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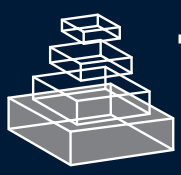
RESEARCH TOPICS

ANTIMICROBIAL PEPTIDES: UTILITY PLAYERS IN INNATE IMMUNITY

Hosted by
Mark W. Robinson, Andrew T. Hutchinson and Sheila Donnelly



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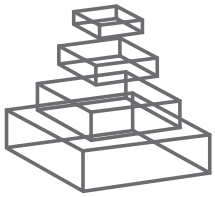

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ANTIMICROBIAL PEPTIDES: UTILITY PLAYERS IN INNATE IMMUNITY

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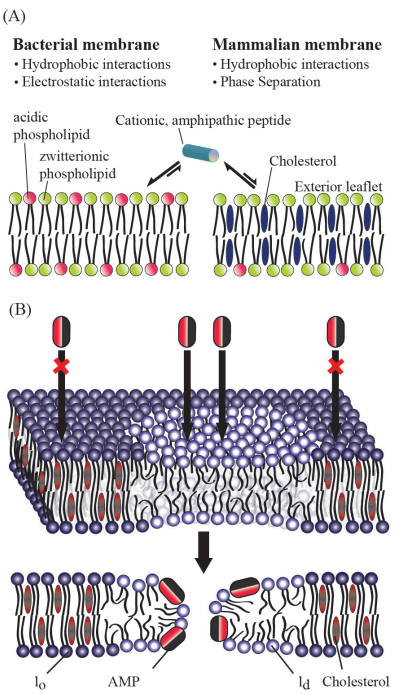

Taken from Brender JR, McHenry AJ and Ramamoorthy A (2012) Does cholesterol play a role in the bacterial selectivity of antimicrobial peptides? *Front. Immun.* 3:195. doi: 10.3389/fimmu.2012.00195

Antimicrobial peptides (AMPs) represent an ancient group of molecules with diverse functions in innate immunity. To date, more than 1000 naturally-occurring AMPs have been identified which display considerable diversity in their primary sequences, lengths, structures and biological activities.

Despite this variability, AMPs are broadly classified according to homologous secondary structures as cathelicidins (linear α-helical peptides), defensins (β-strand peptides connected by disulfide bonds) and bactenecins (loop peptides). Most, but not all, AMPs are cationic with amphipathic faces. These biochemical properties bestow many peptides with potent antimicrobial activity by facilitating interactions with negatively charged microbial cell membrane components, thereby increasing membrane permeability and resulting in microbial death. Other indirect effects on microbial physiology have been reported including inhibition of DNA/RNA synthesis, impaired protein synthesis and folding, disruption of cell wall formation and inhibition of microbial cell metabolism.

Thus, with the spread of antibiotic-resistant microbial pathogens, AMPs have emerged as exciting candidates for next generation anti-infective therapies. However, recent studies suggest that AMPs have evolved other mechanisms of pathogen

clearance. Immunomodulation is a novel approach to antimicrobial therapy that centers on boosting host immunity rather than direct microbial killing. This is also an attractive means to treat sepsis and other immune-mediated diseases. Whilst several cationic peptides are under investigation as antimicrobial agents, a select few show a remarkable ability to protect against lethal endotoxæmia and clinically-relevant bacterial infections including methicillin-resistant *Staphylococcus aureus* (MRSA). The molecular mechanisms responsible for this protection are only beginning to emerge but include prevention of innate cell activation by targeting key stages of bacterial endotoxin-mediated cell signaling.

In this research topic, hosted by *Frontiers in Molecular Innate Immunity*, we aim to highlight key areas of AMP research including peptide diversity, structure-function relationships, antimicrobial activity and mechanisms of immune-modulation. We also aim to stimulate discussion on the emerging therapeutic potential of AMPs including antifungal, antiviral and anticancer applications.

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EDITORIAL
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Antimicrobial peptides: utility players in innate immunity

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Antimicrobial peptides (AMPs) are an ancient group of molecules that are expressed in many species ranging from bacteria to humans. At least 1200 naturally-occurring AMPs have been identified which display considerable diversity in their primary sequences, lengths, structures, and biological activities (Wang et al., 2009). Generally speaking, these peptides have been studied due to their ability to directly kill medically important-microbes. Indeed, the increased demand for novel anti-infective therapies (due to the spread of drug-resistant bacteria) has likely contributed to the expansion of this field of research. However, there has been a definite change of focus in AMP research in light of the recent recognition of the regulatory functions of these molecules in the innate immune system. This has opened up the exciting possibility of developing novel antimicrobial therapies that centre on boosting host immunity rather than direct microbial killing (Zhang and Falla, 2010). In this volume, with emphasis on emerging technologies, mechanism of action studies and roles in disease, we aim to consolidate some of these developments and stimulate discussion on the therapeutic potential of these important molecules.

The first three articles demonstrate how knowledge of AMP structure and amino acid composition is guiding rational peptide design strategies aimed at improving the therapeutic potential of these molecules. Mishra and Wang describe the development of a comprehensive AMP database that is enabling us to link amino acid composition with specific peptide activities (e.g., antibacterial, antifungal, antiviral, antiparasital, insecticidal, spermicidal, anticancer, etc.). The article suggests how this information may be used to develop novel peptides with a desired activity. Next, Scorciapino and Rinaldi discuss how knowledge of the amino acid sequence of naturally-occurring AMPs can be used to dictate the design of antimicrobial peptidomimetics. The authors note that by retaining the biological activity of natural AMPs and improving their pharmacokinetic properties, these novel molecules may allow systemic use of AMPs to treat microbial infections. The third article by Devocelle describes how conjugation of AMPs to targeting moieties can improve delivery to their desired site of action and reduce potential off-target effects. It is clear that the complementary approaches described in these three articles will play an important part in making therapeutic AMPs a reality.

The next two articles describe different, but equally powerful, approaches that may be used to probe the biological functions of AMPs. The development of various "–omics" (genomics, transcriptomics, proteomics, and others) technologies has truly revolutionised biological research. In this regard, Plichta et al. discuss how the integration of various—omics datasets can help us to understand the role of AMPs in varied contexts from resolution of infections, improvement of prognosis for cancer patients to early detection of transplant rejection. The fifth article by Munoz and Read highlights the use of live cell imaging to study AMP function. With emphasis on the various approaches that may be used to label AMPs, this article demonstrates the central role live cell imaging continues to play in the elucidation of AMP function.

The next six articles in this research topic cover recent advances in our understanding of the biological role of AMPs and their mechanism(s) of action. Brender et al. examine the role of cholesterol in dictating the selectivity of AMPs for microbial membranes. Defining the role of cholesterol in AMP-mediated cytotoxicity is important, especially if AMPs are to be used therapeutically. Melo and Castanho discuss the various experimental approaches that may be used to answer this very question. By comparing the use of bacterial membranes with model liposomal membranes, they illustrate the importance of physiological relevance in experimental design. The next two articles in this series illustrate how helminths express AMP homologues that are functionally adapted to either a free-living or parasitic lifestyle. For example, Pujol et al. describe the array of AMPs expressed by the model nematode *Caenorhabditis elelgans* and how they serve to protect the worm against attack by fungi in the free-living environment. Next, Cotton et al. probe the role of AMP homologues that are secreted by medically-important parasitic worms. This interesting family of molecules may hold the key to the evolution of immunomodulatory function in AMPs. In the following two articles, Ulm et al. and Choi and Mookherjee discuss advances in our understanding of the molecular mechanisms by which members of these major AMP families influence immune cell function.

The final two articles in the volume cover the role of AMPs in disease resolution and the outlook for their therapeutic use in humans. The article by David describes how AMPs may fill a gap in our existing therapeutic repertoire. David builds the case for the use of polymyxins to treat Gram-negative sepsis, a condition for which there is currently no single effective therapeutic approach. The article by Berditsch et al. concludes the volume and describes how, perhaps surprisingly, AMPs can stimulate bacterial survival mechanisms leading to persistent infections. This article highlights the importance of understanding how microbial

populations respond to exposure to AMPs before they can be used in the clinic.

In summary, by inviting opinion articles from leading AMP researchers, the aim of this volume was to highlight recent advances in our understanding of the roles of these important molecules. We also hope that the articles compiled will stimulate discussion and further research in this area so that the therapeutic potential of AMPs may be realised.

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The importance of amino acid composition in natural AMPs: an evolutional, structural, and functional perspective

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Antimicrobial peptides (AMPs) are critical components of natural host defense systems against infectious pathogens (Zasloff, 2002; Boman, 2003; Hancock and Sahl, 2006). They are ubiquitous in nature and have been found in nearly all forms of life, ranging from single-celled bacteria to multicellular organisms such as plants and animals. AMPs are short peptides (5–100 amino acids) with an average net charge of +3 (Wang, 2010). They can display broad or narrow-spectrum antimicrobial activities. The fact that AMPs are effective against multidrug resistance pathogens, including suppression of biofilm formation, deserves our attention (Menousek et al., 2012). In addition to direct bacterial elimination, these peptides have regulatory effects on immune systems. Consequently, AMPs are also referred to as host defense peptides (Hancock and Sahl, 2006). To decode the key elements behind the functional diversity of AMPs, we have been taking time and efforts in constructing a comprehensive database that annotates such information. The first version of the Antimicrobial Peptide Database (APD; http://aps.unmc.edu/AP/main.html) was established in 2003 (Wang and Wang, 2004) and the database has since been further developed (Wang et al., 2009). The APD contained 1973 entries as of May 2012. To facilitate our bioinformatic analysis, we will register a peptide into the APD if it is (1) from natural sources; (2) with minimal inhibitory concentration (MIC) of less than 100 μM or 100 μg/mL; (3) less than 100 amino acid residues; and (4) with a characterized amino acid sequence (Wang, 2010). The APD allows users to extract important parameters (e.g., charge, hydrophobicity, motif, and structure) that determine peptide function. In particular, our database enables the generation of the amino acid composition for a select peptide or a family of AMPs with a common feature. This bioinformatic tool thus uncovers the amino acid use in natural AMPs from different sources, with different functions, or three-dimensional structures. This opinion article highlights the critical roles of the amino acid composition in naturally occurring AMPs in terms of evolutional, structural, and functional significance. Moreover, its application in designing and predicting new AMPs will also be discussed.

EVOLUTIONARY PERSPECTIVE

To get an idea of AMPs in different kingdoms, we obtained the amino acid composition profiles of these peptides from bacteria, fungi, plants, insects, fish, amphibia, reptiles, birds, and humans (**Figure 1A**) by performing source search in the APD (Wang et al., 2009). In our database, the 20 standard amino acids are classified into four groups: hydrophobic (I, V, L, F, C, M, A, and W), GP (G and P), polar (T, S, Y, Q, and N), and charged (E, D, H, K, and R; Wang and Wang, 2004). In **Figure 1**, the dominant amino acids (highest percentages) in the four groups are represented as solid bars. For bacterial AMPs (i.e., bacteriocins), alanines (A) are the most preferred hydrophobic amino acid while residues G, S, and K are the most abundant in the other three groups (**Figure 1A**). Similarly, A is also a dominating hydrophobic residue in AMPs from insects or fish. In amphibian AMPs, L is the most abundant hydrophobic residue. In contrast, C is the major hydrophobic residue in AMPs from fungi, plants, and birds, probably due to the dominance of disulfide bonded defensin-like molecules. In human or reptile AMPs, C is comparable to other hydrophobic residues (e.g., L), probably reflecting the diversity in peptide sequences. For example, the known human AMPs are defensins, cathelicidins, histatins, and β-amyloid peptides. Like the case of bacteria, G, S, and K are usually the dominant residues in the other three amino acid groups in **Figure 1A**. Exceptions are as follows. In the case of reptile AMPs, G and P are comparable. For AMPs from fungi and insects, the level of N is higher than or similar to S. Different from other kingdoms, birds select arginines (R) as the main charged amino acid, whereas arginines and lysines (K) are comparable in human AMPs. Based on the above description, it is clear that the dominant hydrophobic amino acids differ in various kingdoms, while residues G, S, and K are generally preferred amino acids in natural AMPs from nearly all the kingdoms (**Figure 1A**). The variations in the dominant amino acids in the hydrophobic group are an important observation and could suggest the preference of specific types of AMPs in certain kingdoms. In addition, one of the most important aspects is the observation made by Torrent et al. (2011) also on the basis of the APD (Wang et al., 2009). They found that higher organisms tend to incorporate R more frequently than K except amphibians (**Figure 1B**). The authors attributed this phenomenon to the possible emergence of the adaptive immune systems and the arginine-rich AMPs may well play an important role in modulating the immune system and in linking the innate and adaptive immune systems.

STRUCTURAL INSIGHT

It is now clear that AMPs can adopt a variety of fascinating scaffolds, ranging from linear to circular. However, there are only four types of structures based on secondary structures: α, β, αβ, and non-αβ (Wang, 2010). The α family consists of AMPs with α-helical structures, while the β family comprises AMPs with a β-sheet structure. Another two families can be understood

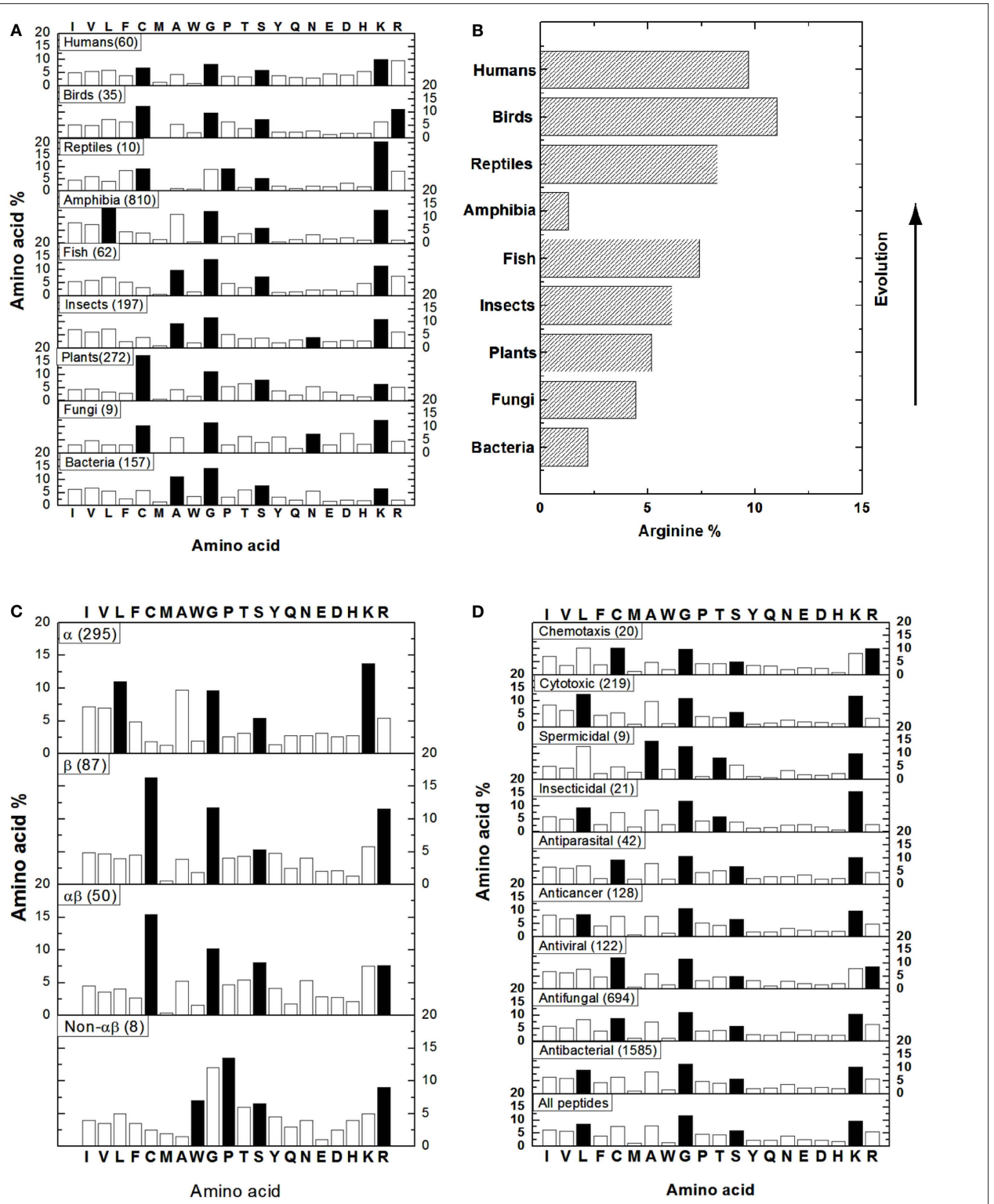

FIGURE 1 | Amino acid composition analysis of natural AMPs based on source (A,B), structure (C), and activity (D). (B) Shows the arginine percentages of AMPs from different life domains. In each case, the number of peptides included in the bioinformatic analysis is given in parentheses. The solid bars represent the most abundant amino acids in the hydrophobic (I, V, L, F, C, M, A, W), GP (G and P), polar (T, S, Y, Q, N), and charged (E, D, H, K, R) groups. Data were obtained in May 2012 (total peptides: 1973) from http://aps.unmc.edu/AP/main.php by using the search and statistical analysis functions of the APD (Wang et al., 2009).

accordingly: αβ = α + β and non-αβ = no α and no β structure. Representative structures for these four families can be viewed at the face page of the APD website above. The APD has also annotated those AMPs with determined 3D structures, which form the basis for our amino acid analysis. The peptides belonging to the α family are widely distributed in bacteria and animals, while most of the plant AMPs, such as cyclotides and defensins, belong to the β family. The αβ members occur in all kingdoms, including bacteria, plants and animals, but the non-αβ AMPs are much less frequent and are only confined to the animal kingdom at present. Depending on the structural family, the dominant amino acid in each amino acid group differs (**Figure 1C**). The α helical AMPs prefer L as the major hydrophobic amino acid while K is selected as the charged amino acid. On contrary, the β stranded AMPs are dominated by C that determines the polypeptide fold. Meanwhile, it prefers R instead of K as the charged amino acid. Likewise, the αβ family has a high content of C as the hydrophobic component required for peptide folding. However, it possesses equal amounts of R and K. Finally, the non-αβ family is generally composed of AMPs that are rich in particular amino acids such as tryptophan (W), proline (P), and R. The G and S are the other two preferred amino acids in all the families (**Figure 1C**). It is evident that amino acid composition is related to 3D structure of natural AMPs (Wang et al., 2009).

FUNCTIONAL IMPLICATIONS

Natural AMPs with either narrow or broad-spectrum activity have been reported. Moreover, there is overlap in the activity spectrum of some AMPs (Zasloff, 2002; Hancock and Sahl, 2006). Such an activity spectrum for each AMP has been annotated in the APD. This includes antibacterial, antifungal, antiviral, antiparasital, insecticidal, spermicidal, anticancer, cytotoxic (e.g., hemolytic), and chemotactic activity (Wang and Wang, 2004; Wang et al., 2009). **Figure 1D** shows the distribution of amino acids based on peptide activity. Except for spermicidal and insecticidal peptides, where T is preferred in the polar group, amino acids G and S are the two representative residues in the GP and polar amino acid groups in all the cases. For hydrophobic residues, L is the dominant amino acid for AMPs with cytotoxic, insecticidal, anticancer, or antibacterial activity. There is a subtle difference between AMPs active against Gram-positive and Gram-negative strains only. AMPs active against Gram-positive bacteria have similar contents of C and L, whereas those against only Gram-negative AMPs have higher L and lower C contents (not shown). Residue C is dominant in the hydrophobic group for AMPs with chemotactic, antiparasital, antiviral, or antifungal activity, suggesting the existence of a significant number of disulfide bonded molecules. In the case of spermicidal AMPs, residue A is the major hydrophobic amino acid. Finally, while lysines are usually the positively charged residue, arginines are clearly important in AMPs with chemotactic and antiviral activities. Therefore, the amino acid composition plays a role in determining peptide activity as well. For example, anticancer peptides are rich in L, G, S, and K, whereas chemotactic peptides have high C, G, T, and R contents.

APPLICATIONS OF ABUNDANT AMINO ACIDS IN PEPTIDE DESIGN

As recently summarized by Wang (2010), there are various methods in designing new AMPs, ranging from template optimization, motif hybridization, sequence shuffling/library screening, to rationale design. It has been recognized that parameters such as charge and hydrophobicity play a tremendous role in determining AMP activity (Zasloff, 2002; Hancock and Sahl, 2006; Wang, 2010). Our opinion is that the abundant residues identified in amino acid composition profiles of AMPs (**Figure 1**) can be used to design a specific peptide with the *desired* activity. Indeed, we succeeded in designing a 19-residue peptide using only residues G, L, and K. GLK-19 is active against *E. coli* but not *S. aureus* (Wang et al., 2009) or HIV-1. Since antiviral AMPs prefer arginines (**Figure 1D**), we obtained an anti-HIV peptide GLR-19 after the conversion of lysines in GLK-19 to arginines (Wang et al., 2010). In addition, because C is the dominant hydrophobic residue in antiviral peptides, we further improved anti-HIV activity of the peptide after introduction of a pair of cysteines to GLR-19 between residues 4 and 16 (Wang et al., 2011). Therefore, we succeeded in modulating peptide activity by varying the amino acid composition. We also propose that the prediction interface of the APD can be improved based on the abundant amino acids identified herein.

A SUMMARY OF OPINIONS

The construction of the APD made it possible for us to extract the amino acid composition information in natural AMPs for the first time. Further classification of AMPs and the update of our database made the extracted parameters more informative (Wang et al., 2009). We propose that the amino acid composition plays an important role in terms of evolution, structure, and function of natural AMPs. The overall picture for natural AMPs is shaped through evolution. For example, the preference of arginines in the AMPs in higher organisms (**Figure 1B**) is proposed to be significant in the emergence of adaptive immune systems (Torrent et al., 2011) and probably also confers the regulatory and integrative role to natural AMPs in host defense. We demonstrated that arginines are more effective in targeting MRSA or HIV-1 (Wang et al., 2010, 2012). The amino acid composition appears to directly determine the various structural scaffolds of natural AMPs (**Figure 1C**). In the case of amphibians, the dominance of G, L, A, and K determines a helical conformation, leading to a natural recombinant library of peptides (up to ~100 in each frog) achieved by presenting varying other amino acids on the same helical structure backbone (Wang et al., 2009). Likewise, plant cyclotides are rich in C, G, T/S, and K that determines a universal β-sheet containing scaffold (Wang, 2010). Again, nature has created a natural recombinant library of cyclotides by introducing other amino acids to various loop regions. The same strategy is now utilized to generate new cyclotides with a desired biological function via segment grafting (Craik et al., 2012). Amino acid compositions may determine the mechanisms of action of natural AMPs. As we noticed previously, plant cyclotides and bacterial lantibiotics have different structural folds but similar amino acid composition profiles (Wang, 2010). Interestingly, certain lantibiotics can bind phosphatidylethanolamines (PE; Zhao, 2011; Ökesli

et al., 2011), so can cyclotides (Henriques et al., 2011). There is now converged view regarding the mechanism of action of proline-rich AMPs. They can cross bacterial membranes and associate with heat-shock proteins (Scocchi et al., 2011). The abundant amino acids elucidated from the APD (**Figure 1**) are helpful for both prediction and design of new AMPs (Wang, 2010). In our opinion, database-guided design is preferred over library screening due to its cost effectiveness by synthesizing only few peptides. Future database annotations and expansion are anticipated to further improve the accuracy of the amino acid composition profiles, thereby opening the door to other potential applications.

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Antimicrobial peptidomimetics: reinterpreting nature to deliver innovative therapeutics

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NATURAL ANTIMICROBIAL PEPTIDES: A PALETTE OF EXTRAORDINARY COLORS

Virtually all multicellular organisms must ward off pathogenic microbes in order to survive and thrive on this planet. To accomplish this, most metazoans rely on gene-encoded antimicrobial peptides (AMPs) as an essential part of their innate immune system. The role played by AMPs – and by the other umoral and cellular components of innate immunity – is particularly crucial in those organisms (the vast majority) that have not developed the more sophisticated adaptive immune system. Even in higher vertebrates as humans, AMPs like defensins and cathelicidins (e.g., LL-37) do not only have direct microbicidal activity, but they also serve as signals which initiate, mobilize, and amplify adaptive immune host defenses, thus functioning as immunomodulatory and immunostimulatory elements (Giuliani and Rinaldi, 2010).

Despite a bewildering variety in their primary sequences, AMPs generally share a cationic character, a length that usually does not exceeds 50 residues (a large proportion of which are generally hydrophobic), and a globally amphipathic fold, with clearly distinguishable hydrophilic and hydrophobic faces. These structural features reflect their mode of action, which is primarily directed to the interaction with and damage of the pathogen cell's plasma membrane, although the evidence that many AMPs may hit other targets is rapidly increasing. These evolutionarily conserved molecules display a broad spectrum antimicrobial activity against bacteria, fungi, protozoans, and even enveloped viruses. AMPs represent key components especially at epithelial surfaces, where the initial contact with pathogens takes place, thus being deployed at the very front line of the defense system, where rapid action is required before the more slowly responding adaptive immune system (if any) can be brought into action.

