. The cytotoxic activities of several AMPs turn this group of molecules into an amazing pool of new templates for anticancer drug development. However, several unmet challenges limit application of peptides in cancer therapy. The mechanism(s) of action of the peptides need better description and understanding, and innovative targets have to be discovered and explored, facilitating drug design and development. In this chapter, we explore the natural occurring AMPs as potential new anticancer peptides (ACPs) for cancer prevention and treatment. Their modes of action, selectivity to tumor compared to normal cells, preferential targets, and applications, but also their weaknesses, are described and discussed.

4.1 Introduction

Even though sharing similar characteristics such as replicative immortality, ability to evade immunosurveillance, and ability to invade surrounding and distant tissues and organs (Wu et al. 2014), tumor cells are still a challenging target in oncology. The development of resistance mechanisms and specific contributions of each tumor microenvironment (TME) contribute for the many difficulties in selectively targeting diseased rather than normal cells. These limitations in oncology treatment

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are the main reason why cancer diseases remain a leading cause of death worldwide.

The term cancer refers to a group of diseases characterized by an uncontrolled growth and by the spread of abnormal cells (Sah et al. 2015). The carcinogenesis process, triggered either by external factors such as radiation or chemicals, but also by internal factors such as mutations and hormones (Tanaka 1997), encompasses many changes on the cells' biochemistry (Tanaka 2009). Advances in oncotherapy need to address the particular biochemical signatures of each tumor. Identifying these signatures and exploiting their vulnerabilities will lead to the development of selective anticancer drugs than can prolong patients' lifetime and delay or prevent tumor metastases.

Conventional therapies including chemotherapy fail in selecting effectively which cells are to be targeted. While delivering a cytotoxic compound to the tumor, either a DNA-alkylating agent or a hormone agonist/antagonist, several constraints determine the fate of both malignant and normal cells. These include the effective localization of the chemotherapeutic drug, but also drug's biodistribution and selectivity determinants (Chen et al. 2014). These therapeutic options have been successful in converting some fatal cancers into chronic diseases that allow patients to survive for many years. However, the secondary effects that eventually arise in this process result in patients' suffering and slow clinical status deterioration with stages of myelosuppression, thrombocytopenia, mucositis, and alopecia (Riedl et al. 2011) before culminating in death.

In this scenario, peptide-based drugs raise renewed hope (Wu et al. 2014). The development of peptide sequences designed to interact with specific molecular markers, receptors or other tumor cell components, has been of value for application in cancer diagnosis, prognosis, and treatment. In this chapter we will review the use of peptides on cancer treatment, with focuses in their natural sources and specificity of their mechanism(s) of action.

4.2 Peptide-Based Strategies for Cancer Treatment—Anticancer Peptides

Peptide-based therapies have many benefits for cancer chemotherapy or supportive care, such as low cytotoxicity, strong specificity, tumor-penetrating ability, small size, and ease of modification (Barras and Widmann 2011; Wu et al. 2014). In fact, peptides have small to intermediate sizes, up to just a few hundreds of amino acids residues, amenable pharmacokinetic profiles, high uptake into tissues, and rapid clearance from blood (Wu et al. 2014). Thus, peptides recognizing and binding to specific membrane proteins or receptors on tumor cells' membranes are potential alternative drugs to overcome the limitations of low tissue penetration and low cellular uptake when using monoclonal antibodies (mAbs), for instance (Wu et al. 2014). Furthermore, peptide's production is of lower complexity when compared to

other protein-based therapies and thus, cost-effective (Fosgerau and Hoffmann 2015).

Antimicrobial peptides (AMPs) are a class of natural occurring peptides with several important targets and activities, from antimicrobial, antiviral, and antifungal (Reddy et al. 2004; Torcato et al. 2013a, b; Mello et al. 2011) to the modulation of the immune response (Silva et al. 2012). As part of immune defense (Iwasaki et al. 2009), AMPs are found in eukaryotic organisms of many different species (Reddy et al. 2004) and their rapid and non-specific interactions with the membrane lipids of the microbial targets results in the pathogen death with very low chance of resistance development (Arouri et al. 2009; Fernebro 2011). This interaction is enhanced by the high proportion of cationic and hydrophobic amino acid residues present in the structure of the peptides (Seo et al. 2012). AMPs are electrostatically attracted to the anionic membrane of the microbe and subsequently insert and disrupt the lipid structures, leading to its permeation (Huang et al. 2014). The changes that the cell machinery should endure for producing a resistant biological membrane, capable of neutralizing the action of AMPs is significant biological effort that has been rarely met until today (Chen et al. 2014).

In addition to their antimicrobial properties, some natural and synthetic AMPs also have antitumor activities with varying degrees of selectivity towards cancer cells (Hoskin and Ramamoorthy 2008). In fact, some of these newly found anticancer peptides (ACPs) have been successful in decreasing the burden of tumors in many animal models (Bhutia and Maiti 2008; Papo and Shai 2005).

The use of ACPs in oncology has been researched either to treat the tumor directly or to prevent formation of metastases; in this way, they are potential alternatives or adjuvant to the current therapies. Peptides can be used as drugs, hormones, or immunization agents (vaccines) (Sah et al. 2015). The biological effects include inhibition of tumor vasculature growth (angiogenesis), alterations in protein–protein interaction, changes in gene expression, and apoptosis, among others (Rosca et al. 2011; Walensky et al. 2004; Zheng et al. 2011).

The main weaknesses of ACPs are poor stability with susceptibility to proteolytic degradation and insufficient membrane permeability (Craik et al. 2013). There are strategies to overcome these limitations and their consequences (Wu et al. 2014), including amino acid substitution (Kohno et al. 2011), fusion of peptides (Yang et al. 2008), and peptide conjugation with chemotherapeutic drugs (Zhao et al. 2012).

4.3 Mechanisms of Action, Cellular Targets and Selectivity of Anticancer Peptides

There is intensive debate on ACPs' modes of action. Reviews available in the literature provide detailed description of the many different mechanisms underlying cancer cell toxicity (Gaspar et al. 2013; Harris et al. 2013; Hoskin and

Ramamoorthy 2008; Papo and Shai 2005; Mulder et al. 2013). Studies on structure–activity relationship have shown that some ACPs share with AMPs the ability to disrupt cell membranes, causing poration or micellization, and additionally inducing necrosis and/or apoptosis (Bhutia and Maiti 2008; Papo and Shai 2005). Additionally, numerous studies suggest that AMPs and ACPs share similar mechanisms of membrane interaction (Al-Benna et al. 2011; Harris et al. 2013; Riedl et al. 2011). This assumption is supported by the structural requirements that attract AMPs and ACPs to their respective microbial and human cell targets. Other membranolytic effects include mitochondrial swelling with cytochrome c release (Mai et al. 2001). However, non-membranolytic mechanisms are expected to be found for other ACPs (Harris et al. 2013; Sharma 1992) and it is frequent to discover that one ACP can have more than one cellular target and thus follow more than one mode of action. The modes of action not involving direct targeting of the cell membrane, such as interference with nucleic acid synthesis, hormonal receptors, or angiogenesis, have been hypothesized to be part of mediated immunity (Gaspar et al. 2013; Kuriyama et al. 2013).

Short linear ACPs fold into amphipathic conformations upon membrane interaction (Chen et al. 2014; Schweizer 2009), depending on hydrophobicity, amphipathicity, net charge, secondary structure, and oligomerization at the membrane level (Harris et al. 2013; Hoskin and Ramamoorthy 2008). Uncovering the details of the molecular mechanisms underlying each ACP mode of action is a technically challenging but rewarding task because the information gathered from these studies can be successfully applied in the development of innovative approaches in cancer treatment (Medina and Schneider 2015). There are ACPs with high specificity and selectivity for their targets. These include matrix metalloproteinases (MMP) such as MMP-2 and MMP-9 (Koivunen et al. 1999), the c-Src signaling pathway involved in tumor angiogenesis (Yi et al. 2009), cyclooxygenase-2 (Vesely et al. 2006), the heat shock protein 90 (Hsp90) and S100P, a marker for differentiating tumor and normal cells (Sah et al. 2015).

The details of the mechanisms of membrane-targeting ACPs are also not fully elucidated. The cellular membrane in tumors is biochemically modified when compared to normal cells (Huang et al. 2014; Schweizer 2009) because cancer cells have an higher content of anionic lipids in the outer surface of cytoplasmic membrane due to the increased fraction of negatively-charged phospholipids such as phosphatidylserine (PS) (Hoskin and Ramamoorthy 2008; Riedl et al. 2011). The loss of membrane asymmetry in the lipid distribution between the inner and outer leaflet of the plasma membrane during cell transformation into a malignant phenotype appears to be the cause for the exposure of PS on the surface of cells, which contributes to the selectivity of ACPs for solid and non-solid tumors (Gaspar et al. 2013). Other anionic components are also present on cancer cells' membrane such as O-glycosylated mucins, heparin sulfate and sialylated gangliosides (Gaspar et al. 2013). Cholesterol content on tumor cells' membrane also modulates cellular fluidity and condition ACPs activity (Schweizer 2009). The higher transmembrane potential and the higher surface area of tumor cells, which promotes contact with an increased number or peptide molecules, further contribute to the preferred action of

ACPs on tumor cells (Chan et al. 1998; Chaudhary and Munshi 1995; Huang et al. 2014). Peptides such as MPI-1 from the venom wasp *Polybia paulista* (Wang et al. 2009a), NK-2 derived from the protein NK-lysin found in porcines' NK- an T-cells (Schroder-Borm et al. 2005) and the synthetic peptide SVS-1 (Gaspar et al. 2012; Sinthuvanich et al. 2012) are a few examples of natural and synthetic peptides that base their preference for solid and hematological tumor cells based on the membrane surface net charge. However, detailed studies using biophysical and imaging techniques have shown that even though net charge has an important role in determining membrane interaction, AMPs and ACPs tend to behave differently in lipid environment (Freire et al. 2015; Gaspar et al. 2012, 2015). SVS-1 and HNP-1 ACPs are examples of this difference (Fig. 4.1). SVS-1 was designed to adopt a β-sheet structure after contact with the negatively-charged cancer cell membrane and is preferentially cytotoxic against lung, epidermal, and breast carcinomas when compared to HUVEC and red blood cells (Gaspar et al. 2012; Sinthuvanich et al. 2012). The mode of action described for SVS-1 is a lytic mechanism involving cell-surface induced folding into a β-hairpin structure capable of forming pores in the cell membrane (Sinthuvanich et al. 2012) (Fig. 4.1). The trigger for the peptide

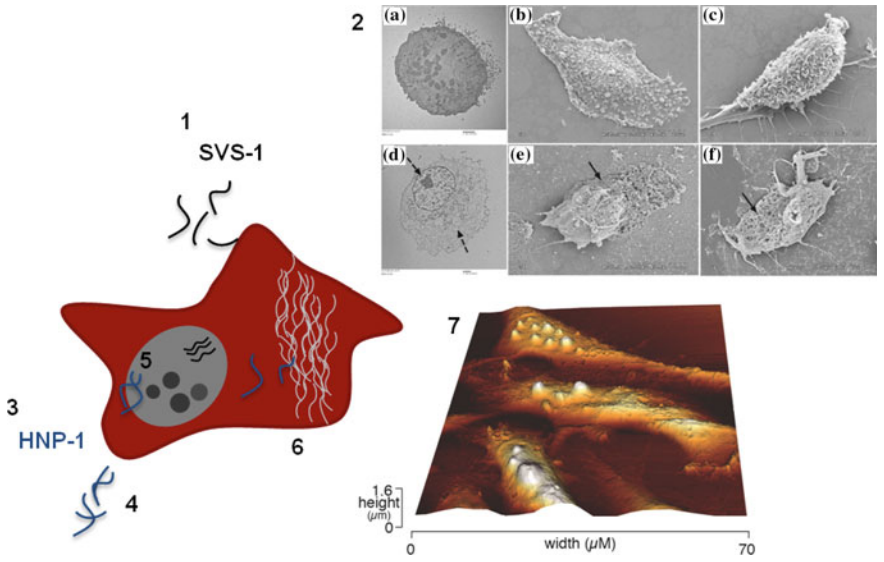

Fig. 4.1 Cell death induced by *SVS-1* and *HNP-1* peptides. *SVS-1* engages electrostatically the cancer cell membrane (*1*) and folds into a β-hairpin structure capable of forming pores (*solid arrow*) with leakage of the cellular contents (*dashed arrow*) as shown by TEM (*2a*, *2d*) and SEM (*2c*, *2e*, *2b*, *2f*) images. Scale bar: 10 μm for SEM, 2 μm for TEM. *HNP-1* (*3*) interacts with the negatively-charged cell membrane of the tumor cell (*4*), translocates into the cell-inducing DNA fragmentation (*6*) and fragilizing the cell's cytoskeleton structure (*6*) resulting in the collapse of the cell as shown in the 3D projection of the AFM height image (*7*). For both SVS-1 and HNP-1, cell death precedes membrane neutralization, in contrast to typical AMP action (Alves et al. 2010, Torcato et al. 2013b). Adapted from references Sinthuvanich et al. (2012) and Gaspar et al. (2015)

folding is the membrane net negative charge but full membrane neutralization is not mandatory for cell death (Gaspar et al. 2012). It was recently found that SVS-1 is able to translocate across the cell membrane into the cytoplasm and into the nucleus when present in concentrations below the minimal inhibitory concentration (MIC_{50}) necessary for lytic action (Medina and Schneider 2015). The combination of SVS-1 with paclitaxel improves SVS-1 aqueous solubility and the peptide is capable of delivering and releasing paclitaxel into cancer cells and tumors *in vivo* without any adjuvant (Medina and Schneider 2015).

On the contrary, studies with AMPs such as BP100 show a neutralization of the bacterial membrane that can be correlated with the minimal inhibitory concentration (MIC) values found to inhibit the growth of *Escherichia coli* bacteria (Alves et al. 2010). Therefore, one should be cautious when translating conclusions on AMPs structure-activity studies to ACPs because not all AMPs behave as ACPs.

4.4 Targeting Cancer Cells Using Natural Peptides

More than 7000 natural peptides have been identified until today (Fosgerau and Hoffmann 2015) and AMPs can be found virtually in all living organisms, from plants and insects to animals (Salas et al. 2015). Table 4.1 lists selected examples of these AMPs with anticancer activity.

Natural products derived from plants have contributed greatly to chemotherapy development. Examples of this are the drugs paclitaxel, vincristine, and vinblastine (Wu et al. 2014). Plants are also great producers of small cysteine-rich AMPs and some of them present cytotoxic activities. Cytotoxic classes are mainly represented by thionins, defensins and cyclotides (Guzman-Rodriguez et al. 2015).

Table 4.1 Selected naturally occurring antimicrobial peptides (AMPs) with anticancer activity

Peptide	Source	Activity	Reference
Pyrularia	Plant	Changes in Ca^{2+} influx	Evans et al. (1989)
NaD1	Plant	Binding to plasma membrane PIP2	Poon et al. (2014)
RA-V	Plant	Mitochondria-mediated apoptosis with PDK1-AKT blocking; Inhibition of cell adhesion and migration through regulation of adhesion molecules, receptors and MMPs expression	Fang et al. (2013); Leung et al. (2015)
Lunasin	Plant	HAT inhibition and cell cycle progression repression	Galvez et al. 2001); Hernandez-Ledesma et al. 2009)
Gomesin	Insect	Ca^{2+} accumulation, loss of mitochondrial potential, pore formation	Rodrigues et al. (2008); Paredes-Gamero et al. (20120
Mastoparan	Insect	Oxidative stress, mitochondrial depolarization and apoptosis	de Azevedo (2015)
HNP-1	Human	DNA breakdown and cell collapse	Gaspar et al. (2015)

Thionins are small cysteine-rich peptides with diversified activities, in addition to being antimicrobial. They help seed maturation and germination and have roles in signal transduction (Stec 2006), and some of them, such as pyrularia, Thi2.1, and β-purothionin have cytotoxic activity against cervical, lung, and breast cancers (Evans et al. 1989; Hughes et al. 2000; Loeza-Angeles et al. 2008). In some cases, anticancer effects are based on changes on Ca^{2+} influx that depolarize the cellular membrane (Evans et al. 1989), but for others remain unknown (Loeza-Angeles et al. 2008).

Plant defensins represent a diversified group in terms of their amino acid sequence but some of the amino acid positions are highly conserved (Guzman-Rodriguez et al. 2015). They present powerful antifungal activity (Mello et al. 2011) and their mode of action is related to membrane destabilization or insertion followed by pore formation and leakage of essential biomolecules (Lacerda et al. 2014). Sesquin was the first plant defensin known to be active against breast cancer and leukemia cells (Wong and Ng 2005b). Other plant defensins, such as lunatusin (Wong and Ng 2005a) and phaseococcin (Ngai and Ng 2005) are also active on breast cancer and leukemia; however, their mode of action and selectivity are still poorly described. Several reports on plant defensins show that this group of peptides might have alternative targets to conventional drugs. This is the case of NaD1 that can act by direct binding to the plasma membrane phospholipid phosphatidylinositol 4,5-biphosphate (PIP2) (Poon et al. 2014).

Cyclotides is another group of Cys-rich peptides derived from plants with cytotoxic activity. These macrocyclic peptides have around 30 amino acid residues in their sequence and a wide range of biological activities (Craik 2012). Their tight cyclic structure is of particular relevance because it confers chemical and biological stability, conferring high pharmaceutical value to the peptides (Guzman-Rodriguez et al. 2015). They are characterized by a cystine knot with an embedded ring formed by two disulfide bonds and connecting backbone segments threaded by one more disulfide bond (Guzman-Rodriguez et al. 2015). Expressed in large quantities by plants of Rubiaceae and Violaceae families, cyclotides are described mainly as host protectors (Craik 2012). However, their activities go much further than biocidal protection and include anti-HIV and anticancer effects (Craik 2012). The mechanism of action described for cyclotides is also very interesting from a therapeutical point of view. For kalatas B1–B9 peptides, the presence of phosphatidylethanolamine (PE) headgroups on the cellular membrane favors peptide binding (Henriques et al. 2012), which is advantageous in the drug design process for increasing peptide's selectivity towards specific cancer cells that express higher contents of this PE phospholipid, for instance. Other described cyclotides include cycloviolacin O2 and Viba 15 and 17 with activities against lymphoma, melanoma, and also cervical and gastric cancers (He et al. 2011; Svangard et al. 2007).

More recently, the cyclopeptide deoxybouvardin, RA-V, derived from *Rubia yunnanensis* has been characterized with antitumor and anti-angiogenesis activity (Fang et al. 2013; Leung et al. 2015). This peptide shows anticancer activity against human and murine breast cancer cells through mitochondria-mediated apoptosis by blocking PDK1 and AKT interaction and consequently apoptosis resistance

(Fang et al. 2013). RA-V peptide is also capable of inhibiting breast cancer cell adhesion and migration through the interference on cofilin signaling and chemokine receptors. This peptide reduces the expression of several adhesion molecules and MMPs (Leung et al. 2015).

Lunasin is another example of natural ACP isolated from plants (Hernandez-Ledesma et al. 2009). This 43-amino acid peptide is found in soy, wheat, barley, and other seeds (Hernandez-Ledesma et al. 2009) and is a chemopreventive agent against oncogenes and chemical carcinogens (Ortiz-Martinez et al. 2014). With an adequate bioavailability following oral administration, lunasin was shown to prevent skin cancer in a mouse model induced by chemical carcinogens (Galvez et al. 2001; Hsieh et al. 2004). An epigenetic mechanism of action proposed for this peptide is the selective killing of newly transformed cancer cells by acting as a histone acetyltransferase (HAT) inhibitor and repressing cell cycle progression (Hernandez-Ledesma et al. 2009).

Insects are also a good natural source of AMPs with anticancer activity. Gomesin is a β-hairpin peptide isolated from the hemolymph of *Acanthoscurria gomesiana*, a Brazilian spider (Rodrigues et al. 2008). This peptide has the ability to form pores and is active as a topical agent against melanoma, breast, and colon carcinomas neuroblastomas and pheochromocytomas (Rodrigues et al. 2008). Gomesin induces membrane permeabilization through a Ca^{2+} dependent pathway which involves particular intracellular events: perturbation of the endoplasmic reticulum, accumulation of Ca^{2+} in organelles, of mitochondrial potential and oxidative stress (Paredes-Gamero et al. 2012). Mastoparan is a 14-amino acid α-helical cell penetrating peptide from the venom of *Vespula lewisii* wasp that has nocive effects on cell membranes (Saar et al. 2005). Mastoparan shows antitumor activity against human erythroleukemia cells and melanoma (Yamada et al. 2005). In the latter, tumor cell death occurs through an induce of programmed cell death by oxidative stress, which causes mitochondrial depolarization (de Azevedo et al. 2015). Apoptosis induced by mastoparan stems from the activation of caspases -9, -12, and -3, cleavage of PARP, up-regulation of pro-apoptotic proteins Bax and Bim and down-regulation of the anti-apoptotic Bcl-XL proteins (de Azevedo et al. 2015).

In animals, AMPs with anticancer activity can be found in the immune, digestive, and central nervous systems (CNS) and also in the heart, bones, muscle, and skin (Wu et al. 2014). Many AMPs from the animal kingdom have been extensively studied, such as LfcinB. This 25-amino acid residues peptide is isolated from cows' milk and causes cell death through at least two mechanisms. LfcinB is active against leukemia cells and diverse solid tumors (Mader et al. 2005) and is capable of binding to glycosaminoglycans (GAGs) present on the membrane surface (Jenssen et al. 2004), inducing apoptosis by mitochondrial pathway, and also lysis of the cellular membrane (Eliassen et al. 2006; Furlong et al. 2008).

One of the most studied groups of peptides derived from humans is the defensins group. These are disulfide-rich peptides, similar to defensin plants, and comprise 29-35 amino acids with three disulfide bonds (Conibear and Craik 2014). Defensins are organized in three classes, α-, β-, and Θ-defensins (Conibear and Craik 2014).

The class of α-defensins includes the human neutrophil peptides 1–4 and the human defensins HD5 and HD6, produced in the Paneth cells of the intestine (Ouellette and Bevins 2001). The human neutrophil peptides, HNPs, possess antitumoral effects trough diversified mechanisms (Wang et al. 2009b). HNP-1 to 3 have been appointed has potential tumor biomarkers (Albrethsen et al. 2005, 2006; Droin et al. 2009). The HNP-1 has been intensively studied for anticancer properties. Produced and stored in the azurophilic granules of human neutrophils, this peptide is released when an inactivation of bacteria and yeast is necessary (Ganz and Lehrer 1998). However, many studies report the importance of this AMP in oncology. The expression of HNP-1 in models of tumors such as breast and colon stimulates an immune response from the host against the tumor (Wang et al. 2009c). In addition, it has been found up-regulated in cancers such as colorectal (Mohri et al. 2009) and other tumors (Albrethsen et al. 2006; Holterman et al. 2006) and to be linked to tumor necrosis when expressed intratumorally (Bateman et al. 1992; Muller et al. 2002). HNP-1 mode of action is believed to involve damage to the cell membrane but also the induction of DNA strand break (Gera and Lichtenstein 1991). A recent study revealed that HNP-1 attacks solid tumors, human prostate cancer in this particular case, after translocating into the cell, following DNA and cytoskeleton damage and final cell collapse (Gaspar et al. 2015) (Fig. 4.1). Cell death occurs without full neutralization of the cancer cell membrane and although HNP-1 interacts with prostate and leukemia cells, differences on the membrane composition of each tumor cells dictate the peptide's preference (Gaspar et al. 2015).

4.5 Final Remarks

Today, hundreds of novel peptide sequences are part of the clinical and preclinical testing of pharmaceutical companies. As peptide-based therapies are on the spotlight, a great number of studies report on the role of AMPs on cancer treatment. With a very particular mode of action involving non-specific interactions, peptides can be expected to meet the selectivity, efficacy, and safety requisites for successful drugs with diminutive resistance barriers. However, drug development optimization is still needed, which requires that the mechanism(s) of action and molecular and cellular targets, as well as potential off-target effects, are researched and described with molecular level detail and correlated to human physiology. The future of peptide applications in oncology and oncotherapeutics depends on how successful basic research will be in this endeavor.

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References

Al-Benna S, Shai Y, Jacobsen F, Steinstraesser L (2011) Oncolytic activities of host defense peptides. Int J Mol Sci 12(11):8027–8051. doi:10.3390/ijms12118027

Albrethsen J, Bogebo R, Gammeltoft S, Olsen J, Winther B, Raskov H (2005) Upregulated expression of human neutrophil peptides 1, 2 and 3 (HNP 1-3) in colon cancer serum and tumours: a biomarker study. BMC Cancer 5:8. doi:10.1186/1471-2407-5-8

Albrethsen J, Moller CH, Olsen J, Raskov H, Gammeltoft S (2006) Human neutrophil peptides 1, 2 and 3 are biochemical markers for metastatic colorectal cancer. Eur J Cancer 42(17):3057–3064. doi:10.1016/j.ejca.2006.05.039

Alves CS, Melo MN, Franquelim HG, Ferre R, Planas M, Feliu L, Bardaji E, Kowalczyk W, Andreu D, Santos NC, Fernandes MX, Castanho MARB (2010) Escherichia coli cell surface perturbation and disruption induced by antimicrobial peptides BP100 and pepR. J Biol Chem 285(36):27536–27544. doi:10.1074/jbc.M110.130955

Arouri A, Dathe M, Blume A (2009) Peptide induced demixing in PG/PE lipid mixtures: A mechanism for the specificity of antimicrobial peptides towards bacterial membranes? Biochimica Et Biophysica Acta-Biomembranes 1788(3):650–659. doi:10.1016/j.bbamem.2008.11.022

Barras D, Widmann C (2011) Promises of apoptosis-inducing peptides in cancer therapeutics. Curr Pharm Biotechnol 12(8):1153–1165

Bateman A, Singh A, Jothy S, Fraser R, Esch F, Solomon S (1992) The levels and biologic action of the human neutrophil granule peptide Hp-1 in lung-tumors. Peptides 13(1):133–139. doi:10.1016/0196-9781(92)90152-S

Bhutia SK, Maiti TK (2008) Targeting tumors with peptides from natural sources. Trends Biotechnol 26(4):210–217. doi:10.1016/j.tibtech.2008.01.002

Chan SC, Hui L, Chen HM (1998) Enhancement of the cytolytic effect of anti-bacterial cecropin by the microvilli of cancer cells. Anticancer Res 18(6A):4467–4474

Chaudhary J, Munshi M (1995) Scanning electron-microscopic analysis of breast aspirates. Cytopathology 6(3):162–167. doi:10.1111/j.1365-2303.1995.tb00469.x

Chen CX, Hu J, Zeng P, Pan F, Yaseen M, Xu H, Lu JR (2014) Molecular mechanisms of anticancer action and cell selectivity of short alpha-helical peptides. Biomaterials 35(5):1552–1561. doi:10.1016/j.biomaterials.2013.10.082

Conibear AC, Craik DJ (2014) The chemistry and biology of theta defensins. Angewandte Chemie-International Edition 53(40):10612–10623. doi:10.1002/anie.201402167

Craik DJ (2012) Host-defense activities of cyclotides. Toxins 4(2):139–156. doi:10.3390/toxins4020139

Craik DJ, Fairlie DP, Liras S, Price D (2013) The future of peptide-based drugs. Chem Biol Drug Des 81(1):136–147. doi:10.1111/cbdd.12055

de Azevedo RA, Figueiredo CR, Ferreira AK, Matsuo AL, Massaoka MH, Girola N, Auada AV, Farias CF, Pasqualoto KF, Rodrigues CP, Barbuto JA, Levy D, Bydlowski SP, de Sa-Junior PL, Travassos LR, Lebrun I (2015) Mastoparan induces apoptosis in B16F10-Nex2 melanoma cells via the intrinsic mitochondrial pathway and displays antitumor activity in vivo. Peptides 68:113–119. doi:10.1016/j.peptides.2014.09.024

Droin N, Hendra JB, Ducoroy P, Solary E (2009) Human defensins as cancer biomarkers and antitumour molecules. J Proteomics 72(6):918–927. doi:10.1016/j.jprot.2009.01.002

Eliassen LT, Berge G, Leknessund A, Wikman M, Lindin I, Lokke C, Ponthan F, Johnsen JI, Sveinbjornsson B, Kogner P, Flaegstad T, Rekdal O (2006) The antimicrobial peptide, Lactoferricin B, is cytotoxic to neuroblastoma cells in vitro and inhibits xenograft growth in vivo. Int J Cancer 119(3):493–500. doi:10.1002/ijc.21886

Evans J, Wang YD, Shaw KP, Vernon LP (1989) Cellular-responses to pyrularia thionin are mediated by $Ca\text{-}2^{+}$ influx and phospholipase-A2 activation and are inhibited by thionin tyrosine iodination. Proc Natl Acad Sci USA 86(15):5849–5853. doi:10.1073/pnas.86.15.5849

Fang XY, Chen W, Fan JT, Song R, Wang L, Gu YH, Zeng GZ, Shen Y, Wu XF, Tan NH, Xu Q, Sun Y (2013) Plant cyclopeptide RA-V kills human breast cancer cells by inducing mitochondria-mediated apoptosis through blocking PDK1-AKT interaction. Toxicol Appl Pharmacol 267(1):95–103. doi:10.1016/j.taap.2012.12.010

Fernebro J (2011) Fighting bacterial infections-future treatment options. Drug Resist Updates 14 (2):125–139. doi:10.1016/j.drup.2011.02.001

Fosgerau K, Hoffmann T (2015) Peptide therapeutics: current status and future directions. Drug Discov Today 20(1):122–128

Freire JM, Gaspar D, Veiga AS, Castanho MARB (2015) Shifting gear in antimicrobial and anticancer peptides biophysical studies: from vesicles to cells. J Pept Sci 21(3):178–185. doi:10.1002/psc.2741

Furlong SJ, Ridgway ND, Hoskin DW (2008) Modulation of ceramide metabolism in T-leukemia cell lines potentiates apoptosis induced by the cationic antimicrobial peptide bovine lactoferricin. Int J Oncol 32(3):537–544

Galvez AF, Chen N, Macasieb J, de Lumen BO (2001) Chemopreventive property of a soybean peptide (lunasin) that binds to deacetylated histones and inhibits acetylation. Cancer Res 61 (20):7473–7478

Ganz T, Lehrer RI (1998) Antimicrobial peptides of vertebrates. Curr Opin Immunol 10(1):41–44. doi:10.1016/S0952-7915(98)80029-0

Gaspar D, Veiga AS, Sinthuvanich C, Schneider JP, Castanho MARB (2012) Anticancer peptide SVS-1: efficacy precedes membrane neutralization. Biochemistry 51(32):6263–6265. doi:10.1021/bi300836r

Gaspar D, Veiga AS, Castanho MA (2013) From antimicrobial to anticancer peptides. A review. Front Microbiol 4:294. doi:10.3389/fmicb.2013.00294

Gaspar D, Freire JM, Pacheco TR, Barata JT, Castanho MARB (2015) Apoptotic human neutrophil peptide-1 anti-tumor activity revealed by cellular biomechanics. Biochimica Et Biophysica Acta-Molecular Cell Res 1853(2):308–316. doi:10.1016/j.bbamcr.2014.11.006

Gera JF, Lichtenstein A (1991) Human neutrophil peptide defensins induce single-strand DNA breaks in target-cells. Cell Immunol 138(1):108–120. doi:10.1016/0008-8749(91)90136-Y

Guzman-Rodriguez JJ, Ochoa-Zarzosa A, Lopez-Gomez R, Lopez-Meza JE (2015) Plant antimicrobial peptides as potential anticancer agents. Biomed Res Int. doi:10.1155/2015/735087

Harris F, Dennison SR, Singh J, Phoenix DA (2013) On the selectivity and efficacy of defense peptides with respect to cancer cells. Med Res Rev 33(1):190–234. doi:10.1002/med.20252

He WJ, Chan LY, Zeng GZ, Daly NL, Craik DJ, Tan NH (2011) Isolation and characterization of cytotoxic cyclotides from Viola philippica. Peptides 32(8):1719–1723. doi:10.1016/j.peptides.2011.06.016

Henriques ST, Huang YH, Castanho MARB, Bagatolli LA, Sonza S, Tachedjian G, Daly NL, Craik DJ (2012) Phosphatidylethanolamine binding is a conserved feature of cyclotide-membrane interactions. J Biol Chem 287(40):33629–33643. doi:10.1074/jbc.M112.372011

Hernandez-Ledesma B, Hsieh CC, de Lumen BO (2009) Lunasin, a novel seed peptide for cancer prevention. Peptides 30(2):426–430. doi:10.1016/j.peptides.2008.11.002

Holterman DA, Diaz JI, Blackmore PF, Davis JW, Schellhammer PF, Corica A, Semmes OJ, Vlahou A (2006) Overexpression of alpha-defensin is associated with bladder cancer invasiveness. Urol Oncol-Semin Original Inv 24(2):97–108. doi:10.1016/j.urolonc.2005.07.010

Hoskin DW, Ramamoorthy A (2008) Studies on anticancer activities of antimicrobial peptides. Biochimica Et Biophysica Acta-Biomembranes 1778(2):357–375. doi:10.1016/j.bbamem.2007.11.008

Hsieh EA, Chai CM, de Lumen BO, Neese RA, Hellerstein MK (2004) Dynamics of keratinocytes in vivo using (H2O)-H-2 labeling: a sensitive marker of epidermal proliferation state. J Inv Dermatol 123(3):530–536. doi:10.1111/j.0022-202X.2004.23303.x

Huang W, Seo J, Willingham SB, Czyzewski AM, Gonzalgo ML, Weissman IL, Barron AE (2014) Learning from host-defense peptides: cationic, amphipathic peptoids with potent anticancer activity. Plos One 9(2). doi:10.1371/journal.pone.0090397

Hughes P, Dennis E, Whitecross M, Llewellyn D, Gage P (2000) The cytotoxic plant protein, beta-purothionin, forms ion channels in lipid membranes. J Biol Chem 275(2):823–827. doi:10.1074/jbc.275.2.823

Iwasaki T, Ishibashi J, Tanaka H, Sato M, Asaoka A, Taylor D, Yamakawa M (2009) Selective cancer cell cytotoxicity of enantiomeric 9-mer peptides derived from beetle defensins depends on negatively charged phosphatidylserine on the cell surface. Peptides 30(4):660–668. doi:10.1016/j.peptides.2008.12.019

Jenssen H, Andersen JH, Uhlin-Hansen L, Gutteberg TJ, Rekdal O (2004) Anti-HSV activity of lactoferricin analogues is only partly related to their affinity for heparan sulfate. Antiviral Res 61(2):101–109. doi:10.1016/j.antiviral.2003.09.001

Kohno M, Horibe T, Haramoto M, Yano Y, Ohara K, Nakajima O, Matsuzaki K, Kawakami K (2011) A novel hybrid peptide targeting EGFR-expressing cancers. Eur J Cancer 47(5):773–783. doi:10.1016/j.ejca.2010.10.021

Koivunen E, Arap W, Valtanen H, Rainisalo A, Medina OP, Heikkila P, Kantor C, Gahmberg CG, Salo T, Konttinen YT, Sorsa T, Ruoslahti E, Pasqualini R (1999) Tumor targeting with a selective gelatinase inhibitor. Nat Biotechnol 17(8):768–774. doi:10.1038/11703

Kuriyama I, Miyazaki A, Tsuda Y, Yoshida H, Mizushina Y (2013) Inhibitory effect of novel somatostatin peptide analogues on human cancer cell growth based on the selective inhibition of DNA polymerase beta. Bioorg Med Chem 21(2):403–411. doi:10.1016/j.bmc.2012.11.024

Lacerda AF, Vasconcelos EA, Pelegrini PB, Grossi de Sa MF (2014) Antifungal defensins and their role in plant defense. Front Microbiol 5:116. doi:10.3389/fmicb.2014.00116

Leung HW, Wang Z, Yue GGL, Zhao SM, Lee JKM, Fung KP, Leung PC, Lau CBS, Tan NH (2015) Cyclopeptide RA-V inhibits cell adhesion and invasion in both estrogen receptor positive and negative breast cancer cells via PI3 K/AKT and NF-kappa B signaling pathways. Biochimica Et Biophys Acta-Mol Cell Res 1853(8):1827–1840. doi:10.1016/j.bbamcr.2015.04.020

Loeza-Angeles H, Sagrero-Cisneros E, Lara-Zarate L, Villagomez-Gomez E, Lopez-Meza JE, Ochoa-Zarzosa A (2008) Thionin Thi2.1 from Arabidopsis thaliana expressed in endothelial cells shows antibacterial, antifungal and cytotoxic activity. Biotechnol Lett 30(10):1713–1719. doi:10.1007/s10529-008-9756-8

Mader JS, Salsman J, Conrad DM, Hoskin DW (2005) Bovine lactoferricin selectively induces apoptosis in human leukemia and carcinoma cell lines. Mol Cancer Ther 4(4):612–624. doi:10.1158/1535-7163.Mct-04-0077

Mai JC, Mi ZB, Kim SH, Ng B, Robbins PD (2001) A proapoptotic peptide for the treatment of solid tumors. Cancer Res 61(21):7709–7712

Medina SH, Schneider JP (2015) Cancer cell surface induced peptide folding allows intracellular translocation of drug. J Controlled Release 209:317–326. doi:10.1016/j.jconrel.2015.05.267

Mello EO, Ribeiro SFF, Carvalho AO, Santos IS, Da Cunha M, Santa-Catarina C, Gomes VM (2011) Antifungal activity of PvD1 defensin involves plasma membrane permeabilization, inhibition of medium acidification, and induction of ROS in fungi cells. Curr Microbiol 62(4):1209–1217. doi:10.1007/s00284-010-9847-3

Mohri Y, Mohri T, Wei W, Qi YJ, Martin A, Miki C, Kusunoki M, Ward DG, Johnson PJ (2009) Identification of macrophage migration inhibitory factor and human neutrophil peptides 1-3 as potential biomarkers for gastric cancer. Br J Cancer 101(2):295–302. doi:10.1038/sj.bjc.6605138

Mulder KCL, Lima LA, Miranda VJ, Dias SC, Franco OL (2013) Current scenario of peptide-based drugs: the key roles of cationic antitumor and antiviral peptides. Frontiers Microbiol 4. doi:10.3389/Fmicb.2013.00321

Muller CA, Markovic-Lipkovski J, Klatt T, Gamper J, Schwarz G, Beck H, Deeg M, Kalbacher H, Widmann S, Wessels JT, Becker V, Muller GA, Flad T (2002) Human alpha-defensins

HNPs-1, -2, and -3 in renal cell carcinoma: influences on tumor cell proliferation. Am J Pathol 160(4):1311–1324. doi:10.1016/S0002-9440(10)62558-8

Ngai PHK, Ng TB (2005) Phaseococcin, an antifungal protein with antiproliferative and anti-HIV-1 reverse transcriptase activities from small scarlet runner beans. Biochem Cell Biol-Biochimie Et Biologie Cellulaire 83(2):212–220. doi:10.1139/O05-037

Ortiz-Martinez M, Winkler R, Garcia-Lara S (2014) Preventive and therapeutic potential of peptides from cereals against cancer. J Proteomics 111:165–183. doi:10.1016/j.jprot.2014.03.044

Ouellette AJ, Bevins CL (2001) Paneth cell defensins and innate immunity of the small bowel. Inflamm Bowel Dis 7(1):43–50. doi:10.1097/00054725-200102000-00007

Papo N, Shai Y (2005) Host defense peptides as new weapons in cancer treatment. Cell Mol Life Sci 62(7–8):784–790. doi:10.1007/s00018-005-4560-2

Paredes-Gamero EJ, Casaes-Rodrigues RL, Moura GE, Domingues TM, Buri MV, Ferreira VH, Trindade ES, Moreno-Ortega AJ, Cano-Abad MF, Nader HB, Ferreira AT, Miranda A, Justo GZ, Tersariol IL (2012) Cell-permeable gomesin peptide promotes cell death by intracellular $Ca(2^+)$ overload. Mol Pharm 9(9):2686–2697. doi:10.1021/mp300251j

Poon IKH, Baxter AA, Lay FT, Mills GD, Adda CG, Payne JAE, Phan TK, Ryan GF, White JA, Veneer PK, van der Weerden NL, Anderson MA, Kvansakul M, Hulett MD (2014) Phosphoinositide-mediated oligomerization of a defensin induces cell lysis. Elife 3. doi:10.7554/eLife.01808

Reddy KVR, Yedery RD, Aranha C (2004) Antimicrobial peptides: premises and promises. Int J Antimicrob Agents 24(6):536–547. doi:10.1016/j.ijantimicag.2004.09.005

Riedl S, Zweytick D, Lohner K (2011) Membrane-active host defense peptides—challenges and perspectives for the development of novel anticancer drugs. Chem Phys Lipids 164(8):766–781. doi:10.1016/j.chemphyslip.2011.09.004

Rodrigues EG, Dobroff ASS, Cavarsan CF, Paschoalin T, Nimrichter L, Mortara RA, Santos EL, Fazio MA, Miranda A, Daffre S, Travassos LR (2008) Effective topical treatment of subcutaneous murine B16F10-Nex2 melanoma by the antimicrobial peptide gomesin. Neoplasia 10(1):61–68. doi:10.1593/neo.07885

Rosca EV, Koskimaki JE, Rivera CG, Pandey NB, Tamiz AP, Popel AS (2011) Anti-angiogenic peptides for cancer therapeutics. Curr Pharm Biotechnol 12(8):1101–1116

Saar K, Lindgren M, Hansen M, Eiriksdottir E, Jiang Y, Rosenthal-Aizman K, Sassian M, Langel U (2005) Cell-penetrating peptides: a comparative membrane toxicity study. Anal Biochem 345(1):55–65. doi:10.1016/j.ab.2005.07.033

Sah BNP, Vasiljevic T, McKechnie S, Donkor ON (2015) Identification of anticancer peptides from bovine milk proteins and their potential roles in management of cancer: a critical review. Comprehensive Rev. Food Sci. Food Safety 14(2):123–138. doi:10.1111/1541-4337.12126

Salas CE, Badillo-Corona JA, Ramirez-Sotelo G, Oliver-Salvador C (2015) Biologically active and antimicrobial peptides from plants. Biomed Res Int. doi:10.1155/2015/102129

Schroder-Borm H, Bakalova R, Andra J (2005) The NK-lysin derived peptide NK-2 preferentially kills cancer cells with increased surface levels of negatively charged phosphatidylserine. FEBS Lett 579(27):6128–6134. doi:10.1016/j.febslet.2005.09.084

Schweizer F (2009) Cationic amphiphilic peptides with cancer-selective toxicity. Eur J Pharmacol 625(1–3):190–194. doi:10.1016/j.ejphar.2009.08.043

Seo MD, Won HS, Kim JH, Mishig-Ochir T, Lee BJ (2012) Antimicrobial peptides for therapeutic applications: a review. Molecules 17(10):12276–12286. doi:10.3390/molecules171012276

Sharma SV (1992) Melittin resistance - a counterselection for ras transformation. Oncogene 7(2):193–201

Silva ON, Porto WF, Migliolo L, Mandal SM, Gomes DG, Holanda HHS, Silva RSP, Dias SC, Costa MP, Costa CR, Silva MR, Rezende TMB, Franco OL (2012) Cn-AMP1: a new promiscuous peptide with potential for microbial infections treatment. Biopolymers 98(4):322–331. doi:10.1002/bip.22071

Sinthuvanich C, Veiga AS, Gupta K, Gaspar D, Blumenthal R, Schneider JP (2012) Anticancer beta-hairpin peptides: membrane-induced folding triggers activity. J Am Chem Soc 134 (14):6210–6217. doi:10.1021/ja210569f

Stec B (2006) Plant thionins—the structural perspective. Cell Mol Life Sci 63(12):1370–1385. doi:10.1007/s00018-005-5574-5

Svangard E, Burman R, Gunasekera S, Lovborg H, Gullbo J, Goransson U (2007) Mechanism of action of cytotoxic cyclotides: cycloviolacin O2 disrupts lipid membranes. J Nat Prod 70 (4):643–647. doi:10.1021/np070007v

Tanaka T (1997) Chemoprevention of human cancer: biology and therapy. Crit Rev Oncol Hematol 25(3):139–174. doi:10.1016/S1040-8428(97)00232-1

Tanaka T (2009) Colorectal carcinogenesis: review of human and experimental animal studies. J Carcinog 8:5

Torcato IM, Huang YH, Franquelim HG, Gaspar D, Craik DJ, Castanho MARB, Henriques ST (2013a) Design and characterization of novel antimicrobial peptides, R-BP100 and RW-BP100, with activity against Gram-negative and Gram-positive bacteria. Biochimica Et Biophysica Acta-Biomembr 1828(3):944–955. doi:10.1016/j.bbamem.2012.12.002

Torcato IM, Huang YH, Franquelim HG, Gaspar DD, Craik DJ, Castanho MARB, Henriques ST (2013b) The antimicrobial activity of Sub3 is dependent on membrane binding and cell-penetrating ability. ChemBioChem 14(15):2013–2022. doi:10.1002/cbic.201300274

Vesely BA, Eichelbaum EJ, Alli AA, Sun Y, Gower WR, Vesely DL (2006) Urodilatin and four cardiac hormones decrease human renal carcinoma cell numbers. Eur J Clin Invest 36(11):810–819. doi:10.1111/j.1365-2362.2006.01721.x

Walensky LD, Kung AL, Escher I, Malia TJ, Barbuto S, Wright RD, Wagner G, Verdine GL, Korsmeyer SJ (2004) Activation of apoptosis in vivo by a hydrocarbon-stapled BH3 helix. Science 305(5689):1466–1470. doi:10.1126/science.1099191

Wang KR, Yan JX, Zhang BZ, Song JJ, Jia PF, Wang R (2009a) Novel mode of action of polybia-MPI, a novel antimicrobial peptide, in multi-drug resistant leukemic cells. Cancer Lett 278(1):65–72. doi:10.1016/j.canlet.2008.12.027

Wang YS, Li D, Shi HS, Wen YJ, Yang L, Xu N, Chen XC, Chen X, Chen P, Li J, Deng HX, Wang CT, Xie G, Huang S, Mao YQ, Chen LJ, Zhao X, Wei YQ (2009b) Intratumoral expression of mature human neutrophil peptide-1 mediates antitumor immunity in mice. Clin Cancer Res 15(22):6901–6911. doi:10.1158/1078-0432.CCR-09-0484

Wang YS, Li D, Shi HS, Wen YJ, Yang L, Xu N, Chen XC, Chen X, Chen P, Li J, Deng HX, Wang CT, Xie G, Huang S, Mao YQ, Chen LJ, Zhao X, Wei YQ (2009c) Intratumoral expression of mature human neutrophil peptide-1 mediates antitumor immunity in mice. Clin Cancer Res 15(22):6901–6911. doi:10.1158/1078-0432.CCR-09-0484

Wong JH, Ng TB (2005a) Lunatusin, a trypsin-stable antimicrobial peptide from lima beans (Phaseolus lunatus L.). Peptides 26 (11):2086–2092. doi:10.1016/j.peptides.2005.03.004

Wong JH, Ng TB (2005b) Sesquin, a potent defensin-like antimicrobial peptide from ground beans with inhibitory activities toward tumor cells and HIV-1 reverse transcriptase. Peptides 26 (7):1120–1126. doi:10.1016/j.peptides.2005.01.003

Wu DD, Gao YF, Qi YM, Chen LX, Ma YF, Li YZ (2014) Peptide-based cancer therapy: opportunity and challenge. Cancer Lett 351(1):13–22. doi:10.1016/j.canlet.2014.05.002

Yamada Y, Shinohara Y, Kakudo T, Chaki S, Futaki S, Kamiya H, Harashima H (2005) Mitochondrial delivery of mastoparan with transferrin liposornes equipped with a pH-sensitive fusogenic peptide for selective cancer therapy. Int J Pharm 303(1–2):1–7. doi:10.1016/j.ijpharm.2005.06.009

Yang WH, Luo DF, Wang SX, Wang R, Chen R, Liu Y, Zhu T, Ma XY, Liu RH, Xu G, Meng L, Lu YP, Zhou JF, Ma D (2008) TMTP1, a novel tumor-homing peptide specifically targeting metastasis. Clin Cancer Res 14(17):5494–5502. doi:10.1158/1078-0432.CCR-08-0233

Yi ZF, Cho SG, Zhao H, Wu YY, Luo J, Li DL, Yi T, Xu X, Wu ZR, Liu M (2009) A novel peptide from human apolipoprotein(a) inhibits angiogenesis and tumor growth by targeting c-Src phosphorylation in VEGF-induced human umbilical endothelial cells. Int J Cancer 124 (4):843–852. doi:10.1002/ijc.24027

Zhao BX, Zhao Y, Huang Y, Luo LM, Song P, Wang X, Chen S, Yu KF, Zhang X, Zhang Q (2012) The efficiency of tumor-specific pH-responsive peptide-modified polymeric micelles containing paclitaxel. Biomaterials 33(8):2508–2520. doi:10.1016/j.biomaterials.2011.11.078

Zheng LH, Wang YJ, Sheng J, Wang F, Zheng Y, Lin XK, Sun M (2011) Antitumor peptides from marine organisms. Marine Drugs 9(10):1840–1859. doi:10.3390/md9101840

Chapter 5
Plant Antimicrobial Peptides

Ravinder K. Goyal and Autar K. Mattoo

Abstract Disease afflicts crop productivity as well as nutritional attributes. Pathogens have the ability to mutate rapidly and thereby develop resistance to pesticides. Despite plant's multilayer of innate defence against pathogens, often the latter are able to penetrate and establish themselves on plant host. The discovery of antimicrobial peptides (AMPs) has the promise of durable defence by quickly eliminating pathogens through membrane lysis. AMPs characteristically are made up of from fewer than 20 amino acids to about 100 amino acids, and yet are structurally diverse. AMPs in plants are classified into cyclotides, defensins, lipid transfer proteins (LTPs), thionins, snakins, hevein-like peptides, knottin-type peptides, and others. It is important to characterize and study mechanism of their action in order to develop a wide range of structures with the potential to provide durable plant immunity against pathogens. We bring together recent information on the mechanisms by which AMPs are able to help the plant to thwart pathogen attack. Although permeabilizing cellular membrane is a major mechanism known for AMP action, new and diverse modes of action have recently been unearthed, including targeting of intracellular function of the pathogen.

5.1 Introduction

A serious impediment to sustainable production and yield of crops is the major loss due to environmental factors be it of abiotic or biotic nature. The latter factors mainly involve pathogens and pests which regularly pose significant threat to food security globally. Pathogens find unique ways to establish themselves on their plant

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hosts, particularly because of their variability, diversity, and ability to mutate. The host plant, in return, also employs a medley of processes in response to pathogen attack, but such multilayer nature of host defence also eventually capitulates. R-gene regulated pathogen resistance is well known in plants (Chisholm et al. 2006). Breeders employed selection pressure for identifying resilient cultivars with robust 'R' resistance factors and incorporated such resistance in high yielding cultivars. However, such strategy works as long as the 'R' resistance does not break down. The other caveat is that it is often a slow process and restricted to closely related species. Thus far, elite breeding lines together with the use of chemical pesticides have contained plant diseases to a large extent. Unfortunately, regular and excessive pesticide use has led to environmental and human health issues.

Considerable attention has also been given to understanding plant-pathogen interactions in order to highlight plant genes that durably respond to a pathogen ingression in order to develop durable disease resistance through 'innate' immunity. In addition to hypersensitive defence response and 'R' resistance proteins, plants also employ barriers through the cell wall and synthesize antimicrobial peptides (AMPs). The defence employed through the AMPs has generated much attention as also the recombinant technology as potential alternative strategies to contain pathogens from devouring their host plants.

5.2 Structure and Classification

Small peptides are generally made in a cell either as precursor proteins or non-precursor proteins. The precursor protein can possess functional significance or be nonfunctional. The term nonfunctional is used for those having no known biological activity, as for nonfunctional precursors. Interestingly, small peptides form, in certain instances, a part of plant proteins, somewhat buried within the long stretch. Such buried peptides have a distinct biological activity. The non-precursor-derived peptides encoded by sORFs are located in or near five regions of a gene (Tavormina et al. 2015). Some antimicrobial peptides (AMPs) are made as precursor proteins that need to be processed to produce a functional peptide.

AMPs are grouped according to their origin, primary and secondary structure, and the presence of disulfide linkages or net charge. Some have either α helical, β sheets or both αβ secondary structures. Majority of AMPs are rich in basic amino acids providing them a net positive charge at physiological pH and are called cationic AMPs. AMPs are made of fewer than 20 amino acids to about 100 amino acids. Details on each AMP category or family of peptides have been reviewed (Stotz et al. 2013; van der Weerden et al. 2013; Nawrot et al. 2014). As mentioned above, peptides have also been classified based on their synthesis as precursor proteins and/or posttranslational processing into mature peptides (Tavormina et al. 2015). A brief description of the prominent families of AMP members is given below. The 3D ribbon structures of representative AMPs categorized according to the prevalent system are illustrated in Fig. 5.1.

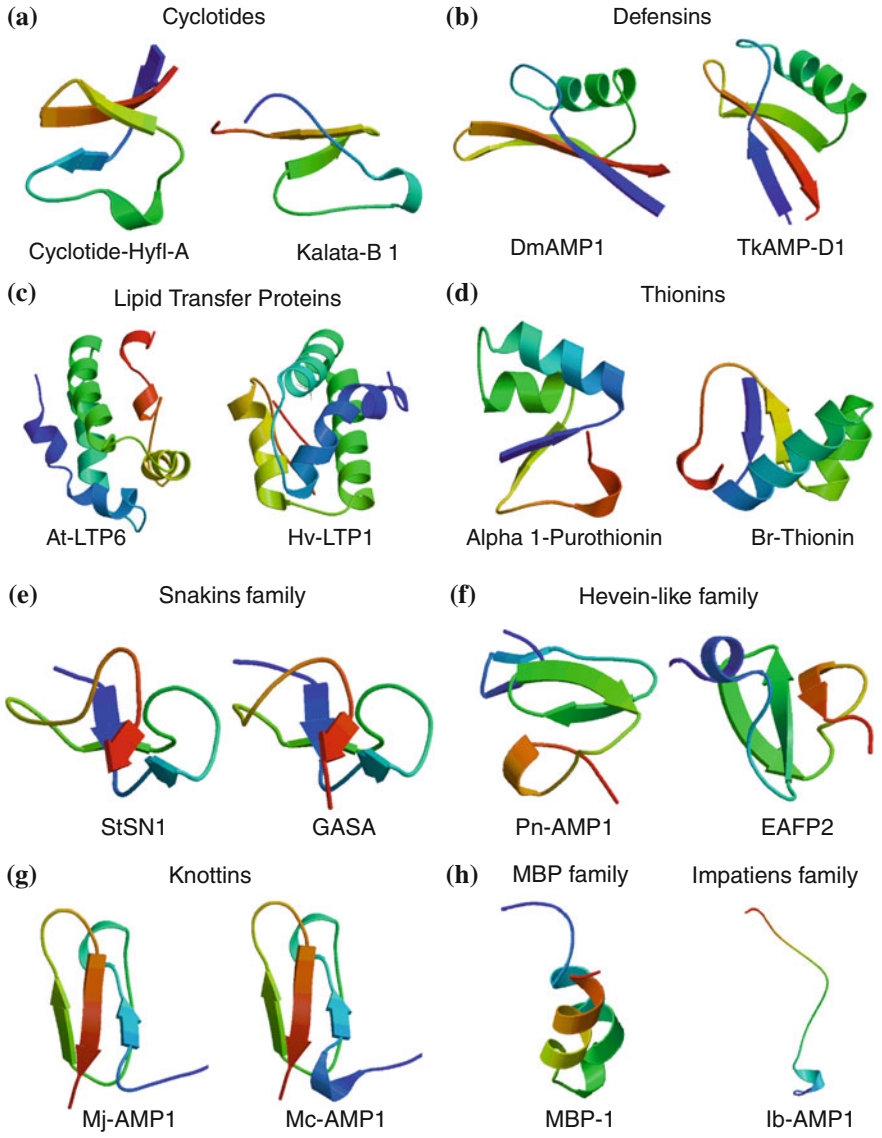

Fig. 5.1 a–h 3D ribbon structures of different family members of plant AMPs. The structures were computed using SWISS-MODEL. Cyclotide Hyfl-A: *Hybanthus floribundus* (P84647), Kalata-B1: *Oldenlandia affinis* (P56254), DmAMP1: *Dahlia merckii* (P0C8Y4), Tk-AMP-D1: *Triticum kiharae* (P84963), At-LTP6: *Arabidopsis thaliana* (Q9LDB4), Hv-LTP1: *Hordeum vulgare* (A8YPK3), Alpha-1-Purothionin: *Triticum aestivum* (P01543), Br-Thionin: *Brassica rapa subsp. pekinensis* (Q9SBK8), StSN1: *Solanum tuberosum* (Q948Z4), GASA: *Fagus sylvatica* (Q0VYL5), Pn-AMP1: *Ipomoea nil* (P81591), EAFP2: *Eucommia ulmoides* (P83596), Mj-AMP1: *Mirabilis jalapa* (P25403), Mc-AMP1: *Mesembryanthemum crystallinum* (O81338), MBP-1: *Zea mays L.* (P28794), Ib-AMP1: *Impatiens balsamina* (O24006). AMP name: plant name (GenBank or UniProt ID)

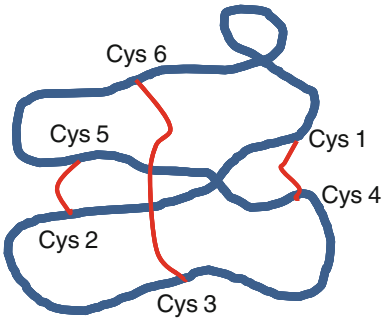

Fig. 5.2 Cyclotide backbone connected through three disulfide bonds at designated cysteine residues to create a CCK motif

5.2.1 Cyclotides

Cyclotides form the largest family of plant AMPs. Violaceae and Rubiaceae families are the richest source of cyclotides but they have also been detected in Fabaceae, Cucurbitaceae, Poaceae, and Solanaceae families. These AMPs are characterized by their unique structure where N- and C-termini are attached through a peptide bond to form a cyclic backbone (Fig. 5.1a). The cyclic structure is made of approximately 30 amino acids, which contains six cysteine residues engaged in three internal disulfide bonds to give it a cyclic cystine knot (CCK) structural motif (Fig. 5.2). The CCK motif provides extraordinary stability to the peptide as also resistance against proteases. Its surface exposed hydrophobic amino acids influence its antimicrobial activity. In addition to cysteine residues, Glu in loop 1 is highly conserved. Its ability to form hydrogen bond contributes to the cyclotides activity. The ribbon model of two cyclotides, kalata B1 and cyclotide-Hvfl A is presented in Fig. 5.1a. The cyclotides are synthesized as precursor molecules with a conserved signal for endoplasmic reticulum (ER) along with pro-region and a highly conserved N-terminal repeat (NTR). The presence of NTR in multiple numbers can lead to multiple molecules of cyclotides. The structure, isolation, and synthesis of cyclotides have been recently reviewed (Burman et al. 2014).

5.2.2 Defensins

Defensins, representing another large family of AMPs, are widely distributed in plant species. Defensins are the best studied Cys-rich peptides. Initially thought to be localized to seeds, their distribution in almost all plant organs has since become apparent. The defensins are synthesized as two types of precursor molecules. Majority of the defensin precursors contain ER sequence and a mature ‘defensin

domain'. In another category, the precursors are larger in size and contain an additional C-terminal prodomain (Aerts et al. 2008). Defensins are rich in cysteine content, carry a net positive charge, and constituted between 45–54 amino acids. The conserved eight cysteine residues with disulfide bridges favour triple-stranded antiparallel β-sheets and one α-helix structure (Fig. 5.1b). One disulfide bond near N- and C-termini provides extraordinary stability to the peptide. Besides four disulfide bonds present in a majority of defensins, an additional Cys–Cys has been noticed in *Petunia hybrida* peptide (PhD1). The core conserved structure of the defensin is maintained even with an additional disulfide bond. In addition to conserved cysteines, glycine residue (near fifth) and second cysteine residue are conserved with a high priority for an aromatic amino acid before second conserved cysteine residue (Fig. 5.3). The integrity of disulfide bonds and structural conformation is essential for antimicrobial activity but the stability of the structure does not directly correlate with the activity. Structure–activity relationship of defensins has been reviewed (Sagaram et al. 2011; Lacerda et al. 2014). A majority of the

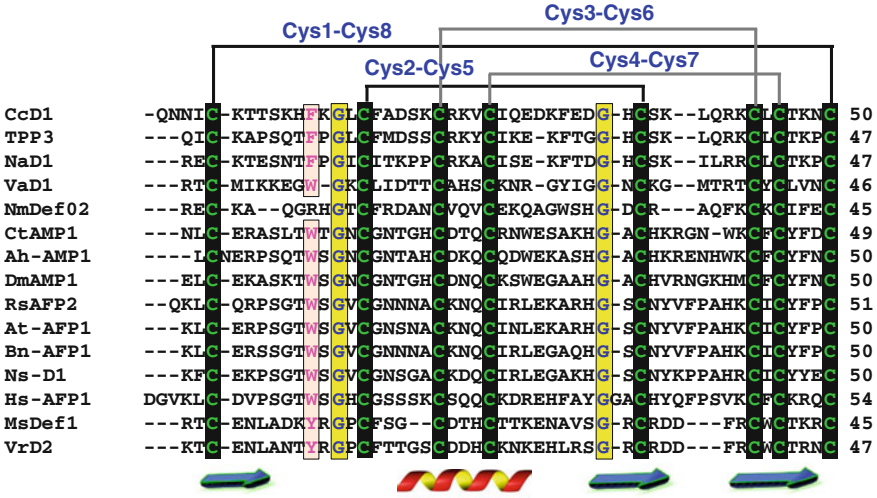

Fig. 5.3 Alignment of amino acid sequence of plant defensins. CcD1: *Capsicum chinense* (Af128239), TPP3: *Lycopersicon esculentum* (4UJ0), NaD1: *Nicotiana tabacum* (P32026), NmDef02: *Nicotiana megalosiphon* (ACR46857), Ct-AMP1: *Clitoria ternatea* (AAB34971), Ah-AMP1: *Aesculus hippocastanum* (Q7M1F3), DmAMP1: *Dahlia merckil* (P0C8Y4), At-AFP1: *Arabidopsis thaliana* (P30224), Bn-AFP1: *Brassica napus* (Q39313), Ns-D1: *Nigella sativa L.* (P86972), Rs-AFP2: *Raphanus sativus* (P30230), Hs-AFP1: *Heuchera sanguinea* (AAB34974), MsDef1: *Medicago sativa* (Q9FPM3), VrD2: *Vigna radiata* (2GL1). The residues enclosed in eight black bars represent highly conserved cys. The *solid lines* indicate disulfide bonds engaging the two cys residues. The two highly conserved gly residues are shown in *greenish yellow*. The preference for an aromatic amino acid before 1st conserved gly is labelled in *pink*. The *arrows* represent β-sheet and a helix represents α-helical secondary structures corresponding to the amino acids above them. AMP name: plant name (GenBank or UniProt ID)

defensins possesses activity against diverse range of fungi and oomycetes, but some members are toxic to bacteria.

5.2.3 Lipid Transfer Proteins

The nonspecific, plant lipid transfer proteins (nsLTPs) were first isolated from potato tubers and later discovered from a wide range of monocotyledonous (monocots) and dicotyledonous (dicots) species. nsLTPs represent small proteins deriving their name from their function of transferring lipids between the different membranes as well as in vitro. They carry lipids nonspecifically, the list includes phospholipids, fatty acids, their acylCoAs or sterols. LTPs with approximately 100 amino acids are relatively larger in size than defensins. Depending on their size, LTPs are subcategorized into LTP1s and LTP2s having a molar mass of 9 and 7 kDa, respectively. These are synthesized with an N-terminal signal sequence directing them to cell walls. Some LTPs possess a C-terminal sequence which enables their posttranslational modification with a glycosylphosphatidylinositol molecule. The latter facilitates the integration of LTP on extracellular side of the plasma membrane. LTPs are structured with eight cysteine residues forming four disulfide bridges like defensins. However, LTPs are distinct in having four α-helices in their tertiary structure (Fig. 5.1c), which carve out a hydrophobic cavity to bind the lipids through hydrophobic interactions. A different arrangement of cysteine residues in disulfide bonds results in two types of folds—Type 1 and Type 2. These folds provide different specificity of lipid binding at the LTP binding site with Type 2 fold relatively more flexible and with lower lipid specificity than Type 1.

5.2.4 Thionins

The first plant thionin AMP was isolated in 1942 from wheat flour and labelled as purothionin. Thionins are yet another class of cysteine-rich peptides that are present in a wide range of plants. They are smaller in size, ~5 kDa containing 45–47 amino acids. Thionins comprise of two distinct groups of plant peptides —α/β-thionins and γ-thionins with distinguished structural features. Based on γ-thionins' more resemblance with defensins than the other group of thionins, it has been suggested that they should be placed along with defensins (Stec 2006). Both groups of peptides share about 25 % sequence similarity. Thionins, rich in basic amino acids providing the peptides a net positive charge, have highly conserved Lys1, Arg10 and Tyr13 in addition to six Cys residues. The secondary structure contains two antiparallel α-helices and an antiparallel β-sheet (Fig. 5.1d). α/β-Thionins are further divided into five sub-types based on the number of disulfide bonds, net charge, length, or the origin. Thionins I and II contain eight Cys residues bonded with each other to make four disulfide bridges. Type I are more

basic and contain 45 amino acids compared to Type II, which have 46–47 amino acids. Type III are 45–46 amino acids long, containing three disulfide bridges and being basic as the Type IIs. Like Type III, the Type IV thionins have three disulfide bonds but possess no charge at neutral pH. Type V are the truncated forms of thionins demonstrating no activity. Thionins are synthesized as precursor molecules and demonstrate antimicrobial activity after acidic C-terminal domain is removed (Ponz et al. 1983). Interestingly, an unprocessed thionin has been identified in Arabidopsis, providing an example of peptides derived from a functional protein, without the involvement of a precursor (Tavormina et al. 2015). Further, a thionin proprotein processing enzyme has been isolated and characterized from barley that releases the acidic domain of leaf-specific thionin (Plattner et al. 2015). Thionins have broad-spectrum antimicrobial activity targeting bacterial, fungal and mammalian cells.

5.2.5 *Snakins*

Snakins too are cysteine-rich peptides differing from other cysteine-abundant AMPs in having relatively more number of disulfide bonds. As the name suggests, there is a structure motif similarity between snakins and the hemotoxic desintegrin-like snake venoms. The first snakin, Snakin-1 (StSN1), was isolated from potato tubers (Segura et al. 1993). The sequence of 63 amino acids long StSN1 did not relate to previously purified protein sequence. Instead, it depicted homology with some sequences deduced from plant cDNAs that were induced by the plant hormone gibberellic acid. This led to their being categorized as GASA (gibberellic acid stimulated in Arabidopsis) protein family. Later, another snakin, Snakin-2 (StSN2) was identified in potato tubers that was inducible by certain phytopathogens. The precursor form of StSN2 is processed into 66 amino acids long peptide that has low identity (38 %) with StSN1. The members of snakin/GASA family contain N-terminal signal sequence of 15–20 residues followed by a variable region both in terms of length, amino acid composition and a region of approximately 60 residues at C-terminus. The latter contain 12 Cys conserved residues forming six disulfide bonds and nine other conserved amino acids. Later studies found snakins expressed in different plant organs and widely spread in both monocots and dicots. Like other AMPs, the mature snakins are enriched in basic amino acids and thus are positively charged. The 3D Swiss-Models of some snakins depicted only β-sheets and the absence of α-helices was conspicuous (Fig. 5.1e).

5.2.6 *Hevein-like Peptides*

Hevein was discovered in the latex of rubber tree (*Hevea brasiliensis*). Due to its chitin-binding property, it inhibits the hyphal growth and confers protection against

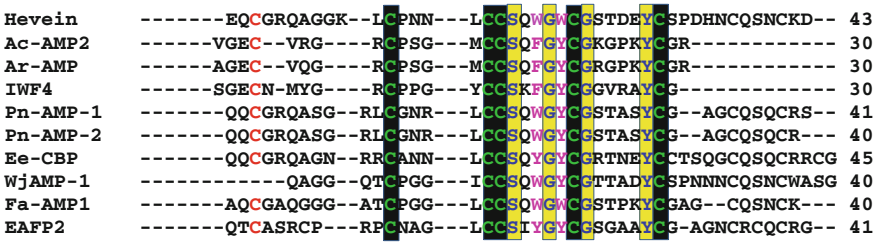

Fig. 5.4 Alignment of amino acid sequences of Hevein-like peptides. Hevein: *Hevea brasiliensis* (P02877), Ac-AMP2: *Amaranthus caudatus* (Q9S8Z7), Ar-AMP: *Amaranthus caudatus* (Q512B2), IWF4: *Beta vulgaris*, Pn-AMP1: *Ipomoea nil* (P81591), Pn-AMP2: *Ipomoea nil* (P81591), Ee-CBP: *Euonymus europaeus* (Q7X9R9), WjAMP-1: *Eutrema wasabi* (Q8H950), Fa-AMP-1: *Fagopyrum esculentum* (P0DKH7), EAFP2: *Eucomia ulmoides* (P83596). The highly conserved cys residues are enclosed in *black bars*. The cys residues labelled in *red* depict a nearly conserved cys at this position. The other conserved amino acids (ser, gly, and tyr) are shown in *greenish yellow*. The preference for an aromatic amino acid before and after conserved gly is labelled in *pink*. AMP name: plant name (GenBank or UniProt ID)

fungal phytopathogens (Van Parijs et al. 1991). Hevein is a relatively small peptide with 43 residues (4.7 kDa) and contains 3–5 disulfide bridges. The other chitin-binding peptides that display similarity in their antifungal activity but differ in amino acid composition from Hevein are grouped in hevein-like peptides. Several hevein-like peptides have been isolated from plants, including *Beta vulgaris* (IWF4), *Pharbitis nil* (Pn-AMP1) and *Eucommia ulmoides* (EAFP2). These peptides contain 6, 8 and 10 Cys residues, respectively. The 3D structure of hevein-like peptides contains three antiparallel β-sheets (Fig. 5.1f). The presence of α-helical turns varies from peptide-to-peptide. The sequence alignment of hevein-like peptides shows five Cys residues that are highly conserved and another towards N-terminus is nearly conserved (Fig. 5.4). The four conserved Cys residues near C-terminus are part of a conserved domain in the peptides, which seems to follow a pattern of Cys–Cys–Ser–X–(aromatic amino acid)–Gly–(aromatic amino acid)–Cys–Gly–X_4–Tyr–Cys. Initially, binding to chitin in fungus cell wall was thought to be an essential property of hevein-like peptides. However, when other hevein-like peptides were isolated (e.g. Pn–AMP1 and EAFP2) these were found to target fungi irrespective of the presence of chitin (see van der Weerden et al. 2013). Pn–AMP1 being highly basic (pI 12.02), with a net positive charge belongs to a broad category of cationic AMPs. The distribution of hevein-like peptides from aerial parts of plants to seeds indicate this group of peptides may contribute to plant immune system.

5.2.7 Knottin-Type Peptides

Knottin peptides contain six Cys residues forming three disulfide bonds with one disulfide bond crossing through the other two like in cyclotides. Like with

cyclotides, the Cys-stabilized structure supports a knotted fold. However, the free N- and C-termini make them distinct from cyclotides (Fig. 5.1g). The first plant knottin-type peptides, Mj-AMP1 and Mj-AMP2, were identified from *Mirabilis jalapa* L. (Cammue et al. 1992), and subsequently from *Phytolacca americana* (PAFP-S) and *Mesembryanthemum crystallinum* (Mc-AMP1). These peptides are synthesized as precursor proteins and after maturation display antimicrobial activity against both fungi and bacteria. The structures of Mj-AMP1 and Mc-AMP1 consist of triple-stranded, antiparallel β-sheets connected through a loop (Nawrot et al. 2014).

5.2.8 Other AMPs

There are several other AMPs that do not relate with the abovementioned categories of peptides but possess unique amino acid composition or secondary structures. Some of them are named after the plant source from where they were isolated. For instance, 1b-AMP1 (Impatiens family) and Shepherin-1 (Shepherin family) were isolated from *Impatiens balsamina* and *Capsella bursa-pastoris* (Shepherds purse), respectively. The others include vicilin-like, 2S albumin peptides, MBPs, puroindolines, hairpinins, β-barrelins, glycine-rich cysteine-free, and glutamic acid-rich peptides. A 3D structure of two such AMPs is shown in Fig. 5.1h. A recently isolated peptide from *Benincasa hispida* seeds called Hispidulin containing 49 residues does not show homology with any known sequence in the database. As we make more discoveries, the number of unique peptides that do not share similarity with known peptides is likely to grow. A wide spectrum of peptides should in the future provide a better classification rationale.

5.3 Mechanism(s) of Action

Most of the AMPs are able to target several different fungal and bacterial pathogens. This broad-spectrum ability suggests that AMPs interfere with the structural and/or functional cellular components essential for the survival and proliferation of a pathogen. The protective cell wall of a microorganism is likely the first contact point with AMPs. The fungal cell wall is complex, assembled in many layers comprising 80 % heteropolysaccharides (Fig. 5.5a). The inner most layer or plasma membrane is composed of lipid bilayer with interspersed proteins surrounded by chitin and β-glucan layers. The outer envelope is made mainly of mannosylated glycoproteins that aid in host cell wall receptor recognition and interaction. A Gram-negative bacterial cell wall contains inner lipid bilayer membrane surrounded by a thin layer of peptidoglycan (Fig. 5.5b). Additionally, there is an outer membrane composed of phospholipids and lipopolysaccharides. The latter are highly charged molecules providing a net negative charge to the membrane surface.

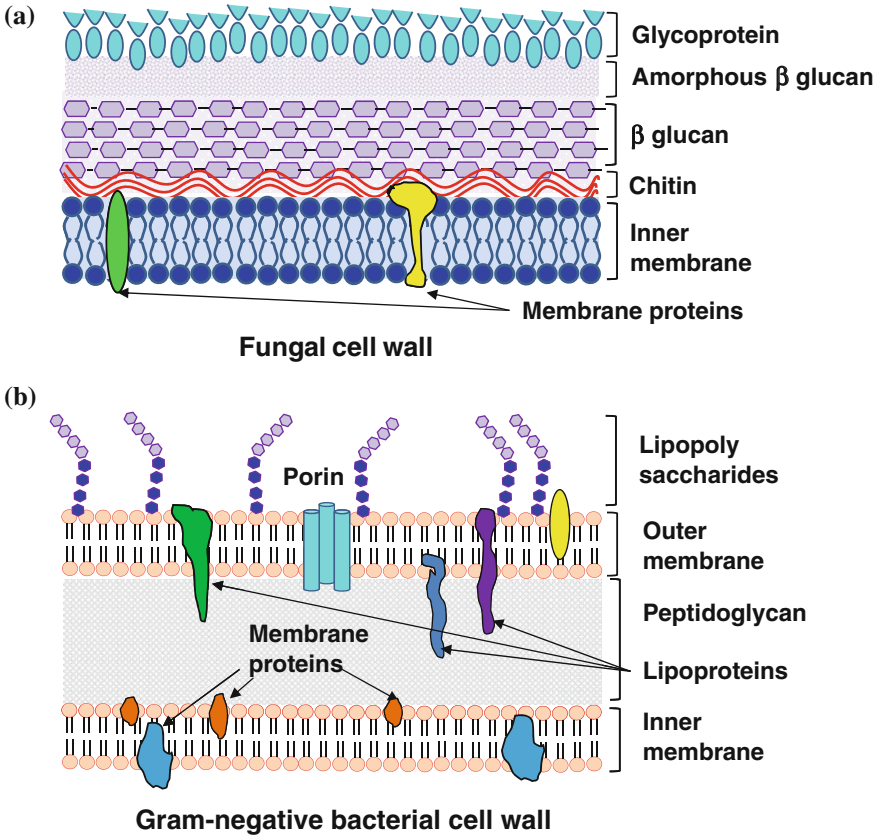

Fig. 5.5 Composition of typical fungal and Gram-negative bacterial cell walls

The Gram-positive bacteria, however, contain only a very thick layer of peptidoglycan adjacent to inner membrane. The enrichment of peptidoglycan layer with acidic polysaccharides such as teichoic and teichuronic acids confers a negative charge to the membrane. The plant cell wall has distinguishable features of cellulose, hemicellulose, lignins and the absence of peptidoglycan, chitin and β-glucan. In mammalian membrane phospholipids, phosphatidylcholine and phosphatidylethanolamine are neutral in charge.

AMPs from plants or mammalian sources can distinguish the host from its microbial targets. The studies indicate that structural disparity of prokaryotic and eukaryotic membranes contribute towards the AMP selectivity (Zasloff 2002; Yeaman and Yount, 2003; Yount and Yeaman 2013). In spite of significant structural differences in prokaryotic organisms, the AMPs are known to establish interaction at the surface of these microbes. The structure of AMPs with hydrophobic regions and net positive charge supports the interaction with negatively-charged polar heads and hydrophobic core of the microbial membranes.

A high positive charge in cationic AMPs forges an electrostatic interaction, thus facilitating their initial binding to the membranes.

The structure of an AMP is critical to its antimicrobial activity. There are several structural parameters such as conformation, charge, hydrophobicity, hydrophobic moment, amphipathicity and polar angle that contribute to the toxicity and target specificity. The topic has been comprehensively reviewed (Yeaman and Yount 2003). Experimental evidence showed the presence of specific binding sites for AMPs on targeted pathogen envelope. For example, mannosylinositol phosphoryl-ceramide, an acidic complex sphingolipid in fungal cell wall, was identified as a high-affinity binding target for a defensin (DmAMP1) from *Dahlia merckii* (Thevissen et al. 2003). Thus, it would mean that the binding of an AMP to a pathogen is not necessarily dictated only by electrostatic interactions but also recognition of specific cell wall component(s). Once the contact has been made via an initial interaction and subsequent binding of an AMP with the target, the toxic effect on the pathogen can then be exerted in two broad ways, membrane permeabilization and impairment of intracellular functions as discussed below.

5.3.1 Membrane Permeabilization

Membrane permeabilization occurs after an AMP interacts with the target site of the pathogen. It results in dissipation of electrochemical gradient across the membrane, membrane fragmentation, leakage of ions and other cellular contents and ultimately cell death (Shai 2002). A threshold concentration of an AMP is required for inducing permeabilization and the phenomenon is time-dependent (Wimley 2010). More structural deformity of the membrane occurs over time. Membrane permeabilization can occur in different ways depending on the interaction dictated by the structure of an AMP. Models have been proposed to explain the disruptive effect of AMPs on the membranes (Fig. 5.6).

5.3.1.1 Barrel-Stave Model

In this model, AMP molecules bind to the target membrane as monomers. After self-aggregation, the molecules get inserted across the membrane to form a transmembrane pore (Fig. 5.6b). The hydrophobic regions of α-helix or β-sheets of an AMP align with the hydrophobic core of target membrane and hydrophilic surfaces form the lining or lumen of the pore. The peptides engaged in a pore are oriented parallel to the lipid bilayer. The pore size may vary depending on the peptide and the degree of aggregation. It can be further expanded in a cooperative manner by assembling more molecules into the pore. A case study with alamethicin, a 20-residue peptide produced by fungus *Trichoderma viride*, lent evidence in favour of the Barrel-Stave pore mechanism.

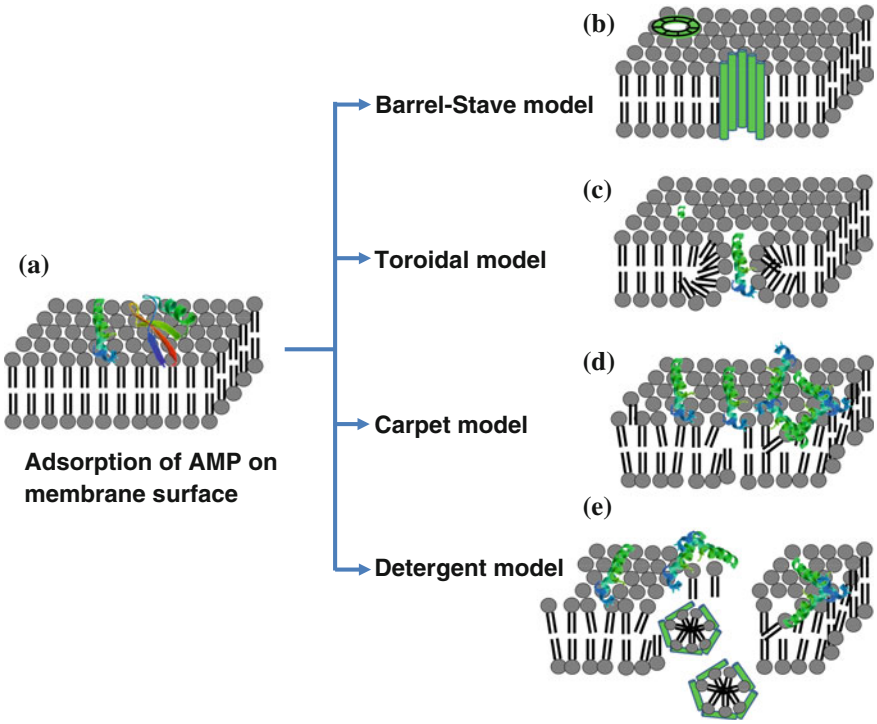

Fig. 5.6 Models of membrane permeabilization by antimicrobial peptides. After initial attachment of an AMP on the membrane surface (**a**), it can disrupt the membrane structure/function either through pore formation (**b, c**) or by other mechanisms (**d, e**)

5.3.1.2 Toroidal Model

The Toroidal model or Wormhole mechanism is also a pore forming way of membrane disruption. Its major difference from the Barrel-Stave model is that the AMP intercalation in lipid bilayer induces positive curvature of phospholipid polar heads perpendicular to the membrane plane. The peptide provides a stronger alternative of both hydrophobic and hydrophilic interactions than intramolecular interactions of lipid molecules. The presence of a peptide, thus, breaks hydrophobic–hydrophobic interactions of lipid molecules and favours their realignment to create toroidal pores (Fig. 5.6c). In contrast to Barrel-Stave model, the lipid headgroups in toroidal pores are exposed to the lumen of a pore. In vitro studies with peptide and membrane vesicles have suggested that the threshold of peptide-to-lipid (P/L) ratio for magainin is 1:30, which is consistent with the micromolar quantities of peptides required for their toxic effect on pathogen membranes.

5.3.1.3 Carpet Model

Carpet model is a non-pore forming mechanism of AMP action. In contrast to forming pores, the peptide does not insert into the hydrophobic core of the membrane as observed in pore inducing models but instead orients itself parallel to the membrane surface and covers it like a carpet. A strong electrostatic interaction between negatively charged phospholipid polar headgroups and the cationic peptide distorts the structure of the membrane and its fluidity (Fig. 5.6d). As the peptide reaches its threshold membrane disintegration or cell lysis is induced. The model was first proposed to explain the toxic effect of a moth hemolymph cecropin P1, which aligns parallel with the membrane surface. Its activity was noticeable only at relatively high concentrations or at high P/L ratio. Dermaseptins from *Phyllomedusa spp* are thought to follow carpet model to induce membrane damage.

5.3.1.4 Detergent Model

This is an extended version of the carpet model of AMP action. The peptide interacts through a mechanism similar to the carpet model, leading to catastrophic collapse of the membrane. The peptide molecules form micelles with the fragmented membrane like a detergent (Fig. 5.6e). The comprehensive breakdown of the membrane cannot hold its contents and results in cell death.

5.3.2 Impairment of Intracellular Functions

Membrane permeabilization is considered an important attribute of antimicrobial activity. However, increasing evidence suggests other modes of AMP action in addition to disruption of membrane functions (Brogden 2005; Muñoz et al. 2013). The degree of permeabilization for some peptides did not correlate with their activity, in some the microorganism survived for an extended time period after membrane disruption. In another study, the active fragments of a bovine peptide Bac7 did not permeabilize the *Escherichia coli* membrane but a 2–5 log reduction in viable cell count was apparent (Gennaro and Zanetti 2000). In vitro studies showed the ability of an AMP to associate with intracellular targets such as nucleic acids, proteins or enzymes, which suggested a mechanism of action other than merely involving the membranes. It is now accepted that AMP action is a combined outcome of membrane permeabilization and inhibition of intracellular functions. To facilitate internalization of the peptide into cytoplasm there could be either transient or permanent disruption of the membrane structure, which may enhance the lethal effect of an AMP. It is not known how much contribution membrane

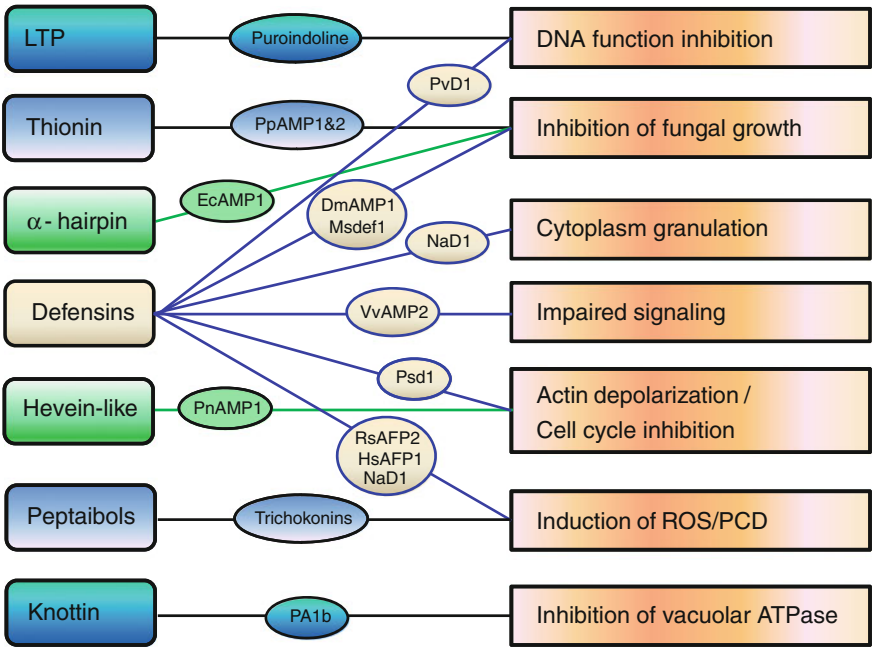

Fig. 5.7 Plant AMPs targeted cellular functions. The name in *oval* shape represents AMPs, which are connected by *solid lines* to the class they belong to (on the *left*) and their intracellular targets (on the *right*)

permeabilization makes to the potency of such peptides. Other possibilities such as passive peptide transport (Henriques et al. 2006) and/or an active energy-dependent process (Kim et al. 2001) are meaningful. Many plant antifungal defensins interact with fungal membrane sphingolipids or phospholipids. Subsequently, some of them are internalized and induce cell death. For example, cellular uptake for MtDef4, NaD1 and Psd1 has been observed.

Mechanistic details of action for some defensins have been reviewed (Vriens et al. 2014). Once inside the cell, an AMP can target cellular organelles and affect their associated functions. Several plant AMPs have been identified that display cellular toxicity through diverse modes of action. Detailed information on the targets and pathogens have been reviewed (Goyal and Mattoo 2014). A brief summary is illustrated in Fig. 5.7. Different members of AMPs interact with diverse intracellular targets and interfere with their functions. Also, different intracellular functions can be targeted by the same class of AMPs. There is a large repertoire of plant AMPs that target a variety of cellular functions (Goyal and Mattoo 2014).

5.4 AMP-Mediated Defence Is Highly Conserved

Protection against life-threatening challenges is a critical determinant of self-survival. Thus defensive mechanisms evolve as organisms combat living in changing environments. Based on organisms and their growth environment some of the defensive mechanisms are widespread although some are unique to a certain class of organisms. In addition to other modes of protection, plants have developed a hypersensitive defence response (HR) against biotic and abiotic stresses. In HR, plants recognize pathogen's presence through structural signatures called pathogen-associated molecular patterns (PAMP) via transmembrane receptors such as receptor-like kinases/proteins. Once the process of recognition is established, a cascade of signalling is initiated to mount a comprehensive defence against the pathogen. The immunity conferred through this mechanism is referred to as PAMP-triggered immunity. In a *tug-of-war* with the pathogen there is an evolutionary trail to keep the effectiveness of defence in place. The plants have evolved strategies to counter the evasion mechanisms developed by the pathogen (Chisholm et al. 2006). It is expected that any type of defence would require allocation of resources in proportion to its magnitude. The comprehensive changes associated with HR defence to pathogens divert the resources that would otherwise be used for growth and development of the plant. Consequently, biotic stresses lead to reduced plant productivity by down-regulation of photosynthetic genes and reduced photosynthetic activity. Thus, in HR defence of plant immunity, there is a fitness cost associated with heightened defence response (Brown 2002; Bolton 2009). Perhaps, this explains why HR, which is an effective response to contain the pathogen, is not all-time-deployment (constitutive) defence feature but activated/induced only in response to a pathogenic or non-pathogenic threat to the plant.

Keeping in view the cost and benefit, the natural selection is likely to favour the retention of a defensive apparatus that has minimal maintenance cost but has high deterrence value. A defence through the deployment of AMPs likely incurs low cost taking into consideration their size and complexity—thus AMPs fit the criterion of a defensive apparatus that has minimal maintenance cost with a high deterrence value. It is therefore not surprising that, in addition to animal and plant sources, AMPs have been identified also in microorganisms including bacteria and fungi (Paiva and Breukink 2013). This reflects the ubiquitous presence of AMPs, ranging from microorganisms to higher eukaryotes.

Like eukaryotic AMPs, bacterial species and members of Archaea domain synthesize peptides with antimicrobial activity involving ribosomal machinery. These peptides are named bacteriocins, which are active against human and animals pathogens. The most commonly known bacteriocin is nisin, which is a 34-amino acid long, cationic and hydrophobic peptide produced by a Gram-positive bacterium. Interestingly, nisin uses membrane disruption of the target through pore formation as observed for eukaryotic AMPs. The negative charge on bacterial membrane lipids facilitates the binding of cationic nisin and subsequently peptide molecules aggregate along with lipids to create a pore. Besides being similar in their

mechanism of action, an evolutionary conservation is apparent in structural relatedness of fungal defensin-like peptides. The first defensin-like peptide, plectasin, identified from a saprophytic fungus, *Pseudoplectania nigrella* is structurally similar to defensins from primitive arthropods and molluscs. Like plant defensins, many fungal defensin-like peptides are cysteine rich and have α-helix and β-sheet structures. Among the six families of defensin-like peptides predicted by computational studies in fungal genome three families display high similarity with insect, invertebrate and plant defensins. A genetic closeness study between certain eukaryotic AMPs revealed a structural conservation in evolutionary divergent group of organisms suggesting minimal speciation events during evolution (Goyal and Mattoo 2014). These observations point to AMPs as integral components of innate immune defence in organisms early during evolution, while the retention of the close-to-basic form suggests their importance in the defence architecture of living organisms. A broad-spectrum activity and protective function across kingdoms highlights the importance of AMP-mediated defence.

5.5 AMP Potency Across Kingdoms

AMPs display effectiveness at low concentrations and relatively within short exposure times against pathogens. Their potency has been assessed through in vitro studies involving a purified candidate peptide and a targeted pathogen grown in culture media, and generally expressed as IC_{50} (a concentration of peptide required to inhibit 50 % growth) or as MIC (minimum inhibitory concentration: a minimum concentration of peptide required to completely inhibit the growth). The IC_{50} values for plant AMPs ranges from <1.0 to >100 μg/ml. For each AMP the IC_{50} value varies from pathogen to pathogen. The composition of growth media also affects the IC_{50} or MIC values. The effective in vivo concentrations of AMP that provide immunity against pathogens, however, are largely unknown. The IC_{50} or MIC values for some AMPs are given in Table 5.1.

AMPs are known to possess broad-spectrum antimicrobial activity. Both in vitro studies and in vivo expression of AMPs in transgenics suggested that the antimicrobial activity of peptides isolated from an organism is not restricted against its own pathogenic population but also well beyond the phylum or kingdom. This characteristic of AMPs is evident from their mode of action where AMPs target microbial cell walls in addition to binding to specific domains. The activity across the kingdoms has been observed in plant isolated AMPs, which showed antimicrobial activity against mammalian pathogens, including human ones. Conversely, AMPs isolated from insects, arthropods, amphibians, humans, etc., display toxicity against a variety of phytopathogens. In cross-kingdom scenarios, the AMPs do not exhibit cytotoxicity to the host cells. This property of AMPs has enhanced the scope of their application in disease management of humans or other mammals of

Table 5.1 Plant AMP IC50 or MIC values against specified microbial pathogens

Peptide	Class/family	Source	Pathogen	IC_{50} or MIC
Circulin-A	Cyclotide	*Chassalia parviflora*	*Staphylococcus aureus*	MIC: 0.19 μM
–	–	–	*Candida kefyr*	MIC: 18.6 μM
Circulin-C	Cyclotide	*Chassalia parviflora*	HIV-1	IC_{50}: 50–275 ηM
Kalata-B1	Cyclotide	*Oldenlandia affinis*	*Staphylococcus aureus*	MIC: 0.26 μM
–	–	–	*Pseudomonas aeruginosa*	MIC: >500 μM
–	–	–	*Candida albicans*	MIC: >500 μM
Rs-AFP2	Defensin	*Raphanus sativus*	*Pyricularia oryzae*	IC_{50}: 0.4 μg/ml
–	–	–	*Verticiliium dahliae*	IC_{50}: 1.5 μg/ml
–	–	–	*Alternaria brassicola*	IC_{50}: 2 μg/ml
Ah-AMP1	Defensin	*Aesculus hippocastanum*	*Bacillus subtilis*	IC_{50}: 100 μg/ml
–	–	–	*Leptosphaeria maculans*	IC_{50}: 0.5 μg/ml
Psd2	Defensin	*Pisum sativum*	*Neurospora crassa*	IC_{50}: <0.5 μg/ml
–	–	–	*Fusarium solani*	IC_{50}: 8.5 μg/ml
Ace-AMP1	LTP	*Allium cepa*	*Alternaria brassicola*	IC_{50}: 2.5 μg/ml
–	–	–	*Verticillium dahliae*	IC_{50}: 0.25 μg/ml
–	–	–	*Botrytis cinerea*	IC_{50}: 3 μg/ml
La-LTP	LTP	*Leonurus artemisia*	*Ralstonia solanacearum*	IC_{50}: 15 μM
–	–	–	*Botrytis cinerea*	IC_{50}: 7.5–15 μM
Cw18	ns-LTP	*Hordeum vulgare*	*Fusarium solani*	MIC: 174 μg/ml
Pp-AMP1	Thionin	*Phyllostachys pubescens*	*Erwinia carotovora*	IC_{50}: 22 μg/ml
–	–	–	*Clavibacter michiganensis*	IC_{50}: 14 μg/ml
–	–	–	*Fusarium oxysporum*	IC_{50}: 2 μg/ml
Tu-AMP1	Thionin	*Tulipa gesneriana*	*Erwinia carotovora*	IC_{50}: 11 μg/ml
–	–	–	*Fusarium oxysporum*	IC_{50}: 2 μg/ml
AX1	Thionin	*Beta vulgaris*	*Cercospora beticola*	MIC: 4 μg/ml
AC-AMP1	Hevein-like	*Amaranthus caudatus*	*Fusarium culmorum*	IC_{50}: 2 μg/ml
–	–	–	*Alternaria brassicola*	IC_{50}: 7 μg/ml
–	–	–	*Bacillus megaterium*	IC_{50}: 40 μg/ml

(continued)

Table 5.1 (continued)

Peptide	Class/family	Source	Pathogen	IC_{50} or MIC
Ee-CBP	Hevein-like	*Euonymus europaeus*	*Botrytis cinerea*	IC_{50}: 0.2 μM
–	–	–	*Alternaria brassicola*	IC_{50}: 0.6 μM
–	–	–	*Pythium ultimum*	IC_{50}: 6.6 μM
Fa-AMP1	Hevein-like	*Fagopyrum esculentum*	*Clavibacter michiganensis*	IC_{50}: 14 μg/ml
–	–	–	*Fusarium oxysporum*	IC_{50}: 19 μg/ml
–	–	–	*Geotrichum candidum*	IC_{50}: 36 μg/ml
StSN1	Snakins	*Solanum tuberosum*	*Listeria monocytogenes*	MIC: 10 μg/ml
–	–	–	*Botrytis cinerea*	IC_{50}: 2 μM
–	–	–	*Colletotrichum graminicola*	IC_{50}: 10 μM
MJ-AMP1	Knottins	*Mirabilis jalapa*	*Bacillus megaterium*	IC_{50}: 6 μg/ml
–	–	–	*Cercospora beticola*	IC_{50}: 10 μg/ml
–	–	–	*Ascochyta pisi*	IC_{50}: 200 μg/ml
Pa-AMP1	Knottins	*Phytolacca americana*	*Staphyanococcus sp.*	IC_{50}: 11 μg/ml
–	–	–	*Fusarium oxysporum*	MIC: 40 μg/ml
Ib-AMP4	Impatiens	*Impatiens balsamina*	*Micrococcus luteus*	IC_{50}: 5 μg/ml
			Penicillium digitatum	IC_{50}: 3 μg/ml
Shepherin I	Shepherin	*Capsella bursa-pastoris*	*E. coli*	IC_{50}: <2.5 μg/ml
–	–	–	*Fusarium culmorum*	IC_{50}: 72 μg/ml
MBP-1	MBP	*Zea maize*	*Fusarium graminearum*	MIC: 60 μg/ml
MiAMP2c-3	Vicilin-like	*Macadamia integrifolia*	*Phytophthora cryptogea*	IC_{50}: 5–10 μg/ml

Source http://phytamp.pfba-lab-tun.org/main.php (Hammani et al. 2009)

commercial interest as well as of plants. Interestingly, plant AMPs have shown promising results in specifically targeting the human cancerous cells. Some of the examples of cross protection by AMPs are illustrated in Fig. 5.8.

5.6 Production of AMPs in Plants

The therapeutic application of AMPs in medicine and their ability to protect plants from host of diseases generated interest in devising strategies to produce AMPs on a mass scale. A good number of peptide-based drugs have been approved by US

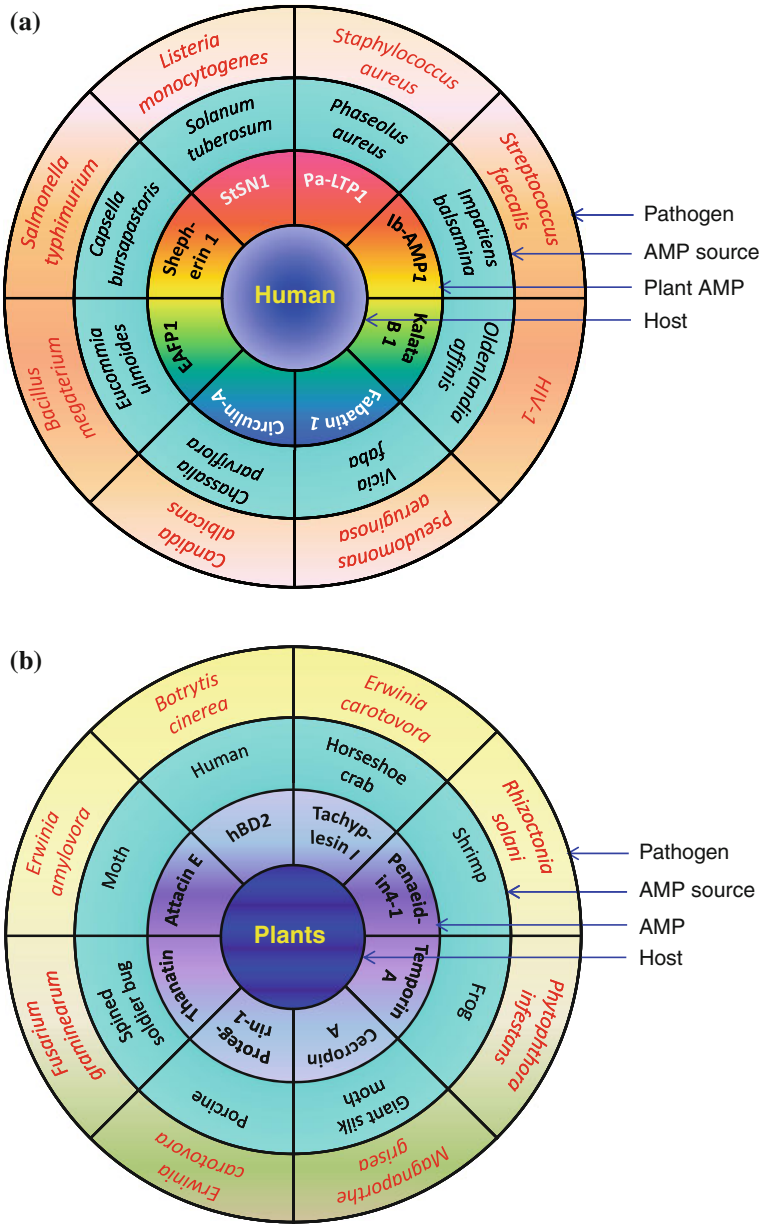

Fig. 5.8 AMPs showing antimicrobial activity in distantly related hosts to their pathogenic microbes. **a** The AMPs from different plant sources (2nd ring from the outside) active against a variety of human pathogens. **b** The AMPs from different animal sources possess activity against plant pathogens

Food and Drug Administration. Currently, majority of these peptides are produced through chemical synthesis using either *solid-phase or solution-phase* syntheses. Despite high cost of production and environmental effects, chemical synthesis continues to dominate the peptide synthesis industry. Chemical synthesis has advantage of incorporating non-natural components or performing other modifications. Alternate eco-friendly strategies such as biological sources have been explored to produce AMPs in large quantities. High yield, stability, solubility, ease of purification of proteins and scalability of the process are generally some of the criteria for any commercial process. Bacteria and yeast meet most of these criteria with the ease of being genetically transformed and therefore provide the most suitable choice to develop platforms for biomolecule production. However, AMPs are cytotoxic to microorganisms, especially bacteria, and this presents challenges for their deployment as biofactories. Nevertheless, when a snakin peptide SN1 was fused with thioredoxin, it was expressed in *E. coli.* The thioredoxin fusion increased the solubility of the expressed AMP while rendering it ineffective as a toxic compound. Yeast offers another avenue for improving AMP yields once the conditions are optimized. For example, an enhancement in yield of a specific peptide was obtained using a constitutive promoter of glyceraldehyde-3-phosphate dehydrogenase, which is essential to carbohydrate metabolism. Also, the protozoic options, though less common, have been explored.

The successful heterologous, ectopic and overexpression of AMPs in plants is another alternative choice to microbial production. A large leaf biomass and high seed or tuber yields provide a strong platform for the large scale production commonly termed as *molecular farming* of biomolecules. Plant-based systems could be cost effective and proteins can be synthesized with post-translational modifications such as disulfide bond formation and glycosylation. Various strategies have been formulated to increase the proportion of AMPs in soluble fraction of host cells. For enhanced expression, AMP-genes are driven by strong constitutive and inducible promoters. The *cauliflower mosaic virus 35S RNA* and *ubiquitin* are among the commonly used constitutive promoters. Some of the inducible promoters tested include wound, *win3.12T*, and pathogenic, *mannopine synthase,* specific and heat shock responsive *Os.hsp82* promoters. Plants offer many opportunities to manipulate the expression of AMPs for desired results. Some of the strategies employed are briefly described here. For a more descriptive review on plant-based expression systems, the readers are suggested to go to these recent ones (Holaskova et al. 2015; Liew and Hair-Bejo 2015).

5.6.1 Sub-cellular Localization of Recombinant Proteins

The recombinant proteins without a signal peptide usually end up in the cytosol. They accumulate at relatively low concentrations in the soluble forms. A major portion of these tend to be present as insoluble form perhaps due to the lack of chaperons or other cellular factors. Also, such ‘free’ proteins tend to become targets

of endogenous proteases. Therefore to enhance their yield and stability, the candidate protein genes are constructed such that they get targeted to sub-cellular locations such as endoplasmic reticulum, chloroplasts, amyloplasts or extracellular spaces. Chloroplasts in particular have received researchers' attention due to their high number in green tissues like leaves. More importantly, these organelles have their own genome that can be suitably transformed with the desired genes. Compared to nuclear genome transformation chloroplasts can potentially generate more than 20,000 copies per cell. Also, chloroplasts being maternal tissue offer a better control on unintended genetic spread of the recombinant gene.

5.6.2 Tissue/Organ Specific Accumulation

Besides leaves that constitute a large biomass, cereal grains, oilseeds, tubers or roots, which are the primary storage organs of plant photosynthates, can also serve as platforms for *molecular farming*. The monoclonal antibody against hepatitis B surface antigen (HBsAg), expressed in tobacco, was the first commercialized plant-derived antibody (Liew and Hair-Bejo 2015). Transformed rice grains are able to accumulate human lysozyme up to 14 % of total soluble protein. A stronger promoter such as the '*rice glutelin 1*' can empower the accumulation of a recombinant protein up to 40 % of total cellular protein. A human lactoferrin protein essential for iron binding was expressed at 25 % of the total proteins. Maize is another cereal crop that has been used for *molecular farming*. Although economically unviable compared to cereals, a higher percentage of recombinant protein can also be obtained in Arabidopsis seeds.

The large genomes of cereal grains have some merits as well as demerits in being employed for protein-making factories. First, it is relatively cumbersome to transform monocots as compared to dicots. The redundancy in the genomes and associated differentially-active regions can limit the expression of the gene especially when a single copy gets inserted into those regions. The large genomes on the other hand are more tolerant to recombinant gene insertions compared to a small genome like in Arabidopsis. The grains offer a better storage medium of the product vis-à-vis other tissues with high moisture content. Like in bacteria and yeast, the stable incorporation of gene in plant genome can be inherited and the seeds then act as mode of continuum propagation. It is estimated that the production cost of a recombinant protein in plants could be 10–50 times less than *E. coli*. Various veterinary vaccines have been expressed in edible portion of plants.

5.6.3 Plant Cell Cultures and Protein Production

Cell suspension cultures (CSC) are rapidly dividing cells in liquid medium with appropriate nutrients. They are maintained in closed environments with control of

light and temperature. The tissue culture resembles with CSC except that the dividing cells grow on solid support producing a mass of cells. The CSC has added advantage of excreting the product into liquid medium with appropriate secretary signals. It makes the product recovery process easier thus cutting down the cost of production. CSC has better control over the growth process leading to high batch-to-batch reproducibility, which helps compliance with good manufacturing practise (cGMP). Both these platforms are more contained in nature than the whole plant system. They are considered safe therapeutically, environmentally and in controlling the proliferation of transgene. These factors make the regulatory approval process easier compared to whole transgenic plants. Taliglucerase alfa (TGA) developed by Protalix and Pfizer was the first plant-made pharmaceutical drug approved by FDA. It is a glucocerebrosidase used to treat Gaucher's Disease. TGA employs carrot cells and its production on commercial scale involves a series of bioreactors that can process thousands of litres of growth media. Like TGA and many more pharmaceutical drugs that are at different stages of commercial production and regulatory approval, AMPs can be synthesized on large scale. The selection of new plant sources and optimization of growth conditions of cell culture media are being explored. A recombinant human serum albumin has been tested on a laboratory scale using rice suspension cells in a simplified bioreactor process leading to sixfold increase in yield. With increasing demand of AMPs as pharmaceuticals, the CSC advances are expected to be extended to commercial production of AMPs.

5.7 AMPs Are More than Just Antimicrobial Compounds

It has become apparent in recent years that the role of AMPs is larger than strictly being toxic to pathogens. These peptides now appear to be involved in different phases of plants' life cycle (Marshall et al. 2011; Stotz et al. 2013; Pelegrini et al. 2011; Goyal and Mattoo 2014). A few examples are: (a). Defensins share structural similarity with nodule specific cysteine-rich peptides (CRPs) and are abundantly expressed in seeds (Graham et al. 2004). That plant defensins are multi-taskers stems from the fact that CRPs are expressed early during bacterial symbiotic relationship, permeable across bacterial membrane, inhibitory to cell division and suppress reproduction, and released by nodule-specific secretary pathway (Marshall et al. 2011; Penterman et al. 2014). Defensin-like polypeptides—LUREs, DEFL, ZmES-1, DEF2—seem involved in one or the other biological process associated with pollen tube (pollination) in plants. LUREs mediate guidance of the pollen tube (Okuda et al. 2009; Takeuchi and Higashiyama 2012); ZmES-4 leads to pollen tube burst, discharging sperms by targeting potassium channel KZM1 (Amien et al. 2010); PCP–A1 and SP11 peptides contribute to self-incompatibility in Brassica pollen (Doughty et al. 1998; Takayama et al. 2001). Forward and reverse genetic manipulation of DEF2 in tomato resulted in traits showing roles of this gene product in pollen viability, seeding, and morphology (Stotz et al. 2009); while

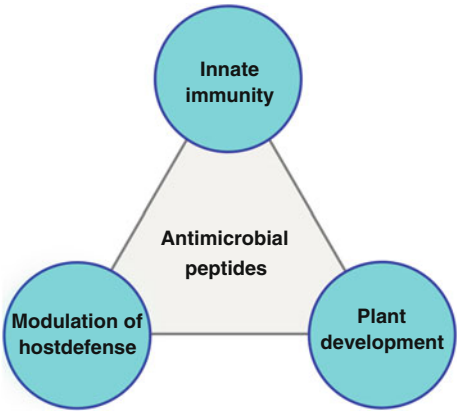

Fig. 5.9 Plant AMPs are multifunctional

silencing of snakin-1 resulted in negative effects on plant development in potato (Nahirñak et al. 2012). (b) LTPs are involved in cuticular wax synthesis, pollen adhesion, guiding the pollen tube towards fertilization, oxylipin-mediated SAR, and cell wall loosening (Molina and García-Olmedo 1997; Park et al. 2000; Nieuwland et al. 2005; Chae et al. 2009; DeBono et al. 2009). (c) A synthetic heterologous AMP, *msrA3*, when expressed in potato was found to alter floral development and mitigate normal plant response to abiotic and biotic stresses (Goyal et al. 2013). The transgenic potato plants were resistant to *Fusarium solani* and the tuber yield was significantly higher than the control plants. Detailed investigation showed suppression of HR, wound-induced JA and ROS, in concert with changes in transcript profiles of related gene markers under both biotic and abiotic stresses (Goyal et al., 2013). Among other functions, AMPs interact with cellular signalling processes include oxidative stress and its components ROS and NO, MAPK signalling, HR, and systemic acquired resistance (SAR) (reviewed in Goyal and Mattoo 2014). Thus, plant AMPs are potent defence molecules while they also have moonlighting functions related to plant development processes, similar to what is known about mammalian AMPs which, in addition to immunomodulating host defence, also modify physiological responses of the cell (Choi et al. 2012; Hilchie et al. 2013). Multifunctional role of plant AMPs is summarized in Fig. 5.9.

5.8 Conclusions

Disease afflicts crop productivity as well as nutritional attributes. Pathogens have the ability to mutate rapidly and thereby develop resistance to pesticides. Despite plant's multilayer of innate defence against pathogens, often the latter are able to penetrate and establish themselves on plant host. The discovery of antimicrobial

peptides (AMPs) has the promise of durable defence by quickly eliminating pathogens through membrane lysis, and positively impacting the host's cellular machinery for development. AMPs characteristically are made up of from fewer than 20 amino acids to about 100 amino acids, structurally diverse, and amenable for higher potency by either alteration of their chemical structure and/or engineering them to produce higher amounts in heterologous systems in order to provide durable plant immunity against pathogens. For achieving this, it will be important to first characterize them, understand their mechanism(s) of action, and develop a wide range of structures. Although permeabilizing cellular membrane is a major mechanism known for AMP action, new and diverse modes of action have recently been unearthed, including targeting of intracellular function of the pathogen.

Crop protection against pathogens is inimical to global food security. Immense focus on the 'R' gene defence for crop survival against pathogens has demonstrated the short half-life of such a strategy and breakdown of such defence. The discovery of antimicrobial peptides (AMPs) as generators of durable plant resistance against target pathogens together with their broad-spectrum activity across kingdoms has shown their promise in enabling crop resistance to disease.

References

Aerts AM, FranÅois IEJA, Cammue BPA, Thevissen K (2008) The mode of antifungal action of plant, insect and human defensins. Cell Mol Life Sci 65:2069–2079

Amien S, Kliwer I, Márton ML, Debener T, Geiger D, Becker D, Dresselhaus T (2010) Defensin-like $ZmES_4$ mediates pollen tube burst in maize via opening of the potassium channel KZM1. PLoS Biol 8:e1000388

Bolton MD (2009) Primary metabolism and plant defense—fuel for the fire. MPMI 22:487–497

Brogden KA (2005) Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat Rev Microbiol 3:238–250

Brown JKM (2002) Yield penalties of disease resistance in crops. Curr Opin Plant Biol 5:1–6

Burman R, Gunasekera S, Strömstedt AA, Göransson U (2014) Chemistry and biology of cyclotides: circular plant peptides outside the box. J Nat Prod 77:724–736

Cammue BP, De Bolle MF, Terras FR, Proost P, Van Damme J, Rees SB, Vanderleyden J, Broekaert WF (1992) Isolation and characterization of a novel class of plant antimicrobial peptides form *Mirabilis jalapa* L seeds. J Biol Chem 267:2228–2233

Chae K, Kieslich CA, Morikis D, Kim SC, Lord EM (2009) A gain-of-function mutation of Arabidopsis lipid transfer protein 5 disturbs pollen tube tip growth and fertilization. Plant Cell 21:3902–3914

Chisholm ST, Coaker G, Day B, Staskawicz BJ (2006) Host-microbe interactions: shaping the evolution of the plant immune response. Cell 124:803–814

Choi KY, Chow LN, Mookherjee N (2012) Cationic host defence peptides: multifaceted role in immune modulation and inflammation. J Innate Immun 4:361–370

DeBono A, Yeats TH, Rose JKC, Bird D, Jetter R, Kunst L, Samuels L (2009) Arabidopsis LTPG is a glycosylphosphatidylinositol-anchored lipid transfer protein required for export of lipids to the plant surface. Plant Cell 21:1230–1238

Doughty J, Dixon S, Hiscock SJ, Willis AC, Parkin IAP, Dickinson HG (1998) PCP–A1, a defensin-like Brassica pollen coat protein that binds the S locus glycoprotein, is the product of gametophytic gene expression. Plant Cell 10:1333–1347

Gennaro R, Zanetti M (2000) Structural features and biological activities of the cathelicidin-derived antimicrobial peptides. Pept Sci 55:31–49
Goyal RK, Mattoo AK (2014) Multitasking antimicrobial peptides in plant development and host defense against biotic/abiotic stress. Plant Sci 228:135–149. doi:10.1016/j.plantsci.2014.05.012
Goyal RK, Hancock REW, Mattoo AK, Misra S (2013) Expression of an engineered heterologous antimicrobial peptide in potato alters plant development and mitigates normal abiotic and biotic responses. PLoS ONE 8:e82838
Graham MA, Silverstein KAT, Cannon SB, VandenBosch KA (2004) Computational identification and characterization of novel genes from legumes. Plant Physiol 135:1179–1197
Hammami R, Hamida JB, Vergoten G, Fliss I (2009) PhytAMP: a database dedicated to plant antimicrobial peptides. Nucleic Acids Res 37:D963–D968. doi:10.1093/nar/gkn655
Henriques ST, Melo MN, Castanho MA (2006) Cell-penetrating peptides and antimicrobial peptides: how different are they? Biochem J 399:1–7
Hilchie AL, Wuerth K, Hancock REW (2013) Immune modulation by multifaceted cationic host defense (antimicrobial) peptides. Nat Chem Biol 9:61–768
Holaskova E, Galuszka P, Frebort I, Oz MT (2015) Antimicrobial peptide production and plant-based expression systems for medical and agricultural biotechnology. Biotechnol Adv 33:1005–1023
Kim DH, Lee DG, Kim KL, Lee Y (2001) Internalization of tenecin 3 by a fungal cellular process is essential for its fungicidal effect on *Candida albicans*. Eur J Biochem 268:4449–4458
Lacerda AF, Vasconcelos ÉAR, Pelegrini PB, Grossi de Sa MF (2014) Antifungal defensins and their role in plant defense. Front Microbiol 5:116. doi:10.3389/fmicb.2014.00116
Liew PS, Hair-Bejo H (2015) Farming of plant-based veterinary vaccines and their applications for disease prevention in animals. Adv Virol 2015:936940. doi:10.1155/2015/936940
Marshall E, Costa LM, Gutierrez-Marcos J (2011) Cysteine-rich peptides (CRPs) mediate diverse aspects of cell-cell communication in plant reproduction and development. J Exp Bot 62:1677–1686
Molina A, García-Olmedo F (1997) Enhanced tolerance to bacterial pathogens caused by the transgenic expression of barley lipid transfer protein LTP2. Plant J 12:669–675
Muñoz A, Gandía M, Harries E, Carmona L, Read ND, Marcos JF (2013) Understanding the mechanism of action of cell-penetrating antifungal peptides using the rationally designed hexapeptide PAF26 as a model. Fungal Biol Rev 26:146–155
Nahirñak V, Almasia NI, Fernandez PV, Hopp HE, Estevez JM, Carrari F, Vazquez-Rovere C (2012) Potato Snakin-1 gene silencing affects cell division, primary metabolism, and cell wall composition. Plant Physiol 158:252–263
Nawrot R, Barylski J, Nowicki G, Broniarczyk J, Buchwald W, Goździcka-Józefiaket A (2014) Plant antimicrobial peptides. Folia Microbiol 59:181–196
Nieuwland J, Feron R, Huisman BAH, Fasolino A, Hilbers CW, Derksen J, Mariani C (2005) Lipid transfer proteins enhance cell wall extension in tobacco. Plant Cell 17:2009–2019
Okuda S, Tsutsui H, Shiina K, Sprunck S, Takeuchi H, Yui R, Kasahara RD, Hamamura Y, Mizukami A, Susaki D, Kawano N, Sakakibara T, Namiki S, Itoh K, Otsuka K, Matsuzaki M, Nozaki H, Kuroiwa T, Nakano A, Kanaoka MM, Dresselhaus T, Sasaki N, Higashiyama T (2009) Defensin-like polypeptide LUREs are pollen tube attractants secreted from synergid cells. Nature 458:357–361
Paiva AD and Breukink E (2013) Antimicrobial peptides produced by microorganisms. In: Hiemstra PS, Zaat SAJ (eds) Antimicrobial peptides and innate immunity—progress in inflammation research. Springer, Basel, pp 53–95. doi:10.1007/978-3-0348-0541-4_2
Park SY, Jauh GY, Mollet JC, Eckard KJ, Nothnagel EA, Walling LL, Lord EM (2000) A lipid transfer-like protein is necessary for lily pollen tube adhesion to an in vitro stylar matrix. Plant Cell 12:151–163
Pelegrini PB, del Sarto RP, Silva ON, Franco OL, Grossi-De-Sa MF (2011) Antibacterial peptides from plants: what they are and how they probably work. Biochem Res Int 2011:250349

Penterman J, Abo RP, De Nisco NJ, Arnold MF, Longhi R, Zanda M, Walker GC (2014) Host plant peptides elicit a transcriptional response to control the *Sinorhizobium meliloti* cell cycle during symbiosis. Proc Nat Acad Sci USA 111:3561–3566

Plattner S, Gruber C, Stadlmann J, Widmann S, Gruber CW, Altmann F, Bohlmann H (2015) Isolation and characterization of a thionin proprotein-processing enzyme from barley. J Biol Chem 290:18056–18067. doi:10.1074/jbc.M115.647859

Ponz F, Paz-Ares J, Hernandez-Lucas C, Carbonero P, Garcia-Olmedo F (1983) Synthesis and processing of thionin precursors in developing endosperm from barley (Hordeum vulgare L.). EMBO J 2:1035–1040

Sagaram US, Pandurangi R, Karu J, Smith TJ, Shah DM (2011) Structure-activity determinants in antifungal plant defensins MsDef1 and MtDef4 with different modes of action against *Fusarium graminearum*. PLoS ONE 6:e18550. doi:10.1371/journal.pone.0018550

Segura A, Moreno M, Madueno F, Garcia-Olmedo F (1993) Snakin-1, a peptide from potato that is active against plant pathogens. MPMI 12:16–23

Shai Y (2002) Mode of action of membrane active antimicrobial peptides. Biopolymers (Pept Sci) 66:236–248

Stec B (2006) Plant thionins—the structural perspective. Cell Mol Life Sci 63:1370–1385

Stotz HU, Spence B, Wang Y (2009) A defensin from tomato with dual function in defense and development. Plant Mol Biol 71:131–143

Stotz HU, Waller F and Wang K (2013) Innate immunity in plants: the role of antimicrobial peptides. In: Hiemstra PS, Zaat SAJ (eds) Antimicrobial peptides and innate immunity, progress in inflammation research, Springer, Basel, pp 29–51. doi:10.1007/978-3-0348-0541-4_2

Takayama S, Shimosato H, Shiba H, Funato M, Che FS, Watanabe M, Iwano M, Isogai A (2001) Direct ligand-receptor complex interaction controls Brassica self-incompatibility. Nature 413:534–538

Takeuchi H, Higashiyama T (2012) A species-specific cluster of defensin-like genes encodes diffusible pollen tube attractants in Arabidopsis. PLoS Biol 10:e1001449. doi:10.1371/journal.pbio.1001449

Tavormina P, Coninck B, Nikonorova N, Smet I, Cammuea BPA (2015) The plant peptidome: an expanding repertoire of structural features and biological functions. Plant Cell 27:2095–2118

Thevissen K, Ferket KKA, François IEJA, Cammue BPA (2003) Interactions of antifungal plant defensins with fungal membrane components. Peptides 24:1705–1712

van der Weerden NL, Bleackley MR, Anderson MA (2013) Properties and mechanisms of action of naturally occurring antifungal peptides. Cell Mol Life Sci 70:3545–3570

Van Parijs J, Broekaert WF, Goldstein IJ, Peumans WJ (1991) Hevein an antifungal protein from rubber-tree (*Hevea braziliensis*) latex. Planta 183:258–264

Vriens K, Cammue BPA, Thevissen K (2014) Antifungal plant defensins: mechanisms of action and production. Molecules 19:12280–12303. doi:10.3390/molecules190812280

Wimley WC (2010) Describing the mechanism of antimicrobial peptide action with the interfacial activity model. ACS Chem Biol 5:905–917

Yeaman MR, Yount NY (2003) Mechanisms of antimicrobial peptide action and resistance. Pharmacol Rev 55:27–55

Yount NY, Yeaman MR (2013) Peptide antimicrobials: cell wall as a bacterial target. Ann N Y Acad Sci 1277:127–138

Zasloff M (2002) Antimicrobial peptides of multicellular organisms. Nature 415:389–395

Part II
Immune Modulation by Host Defense Peptides

Chapter 6
Host Defense Peptides and the Eicosanoid Cascade

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Abstract Host defense peptides (HDPs) and eicosanoids are two important families in host defense and inflammation. Most of the naturally occurring HDPs are cationic and amphipathic short polypeptides with typical length between 15 and 40 amino acid residues. HDPs not only possess potent antimicrobial activity against a variety of pathogens, they are also widely recognized for their multifunctional roles in both the innate and adaptive immune responses. On the other hand, arachidonic acid-derived eicosanoids, including prostaglandins, thromboxanes, leukotrienes and lipoxins, are small lipid molecules with a 20-carbon backbone, which possess potent biological properties and participate in regulation of physiological and pathophysiological processes. In this article, we discuss the biosynthesis and functions of eicosanoids with emphasis on the roles of eicosanoids in host defense and regulation of HDP production. Moreover, we review how HDPs regulate eicosanoid metabolism and conclude that there are positive feedback circuits between HDP and eicosanoid signaling with implications for certain pathological conditions, such as infection and allergy.

6.1 Introduction

Arachidonic acid (AA) is released from phospholipids by phospholipases A_2 (PLA_2), and metabolism of AA leads to several families of lipid mediators collectively known as eicosanoids, including prostaglandins (PGs), thromboxanes, leukotrienes (LTs), and lipoxins (LXs), along two major pathways, the lipoxygenase (LOX) and the cyclooxygenase (COX) pathways (Haeggstrom and Funk

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2011). Eicosanoids are secreted and act locally in an autocrine or paracrine fashion through interaction with specific G-protein coupled receptors (GPCR) to exert their biological effects (Back et al. 2011; Woodward et al. 2011; Serhan 2014). They possess potent biological activities and are involved in fever, pain, maintenance of normal hemostasis, regulation of blood pressure, renal function, and reproduction as well as host defense (Haeggstrom and Funk 2011).

Host defense peptides (HDPs), also known as antimicrobial peptides (AMPs), are evolutionarily conserved molecules of the innate immune system. Their widespread distribution throughout the animal and plant kingdoms suggests that HDPs have served a fundamental role in the successful evolution of complex multicellular organisms (Zasloff 2002). They are not only important molecules in host defense against a very broad spectrum of microorganisms, such as gram negative and gram positive bacteria, fungi, parasites, and viruses, via various mechanisms of action, but also possess diverse immunomodulatory capabilities (Mansour et al. 2014).

Several studies have indicated that inhibition of eicosanoid biosynthesis lethally impairs insect immune reactions (Stanley-Samuelson et al. 1991) and also that eicosanoids play an important role in regulating innate immunity and host defense of mammals (Peters-Golden et al. 2005; Dennis and Norris 2015). The first suggestion that eicosanoids are involved in signaling pathways that lead to HDP production came from a study demonstrating that an eicosanoid biosynthesis inhibitor suppressed two HDP gene expressions in response to bacterial challenge in the fat body of the silkworm, *Bombyx mori* (Morishima et al. 1997). Accumulating evidence from the studies on insects suggested a direct induction of HDPs by AA (Morishima et al. 1997; Sun and Faye 1995), and also a direct functional link between eicosanoids and the production of HDPs induced by LPS (Yajima et al. 2003) or peptidoglycan (Morishima et al. 1997). Interestingly, the studies from our research group and others have also suggested positive feedback loops between eicosanoids and HDP production in mammalian leukocytes (Wan et al. 2011; Sun et al. 2013; Kanda et al. 2010; Bernard and Gallo 2010; Niyonsaba et al. 2001; Kase et al. 2009; Chen et al. 2007).

In this chapter, we will discuss the role of eicosanoids in host defense, and how HDPs and eicosanoids, two important families of innate immunity interact and cooperate to regulate the innate immunity and control infections.

6.2 Eicosanoids in Host Defense

PLA_2 enzymes are crucial for increasing the levels of free AA for eicosanoid biosynthesis under most physiological conditions, but particularly following inflammatory cell activation (Dennis and Norris 2015). Three members of the PLA_2 superfamily have been implicated most strongly in cellular eicosanoid production: cytosolic calcium-dependent PLA_2 ($cPLA_2$), cytosolic calcium independent PLA_2 ($iPLA_2$) and secreted PLA_2 ($sPLA_2$) (Dennis and Norris 2015). Among them, $cPLA_{2\alpha}$ is the only PLA_2 that exhibits preference for hydrolysis of AA from

phospholipid substrates that occurs in cells stimulated with diverse agonists (Leslie 2015). The observation that mice lacking $cPLA_2$ are profoundly depleted of all eicosanoids indicates that this enzyme is indispensable for the liberation of AA in vivo (Fujishima et al. 1999). Because AA is the precursor of eicosanoids, it is generally accepted that $cPLA_{2\alpha}$ plays a major role in inflammatory diseases (Dennis et al. 2011).

Early studies on insects demonstrated that eicosanoids play an important role in bacterial infection by using PLA_2 inhibitor, and the functions suppressed by PLA_2 inhibitor can be rescued by adding exogenous AA (Stanley-Samuelson et al. 1991, 1997; Yajima et al. 2003; Miller et al. 1994). Subsequent studies further showed that AA and eicosanoids promote HDP expression in insects (Morishima et al. 1997; Sun and Faye 1995; Yajima et al. 2003; Hwang et al. 2013). Studies in humans and mammals demonstrated that eicosanoids affect the immune response by modulating cellular differentiation, migration, phagocytosis, and cytokine/chemokine production, and also play an important role in connecting innate and adaptive immunity by acting on cells of both systems (Harizi and Gualde 2005). It has been reported that zymosan and *Candida albicans* induce $cPLA_2$ activation and eicosanoid production in macrophages via different signaling mechanisms (Gijon et al. 2000; Suram et al. 2006, 2010). Moreover, a recent report investigated the functional consequences of $cPLA_{2\alpha}$ activation and the effect of endogenously produced eicosanoids on gene expression in response to *C. albicans* by comparing $cPLA_{2\alpha}^{+/+}$ and $cPLA_{2\alpha}^{-/-}$ resident mouse peritoneal macrophages (RPM), and the results revealed that *C. albicans* killing was impaired in $cPLA_{2\alpha}$ deficient RPM, and *C. albicans*-stimulated $cPLA_{2\alpha}$ activation and the early production of prostanoids promote an autocrine pathway in RPM that affects the expression of genes involved in host defense to dampen inflammation (Suram et al. 2013). In addition, it has also been shown that AA stimulates human neutrophils to release HDPs to strongly impair bacterial growth (Chouinard et al. 2013). Interestingly, evidence has been provided that eicosanoids are involved in lactose and phenylbutyrate (PBA)-induced human cathelicidin expression in human epithelial cell line HT-29 since a PLA_2 inhibitor significantly suppressed lactose/PBA-induced peptide expression (Cederlund et al. 2014).

We will discuss more details on LTs, PGs and LXs in host defense and infections.

6.2.1 Prostanoids

Prostanoids are lipid mediators derived from AA via the COX pathway. COX exists as two isoforms referred to as COX-1 and COX-2. COX-1 is expressed constitutively in most tissues, whereas COX-2 is not detectable in most normal tissues or resting immune cells, but its expression can be induced by factors such as endotoxins, cytokines, growth factors, and carcinogens (Smith et al. 2011). COX enzymes convert AA to the unstable endoperoxide PGH_2, which can be

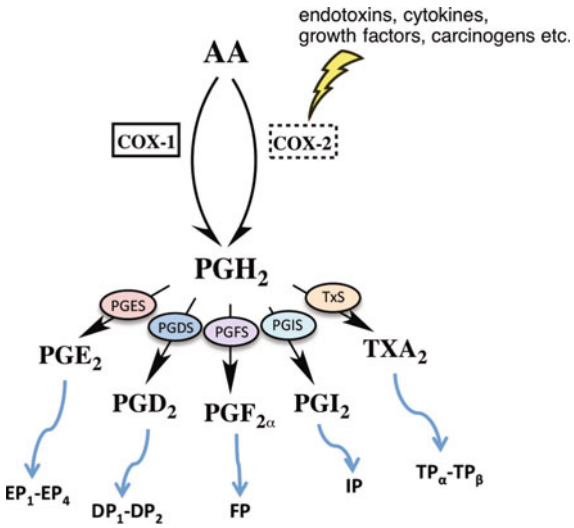

Fig. 6.1 Biosynthesis of prostanoids. Arachidonic acid (AA) can be metabolized by cyclooxygenase (COX) isoforms known as COX-1 and COX-2. Unlike COX-1, which is constitutively expressed in most tissues and cells, COX-2 remains at low expression in resting cells. However, COX-2 can be activated by factors such as endotoxins, cytokines, growth factors and carcinogens. COX isozymes convert AA to the unstable endoperoxide prostaglandin (PG)H_2, which is further metabolized to PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2 (prostacyclin), and thromboxane A_2 (TXA_2) by specific terminal synthases, in a cell-type restricted fashion. PGs exert their functions via specific G-protein coupled receptors (GPCR), i.e., EPs, DPs, FP, IP and TPs in an autocrine or paracrine manner

metabolized by the actions of specific terminal synthases that are expressed in a cell type-selective fashion to produce PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2 (prostacyclin), and thromboxane A_2 (TXA_2), which are collectively called prostanoids (Hirata and Narumiya 2012). The biosynthesis of prostanoids is summarized in Fig. 6.1.

PGE_2 is the most abundant prostanoid in the inflammatory milieu, and it is one of the best known and most well-characterized prostanoids in terms of immunomodulation. PGE_2 exerts its biological functions through four distinct G protein–coupled receptors called E prostanoid (EP) receptors, which are numbered EP1–4 (Woodward et al. 2011). Although PGE_2 has myriads of immunomodulatory effects and induces both pro- and anti-inflammatory effects, the immunosuppressive actions of PGE_2 to limit both the amplitude and duration of immune responses have been extensively studied and reported (Kalinski 2012; Agard et al. 2013). For example, PGE_2 suppresses macrophage phagocytosis (Aronoff et al. 2004; Lee et al. 2009; Serezani et al. 2012), restrains bacterial killing in alveolar macrophages by inhibiting NADPH oxidase (Serezani et al. 2007), exacerbates intrauterine group A *Streptococcal* infections (Mason et al. 2013), dampens antifungal immunity by inhibiting interferon regulatory factor 4 functions and interleukin-17 expression in

T cells (Valdez et al. 2012). Moreover, PGE_2 suppresses antiviral immunity through induction of type I interferon and apoptosis in macrophages (Coulombe et al. 2014), and blockade of PGE_2 signaling improves viral control (Chen et al. 2015). However, it has also been reported that PGE_2 induces resistance to HIV-1 infection by downregulation of the chemokine receptor CCR5 expression to suppress HIV-1 entry into macrophages (Thivierge et al. 1998). Several studies also demonstrate that PGE_2 plays an important role to inhibit *Mycobacterium tuberculosis* replication in vitro and in vivo (Chen et al. 2008; Kaul et al. 2012; Mayer-Barber et al. 2014).

Several lines of evidence indicate that PGs are involved in host defense by modulating HDP expression. Studies from insects have demonstrated that PGs induce the expression of humoral immune-associated genes, including HDP cecropin in the beet armyworm, *Spodoptera exigua* (Shrestha and Kim 2009). In another study on mosquito *Anopheles albimanus* has shown that PGE_2 reduces mRNA synthesis of HDP ambicin and attacin in cultured midguts and fat bodies, while enhancing the cecropin mRNA (Garcia Gil de Munoz et al. 2008). Studies have also demonstrated that PGD_2 induces human β-defensin (hBD)-3 production in human keratinocytes (Kanda et al. 2010), and one possible mechanism for the antimicrobial effects of the antimycotic drugs itraconazole and terbinafine hydrochloride could be to induce hBD-3 in keratinocytes by increasing PGD_2 release from keratinocytes (Kanda et al. 2011). Consistently, Bernard et al. also found that PGD_2 and 15-deoxy-$D^{12,14}$-PGJ_2 (a dehydration product of PGD_2) induces the production of hBD-2 and hBD-3 by human keratinocytes (Bernard and Gallo 2010). However, PGE_2 dramatically suppresses hBD-1 expression in human uterine epithelial cells, and also moderates TNF-α-induced hBD-2 expression in human uterine epithelial cells (Aronoff et al. 2008).

It is well-established that non-steroidal anti-inflammatory drugs (NSAIDs) block prostanoid synthesis by inhibiting COX enzymes and are widely used to treat both acute and chronic inflammation. Some evidence suggests that NSAID use is also linked to modulation of HDP expression. One report showed that the COX inhibitor etodolac enhanced hBD-2 mRNA levels in *Actinobacillus actinomycetemcomitans* infected human gingival epithelial cells (HGEC) (Noguchi et al. 2003). Since etodolac almost suppressed the production of PGE_2 by *A. actinomycetemcomitans* in HGEC, this result indicated that endogenous PGE_2 produced by *A. actinomycetemcomitans* in HGEC suppresses hBD-2 expression (Noguchi et al. 2003). In another report, Bernard et al. demonstrated a critical role for COX-2 in hBD production by human keratinocytes and treatment with a COX-2 inhibitor led to reduced antibacterial activity in these cells (Bernard and Gallo 2010). Interestingly, one recent report also describes that COX inhibitors aspirin or etoricoxib significantly suppress lactose/PBA-induced cathelicidin expression in the human epithelial cell line HT-29 (Cederlund et al. 2014). All these evidences suggest that prostanoid signaling is involved in the regulation of HDP expression.

6.2.2 *Leukotrienes (LTs)*

LT biosynthesis from free unesterified AA is catalyzed by a series of enzymes, starting from 5-lipoxygenase (5-LOX). Upon an increase in intracellular calcium, 5-LOX translocates to the nuclear membrane and associates with 5-LO-activating protein (FLAP) to promote dioxygenation and dehydration of AA (Dixon et al. 1990). This process gives rise to the unstable epoxide LTA_4, the key intermediate in leukotriene biosynthesis, which is converted to LTB_4 through the action of LTA_4 hydrolase (Samuelsson and Funk 1989). LTA_4 can also be conjugated with GSH by LTC_4 synthase to produce LTC_4, which constitutes the parent compound of the cysteinyl LTs (cys-LTs) also including LTD_4 and LTE_4 (Samuelsson et al. 1987). The biosynthesis of LTs is summarized in Fig. 6.2. LT production is cell type specific and largely limited to cells of the myeloid lineage. LT signaling is achieved through a family of GPCR. So far, it has been found that LTB_4 binds to two GPCRs, BLT1 and BLT2 with high and low affinity. Likewise, CysLT1, CysLT2

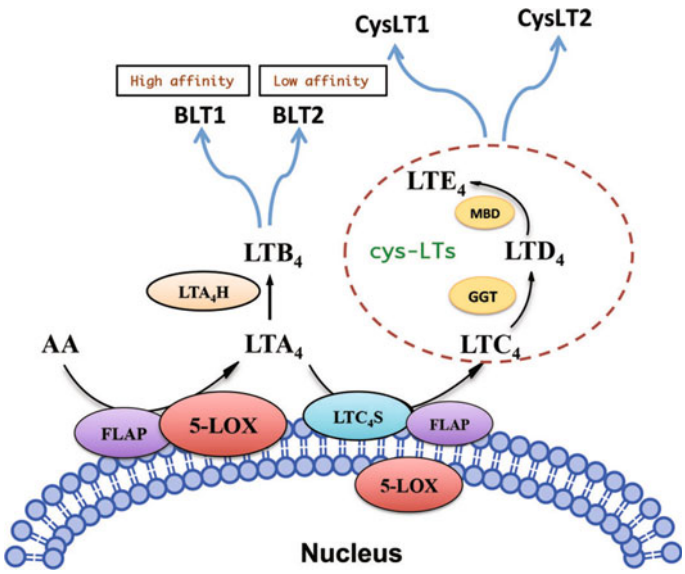

Fig. 6.2 Biosynthesis of leukotrienes (LTs) at the nuclear membrane. Upon certain stimuli, intracellular calcium level increases, leading to the translocation of 5-LOX from cytosol to the nuclear membrane (not depicted in figure). Free AA is presented to 5-LOX by the nuclear membrane integral protein 5-LO-activating protein (FLAP) and converted to LTA_4, the key intermediate in LT biosynthesis. LTA_4 is further converted to LTB_4 by LTA_4H, and the released LTB_4 acts via its high affinity receptor BLT1 or low affinity receptor BLT2 on target cells. Meanwhile, LTA_4 can also be metabolized to LTC_4 by the integral membrane enzyme LTC_4s. LTC_4 is further transformed by extracellularly localized γ-glutamyl transpeptidase (GGT) to LTD_4, and further into LTE_4 by membrane-bound dipeptidase (MBD). LTC_4, LTD_4 and LTE_4, together known as cys-LTs, can activate and bind to two main receptors CysLT1 and CysLT2 for their bioactivities

and a putative receptor for LTE_4 (CysLT3) have been reported to transduce cys-LT signaling (Back et al. 2011).

LTB_4 is best known for its role as a neutrophil chemoattractant, and cys-LTs are famous for their ability to induce bronchoconstriction in asthma (Peters-Golden and Henderson 2007). However, LTs are now recognized as important participants in the host response and they are produced at sites of infection by phagocytes, which promotes killing of microbes, including bacteria (Bailie et al. 1996; Mancuso et al. 1998, 2010; Serezani et al. 2005; Soares et al. 2013), mycobacteria (Peres et al. 2007; Tobin et al. 2010; Peres-Buzalaf et al. 2011; Tobin et al. 2012), fungi (Medeiros et al. 2008; Morato-Marques et al. 2011; Secatto et al. 2012, 2014), parasites (Talvani et al. 2002; Machado et al. 2005; Serezani et al. 2006; Morato et al. 2014; Canavaci et al. 2014) and virus (Flamand et al. 2004; Gosselin et al. 2005; Gaudreault and Gosselin 2008; Bertin et al. 2012).

Accumulating evidence has clearly demonstrated that LTs, particularly LTB_4, plays a significant role in the control of microbial infections through its ability to activate host immunity, for instance by promoting HDP release (Peters-Golden et al. 2005; Le Bel et al. 2014). A study on insects showed that LTB_4 induced expression of humoral immune-associated genes, including HDP cecropin in the beet armyworm, *S. exigua* (Shrestha and Kim 2009). Moreover, studies on human neutrophils demonstrated that LTB_4 induces the release of human HDP, including α-defensins, cathepsin G, elastase, lysozyme C, and LL-37 from human neutrophils via the BLT1 receptor (Wan et al. 2007; Flamand et al. 2007). Furthermore, it has been shown that *i.v.* injection of LTB_4 to monkey and human (Flamand et al. 2004, 2007) induces a dose-dependent plasmatic increase in α-defensins. Meanwhile, evidence has been provided that LTB_4-induced HDP production by neutrophils, transduced via BLT1, plays a role in LTB_4-mediated antiviral activity in vitro and in vivo (Gaudreault and Gosselin 2007, 2008). Recently, it was also reported that AA induces the release of HDPs (α-defensins and LL-37) from human neutrophils through metabolism into LTB_4 and the activation of BLT1 (Chouinard et al. 2013). All these studies further support the notion that LTB_4-induced HDPs play an important role in host defense.

6.2.3 Lipoxins (LXs)

In 1984, Serhan and colleagues discovered a new family of eicosanoids called lipoxins (LXs) (Serhan et al. 1984a, b). LXA_4 and LXB_4 are positional isomers that each possesses potent cellular and in vivo actions, whereas LXA_4 has been more widely studied for its functions. The LXs are generated from AA via sequential action of two or more LOXs during cell–cell interactions by transcellular biosynthetic routes that occur in inflammation and disease pathogenesis (Serhan 2005). Three transcellular pathways for biosynthesis of LXA_4 in human cells are depicted

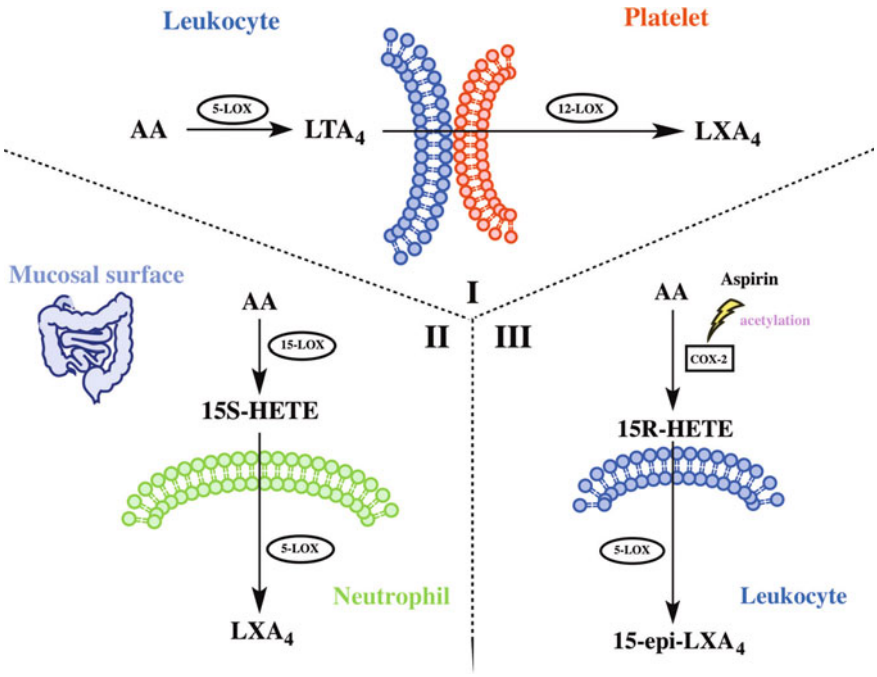

Fig. 6.3 Transcellular biosynthesis of lipoxins (LXs). (**I**) 5-LOX in leukocytes converts AA to LTA_4, which is released from leukocytes and further transformed to LXA_4 via the enzyme 12-LOX in adherent platelets. (**II**) On mucosal surfaces, AA can be transformed via 15-LOX to 15S-hydroxy-eicosatetraenoic acid (15S-HETE), which is rapidly taken up by neutrophils and converted to LXA_4 via 5-LOX. (**III**) aspirin-acetylated COX-2 transforms AA to 15R-HETE, which is taken up by leukocytes and converted via 5-LOX to 15-epi-LXA_4, a product that is also called aspirin-triggered lipoxins (ATL)

in Fig. 6.3. The first pathway involves peripheral blood leukocyte–platelet interactions. The enzyme 5-LOX in leukocytes converts AA to LTA_4, which is released from leukocytes and further transformed by adherent platelets to LXA_4 via 12-LOX (Edenius et al. 1988; Romano and Serhan 1992). The second biosynthetic route is initiated at mucosal surfaces by 15-LOX that transforms AA to 15S-hydroxy-eicosatetraenoic acid (15S-HETE), which is rapidly taken up by neutrophils and subsequently converted via 5-LOX to LXA_4 (Serhan 1997). In addition to these two main routes, it has been discovered that aspirin-acetylated COX-2 can transform AA to 15R-HETE, which is taken up by leukocytes and converted via 5-LOX to 15-epi-LXA_4, also called aspirin-triggered lipoxins (ATL) (Claria and Serhan 1995).

LXs and ATL act at both temporal and spatially distinct sites from other eicosanoids produced during the course of inflammatory responses to actively participate in anti-inflammation and resolution of inflammation (Serhan 2005). LXs and

n-3 polyunsaturated fatty acid (PUFA) eicosapentaenoic acid and docosahexaenoic acid-derived resolvins, protectins and maresins are collectively termed specialized proresolving mediators (SPM). Recent studies demonstrated that SPMs are temporally and differentially regulated during infections (Chiang et al. 2012), and they potently stimulate cessation of PMN infiltration and enhance macrophage uptake of apoptotic cells, debris and microbes (Serhan 2014), aiding a return to tissue homeostasis. Accumulated evidence demonstrated that SPM including LXA_4 play an important role in infectious diseases. Thus, it has been reported that LXA_4 displays protective effects on host defense against fungal pathogen *Cryptococcus neoformans* (Colby et al. 2015), parasite *Toxoplasma gondii* (Aliberti et al. 2002) and *Trypanosoma cruzi* (Molina-Berrios et al. 2013), respiratory viruses (Kim 1990; Shirey et al. 2014) and influenza A virus (Cilloniz et al. 2010), and cerebral malaria (Shryock et al. 2013). Moreover, LXA_4 exhibits beneficial effects on bacteria *Porphyromonas gingivalis*-induced periodontitis (Serhan et al. 2003) and LPS-induced preterm birth (Rinaldi et al. 2015). Interestingly, ATL combined with antibiotics protects mice from *Escherichia coli*-induced sepsis (Ueda et al. 2014). In contrast, LXA_4 seems to exert detrimental effects on *Mycobacterium tuberculosis* infection (Chen et al. 2008; Bafica et al. 2005).

Interestingly, it has been demonstrated that LXA_4 and other SPM also can regulate HDP expression. We have shown that resolvin E1 (RvE1) dampens LTB_4-induced human cathelicidin LL-37 release from human neutrophils via binding to BLT1 (Wan et al. 2011). On the other hand, ATL induces the expression of an antimicrobial peptide/protein called bactericidal permeability-increasing protein in epithelial cells (Canny et al. 2002). Campbell et al. concluded that generation of SPM in the resolution phase elicits the induction of "nonclassical" AMPs to accelerate return to homeostasis via continued bacterial killing, and inhibition of LPS signaling; meanwhile, SPMs can block and/or counteract the release of "classical" AMPs from leukocytes, dampening the proinflammatory signals (Campbell et al. 2011).

It is noteworthy that LXs act as agonists at specific GPCRs, in particular FPR2/ALX, to regulate cellular responses in inflammation and resolution (Fiore et al. 1994). Actually, FPR2/ALX binds both protein and lipid ligands that evoke opposing biological responses. For example, ALX mediates the proinflammatory actions of LL-37 (Wan et al. 2011) and the acute-phase protein serum amyloid A (SAA) (Bozinovski et al. 2012). In contrast, ALX also mediates the anti-inflammatory actions of the lipid LXA_4 (Fiore et al. 1994) and protein annexin A1 (AnxA1) (Perretti and D'Acquisto 2009). Actually, lipid and peptide ligands act with different affinities and bind to distinct pockets on the receptor, thus making a direct competition unlikely (Filep 2013). In one recent report, Cooray et al. identified that AnxA1, but not SAA, stimulated ALX homodimerization and activated the p38 MAPK/MAPKAPK/Hsp27 signaling cascade (Cooray et al. 2013).

6.3 HDPs and Eicosanoid Productions

Although first studied for their antimicrobial activity, HDPs are now widely recognized for their multifunctional roles in both the innate and adaptive immune responses. Their diverse immunomodulatory capabilities include the modulation of pro- and anti-inflammatory responses, chemoattraction, enhancement of extracellular and intracellular bacterial killing, cellular differentiation and activation of the innate and adaptive compartments, wound-healing, and modulation of autophagy as well as apoptosis and pyroptosis (Mansour et al. 2014). Increasing evidence demonstrate that HDPs also promote eicosanoid productions in various cell types to form a positive feedback loop between HDP and eicosanoid production, which plays an important role in several pathophysiological conditions.

6.3.1 Positive Feedback Loops Between HDPs and Eicosanoids

6.3.1.1 HDPs and LTB_4 in Host Defense

Human α-defensins, also known as human neutrophil peptides (HNP), are a major product of activated neutrophils. HNP types 1–4 are abundant in human neutrophils, constituting 5 % of all neutrophil proteins and 30–50 % of the total protein content of the azurophilic granules in neutrophils (Ganz et al. 1985). It has been shown that LTB_4 triggers the release of α-defensins from circulating neutrophils (Flamand et al. 2004; Gaudreault and Gosselin 2008; Flamand et al. 2007). Moreover, HNP promote LTB_4 production in human alveolar macrophages in a dose-dependent manner (Spencer et al. 2004), suggesting α-defensins and LTB_4 could interact through a positive feedback loop. In addition, our research group and others have demonstrated that LTB_4 also induces hCAP18/LL-37 release from neutrophils via the BLT1 receptor (Wan et al. 2007; Flamand et al. 2007). LL-37 is present at high concentrations as the inactive proform hCAP-18 in the secondary granules of neutrophils (Sorensen et al. 1997). Once hCAP18 is secreted from neutrophils, it is processed into the active LL-37 peptide by proteinase 3 that is present in the primary granules of these cells (Gudmundsson et al. 1996; Sorensen et al. 2001). Interestingly, we also identified that LL-37 induces intracellular calcium mobilization, activates p38 MAP kinase, promotes phosphorylation of $cPLA_2$ and translocation of 5-LOX, which promotes LTB_4 release from human neutrophils via the GPCR FPR2/ALX (Wan et al. 2007, 2011), and from human macrophages via $P2X_7R$ (Wan et al. 2014), indicating that a positive feedback loop exists between cathelicidin and LTB_4 production as well.

Considering the importance of LTB_4-induced HDPs from neutrophils in antiviral activity in vitro and in vivo (Flamand et al. 2004, 2007; Gaudreault and Gosselin 2008), the positive feedback loop between LTB_4 and HDPs could be vital to

amplify the host antiviral activity. Recently, Chaves et al. provided evidence that $P2X_7R$ activation in macrophages leads to LTB_4 formation, which is required for *L. amazonensis* elimination (Chaves et al. 2014). In our recent studies, we further demonstrate that LL-37 promotes LTB_4 production in human macrophage via $P2X_7R$ (Wan et al. 2014) and that LL-37 enhances bacterial clearance by macrophages (Wan et al. 2014). Macrophages can also import LL-37 released from LTB_4-challenged neutrophils to promote intracellular bacterial clearance (Tang et al. 2015). All these evidences suggest the presence of positive feedback circuits between HDPs and LTB_4 that are important in host defense against various pathogens.

Intriguingly, we have also shown that anti-inflammatory lipid mediators LXA_4 and RvE1 might counteract the proinflammatory circuit between LL-37 and LTB_4 in human neutrophils by blocking the receptors FPR2/ALX and BLT1, respectively, that signal in a LTB_4/LL-37 positive feedback loop in neutrophils (Wan et al. 2011). It is noteworthy that both LTB_4 and LL-37 are involved in many chronic inflammatory diseases, such as atherosclerosis (Funk 2005; Qiu et al. 2006; Edfeldt et al. 2006), inflammatory bowel diseases (Hawthorne et al. 1992; Schauber et al. 2006) and cancers (Satpathy et al. 2015; von Haussen et al. 2008). Therefore, it appears essential to interfere with and dampen LTB_4/LL-37 co-driven inflammatory responses.

6.3.1.2 HDPs and PGD_2/cys-LTs in Allergy

Human β-defensins (hBDs) are mainly produced by epithelia of several organs including skin, and participate in host defense by killing invading pathogens, as well as promoting both innate and adaptive immune responses (Yang et al. 1999). Among four hBDs identified in epithelium so far, hBD-1 is generally constitutively produced by various epithelial tissues such as those in the urogenital and respiratory tracts, and skin (Valore et al. 1998), whereas the expressions of hBD-2, hBD-3, and hBD-4 are inducible (Singh et al. 1998).

It has been reported that PGD_2 induces hBDs including hBD-2 and hBD-3 production in human keratinocytes (Kanda et al. 2010; Bernard and Gallo 2010). PGD_2 is the major prostaglandin produced by mast cells and is involved in allergic diseases such as asthma (Matsuoka et al. 2000). Interestingly, it has been reported that hBD-2 works as a chemotaxin for mast cells (Niyonsaba et al. 2002), and induces intracellular calcium mobilization, AA release and PGD_2 production in rat peritoneal mast cells via COX-1 (Niyonsaba et al. 2001; Kase et al. 2009). Furthermore, hBD-3 and hBD-4 also act on mast cells and enhances their chemotaxis and degranulation, which results in the release of PGD_2 (Chen et al. 2007). Moreover, cathelicidins can activate mast cells as well. For example, LL-37 was shown to induce intracellular calcium mobilization, AA release and PGD_2 production in rat peritoneal mast cells via COX-1 (Niyonsaba et al. 2001). Furthermore, rat cathelicidin rCRAMP and human cathelicidin LL-37 trigger generation and release of cys-LTs and other pro-inflammatory mediators in mast

cells (Babolewska et al. 2014; Babolewska and Brzezinska-Blaszczyk 2015). Work in our laboratory has also revealed that LL-37 induces synthesis and release of cys-LTs from human eosinophils via FPR2/ALX by enhancing $cPLA_2$ activity and inducing intracellular translocation and assembly of 5-LOX and LTC_4S at perinuclear locations and lipid bodies (Sun et al. 2013). In addition, we could observe that eosinophils from asthmatics express significantly higher hCAP18 protein levels compared with those from healthy subjects, and eosinophils isolated from asthmatics released more hCAP18 upon leukotriene stimulation than did cells from healthy subjects (Sun et al. 2013). Hence, this study suggested that a positive feedback loop also exists between leukotrienes and LL-37 in eosinophils, which might contribute to asthma progress. The results from all these studies indicate that the proinflammatory circuit between HDPs and eicosanoids in mast cells and eosinophils may be undesirable for the host during an antimicrobial process, and could be a potential therapeutic target for allergic diseases, such as asthma.

6.3.2 Other HDPs and Eicosanoid Production

One early study investigated the effects of two magainin peptides originally isolated from the skin of the African claw toad (*Xenopus laevis*) on eicosanoid synthesis by rat peritoneal macrophages stimulated with LPS and lipid A from *Salmonella*. The results showed that depending on the type of peptide used and on its concentration, these two magainin peptides exhibited different effects on LPS or lipid A-induced macrophage eicosanoid synthesis (TXB_2 and 6-keto-$PGF_{1\alpha}$) (Matera et al. 1993). Another family of α-helical HDPs, pleurocidins, originate from fish and are structurally and functionally similar to cathelicidins (Cole et al. 1997; Patrzykat et al. 2003). Chiou et al. demonstrated that cecropin and pleurocidin induce gene expression of IL-1β and COX-2 in a trout macrophage cell line (Peter Chiou et al. 2006), suggesting a proinflammatory role of pleurocidin in the vertebrate immune system. One recent report illustrated that pleurocidins activated human mast cells to induce intracellular calcium mobilization and production of cys-LTs and PGD_2 in human mast cells through the FPR2/ALX receptor (Pundir et al. 2014). Moreover, catestatin, a neuroendocrine antimicrobial peptide, can also activate human mast cells to induce intracellular calcium mobilization and the production of cys-LTs, PGD_2 and PGE_2 (Aung et al. 2011). Another report revealed that hBD-3 and LL-37 function as proinflammatory mediators to up-regulate COX-2 expression and PGE_2 synthesis in human gingival fibroblasts (Chotjumlong et al. 2010, 2013). Furthermore, we also identified that LL-37 elicited a biphasic release of eicosanoids in macrophages with early, Ca^{2+}-dependent formation of LTB_4 and TXA_2 (measured as TXB_2) followed by a late peak of TXA_2 (measured as TXB_2), generated via induction of COX-2 by internalized LL-37 (Wan et al. 2014). Importantly, our findings provide evidence that LL-37 is an endogenous regulator of eicosanoid-dependent inflammatory responses in vivo, since intraperitoneal injection of mice with murine cathelicidin-related antimicrobial peptide (mCRAMP) induces significantly higher levels of LTB_4 and

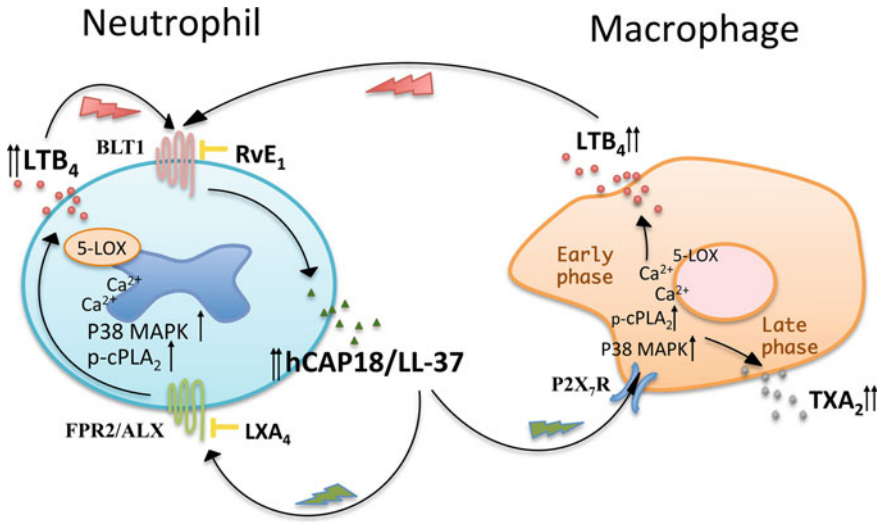

Fig. 6.4 The crosstalk between LL-37, a human HDP, and eicosanoids in leukocytes. Inflammation is induced (e.g., by microbes), and neutrophils are recruited to the sites of inflammation guided by chemoattractants, such as LTB_4. LTB_4 can trigger the release of hCAP18/LL-37 via BLT1. The released LL-37 conversely induces LTB_4 production from neutrophils via FPR2/ALX, or from macrophages via $P2X_7R$, by triggering intracellular calcium mobilization, activating p38 MAP kinase, promoting phosphorylation of $cPLA_2$ and translocation of 5-LOX. Similarly, the released LL-37 also induces LTB_4 production from macrophages. Therefore, there is a positive feedback loop between LL-37 and LTB_4 production in leukocytes, which can be blocked by the anti-inflammatory lipid mediators LXA_4 and RvE_1 by competition with LL-37 or LTB_4 for binding at FPR2/ALX or BLT1, respectively. Moreover, the neutrophil-released LL-37 can also be taken up by macrophages, leading to further eicosanoid production such as TXA_2 or facilitated intracellular bacterial killing

TXA_2 in mouse ascites rich in macrophages. Conversely, cathelicidin-deficient ($Cnlp^{-/-}$) mice produce much less LTB_4 and TXB_2 in vivo in response to TNF-α compared with control mice (Wan et al. 2014). Additionally, we also found that LL-37 induces angiogenesis via PGE_2–EP3 signaling in endothelial cells, and mCRAMP also induced prostaglandin-dependent angiogenesis in vivo, which could be blocked by aspirin (Salvado et al. 2013).

6.4 Conclusion

As two important components in innate immunity and inflammation, plenty of studies on HDPs and eicosanoids have been undertaken. Accumulated evidence demonstrates that HDPs not only kill pathogens directly, but also possess potent immunoregulatory activities including regulation of eicosanoid production. Meanwhile, eicosanoids participate in host defense via various mechanisms, one of

which is to modulate HDP production. The crosstalk between these two families of molecules has been concluded in Fig. 6.4. Considering the involvement of these two families of peptides and lipids in several pathophysiological situations, such as infections and allergy, intervention in positive cross-signaling loops has become a potential strategy for treatments against infection, allergy, and other inflammatory diseases.