With the prospects of an ever-increasing bacterial resistance to conventional antibiotics looming at the horizon, much attention has been devoted to AMPs as a potential source of new anti-infective drugs (Mangoni, 2011; Yeung et al., 2011). However, despite intense research aimed at spotting possible ways for harnessing the therapeutic potential of these intriguing natural compounds, little practical outcome has been generated as yet. Multiple hurdles on this way exist, indeed, related to the fact that naturally occurring AMPs present serious drawbacks that limit their development into clinically applicable antibiotics. These include (but are not limited to) high costs of manufacture, susceptibility to protease degradation, reduced activity in the presence of salts as those present in serum. In addition, given the recognized immunomodulatory and immunostimulatory effect of several AMPs – that is some cases is so evident that the term Host Defense Peptides has been proposed as more indicative of the real role played by these molecules *in vivo* – using AMPs systemically to treat infections should necessarily suppose an advanced knowledge of (and possibility to control) the possible responses these peptides can trigger. Furthermore, as mentioned before, AMPs have been crafted by evolution to be part of the network of interacting and self-reinforcing components of the innate immune systems, thus expecting that they could stand alone as the new "magic bullet" against resistant microbes would be simply overoptimistic.

Given the inherent limitations of naturally occurring AMPs that have so far prevented their transformation into therapeutics, two general approaches have emerged to overcome this major obstacle, i.e., the modification of existing peptide sequences or the *de novo* synthesis of peptides, and the development of synthetic molecules that mimic the properties and activities of AMPs (Giuliani et al., 2008; Brogden and Brogden, 2011; Fjell et al., 2011; Giuliani and Rinaldi, 2011). Here, a few recent, significant examples pertaining to these research avenues will be highlighted.

DESIGNED PEPTIDE ANTIBIOTICS: RATIONALLY BANKING ON NATURE'S WORK

Several computational approaches have been proposed for the design of promising novel peptide antibiotics based on our current understanding of structure–activity relationships of the naturally occurring AMPs. One of the most interesting of such directions was reported by the group led by Davor Juretić at the University of Split, Croatia. By inspecting a self-assembled database of natural AMPs where primary and secondary structures have been linked to the peptides' antimicrobial activity and selectivity (i.e., the ability to discriminate bacterial versus host cells as targets), these researchers have extracted selected physicochemical peptide properties then used to construct a "AMP-Designer" algorithm (Juretić et al., 2009). A peptide (23 residues, glycine-rich) suggested by the algorithm, was synthesized and its activity and selectivity tested. This peptide, adepantin 1 (from "Automatically DEsigned Peptide ANTIbiotic Number 1"), is less than 50% identical to any other AMP, and displayed a potent antibacterial activity

against *Escherichia coli* coupled to a significantly greater selectivity for bacterial cells than the best AMP present in the reference database (Juretić et al., 2009).

Multimeric peptides have proved to be another intriguing variation on the AMPs theme. Recently, several examples of peptide dendrimers endowed with antimicrobial properties were reported. These molecules were designed following the so-called multiple antigenic peptide (MAP) system, introduced many years ago, where multiple peptide sequences can be added using standard solid phase chemistry to a inner core of radially branched lysine residues. Starting from a linear AMP sequence originally identified by selecting a random phage library against whole *E. coli* cells, several cycles of rational modification and optimization led to the tetra-branched peptide known as SB041, found to be especially active against Gram-negative strains and able to strongly bind *E. coli* and *Pseudomonas aeruginosa* lipopolysaccharide (LPS) *in vitro* (Bruschi et al., 2010). More recently, a novel AMP with a dimeric dendrimer scaffold, SB056 (**Figure 1A**), was synthesized and showed high activity against Gram-negative bacteria and some limited activity against Gram-positive bacteria (Scorciapino et al., 2012). Its potency against Gram-negative strains was comparable (on a molar basis) to that of colistin and polymyxin B, with an even broader spectrum of activity than numerous other reference compounds. Biophysical characterization through circular dichroism, NMR, molecular dynamics simulation, and membrane affinity assayed through lipid monolayer surface pressure experiments, revealed that the peptide is membrane-active, and tends to folds into a β-type conformation in a lipid environment (Scorciapino et al., 2012). In general, dendrimeric peptides display increased activity compared to their monomeric counterparts – a fact probably attributable to the higher local concentration of bioactive units for multimeric peptides – as well as greater stability to peptidases and proteases, possibly due to the steric hindrance of the branching core that would limit the cleavage rates of plasma peptidases, thus increasing the peptides' pharmacokinetics properties (Giuliani and Rinaldi, 2011).

Researchers at the University of Zurich and Polyphor Ltd.[1] have used a different approach to come out with a *Pseudomonas* specific antibiotic with a novel mode of action. The lead, termed POL7080, was derived from the so-called PEM (protein epitope mimetic) technology platform, which takes advantage of the fact that molecular recognition involving proteins is often mediated by surface-exposed secondary structure motifs such as β-hairpins and α-helices (Obrecht et al., 2012). Following this path, researchers have developed epitope mimetics of protegrin I – an AMP

[1]www.polyphor.com

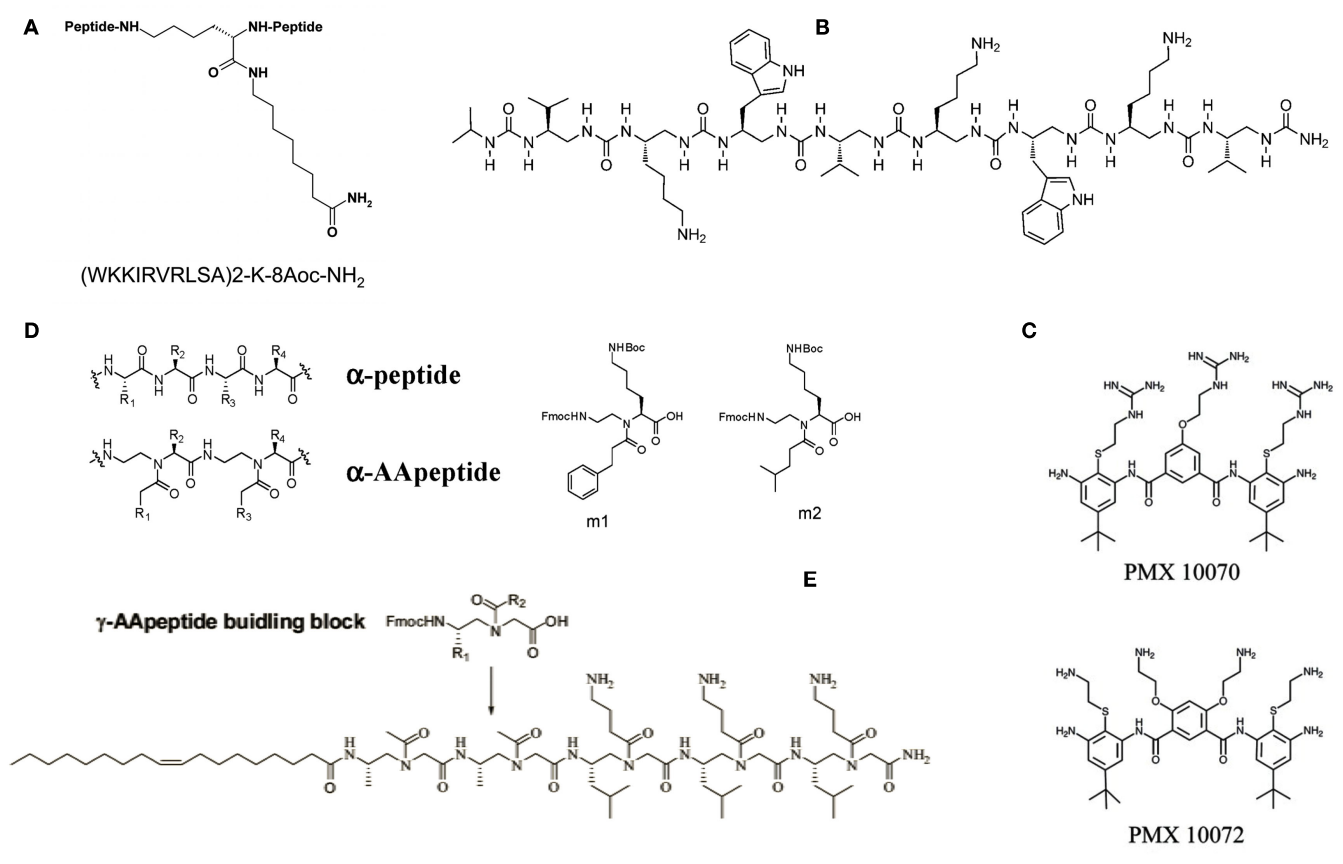

FIGURE 1 | Examples of designed antimicrobial peptides and mimics. (A) The dendrimeric peptide SB056. 8-Aoc is 8-aminooctanoic acid, which is amidated in the peptide construct; **(B)** antimicrobial oligourea; **(C)** arylamide compounds; **(D)** structures of an α-peptide and a corresponding AApeptide, and α-AApeptide building blocks (m1 and m2) used for the preparation of antimicrobial α-AApeptides; **(E)** lipo-γ-AApeptide. See text for more details and original studies.

first discovered in porcine leukocytes – that specifically target *P. aeruginosa* in the nanomolar range via a mechanism of action that is distinct from the membrane-disrupting activity of the parent peptide. Studies have shown that POL7080 rather targets an essential bacterial β-barrel outer membrane protein called LptD, which functions in outer membrane biogenesis (Srinivas et al., 2010). The β-hairpin mimetic POL7080 is currently in a Phase I clinical trial in Europe, and could represent an important drive in the treatment of chronic lung infections in patients with cystic fibrosis where *P. aeruginosa* plays a crucial role.

THE RAISE OF PEPTIDE MIMETICS

Along the years, a number of mimics of AMPs, both (unconventionally) peptidic and non-peptidic in nature, have been explored as for their capacity of working as antimicrobial substances with potential therapeutic interest. These different classes of compounds include oligoacyllysines, ceragenins, peptoids, phenylene-ethynylene oligomers, and others. Some further examples are detailed below. The main idea behind the design of these mimics is to build foldamers that could maintain the amphiphilic character of naturally occurring AMPs while circumventing the major drawbacks that have prevented peptide-based antimicrobials from having significant success in practical terms.

Among the AMP-mimicking synthetic oligomers built with unnatural building blocks, foldamers such as aromatic and aliphatic *N,N′*-linked oligoureas (**Figure 1B**) have received considerable attention (Violette et al., 2006; Claudon et al., 2010). High resistant to proteolytic degradation oligoureas have been shown to adopt a remarkably stable helical fold stabilized by 12- and 14-membered H-bonded rings, with helical propensity being enhanced in the presence of phospholipid vesicles, and to possess significant antibacterial activity against Gram-negative and Gram-positive bacteria (Violette et al., 2006; Claudon et al., 2010). Recently, a detailed solid-state NMR structural investigation was performed on aliphatic oligoureas synthesized with ^{15}N at selected positions (Aisenbrey et al., 2012). This permitted to acquire information on the oligomers' conformation, dynamics, and interactions with model membranes. The acquired data were indicative of an alignment of the oligourea helix parallel to the surface of the phospholipid bilayers, in agreement with the amphipathic character of the foldamer and consistent with previous models explaining the modes of action of AMPs (Aisenbrey et al., 2012).

One of the most interesting approaches to chemically mimicking AMPs, is that of small arylamide foldamers, i.e., an arylamide backbone and various charged and hydrophobic groups yielding a topographically amphiphilic structure. The best known compound belonging to this class is PMX-30063, a "defensin-mimetic" lead compound being developed by PolyMedix for the broad treatment of *Staphylococcus aureus* infections. PMX-30063 has just completed a Phase 2 clinical trial to treat patients with acute bacterial skin and skin structure infections caused by *S. aureus*: the study objectives were met, demonstrating clinical efficacy and safety in all evaluated doses of PMX-30063[2]. A recent investigation on the mechanism of action of two arylamides – namely PMX 10070, which has a 2-ethyl guanidinium charged substitution, and PMX 10072, which has a 2-ethyl amine substitution on the arylamide backbone (**Figure 1C**) – has shown that the arylamide compounds significantly disrupted the permeability of the outer membrane of *E. coli* cells, possibly by binding to the LPS component of cell wall (Mensa et al., 2011). Perturbation of the inner membrane was comparably less dramatic, but arylamide exposure nevertheless led to increased permeability of the inner membrane to small ions and to defects in protein translocation across the membrane, thus contributing to arylamides' lethality, authors concluded. "Compared to several natural AMPs, the membrane permeabilization caused by arylamides is less extensive, which could minimize the risk of detrimental inflammatory responses to leakage of bacterial cellular contents and thus prove advantageous in a therapeutic setting," they remarked (Mensa et al., 2011).

An entire new class of peptide mimetics, namely α-AApeptides, with broad spectrum activity against both Gram-negative and Gram-positive bacteria and fungi, was recently reported. AApeptides are oligomers of *N*-acylated-*N*-aminoethyl amino acids, designed on the principle of globally amphipathic structures as drivers for membrane-active antimicrobial compounds (**Figure 1D**). "Coupled with straightforward solid phase synthesis, virtually limitless structural possibilities, low cost of production, simple tunability and programmability, and resistance to protease hydrolysis, α-AApeptides may lead to a new class of antimicrobial peptidomimetics," remarked authors (Padhee et al., 2011). In their hands, one of the synthesized oligomers displayed minimal inhibitory concentration (MIC) values – defined as the lowest inhibitor concentration that completely inhibits the growth of microbes during a 24 h incubation period at 37°C – as low as 2.1 μg/ml against the Gram-negative *E. coli* and 0.9 μg/ml against the Gram-positive *Bacillus subtilis*, respectively, while being non-hemolytic (Padhee et al., 2011). These levels of activities are equal to or better than those of most natural AMPs and peptidomimetics reported so far. The same group, led by Jianfeng Cai at the University of South Florida in Tampa, FL, USA, lately reported the design and synthesis of lipidated γ-AApeptides (**Figure 1E**) as antimicrobial agents (Niu et al., 2012). According to the authors, the introduction of an unsaturated lipid chain significantly decreased hemolytic activity, thereby enhancing target selectivity. One of the synthesized lipo-γ-AApeptides did not induce drug resistance in methicillin-resistant *S. aureus*, even after 17 passages (Niu et al., 2012).

LOOKING BACK, LOOKING AHEAD

Considering that AMPs, like cecropins and defensins, have been identified some three decades ago, the failure in transforming their potential into therapeutics against resistant microbial infections might be felt as scorchingly disappointing. However, great advances in our understanding of AMPs' physicochemical properties and modes of action are fostering the development of a new wave of peptidomimetics, inspired by naturally occurring examples, that could remove the hurdles that have prevented the adoption of AMPs in clinical settings. The next few years will be crucial in showing whether this view is correct.

[2]www.polymedix.com

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Targeted antimicrobial peptides

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The existence of natural antimicrobial substances, contributing to the mechanisms of host defenses, has been recognized since the late nineteenth century. In 1963, the *in vitro* antibacterial activity of leukocyte extracts was attributed to basic proteins. Since the late 1980s, cationic peptides with antimicrobial properties have been subsequently identified in other host cells and tissues and in virtually every living species (Lehrer, 2004). The properties of these "Nature's antibiotics" and their multiple functions in host defenses of multicellular organisms support the rationale of developing entirely novel peptide-based therapeutics harnessing the effector mechanisms of innate immunity (Hancock and Sahl, 2006). The term antimicrobial peptides covers different forms of natural macromolecules; ribosomally synthesized and non-post-translationally modified innate immunity peptides, or their synthetic analogs, are predominantly considered here. Their antimicrobial and immunomodulatory activities will not be dissociated in general and they will be indistinctively described as (cationic) antimicrobial or host defense peptides.

The main assets of innate immunity peptides originate from their primary activity essentially directed at a universal non-protein target, the bacterial membrane, reinforced by a polypharmacology which includes multiple immunomodulatory and anti-inflammatory activities (Zasloff, 2002; Finlay and Hancock, 2004). Other distinctive and attractive properties in a clinical context comprise their low susceptibility to classical mechanisms of drug resistance, associated with a low propensity to select resistant mutants, their ability to reduce both biofilm and planktonic bacterial counts and to interact with dividing and non-dividing cells by generally targeting a conserved structure which is independent of the proliferative status of the cells (Peschel and Sahl, 2006; Chen et al., 2011; Fjell et al., 2012). Although some mechanisms of bacterial resistance to antimicrobial peptides have been identified, their emergence occurs at significantly lower frequencies than for traditional antibiotics (Peschel and Sahl, 2006). Furthermore, host defense peptides can also act synergistically with these classical antibiotic agents (Giacometti et al., 2000).

Innate immunity peptides are therefore a prospective source of antibiotic candidates with extended clinical lifetimes. They have not been approved for clinical use to date, but have progressed toward commercial development through recent technological advances. About 1,200 to 1,700 antimicrobial peptide sequences have been identified and/or predicted to date. Approximately 15 different peptide-based therapeutic agents are currently in clinical trials for anti-infective and/or anti-inflammatory indications, generally limited to topical administration (Fjell et al., 2012; Yount and Yeaman, 2012). The challenges traditionally associated with the clinical development of host defense peptide candidates for systemic therapies require notably solutions addressing the question of possible toxicity. Owing to their rapid metabolic degradation and/or excretion, high doses of these peptides might be required to maintain therapeutic levels *in vivo*. This may correlate with an improper margin of safety, despite their selectivity for bacterial over mammalian cells (Zasloff, 2002). In addition, the issue of potential immunogenicity should be considered (Mader and Hoskin, 2006). Other concerns may be raised, either inherent to all peptide-based drug candidates, such as low oral bioavailabilities and elevated cost of production, or specific to host defense peptides, in particular their complex pharmacology which could result in uncontrolled off-target toxicity (Pauletti et al., 1997; Hancock and Sahl., 2006; Brown et al., 2007).

Peptide therapeutics are capturing an increasing fraction of the global pharmaceutical pipeline. Advances in peptide modification, formulation, and delivery technologies can overcome some of their pharmacokinetic, bioavailability, and toxicity shortcomings and likewise have been applied to innate immunity peptides. The latter have been modified by either optimizing the length and content of their sequences, to increase their selective antibacterial activity, or by conversion into peptidomimetics, to improve their pharmacokinetic properties. In the first case, minimizing the length of the sequence and systematically substituting each residue with other coded amino acids, can yield peptide candidates with improved antibacterial activity and/or increased activity differentials between prokaryotic and eukaryotic (generally erythrocytes) cells. This work has been performed in parallel with structure activity relationship studies for the rationale design of therapeutic candidates (Fjell et al., 2012). It focused on the direct antibacterial activity of some selected candidates, but immunomodulatory peptides devoid of *in vitro* antimicrobial activity have also been optimized (Hancock et al., 2012). In the second case, peptidomimetics, structures departing from the traditional peptide backbone and/or stereochemistry but reproducing the biological activity of the parent sequence, have been generated as candidates resistant to proteolysis. They include sequences assembled from non-natural amino acids (e.g., D- or

β-amino acids or proteinogenic amino acid analogs with increased hydrophobicity), N- or C-terminally modified with lipophilic chains or groups and also peptoids and non-peptide mimetics (e.g., aminosteroids or amphiphilic polymers; Brogden and Brogden, 2011; Yount and Yeaman, 2012). Other methods for improving the pharmacokinetic properties, preventing the immunogenicity and serum protein inactivation of biopharmaceuticals, such as the pegylation technology, have also been implemented (Harris and Chess, 2003; Imura et al., 2007).

The issue of unknown toxicology for systemically administered innate immunity peptides has also been addressed in a number of these approaches. Alternatively, some modified peptides or peptidomimetics may retain not only the activity, but also the toxicity of the parent peptide. To efficiently and reliably control the toxicity of a therapeutic candidate, a selective delivery technology can be implemented as an alternative method, or in combination with the previous approaches. These methods can confine the activity of innate immunity peptides to the sites of infection and generate thereby targeted antimicrobial peptides. They consist either of peptide sequences conjugated to targeting moieties, modified as inactive precursors which can be selectively activated at a target body site, or loaded in drug delivery systems that can be targeted to their desired site of action. In the former case, the targeting moiety can be an antibody directed against a pathogen-specific antigen. A host defense peptide sequence is in this case conjugated to the sequence of an antibody fragment through the production of a fusion protein containing or not a cleavable linker between the targeting and antimicrobial domains (Peschen et al., 2004; Szynol et al., 2006; Franzman et al., 2009). The resulting immunoconjugate can, for example, confer specific resistance to a fungus in transgenic plants or discriminate a specific periodontal pathogen from other bacteria of the oral commensal flora. The targeting moiety can also be another peptide sequence that can bind selectively to a specific cell surface receptor of a bacterial pathogen, such as a pheromone receptor for instance. A targeted antimicrobial candidate is then generated as a fusion or synthetic peptide comprising the antimicrobial and pheromone sequences (Qiu et al., 2003; Eckert et al., 2006). These chimeric peptides can, for example, discriminate between *Staphylococcus aureus* (MSSA and MRSA strains) and *Staphylococcus epidermidis* or *Streptococcus pneumoniae* and be protective in a mouse model of infection with MRSA, or selectively eliminate the cariogenic bacterium *Streptococcus mutans* in planktonic cultures and multispecies biofilms, without affecting closely related non-cariogenic oral streptococci.

The generation of targeted antimicrobial peptides has also been investigated through the conjugation of a classical antibiotic to a host defense peptide sequence, to increase its selectivity and activity against bacteria expressing the target of the conventional antibiotic. Vancomycin-peptide conjugates of magainin 2 were for example constructed by using the copper(I)-catalyzed azide-alkyne cyclo-addition (Arnusch et al., 2012). This approach yields hybrid antibiotics containing 2 different pharmacophores, which can capitalize on their dual activity to increase their efficiency and delay resistance development, but can also restore the activity of the classical agent against resistant bacteria (Pokrovskaya and Baasov, 2010). Another application of these hybrid antibiotics is in the generation of antimicrobial peptide prodrug candidates, where the classical antibiotic acts as a promoiety rather than as an active agent (Rautio et al., 2008). For example, conjugation of a cephalosporin to a host defense peptide sequence can reversibly modulate one of the activity determinants, the net charge, of the parent peptide (Desgranges et al., 2012). The latter can be selectively released from the conjugate by β-lactamase-mediated hydrolysis of the cephalosporin's lactam ring, a reaction which constitutes the main mechanism of antibiotic resistance in Gram-negative pathogens. A prodrug modification has been proposed as a promising strategy to potentiate the systemic applications of host defense peptides and has been frequently used to overcome the toxicity of low molecular weight drug candidates, but also of the lipopeptide polymyxin E (Hancock, 2001; Stella, 2004). Nature also selected a prodrug approach to regulate and control the activity of some innate immunity peptides (Yeaman and Yount, 2007). This natural mechanism can be synthetically mimicked by generating peptide sequences containing three domains, including an oligo-glutamyl profragment, a linker cleavable by a target disease-associated protease and the parent sequence of a host defense peptide assembled from D-amino acids (Desgranges et al., 2011). Activity and toxicity differentials can be achieved between a neutrophil elastase-dependent propeptide and its parent peptide, although enzymes of bacterial origin that have narrow substrate specificities and no mammalian homologs might have to be targeted for the activation of a propeptide systemically administered. Finally, peptide prodrugs containing a promoiety yielding itself a pharmacologically active entity upon activation, i.e., co-drugs, can target an antimicrobial peptide to a site of bacterial infection or colonization while allowing the co-delivery of an agent with a complementary activity. For example, conjugation through an azo bond of aniline-based agents such as 4-aminophenylacetic acid or 5-aminosalicylic acid, to the N-terminus of a peptide requiring a free N^{α}-amino group, can generate co-drug candidates for colonic delivery of non-steroidal anti-inflammatory and antibiotic agents (Kennedy et al., 2011). The metabolic activation of these two therapeutic candidates can only be performed by azo-reductases, enzymes limited to anaerobic bacteria only, restricting thereby the activity of the peptide to the colon. Alternatively, environmentally sensitive antimicrobial peptides, such as pH-responsive sequences, can inherently have their activity confined to a particular body site (Li et al., 2010).