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References

Agard M, Asakrah S, Morici LA (2013) PGE(2) suppression of innate immunity during mucosal bacterial infection. Front Cell Infect Microbiol 3:45

Aliberti J, Serhan C, Sher A (2002) Parasite-induced lipoxin A4 is an endogenous regulator of IL-12 production and immunopathology in Toxoplasma gondii infection. J Exp Med 196 (9):1253–1262

Aronoff DM, Canetti C, Peters-Golden M (2004) Prostaglandin E2 inhibits alveolar macrophage phagocytosis through an E-prostanoid 2 receptor-mediated increase in intracellular cyclic AMP. J Immunol 173(1):559–565

Aronoff DM et al (2008) Misoprostol impairs female reproductive tract innate immunity against Clostridium sordellii. J Immunol 180(12):8222–8230

Aung G et al (2011) Catestatin, a neuroendocrine antimicrobial peptide, induces human mast cell migration, degranulation and production of cytokines and chemokines. Immunology 132(4):527–539

Babolewska E, Brzezinska-Blaszczyk E (2015) Human-derived cathelicidin LL-37 directly activates mast cells to proinflammatory mediator synthesis and migratory response. Cell Immunol 293(2):67–73

Babolewska E, Pietrzak A, Brzezinska-Blaszczyk E (2014) Cathelicidin rCRAMP stimulates rat mast cells to generate cysteinyl leukotrienes, synthesize TNF and migrate: involvement of PLC/A2, PI3 K and MAPK signaling pathways. Int Immunol 26(11):637–646

Back M et al (2011) International Union of Basic and Clinical Pharmacology. LXXXIV: leukotriene receptor nomenclature, distribution, and pathophysiological functions. Pharmacol Rev 63(3):539–584

Bafica A et al (2005) Host control of Mycobacterium tuberculosis is regulated by 5-lipoxygenase-dependent lipoxin production. J Clin Invest 115(6):1601–1606

Bailie MB et al (1996) Leukotriene-deficient mice manifest enhanced lethality from Klebsiella pneumonia in association with decreased alveolar macrophage phagocytic and bactericidal activities. J Immunol 157(12):5221–5224

Bernard JJ, Gallo RL (2010) Cyclooxygenase-2 enhances antimicrobial peptide expression and killing of Staphylococcus aureus. J Immunol 185(11):6535–6544

Bertin J et al (2012) Leukotrienes inhibit early stages of HIV-1 infection in monocyte-derived microglia-like cells. J Neuroinflamm 9:55

Bozinovski S et al (2012) Serum amyloid A opposes lipoxin A(4) to mediate glucocorticoid refractory lung inflammation in chronic obstructive pulmonary disease. Proc Natl Acad Sci U S A 109(3):935–940

Campbell EL, Serhan CN, Colgan SP (2011) Antimicrobial aspects of inflammatory resolution in the mucosa: a role for proresolving mediators. J Immunol 187(7):3475–3481
Canavaci AM et al (2014) The acute phase of Trypanosoma cruzi infection is attenuated in 5-lipoxygenase-deficient mice. Mediators Inflamm 2014:893634
Canny G et al (2002) Lipid mediator-induced expression of bactericidal/permeability-increasing protein (BPI) in human mucosal epithelia. Proc Natl Acad Sci U S A 99(6):3902–3907
Cederlund A et al (2014) Label-free quantitative mass spectrometry reveals novel pathways involved in LL-37 expression. J Innate Immun 6(3):365–376
Chaves MM et al (2014) Leukotriene B4 modulates P2X7 receptor-mediated Leishmania amazonensis elimination in murine macrophages. J Immunol 192(10):4765–4773
Chen X et al (2007) Antimicrobial peptides human beta-defensin (hBD)-3 and hBD-4 activate mast cells and increase skin vascular permeability. Eur J Immunol 37(2):434–444
Chen M et al (2008) Lipid mediators in innate immunity against tuberculosis: opposing roles of PGE2 and LXA4 in the induction of macrophage death. J Exp Med 205(12):2791–2801
Chen JH et al (2015) Prostaglandin E2 and programmed cell death 1 signaling coordinately impair CTL function and survival during chronic viral infection. Nat Med 21(4):327–334
Chiang N et al (2012) Infection regulates pro-resolving mediators that lower antibiotic requirements. Nature 484(7395):524–528
Chotjumlong P et al (2010) Human beta-defensin-3 up-regulates cyclooxygenase-2 expression and prostaglandin E2 synthesis in human gingival fibroblasts. J Periodontal Res 45(4):464–470
Chotjumlong P et al (2013) Involvement of the P2X7 purinergic receptor and c-Jun N-terminal and extracellular signal-regulated kinases in cyclooxygenase-2 and prostaglandin E2 induction by LL-37. J Innate Immun 5(1):72–83
Chouinard F et al (2013) 2-Arachidonoyl-glycerol- and arachidonic acid-stimulated neutrophils release antimicrobial effectors against E. coli, S. aureus, HSV-1, and RSV. J Leukoc Biol 93 (2):267–276
Cilloniz C et al (2010) Lethal dissemination of H5N1 influenza virus is associated with dysregulation of inflammation and lipoxin signaling in a mouse model of infection. J Virol 84 (15):7613–7624
Claria J, Serhan CN (1995) Aspirin triggers previously undescribed bioactive eicosanoids by human endothelial cell-leukocyte interactions. Proc Natl Acad Sci U S A 92(21):9475–9479
Colby JK et al (2015) Differences in lung Cryptococcus neoformans burden after infection are associated with lipoxin signaling. Am J Respir Cell Mol Biol
Cole AM, Weis P, Diamond G (1997) Isolation and characterization of pleurocidin, an antimicrobial peptide in the skin secretions of winter flounder. J Biol Chem 272(18):12008–12013
Cooray SN et al (2013) Ligand-specific conformational change of the G-protein-coupled receptor ALX/FPR2 determines proresolving functional responses. Proc Natl Acad Sci U S A 110 (45):18232–18237
Coulombe F et al (2014) Targeted prostaglandin E2 inhibition enhances antiviral immunity through induction of type I interferon and apoptosis in macrophages. Immunity 40(4):554–568
Dennis EA, Norris PC (2015) Eicosanoid storm in infection and inflammation. Nat Rev Immunol
Dennis EA et al (2011) Phospholipase A2 enzymes: physical structure, biological function, disease implication, chemical inhibition, and therapeutic intervention. Chem Rev 111(10):6130–6185
Dixon RA et al (1990) Requirement of a 5-lipoxygenase-activating protein for leukotriene synthesis. Nature 343(6255):282–284
Edenius C, Haeggstrom J, Lindgren JA (1988) Transcellular conversion of endogenous arachidonic acid to lipoxins in mixed human platelet-granulocyte suspensions. Biochem Biophys Res Commun 157(2):801–807
Edfeldt K et al (2006) Involvement of the antimicrobial peptide LL-37 in human atherosclerosis. Arterioscler Thromb Vasc Biol 26(7):1551–1557
Filep JG (2013) Biasing the lipoxin A4/formyl peptide receptor 2 pushes inflammatory resolution. Proc Natl Acad Sci U S A 110(45):18033–18034

Fiore S et al (1994) Identification of a human cDNA encoding a functional high affinity lipoxin A4 receptor. J Exp Med 180(1):253–260
Flamand L et al (2004) Release of anti-HIV mediators after administration of leukotriene B4 to humans. J Infect Dis 189(11):2001–2009
Flamand L, Tremblay MJ, Borgeat P (2007) Leukotriene B4 triggers the in vitro and in vivo release of potent antimicrobial agents. J Immunol 178(12):8036–8045
Fujishima H et al (1999) Cytosolic phospholipase A2 is essential for both the immediate and the delayed phases of eicosanoid generation in mouse bone marrow-derived mast cells. Proc Natl Acad Sci U S A 96(9):4803–4807
Funk CD (2005) Leukotriene modifiers as potential therapeutics for cardiovascular disease. Nat Rev Drug Discov 4(8):664–672
Ganz T et al (1985) Defensins. Natural peptide antibiotics of human neutrophils. J Clin Invest 76(4):1427–1435
Garcia Gil de Munoz FL et al (2008) Prostaglandin E2 modulates the expression of antimicrobial peptides in the fat body and midgut of Anopheles albimanus. Arch Insect Biochem Physiol 68(1):14–25
Gaudreault E, Gosselin J (2007) Leukotriene B4-mediated release of antimicrobial peptides against cytomegalovirus is BLT1 dependent. Viral Immunol 20(3):407–420
Gaudreault E, Gosselin J (2008) Leukotriene B4 induces release of antimicrobial peptides in lungs of virally infected mice. J Immunol 180(9):6211–6221
Gijon MA et al (2000) Cytosolic phospholipase A2 is required for macrophage arachidonic acid release by agonists that Do and Do not mobilize calcium. Novel role of mitogen-activated protein kinase pathways in cytosolic phospholipase A2 regulation. J Biol Chem 275(26): 20146–20156
Gosselin J, Borgeat P, Flamand L (2005) Leukotriene B4 protects latently infected mice against murine cytomegalovirus reactivation following allogeneic transplantation. J Immunol 174(3): 1587–1593
Gudmundsson GH et al (1996) The human gene FALL39 and processing of the cathelin precursor to the antibacterial peptide LL-37 in granulocytes. Eur J Biochem 238(2):325–332
Haeggstrom JZ, Funk CD (2011) Lipoxygenase and leukotriene pathways: biochemistry, biology, and roles in disease. Chem Rev 111(10):5866–5898
Harizi H, Gualde N (2005) The impact of eicosanoids on the crosstalk between innate and adaptive immunity: the key roles of dendritic cells. Tissue Antigens 65(6):507–514
Hawthorne AB et al (1992) Colorectal leukotriene B4 synthesis in vitro in inflammatory bowel disease: inhibition by the selective 5-lipoxygenase inhibitor BWA4C. Gut 33(4):513–517
Hirata T, Narumiya S (2012) Prostanoids as regulators of innate and adaptive immunity. Adv Immunol 116:143–174
Hwang J et al (2013) An entomopathogenic bacterium, Xenorhabdus nematophila, suppresses expression of antimicrobial peptides controlled by Toll and Imd pathways by blocking eicosanoid biosynthesis. Arch Insect Biochem Physiol 83(3):151–169
Kalinski P (2012) Regulation of immune responses by prostaglandin E2. J Immunol 188(1):21–28
Kanda N, Ishikawa T, Watanabe S (2010) Prostaglandin D2 induces the production of human beta-defensin-3 in human keratinocytes. Biochem Pharmacol 79(7):982–989
Kanda N et al (2011) The antimycotic drugs itraconazole and terbinafine hydrochloride induce the production of human beta-defensin-3 in human keratinocytes. Immunobiology 216(4):497–504
Kase K et al (2009) Inhibitory action of roxithromycin on histamine release and prostaglandin D2 production from beta-defensin 2-stimulated mast cells. Int J Mol Med 23(3):337–340
Kaul V et al (2012) An important role of prostanoid receptor EP2 in host resistance to Mycobacterium tuberculosis infection in mice. J Infect Dis 206(12):1816–1825
Kim SJ (1990) Elevated formation of lipoxins in viral antibody-positive rat alveolar macrophages. Am J Respir Cell Mol Biol 3(2):113–118
Le Bel M, Brunet A, Gosselin J (2014) Leukotriene B4, an endogenous stimulator of the innate immune response against pathogens. J Innate Immun 6(2):159–168

Lee SP et al (2009) Crosstalk between prostaglandin E2 and leukotriene B4 regulates phagocytosis in alveolar macrophages via combinatorial effects on cyclic AMP. J Immunol 182(1):530–537
Leslie CC (2015) Cytosolic phospholipase A2: physiological function and role in disease. J Lipid Res
Machado ER et al (2005) Leukotrienes play a role in the control of parasite burden in murine strongyloidiasis. J Immunol 175(6):3892–3899
Mancuso P et al (1998) 5-Lipoxygenase reaction products modulate alveolar macrophage phagocytosis of Klebsiella pneumoniae. Infect Immun 66(11):5140–5146
Mancuso P et al (2010) Intrapulmonary administration of leukotriene B4 enhances pulmonary host defense against pneumococcal pneumonia. Infect Immun 78(5):2264–2271
Mansour SC, Pena OM, Hancock RE (2014) Host defense peptides: front-line immunomodulators. Trends Immunol 35(9):443–450
Mason KL et al (2013) Intrauterine group A streptococcal infections are exacerbated by prostaglandin E2. J Immunol 191(5):2457–2465
Matera G et al (1993) Effects of two magainin peptides on eicosanoid release from rat peritoneal macrophages. Antimicrob Agents Chemother 37(3):393–397
Matsuoka T et al (2000) Prostaglandin D2 as a mediator of allergic asthma. Science 287(5460):2013–2017
Mayer-Barber KD et al (2014) Host-directed therapy of tuberculosis based on interleukin-1 and type I interferon crosstalk. Nature 511(7507):99–103
Medeiros AI et al (2008) Leukotrienes are potent adjuvant during fungal infection: effects on memory T cells. J Immunol 181(12):8544–8551
Miller JS, Nguyen T, Stanley-Samuelson DW (1994) Eicosanoids mediate insect nodulation responses to bacterial infections. Proc Natl Acad Sci U S A 91(26):12418–12422
Molina-Berrios A et al (2013) Protective role of acetylsalicylic acid in experimental Trypanosoma cruzi infection: evidence of a 15-epi-lipoxin A(4)-mediated effect. PLoS Negl Trop Dis 7(4):e2173
Morato CI et al (2014) Essential role of leukotriene B4 on Leishmania (Viannia) braziliensis killing by human macrophages. Microbes Infect 16(11):945–953
Morato-Marques M et al (2011) Leukotrienes target F-actin/cofilin-1 to enhance alveolar macrophage anti-fungal activity. J Biol Chem 286(33):28902–28913
Morishima I et al (1997) Eicosanoids mediate induction of immune genes in the fat body of the silkworm, Bombyx mori. FEBS Lett 419(1):83–86
Niyonsaba F et al (2001) Evaluation of the effects of peptide antibiotics human beta-defensins-1/-2 and LL-37 on histamine release and prostaglandin D(2) production from mast cells. Eur J Immunol 31(4):1066–1075
Niyonsaba F et al (2002) Epithelial cell-derived human beta-defensin-2 acts as a chemotaxin for mast cells through a pertussis toxin-sensitive and phospholipase C-dependent pathway. Int Immunol 14(4):421–426
Noguchi T et al (2003) Syntheses of prostaglandin E2 and E-cadherin and gene expression of beta-defensin-2 by human gingival epithelial cells in response to Actinobacillus actinomycetemcomitans. Inflammation 27(6):341–349
Patrzykat A et al (2003) Novel antimicrobial peptides derived from flatfish genes. Antimicrob Agents Chemother 47(8):2464–2470
Peres CM et al (2007) Inhibition of leukotriene biosynthesis abrogates the host control of Mycobacterium tuberculosis. Microbes Infect 9(4):483–489
Peres-Buzalaf C et al (2011) Control of experimental pulmonary tuberculosis depends more on immunostimulatory leukotrienes than on the absence of immunosuppressive prostaglandins. Prostaglandins Leukot Essent Fatty Acids 85(2):75–81
Perretti M, D'Acquisto F (2009) Annexin A1 and glucocorticoids as effectors of the resolution of inflammation. Nat Rev Immunol 9(1):62–70
Peter Chiou P et al (2006) Effects of linear cationic alpha-helical antimicrobial peptides on immune-relevant genes in trout macrophages. Dev Comp Immunol 30(9):797–806

Peters-Golden M, Henderson WR Jr (2007) Leukotrienes. N Engl J Med 357(18):1841–1854
Peters-Golden M et al (2005) Leukotrienes: underappreciated mediators of innate immune responses. J Immunol 174(2):589–594
Pundir P et al (2014) Pleurocidin, a novel antimicrobial peptide, induces human mast cell activation through the FPRL1 receptor. Mucosal Immunol 7(1):177–187
Qiu H et al (2006) Expression of 5-lipoxygenase and leukotriene A4 hydrolase in human atherosclerotic lesions correlates with symptoms of plaque instability. Proc Natl Acad Sci U S A 103(21):8161–8166
Rinaldi SF et al (2015) 15-epi-lipoxin A4 reduces the mortality of prematurely born pups in a mouse model of infection-induced preterm birth. Mol Hum Reprod 21(4):359–368
Romano M, Serhan CN (1992) Lipoxin generation by permeabilized human platelets. Biochemistry 31(35):8269–8277
Salvado MD et al (2013) Cathelicidin LL-37 induces angiogenesis via PGE2-EP3 signaling in endothelial cells, in vivo inhibition by aspirin. Arterioscler Thromb Vasc Biol 33(8): 1965–1972
Samuelsson B, Funk CD (1989) Enzymes involved in the biosynthesis of leukotriene B4. J Biol Chem 264(33):19469–19472
Samuelsson B et al (1987) Leukotrienes and lipoxins: structures, biosynthesis, and biological effects. Science 237(4819):1171–1176
Satpathy SR et al (2015) Crystalline silica-induced leukotriene B4-dependent inflammation promotes lung tumour growth. Nat Commun 6:7064
Schauber J et al (2006) Heterogeneous expression of human cathelicidin hCAP18/LL-37 in inflammatory bowel diseases. Eur J Gastroenterol Hepatol 18(6):615–621
Secatto A et al (2012) 5-Lipoxygenase deficiency impairs innate and adaptive immune responses during fungal infection. PLoS ONE 7(3):e31701
Secatto A et al (2014) The leukotriene B(4)/BLT(1) axis is a key determinant in susceptibility and resistance to histoplasmosis. PLoS ONE 9(1):e85083
Serezani CH et al (2005) Leukotrienes enhance the bactericidal activity of alveolar macrophages against Klebsiella pneumoniae through the activation of NADPH oxidase. Blood 106(3): 1067–1075
Serezani CH et al (2006) Leukotrienes are essential for the control of Leishmania amazonensis infection and contribute to strain variation in susceptibility. J Immunol 177(5):3201–3208
Serezani CH et al (2007) Prostaglandin E2 suppresses bacterial killing in alveolar macrophages by inhibiting NADPH oxidase. Am J Respir Cell Mol Biol 37(5):562–570
Serezani CH et al (2012) PTEN directly activates the actin depolymerization factor cofilin-1 during PGE2-mediated inhibition of phagocytosis of fungi. Sci Signal 5(210):ra12
Serhan CN (1997) Lipoxins and novel aspirin-triggered 15-epi-lipoxins (ATL): a jungle of cell-cell interactions or a therapeutic opportunity? Prostaglandins 53(2):107–137
Serhan CN (2005) Lipoxins and aspirin-triggered 15-epi-lipoxins are the first lipid mediators of endogenous anti-inflammation and resolution. Prostaglandins Leukot Essent Fatty Acids 73(3–4):141–162
Serhan CN (2014) Pro-resolving lipid mediators are leads for resolution physiology. Nature 510(7503):92–101
Serhan CN, Hamberg M, Samuelsson B (1984a) Trihydroxytetraenes: a novel series of compounds formed from arachidonic acid in human leukocytes. Biochem Biophys Res Commun 118(3): 943–949
Serhan CN, Hamberg M, Samuelsson B (1984b) Lipoxins: novel series of biologically active compounds formed from arachidonic acid in human leukocytes. Proc Natl Acad Sci U S A 81(17):5335–5339
Serhan CN et al (2003) Reduced inflammation and tissue damage in transgenic rabbits overexpressing 15-lipoxygenase and endogenous anti-inflammatory lipid mediators. J Immunol 171(12):6856–6865
Shirey KA et al (2014) Role of the lipoxygenase pathway in RSV-induced alternatively activated macrophages leading to resolution of lung pathology. Mucosal Immunol 7(3):549–557

Shrestha S, Kim Y (2009) Various eicosanoids modulate the cellular and humoral immune responses of the beet armyworm, Spodoptera exigua. Biosci Biotechnol Biochem 73(9): 2077–2084
Shryock N et al (2013) Lipoxin A(4) and 15-epi-lipoxin A(4) protect against experimental cerebral malaria by inhibiting IL-12/IFN-gamma in the brain. PLoS ONE 8(4):e61882
Singh PK et al (1998) Production of beta-defensins by human airway epithelia. Proc Natl Acad Sci U S A 95(25):14961–14966
Smith WL, Urade Y, Jakobsson PJ (2011) Enzymes of the cyclooxygenase pathways of prostanoid biosynthesis. Chem Rev 111(10):5821–5865
Soares EM et al (2013) Leukotriene B4 enhances innate immune defense against the puerperal sepsis agent Streptococcus pyogenes. J Immunol 190(4):1614–1622
Sorensen O et al (1997) The human antibacterial cathelicidin, hCAP-18, is synthesized in myelocytes and metamyelocytes and localized to specific granules in neutrophils. Blood 90(7):2796–2803
Sorensen OE et al (2001) Human cathelicidin, hCAP-18, is processed to the antimicrobial peptide LL-37 by extracellular cleavage with proteinase 3. Blood 97(12):3951–3959
Spencer LT et al (2004) Role of human neutrophil peptides in lung inflammation associated with alpha1-antitrypsin deficiency. Am J Physiol Lung Cell Mol Physiol 286(3):L514–L520
Stanley-Samuelson DW et al (1991) Insect immune response to bacterial infection is mediated by eicosanoids. Proc Natl Acad Sci U S A 88(3):1064–1068
Stanley-Samuelson D et al (1997) Eicosanoids mediate microaggregation and nodulation responses to bacterial infections in black cutworms, Agrotis ipsilon, and true armyworms, Pseudaletia unipuncta. J Insect Physiol 43(2):125–133
Sun SC, Faye I (1995) Transcription of immune genes in the giant silkmoth, Hyalophora cecropia, is augmented by H_2O_2 and diminished by thiol reagents. Eur J Biochem 231(1):93–98
Sun J et al (2013) The antimicrobial peptide LL-37 induces synthesis and release of cysteinyl leukotrienes from human eosinophils–implications for asthma. Allergy 68(3):304–311
Suram S et al (2006) Regulation of cytosolic phospholipase A2 activation and cyclooxygenase 2 expression in macrophages by the beta-glucan receptor. J Biol Chem 281(9):5506–5514
Suram S et al (2010) Pathways regulating cytosolic phospholipase A2 activation and eicosanoid production in macrophages by Candida albicans. J Biol Chem 285(40):30676–30685
Suram S et al (2013) Cytosolic phospholipase A(2)alpha and eicosanoids regulate expression of genes in macrophages involved in host defense and inflammation. PLoS ONE 8(7):e69002
Talvani A et al (2002) Leukotriene B(4) induces nitric oxide synthesis in Trypanosoma cruzi-infected murine macrophages and mediates resistance to infection. Infect Immun 70(8):4247–4253
Tang X et al (2015) P2X7 Receptor regulates internalization of antimicrobial peptide LL-37 by human macrophages that promotes intracellular pathogen clearance. J Immunol
Thivierge M et al (1998) Prostaglandin E2 induces resistance to human immunodeficiency virus-1 infection in monocyte-derived macrophages: downregulation of CCR5 expression by cyclic adenosine monophosphate. Blood 92(1):40–45
Tobin DM et al (2010) The lta4 h locus modulates susceptibility to mycobacterial infection in zebrafish and humans. Cell 140(5):717–730
Tobin DM et al (2012) Host genotype-specific therapies can optimize the inflammatory response to mycobacterial infections. Cell 148(3):434–446
Ueda T et al (2014) Combination therapy of 15-epi-lipoxin A4 with antibiotics protects mice from Escherichia coli-induced sepsis*. Crit Care Med 42(4):e288–e295
Valdez PA et al (2012) Prostaglandin E2 suppresses antifungal immunity by inhibiting interferon regulatory factor 4 function and interleukin-17 expression in T cells. Immunity 36(4):668–679
Valore EV et al (1998) Human beta-defensin-1: an antimicrobial peptide of urogenital tissues. J Clin Invest 101(8):1633–1642
von Haussen J et al (2008) The host defence peptide LL-37/hCAP-18 is a growth factor for lung cancer cells. Lung Cancer 59(1):12–23

Wan M et al (2007) Leukotriene B4 triggers release of the cathelicidin LL-37 from human neutrophils: novel lipid-peptide interactions in innate immune responses. FASEB J 21(11):2897–2905

Wan M et al (2011) Leukotriene B4/antimicrobial peptide LL-37 proinflammatory circuits are mediated by BLT1 and FPR2/ALX and are counterregulated by lipoxin A4 and resolvin E1. FASEB J 25(5):1697–1705

Wan M et al (2014a) Cathelicidin LL-37 induces time-resolved release of LTB4 and TXA2 by human macrophages and triggers eicosanoid generation in vivo. FASEB J 28(8):3456–3467

Wan M et al (2014b) Antimicrobial peptide LL-37 promotes bacterial phagocytosis by human macrophages. J Leukoc Biol 95(6):971–981

Woodward DF, Jones RL, Narumiya S (2011) International Union of Basic and Clinical Pharmacology. LXXXIII: classification of prostanoid receptors, updating 15 years of progress. Pharmacol Rev 63(3):471–538

Yajima M et al (2003) A newly established in vitro culture using transgenic Drosophila reveals functional coupling between the phospholipase A2-generated fatty acid cascade and lipopolysaccharide-dependent activation of the immune deficiency (imd) pathway in insect immunity. Biochem J 371(Pt 1):205–210

Yang D et al (1999) Beta-defensins: linking innate and adaptive immunity through dendritic and T cell CCR6. Science 286(5439):525–528

Zasloff M (2002) Antimicrobial peptides of multicellular organisms. Nature 415(6870):389–395

Part III
Development of Resistance

Chapter 7
Bacterial Resistance to Host Defence Peptides

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Abstract Currently, antimicrobial drug resistance is a global problem that threatens to precipitate a 'Post-antibiotic era' in which the ability of common infections and minor injuries to kill is a very real possibility. A potential solution to this problem is the development of host defence peptides, which are endogenous antibiotics that kill microbes via membranolytic action, based in part on the belief that microbes were unlikely to develop resistance to this action. However, the incidence of microbes exhibiting resistance to the action of host defence peptides is growing and an increasingly diverse spectrum of mechanisms is being reported to underpin this resistance. These mechanisms can be broadly categorized as those that either: destroy these peptides, such as through the production of bacterial proteases; intercept/shield these peptides, such as by the release of host cell proteoglycans by bacterial enzymes; or export these peptides, such as via the use of bacterial efflux pumps. Here we give an overview of these mechanisms, with a focus on recent developments in this area, and then discuss the potential of inhibitors of these resistance mechanisms to treat infections due to bacterial pathogens.

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7.1 Introduction

Antimicrobial drug resistance is a global problem, the gravity of which, was highlighted by a recent report issued by the World Health Organization, which stated that: …. *Antimicrobial resistance threatens the effective prevention and treatment of an ever-increasing range of infections caused by bacteria, parasites, viruses and fungi. An increasing number of governments around the world are devoting efforts to a problem so serious that it threatens the achievements of modern medicine. A post-antibiotic era—in which common infections and minor injuries can kill—far from being an apocalyptic fantasy, is instead a very real possibility for the twenty first Century* (World Health Organization 2014).

This rhetoric harks back to the pre-antibiotic era when currently treatable infections such as syphilis were endemic and almost incurable whilst infections such as diarrhoea and pneumonia and those caught post-surgically were the main causes of death (Zaffiri et al. 2012). This era is generally taken to have ended towards the early part of the last century with the discovery and commercial use of the first antibiotics such as Salvarsan, Penicillin and Streptomycin (Pringle 2013; Aminov 2010; Radecka et al. 2014). With the advent of these drugs, so began what came to be known as the 'Golden age of antibiotics' which led to an increase in human life expectancy of circa 8 years (Mills and Dougherty 2012), accompanied by a rapid loss of interest in the therapeutic potential of natural host antibiotics and the importance of this immune defence strategy (Zaffiri et al. 2012; Bentley 2009). However, in the early 1960s, the rise of multi-drug-resistant (MDR) microbial pathogens coupled to the concomitant loss of efficacy by conventional antibiotics signalled the end of this 'Golden age' (Mills and Dougherty 2012; Davies 2006; Katz et al. 2006; Cole 2014; Tremolieres 2010) and prompted a resurgence of interest in the development of host defence peptides (HDPs) (Phoenix et al. 2013a, 2014; Franco and Parachin 2014; Drider and Rebuffat 2011).

Research into HDPs produced by prokaryotes has its origins in the 1920s (Drider and Rebuffat 2011), which accelerated in the 1960s and currently, these peptides have a range of therapeutic and biotechnical applications such as in the areas of food science, pharmaceutics and clinical medicine (Drider and Rebuffat 2011; Balciunas et al. 2013; Nishie et al. 2012; Duquesne et al. 2007; Yang 2014). However, MDR organisms with resistance to the action of these peptides are becoming a major problem (Bastos et al. 2015; Cotter et al. 2013; Hassan et al. 2012; Nawrocki et al. 2014), particularly in the cases of nisin, which is extensively used in food preservation (Zhou et al. 2014; Draper et al. 2015; Kaur et al. 2011), and polymixins and daptomycin, which are regarded as last-resort options in the treatment of Gram-negative and Gram-positive infections, respectively (Stefania et al. 2015; Bayer et al. 2013; Vilhena and Bettencourt 2012; Yu et al. 2015; Olaitan et al. 2014; Bialvaei and Kafil 2015). Research into HDPs from eukaryotes is generally taken to have its origins in the early 1960s, which led to a number of

landmark studies and the first major reports of these peptides in the 1980s (Phoenix et al. 2013). In 1981, Boman and colleagues injected bacteria into the pupae of the silk moth, *Hyalophora cecropia*, and isolated several inducible antimicrobial peptides (Hultmark et al. 1980), which were later characterized and named 'cecropins' (Steiner et al. 1981). In the mid-1980s, antimicrobial peptides were identified by Lehrer and colleagues in rabbits and humans, which are now known as α-defensins (Selsted et al. 1983, 1984, 1985; Ganz et al. 1985). Soon after, in 1987, Zasloff and co-workers isolated and characterized antimicrobial peptides from the African clawed frog, *Xenopus laevis*, and named these peptides magainins after the Hebrew word for 'Shield' thereby acknowledging their defence role (Zasloff 1987). Since these earlier studies, over 2500 HDPs have been reported in the APD2 database (Wang et al. 2009, 2010) and today many of these peptides are undergoing extensive investigation as putative antimicrobials including a number of HDPs that have strong clinical potential and are in clinical trials (Fox 2013; Kang et al. 2014; Ashby et al. 2014). Currently, one of the most promising examples of these HDPs is pexiganan, a homologue of magainin (Locilex®) that is currently in phase III clinical trials as a broad-spectrum, topical treatment for patients with mild infections of diabetic foot ulcers (Fox 2013; ClinicalTrials.gov 2014).

The perceived clinical potential of HDPs in part derives from the view held around the time of their discovery, which was that these peptides had maintained their antimicrobial capacity over evolutionary time (10^8 years), despite their continual presence in microbial environments, and microbes were therefore, unlikely to develop resistance to their action (Zasloff 2002). However, today, although low, the incidence of microbes exhibiting resistance to the action of HDPs is growing and an increasingly diverse spectrum of mechanisms is being reported to underpin this resistance (Nawrocki et al. 2014; Koprivnjak and Peschel 2011; Guilhelmelli et al. 2013; Gruenheid and Le Moual 2012). Moreover, recent in vitro studies showed that bacterial resistance to HDPs could be evolved experimentally within a few hundred generations (Pranting et al. 2008; Habets et al. 2012; Dobson et al. 2013) and that such bacteria can survive better in vivo as demonstrated in an animal model (Dobson et al. 2014). In particular, it has been shown that bacterial resistance to pexiganan can be experimentally evolved (Perron et al. 2006), which in this case also provides cross-resistance to human HDPs that act as key components of the innate immune response to infection (Habets and Brockhurst 2012). Clearly, this situation could severely undermine the ability of the innate immune system to control and clear microbial infections and thereby pose grave potential risks for anti-infective therapies based on HDPs (Dobson et al. 2014). Here we give an overview of mechanisms that underpin bacterial resistance to the action of HDPs with a focus on recent developments in this area. We then discuss the potential of inhibitors of these resistance mechanisms to treat infections due to bacterial pathogens.

7.2 Bacterial Interaction with HDPs

Bacteria resistance to the action of HDPs is an aspect of host–pathogen interactions that result from mutual inhibition, evasion and adaption strategies that have evolved over millions of years and is a part of ongoing highly dynamic co-evolutionary processes (Peschel and Sahl 2006). In this context, the production of HDPs by eukaryotes can also be considered a resistance mechanism to protect the host from bacterial infection (Heimlich et al. 2014). Indeed, the relatively recent discovery of HDPs helped to explain why plants and insects remain free from infections for most of the time (Phoenix et al. 2013a) although they lack adaptive immune systems (Jones and Dangl 2006; Spoel and Dong 2012; Vilcinskas 2013; Lemaitre and Hoffmann 2007). The vast majority of HDPs are cationic to facilitate direct electrostatic interaction with anionic components of the bacterial cell envelope (Guilhelmelli et al. 2013; Phoenix et al. 2013; Cruz et al. 2014) although a number of these peptides are anionic and use varying strategies to interact with these anionic envelope components (Harris et al. 2009, 2011; Phoenix et al. 2013). It is believed that, in general, HDPs engage in low affinity, electrostatic interactions with negatively charged components of the cell envelope to promote their passage through the envelope, although this process is not well understood (Fig. 7.1). It has been suggested that these low affinity interactions result in the formation of polyelectrolyte complexes with peptidoglycan and/or lipopolysaccharide (LPS), which helps these peptides attach to the cell wall and diffuse or migrate to the cytoplasmic membrane (CM) of the target bacteria (Dorner and Lienkamp 2014; Brogden 2005; Phoenix et al. 2013; McPhee et al. 2009). It is generally accepted that an essential step in the antibacterial mechanisms of all HDPs is interaction with the CM (Phoenix et al. 2013; Teixeira et al. 2012; Epand and Epand 2011; Harris et al. 2013), which is rich in anionic lipids. These lipids comprise over 80 % of the total lipid found in the membranes of Gram-positive bacteria and up to 30 % of the total lipid present in membranes of Gram-negative bacteria (Epand and Epand 2011). In most cases, interaction with the CM is the major site of action for HDPs and these interactions generally lead to either lysis or permeabilisation of the membrane (Zasloff 2002; Guilhelmelli et al. 2013; Phoenix et al. 2013a, b, c; Cruz et al. 2014; Harris et al. 2009, 2011, 2013; Teixeira et al. 2012; Epand and Epand 2011; Schmidt and Wong 2013), although in some cases, these peptides are translocated across the bilayer to attack intracellular targets such as DNA (Silhavy et al. 2010; Nicolas 2009; Marcos and Gandia 2009; Splith and Neundorf 2011; Last et al. 2013; Henriques et al. 2006; Wilmes et al. 2011; Anaya-Lopez et al. 2013).

In response, Gram-positive and Gram-negative bacteria have developed sensor systems that are able to detect the presence of both cationic and anionic HDPs and induce adaptive mechanisms of resistance to these peptides (Heimlich et al. 2014; Otto 2009; Poole 2012; Kindrachuk and Napper 2008). A well characterized example in the case of Gram-negative bacteria is the two-component regulatory system, PhoPQ, in which PhoQ, a transmembrane kinase sensor, detects HDPs thereby inducing the phosphorylation and activation of the cytoplasmic

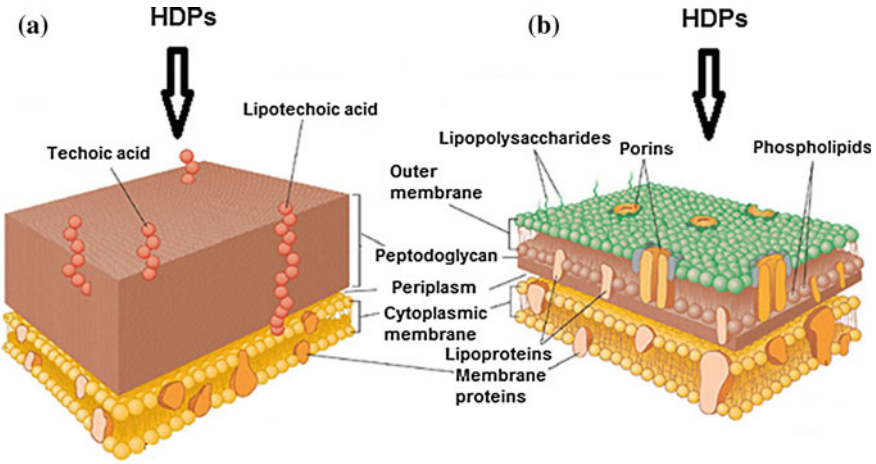

Fig. 7.1 Bacterial cell envelopes. Fig. 7.1 shows the cell envelope for Gram-positive bacteria (a) and Gram-negative bacteria (b). Essentially, the bacterial cell envelope is a complex multilayered structure that serves to protect these organisms from the external environment. The envelope of Gram-negative bacteria comprises three principal layers; the outer membrane (OM), which is predominantly formed from lipopolysaccharide (LPS), a thin peptidoglycan cell wall, and the cytoplasmic membrane (CM), which is primarily formed from phospholipids. The OM and CM delimit the periplasm, which is an aqueous cellular compartment (Fig. 7.1b) (Silhavy et al. 2010). In contrast, the Gram-positive cell envelope possesses no outer membrane and includes a peptidoglycan layer that is many times thicker than that found in Gram-negative bacteria. Threading through this peptidoglycan layer are long polymers, including teichoic acids, which are covalently attached to peptidoglycan, and lipoteichoic acids, which are anchored to the headgroups of membrane lipids. Sandwiched between this peptidoglycan layer and the CM of Gram-positive bacteria is the periplasm, which is much thinner than that of Gram-negative bacteria (Fig. 7.1a). Interaction with the CM of Gram-positive and Gram-negative bacteria to facilitate their antibacterial activity is a requirement of all known HDPs (Guilhelmelli et al. 2013; Phoenix et al. 2013a, c; Cruz et al. 2014; Teixeira et al. 2012; Epand and Epand 2011; Harris et al. 2013). It is believed that these peptides, which are predominantly cationic (Phoenix et al. 2013; Brogden 2005), target bacteria via the overall net negative charge on the outer surface of their cell envelopes, primarily due to the presence of techoic and lipotechoic acids in the case of Gram-positive organisms and LPS in the case of Gram-negative bacteria (Splith and Neundorf 2011). Low affinity, electrostatic interactions between HDPs and negatively charged components of the cell envelope, such as peptidoglycan moieties and/or LPS, then helps these peptides attach to the cell wall and migrate to the CM of the target bacteria (Dorner and Lienkamp 2014; Brogden 2005; Phoenix et al. 2013; McPhee et al. 2009). Interaction with the CM of both Gram-positive and Gram-negative bacteria, which is rich in anionic lipids (Epand and Epand 2011), then generally leads to either lysis or permeabilisation of the membrane, which in most cases is the site of action of HDPs (Zasloff 2002; Guihelmelli et al. 2013; Phoenix et al. 2013a, b, c; Cruz et al. 2014; Harris et al. 2009, 2011, 2013; Teixeira et al. 2012; Epand and Epand 2011; Schmidt and Wong 2013), or the translocation of these peptides across the bilayer to attack intracellular targets such as DNA (Nicolas 2009; Marcos and Gandia 2009; Splith and Neundorf 2011; Last et al. 2013; Henriques et al. 2006; Wilmes et al. 2011; Anaya-Lopez et al. 2013).

transcription factor, PhoP (Otto 2009; Poole 2012; Vasil and Darwin 2012; Groisman 2001). This regulatory system responds to environmental stressors and modulates the expression of genes that contribute to the modification of LPS in the outer membrane (OM) (Fig. 7.1), leading to increased resistance to HDPs (Gruenheid and Le Moual 2012; Needham and Trent 2013; Band and Weiss 2015). In this capacity, the PhoPQ regulatory system has been best described in *Salmonella typhimurium* (Dalebroux and Miller 2014; Bader et al. 2003) but has also been demonstrated to play an analogous role in other pathogens (Heimlich et al. 2014; Poole 2012; Kindrachuk and Napper 2008; Band and Weiss 2015) including: *Pseudomonas aeruginosa* (Breidenstein et al. 2011; Miller et al. 2011), *Escherichia coli* (Alteri et al. 2011; Rubin et al. 2015), *Klebsiella pneumoniae* (Llobet et al. 2011; Cheng et al. 2010), *Erwina amylovora* (Nakka et al. 2010) and *Serratia marcescens* (Lin et al. 2014). Sensor systems for the detection of HDPs in Gram-positive bacteria are less well described than those in Gram-negative bacteria (Otto 2009; Poole 2012) and one of the most intensively studied of these systems is the GraSR regulon of *Staphylococcus aureus* and *Staphylococcus epidermis* (also known as the Aps regulon), which has recently been shown to also involve graX. This gene is also involved in resistance to HDPs and is cotranscribed with graRS, encoding a regulatory cofactor of the GraSR signalling pathway, which effectively constitutes a three-component system, GraXSR (Heimlich et al. 2014; Yang et al. 2012; Li et al. 2007; Muzamal et al. 2014; Falord et al. 2012). In this system, the transmembrane kinase sensor, GraS, detects HDPs, resulting in the phosphorylation and activation of the cytoplasmic transcription factor, GraR, to induce membrane modifications and the use of transport systems that mediate resistance to these peptides (Otto 2009; Poole 2012; Yang et al. 2012; Li et al. 2007; Falord et al. 2011; Joo and Otto 2015; Matsuo et al. 2011). In addition to those induced by PhoPQ and GraXSR, a variety of other bacterial resistance mechanisms to HDPs, both adaptive and intrinsic, are encountered by HDPs as they migrate through the envelope systems of these organisms to interact with the CM (Fig. 7.1) (Nawrocki et al. 2014; Koprivnjak and Peschel 2011; Guilhelmelli et al. 2013; Anaya-Lopez et al. 2013; Band and Weiss 2015; Dorotkiewicz-Jach et al. 2015; German et al. 2008).

7.3 Bacterial Defences to the Action of HDPs

In their quest to attain the CM of bacteria, HDPs must traverse through multiple layers of the cell envelopes possessed by these organisms, including capsules, the outer membrane, peptidoglycan and the periplasm (Fig. 7.1). In response, bacteria have acquired defence mechanisms that are active at multiple points along the passage of these peptides through the cell envelope (Nawrocki et al. 2014; Koprivnjak and Peschel 2011; Guilhelmelli et al. 2013; Anaya-Lopez et al. 2013; Band and Weiss 2015; Dorotkiewicz-Jach et al. 2015) and can be broadly

categorized as those that either destroy, intercept/shield or export these peptides (LaRock and Nizet 2015).

7.3.1 Bacterial Resistance Mechanisms that Destroy HDPs

In relation to the destruction of HDPs, this can be indirect through bacterial modulation of the host expression of these peptides, thereby reducing their concentration and potential for antibacterial action (Guihelmellai et al. 2013). For example, *Shigella flexneri* (Islam et al. 2001; Sperandio et al. 2008), which is a Gram-negative pathogen that causes bacillary dysentery (Lima et al. 2015), was shown to subvert the host immune system by downregulating the expression of human LL-37 and HBD-1. This downregulation of host gene expression appeared to significantly reduce the production of these HDPs thereby facilitating deeper invasion of human intestinal crypts by the organism (Islam et al. 2001; Sperandio et al. 2008).

However, the major mechanism used by bacteria to destroy HDPs is directly through the use of proteases (Nawrocki et al. 2014; Koprivnjak and Peschel 2011; Guihelmelli et al. 2013; Anaya-Lopez et al. 2013; Band and Weiss 2015), which are used by these organisms for a variety of purposes during the infection process (Frees et al. 2013). Based on the active residues that are involved in catalysis, proteases can be divided into six major groups: cysteine proteases, threonine proteases, glutamic proteases, aspartic proteases, metalloproteases and serine proteases with members of these last three of these groups being the most abundant (Barrett et al. 2012). Currently, there appears to be only one major report of a cytostolic protease mediating bacterial resistance to HDPs, which is oligopeptidase B (OpdB), a serine peptidase with unknown physiological function (Coetzer et al. 2008; Mohamed Mustafa et al. 2012; Szeltner and Polgar 2008). It was found that OpdB was able to degrade a variety of proline-rich HDPs with intracellular targets, such as the Bac7(1-35) and Bac7(1-16) derivatives of bovine Bac7, so conferring *E. coli* with reduced sensitivity to their action (Mattiuzzo et al. 2007, 2014).

There is evidence to suggest that the degradation of some HDPs is mediated by periplasmic proteases as in the case of *Burkholderia cenocepacia,* and other species of this genus, which degrade apiarian, melittin and other HDPs using the periplasmic serine protease, MucD (Loutet et al. 2011). A periplasmic serine protease, DegP (HtrA), along with extracellular serine proteases, metalloproteases and aspartic proteases has also been associated with resistance to lactoferrin B in *E. coli* but whereas the proteolytic activity of these extracellular proteases was shown to contribute to this resistance mechanism, the role of DegP, which has a number of physiological functions, is currently unclear (Ulvatne et al. 2002).

However, in most cases, bacterial resistance to HDPs is associated with extracellular proteases (Nawrocki et al. 2014; Koprivnjak and Peschel 2011; Gruenheid and Le Moual 2012; Anaya-Lopez et al. 2013; Band and Weiss 2015; Dorotkiewicz-Jach et al. 2015), which are in close contact with host components

(Frees et al. 2013). For example, *Porphyromonas gingivalis*, which is the Gram-negative pathogen most associated with chronic periodontal disease (Mysak et al. 2014; Bostanci and Belibasakis 2012), is highly proteolytic and secretes three proteases known as gingipains that belong to the cysteine family of proteases and cleave substrates after arginine and lysine residues (Guo et al. 2000; Grenier and La 2011). The gingipains have been shown to degrade a plethora of HDPs, including LL-37, HBD-1, human HBD-2, HBD-3 and histatin 5; magainin 2; ranine dermaseptin and brevinin; arctian cecropin B; and vespine mastoparan (Carlisle et al. 2009; Devine et al. 1999; Gutner et al. 2009; Maisetta et al. 2011). Some Gram-negative pathogens also degrade HDPs through the use of omptins, which is an expanding family of outer membrane (OM) proteases mainly found in *Enterobacteriaceae*. These proteases possess a unique active site that combines elements of both serine and aspartate proteases, and interaction with LPS has been shown to be critical for their activity. High overall levels of sequence homology exist between omptins of different bacteria suggesting that their use by Gram-negative pathogens to degrade HDPs may be common (Gruenheid and Le Moual 2012; Band and Weiss 2015; Lin et al. 2002; Hritonenko and Stathopoulos 2007; Haiko et al. 2009; Kukkonen and Korhonen 2004). As an example, enteropathogenic *E. coli* (EPEC) and enterohaemorrhagic *E. coli* (EHEC) are two clinically important human gastrointestinal pathogens (Wong et al. 2011). Recent studies on the omptin produced by these *E. coli* strains, OmpT, showed that the protease was able to degrade LL-37 in EHEC more efficiently than in EPEC due to differences in the regulation of ompT genes, which resulted in higher levels of the protease in EHEC (Thomassin et al. 2012). A follow-up study suggested that this differential expression of OmpT by EHEC and EPEC was due to these *E. coli* strains colonizing different niches of the human gastrointestinal tract where they would be exposed to different patterns of host HDPs. For example, EHEC most likely developed high OmpT expression to survive the high levels of LL-37 in the large intestine whereas low expression of the protease in EPEC is sufficient to counter the trace amounts of LL-37 in the small intestine (Thomassin et al. 2012). Consistent with these latter studies, work on uropathegenic *E coli* (UPEC), which is the causative agent of most urinary tract infections (Ulett et al. 2013), demonstrated that OmpT from this strain of the organism can degrade LL-37 but only when the protease is expressed at high levels. It was suggested that OmpT was not essential for resistance to LL-37 in the case of UPEC and that resistance to HDPs mediated by this omptin was specific to a given bacterial pathogen (Brannon et al. 2013). More recent work on adherent-invasive *E. coli* (AIEC), which is associated with Crohn's disease (Agus et al. 2014), demonstrated that plasmid-encoded ArlC is a protease belonging to the OmpT family of proteases and appeared to degrade a range of HDPs, including LL-37, HBD-1 and human HNP-5 (McPhee et al. 2014). It was suggested by these latter authors that the degradation of HDPs by AEIC resulted from combinatorial proteolytic activity between ArlC and OmpT (McPhee et al. 2014), which is chromosomally encoded and common to the *E. coli* lineage (Kukkonen and Korhonen 2004). These results were consistent with previous studies on *E. coli*, which demonstrated that the plasmid encoded protease, OmpP,

degraded piscine protamine, which is abasic peptide that serves as an HDP, with an enhanced degradation capacity seen in strains carrying both this omptin and chromosomal OmpT (Hwang et al. 2007). Other work on *E. coli* has also reported that OmpT was able to efficiently degrade protamine (Stumpe et al. 1998) and provide the organism with resistance to HDPs present in urine, which appeared to include HBD-1 to HBD-6, and HNP-1 to HNP-6 (Hui et al. 2010). In relation to Gram-positive bacteria, *Streptococcus pyogenes*, which over the past two decades has caused a resurgence of several infective syndromes such as soft tissue infections (STIs) and scarlet fever (Wong and Yuen 2012), has been shown to produce the cysteine protease, SpeB (Schmidtchen et al. 2002). This protease was shown to degrade LL-37 both in vitro and in vivo in biopsy specimens from patients with severe STIs due to the organism (Schmidtchen et al. 2002; Johansson et al. 2008). SpeB also plays a role in another strategy used by Gram-positive bacteria to resist HDPs, which involves the formation of complexes that retain their proteases near the bacterial surface. In addition to HDPs (Schmidtchen et al. 2002), SpeB cleaves other host proteins such as fibrin and immunoglobulins (Nelson et al. 2011; Rasmussen and Bjorck 2002) and the protease was found to complex with the host proteinase inhibitor, α2-macroglobulin (α2 M), during infection by the organism (Nyberg et al. 2004). These catalytically active complexes were retained on the bacterial cell surface by high affinity association with the *S. pyogenes* G-related α2M-binding protein (GRAB) (Nelson et al. 2011; Rasmussen and Bjorck 2002; Nyberg et al. 2004). Although trapped in this complex, SpeB retained its ability to degrade LL-37 and interestingly, its activity against the peptide was enhanced, as evidenced by the reduced killing of *S. pyogenes* in vitro (Nyberg et al. 2004).

7.3.2 *Bacterial Resistance Mechanisms that Intercept/Shield HDPs*

Bacterial defence mechanisms that intercept HDPs have been developed by both Gram-positive and Gram-negative bacteria and include extracellular or surface-linked proteins and polysaccharides that intercept and directly bind to these peptides, thereby inhibiting their passage through the bacterial envelope and blocking access to the CM (Nawrocki et al. 2014; Koprivnjak and Peschel 2011; Gruenheid and Le Moual 2012; Anaya-Lopez et al. 2013; Band and Weiss 2015; Dorotkiewicz-Jach et al. 2015). These molecules can be derived from host cells as in the case of anionic proteoglycans, which can be cleaved and released from the surface of fibroblasts and epithelial cells by bacterial enzymes to sequester HDPs (Menozzi et al. 2002; Zamfir et al. 2003). A major example of these proteoglycans is decorin and it was found that when this molecule was incubated with a range of bacteria, including *S. pyogenes* and *P. aeruginosa*, it was cleaved by the bacterial proteases, SpeB and this proteolytic action released a number of products, including dermatan sulphate, which was able to bind HNP-1 and render these organisms

resistant to the action of the peptide (Zamfir et al. 2003; Schmidtchen et al. 2001). Similarly, several studies showed that the incubation of *P. aeruginosa* with syndecan-1, which is a proteoglycan found on the surface of epithelial cells, led to cleavage of the molecule and release of its soluble ectodomain (heparin sulphate chains), which was able to bind LL-37 and other HDPs. These studies also showed that in a murine model of lung infection, the shedding of syndecan-1 was activated by *P. aeruginosa* and that either inhibition of this shedding or inactivation of the shed ectodomain's heparan sulphate chains prevented lung infection in the murine model (Park et al. 2000, 2001).

Bacterially produced polysaccharides involved in resistance to HDPs are composed of structurally diverse polymers and can be attached to the cell surface of bacteria through covalent linkages with the cell wall when they are known as capsules or capsular polysaccharides. Other extracellular polysaccharides can be polymers that are loosely attached to the cell surface and are generally referred to as exopolysaccharides (EPS) (Yother 2011; Taylor and Roberts 2005; Nwodo et al. 2012; Schmid et al. 2015). These extracellular polysaccharides play critical roles in bacterial survival strategies, including the promotion of both virulence and host colonization (Mishra and Jha 2013; Ullrich 2009), and there is evidence to suggest that they also mediate resistance to HDPs in both Gram-positive bacteria and Gram-negative bacteria via the binding or electrostatic repulsion of HPDs (Nawrocki et al. 2014; Koprivnjak and Peschel 2011; Guihelmelli et al. 2013; Anaya-Lopez et al. 2013; Band and Weiss 2015; Dorotkiewicz-Jach et al. 2015). Major examples of secreted EPS are polysaccharide intercellular adhesin (PIA), also known as poly-N-acetylglucosamine (PNAG), and poly-γ-glutamic acid (PGA). These molecules are both cationic EPS that are produced by a range of staphylococci, including *S. Epidermidis* and *S. aureus*, and homologous systems have been reported in other bacteria (Nawrocki et al. 2014; Joo and Otto 2015). Several studies on *S. epidermidis* suggested that both PIA and PGA were able to bind HDPs and endow the organism with resistance to LL-37, HBD-3, and the human anionic HDP, dermcidin (Vuong et al. 2004a, b; Kocianova et al. 2005). It was suggested that whilst this protection from HDPs was likely to include electrostatic repulsion, other resistance mechanisms such as those based on the moderation of electrostatic sequestration could also contribute to bacterial resistance to these peptides (Joo and Otto 2015). As an example of a surface-anchored EPS, several studies on *Campylobacter jejuni*, which is an important human foodborne pathogen that causes diarrheal disease (Sahin et al. 2015), showed that truncation of the core of lipooligosaccharide, which is a low molecular weight form of LPS, increased the sensitivity of the organism to HDPs such as LL-37, HNP-5, murine cryptdin-4 and avian fowlicidin-1. It was suggested that truncation of these core regions might remove negatively charged sialic acids, which bind cationic AMPs and thereby endow the organism with resistance to these peptides (Naito et al. 2010; Keo et al. 2011).

One of the first secreted proteins reported to intercept and bind HDPs was the serum inhibitor of complement (SIC), which is produced by *S. pyogenes* and was first characterized for its role protecting the organism against killing by the

membrane attack complex (Åkesson et al. 1996). However, SIC was later found to not only interfere with the activation of the contact system but to also bind and inhibit the action of a range of HDPs generated through complement and contact activation, including LL-37, HNP-1, HBD-1 to HBD-4, and murine CRAMP (mCRAMP). SIC was also shown to enhance the dissemination and attenuate the virulence of *S. pyogenes* in murine models of infection by the organism (Pence et al. 2010; Frick et al. 2003, 2011a, b; Fernie-King et al. 2004). The SIC protein of *S. pyogenes* has also been shown to bind and neutralize the activity of BRAK/CXCL14 and midkine (Frick et al. 2011b), which are chemokines with antibacterial activity (Gela et al. 2014; Wolf and Moser 2012). The same study showed that the FAF surface protein of *Finegoldia magna* (*Peptostreptococcus magnus*), which is a commensal organism of human skin that can act as an opportunistic pathogen (Murphy and Frick 2013), also bound these chemokines but more efficiently than SIC of *S. pyogenes*. It was suggested that this difference in efficiency of binding antibacterial chemokines might be related to the differing requirements of the two organisms for virulence (Frick et al. 2011b). The FAF surface protein is used by *F. magna t*o adhere to the basement membrane of the human epidermis (Murphy et al. 2014) and has previously been shown to bind and inactivate LL-37 in a similar manner to SIC of *S. Pyogenes* (Frick et al. 2008). More recent studies have identified several proteins with homology to SIC that have similar activity against HDPs such as HBD-3 and LL-37, including DRS, which is also found in strains of *S. pyogenes* (Fernie-King et al. 2007), and DrsG, which occurs in *Streptococcus dysgalactiae* (Smyth et al. 2014), an emerging pathogen of fishes and mammals (Abdelsalam et al. 2013).

Both Gram-positive and Gram-negative bacteria are known to use surface-anchored proteins to intercept HDPs (Nawrocki et al. 2014; Koprivnjak and Peschel 2011; Guihelmelli et al. 2013; Anaya-Lopez et al. 2013; Band and Weiss 2015; Dorotkiewicz-Jach et al. 2015), such as the long filamentous, proteinaceous structures known as pili or fimbriae that extend from their surface and are often involved in the initial adhesion of the bacteria to host tissues during colonization. In the former case, these surface structures are formed by the covalent polymerization of adhesive pilin subunits and in the latter case, they are composed of non-covalently associated protein subunits called pilins or fimbrins (Proft and Baker 2009; Danne and Dramsi 2012; Telford et al. 2006; Fronzes et al. 2008). As an example of these protein structures, curli are a fairly recently identified class of pili, which are essentially amyloid fibres formed from repeating subunits of the major pilin CsgA (curlin). Curli are expressed on the outer surfaces of Enterobacteriaceae, which are best characterized in species of *Escherichia* and *Salmonella*. These surface structures have been implicated in a number of biological processes, including biofilm formation, cell aggregation, host cell adhesion and invasion (Proft and Baker 2009; Fronzes et al. 2008; Evans and Chapman 2014; Costa et al. 2015). However, in a recent study, curli expressed by UPEC increased the resistance of the organism to the action of LL-37 by binding the peptide via the overall negative charge carried by the CsgA subunits of the curli (Kai-Larsen et al. 2010). In the case of Gram-positive bacteria, the fimbrial M pili protein is produced by *S. pyogenes*

(Telford et al. 2006) and is multifunctional during infection, being linked to both host tissue adherence and invasive disease (Bisno et al. 2003; Ghosh 2011). Several studies have shown that one M protein variant encoded by the isolates most commonly associated with infection, M1, can protect *S. pyogenes* by directly binding and neutralizing HDPs, such as LL-37 and mCRAMP (Cole et al. 2010; Lauth et al. 2009). Furthermore, the sequestration of LL-37 by M1 also promotes the survival of *S. pyogenes* in neutrophil extracellular traps (NETs) (Cole et al. 2010; Lauth et al. 2009), which are able to engulf and kill microbial pathogens and are formed from networks of extracellular fibres, primarily composed of DNA from neutrophils that are associated with HDPs (Brinkmann and Zychlinsky 2012; Cooper et al. 2000; Halverson et al. 2015). High levels of LL-37 were associated with DNA within these structures but the function of this association appeared to be stabilizing the structure of NETs against bacterial nuclease degradation rather than antibacterial activity, which is lost when the peptide binds to DNA (Neumann et al. 2014). More recent studies have suggested that the sequestration of LL-37 by M1 promotes the survival of *S. pyogenes* by reducing the availability of the peptide to participate in the formation of NETs. It appears that, upon bacterial infection, LL-37 released by host cells translocates to the nucleus of neutrophils, leading to disruption of the nuclear membrane, the release of DNA and the formation of NETs (Neumann et al. 2014; von Koeckritz-Blickwede 2012). Another protective consequence of the M1-mediated sequestration of HDPs may be to inhibit the recently reported ability of these peptides to target the ExPortal of *S. pyogenes* membranes (Vega and Caparon 2012; Port et al. 2014), which is a unique microdomain of these membranes that is specialized for protein secretion and processing (Rosch and Caparon 2005; Rosch et al. 2007; Vega et al. 2013). The disruption of ExPortal organization in the organism's membranes by HDPs leads to a redistribution of ExPortal components into the peripheral membrane and the inhibition of secreted defence molecules including the protease SpeB, described above, but interestingly, not SIC (Vega and Caparon 2012; Port et al. 2014).

Resistance to HDPs can also be mediated by intrinsic properties of the OM and CM such as membrane fluidity, the formation of outer membrane vesicles (OMVs) and lipid receptors in the CM (Nawrocki et al. 2014; Koprivnjak and Peschel 2011; Band and Weiss 2015; Yeaman and Yount 2003; Schaffer 2006). Membrane fluidity encompasses the order, the mobility and the viscoelastic properties of the bilayer and several studies showed that strains of *S. aureus* with elevated levels of longer chain, unsaturated lipids in their CM exhibited higher degrees of fluidity and were more resistant to the action of human thrombin-induced platelet microbicidal protein-1 (tPMP-1), which is a basic peptide released by human platelets and effectively serves as an HDP, in comparison to their wild-type counterparts. (Bayer et al. 2000; Koo et al. 1996; Yeaman et al. 1998). Similarly, increased membrane fluidity and cell wall thickness appeared to mediate the resistance of methicillin-resistant *S. aureus* (MRSA), which is a major nosocomial pathogen that causes severe morbidity and mortality (Batabyal et al. 2012), to tPMP-1 and HNP-1 (Mishra et al. 2012). However, in contrast, work on other strains of *S. aureus* has shown that production of the carotenoid, staphyloxanthin, enhanced resistance to

HNP-1 by increasing order in the fatty acid tails of CM lipids and thereby increasing the rigidity of these membranes (Mishra et al. 2011; Pelz et al. 2005; Katzif et al. 2005). More recently, a number of studies have shown that lysylated phosphatidylglycerol (LysylPG), a cationic lipid found in *S. aureus* (Phoenix et al. 2013; Epand and Epand 2011; van Meer et al. 2008), can induce rigidity of the organism's CM, thereby inhibiting the insertion of magainin 2 (Shireen et al. 2013) and 6W-RP-1, a synthetic HDP (Kilelee et al. 2010). Studies on NK-2, which is also a synthetic HDP and derived from mammalian NK-lysin (Andra et al. 2004), suggested that this lysylPG mediated rigidifying effect, resulted from the binding of cationic HDPs to the CM of *S. aureus via* and the bridging of anionic lipid headgroups, thereby increasing the acyl chain order of the membrane (Andra et al. 2011). Interestingly, a similar lysylPG-mediated rigidifying effect was reported to contribute to the resistance of *S. aureus* to the action of maximin H5 (Dennison et al. 2015), which belongs to a suite of anionic HDPs, found in the skin secretions and brains of toads from the Bombina genus (Lai et al. 2002; Liu et al. 2011). However, studies on the interaction of the peptide with the CM of *S. aureus* showed that maximin H5 was effectively functioning as a cationic AMP via amidated residues at the two termini of its primary structure (Dennison et al. 2015). These amidated terminal residues are relatively accessible to external molecules whereas the anionic residues possessed by the peptide are buried within its α-helical hairpin-type structure (Phoenix et al. 2015; Dennison et al. 2015; Dennison et al. 2013). Studies on maximin H5 also showed that in addition to the rigidifying effect of lysylPG on the CM of the organism, this lipid mediated a protective effect by decreasing the affinity of the peptide for these membranes (Dennison et al. 2015). It is well established that cationic HDPs induce the incorporation of lysylPG into the *S. aureus* membranes via the GraXSR mediated expression of MprF, an enzyme that modifies anionic phospholipids with lysine (or alanine) effectively decreasing the net negative charge of these membranes and shielding them from the action of these peptides (Otto 2009; Li et al. 2007a; b). Resistance to the action of HPDs by the alteration of net charge on bacterial membranes through the modification of both CM and OM lipids has been described for many Gram-positive and Gram-negative bacteria, and has been extensively reviewed elsewhere (Nawrocki et al. 2014; Koprivnjak and Peschel 2011; Goyita et al. 2013; Gruenheid and Le Moual 2012; Anaya-Lopez et al. 2013; Band and Weiss 2015). However, it has been suggested that the bacterially mediated alteration of charge on CM and OM lipids is more likely to contribute to resistance to HDPs via the modulation of the fluidity of these membranes than electrostatic effects (Kilelee et al. 2010; Andra et al. 2011; Dennison et al. 2015). In combination, these studies show that both increases and decreases in membrane fluidity can lead to increased resistance to HDPs and similar results have been reported for other Gram-positive bacteria (Mehla and Sood 2011, 2013; Goyita et al. 2013). In relation to Gram-negative bacteria, some organisms resist HDPs by changing the fluidity of the OM through the modification of LPS, such as by the addition of hydrophobic lipid chains to lipid A phosphates, the glucosamine backbone or existing acyl chains (Koprivnjak and Peschel 2011; Needham and Trent 2013; Band and Weiss 2015). As an example, a

recent study on *Francisella novicida*, which are best known for their intracellular parasitic capabilities (Kingry and Petersen 2014), showed that increasing the length of lipid A acyl chains led to rigidity of the OM and resistance to the action HDPs whereas decreasing the length of these acyl chains led to increased fluidity and susceptibility to these peptides (Li et al. 2012). However, one of the best characterized examples of bacteria that utilize this mechanism to resist the action of HDPs is *S. typhimurium* (Dalebroux and Miller 2014), which employs PhoPQ-mediated changes to the structure of lipid A to reduce the fluidity of the OM, rendering the organism resistant to a range of HPDs (Koprivnjak and Peschel 2011; Band and Weiss 2015). Major examples of these peptides include LL-37, HNP-1, C18G, murine cryptidin 2, porcine protegrin-1 and leporine NP1, NP2 and NP5 (Guo et al. 1998; Belden and Miller 1994; Guina et al. 2000; Miller et al. 1990; Bader et al. 2005; Gunn and Miller 1996). In addition to LPS, another major component of the OM is phospholipid (Silhavy et al. 2010) and in *S. typhimurium*, the PhoPQ mediated addition of palmitoyl groups to phosphatidylglycerol leads to decreased fluidity in the OM and resistance to C18G, which is derived from human protein platelet factor 4, and other HDPs (Dalebroux et al. 2014). It has also been suggested that modulation of CM fluidity may help to protect Gram-negative bacteria from HDPs (Band and Weiss 2015) as in the case of *P. aeruginosa* and *Rhizobium tropici*, which is a soilborne symbiont of several legumes (Ormeno-Orrillo et al. 2012). Several studies showed that the presence of lysylPG in the CM of *P. aeruginosa* and *R. tropici*, helped to protect these organisms from the action of protamine, poly-l-lysine and other HDPs (Sohlenkamp et al. 2007; Arendt et al. 2012). LysylPG and aminoacyl-PG homologues have also been identified in other Gram-negative species (Arendt et al. 2012; Roy 2009) and taken in combination, these results suggested that the regulation of membrane fluidity to induce resistance to HDPs may be widely used by Gram-negative bacteria (Heimlich et al. 2014; Poole 2012; Needham and Trent 2013). It is clear from the above studies that membrane fluidity has a variable effect on bacterial resistance to HDPs and it is still an open question as to why this is the case but it has been proposed that this may reflect the fact that different resistance mechanisms may be specific for different peptides (Koprivnjak and Peschel 2011; Mehla and Sood 2013).

Another recently described intercept mechanism of resistance to HDPs is the production of outer membrane vesicles (OMVs) by Gram-negative bacteria, which are spherical, membrane structures that contain many components found within the OM of the parent organism. Gram-negative bacteria shed OMVs constitutively throughout their normal growth and it has been proposed that they have roles in virulence, inflammation and the envelope stress response of these organisms (Kaparakis-Liaskos and Ferrero 2015; Olsen and Amano 2015; Manning and Kuehn 2013). However, it is becoming increasingly clear that OMVs also play an important role in providing bacteria with resistance to HDPs (Band and Weiss 2015) as shown by studies on *E. Coli* and *S. typhimurium*. In this work, it was demonstrated that HDPs induced the production of OMVs, suggesting a regulated response by these organism, with the result that these peptides were adsorbed, or 'bound' by these vesicles thereby neutralizing their antibacterial action (McBroom

and Kuehn 2007; Manning and Kuehn 2011). A similar mechanism based on OMVs was reported more recently for resistance to the action of HDPs, including the prokaryotic HDP, polymixin B (PmB), *by Vibrio tasmaniensis* (Vanhove et al. 2015; Destoumieux-Garzón et al. 2014), which is a marine pathogen that infects oyster haemocytes (Destoumieux-Garzón et al. 2014; Romalde et al. 2014). Interestingly, studies on *Vibrio cholera*, which causes cholera in humans (Harris et al. 2012), found that OMVs produced by the organism were unable to protect it against a lethal concentration of LL-37. However, growing *V. cholera* in the presence of a sublethal concentration of PmB induced the release of OMVs that were larger in size and the secretion of a biofilm-associated extracellular matrix protein (Bap1) that associated with OmpT in these vesicles. Bap1, which is negatively charged, then bound LL-37 on the surface of these OMVs endowing *V. cholerae* with resistance to the action of the peptide (Destoumieux-Garzón et al. 2014; Duperthuy et al. 2013). In combination, these results clearly showed that the production of OMVs is an inducible mechanism of bacterial defence to HDPs that is able to provide cross-resistance to these peptides in some cases (Band and Weiss 2015). No analogous resistance mechanism to HDPs appears to have been reported for Gram-positive bacteria and Archaebacteria although a similar process of shedding membrane vesicles by these organisms has been described (Olsen and Amano 2015; Manning 2013; Avila-Calderón et al. 2015).

Most recently, studies on *E. coli* identified a previously unreported and novel 'intercept' mechanism of resistance to HDPs that involved the use of a CM receptor (Phoenix et al. 2015; Dennison et al. 2013). As described above, maximin H5 is an anionic amphibian peptide (Lai et al. 2002; Liu et al. 2011) and was shown to possess membranolytic activity towards both *S. aureus* and erythrocytes (Lai et al. 2002; Dennison et al. 2013, 2015). This activity appeared to be primarily driven by the formation of an oblique orientated α-helix by its amidated N-terminal segment, H_2N-ILGPVLGLVS, which induced deep bilayer insertion by the peptide at an angle of *circa* 45° (Fig. 7.2a). The amidated C-terminal segment, VLGIL-NH_2, of maximin H5 was also found to play a key role in its membranolytic activity by forming an intra-peptide hydrogen-bonding network with the N-terminal region of the peptide that stabilized the levels of its oblique-orientated α-helical structure (Fig. 7.2a) (Dennison et al. 2005, 2015; Phoenix et al. 2013). However, further investigations into the antimicrobial action of maximin H5 showed that it had no activity against other Gram-positive bacteria, Gram-negative bacteria, fungi or enveloped viruses (Lai et al. 2002; Dennison et al. 2013, 2015; Wang et al. 2010). A clear difference between these two groups of microbes was the presence of PE in the target membranes of organisms resistant to the action of maximin H5 and the absence of this lipid in the target membranes of microbes susceptible to its membranolytic activity (Epand and Epand 2011; van Meer et al. 2008; van Meer and de Kroon 2011; Lohner and Prenner 1999; Lösel 1900; Ratledge and Wilkinson 1988; Aloia et al. 1993). Studies on *E. coli* showed that maximin H5 had no propensity to partition into membranes derived from this organism, or other PE-containing membranes, and the peptide was predicted by MD simulations to remain bound to the surface of these bilayers via a variety of peptide–lipid interactions and

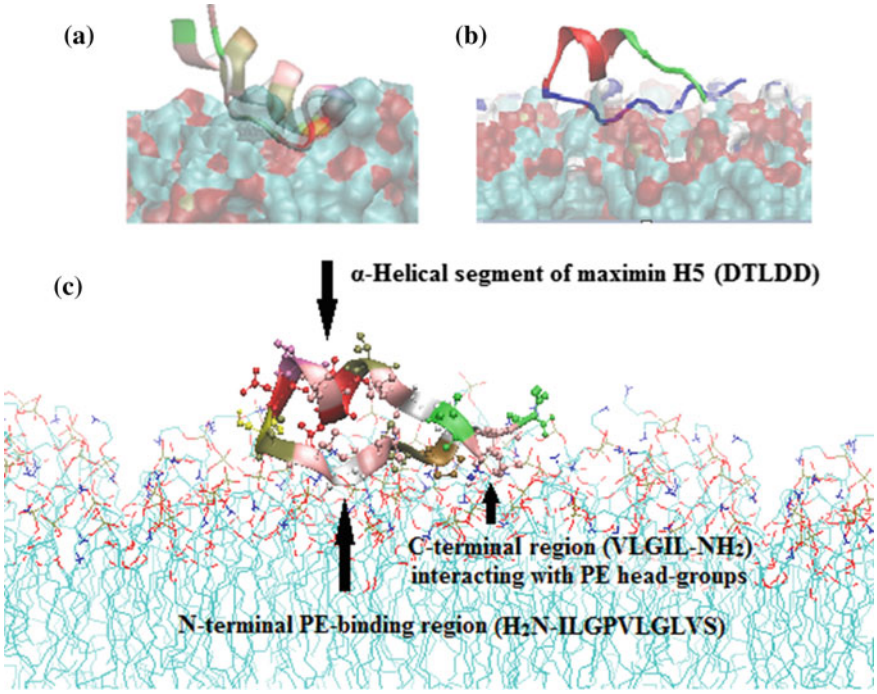

Fig. 7.2 Molecular dynamic simulations of maximin H5–membrane interactions. In Fig. 7.2a the peptide is partitioned into DMPC membranes via an oblique orientated α-helix formed by its amidated N-terminal segment, H2N-ILGPVLGLVS that induces deep bilayer at an angle of circa 45°. The amidated C-terminal segment, VLGIL-NH2, of maximin H5 forms an intra-peptide hydrogen-bonding network with the N-terminal region of the peptide that stabilizes the levels of its oblique-orientated α-helical structure (Dennison et al. 2005, 2015; Phoenix et al. 2013). Figure 7.2b, maximin H5 is bound to the surface of DMPE membranes and has clearly undergone a conformational change in relation to its membranolytic form (Dennison et al. 2015). Figure 7.2c shows a more detailed description of PE-bound maximin H5, indicating that the peptide possesses random coil structure in its N-terminal segment, H2N-ILGPVLGLVS, and there is an absence of intra-peptide interactions between this segment and the C-terminal segment, VLGIL-NH2 (Figs. 7.2b, c) that stabilizes the oblique-orientated α-helical structure observed in the peptide's membranolytic form (Fig. 7.2a) (Phoenix et al. 2015; Dennison et al. 2013).

intra-peptide associations (Figs. 7.2b, c). The major contributions to this peptide–membrane binding came from hydrogen bonding between phosphate and ammonium groups within the PE headgroup and residues in both terminal regions of maximin H5 (Phoenix et al. 2015; Dennison et al. 2013). Essentially, in relation to the membranolytic form of the peptide (Fig. 7.2a) (Dennison et al. 2015), maximin H5 appeared to undergo a conformational change in the presence of PE that led to random coil structure in its N-terminal segment, H_2N-ILGPVLGLVS, and the absence of intra-peptide interactions between this segment and the C-terminal segment, VLGIL-NH_2, of the peptide (Figs. 7.2b, c) (Phoenix et al. 2015; Dennison et al. 2013). Taken in combination, these data suggest that maximin H5 has high

affinity for PE that induces immobilization of the peptide on the surface of membranes, thereby inhibiting the ability of the peptide to adopt the membrane interactive, oblique-orientated α-helical structure necessary for its antimicrobial activity.

7.3.3 *Bacterial Resistance Mechanisms that Export HDPs*

Efflux systems are complexes of mostly membrane bound proteins that serve as energy-dependent transporters to extrude compounds from the cells of organisms across the three domains of life (Gupta et al. 2011; Saier 1998), including eukaryotes, such as humans (Miller 2015; Misaka et al. 2013; Köck and Brouwer 2012; Zhang et al. 2015; Kathawala et al. 2015), fish (Luckenbach et al. 2014; Ferreira et al. 2014) and fungi (Morace et al. 2014; Prasad and Rawal 2014; Viti et al. 2014), archaebacteria (Gudhka et al. 2015; Tanaka et al. 2013) and bacteria (Costa et al. 2015; Viti et al. 2014; Delmar et al. 2014; Sun et al. 2014; Lycklama and Driessen 2012; Palmer and Berks 2012; Davidson et al. 2008; Handzlik et al. 2013; Klein and Lewinson 2011). As is the case for every other living creature, all bacteria possess efflux pumps genes that are highly conserved, with all members of the same bacterial species possessing the same pumps. It is also now known that the expression of efflux pump genes is tightly controlled by various local and global transcriptional regulators, which suggested that drug efflux pumps have physiological functions. Currently, it is believed that bacterial efflux pumps play a general detoxification role in various physiological processes associated with these organisms along with contributions to other processes including cell-to-cell communication, biofilm formation, stress adaptation, development, pathogenesis and virulence (Sun et al. 2014; Alvarez-Ortega et al. 2013; Piddock 2006). However, these efflux pumps have emerged as major elements in the intrinsic and acquired resistance of bacterial pathogens to antibiotics and HDPs (Radecka et al. 2014; Nawrocki et al. 2014; Koprivnjak and Peschel 2011; Guihelmelli et al. 2013; Anaya-Lopez et al. 2013; Band and Weiss 2015; Dorotkiewicz-Jach et al. 2015; German et al. 2008; Handzlik et al. 2013; Andersen et al. 2015; Bhardwaj and Mohanty 2012; Kumar and Schweizer 2005; Blair et al. 2014; Nikaido and Pagès 2012; Poole 2000; Van Bambeke et al. 2003; Fernández and Hancock 2012; Martinez et al. 2009). The efflux pumps possessed by bacteria can be classified as belonging to five different groups depending upon a range of factors, including their amino acid sequence, number of transmembrane spanning regions, component stoichiometry, energy coupling mechanism, substrates, transport mode and phylogeny (Fig. 7.3): the major facilitator superfamily (MFS); the ATP-binding cassette (ABC) superfamily; the small multidrug resistance (SMR) family; the resistance-modulation-division (RND) superfamily; and the multidrug and toxic compound extrusion (MATE) superfamily (Fig. 7.3) (Saier 1998; Sun et al. 2014; Andersen et al. 2015; Paulsen 2003; Zgurskaya 2009; Putman et al. 2000; Li and Nikaido 2004, 2009; Collu and Cascella 2013). Except for the RND superfamily, which is only found in Gram-negative bacteria (Fernando and Kumar 2013; Anes

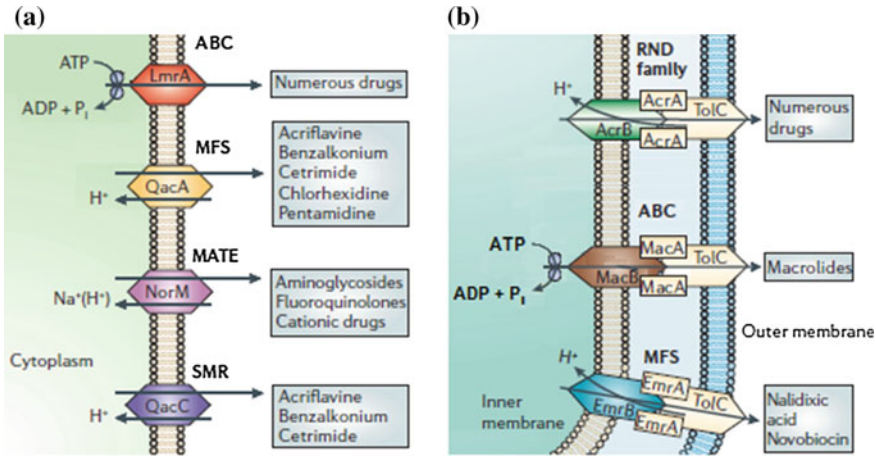

Fig. 7.3 The membrane location of bacterial efflux pumps. **a** Gram-positive bacteria. **b** Gram-negative bacteria. Figure 7.3 was revised from Piddock (2006) and shows the membrane location and typical substrates of major bacterial efflux pumps involved in multiple drug resistance. Shown in Fig. 7.3a are monopartite systems of the MFS (Major facilitator superfamily), the SMR (Small multidrug resistance) and MATE (multidrug and toxic compound extrusion) superfamilies. Representative examples of these monopartite systems include: LmrA from *Lactococcus lactis*, an ABC transporter (Poelarends et al. 2002); QacA from *S. aureus*, an MFS transporter (Brown and Skurray 2002); NorM from *Vibrio parahaemolyticus*, a MATE transporter (Morita et al. 2000); and QacC (also known as smr) from *S. aureus* (Costa et al. 2013). Shown in Fig. 7.3b are tripartite systems of the RND (resistance-modulation-division), ABC and MFS superfamilies, which are represented by the *E. coli* transporter complexes: AcrAB–TolC (Nikaido and Zgurskaya 2001), MacAB–TolC (Kobayashi et al. 2001) and EmrAB–TolC (Lewis 2000). These efflux systems differ in their energy sources with ABC transporters driven by ATP hydrolysis, MFS and SMR and RND transporters utilizing energy derived from H+ gradients and MATE transporters employing energy derived from Na^+/H^+ gradients (Piddock 2006).

et al. 2015), efflux systems of the remaining four superfamilies are widely distributed across both these latter organisms (Nawrocki et al. 2014; Band and Weiss 2015; German et al. 2008; Zgurskaya 2009) and Gram-positive bacteria (Handzlik et al. 2013). Most recently, a novel group of efflux pumps have been identified in Gram-negative bacteria, which, as yet, is largely uncharacterized and has been designated the PACE (proteobacterial antimicrobial compound efflux) family (Hassan et al. 2013; 2015). ABC efflux systems form the only multidrug pump superfamily that are primary transporters with extrusion powered by the direct hydrolysis of ATP by the transporter itself. The remaining four efflux systems are secondary transporters and extrude multiple drugs utilizing energy derived from H^+ gradients in the case of the RND, MFS and SMR superfamilies and Na^+/H^+ gradients in the case of the MATE superfamily (Fig. 7.3) (Saier 1998; Sun et al. 2014; Andersen et al. 2015; Paulsen 2003; Zgurskaya 2009; Putman et al. 2000; Li and Nikaido 2004, 2009).

Efflux systems can occur as monopartite systems as in the case of ABC, MFS, MATE and SMR transporters in the membranes of Gram-positive bacteria (Fig. 7.3). The MFS superfamily is one of the biggest groups of secondary transporters and includes a number of examples of these monopartite systems (Kumar et al. 2013; Reddy et al. 2012; Law et al. 2008; Yan 2013, 2015; Saidijam et al. 2006). For example, in Gram-positive bacteria, LmrP of *Lactococcu lactis* appears to capture its substrates from within the membrane or from the cytoplasm of these organisms, and then use H^+ antiport to transport them to the extracellular space (Poelarends et al. 2002; Masureel et al. 2014). In Gram-negative bacteria, the MFS transporter, EmrD of *E. coli,* is located in the CM of the organism and is believed to use H^+ antiport to carry captured substrates across these membranes to the periplasmic space (Yin et al. 2006; Baker et al. 2012). In the case of Gram-negative bacteria, to accommodate the double membrane systems of these organisms, efflux pumps can participate in tripartite assemblies that span these membrane systems (Fig. 7.3). In these tripartite assemblies, RND ABC and MFS transporter proteins located in the CM of Gram-negative bacteria are linked by periplasmic adaptor proteins (PAPs) to discrete channels in the outer membrane to create continuous conduits from the cytoplasm to the extracellular space (Li and Nikaido 2009; Hinchliffe et al. 2013; Bavro et al. 2015; Yamaguchi et al. 2015; Zgurskaya et al. 2015; Lin et al. 2014; Huang et al. 2013). Using the RND efflux pump, AcrA/AcrB/TolC, from *E. coli* as an example (Fig. 7.3), it is believed that the transporter protein, AcrB, captures its substrates either from within the CM or from the cytoplasm, and then transports them to the extracellular space through the outer membrane channel formed by TolC. Cooperation between AcrB and TolC is mediated by the PAP, AcrA, and efflux through RND systems is driven by energy derived from H^+ gradients (Sun et al. 2014; Amaral et al. 2012). A detailed description of current understanding of mechanisms underpinning the activity of these efflux systems is beyond the scope of this review but it is believed that they all essentially use variants of the 'alternating access' model (Slotboom 2014; Forrest et al. 2011; Jardetzky 1966), which involves substrate binding in the membrane embedded region of MATE, SMR, MFS and ABC transporters and in the periplasmic domain of RND transporters (Sun et al. 2014; Murakami 2008; Wong et al. 2014; Yu et al. 2013; Wilkens 2015; Radestock and Forrest 2011; Madej 2014). The physiological substrates of these efflux systems are believed to be primarily endogenous metabolites that are noxious to the host organism and virulence determinants that are secreted during stress adaptation, development and pathogenesis (Sun et al. 2014; Piddock 2006; Martinez et al. 2009; Piddock 2006; Poole 2008). However, the generally broad-range specificity of these efflux systems also enables them to extrude many non-physiological substrates (Sun et al. 2014; Nikaido and Pagès 2012; Wong et al. 2014; Yu et al. 2013; Elkins and Mullis 2006), which appears to underpin the role of these systems in the multiple resistance shown by bacteria to antibiotics (Radecka et al. 2014; Handzlik et al. 2013; Andersen et al. 2015; Bhardwaj and Mohanty 2012; Kumar and Schweizer 2005; Blair et al. 2014; Nikaido and Pagès 2012; Poole 2000; Van Bambeke et al. 2003; Fernández and Hancock 2012) and, as is becoming increasingly clear, their

defences against the action of HDPs (Nawrocki et al. 2014; Koprivnjak and Peschel 2011; Guihelmelli et al. 2013; Anaya-Lopez et al. 2013; Band and Weiss 2015; Dorotkiewicz-Jach et al. 2015; German et al. 2008).

7.3.3.1 MFS Efflux Pumps

Studies on *S. aureus* appeared to show that the efflux system, QacA, which is carried on the MDR plasmid pSK1, endowed the organism with resistance to the leporine tPMP-1, which is released from thrombin-stimulated rabbit platelets and serves as an HDP. However, this efflux pump did not confer the organism with resistance to other structurally unrelated HDPs, including HNP-1and protamine, suggesting that this resistance may be specific to tPMP-1 (Kupferwasser et al. 1999). QacA-dependent resistance to the action of tPMP-1 was found to confer a survival advantage upon *S. aureus* in animal models of infection and also to correlate with the diagnosis of endocarditis in humans (Bayer et al. 1998; Dhawan et al. 1998; Kupferwasser et al. 2002). However, later studies suggested that the mechanism used by the organism to resist the action of tPMP-1 may not relate to efflux function of QacA but may result from the impact of the transporter upon membrane structure or fluidity, or some function unrelated to H^+ dependent peptide efflux (Bayer et al. 2006). More recently, orthologs of QacA have been identified in other staphylococci, as well as in species of *Enterococcus* and *Bacillus* and Qac efflux systems of the SMR superfamily have been reported in both *S. aureus* and *S. epidermis*, which is a frequent cause of nosocomial infections (Gomes et al. 2014), although in most cases, the ability of these proteins to transport HDPs has yet to be investigated (Fernández-Fuentes et al. 2014; Solheim et al. 2007; Wassenaar et al. 2015). Most recently, studies on *S. aureus* showed that the efflux system, NorA, did not appear to endow the organism with resistance to a range of human HDPs, including HNP-1, HNP-2, HNP-3, HNP-5, HBD-2, HBD-3 and LL-37 (Rieg et al. 2009). *Yersinia enterocolitica* is a human gastrointestinal pathogen which causes yersiniosis, an illness characterized by diarrhoea, ileitis and mesenteric lymphadenitis (Gupta et al. 2015) and was found to possess resistance to the HDPs, arctian cecropin P1 and melittin, which appeared to involve the MFS transporter, RosAB (Bengoechea and Skurnik 2000). This pump, which acts as a potassium antiporter, is induced by the presence of HDPs and is activated at 37 °C (Bengoechea and Skurnik 2000), which is similar to conditions encountered within the host during infection (Straley and Perry 1995). It was proposed that the RosAB system protects host bacteria by acidifying the cytoplasm to inhibit the action of HDPs (Bengoechea and Skurnik 2000).

7.3.3.2 RND Efflux Pumps

The efflux pump, MtrCDE, of *Neisseria gonorrhoeae* was the first one demonstrated to export HDPs to the extracellular milieu (Shafer et al. 1998) and along

with the analogous efflux pump in *Neisseria meningitidis* mediates resistance to an array of these peptides, including protegrin-1, LL-37 and cancrine tachyplesin-1. These results indicated that MtrCDE was able to recognize structurally different HDPs, for example protegrin-1 and tachyplesin-1 adopt β-type structures whereas LL-37 assumes an α-helical conformation (Shafer et al. 1998; Tzeng et al. 2005; Wang 2008; Johansson et al. 1998; Bellm et al. 2000; Nakamura et al. 1988). The importance of the MtrCDE, efflux pump to the resistance of *N. gonorrhoeae* to HDPs was emphasized by in vivo studies using a murine model of female gonococcal genital tract infection. These studies showed that mutant forms of the organism, which lacked MtrCDE were more susceptible to the host immune system and it was hypothesized that CRAMP-38, which is an alternatively processed form of mCRAMP, was likely to be present on urogenital mucosae, would select for these mutants (Jerse et al. 2003). More recently, studies were conducted on clinical and murine vaginal isolates of *N. gonorrhoeae* with mutations in *mtrR*, which encodes a repressor forMtrCDE. These studies showed that mutations upstream of *mtrC,* on the promoter of *mtr*, or in the binding site of the *mtr* gene product, MtrR, increased the resistance of the organism to LL-37 and CRAMP-38 by increasing the levels of MtrE and *mtrC* RNA stability. Additionally, these studies showed that these mutants had increased *in vivo* fitness in female murine genital tract infection, as compared to type strains of *N. gonorrhoeae*, which was ascribed in part to increased resistance to CRAMP-38 (Warner et al. 2008). *Vibrio parahaemolyticus*is a marine pathogen that causes vibriosis in fish and gastroenteritis of humans through the consumption of uncooked contaminated seafoods (Wang et al. 2015), and the use of proteonomic-based analyses suggested that AcrAB/TolC and other efflux systems bestowed the organism with resistance to a variety of synthetic HDPs and piscine pleurocidin (Shen et al. 2010). *K. pneumonia* is a prevalent pathogen able to cause a range of diseases from pneumonia to upper respiratory tract infections (Hawkey 2015). Work on the organism showed that AcrAB–TolC, endowed it with resistance to HDPs present in the human lung, including HNP-1, HBD-1 and HBD-2, which was supported by experiments on a murine model of pneumonia whose lungs were infected by *K. pneumonia* (Padilla et al. 2010). More recently, studies on *E. coli* have shown that AcrAB/TolC plays a role in the resistance of the organism to LL-37 and a number of synthetic HDPs (Goldberg et al. 2013) whilst this latter transporter along with EmrAB/TolC was found to play a major role in the resistance of the organism to protamine (Weatherspoon-Griffin et al. 2014). This observation contrasts with other investigations showing that neither AcrAB/TolC in *E. coli* (Rieg et al. 2009) nor the efflux pumps MexAB/OprM, MexCD/OprJ and MexGHI/OpmD in *P. aeruginosa* endowed these organisms with resistance to human HDPs such as LL-37, HNP-1, HNP-3, HD-5, HBD-2 and HBD-3. In combination, these results indicate that the specificity of RND efflux pumps for HDPs can vary between not only bacterial species but also between strains of the same species and as yet more work is required to understand the exact mode of resistance (Rieg et al. 2009; Strempel et al. 2013).

7.3.3.3 3ABC Efflux Pumps

A recent study suggested that the ABC transporter, VraFG, was involved in the resistance of *S. aureus* to tPMP-1 but not HNP-1 (Yang et al. 2012) whilst work on this latter organism and *S. epidermis* also suggested that the transporter was involved in resistance to LL-37, HBD-3 and bovine indolicidin, but not dermcidin. However, although these peptides were able to induce the expression of VraFG in the case of both organisms, this ability varied between *S. aureus* and *S. epidermidis* due to structural differences between the GraXRS sensors used by these bacteria to detect HDPs (Li et al. 2007a, b). Studies on *Streptococcus pneumoniae* showed that LL-37, CRAMP38 and CRAMP39, which is another alternatively processed form of mCRAMP, were also able to induce the expression of MefE/Mel, which is a half ABC transporter, and thereby confer the organism with resistance to the action of these peptides (Zähner et al. 2010). In these studies on Gram-positive bacteria, the mechanisms underpinning the transport of HDPs were not determined but, in addition to acting as efflux systems, ABC transporters are known to serve as importers, such as in the case of BceAB-type transporters (Davidson et al. 2008; Winter and Lawrence 2011), which includes VraFG (Nawrocki et al. 2014). ABC importers are believed to transport HDPs from their site of action, such as the periplasm/CM interface, to the cytoplasm where they are inactivated, most likely through proteolytic degradation to be recycled as nutrients (German et al. 2008; Gebhard 2012).

There has been considerable research in the use of ABC transporters by Gram-negative bacteria to mediate resistance to HDPs and there is evidence for import mechanisms in a number of cases (Gruenheid and Le Moual 2012; Costa et al. 2015; Lewis et al. 2012). For example, *S. typhimurium* causes a systemic disease in mice that serves as an animal model of typhoid caused by the organism in humans (Wick 2011). A recent study on the organism suggested that a compromised ability to proliferate inside activated macrophages and decreased virulence in a murine typhoid model was related to the activity of an ABC-type transport system, YejABEF, which putatively imports HDPs into the organism. It was found that inactivation of the ATPase component of the transporter led to increased sensitivity to protamine, melittin, HBD-1 and HBD-2 (Eswarappa et al. 2008). However, the strongest evidence for the import of HDPs into bacterial cells by ABC transporters would seem to come from SapABCDF, which is found in a number of Gram-negative bacteria (Gruenheid and Le Moual 2012; Lewis et al. 2012). The operon coding for SapABCDF was first identified in studies on *S. typhimurium*, which led to the suggestion that this transporter mediated the resistance of the organism to HDPs, such as protamine and melittin (Parra-Lopez et al. 1993). Strongly supporting this suggestion, more recent studies on *Salmonella typhi*, which is the major cause of typhoid (Kariuki et al. 2015), showed that some naturally occurring strains of the organism had genetic islands inserted within the SapABCDF operon that rendered *S. typhi* susceptible to protamine (Rodas et al. 2010). Most recently, studies on UPEC showed that loss of genes in the SapABCDF operon led to increased susceptibility to HDPs by the organism

(Subashchandrabose et al. 2013). A number of studies have shown that SapABCDF has a variable specificity for HDPs as in the case of *Erwinia chrysanthemi* (*Dickeya dadantii*), which causes soft rot diseases in a wide range of crops (Reverchon and Nasser 2013), where SapABCDF rendered the organism resistant to the plant HDPs α-thionin and snakin-1 but not defensin-Pth1 (Lopez-Solanilla et al. 1998). In *Haemophilus ducreyi*, which causes the genital infection chancroid (Lewis 2003), SapABCDF mediated resistance to LL-37 but not other HDPs such as HNP-1 and HBD-4 (Mount et al. 2010). Most recently, studies on *Campylobacter jejuni* identified SapABCDF as a mediator of resistance to the avian HDP, fowlicidin-1 but not arctian cecropin A or ranine magainin (Hoang et al. 2012). In contrast, SapABCDF did not appear to confer *Vibrio fischeri*, which is a marine foodborne pathogen (Norsworthy and Visick 2013), with resistance to any HDPs investigated, including LL-37 (Lupp et al. 2002). Clearly, these studies suggested that SapABCDF does not endow all bacteria expressing the transporter with resistance to HDPs and that the specificity of the transporter for these peptides.

Work on *S. typhimurium* first revealed that SapA, the periplasmic-binding component of the SapABCDF system, was required for resistance to HDPs and suggested that these peptides bind directly to SapA with subsequent import into the cytoplasm mediated by the transporter (Parra-Lopez et al. 1993). It was speculated that a similar mechanism of SapA mediated import into the cytoplasm may underpin the resistance shown by *E. chrysanthemi* to HDPs based on the conservation of gene order and the sequence similarity between SapABCDF of the latter organism and that of *S. Typhimurium* (Lopez-Solanilla et al. 1998). Non-typeable *Haemophilus influenzae* (NTHI) is a leading cause of otitis media (OD), or infection of the middle ear (Thanavala and Lugade 2011), and studies employing a chinchilla model of OD showed that inactivation of SapA in the SapABCDF transporter conferred NTHI with increased sensitivity to killing by β-defensin 1 from the host animal (Mason et al. 2005). In a more recent study on NTHI it was shown that SapABCDF mediated haeme transport into the cytoplasm of the organism via SapA binding and that HDPs, including HNP-1, HBD-2, HBD-3, LL-37 and melittin, competed with haeme for binding to the SapA protein (Mason et al. 2011). Taken together, these studies clearly suggested that SapABCDF served as an importer when mediating bacterial resistance to HDPs, which was confirmed by a recent investigation and led to the proposal of a model to describe the process (Fig. 7.4) (Shelton et al. 2011). According to this model, breaching of the outer membrane by HDPs such as protamine through their antimicrobial action leads to an increase in the local concentration of these peptides and elevated expression of the SapABCDF transporter. In turn, this leads to the binding of HDPs to SapA in the periplasmic space and transport of these peptides across the cytoplasmic membrane by SapABCDF transporter to the cytosol where they are subject to proteolytic degradation. A reduction in the critical threshold concentration of HDPs in the periplasm returns the organism to a homestatic state of innate immune resistance (Fig. 7.4). A number of studies have suggested that this return to homeostatic immune resistance may involve other Sap proteins, including SapG, SapJ and SapD, in Sap-dependent K^+ transport. (Parra-Lopez et al. 1994; Mason et al. 2006). A study on *Vibrio vulnificus*, which is a

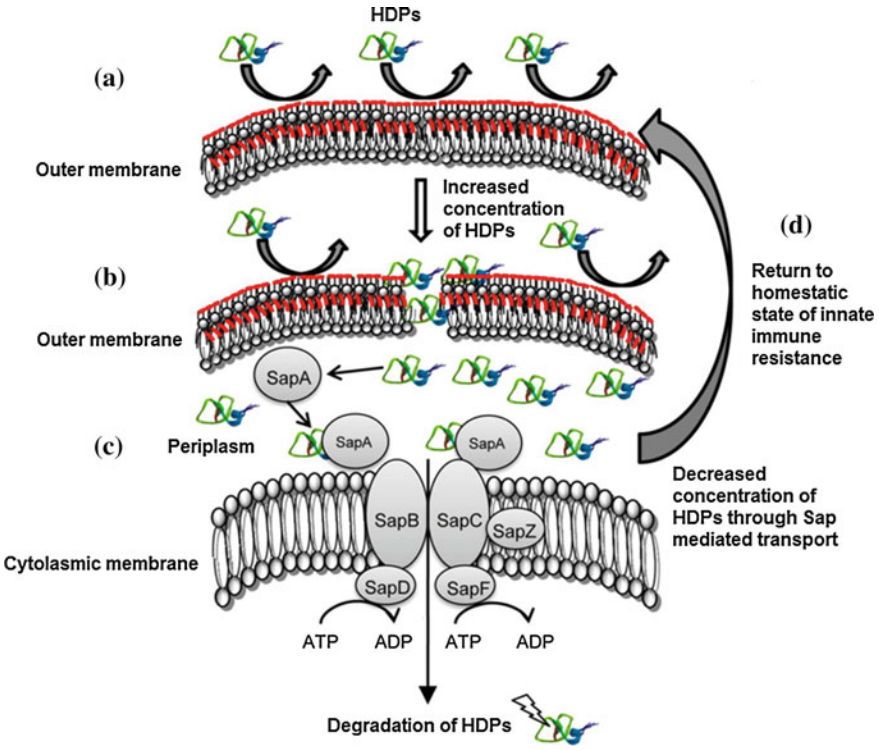

Fig. 7.4 SapABCDF mediated uptake of HDPs in Gram-negative bacteria. Figure 7.4 was revised from Shelton et al. (2011) and shows a proposed model for the SapABCDF mediated uptake of HDPs by Gram-negative bacteria. According to this model, low concentrations of HDPs are resisted by defence mechanism associated with the outer membrane (OM; ChoP and Lipid A acylation, red) **a** However, as HDPs breach the outer membrane through their antimicrobial action **b** an increase in the local concentrations of these peptides elevates the production of the SapABCDF transporter. This leads to the binding of HDPs to SapA in the periplasmic space and transport of these peptides across the cytoplasmic membrane where they are susceptible subject to proteolytic degradation **c** A reduction in the critical threshold concentration of HDPs in the periplasm returns the organism to a homestatic state of innate immune resistance **d**

highly virulent foodborne pathogen that can cause swound infections and septicaemia (Oliver 2015), led to the identification TrkA, which is highly homologous to SapG and appears to be component of a Trk K^+ uptake transporter that mediated resistance to protamine by the organism (Chen et al. 2004). It was hypothesized that protamine forms channels in the membrane system of *V. vulnificus* through which K^+ leaks from the cell and in response, TrkA-mediated activity rapidly pumps K^+ into the cell to restore homeostasis, thereby preventing death of the organism until it is detoxified from protamine (Chen et al. 2004).

7.4 Discussion

This chapter has shown that both Gram-positive bacteria and Gram-negative bacteria have a formidable arsenal of defence mechanisms to combat the action of HDPs from a diverse range of eukaryotes ranging from humans and amphibians to rodents and insects. However, it is generally accepted that Gram-positive bacteria are less well characterized in this capacity than Gram-negative bacteria and it seems likely that continued research on the former organisms will reveal new mechanisms of resistance to HDPs (Nawrocki et al. 2014). Indeed, a number of the resistance mechanisms discussed in this chapter have been only reported relatively recently, such as use of the M pili protein of *S. pyogenes* to bind HDPs and protect the organism from both NETs and attack on its CM Exportal domain by these peptides. In the case of Gram-negative bacteria, curli expressed by *E. Coli* are also able to sequester HDPs, impeding the progress of these peptides into the cell envelope whilst PE receptors in the CM of the organism are able to bind and immobilize HDPs, inhibiting their ability to insert into these membranes. Also recently, Gram-negative bacteria have been reported to employ OMVs to sequester HDPs and a mechanism has been presented to help explain the use of ABC transporters by these bacteria to mediate the uptake and proteolytic degradation of these peptides. Most recently, the first new family of multidrug efflux pumps to be described in 15 years was reported, namely the PACE group of transporters, which were identified in Gram-negative bacteria. These transporters appear to be widespread in Gram-negative pathogens and their known substrates only currently include biocides, such as chlorhexidine. Whether PACE transporters will provide their host bacteria with resistance to HDPs or not is awaiting investigation (Hanson et al. 2015).

This chapter has also shown that a given bacterium can use multiple mechanisms to resist HDPs, illustrated by *S. aureus* which utilizes efflux pumps and EPS along with changes in both the fluidity and surface charge of the CM for this purpose. However, for a given organism, the respective contribution made by each resistance mechanism to combatting the action of HDPs can be unclear. For example, in the case of *S. aureus*, the relative importance of changes to CM fluidity and surface charge to resisting HDPs appears to vary according to the nature of the HDPs concerned. Moreover, a high level of heterogeneity exists between bacterial species in relation to the relative importance of each these resistance mechanisms. Indeed, the relative importance of resistance mechanisms to HDPs can vary between different strains of the same species as in the case of EHEC and EPEC. Both of these *E. coli* strains use an OM protease to degrade HDPs but the expression of this protease is differentially regulated according to the profile of the peptides encountered by each strain in its host niche (Thomassin et al. 2012).

As described above, resistance to HDPs often accompanies and increases the virulence of both Gram-positive and Gram-negative pathogens, which makes these pathogens highly dangerous in the clinical environment. Clearly then, a better understanding of the mechanisms used by bacteria to resist HDPs will enable

illumination of the interaction between bacterial pathogens and their hosts, and help facilitate the many current efforts to exploit HDPs for therapeutic and other purposes (Ashby et al. 2014; Eckert 2011; da Costa et al. 2015; Mohammad et al. 2015). However, knowledge of these bacterial resistance mechanisms can also provide an alternative approach to the therapeutic development of HDPs, which is to develop compounds that target these resistance mechanisms themselves. Ideally, these compounds would target resistance mechanisms to HDPs that are conserved across bacterial pathogens such as MprF, which as described above is found in both Gram-positive and Gram-negative pathogens. It has previously been shown that the inactivation of MprF renders bacteria susceptible to not only HDPs but also daptomycin, regarded as a last-resort option in the treatment of some infections (Ernst and Peschel 2011). Based on these observations it has been suggested that inhibitors of MprF have great potential for either complementing or replacing conventional antibiotic therapies (Escaich 2010; Weidenmaier et al. 2003). It has also been suggested that a similar purpose could be served by inhibitors of enzymes that are responsible for the D-alanylation of cell wall teichoic acids in Gram-positive bacteria (Koprivnjak and Peschel 2011; Weidenmaier et al. 2003). Similarly to MprF, DltABCD endows bacteria with resistance to HDPs by reducing the net negative surface charge of their membranes (Nawrocki et al. 2014; Anaya-Lopez et al. 2013) and it has previously been shown that inhibitors of DltA, which is involved in the first step of D-alanylation, were able to inhibit the growth of Gram-positive bacteria, especially when used in combination with other antibiotics (May et al. 2005). A more recent study has suggested that combinations of compounds that target D-alanylation and other processes related to the teichoic acid pathway are attractive propositions as antibacterial agents. Potential advantages of this combination therapy are that its targets are absent from humans and it may have the capacity to treat infections due to MRSA (Santa Maria et al. 2014).

In relation to Gram-negative bacteria, LPS mediates a number of mechanisms of resistance to HDPs, such as through the modulation of OM fluidity as discussed above (Gruenheid and Le Moual 2012; Band and Weiss 2015). Recent advances in understanding of the LPS biosynthetic pathway has led to the proposal that enzymes involved in this pathway are optimal targets for antibacterial agents as they are conserved among diverse, clinically relevant bacteria and have no counterpart in humans (Cipolla et al. 2011). Based on these observations, a major focus in the development of novel agents against Gram-negative bacteria has been designing inhibitors of different aspects of LPS biosynthesis, such as the production of lipid A and sugar (Kdo) moieties found in the core domain of the lipid (Gabrielli et al. 2012). For example, lipid A anchors LPS to membranes and is essential for the assembly of LPS in most Gram-negative bacteria and more recent studies on the biosynthesis of lipid A have shown that the first three enzymes in its biosynthesis show promise as targets for antibacterial agents (Lee et al. 2013). Most recently, an inhibitor of LpxC, the enzyme responsible for the first committed step in the biosynthesis of lipid A was reported to be efficacious against *P. aeruginosa* and members of the Enterobacteriaceae both in vitro and various models of bacterial infection (Tomaras et al. 2014).

As described above, efflux pumps are ubiquitous in bacteria and constitute a major mechanism in the resistance of these organisms to HDPs thus making them obvious potential targets for the development of antibacterial agents. The three-dimensional structures of some bacterial efflux pumps are available, which in these cases potentially permits the rational design of efflux pump inhibitors (EPIs) to block their function (Delmar et al. 2014; Opperman and Nguyen 2015). A variety of other approaches to identifying EPIs have been reported and a number of naturally occurring compounds able to function in this capacity are known for both Gram-positive and Gram-negative bacteria (Fernández and Hancock 2012; Mandal et al. 2014). For example, recent work has isolated a compound from a plant in the Lauraceae family, Persea lingue Nees, and identified a series of different chemical molecules through virtual screening that were able to inhibit the NorA efflux pump of *S. aureus* (Holler et al. 2012; Brincat et al. 2011). It has also been shown that efflux pumps play a major role in the resistance to HDPs exhibited by biofilms, which are communities of microorganisms that adhere to surfaces and are generally embedded within a self-produced matrix of extracellular polymeric substance (Guihelmelli et al. 2013; Otto 2006). In response, some EPIs have been shown able to inhibit biofilm formation making them attractive targets for development as anti-biofilm agents (Soto 2013), which could be of great advantage in the medical arena as biofilms are far more recalcitrant to antimicrobial agents than their planktonic counterparts and they dominate the occurrence of chronic bacterial infections (Bjarnsholt 2013; Wu et al. 2015).

In conclusion, this chapter has shown that bacteria have acquired a multiplicity of resistance mechanisms to the action of HDPs but in the ongoing war between bacteria and mankind, this may be their undoing by providing targets for antibacterials with the potential to act in their own right or complement the action of HDPs, antibiotics, biocides and other anti-infectives.

References

Abdelsalam M, Asheg A, Eissa AE (2013) Streptococcus dysgalactiae: An emerging pathogen of fishes and mammals. Int J Vet Sci Med 1(1):1–6

Agus A et al (2014) Understanding host-adherent-invasive *Escherichia coli* interaction in crohn's disease: opening up new therapeutic strategies. Biomed Res Int 2014:16

Åkesson P, Sjöholm AG, Björck L (1996) Protein SIC, a novel extracellular protein of *Streptococcus pyogenes* interfering with complement function. J Biol Chem 271(2):1081–1088

Aloia RC, Tian H, Jensen FC (1993) Lipid composition and fluidity of the human immunodeficiency virus envelope and host cell plasma membranes. Proc Natl Acad Sci 90(11):5181–5185

Alteri CJ et al (2011) The broadly conserved regulator PhoP links pathogen virulence and membrane potential in *Escherichia coli*. Mol Microbiol 82(1):145–163

Alvarez-Ortega C, Olivares J, Martinez JL (2013) RND multidrug efflux pumps: what are they good for? Front Microbiol 4

Amaral L et al (2012) Structure, genetic regulation, physiology and function of the AcrAB-TolC efflux pump of *Escherichia coli* and Salmonella. In: Tegos G, Mylonakis E (ed) Antimicrobial drug discovery: emerging strategies, pp 44–61

Aminov RI (2010) A brief history of the antibiotic era: lessons learned and challenges for the future. Front Microbiol 1:134

Anaya-Lopez JL, Lopez-Meza JE, Ochoa-Zarzosa A (2013) Bacterial resistance to cationic antimicrobial peptides. Crit Rev Microbiol 39(2):180–195

Andersen JL et al (2015) Multidrug efflux pumps from enterobacteriaceae, vibrio cholerae and *Staphylococcus aureus* bacterial food pathogens. Int J Environ Res Public Health 12(2):1487–1547

Andra J et al (2004) Biophysical characterization of endotoxin inactivation by NK-2, an antimicrobial peptide derived from mammalian NK-lysin. Antimicrob Agents Chemother 48 (5):1593–1599

Andra J et al (2011) Multiple peptide resistance factor (MprF)-mediated resistance of *Staphylococcus aureus* against antimicrobial peptides coincides with a modulated peptide interaction with artificial membranes comprising lysyl-phosphatidylglycerol. J Biol Chem 286 (21):18692–18700

Anes J et al (2015) The ins and outs of RND efflux pumps in *Escherichia coli*. Front Microbiol 6:14

Arendt W et al (2012) Resistance phenotypes mediated by aminoacyl-phosphatidylglycerol synthases. J Bacteriol 194(6):1401–1416

Ashby M, Petkova A, Hilpert K (2014) Cationic antimicrobial peptides as potential new therapeutic agents in neonates and children: a review. Curr Opin Infect Dis 27(3):258–267

Avila-Calderón E et al (2015) Roles of bacterial membrane vesicles. Arch Microbiol 197(1):1–10

Bader MW et al (2003) Regulation of *Salmonella typhimurium* virulence gene expression by cationic antimicrobial peptides. Mol Microbiol 50(1):219–230

Bader MW et al (2005) Recognition of antimicrobial peptides by a bacterial sensor kinase. Cell 122(3):461–472

Baker J, Wright SH, Tama F (2012) Simulations of substrate transport in the multidrug transporter EmrD. Proteins Struct Funct Bioinf 80(6):1620–1632

Balciunas EM et al (2013) Novel biotechnological applications of bacteriocins: a review. Food Control 32(1):134–142

Band VI, Weiss DS (2015) Mechanisms of antimicrobial peptide resistance in Gram-negative bacteria. Antibiotics (Basel) 4(1):18–41

Barrett AJ, Rawlings ND, Woessner JF (2012) Handbook of proteolytic enzymes, Elsevier Science

Bastos MDD, Coelho MLV, Santos OCD (2015) Resistance to bacteriocins produced by Gram-positive bacteria. Microbiol SGM 161:683–700

Batabyal B, Kundu GKR, Biswas S (2012) Methicillin-resistant *Staphylococcus aureus*: a brief review. International Research Journal of Biological Sciences 1:65–71

Bavro VN, Marshall RL, Symmons MF (2015) Architecture and roles of periplasmic adaptor proteins in tripartite efflux assemblies. Front Microbiol 6

Bayer AS et al (1998) In vitro resistance to thrombin-induced platelet microbicidal protein among clinical bacteremic isolates of *Staphylococcus aureus* correlates with an endovascular infectious source. Antimicrob Agents Chemother 42(12):3169–3172

Bayer AS et al (2000) In vitro resistance of *Staphylococcus aureus* to thrombin-induced platelet microbicidal protein is associated with alterations in cytoplasmic membrane fluidity. Infect Immun 68(6):3548–3553

Bayer AS et al (2006) Low-level resistance of *Staphylococcus aureus* to thrombin-induced platelet microbicidal protein 1 in vitro associated with qacA gene carriage is independent of multidrug efflux pump activity. Antimicrob Agents Chemother 50(7):2448–2454

Bayer AS, Schneider T, Sahl HG (2013) Mechanisms of daptomycin resistance in *Staphylococcus aureus*: role of the cell membrane and cell wall. Ann N Y Acad Sci 1277:139–158

Belden WJ, Miller SI (1994) Further characterization of the PhoP regulon—identification of new PhoP-activated virulence loci. Infect Immun 62(11):5095–5101

Bellm L, Lehrer RI, Ganz T (2000) Protegrins: new antibiotics of mammalian origin. Expert Opin Investig Drugs 9(8):1731–1742
Bengoechea JA, Skurnik M (2000) Temperature-regulated efflux pump/potassium antiporter system mediates resistance to cationic antimicrobial peptides in Yersinia. Mol Microbiol 37 (1):67–80
Bentley R (2009) Different roads to discovery; Prontosil (hence sulfa drugs) and penicillin (hence beta-lactams). J Ind Microbiol Biotechnol 36(6):775–786
Bhardwaj AK, Mohanty P (2012) Bacterial efflux pumps involved in multidrug resistance and their inhibitors: rejuvinating the antimicrobial chemotherapy. Recent Pat Anti-Infect Drug Discov 7 (1):73–89
Bialvaei AZ, Kafil HS (2015) Colistin, mechanisms and prevalence of resistance. Curr Med Res Opin 31(4):707–721
Bisno AL, Brito MO, Collins CM (2003) Molecular basis of group A streptococcal virulence. Lancet Infect Dis 3(4):191–200
Bjarnsholt T (2013) The role of bacterial biofilms in chronic infections. APMIS 121:1–58
Blair JMA, Richmond GE, Piddock LJV (2014) Multidrug efflux pumps in Gram-negative bacteria and their role in antibiotic resistance. Future Microbiology 9(10):1165–1177
Bostanci N, Belibasakis GN (2012) Porphyromonas gingivalis: an invasive and evasive opportunistic oral pathogen. FEMS Microbiol Lett 333(1):1–9
Brannon JR et al (2013) Role of uropathogenic *Escherichia coli* OmpT in the resistance against human cathelicidin LL-37. FEMS Microbiol Lett 345(1):64–71
Breidenstein EBM, de la Fuente-Nunez C, Hancock REW (2011) *Pseudomonas aeruginosa*: all roads lead to resistance. Trends Microbiol 19(8):419–426
Brincat JP et al (2011) Discovery of novel inhibitors of the NorA multidrug transporter of *Staphylococcus aureus*. J Med Chem 54(1):354–365
Brinkmann V, Zychlinsky A (2012) Neutrophil extracellular traps: Is immunity the second function of chromatin? J Cell Biol 198(5):773–783
Brogden KA (2005) Antimicrobial peptides: Pore formers or metabolic inhibitors in bacteria? Nat Rev Microbiol 3(3):238–250
Brown MH, Skurray RA (2002) Structure, function and regulation of the staphylococcal multidrug efflux protein QacA. In: Paulsen IT, Lewis K (eds) JMMB symposium series, microbial multidrug efflux, vol 4, pp 49–66
Carlisle MD, Srikantha RN, Brogden KA (2009) Degradation of human alpha- and beta-defensins by culture supernatants of Porphyromonas gingivalis strain 381. J Innate Immun 1(2):118–122
Chen YC et al (2004) A K + yptake protein, TrkA, is required for serum, protamine, and polymyxin B resistance in Vibrio vulnificus. Infect Immun 72(2):629–636
Cheng H-Y, Chen Y-F, Peng H-L (2010) Molecular characterization of the PhoPQ-PmrD-PmrAB mediated pathway regulating polymyxin B resistance in *Klebsiella pneumoniae* CG43. J Biomed Sci 17
Cipolla L et al (2011) New targets for antibacterial design: Kdo biosynthesis and LPS machinery transport to the cell surface. Curr Med Chem 18(6):830–852
ClinicalTrials.gov (2014) Pexiganan versus placebo control for the treatment of mild infections of diabetic foot ulcers (OneStep-1). Accessed 6 April 15. https://clinicaltrials.gov/ct2/show/NCT01590758
Coetzer THT, Goldring JPD, Huson LEJ (2008) Oligopeptidase B: A processing peptidase involved in pathogenesis. Biochimie 90(2):336–344
Cole ST (2014) Who will develop new antibacterial agents? Philos Trans R Soc B Biol Sci 369 (1645):7
Cole JN et al (2010) M protein and hyaluronic acid capsule are essential for in vivo selection of covRS mutations characteristic of invasive serotype M1T1 group A Streptococcus. MBio 1(4)
Collu F, Cascella M (2013) Multidrug resistance and efflux pumps: insights from molecular dynamics simulations. Curr Top Med Chem 13(24):3165–3183
Cooper PR, Palmer LJ, Chapple ILC (2000) Neutrophil extracellular traps as a new paradigm in innate immunity: friend or foe? Periodontology 2000, 2013. 63(1):165–197

Costa SS et al (2013) Multidrug efflux pumps in *Staphylococcus aureus*: an update. Open Microbiol J 7:59–71
Costa TRD et al (2015) Secretion systems in Gram-negative bacteria: structural and mechanistic insights. Nat Rev Micro 13(6):343–359
Cotter PD, Ross RP, Hill C (2013) Bacteriocins—a viable alternative to antibiotics? Nat Rev Microbiol 11(2):95–105
Cruz J et al (2014) Antimicrobial peptides: promising compounds against pathogenic microorganisms. Curr Med Chem 21(20):2299–2321
da Costa JP et al (2015) Antimicrobial peptides: an alternative for innovative medicines? Appl Microbiol Biotechnol 99(5):2023–2040
Dalebroux ZD, Miller SI (2014) Salmonellae PhoPQ regulation of the outer membrane to resist innate immunity. Curr Opin Microbiol 17:106–113
Dalebroux ZD et al (2014) PhoPQ regulates acidic glycerophospholipid content of the *Salmonella typhimurium* outer membrane. Proc Natl Acad Sci USA 111(5):1963–1968
Danne C, Dramsi S (2012) Pili of Gram-positive bacteria: roles in host colonization. Res Microbiol 163(9–10):645–658
Davidson AL et al (2008) Structure, function, and evolution of bacterial ATP-binding cassette systems. Microbiol Mol Biol Rev 72(2):317–364
Davies J (2006) Where have all the antibiotics gone? Can J Infect Dis Med Microbiol (Journal canadien des maladies infectieuses et de la microbiologie medicale/AMMI Canada) 17(5):287–90
Delmar JA, Su C-C, Yu EW (2014) Bacterial multidrug efflux transporters. Annu Rev Biophys 43 (1):93–117
Dennison SR, Harris F, Phoenix DA (2005) Are oblique orientated alpha-helices used by antimicrobial peptides for membrane invasion? Protein Pept Lett 12(1):27–29
Dennison SR et al (2013) A novel form of bacterial resistance to the action of eukaryotic host defense peptides, the use of a lipid receptor. Biochemistry 52(35):6021–6029
Dennison SR et al (2015) Use of the antimicrobial peptide, maximin H5 to protect against *Staphylococcus aureus*. In: 5th international meeting of antimicrobial peptides 2015. Royal Society for Chemistry, London
Dennison SR et al (2015) The role of C-terminal amidation in the membrane interactions of the anionic antimicrobial peptide, maximin H5. Biochim Biophys Acta (BBA) Biomembr 1848 (5):1111–1118
Destoumieux-Garzón D et al (2014) Resistance to antimicrobial peptides in vibrios. Antibiotics 3 (4):540–563
Devine DA et al (1999) Modulation of antibacterial peptide activity by products of Porphyromonas Gingivalis and Prevotella spp. Microbiol UK 145:965–971
Dhawan VK, Bayer AS, Yeaman MR (1998) In vitro resistance to thrombin-induced platelet microbicidal protein is associated with enhanced progression and hematogenous dissemination in experimental *Staphylococcus aureus* infective endocarditis. Infect Immun 66(7):3476–3479
Dobson AJ et al (2013) Comparing selection on *S. aureus* between antimicrobial peptides and common antibiotics. PLoS ONE 8(10):5
Dobson AJ, Purves J, Rolff J (2014) Increased survival of experimentally evolved antimicrobial peptide-resistant *Staphylococcus aureus* in an animal host. Evol Appl 7(8):905–912
Dorner F, Lienkamp K (2014) Chapter 5 polymer-based synthetic mimics of antimicrobial peptides (SMAMPs)—a new class of nature-inspired antimicrobial agents with low bacterial resistance formation potential, in polymeric materials with antimicrobial activity: from synthesis to applications. R Soc Chem 97–138
Dorotkiewicz-Jach A et al (2015) Modern therapeutic approaches against *Pseudomonas aeruginosa* infections. Curr Med Chem 22(14):1642–1664
Draper LA et al (2015) Lantibiotic resistance. Microbiol Mol Biol Rev MMBR 79(2):171–191
Drider D, Rebuffat S (2011) Prokaryotic antimicrobial peptides: from genes to applications. Springer, New York

Duperthuy M et al (2013) Role of the *Vibrio cholerae* matrix protein Bap1 in cross-resistance to antimicrobial peptides. PLoS Pathog 9(10):e1003620
Duquesne S et al (2007) Microcins, gene-encoded antibacterial peptides from enterobacteria. Nat Prod Rep 24(4):708–734
Eckert R (2011) Road to clinical efficacy: challenges and novel strategies for antimicrobial peptide development. Future Microbiol 6(6):635–651
Elkins CA, Mullis LB (2006) Mammalian steroid hormones are substrates for the major RND- and MFS-type tripartite multidrug efflux pumps of *Escherichia coli*. J Bacteriol 188(3):1191–1195
Epand RM, Epand RF (2011) Bacterial membrane lipids in the action of antimicrobial agents. J Pept Sci 17(5):298–305
Ernst CM, Peschel A (2011) Broad-spectrum antimicrobial peptide resistance by MprF-mediated aminoacylation and flipping of phospholipids. Mol Microbiol 80(2):290–299
Escaich S (2010) Novel agents to inhibit microbial virulence and pathogenicity. Expert Opin Ther Pat 20(10):1401–1418
Eswarappa SM et al (2008) The yejABEF operon of Salmonella confers resistance to antimicrobial peptides and contributes to its virulence. Microbiology 154(2):666–678
Evans ML, Chapman MR (2014) Curli biogenesis: Order out of disorder. Biochim Biophys Acta Mol Cell Res 1843(8):1551–1558
Falord M et al (2011) Investigation of the *Staphylococcus aureus* GraSR regulon reveals novel links to virulence, stress response and cell wall signal transduction pathways. PLoS ONE 6(7): e21323
Falord M et al (2012) GraXSR proteins interact with the VraFG ABC transporter to form a five-component system required for cationic antimicrobial peptide sensing and resistance in *Staphylococcus aureus*. Antimicrob Agents Chemother 56(2):1047–1058
Fernández L, Hancock REW (2012) Adaptive and mutational resistance: role of porins and efflux pumps in drug resistance. Clin Microbiol Rev 25(4):661–681
Fernández-Fuentes MA et al (2014) Genetic determinants of antimicrobial resistance in Gram positive bacteria from organic foods. Int J Food Microbiol 172:49–56
Fernando D, Kumar A (2013) Resistance-nodulation-division multidrug efflux pumps in Gram-negative bacteria: role in virulence. Antibiotics 2(1):163
Fernie-King BA, Seilly DJ, Lachmann PJ (2004) The interaction of streptococcal inhibitor of complement (SIC) and its proteolytic fragments with the human beta defensins. Immunology 111(4):444–452
Fernie-King BA et al (2007) Streptococcal DRS (distantly related to SIC) and SIC inhibit antimicrobial peptides, components of mucosal innate immunity: a comparison of their activities. Microbes Infect 9(3):300–307
Ferreira M, Costa J, Reis-Henriques MA (2014) ABC transporters in fish species: a review. Front Physiol 5
Forrest LR, Krämer R, Ziegler C (2011) The structural basis of secondary active transport mechanisms. Biochim Biophys Acta (BBA) Bioenerg 1807(2):167–188
Fox JL (2013) Antimicrobial peptides stage a comeback. Nat Biotechnol 31(5):379–382
Franco OL, Parachin NS (2014) New edge of antibiotic development: antimicrobial peptides and corresponding resistance. Frontiers E-books
Frees D, Brøndsted L, Ingmer H (2013) Bacterial proteases and virulence. In: Dougan DA (ed) Regulated proteolysis in microorganisms. Springer, Netherlands, pp 161–192
Frick I-M et al (2003) SIC, a secreted protein of *Streptococcus pyogenes* that inactivates antibacterial peptides. J Biol Chem 278(19):16561–16566
Frick I-M et al (2008) Identification of a novel protein promoting the colonization and survival of Finegoldia magna, a bacterial commensal and opportunistic pathogen. Mol Microbiol 70 (3):695–708
Frick I-M et al (2011a) Antibacterial activity of the contact and complement systems is blocked by SIC, a protein secreted by *Streptococcus pyogenes*. J Biol Chem 286(2):1331–1340

Frick I-M et al (2011b) Constitutive and inflammation-dependent antimicrobial peptides produced by epithelium are differentially processed and inactivated by the commensal finegoldia magna and the pathogen *Streptococcus pyogenes*. J Immunol 187(8):4300–4309
Fronzes R, Remaut H, Waksman G (2008) Architectures and biogenesis of non-flagellar protein appendages in Gram-negative bacteria. The EMBO J 27(17):2271–2280
Gabrielli L et al (2012) Recent approaches to novel antibacterials designed after LPS structure and biochemistry. Curr Drug Targets 13(11):1458–1471
Ganz T et al (1985) Defensins—natural peptide antibiotics of human-neutrophils. J Clin Invest 76 (4):1427–1435
Gebhard S (2012) ABC transporters of antimicrobial peptides in Firmicutes bacteria—phylogeny, function and regulation. Mol Microbiol 86(6):1295–1317
Gela A et al (2014) Midkine in host defence. Br J Pharmacol 171(4):859–869
German N, Wei P, Kaatz GW, Kerns RJ (2008) Synthesis and evaluation of fluoroquinolone derivatives as substrate-based inhibitors of bacterial efflux pumps. Eur J Med Chem. 43 (11):2453–63
Ghosh P (2011) The nonideal coiled coil of M protein and its multifarious functions in pathogenesis. In: Advances in Experimental Medicine and Biology, pp 197–211
Goldberg K et al (2013) Sensitization of gram-negative bacteria by targeting the membrane potential. Faseb Journal 27(9):3818–3826
Gomes F, Teixeira P, Oliveira R (2014) Mini-review: *Staphylococcus epidermidis* as the most frequent cause of nosocomial infections: old and new fighting strategies. Biofouling 30 (2):131–141
Goyita M, Kandler JL, Shafer WM (2013) Mechanisms and significance of bacterial resistance to human cationic antimicrobial peptides. In: Hiemstra PS, Zaat SAJ (eds) Antimicrobial peptides and innate immunity. Springer, Basel, pp 219–255
Grenier D, La VD (2011) Proteases of *Porphyromonas gingivalis* as important virulence factors in periodontal disease and potential targets for plant-derived compounds: a review article. Curr Drug Targets 12(3):322–331
Groisman EA (2001) The pleiotropic two-component regulatory system PhoP-PhoQ. J Bacteriol 183(6):1835–1842
Gruenheid S, Le Moual H (2012) Resistance to antimicrobial peptides in Gram-negative bacteria. FEMS Microbiol Lett 330(2):81–89
Gudhka RK, Neilan BA, Burns BP (2015) Adaptation, ecology, and evolution of the halophilic stromatolite archaeon *Halococcus hamelinensis* inferred through genome analyses. Archaea (Vancouver, B.C.) 2015:241608–241608
Guilhelmelli F et al (2013) Antimicrobial development challenges: the various mechanisms of action of antimicrobial peptides and of bacterial resistance. Front Microbiol 4
Guina T et al (2000) A PhoP-regulated outer membrane protease of *Salmonella enterica* serovar typhimurium promotes resistance to alpha-helical antimicrobial peptides. J Bacteriol 182 (14):4077–4086
Gunn JS, Miller SI (1996) PhoP-PhoQ activates transcription of pmrAB, encoding a two-component regulatory system involved in *Salmonella typhimurium* antimicrobial peptide resistance. J Bacteriol 178(23):6857–6864
Guo L et al (1998) Lipid a acylation and bacterial resistance against vertebrate antimicrobial peptides. Cell 95(2):189–198
Guo Y, Nguyen K-A, Potempa J (2000) Dichotomy of gingipains action as virulence factors: from cleaving substrates with the precision of a surgeon's knife to a meat chopper-like brutal degradation of proteins. Periodontology 2010(54):15–44
Gupta S et al (2011) DbMDR: a relational database for multidrug resistance genes as potential drug targets. Chem Biol Drug Des 78(4):734–738
Gupta V et al (2015) Detection of *Yersinia enterocolitica* in food: an overview. Eur J Clin Microbiol Infect Dis 34(4):641–650
Gutner M et al (2009) Saliva enables the antimicrobial activity of LL-37 in the presence of proteases of *Porphyromonas gingivalis*. Infect Immun 77(12):5558–5563

Habets MGJL, Brockhurst MA (2012) Therapeutic antimicrobial peptides may compromise natural immunity. Biol Lett 8(3):416–418
Habets M, Rozen DE, Brockhurst MA (2012) Variation in *Streptococcus pneumoniae* susceptibility to human antimicrobial peptides may mediate intraspecific competition. Proc R Soc B Biol Sci 279(1743):3803–3811
Haiko J et al (2009) Invited review: breaking barriers—attack on innate immune defences by omptin surface proteases of enterobacterial pathogens. Innate Immun 15(2):67–80
Halverson TWR et al (2015) DNA is an antimicrobial component of Neutrophil Extracellular Traps. Plos Pathog 11(1)
Handzlik J, Matys A, Kieć-Kononowicz K (2013) Recent advances in Multi-Drug Resistance (MDR) efflux pump inhibitors of Gram-positive bacteria *S. aureus*. Antibiotics 2(1):28
Harris F, Dennison SR, Phoenix DA (2009) Anionic antimicrobial peptides from eukaryotic organisms. Curr Protein Pept Sci 10(6):585–606
Harris F, Dennison S, Phoenix D (2011) Anionic antimicrobial peptides from eukaryotic organisms and their mechanisms of action. Curr Chem Biol 5(2):142–153
Harris JB et al (2012) Cholera. Lancet 379(9835):2466–2476
Harris F et al (2013) On the selectivity and efficacy of defense peptides with respect to cancer cells. Med Res Rev 33(1):190–234
Hassan M et al (2012) Natural antimicrobial peptides from bacteria: characteristics and potential applications to fight against antibiotic resistance. J Appl Microbiol 113(4):723–736
Hassan KA et al (2013) Transcriptomic and biochemical analyses identify a family of chlorhexidine efflux proteins. Proc Natl Acad Sci USA 110(50):20254–20259
Hassan KA et al (2015a) Homologs of the *Acinetobacter baumannii* aceI transporter represent a new family of bacterial multidrug efflux systems. mBio 6(1)
Hassan KA et al (2015b) An ace up their sleeve: a transcriptomic approach exposes the AceI efflux protein of *Acinetobacter baumannii* and reveals the drug efflux potential hidden in many microbial pathogens. Front Microbiol 6
Hawkey PM (2015) Multidrug-resistant Gram-negative bacteria: a product of globalization. J Hosp Infect 89(4):241–247
Heimlich D, Harrison A, Mason K (2014) Host antimicrobial peptides in bacterial homeostasis and pathogenesis of disease. Antibiotics 3(4):645
Henriques ST, Melo MN, Castanho MARB (2006) Cell-penetrating peptides and antimicrobial peptides: how different are they? Biochem J 399:1–7
Hinchliffe P et al (2013) Structure and operation of bacterial tripartite pumps. Annu Rev Microbiol 67(67):221–242
Hoang KV, Wang Y, Lin J (2012) Identification of genetic loci that contribute to Campylobacter resistance to fowlicidin-1, a chicken host defense peptide. Front Cell Infect Microbiol 2:32
Holler JG et al (2012) Novel inhibitory activity of the *Staphylococcus aureus* NorA efflux pump by a kaempferol rhamnoside isolated from Persea lingue Nees. J Antimicrob Chemother 67 (5):1138–1144
Hritonenko V, Stathopoulos C (2007) Omptin proteins: an expanding family of outer membrane proteases in Gram-negative Enterobacteriaceae. Mol Membr Biol 24(5–6):395–406
Huang YW et al (2013) Characterization of a major facilitator superfamily (MFS) tripartite efflux pump EmrCABsm from *Stenotrophomonas maltophilia*. J Antimicrob Chemother 68 (11):2498–2505
Hui C-Y et al (2010) *Escherichia coli* outer membrane protease OmpT confers resistance to urinary cationic peptides. Microbiol Immunol 54(8):452–459
Hultmark D et al (1980) Insect immunity - purification and properties of 3 inducible bactericidal proteins from hemolymph of immunized pupae of hyalophora-cecropia. Eur J Biochem 106 (1):7–16
Hwang B-Y et al (2007) Substrate specificity of the *Escherichia coli* outer membrane protease OmpP. J Bacteriol 189(2):522–530
Islam D et al (2001) Downregulation of bactericidal peptides in enteric infections: a novel immune escape mechanism with bacterial DNA as a potential regulator. Nat Med 7(2):180–185

Jardetzky O (1966) Simple allosteric model for membrane pumps [27]. Nature 211(5052):969–970
Jerse AE et al (2003) A gonococcal efflux pump system enhances bacterial survival in a female mouse model of genital tract infection. Infect Immun 71(10):5576–5582
Johansson J et al (1998) Conformation-dependent antibacterial activity of the naturally occurring human peptide LL-37. J Biol Chem 273(6):3718–3724
Johansson L et al (2008) Cathelicidin LL-37 in severe *Streptococcus pyogenes* soft tissue infections in humans. Infect Immun 76(8):3399–3404
Jones JDG, Dangl JL (2006) The plant immune system. Nature 444(7117):323–329
Joo H-S, Otto M (2015) Mechanisms of resistance to antimicrobial peptides in staphylococci. Biochim Biophys Acta (BBA) Biomembr
Kai-Larsen Y et al (2010) Uropathogenic *Escherichia coli* modulates immune responses and its curli fimbriae interact with the antimicrobial peptide LL-37. PLoS Pathog 6(7):e1001010
Kang S-J et al (2014) Antimicrobial peptides: therapeutic potentials. Expert Rev Anti Infect Ther 12(12):1477–1486
Kaparakis-Liaskos M, Ferrero RL (2015) Immune modulation by bacterial outer membrane vesicles. Nat Rev Immunol 15(6):375–387
Kariuki S et al (2015) Antimicrobial resistance and management of invasive Salmonella disease. Vaccine 33(Supplement 3):C21–C29
Kathawala RJ et al (2015) The modulation of ABC transporter-mediated multidrug resistance in cancer: a review of the past decade. Drug Resist Updates 18:1–17
Katz ML et al (2006) Where have all the antibiotic patents gone? Nat Biotechnol 24(12):1529–1531
Katzif S et al (2005) CspA regulates pigment production in *Staphylococcus aureus* through a SigB-dependent mechanism. J Bacteriol 187(23):8181–8184
Kaur G et al (2011) Nisin and class IIa bacteriocin resistance among listeria and other foodborne pathogens and spoilage bacteria. Microbial Drug Resist 17(2):197–205
Keo T et al (2011) Campylobacter capsule and lipooligosaccharide confer resistance to serum and cationic antimicrobials. Virulence 2(1):30–40
Kilelee E et al (2010) Lysyl-phosphatidylglycerol attenuates membrane perturbation rather than surface association of the cationic antimicrobial peptide 6 W-RP-1 in a model membrane system: implications for daptomycin resistance. Antimicrob Agents Chemother 54(10):4476–4479
Kindrachuk J, Napper S (2008) Host and pathogen sensory systems as targets for therapeutic intervention, VDM Publishing
Kingry LC, Petersen JM (2014) Comparative review of *Francisella tularensis* and *Francisella novicida*. Front Cell Infect Microbiol 4:35
Klein JS, Lewinson O (2011) Bacterial ATP-driven transporters of transition metals: physiological roles, mechanisms of action, and roles in bacterial virulence. Metallomics 3(11):1098–1108
Kobayashi N, Nishino K, Yamaguchi A (2001) Novel macrolide-specific ABC-type efflux transporter in *Escherichia coli*. J Bacteriol 183(19):5639–5644
Kocianova S et al (2005) Key role of poly-γ-DL-glutamic acid in immune evasion and virulence of *Staphylococcus epidermidis*. J Clin Invest 115(3):688–694
Köck K, Brouwer KLR (2012) A perspective on efflux transport proteins in the liver. Clin Pharmacol Ther 92(5):599–612
Koo SP et al (1996) Staphylocidal action of thrombin-induced platelet microbicidal protein is not solely dependent on transmembrane potential. Infect Immun 64(3):1070–1074
Koprivnjak T, Peschel A (2011) Bacterial resistance mechanisms against host defense peptides. Cell Mol Life Sci 68(13):2243–2254
Kukkonen M, Korhonen TK (2004) The omptin family of enterobacterial surface proteases/adhesins: from housekeeping in *Escherichia coli* to systemic spread of *Yersinia pestis*. Int J Med Microbiol 294(1):7–14
Kumar A, Schweizer HP (2005) Bacterial resistance to antibiotics: active efflux and reduced uptake. Adv Drug Deliv Rev 57(10):1486–1513

Kumar S et al (2013) Bacterial antimicrobial efflux pumps of the MFS and MATE transporter families: a review. In: Pandali S (ed) Recent research developments in antimicrobial agents & chemotherapy. Research Signpost Inc., Kerala, India, pp 1–21

Kupferwasser LI et al (1999) Plasmid-mediated resistance to thrombin-induced platelet microbicidal protein in staphylococci: role of the qacA locus. Antimicrob Agents Chemother 43(10):2395–2399

Kupferwasser LI et al (2002) In vitro susceptibility to thrombin-induced platelet microbicidal protein is associated with reduced disease progression and complication rates in experimental *Staphylococcus aureus* endocarditis: microbiological, histopathologic, and echocardiographic analyses. Circulation 105(6):746–752

Lai R et al (2002) An anionic antimicrobial peptide from toad *Bombina maxima*. Biochem Biophys Res Commun 295(4):796–799

LaRock CN, Nizet V (2015) Cationic antimicrobial peptide resistance mechanisms of streptococcal pathogens. Biochim Biophys Acta

Last NB, Schlamadinger DE, Miranker AD (2013) A common landscape for membrane-active peptides. Protein Sci 22(7):870–882

Lauth X et al (2009) M1 protein allows group a streptococcal survival in phagocyte extracellular traps through cathelicidin inhibition. J Innate Immun 1(3):202–214

Law CJ, Maloney PC, Wang D-N (2008) Ins and outs of major facilitator superfamily, antiporters. Annu Rev Microbiol 62:289–305

Lee CR et al (2013) Lipid a biosynthesis of multidrug-resistant pathogens—a novel drug target. Curr Pharm Des 19(36):6534–6550

Lemaitre B, Hoffmann J (2007) The host defense of *Drosophila melanogaster*. Annu Rev Immunol 25:697–743

Lewis K (2000) Translocases: a bacterial tunnel for drugs and proteins. Curr Biol 10(18):R678–R681

Lewis DA (2003) Chancroid: clinical manifestations, diagnosis, and management. Sex Transm Infect 79(1):68–71

Lewis VG, Ween MP, McDevitt CA (2012) The role of ATP-binding cassette transporters in bacterial pathogenicity. Protoplasma 249(4):919–942

Li X-Z, Nikaido H (2004) Efflux-mediated drug resistance in bacteria. Drugs 64(2):159–204

Li X-Z, Nikaido H (2009) Efflux-mediated drug resistance in bacteria: an update. Drugs 69 (12):1555–1623

Li M et al (2007a) The antimicrobial peptide-sensing system aps of *Staphylococcus aureus*. Mol Microbiol 66(5):1136–1147

Li M et al (2007b) Gram-positive three-component antimicrobial peptide-sensing system. Proc Natl Acad Sci USA 104(22):9469–9474

Li Y et al (2012) LPS remodeling is an evolved survival strategy for bacteria. Proc Natl Acad Sci 109(22):8716–8721

Lima IFN, Havt A, Lima AAM (2015) Update on molecular epidemiology of Shigella infection. Curr Opin Gastroenterol 31(1):30–37

Lin J, Huang S, Zhang Q (2002) Outer membrane proteins: key players for bacterial adaptation in host niches. Microbes Infect 4(3):325–331

Lin QY et al (2014a) *Serratia marcescens* arn, a PhoP-regulated locus necessary for polymyxin B resistance. Antimicrob Agents Chemother 58(9):5181–5190

Lin YT et al (2014b) MacABCsm, an ABC-type tripartite efflux pump of *Stenotrophomonas maltophilia* involved in drug resistance, oxidative and envelope stress tolerances and biofilm formation. J Antimicrob Chemother 69(12):3221–3226

Liu R et al (2011) There are abundant antimicrobial peptides in brains of two kinds of bombina toads. J Proteome Res 10(4):1806–1815

Llobet E et al (2011) Analysis of the networks controlling the antimicrobial-peptide-dependent induction of *Klebsiella pneumoniae* virulence factors. Infect Immun 79(9):3718–3732

Lohner K, Prenner EJ (1999) Differential scanning calorimetry and X-ray diffraction studies of the specificity of the interaction of antimicrobial peptides with membrane-mimetic systems. Biochim Biophys Acta (BBA) 1462(1–2):141–156
Lopez-Solanilla E, Garcia-Olmedo F, Rodriguez-Palenzuela P (1998) Inactivation of the sapA to sapF locus of *Erwinia chrysanthemi* reveals common features in plant and animal bacterial pathogenesis. Plant Cell 10(6):917–924
Lösel DM (1900) Lipids in the structure and function of fungal membranes. In: Kuhn P et al (ed) Biochemistry of cell walls and membranes in fungi. Springer, Berlin, pp 119–133
Loutet SA et al (2011) A two-tier model of polymyxin B resistance in *Burkholderia cenocepacia*. Environ Microbiol Rep 3(2):278–285
Luckenbach T, Fischer S, Sturm A (2014) Current advances on ABC drug transporters in fish. Comp Biochem Physiol C Toxicol Pharmacol 165:28–52
Lupp C, Hancock RW, Ruby E (2002) *The Vibrio fischeri* sapABCDF locus is required for normal growth, both in culture and in symbiosis. Arch Microbiol 179(1):57–65
Lycklama ANJA, Driessen AJ (2012) The bacterial sec-translocase: structure and mechanism. Philos Trans R Soc Lond B Biol Sci 367(1592):1016–1028
Madej MG (2014) Function, structure, and evolution of the major facilitator superfamily: the LacY manifesto. Adv Biol 2014:20 Article ID 523591. doi: 10.1155/2014/523591
Maisetta G et al (2011) Gingipains produced by *Porphyromonas gingivalis* ATCC49417 degrade human-β-defensin 3 and affect peptide's antibacterial activity in vitro. Peptides 32(5):1073–1077
Mandal SM et al (2014) Challenges and future prospects of antibiotic therapy: from peptides to phages utilization. Front Pharmacol 5
Manning AJ (2013) Outer membrane vesicles: a new paradigm of bacterial innate immunity. PhD thesis, Department of biochemistry, Duke University, Durham, USA
Manning AJ, Kuehn MJ (2011) Contribution of bacterial outer membrane vesicles to innate bacterial defense. Bmc Microbiol 11
Manning AJ, Kuehn MJ (2013) Functional advantages conferred by extracellular prokaryotic membrane vesicles. J Mol Microbiol Biotechnol 23(1–2):131–141
Marcos JF, Gandia M (2009) Antimicrobial peptides: to membranes and beyond. Expert Opin Drug Discov 4(6):659–671
Martinez JL et al (2009) Functional role of bacterial multidrug efflux pumps in microbial natural ecosystems. FEMS Microbiol Rev 33(2):430–449
Mason KM, Munson RS, Bakaletz LO (2005) A Mutation in the sap operon attenuates survival of nontypeable *Haemophilus influenzae* in a Chinchilla Model of Otitis Media. Infect Immun 73 (1):599–608
Mason KM et al (2006) The non-typeable *Haemophilus influenzae* Sap transporter provides a mechanism of antimicrobial peptide resistance and SapD-dependent potassium acquisition. Mol Microbiol 62(5):1357–1372
Mason KM et al (2011) Heme utilization by nontypeable *Haemophilus influenzae* is essential and dependent on sap transporter function. J Bacteriol 193(10):2527–2535
Masureel M et al (2014) Protonation drives the conformational switch in the multidrug transporter LmrP. Nat Chem Biol 10(2):149–155
Matsuo M et al (2011) Growth-phase dependence of susceptibility to antimicrobial peptides in *Staphylococcus aureus*. Microbiology-Sgm 157:1786–1797
Mattiuzzo M et al (2007) Role of the *Escherichia coli* SbmA in the antimicrobial activity of proline-rich peptides. Mol Microbiol 66(1):151–163
Mattiuzzo M et al (2014) Proteolytic activity of *Escherichia coli* oligopeptidase B against proline-rich antimicrobial peptides. J Microbiol Biotechnol 24(2):160–167
May JJ et al (2005) Inhibition of the D-alanine:D-alanyl carrier protein ligase from Bacillus subtilis increases the bacterium's susceptibility to antibiotics that target the cell wall. FEBS J 272 (12):2993–3003
McBroom AJ, Kuehn MJ (2007) Release of outer membrane vesicles by Gram-negative bacteria is a novel envelope stress response. Mol Microbiol 63(2):545–558

McPhee JB et al (2009) Antibiotic resistance due to reduced uptake. In: Mayers D (ed) Antimicrobial drug resistance, Humana Press, pp 97–110
McPhee JB et al (2014) Host defense peptide resistance contributes to colonization and maximal intestinal pathology by crohn's disease-associated adherent-invasive *Escherichia coli*. Infect Immun 82(8):3383–3393
Mehla J, Sood SK (2011) Substantiation in *Enterococcus faecalis* of dose-dependent resistance and cross-resistance to pore-forming antimicrobial peptides by use of a polydiacetylene-based colorimetric assay. Appl Environ Microbiol 77(3):786–793
Mehla J, Sood SK (2013) Connecting membrane fluidity and surface charge to pore-forming antimicrobial peptides resistance by an ANN-based predictive model. Appl Microbiol Biotechnol 97(10):4377–4384
Menozzi FD et al (2002) Enhanced bacterial virulence through exploitation of host glycosaminoglycans. Mol Microbiol 43(6):1379–1386
Miller DS (2015) Regulation of ABC transporters blood-brain barrier: the good, the bad, and the ugly. Adv Cancer Res 125:43–70
Miller SI et al (1990) Characterization of defensin resistance phenotypes associated with mutations in the phoP virulence regulon of *Salmonella typhimurium*. Infect Immun 58(11):3706–3710
Miller AK et al (2011) PhoQ mutations promote lipid A modification and polymyxin resistance of *Pseudomonas aeruginosa* found in colistin-treated cystic fibrosis patients. Antimicrob Agents Chemother 55(12):5761–5769
Mills SD, Dougherty TJ (2012) Cell-based screening in antibacterial discovery. In: Dougherty TJ, Pucci MJ (eds) Antibiotic discovery and development, vols 1 and 2. Springer, New York, pp 901–929
Misaka S, Muller F, Fromm MF (2013) Clinical relevance of drug efflux pumps in the gut. Curr Opin Pharmacol 13(6):847–852
Mishra A, Jha B (2013) Microbial exopolysaccharides. In: Rosenberg E et al (ed) The Prokaryotes. Springer, Berlin, pp 179–192
Mishra NN et al (2011) Carotenoid-related alteration of cell membrane fluidity impacts *Staphylococcus aureus* susceptibility to host defense peptides. Antimicrob Agents Chemother 55(2):526–531
Mishra NN et al (2012) Differential adaptations of methicillin-resistant *Staphylococcus aureus* to serial in vitro passage in daptomycin: evolution of daptomycin resistance and role of membrane carotenoid content and fluidity. Int J Microbiol 2012:6
Mohamed Mustafa MS et al (2012) Assessment of substrate inhibition of bacterial oligopeptidase B. Biol Pharm Bull 35(11):2010–6
Mohammad H, Thangamani S, Seleem MN (2015) Antimicrobial peptides and peptidomimetics—potent therapeutic allies for staphylococcal infections. Curr Pharm Des 21(16):2073–2088
Morace G, Perdoni F, Borghi E (2014) Antifungal drug resistance in Candida species. J Global Antimicrob Resist 2(4):254–259
Morita Y et al (2000) NorM of vibrio parahaemolyticus is an Na+-driven multidrug efflux pump. J Bacteriol 182(23):6694–6697
Mount KLB et al (2010) *Haemophilus ducreyi* SapA contributes to cathelicidin resistance and virulence in humans. Infect Immun 78(3):1176–1184
Murakami S (2008) Multidrug efflux transporter, AcrB—the pumping mechanism. Curr Opin Struct Biol 18(4):459–465
Murphy EC, Frick IM (2013) Gram-positive anaerobic cocci—commensals and opportunistic pathogens. FEMS Microbiol Rev 37(4):520–553
Murphy EC et al (2014) Identification of molecular mechanisms used by *Finegoldia magna* to penetrate and colonize human skin. Mol Microbiol 94(2):403–417
Mysak J et al (2014) *Porphyromonas gingivalis*: major periodontopathic pathogen overview. J Immunol Res 2014:8
Muzamal U et al (2014) Diversity of two-component systems: insights into the signal transduction mechanism by the *Staphylococcus aureus* two-component system GraSR [version 1; referees: 2 approved with reservations], vol 3

Naito M et al (2010) Effects of sequential *Campylobacter jejuni* 81-176 lipooligosaccharide core truncations on biofilm formation, stress survival, and pathogenesis. J Bacteriol 192(8):2182–2192

Nakamura T et al (1988) Tachyplesin, a class of antimicrobial peptide from the hemocytes of the horseshoe crab (*Tachypleus tridentatus*). Isolation and chemical structure. J Biol Chem 263 (32):16709–16713

Nakka S, Qi M, Zhao Y (2010) The *Erwinia amylovora* PhoPQ system is involved in resistance to antimicrobial peptide and suppresses gene expression of two novel type III secretion systems. Microbiol Res 165(8):665–673

Nawrocki KL, Crispell EK, McBride SM (2014) Antimicrobial peptide resistance mechanisms of Gram-positive bacteria. Antibiotics (Basel) 3(4):461–492

Needham BD, Trent MS (2013) Fortifying the barrier: the impact of lipid A remodelling on bacterial pathogenesis. Nat Rev Microbiol 11(7):467–481

Nelson DC, Garbe J, Collin M (2011) Cysteine proteinase SpeB from *Streptococcus pyogenes*—a potent modifier of immunologically important host and bacterial proteins. Biol Chem 392 (12):1077–1088

Neumann A et al (2014a) Novel role of the antimicrobial peptide LL-37 in the protection of neutrophil extracellular traps against degradation by bacterial nucleases. J Innate Immun 6 (6):860–868

Neumann A et al (2014b) The antimicrobial peptide LL-37 facilitates the formation of neutrophil extracellular traps. Biochem J 464:3–11

Nicolas P (2009) Multifunctional host defense peptides: intracellular-targeting antimicrobial peptides. FEBS J 276(22):6483–6496

Nikaido H, Pagès J-M (2012) Broad-specificity efflux pumps and their role in multidrug resistance of Gram-negative bacteria. FEMS Microbiol Rev 36(2):340–363

Nikaido H, Zgurskaya HI (2001) AcrAB and related multidrug efflux pumps of *Escherichia coli*. J Mol Microbiol Biotechnol 3(2):215–218

Nishie M, Nagao J, Sonomoto K (2012) Antibacterial peptides "bacteriocins": an overview of their diverse characteristics and applications. Biocontrol Sci 17(1):1–16

Norsworthy AN, Visick KL (2013) Gimme shelter: how *Vibrio fischeri* successfully navigates an animal's multiple environments. Front Microbiol 4

Nwodo UU, Green E, Okoh AI (2012) Bacterial exopolysaccharides: functionality and prospects. Int J Mol Sci 13(11):14002–14015

Nyberg P, Rasmussen M, Bjorck L (2004) alpha2-Macroglobulin-proteinase complexes protect *Streptococcus pyogenes* from killing by the antimicrobial peptide LL-37. J Biol Chem 279 (51):52820–52823

Olaitan AO, Morand S, Rolain J-M (2014) Mechanisms of polymyxin resistance: acquired and intrinsic resistance in bacteria. Front Microbiol 5:643

Oliver JD (2015) The Biology of *Vibrio vulnificus*. Microbiol Spectr 3(3)

Olsen I, Amano A (2015) Outer membrane vesicles—offensive weapons or good Samaritans? 7

Opperman TJ, Nguyen S (2015) Recent advances toward a molecular mechanism of efflux pump inhibition. Front Microbiol 6

Ormeno-Orrillo E et al (2012) Genomic basis of broad host range and environmental adaptability of *Rhizobium tropici* CIAT 899 and Rhizobium sp PRF 81 which are used in inoculants for common bean (Phaseolus vulgaris L.). Bmc Genomics 13

Otto M (2006) Bacterial evasion of antimicrobial peptides by biofilm formation. In: Shafer W (ed) Antimicrobial peptides and human disease. Springer, Berlin, pp 251–258

Otto M (2009) Bacterial sensing of antimicrobial peptides. In: Collin M, Schuch R (ed) Bacterial sensing and signaling, pp 136–149

Padilla E et al (2010) *Klebsiella pneumoniae* AcrAB efflux pump contributes to antimicrobial resistance and virulence. Antimicrob Agents Chemother 54(1):177–183

Palmer T, Berks BC (2012) The twin-arginine translocation (Tat) protein export pathway. Nat Rev Microbiol 10(7):483–496

Park PW et al (2000) Syndecan-1 shedding is enhanced by LasA, a secreted virulence factor of *Pseudomonas aeruginosa*. J Biol Chem 275(5):3057–3064
Park PW et al (2001) Exploitation of syndecan-1 shedding by *Pseudomonas aeruginosa* enhances virulence. Nature 411(6833):98–102
Parra-Lopez C, Baer MT, Groisman EA (1993) Molecular genetic analysis of a locus required for resistance to antimicrobial peptides in *Salmonella typhimurium*. The EMBO Journal 12 (11):4053–4062
Parra-Lopez C et al (1994) A Salmonella protein that is required for resistance to antimicrobial peptides and transport of potassium. The EMBO J 13(17):3964–3972
Paulsen IT (2003) Multidrug efflux pumps and resistance: regulation and evolution. Curr Opin Microbiol 6(5):446–451
Pelz A et al (2005) Structure and biosynthesis of staphyloxanthin from *Staphylococcus aureus*. J Biol Chem 280(37):32493–32498
Pence MA et al (2010) Streptococcal inhibitor of complement promotes innate immune resistance phenotypes of invasive M1T1 group A Streptococcus. J Innate Immun 2(6):587–595
Perron GG, Zasloff M, Bell G (2006) Experimental evolution of resistance to an antimicrobial peptide. Proc R Soc B Biol Sci 273(1583):251–256
Peschel A, Sahl H-G (2006) The co-evolution of host cationic antimicrobial peptides and microbial resistance. Nat Rev Micro 4(7):529–536
Phoenix DA, Dennison SR, Harris F (2013a) Antimicrobial peptides: their history, evolution, and functional promiscuity. In Antimicrobial Peptides, Wiley-VCH Verlag GmbH & Co. KGaA, pp 1–37
Phoenix DA, Dennison SR, Harris F (2013b) Cationic antimicrobial peptides, in antimicrobial peptides. Wiley-VCH Verlag GmbH & Co. KGaA, pp 39–81
Phoenix DA, Dennison SR, Harris F (2013c) Anionic antimicrobial peptides, in antimicrobial peptides. Wiley-VCH Verlag GmbH & Co. KGaA, pp 83–113
Phoenix DA, Dennison SR, Harris F (2013d) Models for the membrane interactions of antimicrobial peptides, in antimicrobial peptides. Wiley-VCH Verlag GmbH & Co. KGaA, pp 145–180
Phoenix DA, Dennison SR, Harris F (2013e) Graphical techniques to visualize the amphiphilic structures of antimicrobial peptides. In: Antimicrobial peptides. Wiley-VCH Verlag GmbH & Co. KGaA, pp 115–144
Phoenix DA, Harris F, Dennison SR (2014) Novel antimicrobial agents and strategies. Wiley, Weinheim
Phoenix DA et al (2015) The increasing role of phosphatidylethanolamine as a lipid receptor in the action of host defence peptides. Prog Lipid Res 59:26–37
Piddock LJV (2006a) Multidrug-resistance efflux pumps? not just for resistance. Nat Rev Micro 4 (8):629–636
Piddock LJV (2006b) Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. Clin Microbiol Rev 19(2):382–402
Poelarends GJ, Mazurkiewicz P, Konings WN (2002) Multidrug transporters and antibiotic resistance in *Lactococcus lactis*. Biochim Biophys Acta (BBA) Bioenerg 1555(1–3):1–7
Poole K (2000) Efflux-mediated resistance to fluoroquinolones in Gram-positive bacteria and the mycobacteria. Antimicrob Agents Chemother 44(10):2595–2599
Poole K (2008) Bacterial multidrug efflux pumps serve other functions. Microbe 3(4):179–185
Poole K (2012) Bacterial stress responses as determinants of antimicrobial resistance. J Antimicrob Chemother. doi:10.1093/jac/dks196
Port GC et al (2014) *Streptococcus pyogenes* polymyxin B-resistant mutants display enhanced exPortal integrity. J Bacteriol 196(14):2563–2577
Pranting M et al (2008) Mechanism and fitness costs of PR-39 resistance in salmonella enterica serovar typhimurium LT2. Antimicrob Agents Chemother 52(8):2734–2741
Prasad R, Rawal MK (2014) Efflux pump proteins in ant fungal resistance. Front Pharmacol 5:202. doi:10.3389/fphar.2014.00202

Pringle P (2013) Experiment eleven: deceit and betrayal in the discovery of the cure for tuberculosis. Bloomsbury Publishing PLC, London
Proft T, Baker EN (2009) Pili in gram-negative and Gram-positive bacteria—structure, assembly and their role in disease. Cell Mol Life Sci 66(4):613–635
Putman M, van Veen HW, Konings WN (2000) Molecular properties of bacterial multidrug transporters. Microbiol Mol Biol Rev 64(4):672–692
Radecka I, Martin C, Hill D (2014) The problem of microbial drug resistance, in novel antimicrobial agents and strategies. Wiley-VCH Verlag GmbH & Co. KGaA, pp 1–16
Radestock S, Forrest LR (2011) The alternating-access mechanism of MFS transporters arises from inverted-topology repeats. J Mol Biol 407(5):698–715
Rasmussen M, Bjorck L (2002) Proteolysis and its regulation at the surface of *Streptococcus pyogenes*. Mol Microbiol 43(3):537–544
Ratledge C, Wilkinson SG (1988) Microbial lipids. Academic Press, London
Reddy VS et al (2012) The major facilitator superfamily (MFS) revisited. FEBS J 279(11):2022–2035
Reverchon S, Nasser W (2013) Dickeya ecology, environment sensing and regulation of virulence programme. Environ Microbiol Rep 5(5):622–636
Rieg S et al (2009) Resistance against antimicrobial peptides is independent of *Escherichia coli* AcrAB, *Pseudomonas aeruginosa* MexAB and *Staphylococcus aureus* NorA efflux pumps. Int J Antimicrob Agents 33(2):174–176
Rodas PI, Contreras I, Mora GC (2010) Salmonella enterica serovar Typhi has a 4.1 kb genetic island inserted within the sapABCDF operon that causes loss of resistance to the antimicrobial peptide protamine. J Antimicrob Chemother 65(8):1624–1630
Romalde JL et al (2014) New Vibrio species associated to molluscan microbiota: a review. Front Microbiol 4:413. doi:10.3389/fmicb.2013.00413
Rosch JW, Caparon MG (2005) The ExPortal: an organelle dedicated to the biogenesis of secreted proteins in *Streptococcus pyogenes*. Mol Microbiol 58(4):959–968
Rosch JW, Hsu FF, Caparon MG (2007) Anionic lipids enriched at the ExPortal of *Streptococcus pyogenes*. J Bacteriol 189(3):801–806
Roy H (2009) Tuning the properties of the bacterial membrane with aminoacylated phosphatidylglycerol. IUBMB Life 61(10):940–953
Rubin EJ et al (2015) PmrD is required for modifications to *Escherichia coli* endotoxin that promote antimicrobial resistance. Antimicrob Agents Chemother 59(4):2051–2061
Sahin O et al (2015) Campylobacter in poultry: ecology and potential interventions. Avian Dis 59 (2):185–200
Saidijam M et al (2006) Microbial drug efflux proteins of the major facilitator superfamily. Curr Drug Targets 7(7):793–811
Saier Jr MH (1998) Molecular phylogeny as a basis for the classification of transport proteins from bacteria, archaea and eukarya. In: Poole RK (ed) Advances in microbial physiology, Academic Press, pp 81–136
Santa Maria JP et al (2014) Compound-gene interaction mapping reveals distinct roles for *Staphylococcus aureus* teichoic acids. In: Proceedings of the national academy of sciences 111 (34):12510–12515
Schaffer W (2006) Antimicrobial peptides and human disease. Springer, Berlin
Schmid J, Sieber V, Rehm B (2015) Bacterial exopolysaccharides: biosynthesis pathways and engineering strategies. Front Microbiol 6:496. doi:10.3389/fmicb.2015.00496
Schmidt NW, Wong GCL (2013) Antimicrobial peptides and induced membrane curvature: geometry, coordination chemistry, and molecular engineering. Curr Opin Solid State Mater Sci 17(4):151–163
Schmidtchen A, Frick IM, Bjorck L (2001) Dermatan sulphate is released by proteinases of common pathogenic bacteria and inactivates antibacterial alpha-defensin. Mol Microbiol 39 (3):708–713
Schmidtchen A et al (2002) Proteinases of common pathogenic bacteria degrade and inactivate the antibacterial peptide LL-37. Mol Microbiol 46(1):157–168

Selsted ME et al (1983) Primary structures of MCP-1 and MCP-2, natural peptide antibiotics of rabbit lung macrophages. J Biol Chem 258(23):4485–4489
Selsted ME, Szklarek D, Lehrer RI (1984) Purification and antibacterial activity of antimicrobial peptides of rabbit granulocytes. Infect Immun 45(1):150–154
Selsted ME et al (1985) Primary structures of 6 antimicrobial peptides of rabbit peritoneal neutrophils. J Biol Chem 260(8):4579–4584
Shafer WM et al (1998) Modulation of *Neisseria gonorrhoeae* susceptibility to vertebrate antibacterial peptides due to a member of the resistance/nodulation/division efflux pump family. Proc Natl Acad Sci USA 95(4):1829–1833
Shelton, C.L., et al., Sap Transporter Mediated Import and Subsequent Degradation of Antimicrobial Peptides in Haemophilus. Plos Pathogens, 2011. **7**(11)
Shen CJ et al (2010) Proteomic identification of membrane proteins regulating antimicrobial peptide resistance in Vibrio parahaemolyticus. J Appl Microbiol 108(4):1398–1407
Shireen T et al (2013) Differential adaptive responses of *Staphylococcus aureus* to in vitro selection with different antimicrobial peptides. Antimicrob Agents Chemother 57(10):5134–5137
Silhavy TJ, Kahne D, Walker S (2010) The bacterial cell envelope. Cold Spring Harb Perspect Biol 2(5), a000414:1-16
Slotboom DJ (2014) Structural and mechanistic insights into prokaryotic energy-coupling factor transporters. Nat Rev Microbiol 12(2):79–87
Smyth D et al (2014) DrsG from *Streptococcus dysgalactiae* subsp. *equisimilis* inhibits the antimicrobial peptide LL-37. Infect Immun 82(6):2337–2344
Sohlenkamp C et al (2007) The lipid lysyl-phosphatidylglycerol Is present in membranes of *Rhizobium tropici* CIAT899 and confers increased resistance to polymyxin B under acidic growth conditions. Mol Plant Microbe Interact 20(11):1421–1430
Solheim M et al (2007) Transcriptional responses of *Enterococcus faecalis* V583 to bovine bile and sodium dodecyl sulfate. Appl Environ Microbiol 73(18):5767–5774
Soto SM (2013) Role of efflux pumps in the antibiotic resistance of bacteria embedded in a biofilm. Virulence 4(3):223–229
Sperandio B et al (2008) Virulent *Shigella flexneri* subverts the host innate immune response through manipulation of antimicrobial peptide gene expression. J Exp Med 205(5):1121–1132
Splith K, Neundorf I (2011) Antimicrobial peptides with cell-penetrating peptide properties and vice versa. Eur Biophys J 40(4):387–397
Spoel SH, Dong X (2012) How do plants achieve immunity? Defence without specialized immune cells. Nat Rev Immunol 12(2):89–100
Stefani S et al (2015) Insights and clinical perspectives of daptomycin resistance in *Staphylococcus aureus*: a review of the available evidence. Int J Antimicrob Agents 46:278–289
Steiner H et al (1981) Sequence and specificity of 2 anti-bacterial proteins involved in insect immunity. Nature 292(5820):246–248
Straley SC, Perry RD (1995) Environmental modulation of gene-expression and pathogenesis in Yersinia. Trends Microbiol 3(8):310–317
Strempel N et al (2013) Human host defense peptide LL-37 stimulates virulence factor production and adaptive resistance in *Pseudomonas aeruginosa*. PLoS ONE 8(12):12
Stumpe S et al (1998) Identification of OmpT as the protease that hydrolyzes the antimicrobial peptide protamine before it enters growing cells of *Escherichia coli*. J Bacteriol 180(15):4002–4006
Subashchandrabose S et al (2013) Genome-wide detection of fitness genes in uropathogenic *Escherichia coli* during systemic infection. PLoS Pathog 9(12):15
Sun J, Deng Z, Yan A (2014) Bacterial multidrug efflux pumps: mechanisms, physiology and pharmacological exploitations. Biochem Biophys Res Commun 453(2):254–267
Szeltner Z, Polgar L (2008) Structure, function and biological relevance of prolyl oligopeptidase. Curr Protein Pept Sci 9(1):96–107
Tanaka Y et al (2013) Structural basis for the drug extrusion mechanism by a MATE multidrug transporter. Nature 496(7444):247–251

Taylor CM, Roberts IS (2005) Capsular polysaccharides and their role in virulence. In: Russell W, Herwald H (ed) Contributions to microbiology, pp 55–66
Teixeira V, Feio MJ, Bastos M (2012) Role of lipids in the interaction of antimicrobial peptides with membranes. Prog Lipid Res 51(2):149–177
Telford JL et al (2006) Pili in Gram-positive pathogens. Nat Rev Micro 4(7):509–519
Thanavala Y, Lugade AA (2011) Role of nontypeable *Haemophilus influenzae* in otitis media and chronic obstructive pulmonary disease. In: Harabuchi Y, Hayashi T, Katada A (eds) Recent advances in tonsils and mucosal barriers of the upper airways, pp 170–175
Thomassin J-L et al (2012a) OmpT outer membrane proteases of enterohemorrhagic and enteropathogenic *Escherichia coli* contribute differently to the degradation of human LL-37. Infect Immun 80(2):483–492
Thomassin J-L et al (2012b) Enterohemorrhagic and enteropathogenic *Escherichia coli* evolved different strategies to resist antimicrobial peptides. Gut Microbes 3(6):556–561
Tomaras AP et al (2014) LpxC Inhibitors as New Antibacterial Agents and Tools for Studying Regulation of Lipid A Biosynthesis in Gram-Negative Pathogens. mBio 5(5):e01551-14
Tremolieres F (2010) When the antibiotic miracle turns into a nightmare. MS Med Sci 26(11):925–929
Tzeng Y-L et al (2005) Cationic antimicrobial peptide resistance in *Neisseria meningitidis*. J Bacteriol 187(15):5387–5396
Ulett GC et al (2013) Uropathogenic *Escherichia coli* virulence and innate immune responses during urinary tract infection. Curr Opin Microbiol 16(1):100–107
Ullrich M (2009) Bacterial polysaccharides: current innovations and future trends, Caister Academic Press
Ulvatne H et al (2002) Proteases in *Escherichia coli* and *Staphylococcus aureus* confer reduced susceptibility to lactoferricin B. J Antimicrob Chemother 50(4):461–467
Van Bambeke F et al (2003) Antibiotic efflux pumps in prokaryotic cells: occurrence, impact on resistance and strategies for the future of antimicrobial therapy. J Antimicrob Chemother 51 (5):1055–1065
van Meer G, de Kroon AIPM (2011) Lipid map of the mammalian cell. J Cell Sci 124(1):5–8
van Meer G, Voelker DR, Feigenson GW (2008) Membrane lipids: where they are and how they behave. Nat Rev Mol Cell Biol 9(2):112–124
Vanhove AS et al (2015) Outer membrane vesicles are vehicles for the delivery of *Vibrio tasmaniensis* virulence factors to oyster immune cells. Environ Microbiol 17(4):1152–1165
Vasil ML, Darwin AJ (2012) Regulation of bacterial virulence, ASM Press
Vega LA, Caparon MG (2012) Cationic antimicrobial peptides disrupt the *Streptococcus pyogenes* ExPortal. Mol Microbiol 85(6):1119–1132
Vega LA, Port GC, Caparon MG (2013) An association between peptidoglycan synthesis and organization of the *Streptococcus pyogenes* ExPortal. Mbio 4(5):9
Vilcinskas A (2013) Evolutionary plasticity of insect immunity. J Insect Physiol 59(2):123–129
Vilhena C, Bettencourt A (2012) Daptomycin: a review of properties, clinical use, drug delivery and resistance. Mini Rev Med Chem 12(3):202–209
Viti C et al (2014) Molecular mechanisms of Cr(VI) resistance in bacteria and fungi. FEMS Microbiol Rev 38(4):633–659
von Koeckritz-Blickwede M et al (2012) Novel role of the antimicrobial peptide LL37 in the formation and stabilization of neutrophil extracellular traps. Faseb J 26
Vuong C et al (2004a) A crucial role for exopolysaccharide modification in bacterial biofilm formation, immune evasion, and virulence. J Biol Chem 279(52):54881–54886
Vuong C et al (2004b) Polysaccharide intercellular adhesin (PIA) protects *Staphylococcus epidermidis* against major components of the human innate immune system. Cell Microbiol 6 (3):269–275
Wang G (2008) Structures of human host defense cathelicidin LL-37 and its smallest antimicrobial peptide KR-12 in lipid micelles. J Biol Chem 283(47):32637–32643
Wang G, Li X, Wang Z (2009) APD2: the updated antimicrobial peptide database and its application in peptide design. Nucleic Acids Res 37:D933–D937

Wang G, Li X, Zasloff M (2010a) A database view of naturally occurring antimicrobial peptides: nomenclature, classification and amino acid sequence analysis. In: Wang G (ed) Antimicrobial peptides: discovery, design and novel therapeutic strategies, pp 1–21
Wang G et al (2010b) Identification of novel human immunodeficiency virus type 1-inhibitory peptides based on the antimicrobial peptide database. Antimicrob Agents Chemother 54 (3):1343–1346
Wang RZ et al (2015) The pathogenesis, detection, and prevention of *Vibrio parahaemolyticus*. Frontiers in Microbiology 6(13):144
Warner DM, Shafer WM, Jerse AE (2008) Clinically relevant mutations that cause derepression of the *Neisseria gonorrhoeae* MtrC-MtrD-MtrE efflux pump system confer different levels of antimicrobial resistance and in vivo fitness. Mol Microbiol 70(2):462–478
Wassenaar TM et al (2015) Review and phylogenetic analysis of qac genes that reduce susceptibility to quaternary ammonium compounds in Staphylococcus species. Eur J Microbiol Immunol 5(1):44–61
Weatherspoon-Griffin N et al (2014) The CpxR/CpxA two-component regulatory system up-regulates the multidrug resistance cascade to facilitate *Escherichia coli* resistance to a model antimicrobial peptide. J Biol Chem 289(47):32571–32582
Weidenmaier C, Kristian SA, Peschel A (2003) Bacterial resistance to antimicrobial host defenses–an emerging target for novel antiinfective strategies? Curr Drug Targets 4(8):643–649
Wick MJ (2011) Innate immune control of *salmonella enterica serovar typhimurium*: mechanisms contributing to combating systemic salmonella infection. J Innate Immun 3(6):543–549
Wilkens S (2015) Structure and mechanism of ABC transporters. F1000prime reports 7:14–14
Wilmes M et al (2011) Antibiotic activities of host defense peptides: more to it than lipid bilayer perturbation. Nat Prod Rep 28(8):1350–1358
Winter M, Lawrence C (2011) Asperger syndrome—What teachers need to Know, 2nd edn. Jessica Kingsley Publishers
Wolf M, Moser B (2012) Antimicrobial activities of chemokines: not just a side-effect? Front Immunol 3:213
Wong SSY, Yuen K-Y (2012) *Streptococcus pyogenes* and re-emergence of scarlet fever as a public health problem. Emerg Microbes Infect 1:e2
Wong AR et al (2011) Enteropathogenic and enterohaemorrhagic *Escherichia coli*: even more subversive elements. Mol Microbiol 80(6):1420–1438
Wong K et al (2014) Towards understanding promiscuity in multidrug efflux pumps. Trends Biochem Sci 39(1):8–16
World Health Organization (2014) Antimicrobial resistance: global report on surveillance. Switzerland, Geneva
Wu H et al (2015) Strategies for combating bacterial biofilm infections. J Oral Sci 7(1):1–7
Yamaguchi A, Nakashima R, Sakurai K (2015) Structural basis of RND-type multidrug exporters. Front Microbiol 6:327. doi:10.3389/fmicb.2015.00327
Yan N (2013) Structural advances for the major facilitator superfamily (MFS) transporters. Trends Biochem Sci 38(3):151–159
Yan N (2015) Structural biology of the major facilitator superfamily transporters. Annu Rev Biophys 44(44):257–283
Yang S-J et al (2012) The *Staphylococcus aureus* two-component regulatory system, GraRS, senses and confers resistance to selected cationic antimicrobial peptides. Infect Immun 80 (1):74–81
Yang S-C et al (2014) Antibacterial activities of bacteriocins: application in foods and pharmaceuticals. Front Microbiol 5:241
Yeaman MR, Yount NY (2003) Mechanisms of antimicrobial peptide action and resistance. Pharmacol Rev 55(1):27–55
Yeaman MR et al (1998) Platelet microbicidal proteins and neutrophil defensin disrupt the *Staphylococcus aureus* cytoplasmic membrane by distinct mechanisms of action. J Clin Investig 101(1):178–187

Yin Y et al (2006) Structure of the multidrug transporter EmrD from *Escherichia coli*. Science 312 (5774):741–744

Yother J (2011) Capsules of *Streptococcus pneumoniae* and other bacteria: paradigms for polysaccharide biosynthesis and regulation. Annu Rev Microbiol 65(65):563–581

Yu EW et al (2013) Microbial efflux pumps: current research. Caister Academic Press

Yu Z et al (2015) Antibacterial mechanisms of polymyxin and bacterial resistance. Biomed Res Int 2015:11

Zaffiri L, Gardner J, Toledo-Pereyra LH (2012) History of antibiotics. From salvarsan to cephalosporins. J Invest Surg 25(2):67–77

Zähner D et al (2010) Human antimicrobial peptide LL-37 induces MefE/Mel-mediated macrolide resistance in *Streptococcus pneumoniae*. Antimicrob Agents Chemother 54(8):3516–3519

Zamfir A et al (2003) Structural investigation of chondroitin/dermatan sulfate oligosaccharides from human skin fibroblast decorin. Glycobiology 13(11):733–742

Zasloff M (1987) Magainins, a class of antimicrobial peptides from xenopus skin—isolation, characterization of 2 active forms, and partial cdna sequence of a precursor. Proc Natl Acad Sci USA 84(15):5449–5453

Zasloff M (2002) Antimicrobial peptides of multicellular organisms. Nature 415(6870):389–395

Zgurskaya HI (2009) Multicomponent drug efflux complexes: architecture and mechanism of assembly. Future microbiology 4:919–932

Zgurskaya HI et al (2015) Mechanism of coupling drug transport reactions located in two different membranes. Front Microbiol 6:100. doi:10.3389/fmicb.2015.00100

Zhang YK et al (2015) Multidrug Resistance Proteins (MRPs) and cancer therapy. AAPS J 17 (4):802–812

Zhou H et al (2014) Mechanisms of nisin resistance in Gram-positive bacteria. Ann Microbiol 64 (2):413–420

Chapter 8
Engineered OAKs Against Antibiotic Resistance and for Bacterial Detection

Amram Mor

Abstract As bacterial resistance to antibiotics continues to threaten modern healthcare worldwide, the need for new approaches that control bacterial infections becomes evermore urgent. Membrane-active compounds (MACs) are currently gaining interest for their potential to address various antibiotic resistance challenges. Since MACs are able to target multiple vital bacterial functions simultaneously, they may have the advantage of fighting the infection while avoiding many of the known resistance mechanisms. This chapter reviews current data regarding the attempts to use oligomers of acylated cations (OACs) as a platform for optimizing the hydrophobic/cationic balance required for selective nonspecific membrane interactions of MACs, under in vitro and in vivo conditions. With the perspective gained over nearly a decade after their conception and after a few dozen investigations involving several hundreds of analogs, we describe the properties of a few representative lysyl-based OAC (OAK) sequences. These sequences reflect the OAC concept evolution from the original focus on bactericidal MACs that later shifted onto bacteriostatic derivatives and presently concentrates on seemingly inactive analogs that nonetheless improve the control of bacterial infections. Collectively, the current data appear to substantiate the potential of OAC-based MACs as a valuable resource for therapeutic antibacterial development, including for systemic applications.

8.1 Introduction

The continuous escalation of multidrug resistant (MDR) bacteria (Schaberle and Hack 2014; Eckert 2011) is inevitably leading to the dwindling supply of clinical treatment options (Haney and Hancock 2013). Thus, along with the multitude of strategies currently employed in an attempt to maintain an effective arsenal of

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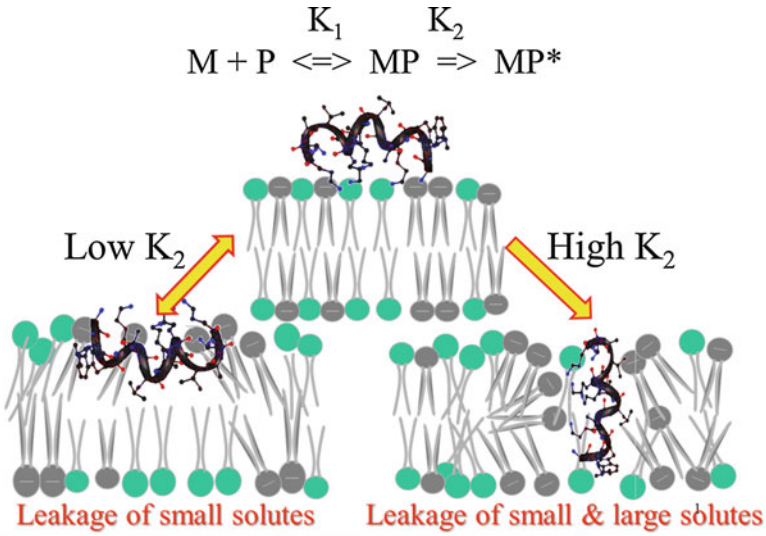
available chemotherapeutic products (Kinch et al. 2014; Silver 2011; Fischbach and Walsh 2009), there is a genuine need for new infection control approaches in order to meet the formidable capacity of bacteria to challenge new and old generations of antibiotics (Blair et al. 2015; McCallum et al. 2010; Poole 2012). In this respect, membrane active compounds (MACs) are presently gaining a renewed interest for their potential to control MDR infections (Klitgaard et al. 2008; Hurdle et al. 2011; Allen et al. 2014) by affecting critical processes that rely on common principles such as in bacterial sensing/communication (Gooderham and Hancock 2009; Daly et al. 2015), membrane proteins localization during division (Strahl and Hamoen 2010) and virulence (Daly et al. 2015; Sully et al. 2014). The molecular basis for these effects are relatively ill understood, however.