Antimicrobial peptides can also be targeted through their loading in nanoparticulate systems with selective delivery capacity. They include liposomes, including stealth liposomes, polymeric structures, including hydrogels and dendritic polymers, nanospheres, and nanocapsules, carbon nanotubes and DNA cages (Urbán et al., 2012). Their nanoscale size determines their drug loading capacities, but prolongs their circulation times. Their structure can protect the cargo from metabolic degradation and limit its toxicity by preventing its interaction with plasma proteins and host cell surfaces. Furthermore, the release of the cargo can be environmentally controlled or the surfaces of these nano-carriers can be modified with targeting moieties to allow their selective delivery to specific cells or tissues and even through the blood-brain barrier. Some of these drug delivery systems (e.g., liposome,

protein, and polymer carriers) have already been investigated with host defense peptides as cargoes (McClanahan et al., 2011; Yount and Yeaman, 2012).

Finally, the concern related to the elevated cost of production of these candidates is now moderated by the advances in their production methods, such as the recombinant expression in heterologous microbial systems (Mygind et al., 2005; Li, 2011). Applicable to the production of peptides assembled from natural amino acids, it is also complemented by the solid phase synthetic approach which allows the cost-effective production on a multi-tonne per year scale of peptides, but also modified peptides and peptidomimetics, that can meet the requirements of regulatory agencies (Bray, 2003).

The therapeutic potential of host defense peptides can be extended beyond the anti-infective and anti-inflammatory applications to cancer therapy. Indeed, innate immunity peptides can be active against prokaryotic and neoplastic eukaryotic cells, according to the high anionic lipid content of the bacterial, malignant cells and mitochondrial membranes and to structural differences between the bacterial or cancer cell membranes and the plasma membrane of normal eukaryotic cells (Papo and Shai, 2005; Mader and Hoskin, 2006). Some of the approaches developed in the anti-infective and anticancer areas to address, separately or collectively, the limitations of antimicrobial peptides, could address the clinical shortcomings associated with these candidates and the optimization and/or combination of these approaches, together with the advances in production and purification methods, could ultimately realize the full therapeutic potential of antimicrobial peptides.

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Integrating "omics" technologies to conceptualize dynamic antimicrobial peptide responses

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INTRODUCTION

Rarely in human disease can a single host factor be identified as the primary causal factor of a disease, syndrome, or disorder. Rather, the clinical manifestations that culminate in a symptomatic state are usually triggered by a perturbation of multiple factors derived from several distinct, but integrated, domains of cellular regulation. Host immune responses, such as antimicrobial peptide (AMP) production and regulation, are the result of interdependent dynamic interactions between host cells, extracellular milieu, and the external environment. In order to truly elucidate disease pathogenesis, it is imperative to consider the entire system at play from a global perspective, which is the promise of "omics" technology. The term "omics" was originally coined as a discipline in molecular biology that examines global sets of biological molecules (Micheel et al., 2012), which consequently stimulated an "Omics Revolution" throughout the scientific community. Genomics emerged following the sequencing of the human genome in the 1990s and early 2000s. This subsequently led to proteomics, which includes the entire complement of proteins and their related structure, modifications, and function. Pauling et al. (1971) proposed that estimating the abundance of metabolites in biological fluids may indicate the functional status of a specific biological system. Consequently, metabolomics has recently surfaced as an approach to comprehensively identify and quantify low molecular weight exogenous and endogenous metabolites and compounds in a biological system using high-throughput methods. Using 16S rRNA gene sequencing tools originally developed in the field of environmental microbiology, we can now generate a site-specific microbiome profile for any given patient. The Human Microbiome Project and numerous other projects aim to discern relationships between human-associated microbes and health and disease (Zoetendal et al., 2006). Such research has generated massive and complex data sets, which requires further advancement and refinement of bioinformatics and biostatistical applications. Often, the data is used to produce computational models to distinguish specific characteristics of a given population. Due to the unavoidable complexities and required statistical sophistication, few medical fields have developed clinically useful applications for the resulting data. Thus, AMPs are an ideal set of biological molecules to assess, since there biological role spans numerous areas of clinical research.

Due to their diverse microbicidal and immunomodulatory functions (Steinstraesser et al., 2011), AMPs comprise a major aspect of the host innate immune response (Nakatsuji and Gallo, 2012). Alterations in AMP production and/or localization are associated with several cutaneous dermatoses. Patients diagnosed with atopic dermatitis and psoriasis ultimately develop epidermal barrier defects and microbial susceptibility, or a hyperproliferative/inflammatory epidermis, respectively (Schauber and Gallo, 2008; Schroder, 2011). The contributions of AMPs to tissue integrity and epithelial defense was also established in the brain (Schluesener et al., 2012; Williams et al., 2012), urinary tract (Saemann et al., 2007; Zasloff, 2007; Ali et al., 2009), gastrointestinal tract (Lisitsyn et al., 2012), and certain malignancies (Kesting et al., 2012; Scola et al., 2012). However, altered AMP regulation does not occur exclusively, but instead embodies dynamic transformations that emerge as a disturbed system. The integration of several different "omics" techniques may provide a more complete understanding of the diverse factors contributing to a specific disease state, responses to pharmacologic agents, or novel alternatives to current treatment modalities.

AMPs AS A DISEASE MARKER

The appeal of "omics" research lies in the possibility of identifying an AMP profile that distinguishes one particular patient in a clinically useful manner to categorize them as high- or low-risk for developing a post-operative infection (e.g., pneumonia). Several studies have demonstrated the importance of cathelicidin (Braff et al., 2007; Kovach et al., 2012), beta-defensins (Chong et al., 2008; Scharf et al., 2012), and several other AMPs in regulating epithelial immunity (Cole and Waring, 2002; Tecle et al., 2010). Consequently, it is feasible that integrating these variables simultaneously may reveal unexplored or intriguing connections, and likely identify invaluable correlations that are clinically significant.

Antimicrobial peptides have been extensively evaluated for their role in inflammation (Lai and Gallo, 2009), while little research has investigated their potential role in rejection following organ transplantation. Although these patients are typically immunosuppressed, abnormal alterations in AMPs following transplantation may contribute to or serve as a marker of inflammation and rejection. A diagnostic tool to yield a molecular AMP profile of a transplant patient could serve as a prognostic indicator of organ failure. Nuclear magnetic resonance (NMR) based metabolomic technologies have also attempted to identify other urine biomarkers as an indicator of chronic renal failure and renal transplant

function (Bell et al., 1991; Foxall et al., 1993). Similar technologies could be employed to identify how AMPs may correlate with clinical outcomes in transplant patients. In comparison, trauma and burn patients exhibit profound defects in immune regulation following injury, including perturbations in AMPs (Steinstraesser et al., 2004; Bhat and Milner, 2007). A diagnostic AMP profile may again provide invaluable data to predict healing, immune integrity, or graft survival. The clinical utility of such targeted profiles could undoubtedly be applied to numerous disease states involving infection and/or inflammation, to serve as markers of prognosis.

Currently, NMR and Mass Spectrometry are two major platforms by which metabolomic analyses are evaluated via bioinformatic tools. Several skin AMPs were recently implicated in tumorigenesis of cutaneous squamous cell carcinoma (Scola et al., 2012). Significant metabolic alterations usually ensue as normal cells are transformed into a malignant phenotype. Using a limited rationale, AMPs may simply reflect alterations in the local environment from the presence of the malignancy, or may simply be irrelevant to the malignancy. A more sophisticated rationale suggests that alterations in AMPs may serve as a biomarker for disease severity and/or progression, and denote a significant underlying process that contributes to a malignancy. Interestingly, the wound repair process and cancer progression are both associated with alterations in the inflammatory/immune microenvironment. During wound repair, AMPs are released from epithelial and infiltrating immune cells to stimulate re-epithelialization, new vessel formation, and extracellular matrix (ECM) remodeling (Radek and Gallo, 2007). However, the dynamics of cancer progression and tissue repair differ in that wound healing is a self-limiting process, while tumor formation is characterized by a continuous, uncontrolled activation of similar pathways that facilitate tumor growth and metastasis. One key observation is that prominent associations exist between the cytokines, chemokines, and growth factors present in healing wounds and wound fluid, as compared to tumors. In parallel, a striking difference in the temporal regulation of these factors was determined by the combination of several genomic technologies (Pedersen et al., 2003). Furthermore, proteomics and genomic methods are now being employed through a multidisciplinary translational research approach to improve the bioactive components in matrix therapies for non-healing wounds to specifically modulate the temporal and local release of these micromolecules (Sweitzer et al., 2006). Degradomics is emerging in the wound healing field as a new technology that assimilates the current knowledge database of ECM regulation and deciphers the complex interactions between proteases and their respective inhibitors using systems biology as a means to improve wound integrity in chronic wounds (Hermes et al., 2011). Since AMPs are an integral part of wound healing and inflammation, the knowledge gained from utilizing these evolving "omics" technologies may be extrapolated to other dimensions of data analysis that span other disease states which share similar mechanisms of disease progression.

Antimicrobial peptide regulation can clearly modulate and be influenced by the composition of the microbial flora of the human host. Several AMPs are induced in response to both invasive pathogens, as well as commensal strains of bacteria to generate specific down-stream innate or adaptive immune signaling events. For instance, the cutaneous commensal *Staphylococcus epidermidis* induces human β-defensin-2 and -3 via a TLR-2 signaling dependent mechanism (Lai et al., 2010). This interaction is beneficial for both the host and microbe by facilitating the eradication of pathogens on the skin via AMP induction, while simultaneously allowing *S. epidermidis* to further proliferate with fewer competitors for metabolic resources. Further complicating these interactions, microbes have evolved several mechanisms to evade host AMPs via altered cell surface charge, efflux transporters, proteases, or trapping proteins, and direct adaptations of host cellular processes (Nizet, 2006). These dynamic interactions between the host and the resident microbiota can significantly influence the overall homeostatic balance.

The integration of multiple "omics" disciplines is applicable to several tangible clinical situations where infection is a risk factor. For example, identification of patients most at risk for a urinary tract infection (UTI) would improve prophylactic therapies for susceptible patient populations, including burn-injured, surgical, or bedridden individuals. Recent studies deliver a long overdue confirmation that urine is not sterile, which challenges the current dogma (Nelson et al., 2010; Dong et al., 2011; Wolfe et al., 2012). Thus, integration of multiple "omics" technologies, such as 16S rRNA gene sequencing and proteomics, may identify correlations between specific AMPs and distinct genera of bacteria to identify unique patterns that could be employed as a diagnostic tool to predict those individuals who may be at a higher risk for a UTI. Furthermore, the development of a rapid, high-throughput assay that integrates multiple "omics" technologies to correlate AMPs with tissue specific microbiota would be invaluable to clinicians for prediction of UTI or other disease states.

AMPs AS A THERAPEUTIC TARGET

Aside from patient-centered applications, AMP-related "omics" research is also being utilized in drug discovery, as AMPs have been targeted as a potential alternative to conventional antibiotics by serving as adjuncts and/or replacements to traditional antibiotics, although more research is clearly needed to develop these promising tools (Hirsch et al., 2008; Baltzer and Brown, 2011; Ahmad et al., 2012; Hassan et al., 2012). Infection remains a leading cause of death in the US, with influenza/pneumonia and septicemia both ranking in the top 15 (Murphy et al., 2012). Several publicly available databases have already been established in order to collect relevant information related focused on AMPs (Brahmachary et al., 2004; Wang and Wang, 2004; Wang et al., 2009; Seshadri Sundararajan et al., 2012) in order to construct models based on clustering and analyzing AMP sequences, which allow accurate recognition of specific antimicrobial classes (Fjell et al., 2007). Recently, a bioinformatics strategy using peptide sequences was employed to classify synthetic and endogenous AMPs based on their physiochemical properties to identify active peptides and assess antimicrobial potency (Kumari et al., 2012; Torrent et al., 2012). Despite promising preliminary data, AMPs have yet to prove their full potential in clinical trials.

RESEARCH LIMITATIONS

It is evident that less costly and efficient high-throughput technologies are undoubtedly needed to fully explore the under-utilized

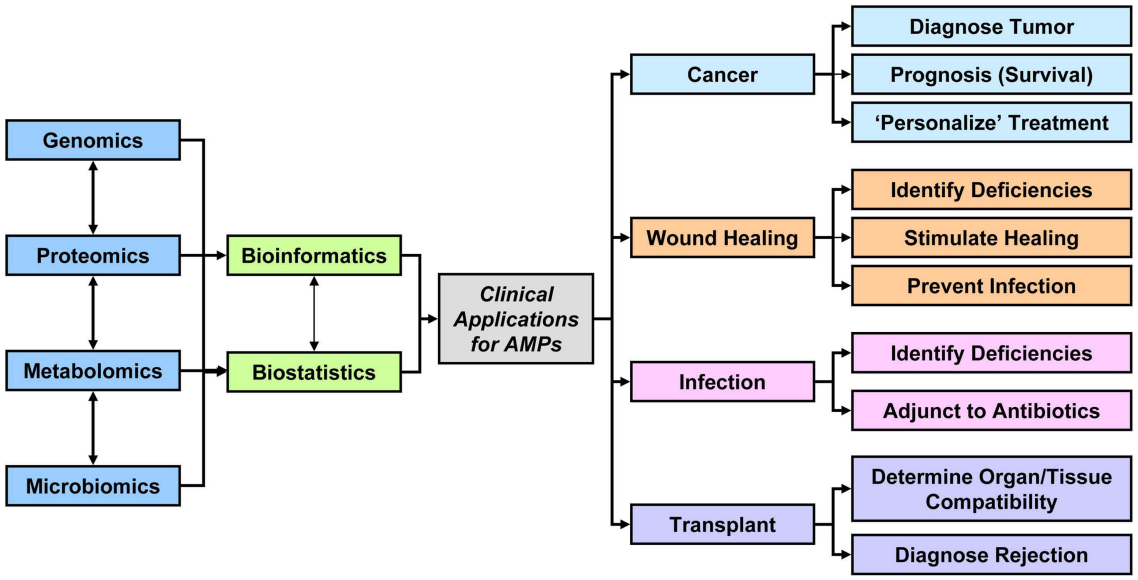

FIGURE 1 | Data acquired from the combination of genomics, proteomics, metabolomics, microbiomics, and other omics technologies either directly investigating AMPs or examining related parameters may be integrated to design better diagnostic tools, therapeutic options, or prognostic indicators for patients with cancer, wounds, infections, or transplant.

and under-recognized potential of AMPs for therapeutic applications. Although it is feasible to capture a given patient's molecular profile, the interpretation of those findings remains a challenge. Given the enormous data sets being generated by "omics" research, the development of useful computational models has been limited by several factors. Primarily, analyses of high-dimensional data are prone to overfitting of the models to the study samples, thus yielding inaccurate results in subsequent follow-up studies. Therefore, replication and reproducible verification become imperative when performing such research. Prospective technological advancement merged with more robust bioinformatic tools and greater data analysis capacity will help surmount the existing limitations to allow for the complete integration of micromolecules with systems biology.

CONCLUDING REMARKS

While the "Omics Revolution" continues to rapidly expand and mature, it is apparent that its maximal applicability and utility remain to be fully elucidated. "Omics" research, either directly examining AMPs or researching AMPs in the context of related factors such as the metabalome or the microbiome, could potentially identify significant and crucial relationships between various molecular signatures and human disease (**Figure 1**). These enormous data sets may serve as the foundation for the evolution of computational models that could predict disease, complications, or even prognosis based on a specific AMP profile. Furthermore, modified AMPs have the potential to replace current antibiotic therapies, as drug discovery begins incorporating the latest technology into their pharmaceutical development pipelines. Ultimately, the new approach to personalize healthcare can foster novel applications to refine the characterization of a disease phenotype, identify predictive biomarkers, determine the efficacy of various therapies, or determine the susceptibility to drug toxicity for each individual patient.

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Live-cell imaging and analysis shed light on the complexity and dynamics of antimicrobial peptide action

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Fungi and bacteria cause many serious and sometimes devastating diseases in humans, animals, and crops. Furthermore, resistance against antimicrobial drugs is steadily increasing. There is thus an urgent need to discover new antimicrobial drugs and drug targets. Antimicrobial peptides (AMPs) are widespread in nature and produced naturally by animals, plants, fungi, and bacteria (Zasloff, 2002). These secreted peptides typically act as broad spectrum antibiotics as part of the innate immune systems of these organisms. AMPs that possess high activity against microbial pathogens are attracting great interest for use as novel therapeutic agents to prevent and treat microbial diseases (Brogden, 2005; Hancock and Sahl, 2006). The cell-penetrating properties of many AMPs facilitate them reaching intracellular targets which, in most cases, are unknown and may be novel (Marcos and Gandía, 2009; Nicolas, 2009). Numerous candidate AMP-based drugs for use in humans, animals, and crops will undoubtedly appear over the next decade. A mechanistic understanding of their mode-of-action will be essential to underpin their use as new antimicrobial drugs, to identify novel drug microbial targets, and assist the rational design of more powerful and specific AMPs and peptidomimetics.

LIVE-CELL IMAGING OF INDIVIDUAL CELLS

Live-cell imaging techniques have become powerful tools for understanding the dynamic modes-of-action of AMPs. They complement methods in which AMP- or peptoid-treated cells are typically fixed and then processed for immunolocalization (e.g., Theis et al., 2005), electron microscopy (e.g., Friedrich et al., 2000), atomic force microscopy (e.g., Alves et al., 2010), or X-ray tomography (Uchida et al., 2009).

Live-cell imaging has been used primarily to analyze the effects of AMPs on the morphology and growth of bacteria and fungi. Fluorescent dyes have sometimes been used in these studies (e.g., as reporters of plasma membrane permeabilization, cell death, or to label cell walls). However, most of these studies have been restricted to imaging cells at a specific time point after treatment rather than performing time-lapse imaging and measurements on the same cells to monitor their dynamic changes in response to AMPs. Nevertheless, these studies provide useful information relating to the effects of AMPs on microbial cells, including thickening or weakening of cell walls, cell enlargement/shrinkage, or alterations in cell growth/branching patterns, as well as cell permeabilization and killing. These responses can be related to the stress the cell is sensing but also as defense responses to counteract peptide action. Most studies using live-cell imaging and AMPs have been done with fungi rather than bacteria, due, in part, to the advantages of fungal cells (larger cells, easy visualization, and non-motile). Morphological studies of the effects of plant defensins on fungi, for instance, have resulted in these AMPs being divided into two different subgroups, referred to as *morphogenic* and *non-morphogenic*, according to the type of morphological changes they induce in defensin-sensitive fungi (Thomma et al., 2002). Morphogenic defensins inhibit hyphal growth with a concomitant increase in hyphal branching, whereas non-morphogenic defensins inhibit growth without causing marked changes in cell morphology (Terras et al., 1992; Broekaert et al., 1995). Recently, the γ-core motif within the related *Medicago* defensins MsDef1 and MtDef4 has been shown to contain the major determinants which contribute to their morphogenicity and antifungal activity against the phytopathogenic fungus *Fusarium graminearum* (Sagaram et al2011). Another plant defensin, RsAFP2, has been shown to induce septin mislocalization and to impair the yeast-to-hypha transition in the human pathogen *Candida albicans* (Thevissen et al., 2012). In the phytopathogen *Penicillium digitatum*, we have reported alterations in cell morphology, conidiophore formation, and cell wall structure following exposure to the rationally designed peptide PAF26 (Muñoz et al., 2006) and cationic Lactoferricin-derived peptides (Muñoz and Marcos, 2006). Here it was shown, using the chitin-binding fluorophore calcofluor white with the membrane permeabilization reporter dye Sytox Green, that the peptides when used at sub-inhibitory concentrations caused abnormalities in cell morphology and growth pattern without permeabilizing the plasma membrane.

The real innovation for visualizing the dynamics of AMP–microbe interactions in recent years has been achieved with the use of fluorescently labeled peptides (or peptidomimetics) in combination with live-cell imaging, particularly using confocal microscopy. Conveniently, both natural and synthetic AMPs can be fluorescently labeled using commercially available protein tagging protocols (e.g., Lobo et al., 2007; van der Weerden et al., 2008). AMPs can also be chemically synthesized with fluorescent labels (e.g., fluorescein-, rhodamine-, BODIPY-, or Alexa fluor-based dyes, or quantum dots) with the fluorescent group at the N or C terminus of the peptide or peptidomimetic (e.g., Muñoz et al., 2006, 2012;

Mochon and Liu, 2008; Jang et al., 2010; Mania et al., 2010; Srinivas et al., 2010). The fluorescent AMPs commonly retain their antifungal activity although this may be reduced, often minimally, compared with the unlabelled AMPs. Most importantly, is that these fluorescent AMPs can be directly observed by live-cell imaging in time course experiments. The use of these AMP conjugates is greatly increasing our knowledge of their interaction, method of penetration, pathways of intracellular transport, and sites of antimicrobial action within living microorganisms. Furthermore, live-cell imaging gives an improved understanding of how the dynamic intracellular localization of the peptide influences the dynamic morphogenesis and physiology of individual microbial cells.

Various antifungal peptides and plant defensins have been shown to be internalized by living fungal cells (Oberparleiter et al., 2003; Theis et al., 2003; Moreno et al., 2006; van der Weerden et al., 2008; Mania et al., 2010; Binder et al., 2011; Maurya et al., 2011). For instance, the antifungal protein AFP from *Aspergillus giganteus* and the plant defensin Psd1 exhibited co-localization with Sytox Green- and DAPI-stained fungal nuclei, respectively (Moreno et al., 2006; Lobo et al., 2007). Nevertheless, in all these examples the mechanism of AMP internalization or their intracellular targets remain unknown. Histatin-5 is an antifungal AMP whose internalization mechanism coupled with activity has been studied in most detail in the pathogen *C. albicans*. Two distinct pathways for its intracellular trafficking have been postulated: slower endocytic internalization and a more rapid energy-independent uptake into the cytoplasm and vacuole (Mochon and Liu, 2008; Jang et al., 2010; Jang and Edgerton, 2012). In parallel, we have recently described a concentration-dependent mechanism of cell penetration and killing by the *de novo* designed hexapeptide PAF26 in the fungus *Neurospora crassa* (Muñoz et al., 2012). This peptide was shown to be endocytically internalized at low fungicidal concentrations, accumulating in vacuoles that expanded, and then was actively transported into the cytoplasm, which coincided with cell death. This study used a combination of vital fluorescent dyes (the membrane selective dye, FM4-64; the vacuolar dye, cDFFDA, and the cell death reporter propidium iodide), nuclei labeled with GFP, and either FITC- or TMR-labeled PAF26. These analyses demonstrated the advantages of how the direct observation of a peptide can be related to its subcellular effects at different stages in its antimicrobial action in individual cells. Even though the synthetic PAF26 and natural histatin-5 are cationic peptides, they are structurally unrelated (e.g., PAF26 is a hexapeptide and histatin-5 possesses 24 amino acids), they nevertheless seem to exhibit similar concentration-dependent pathways of internalization. As a result, we have proposed that PAF26 may be used as a simple model for mode-of-action studies of cationic antifungal AMPs such as histatin-5, as well as understanding how different aspects of its activity (e.g., endocytic and passive internalization, intracellular trafficking, and cell killing) are determined by individual residues or domains within its six amino acid sequence (Muñoz et al., 2012). Live-cell imaging and analytical techniques will provide novel insights into these processes.

Fluorescently labeled AMPs have only been used in a few live-cell imaging studies on bacteria. An interesting recent study by Sochacki et al (2011) showed the dynamic killing by the human AMP LL-37 of single *Escherichia coli* cells using time-lapse imaging. Rhodamine labeled LL-37 was monitored in combination with periplasmic GFP and the dye Sytox green to demonstrate that disruption of the cytoplasmic membrane by the peptide was not the growth-inhibiting mechanism, but rather this was caused by translocation across the outer membrane and access of the peptide into the periplasmic space. Leptihn et al. (2009) investigated the mode-of-action of the S1 peptide using fluorescence correlation spectroscopy (FCS) and single molecule tracking using quantum-dot labeled peptide. Using this approach they elucidated a temporal and spatial perspective of the bactericidal events involved in S1 peptide action.

LIVE-CELL ANALYSIS OF CELL POPULATIONS

The use of live-cell probes to measure and analyze various physiological parameters in AMP-treated populations of living cells has also proven very useful. For these studies, multiwell plate fluorimetry/luminometry or flow cytometry have been used. 96- or 384-microtiter well plate assays provide average measurements across a whole cell population. They are well suited for high throughput analysis and for monitoring changes in various physiological parameters [e.g., membrane potential and permeability, reactive oxygen species (ROS), intracellular calcium] with high temporal resolution. Flow cytometry, on the other hand, is able to generate one or more fluorescence measurements of a physiological or other cell parameter at a single time point for each cell in population. It is thus not suited for dynamic measurements of cell physiology but provides detailed information on the heterogeneity of responses within a cell population. Potentiometric dyes have been used to measure the plasma membrane potential in response to treatment with plant defensins (Thevissen et al., 1996), the protein PAF from *Penicillium chrysogenum* (Leiter et al., 2005), histatin-5 (Helmerhorst et al., 2001b), or PAF26 (Muñoz et al., 2012). Fluorescent dyes have been employed to detect ROS formation following treatment with histatin-5 (Helmerhorst et al., 2001a), VS2 and VS3 (Maurya et al., 2011), PAF26 (Carmona et al., 2012), PAF protein (Leiter et al., 2005), or Lactoferrin (Andrés et al., 2008). The genetically encoded calcium-sensitive, bioluminescent protein aequorin has been used to measure changes in intracellular calcium in response to treatment with the PAF protein (Binder et al., 2010, 2011) or PAF26 (Muñoz et al., 2012). Kim and Cha (2006) used a Förster resonance energy transfer (FRET)-based assay to quantify AMP-induced membrane disruption in *E. coli* by measuring changes in the FRET efficiency of a cytosolic protein when it became released into the lower pH environment of the external medium.