Based on their hydrophobic attributes MACs are dividable into two main classes; MACs having a pronounced hydrophobic character and borderline hydrophobic MACs. Members of the first class, tend to disrupt the bilayer structure abruptly, following deep insertion within the cytoplasmic membrane (Epand and Vogel 1999; Epand et al. 2010; Westerhoff et al. 1989) which, often culminates in a rapid bactericidal outcome at low micromolar concentrations (Hancock and Chapple 1999; Rotem and Mor 2009). Members of the second class are subject to more superficial membrane interactions and consequently believed to cause milder structural damages at the same low concentrations. As many bacteria can readily repair these damages (Hicks et al. 1994; Padan et al. 2005), such MACs might be considered altogether inactive molecules, although they can exhibit a minimal inhibitory concentration (MIC) at higher doses or display a bacteriostatic mode of action. Thus, even though transient, such superficial membrane damages appear nonetheless to inflict crippling injuries that clearly bare high cost on bacterial metabolism. For instance, the ordered packing of phospholipid can be distorted by the steric hindrance of bulky MACs, to the point that allows leakage of small ions such as protons, thereby leading to loss of the transmembrane potential (TMP). The repair process therefore, can be an exploitable window of opportunity toward controlling bacterial infections since their energy sources become depleted following membrane depolarization, thereby inhibiting vital bioenergetics and transport functions. Thus, despite maintaining near-normal proliferation rates, the penalties for bacteria can be devastating since, by inhibiting efflux pumps or export of resistance factors, such MACs might in fact sensitize bacteria to efflux substrate antibiotics (as they can now accumulate in the cytoplasm and exert their toxic effect) or restore sensitivity to formerly efficient antibiotics (for lack of resistance factors), respectively. By extension, such MACs might also significantly affect bacterial communication and virulence, as discussed in Sect. 8.2.

Host defense peptides (HDPs) can include both classes of MACs, as illustrated in Fig. 8.1. One might wonder which of these MAC classes are preferable for the developing therapeutic drugs. As the issue is out of the scope of this review, we briefly illustrate the debate with two opposing arguments: on one hand, the latter compounds might be advantageous since their milder action reduces the risk for complications associated with endotoxins released by bactericidal counterparts (Marr et al. 2006; Schuerholz et al. 2012). On the other hand, exposure of bacteria to sublethal drug



Cationic MAC	K_1 (M^{-1}) Adhesion	K_2 Insertion
$C_{12}K\text{-}7\alpha_8$	0.6×10^5	1.7
$C_{12(\omega 7)}K\text{-}\beta_{12}$	8.6×10^5	0.006

Fig. 8.1 Hypothetical interactions between a MAC and a mixed phospholipid bilayer. At the top is an equation describing the interaction between a MAC (M) and the phospholipid membrane (P). The cartoon underneath, illustrates the idea that electrostatic attraction is the initial force driving adhesion between a cationic M and anionic P to form a reversible complex (MP). This complex can reorganize to form different types of a more stable complex MP*, thereby perturbing the membrane structure in a manner that depends mainly on M's insertion within the membrane. The table at the bottom, lists parameters describing the binding of representative OAKs to a P composed of POPE:PG (3:1) as determined by SPR and analyzed by the 2-step model (Gaidukov et al. 2003). Our interpretation of these data, envisions that the apparent affinity constant describing the global interaction is the product of the individual constants for each step (K_1 and K_2), respectively, describing the kinetic ratio (k_{on}/k_{off}) for the adhesion and the insertion steps. In that case, the observed values suggest that despite the fact that roughly 10 times less $C_{12}K\text{-}7\alpha_8$ molecules adhere to the membrane (compared with $C_{12\omega 7}K\text{-}\beta_{12}$), their tendency for insertion is much stronger. High tendency for insertion, in turn, can be correlated to massive bilayer disturbances (Rotem et al. 2008a). In contrast, $C_{12\omega 7}K\text{-}\beta_{12}$ molecules tendency for insertion is very low and hence likely to remain stuck in superficial interactions that cause milder damages (e.g., membrane depolarization) (Sarig et al. 2010)

concentrations is not without potential detrimental effects, as various multicomponent sensory systems were implicated in bacterial resistance to MACs. For instance, Gram-positive bacteria (GPB) sense sub-MIC levels of antimicrobial peptides and confer resistance to these peptides in a GraRS–VraFG pathway-dependent manner

(Yang et al. 2012; Koprivnjak and Peschel 2011). The subsequent cell modifications can reduce the cytoplasmic membrane net negative charge (e.g., by addition of a lysine or alanine to phosphatidyl glycerol and lipoteichoic acid, respectively (Andra et al. 2011; Fedtke et al. 2004)), thereby reducing the electrostatic attraction between bacteria and cationic MACs. The fact that this induction occurred on exposure to polymyxin B and to RP-1 but not to daptomycin or hNP-1 (Yang et al. 2012), suggests that it might concern only certain antimicrobial peptides. In Gram-negative bacteria (GNB), several similar two-component systems for magnesium ions (Gooderham and Hancock 2009; Fernandez et al. 2010) were shown to be activated by a variety of HDPs (Fernandez et al. 2010; Shprung et al. 2012) and resulted in the modification of lipid A by addition of amino arabinose and phosphoethanolamine (Koprivnjak and Peschel 2011).

Many antibacterial HDPs increase outer membrane permeability through perturbation of the lipopolysaccharides (LPS) structure/function of Gram-negative species (Vaara et al. 2008; Zhang et al. 2000; Sawyer et al. 1988). These peptides can ultimately alter functions of the cytoplasmic membrane such as the permeability barrier (Epand et al. 2010; Ruhr and Sahl 1985; Zasloff 2002; Hancock 2005) and cell wall synthesis (Reisinger et al. 1980; Sass et al. 2010), namely as a consequence of charge clustering (Epand et al. 2010, 2008a; Epand and Epand 2009; Jean-Francois et al. 2008). Similarly, various chemical mimics of HDPs also interact with LPS (Jahnsen et al. 2013; Rotem et al. 2008a) and perturb the outer (Mensa et al. 2011) and cytoplasmic membranes, even at sub-MIC (Jammal et al. 2015; Goldberg et al. 2013; Livne et al. 2010), suggesting that certain membrane damages, such as those sustained at sub-MIC conditions, may underlie bacterial sensitization to antibiotics, as illustrated in Fig. 8.2.

Furthermore, as evident in current literature, there is an emerging interest in developing new combination therapies involving mixtures of classical antibiotics and antimicrobial HDPs (Dhand et al. 2011; Sakoulas et al. 2014; Paul et al. 2014; Li et al. 2014). However, despite their promising attributes, some HDPs can suffer from shortcomings such as protease sensitivity, systemic toxicity and/or high production costs, which hamper their systemic therapeutic potential. Therefore, at least theoretically, de novo designed chemical mimics of HDPs may be better adapted in addressing some of these challenges (Rotem and Mor 2009; Jahnsen et al. 2013; Jammal et al. 2015; Goldberg et al. 2013; Livne et al. 2010; Kaneti et al. 2013; Liu et al. 2004). HDP-mimics may better promote efficient systemic therapies owing to their improved pharmacokinetics (Jammal et al. 2015; Radzishevsky et al. 2007; Choi et al. 2009) whereas their structural simplicity should better support fine-tuning mechanistic studies. In the following sections, the review will focus on attempts to mimic natural MACs using oligomers of acylated cations (OACs). Table 8.1 lists a few representative lysyl-based OACs (OAKs) that will be emphasized throughout the review as they reflect the actual evolution of the concept, which originally concentrated on bactericidal MACs, moved on to bacteriostatic derivatives and ended up converging on seemingly inactive but promising analogs.

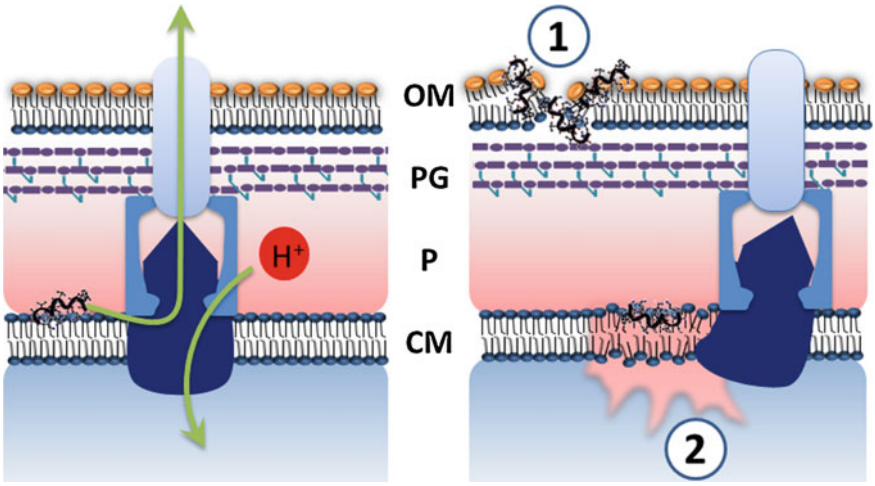


Fig. 8.2 Potential membrane damages affecting permeability and efflux functions. The *left panel* is a cartoon representation of a typical efflux pump, AcrAB-TolC, a member of the resistance nodulation division (RND) family, exclusively found in Gram-negative bacteria. It is able to extrude an antibiotic (or an HDP) from the cytoplasm or periplasm (P) in exchange for proton influx (Paulsen et al. 1996). The *right panel* illustrates two potential MAC-induced damages: (1) Cations (e.g., Ca^{++}) that normally stabilize the negative phosphate charges of the outer membrane (OM) LPS layer, are displaced by a MAC, owing to its higher affinity to LPS. However, due to its bulkier size, the MAC distorts the ordered packing of LPS molecules, thereby leading to cracks that allow entry of solutes (including of MACs), to the peptidoglycan (PG) layer, as described by the self-promoted uptake theory (Hancock and Chapple 1999). Option (2) illustrates potential fates of a moderately hydrophobic MAC (such as $C_{12\omega 7}K\text{-}\beta_{12}$ from Fig. 8.1) that adheres to anionic phospholipids of the cytoplasmic membrane (CM), a step likely facilitated by the negative inside electrochemical difference of potential existing across the CM. Such a MAC is predicted to assume only a superficial position on the CM outer leaflet (Sarig et al. 2010). Nonetheless, MAC accumulation over the CM might distort (again) the phospholipids ordered packing, to the point that allows leakage of small ions (such as protons) thereby leading to loss of the transmembrane potential and consequently loss of the energy source driving the function of efflux pumps and many other membrane proteins. Moreover, such MACs are also likely to alter the lipid environment of membrane proteins whose function relies on specific chemophysical characteristics (such as charge, fluidity, or bilayer thickness), or modify the proteins relative positions (see, for instance, the distorted alignment between AcrB and TolC in the CM and OM, respectively) which could also lead to a malfunctioning complex

8.2 Membrane-Active Antibacterial OAKs

Unlike animal cells whose cytoplasmic membrane contains a very low amount of anionic lipids, bacterial membranes are typically rich in anionic phospholipids whose relative proportion can reach 20–30 % in Gram-negative bacteria and nearly 100 % in Gram-positive bacteria (Ratledge and Wilkinson 1988; Yeaman and Yount 2003). As the OAC platform consists exclusively of fatty acyls and amide-linked

Table 8.1 Biophysical properties of representative OAKs

Designation	Sequence	Q	H	[e]MIC_{90} (μM)	Known damage in vitro	MOA at MIC	Systemic efficacy
[a]$C_{12}K\text{-}7\alpha_8$	$C_{12}\mathbf{K}\text{-}c_8\mathbf{K}c_8\mathbf{K}c_8\mathbf{K}c_8\mathbf{K}c_8\mathbf{K}c_8\mathbf{K}c_8\mathbf{K}_{NH2}$	8	47	GNB: 5 GPB: ≥50	CM charge clustering	Bactericidal	Only in combination therapy and after encapsulation
[b]$C_{12}K\text{-}3\beta_{10}$	$C_{12}\mathbf{K}\text{-}\mathbf{K}c_{10}\mathbf{K}\mathbf{K}c_{10}\mathbf{K}\mathbf{K}c_{10}\mathbf{K}_{NH2}$	7	45	GNB: 5 GPB: 5	CM charge clustering	Bactericidal	Yes
[c]$C_{12\omega7}K\text{-}\beta_{12}$	$C_{12\omega7}\mathbf{K}\text{-}\mathbf{K}c_{12}\mathbf{K}_{NH2}$	3	49	GNB: ≥50 GPB: 5	CM depolarization	Bacteriostatic	Only in combination therapy (without encapsulation)
[d]$C_{10}K\text{-}\beta_{12}$	$C_{10}\mathbf{K}\text{-}\mathbf{K}c_{12}\mathbf{K}_{NH2}$	3	46	GNB: >50 GPB: >50	CM depolarization	Inactive	Only in combination therapy (without encapsulation)

[a]Dodecanoyllysysl-[aminooctanoyllysyl]$_7$; [b]Dodecanoyllysysl-[lysylaminodecanoyllysyl]$_3$; [c]ω7-dodecenoyllysysl-lysylaminododecanoyl lysyl; [d]Decanoyl-lysysl-lysylaminododecanoyllysyl; [e]MIC_{90}, as determined on 90 % of at least 50 bacterial strains belonging to at least 10 different species; C, N-terminal acyl; c, aminoacyl. Q, Charge at physiological pH; H, Hydrophobicity as estimated by HPLC, reflecting percent acetonitrile/water required for elution from a C18 column; GNB, Gram-negative bacteria; GPB, Gram-positive bacteria; CM, Cytoplasmic membrane; MOA, Mode of action

cationic aminoacyls (Radzishevsky et al. 2007; Livne et al. 2009; Radzishevsky et al. 2008), it is particularly suitable for engineering high-affinity MACs.

One of the first sequences investigated for its membrane-active properties was dodecanoyllysysl-[aminooctanoyllysyl]$_7$ referred to as $C_{12}K\text{-}7\alpha_8$, (Radzishevsky et al. 2007) that preferentially targeted Gram-negative bacteria by exerting a rapid biocidal effect. The bactericidal outcome was proposed to stem from the peptides high binding affinity to the cytoplasmic membrane phospholipids, despite strong interactions with cell wall components as well. Various mechanistic studies support the view that $C_{12}K\text{-}7\alpha_8$ causes rapid bacterial death through disruption of the cytoplasmic membrane (Radzishevsky et al. 2007; Rotem et al. 2008b; Epand et al. 2008b). Conversely, a shorter analog ($C_{12}K\text{-}5\alpha_8$) having significantly lower aptitude for disrupting the membrane and displaying significantly slower time-kill curves, was proposed to rather inhibit the biosynthetic process. Being more hydrophobic but less cationic, $C_{12}K\text{-}5\alpha_8$ was allegedly able to reduce many electrostatic interactions on the way from the cell wall to the cytoplasmic membrane. Hence, unlike the case of $C_{12}K\text{-}7\alpha_8$, the functional transmembrane potential difference might actually promote internalization of $C_{12}K\text{-}5\alpha_8$, thereby enabling its interaction with intracellular targets, as exemplified with nucleic acids (Rotem et al. 2008b).

Two different studies have used either isothermal titration calorimetry (ITC (Epand et al. 2008b)) or surface plasmon resonance (SPR (Rotem et al. 2008b)) technologies to compare the binding properties of these analogs to model phospholipid membranes. The studies independently confirmed the higher binding affinity of $C_{12}K\text{-}7\alpha_8$ (also observed using DSC and NMR studies) while moreover indicating that only $C_{12}K\text{-}7\alpha_8$ had the ability to induce the clustering of anionic lipids. This clustering effect may lead to the lateral segregation of domains rich in anionic versus zwitterionic lipids, producing phase boundary defects that ultimately breach the permeability barrier of the cytoplasmic membrane.

Further studies of this ability to induce clustering of anionic lipids eventually led to the idea to exploit this property for co-encapsulation of synergistic drugs in lipid-based stable structures, called cochleates, whose aim would be to shield initially, and ultimately co-deliver the drugs. Inspired by reports on antimicrobial peptides that exhibited synergistic action with conventional antibiotics (Livne et al. 2010), such a potential role for $C_{12}K\text{-}7\alpha_8$ was investigated toward fighting MDR phenomena in Gram-negative bacteria. MIC determination against multiple *E. coli* MDR strains revealed combinations with sub-MIC OAK levels that acted synergistically with several antibiotics, thus lowering their MICs by several orders of magnitude. Attempts to shed light into the molecular basis for this synergism suggested that bacterial sensitization to antibiotics was derived mainly from the OAK's capacity to overcome the efflux-enhanced resistance mechanism, by promoting backdoor entry of otherwise excluded antibiotics (Fig. 8.2). Synergistic action between distinct molecular entities is, however, likely to suffer from numerous challenges during systemic therapy (namely owing to differential pharmacokinetics, body distribution, or tissue penetration), that might challenge the sensitization effect observed in vitro. Consequently, a follow-up work has aimed to

facilitate the simultaneous delivery of the synergistic drugs to the infection site while co-encapsulated within a delivery system. Out of several systems screened, OAK-based cochleates turned out to be quite remarkable in their capacity for rapid, stable, and high capacity co-encapsulation of drugs (Sarig et al. 2011; Epand et al. 2011). Such cochleates have also demonstrated advantages in systemic therapeutic efficacy in treating severe murine bacterial infection, as elaborated in Sect. 8.3.

While $C_{12}K\text{-}7\alpha_8$ is preferentially active on Gram-negative bacteria, its analog dodecanoyllysysl-[lysylaminodecanoyllysyl]$_3$ (referred to as $C_{12}K\text{-}3\beta_{10}$) presents an indiscriminate activity over many more bacterial species (MIC_{90} = 5 μM), yet displays low hemotoxicity, namely at concentrations as high as >100 μM. The main difference between these closely related OAKs (i.e., having similar HQ properties as shown in Table 8.1) pertains to their building blocks in that the acyl-lysyl (α) subunits were replaced with lysyl-acyl-lysyl (β) subunits. Consequently, subunits juxtaposition creates a structural motive (lysyl-lysyl) that is absent in the α-OAKs. This motive turned out to be critical in broadening the spectrum of activity, namely to include a wide range of Gram-positive bacteria (Livne et al. 2009) and of cancer cells (Held-Kuznetsov et al. 2009).

Interestingly, although $C_{12}K\text{-}3\beta_{10}$ exerts an essentially bactericidal effect, *E. coli* bacteria, are killed faster than *S. aureus* (i.e., within minutes versus hours), suggesting the involvement of different mechanisms of action. This contrasted with data obtained from SPR analysis that compared the OAK's binding properties using POPG:PE and POPG:CL bilayers (respective molar ratio of 20:80 and 60:40, to mimic the cytoplasmic membranes of the investigated bacteria), suggesting that the OAK presented quite similar membrane binding affinities (i.e., K_{app} = 1.1 and 0.9×10^6 M^{-1}, respectively). Mechanistic studies addressing this discrepancy suggested a peculiar mode of action involving OAK accumulation in the cell wall due to its differential affinity to GPB cell wall specific components. This blocks the advancement of OAK (and other) molecules toward the cytoplasmic membrane and leads to the observed outcome where *S. aureus* bacteria have undergone a transient rapid bactericidal stage that over time converted to a bacteriostatic effect (Livne et al. 2009).

Contrasting with the broad-spectrum activity of $C_{12}K\text{-}3\beta_{10}$, its shorter version ω7-dodecenoyl-lysyl-lysyl-aminododecanoyl-lysyl (referred to as $C_{12\omega7}K\text{-}\beta_{12}$) is principally active on Gram-positive bacteria only (Sarig et al. 2010). Additionally, while maintaining a similar potency in inhibiting bacterial growth (MIC_{90} = 5 μM), $C_{12\omega7}K\text{-}\beta_{12}$ is no longer endowed with the capability for rapid killing of bacteria, even at concentrations of several MIC multiples. These characteristics of $C_{12\omega7}K\text{-}\beta_{12}$ antibacterial activity correlate well with its membrane binding properties, exhibiting clearly lesser binding affinity to model bilayers mimicking the cytoplasmic membrane of Gram-negative compared with Gram-positive bacteria, i.e., $K_{app} = 5 \times 10^3$ versus 2×10^7 M^{-1}, respectively, as determined by SPR (Sarig et al. 2010). Here again, the tempting option to conclude for a causative relationship, is counterargued by the finding that, $C_{12\omega7}K\text{-}\beta_{12}$ was potently active on the isogenic mutant strains where efflux pump components were deleted (Goldberg et al. 2013). This suggested

that the OAK's inactivity rather resulted from the GNB capacity for rapid extrusion of this OAK through efflux pumps, unlike the previous analogs (i.e., $C_{12}K\text{-}7\alpha_8$ and $C_{12}K\text{-}3\beta_{10}$).

Otherwise, $C_{12\omega7}K\text{-}\beta_{12}$ is believed to exert a bacteriostatic effect over GPB owing to its distinct interactions with their cytoplasmic membrane. Once the OAK has adhered to the bilayer outer leaflet (e.g., in *S. aureus*), the deep insertion within the bilayer (observed for bactericidal OAKs such as $C_{12}K\text{-}7\alpha_8$ and $C_{12}K\text{-}3\beta_{10}$) is prevented by its particular chemophysical attributes, thereby limiting the extent of membrane damage that this OAK can inflict (Sarig et al. 2010). In support of this view is the fact that its hydrophobic analogs such as $C_{16\omega7}K\text{-}\beta_{12}$ are rapidly bactericidal to GPB (Sarig et al. 2008). This superficial interaction of $C_{12\omega7}K\text{-}\beta_{12}$ with the cytoplasmic membrane can nonetheless drastically alter various membrane protein functions that rely on its specific chemophysical characteristics (e.g., charge and/or fluidity) for carrying out a function, such as during signal transduction. Moreover, small solutes might leak out owing to the steric hindrance introduced by the OAK that distorts the membrane and induces its depolarization, which in turn also affects additional membrane functions, such as efflux.

Although exhibiting lesser binding affinity for GNB cytoplasmic membrane mimics, various assays assessing membrane damages provide evidence for the ability of $C_{12\omega7}K\text{-}\beta_{12}$ to induce membrane depolarization at low micromolar concentrations (Goldberg et al. 2013). As mentioned above, $C_{12\omega7}K\text{-}\beta_{12}$ seems to be a good substrate for GNB efflux pumps, unlike $C_{12}K\text{-}7\alpha_8$ or $C_{12}K\text{-}3\beta_{10}$, hence, its high MIC over these species ($MIC_{90} \geq 50$ μM). From both respects therefore, membrane depolarization of GNB by $C_{12\omega7}K\text{-}\beta_{12}$, is unexpected (Goldberg et al. 2013). Moreover, the literature often reports that depolarization is associated with bacterial death that normally occurs shortly thereafter (Silverman et al. 2003). It is therefore surprising (again), why is depolarization dissociated from bacterial death, since the number of colony-forming units remains unchanged over time, for at least several hours (Sarig et al. 2010). A possible explanation for these discrepancies maybe directly related to $C_{12\omega7}K\text{-}\beta_{12}$ binding properties to GNB versus GPB cytoplasmic membranes, as determined by SPR. The binding parameters suggest not only a lower apparent binding affinity (recall, $K_{app} = 5 \times 10^3$ versus 2×10^7 M^{-1}, respectively), they also suggested a lower propensity for insertion within the membrane (i.e., $K_{insertion} = k2_{on}/k2_{off} < 1$ versus >1, respectively, as illustrated in Fig. 8.1). Consequently, the events taking place upon adhesion to the cytoplasmic membrane of GNB are probably only slightly deviant from those described above for GPB, summarized as follows: The OAK can readily translocate across the outer membrane, like many HDPs (Hancock and Chapple 1999) as illustrated in Fig. 8.2. After reaching the periplasmic space, the OAK molecules undergo partitioning as they are simultaneously attracted to the inner membrane phospholipids and the efflux pumps. Since the OAK's tendency for insertion within the bilayer is quite low (as illustrated in Fig. 8.1), it is likely to be extruded by RND pumps, unlike $C_{12}K\text{-}7\alpha_8$ for instance, whose deeper insertion in the bilayer likely contributes to its ability to evade extrusion by the efflux pump. Therefore, the observable rapid membrane

depolarization of Gram-negative bacteria by $C_{12\omega7}K\text{-}\beta_{12}$, must be due to the action of the partitioned fraction of OAK molecules that managed to escape efflux.

Furthermore, membrane depolarization itself was associated with the sensitization of various MDR pathogenic species (e.g., Pseudomonas *aeruginosa*, *Klebsiella pneumoniae*, *Salmonella enterica,* and *Escherichia coli*) to ribosome-targeting antibiotics such as erythromycin, an excellent substrate of RND pumps. On this basis, sensitization was proposed to be TMP dependent since it correlated well with inhibition of their efflux pumps (Goldberg et al. 2013), that require a working membrane potential as energy source (Paulsen et al. 1996; Poole 2005). Thus, besides illustrating an interesting case of a highly potent synergistic antibacterial activity that emanated from combination of individually inactive compounds, this study has moreover highlighted the ability of MACs to overcome innate resistance to erythromycin and alike antibiotics.

Further investigation of this sub-MIC bacterial sensitization effect and its pertinence to the TMP revealed additional consequences in Gram-positive bacteria. As for GNB, $C_{12(\omega7)}K\text{-}\beta_{12}$ was able to simultaneously overcome multiple resistance mechanisms in multidrug resistant clinical isolates of *Staphylococcus aureus* strains, which became significantly sensitive to several antibiotics including cell wall-targeting β-lactams (e.g., oxacillin, piperacillin, and penicillin G) as well as ciprofloxacin and tetracycline, respectively, targeting DNA and ribosomes. However, while sensitization of the cytoplasm targeting drugs might be attributed to proton motive force-dependent efflux pumps (e.g., norA and tetK), this could not be the case for *S. aureus* sensitization to β-lactams, whose resistance mechanism is more likely to involve alterations in processing enzymes such as β-lactamase and/or penicillin binding protein 2a. Also noteworthy is the fact that, in addition to the OAC's ability to reduce the β-lactams MIC by up to three orders of magnitude (Kaneti et al. 2013), the study revealed that the rate at which *S. aureus* acquired resistance to β-lactams was considerably delayed in the presence of $C_{12(\omega7)}K\text{-}\beta_{12}$. Thus, the OAC interaction with *S. aureus* has achieved a double score: (a) resensitization to an antibiotic and (b) prevention of developing renewed resistance to that antibiotic. Importantly, antibiotic sensitization was shown to prevail under in vivo conditions, as well (Kaneti et al. 2013) as elaborated in Sect. 8.3.

Studies attempting to shed light into the molecular basis for this remarkable phenomenon suggest that the OAC's ability to resensitize *S. aureus* to β-lactam antibiotics is linked to inhibition of signal transduction cascades, as follows. Binding of a β-lactam antibiotic to its receptor extracellular domain induces a conformational change in the intracellular domain thereby allowing it to function as a protease that cleaves the β-lactamase gene repressor and consequently permits transcription of the Bla divergon (Wilke et al. 2004). Accordingly, qPCR was used to show that bacterial exposure to a β-lactam has indeed induced the signal transduction cascade for both *blaZ* and *mecA* (respectively encoding for β-lactamase and penicillin binding protein 2a). In contrast, addition of sub-MIC OAK has significantly reduced expression of both resistance factors, thereby providing support to the view that $C_{12(\omega7)}K\text{-}\beta_{12}$ interactions with the plasma membrane led to superficial damages (as evidenced by the depolarization assay) which in turn inhibited the signal transduction cascade.

A similar sensitization effect was obtained for bacteria exposed to sub-MIC of another MAC, the ionophore carbonylcyanide 3-chloro-phenylhydrazone (CCCP), in the presence of oxacillin, thereby further supporting the causative relationship between membrane damages (as reflected by depolarization) and the synergistic effects observed with the OAK (Kaneti et al. 2013).

This ability to interfere with expression of resistance factors has motivated additional investigations as to the effects of $C_{12(\omega 7)}K\text{-}\beta_{12}$ on MRSA, namely with respect to the occurrence of additional important signal transduction systems that are disrupted by sub-MIC OAK. The findings argue for the ability of sub-MIC OAK to inhibit quorum sensing (QS)-mediated lipolytic activity and activities of various virulence factors such as α-hemolysin and phenol-soluble modulins (PSM)-mediated cytotoxicity to erythrocytes and neutrophils (unpublished data). Similar effects were observed for the cyclodepsipeptide solonamide B, that was reported to reduce expression of RNAIII, the effector molecule of the *agr* quorum sensing system (Nielsen et al. 2014). Solonamide B too did not exhibit antimicrobial activity but displayed specific QS inhibitory traits that reduced the *S. aureus* cytotoxicity toward human neutrophils and rabbit erythrocytes in a dose-dependent manner. The authors have concluded that solonamide B interferes with *agr* activation by binding to the transmembrane (AgrC) sensor histidine kinase and thereby preventing interactions between AgrC and the auto-inducing peptides. The similarities observed between $C_{12(\omega 7)}K\text{-}\beta_{12}$ and solonamide B suggest a similar mode of action whereby $C_{12(\omega 7)}K\text{-}\beta_{12}$ like solonamide B may inhibit AgrC function through direct binding, or that both compounds indirectly interfere with the signal transduction as MACs do. Future investigation might resolve this issue.

These findings also prompted the undertaking of a structure–activity relationships (SAR) study focusing on the sequence $C_{12(\omega 7)}K\text{-}\beta_{12}$, aiming to assess the ability of OAKs to generate MACs that are devoid of antibiotic activity per se, but whose membrane perturbing properties might enhance the potency of some other antibacterial entity. Such a compound could be exploited for widening the sensitivity spectrum of GNB to include excellent antibiotics that are excluded by the outer membrane, namely due to their hydrophobicity. Also, the established inactivity of such a compound would have a mechanistic advantage in clarifying the issue of "who is doing what" during combination studies. This study revealed an analog ($C_{10}K\text{-}\beta_{12}$) that was a very good substrate of the RND family of efflux pumps and therefore inefficient on its own, in affecting the growth of Gram-negative bacteria (actually, even less efficient than $C_{12(\omega 7)}K\text{-}\beta_{12}$, being less hydrophobic). Yet, these analogs have also exhibited similar MAC properties, inducing membrane damages at sub-MIC (e.g., at 1–2 micromolar), including permeabilization of the outer membrane and depolarization of the cytoplasmic membrane (Jammal et al. 2015). In fact, $C_{10}K\text{-}\beta_{12}$ has enabled erythromycin and rifampicin to, respectively, exert their mode of action (i.e., bacteriostatic and bactericidal, respectively), likely by permeabilizing the outer membrane to rifampin and the cytoplasmic membrane to erythromycin. This study, therefore, provided strong arguments for the capacity of an OAK that is devoid of antibiotic activity to

sensitize GNB to rifampicin, reducing its MIC by up to four orders of magnitude, which was significantly higher than for the gold standard polymixin B. Possibly, this is due to the multiplicity of the types of simultaneous damages inflicted by such OAKs, including their ability to avoid interactions with cell wall components such as LPS, as well as the reciprocal drug's ability (OAK and antibiotic) to potentiate each other. Intriguingly, in the absence of exogenous antibiotics, $C_{10}K\text{-}\beta_{12}$ exhibited an improved capacity to control infection in vivo, as discussed below.

8.3 OAKs In vivo Properties

Several OAKs have shown efficacy in various mice models of infection. The sequence $C_{12}K\text{-}7\alpha_8$ was one of the first investigated for its in vivo properties using the peritonitic sepsis model (intraperitoneal (ip) treatment an hour after ip infection of neutropenic mice and the effect evaluated by monitoring survival for 6 days). In this model, the infecting bacterial inoculum ($4 \pm 1 \times 10^6$ CFU of extended spectrum beta-lactamase producing *E. coli*) corresponded to 2–3 times the LD_{50} (lethal dose at which half the animals are killed) and survival was as low as 0 % in the vehicle-treated groups. Under these conditions $C_{12}K\text{-}7\alpha_8$ prevented mortality to a similar extent as ciprofloxacin which increased the survival rates by up to 100 % after either single or multiple doses (1 or 4 mg/kg). As the lowest therapeutic dose was 2 mg/kg/day these results predict a therapeutic index (ratio of toxic to therapeutic dose >10) (Rotem et al. 2008a; Radzishevsky et al. 2007). Analysis of mice blood after single-dose ip administration revealed that $C_{12}K\text{-}7\alpha_8$ was present in the bloodstream within minutes but did not exceed the low micromolar level. *A propos*, noteworthy is the fact that a short version of this OAK (i.e., $C_{12}K\text{-}2\alpha_8$) demonstrated a maximal level of about 5 μM in mice blood upon ip administration of 5 mg/kg of body weight, whereas sustainable significantly higher concentrations in blood were also achievable (e.g., nearly 0.1 mM at 25 mg/kg). While $C_{12}K\text{-}2\alpha_8$ was devoid of antibacterial activity, it showed efficacy in experimental malaria where the blood stage of the disease revealed to be quite sensitive. Thus, in *Plasmodium vinckei*-infected mice, $C_{12}K\text{-}2\alpha_8$ presented an ED_{50} (50 % effective dose) of 22 mg/kg while toxicity emerged at the dose 4 × 50 mg/kg/day (Zaknoon et al. 2011). Interestingly, $C_{12}K\text{-}2\alpha_8$ inhibited in vitro parasite growth at submicromolar concentrations IC_{50} (50 % inhibitory concentration) was 0.3 ± 0.1 μM, but was devoid of hemolytic activity (i.e., displaying <1 % hemolysis at a concentration 1000-fold higher than IC_{50}). The fact that the early (ring) stage of the parasite developmental cycle was more sensitive (by 4- to 5-fold) than the intracellular feeding stage (trophozoite), further supports the view that the antiplasmodial mechanism was non-membranolytic to the host red blood cells.

Another antibacterial study of $C_{12}K\text{-}7\alpha_8$ used a pneumonia infection model, where mice were infected with *Pseudomonas aeruginosa* and treated by inhalation (25 μg per mouse). The OAK was similarly efficient as the antipseudomonal antibiotic tobramycin, reducing the lung bacterial population by up to 2 log units as

compared to inoculated vehicle controls (unpublished data). Collectively, these studies seem to indicate that $C_{12}K\text{-}7\alpha_8$ might be useful in the treatments of severe infections caused by Gram-negative pathogens. However, when tested against the thigh infection model (e.g., ip treatment an hour after intramuscular infection of neutropenic mice and the effect evaluated by monitoring viable bacteria extracted from the thigh 24 h post treatment), $C_{12}K\text{-}7\alpha_8$ failed to reduce bacterial load significantly, suggesting a rather poor potential for systemic efficacy probably due to poor tissue penetration.

Given its in vitro synergistic action with conventional antibiotics (Livne et al. 2010), a potential role of $C_{12}K\text{-}7\alpha_8$ in systemic therapy was investigated against Gram-negative bacteria (Epand et al. 2011). As mentioned above (Sect. 8.2) co-encapsulation of synergistic drugs in OAK-based cochleates may offer advantages toward overcoming potential problems arising during a systemic combination therapy, such as attenuating toxicity, shielding from undesired interactions and/or rectifying differential pharmacokinetic traits of the synergistic drugs. The maximal tolerated dose (MTD) of free $C_{12}K\text{-}7\alpha_8$ was compared by single IV administration to ICR mice of free or cochleated $C_{12}K\text{-}7\alpha_8$. While the MTD of free OAK was estimated at 5 mg OAK/kg of mouse weight, the MTD observed for the cochleated version was estimated at least 5-fold higher, as no detectable signs of toxicity were apparent at the highest tested dose (i.e., 20 mg/kg), indicating that encapsulation of $C_{12}K\text{-}7\alpha_8$ has significantly reduced its systemic toxicity. As mention above, previous attempts aiming to assess the therapeutic potential of $C_{12}K\text{-}7\alpha_8$ have shown encouraging outcome in topical but not in genuine systemic treatment models. While not necessarily promising an improved outcome, these acute toxicity results open the possibility for increasing the administrated doses beyond the free MTD, which in turn might achieve an improved therapeutic outcome. Regardless of this issue, the cochleates approach demonstrated moreover, that systemic treatments using single-dose administrations of co-encapsulated $C_{12}K\text{-}7\alpha_8$ and erythromycin, have significantly increased the therapeutic efficacy and protected mice from lethal bacterial infections in a dose-dependent manner (Livne et al. 2010; Sarig et al. 2011).

The use of lysyl-acyl-lysyl (β) building blocks in OAKs design also appears beneficial. We already mentioned that it enabled to broaden the spectrum of activity, namely to include a wide range of Gram-positive and Gram-negative bacteria. As it turned out, this structural motif seems also to improve the OAK's bioavailability since at least from preliminary efficacy studies using the thigh infection model, various β-OAKs demonstrated the ability to significantly affect the colony forming units (CFU) upon systemic administration. Thus, unlike α-OAKs (e.g., $C_{12}K\text{-}7\alpha_8$ and $C_{12}K\text{-}5\alpha_8$) that exhibited in vivo antibacterial efficacy only upon using topical (or semi-topical such as ip-ip) applications, $C_{12}K\text{-}3\beta_{10}$ and $C_{12(\omega7)}K\text{-}\beta_{12}$ were efficient at 2 mg/kg in reducing the viability of *Staphylococcus aureus*, an important human and animal pathogen (Zetola et al., 2005), albeit, they were assessed under somewhat different conditions (i.e., using normal ICR mice infected with *S. aureus* ATCC 29213). However, assessment of the shorter β-OAK version ($C_{12(\omega7)}K\text{-}\beta_{12}$) in the thigh infection model indicated quite comparable

efficacies on using different systemic routes for administrating the OAK (including ip, subcutanous (sc) and intreavenous (iv)), where the OAK has reduced bacterial load similarly to vancomycin. The MTD for iv and ip routes were 5 and 10 mg/kg, respectively, whereas the sc route was well tolerated at least up to 20 mg/kg. It is estimated that $C_{12(\omega7)}K\text{-}\beta_{12}$ rapidly enters circulation and remains stable for several hours.

Another antibacterial β-OAK worth mentioning is the sequence $C_{12}K\text{-}2\beta_{12}$ that demonstrated in vitro and in vivo efficacies against *Helicobacter pylori*, namely when using an experimental infection of Mongolian gerbils treated orogastrically (Makobongo et al. 2012), suggesting that the OAK concept may be a valuable resource for therapeutic treatment of *H. pylori* infection, as well. Together, these studies suggest that the potential of β-OAKs for antibacterial therapeutic development includes systemic monotherapy.

$C_{12(\omega7)}K\text{-}\beta_{12}$ was also investigated for its potential in combination therapy. As the OAK was able to overcome resistance of *S. aureus* clinical isolates to β-lactam antibiotics (e.g., oxacillin, piperacillin, and penicillin G) under in vitro conditions, it was verified whether this resensitization effect could prevail under in vivo conditions as well. Using the ip-ip version of the mouse peritonitis-sepsis model, various single doses of oxacillin and OAK combinations were able to prevent death induced by a lethal infection, in a synergistic dose-dependent manner (Kaneti et al. 2013).

Another study targeting GNB by combining $C_{12(\omega7)}K\text{-}\beta_{12}$ and erythromycin, tested their ability to affect disease course systemically, using the mouse thigh infection model in neutropenic mice that were inoculated intramuscularly with a clinically isolated MDR strain of *E. coli* and treated subcutaneously. Unlike individual treatments with OAK or erythromycin, treatments with the combined drugs have significantly enhanced growth inhibition of *E. coli* in most mice (Goldberg et al. 2013). Collectively, these findings suggest a potentially useful approach for expanding the antibiotics sensitivity spectrum of MDR Gram-negative bacteria to include efflux substrates. Another important outcome of this study is the realization that in vivo antibiotic sensitization of bacteria can prevail without the requirement for encapsulation and delivery of the synergistic drugs.

Possibly more interesting is the combination of two virtually inactive drugs on GNB such as rifampin and $C_{10}K\text{-}\beta_{12}$, whose systemic efficacy was further challenged by distinct modes of administration (oral and subcutaneous, respectively) without encapsulation (Jammal et al. 2015). Vehicle treatment of neutropenic mice inoculated with *K. pneumoniae* resulted in rapid death of most mice (20 and 10 % survival) within 1–2 days. Under these conditions, single dose treatments with rifampin, $C_{12(\omega7)}K\text{-}\beta_{12}$ or $C_{10}K\text{-}\beta_{12}$, were unable to significantly improve the survival rates, as they yielded 10, 20 and 25 % survivors at day 7, respectively. In contrast, administration of rifampin combined with $C_{12(\omega7)}K\text{-}\beta_{12}$ increased mice survival from 20 to 40 %, while the combination including $C_{10}K\text{-}\beta_{12}$ has further increased the survival rate to 60 %. The improved in vivo performance of $C_{10}K\text{-}\beta_{12}$ compared with $C_{12(\omega7)}K\text{-}\beta_{12}$ were attributed to two factors: a better bioavailability and a higher capacity to permeabilize the outer membrane of GNB.

Noteworthy is the most recent SAR study that revealed a closely related analog of $C_{10}K\text{-}\beta_{12}$ displaying quite intriguing in vivo properties (unpublished data). Namely, it is the first OAK to exhibit potent systemic efficacy against GNB using single dose monotherapy. It is intriguing not only because the systemic efficacy was the highest achieved in the OACs history thus far, but also because this outcome was achieved despite lack of antibiotic activity in vitro. Thus, using neutropenic mice infected ip with *E. coli* or *K. pneumonia*, the new analog increased the number of surviving mice in the sepsis-peritonitis model (by up to 90 %) and fully inhibited the number of viable bacteria in the thigh infection model after subcutaneous OAC administrations. The still ongoing mechanistic studies suggest that the new analog is endowed with improved bioavailability, as its free concentration in mice blood was higher than that of $C_{10}K\text{-}\beta_{12}$ (for instance, achieving 12 versus 5 micromolar, 60 min after administration of 12 mg/kg). However, while this quantitative information might justify the higher potency, the cause for the antibiotic effect remains to be determined since the MIC is consistently >50 micromolar (in culture medium, this concentration inhibited growth of some strains by about 10 %, at most). One direction taken is to verify the possibility of an OAK-mediated recruitment of the immune system.

8.4 Resin-Linked OAKs

Besides investigating OAKs potential in controlling bacterial infections, their ability to capture bacteria in the resin-linked (ROAK) form, was also investigated (Rotem et al. 2010; Marjieh et al. 2015). The first idea examined was whether OAK's binding affinity to bacteria might be exploited toward bacterial filtration from liquid media and/or eventual additional downstream applications. Having established their capacity to capture bacteria under different environmental conditions, it was next attempted to improve that capacity by investigating the SAR involved. Subsequently, the ROAKs aptitude to release the captured bacteria was examined and finally, the potential use of ROAKs in downstream applications was assessed by exploiting the capture/release capacities.

Following a preliminary SAR study, the sequence $K\text{-}7\alpha_{12}$ was initially selected for this investigation as its charge and hydrophobic characteristics were clearly implicated in bacterial capture by ROAK beads (Rotem et al. 2010). Using confocal microscopy for visualization of ROAK-bound bacteria (Fig. 8.3), and SPR technology for measuring bacterial binding to an OAK-linked chip, it was concluded that ROAKs are highly apt for rapid capture of various pathogens in different media, under incubation or continuous flow conditions. A single ROAK bead (average diameter of 50–100 μm) is estimated to capture >1,000 bacteria in contaminated culture medium, saline, or tap water. Moreover, after a brief ethanol treatment/elution, the ROAK-bound bacteria were readily identifiable by real-time PCR.

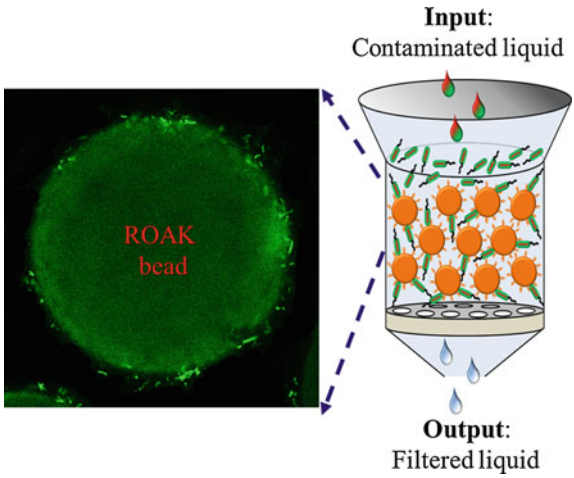


Fig. 8.3 Bacterial filtration using a ROAK column. The right panel is cartoon illustrating the principle of a ROAK-packed column used for continuous flow analysis of bacterial contaminations. Typically, 10 mg ROAK beads are packed in a glass pipette (restrained by glass fibers) and preconditioned in saline. Contaminated liquid media (e.g., tap water, saline, buffered solutions, or biological fluids) are passed through the column at a flow rate of 2.5 ml/min using a peristaltic pump. The captured bacteria can be released using a minimal elution solution (typically 2 ml of 70 % ethanol or 0.5 M $CaCl_2$ in water, to obtain dead or live free bacteria, respectively) for downstream analysis such as bacterial quantification by qPCR or determination of the numbers of colony forming units (Marjieh et al. 2015). The left panel is a "zoom" image showing GFP-expressing bacteria associated with a ROAK bead as analyzed by confocal fluorescence microscopy (Rotem et al. 2010)

Further characterization of bacterial capture by ROAKs in a recent follow-up study is summarized as follows:

(1) ROAKs maintained high-capture efficacy (80–100 %) for various representative species including medically relevant bacteria, while using inoculums differing by several orders of magnitude, starting from 1×10^4 CFU per milliliter medium;
(2) Bacterial capture in water and in the presence of salts at concentrations at least up to 100 mM was essentially similar, whereas only molar concentrations achieved significant levels of inhibition, bivalent salts being more potent inhibitors;
(3) No significant interference was detected at pH range 3–9, reflecting the hydrophobic forces at play;
(4) Partial bacterial capture (up to 23 %) occurred in contaminated whole blood, whereas 10-fold blood dilution enabled to increase the captured fraction to 50 %. These findings stand in line with previous data demonstrating efficient bacterial capture in wastewaters (Rotem et al. 2010), thereby consolidating the view of a high-affinity interaction between bacteria and ROAK beads;

(5) Free bacteria can be recovered nonetheless, as demonstrated after washing ROAK-bound *E. coli* with an eluting agent (for instance, ethanol, NaCl and $CaCl_2$, respectively yielding <1, 5 and 17 %, recovery).

Attempts to establish the minimal requirements for effective bacterial capture, point to an N-terminal lysyl residue being critical for maintaining significant capture activity, whereas OAK sequences composed of 3–4 acyl-lysyl subunits are sufficient for efficient capture. However, the data also suggest that such optimum maybe species- and/or strain dependent. For instance, K-4α_{12} was the shortest sequence to maintain similar capture of *Pseudomonas aeruginosa* as its parent sequence K-7α_{12}, whereas for *Escherichia coli* or *Klebsiella pneumonia*, it was K-3α_{12}. Thus, further studies are needed to validate this notion.

Based on these data, the capture and release capabilities were exploited for active filtration of bacteria-contaminated liquids in column chromatography. For this purpose, a glass pipette loaded with ROAK beads was utilized (as illustrated in Fig. 8.3) to filter saline inoculums spiked with a constant number of *E. coli* bacteria (6.0 ± 0.5 log CFU). Both the K-7α_{12} and K-3α_{12} ROAKs maintained a high capacity for bacterial capture under these continuous flow conditions, however, the elution yield from the K-3α_{12} column was substantially higher. The data therefore argue that the K-3α_{12} ROAK column assay represents a rapid bacterial enrichment procedure since bacterial counts were increased by a factor of about 7. Moreover, by applying a higher inoculum volume (100 ml, as often required in standard tests (Guidelines for drinking water quality. WHO 2008; (Rompre et al. 2002)), the concentration factor was increased to about 20-fold, a number that, at least theoretically, should further increase with increasing sample volumes. Collectively, these data provide evidence for the ability of ROAKs to deplete a sample of bacteria using extremely high-affinity sequences (e.g., K-7α_{12}) or, the ability to improve the sensitivity of qPCR-based bacterial detection by using moderate affinity OAKs (e.g., K-3α_{12}). Thus, in addition to its compositional simplicity and robustness, the new attributes highlight potential advantages of the OAK approach over approaches that use antibodies (Iqbal et al. 2000) or AMPs (Mannoor et al. 2010), including in terms of how environmental conditions (pH, ionic strength, and complexity) might affect their performances.

8.5 Concluding Remarks

Various studies sustain the notion that combination therapies targeting the membrane potential may represent an advantageous approach for controlling bacterial infections by disabling the devastating effects related to both antibiotic resistance and virulence factors. This notion was illustrated herein, through MAC investigations using the OAC platform. Together, the data show promise as to the concept's capacity to generate small molecules that simultaneously affect multiple membrane functions, control systemic bacterial infections in single and combination therapy

and overcome (and/or delay) innate and acquired resistance to antibiotics. In the future, therefore, side-by-side with conventional antibiotics development programs, we anticipate to witness an increased interest for exploring MACs that target signal transduction. In particular, owing to their simplicity and robustness, new OAC generations maybe also useful in elucidating the mechanisms involved in the alleged inhibition of quorum sensing and possibly for immune modulation.

Similarly, ROAKs were able to deplete liquid samples of bacterial content after incubation and during flow settings, illustrating the efficient capture of different bacterial species under a wide range of ionic strength and pH conditions. The studies also showed circumstances for the significant release of captured bacteria, live or dead, toward further analysis. The data therefore support the potential usefulness of this simple, robust, and efficient approach for rapid capture/analysis of bacteria from tap water and, possibly, from more complex media. As an effective tool for dissecting the relative roles of parameters considered most crucial for antimicrobial activities (i.e., charge and hydrophobicity), new OAC-linked surfaces might also pave the way to new potential applications (e.g., as biosensors, magnetic beads, etc.), so as to allow sensitive detection of bacteria in water and foods and/or their filtration from biological fluids such as blood.

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References

Allen RC, Popat R, Diggle SP, Brown SP (2014) Targeting virulence: can we make evolution-proof drugs? Nat Rev Microbiol 12:300–308

Andra J, Goldmann T, Ernst CM, Peschel A, Gutsmann T (2011) Multiple peptide resistance factor (MprF)-mediated resistance of Staphylococcus aureus against antimicrobial peptides coincides with a modulated peptide interaction with artificial membranes comprising lysyl-phosphatidylglycerol. J Biol Chem 286:18692–18700

Blair JM, Webber MA, Baylay AJ, Ogbolu DO, Piddock LJ (2015) Molecular mechanisms of antibiotic resistance. Nat Rev Microbiol 13:42–51

Choi S, Isaacs A, Clements D, Liu D, Kim H, Scott RW, Winkler JD, DeGrado WF (2009) De novo design and in vivo activity of conformationally restrained antimicrobial arylamide foldamers. Proc Natl Acad Sci USA 106:6968–6973

Daly SM, Elmore BO, Kavanaugh JS, Triplett KD, Figueroa M, Raja HA, El-Elimat T, Crosby HA, Femling JK, Cech NB, Horswill AR, Oberlies NH, Hall PR (2015) ω-Hydroxyemodin limits staphylococcus aureus quorum sensing-mediated pathogenesis and inflammation. Antimicrob Agents Chemother 59:2223–2235

Dhand A, Bayer AS, Pogliano J, Yang SJ, Bolaris M, Nizet V, Wang G, Sakoulas G (2011) Use of antistaphylococcal beta-lactams to increase daptomycin activity in eradicating persistent bacteremia due to methicillin-resistant Staphylococcus aureus: role of enhanced daptomycin binding. Clin Infect Dis 53:158–163

Eckert R (2011) Road to clinical efficacy: challenges and novel strategies for antimicrobial peptide development. Future Microbiol 6:635–651

Epand RM, Epand RF (2009) Lipid domains in bacterial membranes and the action of antimicrobial agents. Biochim Biophys Acta 1788:289–294

Epand RM, Vogel HJ (1999) Diversity of antimicrobial peptides and their mechanisms of action. Biochim Biophys Acta 1462:11–28

Epand RM, Rotem S, Mor A, Berno B, Epand RF (2008a) Bacterial membranes as predictors of antimicrobial potency. J Am Chem Soc 130:14346–14352

Epand R, Rotem S, Mor A, Berno B, Epand R (2008b) Bacterial membranes as predictors of antimicrobial potency. J Am Chem Soc 130:14346–14352

Epand RF, Maloy WL, Ramamoorthy A, Epand RM (2010) Probing the "charge cluster mechanism" in amphipathic helical cationic antimicrobial peptides. Biochemistry 49:4076–4084

Epand RF, Sarig H, Ohana D, Papahadjopoulos-Sternberg B, Mor A, Epand RM (2011) Physical properties affecting cochleate formation and morphology using antimicrobial oligo-acyl-lysyl peptide mimetics and mixtures mimicking the composition of bacterial membranes in the absence of divalent cations. J Phys Chem B 115:2287–2293

Fedtke I, Gotz F, Peschel A (2004) Bacterial evasion of innate host defenses-the Staphylococcus aureus lesson. Int J Med Microbiol 294:189–194

Fernandez L, Gooderham WJ, Bains M, McPhee JB, Wiegand I, Hancock RE (2010) Adaptive resistance to the "last hope" antibiotics polymyxin B and colistin in Pseudomonas aeruginosa is mediated by the novel two-component regulatory system ParR-ParS. Antimicrob Agents Chemother 54:3372–3382

Fischbach MA, Walsh CT (2009) Antibiotics for emerging pathogens. Science 325:1089–1093 (New York, N.Y.)

Gaidukov L, Fish A, Mor A (2003) Analysis of membrane-binding properties of dermaseptin analogues: relationships between binding and cytotoxicity. Biochemistry 42:12866–12874

Goldberg K, Sarig H, Zaknoon F, Epand RF, Epand RM, Mor A (2013) Sensitization of gram-negative bacteria by targeting the membrane potential. FASEB J 27:3818–3826

Gooderham WJ, Hancock RE (2009) Regulation of virulence and antibiotic resistance by two-component regulatory systems in Pseudomonas aeruginosa. FEMS Microbiol Rev 33:279–294

Hancock RE (2005) Mechanisms of action of newer antibiotics for Gram-positive pathogens. Lancet Infect Dis 5:209–218

Hancock RE, Chapple DS (1999) Peptide antibiotics. Antimicrob Agents Chemother 43:1317–1323

Haney EF, Hancock RE (2013) Peptide design for antimicrobial and immunomodulatory applications. Biopolymers 100:572–583

Held-Kuznetsov V, Rotem S, Assaraf YG, Mor A (2009) Host-defense peptide mimicry for novel antitumor agents. FASEB J 23:4299–4307

Hicks DB, Cohen DM, Krulwich TA (1994) Reconstitution of energy-linked activities of the solubilized F1F0 ATP synthase from Bacillus subtilis. J Bacteriol

Hurdle JG, O'Neill AJ, Chopra I, Lee RE (2011) Targeting bacterial membrane function: an underexploited mechanism for treating persistent infections. Nat Rev Microbiol 9:62–75

Iqbal SS, Mayo MW, Bruno JG, Bronk BV, Batt CA, Chambers JP (2000) A review of molecular recognition technologies for detection of biological threat agents. Biosens Bioelectron 15:549–578

Jahnsen RD, Haney EF, Franzyk H, Hancock RE (2013) Characterization of a proteolytically stable multifunctional host defense peptidomimetic. Chem Biol 20:1286–1295

Jammal J, Zaknoon F, Kaneti G, Goldberg G, Mor A (2015) Sensitization of Gram-negative bacteria to rifampin and OAK combinations. Scientific Reports 5

Jean-Francois F, Castano S, Desbat B, Odaert B, Roux M, Metz-Boutigue MH, Dufourc EJ (2008) Aggregation of cateslytin beta-sheets on negatively charged lipids promotes rigid membrane domains. A new mode of action for antimicrobial peptides? Biochemistry 47:6394–6402

Kaneti G, Sarig H, Marjieh I, Zaknoon F, Mor A (2013) Simultaneous breakdown of multiple antibiotic resistance mechanisms in S. aureus. FASEB J

Kinch MS, Haynesworth A, Kinch SL, Hoyer D (2014) An overview of FDA-approved new molecular entities: 1827–2013. Drug Discov Today 19:1033–1039

Klitgaard JK, Skov MN, Kallipolitis BH, Kolmos HJ (2008) Reversal of methicillin resistance in Staphylococcus aureus by thioridazine. J Antimicrob Chemother 62:1215–1221
Koprivnjak T, Peschel A (2011) Bacterial resistance mechanisms against host defense peptides. Cell Mol Life Sci 68:2243–2254
Li SA, Liu J, Xiang Y, Wang YJ, Lee WH, Zhang Y (2014) Therapeutic potential of the antimicrobial peptide OH-CATH30 for antibiotic-resistant Pseudomonas aeruginosa keratitis. Antimicrob Agents Chemother 58:3144–3150
Liu D, Choi S, Chen B, Doerksen RJ, Clements DJ, Winkler JD, Klein ML, DeGrado WF (2004) Nontoxic membrane-active antimicrobial arylamide oligomers. Angew Chem Int Ed Engl 43:1158–1162
Livne L, Kovachi T, Sarig H, Epand RF, Zaknoon F, Epand RM, Mor A (2009) Design and characterization of a broad-spectrum bactericidal acyl-lysyl oligomer. Chem Biol 16:1250–1258
Livne L, Epand RF, Papahadjopoulos-Sternberg B, Epand RM, Mor A (2010) OAK-based cochleates as a novel approach to overcome multidrug resistance in bacteria. FASEB J 24:5092–5101
Makobongo MO, Gancz H, Carpenter BM, McDaniel DP, Merrell DS (2012) The oligo-acyl lysyl antimicrobial peptide C(1)(2)K-2beta(1)(2) exhibits a dual mechanism of action and demonstrates strong in vivo efficacy against Helicobacter pylori. Antimicrob Agents Chemother 56:378–390
Mannoor MS, Zhang S, Link AJ, McAlpine MC (2010) Electrical detection of pathogenic bacteria via immobilized antimicrobial peptides. Proc Natl Acad Sci USA 107:19207–19212
Marjieh I, Meir O, Zaknoon F, Mor A (2015) Improved bacterial detection using immobilized acyl-lysyl oligomers. Appl Environ Microbiol 81:74–80
Marr AK, Gooderham WJ, Hancock RE (2006) Antibacterial peptides for therapeutic use: obstacles and realistic outlook. Curr Opin Pharmacol 6:468–472
McCallum N, Berger-Bächi B, Senn MM (2010) Regulation of antibiotic resistance in Staphylococcus aureus. Int J Med Microbiol 300:118–129
Mensa B, Kim YH, Choi S, Scott R, Caputo GA, DeGrado WF (2011) Antibacterial mechanism of action of arylamide foldamers. Antimicrob Agents Chemother 55:5043–5053
Nielsen A, Mansson M, Bojer MS, Gram L, Larsen TO, Novick RP, Frees D, Frøkiær H, Ingmer H (2014) Solonamide B inhibits quorum sensing and reduces staphylococcus aureus mediated killing of human neutrophils. PLoS One 9
Padan E, Bibi E, Ito M, Krulwich TA (2005) Alkaline pH homeostasis in bacteria: new insights. Biochim Biophys Acta 1717:67–88
Paul M, Carmeli Y, Durante-Mangoni E, Mouton JW, Tacconelli E, Theuretzbacher U, Mussini C, Leibovici L (2014) Combination therapy for carbapenem-resistant Gram-negative bacteria. J Antimicrob Chemother 69:2305–2309
Paulsen IT, Brown MH, Skurray RA (1996) Proton-dependent multidrug efflux systems. Microbiol Rev 60:575–608
Poole K (2005) Efflux-mediated antimicrobial resistance. J Antimicrob Chemother 56:20–51
Poole K (2012) Bacterial stress responses as determinants of antimicrobial resistance. J Antimicrob Chemother 67:2069–2089
Radzishevsky IS, Rotem S, Bourdetsky D, Navon-Venezia S, Carmeli Y, Mor A (2007) Improved antimicrobial peptides based on acyl-lysine oligomers. Nat Biotechnol 25:657–659
Radzishevsky IS, Kovachi T, Porat Y, Ziserman L, Zaknoon F, Danino D, Mor A (2008) Structure-activity relationships of antibacterial acyl-lysine oligomers. Chem Biol 15:354–362
Ratledge C, Wilkinson SG(1988) Microbial lipids. Academic Press, London
Reisinger P, Seidel H, Tschesche H, Hammes WP (1980) The effect of nisin on murein synthesis. Arch Microbiol 127:187–193
Rompre A, Servais P, Baudart J, de-Roubin MR, Laurent P (2002) Detection and enumeration of coliforms in drinking water: current methods and emerging approaches. J Microbiol Methods 49:31–54

Rotem S, Mor A (2009) Antimicrobial peptide mimics for improved therapeutic properties. Biochim Biophys Acta 1788:1582–1592

Rotem S, Radzishevsky IS, Bourdetsky D, Navon-Venezia S, Carmeli Y, Mor A (2008a) Analogous oligo-acyl-lysines with distinct antibacterial mechanisms. FASEB J 22:2652–2661

Rotem S, Radzishevsky IS, Bourdetsky D, Navon-Venezia S, Carmeli Y, Mor A (2008b) Analogous oligo-acyl-lysines with distinct antibacterial mechanisms. FASEB J 22:2652–2661

Rotem S, Raz N, Kashi Y, Mor A (2010) Bacterial capture by peptide-mimetic oligoacyllysine surfaces. Appl Environ Microbiol 76:3301–3307

Ruhr E, Sahl HG (1985) Mode of action of the peptide antibiotic nisin and influence on the membrane potential of whole cells and on cytoplasmic and artificial membrane vesicles. Antimicrob Agents Chemother 27:841–845

Sakoulas G, Okumura CY, Thienphrapa W, Olson J, Nonejuie P, Dam Q, Dhand A, Pogliano J, Yeaman MR, Hensler ME, Bayer AS, Nizet V (2014) Nafcillin enhances innate immune-mediated killing of methicillin-resistant Staphylococcus aureus. J Mol Med 92:139–149 (Berlin, Germany)

Sarig H, Rotem S, Ziserman L, Danino D, Mor A (2008) Impact of self-assembly properties on antibacterial activity of short acyl-lysine oligomers. Antimicrob Agents Chemother 52:4308–4314

Sarig H, Livne L, Held-Kuznetsov V, Zaknoon F, Ivankin A, Gidalevitz D, Mor A (2010) A miniature mimic of host defense peptides with systemic antibacterial efficacy. FASEB J 24:1904–1913

Sarig H, Ohana D, Epand R, Mor A, Epand R (2011) Functional studies of cochleate assemblies of an oligo-acyl-lysyl with lipid mixtures for combating bacterial multidrug resistance. FASEB J 25:3336–3343

Sass V, Schneider T, Wilmes M, Korner C, Tossi A, Novikova N, Shamova O, Sahl HG (2010) Human beta-defensin 3 inhibits cell wall biosynthesis in Staphylococci. Infect Immun 78:2793–2800

Sawyer JG, Martin NL, Hancock RE (1988) Interaction of macrophage cationic proteins with the outer membrane of Pseudomonas aeruginosa. Infect Immun 56:693–698

Schaberle TF, Hack IM (2014) Overcoming the current deadlock in antibiotic research. Trends Microbiol 22:165–167

Schuerholz T, Brandenburg K, Marx G (2012) Antimicrobial peptides and their potential application in inflammation and sepsis. Crit Care 16:207

Shprung T, Peleg A, Rosenfeld Y, Trieu-Cuot P, Shai Y (2012) Effect of PhoP-PhoQ activation by broad repertoire of antimicrobial peptides on bacterial resistance. J Biol Chem 287:4544–4551

Silver LL (2011) Challenges of antibacterial discovery. Clin Microbiol Rev 24:71–109

Silverman JA, Perlmutter NG, Shapiro HM (2003) Correlation of daptomycin bactericidal activity and membrane depolarization in Staphylococcus aureus. Antimicrob Agents Chemother 47:2538–2544

Strahl H, Hamoen W (2010) Membrane potential is important for bacterial cell division. Proc Natl Acad Sci USA 107:12281–12286

Sully EK, Malachowa N, Elmore BO, Alexander SM, Femling JK, Gray BM, DeLeo FR, Otto M, Cheung AL, Edwards BS, Sklar LA, Horswill AR, Hall PR, Gresham HD (2014) Selective chemical inhibition of agr quorum sensing in staphylococcus aureus promotes host defense with minimal impact on resistance, PLoS Pathogens 10

Vaara M, Fox J, Loidl G, Siikanen O, Apajalahti J, Hansen F, Frimodt-Møller N, Nagai J, Takano M, Vaara T (2008) Novel polymyxin derivatives carrying only three positive charges are effective antibacterial agents. Antimicrob Agents Chemother 52:3229–3236

Westerhoff HV, Juretic D, Hendler RW, Zasloff M (1989) Magainins and the disruption of membrane-linked free-energy transduction. Proc Natl Acad Sci USA 86:6597–6601

Wilke MS, Hills TL, Zhang HZ, Chambers HF, Strynadka NC (2004) Crystal structures of the Apo and penicillin-acylated forms of the BlaR1 beta-lactam sensor of Staphylococcus aureus. J Biol Chem 279:47278–47287

Yang SJ, Bayer AS, Mishra NN, Meehl M, Ledala N, Yeaman MR, Xiong YQ, Cheung AL (2012) The Staphylococcus aureus two-component regulatory system, GraRS, senses and confers resistance to selected cationic antimicrobial peptides. Infect Immun 80:74–81

Yeaman M, Yount N (2003) Mechanisms of antimicrobial peptide action and resistance. Pharmacol Rev 55:27–55

Zaknoon F, Wein S, Krugliak M, Meir O, Rotem S, Ginsburg H, Vial H, Mor A (2011) Antiplasmodial properties of acyl-lysyl oligomers in culture and animal models of malaria. Antimicrob Agents Chemother 55:3803–3811

Zasloff M (2002) Antimicrobial peptides of multicellular organisms. Nature 415:389–395

Zhang L, Dhillon P, Yan H, Farmer S, Hancock RE (2000) Interactions of bacterial cationic peptide antibiotics with outer and cytoplasmic membranes of Pseudomonas aeruginosa. Antimicrob Agents Chemother 44:3317–3321

Part IV
Recent Advances in Engineering Host Defense Peptides

Chapter 9
Structural Analysis of Amphibian, Insect, and Plant Host Defense Peptides Inspires the Design of Novel Therapeutic Molecules

Guangshun Wang

Abstract Host defense antimicrobial peptides (AMPs) are the key components of innate immune systems of both invertebrates and vertebrates. They play an important role in preventing microbial invasion and regulating immune response. This chapter intends to identify nature's peptide design strategies based on a structural analysis of select AMPs from amphibians, insects, and plants. The plant kingdom and amphibian/insect classes have 250–1000 peptide entries in the antimicrobial peptide database. Both insects and plants deploy AMPs with a variety of structural scaffolds (α-helix, β-sheet, αβ-structure, and non-αβ structure). In contrast, amphibians make numerous defense peptides (combinatory libraries) based almost solely on the classic amphipathic α-helix structure. Thus, these 3-D structures suggest two general strategies for peptide discovery: (1) screening a library of peptides with a fixed backbone structure and (2) rational design by selecting a structural scaffold with a known target (e.g., cell walls, membranes, ribosomes, or nucleic acids). It is demonstrated that peptide library screening can be combined with structure-based design to better achieve the molecular design goals.

9.1 Introduction

The discovery of cecropins in insects, magainins in frogs, and defensins from humans in the 1980s (Selsted et al. 1985; Steiner et al. 1981; Zasloff 1987) led to a rapid increase in the number of antimicrobial peptides (AMPs) identified from bacteria, archeae, protists, fungi, plants, and animals (Wang 2013; Wang et al. 2015). As of August 20, 2015, there are 2,600 AMPs in the antimicrobial peptide database (APD) (Wang 2015a; Wang and Wang 2004; Wang et al. 2009). These host defense peptides are key components of the innate immune systems of all life forms (i.e., innate immune peptides). AMPs are expressed in all the exposed tissues

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where pathogens may have a chance to invade. These small proteins play a big role in a variety of biological systems. Not only do they kill invading bacteria, viruses, fungi, parasites, but also participate in other biological processes, including antioxidant, protease inhibition, apoptosis, immune modulation, and wound healing (Zasloff 2002; Fjell et al. 2012; Boman 1995; Wang 2010; Tossi and Sandri 2002; Haney et al. 2009; Marsh and Goode 1994; Lai and Gallo 2009).

The aim of this chapter is to compare the structural scaffolds of AMPs from amphibians, insects, and plants so that useful strategies may be derived for peptide design. These kingdoms/groups were chosen because of a large number of peptides in each (253–994 entries in the APD database, Wang and Wang 2004; Wang et al. 2009). In addition, there is a food chain relationship between these organisms. While insects can feed on plants, frogs and toads can engulf insects. Of course, amphibians can also become the food of other organisms such as reptiles. Here AMPs might serve as poisons to retard the action of predators. Indeed, plant AMPs can be insecticidal. Multiple AMPs and other poisons on the skin of frogs can scare away predators.

This chapter starts with a systematic classification of 3-D structures of AMPs. Based on this classification, structural studies of natural AMPs from insects, amphibians, and plants are highlighted. Unlike insects and plants, amphibians use almost exclusively helical structure for defense. In an era of antibiotic resistance, elucidation of nature's peptide design strategies inspires the discovery of novel antimicrobials to combat antibiotic resistant bacteria (superbugs).

9.2 Structural Classification of Host Defense Peptides

9.2.1 The Dominant Role of NMR Spectroscopy

It is well appreciated that 3-D structure can provide in-depth insight into biological functions of polypeptides. X-ray diffraction and nuclear magnetic resonance (NMR) spectroscopy are two established methods for structural determination to atomic resolution. The X-ray diffraction method was established earlier than NMR. The crystal structure of myoglobin was solved in 1958 (Perutz 1962), while the first NMR structure for bull seminal proteinase inhibitor (BUSI) was not determined until 1985 (Wüthrich 2002). The structural data were initially scattered in the literature or in different laboratories. It was clear that a centralized platform could benefit development of the science itself and the public. The Protein Data Bank (PDB) is such are source that collects structures of biomolecules with an ultimate goal of construction of a structural view for life (Rose et al. 2015). While the journals require the deposition of data, not all structures are deposited. As of August 20, 2015, there are 99,264 structures determined by X-ray diffraction and 11,100 structures solved by NMR indicating the majority of the structures originated from the X-ray method.

The APD (Wang and Wang 2004; Wang et al. 2009) is a widely used resource that annotates biological source, activity, sequence, 3-D structure, and literature of

Table 9.1 3-D structural statistics in the PDB[a] and APD[b]

Database	PDB	APD
X-ray	99,264 (89.9 %)	43 (12.3 %)
NMR	11,100 (10.1 %)	307 (87.7 %)
Sum	110,364	350

[a]Data obtained from the PDB (http://www.rcsb.org/pdb/home/home.do); [b]Data obtained from the APD (http://aps.unmc.edu/AP). Accessed on August 20, 2015

AMPs. Both crystal and NMR structures are annotated including those not deposited into the PDB. When there are multiple structures solved for a single AMP under different conditions, the APD usually provides a link to the high resolution structure in the PDB. Of a total of 350 unique structures annotated in the APD, 307 are determined by NMR spectroscopy and 43 by X-ray diffraction (Table 9.1). Hence, NMR is the major player in structural studies of AMPs.

It can be helpful to make a comment on the major role of NMR. Most of the linear peptides do not have a defined structure in aqueous solution, making it difficult to grow crystals, a prerequisite for structural determination by X-ray crystallography. However, it was possible to crystallize some disulfide bond stabilized peptides such as defensins (Lehrer and Lu 2012) because they do have a folded structure in aqueous solution. Another factor is that the majority of AMPs are likely to target membranes and growing crystals for membrane-associated peptides is a challenging task (Wang 2008a; Brown and Ladizhansky 2015). All these explain the paucity of crystal structures of AMPs in the APD (Wang et al. 2009).

It was a perfect time for NMR spectroscopy to be developed into a tool for structural determination in the 1980s when the AMP field started to grow rapidly. Fortunately, there is no need to grow crystal for NMR studies. Direct measurements in solution made it convenient for NMR to follow the conformational change of a peptide with the solution conditions (Wang 2010, 2008b). In particular, membrane-mimetic models can be used to mimic bacterial membranes. In complex with membranes, many linear AMPs become structured, allowing for structural determination. For structural determination of AMPs by solution NMR spectroscopy, the commonly used membrane-mimicking models are organic solvents or detergent micelles. In addition, the lipid bilayer model is suitable for solid-state NMR studies (Wang 2008a; Brown and Ladizhansky 2015; Wüthrich 1986).

In the case of large complexes there is a clear advantage to utilize the X-ray diffraction technique. Recently, the structures of proline-rich peptides in complex with bacterial ribosomes have been determined (Seefeldt et al. 2015; Roy et al. 2015). When used simultaneously, the two techniques could provide complementary information. Crystal structure is known to provide higher resolution, whereas NMR can identify the crystal form more resembling that measured under physiological conditions. More importantly, NMR can shed light on polypeptide motions

on wide time scales, ranging from nanoseconds to days (Wang et al. 2005a). In addition, solid-state NMR studies can provide insight into peptide oligomerization, orientation and dynamics in the lipid bilayer model (Brown and Ladizhansky 2015; Strandberg and Ulrich 2015; Hong 2006). Collectively, these biophysical methods could uncover structure and dynamics of AMPs in complex with different molecular targets.

9.2.2 *Structural Classification of AMPs*

Boman classified AMPs into three classes: α-helical, β-sheet (usually disulfide bond connected), and rich in amino acids such as Pro, His, Arg, and Trp (Boman 1995). Others have added looped structures or circular peptides as a new class (Fjell et al. 2012; Vale et al. 2014). Based on whether there are α and β structures in the peptides, Wang classified the 3-D structures of natural AMPs into four major families: α, β, αβ, and non-αβ (Wang 2010). The α family consists of helical AMPs, while the β family contains a collection of AMPs with β-sheet structures. AMPs in the αβ family contain a mixture of α and β structures, which may, or may not, be packed into a single protein fold. Peptides in the non-αβ family do not have a well-defined α or β structure. Representative structures can be viewed in the APD face page (http://aps.unmc.edu/AP). Each structural family of peptides may be further classified based on the number of α-helices or β-strands (Wang 2015a; Fjell et al. 2012).

Table 9.2 provides the statistics for the known AMP structures from insects, amphibians, and plants annotated in the APD (Wang 2015a). There are 46 AMP structures from amphibians, 34 from insects, and 40 from plants. It is evident that different structural scaffolds have been found for insect and plant AMPs. However, the known 3-D structures from amphibian AMPs are entirely helical (Table 9.2). In the following three sections I highlight representative structures from these organisms.

Table 9.2 Structural classes of amphibian, insect, and plant antimicrobial peptides[a]

Source	Total peptides	NMR structures	α-helix	β-sheet	αβ-structure	Non-αβ structure
Amphibians	994	46	46	0	0	0
Insects	252	34	19	2	11	2
Plants	320	40	2	10	25	0

[a]Data obtained from the APD (http://aps.unmc.edu/AP) on August 20, 2015 (Wang and Wang 2004; Wang et al. 2009) based on the Wang classification (Wang 2010)

9.3 Structures of Amphibian Antimicrobial Peptides

Amphibians are famous for abundance in biologically active peptides (Zasloff 2002; Marsh and Goode 1994; Xu and Lai 2015; Mangoni 2006; Bowie et al. 2012; Conlon et al. 2004). Currently, the APD collected 994 amphibian AMPs, accounting for 38.2 % of the 2,600 entries (Wang et al. 2009). The major families are magainins, brevinins, esculentins, temporins, ranalexins, ranatuerins, palustrins, tigerinins, japonicins, and nigrocins (Conlon et al. 2004). These AMPs can have different biological activities. In the APD, 889 are annotated to be antibacterial, 38 antiviral, 397 antifungal, 29 antiparasitic, 15 antioxidant, and 66 anticancer. However, only 46 of the amphibian AMPs have known 3-D structures (Table 9.2). The sequence and activity of representative amphibian AMPs with known 3-D structure are given in Table 9.3. It is clear that amphibian AMPs varying activity spectrum. In addition, amphibian AMPs, such as magainin 2 and PGLa, can also work together to achieve a synergistic effect (Marsh and Goode 1994).

Magainins (Table 9.3) were the first AMPs identified from the African clawed frogs *Xenopuslaevis* (Zasloff 1987). They have demonstrated to have antibacterial, antiviral, antifungal, and antiparasitic activities (Zasloff 2002). Using solution NMR methods, Opella and colleagues found a similar helical structure for magainin 2 in several membrane-mimetic models, including trifluoroethanol (TFE), deuterated sodium dodecylsulfate (SDS), and dodecylphosphocholine (DPC) (Gesell et al. 1997). Therefore, the structure of this peptide does not appear to be influenced by such environmental conditions. The orientation of the peptide in a lipid bilayer model was also determined by solid-state NMR. The amphipathic helix was found

Table 9.3 Structure and activity of representative AMPs from amphibians

APD ID	Name	Peptide sequence	Structure[a]	Activity[b]	References
144	Magainin 2	GIGKFLHSAKKFGK AFVGEIMNS	α	G, F, C	Zasloff (1987), Gesell et al. (1997)
13	Aurein 1.2	GLFDIIKKIAESF-amide	α	G, V, F, C	Rozek et al. (2000), Wang et al. (2005b)
1534	Temporin-SHf	FFFLSRIF-amide	α	G, F	Abbassi et al. (2010)
1656	Esculentin-1c	GIFSKLAGKKIKNLISGLKNIGK EVGMDVVRTGIDIAGCKIKGEC	α	G	Kang et al. (2010)
493	Distinctin	Chain 1: **ENRE**VPPGFTALIKTL RKCII; Chain 2: NLVSGLIEARKYL EQLHRKLKNCKV	α	G	Dalla Serra et al. (2008)
764	Dermaseptin S9	GLRSKIWLWVLLMIW QESNKFKKM	α	G	Lequin et al. (2006)
308	Buforin II	TRSSRAGLQFPVGRVHRLLRK	α	G, F, C	Yi et al. (1996)

[a]Except for distinctin, the structures were determined in membrane-mimetic environments such as TFE, SDS, and DPC. [b]Antimicrobial activity as annotated in the APD: G, bacteria; F, fungi; V, viruses; C, cancer cells; and I, insects (Wang and Wang 2004; Wang et al. 2009)

to be located on the surface of the membrane (Bechinger et al. 1991). However, it was also proposed that this peptide could create a pore in membranes based on other biophysical studies (Tamba et al. 2010). Such a conflict may reflect the difference in the model membrane environments or experimental conditions. Thus, it is not always trivial to link structure to mechanism of action (Haney et al. 2009).

Aureins were isolated from Australian frogs (Bowie et al. 2012). Among them, aurein 1.2 (Table 9.3) is one of the shortest antibacterial and anticancer peptides (Rozek et al. 2000). It showed a good activity against *Leuconostoclactis* and *Listeria innocua* with a minimal inhibitory concentration (MIC) at 6 μg/ml and moderate to weak activities against *Bacillus cereus, Staphylococcus epidermidis, Streptococcus uberis*, and *Micrococcus luteus* (50–100 μg/ml). The 3-D structure of this peptide was determined in TFE (Rozek et al. 2000). A high quality structure was also solved in SDS micelles (Wang et al. 2005b). The micelle-bound structure (Fig. 9.1a) was refined using dihedral angles derived from ^{13}C and ^{15}N chemical shifts measured at natural abundance (Wang et al. 2005b; Li et al. 2006a). In this high quality structure, the C-terminal aromatic ring of F13 bends toward the hydrophobic surface as a result of direct NOE cross peaks between the side chains of F13 and I9 (Fig. 9.1a). Furthermore, NMR studies of aurein 1.2 in the dioctanoyl phosphatidylglycerol (D8PG) model provide novel insights into peptide-PG interactions. There are direct contacts between the aromatic rings (both F3 and F13) and the anionic lipid (Wang et al. 2005b). Solid-state NMR studies revealed a preferred binding to anionic membranes and a 50° tilt of the helix axis relative to the membrane surface (Marcotte et al. 2003). At elevated concentrations, aurein 1.2 generated an isotropic ^{31}P NMR peak in a lipid system mimicking the *E. coli* membranes (Wang et al. 2005b). An isotropic membrane phase involving aurein 1.2 was also suggested by the solid-state ^{15}N NMR study (Boland and Separovic 2006). These results suggest that aurein 1.2 works like a detergent and can disrupt bacterial membranes by the carpet mechanism (Fernandez et al. 2012).

Temporins are a family of amphibian AMPs (Mangoni 2006) with very short peptide sequences (8–17 amino acid residues for 110 temporins in the APD Wang 2015a). A unique feature of these peptides is that they usually have only few basic residues. Temporin-SHf, with eight residues (Table 9.3), is the shortest temporin from the African frog *Pelophylaxsaharica* (Abbassi et al. 2010). It is active against B. *megaterium* (MIC 3 μM), *S. aureus* ATCC 25,923 (12.5 μM), *E. faecalis* ATCC 29,212 (50 μM), *E. coli* ML-35p (30 μM), and *E. coli* ATCC 25,922 (25 μM), and *E. coli* D21 (100 μM). Residues 3–8 adopted a non-amphipathic helical structure in either SDS or DPC micelles. Of the eight residues, 50 % are aromatic Phe. Some of these Phe aromatic rings may interdigitate into the membrane in a manner similar to those in aurein 1.2 (F3 and F13 in Fig. 9.1a).

Many amphibian AMPs in the APD (34 %) are known to be amidated at the C-terminus. This amidation can be essential for peptide activity and helicity (Dennison et al. 2012). Approximately another one third of amphibian AMPs potentially forms a “Rana box” usually in the C-terminal region. A Rana box is characterized by a set of residues sandwiched between a pair of Cys residues that form a disulfide bond (Clark et al. 1994). It appears that such a local structure

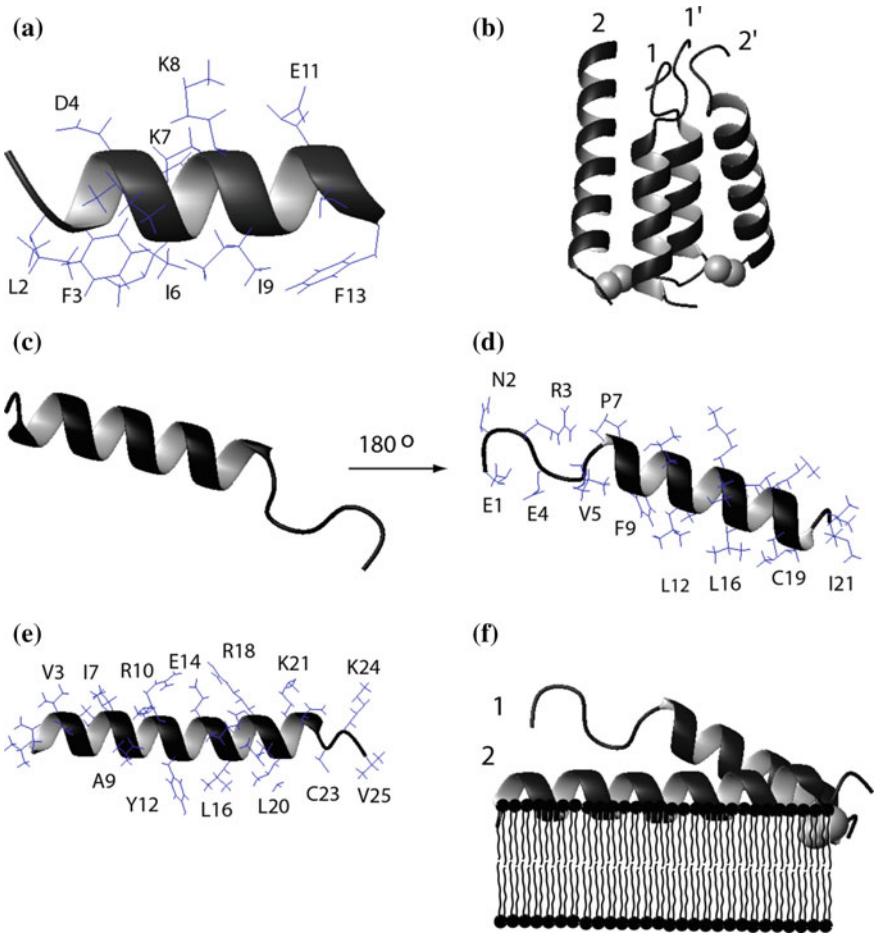

Fig. 9.1 Structures of amphibian antimicrobial peptides. **a** Aurein 1.2 (PDB entry: 1VM5) (Wang et al. 2005b), **b** distinctin (PDB entry: 1XKM) (Dalla Serra et al. 2008), **c** the orientation of chain 1 of distinctin in lipid bilayers (Resende et al. 2009), **d** an alternative orientation of chain 1 of distinctin in lipid bilayers obtained by a 180° rotation of the model in panel C as indicated, **e** the orientation of chain 2 of distinctin in lipid bilayers determined by solid-state NMR, and **f** a proposed membrane-binding model for distinctin in a lipid bilayer. A similar bilayer, although not displayed, is assumed to be underneath the structures in panels C, D, and E. Chains 1 and 2 of distinctin (sequences in Table 9.3) are labeled in panels B and F. Disulfide bonds are represented with a pair of balls and are recently proposed to be important for membrane binding (Wang and Wang 2015) as shown in panel F

provides an alternative way to stabilize the C-terminal end of the peptide. In addition, the hydrophobic nature of such a disulfide bond should not be ignored (Wang and Wang 2015). For nigrocin-2, the helical conformation (residues 3–18) was found to extend into the Rana box region (between Cys15 and Cys21), allowing the C-terminal Cys21 to bend over to form a disulfide bond with Cys15

(Park et al. 2001). In the case of gaegurin-4, the Rana box (C31-C37) appeared to extend beyond the helical regions of the peptide (Park et al. 2000a). These authors found two helical regions (residues 2–10 and 16–32) for gaegurin-4 in TFE. A subsequent NMR study using ^{15}N-labeled peptide revealed the same structure in SDS micelles (Park et al. 2007). Structurally, the middle region of the peptide was proposed to be "flexible". However, such a motion picture is not consistent with heteronuclear ^{15}NNOE measurements, which indicate a rigid conformation spanning the entire peptide backbone. Interestingly, another study suggests two helical regions covering residues 2–23 and 25–34, which better agree with the heteronuclear NOE values above (Chi et al. 2007). Due to the spectral resolution issue, NMR studies of longer amphibian peptides were carried out either in organic solvents by 2-D homonuclear NMR (Kang et al. 2010) or in micelles by heteronuclear 3-D NMR methods at elevated temperatures e.g., human cathelicidin LL-37 (Wang 2008b). It should be pointed out that some amphibian AMPs did show subtle conformational differences in different models. In the case of maximin-4, a major difference was observed in the break region, although the overall helix-break-helix structure is similar in SDS micelles or in 50 % methanol (Toke et al. 2011). Clearly, it is important to determine the structure of an AMP in a relevant model. In this regard, ^{19}F, which is usually not in natural products, provides a unique probe for studying the structure and dynamics of AMPs in *native* membranes by NMR (Koch et al. 2012).