FUTURE PROSPECTS

The potential of using fluorescent labeled AMPs in combination with multiwell plate measurements or flow cytometry has been explored to only a limit extent. For example, Benincasa et al. (2009) used flow cytometry to distinguish an AMP that was internalized by bacterial cells from another that was membrane active by using cell impermeant trypan blue to quench the fluorescence of fluorescently labeled AMP that was on the cell surface. In the future, the use of "smart probes" to label AMPs will be useful where the fluorescence spectral characteristics

of the label changes depending on the cell compartment that the AMP is in. For example, a label that is pH-sensitive could be used to report the transport of the AMP into an acidic organelle such as a vacuole. The targeting of genetically encoded physiological reporters (e.g., ROS-sensitive, pH-sensitive, or calcium-sensitive GFP-based indicators) to different cell compartments or organelles will also be extremely useful to monitor the effects of AMPs on cell populations using multiwell plate fluorimetry or flow cytometry.

There are also now a wide range of advanced, live-cell imaging technologies which are commercially available and that need to be explored with regard to analyzing the influence of fluorescently labeled AMPs on living cells. These include: fluorescence lifetime imaging microscopy and/or FRET microscopy to image and measure interactions between fluorescently labeled AMPs and other molecules (Sekar and Periasamy, 2003; Becker, 2012); fluorescence recovery after photobleaching (FRAP) to visualize and measure processes such as the rate of AMP diffusion or trafficking in cells (Lippincott-Schwartz et al., 2003); FCS to measure binding constants between peptides and other molecules (Bacia and Schwille, 2007); various super-resolution microscopic techniques that allow the spatial resolution achievable with fluorescence microscopy to be significantly increased with living cells (Chi, 2009); and high content, multiparameter imaging which are designed for ultra high throughput analysis of living cells in multiwell plates (Taylor and Haskins, 2007).

In summary, live-cell imaging and analytical techniques are extremely powerful methods that can provide direct, high spatiotemporal resolution information on the dynamics and complexity of the modes-of-action of AMPs, and also novel peptidomimetics (Scorciapino and Rinaldi, 2012). In the future, these approaches will undoubtedly have a profound impact on our understanding of the ways in which AMPs work which should greatly assist the rational design of new and more effective antimicrobial drugs.

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Does cholesterol play a role in the bacterial selectivity of antimicrobial peptides?

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ANTIMICROBIAL PEPTIDES ARE PROMISING ANTIBIOTIC COMPOUNDS

The development of novel methods to overcome the inevitable resistance that develops with common antibiotics is an important area of current research. Recent studies have shown that antimicrobial peptides (AMPs) have the potential to become excellent antibiotic compounds toward a broad-spectrum of Gram-positive and Gram-negative bacteria with less potential for bacterial resistance than conventional antibiotics (Shai, 2004). Because these compounds are highly selective toward bacteria and bacteria have difficulty in developing resistance to their effects, a large number of studies have focused on designing potent AMPs for potential pharmaceutical applications (Maloy Biopolymers; Marsh et al., 2009). One of the designed peptides, MSI-78 (also known as pexiganan), rose successfully to phase II clinical trials for treating infection in the case of diabetic foot ulcer (Gottler and Ramamoorthy, 2009).

COMPOSITION OF MEMBRANES IS KEY TO AMP SELECTIVITY

Bacteria have difficulty in developing resistance to AMPs because the toxicity of AMP is mostly mediated by a non-specific process rather than by an interaction with a specific protein target. Most AMPs lyse bacteria by directly interacting with the lipid bilayer of the bacterial cell membrane and disrupting the lipid bilayer structure (Oren and Shai, 1998; Epand and Vogel, 1999; Shai, 2002; Bechinger, 2011). The development of more potent and selective AMPs requires that the molecular basis of activity and selectivity be understood. Substantial progress has been made in recent years in this area, particularly using cutting-edge solid-state NMR spectroscopy to provide insights into the mechanisms of membrane disruption by AMPs (Bechinger, 1999; Durr et al., 2006; Bhattacharjya and Ramamoorthy, 2009; Ramamoorthy, 2009; Nguyen et al., 2011). For example, the high-resolution 3D structure, membrane orientation, and mechanism of membrane disruption are reported for several important peptides including LL-37 (Wildman et al., 2003; Porcelli et al., 2008), MSI-78 (Hallock et al., 2003), MSI-594 (Ramamoorthy et al., 2006; Bhunia et al., 2009), and pardaxin (Hallock et al., 2002; Porcelli et al., 2004; Bhunia et al., 2010). Biophysical studies have also revealed the role of anionic lipids, (Thennarasu et al., 2010) cholesterol, and lipopolysaccharides (Bhunia et al., 2009, 2010; Domadia et al., 2010) in Gram-negative bacteria on the antimicrobial activities of these AMPs. In addition, substantial progress has been in understanding the molecular determinants of AMP activity. For example, recent studies have shown the ability to form oligomeric aggregates in the cell membrane enhances the potency of an AMP (Toke et al., 2004; Tremouilhac et al., 2006; Marquette et al., 2008; Ramamoorthy et al., 2008; Strandberg et al., 2008). Studies have also shown that the presence of D-amino acids (Mangoni et al., 2006) and disulfide bridges (Dhople et al., 2006) can enhance resistance against proteolytic degradation without affecting the antimicrobial activity.

From these studies, a picture of how AMPs preferentially target bacteria has begun to emerge. The selectivity of AMPs therefore largely lies in their ability to distinguish between prokaryotic and eukaryotic membranes (Glukhov et al., 2005; Epand et al., 2006b). Biophysical studies have shown the importance of two factors in the membrane selectivity of an AMP (**Figure 1A**): (a) the electrostatic interaction between a cationic AMP and the acidic bacterial membrane which is composed of about ~25% anionic lipids (POPS, POPG, and/or cardiolipin; Glukhov et al., 2005; van Meer et al., 2008; Epand et al., 2010) and (b) the presence of a large amount of cholesterol in a eukaryotic cell membrane which inhibits membrane disruption by rigidifying the lipid bilayer structure (Benachir et al., 1997; Matsuzaki, 1999; Glukhov et al., 2005; Epand et al., 2006a; Verly et al., 2008). These factors controlling the membrane selectivity of AMPs can also be exploited for other pharmaceutical targets. For example, several AMPs have been shown to have anticancer activities; this property has been attributed to the presence of anionic lipids in the outer leaflet of the cancer cell plasma membrane (Hoskin and Ramamoorthy, 2008). Similarly, most AMPs also kill fungi, protozoa, and even enveloped viruses, which all show a lipid distribution different than a normal eukaryotic cell (Oren and Shai, 1998; Epand and Vogel, 1999; Shai, 2002; Bechinger, 2011; Nguyen et al., 2011; Pius et al., 2012). Despite this progress in understanding the molecular determinants of AMP activity, there are still unresolved questions, particularly with regards to the preferential targeting of bacterial membranes. While the role of anionic lipids in membrane targeting of AMPs is well established, the role of cholesterol is still not clear. Accordingly, this opinion article focuses on the distinct roles of cholesterol in homogenous versus heterogeneous lipid bilayers.

CHOLESTEROL IS BELIEVED TO PLAY A ROLE IN BACTERIAL SELECTIVITY OF AMPs

One of the major differences between bacterial and eukaryotic cell membranes is the presence of a large amount of cholesterol in eukaryotic cell membranes and a complete absence in bacterial cell membranes

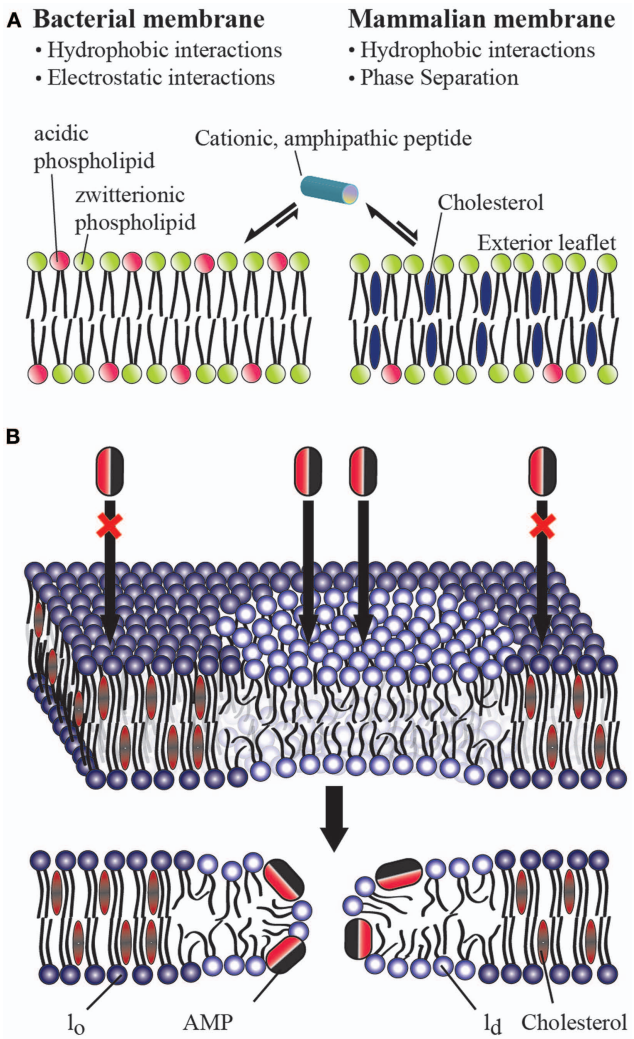

FIGURE 1 | (A) Role of cholesterol on the bacterial selectivity of antimicrobial peptides. Lipid bilayers mimicking bacterial **(A)** and eukaryotic **(B)** cell membranes are commonly used in *in vitro* studies on AMPs. In eukaryotic cell membranes, the outer leaflet consists primarily of zwitterionic phosphatidylcholine lipids (such as POPC), and cholesterol (~25%) while the inner leaflet contains anionic lipids (such as POPS). Bacterial cell membranes typically lack cholesterol and contain ~25% acidic lipids (like POPG and cardiolipin), and ~55% phosphatidylethanolamine (POPE). AMPs have been shown to directly interact with the lipid bilayer of bacterial cell membranes and lyse the cell by disrupting the membrane via one of the several proposed mechanisms including barrel-stave, toroidal-pore, and detergent-type disturbances. The presence of cholesterol in the eukaryotic cell membrane enhances the rigidity of lipid bilayers to inhibit the membrane disruption activities of antimicrobial peptides. The electrostatic interaction between a cationic antimicrobial peptide and the anionic lipids (POPS) present in the outer leaflet of bacterial membranes plays a vital role in bacterial selectivity and the absence of cholesterol makes the membrane disruption by an AMP easier. In the case of Gram-negative bacteria, the presence of anionic lipopolysaccharides attracts cationic AMPs. **(B)** Mechanism of action of an antimicrobial peptide in a raft-containing membrane. In a heterogeneous mixture of lipids, the presence of cholesterol in the raft domain (l_o) resists the permeation of an antimicrobial peptide while the disordered (l_d) lipid domain is easily disrupted by an antimicrobial peptide.

(**Figure 1A**). Cholesterol has been shown to protect human erythrocytes from attack by magainin 2 (Matsuzaki et al., 1995b). Similar studies on model membranes have demonstrated that the presence of cholesterol reduces AMP binding and suppresses the disruption of lipid bilayer structure by AMPS (Feigin et al., 1995; Matsuzaki et al., 1995a; Tytler et al., 1995; Raghuraman and Chattopadhyay, 2004; Glukhov et al., 2005; Verly et al., 2008; Wu et al., 2010). Solid-state NMR studies have provided high-resolution insights into the role of cholesterol against the function of several AMPs (Benachir et al., 1997; Wildman et al., 2003; Ramamoorthy et al., 2010). Cholesterol is known to increase membrane cohesion and mechanical stiffness (Evans and Waugh, 1977; Henriksen et al., 2006) which may resist the membrane bending required for many AMPs to function (Allende et al., 2005). This interaction reduces the tilt of the paradaxin helix relative to the bilayer normal, which in turn reduces the stability of the paradaxin pore (Ramamoorthy et al., 2010). However, for most AMPs a noticeable inhibitory effect of cholesterol is only noticeable after the formation of liquid ordered lipid phase at high concentrations of cholesterol (~20%; McHenry et al., submitted) which suggests it may be due to an indirect effect due to a modulation of membrane properties rather than a direct interaction (Feigin et al., 1995). Despite these advances, the actual reason for the reduced affinity of many AMPs for cholesterol containing membranes is not fully understood. As noted above, this is traditionally been interpreted as a consequence of the increased acyl chain order in the liquid ordered phase of cholesterol containing membranes. In this context, it is interesting to compare cholesterol's effects on AMPs which do not clearly prefer the disordered liquid crystalline lipid phase or ordered gel phase. Surprisingly, cholesterol still strongly inhibits these peptides, which suggests an additional factor, such as dehydration of the headgroup region (M'Baye et al., 2008) is partially responsible for cholesterol's effect.

CHOLESTEROL LOSES ITS EFFECTIVENESS IN INHIBITING AMPs WHEN INCORPORATED INTO RAFT-LIKE DOMAINS

While biophysical studies have shown the ability of cholesterol to suppress the action of an AMP against a homogeneous lipid bilayer, recent studies have revealed that cholesterol does not have this same effect in heterogeneous lipid systems (Pokorny and Almeida, 2005; Pokorny et al., 2006). Though few studies have looked at membrane disruption by AMPs in heterogeneous systems with phase separation [particularly in liquid ordered (l_o) liquid-disordered (l_d) domain coexistences often referred to as "lipid rafts"], two studies by the Almeida

group demonstrated the permeabilizing activity of δ-lysin in raft-like palmitoyl-2-oleoylphosphatidylcholine/cholesterol/sphingomyelin (POPC/Chol/SM) mixtures (Pokorny and Almeida, 2005; Pokorny et al., 2006). These studies revealed that membrane permeabilization by δ-lysin occurs exclusively in the l_d phase in membranes with $l_d - l_o$ phase segregation and that the localization of δ-lysin to the l_d phase results in greater membrane disruption than would be expected in the absence of phase segregation. Our own group recently demonstrated that this important effect occurs among a diverse set of AMPs (MSI-78, MSI-594, MSI-843, and MSI-367) encompassing several membrane disruptive mechanisms (McHenry et al., submitted). These combined results indicate that the phase separation naturally occurring in eukaryotic membranes is likely to nullify the effect of cholesterol against membrane disruption by AMPs. This surprising result suggests either cholesterol is not as important in determining the selectivity of AMPs toward bacterial membranes as once supposed, or unknown additional factors mitigate this effect in eukaryotic cells.

The mechanism of action of an AMP in a heterogeneous lipid system is depicted in **Figure 1B**. These results suggest that raft formation localizes the concentration of cholesterol in the cell membrane in such a way that non-raft domains of the cell membrane can be easily disrupted by AMPs and toxins. It is also possible that the phase behavior of the membrane and the physicochemical properties of the boundaries connecting the ordered and disordered domains play important roles in the membrane disruption process by AMPs. For instance, paradaxin has been shown to segregate a homogeneous membrane into cholesterol rich and cholesterol poor domains (Epand et al., 2006a). While the AMPs that have been investigated so far function by the non-specifically mechanically disrupting the membranes (carpet, detergent-type, or toroidal-pore formation) mechanism (**Figure 1A**), it is unclear how AMPs resembling more traditional ion channels (barrel-stave mechanism) would interact with heterogeneous lipid systems (**Figure 1B**). Therefore, it is important to further investigate the interaction of a variety of AMPs with more heterogeneous lipid systems.

FUTURE SCOPE

While the development of AMPs for antibiotic applications is highly important, it is essential to understand the origin of their bacterial selectivity. As mentioned above, recent studies have shown that AMPs easily disrupt the structure of heterogeneous lipid systems, and therefore cholesterol is unlikely to play a major role in reducing the toxicity or increasing the selectivity of AMPs. Since a natural eukaryotic cell membrane contains heterogeneous lipid systems and domains, cholesterol poor domains must be easily disruptable by an AMP. Further studies probing the role of cholesterol in different types of lipid bilayers with a variety of AMPs are essential to better understand the exact role of cholesterol on the toxicity and selectivity of AMPs. Such studies would aid in the design of more efficient AMPs.

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The mechanism of action of antimicrobial peptides: lipid vesicles vs. bacteria

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OVERVIEW

AMPs AND THEIR MICs

Antimicrobial peptides (AMPs) are a class of antibiotics that is part of the innate immune system of virtually all organisms. This is a broadly defined class, with many common characteristics – and many exceptions to those. The reader will find detailed reviews of such characteristics and proposed subclassifications of the AMPs (see Yeaman and Yount, 2003, for example) but, very generally, these are short, cationic peptides that kill bacteria (sometimes only Gram-positives, sometimes only Gram-negatives, sometimes both) at concentrations typically in the low micromolar range. The clinical interest of some AMPs stems from a low toxicity to mammalian cells, together with the fact that bacterial resistance to these antibiotics seems inherently difficult to acquire (Bell and Gouyon, 2003; Perron et al., 2006).

Of importance to the discussion later on is the method and conditions through which MICs are established for a given peptide against a given bacterial strain. A widely adopted protocol to this end is the one provided by the lab of Robert Hancock (Giacometti et al., 2000); it consists in monitoring the growth of quite dilute bacterial suspensions after administration of different peptide doses. The MIC*n* is then defined as the peptide concentration that causes a reduction of *n*% in growth relative to control (MIC_{50} and MIC_{90} being the most commonly reported) after a set time interval.

LIPOSOMES AS BIOPHYSICAL MEMBRANE MODELS

AMPs make good test subjects for biophysical methodologies. Accounting for this is the peptides' small size, and the ease with which one can influence their molecular-level properties simply by changing the amino acid sequence. In addition, an early recognition of the importance of membrane interaction and disruption for the action of AMPs (Lehrer et al., 1989) allowed the coupling of the mature field of membrane biophysics to study the latter.

With the exception of certain methodologies, lipid vesicles have been the preferred biophysical model for mimicking biological membranes – bacterial or otherwise (Matsuzaki et al., 1995; Willumeit et al., 2005). In the three decades of AMP studies a wealth of biophysical data has been collected from these peptide-vesicle systems. These data include structural features – such as peptide structuring, oligomerization, depth of insertion, etc. – thermodynamic properties – such as peptide-membrane affinity and functional aspects – typically membrane disruption events such as poration or lysis, but also peptide translocation and lipid charge neutralization (Shai, 1999).

MEMBRANE DISRUPTION AND HIGH PEPTIDE-TO-LIPID RATIOS

It came to our attention, first from our own studies and then from others' reports (Melo et al., 2009), that several membrane disruptive events were often observed at concentrations where the vesicle membrane would be almost completely covered by the peptide–peptide-to-lipid (P:L) ratios higher than 1:25. It is common to read remarks on the unphysiological character of results obtained using too high such ratios (Zhang et al., 2001; Hancock and Rozek, 2002; Nicolas, 2009) but a sound basis for this assumption is yet to be provided.

It is easy to see the reasons behind the intuitive notion of the unphysiological character of high P:L ratios: first, peptides are nanometer-scale entities, typically present at micromolar concentrations, that seem impossible to exist in enough numbers to cover a (relatively) macroscopic entity such as a bacterium. Second, laboratory research often requires the use of peptide and lipid concentrations equivalent to many times those present in MIC assays; this further contributes to the notion that any events thus observed are only achievable with the extreme concentrations available *in vitro*, even though the bound P:L ratios may actually be the same as in much more dilute conditions.

Conversely to these considerations, already in 2000 Tossi et al. had pointed out the great excess of peptide to bacterial lipids at typical AMP MIC conditions. We further developed that consideration by taking into account measured affinities of AMPs to bacterial membrane mimics. From there we arrived at expected peptide-to-lipid ratios in the bacterial membrane that fall precisely in the range where liposomal disruption is commonly observed (Melo et al., 2009, 2011), and that many insist on calling "unphysiological." Furthermore, attempts at directly quantifying bacterium-bound AMPs – though scarce – again point to very high degrees of bacterial surface coverage (Steiner et al., 1988; Albrecht et al., 2002; Tran et al., 2002; Ding et al., 2003).

FROM BIOPHYSICS TO BIOLOGY

BACTERIAL MEMBRANE – HOW MUCH OF IT IS THERE?

One of the main issues when trying to assess the validity of high P:L ratios comes from the fact that it is not readily measurable how much membrane a bacterial suspension has available for interaction. To estimate a peptide-to-lipid proportion Tossi et al. needed to find an approximate value for this amount of available lipid. They did so

using a geometrical approach, taking into account the average areas of the bacterial surface and of a membrane phospholipid. After factoring in the bacterial titer a lipid concentration of 25 nM was estimated (Tossi et al., 2000). Blazyk et al. (2001) later followed a similar reasoning, estimating a value of 66 nM. We carried out a different calculation, using the bacterial dry weight, its fraction that are phospholipids, and an average phospholipid mass. The value of 58 nM to which we arrived (Melo et al., 2011) is in good agreement with the two previous estimates.

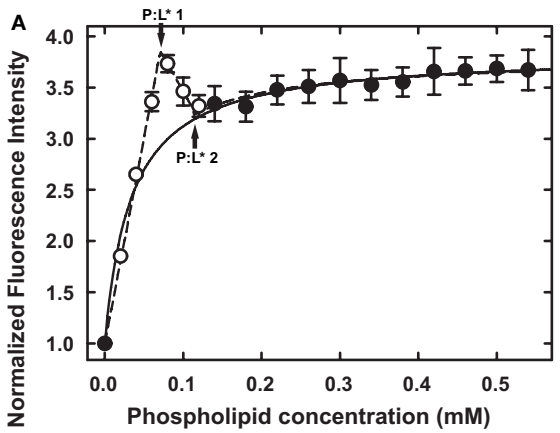
The bottom line from these numbers is that under MIC assay conditions membrane lipids are present in concentrations in the range of tens of nanomolars, whereas there are about two orders of magnitude more peptide available to bind it.

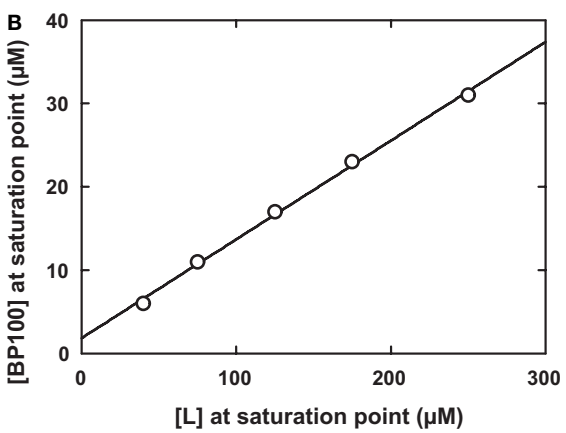
HOW MUCH PEPTIDE BINDS?

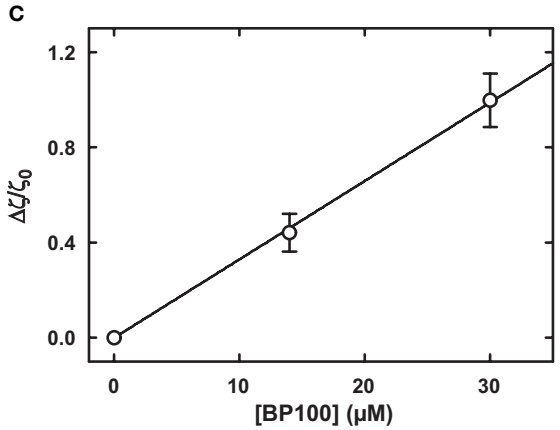
Not all the peptide in solution will bind the available membrane lipids, as binding is a reversible process subject to an equilibrium constant (Santos et al., 2003). How much peptide does bind can be quantitatively calculated since those equilibrium constants are often measured, usually in the form of membrane binding or partition (K_p) constants. We have shown (Melo et al., 2011) that typical partition constants for AMPs, though quite high, will drive less than 1% of the total peptide to the membrane; this is still enough, however, for a typical AMP to reach P:L ratios between 1:20 and 1:10 at global concentrations close to their MIC. These calculations provide theoretical significance to the aforementioned observations of disruptive events at high P:L ratios. This is not to say that all AMPs only become active at high degrees of membrane coverage, but it is plausible to say that many might behave thus, and therefore that no observations at high P:L ratios should be discarded.

BRIDGING THE GAP: MIC PREDICTION

The simple relationships that were used to establish the plausibility of high P:L ratios could be used in the reverse direction: by







D

MIC estimation[a,b] by eq. 1 from different parameters of BP100

K_p \ P:L*	*Panel A* (P:L* 1)	*Panel A* (P:L* 2)	*Panel B*	*Neutralization assumption*[c]
Panel A[d]	3.18 ± 1.00	1.91 ± 0.63	3.39 ± 1.03	3.18 ± 0.96
Panel C	2.58 ± 0.49	1.55 ± 0.33	2.75 ± 0.46	2.58 ± 0.43
Direct MIC estimate[e]	1.84 ± 0.51			

[a]Values in μM.
[b]A γ_L value of 0.763 M^{-1} was used.
[c]Estimated P:L* of 1:9 as per eq. 2 for BP100 in a 2:1 POPG:POPC system.
[d]Only the K_p value from the multiple-state fit was used.
[e]Estimate from the intercept of the regression in panel B.

FIGURE 1 | Different experimental methods to determine K_p and P:L* for a peptide-vesicle system, exemplified with the peptide BP100 with liposomes of 2:1 anionic-to-zwitterionic lipid constitution (Ferre et al., 2009). (A) Partition curve with an obvious deviation from hyperbolic behavior at low lipid concentrations; a K_p of $(39.3 \pm 14.4) \times 10^3$ was obtained from a simple fit to the filled data points (Ferre et al., 2009); a fit to all data points was possible by assuming the coexistence of different bound states (Melo and Castanho, 2007), yielding a K_p of $(45.8 \pm 13.8) \times 10^3$ and two P:L* values, of 0.111 ± 0.01 and 0.067 ± 0.008, respectively (indicated by arrows). **(B)** Membrane saturation points obtained at different peptide and lipid concentrations; the linear fit (Ferre et al., 2009) yielded a P:L* of 0.118 ± 0.003; the intercept of (1.84 ± 0.51) μM is a direct estimate of the MIC as per Eq. 1 (Melo et al., 2011). **(C)** Normalized ζ-potential measurements from where a K_p of $(56.4 \pm 9.4) \times 10^3$ was extracted (Freire et al., 2011). **(D)** Summary of the possible MIC calculations, by Eq. 1, from different parameters (estimated – see Eq. 2 – or from panels **A–C**); these overlap nicely with the 2.5–7.5 μM range where BP100 is active against different Gram-negatives (Ferre et al., 2009; Alves et al., 2010).

knowing a critical P:L ratio at which an AMP becomes disruptive against a given membrane model we can calculate the global AMP concentration required for it to reach a similar P:L ratio in a bacterium. This concentration will be a MIC estimate. A very simple equation (Melo et al., 2011) summarizes the relationship between MIC, critical P:L ratio (P:L*), and membrane affinity(K_p):

$$\mathrm{MIC} = \frac{\mathrm{P{:}L}^*}{K_p \cdot \gamma_L} \quad (1)$$

where γ_L is the lipid molar volume, a known value for artificial lipid fluid bilayers (Chiu et al., 1999).

Equation 1 does require the determination of the P:L* and K_p parameters. We provide here a summary of methods to their determination using model membranes, as well as collected examples with the peptide BP100 in **Figure 1**:

Partition data

The value for K_p can be extracted from experiments where the fraction of bound peptide is determined at different concentrations of lipid (**Figure 1A**; see Santos et al., 2003, for an overview on K_p extraction from these curves). Oftentimes data points at low lipid concentrations – the conditions at which the peptide is most concentrated in the membrane – will deviate from the expected hyperbolical relationship. By fitting a model that accounts for different membrane-bound states (Melo and Castanho, 2007) a value for K_p and two critical P:L ratios can be recovered (**Figure 1A**).

Other setups similar to the described above, but not necessarily involving a lipid titration, can be used to determine K_p: isothermal titration calorimetry (Bastos et al., 2008) is an accurate alternative; peptide quantification after separation by ultracentrifugation has also been successfully applied (Cirac et al., 2011); and a method has been recently put forth that allows K_p extraction from ζ-potential data (**Figure 1C**; Freire et al., 2011).

Threshold points

There is a linear relationship between global peptide and lipid concentrations for a threshold point to be reached (**Figure 1B**; Pott et al., 1998; Melo and Castanho, 2007). P:L* can be recovered from the slope of this peptide vs. lipid relationship, whereas the intercept will be itself a direct estimate of the MIC (this is so because the limit of zero lipid concentration parallels the very low lipid concentration in a MIC assay; Melo et al., 2011).

Neutralization assumption

For some peptides it may be plausible to assume that P:L* coincides with, or is triggered by, electrostatic neutralization of the membrane (Ferre et al., 2009). In the case of a one-to-one peptide-lipid charge neutralization it can be seen that P:L* will be given by:

$$\mathrm{P{:}L}^* = \frac{f_C \cdot z_L}{z_P} \quad (2)$$

where f_C is the fraction of charged lipids, z_L the absolute formal charge per charged lipid, and z_p the absolute formal charge on the peptide (assuming peptide and lipid charges have opposite signs; extra terms must be introduced in case of multiple lipids of different charges). This assumption provides an easy shortcut to a potentially relevant threshold, if no other data are available.

Equation 1 is not only simple and straightforward to use, but was also successfully applied to data for peptides BP100 (**Figure 1D**) and omiganan (Melo et al., 2011). Furthermore, it should be remarked that although the involved research was spurred by such observations the applicability of Eq. 1 does not depend on P:L* actually being very high. Nor is Eq. 1 restricted to bacterial death: it may be applicable to systems with relevant membrane threshold events, such as hemolysis (Melo et al., 2011).

IMPROVING THE MICs OF AMPs

It is interesting to analyze the issue of AMP activity optimization under the light of Eq. 1. It can be seen that lower MICs can be achieved either by having peptides with a low P:L* (i.e., requiring very little membrane-bound concentration to trigger bactericidal action) or with a high K_p (i.e., having a very strong affinity for the membrane). Designing peptides with lowered P:L* is not straightforward as the cooperative mechanisms involved in disruption are not yet fully understood. On the other hand, improving K_p is an apparently easier task: one would need only increase the charge density on the peptide as much as possible, which will then boost the affinity for the anionic bacterial membrane. However, peptide charge density is a double-edged sword: too much of it will cause surface neutralization at low bound concentrations – perhaps too low to trigger activity (Alves et al., 2010) – and subsequent peptide binding will no longer be favored after the electrostatic driving forces have been quenched. Given this, it may be the case that typical AMPs have evolved to have the highest charge density that does not compromise their accumulation in the membrane. Surface charge neutralization at membrane disruption would then arise not from a cause-effect relationship but rather as a consequence of optimal antibacterial peptide properties.

CONCLUSION

The path that bridges biophysical data with macroscopic physiological observations is paved with assumptions that are sometimes taken for granted. We started this work by challenging the notion of unphysiologically high AMP P:L ratios. By then taking a modeling approach we not only demystified the issue, but also achieved new perspectives on AMP action that ultimately led to a predictive model – interestingly, one that does not require specifically high P:L ratios and is therefore even compatible with more conservative views.

Just as there are exceptions to almost any characteristic a typical AMP is said to possess, so do we expect there to be several cases of peptides that will not conform to our predictions. At any rate, a simple new approach to AMP action is provided that we hope will spur novel developments on the field.

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The origin and function of anti-fungal peptides in *C. elegans*: open questions

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While innate immunity has been studied in insects since the time of Pasteur and Metchnikoff (Brey, 1998), research into nematode immune defenses was initiated only comparatively recently (Kurz and Ewbank, 2003). In the mid 1990s, through a biochemical approach, Yusuke Kato was able to isolate an antibacterial activity from the body fluid of the parasitic nematode *Ascaris suum*. This activity was ascribed to *A. suum* antibacterial factor (ASABF), a peptide that is particularly potent against Gram-positive bacteria (Kato, 1995). The subsequent molecular characterization of ASABF allowed the identification of six orthologous "ABF" peptides in the model nematode *C. elegans*, one of which, ABF-2 displays *in vitro* microbicidal activity against a range of bacteria and fungi (Kato and Komatsu, 1996; Kato et al., 2002; Zhang and Kato, 2003). The six ABF peptides were immediately recognized as sharing features with defensins, placing them in the class of cysteine-stabilized α-helix and β-sheet (CSαβ) peptides, the most wide-spread and conserved class of antimicrobial peptides (AMPs; Zhu, 2008). The constitutive expression of *abf-1* and *abf-2*, the best characterized of the six corresponding genes in *C. elegans*, overlaps in the pharynx (Kato et al., 2002); they are likely to contribute to the breakdown of the microbes that form the nematode's normal diet. Although they undoubtedly act upon both bacteria and fungi, here, we consider only their potential role in anti-fungal defenses. Both *abf-1* and *abf-2* are up-regulated by infection with the fungus *Cryptococcus neoformans* (Means et al., 2009), while the expression of *abf-1* but not of *abf-2* is induced by infection with the natural fungal pathogens *Drechmeria coniospora* and *Harposporium* sp. (Engelmann et al., 2011). Conversely, *abf-2* is up-regulated by *Candida albicans*. It is not clear what the underlying regulatory pathways governing *abf* gene expression are (Pukkila-Worley et al., 2011), but one can speculate that their differential regulation reflects both the various modes of pathogen infection (Labed and Pujol, 2011), and also their potentially distinct spectra of antimicrobial activities.

This conserved family of defensin-like peptides is something of an exception, since overall, *C. elegans* possesses a highly derived innate immune system. It has no equivalent of NF-κB, central to immunity in many animals, and also lacks orthologs of most of the receptors known from other species to be important for triggering host defenses (Pujol et al., 2001; Gravato-Nobre and Hodgkin, 2005). Indeed, as explained more in detail below, several classes of AMPs implicated in anti-fungal defense appear to be restricted to certain nematode species, and are controlled by signal transduction cascades with a very limited phylogenetic range (Dierking et al., 2011; Labed et al., 2012). Different fungal pathogens infect either via the intestine following ingestion, or via the epidermis. They influence AMP gene expression via distinct signaling cascades, but a detailed discussion of these regulatory mechanisms is beyond the scope of this short article.

Unlike *A. suum*, which can grow to a length of 40 cm, an adult *C. elegans* measures barely 1 mm, making extraction of body fluid technically almost impossible. Many of the other putative AMP genes in *C. elegans* were initially identified on the basis of their differential regulation following *D. coniospora* infection. The first such genes were members of the *nlp* (for neuropeptide-like protein) and *cnc* (caenacin) families (Couillault et al., 2004). At the time, the former had been tentatively annotated as neuropeptides, based on their limited sequence similarity with known neuropeptides. It was, however, observed that these genes were not generally expressed in the nervous system, but rather in the epidermis (Nathoo et al., 2001). This ties in with the fact that *D. coniospora* spores attach to the nematode's cuticle and then germinate, penetrating into the body of the worm via the epidermis. Much, but not all, of the response to infection is a cell-autonomous mechanism acting in the epidermis; reviewed in Labed and Pujol (2011). The infection-induced *nlp* genes are structurally related to the *cnc* genes. The two groups further share the property of being found in clusters in the genome. Interestingly, comparison of the syntenic regions in two other *Caenorhabditis* species, *C. briggsae* and *C. remanei* revealed that these genes are undergoing relatively rapid evolution, with clear evidence for gene duplication and gene loss, and appear to be under positive selective pressure. Indeed, over-expression of either the *nlp* or *cnc* AMPs leads to somewhat increased resistance to *D. coniospora* infection (Pujol et al., 2008; Zugasti and Ewbank, 2009), suggesting that they play a direct role in host defense against invasive fungi. This is further reinforced by the finding that the *nlp* and *cnc* AMP genes feature prominently among the few genes commonly up-regulated by *D. coniospora* and the fungi *Harposporium* sp. (Engelmann et al., 2011). The expression of *cnc-4* and *cnc-7* is also induced by virulent *C. albicans* (Pukkila-Worley et al., 2011), which like *Harposporium* sp. infects *C. elegans* via the intestine.

Since the initial phylogenetic studies, more nematode genome sequences have become available. Comparative analyses reinforce the notion of rapid gene evolution. For example, the single copy *C. remanei* gene CRE-*nlp-27* corresponds to a cluster of 5 paralogs in *C. elegans* (Pujol et al., 2008), but 10 predicted paralogs in *C. japonica* (**Figures 1A,B**). On the other hand, there apparently has been no expansion of the *cnc* genes in *C. japonica*, whereas multiple paralogs are found in the other *Caenorhabditis* species (**Figure 1C**). We have not found orthologous genes in several other nematode species for which genome

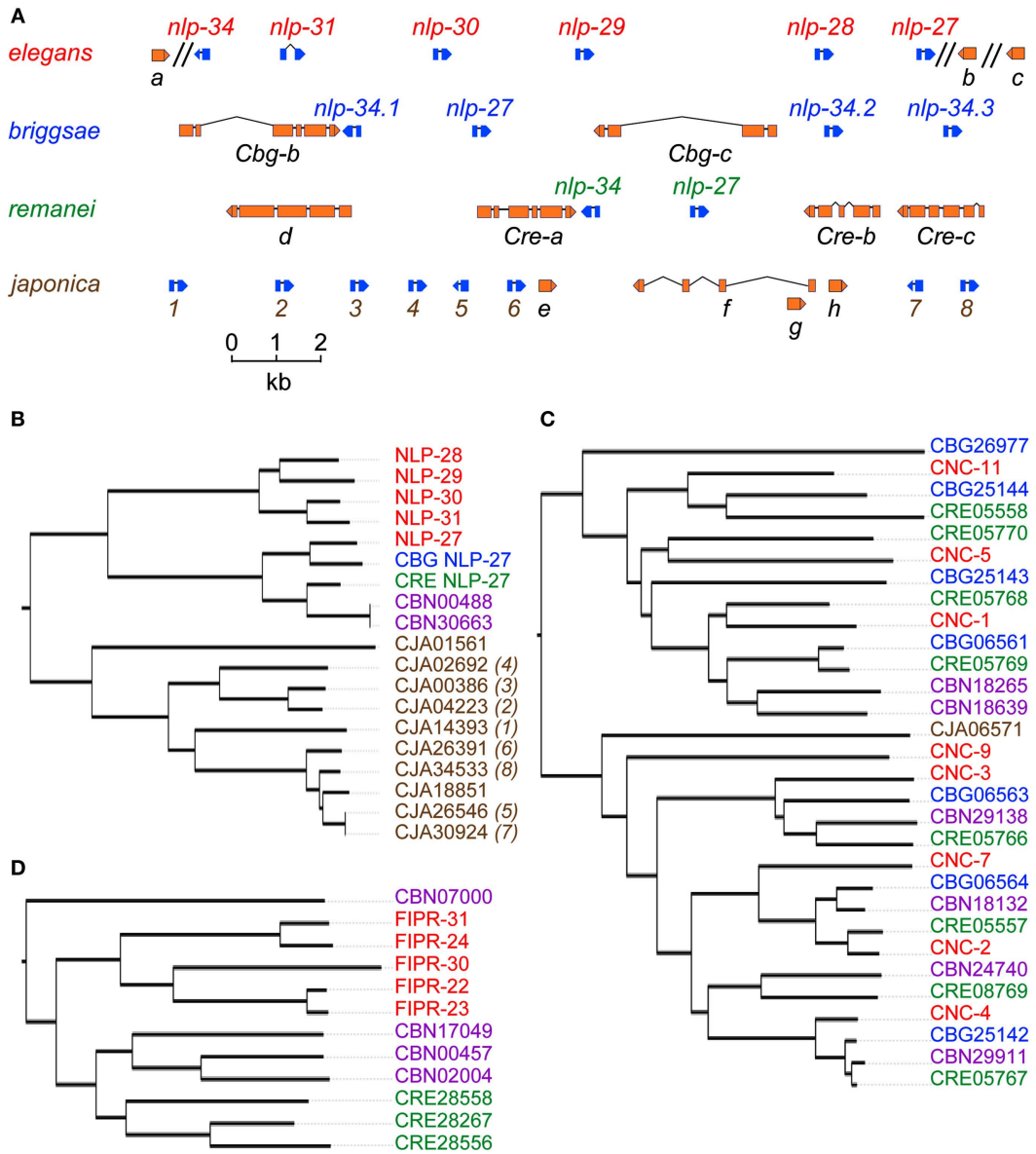

FIGURE 1 | (A) The *nlp-29* cluster in *C. elegans* together with syntenic regions from *C. brigssae* (Cbg), *C. remanei* (Cre), and *C. japonica*. AMP genes of the *nlp* class are shown in blue; there has been a marked expansion in *C. japonica*. The genes labeled "*a*" and "*c*" in *C. elegans*, and their orthologs (*Cbg-a*, *Cbg-c*, *Cre-a*, and *Cre-c*) are predicted to encode serpentine receptors; "*b*" in *C. elegans* is K09D9.9. Its ortholog in *C. japonica* is ca. 60 kb 3′ of the locus. Immediately 3′ of the gene labeled "*e*" in *C. japonica* there is the remnant of a degenerate paralog of K09D9.9. The figure is adapted from data in Wormbase WS230; it does not reproduce the predicted fusion of *Cre-b* and *Cre-c*, as this does not appear to be probable. Only the 3′ extremities of *a*, *b*, and *c* are shown. **(B–D)** Phylogenetic trees for selected members of the NLP **(B)**, CNC **(C)**, and FIPR **(D)** family peptides, including homologs in *C. brenneri* (CBN). A distance matrix analysis was performed using the alignment program clustalw2 to generate a guide tree via pairwise and subsequent multiple sequence alignment. This guide tree was then used to produce a true phylogenetic tree that was loaded into the Interactive Tree Of Life v2 software suite (Letunic and Bork, 2011). Partial rooted trees were extracted that corresponding to interesting features within the complete FIP/FIPR/NLP/CNC tree. For the non-*elegans* peptides, with the exception of CBG NLP-27 and CRE NLP-27, the corresponding gene identifier is given. The numbers in brackets for the *C. japonica* gene names match the corresponding genes in **(A)**.

sequences are available, including *Brugia malayi* (Ghedin et al., 2007), *Meloidogyne incognita* (Abad et al., 2008), and *A. suum* (Jex et al., 2011). It is possible that these parasitic nematodes have a natural environment that shields them from fungal pathogens throughout their life cycle, so that they have been able to dispense with these AMP genes (Abad et al., 2008). On the other hand, there do appear to be two similar AMP-encoding genes in *Drosophila*, CG7738, now called CG34227, and CG17738 (Couillault et al., 2004). It is notable that the former was found to be up-regulated by fungal infection (De Gregorio et al., 2002), and the corresponding peptide was identified via the biochemical characterization of hemolymph from septically injured flies (Verleyen et al., 2006). The extremely repetitive nature of these peptides, characterized by the presence of multiple GGY and/or GGW triplets (Couillault et al., 2004), does makes identification of orthologs in more distant species problematic, and it is currently unclear how evolutionary ancient are the NLP/CNC AMPs as defense molecules. Nevertheless, the existence of CNC-like peptides in insects supports the idea that the parasitic *A. suum* lost this part of its immune defenses, while retaining defensin-like peptides that may be important in the bacteria-rich intestinal tract of its vertebrate hosts, and also that the primary target of CNCs may be fungi.

The ABFs, NLPs, and CNCs are not the only predicted AMPs in *C. elegans*. Microarray-based transcriptionally profiling of infected worms also led to the identification of seven *fip* (fungus-induced peptide) genes that not only were strongly up-regulated by *D. coniospora*, but that also fulfilled at least three of the four following criteria: (i) predicted to encode a protein of less than 100 amino acids, (ii) with a predicted signal peptide, (iii) judged by inspection to have a simple primary structure (iv) having a homolog in the immediate genomic proximity, or similar in sequence to more than one structurally related protein encoded by clustered genes. Although there is currently no direct evidence to indicate that these genes do encode genuine AMPs, the combination of the above criteria make it highly likely. A larger class of 29 genes, called *fipr* (*fip*-related) was also created for genes that shared these characteristics, but for which there was no indication of any transcriptional up-regulation after *D. coniospora* infection.

This distinction subsequently turned out to be somewhat premature, since an RNAseq-based analysis of the transcriptional response to infection showed that *fipr-22*, *fipr-23*, and *fipr-26* are up-regulated by *D. coniospora*, while the expression of five others (*fipr-1*, *2*, *16*, *17*, and *20*) is induced by the fungus *Harposporium* sp. (Engelmann et al., 2011); *fipr-22* and *fipr-23* are also induced by *C. albicans* (Pukkila-Worley et al., 2011). Because of their large number, and their relatively simple structure, establishing solid phylogenetic relationships both within the nematode clade and beyond is a difficult, and on-going task. It is already clear, however, that these genes too are undergoing relatively rapid evolution, presumably a consequence of their role in host defense. For example, in *C. elegans* there are five paralogous *fipr* genes (including the infection-regulated *fipr-22* and *fipr-23*) clustered together, for which orthologs have not been identified in *C. japonica* or *C. briggsae* (**Figure 1D**). A very recent study has revealed marked differences in the seasonal abundance of proliferating *C. elegans* and *C. briggsae* populations in Northern France, with *C. elegans* being much more prevalent in the cooler autumn than in summer, when *C. briggsae* predominates (Felix and Duveau, 2012). There is therefore every reason to expect that the two nematode species will not be exposed to exactly the same range of natural pathogens, since microbial communities change with the seasons, perhaps explaining the divergent evolution of their AMP gene repertoire. There is increasing evidence that the commonly used strain of *C. elegans*, N2, underwent a number of changes to adapt to culture under laboratory conditions (see for example, Duveau and Felix, 2012). In the future, it will be interesting to see whether wild isolates of *C. elegans* present variations in their AMP genes.

Most *nlp* genes not regulated by fungal infection encode peptides that are either known to have an endocrine signaling function, or are hypothesized to; certain have been matched to specific G-protein coupled receptors (Husson et al., 2007; Janssen et al., 2010). One cannot exclude the possibility that some of the putative AMPs from the CNC and NLP families also exert a regulatory function during the innate immune response to fungal infection. The metabolism and activity of several classes of neuropeptide, both in *C. elegans* and other species are influenced by neprilysins (Husson et al., 2007). Typically, these zinc metallopeptidases are found on the outer surface of animal cells. They cleave small signaling peptides and thereby block their action. Interestingly, 13 of the 27 neprilysins genes in *C. elegans* are down-regulated upon infection with *D. coniospora* or by *Harposporium* sp. It remains to be determined whether they act on the infection-induced NLPs, or on other classes of peptides, such as insulin-like peptides, which are also transcriptionally regulated upon fungal infection (Engelmann et al., 2011). If they do, this would add a further level of complexity to the regulation of the host response to pathogens.

In conclusion, the last decade has seen considerable advances in our understanding of the role and evolution of AMPs in *C. elegans*. Future studies should yield more insights into their evolutionary origins and conservation, as well as their precise mode of action and the details of their complex regulation.

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Defense peptides secreted by helminth pathogens: antimicrobial and/or immunomodulator molecules?

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Host defense peptides (HDPs) are an evolutionarily conserved component of the innate immune response found in all living species. They possess antimicrobial activities against a broad range of organisms including bacteria, fungi, eukaryotic parasites, and viruses. HDPs also have the ability to enhance immune responses by acting as immunomodulators. We discovered a new family of HDPs derived from pathogenic helminth (worms) that cause enormous disease in animals and humans worldwide. The discovery of these peptides was based on their similar biochemical and functional characteristics to the human defense peptide LL-37. We propose that these new peptides modulate the immune response via molecular mimicry of mammalian HDPs thus providing a mechanism behind the anti-inflammatory properties of helminth infections.

Keywords: defense peptides, helminths, trematodes, parasites, antimicrobial peptides, immunomodulation, innate immune system

INTRODUCTION

Host defense peptides (HDPs) are conserved in all living species as a primitive component of the innate immune response (Hancock and Diamond, 2000; Bowdish et al., 2004) and have a broad-spectrum of activity against bacteria, fungi, eukaryotic parasites, and viruses (Mookherjee and Hancock, 2007; Andes et al., 2009; Hsu et al., 2009). The abilities of HDPs to suppress infections are mediated by direct antimicrobial properties, modulation of host immune responses, or both (Bommarius et al., 2010). Initially called antimicrobial peptides (AMPs) because of their capacity of directly killing microbes, these peptides are now referred as HDPs due to their added immunomodulatory properties (Hancock and Sahl, 2006).