Distinctin represents an interesting and unique case where two peptide chains are linked via one disulfide bond (Table 9.3). Unlike the majority of amphibian AMPs, this peptide spontaneously dimerizes and adopts a helix bundle structure in water (Fig. 9.1b). Interestingly, such a structure is critical for peptide stability to proteases but not antimicrobial activity (Dalla Serra et al. 2008). However, the helix bundle structure did not persist in the presence of membranes (Resende et al. 2009). A recent solid-state NMR study determined the orientation of both chains of distinctin (Verardi et al. 2011). Consistent with a previous solid-state NMR study (Resende et al. 2009), the short helix in chain 1 was more tilted at an angle of 24° (Fig. 9.1c), the longer helix (chain 2) is nearly parallel to the membrane surface (Fig. 9.1e). Chain 1 was proposed to insert into the membrane with the charged and disordered region in the membrane (Fig. 9.1c) (Verardi et al. 2011). This is unlikely because burying the N-terminal charged moiety of the peptide (bold in Table 9.3 and Fig. 9.1) into the membrane is energetically unfavorable. Therefore, I propose an alternative model for chain 1, where chain 1 is inverted (i.e., 180°). In this model (Fig. 9.1d), the hydrophobic face retains its membrane-bound state, while the disordered region is now exposed to the aqueous phase. Such a chain 1 orientation is consistent with angle dependent solid-state NMR measurements, which have multiple solutions owing to the $\cos^2\theta-1$ relationship. Remarkably, half of the structure determined in the solution state (Dalla Serra et al. 2008) is fully consistent with the solid-state NMR measurements, where chain 1 is more tilted if chain 2 sits on the membrane surface (Fig. 9.1f). The alternative membrane-binding model proposed here for distinctin enables both helices to attach to the lipid bilayer through their hydrophobic surfaces (see Fig. 9.1d, e). This allows the helix bundle

structure (Fig. 9.1b) to directly open into two on bacterial membrane surfaces (Fig. 9.1f). Thus, distinctin provides an excellent example that illustrates the complementary nature of solution and solid-state NMR methods. The solid-state NMR studies (Resende et al. 2009; Verardi et al. 2011) shed light on how the helix bundle structure might open and bind to membranes (Fig. 9.1).

Not all amphibian peptides are located on the membrane surface, however. Dermerceptin S9, which was identified from the South African hylid frog *Phyllomedusa sauvagei*, is such an example that can form a transmembrane helix (Lequin et al. 2006). This was made possible by a string of hydrophobic residues in the middle of the peptide sequence (Table 9.3). Such a sequence differs from those that form an amphipathic helix structure.

Beyond membranes, can an amphibian AMP cross cellular membranes and work on an intracellular target? The answer is yes. Buforin II is a well characterized example for this. This peptide actually binds to DNA or RNA (Cho and Kim 2010). Its 3-D structure was determined in TFE (Yi et al. 1996) rather than in complex with DNA. The structure of buforin II helped identify the importance of the amino acid Pro in membrane penetration and cellular entrance (Table 9.3). This structure inspires the design of cell-penetrating peptides.

In summary, the helical structure is universal in amphibian AMPs. Interestingly, these helical AMPs can kill pathogens by different mechanisms. Many target bacterial membranes. Some peptides are proposed to form a pore in bacterial membranes (Tamba et al. 2010), whereas other AMPs, such as aurein 1.2 and temporin-SHf, are too short (8–13 residues) to form a transmembrane pore by themselves. Therefore, such short AMPs may disrupt bacterial membranes like a detergent to disintegrate bacterial membranes (Boland and Separovic 2006). Remarkably, some AMPs such as buforin II could, however, cross the membrane and associate with internal targets such as nucleic acids. These results imply that *one can design AMPs with different activities and even mechanisms of action by altering amino acids on the same helical backbone*.

9.4 Structures of Insect Antimicrobial Peptides

Insects are invertebrates that do not have adaptive immune systems. The discovery of AMPs in insects is a milestone, establishing their fundamental role in innate immunity for survival of these organisms during microbial infections (Lemaitre and Hoffmann 2007). Cecropins were the first AMPs identified from insects in 1980 (Steiner et al. 1981). Currently, there are 253 insect AMPs in the APD (Wang et al. 2009). Among them, 227 have known antibacterial activities. Insect AMPs can also have antiviral (9 entries in the APD), antifungal (91 peptides), and antiparasitic (9 entries) activities. As summarized in Table 9.2, insect AMPs could adopt various structural scaffolds. Of the 34 known structures, 19 insect AMPs are α-helical and two form β-sheet structures. Another 11 peptides contain both α and β structure (i.e., αβ structure). Finally, there are two insect AMPs with a non-αβ structure.

9.4.1 The α-Helical Family

The structure of cecropin A was first studied by 2-D NMR in 1988 (Holak et al. 1988). In 15 % 1, 1, 1, 3, 3, 3-hexafluoro-2-isopropanol (HFIP), two helical regions (α1: residues 15–21 and α2: residues 24–37) were found. The angle between the two helices could not be determined because of the paucity in NOE restraints for that region. Likewise, sarcotoxin IA has a helix-hinge-helix structure (α1: 3–23 and α2: 28–38) (Iwai et al. 1993). Helical structures have also been found for other insect AMPs, including *Spodopteralitura* (Sl) moricin (Oizumi et al. 2005), mastoparan B (Yu et al. 2000), spinigerin (Landon et al. 2006), stomoxyn (Landon et al. 2006), and lassiglossins (Cerovský et al. 2009). Most of these NMR studies were conducted using organic solvents as a membrane-mimetic model. The helix-promoting ability of these solvent systems is in the following order: acetonitrile < H_2O < methanol < HFIP. This trend explains why HFIP was chosen in some cases as a helix-promoting agent. For spinigerin (25 residues), CD studies suggest a similar helical structure in SDS, DPC, and TFE. In SDS micelles, the helical region of spinigerin was mapped to residues 4–23 by NMR (Lee et al. 2003).

9.4.2 The β-Sheet Family

The structures of thanatin and alo3 belong to the β-sheet family. Thanatin ("thanato" means death) is an inducible insect AMP with a broad activity spectrum against both bacteria and fungi (Table 9.4). Structurally, thanatin forms a hairpin structure with an antiparallel β-sheet from residue 8 to the C-terminus (Fig. 9.2a). The two β-strands are located between residues 8–12 and 17–21, while a short helical turn spans residues 13–15 (Mandard et al. 1998). The only disulfide (S–S) bond between Cys11 and Cys18 is indispensable for antimicrobial activity (Imamura et al. 2008). This S–S bond is also important for salt tolerance and pore formation in membranes. However, C-terminal amidation had no effect on the activity of thanatin. An alternative form of thanatin synthesized in D-amino acids retained activity against Gram-positive bacteria and fungi, but not Gram-negative *E. coli*. Among the three alo peptides identified from the insect *Acrocinus longimanus*, alo-3 is most potent against fungi. It contains three pairs of S–S bonds (see Fig. 9.2b) and a triple-stranded antiparallel β-sheet (β1: residues 7–9; β2: 22–23; and β3: 31–33) (Barbault et al. 2003). Alo-3 has a unique fold belonging to the cystine knot family.

9.4.3 The αβ Structural Family

Drosomycin is the first inducible antifungal peptide from insects (Fehlbaum et al. 1994). The inducing agent can be microbes or injury. The expression of this AMP is

Table 9.4 Structure and activity of select insect antimicrobial peptides

APD ID	Name	Peptide sequence	Structure[a]	Activity[b]	References
139	Cecropin A	KWKLFKKIEKVGQNIRDGIIK AGPAVAVVGQATQIAK	α	G, V	Holak et al. (1988)
230	Sarcotoxin IA	GWLKKIGKKIERVGQHTRDA TIQGLGIAQQAANVAATAR	α	G	Iwai et al. (1993)
554	SlMoricin	GKIPVKAIKKAGAAIGKGL RAINIASTAHDVYSFFKPKHKKK	α	G	Oizumi et al. (2005)
200	Mastoparan B	LKLKSIVSWAKKVL	α	G+, C	Yu et al. (2000)
399	Spinigerin	HVDKKVADKVLLLKQLRIMRLL TRL	α	G, V, F	Landon et al. (2006), Lee et al. (2003)
484	Stomoxyn	RGFRKHFNKLVKKVKHTISETAHV AKDTAVIAGSGAAVVAAT	α	G, F	Landon et al. (2006)
1465	Lasioglossin LL-I	VNWKKVLGKIIKVAK	α	G	Lee et al. (2003)
102	Thanatin	GSKKPVPIIYCNRRTGKCQRM	β	G, F	Mandard et al. (1998)
813	Alo-3	CIKNGNGCQPNGSQNGCCSGY CHKQPGWVAGYCRRK	β	F	Barbault et al. (2003)
672	Drosomycin	DCLSGRYKGPCAVWDNETCRR VCKEEGRSSGHCSPSLKCWCEGC	αβ	F	Landon et al. (1878)
31	Heliomicin	DKLIGSCVWGAVNYTSDCNGECK RRGYKGGHCGSFANVNCWCET	αβ	F	Lamberty et al. (2001)
216	Phormiadefensin A	ATCDLLSGTGINHSACAAHCLL RGNRGGYCNGKGVCVCRN	αβ	G+	Cornet et al. (1995)
1166	Diapause-specific peptide (DSP)	AVRIGPCDQVCPRIVPERHECC RAHGRSGYAYCSGGGMYCN	αβ	F	Kouno et al. (2007)
172	Drosocin	GKPRPYSPRPTSHPRPIRV	Non-αβ	G	McManus et al. (1999a)
170	Pyrrhocoricin	VDKGSYLPRPTPPRPIYNRN	Non-αβ	G	Rosengren et al. (2004)

[a, b]Same as in Table 9.3

mediated through the Toll pathway (Lemaitre et al. 1996). The 3-D structure of drosomycin was solved by NMR (Landon et al. 1997). It consists of one α-helix (residues 30–34) and three β-strands (β1: residues 2–3; β2: 30–34; and β3: 38–42) stabilized by four S–S bonds (see Fig. 9.2c), leading to a cysteine-stabilized alpha beta (CSαβ) motif. The CSαβ motif is also observed in heliomicin (Lamberty et al. 2001) and phormia defensin A (Cornet et al. 1995). Similar to drosomycin, heliomicin is active against a large number of fungi. Importantly, heliomicin remained active in the presence of >100 mM NaCl. Although ARD1 (Landon et al. 2004) differs from heliomicin (Lamberty et al. 2001) by only two residues (N17D and A20G), it is more active against the human pathogens *Aspergillus fumigatus* and *Candida albicans*. Based on sequence alignment with defensins from the insect family, residues K23 and R24 at the end of the helix of heliomicin were mutated to leucines, leading to a gain in antibacterial activity due to the generation of an amphipathic helix. The gain in activity is not due to a conformational change. Like some plant defensins, heliomicin also binds specifically to glucosylceramides in fungal membranes (Vriens et al. 2014; Thevissen et al. 2004).

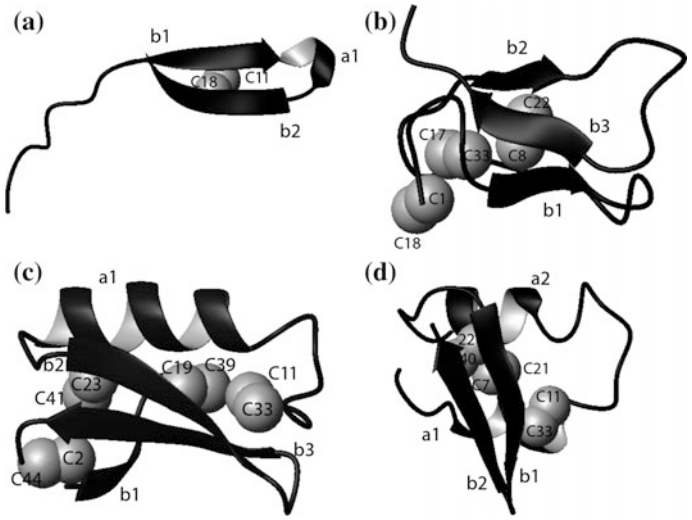

Fig. 9.2 Structures of insect antimicrobial peptides. **a** thanatin (PDB entry: 8TFV) (Mandard et al. 1998), **b** alo-3 (PDB entry: 1Q3 J) (Barbault et al. 2003), **c** drosomycin (PDB entry: 1MYN) (Landon et al. 1878), and **d** DSP (PDB entry: 2E2F) (Kouno et al. 2007). Secondary structures (α and β) are labeled and disulfide bonds are represented with a pair of balls to emphasize the hydrophobic nature of the bond

In addition to antifungal activity, a 41-residue dispause-specific peptide (DSP) from the leaf beetle can block the Ca^{2+} channel. The structure of DSP is composed of two α-helices (α1: 5–9 and α2: 19–24) and two β-strands (β1: 30–34 and β2: 37–40). The arrangement of the three S–S bonds in DSP (Fig. 9.2d) differs from those in other antifungal peptides and conotoxins. However, they have *similar surface residues for channel inhibition*. Except for one additional helix following β1, the overall structure of DSP is very similar to those of the insect- and plant-derived antifungal peptides. In particular, *a similar hydrophobic patch bordered by a basic residue* is required for antifungal activity. Therefore, the dual activity of DSP is determined by the 3-D structure (Kouno et al. 2007). This finding has an important implication in peptide design.

9.4.4 The Non-αβ Structural Family

Some insect AMPs are rich in prolines. For example, drosocin (Table 9.4) is a 19-residue Pro-rich peptide that can be glycosylated at T11. This glycosylation greatly reduced peptide activity. NMR studies found that drosocin was largely random coiled in water, but adopted some turn structures near residues 4–7, 10–13, 17, and 18 in the presence of 50 % TFE. However, glycosylation only had a subtle effect on the overall conformation of drosocin and the pKa of His13 (McManus et al.

1999a). Although the structural basis is not yet clear, glycosylation could reduce the interaction of the peptide with bacteria. Also, the linear and cyclic forms of pyrrhocoricin (Table 9.4) were investigated. A similar turn structure was found for both forms of peptides (Rosengren et al. 2004). Different from traditional membrane-targeting AMPs, these Pro-rich peptides can enter cells and bind to heat shock proteins (reviewed in Cho and Kim 2010). The 3-D structure between a portion of heat shock protein DnaKand a Pro-rich peptide oncocin was determined by X-ray crystallography (Knappe et al. 2011). However, recent studies suggest that these peptides inhibit protein translation by binding to ribosomes (Krizsan et al. 2014; Mardirossian et al. 2014). Based on the structure of the peptide in complex with ribosomes (Seefeldt et al. 2015; Roy et al. 2015), one may design better peptide therapeutics.

In summary, insect AMPs are capable of adopting a plethora of structural scaffolds (Fig. 9.2). While the helical structure prefers bacterial membranes, defensins can recognize specific lipids in fungal membranes or even block ion channels (Vriens et al. 2014). As discussed recently (Wang et al. 2015), proline-rich peptides, previously found to bind to heat shock proteins (Cho and Kim 2010), actually associate with ribosomes to shut down the bacterial machinery (Krizsan et al. 2014; Mardirossian et al. 2014). Chemical modifications such as glycosylation can also modulate peptide activity. It appears that the structural diversity of insect AMPs provides a basis for functioning by different mechanisms. This observation implies that our design can start from a known peptide scaffold with desired activity.

9.5 Structures of Plant Antimicrobial Peptides

Plant AMPs are expressed in roots, flowers, leaves, and stems. There are 320 plant AMPs in the APD (Wang et al. 2009). Among them, 135 peptides are known to inhibit fungi and 92 have an effect on bacteria. 30 plant AMPs are active against viruses, while 11 peptides are toxic against parasites. In addition, at least seven plant AMPs showed insecticidal effects. Importantly, some of these peptides are not only active against phytopathogens but also human pathogens. Such an activity spectrum indicates the important role plant AMPs play in innate immunity. Plant AMPs may find potential applications in agriculture for pest control and human health as therapeutic agents (Vriens et al. 2014; Kaas et al. 2010; Nawrot et al. 2014; Wang 2015b). Here I highlight representative plant AMPs from each structural class (Table 9.5).

9.5.1 The α-Helical Family

In contrast to amphibians, helical AMPs are rarely found in plants. However, a few interesting members appeared. Maize Basic Peptide 1 (MBP-1) is the first such

Table 9.5 Select plant AMPs with known 3-D structures or unique sequences

APD ID	Name	Sequence	Structure[a]	Activity[b]	References
1760	EcAMP1	GSGRGSCRSQCMRRHED EPWRVQECVSQCRRRRGGGD	α	F	Nolde et al. (2011)
2377	Tk-AMP-X2	ADDRCERMCQRYHDRREKK QCMKGCRYG	α	F	Berkut et al. (2014)
913	Ib-AMP1	QWGRRCCGWGPGRRYCVRWC	β (2S-S)	G, F	Tailor et al. (1997)
729	Kalata B1	GLPVCGETCVGGTCNTPGCTC SWPVCTRN	β (3S-S)	G+, V, I	Tam et al. (1999)
479	PAFP-S	AGCIKNGGRCNASAGPPYCCSS YCFQIAGQSYGVCKNR	β (3S-S)	F	Marcus et al. (1997)
428	MiAMP1	SAFTVWSGPGCNNRAERYSKCG CSAIHQKGGYDFSYTGQTAALYNQ AGCSGVAHTRFGSSARACNPFG WKSIFIQC	β (3S-S)	G+, F	Shao et al. (1999)
2041	β-Purothionin	KSCCKSTLGRNCYNLCRARGAQ KLCANVCRCKLTSGLSCPKDFPK	αβ (3S-S)	G, F	Hernandez-Lucas et al. (1974)
979	NaD1	RECKTESNTFPGICITKPPCRKA CISEKFTDGHCSKILRRCLCTKPC	αβ (4S-S)	F, I	Lay et al. (2003)
483	PsD1	KTCEHLADTYRGVCFTNASCDD HCKNKAHLISGTCHNWKCFCTQNC	αβ (4S-S)	F	Almeida et al. (2002)
981	PhD1	ATCKAECPTWDSVCINKKPCVAC CKKAKFSDGHCSKILRRCLCTKEC	αβ (5S-S)	F	Janssen et al. (2003)
511	Shepherin I	GYGGHGGHGGHGGHGGHGGH GHGGGGHG	Non-αβ (Gly-rich)	G–, F	Park et al. (2000b)
512	Shepherin II	GYHGGHGGHGGGYNGGGGH GGHGGGY NGGGHHGGGGHG	Non-αβ (Gly-rich)	G–, V, F	Park et al. (2000b)

[a]Structural classification based on the presence or absence of α-helices and β-sheet (Wang 2010). [b]G, antibacterial; G+, active against Gram-positive bacteria; G-, active against Gram-negative bacteria; F, antifungal; I, insecticidal; and V, virucidal. Data taken from the APD (Wang et al. 2009)

AMP, which is active against both bacteria and fungi. The basic nature of the peptide results primarily from the abundance of arginines (33.3 %) in the sequence. MBP-1 in phosphate buffer is helical based on CD spectra. Synthetic peptide, after oxidation, has a similar activity to the native peptide (Duvick et al. 1992). Sequence analysis in the APD revealed 59.4 % similarity to EcAMP1, another arginine-rich peptide recently found in plants (Nolde et al. 2011). NMR studies found a unique helical hairpin structure stabilized by two disulfide bonds (C7-C29 and C11-C25) (Fig. 9.3a). EcAMP1 is able to inhibit the growth of several plant fungal pathogens, including *F. graminearum* (IC_{50} 4.5 μM), *F. oxysporum* (8.5 μM), and *B. sorokiniana* (18.2 μM). EcAMP1 binds to fungal cell surface followed by internalization without membrane disruption. Tk-AMP-X2, a third member of the α-helical hairpin structure, was reported in 2013 (Berkut et al. 2014). This helical hairpin structure is an attractive template for designing peptide drugs. Based on structural similarity between Tk-AMP-X2 and scorpion potassium channel blockers, amino acid mutations conferred Kv1.3 channel blocking property to a Tk-AMP-X2 variant. Again, we saw peptide examples that could share the same structural scaffold but not identical activity.

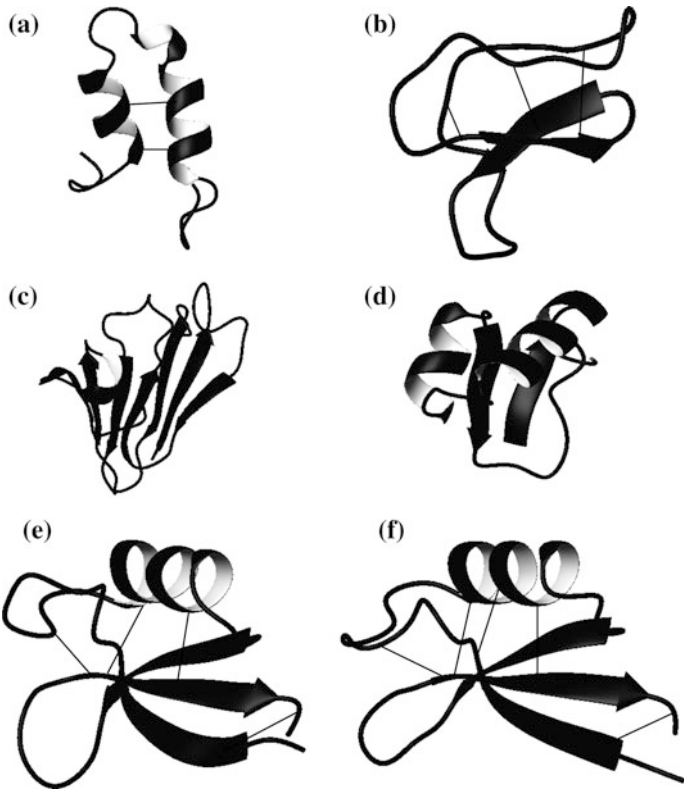

Fig. 9.3 Structures of plantantimicrobial peptides. **a** EcAMP1 (PDB entry: 2L2R) (Nolde et al. 2011), **b** kalata B1 (PDB entry: 4TTM) (Wang et al. 2014), **c** MiAMP1 (PDB entry: 1C01) (McManus et al. 1999), **d** β-purothionin (PDB entry: 1BHP) (Stec et al. 1995), **e** NaD1 (PDB entry: 1MR4) (Lay et al. 2012), and **f** PhD1 (PDB entry: 1N4 N) (Janssen et al. 2003). For clarity, disulfide bonds are only displayed in panels A, B, E, and F

9.5.2 The β-Sheet Family

In the APD, 10 peptides are annotated to have a β-sheet structure determined by NMR. I. balsamina antimicrobial peptides (Ib-AMPs) contains two disulfide bonds (C6-C16 and C7-C20) (Tailor et al. 1997). The NMR study of Ib-AMP1 was reported (Patel et al. 1998). This low resolution structure displays some feature resembling gomesin with a β-hairpin structure (Silva et al. 2000).

Circular AMPs have been found from bacteria, plants, and animals (Wang 2012). A large number of circular peptides called cyclotides exists in plants. Approximately 50 % of plant AMPs collected in the APD (Wang et al. 2009) are cyclotides. Not all of them are well characterized in terms of structure and activity. However, kalata B1 has been widely studied as a model peptide. It was initially isolated by Gran in 1973 as a uterotonic compound (Gran 1973). Antimicrobial

activity was demonstrated in 1999 (Tam et al. 1999). The peptide was most active against *S. aureus* (0.26 μM) and antibacterial activity was salt dependent. The circular structure of kalata B1 was established in 1995 (Saether et al. 1995). This results from a peptide bond formation between the N and C-termini of the peptide. The three pairs of disulfide bonds define the folding of the polypeptide chain in space. This overall fold is important for anti-HIV activity, since the acyclic permutant is inactive. Kalata B1 also has insecticidal activity (natural pesticide). A crystal structure was also solved using the racemic crystallization method (Fig. 9.3b) (Wang et al. 2014). NMR studies of the ternary complex identified amino acid residues in loop 5 (W23, P24, and V25) and loop 6 (L2, P3, and V4) are important for membrane binding (Shenkarev et al. 2006). It binds to phosphatidylethanolamine (PE) and leads to pore formation (41–70 Å) in lipid bilayers (Huang et al. 2009). Oligomerization on the membrane surface could be important for this process. Because of the stability of the cyclotides, there is great interest to make use of this scaffold. For example, efforts have been made to graft a peptide segment to the loop 5 or 6 region to confer desired functions (Northfield et al. 2014; Aboye et al. 2012).

There are also other types of β-sheet folds stabilized by three disulfide bonds. MiAMP1 is the first AMP isolated from *M. integrifolia* (Marcus et al. 1997). It showed a good antifungal activity against A. *heliarzthi*, C. *gloeosporioides*, F. *oxysporum*, L. *maculans*, P. *grunzinicolu, S. sclerotiorum, V. dahlia, and S. cerevisiae*. This small protein contains three disulfide bonds: C1-C64, C21-C76, and C23-C49 and adopts a unique Greek keyβ-barrel fold (Fig. 9.3c) (McManus et al. 1999). This two-layer β-sheet fold was proposed to be important for protein stability but not activity (Stephens et al. 2005). Another antifungal peptide, PAFP-S isolated from the seeds of *Phytolacca Americana,* also contains three disulfide bonds: C3-C20, C10-C24, and C19-C35 (Shao et al. 1999). The global fold involves a cystine knotted three-stranded antiparallel β-sheet (residues 8–10, 23–27, 32–36), a flexible loop (residues 14–19), and four β-reverse turns (residues 4–8, 11–14, 19–22, 28–32). This structure features all the characteristics of the knottin fold. A hydrophobic surface, comprising Y23, F25, I27, Y32, and V34, is bordered by basic R9, K36, and R38. Such an amphipathic surface may be important for antimicrobial activity (Gao et al. 2001).

9.5.3 The Mixed αβ Structural Family

There are 33 plant AMP structures in the APD that contain both α and β structures (Wang et al. 2009). These αβ structures can be further classified based on the number of disulfide bonds. Seventeen such peptides possess three disulfide bonds, while another 13 are stabilized by four disulfide bonds. The rest three peptides are stabilized by five disulfide bonds. Selected structures are described below.

Beta-purothionin is an early and representative member of plant thionins with three disulfide bonds. Antimicrobial activity was noticed long time ago based on a

mixture of α and β-thionin proteins. In 1974, antimicrobial activities against plant pathogens for the purified component proteins were observed (Hernandez-Lucas et al. 1974). The primary and tertiary structures of this small protein were determined in 1976 and 1995, respectively (Mak and Jones 1976; Stec et al. 1995). In the 3-D structure of β-purothionin, there are two helices and two β-strands (Fig. 9.3d). This fold is shared by viscotoxin A3 (Romagnoli et al. 2000) and some other thionins (Kaas et al. 2010). It appears that the differences in the potential surface determine different activities of these AMPs with the same fold.

NaD1 is a *Nicotianaalata* defensin stabilized by four disulfide bonds. It showed activity against *F. oxysporum, B. cinerea, A. niger*, Cryptococcus species, as well as the yeasts *S. cerevisiae* and *C. albicans* (Lay et al. 2003). In addition, it displayed insecticidal activity. Like RsAMP2 (which recognizes fungal glucosylceramides and induces apoptosis in *Candida albicans*) (Thevissen et al. 2012), NaD1 is unable to disrupt lipid vesicles. The antifungal activity involves specific interactions with the fungal cell wall (surface protein) followed by membrane permeation and entry of the cell to affect internal targets (van der Weerden et al. 2008, 2010). NaD1 itself forms a dimer in the crystal and oligomerization increases peptide activity (Lay et al. 2012). Each monomer contains a three-stranded β-sheet (Fig. 9.3e). In 2014, the crystal structure of the NaD1:PIP2 complex was solved. Remarkably, seven dimers of NaD1 (14 monomers) oligomerize into an arch configuration to cooperatively bind the anionic head groups of 14 phosphatidylinositol 4, 5-bisphosphate (PIP2) molecules (Poon et al. 2014). Recently, tomato defensin TPP3 was found to oligomerize and bind to PIP2 in the same manner (Baxter et al. 2015). Finally, several other plant AMPs were found to share the same structural fold in the monomeric state. Examples include PsD1 (Almeida et al. 2002), VrD2 (Lin et al. 2007), and MtDef4 (Sagaram et al. 2013). PhD1 is the first member of plant defensins that shares the same cysteine-stabilized αβ motif (Janssen et al. 2003) as NaD1 (Fig. 9.3e) yet is stabilized by five disulfide bonds (Fig. 9.3f). Additional disulfide bonds could be responsible for the increased stability of the peptide.

9.5.4 The Non-αβ Structural Family

There are no 3-D structures in this category for plant AMPs in the current APD. However, we may predict that glycine-rich peptides could be the members in this structural family. Shepherins I and II are such peptide isolated from plants (Park et al. 2000b). In these peptides, a string of glycines is split by histidines (Table 9.5). These Gly-rich peptides are active against Gram-negative bacteria and fungi [refs]. Gly-rich AMPs against Gram-negative bacteria have also been found in spiders (Wang and Wang 2015; Lorenzini et al. 2003). Different from plant counterparts, these spider peptides are longer and contain more amino acid types such as tyrosines, glutamines, arginines, lysines, and leucines. Thus, these two types of Gly-rich AMPs may have different targets yet to be elucidated. Recently, a Gly-rich cathelicidin has been found in amphibians (Hao et al. 2012). This design has been

utilized by other organisms as well, indicating a common defense strategy in nature (Wang 2014).

In summary, the majority structures of plant AMPs contain β-sheets stabilized by disulfide bonds. Surprisingly, there are α-helical hairpin structures, also stabilized by disulfide bonds. In addition, Gly-rich peptides have been found in plants. Therefore, plants deploy defense peptides with a variety of structures (Fig. 9.3).

9.6 Peptide Design Strategies and Therapeutic Potential

To humans, AMPs constitute a rich resource for designing useful molecules. The APD has annotated 2,600 such peptides, primarily from natural sources. There are 2,149 entries known to be antibacterial, 170 antiviral, 943 antifungal, and 80 antiparasitic (Wang and Wang 2004; Wang et al. 2009). Consequently, efforts are spent on the design of novel antimicrobial agents urgently needed to combat pathogenic bacteria, viruses, fungi, and parasites. Unlike traditional antibiotics, resistance to AMPs is rare or has not been observed (Zasloff 2002; Fjell et al. 2012; Wang 2010). Such observations further stimulated the research in this field.

This chapter presents a structural view for AMPs from amphibians, insects, and plants, three eukaryotic groups with abundant peptides in the APD (Wang et al. 2009; Wang 2015a). Structural studies of AMPs inspired most of the peptide design if not all. The structural analysis in this chapter uncovers two general peptide design strategies for host defense. On one hand, the classic amphipthic helix motif is universal in amphibian AMPs. Hence, amphibians are master in generating multiple defense molecules based on the same helix backbone, leading to a natural combinatorial library. The diversity is achieved by deploying different types of side chains along the peptide backbone, enabling them to act on bacterial membranes or to target DNA (Table 9.6). This is remarkable considering a single frog may deploy up to one hundred such peptides. Such a combinatory peptide library generates a set of molecular devices that enable the execution of numerous functional roles for the survival of frogs, including antimicrobials, protease inhibition, antioxidant, wound healing, and immune modulation (Xu and Lai 2015). Different from amphibians, plants utilize primarily β-sheet scaffolds (usually involving 2–6 disulfide bonds) to generate numerous defense molecules. It is estimated that 50,000 cyclotides exist in plants (Kaas et al. 2010). These cyclotides, which share the same structural fold, gain distinct properties by presenting different amino acids at the loop regions (i.e., another natural combinatorial library). These examples from both amphibians and plants underscore a fundamental design strategy for host defense in nature–*the same peptide scaffold can be duplicated many times to achieve functional diversity by deploying different side chains on the binding surfaces*.

On the other hand, insects, however, have come up with a different defense strategy, where multiple AMPs with various structural scaffolds (Table 9.4) are designed perhaps to recognize different targets (Table 9.6). Insect Pro-rich peptides are now established to bind to ribosomes (Seefeldt et al. 2015; Roy et al. 2015).

Table 9.6 Select antimicrobial peptides and their proposed targets

Source	AMP	Structure	Molecular target
Frogs	Magainins	α	Bacterial membrane (pore?)
Frogs	Buforin	α	Bacterial DNA/RNA
Insects	Pro-rich AMPs	Non-αβ	Bacterial ribosomes
Insects	Defensins	αβ	Glucosylceramides in fungal membranes
Plants	Kalata B1	β	Phosphatidylethanolamine (PE)
Bacteria	MccJ25	β	Inhibition of RNA polymerase
Bacteria	Nisin	Non-αβ	Bacterial cell wall (lipid II)

Likewise, humans also deploy different AMPs with varying structural scaffolds (Wang 2014). Remarkably, bacteriocins (i.e., bacterial AMPs) vary substantially in both structural scaffold and posttranslational modification (Perez et al. 2014). These peptides with different structural scaffolds may kill the same bacterium by different mechanisms (Table 9.6). For example, lantibiotics such as nisin usually target cell wall (Hsu et al. 2004), whereas microcin J25 (MccJ25) can inhibit RNA polymerase (Mukhopadhyay et al. 2004). To humans, these natural molecules with desired functions may be directly utilized to improve life quality. For example, nisin has been long employed as a food preservative. Daptomycin and colistin are peptide antibiotics (i.e., AMPs) already in clinical use. In addition, numerous 3-D structures of AMPs serve as excellent starting templates for us to design novel therapeutic peptides. One of the important design goals is to achieve peptide stability to proteases so that the peptide becomes more druggable. Perhaps humans have been inspired by the amphibian Rana box in designing the helix staple motif that enhances peptide stability. The Rana box consists of a string of amino acids bracketed by a disulfide bond (Xu and Lai 2015), whereas the helix staple motif connects two side chains via a chemical bridge (Walensky and Bird 2014; Muñoz et al. 1995). The helical hairpin structure is a surprise to us because the majority of plant defense peptides contain a β-sheet structural unit. Interestingly, Tk-AMP-X2, an antimicrobial helical hairpin molecule can be engineered to block channels (Berkut et al. 2014). There is now also high interest in harnessing plant cyclotide scaffolds owing to its known stability to proteases. In particular, new therapeutic molecules can be generated via grafting a "drug epitope" for a specific biological function such as receptor binding (Northfield et al. 2014; Aboye et al. 2012). Thus, the use of AMPs is not limited to development of novel antimicrobials to combat superbugs.

The optimization of a therapeutic peptide is a demanding task due to multiple parameters such as potency, stability, and low cytotoxicity. Computer software (Fjell et al. 2012) as well as database (Wang 2013) can be helpful. Recently, we found it useful to combine library screening with structure-based design. Library screening led to the identification of a chymotrypsin-resistant template designed based on human cathelicidin LL-37 (Li et al. 2006b), whereas structure-based design enhanced its potency against MRSA (Wang et al. 2014). 17BIPHE2, one of

the designed peptides in the family, showed antimicrobial activity against the ESKAPE pathogens, which include *Enterococcus faecium*, *S. aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanii*, *Pseudomonas aeruginosa*, and *Enterobacter* species. In vivo, the peptide prevented biofilm formation and reversed the chemokine pattern found for the *S. aureus* biofilms (Wang et al. 2014). Such a combined therapeutic benefit, namely, antimicrobial and immune boosting is exactly what patients need.

In conclusion, structure studies revealed two major strategies for peptide discovery: combinatory library screening and structure-based rational design. One may also combine peptide library screening with structure-based design to achieve multiple design goals. In addition, two compounds may be jointly utilized to eliminate established biofilms that cannot be removed by a single antibiotic (Wang et al. 2015). Indeed, the APD (Wang 2015a) has annotated five bacterial AMPs (e.g., nisin, pediocin PA-1, circular gramicidin S, polymyxin E/colistin, and daptomycin) already in use, and additional candidates (leads) are under developments or in clinical trials (Zasloff 2002; Fjell et al. 2012). Thus, the AMP field will continue to inspire the design of novel antimicrobials as well as other therapeutic molecules.

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References

Abbassi F, Lequin O, Piesse C, Goasdoue N, Foulon T, Nicolas P, Ladram A (2010) J Biol Chem 285:16880

Aboye TL, Ha H, Majumder S, Christ F, Debyser Z, Shekhtman A, Neamati N, Camarero JA (2012) J Med Chem 55:10729–10734

Almeida MS, Cabral KM, Kurtenbach E, Almeida FC, Valente AP (2002) J Mol Biol 315:749–757

Barbault F, Landon C, Guenneugues M, Meyer JP, Schott V, Dimarcq JL, Vovelle F (2003) Biochemistry 42:14434

Baxter AA, Richter V, Lay FT, Poon IK, Adda CG, Veneer PK, Phan TK, Bleackley MR, Anderson MA, Kvansakul M, Hulett MD (2015) Mol Cell Biol 35:1964–1978

Bechinger B, Kim Y, Chirlian LE, Gesell J, Neumann JM, Montal M, Tomich J, Zasloff M, Opella SJ (1991) J Biomol NMR 1:167–173

Berkut AA, Usmanova DR, Peigneur S, Oparin PB, Mineev KS, Odintsova TI, Tytgat J, Arseniev AS, Grishin EV, Vassilevski AA (2014) J Biol Chem 289:14331–14340

Boland MP, Separovic F (2006) Biochim Biophys Acta 1758:1178

Boman HG (1995) Annu Rev Immunol 13:61

Bowie JH, Separovic F, Tyler MJ (2012) Peptides 37:174

Brown LS, Ladizhansky V (2015) Protein Sci. doi:10.1002/pro.2700

Cerovský V, Budesínský M, Hovorka O, Cvacka J, Voburka Z, Slaninová J, Borovicková L, Fucík V, Bednárová L, Votruba I, Straka J (2009) ChemBioChem 10:2089

Chi SW, Kim JS, Kim DH, Lee SH, Park YH, Han KH (2007) Biochem. Biophys. Res. Commun. 352:592

Cho JH, Kim SC (2010) In: Wang G (ed) Antimicrobial peptides: discovery, design and novel therapeutic strategies. CABI, England
Clark DP, Durell S, Maloy WL, Zasloff M (1994) J Biol Chem 269:10849
Conlon JM, Kolodziejek J, Nowotny N (2004) Biochim Biophys Acta 1696:1–14
Cornet B, Bonmatin JM, Hetru C, Hoffmann JA, Ptak M, Vovelle F (1995) Structure 3:435–448
Dalla Serra M, Cirioni O, Vitale RM, Renzone G, Coraiola M, Giacometti A, Potrich C, Baroni E, Guella G, Sanseverino M, de Luca S, Scalise G, Amodeo P, Scaloni A (2008) Biochemistry 47:7888
Dennison SR, Morton LH, Phoenix DA (2012) Protein Pept Lett 19:586–591
Duvick JP, Rood T, Rao AG, Marshak DR (1992) J Biol Chem 267:18814–18820
Fehlbaum P, Bulet P, Michaut L, Lagueux M, Broekaert WF, Hetru C, Hoffmann JA (1994) J Biol Chem 269:33159
Fernandez DI, Le Brun AP, Whitwell TC, Sani MA, James M, Separovic F (2012) PhysChemChem Phys 14:15739–15751
Fjell CD, Hiss JA, Hancock REW, Schneider G (2012) Nat Rev Drug Discov 11:37–51
Gao GH, Liu W, Dai JX, Wang JF, Hu Z, Zhang Y, Wang DC (2001) Biochemistry 40:10973–10978
Gesell J, Zasloff M, Opella SJ (1997) J Biomol NMR 9:127
Gran L (1973) Acta Pharmacol Toxicol (Copenh) 33:400–408
Haney EF, Hunter HN, Matsuzaki K, Vogel HJ (2009) Biochim Biophys Acta 1788:1639
Hao X, Yang H, Wei L, Yang S, Zhu W, Ma D, Yu H, Lai R (2012) Amino Acids 43:677–685
Hernandez-Lucas C, Fernandez de Caleya R, Carbonero P (1974) Appl Microbiol 28:165–168
Holak TA, Engstroem A, Kraulis PJ, Lindeberg G, Bennich H, Jones TA, Gronenborn AM, Clore GM (1988) Biochemistry 27:7620
Hong M (2006) Acc Chem Res 39:176
Hsu ST, Breukink E, Tischenko E, Lutters MA, de Kruijff B, Kaptein R, Bonvin AM, van Nuland NA (2004) Nat Struct Mol Biol 11:963–967
Huang YH, Colgrave ML, Daly NL, Keleshian A, Martinac B, Craik DJ (2009) J Biol Chem 284:20699–20707
Imamura T, Yamamoto N, Tamura A, Murabayashi S, Hashimoto S, Shimada H, Taguchi S (2008) Biochem Biophys Res Commun 369:609
Iwai H, Nakajima Y, Natori S, Arata Y, Shimada I (1993) Eur J Biochem 217:639
Janssen BJ, Schirra HJ, Lay FT, Anderson MA, Craik DJ (2003) Biochemistry 42:8214–8222
Kaas Q, Westermann JC, Henriques ST, Craik DJ (2010) In: Wang G (ed) Antimicrobial peptides: discovery, design and novel therapeutic strategies. CABI, England
Kang SJ, Son WS, Han KD, Mishig-Ochir T, Kim DW, Kim JI, Lee BJ (2010) Mol Cells 30:435
Knappe D, Zahn M, Sauer U, Schiffer G, Strater N, Hoffmann R (2011) ChemBioChem 12:874
Koch K, Afonin S, Ieronimo M, Berditsch M, Ulrich AS (2012) Top Curr Chem 306:89–91
Kouno T, Mizuguchi M, Tanaka H, Yang P, Mori Y, Shinoda H, Unoki K, Aizawa T, Demura M, Suzuki K, Kawano K (2007) Biochemistry 46:13733
Krizsan A, Volke D, Weinert S, Strater N, Knappe D, Hoffmann R (2014) Angew Chem Int Ed Engl 53:12236–12239
Lai Y, Gallo RL (2009) Trends Immunol 30:131–141
Lamberty M, Caille A, Landon C, Tassin-Moindrot S, Hetru C, Bulet P, Vovelle F (2001) Biochemistry 40:11995
Landon C, Sodano P, Hetru C, Hoffmann J, Ptak M (1997) Protein Sci 6:1878
Landon C, Guenneugues M, Barbault F, Legrain M, Menin L, Schott V, Vovelle F, Dimarcq JL (2004) Protein Sci 13:703
Landon C, Meudal H, Boulanger N, Bulet P, Vovelle F (2006) Biopolymers 81:92
Lay FT, Schirra HJ, Scanlon MJ, Anderson MA, Craik DJ (2003) J Mol Biol 325:175–188
Lay FT, Mills GD, Poon IK, Cowieson NP, Kirby N, Baxter AA, van der Weerden NL, Dogovski C, Perugini MA, Anderson MA, Kvansakul M, Hulett MD (2012) J Biol Chem 287:19961–19972

Lee KH, Shin SY, Hong JE, Yang ST, Kim JI, Hahm KS, Kim Y (2003) Biochem Biophys Res Commun 309:591
Lehrer RI, Lu W (2012) Immunol Rev 245:284
Lemaitre B, Hoffmann J (2007) Annu Rev Immunol 25:697–743
Lemaitre B, Nicolas E, Michaut L, Reichhart JM, Hoffmann JA (1996) Cell 86:973
Lequin O, Ladram A, Chabbert L, Bruston F, Convert O, Vanhoye D, Chassaing G, Nicolas P, Amiche M (2006) Biochemistry 45:468
Li X, Li Y, Peterkofsky A, Wang G (2006a) Biochim Biophys Acta 1758:1203
Li X, Li Y, Han H, Miller DW, Wang G (2006b) J Am Chem Soc 128:5776–5785
Lin KF, Lee TR, Tsai PH, Hsu MP, Chen CS, Lyu PC (2007) Proteins 68:530–540
Lorenzini DM, da Silva PI Jr, Fogaça AC, Bulet P, Daffre S (2003) Dev Comp Immunol 27:781–791
Mak AS, Jones BL (1976) Can J Biochem 54:835–842
Mandard N, Sodano P, Labbe H, Bonmatin JM, Bulet P, Hetru C, Ptak M, Vovelle F (1998) Eur J Biochem 256:404
Mangoni ML (2006) Cell Mol Life Sci 63:1060
Marcotte I, Wegener KL, Lam YH, Chia BC, de Planque MR, Bowie JH, Auger M, Separovic F (2003) ChemPhys Lipids 122:107–120
Marcus JP, Goulter KC, Green JL, Harrison SJ, Manners JM (1997) Eur J Biochem 244:743–749
Mardirossian M, Grzela R, Giglione C, Meinnel T, Gennaro R, Mergaert P, Scocchi M (2014) Chem Biol 21:1639–1647
Marsh J, Goode JA (eds) (1994) Antimicrobial Peptides (ciba foundation symposium 186). Wiley, Chichester
McManus AM, Otvos L Jr, Hoffmann R, Craik DJ (1999a) Biochemistry 38:705
McManus AM, Nielsen KJ, Marcus JP, Harrison SJ, Green JL, Manners JM, Craik DJ (1999b) J Mol Biol 293:629–638
Mukhopadhyay J, Sineva E, Knight J, Levy RM, Ebright RH (2004) Mol Cell 14:739–751
Muñoz V, Blanco FJ, Serrano L (1995) Nat Struct Biol 2:380–385
Nawrot R, Barylski J, Nowicki G, Broniarczyk J, Buchwald W, Goździcka-Józefiak A (2014) Folia Microbiol (Praha) 59:181–196
Nolde SB, Vassilevski AA, Rogozhin EA, Barinov NA, Balashova TA, Samsonova OV, Baranov YV, Feofanov AV, Egorov TA, Arseniev AS, Grishin EV (2011) J Biol Chem 286:25145–25153
Northfield SE, Wang CK, Schroeder CI, Durek T, Kan MW, Swedberg JE, Craik DJ (2014) Eur J Med Chem 77:248–257
Oizumi Y, Hemmi H, Minami M, Asaoka A, Yamakawa M (2005) Biochim Biophys Acta 1752:83
Park SH, Kim YK, Park JW, Lee B, Lee BJ (2000a) Eur J Biochem 267:2695
Park CJ, Park CB, Hong SS, Lee HS, Lee SY, Kim SC (2000b) Plant Mol Biol 44:187–197
Park S, Park SH, Ahn HC, Kim S, Kim SS, Lee BJ, Lee BJ (2001) FEBS Lett 507:595
Park S, Son WS, Kim YJ, Kwon AR, Lee BJ (2007) J Biochem Mol Biol 40:261
Patel SU, Osborn R, Rees S, Thornton JM (1998) Biochemistry 37:983–990
Perez RH, Zendo T, Sonomoto K (2014) Microb Cell Fact 13(Suppl1):S3
Perutz MF (1962) Nobel lecture: X-ray analysis of haemoglobin
Poon IK, Baxter AA, Lay FT, Mills GD, Adda CG, Payne JA, Phan TK, Ryan GF, White JA, Veneer PK, van der Weerden NL, Anderson MA, Kvansakul M, Hulett MD (2014) Elife 3: e01808
Resende JM, Moraes CM, Munhoz VH, Aisenbrey C, Verly RM, Bertani P, Cesar A, Pilo-Veroso D, Bechinger B (2009) Proc Natl Acad Sci USA 106:16639
Romagnoli S, Ugolini R, Fogolari F, Schaller G, Urech K, Giannattasio M, Ragona L, Molinari H (2000) Biochem J 350(Pt 2):569–577
Rose PW, Prlić A, Bi C, Bluhm WF, Christie CH, Dutta S, Green RK, Goodsell DS, Westbrook JD, Woo J, Young J, Zardecki C, Berman HM, Bourne PE, Burley SK (2015) Nucleic Acids Res 43(Database issue):D345–D356

Rosengren KJ, Göransson U, Otvos L Jr, Craik DJ (2004) Biopolymers 76:446
Roy RN, Lomakin IB, Gagnon MG, Steitz TA (2015) Nat Struct Mol Biol 22:466–469
Rozek T, Wegener KL, Bowie JH, Olver IN, Caver JA, Wallace JC, Tyler MJ (2000) Eur J Biochem 267:5330
Saether O, Craik DJ, Campbell ID, Sletten K, Juul J, Norman DG (1995) Biochemistry 34:4147–4158
Sagaram US, El-Mounadi K, Buchko GW, Berg HR, Kaur J, Pandurangi RS, Smith TJ, Shah DM (2013) PLoS One 8:e82485
Seefeldt AC, Nguyen F, Antunes S, Pérébaskine N, Graf M, Arenz S, Inampudi KK, Douat C, Guichard G, Wilson DN, Innis CA (2015) Nat Struct Mol Biol 22:470–475
Selsted ME, Harwig SS, Ganz T, Schilling JW, Lehrer RI (1985) J Clin Invest 76:1436
Shao F, Hu Z, Xiong YM, Huang QZ, Wang CG, Zhu RH, Wang DC (1999) Biochim Biophys Acta 1430:262–268
Shenkarev ZO, Nadezhdin KD, Sobol VA, Sobol AG, Skjeldal L, Arseniev AS (2006) FEBS J 273:2658–2672
Silva PI Jr, Daffre S, Bulet P (2000) J Biol Chem 275:33464–33470
Stec B, Rao U, Teeter MM (1995) Acta Crystallogr D Biol Crystallogr 51(Pt 6):914–924
Steiner H, Hultmark D, Engström A, Bennich H, Boman HG (1981) Nature 292:246
Stephens C, Kazan K, Goulter KC, Maclean DJ, Manners JM (2005) FEMS Microbiol Lett 243:205–210
Strandberg E, Ulrich AS (2015) BiochimBiophysActa 1848:1944
Tailor RH, Acland DP, Attenborough S, Cammue BP, Evans IJ, Osborn RW, Ray JA, Rees SB, Broekaert WF (1997) J Biol Chem 272:24480–24487
Tam JP, Lu YA, Yang JL, Chiu KW (1999) Proc Natl Acad Sci USA 96:8913–8918
Tamba Y, Ariyama H, Levadny V, Yamazaki M (2010) J Phys Chem B 114:12018
Thevissen K, Warnecke DC, François IE, Leipelt M, Heinz E, Ott C, Zähringer U, Thomma BP, Ferket KK, Cammue BP (2004) J Biol Chem 279:3900
Thevissen K, de Mello Tavares P, Xu D, Blankenship J, Vandenbosch D, Idkowiak-Baldys J, Govaert G, Bink A, Rozental S, de Groot PW, Davis TR, Kumamoto CA, Vargas G, Nimrichter L, Coenye T, Mitchell A, Roemer T, Hannun YA, Cammue BP (2012) Mol Microbiol 84:166–180
Toke O, Bánóczi Z, Király P, Heinzmann R, Bürck J, Ulrich AS, Hudecz F (2011) Eur Biophys J 40:447–462
Tossi A, Sandri L (2002) Curr Pharm Des 8:743
Vale N, Aguiar L, Gomes P (2014) Front Pharmacol 57:275
van der Weerden NL, Lay FT, Anderson MA (2008) J Biol Chem 283:14445–14452
van der Weerden NL, Hancock RE, Anderson MA (2010) J Biol Chem 285:37513–37520
Verardi R, Traaseth NJ, Shi L, Porcelli F, Monfregola L, De Luca S, Amodeo P, Veglia G, Scaloni A (2011) Biochim Biophys Acta 1808:34
Vriens K, Cammue BP, Thevissen K (2014) Molecules 19:12280–12303
Walensky LD, Bird GH (2014) J Med Chem 57:6275–6288
Wang G (2008a) Curr Protein Pept Sci 9:50
Wang G (2008b) J Biol Chem 283:32637
Wang G (2010) Antimicrobial peptides: discovery, design and novel therapeutic strategies. CABI, Oxfordshire, England
Wang G (2012) Curr Biotechnol 1:72–79
Wang G (2013) Pharmaceuticals 6:728–758
Wang G (2014) Pharmaceuticals 7:545–594
Wang G (2015a) Methods Mol Biol 1268:43
Wang G (2015b) In: Chen CY, Yan X, Jackson CR (eds) Antimicrobial resistance and food safety: methods and techniques. Academic Press, Boston
Wang Z, Wang G (2004) Nucleic Acids Res 32:D590
Wang X, Wang G (2015) Protein Pept Lett, In press

Wang G, Hanke ML, Mishra B, Lushnikova T, Heim CE, Chitterzhan Thomas V, Bayles KW, Kielian T (2014) ACS Chem Biol 9:1997
Wang G, Peterkofsky A, Keifer PA, Li X (2005a) Protein Sci 14:1082
Wang G, Li Y, Li X (2005b) J Biol Chem 280:5803
Wang G, Li X, Wang Z (2009) Nucleic Acids Res 37:D933
Wang CK, King GJ, Northfield SE, Ojeda PG, Craik DJ (2014) Angew Chem Int Ed Engl 53:11236–11241
Wang G, Mishra B, Lau K, Lushnikova T, Golla R, Wang X (2015) Pharmaceuticals 8:123–150
Wüthrich K (1986) NMR of proteins and nucleic acids. Wiley, New York
Wüthrich K (2002) Nobel lecture: NMR studies of structure and function of biological macromolecules 8
Xu X, Lai R (2015) Chem Rev 115:1760
Yi G-S, Park CB, Kim SC, Cheong C (1996) FEBS Lett 398:87
Yu K, Kang S, Park N, Shin J, Kim Y (2000) J Pept Res 55:51
Zasloff M (1987) Proc Natl Acad Sci USA 84:5449
Zasloff M (2002) Nature 415:389

Chapter 10
How to Teach Old Antibiotics New Tricks

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Abstract Antimicrobial peptides (AMPs), or more generally host defense peptides, have broad-spectrum antimicrobial activity and use nonspecific interactions to target generic features common to the membranes of many pathogens. As a result, development of resistance to such natural defenses is inhibited compared to conventional antibiotics. The disadvantage of AMPs, however, is that they are often not very potent. In contrast, traditional antibiotics typically have strong potency, but due to a broad range of bacterial defense mechanisms, there are many examples of resistance. Here, we explore the possibility of combining these two classes of molecules. In the first half of this chapter, we review the fundamentals of membrane curvature generation and the various strategies recently used to mimic this membrane activity of AMPs using different classes of synthetic molecules. In the second half, we show that it is possible to impart membrane activity to molecules with no previous membrane activity, and summarize some of our recent works which aim to combine advantages of traditional antibiotics and AMPs into a single molecule with multiple mechanisms of killing as well as multiple mechanisms of specificity.

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10.1 Introduction

The emergence of antibiotic resistance poses a serious and growing global health problem with at least 2 million illnesses and 23,000 deaths in the United States every year. Resistance essentially arises from antibiotic misuse and overuse both within human and veterinary medicine, especially in livestock. Approximately 70 % of hospital acquired infections in the United States are resistant to at least one antibiotic. Despite the important need for new antimicrobial agents, the antibiotic pipeline is running dry. The growing gap between the decline of antibiotic development and the escalating evolution of drug resistance has become one of the most urgent challenges for global health.

Antimicrobial peptides (AMPs), or more generally, host defense peptides, have coevolved with prokaryotes for millennia. AMPs have broad-spectrum antimicrobial activity and utilize nonspecific interactions to target generic features common to the outer membranes of many pathogens, hence development of resistance to such natural defenses is inhibited compared to conventional antibiotics. However, the disadvantage of AMPs is that they are not usually potent. In contrast, traditional antibiotics, such as the aminoglycosides, which inhibits of protein synthesis by ribosomal binding, are quite potent as antibiotics, but due to a broad range of bacterial mechanisms, there are many examples of resistance (Anguita-Alonso et al. 2005; Magnet and Blanchard 2005; Mingeot-Leclercq et al. 1999; Vakulenko and Mobashery 2003). In this review, we summarize some of our recent works which try to combine advantages of these two classes of compounds, and explore situations where the antibacterial actions of these compounds may be synergistic.

10.2 Natural Host Defense Peptides and How They Work: The Importance of Membrane Activity

Antimicrobial peptides (AMPs) comprise an important part of innate host defense (Zasloff 2002; Brogden 2005a; Shai 1999; Hancock and Lehrer 1998; Hancock and Sahl 2006; Yeaman and Yount 2003). Here we present only a cursory introduction since AMPs are described in more detail elsewhere in this volume. AMPs act as natural antibiotics to protect the host from bacterial infections. Collectively, AMPs display broad-spectrum antimicrobial activity and target organisms ranging from viruses to parasites (Zasloff 2002; Brogden 2005a; Hancock and Sahl 2006). To date, well over 1,000 AMPs have been discovered in both prokaryotic and eukaryotic cells. In animals, the tissues and organs exposed to airborne pathogens tend to produce most AMPs. For example, whereas our skin is constantly in contact with large numbers of microorganisms, it is rarely infected because many cell types residing in the skin (including keratinocytes, sebocytes, eccrine glands, and mast cells) produce AMPs in response to microbial invasion (Schauber and Gallo 2008).

Whereas many commonly used antibiotics like β-lactams, quinolones, macrolides, and tetracyclines have core structural features that are responsible for their antibacterial activities, AMP sequences are highly diverse and do not have a common core structure. We know that most AMPs tend to be relatively short (<50 amino acids) and share two fundamental features: net cationic charge (+2 to +9) and amphiphilicity (Zasloff 2002; Brogden 2005a; Shai 1999; Hancock and Lehrer 1998; Hancock and Sahl 2006; Yeaman and Yount 2003). AMPs are often classified according to their secondary structures (Table 10.1). The α-helical AMPs include magainins (Zasloff 1987) from frogs and LL-37 cathelicidins (Dürr et al. 2006) from humans, and the β-sheet AMPs include protegrins (Kokryakov et al. 1993) from pigs and defensins (Ganz 2003; Lehrer 2004; Selsted and Ouellette 2005) from mammals. A third group is categorized by extended linear peptides with sequences dominated by a few amino acid species, like the tryptophan-rich indolicidin (Selsted et al. 1992) from cattle, and the arginine- and proline-rich PR-39 (Agerberth et al. 1991) from pigs.

The role of AMPs is not restricted to acting as direct microbicides, many host defense peptides execute important defensive and regulatory functions in their hosts

Table 10.1 Examples of antimicrobial peptides, classified according to their secondary structure

Peptide	Source	Peptide sequence
α-helix		
Magainin-1	Frog	GIGKFLHSAGKFGKAFVGEIMKS
Magainin-2	Frog	GIGKFLHSAKKFGKAFVGEIMNS
LL-37 (cathelicidin)	Human	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES
Cecropin A	Silk moth	KWKLFKKIEKVGQNIRDGIIKAGPAVAVVGQATQIAK-NH_2
Melittin	Honey bee	GIGAVLKVLTTGLPALISWIKRKRQQ-NH_2
PMAP-23 (cathelicidin)	Porcine	RIIDLLWRVRRPQKPKFVTVWVR
Dermaseptin S1	Frog	ALWKTMLKKLGTMALHAGKAALGAAADTISQGTQ
β-sheet		
Protegrin-1	Porcine	RGGRLC$_1$YC$_2$RRRFC$_2$VC$_1$VGR-NH_2
α-Defensin (HNP3)	Human	DC$_1$YC$_2$RIPAC$_3$IAGERRYGTC$_2$IYQGRLWAFC$_3$C$_1$
β-Defensin (TAP)	Bovine	NPVSC$_1$VRNKGIC$_2$VPIRC$_3$PGSMKQIGTC$_2$VGRAVKC$_1$C$_3$RKK
θ-defensin	Monkey	GFC$_1$RC$_2$LC$_3$RRGVC$_3$RC$_2$IC$_1$TR (cyclic)
Lactoferricin B	Bovine	FKCRRWQWRMKKLGAPSITCVRRAF
Bactenecin	Bovine neutrophils	RLCRIVVIRVCR (cyclic)
PR-39	Porcine	RRRPRPPYLPRPRPPPFFPPRLPPRIPPGFPPRFPPRFP-NH_2
Indolicidin (cathelicidin)	Bovine neutrophils	ILPWKWPWWPWRR-NH_2

Cysteines paired in disulphide linkages are noted by common numerical subscripts. The *C*-terminal protecting group abbreviation NH_2 is for carboxyamide

including immunomodulatory, and anti-inflammatory properties systems, which are not found in traditional antibiotics. AMPs have been shown to interact with components of the innate and adaptive immune systems (Bowdish et al. 2005; Schmidt et al. 2015a). Recent work has even shown that the AMP LL-37 plays a role in autoimmune disorders such as lupus (Lande et al. 2011) and psoriasis (Lande et al. 2007).

The general mechanism of AMP activity is believed to involve the selective disruption and permeabilization of microbial membranes causing leakage of cellular components, breakdown of membrane potential, and cell death. While some AMPs employ alternative modes of action or act upon multiple bacterial cell targets, most of them interact strongly with lipid bilayers and can alter their structure. The preferential action of AMPs against bacterial membranes and not against eukaryotic membranes is thought to be rooted in the compositional differences between bacterial cell membranes and eukaryotic cell membranes (Zasloff 2002; Brogden 2005a; Shai 1999; Hancock and Sahl 2006; Matsuzaki 1999; Matsuzaki et al. 1998; Huang 2000). Microbial cell surfaces are decorated with polyanionic molecules like lipopolysaccarides in Gram-negative bacteria, and lipoteichoic acids in Gram-positive bacteria (Hancock and Sahl 2006). Additionally, the outer leaflet of bacterial plasma membranes contains large amounts of anionic lipids such as those with phosphatidylglycerol (PG) and cardiolipin (CL) head groups. The membranes of the Gram-positive bacteria *Staphylococcus aureus* and *Streptococcus pneumoniae* are composed primarily of PG and CL lipids. Phosphatidylethanolamine (PE) is the principle zwitterionic phospholipid found in Gram-negative bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella typhimurium* (Epand and Epand 2011). The lipid compositions of animal cell membranes differ from bacteria plasma membranes in a number of ways (Zasloff 2002; Zachowski 1993; van Meer et al. 2008): mammalian cell membranes have more lipids with neutral zwitterionic head groups such as phosphatidylcholine (PC) and sphingomyelin (SM). Moreover, their lipid compositions are asymmetrically distributed between the inner and outer bilayer leaflets. PC and SM are found in the outer leaflet of human erythrocytes, while PE and the anionic lipids phosphatidylserine (PS), and phosphatidylinositol (PI) are found on the inner leaflet. Finally, sterols such as cholesterol constitute a major component (for cholesterol $\sim$30 % by mole) of animal plasma membranes. Consistent with the above differences, in vitro studies on both natural and synthetic cationic membrane-active antimicrobials have shown that the presence of anionic lipids increases membrane disruption and permeabilization (Shai 1999).

To interact with bacterial cells, AMPs must first be attracted to their membrane surfaces, which is believed to occur through nonspecific electrostatic binding between the cationic region of the peptide and anionic components on the surface. These peptides adsorb and orient parallel to the surface, and are then partitioned into the amphiphilic interface (between the hydrophilic and hydrophobic regions of the membrane). This process is driven by the hydrophobic interactions that control hydrophobic insertion. The amphipathic nature of AMPs is essential for this behavior, as the hydrophobic region of the peptide allows direct interaction with the lipid components of the membrane. To understand peptide–membrane interactions better, we review several biophysical aspects of membranes.

10.2.1 Membrane Curvature: Definition of Terms

Adsorption of antimicrobial peptides (AMPs) onto a membrane can alter the membrane curvature and remodel the membrane. Here, we briefly review the membrane curvature generation by AMPs, and describe how we can leverage recent work to design molecules that permeate membranes, but have in addition other functions besides permeation of membranes.

We begin with a description of recent work on AMPs, which shows how different peptide-induced effects combine to form complex curvatures. Central to this approach is the idea that bulk phase diagrams can be quite informative in identifying structural tendencies in peptide–membrane interactions and resultant induced curvature. We illustrate the approach by identifying some basic sequence principles for peptides that permeate membranes based on induced curvature considerations, and apply these principles to impart membrane-permeability to nonmembrane-active molecules, such as traditional antibiotics that do not have membrane permeating functions.

Membrane deformations can be described using geometric concepts. There are many good reviews and books on this topic, so we include a cursory treatment to acquaint the reader with the basic concepts useful in thinking about peptide-induced membrane curvature, rather than a unified and rigorous treatment. On a 2D membrane surface that exists in 3D space, the curvature at any point on the surface can be defined by a tangent plane at that point. Planes that are perpendicular to this tangent plane intersect the surface as a normal section, and each normal section is associated with a curvature at the tangent point, defined as $c = 1/R$, with R being the radius of curvature. One can see that there are many different possible normal sections, and many different curvatures that can be defined through that point. The maximum and minimum curvatures that go through the point correspond to normal sections that are orthogonal to one another. These curvatures are called the *principal curvatures*. The two principal curvatures can therefore be defined as, $c_1 = 1/R_{max}$ and $c_2 = 1/R_{min}$ (with R_{max} and R_{min} being the principal radii of curvature) (Fig. 10.1).

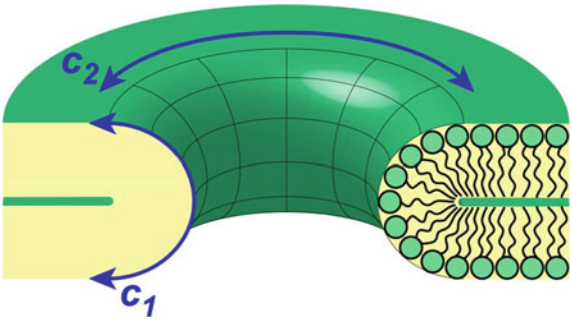

Fig. 10.1 The membrane of a pore features a curved surface with a saddle shape, which is characterized by principal curvatures c_1 and c_2 (directions indicated by *arrows*) that are opposite in sign and together create negative Gaussian curvature (NGC)

These quantities can be used to construct two types of curvatures that are useful in describing the shape of a surface, the mean curvature H and the Gaussian curvature K:

$$H = \frac{1}{2}(c_1 + c_2)$$
$$K = c_1 c_2$$

By convention, a membrane monolayer that bends to form a convex hydrophilic surface is said to have positive curvature, while a monolayer that bends in the opposite direction to form a concave hydrophilic surface is said to have negative curvature. The lipid molecules that make up a bilayer have a specific "shape" and can contribute to the membrane curvature. (Here we point out that "molecular shape" can be determined by different effects. In addition to steric shapes, patterns of hydrogen bonding can also be important, for example.) Israelachvili et al. have described how the properties of a specific amphiphile can impact packing and therefore the morphology of self-assembled system (Israelachvili et al. 1980). This model describes an average geometric shape for a lipid molecule via a dimensionless packing parameter, S, which depends on the repulsive steric and electrostatic interactions between the polar lipid head groups and the attractive hydrophobic interactions and repulsive steric forces experienced by the lipid tails:

$$S = \frac{V}{A_0 L_c}$$

where V is the molecular volume of the lipid tails, A_0 is the optimum area occupied by the lipid head group, and L_c is the length of the lipid tails. Lipids tend to form specific aggregates of different geometries as a function of their packing parameters. Generally, $S < \frac{1}{3}$ forms spherical aggregates, $\frac{1}{3} < S < \frac{1}{2}$ forms cylindrical aggregates, $\frac{1}{2} < S < 1$ forms planar aggregates, and $S > 1$ forms inverted aggregates. For example, a lipid molecule with $S < \frac{1}{2}$ tends to be cone- or wedge shaped and has positive intrinsic curvature, whereas a lipid molecule with $S > 1$ is shaped like an inverted cone and tends to have negative intrinsic curvature. Clearly, insertion of objects of different shapes into the lipid membrane can impact geometric packing and thereby induce different curvatures.

Deforming a membrane away from its native state is associated with an energetic cost determined by the elasticity of the membrane. The energetics of membrane shape changes have been described by Helfrich (1973). Deformation of the membrane would have an energetic cost that is dependent on the mean and Gaussian curvatures, so that the curvature elasticity energy per unit area of bending a membrane is given by

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

where c_0 is the spontaneous curvature (or equivalently intrinsic curvature). The energy cost per unit area is controlled by the bending modulus κ, and the Gaussian modulus κ_G, which are elastic constants. These constants describe the bending elasticity of the membrane and the resistance of the membrane to topological transitions, respectively. The total elastic energy of a symmetric membrane ($c_0 = 0$) is obtained by integrating the Helfrich energy density over the membrane surface:

$$F = \int \mathrm{d}A(2\kappa H^2 + \kappa_G K)$$

The first term is due to the energetic cost of bending the membrane, while the second term accounts for energetic costs of distortions related to topology. To motivate this qualitatively, one can use the Gauss–Bonnet theorem:

$$\int K\mathrm{d}A = 4\pi(1 - g)$$

where the integer g is the genus of the surface, often described as the number of "holes." Qualitatively, a sphere has plenty of curvature, but it has no holes: A sphere is a $g = 0$ surface. On the other hand, a "donut," which has a single hole, is a surface with $g = 1$ (Siegel and Kozlov 2004). In fact, for each additional hole, the genus increases by one. One can see that the more negative the total integrated Gaussian curvature is on the surface, the "holier" the surface is. In contrast, closed surfaces with no holes have a total Gaussian curvature on the surface that is positive.

The Helfrich approach is almost always used as a starting point in the context of curvature induced by protein–membrane interactions. It should be noted that the Helfrich formalism is not the only way to parametrize a curvature energy functional, due to the Gauss–Bonnet theorem (Deserno 2009). Moreover, Helfrich theory is based on the assumption of small membrane curvatures. For example, a curvature is considered small if the corresponding radius of curvature is larger than the membrane thickness, which is approximately 4 nm. Therefore, this type of elastic theory approaches its limits as the radius of curvature becomes comparable to the membrane thickness (Zimmerberg and Kozlov 2006; Shearman et al. 2006). This can be seen when we consider a saddle-like membrane shape, which can be found in bicontinuous cubic phases. Because the mean curvature is zero, the Helfrich elastic energy per unit area is the product of the Gaussian modulus and the Gaussian curvature, which typically yields a positive value for minimal surfaces (Siegel and Kozlov 2004). In this case, minimization of the curvature elastic energy would reduce the lattice parameter to an arbitrarily small value, which is not observed experimentally (Shearman et al. 2006). Such nonphysical behavior can be corrected by including higher order terms in the expression for curvature elastic energy, which allows for cubic phase stability (Ljunggren and Eriksson 1992; Seddon and Templer 1993).

10.2.2 Empirical Observations of AMP-Induced Membrane Curvature

Now that we have a biophysical description of membranes, we can examine what happens when AMPs interact with membranes. Much of the pioneering work on AMPs has focused on membrane permeation (often via pore formation), and the formulation of models for such permeation, such as "barrel stave" (Yeaman and Yount 2003; Shai and Oren 2001; Yang et al. 2001; Ehrenstein and Lecar 1977; Spaar et al. 2004; Jenssen et al. 2006), "toroidal pore" (Matsuzaki et al. 1998, 1996, 1997; Yang et al. 2001; Jenssen et al. 2006; Bechinger 2009; Ludtke et al. 1996; Tang et al. 2007), and "carpet" models (Shai and Oren 2001; Jenssen et al. 2006; Brogden 2005b; Bechinger et al. 1991; Pouny et al. 1992; Gazit et al. 1994; Matsuzaki et al. 1994) (Fig. 10.2). However, empirically, it is known that there are many diverse ways for AMPs to act against bacterial membranes beyond these basic models. Scanning Electron Microscopy (SEM) reveals the formation of blebs on *P. aeruginosa* membranes after exposure to sheep cathelicidin SMAP29 (Saiman et al. 2001; Kalfa et al. 2001). Micrographs of polymyxin Btreated *E. coli* showed surface vesicularization (Falagas et al. 2005), which are finger-like membrane protrusions that fragment into small vesicles in ways that are analogous to a Raleigh instability. Similar protrusions are seen in EM images of *E. coli* membranes treated with protegrin-1 (Gidalevitz et al. 2003). Similar effects are qualitatively observed in AFM studies on supported lipid bilayers treated with protegrin-1 (Lam et al. 2006). Moreover, recent work shows that the pore-forming AMP melittin can induce budding by promoting lipid phase separation (Yu et al. 2010). This diversity

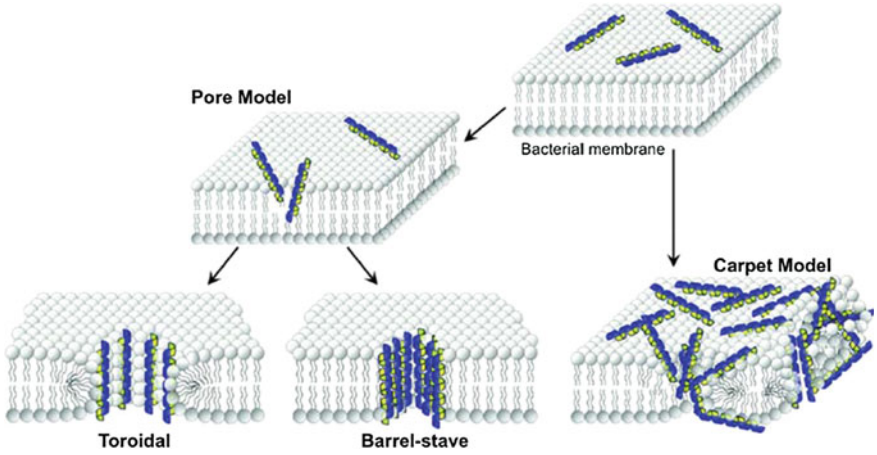

Fig. 10.2 Mechanisms of AMP-induced pore formation in bacterial membranes: "barrel stave" model, "carpet" model, and "toroidal" pore model. Cationic residues of peptides are colored *blue* while hydrophobic residues are *yellow*. Reprinted with permission from Palermo et al. (2013). Copyright © 2013 American Chemical Society

of structural outcomes suggests that there are many ways to destabilize membranes beyond pore formation. AMPs can permeate membranes and compromise barrier function through various processes including blebbing, budding, and vesicularization. Furthermore, the resultant membrane destabilization mechanism is due to interplay between the unique properties of *both* the AMP and membrane. Rather than debate whether a specific AMP uses a particular mechanism, an interesting alternate question to ask is what these different membrane permeation mechanisms (pore formation, blebbing, budding, and vesicularization) have in common.

From the survey on membrane curvature generation above, it can be seen that the biophysics of AMPs and cell membranes can cooperatively lead to a broad range of local membrane distortions, specific combinations of which can be topologically active and lead to membrane destabilization. Induction of positive Gaussian curvature can be seen in the generation of micelles from flat membranes. Negative Gaussian curvature (NGC) seen in lyotropic cubic phases are expected to be especially disruptive since this type of curvature is geometrically required (Gelbart et al. 1994) for pore formation and many known AMP-induced membrane destabilizing processes (Schmidt and Wong 2013; Schmidt et al. 2010) (Fig. 10.3). For example, NGC can be seen in the lining of a transmembrane pore, the neck of a bud, and the base of a bleb.

As a prototypical example, we examine defensins, which are a potent class of membrane-disruptive AMPs that have been extensively studied. In our recent work, we used synchrotron small-angle X-ray scattering (SAXS) to measure the curvature deformation modes induced in model cell membranes of various lipid compositions

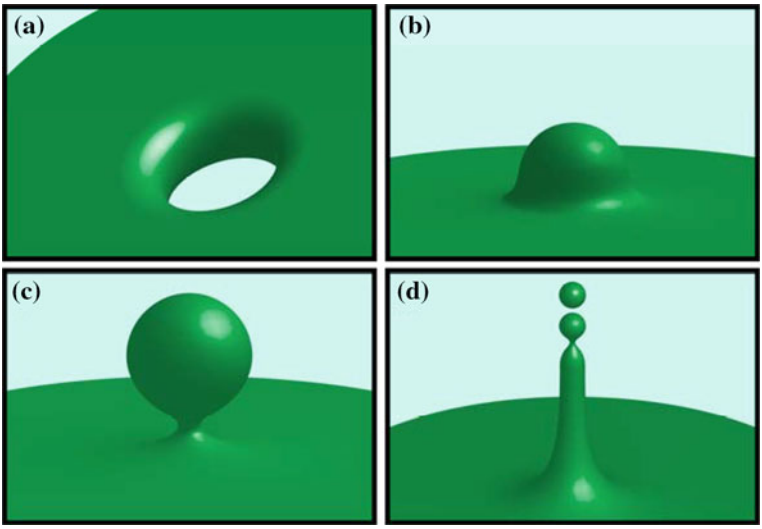

Fig. 10.3 Saddle-splay curvature manifests in different membrane destabilizing processes, such as in the interior of a transmembrane pore (**a**), at the bases of tubules (**b**) and blebs (**c**), and at the necks of vesicularization and budding events (**b**, **d**)

by these AMPs, and elucidate the relationships between induced curvature and membrane disruption processes. In particular, SAXS is used to characterize the phase behavior of the peptide–lipid complexes generated by representative defensins from each of the three defensin families, α-defensins (Crp-4), β-defensins (HBD-2, HBD-3), and θ-defensins (RTD-1, BTD-7). Specifically, we assayed the effects of negative intrinsic curvature lipids on phase behavior, as bacterial membranes generally have higher concentrations of negative intrinsic curvature lipids (PE, CL) compared to eukaryotic cell membranes. These results can be best summarized in phase diagrams (Schmidt et al. 2011). Defensins typically generate NGC in model bacterial membranes rich in negative intrinsic curvature lipid PE. Moreover, when the concentration of PE is decreased, so that the target lipid composition is closer to those of mammalian membranes, this tendency to generate NGC is drastically reduced. These observations are consistent with vesicle leakage assays, and with the known activity profile of defensins, which permeate bacterial membranes but not eukaryotic membranes.

This observed trend of selective NGC generation and permeation in bacterial membranes but not in eukaryotic membranes is quite general, and can be observed in other membrane permeating molecules, natural AMPs, AMP mutants, and synthetic mimics of AMPs (Schmidt and Wong 2013; Schmidt et al. 2011, 2012a, b; Mishra et al. 2011a; Hu et al. 2013; Lee et al. 2014) (and unpublished data). Generation of NGC is also observed in a broad range of CPPs and transporter sequences (Schmidt et al. 2010; Mishra et al. 2008a, 2011b; Zhao et al. 2012). In systems where the activity is strongly determined by membrane permeation, the correlation between NGC generation and mutant activity is striking: AMP defensin mutants (Schmidt et al. 2012a) with decreased activity are correlated with reduced NGC generation across the phase diagram. Circular transporter sequences with increased activity over their linear counterparts also have increased NGC generation (Zhao et al. 2012a).

10.2.3 Composite Mechanisms of Curvature Generation and the Action of AMPs

One interesting question to ask is how AMPs generate NGC. This is a deep question that requires much additional rigorous work beyond what is available today to answer fully. However, in the compass of this review, we can sketch out a qualitative answer based on some of our recent studies.

AMPs are cationic and hydrophobic. Target bacterial membranes are anionic, as previously mentioned. This will lead to a thermodynamic driving force toward membrane wrapping to optimize charge compensation, which allows a large entropy gain from counterion release. Therefore, if considered alone, electrostatic interactions between a cationic AMP and an anionic membrane will generally result in membrane wrapping and negative curvature generation in the target membrane.

Embedding the hydrophobic portions of an AMP into the membrane requires displacing lipids in order to accommodate the peptide sterically. The required amount of displacement will be related to details such as the amount and type of hydrophobicity. In general, however, the above can lead to two consequences. One is membrane thinning, which has been well studied. The other is an increase of hydrophobic volume in the perturbed outer monolayer leaflet (McMahon and Gallop 2005; Campelo et al. 2008; Drin and Antonny 2010). This differential gain in hydrophobic volume of the outer leaflet compared to the inner leaflet has the effect of bending the membrane. When considered in this way, hydrophobic interactions between an amphipathic AMP and a membrane will generally result in positive membrane curvature generation. The ability of AMPs to generate both negative and positive membrane curvatures near the same nanoscopic location (in a manner that does not mutually cancel) is important for its ability to destabilize membranes.

In our recent work, we found that different types of cationic amino acids in AMPs can favor different types of membrane curvatures. Lysine and arginine comprise the majority of cationic amino acids in AMPs, and they can both generate negative curvature with an anionic membrane via membrane wrapping. To understand the differences between lysine and arginine, we used density functional calculations to investigate interactions between phosphates of lipid head groups with guanidinium (side chain of arginine), and compare with the behavior between phosphates and amines (side chain of lysine) (Schmidt et al. 2012b). Differences between these two cationic side chains are the most apparent when multiple side chains are packed close to one another, as they are in AMPs. The guanidinium group can rigidly coordinate two phosphates along the planar Y-shape of the group. By stacking their guanidinium groups "face to face," arginine side chains can maintain a diphosphate coordination. In contrast, the I shape of the amine group along with its monodentate hydrogen-bonding abilities cannot organize phosphates in this way, which results in a significant energetic penalty when amine groups are placed in close proximity. These results suggest that arginines in AMPs can undergo stable bidentate hydrogen bonding with lipid head groups, thereby "cross-linking" lipid head groups into an effectively larger head group area, and thereby generating positive curvature along the peptide chain. This effect is analogous to the molecular crowding mechanism observed when protein aggregation is on the membrane surface, which has been demonstrated by recent studies to induce high membrane curvature (Baumgart et al. 2011; Stachowiak et al. 2010, 2012; Sens et al. 2008). The crowding mechanism creates lateral steric pressure to drive membrane bending and aids in the formation of lipid buds and tubules (Stachowiak et al. 2010, 2012; Farsad and Camilli 2003). Molecular dynamics simulations on the interactions of arginine and lysine homopolymers with lipid membranes showed the above behavior qualitatively (Wu et al. 2013). This positive curvature from lipid head crowding is induced in a direction perpendicular to the negative curvature from electrostatic wrapping. Therefore, arginine produces NGC, in agreement with experiments (Mishra et al. 2008a, 2011b). Lysines, in contrast, can only undergo monodentate hydrogen bonding with lipid head groups, and cannot generate this

additional positive curvature through steric interactions. Lysines therefore only produce negative mean curvature, which is also in agreement with experiments.

The model described above is consistent with a number of empirical observations that are otherwise difficult to explain. We find that increasing the spacing between side chains in guanidinium homopolymers decreases the amount of NGC they can generate, since increasing the average distance between guanidinium groups relieves the stress from head group steric interactions that lead to positive curvature generation (Schmidt et al. 2012b; Mishra et al. 2011b). Furthermore, a critical number of arginines are needed in polyarginine before the peptide can generate NGC; tetraarginine (R4) produced only inverted hexagonal phases with negative mean curvature, and at least five arginine residues are necessary before cubic phases are generated (Mishra et al. 2011b). Taken together, lipid head group organization by arginine and lysine generates distinct types of membrane curvature deformations: Arginine generates NGC (positive and negative curvatures along the two perpendicular principal directions), whereas lysine generates negative curvature along one direction only. Membrane curvature generation based on head group reorganization are inherently sensitive to head group chemistry, therefore such mechanisms can be the basis for specific peptide–lipid interactions.

Many membrane disruption mechanisms require NGC, as noted above. Since AMPs need to disrupt membranes, the topological requirement to generate NGC places constraints on the arginine, lysine, and hydrophobic contents of AMPs. For example, there will be a compositional trade-off between the relative amounts of arginine and lysine plus hydrophobicity used in an AMP sequence. Arginine can generate NGC (both positive and negative curvature in perpendicular directions); lysine generates negative curvature only; hydrophobicity generates positive curvature only. These observations suggest that we may be able to mimic the NGC-generating abilities of arginine using a combination of lysine and hydrophobic amino acids: A decrease in arginine content in an AMP sequence can be offset by an increase in both lysine and hydrophobic content. This trade-off in composition is indeed observed when we analyze the sequences of 1080 cationic AMPs in the antimicrobial peptide database (Schmidt et al. 2011; Wang and Wang 2004). To show that this principle of trade-off is robust and independent of detailed definitions of physical chemical parameters such as hydrophobicity, AMP hydrophobicity was determined using three distinct, widely used hydrophobicity scales: the Kyte–Doolittle scale (Kyte and Doolittle 1982), the Eisenberg Consensus scale (Eisenberg et al. 1982), and the Wimley–White biological scale (Hessa et al. 2005). All three scales show a strong positive trend between the average hydrophobicity and lysine content in AMPs (Schmidt et al. 2011). Interestingly, a similar trend was found for cell-penetrating peptides (CPPs) (Mishra et al. 2011b). For CPPs the same qualitative trade-off is found, but with significantly less hydrophobicity compared to AMPs (Fig. 10.4). We hypothesize that this is related to the shorter membrane residence times of CPPs, since CPPs cross membranes rather than stay on the membrane and kill the cell.