Helminths comprise a variety of parasitic worms, including nematodes, cestodes and trematodes. We discovered a novel family of HDPs derived from pathogenic trematodes including Fasciola, Schistosoma, Opisthorcis, Paragonimus, and Clonorchis species that cause enormous disease in animals and humans in many parts of the world, particularly in poorer regions. We characterized them as HDPs based on their similar biochemical and functional characteristics to human defense peptides, particularly LL-37 (Robinson et al., 2011). We suggested that these new peptides modulate the immune response via molecular mimicry of HDPs thus providing a mechanism for the anti-inflammatory properties commonly observed in these helminth infections.

STRUCTURAL CHARACTERISTICS AND PROPERTIES OF HDPs

HDPs are defined as short peptides of 12–50 amino acids with an overall positive charge of +2 to +9 due to the predominance of basic amino acids (arginine, lysine, and histidine) over acidic amino acids (Hancock and Chapple, 1999). They also have the property of folding into amphipathic structures where the charged and hydrophilic portions are segregated from the hydrophobic portions. In general, at least 50% of the amino acids are hydrophobic, allowing interaction with bacterial membranes as a part of HDP mechanism of action (Hancock and Chapple, 1999). In aqueous solution, HDPs remain unstructured but adopt the amphipathic structure upon interaction with membranes (McPhee and Hancock, 2005), an attribute that may be crucial for their activity and for reducing general cytotoxicity (Kindrachuk et al., 2010). Despite their small size and common physico-chemical features, HDPs are classified according to their 3-D structures.

In mammals, the two major families of HDPs include the α- and β-defensins and the cathelicidin. The defensins are characterized by a β-sheet globular structure stabilized by intramolecular disulfide bridges. In human skin, defensins are principally expressed by keratinocytes, neutrophils, and sudoriferous and sebaceous glands (Koczulla and Bals, 2003) where they can be produced constitutively or in response to an inflammatory stimulus. Their expression was reported in other cell types such as tissue macrophages, small intestinal epithelial cells as well as cardiomyocytes. The second group is formed by the cathelicidins which are distinguishable by their linear α-helical structure. All members of the cathelicidin category contain a structurally variable cationic C-terminal portion and a highly conserved N-terminal cathelin domain that must be cleaved to release the active C-terminal peptide (Ramanathan et al., 2002). Cathelicidins are usually expressed by myeloid precursor cells but they are also found in neonatal lymphoid tissue and in mature circulating neutrophils in some species (Zanetti, 2004). In humans, they are produced in epithelial cells and in different tissues and corporal

fluids like gastric juices, saliva, semen, sweat, plasma, airway surface liquid, and breast milk (Bals et al., 1998; Murakami et al., 2002; Hase et al., 2003). They are stored in their inactive forms in specific granules and processed exclusively upon stimulation, releasing the active HDPs into the extracellular fluid (Scott et al., 2002; Zanetti, 2004).

The best-characterized cathelicidin is the human LL-37, a cationic (+6) peptide of 37 residues with a molecular mass of 4.5 kDa. It adopts an amphipathic α-helix structure and possesses a broad spectrum antimicrobial activity. This peptide is contained within an inactive secreted precursor protein termed hCAP-18 (human cationic antimicrobial protein 18 kDa; the actual molecular weight is 16 kDa) which is cleaved by endogenous serine proteinase three to release the C-terminal active 37-residue peptide (Agerberth et al., 1995; Gudmundsson et al., 1996). It is produced by neutrophils, macrophages and mucosal epithelial cells upon stimulation by microorganisms and pro-inflammatory mediators (Durr et al., 2006; Mookherjee et al., 2007). Upon injury or infection, there is a strong up-regulation of hCAP-18/LL-37, suggesting the involvement of LL-37 in assisting the immune system. In contrast, several diseases have been associated with the down-regulation of LL-37 such as chronic periodontal disease (Putsep et al., 2002), atopic dermatitis (Ong et al., 2002), chronic ulcers (Heilborn et al., 2003) and an increase of the risk for skin infections. LL-37 also plays a central role in innate immune responses and inflammation. It is known as a potent chemoattractant for mast cells (Niyonsaba et al., 2002), monocytes, T lymphocytes and neutrophils (Yang et al., 2000) through the receptor FPRL1 (formyl peptide receptor-like 1). It promotes wound healing (Heilborn et al., 2003) probably through re-epithelialization and vascularization (Ramos et al., 2011), angiogenesis and arteriogenesis (Ramos et al., 2011) and acts as immune adjuvant (Kurosaka et al., 2005). LL-37 is also known to bind to lipopolysaccharide (LPS) and neutralize its biological actions by preventing its interaction with LPS-binding protein (Larrick et al., 1995; Kirikae et al., 1998; Nagaoka et al., 2001).

BIOLOGICAL ACTIVITIES OF HDPs

The HDPs are multifunctional molecules involved in the direct killing of microbes and in the mediation of various host responses. It is well recognized that HDPs exhibit potent activity against microbes as part of the innate immune system (Auvynet and Rosenstein, 2009). Recent studies also evoke their importance in the regulation of innate immune responses and in protecting against the detrimental effects of an excessive innate inflammatory response (Tecle et al., 2007, 2010; Miles et al., 2009; Murakami et al., 2009; Giuliani et al., 2010).

The antimicrobial activity of HDPs is driven by the charge. The bacterial cell membranes are composed of a high proportion of acidic phospholipids, conferring a negative charge to the surface (Matsuzaki, 1999). The cationic nature of the HDPs is attracted by electrostatic forces to the negative surface of the bacteria, facilitating the direct lysis of the cell through the permeabilization of the membranes (Lehrer et al., 1993). The absence of cholesterol in bacterial membranes also increases the activity of HDPs (Zasloff, 2002). In contrast, the phospholipids in eukaryotic cell membranes are predominantly sequestered in the inner leaflet of the lipid bilayer, leaving the outer leaflet with no or little net charge. Cholesterol is an essential lipid in the composition of eukaryotic membranes, preventing membrane damage. These elements explain why concentrations of HDPs found *in vivo* do not cause host-damage (Boman, 2003). HDPs are usually secreted as cocktails at the site of infection and/or inflammation and act synergistically to increase their effectiveness of antimicrobial activity (Doss et al., 2010; Tecle et al., 2010). While several models on how AMPs actually kill microbes have been proposed (Bierbaum and Sahl, 1985; Westerhoff et al., 1989; Matsuzaki, 1999; Yang et al., 2000; Kragol et al., 2001; Brogden, 2005), direct antimicrobial action is probably not the most important role of HDPs since they present low antimicrobial activities under serum and tissue conditions (Hancock and Diamond, 2000; Hancock, 2001). In fact, it has been reported that some HDPs are inactivated by physiological concentrations of salt and cations when tested *in vitro* and that the physiological concentrations of HDPs are far lower than those required to exert antimicrobial activity *in vitro* (Yang et al., 2002; Boman, 2003; Bowdish et al., 2005).

In addition to their bactericidal activity, accumulating evidences are showing that HDPs also have a key modulatory role in the innate immune response and are an important link between the innate and adaptive immune responses under physiological conditions (Zasloff, 2002). During a microbial invasion, the macrophages and dendritic cells (DCs) of the innate immune system detect the presence of microorganisms through the recognition of specific pathogen-associated molecular patterns (PAMPS) such as the gram-negative LPS endotoxin. An early immune response is driven by the interaction between cell receptors and the PAMPS, leading to the production of potent pro-inflammatory cytokines such as IL-6, IL-12 and TNF (Medzhitov, 2007). The production of these cytokines as well as the up-regulation of co-stimulatory molecules on DCs, macrophages, granulocytes and mast cells, are crucial points in the establishment of a protective adaptive immune response. However, an excessive inflammatory response can lead to sepsis, septic shock and also death (Castellheim et al., 2009; Giuliani et al., 2010). HDPs are known to neutralize LPS-mediated responses (Murakami et al., 2009; Giuliani et al., 2010). They have affinity for LPS and can prevent lethal endotoxemia by suppressing cytokine production by macrophages in the presence of bacteria or other non-specific inflammatory stimuli (Gough et al., 1996; Miles et al., 2009; Tecle et al., 2010). These peptides also participate in the inflammatory response by acting as chemotaxins for immune cells, including the recruitment of neutrophils by an increase of IL-8 production, the mobilization of immunocompetent T-cells and the enhancement of cellular adhesion and the subsequent cellular transepithelial migration (Chertov et al., 1996; Van Wetering et al., 1997; Hata and Gallo, 2008). HDPs promote phagocytosis while inhibiting oxidant responses of neutrophils or monocytes (Tecle et al., 2007; Miles et al., 2009). They also stimulate wound healing and angiogenesis through direct action on epithelial and endothelial cell proliferation (Koczulla and Bals, 2003; Li et al., 2006; Otte et al., 2008). Other activities of HDPs include the modulation of pathways regulating cell survival and apoptosis in various

cell types, the induction of chemokines and other immune mediators (Scott et al., 2002; Tjabringa et al., 2003; Bowdish et al., 2004; Mookherjee et al., 2006) and the stimulation of leukocyte degranulation and other microbicidal activities. HDPs have a unique ability to suppress hyperinflammatory responses while maintaining protective effector functions of the immune response.

FEATURES OF HELMINTH-INDUCED IMMUNE RESPONSES

Although each helminth pathogen triggers characteristic infections associated with the biology of the specific parasite, they all evoke immune responses that share common patterns. The first conserved feature of helminth infection is a T helper (Th) 2-type dominated immune response. Th2-type immunity is typically characterized by an increase in the levels of interleukin-4 (IL-4) and other Th2-type cytokines (including IL-5, IL-9, IL-13, and IL-21), activation and expansion of $CD4^+$ Th2 cells, plasma cells secreting immunoglobulin E (IgE), eosinophils, mast cells, and basophils, all of which can produce various types of Th2-type cytokines (Jenkins et al., 2011). The other recurring immunological characteristic of helminth infection is the down regulation of the Th1-type and Th17-type responses and their associated inflammation. Th1-type responses are characterized with increases in the number of Th1 cells, cytotoxic CD8+ T cells, neutrophils and macrophages. Various cytokines that are expressed during Th2-type responses, including IL-4, IL-13, and IL-21 can also downregulate Th1-type and Th17-type.

A further important dimension in helminth infection is the differentiation of alternatively activated macrophages (aaMφs) under the influence of Th2-type cytokines. While aaMφs are recruited to the site of infection and are implicated as functional effectors, they also have strong anti-inflammatory properties and highly express genes whose functions relate to the repair of extracellular matrices, wound healing and fibrosis. The overall outcome of a helminth infection may then be an environment with down-regulated proinflammatory responsiveness, activated damage repair mechanisms and a tightly controlled development of Th2 anti-parasite effector responses (see Cook et al., 2012).

Hence, helminth parasites are master regulators of immune responses utilizing complex mechanisms to favor long-term persistence in the host. Mechanistically, parasite modulation of the immune system is likely to be effected through the release of soluble mediators which ligate, degrade or otherwise interact with host immune cells and molecules (Lightowlers and Rickard, 1988). Much of the earlier literature on immunological effects of helminth products depended on crude extract (such as SEA schistosome eggs antigen), although the degree to which the host is exposed to constituent molecules was uncertain. While both somatically derived and secreted products are known to have immunological activity (Johnston et al., 2009), the secreted helminth modulators are those most likely to be physiological actors at the interface between live parasites and the host (Hewitson et al., 2009). For that reason, research has focused on identifying "excretory-secretory" (ES) products released by live worms with immunomodulatory properties (for reviews on the subject: Maizels and Yazdanbakhsh, 2003; Thomas and Harn, 2004; Dzik, 2006).

DEFENSE PEPTIDES SECRETED BY HELMINTH PATHOGENS

Prospecting of helminth ES products for molecules with immunomodulatory effects has led us to the discovery of a novel family of proteins, the Helminth Defense Molecules (HDMs). The HDMs were termed following their characterization: they exhibit similar functional and biochemical properties to the human defense peptides, defensins and cathelicidins. To date, the best studied HDM is the 8 kDa protein (FhHDM-1) secreted by the trematode, *Fasciola hepatica*, which causes liver fluke diseases in animals and humans. FhHDM-1 can be grouped in the cathelicidin family as it has a high propensity to adopt α-helical secondary structure (**Figure 1**). In addition, similar to hCAP18, the secreted FhHDM-1 undergoes cleavage by an endogenous protease (the major cysteine protease from *F. hepatica*, cathepsin L1) to release a C-terminal fragment. The 34-residue C-terminal peptide of FhHDM-1 contains a 21-residue amphipathic helix which exhibits a marked structural parallel with the bioactive human LL-37 peptide. The amphipathic helix of LL-37 anchors the peptide to phospholipid membranes through interaction with hydrophobic face (Agerberth et al., 1995; Porcelli et al., 2008) and is important for its antimicrobial activity (Giuliani et al., 2010). The same amphipathic helix of the cathelicidin hCAP18-derived peptide has also been suggested to be responsible for interacting with LPS (Hoess et al., 1993; Porro, 1994). Like LL-37, the amphipathic helix of the C-terminal peptide of FhHDM-1 binds *E. coli* LPS; it is a key functional determinant necessary for its biological properties (Robinson et al., 2011). Phylogenetic/bioinformatic studies revealed that a family of related HDMs are expressed by several major animal and human trematodes that inhabit various tissues of the host including the mesenteric blood vessels (Schistosoma), the liver (Fasciola, Opisthorcis, Clonorchis) and lungs (Paragonimus).

WHY WOULD PARASITES HAVE A NEED FOR DEFENSE PEPTIDE?

It is well known that intestinal injury and systemic endotoxemia are two factors leading to morbidity in helminth infected mice (Herbert et al., 2004; Leeto et al., 2006). Loss of gut barrier function and consequently the migration of luminal antigens (bacteria and their toxic products) into the systemic circulation are frequent in helminth infection. Accordingly, enteric nematode infection is characterized by enhanced permeability of the intestinal epithelium, primarily mediated by activated mast cells (McDermott et al., 2003), which contributes to parasite rejection but may lead to the translocation of bacterial LPS into the portal circulation (Farid et al., 2008). The same phenomena can be observed in non-enteric worms. For instance, both *Schistosoma mansoni* (a trematode that resides in the mesenteric vein) and *F. hepatica* (a trematode that lives in the bile ducts) cause damages leading to the systemic movement of bacteria (Ogunrinade and Adegoke, 1982; Herbert et al., 2004; Ferraz et al., 2005; Valero et al., 2006). Despite this translocation of enteric microbes, fatal endotoxemia during infection with trematodes is not a frequent situation (Onguru et al., 2011). Additionally, in endemic

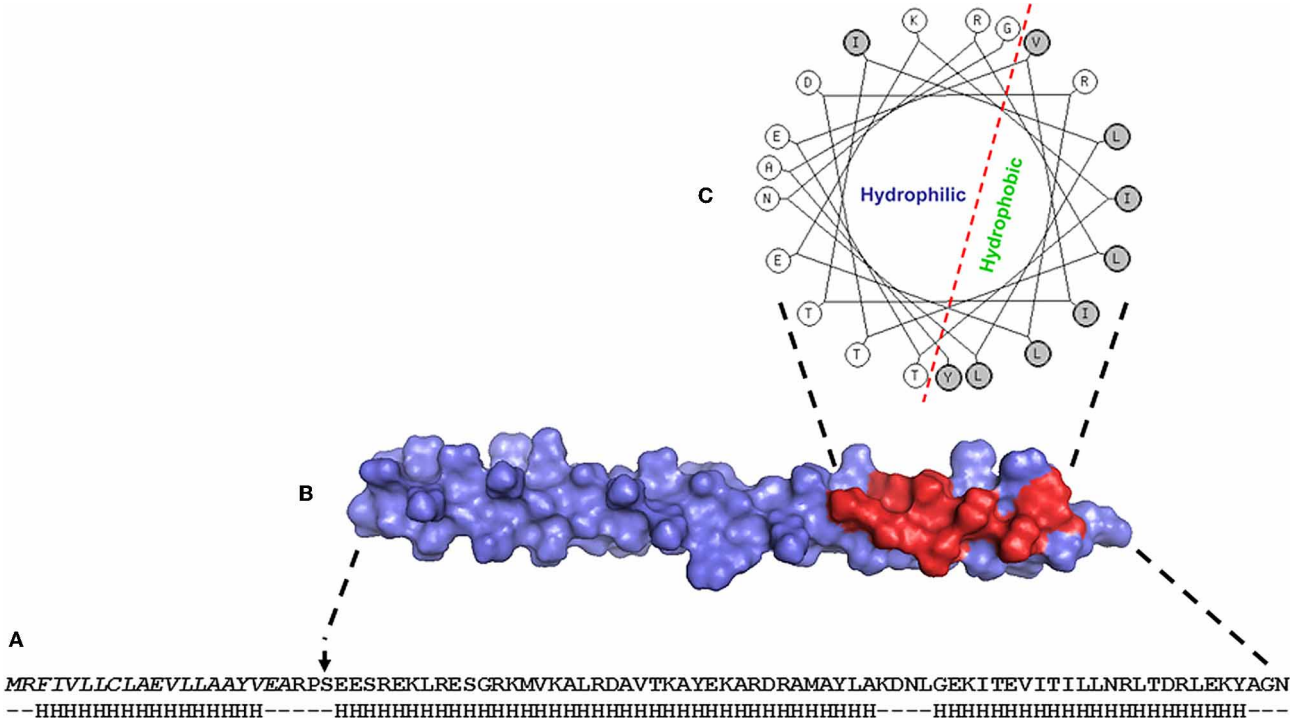

FIGURE 1 | (A) Primary amino acid sequence of the archetypal HDM secreted by *Fasciola hepatica*, FhHDM-1. The N-terminal signal peptide is shown in italics and the predicted secondary structure (predominantly alpha helix) is shown below. **(B)** Model structure of FhHDM-1 with the residues forming the hydrophobic face of the molecule shown in red. **(C)** Helical wheel analysis shows that the C-terminal region of FhHDM-1 forms a distinct amphipathic helix.

areas for helminth parasites, co-infection with gram-negative bacteria, most commonly *Salmonella* sp, is frequent (Melhem and Loverde, 1984). The mechanisms of resistance to septicaemia during helminth infection are not fully understood. One explanation proposed by Robinson et al. (2011) is that the active secretion of HDM by parasites during their lifespan in the mammalian host ensures that potentially lethal LPS, either from intestinal flora or from microbial co-infections, is neutralised and that LPS-mediated activation of macrophages is controlled. In fact, bacterial LPS is known to be a key molecule in the pathogenesis of endotoxin shock associated with gram-negative bacterial infections (Lehmann et al., 1987; Morrison et al., 1994; Castellheim et al., 2009). As mentioned in Section "Structural characteristics and properties of HDPs," LL-37 neutralises the biological activities of LPS by binding to the microbial molecule (Kirikae et al., 1998). The transfer of LPS to cellular CD14 by serum LPS-binding protein (LBP) is the first event in the recognition of microbial infection. This bimolecular complex then initiates downstream signaling via interaction with cellular TLRs (Beutler et al., 2003), which results in the secretion of inflammatory mediators. Just like human LL-37, the direct binding of FhHDM-1 to LPS blocks the interaction of LPS with LBP, thus reducing the number of LPS molecules that are targeted to the TLR signaling complex on the macrophage cell surface. This in turn prevents LPS-induced activation of macrophages. Therefore, FhHDM-1 impairs LPS signaling and protect against harmful immune responses by reducing the release of inflammatory mediators from macrophages.

CONCLUSION

HDPs are an evolutionarily conserved component of the innate immune response and are found among all classes of life. They have been demonstrated to possess antimicrobial activities on a broad range of organism, killing Gram negative and Gram positive bacteria, mycobacteria, enveloped viruses, fungi and even transformed or cancerous cells. It also appears that HDPs have the ability to enhance immunity by functioning as immunomodulators. The discovery of a family of defense peptides that is conserved amongst medically-important trematode pathogens has raised the question of their utility for helminths. Why would parasitic worms need HDPs? Helminth parasites are master regulators of immune responses in order to ensure life-long persistence in the host. One strategy of immune regulation is the secretion of a wide range of immunoregulatory molecules, which are able to target various host cells and alter them to induce a highly directed host response known as a "modified Th2-type response." Our recent finding of a family of HDMs that modulate the immune response via molecular mimicry of HDPs provides a common mechanism for the anti-inflammatory properties of helminth infection (Robinson et al., 2011). By targeting key stages in LPS-mediated cell signaling, the helminth parasite prevents the activation of innate immune response and enhances its longevity by increasing the survival of the host.

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Antimicrobial host defensins – specific antibiotic activities and innate defense modulation

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Current treatment of bacterial and fungal infections heavily relies on strategies which aim to inhibit and kill pathogens with high specificity. These strategies are very successful and antibiotics have contributed to increasing human life expectancy more than any other class of therapeutic drugs. However, antibiotics are losing efficacy as a result of high selection pressure and rapid resistance development. Thus, strategies that rely on boosting natural host defenses are gaining more attention, since compounds targeting host mechanisms should control infections regardless of the antibiotic resistance levels of pathogens. Antimicrobial peptides (AMPs) are considered as ideal candidates for such novel anti-infective strategies since they combine direct antibiotic activities with modulation of immune responses (**Figure 1**). However, AMPs frequently lack specific molecular targets and tend to have membrane disruptive activities, bearing risks of cytotoxicity. For anti-infective drug development, AMPs should ideally inhibit specific microbial targets without impacting on membranes; peptides with such properties were recently identified in a large subfamily of AMPs, the defensins.

All multicellular organisms produce AMPs to protect surfaces and tissues from invading pathogens. These peptides have been referred to as AMPs and more recently as host defense peptides (HDPs). HDPs are ancient effector molecules of innate immunity with multiple functions. They do not share specific sequence similarities, but can be generally defined as amphiphatic cationic peptides consisting of 12–50 amino acids. They are either linear (e.g., LL-37, magainin, and indolicidin) or have tertiary structures stabilized by disulfide bonds (Hancock and Lehrer, 1998; Shai, 2002; Zasloff, 2002). Defensins *sensu stricto* belong to the latter class and were first isolated from mammals, and subsequently also found in invertebrates and plants.

Plant, fungal, and invertebrate defensins share a common structural motif, the cysteine-stabilized αβ-motif that is composed of an α-helix linked to an antiparallel β-sheet with three or four disulfide bonds; they display either antifungal or antibacterial activity. Recently, it has been demonstrated that antibacterial defensins of fungi and invertebrates bind with high affinity to the bacterial cell wall precursor lipid II. They form an equimolar stoichiometric complex with lipid II, thereby inhibiting the incorporation of the cell wall building-block into the nascent peptidoglycan network (Schmitt et al., 2010; Schneider et al., 2010). NMR-based modeling of the plectasin-lipid II complex indicated that the fungal defensin interacts with the pyrophosphate moiety of lipid II by forming four hydrogen bonds (involving residues F2, G3, C4, and C37). Additionally, a salt bridge between the N-terminus (His18) and the D-glutamic acid in position 2 of the lipid II stem peptide is important for binding (Schneider et al., 2010). Interestingly, the amino acid residues involved in the lipid II binding of plectasin are also present in many other fungal and invertebrate defensins, suggesting a conserved lipid II binding motif.

Cell wall biosynthesis is a prominent target of clinically used antibiotics. For example, the glycopeptide vancomycin, a last-resort antibiotic for treatment of infections with multi-resistant Gram-positive bacteria, binds to the *D*-alanyl-*D*-alanine terminus of the lipid II pentapeptide. However, cross-resistance between vancomycin and plectasin could not be observed and also the presence of *D*-alanine-*D*-lactate found in vancomycin-resistant bacteria did not affect the activity of plectasin (Schneider et al., 2010). In general, only modest resistance development toward HDPs has been observed under *in vitro* selection pressure (Zhang et al., 2005). The lipid II isoprenoid anchor ($C_{55}P$) is also involved in the biosynthesis of other major cell envelope polymers (e.g., wall teichoic acid, capsules). Synthesis of $C_{55}P$-anchored molecules always starts with the transfer of a sugar moiety to the lipid carrier, forming a pyrophosphate linkage. This structural motif is highly conserved, as it is part of several essential building blocks and therefore cannot be easily modified to confer resistance.

The antifungal action of plant and invertebrate defensins also appears to be highly specific and is based on interaction with particular sphingolipids in membranes and cell walls of susceptible fungi. For example, the interaction of RsAFP2 (from radish seeds) with fungal glucosylceramides causes the production of radical oxygen species and apoptosis as well as cell wall stress, septin delocalization, and ceramide accumulation (Thevissen et al., 2012). Other plant defensins such as DmaMp1 (from *Dahlia merckii*) bind specifically to inositol phosphoryl-containing sphingolipids leading to membrane permeabilization and ion efflux (Thevissen et al., 1996, 2003).

In contrast, the activity of vertebrate defensins may be of intermediate specificity for microbial targets with a broader activity spectrum. Vertebrate defensins comprise three subfamilies, α-, β-, and θ-defensins, which differ in their pairing of

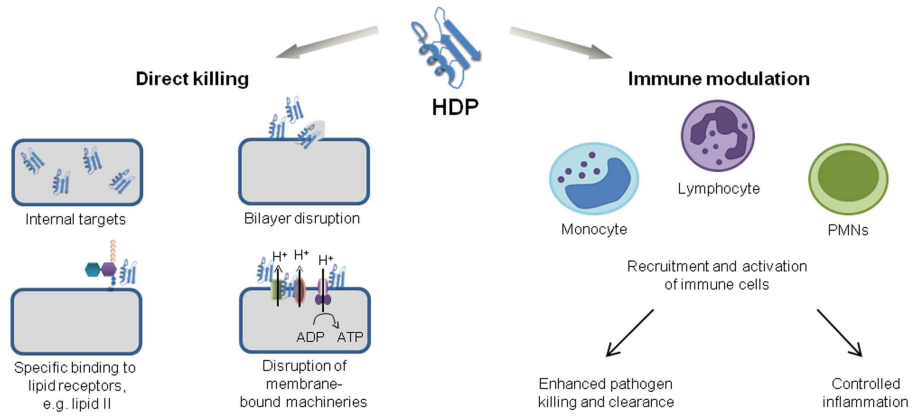

FIGURE 1 | Functions of HDPs.

the six conserved cysteine residues. They are composed of three antiparallel β-sheets and exhibit a broad-spectrum activity against Gram-positive and Gram-negative bacteria, fungi, and some enveloped viruses. α-Defensins have been isolated from the granules of neutrophils and small intestinal Paneth cells whereas β-defensins are mainly expressed in epithelial tissues. The cyclized θ-defensins are found exclusively in leukocytes and bone marrow of Old World monkeys and arose from a pre-existing α-defensin (Ganz, 2003; Schneider et al., 2005). Lipid II binding has also been reported for the vertebrate α-defensin human neutrophil peptide 1 (HNP1) and human-beta defensin 3 (hBD3). However, the affinity of HNP1 to the cell wall precursor is significantly lower compared to that of the fungal peptide plectasin (plectasin-lipid II: 1.8×10^{-7} M; HNP1-lipid II: 2.19×10^{-6} M; de Leeuw et al., 2010; Sass et al., 2010; Schneider et al., 2010). Besides lipid II sequestration, hBD3 additionally seems to have more generalized effects on membrane bound processes such as electron transport (Sass et al., 2008). These findings indicate that the specificity of lipid II binding correlates to some extent with the antimicrobial spectrum. Defensins with high affinity for lipid II may have evolved to mainly act against Gram-positive bacteria, whereas defensins with lower lipid II affinity may have retained the capacity to interact with additional targets and therefore have a broader antimicrobial spectrum, including Gram-negative bacteria or fungi.

The combination of highly targeted antimicrobial activity with the capacity to positively modulate the immune response is highly attractive as anti-infective strategy. Mammalian HDPs are expressed either constitutively or are inducible in various tissues and cell types, including immune cells like neutrophils or macrophages, as well as keratinocytes and epithelial cells. The expression of these peptides is triggered by conserved microbial structures [lipopolysaccharide (LPS), lipoteichoic acid, CpG oligonuclecotides; via Toll-like receptors (TLRs)] or inflammatory effectors such as cytokines (TNF-α, IL-1β; Zasloff, 2002; Lehrer, 2004; Brown and Hancock, 2006). HDPs have been demonstrated to provide an important link between innate and adaptive immune response, acting as both pro- and anti-inflammatory mediators. They enhance beneficial immune responses and dampen harmful ones, enabling the host to control infections. HDPs modulate the expression of hundreds of genes in immune cells and epithelia, influencing processes like maturation of immune cells, cross-regulation of cytokines/chemokines, wound healing, and angiogenesis. The α-defensins HNP1-3 which are released by tissue invading granulocytes, have been shown to trigger secretion of TNF-α and IFN-γ from macrophages. The cytokine release stimulates the phagocytotic macrophage activity via an autocrine loop, thereby enhancing clearance of opsonized bacteria, as observed *in vitro* and in an murine model (Soehnlein et al., 2008). The β-defensin hBD3 activates professional antigen presenting cells (monocytes, dendritic cells) via TLRs 1 and 2 and thereby stimulates adaptive immune responses (Funderburg et al., 2007). Various defensins recruit immune cells by direct binding to chemokine receptors (CCRs). α-Defensins, for example, enhance the migration of T-cells, while β-defensins exhibit chemoattractant functions for immature dendritic cells, monocytes/macrophages, and mast cells (Yang et al., 2000; Niyonsaba et al., 2002; McDermott, 2004). Furthermore, defensins dampen endotoxin-induced secretion of pro-inflammatory cytokines by neutralization of extracellular LPS as well as modulation of intracellular signaling pathways (Scott et al., 2002; Mookherjee et al., 2006). Defensins aid in wound healing not only by direct killing of pathogens and boosting of host defense mechanisms, but moreover through stimulation of processes involved in tissue organization. HBD1-4 have been shown to enhance humane keratinocyte migration and proliferation through epidermal growth factor receptor signaling (Niyonsaba et al., 2007). Gene transfer and exogenous expression of hBD3 accelerated closure of infected diabetic wounds in a porcine model (Hirsch et al., 2009), suggesting a therapeutic potential for defensins in wound healing.

Bacterial peptides sharing the overall features of HDPs, i.e., cationic amphiphilicity, such as gramicidin S and polymyxin B have been used in clinics as topical agents. In contrast, no AMP of eukaryotic origin has so far been approved for the treatment of patients. In clinical phase III studies, the HDP-derivatives pexiganan (from *Xenopus laevis* magainin) and iseganan (from porcine protegrin-1) have been shown effective in the prevention of diabetic food ulcer and irradiation-induced oral mucositis, respectively (Trotti et al., 2004; Lipsky et al., 2008). Nevertheless, these substances were not

approved for medical use by the US Food and Drug administration. Various other synthetic HDPs are in clinical phase I or II trials, which do not only aim at exploiting the direct antimicrobial features of these peptides, but also their ability to modulate the human immune system (Yeung et al., 2011).

The cationic amphiphilic and peptidic nature of AMPs is often considered unfavorable for development of systemic drugs. However, protease lability, contributing to low serum half-life, may be overcome by different approaches, including the use of peptidomimetics, peptides composed of unusual or D-amino acids (instead of natural L-amino acid), and formulation (e.g., in liposomes; Oren et al., 1997; McPhee et al., 2005). Peptides based on defensin templates have not been investigated in clinical studies so far. Defensins are more protease-resistant due to their disulfide-stabilized structure (Wu et al., 2003; Maemoto et al., 2004), and therefore can have a higher serum half-life as other HDPs mentioned above; e.g., plectasin and its improved derivative NZ2114 showed potent activity in animal models, enhanced serum-stability, and extended *in vivo* half-life (Andes et al., 2009). Also, the plectasin example demonstrates that difficulties associated with high yield production of defensins and with correct cysteine-pairing, can be solved. The use of chemically modified prodrugs, could also improve pharmacokinetics and/or lower toxicity, as in the case of the parental antibiotic colistin (methane sulfonate derivative of polymyxin B; Falagas and Kasiakou, 2006).

Antimicrobial mechanisms based on defined target molecules such as lipid II reduce the risk of unspecific membrane disruption and cytotoxic activities, although HDPs clearly have some specificity for microbial membranes; eukaryotic membranes may be less susceptible due to the absence of anionic lipids on the lipid bilayer surface, the lack of a strong membrane potential gradient and the presence of cholesterol (Hancock and Sahl, 2006). However, it cannot be ignored that certain HDPs display potential harmful effects like degranulation of mast cells and enhancement of apoptosis (Niyonsaba et al., 2001; Barlow et al., 2006). It has been reported that hBD3 promotes the proliferation of oral carcinoma and osteosarcoma cells acting as a potential proto-oncogene (Kesting et al., 2009; Kraus et al., 2012). HDPs are reminiscent of peptides with nuclear localization signals and many peptides can migrate into the cell core; the cathelicidin LL-37 was even demonstrated to have nuclear translocation ability regarding DNA plasmids (Sandgren et al., 2004). Thus, it is obvious that such activities need to be extensively studied and taken into account for any drug development program.

The increasing knowledge of the importance of immunomodulatory HDP functions, has led to the synthesis of so called innate defense regulator peptides (IDRs; Easton et al., 2009). These are small synthetic peptides derived from HDP templates, which were designed to selectively modulate the innate immune system without the detrimental activities displayed by certain natural HDPs (see above). Several recent studies focused on cathelicidin-derived IDRs (Choi et al., 2012). The synthetic peptides IDR-1 and IDR-1002 (from bovine bactenecin), despite lacking direct antimicrobial activity, were shown to confer protection against systemic bacterial infection in mouse models challenged with methicillin-resistant *S. aureus* and vancomycin-resistant enterococci. Notably, these IDRs combine anti-infective and anti-inflammatory properties. IDR-1 and IDR-1002 contribute to bacterial clearance by inducing chemokines secretion and enhancing leukocyte recruitment. Moreover, the peptides suppress the induction of several proinflammatory cytokines, thereby dampening immune-mediated inflammation and preventing tissue damage (Scott et al., 2007; Nijnik et al., 2010; Wieczorek et al., 2010; Turner-Brannen et al., 2011). IMX-942, which is based on IDR-1, is tested for its ability to help combat nosocomial infections in immune-suppressed cancer patients, and has recently completed clinical phase I trials[1]. The HLA-I-derived decapeptide RDP58 inhibits the synthesis of proinflammatory cytokines like TNF-α, IL-2, IL-12, and IFN-γ by interfering with MyD88-signaling (Travis et al., 2005). RDP58 has proven safety and efficacy in clinical phase II studies with inflammatory bowel disease patients[2].

[1]http://www.inimexpharma.com

[2]http://www.genzyme.com

Taken together, it appears a most promising approach to design future anti-infective drugs that target host defenses and may combine this with targeted antibiotic activities, even more since classic antibiotics such as macrolides also appear to have immune modulatory properties (Tauber and Nau, 2008). On the other hand, it is obvious that for systematic exploitation of this concept, we need to know more about both, the molecular mechanisms underlying the immune modulation and about specific, targeted antibiotic activities of HDPs – it would be rather surprising if these would occur only with defensins.

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Multiple immune-modulatory functions of cathelicidin host defense peptides

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An essential function of innate immunity is initiating inflammatory responses to limit the spread of invading pathogens, followed by regulatory mechanisms aimed at resolving inflammation and returning the immune system to homeostasis. These meticulously regulated processes are triggered by various pathogen-associated molecular patterns or endogenous damage-associated molecular patterns. A group of natural peptides that have gained notoriety in recent years as immune-modulatory molecules contributing to both resolution of infections and inflammation, thus playing a role in maintaining homeostasis are host defense peptides (HDPs). These are gene-encoded cationic short peptides, less than 50 amino acids with an overall positive charge of +2 to +7, and ≥30% of hydrophobic residues. The two most well characterized families of HDPs in mammals are cathelicidins and defensins. We will focus on cathelicidin HDPs in this review.

Genes encoding cathelicidin HDPs have a highly conserved N-terminal cathelin domain in their precursor protein (Tomasinsig and Zanetti, 2005). This conserved cathelin domain has helped in the discovery of new cathelicidins from diverse species including non-mammalian species such as chicken, trout, and hagfish. The biological active mature mammalian cathelicidin peptides are processed from their precursor protein by proteolytic cleavage by serine proteases, e.g., proteinase 3, elastase, and kallikrein (Shinnar et al., 2003; Morizane et al., 2010). The biologically active mature cathelicidins are diverse in their sequence and structure, and can be classified into four structural groups; amphipathic α-helices (e.g., murine CRAMP), β-hairpin molecules (e.g., porcine protegrin 1), those with extended structures (e.g., bovine indolicidin), and cyclic peptides (e.g., bactenecin). Mammalian cathelicidins are found in granules of neutrophils as well as other cell types such as myeloid precursors, epithelial cells, mast cells, lymphocytes, and keratinocytes. These peptides are expressed in a wide variety of tissues (oral cavity, skin, intestine, lungs, cervix, etc.) and found in body fluids such as plasma, breast milk, saliva, gastric juice, semen, sweat, and bronchoalveolar fluid (Nijnik and Hancock, 2009). The biologically active mature cathelicidin peptides have been demonstrated to mediate a wide range of activity from antimicrobial to immune-modulatory (discussed below). Some of the most well studied cathelicidins are human LL-37, murine CRAMP, bovine BMAP-28, and porcine PR-39.

PROTECTION AGAINST INFECTIONS

Decreased expression of cathelicidin HDPs has been associated with increased susceptibility to infections. For example, decreased expression of human cathelicidin LL-37 is linked to increased susceptibility to skin infections, frequent oral bacterial infections, severe periodontal disease, and cutaneous infections by the parasitic protozoan leishmania (Bowdish et al., 2005; Kulkarni et al., 2011). A recent study has demonstrated that the active metabolite of vitamin D induces the expression of LL-37 thus contributing to protection against tuberculosis (Jo, 2010). Similarly, the murine cathelicidin CRAMP is known to provide protection against streptococcus infections (Nizet et al., 2001). Thus, it is not surprising that research in this area was propelled by the interest of developing these peptides as alternate antibiotic-like therapy for infectious disease. However, it has been shown that the direct microbicidal activity of these peptides is often antagonized in the presence of physiological salt concentration, heparin, and serum factors (Bowdish et al., 2005). Also, microbial factors such as polysaccharides released by certain pulmonary bacterial pathogens antagonize the direct microbial killing property of these peptides (Benincasa et al., 2009). However, the fact remains that cathelicidins can indeed protect against a wide range of infections from bacteria, viruses, and parasites. As cathelicidins have been also demonstrated to stimulate both innate and adaptive immune responses (discussed below), the ability of cathelicidins to contribute to resolution of infections is now thought to be primarily due to their role in host immunity.

ROLE IN IMMUNE-MEDIATED INFLAMMATORY DISORDERS

Altered expressions of cathelicidins have also been reported in auto-inflammatory or auto-immune diseases. Human LL-37 is found to be suppressed in atopic dermatitis and Crohn's disease (Ong et al., 2002; Wehkamp et al., 2007), but shown to be elevated in systemic lupus erythematosus (SLE) and rheumatoid arthritis (Paulsen et al., 2002; Sun et al., 2011). It has been proposed that LL-37 can complex with self DNA activating dendritic cells to contribute to the pathogenesis of SLE (Lande et al., 2011). Whereas, in psoriasis it has been hypothesized that LL-37 can act both as an effector and a regulator (Kanda et al., 2010). Furthermore, a recent study has shown that LL-37 can interfere in the activation of inflammasome contributing to suppression of pro-inflammatory responses in psoriasis (Dombrowski et al., 2011). Consistent with this, it has been hypothesized that LL-37 can protect against auto-immune diseases, which may be in part mediated by vitamin D (Bartley, 2010). Even though studies have demonstrated that altered cathelicidin expression is associated with chronic inflammatory auto-immune diseases, clarification of their specific roles in immune-mediated inflammation will require further investigations. Research in the last decade has established that cathelicidins mediate a wide range of immune functions including promotion of barrier repairs, chemokine and cytokine production, modulation of dendritic cell differentiation, and T-cell polarization, as well as demonstrate potent anti-sepsis and anti-inflammatory properties (discussed below). Elucidating the differential effects

of pro- and anti-inflammatory functions mediated by these peptides resulting in both immune activation and control of inflammation represents an exciting area of research.

CATHELICIDIN-MEDIATED IMMUNE ACTIVATION

A primary innate immune function mediated by cathelicidins is the facilitation of immune cell recruitment, either by direct chemoattractant properties or indirectly by inducing the production of chemokines. For example, human LL-37 and porcine PR-39 are direct chemoattractants for neutrophils, monocytes, T-cells, mast cells, etc. (Yang et al., 2001; Niyonsaba et al., 2002; Tjabringa et al., 2006). In addition, cathelicidins such as LL-37 can also promote chemotaxis by inducing the production of chemokines, e.g., MCP-1, RANTES, Gro-α, and IL-8 from both immune cells and other cell types such as epithelial cells and gingival fibroblasts (Mookherjee et al., 2006; Montreekachon et al., 2011). LL-37 also up-regulates the expression of chemokine receptors such as CXCR4, CCR2, and IL-8RB in macrophages (Scott et al., 2002). Mediating recruitment of leukocytes and thus aiding in phagocytosis to enhance clearance of pathogens is one of the critical innate immune functions of cathelicidin peptides. Furthermore, cathelicidins such as LL-37 synergistically enhances anti-infective immune responses in the presence of certain critical cytokines such as IL-1β and GM-CSF (Yu et al., 2007). Other innate immune activating properties of cathelicidins are (i) mediating protection against mycobacterium by promoting autophagy in macrophages (Yuk et al., 2009), (ii) prolonging the life span of neutrophils by inducing the expression of anti-apoptotic protein BcL-X_L and inhibiting caspase-3 activity, thus enhancing phagocytosis by neutrophils (Nagaoka et al., 2006), and (iii) enhancing epithelial wound healing (Carretero et al., 2008).

Cathelicidins such as human LL-37 appear to be a link between the innate and adaptive immune responses by influencing dendritic cell activiation and polarization of T-lymphocytes (Davidson et al., 2004; Bandholtz et al., 2006). LL-37 up-regulates the endocytic capacity of dendritic cells and enhances the secretion of cytokines that aid in the polarization of a Th1 immune response (Davidson et al., 2004). Apart from influencing the initiation of the adaptive immune response, recent studies have shown that cathelicidins also have a direct impact on lymphocytes. For example, murine CRAMP can directly alter T- and B-cell responses, promote and regulate humoral and cellular antigen-specific adaptive immune responses (Kurosaka et al., 2005; Kin et al., 2011). Overall, cathelicidins have a direct effect on immune cells influencing both innate and adaptive immunity against pathogenic attack. Most of the above mentioned immunity-related activity mediated by cathelicidins can be described as pro-inflammatory functions required for resolution of infections. However, these peptides also exhibit anti-inflammatory properties, thus playing a significant role in balancing inflammation and maintaining homeostasis.

CONTROL OF INFLAMMATION BY CATHELICIDINS

Peptides such as LL-37 and CRAMP have been demonstrated to confer protection in animal models of pathogenic sepsis (Cirioni et al., 2006), and have anti-inflammatory effects in "sterile" inflammatory diseases such as ulcerative colitis (Wong et al., 2012). Consistent with this, cathelicidin knock out mice show increased inflammatory responses compared to wild type (Morioka et al., 2008). Recent studies have also shown that synthetic cationic peptides derived from cathelicidins can indeed suppress inflammation in infection models (Scott et al., 2007) and in immune-mediated inflammatory models (Turner-Brannen et al., 2011).

Mechanistic studies have demonstrated that cathelicidins intervene at multiple points within the inflammatory cascade to suppress inflammation in a targeted fashion. For example, human and bovine cathelicidins alter the TLR-to-NF-κB pathway in the presence of exogenous inflammatory stimuli (e.g., endotoxin) and selectively suppress specific pro-inflammatory cellular responses such as TNF-α, TNFAIP2, IL-1β, and NFκB1 (Mookherjee et al., 2006). In contrast, anti-inflammatory mediators such as IL-10, TNFAIP3, NF-κB inhibitor NFκBIA are enhanced by these peptides (Mookherjee et al., 2006; Brown et al., 2011). It has also been shown that LL-37 can inhibit cellular immune responses triggered by IFN-γ, which is a key cytokine for polarization of Th1-responses (Nijnik et al., 2009). Another mechanism of inflammatory control demonstrated for cathelicidin peptides, e.g., PR-39, is by proteosome-mediated degradation of IκBα and subsequent inhibition of activation of NF-κB (Bao et al., 2001).

Intracellular uptake has been shown to be important for immunomodulatory activity of cathelicidins (Lau et al., 2005). This is mediated either by putative surface receptors (De et al., 2000; Lau et al., 2005) or by atypical endocytic pathways followed by interaction with intracellular receptor, e.g., GAPDH (Mookherjee et al., 2009). Specific receptor interaction for cathelicidin peptides and how this influences different immune-modulatory functions is yet to be completely resolved. The molecular mechanism of the anti-inflammatory activity of cathelicidins appears to be very complex, requires intracellular uptake, interaction with either putative surface or intracellular receptors, leading to the alteration of various signaling pathways (e.g., NF-κB, p38 and JNK MAPK, PI3K) with different kinetics.

The overall result of immunomodulatory functions of cathelicidin HDPs is the net balancing or control of inflammation without compromising immune responses that are required for resolution of infections. This duality or pro- and anti-inflammatory biological activities of these peptides has made them attractive agents to be explored as therapeutics. The distinct advantages of developing cathelicidins and their derivatives as therapeutics are (i) the ability to control inflammation without compromising the immune functions required to clear infections, and (ii) the unlikelihood of developing microbial resistances against these peptides, since most of these peptides influence the immune system under physiological conditions to control infections rather than direct microbicidal activity (Afacan et al., 2012).

SUMMARY

Cathelicidins have been defined to play multiple roles in immunity contributing to both resolution of infections and inflammation. The molecular mechanisms governing the multiple functions of these peptides in immunity is very complex, involving various signaling pathways and multiple transcription factors, and is influenced by

the cellular environment and extracellular signals. Moreover, the process of endocytic uptake or interaction with specific receptors and how this mediates the diverse immunomodulatory functions of cathelicidins is not completely understood. However, evidence in the last decade revealing the duality of pro- and anti-inflammatory functions of cathelicidins, resulting in the promotion of immune responses required for resolution of infections and at the same time controlling inflammation, has captured the interest of biomedical researchers. Consequently, there has been a keen interest in the development of these peptides, in particular their synthetic derivatives as therapeutics; as antimicrobials, anti-inflammatory agents, adjuvants, and in wound healing. The challenges however are limited pharmacokinetic or toxicology data, bioavailability, and high manufacturing costs. Establishing the immune-regulatory properties of HDPs such as cathelicidins and exploring their potential as immunomodulatory therapeutics represents an exciting avenue of research that is growing rapidly.

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Antimicrobial peptides for Gram-negative sepsis: a case for the polymyxins

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Sepsis, or "blood poisoning" in lay terminology, is a common and serious clinical problem. While fewer than 100 cases were reported prior to 1920 (Felty and Keefer, 1924), it is now the 13th leading cause of overall mortality (Gelfand and Shapiro, 1993) and the number one cause of deaths in the intensive care unit accounting for some 200,000 fatalities in the US annually. The incidence continues to rise in the US (Martin et al., 2003; **Figure 1**) and worldwide (Moss and Martin, 2004), perhaps due to increased invasive procedures, immunosuppression, and cytotoxic chemotherapy. Mortality associated with sepsis, unfortunately, has essentially remained unchanged at about 45% (Cross and Opal, 1994), despite tremendous strides in antimicrobial chemotherapy, pointing to the absence of therapeutic strategies aimed specifically at the pathophysiology of sepsis. The pathophysiology of the disease is characterized by a systemic inflammatory response syndrome (SIRS), culminating in its frequently fatal sequel, multiple organ dysfunction syndrome (MODS). The systemic inflammatory response is a consequence of dysregulated activation of innate immune effector mechanisms (Castellheim et al., 2009). Counterregulatory mechanisms that are subsequently deployed to dampen the initial overexuberant systemic inflammatory responses are also thought to contribute to the pathophysiology due to late-stage immunosuppressive (hypoinflammatory) phenomena, which render the host unable to eradicate the offending pathogen (Hotchkiss and Karl, 2003; Hotchkiss et al., 2009).

The primary trigger of SIRS in the Gram-negative septic shock syndrome is thought to be endotoxin, a constituent of the outer membrane of all Gram-negative bacteria. Endotoxins consist of a polysaccharide portion and a lipid called lipid A, and are therefore also called lipopolysaccharides (LPS). The polysaccharide portion consists of an O-antigen-specific polymer of repeating oligosaccharide units, the composition of which is highly varied among Gram-negative bacteria. A relatively well-conserved core hetero-oligosaccharide covalently bridges the O-antigen-specific chain with lipid A (Rietschel et al., 1994). Total synthesis of the structurally highly conserved lipid A has been shown to be the active moiety of LPS (Rietschel et al., 1987).