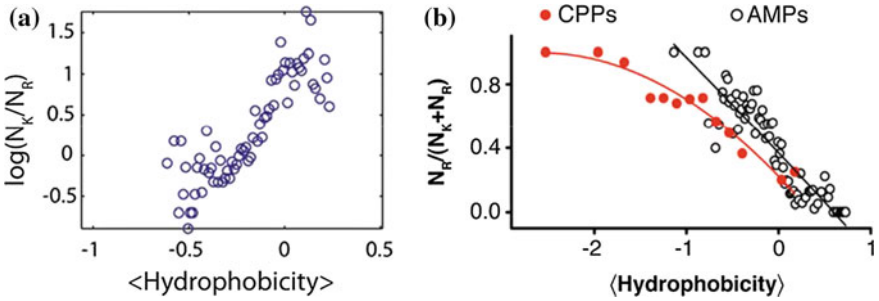

Fig. 10.4 The trade-off between arginine content and lysine plus hydrophobicity content is observed among the sequences of cationic AMPs and CPPs. Generally, as the average hydrophobicity (<Hydrophobicity>) of an AMP or CPP increases, the number of arginines (NR) decreases relative to the number of lysines (NK). **a** Reprinted with permission from Schmidt et al. (2011). Copyright © 2011 American Chemical Society. **b** Reprinted from Mishra et al. (2011b) with permission from Proceedings of the National Academy of Sciences

10.3 Synthetic Mimics to Address Limitations of Natural AMPs

Despite their remarkable antibacterial properties and their low susceptibility to multidrug resistant mechanisms, AMPs suffer from several drawbacks such as susceptibility to proteolysis, toxicity, poor tissue distribution, and cost of production, thus only few AMPs have been found suitable for pharmaceutical applications. To address these limitations, efforts have been made to develop artificial systems mimicking AMPs. Synthetic mimics of AMPs (SMAMPs) consist of a broad family based on AMP properties (e.g., amphiphilicity) but whose backbone is not solely composed of α-amino acids. Scientists have optimized SMAMPs design strategy to improve their stability in physiological conditions, and lower their synthesis cost while ensuring high antimicrobial potency. SMAMPs have been extensively reviewed (Li et al. 2012; Henderson and Lee 2013; Dorner and Lienkamp 2014; Som et al. 2008a; Scott et al. 2008; Rotem and Mor 2009; Giuliani and Rinaldi 2011a; Engler et al. 2012). For the reader's convenience, we will summarize some important facts before focusing on the most recent advances in the design of SMAMPs.

10.3.1 Prerequisites for Antimicrobial Activity

A unique feature of natural AMPs is their amphiphilic secondary structure where hydrophobic and hydrophilic residues are spatially segregated to opposite sides of the α-helical axis such that the nonpolar face interacts with the bilayer core whilst the polar face is engaged in electrostatic interactions with the membrane lipid

headgroups. Many examples in the literature have reported a direct correlation between the membrane-disruptive ability of AMPs and their conformation and it was first thought that a highly defined secondary structure was a crucial prerequisite for antimicrobial activity (Chen et al. 2005; Patch and Barron 2003; Sato and Feix 2006). However, researchers discovered later that peptides of L- and D-amino acids, which did not have α-helical structure, were still highly active (Shai and Oren 2001; Hong et al. 1999; Oren and Shai 1997). While a regular conformation (compared to the structural precision of α-helices or β-sheets) is not absolutely required, a facial amphiphilic structure seems to be critical to obtain high antimicrobial activity. Indeed, their ability to bind membrane depends on a subtle balance of hydrophobicity and charge distribution so they can self-organize to a globally amphiphilic conformation when they are in contact with bacterial membranes.

To be considered as SMAMPs, the synthetic candidates need to demonstrate good selectivity for bacteria over mammalian cells. Because of their positive charges, natural AMPs can selectively discriminate between negatively charged bacterial membranes and mammalian cells that are mostly made of zwitterionic phospholipids (neutral overall charge). The selective toxicity (selectivity) can be quantified by comparing the hemolytic activity to the antimicrobial activity. Thus, the selectivity is defined as the ratio of the hemolytic concentration against erythrocytes (HC_{50}) and the minimum inhibitory concentration (MIC) against bacteria where HC_{50} is the concentration to lyse 50 % of human red blood cells and MIC is the lowest concentration to inhibit bacterial growth.

10.3.2 AMP-like Peptides

Peptide derivatives are the closest, structurally, to natural AMPs, and include aliphatic oligoamides (of which β-peptides are the best known), aromatic oligoamides, peptoids, AA peptides (which are named for their *N*-**a**cylated-*N*-**a**minoethyl amino acid unit), and oligoacyllysines. Due to their similarity to natural peptides, similar synthetic approaches (such as solid phase synthesis) can be used to produce these analogs. Compared to natural peptides, aliphatic oligoamides, aromatic oligoamides, peptoids, AA peptides, and oligoacyllysines may exhibit increased resistance to proteolysis in vivo, prolonging their lifetime and activity. Representative structures are depicted in Fig. 10.5. Each of these classes of peptidomimics has been reviewed (Giuliani and Rinaldi 2011b; Matsuzaki 2009; Som et al. 2008b; Vaara 2009; Mendez-Samperio 2014; Mojsoska and Jensses 2015; Gangloff et al. 2015), therefore only a few recent and outstanding examples are highlighted herein.

Aliphatic oligoamides are constructed from derivatives of amino acids in which the amino group resides on a carbon other than the carbon *alpha* to the carbonyl group (as is the case for natural peptides, α-peptides). For example, β-peptides are constructed from β-amino acids, in which the amino group is bonded to the carbon *beta* to the carbonyl, rather than the carbon *alpha* to the carbonyl (standard amino acids/peptides). Likewise, γ-peptides are constructed from amino acids in which the

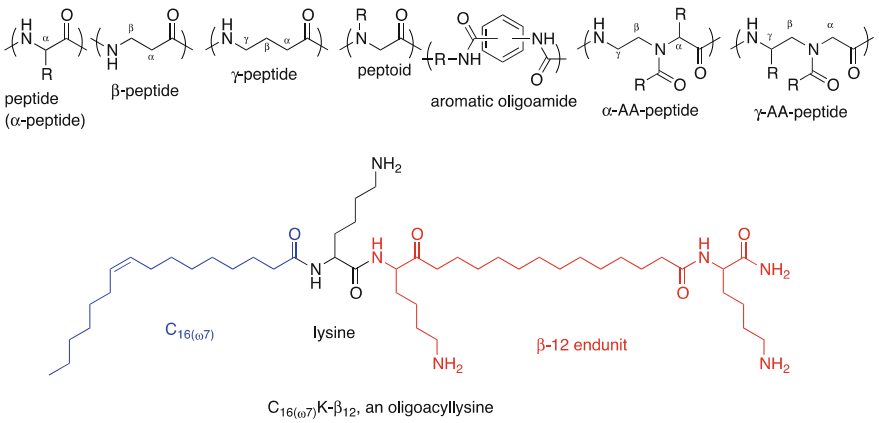

Fig. 10.5 General structures of *α*-peptides, *β*-peptides, *γ*-peptides, aromatic oligoamides, peptoids, *α*-AA peptides and *γ*-AA peptides, and an oligoacyllysine, C16(ω7)K-*β*12

amino group is bonded to the carbon *gamma* to the carbonyl. These aliphatic oligoamides may be homogenous (i.e., all *β*-linkages) or mixed (i.e., *β*-*γ* peptides). Additional carbon atoms are introduced into the peptide backbone in these peptide homologs, which introduces torsional flexibility. Most reported *β*-peptides adopt helical structures, although other structures such as antiparallel hairpin and sheet-like structures have also been reported. The structures of aliphatic oligoamides, like *α*-peptides, are stabilized by intra- and/or interchain hydrogen bonding. Side-chain interactions can also stabilize 3D structures. *β*-peptides are the most widely investigated type of aliphatic oligoamides. In 2000, Gellman's group reported *β*-17, a beta-peptide comprising hydrophobic (*R*, *R*)-*trans*-2-aminocyclopentane carboxylic acid (ACPC) and cationic (*3R,4S*)-trans-4-aminopyrrolidine-3-carboxylic acid (APC) repeat units (Porter et al. 2000). Thus, the structure of *β*-17 mimics the amphipathic, helical structure of natural antimicrobials. Compared to a derivative of magainin, GIGKFLHAAKKFAKAFVAEIMNS-NH_2, *β*-17 exhibited similar activity (defined as comparable MIC and MBC values) against clinical isolates of vancomycin-resistant *E. faecium* and methicillin-resistant *S. aureus*, as well as nonpathogenic laboratory strains of *B. subtilis* and *E. coli*. Furthermore, *β*-17 exhibited lower hemolytic activity compared to the magainin derivative. Since this seminal report, other magainin analogs have been investigated (Liu and DeGrado 2001). While these compounds had similar antimicrobial activity to *β*-17, they also exhibited higher hemolytic activity. In general, *β*-peptides exhibit similar potency to natural AMPs (1 μg/mL range) (Porter et al. 2005). More recently, mixed *α*-/*β*-peptides have been investigated. In one approach, *β*-amino acids substituted at the 2-position with lipophilic side groups were combined with a *C*-terminus *α*-arginine (Hansen et al. 2010). In another approach, the two *β*-amino acids utilized by Gellman, ACPC, and APC were combined with *α*-leucine and *α*-lysine (Schmitt et al. 2004). In both studies, the antimicrobial and hemolytic activity was not very different from earlier *β*-peptide investigations.

Aromatic oligoamides contain aromatic or heteroaromatic rings linked by amide bonds. Similar to aliphatic oligoamides, their 3D structures are stabilized by hydrogen bonds. In contrast to aliphatic oligoamides, the rigid structure of the aromatic group may limit torsional flexibility along the backbone by restricting bond rotation. This conformational restriction allows aromatic oligoamides to recapture the amphipathic secondary structure of natural AMPs. The first oligoarylamides reported were alternating oligomers of 1, 3-phenylene diamine and isophthalic acid (Tew et al. 2002). These oligoarylamides exhibited good antimicrobial activity and good selectivity for prokaryotes over eukaryotes (red blood cells). Additional structural modifications were made to increase the backbone rigidity followed, although the activity of the original phenethylene oligoarylamides was sufficiently high. These oligoarylamides cause changes in permeability of the outer membrane of *E. coli*, although the permeabilization of the inner membrane was more limited (Mensa et al. 2011). Nonetheless, treatment of *E. coli* resulted in activation of genes related to membrane stress, and combined with morphological changes observed by electron microscopy, interaction of the oligoarylamides with the membrane is implicated in their antimicrobial activity.

Peptoids are *N*-substituted oligo/polyglycines; due to their structure, these compounds lack backbone chirality and backbone amide hydrogen bonding. As a result, the 3D structure of peptoids is dictated by the side-chain chemistry. Incorporation of bulky chiral monomers or aromatic side chains allows the formation of a stable α-helical structure (Wu et al. 2001). Several groups investigated the structural requirements for biological activity of peptoids (Nandel and Saini 2007; Fowler and Blackwell 2009; Masip et al. 2005). In 2008, Barron's group reported a library of 15 peptoids that mimic natural helical AMPs. The *N*-side chains in the peptoids included cationic residues analogous to lysine and histidine, hydrophobic residues incorporating phenylethyl, naphthylethyl, methylbutyl, and sec-butyl groups, and an anionic group analogous to glutamic acid. Ten of the peptoids exhibited low MICs against *E. coli* and *B. subtilis*, comparable to a magainin derivative and melittin (Chongsiriwatana et al. 2008). Notably, the peptoids containing negatively charged residues exhibited poor antimicrobial activity, as did shorter sequences. Six of the ten peptoids with MICs comparable to melittin exhibited lower hemolytic activity (greater selectivity for prokaryotes) than melittin. Kirshenbaum compared the antimicrobial activity of linear peptoids to cyclic peptoids, and found that macrocyclization further enhanced peptoid activity (Huang et al. 2012). Generally, peptoids exhibit superior membrane permeability compared to peptides (Kwon and Kodadek 2007).

AA peptides, like aliphatic oligoamides, may be connected at different carbon groups. Indeed, α- and γ-AA peptides have been reported (Ishitsuka et al. 2006; Claudon et al. 2010; Hu et al. 2011; Niu et al. 2011). These peptides have been shown to adopt amphipathic conformations in contact with bacterial membranes, analogous to AMPs, and generally exhibit activity comparable to or exceeding that of magainin. Initial efforts focused on the synthesis of libraries of linear AA peptides. Cyclic AA peptides have also been reported (James et al. 2011; Walsh et al. 2013). Compared to their linear counterparts, the cyclic AA peptides have a more

stable amphipathic structurally, no doubt owing to the more restricted backbone conformation. Interestingly, recent work on homologous linear and cyclic membrane-active peptides suggests that the cyclic topology is more efficient in generating negative Gaussian curvature in PE-rich membranes, which is necessary for membrane permeation, as mentioned above (Zhao et al. 2012b). Generally, the antimicrobial activity of AA peptides is enhanced by increasing hydrophobicity, but this also tends to increase hemolytic activity unless balanced by an increase in cationic residues. Interestingly, Li et al. recently reported a library of AA peptides functionalized with lipid tails (to increase hydrobicity) that exhibit high activity against a broad range of both Gram-positive and Gram-negative bacteria without exhibiting significant hemolytic activity (Li et al. 2014)

Oligoacyllysines (OAKs) comprise alternating acyl chains and cationic amino acids. The originally reported OAKs contained fatty acid chains with either four or eight carbons alternated with a lysine (Radzishevsky et al. 2007). Compared to other peptidomimics, OAKs have a relatively simple structure, and their design deliberately prevents the formation of stable secondary structures. The structure is clearly amphipathic and possesses significant flexibility about the backbone due to the large number of C–C bonds. In the seminal report, only the octanoyl-based OAKs inhibited the growth of *E. coli*. However, modification of *N*-terminal acyl group with a twelve-carbon fatty acid chain (dodecanoyl) not only enhanced the activity of the octanoyl-based OAKs, but also imparted activity to the butyroyl-based OAKs. In addition to inhibiting the growth of bacteria such as *E. coli*, *Acinetobacter*, *Klebsiella*, and *Pseudomonas*, their lead compound $C_{12}K\text{-}7\alpha_8$ (representing dodecyl, lysine, and acyl-linked octanoyl) exhibited virtually no hemolytic activity. Since this initial report, OAKs have evolved to include variations in number of cationic residues, length of fatty acid chain, and unsaturation in the fatty acid chain. A representative example, $C_{16(\omega 7)}K\text{-}\beta_{12}$ is depicted in Fig. 10.5 (Sarig et al. 2008), and exhibits an average MIC of 6 ± 5 μg/mL against a panel of $\sim$50 different bacteria.

10.3.3 AMP-like Polymers

Most peptide synthesis is carried out in the solid phase, which is an expensive technique. This situation led scientists to find alternative routes and move to synthetic polymers with diverse backbones including poly(norbornenes) (Ilker et al. 2004; Altay et al. 2015), poly(methacrylates) (Locock et al. 2014), nylon-3 copolymers (Liu et al. 2015; Chakraborty et al. 2014), polyacrylamide (Palermo et al. 2009), polyolefin (Song et al. 2011), polyvinylpyridines (Sambhy et al. 2008), polyanilines (Gizdavic-Nikolaidis et al. 2011), polycarbonate (Nederberg et al. 2011; Ng et al. 2014; Engler et al. 2013), and poly(vinylether) (Oda et al. 2011). Some excellent reviews have covered the advent of the field of antimicrobial polymers (Dorner and Lienkamp 2014; Palermo and Kuroda 2010; Muñoz-Bonilla and Fernández-García 2012; Kuroda and Caputo 2013).

Contrary to peptide-like oligomers, polymeric SMAMPs have random sequences of hydrophobic and cationic groups along their backbone and a molecular weight distribution. These heterogeneities make them unlikely to form defined secondary conformations but more presumably random coils that self-organize in an irregular yet globally amphiphilic structure when in contact with bacterial membranes. Polymer-based SMAMPs exhibited potent antimicrobial activity suggesting that their activity relies on their physicochemical properties, rather than specific-defined sequence and secondary structure (Dorner and Lienkamp 2014; Palermo et al. 2013).

The molecular and physical properties of synthetic polymers need to be easily tuned to achieve proper amphiphilic balance that would induce good antimicrobial activity and cell selectivity. Nylon-3 polymers offer considerable possibilities in that regard. Nylon-3 polymers are synthetic polyamides with a β-amino acid skeleton similar to the polyamide backbone of proteins and are highly tunable in terms of physicochemical properties (Fig. 10.6). Gellman and coworkers synthesized the first nylon-3 polymer-based SMAMP in 2007 by anionic ring-opening polymerization (AROP) of β-lactams (Mowery et al. 2007). Since then, Gellman's group investigated how tuning the amphiphilicity balance affects the antimicrobial activity and cell selectivity of the polymers (Epand et al. 2008; Zhang et al. 2012; Liu et al. 2012a, b, 2014; Dohm et al. 2010). To do so, the chain length, the structure, and the ratio of hydrophobic and cationic units, and the nature of the *C*- and *N*-terminal were varied. A general trend was observed: increasing the number of hydrophobic units enhances the antimicrobial activity reaching a minimal MIC value after which the MIC goes up again. Indeed, above a certain degree of hydrophobicity, the polymers become less soluble forming aggregates that do not permeate the bacterial membranes but can still insert erythrocytes; so polymers become less active and less selective against bacteria leading to toxicity issues. Kuroda's group reached similar conclusion with random amphiphilic poly (methacrylates) (Kuroda and DeGrado 2005; Kuroda et al. 2009). An optimal balance between hydrophobicity and solubility is crucial to achieve high antimicrobial activity while retaining high selectivity. Most polymer-based SMAMPs are binary copolymers in which one subunit is hydrophobic and the other cationic conferring them higher charge density and higher level of hydrophobicity than

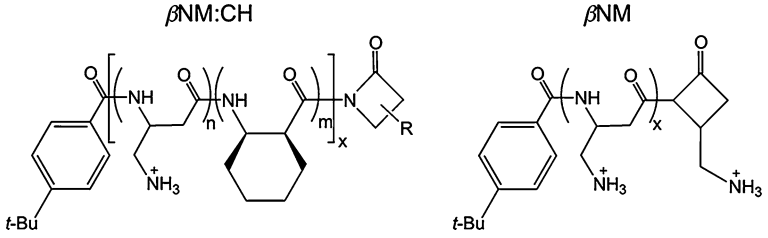

Fig. 10.6 Examples of nylon-3 polymers. R represents the side-chain group for βNM:CH copolymers. Reprinted with permission from Liu et al. (2015). Copyright © 2015 American Chemical Society

natural AMPs. With the intent of mimicking AMPs with higher fidelity, Wong and Gellman have prepared ternary nylon-3 copolymers with either a serine-like or glycine-like subunit (Fig. 10.7) and have shown that partial replacement of the hydrophobic subunit with a "neutral" component reduced hemolysis without affecting the antibacterial activity (Chakraborty et al. 2014). Wong and Gellman's groups also demonstrated that the antibacterial activity of nylon-3 polymers was correlated with two interdependent mechanisms of action: membrane permeation and DNA binding (Lee et al. 2014). Indeed, nylon-3 polymers can induce NGC, which is topologically required for membrane-disruptive process and cellular entry as mentioned above. Nevertheless, their membrane permeation ability was shown to be concentration dependent. At low concentrations, the polymers can cross cell membranes without total membrane disruption and kill bacteria by binding to DNA, while at higher concentrations they can completely disrupt membranes.

Besides hydrophobicity, cationic charges also play a major role in antimicrobial activity and cell selectivity as they allow polymers to bind to the negatively charged bacterial membranes. It has been demonstrated that primary amines are more favorable to confer high antimicrobial properties than secondary or tertiary amines, as they are partially protonated at physiological pH (Palermo and Kuroda 2009; Palermo et al. 2011). Interestingly, SMAMPs with permanently charged quaternary ammonium groups were less or not active suggesting that the chemical structure of the cationic groups, and not uniquely the number of cationic charges, also impacts the complexation with phosphate lipid headgroups by a combination of hydrogen bonding and electrostatic interactions. Although protonoted positively charged amines enhance the initial binding of SMAMPs to the outer leaflet of bacterial cell membranes, it has been speculated that the presence of deprotonated amine groups at physiological pH might facilitate the insertion of the polymers into the inner leaflet of cell membrane (Palermo et al. 2011). However, highly charged polymers may not be suitable for biomedical applications as they seem to favor hemolysis and/or hemagglutination (Palermo and Kuroda 2009; Sovadinova et al. 2011a). In the view of reducing hemolysis, Punia et al. incorporated nonionic and hydrophilic poly(ethylene glycol) (PEG) side chains to acrylic copolymers to reduce hydrophobic interactions of copolymers with red blood cells (RBCs) (Punia et al. 2015). Indeed, PEG is known to interact with RBCs through hydrogen bonds and

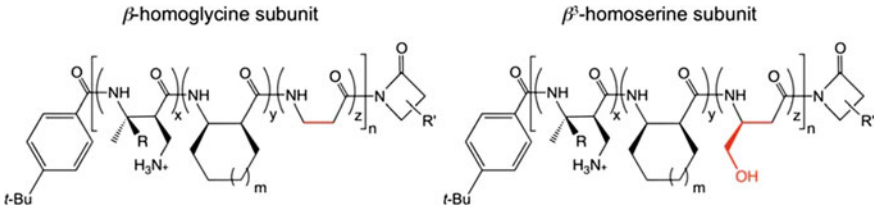

Fig. 10.7 Ternary nylon-3 copolymers that contain either a serine-like or glycine-like subunit, along with a hydrophobic subunit and a cationic subunit. Reproduced with permission from Chakraborty et al. (2014) (http://pubs.acs.org/doi/pdf/10.1021/ja507576a)

weakly adsorb on their surface protecting them from foreign body contact. Thus, a series of copolymers with hexamethyleneamine (6-carbon long lipophilic spacer arm with pendant cationic groups) and PEG side chains were synthesized. While homopolymers of hexamethyleneamine were highly hemolytic, the addition of 33 mol% of hydrophilic PEG side groups led to >1300 times reduction in hemolytic activity while maintaining high antibacterial activity. However, too much PEG induced a drastic loss of antibacterial activity due to the shielding effect of the PEG preventing the hexamethyleneamine units from interacting with the bacterial cell surface.

A number of studies have also investigated the incorporation of functional groups that mimic specific amino acids such as arginine (Budhathoki-Uprety et al. 2012; Locock et al. 2013; Gabriel et al. 2008) and tryptophan (Trp) (Locock et al. 2014) instead of the traditional mimics of lysine (amine-based cationic groups). Trp residue has been identified at high concentrations in various AMPs including indolicidin, tritrpticin, and lactoferrampin and has the unique ability to insert into membranes and associate with the positively charged choline headgroups of the lipid bilayer (Chan et al. 2006). Locock et al. synthesized polymethacrylates with indole pendant groups to mimic tryptophan-rich cationic peptides (Locock et al. 2014). The copolymers combined a Trp-like momoner with either 2-aminoethylmethacrylate as a mimic of lysine or 2-guanidinylmethacrylate as a mimic of arginine (Fig. 10.8). All polymers displayed antibacterial activities against both *Staphylococcus epidermidis* and methicillin-resistant *Staphylococcus aureus* (MRSA). The most potent and the least hemolytic polymers were those with the lowest indole content which could indicate that no lipophilic component is required for potent antimicrobial activity; nevertheless cationic homopolymers with no indole group showed moderate to strong hemagglutination suggesting that a minimal level of hydrophobicity is necessary to achieve low agglutination-based toxicity while retaining high antimicrobial activity and high selectivity. The trend observed here (decreasing level of hydrophobicity giving higher antimicrobial activity) is in contradiction with most studies reported in the literature on this topic indicating that each system is unique and the nature of the cationic and hydrophobic groups ensure varying types and

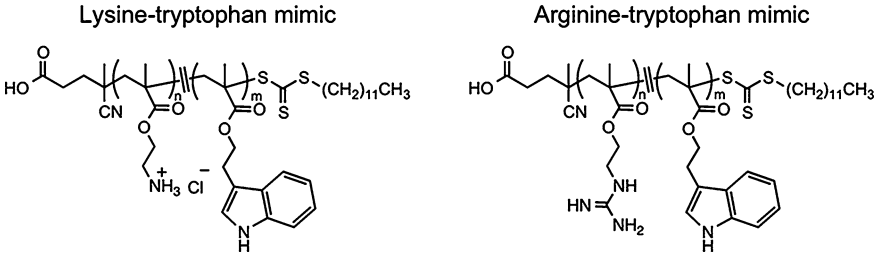

Fig. 10.8 Random copolymers containing amine–indole side chains as lysine-tryptophan mimics and guanidine–indole side chains as arginine–tryptophan mimics. Reprinted with permission from Locock et al. (2014). Copyright © 2014 American Chemical Society

degrees of amphiphilicity and a direct comparison with other polymeric systems is not necessarily relevant.

Clearly, there has been much progress in making synthetic molecules that mimic AMPs. One interesting question to ask is whether there is anything to be gained to move beyond mimesis of AMPs by synthetic molecules. At the beginning of the review, we mentioned that it may be possible to combine the different advantages of AMPs and traditional antibiotics. We describe an example below.

10.4 Engineered Hybrid Antibiotics to Address Limitations of Both AMPs and Traditional Antibiotics

From the discussion above it is apparent that the permeation activity of membrane-active antimicrobials is derived from a fundamental motif of net cationic charge and amphiphilicity, not from conserved core structures. Collectively, it is generally not necessary for natural AMPs and AMP mimics to have specific structural features, or for them to adopt certain conformations, although these characteristics can improve potency and selectivity. Synthetic AMPs can be constructed using a variety of chemistries, and designed with diverse chemical compositions. One consequence of this chemical flexibility is that synthetic AMPs do not have to be solely made of peptides or peptide-like polymers but can also be hybrids. Indeed, synthetic hybrids can be rationally designed by combining two or more entities in order to generate synergistic effects into a single platform and promote desired properties such as better biocompatibility, greater antimicrobial potency, higher bacterial selectivity, and better cellular uptake. This field has been previously reviewed elsewhere (Li et al. 2012). The principal constraint is the antimicrobial which must have the correct proportions of cationic amino acids (arginine and lysine) and hydrophobicity so that it can generate the requisite NGC curvature to compromise the barrier function of bacterial membranes by permeation (also referred to as permeabilization). This raises the exciting possibility that many different molecules can be equipped with membrane activity through chemical modification. In this section we focus on our recent work on developing an aminoglycoside-AMP hybrid and how this approach allows antibiotics to enter bacteria through an entirely new mechanism relative to those used by traditional aminoglycosides.

Most traditional antibiotics such as β-lactams, quinolones, tetracyclines, macrolides, sulphonamides, aminoglycosides, phenicols, do not permeabilize bacterial membranes (Hancock and Bell 1988). β-lactam antibiotics inactivate the series penicillin-binding proteins responsible for building the cell wall (Hancock and Bell 1988; Kohanski et al. 2010). This peptidoglycan network is externally located in Gram-positive bacteria and confined to the periplasm in Gram-negative bacteria. β-lactams, therefore, do not need to cross the cytoplasmic membrane, and they are

believed to reach the periplasm by passing through porins in the outer membranes of Gram-negative bacteria (Hancock and Bell 1988; Pagès et al. 2008; Nikaido 2003). The other classes of antibiotics act on intracellular targets. The tetracyclines, macrolides, aminoglycosides, and phenicols disrupt translation by binding to the ribosome, whereas the quinolones and sulfonamides inhibit DNA synthesis by targeting DNA gyrase, and enzymes responsible for tetrahydrofolic acid synthesis, respectively (Kohanski et al. 2010). All of these drugs must enter the cells to be effective antimicrobials. Like for the β-lactams, the drugs are believed to be transported though the outer membrane by porins (Hancock and Bell 1988; Pagès et al. 2008; Nikaido 2003). While traversing the cytoplasmic membrane is complex and may be accomplished through distinct, parallel systems that can be antibiotic-specific, it is generally accepted that the lipophilic antibiotics can cross directly through cytoplasmic membrane by passive diffusion (Hancock and Bell 1988). The aminoglycosides are polycationic so they cannot simply diffuse through membranes, however, and our understanding of how aminoglycosides cross the cytoplasmic membranes of bacteria is incomplete (Taber et al. 1987; Allison et al. 2011). In general, aminoglycoside uptake is proposed to occur through an active transport mechanism that is energy dependent and relies on the proton motive force (PMF) (Taber et al. 1987; Allison et al. 2011). The polycationicity, action on an intracellular target, and distinct uptake mechanism make aminoglycosides good candidate molecules for "weaponizing" them with the membrane permeation ability of AMPs.

To understand the benefits building aminoglycoside-AMP hybrids it is helpful to compare aminoglycosides and AMPs both in terms of their mechanisms of action and antimicrobial profiles. Aminoglycosides are potent antibiotics that have a specific mechanism of action and a well-defined target. They bind the decoding aminoacyl site on the 16S rRNA component of the 30S ribosomal subunit, which causes mistranslation and inhibition of the bacterial ribosome leading to cell death (Fourmy et al. 1996; Vicens and Westhof 2002). Common bacteria-resistance mechanisms include target modification through posttranscriptional methylation of critical binding positions in the 16S rRNA, and drug modification by aminoglycoside phosphotransferases, acetyltransferases, and adenylytransferases. Plasmid- or chromosomally encoded enzymes are responsible for the modifications (Magnet and Blanchard 2005). Bacteria also limit the concentration of aminoglycosides in cells by energy-dependent drug efflux using pumps, and by reducing uptake across the cytoplasmic membrane (Magnet and Blanchard 2005). Poor uptake into cells is implicated as the reason why aminoglycosides are not active against a wide variety of microbes, including ones considered intrinsically resistant such as anaerobic bacteria (Taber et al. 1987; Davis 1987), as well as persistent bacteria, a slow or nongrowing bacterial phenotype characterized by negligible susceptibility to many clinical antibiotics (Cohen et al. 2013; Lewis 2007; Lewis 2010). As described above, AMPs collectively have broad-spectrum antimicrobial activity and utilize nonspecific interactions to target generic features common to membranes of microbes. Through coevolution with multicellular hosts bacteria have well-developed mechanisms which mitigate the effects of AMPs. Defense mechanisms often involve membrane modification. For example, in response to cationic

peptides, the *staphylocci* decrease the net negative charge of their cell envelope through D-alanylation of cell surface teichoic acids and lysylation of phosphatidylglycerol (Peschel et al. 2001; Li et al. 2007a, b), and a variety of Gram-negative bacteria can modify their cell surface via lipopolysaccharide (LPS) remodeling (Guo et al. 1997; Derzelle et al. 2004; Rebeil et al. 2004). While these responses can reduce the susceptibility of bacteria to AMPs, it is believed that absolute resistance is difficult to attain since this would require large-scale changes in membrane composition (Zasloff 2002; Perron et al. 2006). Indeed, observations from serial passage studies showed bacteria that exhibited a slower upward "creep" in MIC from repeated exposure to synthetic AMPs, whereas resistance to conventional antibiotics occurred at a much faster pace (Sovadinova et al. 2011b). Furthermore, membrane-active antimicrobials may retain activity against persister cells, since an intact membrane is required for cell viability regardless of metabolic state (Hurdle et al. 2011). Despite their broad-spectrum activity, a major drawback of AMPs is that they often display moderate potency in vitro, which can vary widely among AMPs, and different types of bacteria display different degrees of susceptibility (Fjell et al. 2012). In principle, aminoglycoside-AMP hybrid molecules have the ability to overcome the deficiencies of both aminoglycosides and AMPs. Deterministic design of these molecules requires a general methodology for combining their distinct antimicrobial functions without mutual interference.

Using above fundamental principles that govern how peptides generate saddle-splay membrane curvature, we can accelerate our process of antibiotic discovery. We hypothesized that we can impart aminoglycoside antibiotics with membrane permeability by conjugating a peptide sequence so the resulting aminoglycoside–peptide conjugate acts as an AMP. We chose tobramycin because it is a potent aminoglycoside that is used in the clinic. Tobramycin is the standard of care as an aerosol therapy for suppressing infections in the lungs of patients with cystic fibrosis (Döring et al. 2012), and it is among the most common antibiotics impregnated into the bone cements used for arthroplasty revisions in joint implants (Zimmerli et al. 2004). In addition, the sole primary hydroxyl group in tobramycin is amenable to conjugation and this hydroxyl group is not essential for RNA binding (Asensio et al. 2005; Hanessian et al. 1977). The peptide sequence was derived from ANTP penetratin (Derossi et al. 1994) a well-known cationic, amphiphilic cell-penetrating peptide (CPP) (Derossi et al. 1998), which generates saddle-splay curvature (Mishra et al. 2008b, 2011c). CPPs also compromise the barrier function of cell membranes and enter cells, but they are less lytic than AMPs (Mishra et al. 2011c). Furthermore, a number of AMPs including indolicidin, buforin, and tachyplesin are proposed to use membrane activity to cross bacterial membranes and kill bacteria by binding to intracellular targets (Hsu et al. 2005; Park et al. 1998; Yonezawa et al. 1992). We designed a 12-amino-acid peptide, called Pen, and connected it to tobramycin to produce the membrane-active aminoglycoside–peptide conjugate (MAAPC) hybrid, Pentobra (Fig. 10.9a). Small-angle X-ray scattering experiments on small unilamellar vesicles with lipid compositions mimicking bacterial cytoplasmic membranes showed treatment with Pentobra restructured the vesicles into bicontinuous cubic phases, indicating that Pentobra can generate

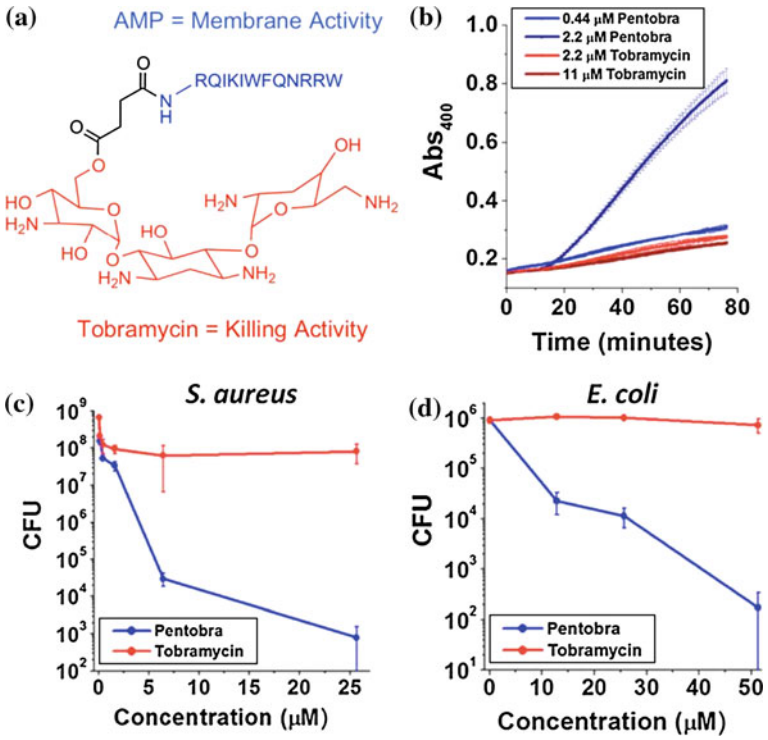

Fig. 10.9 **a** Structure of Pentobra that combines the ribosomal activity of tobramycin and the membrane activity of an AMP. **b** Pentobra permeabilizes *E. coli* ML35 inner membranes. Pentobra displays dose-dependent killing activity against persister cells. **c** *S. aureus* S113 and **d** *E. coli* Dh5 α. Reproduced with permission from Schmidt et al. (2014) (http://pubs.acs.org/doi/pdf/10.1021/nn502201a)

saddle-splay curvature in model bacterial membranes (Schmidt et al. 2014), in a manner similar to AMPs. Conversely, tobramycin could not induce cubic phase formation in vesicles. These results were consistent with permeabilization experiments on bacteria (Schmidt et al. 2014). Single micromolar concentrations of Pentobra readily permeabilized the cytoplasmic membranes of *E. coli*, whereas permeabilization profiles from concentrations of tobramycin well above the MIC (>10×) were indistinguishable from background (Fig. 10.9b). The generation of saddle-splay membrane curvature and permeabilization of bacteria membranes by Pentobra supports the approach of using saddle-splay curvature design rules to renovate existing antibiotics by giving them the additional function of membrane activity.

The ability of Pentobra to permeate bacterial membranes suggests that MAAPCs may have therapeutic value in situations where aminoglycoside antibiotics are not effective. One significant health problem is that bacterial communities almost always contain subpopulations of cells, known as persisters that are not susceptible to conventional antibiotics (Lewis 2007; Balaban et al. 2004; Gefen and Balaban

2009). These bacteria are usually slow growing or nongrowing whose reduced metabolism allows them to survive antibiotic treatment, since most drugs target active growth processes (Lewis 2010). While a small fraction of cells in actively growing cell cultures are persistent, under conditions of limited nutrients and in biofilms the fraction of persistent bacteria can reach a substantial portion of the cell population (Keren et al. 2004). Stationary cultures of *S. aureus* are entirely composed of persisters (Allison et al. 2011; Keren et al. 2004). Once antibiotic treatment has ceased persisters can become active and renew infection (Keren et al. 2004; Bigger 1944), and continued exposure of bacteria to antibiotics increases the likelihood for the emergence of genetic resistance (Lewis 2010; Keren et al. 2004). Drug tolerance is believed to contribute to chronic infections (Lewis 2010; Mulcahy et al. 2010), and there are scarce potential therapeutic strategies against persistent bacteria (Allison et al. 2011; Conlon et al. 2013). To examine whether MAAPCs might have therapeutic value we conducted plate killing assays comparing the activities of Pentobra and tobramycin against persistent bacteria. Remarkably, Pentobra showed dose-dependent bactericidal activity against both model Gram-negative (*E. coli*) (Fig. 10.9c) and Gram-positive (*S. aureus*) (Fig. 10.9d) persisters with reductions reaching 4–6 logs at the highest drug concentrations tested, while equivalent molar concentrations of tobramycin were not bactericidal (Schmidt et al. 2014). Follow-up experiments in our lab indicate that Pentobra outperforms tobramycin to a similar degree against persistent *P. aeruginosa* cells, and Pentobra is superior to both Pen peptide alone and the simple mixture of Pen peptide plus tobramycin (Deshayes et al. unpublished). This last result, in particular, demonstrates the benefit of using drugs that are multifunctional as opposed to cocktails of single function antibiotics for synergistic activity. Moreover, Pentobra is noncytotoxic to eukaryotic cells (Schmidt et al. 2014, 2015b). These results show that equipping aminoglycosides with membrane activity is a promising approach to eradicate pathogenic organisms that are recalcitrant to existing therapies.

Another shortcoming of aminoglycoside antibiotics is that, in vitro, they are not active against anaerobic bacteria. It is hypothesized that the lower PMF across the membranes of anaerobes impairs internalization (Taber et al. 1987; Davis et al. 1986), since aminoglycosides bind the ribosomes of anaerobic bacteria and ones that use oxygen with similar affinities (Bryan et al. 1979). The issue is thought to be drug uptake. To determine if additional membrane activity could expand the activity spectrum of aminoglycosides to anaerobic bacteria we examined the activity of Pentobra against *Propionibacterium acnes* (Wang et al. 1977; Dréno et al. 2004), a major etiological agent in acne (Ross et al. 2003; McInturff et al. 2005) and the dominant pathogen in prosthetic shoulder implant infections (Achermann et al. 2014). Pentobra showed potent bactericidal activity (4–6 log unit decrease in cfu) against *P. acnes* clinical isolates in standard liquid culture killing assays and in assays utilizing human comedone extracts that are more representative of the lipid-rich environment of human skin (Fig. 10.10a–d) (Schmidt et al. 2015b). Conversely, both Pen peptide and tobramycin alone were much less effective. These results suggest that MAAPCs might have therapeutic value against anaerobic pathogens and they have potential as topical antimicrobial agents.

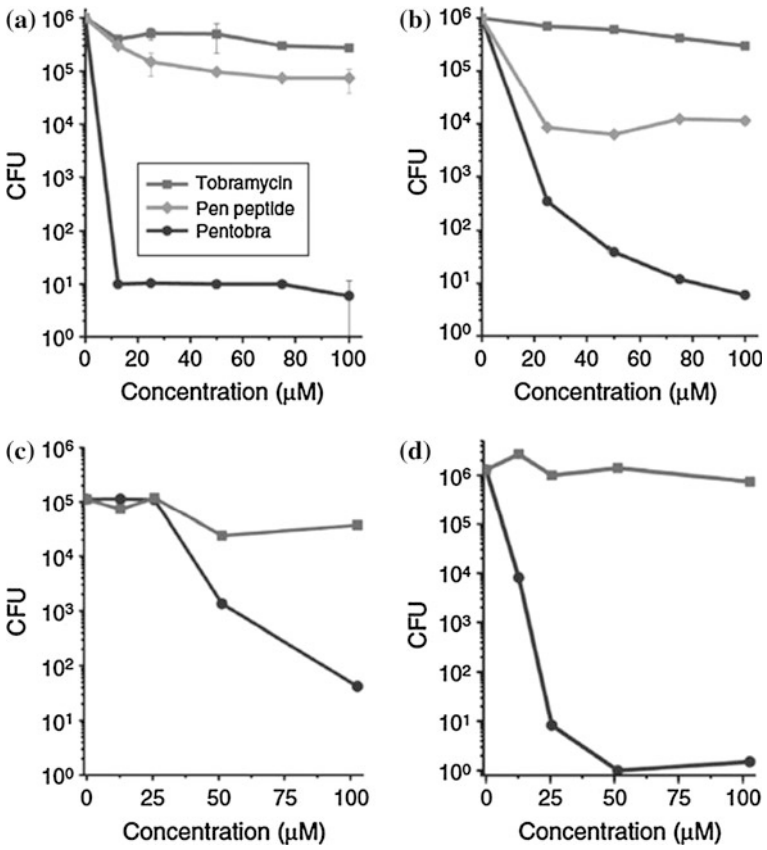

Fig. 10.10 Antimicrobial activity of Pentobra, tobrmaycin, and Pen peptide against *P. acnes* in the microcomedones isolated from (**a**–**d**) four donors' faces using deep cleaning pore strips. Reproduced with permission from Schmidt et al. (2015b). Copyright Elsevier (2015)

10.5 Clinical Development

This section summarizes recent clinical developments in the field of natural and synthetic AMPs. As mentioned above, several drawbacks have hindered the clinical development of AMPs such as poor in vivo activity, poor bioavailability, high cost of manufacturing, low stability, and systemic toxicity. Whereas the development of AMPs for clinical use is challenging, and to date, only nisin, gramicidin, daptomycin, and polymyxins have been granted Food and Drug Administration (FDA) approval (Carmona-Ribeiro and de Melo 2014), many efforts are devoted to accelerate their clinical development in order to face the decline in new antibiotics and the alarming rise in antibiotic resistance. For instance, while pexiganan (a 22-amino-acid synthetic analog of magainin isolated from the skin of the African

Clawed Frog) did not receive FDA approval for the topical treatment of diabetic foot ulcers in 1999, it is currently 50 % enrolled in two Pivotal Phase III clinical studies for the treatment of mild infections of diabetic foot ulcers, thanks to the tenacity of the company Dipexium Pharmaceuticals (ClinicalTrials.gov registration numbers NCT01594762 and NCT01590758). Omiganan (a bovine indolicidin-based peptide) is another example of AMPs that has first failed but has recently reentered in clinical studies. Indeed, the phase III clinical trial of omiganan 1 % gel, named Omigard (as a topical antimicrobial agent for the prevention of catheter-related infections sponsored by Carrus Capital Corporation) has been discontinued because no additional effect was observed over existing therapies. However, Cutanea Life Sciences Inc. has initiated enrolment in a phase II trial for acne vulgaris (NCT02571998) and plans a phase III trial for rosacea (NCT02576860). Other synthetic AMPs have demonstrated promising outcomes in human clinical trials. hLF1-11 (the *N*-terminal peptide of human lactoferrin) has shown efficacy and safety in clinical trials (phase I/II) for the treatment of various infectious (Brouwer et al. 2011) and it has also been tested in the clinic for its immunomodulatory activity (Yeung et al. 2011; van der Does et al. 2010). OP-145 (previously named P60.4Ac), a synthetic 24-mer peptide derived from LL-37, has been proven to be safe and effective in patients with chronic otitis media (chronic middle ear infection) in a clinical phase I/II trial (Malanovic et al. 2015; Peek et al. 2009). LTX-109 (Lytix Biopharma), a topical AMP, has successfully completed phase I/II clinical trials and exhibited safety and potency in the treatment of impetigo and in patients nasally colonized with methicillin-resistant/-sensitive *staphylococcus aureus* (MRSA/MSSA) (Nilsson et al. 2015).

It is worth noting that the majority of AMPs currently in clinical trials are natural or analogs of natural AMPs with all the associated obstacles. However, the new classes of synthetic mimics of AMPs (SMAMPs) have the potential to circumvent many AMP limitations. In contrast to the isolation and/or modification (substitution, deletion, or addition of amino acids) of natural AMPs, SMAMPs are designed with optimized physicochemical characteristics that make them more robust, more active, and more selective. Among the new SMAMPs, the engineered hybrids are very promising at present as they combine multiple functional fragments. For instance, Pentobra leverages the benefits of an AMP with those of a conventional antibiotic while limiting the likelihood for bacteria to acquire resistance.

10.6 Conclusion and Outlook

Unlike conventional antibiotics which pathogens readily evade by resistance mutations, antimicrobial peptides (AMPs) are believed to be less affected by resistance phenomena due to their inherently hybrid mode of action. Indeed, AMPs kill microorganisms by permeating membranes, thus in order for bacteria to acquire systematic resistance against AMPs, they would need to change their whole membrane composition, which is known to be difficult since AMPs have remained effective against bacteria for millenia.

However, a major problem limiting the success of AMPs in clinical application is their susceptibility to proteolytic degradation reducing their activity in vivo. In order to curb this problem and based on the observation that AMP usually adopt an amphiphilic behavior essential for selective disruption and permeabilization of microbial membranes, many artificial systems (SMAMPs) including β-peptides, peptoids, AMP-like polymers, and hybrids have been designed to mimic AMPs with greater stability. To take it to the next level, our recent work suggests that we can identify basic sequence principles for peptides to permeate membranes based on induced curvature, and inform strategies to impart membrane-permeability to nonmembrane-active molecules with diverse functions, as example by our recent design Pentobra, which leverages the benefits of an AMP with those of a conventional antibiotic (tobramycin) and offers unique synergistic antibacterial actions that allow significantly greater potency against recalcitrant bacteria than the parent drug or the mixture of both entities.

References

Achermann Y, Goldstein EJ, Coenye T, Shirtliff ME (2014) Propionibacterium acnes: from commensal to opportunistic biofilm-associated implant pathogen. Clin Microbiol Rev 27(3):419–440

Agerberth B, Lee J-Y, Bergman T, Carlquist M, Boman HG, Mutt V, Jörnvall H (1991) Amino acid sequence of pr-39. Eur J Biochem 202(3):849–854

Allison KR, Brynildsen MP, Collins JJ (2011) Metabolite-enabled eradication of bacterial persisters by aminoglycosides. Nature 473(7346):216–220

Altay E, Yapaöz MA, Keskin B, Yucesan G, Eren T (2015) Influence of alkyl chain length on the surface activity of antibacterial polymers derived from romp. Colloids Surf B 127:73–78

Anguita-Alonso P, Hanssen A, Osmon D, Trampuz A, Steckelberg J, Patel R (2005) High rate of aminoglycoside resistance among staphylococci causing prosthetic joint infection. Clin Orthop Relat Res 439:43–47

Asensio JL, Hidalgo A, Bastida A, Torrado M, Corzana F, Chiara JL, Garcia-Junceda E, Canada J, Jimenez-Barbero J (2005) A simple structural-based approach to prevent aminoglycoside inactivation by bacterial defense proteins. Conformational restriction provides effective protection against neomycin-b nucleotidylation by ant4. J Am Chem Soc 127(23):8278–8279

Balaban NQ, Merrin J, Chait R, Kowalik L, Leibler S (2004) Bacterial persistence as a phenotypic switch. Science 305(5690):1622–1625

Baumgart T, Capraro BR, Zhu C, Das SL (2011) Thermodynamics and mechanics of membrane curvature generation and sensing by proteins and lipids. Annu Rev Phys Chem 62(1):483–506

Bechinger B (2009) Rationalizing the membrane interactions of cationic amphipathic antimicrobial peptides by their molecular shape. Curr Opin Colloid Interface Sci 14(5):349–355

Bechinger B, Kim Y, Chirlian LE, Gesell J, Neumann JM, Montal M, Tomich J, Zasloff M, Opella SJ (1991) Orientations of amphipathic helical peptides in membrane bilayers determined by solid-state nmr spectroscopy. J Biomol NMR 1(2):167–173

Bigger J (1944) Treatment of staphylococcal infections with penicillin by intermittent sterilisation. Lancet 244(6320):497–500

Bowdish DME, Davidson DJ, Hancock REW (2005) A re-evaluation of the role of host defence peptides in mammalian immunity. Curr Protein Pept Sci 6(1):35–51

Brogden KA (2005a) Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat Rev Micro 3(3):238–250

Brogden KA (2005b) Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat Rev Microbiol 3(3):238–250

Brouwer CPJM, Rahman M, Welling MM (2011) Discovery and development of a synthetic peptide derived from lactoferrin for clinical use. Peptides 32(9):1953–1963

Bryan L, Kowand S, Van Den Elzen H (1979) Mechanism of aminoglycoside antibiotic resistance in anaerobic bacteria: clostridium perfringens and bacteroides fragilis. Antimicrob Agents Chemother 15(1):7–13

Budhathoki-Uprety J, Peng L, Melander C, Novak BM (2012) Synthesis of guanidinium functionalized polycarbodiimides and their antibacterial activities. ACS Macro Lett 1(3):370–374

Campelo F, McMahon HT, Kozlov MM (2008) The hydrophobic insertion mechanism of membrane curvature generation by proteins. Biophys J 95(5):2325–2339

Carmona-Ribeiro A, de Melo Carrasco L (2014) Novel formulations for antimicrobial peptides. Int J Mol Sci 15(10):18040

Chakraborty S, Liu R, Hayouka Z, Chen X, Ehrhardt J, Lu Q, Burke E, Yang Y, Weisblum B, Wong GCL, Masters KS, Gellman SH (2014) Ternary nylon-3 copolymers as host-defense peptide mimics: beyond hydrophobic and cationic subunits. J Am Chem Soc 136(41):14530–14535

Chan DI, Prenner EJ, Vogel HJ (2006) Tryptophan- and arginine-rich antimicrobial peptides: structures and mechanisms of action. Biochimica et Biophysica Acta (BBA)-Biomembranes 1758(9):1184–1202

Chen Y, Mant CT, Farmer SW, Hancock REW, Vasil ML, Hodges RS (2005) Rational design of α-helical antimicrobial peptides with enhanced activities and specificity/therapeutic index. J Biol Chem 280(13):12316–12329

Chongsiriwatana NP, Patch JA, Czyzewski AM, Dohm MT, Ivankin A, Gidalevitz D, Zuckermann RN, Barron AE (2008) Peptoids that mimic the structure, function, and mechanism of helical antimicrobial peptides. Proc Natl Acad Sci USA 105(8):2794–2799

Claudon P, Violette A, Lamour K, Decossas M, Fournel S, Heurtault B, Godet J, Mely Y, Jamart-Gregoire B, Averlant-Petit MC, Briand JP, Duportail G, Monteil H, Guichard G (2010) Consequences of isostructural main-chain modifications for the design of antimicrobial foldamers: helical mimics of host-defense peptides based on a heterogeneous amide/urea backbone. Angew Chem Int Ed 49(2):333–336

Cohen NR, Lobritz MA, Collins JJ (2013) Microbial persistence and the road to drug resistance. Cell Host Microbe 13(6):632–642

Conlon BP, Nakayasu ES, Fleck LE, LaFleur MD, Isabella VM, Coleman K, Leonard SN, Smith RD, Adkins JN, Lewis K (2013) Activated clpp kills persisters and eradicates a chronic biofilm infection. Nature 503(7476):365–370

Davis BD (1987) Mechanism of bactericidal action of aminoglycosides. Microbiol Rev 51(3):341

Davis BD, Chen L, Tai PC (1986) Misread protein creates membrane channels: an essential step in the bactericidal action of aminoglycosides. Proc Natl Acad Sci 83(16):6164–6168

Derossi D, Joliot AH, Chassaing G, Prochiantz A (1994) The third helix of the antennapedia homeodomain translocates through biological membranes. J Biol Chem 269(14):10444–10450

Derossi D, Chassaing G, Prochiantz A (1998) Trojan peptides: the penetratin system for intracellular delivery. Trends Cell Biol 8(2):84–87

Derzelle S, Turlin E, Duchaud E, Pages S, Kunst F, Givaudan A, Danchin A (2004) The phop-phoq two-component regulatory system of photorhabdus luminescens is essential for virulence in insects. J Bacteriol 186(5):1270–1279

Deserno M (2009) Membrane elasticity and mediated interactions in continuum theory: a differential geometric approach. In: Faller R, Longo ML, Risbud SH, Jue T (eds) Biomembrane frontiers. Handbook of modern biophysics. Humana Press, pp 41–74

Dohm MT, Mowery BP, Czyzewski AM, Stahl SS, Gellman SH, Barron AE (2010) Biophysical mimicry of lung surfactant protein b by random nylon-3 copolymers. J Am Chem Soc 132(23):7957–7967

Döring G, Flume P, Heijerman H, Elborn JS, Group CS (2012) Treatment of lung infection in patients with cystic fibrosis: current and future strategies. J Cyst Fibros 11(6):461–479

Dorner F, Lienkamp K (2014) Polymer-based synthetic mimics of antimicrobial peptides (smamps)—a new class of nature-inspired antimicrobial agents with low bacterial resistance formation potential. In: Munoz-Bonilla A, Cerrada ML, Fernandez-Garcia M (eds) Polymeric materials with antimicrobial activity: from synthesis to applications, vol 10. R Soc Chem, pp 97–138

Dréno B, Bettoli V, Ochsendorf F, Layton A, Mobacken H, Degreef H (2004) European recommendations on the use of oral antibiotics for acne. Eur J Dermatol 14(6):391–399

Drin G, Antonny B (2010) Amphipathic helices and membrane curvature. FEBS Lett 584(9):1840–1847

Dürr UHN, Sudheendra US, Ramamoorthy A (2006) Ll-37, the only human member of the cathelicidin family of antimicrobial peptides. Biochimica et Biophysica Acta (BBA)-Biomembranes 1758(9):1408–1425

Ehrenstein G, Lecar H (1977) Electrically gated ionic channels in lipid bilayers. Q Rev Biophys 10(01):1–34

Eisenberg D, Weiss RM, Terwilliger TC, Wilcox W (1982) Hydrophobic moments and protein structure. Faraday Symp Chem Soc 17:109–120

Engler AC, Wiradharma N, Ong ZY, Coady DJ, Hedrick JL, Yang Y-Y (2012) Emerging trends in macromolecular antimicrobials to fight multi-drug-resistant infections. Nano Today 7(3):201–222

Engler AC, Tan JPK, Ong ZY, Coady DJ, Ng VWL, Yang YY, Hedrick JL (2013) Antimicrobial polycarbonates: investigating the impact of balancing charge and hydrophobicity using a same-centered polymer approach. Biomacromolecules 14(12):4331–4339

Epand RM, Epand RF (2011) Bacterial membrane lipids in the action of antimicrobial agents. J Pept Sci 17(5):298–305

Epand RF, Mowery BP, Lee SE, Stahl SS, Lehrer RI, Gellman SH, Epand RM (2008) Dual mechanism of bacterial lethality for a cationic sequence-random copolymer that mimics host-defense antimicrobial peptides. J Mol Biol 379(1):38–50

Falagas ME, Kasiakou SK, Saravolatz LD (2005) Colistin: The revival of polymyxins for the management of multidrug-resistant gram-negative bacterial infections. Clin Infect Dis 40(9):1333–1341

Farsad K, Camilli PD (2003) Mechanisms of membrane deformation. Curr Opin Cell Biol 15(4):372–381

Fjell CD, Hiss JA, Hancock RE, Schneider G (2012) Designing antimicrobial peptides: form follows function. Nat Rev Drug Discovery 11(1):37–51

Fourmy D, Recht MI, Blanchard SC, Puglisi JD (1996) Structure of the ä site of escherichia coli 16 s ribosomal rna complexée! With an aminoglycoside antibiotic. Science 274:1367–1371

Fowler SA, Blackwell HE (2009) Structure-function relationships in peptoids: recent advances toward deciphering the structural requirements for biological function. Org Biomol Chem 7(8):1508–1524

Gabriel GJ, Madkour AE, Dabkowski JM, Nelson CF, Nüsslein K, Tew GN (2008) Synthetic mimic of antimicrobial peptide with nonmembrane-disrupting antibacterial properties. Biomacromolecules 9(11):2980–2983

Gangloff N, Ulbricht J, Lorson T, Schlaad H, Luxenhofer R (2015) Peptoids and polypeptoids at the frontier of supra- and macromolecular engineering. Chem Rev (Washington, DC, US)

Ganz T (2003) Defensins: antimicrobial peptides of innate immunity. Nat Rev Immunol 3(9):710–720

Gazit E, Lee W-J, Brey PT, Shai Y (1994) Mode of action of the antibacterial cecropin b2: a spectrofluorometric study. Biochemistry 33(35):10681–10692

Gefen O, Balaban NQ (2009) The importance of being persistent: heterogeneity of bacterial populations under antibiotic stress. FEMS Microbiol Rev 33(4):704–717

Gelbart WM, Ben-Shaul A, Roux D (eds) (1994) Micelles, membranes, microemulsions, and monolayers. Partially ordered systems. Springer

Gidalevitz D, Ishitsuka Y, Muresan AS, Konovalov O, Waring AJ, Lehrer RI, Lee KYC (2003) Interaction of antimicrobial peptide protegrin with biomembranes. Proc Natl Acad Sci 100(11):6302–6307

Giuliani A, Rinaldi A (2011a) Beyond natural antimicrobial peptides: multimeric peptides and other peptidomimetic approaches. Cell Mol Life Sci 68(13):2255–2266

Giuliani A, Rinaldi AC (2011b) Beyond natural antimicrobial peptides: multimeric peptides and other peptidomimetic approaches. Cell Mol Life Sci 68(13):2255–2266

Gizdavic-Nikolaidis MR, Bennett JR, Swift S, Easteal AJ, Ambrose M (2011) Broad spectrum antimicrobial activity of functionalized polyanilines. Acta Biomater 7(12):4204–4209

Guo L, Lim KB, Gunn JS, Bainbridge B, Darveau RP, Hackett M, Miller SI (1997) Regulation of lipid a modifications by salmonella typhimurium virulence genes phop-phoq. Science 276(5310):250–253178

Hancock R, Bell A (1988) Antibiotic uptake into gram-negative bacteria. Eur J Clin Microbiol Infect Dis 7(6):713–720

Hancock REW, Lehrer R (1998) Cationic peptides: a new source of antibiotics. Trends Biotechnol 16(2):82–88

Hancock REW, Sahl H-G (2006) Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. Nat Biotech 24(12):1551–1557

Hanessian S, Masse R, Capmeau M-L (1977) Aminoglycoside antibiotics: synthesis of 5"-amino-5"-deoxyneomycin and 5"-amino-5"-deoxyparomomycin. J Antibiot 30(10):893–896

Hansen T, Alst T, Havelkova M, Strom MB (2010) Antimicrobial activity of small beta-peptidomimetics based on the pharmacophore model of short cationic antimicrobial peptides. J Med Chem 53(2):595–606

Helfrich W (1973) Elastic properties of lipid bilayers: theory and possible experiments. Zeitschrift für Naturforschung C 28(11):693–703

Henderson MJ, Lee KYC (2013) Promising antimicrobial agents designed from natural peptide templates. Curr Opin Solid State Mater Sci 17(4):175–192

Hessa T, Kim H, Bihlmaier K, Lundin C, Boekel J, Andersson H, Nilsson I, White SH, von Heijne G (2005) Recognition of transmembrane helices by the endoplasmic reticulum translocon. Nature 433(7024):377–381

Hong J, Oren Z, Shai Y (1999) Structure and organization of hemolytic and nonhemolytic diastereomers of antimicrobial peptides in membranes. Biochemistry 38(51):16963–16973

Hsu C-H, Chen C, Jou M-L, Lee AY-L, Lin Y-C, Yu Y-P, Huang W-T, Wu S-H (2005) Structural and DNA-binding studies on the bovine antimicrobial peptide, indolicidin: evidence for multiple conformations involved in binding to membranes and DNA. Nucleic Acids Res 33(13):4053–4064

Hu YG, Li XL, Sebti SM, Chen JD, Cai JF (2011) Design and synthesis of aapeptides: a new class of peptide mimics. Bioorg Med Chem Lett 21(5):1469–1471

Hu K, Schmidt NW, Zhu R, Jiang Y, Lai GH, Wei G, Palermo EF, Kuroda K, Wong GCL, Yang L (2013) A critical evaluation of random copolymer mimesis of homogeneous antimicrobial peptides. Macromolecules 46(5):1908–1915

Huang HW (2000) Action of antimicrobial peptides: two-state model. Biochemistry 39(29): 8347–8352

Huang ML, Shin SBY, Benson MA, Torres VJ, Kirshenbaum K (2012) A comparison of linear and cyclic peptoid oligomers as potent antimicrobial agents. ChemMedChem 7(1):114–122

Hurdle JG, O'Neill AJ, Chopra I, Lee RE (2011) Targeting bacterial membrane function: an underexploited mechanism for treating persistent infections. Nat Rev Microbiol 9(1):62–75

Ilker MF, Nüsslein K, Tew GN, Coughlin EB (2004) Tuning the hemolytic and antibacterial activities of amphiphilic polynorbornene derivatives. J Am Chem Soc 126(48):15870–15875

Ishitsuka Y, Arnt L, Majewski J, Frey SL, Ratajczak M, Kjaer K, Tew GN (2006) Lee KYC (2008) Amphiphilic poly(phenyleneethynylene)s can mimic antimicrobial peptide membrane disordering effect by membrane insertion (vol 128, p 13123). J Am Chem Soc 130(7):2372

Israelachvili JN, Marčelja S, Horn RG (1980) Physical principles of membrane organization. Q Rev Biophys 13(02):121–200

James WH, Buchanan EG, Muller CW, Dean JC, Kosenkov D, Slipchenko LV, Guo L, Reidenbach AG, Gellman SH, Zwier TS (2011) Evolution of amide stacking in larger gamma-peptides: triamide h-bonded cycles. J Phys Chem A 115(47):13783–13798

Jenssen H, Hamill P, Hancock REW (2006) Peptide antimicrobial agents. Clin Microbiol Rev 19(3):491–511

Kalfa VC, Jia HP, Kunkle RA, McCray PB, Tack BF, Brogden KA (2001) Congeners of smap29 kill ovine pathogens and induce ultrastructural damage in bacterial cells. Antimicrob Agents Chemother 45(11):3256–3261

Keren I, Kaldalu N, Spoering A, Wang Y, Lewis K (2004) Persister cells and tolerance to antimicrobials. FEMS Microbiol Lett 230(1):13–18

Kohanski MA, Dwyer DJ, Collins JJ (2010) How antibiotics kill bacteria: from targets to networks. Nat Rev Microbiol 8(6):423–435

Kokryakov VN, Harwig SSL, Panyutich EA, Shevchenko AA, Aleshina GM, Shamova OV, Korneva HA, Lehrer RI (1993) Protegrins: leukocyte antimicrobial peptides that combine features of corticostatic defensins and tachyplesins. FEBS Lett 327(2):231–236

Kuroda K, Caputo GA (2013) Antimicrobial polymers as synthetic mimics of host-defense peptides. Wiley Interdiscip Rev Nanomed Nanobiotechnol 5(1):49–66

Kuroda K, DeGrado WF (2005) Amphiphilic polymethacrylate derivatives as antimicrobial agents. J Am Chem Soc 127(12):4128–4129

Kuroda K, Caputo GA, DeGrado WF (2009) The role of hydrophobicity in the antimicrobial and hemolytic activities of polymethacrylate derivatives. Chem Eur J 15(5):1123–1133

Kwon YU, Kodadek T (2007) Quantitative evaluation of the relative cell permeability of peptoids and peptides. J Am Chem Soc 129(6):1508-+

Kyte J, Doolittle RF (1982) A simple method for displaying the hydropathic character of a protein. J Mol Biol 157(1):105–132

Lam KLH, Ishitsuka Y, Cheng Y, Chien K, Waring AJ, Lehrer RI, Lee KYC (2006) Mechanism of supported membrane disruption by antimicrobial peptide protegrin-1. J Phys Chem B 110(42):21282–21286

Lande R, Gregorio J, Facchinetti V, Chatterjee B, Wang Y-H, Homey B, Cao W, Wang Y-H, Su B, Nestle FO, Zal T, Mellman I, Schroder J-M, Liu Y-J, Gilliet M (2007) Plasmacytoid dendritic cells sense self-DNA coupled with antimicrobial peptide. Nature 449(7162):564–569

Lande R, Ganguly D, Facchinetti V, Frasca L, Conrad C, Gregorio J, Meller S, Chamilos G, Sebasigari R, Riccieri V, Bassett R, Amuro H, Fukuhara S, Ito T, Liu Y-J, Gilliet M (2011) Neutrophils activate plasmacytoid dendritic cells by releasing self-DNA–peptide complexes in systemic lupus erythematosus. Sci Trans Med 3(73):73ra19–73ra19

Lee MW, Chakraborty S, Schmidt NW, Murgai R, Gellman SH, Wong GCL (2014) Two interdependent mechanisms of antimicrobial activity allow for efficient killing in nylon-3-based polymeric mimics of innate immunity peptides. Biochimica et Biophysica Acta (BBA)-Biomembranes 1838(9):2269–2279

Lehrer RI (2004) Primate defensins. Nat Rev Micro 2(9):727–738

Lewis K (2007) Persister cells, dormancy and infectious disease. Nat Rev Microbiol 5(1):48–56

Lewis K (2010) Persister cells. Annu Rev Microbiol 64:357–372

Li M, Lai Y, Villaruz AE, Cha DJ, Sturdevant DE, Otto M (2007a) Gram-positive three-component antimicrobial peptide-sensing system. Proc Natl Acad Sci 104(22):9469–9474

Li M, Cha DJ, Lai Y, Villaruz AE, Sturdevant DE, Otto M (2007b) The antimicrobial peptide-sensing system aps of staphylococcus aureus. Mol Microbiol 66(5):1136–1147

Li P, Li X, Saravanan R, Li CM, Leong SSJ (2012) Antimicrobial macromolecules: synthesis methods and future applications. RSC Adv 2(10):4031–4044

Li YQ, Smith C, Wu HF, Teng P, Shi Y, Padhee S, Jones T, Nguyen AM, Cao CH, Yin H, Cai JF (2014) Short antimicrobial lipo-alpha/gamma-aa hybrid peptides. ChemBioChem 15(15):2275–2280

Liu DH, DeGrado WF (2001) De novo design, synthesis, and characterization of antimicrobial beta-peptides. J Am Chem Soc 123(31):7553–7559

Liu R, Masters KS, Gellman SH (2012a) Polymer chain length effects on fibroblast attachment on nylon-3-modified surfaces. Biomacromolecules 13(4):1100–1105

Liu R, Vang KZ, Kreeger PK, Gellman SH, Masters KS (2012b) Experimental and computational analysis of cellular interactions with nylon-3-bearing substrates. J Biomed Mater Res Part A 100A(10):2750–2759

Liu R, Chen X, Chakraborty S, Lemke JJ, Hayouka Z, Chow C, Welch RA, Weisblum B, Masters KS, Gellman SH (2014) Tuning the biological activity profile of antibacterial polymers via subunit substitution pattern. J Am Chem Soc 136(11):4410–4418

Liu R, Chen X, Falk SP, Masters KS, Weisblum B, Gellman SH (2015) Nylon-3 polymers active against drug-resistant candida albicans biofilms. J Am Chem Soc 137(6):2183–2186

Ljunggren S, Eriksson JC (1992) Minimal surfaces and winsor iii microemulsions. Langmuir 8(5):1300–1306

Locock KES, Michl TD, Valentin JDP, Vasilev K, Hayball JD, Qu Y, Traven A, Griesser HJ, Meagher L, Haeussler M (2013) Guanylated polymethacrylates: a class of potent antimicrobial polymers with low hemolytic activity. Biomacromolecules 14(11):4021–4031

Locock KES, Michl TD, Stevens N, Hayball JD, Vasilev K, Postma A, Griesser HJ, Meagher L, Haeussler M (2014) Antimicrobial polymethacrylates synthesized as mimics of tryptophan-rich cationic peptides. ACS Macro Letters 3(4):319–323

Ludtke SJ, He K, Heller WT, Harroun TA, Yang L, Huang HW (1996) Membrane pores induced by magainin. Biochemistry 35(43):13723–13728

Magnet S, Blanchard JS (2005) Molecular insights into aminoglycoside action and resistance. Chem Rev (Washington, DC, US) 105(2):477–498

Malanovic N, Leber R, Schmuck M, Kriechbaum M, Cordfunke RA, Drijfhout JW, de Breij A, Nibbering PH, Kolb D, Lohner K (2015) Phospholipid-driven differences determine the action of the synthetic antimicrobial peptide op-145 on gram-positive bacterial and mammalian membrane model systems. Biochimica et Biophysica Acta (BBA)-Biomembranes 1848(10, Part A):2437–2447

Masip I, Perez-Paya E, Messeguer A (2005) Peptoids as source of compounds eliciting antibacterial activity. Comb Chem High Throughput Screening 8(3):235–239

Matsuzaki K (1999) Why and how are peptide–lipid interactions utilized for self-defense? Magainins and tachyplesins as archetypes. Biochimica et Biophysica Acta (BBA)-Biomembranes 1462(1–2):1–10

Matsuzaki K (2009) Control of cell selectivity of antimicrobial peptides. Biochimica Et Biophysica Acta-Biomembranes 1788(8):1687–1692

Matsuzaki K, Murase O, Tokuda H, Funakoshi S, Fujii N, Miyajima K (1994) Orientational and aggregational states of magainin 2 in phospholipid bilayers. Biochemistry 33(11):3342–3349

Matsuzaki K, Murase O, Fujii N, Miyajima K (1996) An antimicrobial peptide, magainin 2, induced rapid flip-flop of phospholipids coupled with pore formation and peptide translocation. Biochemistry 35(35):11361–11368

Matsuzaki K, Sugishita K, Harada M, Fujii N, Miyajima K (1997) Interactions of an antimicrobial peptide, magainin 2, with outer and inner membranes of gram-negative bacteria. Biochimica et Biophysica Acta (BBA)-Biomembranes 1327(1):119–130

Matsuzaki K, Sugishita K-I, Ishibe N, Ueha M, Nakata S, Miyajima K, Epand RM (1998) Relationship of membrane curvature to the formation of pores by magainin 2. Biochemistry 37(34):11856–11863

McInturff JE, Wang S-J, Machleidt T, Lin TR, Oren A, Hertz CJ, Krutzik SR, Hart S, Zeh K, Anderson DH, Gallo RL, Modlin RL, Kim J (2005) Granulysin-derived peptides demonstrate antimicrobial and anti-inflammatory effects against propionibacterium acnes. J Invest Dermatol 125(2):256–263

McMahon HT, Gallop JL (2005) Membrane curvature and mechanisms of dynamic cell membrane remodelling. Nature 438(7068):590–596

Mendez-Samperio P (2014) Peptidomimetics as a new generation of antimicrobial agents: current progress. Infect Drug Resist 7:229–237

Mensa B, Kim YH, Choi S, Scott R, Caputo GA, DeGrado WF (2011) Antibacterial mechanism of action of arylamide foldamers. Antimicrob Agents Chemother 55(11):5043–5053

Mingeot-Leclercq M-P, Glupczynski Y, Tulkens PM (1999) Aminoglycosides: Activity and resistance. Antimicrob Agents Chemother 43(4):727–737

Mishra A, Gordon VD, Yang L, Coridan R, Wong GCL (2008a) Hiv tat forms pores in membranes by inducing saddle-splay curvature: potential role of bidentate hydrogen bonding. Angew Chem Int Ed 47(16):2986–2989

Mishra A, Gordon VD, Yang L, Coridan R, Wong GC (2008b) Hiv tat forms pores in membranes by inducing saddle-splay curvature: potential role of bidentate hydrogen bonding. Angew Chem Int Ed 47(16):2986–2989

Mishra A, Tai KP, Schmidt NW, Ouellette AJ, Wong GCL (2011a) Chapter four—small-angle X-ray scattering studies of peptide–lipid interactions using the mouse paneth cell α-defensin cryptdin-4. In: Michael L, Johnson JMH, Gary KA (eds) Methods in enzymology, vol 492. Academic Press, pp 127–149

Mishra A, Lai GH, Schmidt NW, Sun VZ, Rodriguez AR, Tong R, Tang L, Cheng J, Deming TJ, Kamei DT, Wong GCL (2011b) Translocation of hiv tat peptide and analogues induced by multiplexed membrane and cytoskeletal interactions. Proc Natl Acad Sci 108(41):16883–16888

Mishra A, Lai GH, Schmidt NW, Sun VZ, Rodriguez AR, Tong R, Tang L, Cheng J, Deming TJ, Kamei DT (2011c) Translocation of hiv tat peptide and analogues induced by multiplexed membrane and cytoskeletal interactions. Proc Natl Acad Sci 108(41):16883–16888

Mojsoska B, Jensses H (2015) Peptides and peptidomimetics for antimicrobial drug design. Pharmaceuticals 8:366–415

Mowery BP, Lee SE, Kissounko DA, Epand RF, Epand RM, Weisblum B, Stahl SS, Gellman SH (2007) Mimicry of antimicrobial host-defense peptides by random copolymers. J Am Chem Soc 129(50):15474–15476

Mulcahy LR, Burns JL, Lory S, Lewis K (2010) Emergence of pseudomonas aeruginosa strains producing high levels of persister cells in patients with cystic fibrosis. J Bacteriol 192(23):6191–6199

Muñoz-Bonilla A, Fernández-García M (2012) Polymeric materials with antimicrobial activity. Prog Polym Sci 37(2):281–339

Nandel FS, Saini A (2007) Conformational study of short peptoid models for future applications as potent antimicrobial compounds. Macromol Theory Simul 16(3):295–303

Nederberg F, Zhang Y, Tan JPK, Xu K, Wang H, Yang C, Gao S, Guo XD, Fukushima K, Li L, Hedrick JL, Yang Y-Y (2011) Biodegradable nanostructures with selective lysis of microbial membranes. Nat Chem 3(5):409–414

Ng VWL, Tan JPK, Leong J, Voo ZX, Hedrick JL, Yang YY (2014) Antimicrobial polycarbonates: investigating the impact of nitrogen-containing heterocycles as quaternizing agents. Macromolecules 47(4):1285–1291

Nikaido H (2003) Molecular basis of bacterial outer membrane permeability revisited. Microbiol Mol Biol Rev 67(4):593–656

Nilsson AC, Janson H, Wold H, Fugelli A, Andersson K, Håkangård C, Olsson P, Olsen WM (2015) Ltx-109 is a novel agent for nasal decolonization of methicillin-resistant and-sensitive staphylococcus aureus. Antimicrob Agents Chemother 59(1):145–151

Niu YH, Hu YG, Li XL, Chen JD, Cai JF (2011) Gamma-aapeptides: design, synthesis and evaluation. New J Chem 35(3):542–545

Oda Y, Kanaoka S, Sato T, Aoshima S, Kuroda K (2011) Block versus random amphiphilic copolymers as antibacterial agents. Biomacromolecules 12(10):3581–3591

Oren Z, Shai Y (1997) Selective lysis of bacteria but not mammalian cells by diastereomers of melittin: structure—function study. Biochemistry 36(7):1826–1835

Pagès J-M, James CE, Winterhalter M (2008) The porin and the permeating antibiotic: a selective diffusion barrier in gram-negative bacteria. Nat Rev Microbiol 6(12):893–903

Palermo EF, Kuroda K (2009) Chemical structure of cationic groups in amphiphilic polymethacrylates modulates the antimicrobial and hemolytic activities. Biomacromolecules 10(6):1416–1428

Palermo E, Kuroda K (2010) Structural determinants of antimicrobial activity in polymers which mimic host defense peptides. Appl Microbiol Biotechnol 87(5):1605–1615

Palermo EF, Sovadinova I, Kuroda K (2009) Structural determinants of antimicrobial activity and biocompatibility in membrane-disrupting methacrylamide random copolymers. Biomacromolecules 10(11):3098–3107

Palermo EF, Lee D-K, Ramamoorthy A, Kuroda K (2011) Role of cationic group structure in membrane binding and disruption by amphiphilic copolymers. J Phys Chem B 115(2):366–375

Palermo EF, Vemparala S, Kuroda K (2013) Antimicrobial polymers: Molecular design as synthetic mimics of host-defense peptides. In: Scholz C, Kressler J (eds) Tailored polymer architectures for pharmaceutical and biomedical applications, vol 1135. Acs symposium series, vol 1135. Am Chem Soc, pp 319–330

Park CB, Kim HS, Kim SC (1998) Mechanism of action of the antimicrobial peptide buforin ii: buforin ii kills microorganisms by penetrating the cell membrane and inhibiting cellular functions. Biochem Biophys Res Commun 244(1):253–257

Patch JA, Barron AE (2003) Helical peptoid mimics of magainin-2 amide. J Am Chem Soc 125(40):12092–12093

Peek F, Nell MJ, Brand R, Jansen-Werkhoven T, Van Hoogdalem E, Frijns J (2009) Double-blind placebo-controlled study of the novel peptide drug p60.4ac in cronic middle ear infection. ICAAC:L1–L337

Perron GG, Zasloff M, Bell G (2006) Experimental evolution of resistance to an antimicrobial peptide. Proc R Soc Lond B Biol Sci 273(1583):251–256

Peschel A, Jack RW, Otto M, Collins LV, Staubitz P, Nicholson G, Kalbacher H, Nieuwenhuizen WF, Jung G, Tarkowski A (2001) Staphylococcus aureus resistance to human defensins and evasion of neutrophil killing via the novel virulence factor mprf is based on modification of membrane lipids with l-lysine. J Exp Med 193(9):1067–1076

Porter EA, Wang XF, Lee HS, Weisblum B, Gellman SH (2000) Antibiotics-non-haemolytic beta-amino-acid oligomers. Nature 404(6778):565

Porter EA, Weisblum B, Gellman SH (2005) Use of parallel synthesis to probe structure-activity relationships among 12-helical beta-peptides: evidence of a limit on antimicrobial activity. J Am Chem Soc 127(32):11516–11529

Pouny Y, Rapaport D, Mor A, Nicolas P, Shai Y (1992) Interaction of antimicrobial dermaseptin and its fluorescently labeled analogs with phospholipid membranes. Biochemistry 31(49):12416–12423

Punia A, Mancuso A, Banerjee P, Yang N-L (2015) Nonhemolytic and antibacterial acrylic copolymers with hexamethyleneamine and poly(ethylene glycol) side chains. ACS Macro Lett 4(4):426–430

Radzishevsky IS, Rotem S, Bourdetsky D, Navon-Venezia S, Carmeli Y, Mor A (2007) Improved antimicrobial peptides based on acyl-lysine oligomers. Nat Biotechnol 25(6):657–659

Rebeil R, Ernst RK, Gowen BB, Miller SI, Hinnebusch BJ (2004) Variation in lipid a structure in the pathogenic yersiniae. Mol Microbiol 52(5):1363–1373

Ross J, Snelling A, Carnegie E, Coates P, Cunliffe W, Bettoli V, Tosti G, Katsambas A, Pulgar Galvan Perez Del, Rollman O (2003) Antibiotic-resistant acne: lessons from europe. Brit J Dermatol 148(3):467–478

Rotem S, Mor A (2009) Antimicrobial peptide mimics for improved therapeutic properties. Biochimica et Biophysica Acta (BBA)-Biomembranes 1788(8):1582–1592

Saiman L, Tabibi S, Starner TD, San Gabriel P, Winokur PL, Jia HP, McCray PB, Tack BF (2001) Cathelicidin peptides inhibit multiply antibiotic-resistant pathogens from patients with cystic fibrosis. Antimicrob Agents Chemother 45(10):2838–2844

Sambhy V, Peterson BR, Sen A (2008) Antibacterial and hemolytic activities of pyridinium polymers as a function of the spatial relationship between the positive charge and the pendant alkyl tail. Angew Chem Int Ed 47(7):1250–1254

Sarig H, Rotem S, Ziserman L, Danino D, Mor A (2008) Impact of self-assembly properties on antibacterial activity of short acyl-lysine oligomers. Antimicrob Agents Chemother 52(12):4308–4314

Sato H, Feix JB (2006) Peptide–membrane interactions and mechanisms of membrane destruction by amphipathic α-helical antimicrobial peptides. Biochimica et Biophysica Acta (BBA)-Biomembranes 1758(9):1245–1256

Schauber J, Gallo RL (2008) Antimicrobial peptides and the skin immune defense system. J Allergy Clin Immunol 122(2):261–266

Schmidt NW, Wong GCL (2013) Antimicrobial peptides and induced membrane curvature: geometry, coordination chemistry, and molecular engineering. Curr Opin Solid State Mater Sci 17(4):151–163

Schmidt N, Mishra A, Lai GH, Wong GCL (2010) Arginine-rich cell-penetrating peptides. FEBS Lett 584(9):1806–1813

Schmidt NW, Mishra A, Lai GH, Davis M, Sanders LK, Tran D, Garcia A, Tai KP, McCray PB, Ouellette AJ, Selsted ME, Wong GCL (2011) Criterion for amino acid composition of defensins and antimicrobial peptides based on geometry of membrane destabilization. J Am Chem Soc 133(17):6720–6727

Schmidt NW, Tai KP, Kamdar K, Mishra A, Lai GH, Zhao K, Ouellette AJ, Wong GCL (2012a) Arginine in α-defensins: differential effects on bactericidal activity correspond to geometry of membrane curvature generation and peptide-lipid phase behavior. J Biol Chem 287(26):21866–21872

Schmidt NW, Lis M, Zhao K, Lai GH, Alexandrova AN, Tew GN, Wong GCL (2012b) Molecular basis for nanoscopic membrane curvature generation from quantum mechanical models and synthetic transporter sequences. J Am Chem Soc 134(46):19207–19216

Schmidt NW, Deshayes S, Hawker S, Blacker A, Kasko AM, Wong GC (2014) Engineering persister-specific antibiotics with synergistic antimicrobial functions. ACS Nano 8 (9):8786–8793

Schmidt NW, Jin F, Lande R, Curk T, Xian W, Lee C, Frasca L, Frenkel D, Dobnikar J, Gilliet M, Wong GCL (2015a) Liquid-crystalline ordering of antimicrobial peptide-DNA complexes controls tlr9 activation. Nat Mater 14(7):696–700

Schmidt NW, Agak GW, Deshayes S, Yu Y, Blacker A, Champer J, Xian W, Kasko AM, Kim J, Wong GC (2015b) Pentobra: a potent antibiotic with multiple layers of selective antimicrobial mechanisms against propionibacterium acnes. J Invest Dermatol 135(6):1581–1589

Schmitt MA, Weisblum B, Gellman SH (2004) Unexpected relationships between structure and function in alpha, beta-peptides: antimicrobial foldamers with heterogeneous backbones. J Am Chem Soc 126(22):6848–6849

Scott RW, DeGrado WF, Tew GN (2008) De novo designed synthetic mimics of antimicrobial peptides. Curr Opin Biotechnol 19(6):620–627

Seddon JM, Templer RH (1993) Cubic phases of self-assembled amphiphilic aggregates. Philos Trans R Soc Lond A Math Phys Eng Sci 344(1672):377–401

Selsted ME, Ouellette AJ (2005) Mammalian defensins in the antimicrobial immune response. Nat Immunol 6(6):551–557

Selsted ME, Novotny MJ, Morris WL, Tang YQ, Smith W, Cullor JS (1992) Indolicidin, a novel bactericidal tridecapeptide amide from neutrophils. J Biol Chem 267(7):4292–4295

Sens P, Johannes L, Bassereau P (2008) Biophysical approaches to protein-induced membrane deformations in trafficking. Curr Opin Cell Biol 20(4):476–482

Shai Y (1999) Mechanism of the binding, insertion and destabilization of phospholipid bilayer membranes by α-helical antimicrobial and cell non-selective membrane-lytic peptides. Biochimica et Biophysica Acta (BBA)-Biomembranes 1462(1–2):55–70

Shai Y, Oren Z (2001) From "carpet" mechanism to de-novo designed diastereomeric cell-selective antimicrobial peptides. Peptides 22(10):1629–1641

Shearman GC, Ces O, Templer RH, Seddon JM (2006) Inverse lyotropic phases of lipids and membrane curvature. J Phys Condens Matter 18(28):S1105

Siegel DP, Kozlov MM (2004) The gaussian curvature elastic modulus of n-monomethylated dioleoylphosphatidylethanolamine: relevance to membrane fusion and lipid phase behavior. Biophys J 87(1):366–374

Som A, Vemparala S, Ivanov I, Tew GN (2008a) Synthetic mimics of antimicrobial peptides. Pept Sci 90(2):83–93

Som A, Vemparala S, Ivanov I, Tew GN (2008b) Synthetic mimics of antimicrobial peptides. Biopolymers 90(2):83–93

Song A, Walker SG, Parker KA, Sampson NS (2011) Antibacterial studies of cationic polymers with alternating, random, and uniform backbones. ACS Chem Biol 6(6):590–599

Sovadinova I, Palermo EF, Huang R, Thoma LM, Kuroda K (2011a) Mechanism of polymer-induced hemolysis: nanosized pore formation and osmotic lysis. Biomacromolecules 12(1):260–268

Sovadinova I, Palermo EF, Urban M, Mpiga P, Caputo GA, Kuroda K (2011b) Activity and mechanism of antimicrobial peptide-mimetic amphiphilic polymethacrylate derivatives. Polymers 3(3):1512–1532

Spaar A, Münster C, Salditt T (2004) Conformation of peptides in lipid membranes studied by X-ray grazing incidence scattering. Biophys J 87(1):396–407

Stachowiak JC, Hayden CC, Sasaki DY (2010) Steric confinement of proteins on lipid membranes can drive curvature and tubulation. Proc Natl Acad Sci 107(17):7781–7786

Stachowiak JC, Schmid EM, Ryan CJ, Ann HS, Sasaki DY, Sherman MB, Geissler PL, Fletcher DA, Hayden CC (2012) Membrane bending by protein–protein crowding. Nat Cell Biol 14(9):944–949

Taber HW, Mueller J, Miller P, Arrow A (1987) Bacterial uptake of aminoglycoside antibiotics. Microbiol Rev 51(4):439

Tang M, Waring AJ, Hong M (2007) Phosphate-mediated arginine insertion into lipid membranes and pore formation by a cationic membrane peptide from solid-state nmr. J Am Chem Soc 129(37):11438–11446

Tew GN, Liu DH, Chen B, Doerksen RJ, Kaplan J, Carroll PJ, Klein ML, DeGrado WF (2002) De novo design of biomimetic antimicrobial polymers. Proc Natl Acad Sci USA 99(8):5110–5114

Vaara M (2009) New approaches in peptide antibiotics. Curr Opin Pharmacol 9(5):571–576

Vakulenko SB, Mobashery S (2003) Versatility of aminoglycosides and prospects for their future. Clin Microbiol Rev 16(3):430–450

van der Does AM, Bogaards SJP, Ravensbergen B, Beekhuizen H, van Dissel JT, Nibbering PH (2010) Antimicrobial peptide hlf1-11 directs granulocyte-macrophage colony-stimulating factor-driven monocyte differentiation toward macrophages with enhanced recognition and clearance of pathogens. Antimicrob Agents Chemother 54(2):811–816

van Meer G, Voelker DR, Feigenson GW (2008) Membrane lipids: where they are and how they behave. Nat Rev Mol Cell Biol 9(2):112–124

Vicens Q, Westhof E (2002) Crystal structure of a complex between the aminoglycoside tobramycin and an oligonucleotide containing the ribosomal decoding a site. Chem Biol 9(6):747–755

Walsh PS, Kusaka R, Buchanan EG, James WH, Fisher BF, Gellman SH, Zwier TS (2013) Cyclic constraints on conformational flexibility in gamma-peptides: conformation specific ir and uv spectroscopy. J Phys Chem A 117(47):12350–12362

Wang Z, Wang G (2004) Apd: The antimicrobial peptide database. Nucleic Acids Res 32(suppl 1): D590–D592

Wang WLL, Everett ED, Johnson M, Dean E (1977) Susceptibility of propionibacterium acnes to seventeen antibiotics. Antimicrob Agents Chemother 11(1):171–173

Wu CW, Sanborn TJ, Huang K, Zuckermann RN, Barron AE (2001) Peptoid oligomers with alpha-chiral, aromatic side chains: sequence requirements for the formation of stable peptoid helices. J Am Chem Soc 123(28):6778–6784

Wu Z, Cui Q, Yethiraj A (2013) Why do arginine and lysine organize lipids differently? Insights from coarse-grained and atomistic simulations. J Phys Chem B 117(40):12145–12156

Yang L, Harroun TA, Weiss TM, Ding L, Huang HW (2001) Barrel-stave model or toroidal model? a case study on melittin pores. Biophys J 81(3):1475–1485

Yeaman MR, Yount NY (2003) Mechanisms of antimicrobial peptide action and resistance. Pharmacol Rev 55(1):27–55

Yeung AY, Gellatly S, Hancock RW (2011) Multifunctional cationic host defence peptides and their clinical applications. Cell Mol Life Sci 68(13):2161–2176

Yonezawa A, Kuwahara J, Fujii N, Sugiura Y (1992) Binding of tachyplesin i to DNA revealed by footprinting analysis: significant contribution of secondary structure to DNA binding and implication for biological action. Biochemistry 31(11):2998–3004

Yu Y, Vroman JA, Bae SC, Granick S (2010) Vesicle budding induced by a pore-forming peptide. J Am Chem Soc 132(1):195–201

Zachowski A (1993) Phospholipids in animal eukaryotic membranes: transverse asymmetry and movement. Biochem J 294(1):1–14

Zasloff M (1987) Magainins, a class of antimicrobial peptides from xenopus skin: Isolation, characterization of two active forms, and partial cdna sequence of a precursor. Proc Natl Acad Sci 84(15):5449–5453

Zasloff M (2002) Antimicrobial peptides of multicellular organisms. Nature 415(6870):389–395

Zhang J, Markiewicz MJ, Mowery BP, Weisblum B, Stahl SS, Gellman SH (2012) C-terminal functionalization of nylon-3 polymers: effects of c-terminal groups on antibacterial and hemolytic activities. Biomacromolecules 13(2):323–331

Zhao K, Choe U-J, Kamei DT, Wong GCL (2012a) Enhanced activity of cyclic transporter sequences driven by phase behavior of peptide-lipid complexes. Soft Matter 8(24):6430–6433

Zhao K, Choe U-J, Kamei DT, Wong GCL (2012b) Enhanced activity of cyclic transporter sequences driven by phase behavior of peptide-lipid complexes. Soft Matter 8:6430–6433

Zimmerberg J, Kozlov MM (2006) How proteins produce cellular membrane curvature. Nat Rev Mol Cell Biol 7(1):9–19

Zimmerli W, Trampuz A, Ochsner PE (2004) Prosthetic-joint infections. N Engl J Med 351(16):1645–1654

Part V
Therapeutic Perspectives

Chapter 11
Antimicrobial Peptides and Preterm Birth

Catherine P. James and Mona Bajaj-Elliott

Abstract Preterm birth (delivery before 37 completed weeks of pregnancy) is a major problem worldwide, leading to high mortality and significant long-term morbidity. A complex interaction between ascending lower genital tract infection and the maternal immune system is a likely underlying component of pathogenesis. In this chapter we consider the ways in which expression of antimicrobial peptides in the maternal genital tract may modulate the risk of ascending genital tract infection and thus the risk of preterm birth.