Whereas LPS itself is chemically inert, the presence of LPS in blood (endotoxemia), often a consequence of antibiotic therapy of preexisting bacterial infections (Holzheimer, 2001), is recognized by Toll-like receptor 4 (TLR4; Beutler and Poltorak, 2001; Palsson-McDermott and O'Neill, 2004; Hennessy et al., 2010), a member of a large super-family of pattern recognition receptors (Jounai et al., 2012; Newton and Dixit, 2012; Olive, 2012). Endotoxemia and its sequelae may arise even in the absence of Gram-negative bacterial infections, conditions such as trauma (Saadia et al., 1990), burns (Jones II et al., 1991), and splanchnic ischemia during cardiac surgery (Rocke et al., 1987) increase intestinal permeability, resulting in the spill-over into the portal circulation of LPS from the colon which is abundantly colonized by Gram-negative bacteria. The sensing of LPS, "read by our tissues as the very worst of bad news" (Thomas, 1975), results in a cascade of exaggerated host responses, manifesting in the clinical syndrome characterized by endothelial damage, coagulopathy, loss of vascular tone, myocardial dysfunction, tissue hypoperfusion, and multiple-system organ failure (Balk and Bone, 1989; Bone et al., 1992; Bone, 1993). LPS activates almost every component of the cellular and humoral (plasma protein) limbs of the immune system, resulting in the production of a plethora of proinflammatory mediators, important among which are not only early-phase cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and IL-6 (Dinarello, 1991, 1996) but also late-phase endogenous mediators such as high mobility group box 1 protein (HMGB1; (Wang et al., 1999; Andersson and Tracey, 2011). These cytokines and other mediators act in concert, amplifying the resultant generalized inflammatory processes.

Our understanding of basic mechanisms underlying the cellular response to LPS has increased vastly in recent years. These advances will likely offer novel therapeutic possibilities in the future. However, after more than two decades of intensive effort at evaluating more than 30 investigational compounds, specific therapeutic options for sepsis have remained elusive. Drotrecogin alfa (Xigris™, recombinant human activated protein C), an anticoagulant that ameliorates disseminated intravascular coagulation was approved in November 2001 by the FDA, but recently withdrawn due to lack of efficacy (Ranieri et al., 2012; Wenzel and Edmond, 2012). Clinical trials aimed at blocking various proinflammatory mediators including TNF-α, IL-1β, platelet-activating factor, and prostaglandins produced by the activated cellular components have all been disappointing (Zeni et al., 1997), suggesting that targeting downstream cellular inflammatory processes once immune activation has already progressed is unlikely to be of benefit.

It follows, therefore, that the paradigm of proximal, upstream intervention using molecules that specifically block the recognition of LPS by TLR4 would offer attractive therapeutic targets. As mentioned

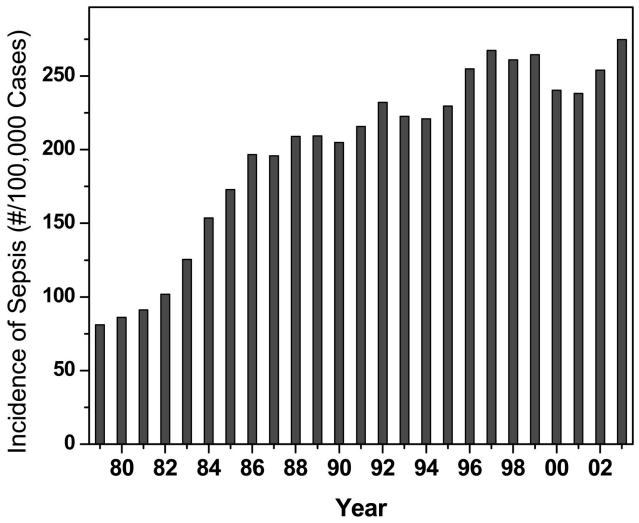

FIGURE 1 | Incidence of sepsis in the US Data provided by Greg Martin.

earlier, the polysaccharide portion of LPS is highly variable and serologically distinct for each strain of the same species of Gram-negative organisms. Although anti-O-polysaccharide antibodies afford protection in experimental models where animals are challenged with homologous bacteria (Kim et al., 1988; Siegel, 1995), these are not likely to be of significant clinical value since sepsis runs an acute course before the pathogen is identified and appropriate specific immunotherapy is instituted. The biologically active part of LPS, lipid A, as well as the core oligosaccharide portion are structurally highly conserved across Gram-negative genera, and thus are attractive targets for sequestration, and elimination of circulating LPS would, in principle, prevent the activation of inflammatory cascades (Ziegler et al., 1982; Ziegler, 1988; Ziegler and Smith, 1992). Experimental studies as early as 1968 suggested that antibodies directed toward epitopes in the core region of LPS may be broadly cross-protective against a range of Gram-negative organisms (Chedid et al., 1968). However, neither human (HA-1A; (Ziegler et al., 1991) nor murine (E5; (Bone et al., 1995) anti-lipid A monoclonal antibodies afforded significant protection in large, multiple, placebo-controlled clinical trials (Cross and Opal, 1994). Similarly disappointing results were obtained with core region-directed antibodies (Di Padova et al., 1993; Le Roy et al., 1999). Taken together, these failures could point to intrinsic problems with lipid antigens: poor immunogenicity, inaccessibility of neutralizing epitopes, the generation of non-specific cross-reactive antibodies against irrelevant hydrophobic epitopes (Vaarala et al., 1988), and potential problems with the antibody molecule itself: predominant intravascular compartmentalization, and possible tissue damage induced by activation of complement. Non-immunological blockade of LPS recognition using TLR4 "blockers" is therefore an alternative strategy, a premise that has indeed been explored with molecules structurally related to lipid A, but acting as TLR4-specific antagonists (Christ et al., 1995; Kawata et al., 1999; Wittebole et al., 2010; Ehrentraut et al., 2011; Tidswell and Larosa, 2011), but unfortunately, do not appear promising (Williams, 2012). It is not known whether the lack of efficacy is attributable to its physicochemical properties (high lipophilicity; (Christ et al., 1995) and consequent partitioning into plasma lipoproteins, with loss of activity (Rose et al., 2000).

As mentioned earlier, the structurally invariant and biologically active center of LPS, lipid A, is a logical therapeutic target for neutralization. Lipid A is composed of a hydrophilic, negatively charged *bis*-phosphorylated di-glucosamine backbone, and a hydrophobic domain of six (*E. coli)* or seven (*Salmonella*) acyl chains. The anionic amphiphilic nature of lipid A enables it to interact with a variety of cationic hydrophobic ligands (Vaara and Vaara, 1983; Peterson et al., 1985; Rocque et al., 1988). Polymyxin B (PMB), a cationic amphiphilic cyclic decapeptide antibiotic isolated from *Bacillus polymyxa* (Storm and Rosenthal, 1977), has long been recognized to bind lipid A (Morrison and Jacobs, 1976), and neutralize its toxicity in animal models of endotoxemia (Stokes et al., 1989; Durando et al., 1994; Yao et al., 1995). Although PMB is a commonly used topical antibiotic, it is nephro- and oto-toxic, which, while hitherto precluding its use as an LPS-neutralizer in patients with sepsis, has stimulated the search for non-toxic PMB analogs (Rustici et al., 1993; Porro et al., 1998), PMB derivatives (Vaara, 1983; Viljanen et al., 1991), as well as other structurally diverse cationic amphiphilic peptides (Rustici et al., 1993; Porro, 1994; Iwagaki et al., 2000; Scott et al., 2000; Jerala and Porro, 2004) as candidate LPS-binding agents. Notably, a hemoperfusion cartridge based on PMB covalently immobilized via one of its NH_2 groups to a polystyrene based fiber became available in Japan in late 2000 for clinical use ("Toraymyxin™," Toray Industries Inc., Tokyo; (Nakamura et al., 1999, 2002, 2003). In the EUPHAS randomized clinical trial, PMB hemoperfusion alongside conventional therapy was found to improve organ dysfunction and reduce 28-days mortality in subsets of patients with sepsis arising from intra-abdominal Gram-negative infections (Cruz et al., 2009). Whilst the utility of Toraymyxin provides a clinically validated proof-of-principle for the value of sequestering circulating LPS (Rimmele and Kellum, 2011), opportunities for extracorporeal hemoperfusion may be infrequent due to unfavorable hemodynamic parameters.

Given that the only encouraging lead for the management of sepsis to date appears to be PMB hemofiltration, it is perhaps useful to re-examine PMB itself, as well as its structurally closely related congener, polymyxin E (or colistin). PMB and polymyxin E differ one from the other by a single amino acid (D-Phe in PMB; D-Leu in PME; (Kwa et al., 2007), and are similar in their *in vitro* antimicrobial activity, clinical efficacy, and toxicity (Oliveira et al., 2009). Although banished to a topical-use-only status on account of its systemic toxicity, the polymyxins are rapidly re-emerging as last-resort parenteral antibiotics for the management of infections with extensive drug resistant

strains of *Pseudomonas aeruginosa* (Zavascki et al., 2007; Michalopoulos and Falagas, 2008; Giamarellou and Poulakou, 2009), *Acinetobacter baumannii* (Gales et al., 2011; Fitzpatrick et al., 2012), and *Burkholderia* species (Gales et al., 2011). The increased use of these peptide antibiotics has led to a careful re-examination of its purported toxicity (Falagas and Kasiakou, 2006). Evaluation in diverse clinical settings (Ramasubban et al., 2008; Nation and Li, 2009; Lim et al., 2010; Durakovic et al., 2011; Spapen et al., 2011; Cho et al., 2012) indicate that tolerability of the antibiotic is acceptable, and the toxicity is less than previously thought. Of particular interest is a recent report (Mizuyachi et al., 2011) detailing the safety and pharmacokinetic evaluation of intravenous colistin as the methanesulfonate prodrug (Bergen et al., 2006) in healthy human volunteers. Urinary *N*-acetyl-β-D-glucosaminidase (a biomarker for early renal damage) showed only transient and reversible increases at doses eliciting plasma concentrations (free area under the concentration-time curve/MIC) of drug that were predicted to be bactericidal (Mizuyachi et al., 2011). Since the antimicrobial effects of the polymyxins are a direct consequence of their binding to lipopolysaccharide (Morrison and Jacobs, 1976; Nikaido and Vaara, 1985), it is reasonable to assume that the plasma concentration of the drug would be of such magnitude as to be sufficient for sequestering LPS.

Erasmus' adage, "*Malo nodo, malus quærendus cuneus*" (for a hard knot a hard tool must be sought), rings perhaps particularly true for sepsis for which a single tractable lead toward a validated therapeutic approach is yet to be found, mortality continues to be unacceptably high, and a survey of the research landscape in the field conjures up images of a bleak battlefield strewn with the corpses of many a failed approach. The venerable polymyxins, never subjected to modern regulatory requirements when they were first introduced, and exiled for the last 50 years, are making their way back into the clinic as parenteral antibiotics. Much is being learned as they are re-examined with the rigor and precision of modern methods of pharmacokinetic (Couet et al., 2012) and pharmacodynamic (Mizuyachi et al., 2011) analyses. Perhaps there is a case to be made for a careful risk-benefit evaluation of the polymyxins in Gram-negative sepsis.

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Antimicrobial peptides can enhance the risk of persistent infections

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Bacterial resistance against conventional antibiotics is an escalating problem in modern medicinal treatment of infectious diseases, as a growing number of immunocompromised patients are suffering from hospital-acquired bacterial colonization. The spreading of genetic bacterial resistance has stimulated the interest in natural antimicrobial peptides (AMPs) as promising drugs against pathogenic species (Giuliani et al., 2007; Zaiou, 2007; Fjell et al., 2012). These peptides are a fundamental component of the innate immune system and represent the first line of defense against a broad spectrum of microorganisms (Radek and Gallo, 2007). Owing to their intrinsic amphiphilic character and usually positive net charge, AMPs bind strongly to bacterial plasma membranes. These receptor-independent interactions, which are present already at sub-lethal concentrations, can induce lipid flip-flop, cause the formation of stable or transient pores, and/or lead to membrane depolarization and permeabilization (Spindler et al., 2011). Elevated AMP concentrations tend to fully disrupt the lipid bilayer barrier, thereby exhibiting an immediate detergent-like action that kills the cells. In the latter scenario, it is impossible for the bacteria to develop any resistance or tolerance against AMPs. In addition to these primary effects on the plasma membrane, several AMPs were also shown to translocate across the lipid bilayer and interact with various intracellular targets. Such alternative targets include membrane respiratory proteins, nucleic acids, as well as machineries of cell wall and protein biosynthesis (Epand and Vogel, 1999; Zhang et al., 2001; Brogden, 2005; Giuliani et al., 2007; Mogi and Kita, 2009; Spindler et al., 2011).

Unlike conventional antibiotics, AMPs do not tend to operate in a stereospecific manner. Nevertheless, some resistance mechanisms have evolved, such as a reduction of the net negative charge in the bacterial envelope, the active efflux removal of AMPs, or their proteolytic destruction (Peschel, 2002; Otto, 2009). However, most pathogenic Gram-positive and Gram-negative bacteria still remain susceptible to AMPs, and much promise is associated with their further development and application. Currently, the high production costs of natural mammalian AMPs and their low molecular stability are considered to be a drawback for reaching the drug market (Zaiou, 2007). Here, we demonstrate that another, as yet unknown problem may emerge in the use of AMPs. Namely, some AMPs can cause bacterial persistence, a phenomenon known to be associated with the formation of biofilms responsible for chronic diseases (Lewis, 2010). These biofilms, in turn, have a high tolerance against conventional antibiotics and represent a dangerous growth form of pathogenic bacteria that should be avoided by all means (Stewart and Costerton, 2001).

Amongst the best-studied AMPs are Magainin-2 (Mag2) and PGLa from *X. laevis*, commonly both denominated as "magainins". These cationic peptides are produced in the granular glands of the frog skin and acquire an amphiphilic α-helical structure when bound to lipid membranes. Both peptides are moderately active *per se*, but a notable feature is their synergistically enhanced action in a 1:1 mixture (Matsuzaki et al., 1998; Strandberg et al., 2009). Magainins were used as a blueprint to design an ideal α-helical "model amphiphilic peptide" MAP (Oehlke et al., 1998), which also exhibits an antibacterial effect (Palm et al., 2006). All three helical molecules (with a length of around 20 amino acids) bind to lipid bilayers, where they can be surface-bound or obliquely immersed, depending on peptide concentration (Glaser et al., 2005; Bürck et al., 2008; Strandberg et al., 2009) and temperature (Afonin et al., 2008b). A fully inserted transmembrane state of these peptides has been associated with the formation of transient pores (Afonin et al., 2008b; Ieronimo et al., 2010). These have been shown to allow the escape of small metabolites and ions (Matsuzaki, 1998), thereby also decreasing the transmembrane proton gradient and as a result ATP generation. Mag2 was found to enhance the uncoupling and depolarizing activity of PGLa in liposomes containing cytochrome oxidase (Westerhoff et al., 1995). However, it is important to note that pore formation *per se* does not result in complete lysis of the membrane, and it does not kill bacteria (Epand and Vogel, 1999; Zhang et al., 2001). Here, we demonstrate that amphiphilic AMPs can actually stimulate survival mechanisms in bacteria instead of eradicating them.

For many pathogenic bacteria is known that under stress conditions (e.g., high bacterial density, depletion of nutrients and oxygen, temperature shift, osmotic shock, or selective pressure of antibiotics), cell populations produce small and temporary subpopulations of dormant cells. They are called persisters, because they have adapted to a long-term survival by a reduced level of metabolic activity, diminished protein synthesis, multidrug tolerance to antibiotics, and an enhanced ability to grow as surface-adherent biofilms. This general survival strategy is explained by an expression of starvation-related (Fux et al., 2005) or persister genes (Lewis, 2010). Due to

their reduced growth rate, these reversible bacterial states are known as non-growing and "viable but non-culturable" (Oliver, 2005), or as slow growing phenotypes. The latter ones manifest on agar media as small colony variants (SCVs; von Eiff et al., 2006; Wellinghausen et al., 2009). Clinical isolates of *S. aureus* SCVs are characterized by deficiencies in transmembrane electron transport and ATP generation, and by an increased expression of adhesins instead of other virulence factors. These metabolic alterations facilitate the protective internalization of the bacteria into host cells and thereby persistent and recurrent infections (Proctor et al., 2006). The first identified molecular mechanism of a cellular stress response, which has been recently described in *E. coli*, explains how cells can convert into a dormant state. In the presence of stress factors, such as ciprofloxacin, which inhibits biosynthesis of DNA, the cells express the small autotoxic protein TisB. Interaction of this 29 amino acid peptide with the plasma membrane decreases the protonmotive force, reduces ATP synthesis, and thereby transforms bacteria into an isogenic dormant state (Dorr et al., 2010). Notably, in both modes of stress response (i.e., in slow growing auxotrophic clinical isolates of *S. aureus*, as well as in *E. coli* expressing TisB) the key feature is the disturbance of the proton gradient. Either by a deficiency in its generation or by its active dissipation, the reduced protonmotive force leads to a lower level of ATP generation by oxidative phosphorylation. The need for ATP production can be compensated by substrate-level phosphorylation, e.g., via glycolysis or via the arginine deiminase pathway. This general metabolic switch is associated with the characteristic slow growth in the biofilm mode (Proctor et al., 2006).

Our key idea, which elicited the present study, was the realization that TisB has a remarkably similar amphiphilic α-helical structure when compared to the antimicrobial magainin peptides described above (see **Figure 1B**). After all, both types of peptides increase the proton permeability of lipid bilayers. The stress response peptide TisB has been shown to localize to the inner membrane of *E. coli* (Unoson and Wagner, 2008), where it gets inserted in a transmembrane alignment (Steinbrecher et al., in revision). We thus wanted to find out whether membrane-active AMPs would also be able to trigger the formation of persister cells via their known depolarizing effect on bacteria. We monitored bacterial growth using the redox indicator resazurin (alamarBlue™), which changes its color from blue to pink upon reduction, as an indicator of cellular respiration. In this way, we carried out twofold microdilution assays with several different AMPs to determine their minimum inhibitory concentration (MIC) values from the bacterial respiratory activities. Subsequently, the wells of the microtiter plates were inspected with a microscope to see whether pinpoint colonies or biofilms had formed. Besides the α-helical PGLa, Mag2, a 1:1 mixture of PGLa/Mag2, and MAP, we also included two cyclic AMPs in this study. Gramicidin S (GS) and polymyxin B (PmB) have a very different molecular structure compared to the magainins, and they are rather

A

Medium*	Mag2	PGLa	PGLa/Mag2	MAP	GS	PmB
	AMPs [MIC value in µg/ml]					
	S. aureus ATCC 25923					
LB	[32]	[2]	[1]	[4]	[1]	[4]
MH	[128]	[16]	[8]	[4]	[2]	[16]
	E. faecalis ATCC 29212					
LB	[128]	[32]	[1]	[8]	[1]	[16]
MH	[128]	[128]	[64]	[8]	[2]	[64]
	E. coli ATCC 25922					
LB	[32]	[8]	[4]	[32]	[8]	[1]
MH	[128]	[64]	[8]	[64]	[32]	[2]
	P. aeruginosa ATCC 27853					
LB	[32]	[16]	[8]	[8]	[16]	[1]
MH	[64]	[128]	[64]	[32]	[32]	[2]
	++++	+++	++	+	-	

*LB – salt free Luria-Bertani medium; MH – salt free Mueller-Hinton medium.

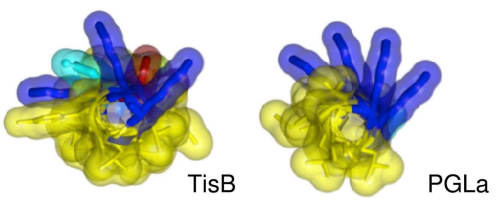

FIGURE 1 | (A) Emergence of small colonies at sub-MIC concentrations of AMPs. MIC values are indicated in square brackets, as determined after 24 of exposure to the AMP by means of a resazurin color change. Pinpoint colonies were detected in the wells with concentrations corresponding to 1/2 MIC for all peptides, except for MAP, which was effective even at 1/4 and 1/8 of MIC. Occurrence of pinpoint colonies was observed as an average of at least two independent experiments; **(B)** Molecular structures of TisB (left) and PGLa (right); **(C)** Pinpoint colonies of *S. aureus* in the presence of MAP were detected on the bottom of 96-wells microtiter plates with an inverted microscope (Leica DM IL) at 100-fold magnification – two individual microcultures under identical conditions are shown as examples.

selective against either Gram-positive or Gram-negative strains, respectively. PmB has a high affinity to Lipid A and allows rapid detoxification of LPS, thus exerting an immediate anti-inflammatory effect. It also inhibits respiratory proteins directly, such as an alternative NADH dehydrogenase and malate-quinone oxidoreductase (Mogi and Kita, 2009). PmB has been shown to kill *P. aeruginosa* already at peptide concentrations below the level that is needed to cause cellular depolarization (Zhang et al., 2000). The antibacterial action of GS is even more multifaceted. In addition to non-lethal depolarization, presumably via formation of transient pores at sub-MIC concentrations (Zhang et al., 2001; Afonin et al., 2008a), and membrane disruption at supra-MIC (Hartmann et al., 2010), GS also inhibits membrane-associated proteins such as cytochrome *bd* quinol oxidase and an alternative NADH dehydrogenase (Mogi and Kita, 2009), as well as various membrane-bound ATPases (Zhao and Dhalla, 1991). In addition, all ATP-dependent processes should be indirectly affected, since cyclic peptide GS has a particularly high affinity to bind ATP molecules (Krauss and Chan, 1983).

As anticipated from our structural and functional comparison of TisB with AMPs, we indeed found that tested bacteria formed small colonies upon treatment with AMPs. The gray scale of the shading in the table of **Figure 1A** summarizes the observed occurrence of adherent colonies in the liquid microcultures of various Gram-positive and Gram-negative strains. Represented here are the findings at sub-lethal concentrations of the different AMPs, usually taken at 1/2 of the corresponding MIC value (the actual MIC values are listed in square brackets). TisB was used as a comparison and lead to intensive biofilm-like growth in both Gram-positive strains and in *E. coli* K12 (data not shown). At sub-MIC levels of the AMPs, it is clear that some cells will survive in the original 10^5–10^6 CFU/ml inoculation dosis of the planctonic microcultures. However, the formation of small adherent colonies in the liquid medium, as illustrated in **Figure 1C**, suggests that these surviving bacteria have changed their phenotype due to the exposure to AMPs. Remarkably, all of the tested AMPs showed this effect, though to a different extent. MAP was the most effective, leading to pinpoint colonies in *S. aureus*, *E. faecalis*, and *P. aeruginosa*. Besides a notable dependence on the cultivation medium, the helical peptides (PGLa, Mag2, PGLa/Mag2, MAP) were generally found to be more effective than the cyclic GS and PmB. Interestingly, for the synergistic pair Mag2/PGLa, the occurrence of SCVs exceeded the corresponding effect of the individual peptides, thereby demonstrating a pronounced synergy in the promotion of bacterial survival. Pinpoint colonies were not found for *E. coli* ATCC 25922, which suggests a species dependent variability in the bacterial response.

It is known that sub-lethal levels of conventional bactericidal agents can induce mutagenesis (Kohanski et al., 2010), but can also enhance bacterial adherence to the host cells (Zhanel and Nicolle, 1992) and biofilm formation (Kaplan, 2011). The induction of survival mechanisms upon incubation with membrane-depolarizing AMPs, as demonstrated here for the first time, shows that these mechanisms are universal and can lead to an emergence of tolerance which does not imply genetic resistance. It might be considered as a general mechanism of evolutionary significance that the passage of protons across the plasma membrane reduces metabolic activity at stress conditions and converts bacteria into dormancy. This way, bacterial genomes are preserved from extinction as a biological species. Our findings have shown that sub-lethal AMP concentrations, at which there is no membranolytic effect, can trigger the transition of bacteria into dormancy, so that they can survive and grow in an adherent form even in liquid media. An alternative possibility to explain the emergence of the small pinpoint colonies could be the inability of the AMPs to kill existing dormant cells that might have been present in the inoculum. Both possibilities require further studies of the phenomenon. Independent of the mechanism, we can conclude that exposure to sub-lethal concentrations of AMPs, especially in the case of amphiphilic α-helical peptides, can enhance the risk of persistent bacterial infections.

Our findings do not imply, however, that AMPs cannot be used as antibacterial drugs in general. Since different types of peptides show different modes of membrane perturbation (c.f. cyclic GS and PmB), they are likely to bear different risks of triggering persistence and/or biofilm formation. Furthermore, higher organisms have evolutionarily optimized their strategies to cope with the universal bacterial stress response. For example, in the case of injury, the immune cells that express high levels of defensins and cathelicidins are attracted directly to the wound. This way, the AMPs are delivered at very high local concentration to the site of potential infection (Radek and Gallo, 2007; Zaiou, 2007; Hancock et al., 2012). Analogous local or topical applications of AMPs have helped and will continue to help the human organism, while avoiding an unfavorable imbalance in the usual, e.g., intestinal microbiota. We believe that medical approaches show most promise, when they can provide high local concentrations of AMPs directly at the site of infection. For instance, cationic AMPs can be delivered into the respiratory tract with an ultrasonic nebulizer (Falagas et al., 2010), and tissue-specific local drug delivery is possible via medical electrophoresis or using polymeric nanosphere gels (Batheja et al., 2011).

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