11.1 Preterm Birth and Ascending Lower Genital Tract Infection

Preterm birth (PTB, delivery before 37 completed weeks of gestation) is a major problem in the United Kingdom and worldwide, leading to a high mortality rate and long-term morbidity in babies who survive—particularly those born before 32 weeks (Marlow et al. 2005; Moore et al. 2012). There are currently no proven strategies that both prevent PTB and improve neonatal outcome, making the search for new preventative therapies a priority (Iams et al. 2008). Prematurity is the single largest direct cause of neonatal deaths worldwide (one million, or 35 % of all deaths) and contributes to 50 % of all neonatal deaths (Blencowe et al. 2012): PTB kills more babies than any other single cause).

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The processes leading to preterm delivery are poorly understood. The pathogenesis of PTB is multifactorial, and many mechanisms have been proposed. There is increasing evidence that intrauterine infection in a significant proportion (40–70 % cases of preterm deliveries) is associated with chorioamnionitis (inflammation of the fetal membranes) (Tita and Andrews 2010) Two main models of intrauterine infection leading to PTB have been proposed: (a) pathogens from either a systemic infection or a remote localized infective focus disseminate hematogenously and initiate an immune response in the intrauterine cavity, and/or (b) pathogens from the extensive vaginal microbiome may gain access to the relatively sterile intrauterine cavity via the cervical canal.

The intrauterine cavity is separated from the prolific bacterial load of the vagina by the three centimeters or so of simple columnar epithelium that make up the endocervical canal. As the intrauterine cavity is demonstrably either sterile or has only minimal bacteria present in uncomplicated pregnancies at term (Jones et al. 2009), it is clear that under normal conditions the cervix must act as an effective barrier to the migration of bacteria from the vagina.

11.2 The Cervix as an Antimicrobial Barrier

The integrity of the cervical canal as an antimicrobial barrier is likely to rely on a number of interrelated physical and chemical factors; these include the apical surface lining of the endocervical columnar epithelium and provision of innate and cellular immunity from resident and recruited immune (e.g., macrophages and T-cells) and nonimmune (epithelium/fibroblast) cells via the production of cytokines and chemokines. Many of the "gate-keeper" functions of the cervix are coordinated by the cervical mucus plug (CMP), a large (c.10 g), viscous structure which fills the cervical canal during pregnancy. The CMP is a dynamic structure unique to pregnancy. Scanning electron microscopy reveals that the ultrastructure undergoes significant change during pregnancy, from a largely porous mesh early in the first trimester, to a dense and highly compact mesh at later gestations (Becher et al. 2009).

Molecularly, the CMP is an anionic mucinous glycoprotein skeleton. This serves as a ligand for a variety of molecules, including cytokines and water. In addition, the complex network inhibits the diffusion of larger molecules and pathogens through the CMP by steric exclusion (Becher et al. 2009). The anionic charge of the mucin skeleton also allows the CMP to retain positively charged molecules, including the many highly cationic antimicrobial peptides (AMPs) of the innate immune system.

More than 800 unique AMPs have been identified in a variety of species, but the two main classes of AMP in mammals are cathelicidins and defensins. Despite expressing only one cathelicidin, humans express a range of defensins; these are classified as alpha defensins or beta defensins depending on the cysteine motif of their beta sheet secondary structure (Fellermann and Stange 2001).

Table 1 The antimicrobial spectrum of HBD1, HBD2, and HBD3

HBD1	HBD2	HBD3
Escherichia coli	*Escherichia coli*	*Escherichia coli*
Pseudomonas aeruginosa	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*
Klebsiella pneumoniae	*Klebsiella pneumoniae*	*Klebsiella pneumoniae*
Staphylococcus aureus	*Staphylococcus aureus*	*Staphylococcus aureus*
HIV-1	*Streptococcus pneumoniae*	*Streptococcus pneumoniae*
–	*Candida albicans*	*Streptococcus pyogenes*
–	*Candida parapsilosis*	*Staphylococcus carnosus*
–	*Candida krusei*	*Burkholderia cepacia*
–	*Enterococcus faecalis*	*Saccharomyces cerevisiae*
–	HIV-1	*Candida albicans*
–	–	*Candida parapsilosis*
–	–	*Candida krusei*
–	–	*Enterococcus faecalis*
–	–	HIV-1

Alpha defensins, also called human neutrophil peptides (HNPs), are small peptides with broad spectrum antimicrobial and/or immunoregulatory properties and are mainly found in the azurophilic granules of neutrophils and in the Paneth cells of the small intestine (Wiesner and Vilcinskas 2010). In contrast, beta defensin expression is not restricted as they can be expressed by all epithelia (Pazgier et al. 2006) and reports indicate expression in circulating immune cells (Jansen et al. 2009).

Defensins were originally described as endogenously occurring antimicrobials. Indeed, collectively they exhibit a considerable antimicrobial spectrum against a range of Gram positive and Gram negative pathogens (Table 11.1). Their mechanism(s) of antimicrobial activity is related to their amphipathic design, in which clusters of hydrophobic and cationic amino acids are spatially organized in discrete sections of the molecule (Zasloff 2002). This design allows the interaction of the cationic, hydrophilic surface of the peptide with the (anionic) bacterial membrane, during which there is displacement of the membrane lipids and subsequent alteration of membrane structure. Many mechanisms of bacterial killing have been proposed, all of which hinge on this single premise of charge-dependent interaction; suggested mechanisms include membrane depolarisation, leakage of intracellular components through compromised bacterial membranes or other disturbances of bacterial membrane function (Zasloff 2002).

Peptides using charge-dependent mechanisms of bacterial killing are able to execute activity in the micromolar concentration range. This capacity is inhibited under conditions of increased ionic strength, for example in a salty environment. HBD3 alone is able to kill in a relatively salt independent fashion (Harder et al. 2001). In addition to this salt insensitivity, HBD3 has potent antimicrobial activity against viruses and fungi (Pazgier et al. 2006). Although the precise mechanisms underlying these properties are not yet clear, the reader is encouraged to read the

accompanying chapters that provide an update to our current understanding of AMP action and function.

In addition to their antimicrobial capacity, HBDs also play an important immunoregulatory role. HBD1 is constitutively expressed but may be upregulated in the context of infection or inflammation (Bajaj-Elliott et al. 2002). This may be IFN-γ dependent (Prado-Montes de Oca 2010). HBD1 induces CCL5/RANTES production by peripheral blood monocytes and, in common with HBD2, can act as a chemoattractant for immature dendritic cells (iDCs) and memory T-cells via CCR6, effectively mediating innate-adaptive immune signaling (Pazgier et al. 2007). HBD3 modulates HIV-1 infectivity via CXCR4, induces antigen presenting cell maturation via TLR1 and TLR2 and is chemoattractant for CCR2 (Röhrl et al. 2010). In addition, HBD3 has been shown to compete with melanocyte stimulating hormone alpha (MSHα), the ligand of the melanocortin 1 receptor (MC1r), in myeloid cells; this competition inhibits the induction of the anti-inflammatory cytokine IL-10 in cells expressing MC1r (Prado-Montes de Oca 2010).

Further immunomodulatory properties of HBD3 include the activation of monocytes via TLR1 and TLR2 to produce IL-8, IL-6 and IL-1β but not IL-10. In contrast to these largely proinflammatory properties, HBD3 can also neutralize lipopolysaccharide (LPS) and inhibit TNFα and IL-6 accumulation (Semple et al. 2010, 2011). The net effect of HBD3 action is therefore difficult to discern as it can be proinflammatory and/or anti-inflammatory. The available information raises the hypothesis that HBD3 pro- and/or anti-inflammatory function in vivo may be context dependent.

11.3 Antimicrobial Peptides and Preterm Birth

The potential role of AMPs, secretory leukocyte protease inhibitor (SLPI), and elafin in the pathogenesis of PTB has been investigated. The CMP itself displays direct antimicrobial activity in vitro, and both peptides have been identified as components of the CMP (Hein et al. 2002; Stock et al. 2009). Low cervicovaginal levels of elafin have been associated with bacterial vaginosis (BV) (Stock et al. 2009); conversely, it has been suggested that high concentrations of elafin in cervicovaginal fluid are associated with cervical shortening and may be predictive of PTB (Abbott et al. 2014). High cervicovaginal concentrations of the human cathelicidin LL37 have also been associated with bacterial vaginosis in pregnancy (a risk factor for PTB) (Frew et al. 2014).

HBDs have also been identified in the CMP (Frew and Stock 2011). Numerous studies (Cobo et al. 2011; Polettini et al. 2011; Buhimschi et al. 2009) have focused on the expression of HBDs in the amniotic fluid, fetal membranes, and the placenta in women who deliver preterm. Although these studies suggest that there may be an association between increased expression (transcriptional and translational) of these peptides and PTB, currently no mechanistic studies have been reported. There are

also no reports detailing cervicovaginal HBD expression in women at increased risk of PTB, although two studies report an association between increased alpha defensins in cervicovaginal fluid and PTB (Xu et al. 2008; Lucovnik et al. 2011).

11.4 Progesterone, AMPs and Preterm Birth

Significant data showing that progesterone can prolong pregnancy in women at risk of PTB have raised questions about its mode of action (Maggio and Rouse 2014). The gestation extending effects of progesterone are most pronounced in those women who have both a history of prior preterm deliveries and a reduced cervical length (below 25 mm when measured by transvaginal ultrasound) in the pregnancy in question. It is clear that women with a reduced cervical length will also have a reduced surface area of endocervical epithelium. The precise mechanism(s) by which progesterone treatment may compensate for this reduced surface area remains ambiguous, and the long-term outcome of children born to women treated with progesterone has yet to be determined.

The risk of ascending genital tract infection is highest when serum progesterone is at its lowest in the menstrual cycle (Wira et al. 2015), and limited data suggest that progesterone may modulate HBD protein expression in primary endometrial cells and transformed cell cultures (King et al. 2003). Furthermore, vaginal progesterone has been shown to increase the expression of HBD1 in mice, albeit not at the protein level (Nold et al. 2013). This has clear implications for the regulation of mucosal immunity in pregnancy. Progesterone receptors A and B are expressed by the cervix in vivo, and it has recently been shown that the ectocervical cell line Ect1/E6E7 and vaginal cell line VK2/E6E7 also express these receptors (Africander et al. 2011). It therefore seems probable that HBD expression by cervical epithelia may also be progesterone sensitive. It is probable that the explanation for the gestation extending effects of progesterone will include a combination of actions, but this limited evidence suggests that regulation of lower genital tract antimicrobial peptide expression may play a role, perhaps by reducing the risk of ascending infection.

11.5 Conclusion

Emerging evidence is providing a tantalizing glimpse linking increased risk of ascending infection and preterm birth. In addition to the mother's adaptive and innate immune response, the cervical antimicrobial barrier is likely to be the key determinant of the status quo. Further studies are needed to confirm the potential protective role of AMPs in reducing the risk of ascending infection in susceptible (those with a history of preterm births) individuals. If confirmed, manipulation of AMP expression may be a viable future therapeutic option.

References

Abbott DS, Chin-Smith EC, Seed PT, Chandiramani M, Shennan AH, Tribe RM (2014) Raised trappin2/elafin protein in cervico-vaginal fluid is a potential predictor of cervical shortening and spontaneous preterm birth. PLoS ONE 9(7):e100771

Africander D, Louw R, Verhoog N, Noeth D, Hapgood JP (2011) Differential regulation of endogenous pro-inflammatory cytokine genes by medroxyproges-terone acetate and norethis-terone acetate in cell lines of the female genital tract. Contraception 84(4):423–435

Bajaj-Elliott M, Fedeli P, Smith GV, Domizio P, Maher L, Ali RS, Quinn AG, Farthing MJ (2002) Modulation of host antimicrobial peptide (beta-defensins 1 and 2) expression during gastritis. Gut 51(3):356–361

Becher N, Adams Waldorf K, Hein M, Uldbjerg N (2009) The cervical mucus plug: structured review of the literature. Acta Obstet Gynecol Scand 88(5):502–513

Blencowe H, Cousens S, Oestergaard MZ, Chou D, Moller AB, Narwal R, Adler A, Vera Garcia C, Rohde S, Say L, Lawn JE (2012) National, regional, and worldwide estimates of preterm birth rates in the year 2010 with time trends since 1990 for selected countries: a systematic analysis and implications. Lancet 379(9832):2162–2172

Buhimschi CS, Dulay AT, Abdel-Razeq S, Zhao G, Lee S, Hodgson EJ, Bhandari V, Buhimschi IA (2009) Fetal inflammatory response in women with proteomic biomarkers characteristic of intra-amniotic inflammation and preterm birth. BJOG 116(2):257–267

Cobo T, Palacio M, Martinez-Terron M, Navarro-Sastre A, Bosch J, Filella X, Gratacos E (2011) Clinical and inflammatory markers in amniotic fluid as predictors of adverse outcomes in preterm premature rupture of membranes. Am J Obstet Gynecol 205(2):1–8

Fellermann K, Stange EF (2001) Defensins—innate immunity at the epithelial frontier. Eur J Gastroenterol Hepatol 13(7):771–776

Frew L, Stock SJ (2011) Antimicrobial peptides and pregnancy. Reproduction 141(6):725–735

Frew L, Makieva S, McKinlay AT, McHugh BJ, Doust A, Norman JE, Davidson DJ, Stock SJ (2014) Human cathelicidin production by the cervix. PLoS ONE 9(8):e103434

Harder J, Bartels J, Christophers E, Schroder JM (2001) Isolation and characterization of human beta-defensin-3, a novel human inducible peptide antibiotic. J Biol Chem 276(8):5707–5713

Hein M, Valore EV, Helmig RB, Uldbjerg N, Ganz T (2002) Antimicrobial factors in the cervical mucus plug. Am J Obstet Gynecol 187(1):137–144

Iams JD, Romero R, Culhane JF, Goldenberg RL (2008) Primary, secondary, and tertiary interventions to reduce the morbidity and mortality of preterm birth. Lancet 371 (9607):164–175

Jansen PA, Rodijk-Olthuis D, Hollox EJ, Kamsteeg M, Tjabringa GS, de Jongh GJ, van Vlijmen-Willems IM, Bergboer JG, van Rossum MM, de Jong EM, den Heijer M, Evers AW, Bergers M, Armour JA, Zeeuwen PL, Schalkwijk J (2009) Beta-defensin-2 protein is a serum biomarker for disease activity in psoriasis and reaches biologically relevant concentrations in lesional skin. PLoS ONE 4(3):e4725

Jones HE, Harris KA, Azizia M, Bank L, Carpenter B, Hartley JC, Klein N, Peebles D (2009) Differing prevalence and diversity of bacterial species in fetal membranes from very preterm and term labor. PLoS ONE 4(12):e8205

King AE, Fleming DC, Critchley HO, Kelly RW (2003) Differential expression of the natural antimicrobials, beta-defensins 3 and 4, in human en-dometrium. J Reprod Immunol 59(1):1–16

Lucovnik M, Kornhauser-Cerar L, Premru-Srsen T, Gmeiner-Stopar T, Derganc M (2011) Neutrophil defensins but not interleukin-6 in vaginal fluid after preterm premature rupture of membranes predict fetal/neonatal inflammation and infant neurological impairment. Acta Obstet Gynecol Scand 90(8):908–916

Maggio L, Rouse DJ (2014) Progesterone. *Clin Obstet Gynecol* 57(3):547–556

Marlow N, Wolke D, Bracewell MA, Samara M (2005) Neurologic and developmental disability at six years of age after extremely preterm birth. N Engl J Med 352(1):9–19

Moore T, Hennessy EM, Myles J, Johnson SJ, Draper ES, Costeloe KL, Marlow N (2012) Neurological and developmental outcome in extremely preterm children born in England in 1995 and 2006: the EPICure studies. BMJ 345:e7961

Nold C, Maubert M, Anton L, Yellon S, Elovitz MA (2013) Prevention of preterm birth by progestational agents: what are the molecular mechanisms? Am J Obstet Gynecol 208(3):1–7

Pazgier M, Hoover DM, Yang D, Lu W, Lubkowski J (2006) Human beta-defensins. Cell Mol Life Sci 63(11):1294–1313

Pazgier, M, Prahl, A, Hoover, DM, Lubkowski, J (2007) Studies of the biological properties of human beta-defensin 1. *J Biol Chem* 19, 282(3):1819–29

Polettini J, Takitane J, Peracoli JC, Silva MG (2011) Expression of defensins 1, 3 and 4 in chorioamniotic membranes of preterm pregnancies complicated by chorioamnionitis. Eur J Obstet Gynecol Reprod Biol 157(2):150–155

Prado-Montes de Oca E (2010) Human beta-defensin 1: a restless warrior against allergies, infections and cancer. Int J Biochem Cell Biol 42(6):800–804

Röhrl J, Yang D, Oppenheim JJ, Hehlgans T (2010) Human beta-defensin 2 and 3 and their mouse orthologs induce chemotaxis through interaction with CCR2. *J Immunol* 15, 184(12):6688–94

Semple F, Webb S, Li HN, Patel HB, Perretti M, Jackson IJ, Gray M, Davidson DJ, Dorin JR (2010) Human beta-defensin 3 has immunosuppressive activity in vitro and in vivo. Eur J Immunol 40(4):1073–1078

Semple F, MacPherson H, Webb S, Cox SL, Mallin LJ, Tyrrell C, Grimes GR, Semple CA, Nix MA, Millhauser GL, Dorin JR (2011) Human—defensin 3 affects the activity of pro-inflammatory pathways associated with MyD88 and TRIF. Eur J Immunol 41 (11):3291–3300

Stock SJ, Duthie L, Tremaine T, Calder AA, Kelly RW, Riley SC (2009) Elafin (SKALP/Trappin-2/proteinase inhibitor-3) is produced by the cervix in pregnancy and cervicovaginal levels are diminished in bacterial vaginosis. Reprod Sci 16(12):1125–1134

Tita AT, Andrews WW (2010) Diagnosis and management of clinical chorioamnionitis. Clin Perinatol 37(2):339–354

Wiesner J, Vilcinskas A (2010) Antimicrobial peptides: the ancient arm of the human immune system. Virulence 1(5):440–464

Wira CR, Rodriguez-Garcia M, Patel MV (2015) The role of sex hormones in immune protection of the female reproductive tract. Nat Rev Immunol 15(4):217–230

Xu J, Holzman CB, Arvidson CG, Chung H, Goepfert AR (2008) Midpreg- nancy vaginal fluid defensins, bacterial vaginosis, and risk of preterm delivery. Obstet Gynecol 112(3):524–531

Zasloff M (2002) Antimicrobial peptides of multicellular organisms. Nature 415(6870):389–395

Chapter 12
Host Defense Peptides and Their Advancements in Translational *Staphylococcus aureus* Research

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Abstract *Staphylococcus aureus* is responsible for a multitude of infections ranging from skin and soft tissue infections to more severe invasive diseases. In response to *S. aureus*, host defense peptides (HDPs) are produced as nature's own sentinel effector molecules. HDPs are small, often cationic, molecules that possess numerous biological activities, such as antimicrobial activity, cellular recruitment, anti-inflammatory properties, and wound healing, all of which play a role in controlling *S. aureus* infections. In hopes of capitalizing on the powerful anti-infective functions of HDPs, there has been a considerable amount of interest in deriving HDP-based therapeutics. Here, we highlight current advancements in HDP research, constraints to commercial development, and solutions for safer and more feasible HDP-based therapies against *S. aureus*.

12.1 Staphylococcal Species and Colonization

Staphylococci are the most abundant bacterial inhabitants of the human skin microbiome. Humans are generally colonized with many different Staphylococcal species, with *S. epidermidis*, a coagulase-negative *Staphylococcus* (CoNS), being the most universal and dominant colonizer. The skin is colonized with many other CoNS species including *S. haemolyticus*, *S. saprophyticus*, *S. capitis*, *S. hominis*, *S. warneri*, *S. cohnii*, and *S. simulans* (Coates et al. 2014). Staphylococcal species differ in their ability to cause disease. Most CoNS are commensal, causing opportunistic infections in immune-compromised individuals (Otto 2010).

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Coagulase-positive *S. aureus*, however, has received considerable attention for its ability to cause disease even in healthy individuals. Colonization is known to be a risk factor for *S. aureus* infection and 20 % of the population is persistently colonized while 30 % carry *S. aureus* transiently. *S. aureus* causes a wide spectrum of diseases ranging from mild skin and soft tissue infections (boils, abscess, impetigo) to life-threatening diseases, such as severe sepsis, pneumonia, toxic shock syndrome, and endocarditis. *S. aureus* is also capable of forming multicellular communities or recalcitrant biofilms, resulting in a high incidence of indwelling device and catheter-related infections.

Adding to its severity, *S. aureus* is a highly adaptable human pathogen. In fact, resistance to methicillin was reported in 1961, only 1 year after it was first introduced. Initially confined to hospitals and other health care facilities, methicillin-resistant *Staphylococcus aureus* (MRSA) affected mainly immune-compromised individuals. Since the mid 1990s, however, there has been an explosion in the number of MRSA cases reported in the community, affecting healthy individuals that may have not been previously exposed to the healthcare environment (Herold et al. 1998, David and Daum 2010). Moreover, the incidence of vancomycin-intermediate and vancomycin-resistant *S. aureus* (VISA and VRSA, respectively) have increased (Tiwari and Sen 2006). Alarmingly, resistance to almost all clinically available antibiotics has emerged (Waldvogel 1999; Levin et al. 2005; Gu et al. 2013).

During the past half-century, very few new antibiotic classes have been developed that can effectively target *S. aureus*. To highlight this urgency, MRSA infections have claimed more lives than human immunodeficiency virus/acquired immune deficiency syndrome (HIV/AIDS) in the western world (Klevens et al. 2007). With the antibiotic pipeline running dry and multi-drug resistance on the rise, there has been considerable interest to exploit HDPs for the treatment of *S. aureus* infections. In this chapter, we will explore advancements in anti-*S. aureus* HDP research and HDP-directed translational breakthroughs.

12.2 Important Anti-staphylococcal HDPs

Host defense peptides (HDPs; also often referred to as antimicrobial peptides, AMPs) are an essential part of the innate defense response against *S. aureus*. HDPs are generally cationic and range in size from 12 to 50 amino acids, roughly 50 % of which are hydrophobic resulting in an overall amphipathic structure. In vitro, the bactericidal activity exhibited by HDPs is mediated by integration within the cytoplasmic membrane, resulting in pore formation and subsequent lysis.

HDPs are produced by a variety of cell types, either constitutively as a constant line of defense, or inducibly upon infection. Generally speaking, two major classes of HDPs, cathelicidins and defensins, have been described. However, other HDPs

such as RNase7 and dermcidin are important members of the constitutive cutaneous defense mechanism protecting us against initial *S. aureus* colonization.

12.2.1 Defensins

Humans express α- and β-defensins, small cysteine-rich amphipathic peptides that are 18-45 amino acid residues long and stabilized by three disulfide bonds. All defensins are initially synthesized as prepropeptides and become propeptides after cleavage of a signal peptide. Proper folding and activation of the defensin requires proteolytic cleavage of the anionic propiece, a segment of the peptide considered important for maintaining charge balance and minimizing host toxicity.

Four α-defensins (HNP1-4) are produced in the azurophilic granules of human neutrophils as well as epithelial and certain hematopoietic cells (natural killer cells and monocytes). Despite accounting for almost half of the degranulating neutrophil proteins, α-defensins do not effectively kill *S. aureus* (Lehrer 2007). Instead, certain HNPs have shown to neutralize *S. aureus* toxins. For example, HNP3 was shown to bind to both Panton–Valentine leukocidin subunits, LukS-PV, and Luk-PV, partially reducing pore formation and neutrophil lysis (Cardot-Martin et al. 2015).

In humans, four types of β-defensins have been characterized (hβD1-4), and are subcategorized based on the number and location of their disulfide bridges. hβD1 was first isolated from human plasma; and hβD2 and hβD3 were subsequently isolated from psoriatic scale extracts (Harder et al. 1997, 2001). hβ4has been identified in lung tissue and is produced by bronchial and bronchiolar epithelium (Yanagi et al. 2005). β-defensins are expressed broadly by epithelial cells such as keratinocytes as well as certain leukocytes. In most tissues, hβD1 is expressed constitutively, while expression of hβD2 and hβD3 is induced upon pro-inflammatory stimuli with cytokines, various microorganisms and microbial products as well as upon tissue injury (Menzies and Kenoyer 2006). Of all the β-defensins, hβD-3 represents the most potent anti-staphylococcal peptide, retaining the highest antimicrobial activity in vivo (Kisich et al. 2007).

β-defensin expression is mediated by recognition of pathogen-associated molecular patterns (PAMPs) by toll like receptors (TLRs). TLR2 recognizes common cell wall constituents from *S. aureus* such as peptidoglycan, lipopeptides and lipotechoic acid, and instigates a number of pro-inflammatory intracellular signaling events. Specifically, *S. aureus* activates the TLR2-mediated p38 MAPK signaling cascade, which regulates the expression of a number of inflammatory mediators (through AP-1 transcription factors) such as cytokines, chemokines and certain HDPs such as hβD2 and hβD3 (Menzies and Kenoyer 2006). Moreover, internalized *S. aureus* has shown to activate the nucleotide-binding oligomerization domain 2 (NOD-2), an intracellular receptor that recognizes muramyl dipeptide in

peptidoglycan, and may by responsible for hβD2 production by keratinocytes (Voss et al. 2006). Lastly, wounding of human skin results in the activation of the epidermal growth factor receptor, which leads to the enhanced production of hβD3 (Sorensen et al. 2006).

12.2.2 Cathelicidin

The human cathelicidin (hCAP-18) is an α-helical amphipathic cationic peptide constitutively expressed in the phagosomes of neutrophils, and inducibly in the mucosal epithelia and keratinocytes. Recent studies by Zhang et al. have shown that dermal adipocytes are also an important source of cathelicidin during cutaneous *S. aureus* infection, as mice defective in adipogenesis produced lower levels of this HDP (Zhang et al. 2015).

Upon release, hCAP-18 becomes proteolytically cleaved by proteinase 3, releasing the inactive N-terminal cathelin domain and generating the active peptide LL-37. LL-37 is 37 amino acid residues in length and is produced in a vitamin D-dependent manner. Moreover, two other cleavage products, RK-31 and KS-30 have been identified, and demonstrates even greater antimicrobial activity compared to LL-37 (Murakami et al. 2002).

12.2.3 RNAse 7

In 2002, RNase7 was first characterized in human skin as a peptide that exhibits broad-spectrum antimicrobial activity (Harder and Schroder 2002). RNAse7 is constitutively expressed in epidermis and in the stratum corneum of healthy skin, however, can be further induced upon bacterial challenge as well as stimulation by IL-1β and IFN-γ (Harder and Schroder 2002). Importantly, RNAse7 is considered an important part of the constitutive host defense, preventing colonization and infection. For example, after 2 h of *S. aureus* bacterial challenge, RNAse7 levels in the stratum corneum of human skin explants are significantly up-regulated at levels high enough to prevent *S. aureus* colonization (Simanski et al. 2010).

12.2.4 Dermcidin

Dermcidin is expressed constitutively in eccrine sweat glands and secreted into sweat onto the epidermal surface, preventing bacterial colonization and serving as an integral cutaneous defense mechanism (Schittek et al. 2001). Post-secretory

proteolytic processing of dermcidin protein (110 amino acid long) in sweat, results in the 47-aa peptide (DCD-1), 48-aa peptide (DCD-IL) or truncated peptide SSL-46, which all display broad-spectrum activity against *Escherichia coli*, *Enterococcus faecalis*, *Candida albicans* and *S. aureus* (Flad et al. 2002). Within human sweat, DCD-1 is found at concentrations (1–10 μg/mL) sufficiently high to kill microorganisms, and its activity is retained in pH and salt conditions that are characteristic of sweat (Schittek et al. 2001). Interestingly, dermcidin does not possess any homology to known HDPs. Notably, in contrast to most AMPs, it possesses a net negative charge. This suggests that the mode of action of dermcidin might be different from other HDPs that rely on electrostatic interactions. While the mode of action of dermcidin remained obscure for a long time, it has recently been shown to form pores (Song et al. 2013).

12.3 HDP-Related Diseases

A growing body of evidence has demonstrated that deficiency of certain HDPs predisposes individuals to *S. aureus* infection. For example, lower induction of hβD-3 in *S. aureus*-infected human skin explants, has been correlated to more severe *S. aureus* skin infection as well as greater susceptibility to reoccurring infection (Zanger et al. 2010). Moreover, lesions taken from individuals with atopic dermatitis (AD), a skin disease that is correlated to increased susceptibility to *S. aureus*, produced significantly lower levels of hβD-2, hβD-3 and LL-37 (Ong et al. 2002). Furthermore, reduced production of dermcidin has been shown to contribute to the propensity of AD patients to recurrent bacterial skin infections (Rieg et al. 2005). This deficiency of dermcidin in sweat is correlated to reduced antimicrobial activity against *S. aureus* in vivo (Flad et al. 2002). Interestingly, A/J mice, which are more susceptible to *S. aureus* bacteremia compared to C57BL6 mice, have polymorphisms in their defensin genes (Ahn et al. 2010). Furthermore, mice deficient of cathelicidin-related antimicrobial peptide (CRAMP) produced larger lesions when injected with Group A *Streptococcus* compared to normal littermates (Nizet et al. 2001).

Conversely, elevated levels of certain HDPs have been linked with increased resistance to *S. aureus*. For example, elevated levels of hβD-2 and LL-37 found in psoriatic lesions prevent *S. aureus* colonization (Ong et al. 2002). Moreover, high baseline levels of RNAse7 expression in the healthy skin confer protection against *S. aureus* skin infections (Zanger et al. 2009).

HDP deficiency can be attributed to an impairment of particular T cell responses. T cell responses, specifically those associated with Th17 cells, are especially important for HDP production. Th17 cytokines IL-17A and IL-22 were shown to up-regulate antimicrobial peptide expression namely, hβD-2, hβD-3 and cathelicidin, in keratinocytes (Liang et al. 2006). Further evidence that supports a role of

Th17 cells in cutaneous immunity is derived from studies, in which mice deficient in IL-17-producing epidermal γδT cells demonstrated higher susceptibility to *S. aureus* skin infection (Cho et al. 2010). In patients with autosomal dominant hyper-IgE syndrome, impaired Th17 cell differentiation, caused by a mutation in signal transducer and activator of transcription 3 (STAT3) (Renner et al. 2008), was linked to a variety of recurrent bacterial including *S. aureus* infections (Milner et al. 2008).

Furthermore, a predominance of Th2 cytokines can specifically impair the production of hβD-2 and hβD-3 (Nomura et al. 2003). Elevated levels of Th2 cytokines in skin lesions from AD patients are thought to promote *S. aureus* colonization by enhancing *S. aureus* binding to fibronectin and fibrinogen (Cho et al. 2001). Moreover, specific *S. aureus*-secreted super antigens such as enterotoxins A and B as well as toxic shock syndrome toxin-1 (TSST-1) elicit dermal infiltration of eosinophils and mononuclear cells, which skews the immune response toward a Th2 environment, exacerbating *S. aureus*-infected skin lesions in AD patients (Laouini et al. 2003).

12.4 Immune-Regulatory Roles of HDPs

Over the years, the immune-regulatory roles of HDPs have become more appreciated. HDPs exert a broad range of activities that refine host defenses to respond to infection, such as chemoattraction, suppression of pro-inflammatory mediators, and wound healing (Mansour et al. 2014). As the antimicrobial activities of many HDPs are significantly dampened at physiological conditions, some would argue that the immune-modulatory activities of HDPs are comparatively more relevant.

In fact, many of the studies highlighting antimicrobial activity are based on in vitro experiments using purified HDP extracts. Moreover, many experiments are performed under conditions (i.e., low ionic strength, neutral pH) that allow for optimal killing, while conditions that would better represent the physiological situation, such as by inclusion of divalent cations, anions, serum components, glycosaminoglycans, mucin and 150 mM NaCl, antagonize peptide activity. For example, when minimal inhibitory concentrations (MIC) assays are conducted on LL-37 under low salt conditions (often ≤20 mM NaCl), the MIC against a number of common bacteria is between 1 and 30 μg/ml. However, in the presence of more relevant ionic conditions (100 mM NaCl), the antimicrobial activity of LL-37 is 2–8 fold lower (Turner et al. 1998) with essentially no activity against *S. aureus* at concentrations as high as 100 μg/ml. Moreover, under these same conditions, the antimicrobial activity of hβD-1 and hβD2 is completely lost. However, the importance of HDPs has been validated in the aforementioned human and animal studies (See Sect. 12.3). These findings have led many to believe that the most

important function of HDPs is to refine the host responses. Here we will highlight the diverse immune-modulatory roles of HDPs that enhance host defenses against *S. aureus*.

12.4.1 Chemoattraction

Interestingly, certain HDPs and chemokines share similar structure as they are both amphipathic and cationic. In fact, an evolutionary relationship between chemokines and HDPs have been inferred based on their strong involvement in the host innate immune response (Yount and Yeaman 2006). Specifically, HDPs were shown to possess a number of chemoattractant capabilities, assisting in the recruitment of immune cells that are important for resolving infection. When produced at sufficiently high concentrations, HDPs can directly act as chemokines, whereas at lower concentrations, they can promote the release of cytokines from other leukocytes (Mansour et al. 2014). For instance, if induced at high enough concentrations, LL-37 can directly attract neutrophils and eosinophils via interactions with formyl-peptide receptors (Tjabringa et al. 2006). However, since these activities are only promoted at LL-37 concentrations above physiological conditions, it is unlikely they possess this direct activity in vivo. On the other hand, LL-37 can indirectly promote chemoattraction by stimulating epithelial cells to release IL-8, an important chemokine for neutrophils and monocytes. Similarly, when human peripheral blood mononuclear cells (PBMCs) are stimulated by LL-37 in culture, they produce neutrophil chemokine IL-8, as well as monocyte chemoattractant protein-1 (MCP-1) and MCP-3 (Davidson et al. 2004). In mice, injection of cathelicidin-related antimicrobial peptide (CRAMP) results in the recruitment of neutrophils and monocytes (Kurosaka et al. 2005). This enhanced chemokine production may have important therapeutic implications, as local application of MCP-1 has been shown to reduce *S. aureus* infection in an osteomyelitis rat model by increasing the number of neutrophils, the first responders to *S. aureus* infection (Li et al. 2010).

Human defensins display a variety of chemotactic roles inducing the migration of immature dendritic cells and lymphocytes, promoting adaptive immunity. For example, hβD2 is capable of enhancing mobility of immature dendritic and memory T cells via interactions with chemokine CCL-20 receptor CCR6 (Yang et al. 1999). Along with CCR6, β-defensins bind to chemokine receptor CCR2 expressed on monocytes, dendritic cells, and certain macrophage subsets. Importantly, CCR2-mediated recruitment of monocytes is essential for innate immune defense and clearance of bacteria in vivo (Kurihara et al. 1997; Jia et al. 2008). Moreover, HNP1 and HNP2 have been shown to serve as a chemotactic for CD4 and CD8 T cells as well as immature dendritic cells via unidentified receptors (Yang et al. 2000).

12.4.2 Wound Healing

LL-37 plays a role in the re-epithelialization of skin wounds and wound closure. Upon skin injury, high levels of LL-37 precursor protein, hCAP18, is produced in the wound bed and it has been suggested that LL-37 plays a role in epithelial cell proliferation. Similarly, low levels of LL-37 precursor protein hCAP18, have been correlated with delayed wound closure and chronic ulcers. Moreover, treatment of anti-LL-37 antibodies inhibited epithelial healing in an ex vivo wound healing model in a dose-dependent manner (Heilborn et al. 2010). Furthermore, hβD3 accelerated wound healing in an *S. aureus*-infected porcine diabetic wound.

12.4.3 Anti-inflammatory

Certain HDPs are important for protecting the host from a harmful cytokine storm that is imposed during bacterial infection. For example, LL-37 has shown to attenuate the production of pro-inflammatory mediators (IL-1β, IL-6, IL-8, and TNF-α) in neutrophils when subjected to *S. aureus* (Alalwani et al. 2010). Likewise, in PBMCs, production of anti-inflammatory IL-10 is up-regulated. This effect on cytokine release is mediated by LL-37 binding directly to an internal signaling molecule, GAPDH, impairing p38 MAPK signaling and subsequent transcription of chemokines and cytokines (Mookherjee et al. 2009). As inflammation has shown to exacerbate conditions such as pneumonia and early stage sepsis, anti-inflammatory activities of HDPs can be considered an asset provided bacterial clearance is not compromised. Fortunately, certain HDPs such as LL-37 enhance neutrophil-killing capacity by increasing *S. aureus* phagocytosis and enhancing ROS production (Alalwani et al. 2010). Similarly, mice deficient in cathelicidin-related antimicrobial peptide (CRAMP) or murine cathelicidin showed significantly less bactericidal activity against *S. aureus*.

12.5 *S. aureus* HDP Evasion Techniques

Despite the numerous anti-infective roles of HDPs, *S. aureus* can tolerate relatively high concentrations of these peptides (Peschel et al. 1999). As with other innate and adaptive immune responses, *S. aureus* has developed multiple HDP evasion mechanisms (summarized in Fig. 12.1). It is thought that cationic HDPs and HDP-resistance mechanisms have co-evolved to allow *S. aureus* to quickly adapt to these integral and ancient host defense components (Peschel et al. 1999).

S. aureus can recognize challenges by various HDPs using the antimicrobial peptide sensor (Aps) system, which induces several resistance mechanisms (Li et al. 2007a, b). One major Aps-regulated mechanism is the activation of the *dltABCD*

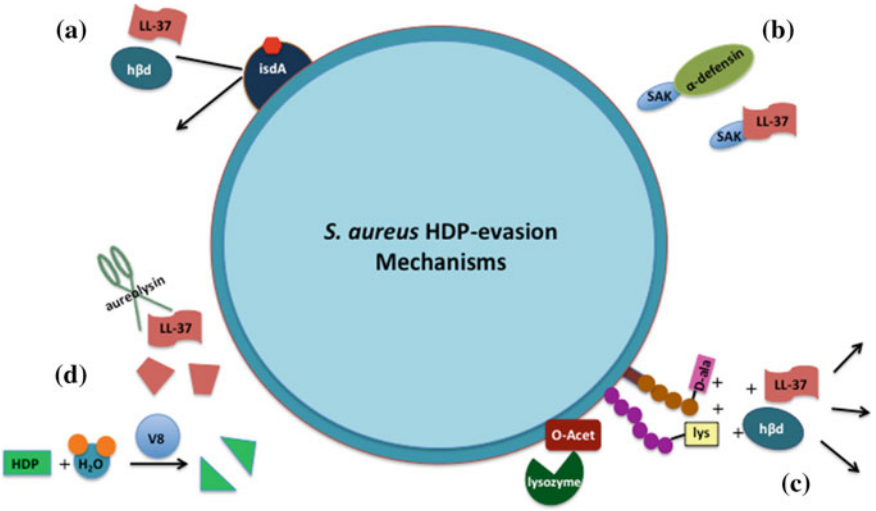

Fig. 12.1 ***S. aureus*** **HDP immune evasion mechanisms**. **a. HDP repulsion via hydrophobic surface molecules**. Cell-wall bound proteins such as IsdA, decrease bacterial hydrophobicity, resulting in increased resistance to certain HDPs. **b**. **HDP inactivity via staphylokinase (SAK) interference**. SAK has shown to form a complex with alpha-defensins, abrogating their activity. Also, SAK binds directly to cathelicidin, enhancing SAK-dependent plasminogen activation, fibrinolysis and bacterial dissemination. **c**. **Cell surface modifications repel HDPs**. Activation of the *dltABCD* operon and expression of MprF, incorporate positively charged amino acids to cell surface constituents, causing repulsion of positively charged HDPs. Steric hindrance is created via theO-acetylation of peptidoglycan muramic acid preventing lysozyme from targeting *S. aureus*. **d. Degradation of HDPs by secreted enzymes**. Aureolysin and V8 protease are two major *S. aureus*-secreted proteases that cleave and inactivate HDPs

operon, which incorporates positively charged D-alanine onto *S. aureus* anionic teichoic acids, conferring a greater positive charge and ultimately repelling HDPs such as defensins and LL-37 (Peschel et al. 1999). Moreover, to reduce anionic charge, *S. aureus* expresses multiple peptide resistance factor, MprF, which transfers lysine to anionic membrane lipid phosphatidylglycerol (PG), and translocates the lysyl-PG to the outer membrane leaflet conferring a net +1 charge and resulting in α-defensins and LL-37 repulsion (Peschel et al. 2001). Indeed, MprF contributes to clinical failures of daptomycin, the only currently available AMP therapy used to combat *S. aureus* infections (Jones et al. 2008). Interestingly, MRSA strains are more resistant to LL-37 compared to less positively net-charged methicillin-susceptible *S. aureus* (MSSA) isolates, verifying electrostatic repulsion as a major resistance mechanism (Ouhara et al. 2008).

S. aureus secretes an array of factors that contribute to resistance against the innate immune system and HDPs. LL-37 is susceptible to proteolytic degradation by two major *S. aureus*-secreted proteases, aureolysin and V8 protease. Specifically, V8 protease is involved in hydrolyzing particular peptide bonds, while aureolysin cleaves LL-37, abolishing its antimicrobial activity (Sieprawska-Lupa et al. 2004).

Furthermore, the exoprotein staphylokinase (SAK) has been shown to form a complex with alpha-defensins, abrogating the effect of these peptides in vitro and in vivo (Jin et al. 2004). *S. aureus* also exploits the production of cathelicidin as it binds directly to staphylokinase, which enhances SAK-dependent plasminogen activation and fibrinolysis, resulting in bacterial dissemination and enhanced virulence (Braff et al. 2007). Dermcidin has also been shown to induce global regulatory changes, resulting in the expression of dermcidin-degrading proteases in *S. epidermidis* and *S. aureus* (Lai et al. 2007). Furthermore, *S. aureus* expresses IsdA, a cell-wall bound heme-binding protein that decreases bacterial hydrophobicity, resulting in increased resistance to bactericidal human skin fatty acids as well as cathelicidins and β-defensins (Clarke et al. 2007).

Growth states have also been associated with increased resistance to HDPs. For example, *S. aureus* small colony variants (SCVs), which have been associated with slow growth and complex physiological and metabolic changes, are less susceptible to RNase 7, hβD2, hβD3, and LL-37 (Glaser et al. 2014).

Finally, peptidoglycan O-acetyltransferase OatO, is a major determinant of lysozyme resistance in *S. aureus*. The enzymatic activity of OatA results in the O-acetylation of the C6 hydroxyl group of muramic acid found in peptidoglycan, creating steric hindrance and preventing lysozyme binding to peptidoglycan (Bera et al. 2005).

12.6 Therapeutics

HDPs demonstrate modest direct antimicrobial activity with concomitant immune-modulatory activities, making them ideal starting points for deriving new therapies to treat multi-drug resistant *S. aureus* infections. Furthermore, while antibiotics typically impair a specific and essential bacterial process, HDPs target multiple hydrophobic and anionic bacterial targets, making it more difficult for bacteria to develop resistance. Much work has been performed to exploit the powerful activities of natural HDPs through the creation of synthetic mimetics. This has led to the creation of peptides with improved anti-infective and anti-inflammatory activities. For example, innate defense regulator-1 (IDR-1) was derived conceptually from the bovine AMP, bactenecin. In an invasive peritonitis model, IDR-1 was protective against MRSA by enhancing levels of monocyte chemokines, thereby recruiting monocytes and macrophages to combat infection. Moreover, IDR-1 reduced levels of harmful pro-inflammatory cytokine responses, which also aided in resolving the infection (Scott et al. 2007). High-throughput screening of bactenecin derivatives led to the identification of IDR-1002, a peptide that demonstrated enhanced chemokine induction and greater protection compared to IDR-1 in an *S. aureus* peritonitis murine infection model (Nijnik et al. 2010). This heightened anti-infective activity was attributed to increased recruitment of neutrophils and monocytes to the site of the infection. Synergistic therapy combining two peptides has also demonstrated efficacy in live infection models. For instance, combining

LL-37 and IDR-1ameliorated MRSA-induced pneumonia by significantly attenuating anti-inflammatory cytokine release (mainly TNF-α and IL-6) in bronchoalveolar lavage fluid, reducing pulmonary tissue damage. Furthermore, another 12-amino acid bactenecin derivative, IDR-1018, possessed very potent immune-modulatory properties through the induction of chemokine and suppression of pro-inflammatory responses. Along with these activities, IDR-1018 demonstrated accelerated wound healing in an *S. aureus*-infected porcine wound model compared to LL-37 (Steinstraesser et al. 2012). Interestingly, this activity was not conserved in an immune-compromised infection model, demonstrating the importance of a functional immune state for peptide activity. Furthermore, in an orthopedic implant *S. aureus* murine infection model, IDR-1018 accelerated the clearance of *S. aureus* by enhancing the recruitment of macrophages (Choe et al. 2015). In this model, IDR-1018 also enhanced osseointegration, which is typically impaired with *S. aureus* bone infection.

12.7 Constraints to HDP Therapeutic Development

Their numerous anti-infective roles make HDPs intriguing candidates for *S. aureus* infections. Nevertheless, there are many hurdles that must be overcome before they may become commercially available. Here, we will highlight these issues, which include toxicity, immunogenicity, susceptibility to proteolytic degradation, and cost.

12.7.1 Toxicity

Unfortunately, a major impediment for the pharmaceutical exploitation of HDPs is their toxicity. This toxicity can be due to many reasons, one of which is the potential of HDPs to stimulate immune responses, which can have unforeseen consequences. In fact, high levels of HDPs have been previously linked to diseases. For example, in humans, abnormally high levels of cathelicidin in facial skin have been linked to rosacea (Yamasaki et al. 2007). In mice, injection of LL-37 in mouse skin induced inflammatory hallmarks of rosacea, such as erythema and vascular dilatation (Yamasaki et al. 2007). This coincided with an increase in IL-8 production and neutrophil infiltration. Moreover, significantly elevated levels of LL-37 have been identified in psoriatic lesional skin (Morizane et al. 2012), where heightened levels of LL-37 were shown to increase the expression of TLR9 on keratinocytes. The enhanced TLR9 expression sensitizes keratinocytes to their ligands, CpG or genomic DNA, resulting in the increased production of type I IFNs, which exacerbate psoriasis (Morizane et al. 2012). Furthermore, LL-37 and hβD2 were shown to stimulate histamine release from mast cells (Niyonsaba et al. 2001).

Interestingly, LL-37-induced mast cell activation results in the release of β-tryptase, a LL-37 degrading enzyme (Duplantier and van Hoek 2013).

Hydrophobic cores of HDPs and synthetic mimetics have been shown to self-associate, aggregate, integrate into zwitterionic membranes, and cause hemolysis (Yin et al. 2012). Although not much is known about whether aggregation is responsible for the observed immune-modulatory effects displayed by HDPs, hydrophobicity (within a certain window) is required for bacterial membrane insertion. Therefore, these properties must be tampered with carefully, preventing peptide inactivation or increase of toxicity (Bahar and Ren 2013).

It is thought that since HDPs have been conserved throughout evolution, they should represent feasible anti-infective strategies (Mansour et al. 2014). In addition, HDPs can be altered to reduce toxicity. For instance, judicious formulation such as nanoparticle encapsulations can alter charge distribution and minimize toxic aggregation. Peptide lengths can be adjusted to minimize toxicity. For example, a shortened 15-amino acid derivative of melittin proved to be 300 times less toxic than melittin (Subbalakshmi et al. 1999). Simple high-throughput screening methodologies that measure cytolytic properties can be used to select for new peptide candidates with reduced toxicity (Haney et al. 2015). Moreover, after HDP targets are identified, computer-aided system biology approaches can be used to predict molecular pathways that may be interrupted, preventing undesirable off-targets of new HDP therapies.

12.7.2 Degradation

Another potential constraint that would reduce efficacy of HDP therapy is the risk of proteolytic degradation. As previously indicated, HDPs are inactivated and degraded by *S. aureus*-secreted enzymes and proteases (See Sect. 12.5). Furthermore, endogenous host enzymes readily degrade HDPs. Notably, digestive enzymes such as trypsin and chemotrypsin cleave peptides at basic and hydrophobic residues altering important structural and functional features of HDPs (Kim et al. 2014).

Many measures can be taken to tackle degradation issues of HDP therapy. Since host proteases cleave peptide bonds between naturally occurring residues, incorporating non-proteinogenic amino acids such as ornithine and β-didehydrophenylalanine into HDP structures can potentially improve metabolic stability (Bahar and Ren 2013). Moreover, cyclization of linear peptides has been shown to increase protease resistance of HDPs (Rozek et al. 2003). As host proteases only cleave peptides with L-enantiomeric backbones, the incorporation of D-amino acids in peptides can drastically reduce degradation, as has been shown with LL-37 (Wang et al. 2014). Presumably due to increased stability, D-amino acid peptides demonstrated greater protective activity compared to their L-counterparts against lethal multi-drug resistant bacteria (de la Fuente-Nunez et al. 2015). However, little is known regarding the pharmacokinetics and toxicity surrounding D-peptide

therapies and whether such manipulations would affect immune-modulatory targets. Furthermore, such modifications in peptide structure and composition are likely to increase the costs of peptide synthesis. Kim et al., described a more economical approach to designing peptides with residues that are systemically arranged to avoid protease-targeted sites (Kim et al. 2014). By using this method and maintaining the structural features that are important for HDP activity (amphipathy, cationic character, helicity, etc.), peptides with retained antimicrobial activity but heightened stability against host proteases could be derived.

12.7.3 Immunogenicity

As mentioned, HDPs are capable of interacting with cells of both the innate and adaptive arms of the immune system, eliciting a number of immunomodulatory roles. While these characteristics may serve as the basis for their protection, peptides may also prove to be immunogenic, stimulating an undesirable humoral response.

Immunogenicity, specifically through the development of anti-therapeutic antibodies, has been reported in a number of current FDA-approved antibody and protein-based therapies (Baker et al. 2010). Initially, these events can be triggered when the foreign peptide is recognized by an antigen-presenting cell (APC). For example, scavenging dendritic cells can phagocytose and present the foreign peptides via the exogenous MHC class II processing pathway, ultimately priming T cell responses. Capturing of antigen by dendritic cells is typically mediated by the FcR receptors that can interact with peptide-immune complexes. Indeed, LL-37 and certain defensins have also been shown to interact with TLRs on the surface of dendritic cells. These interactions can further activate the cells, resulting in the maturation and expression of lymphocyte co-stimulatory receptors initiating a T-cell dependent anti-peptide response (Yang et al. 2009). T-cell independent anti-peptide responses are speculated to be triggered by aggregated peptides that effectively cross-link B cell receptors, enabling the activation of B cells. Such responses can result in the generation of neutralizing antibodies or in some cases, antibodies that cross-react with endogenous proteins. Overall, immunogenicity may result in ineffective peptides or occasionally, autoimmune diseases.

Some proof of concept methods for reducing immunogenicity of protein therapies has involved the use of immunosuppressive therapies (Reding 2006) or slowly inducing tolerance to therapies. Furthermore, removal of T cell epitopes through rational sequence design and targeted amino acid substitutions can lower the development of immunogenicity of protein therapies (Jones et al. 2009). Immunogenicity of peptide derivatives can be assayed using various in vitro T cell assays.

12.7.4 Cost

Another constraint that has hindered mass production of HDPs for therapeutic use is the high cost of synthesis. Peptide synthesis using classic fluorenylmethoxycarbonyl (F-moc) chemistry can range between $50 and $400 per gram of peptide, making widespread clinical usage unrealistic. As peptides are antagonized by physiological conditions and degraded by host or bacterial proteases, relatively high doses may be required to attain the desired therapeutic effects. Moreover, deriving large peptide libraries (>100 peptides) to screen for optimized peptide candidates can become exorbitantly expensive.

Although cost of peptide synthesis has remained one of the major barriers to use of HDPs, several solutions have been proposed in the literature. For example, deriving synthetic HDP mimetics with shorter sequences can tackle the high cost of production. For example, efforts to re-engineer GF-17, a short and active fragment of LL-37, has led to the development of 17BIPHE2, a peptide with enhanced activity against *S. aureus*, which has also shown anti-biofilm properties in a mouse model of catheter-related *S. aureus* infection (Wang et al. 2014). Importantly, peptides must contain a minimum of 7–8 amino acids to maintain amphipathic structures, and for α-helical AMPs, 22 amino acids are required to completely span the bacterial lipid bilayer (Bahar and Ren 2013). Furthermore, exceptional anti-infective activity of synthetic mimetics can potentially lower dosage and thus cost of peptide therapy. The classical approach to optimize peptide activity is through rational design of large synthetic peptide libraries via systematic single amino acid substitutions of short HDP backbone templates (Haney et al. 2015). High-throughput screening methodologies can then select peptides that are optimized for various biological properties. For example, screening for anti-*S. aureus* biofilm activity, chemokine induction and endotoxin suppression, led to the selection of the optimized peptide IDR-2009 (Haney et al. 2015). Lastly, exploiting biological expression systems to derive recombinant fusion peptides serves as a scalable and low cost alternative for peptide synthesis (Li 2009). However, purification of peptides from prokaryotic systems must be carried out carefully to prevent endotoxin and bacterial contamination.

12.8 The Future of HDP Therapy

12.8.1 Synergistic Cocktails

Many of the aforementioned issues hindering widespread clinical use of HDPs can be tackled by administrating synergistic drug cocktails. Indeed, HDPs have previously shown to act synergistically with each other as well as with conventional antibiotics. For example, studies have demonstrated that when β-defensins are combined at sub-effective concentrations with human cathelicidin (LL-37) and

lysozyme, they exhibit additive or synergistic activity, which may be important for retaining in vivo activity (Midorikawa et al. 2003; Chen et al. 2005). Likewise, HDPs and synthetic mimetics display strong synergistic interactions with a number of conventional antibiotics (Reffuveille et al. 2014). Presumably, synergistic combination therapy would drastically lower the dosage of peptide and antibiotic required, reducing the overall cost of peptide therapy and toxicity.

12.8.2 Stimulating Natural HDP Production

Enhancing nature's own arsenal may represent a safer and more feasible method for HDP-directed therapies. Studies have demonstrated that inducing LL-37 by stimulating keratinocytes with both butyrate and vitamin D3 increases antimicrobial activity of keratinocytes against *S. aureus* (Schauber et al. 2008). Furthermore, topical treatment with the vitamin D analog, calcipotriol, following acute skin infection enhanced levels of hCAP18/LL-37 in human skin (Heilborn et al. 2010). As LL-37 has been shown to promote re-epithelialization and tissue repair, therapies that promote LL-37 production may serve as effective wound treatments. However, as previously noted (Sect. 12.7.1), LL-37 displays a wide variety of immune-regulatory activities and levels must be carefully tuned to avoid any adverse consequences.

12.8.3 Impairing CAMP Resistance Mechanisms

An interesting and unexplored avenue for HDP-targeted therapy is to inactivate CAMP resistance mechanisms to sensitize *S. aureus* to HDPs. Studies by Li et al. have shown that mutants of the Aps HDP-sensing system are less virulent in an *S. aureus* murine infection model (Li et al. 2007a, b). Likewise, inactivating Dlt reduces the D-alanine addition to teichoic acids, thereby increasing *S. aureus* susceptibility to HDPs (Peschel et al. 1999). Consistent with these findings, mutants lacking *dltA* and *mprF* are more susceptible to HDPs and have attenuated virulence in vivo (Peschel et al. 1999; Collins et al. 2002; Kristian et al. 2003; Weidenmaier et al. 2005). Moreover, targeting secreted proteases that degrade HDPs may help to enhance the activity of HDPs.

12.9 Conclusions

HDPs serve as one of the first lines of defense against *S. aureus* infections. Specifically, defensins, cathelicidins, dermcidins, and RNAse 7 display potent anti-staphylococcal activity along with a number of immune-modulatory roles.

Despite these various biological activities, *S. aureus* has developed numerous HDP evasion techniques that function for example by repelling positively charged HDPs and abrogating peptide activity. Nevertheless, many HDPs as well as synthetic peptidomimetics have demonstrated exceptional anti-*S. aureus* activity. Scalable and commercial use of HDPs or their synthetic counterparts is, however, constrained by the cost of production, toxicities, immunogenicity and degradation. The future of HDP therapeutic development relies on re-engineering peptides with greater biological activity and reduced toxicities, deriving synergistic cocktails, identifying new HDP-stimulants and sensitizing bacteria to HDPs.

References

Ahn SH, Deshmukh H, Johnson N, Cowell LG, Rude TH, Scott WK, Nelson CL, Zaas AK, Marchuk DA, Keum S, Lamlertthon S, Sharma-Kuinkel BK, Sempowski GD, Fowler VG Jr (2010) Two genes on A/J chromosome 18 are associated with susceptibility to Staphylococcus aureus infection by combined microarray and QTL analyses. PLoS Pathog 6(9):e1001088

Alalwani SM, Sierigk J, Herr C, Pinkenburg O, Gallo R, Vogelmeier C, Bals R (2010) The antimicrobial peptide LL-37 modulates the inflammatory and host defense response of human neutrophils. Eur J Immunol 40(4):1118–1126

Bahar AA, Ren D (2013) Antimicrobial peptides. Pharmaceuticals (Basel) 6(12):1543–1575

Baker MP, Reynolds HM, Lumicisi B, Bryson CJ (2010) Immunogenicity of protein therapeutics: the key causes, consequences and challenges. Self Nonself 1(4):314–322

Bera A, Herbert S, Jakob A, Vollmer W, Gotz F (2005) Why are pathogenic staphylococci so lysozyme resistant? The peptidoglycan O-acetyltransferase OatA is the major determinant for lysozyme resistance of Staphylococcus aureus. Mol Microbiol 55(3):778–787

Braff MH, Jones AL, Skerrett SJ, Rubens CE (2007) Staphylococcus aureus exploits cathelicidin antimicrobial peptides produced during early pneumonia to promote staphylokinase-dependent fibrinolysis. J Infect Dis 195(9):1365–1372

Cardot-Martin E, Casalegno JS, Badiou C, Dauwalder O, Keller D, Prevost G, Rieg S, Kern WV, Cuerq C, Etienne J, Vandenesch F, Lina G, Dumitrescu O (2015) alpha-Defensins partially protect human neutrophils against Panton-Valentine leukocidin produced by Staphylococcus aureus. Lett Appl Microbiol 61(2):158–164

Chen X, Niyonsaba F, Ushio H, Okuda D, Nagaoka I, Ikeda S, Okumura K, Ogawa H (2005) Synergistic effect of antibacterial agents human beta-defensins, cathelicidin LL-37 and lysozyme against Staphylococcus aureus and Escherichia coli. J Dermatol Sci 40(2):123–132

Cho JS, Pietras EM, Garcia NC, Ramos RI, Farzam DM, Monroe HR, Magorien JE, Blauvelt A, Kolls JK, Cheung AL, Cheng G, Modlin RL, Miller LS (2010) IL-17 is essential for host defense against cutaneous Staphylococcus aureus infection in mice. J Clin Invest 120(5):1762–1773

Cho SH, Strickland I, Tomkinson A, Fehringer AP, Gelfand EW, Leung DY (2001) Preferential binding of Staphylococcus aureus to skin sites of Th2-mediated inflammation in a murine model. J Invest Dermatol 116(5):658–663

Choe H, Narayanan AS, Gandhi DA, Weinberg A, Marcus RE, Lee Z, Bonomo RA, Greenfield EM (2015) Immunomodulatory peptide IDR-1018 decreases implant infection and preserves osseointegration. Clin Orthop Relat Res 473(9):2898–2907

Clarke SR, Mohamed R, Bian L, Routh AF, Kokai-Kun JF, Mond JJ, Tarkowski A, Foster SJ (2007) The Staphylococcus aureus surface protein IsdA mediates resistance to innate defenses of human skin. Cell Host Microbe 1(3):199–212

Coates R, Moran J, Horsburgh MJ (2014) Staphylococci: colonizers and pathogens of human skin. Future Microbiol 9(1):75–91

Collins LV, Kristian SA, Weidenmaier C, Faigle M, Van Kessel KP, Van Strijp JA, Gotz F, Neumeister B, Peschel A (2002) Staphylococcus aureus strains lacking D-alanine modifications of teichoic acids are highly susceptible to human neutrophil killing and are virulence attenuated in mice. J Infect Dis 186(2):214–219

David MZ, Daum RS (2010) Community-associated methicillin-resistant Staphylococcus aureus: epidemiology and clinical consequences of an emerging epidemic. Clin Microbiol Rev 23 (3):616–687

Davidson DJ, Currie AJ, Reid GS, Bowdish DM, MacDonald KL, Ma RC, Hancock RE, Speert DP (2004) The cationic antimicrobial peptide LL-37 modulates dendritic cell differentiation and dendritic cell-induced T cell polarization. J Immunol 172(2):1146–1156

de la Fuente-Nunez C, Reffuveille F, Mansour SC, Reckseidler-Zenteno SL, Hernandez D, Brackman G, Coenye T, Hancock RE (2015) D-enantiomeric peptides that eradicate wild-type and multidrug-resistant biofilms and protect against lethal Pseudomonas aeruginosa infections. Chem Biol 22(2):196–205

Duplantier AJ, van Hoek ML (2013) The human cathelicidin antimicrobial peptide LL-37 as a potential treatment for polymicrobial infected wounds. Front Immunol 4:143

Flad T, Bogumil R, Tolson J, Schittek B, Garbe C, Deeg M, Mueller CA, Kalbacher H (2002) Detection of dermcidin-derived peptides in sweat by ProteinChip technology. J Immunol Methods 270(1):53–62

Glaser R, Becker K, von Eiff C, Meyer-Hoffert U, Harder J (2014) Decreased susceptibility of Staphylococcus aureus small-colony variants toward human antimicrobial peptides. J Invest Dermatol 134(9):2347–2350

Gu B, Kelesidis T, Tsiodras S, Hindler J, Humphries RM (2013) The emerging problem of linezolid-resistant Staphylococcus. J Antimicrob Chemother 68(1):4–11

Haney EF, Mansour SC, Hilchie AL, de la Fuente-Nunez C, Hancock RE (2015) High throughput screening methods for assessing antibiofilm and immunomodulatory activities of synthetic peptides. Peptides

Harder J, Schroder JM (2002) RNase 7, a novel innate immune defense antimicrobial protein of healthy human skin. J Biol Chem 277(48):46779–46784

Harder J, Bartels J, Christophers E, Schroder JM (1997) A peptide antibiotic from human skin. Nature 387(6636):861

Harder J, Bartels J, Christophers E, Schroder JM (2001) Isolation and characterization of human beta -defensin-3, a novel human inducible peptide antibiotic. J Biol Chem 276(8):5707–5713

Heilborn JD, Weber G, Gronberg A, Dieterich C, Stahle M (2010) Topical treatment with the vitamin D analogue calcipotriol enhances the upregulation of the antimicrobial protein hCAP18/LL-37 during wounding in human skin in vivo. Exp Dermatol 19(4):332–338

Herold BC, Immergluck LC, Maranan MC, Lauderdale DS, Gaskin RE, Boyle-Vavra S, Leitch CD, Daum RS (1998) Community-acquired methicillin-resistant Staphylococcus aureus in children with no identified predisposing risk. JAMA 279(8):593–598

Jia T, Serbina NV, Brandl K, Zhong MX, Leiner IM, Charo IF, Pamer EG (2008) Additive roles for MCP-1 and MCP-3 in CCR2-mediated recruitment of inflammatory monocytes during Listeria monocytogenes infection. J Immunol 180(10):6846–6853

Jin T, Bokarewa M, Foster T, Mitchell J, Higgins J, Tarkowski A (2004) Staphylococcus aureus resists human defensins by production of staphylokinase, a novel bacterial evasion mechanism. J Immunol 172(2):1169–1176

Jones T, Yeaman MR, Sakoulas G, Yang SJ, Proctor RA, Sahl HG, Schrenzel J, Xiong YQ, Bayer AS (2008) Failures in clinical treatment of Staphylococcus aureus Infection with daptomycin are associated with alterations in surface charge, membrane phospholipid asymmetry, and drug binding. Antimicrob Agents Chemother 52(1):269–278

Jones TD, Crompton LJ, Carr FJ, Baker MP (2009) Deimmunization of monoclonal antibodies. Methods Mol Biol 525:405–423, xiv

Kim H, Jang JH, Kim SC, Cho JH (2014) De novo generation of short antimicrobial peptides with enhanced stability and cell specificity. J Antimicrob Chemother 69(1):121–132

Kisich KO, Howell MD, Boguniewicz M, Heizer HR, Watson NU, Leung DY (2007) The constitutive capacity of human keratinocytes to kill Staphylococcus aureus is dependent on beta-defensin 3. J Invest Dermatol 127(10):2368–2380

Klevens RM, Morrison MA, Nadle J, Petit S, Gershman K, Ray S, Harrison LH, Lynfield R, Dumyati G, Townes JM, Craig AS, Zell ER, Fosheim GE, McDougal LK, Carey RB, Fridkin SK, M.I. Active Bacterial Core surveillance (2007) Invasive methicillin-resistant Staphylococcus aureus infections in the United States. JAMA 298(15):1763–1771

Kristian SA, Lauth X, Nizet V, Goetz F, Neumeister B, Peschel A, Landmann R (2003) Alanylation of teichoic acids protects Staphylococcus aureus against Toll-like receptor 2-dependent host defense in a mouse tissue cage infection model. J Infect Dis 188(3):414–423

Kurihara T, Warr G, Loy J, Bravo R (1997) Defects in macrophage recruitment and host defense in mice lacking the CCR2 chemokine receptor. J Exp Med 186(10):1757–1762

Kurosaka K, Chen Q, Yarovinsky F, Oppenheim JJ, Yang D (2005) Mouse cathelin-related antimicrobial peptide chemoattracts leukocytes using formyl peptide receptor-like 1/mouse formyl peptide receptor-like 2 as the receptor and acts as an immune adjuvant. J Immunol 174 (10):6257–6265

Lai Y, Villaruz AE, Li M, Cha DJ, Sturdevant DE, Otto M (2007) The human anionic antimicrobial peptide dermcidin induces proteolytic defence mechanisms in staphylococci. Mol Microbiol 63(2):497–506

Laouini D, Kawamoto S, Yalcindag A, Bryce P, Mizoguchi E, Oettgen H, Geha RS (2003) Epicutaneous sensitization with superantigen induces allergic skin inflammation. J Allergy Clin Immunol 112(5):981–987

Lehrer RI (2007) Multispecific myeloid defensins. Curr Opin Hematol 14(1):16–21

Levin TP, Suh B, Axelrod P, Truant AL, Fekete T (2005) Potential clindamycin resistance in clindamycin-susceptible, erythromycin-resistant Staphylococcus aureus: report of a clinical failure. Antimicrob Agents Chemother 49(3):1222–1224

Li M, Cha DJ, Lai Y, Villaruz AE, Sturdevant DE, Otto M (2007a) The antimicrobial peptide-sensing system aps of Staphylococcus aureus. Mol Microbiol 66(5):1136–1147

Li M, Lai Y, Villaruz AE, Cha DJ, Sturdevant DE, Otto M (2007b) Gram-positive three-component antimicrobial peptide-sensing system. Proc Natl Acad Sci USA 104 (22):9469–9474

Li Y (2009) Carrier proteins for fusion expression of antimicrobial peptides in Escherichia coli. Biotechnol Appl Biochem 54(1):1–9

Li B, Jiang B, Dietz MJ, Smith ES, Clovis NB, Rao KM (2010) Evaluation of local MCP-1 and IL-12 nanocoatings for infection prevention in open fractures. J Orthop Res 28(1):48–54

Liang SC, Tan XY, Luxenberg DP, Karim R, Dunussi-Joannopoulos K, Collins M, Fouser LA (2006) Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. J Exp Med 203(10):2271–2279

Mansour SC, Pena OM, Hancock RE (2014) Host defense peptides: front-line immunomodulators. Trends Immunol 35(9):443–450

Menzies BE, Kenoyer A (2006) Signal transduction and nuclear responses in Staphylococcus aureus-induced expression of human beta-defensin 3 in skin keratinocytes. Infect Immun 74 (12):6847–6854

Midorikawa K, Ouhara K, Komatsuzawa H, Kawai T, Yamada S, Fujiwara T, Yamazaki K, Sayama K, Taubman MA, Kurihara H, Hashimoto K, Sugai M (2003) Staphylococcus aureus susceptibility to innate antimicrobial peptides, beta-defensins and CAP18, expressed by human keratinocytes. Infect Immun 71(7):3730–3739

Milner JD, Brenchley JM, Laurence A, Freeman AF, Hill BJ, Elias KM, Kanno Y, Spalding C, Elloumi HZ, Paulson ML, Davis J, Hsu A, Asher AI, O'Shea J, Holland SM, Paul WE, Douek DC (2008) Impaired T(H)17 cell differentiation in subjects with autosomal dominant hyper-IgE syndrome. Nature 452(7188):773–776

Mookherjee N, Lippert DN, Hamill P, Falsafi R, Nijnik A, Kindrachuk J, Pistolic J, Gardy J, Miri P, Naseer M, Foster LJ, Hancock RE (2009) Intracellular receptor for human host defense peptide LL-37 in monocytes. J Immunol 183(4):2688–2696

Morizane S, Yamasaki K, Muhleisen B, Kotol PF, Murakami M, Aoyama Y, Iwatsuki K, Hata T, Gallo RL (2012) Cathelicidin antimicrobial peptide LL-37 in psoriasis enables keratinocyte reactivity against TLR9 ligands. J Invest Dermatol 132(1):135–143

Murakami M, Ohtake T, Dorschner RA, Schittek B, Garbe C, Gallo RL (2002) Cathelicidin anti-microbial peptide expression in sweat, an innate defense system for the skin. J Invest Dermatol 119(5):1090–1095

Nijnik A, Madera L, Ma S, Waldbrook M, Elliott MR, Easton DM, Mayer ML, Mullaly SC, Kindrachuk J, Jenssen H, Hancock RE (2010) Synthetic cationic peptide IDR-1002 provides protection against bacterial infections through chemokine induction and enhanced leukocyte recruitment. J Immunol 184(5):2539–2550

Niyonsaba F, Someya A, Hirata M, Ogawa H, Nagaoka I (2001) Evaluation of the effects of peptide antibiotics human beta-defensins-1/-2 and LL-37 on histamine release and prostaglandin D(2) production from mast cells. Eur J Immunol 31(4):1066–1075

Nizet V, Ohtake T, Lauth X, Trowbridge J, Rudisill J, Dorschner RA, Pestonjamasp V, Piraino J, Huttner K, Gallo RL (2001) Innate antimicrobial peptide protects the skin from invasive bacterial infection. Nature 414(6862):454–457

Nomura I, Goleva E, Howell MD, Hamid QA, Ong PY, Hall CF, Darst MA, Gao B, Boguniewicz M, Travers JB, Leung DY (2003) Cytokine milieu of atopic dermatitis, as compared to psoriasis, skin prevents induction of innate immune response genes. J Immunol 171(6):3262–3269

Ong PY, Ohtake T, Brandt C, Strickland I, Boguniewicz M, Ganz T, Gallo RL, Leung DY (2002) Endogenous antimicrobial peptides and skin infections in atopic dermatitis. N Engl J Med 347 (15):1151–1160

Otto M (2010) Staphylococcus colonization of the skin and antimicrobial peptides. Expert Rev Dermatol 5(2):183–195

Ouhara K, Komatsuzawa H, Kawai T, Nishi H, Fujiwara T, Fujiue Y, Kuwabara M, Sayama K, Hashimoto K, Sugai M (2008) Increased resistance to cationic antimicrobial peptide LL-37 in methicillin-resistant strains of Staphylococcus aureus. J Antimicrob Chemother 61(6):1266–1269

Peschel A, Jack RW, Otto M, Collins LV, Staubitz P, Nicholson G, Kalbacher H, Nieuwenhuizen WF, Jung G, Tarkowski A, van Kessel KP, van Strijp JA (2001) Staphylococcus aureus resistance to human defensins and evasion of neutrophil killing via the novel virulence factor MprF is based on modification of membrane lipids with l-lysine. J Exp Med 193(9):1067–1076

Peschel A, Otto M, Jack RW, Kalbacher H, Jung G, Gotz F (1999) Inactivation of the dlt operon in Staphylococcus aureus confers sensitivity to defensins, protegrins, and other antimicrobial peptides. J Biol Chem 274(13):8405–8410

Reding MT (2006) Immunological aspects of inhibitor development. Haemophilia 12(6):30–35; discussion 35–36

Reffuveille F, de la Fuente-Nunez C, Mansour S, Hancock RE (2014) A broad-spectrum antibiofilm peptide enhances antibiotic action against bacterial biofilms. Antimicrob Agents Chemother 58(9):5363–5371

Renner ED, Rylaarsdam S, Anover-Sombke S, Rack AL, Reichenbach J, Carey JC, Zhu Q, Jansson AF, Barboza J, Schimke LF, Leppert MF, Getz MM, Seger RA, Hill HR, Belohradsky BH, Torgerson TR, Ochs HD (2008) Novel signal transducer and activator of transcription 3 (STAT3) mutations, reduced T(H)17 cell numbers, and variably defective STAT3 phosphorylation in hyper-IgE syndrome. J Allergy Clin Immunol 122(1):181–187

Rieg S, Steffen H, Seeber S, Humeny A, Kalbacher H, Dietz K, Garbe C, Schittek B (2005) Deficiency of dermcidin-derived antimicrobial peptides in sweat of patients with atopic dermatitis correlates with an impaired innate defense of human skin in vivo. J Immunol 174 (12):8003–8010

Rozek A, Powers JP, Friedrich CL, Hancock RE (2003) Structure-based design of an indolicidin peptide analogue with increased protease stability. Biochemistry 42(48):14130–14138

Schauber J, Oda Y, Buchau AS, Yun QC, Steinmeyer A, Zugel U, Bikle DD, Gallo RL (2008) Histone acetylation in keratinocytes enables control of the expression of cathelicidin and CD14 by 1,25-dihydroxyvitamin D3. J Invest Dermatol 128(4):816–824

Schittek B, Hipfel R, Sauer B, Bauer J, Kalbacher H, Stevanovic S, Schirle M, Schroeder K, Blin N, Meier F, Rassner G, Garbe C (2001) Dermcidin: a novel human antibiotic peptide secreted by sweat glands. Nat Immunol 2(12):1133–1137

Scott MG, Dullaghan E, Mookherjee N, Glavas N, Waldbrook M, Thompson A, Wang A, Lee K, Doria S, Hamill P, Yu JJ, Li Y, Donini O, Guarna MM, Finlay BB, North JR, Hancock RE (2007) An anti-infective peptide that selectively modulates the innate immune response. Nat Biotechnol 25(4):465–472

Sieprawska-Lupa M, Mydel P, Krawczyk K, Wojcik K, Puklo M, Lupa B, Suder P, Silberring J, Reed M, Pohl J, Shafer W, McAleese F, Foster T, Travis J, Potempa J (2004) Degradation of human antimicrobial peptide LL-37 by Staphylococcus aureus-derived proteinases. Antimicrob Agents Chemother 48(12):4673–4679

Simanski M, Dressel S, Glaser R, Harder J (2010) RNase 7 protects healthy skin from Staphylococcus aureus colonization. J Invest Dermatol 130(12):2836–2838

Song C, Weichbrodt C, Salnikov ES, Dynowski M, Forsberg BO, Bechinger B, Steinem C, de Groot BL, Zachariae U, Zeth K (2013) Crystal structure and functional mechanism of a human antimicrobial membrane channel. Proc Natl Acad Sci USA 110(12):4586–4591

Sorensen OE, Thapa DR, Roupe KM, Valore EV, Sjobring U, Roberts AA, Schmidtchen A, Ganz T (2006) Injury-induced innate immune response in human skin mediated by transactivation of the epidermal growth factor receptor. J Clin Invest 116(7):1878–1885

Steinstraesser L, Hirsch T, Schulte M, Kueckelhaus M, Jacobsen F, Mersch EA, Stricker I, Afacan N, Jenssen H, Hancock RE, Kindrachuk J (2012) Innate defense regulator peptide 1018 in wound healing and wound infection. PLoS One 7(8):e39373

Subbalakshmi C, Nagaraj R, Sitaram N (1999) Biological activities of C-terminal 15-residue synthetic fragment of melittin: design of an analog with improved antibacterial activity. FEBS Lett 448(1):62–66

Tiwari HK, Sen MR (2006) Emergence of vancomycin resistant Staphylococcus aureus (VRSA) from a tertiary care hospital from northern part of India. BMC Infect Dis 6:156

Tjabringa GS, Ninaber DK, Drijfhout JW, Rabe KF, Hiemstra PS (2006) Human cathelicidin LL-37 is a chemoattractant for eosinophils and neutrophils that acts via formyl-peptide receptors. Int Arch Allergy Immunol 140(2):103–112

Turner J, Cho Y, Dinh NN, Waring AJ, Lehrer RI (1998) Activities of LL-37, a cathelin-associated antimicrobial peptide of human neutrophils. Antimicrob Agents Chemother 42(9):2206–2214

Voss E, Wehkamp J, Wehkamp K, Stange EF, Schroder JM, Harder J (2006) NOD2/CARD15 mediates induction of the antimicrobial peptide human beta-defensin-2. J Biol Chem 281 (4):2005–2011

Waldvogel FA (1999) New resistance in Staphylococcus aureus. N Engl J Med 340(7):556–557

Wang G, Hanke ML, Mishra B, Lushnikova T, Heim CE, Chittezham Thomas V, Bayles KW, Kielian T (2014) Transformation of human cathelicidin LL-37 into selective, stable, and potent antimicrobial compounds. ACS Chem Biol 9(9):1997–2002

Weidenmaier C, Peschel A, Kempf VA, Lucindo N, Yeaman MR, Bayer AS (2005) DltABCD- and MprF-mediated cell envelope modifications of Staphylococcus aureus confer resistance to platelet microbicidal proteins and contribute to virulence in a rabbit endocarditis model. Infect Immun 73(12):8033–8038

Yamasaki K, Di Nardo A, Bardan A, Murakami M, Ohtake T, Coda A, Dorschner RA, Bonnart C, Descargues P, Hovnanian A, Morhenn VB, Gallo RL (2007) Increased serine protease activity and cathelicidin promotes skin inflammation in rosacea. Nat Med 13(8):975–980

Yanagi S, Ashitani J, Ishimoto H, Date Y, Mukae H, Chino N, Nakazato M (2005) Isolation of human beta-defensin-4 in lung tissue and its increase in lower respiratory tract infection. Respir Res 6:130

Yang D, Chertov O, Bykovskaia SN, Chen Q, Buffo MJ, Shogan J, Anderson M, Schroder JM, Wang JM, Howard OM, Oppenheim JJ (1999) Beta-defensins: linking innate and adaptive immunity through dendritic and T cell CCR6. Science 286(5439):525–528

Yang D, Chen Q, Chertov O, Oppenheim JJ (2000) Human neutrophil defensins selectively chemoattract naive T and immature dendritic cells. J Leukoc Biol 68(1):9–14

Yang D, de la Rosa G, Tewary P, Oppenheim JJ (2009) Alarmins link neutrophils and dendritic cells. Trends Immunol 30(11):531–537

Yin LM, Edwards MA, Li J, Yip CM, Deber CM (2012) Roles of hydrophobicity and charge distribution of cationic antimicrobial peptides in peptide-membrane interactions. J Biol Chem 287(10):7738–7745

Yount NY, Yeaman MR (2006) Structural congruence among membrane-active host defense polypeptides of diverse phylogeny. Biochim Biophys Acta 1758(9):1373–1386

Zanger P, Holzer J, Schleucher R, Scherbaum H, Schittek B, Gabrysch S (2010) Severity of Staphylococcus aureus infection of the skin is associated with inducibility of human beta-defensin 3 but not human beta-defensin 2. Infect Immun 78(7):3112–3117

Zanger P, Holzer J, Schleucher R, Steffen H, Schittek B, Gabrysch S (2009) Constitutive expression of the antimicrobial peptide RNase 7 is associated with Staphylococcus aureus infection of the skin. J Infect Dis 200(12):1907–1915

Zhang LJ, Guerrero-Juarez CF, Hata T, Bapat SP, Ramos R, Plikus MV, Gallo RL (2015) Innate immunity. Dermal adipocytes protect against invasive Staphylococcus aureus skin infection. Science 347(6217):67–71